Molecular classification of myelodysplastic syndromes

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Hematology Education: the education program for the annual congress of the European Hematology Association

2015;9:209-218

A B S T R A C T

The current approach adopted by the World Health Organization (WHO) for the classification of tumors of hematopoietic and lymphoid tissues is based on a combination of morphology, immunophenotype, and genetic and clinical features to define distinct clinico-pathological disease entities. This classification is providing the best diagnostic approach to myeloid neoplasms with myelodysplasia. However, since its first proposal, biological and analytical limitations have emerged, in particular, the scarce reproducibility of morphological analysis of dysplasia and the poor specificity of dysplastic changes. In the last few years, our understanding of the genetic basis of myelodysplastic syndromes and related disorders has widened and recurrent somatic mutations have been identified in several genes. In 2001, the WHO classification recognized myelodysplastic syndrome with isolated del(5q) as a distinct category, representing the first subtype of myelodysplastic syndrome defined by a genetic abnormality. The available evidence suggests that the incorporation of genetic information may potentially improve the current classification system by identifying biologically homogeneous entities and providing objective and reproducible biomarkers for recognition of specific entities, and may open new avenues of research that could lead to the development of novel therapeutic options.

Learning goals

At the conclusion of this activity, participants should know that:

- the current approach adopted by the World Health Organization (WHO) classification of tumors of hematopoietic and lymphoid tissues aims to define distinct clinico-pathological disease entities, and is currently providing the best diagnostic approach to myeloid neoplasms with myelodysplasia;
- the WHO classification of myeloid neoplasms with myelodysplasia is limited by the scarce reproducibility of morphological analysis of dysplasia and the poor specificity of dysplastic changes;
- the available evidence suggests that the incorporation of genetic information may potentially improve the current classification system of myeloid neoplasms with myelodysplasia by identifying biologically homogeneous entities and providing objective and reproducible biomarkers for recognition of specific entities.

Introduction

Classification is a fundamental process of medicine that aims to distinguish disease entities that are clearly defined, clinically distinctive, non-overlapping, and collectively exhaustive (i.e. that collectively account for all known entities).[1] Classification represents the frame for diagnosis and treatment of diseases, and uniform definitions and terminology are the basis for designing and interpreting clinical and translational studies, as well as for investigation into the genetic basis of neoplasms. There are two elements to the classification process: class discovery (the process of identifying categories of diseases through basic, translational and clinical investigations) and class prediction (the process of determining which category a case belongs to), which is a critical step in the diagnosis of each individual patient.

The current approach adopted by the World Health Organization (WHO) in its classification of tumors of hematopoietic and lymphoid tissues is based on a combination of morphology, immunophenotype, genetic and clinical features to define distinct clinico-pathological disease entities.[1] In principle, morphological criteria may reflect the underlying pathophysiology; however, several factors may affect the intricate circuitry that links the genetic lesions to the clinical phenotype of a cancer, thus introducing an intrinsic source of heterogeneity in the classification process. In the last few years, advances have been made in our understanding of the genetic basis of myeloid malignancies, including myelodysplastic syndromes (MDS) and related disorders, and recurrent somatic mutations have been identified in several genes.[2-9] In addition, gene expression profiling has uncovered many systematic differences between cancer cells and normal cells, and has enabled new, clinically relevant disease subtypes to be defined.[10-16] All this information has the potential to improve the classification process, most importantly by identifying biologically homogeneous entities, and providing objective and reproducible biomarkers for recognition of specific entities, thus impacting on both the processes of class discovery and of class prediction.

Current WHO classification of myeloid neoplasms with myelodysplasia

Myelodysplasia is a term used in pathology for describing morphological abnormalities, or dysplasia, in one or more of the major myeloid cell lines of hematopoiesis, and is a typical feature of MDS. Myelodysplasia is not restricted to MDS but may be found also in other myeloid neoplasms of the WHO classification, including myelodysplastic/myeloproliferative neoplasms (MDS/MPN) and acute myeloid leukemia (AML) with myelodysplasia-related changes.[17-19]

The diagnostic approach to MDS includes morphological studies of peripheral blood and bone marrow to evaluate abnormalities of peripheral blood cells and hematopoietic precursors; bone marrow biopsy to assess marrow cellularity, fibrosis, and topography; and cytogenetics to identify non-random chromosomal abnormalities. Additional investigations are also recommended, including flow cytometry immunophenotyping and fluorescence *in situ* hybridization (FISH).[20]

Myelodysplastic syndromes are currently categorized according to the percentage of blasts on the bone marrow and the peripheral blood, the number of dysplastic lineages, and the presence of ring sideroblasts (Table 1).[21] This classification is a useful instrument for defining the different subtypes of MDS, and also provides clinicians with prognostic information. In fact, several retrospective and prospective studies demonstrated that the WHO classification has important prognostic information. In particular, among patients with MDS without excess blasts, isolated involvement of the erythroid lineage rather than bi- or trilineage marrow dysplasia is associated with a significantly better prognosis. In addition, the definition of two categories of refractory anemia with excess blasts identifies two groups of patients with significantly different survival and risk of leukemic evolution.[22,23] It has been shown that the WHO classification can predict not only the natural history of the disease, but also outcome after disease-modifying treatments, including allogeneic hematopoietic stem cell transplantation.[24]

The WHO classification provides the best diagnostic approach to myeloid neoplasms with myelodysplasia. However, since its proposal in 2001 and its first revision in 2008, biological and analytical limitations have emerged. The most important difference between the WHO and FAB classifications was the lowering of the threshold for the diagnosis of AML from 30% to 20% blasts in the blood or bone marrow.[19,25] In fact, several studies suggested that patients with 20%-29% blasts often have clinical features, including response to therapy and survival times, similar to patients with 30% or more blasts.[26] According to the WHO proposal, these cases are classified as AML with multilineage dysplasia, a category that includes patients with a prior history of MDS, as well as patients who present initially with AML and dysplasia in multiple marrow cell lineages.[19] However, some patients with prior MDS and 20%-29% bone marrow blasts may show a clinical behavior that is more similar to MDS than to AML. Thus, although bone marrow blast count undoubtedly reflects the biology of the disease, it has become clear that in myeloid neoplasms with 20%-29% blasts, the percentage of blasts *per se* is not enough to clearly define distinct clinical entities, and additional data should be taken into account and integrated into the classification and clinical decision making.[20]

Table 1. World Health Organization 2008 classification of myelodysplastic syndromes.

Disease	Blood findings	Bone marrow findings
Refractory cytopenia with unilineage dysplasia (RCUD): refractory anemia (RA), refractory neutropenia (RN), refractory thrombocytopenia (RT)	Single lineage cytopenia, no or rare blasts (<1%), bicytopenia may be occasionally observed*	Unilineage dysplasia (≥10% of the cells in one myeloid lineage) <5% blasts, <15% ring sideroblasts within erythroid precursors
Refractory anemia with ring sideroblasts (RARS)	Anemia, no blasts	Erythroid dysplasia only, <5% blasts, ≥15% ring sideroblasts within erythroid precursors
Refractory cytopenia with multilineage dysplasia (RCMD)	Cytopenia(s), no or rare blasts (<1%), no Auer rods, $<1x10^9$/L monocytes	Dysplasia in ≥10% of cells in 2 or more myeloid cell lineages, <5% blasts, no Auer rods (the percentage of ring sideroblasts is irrelevant)
Refractory anemia with excess blasts-1 (RAEB-1)	Cytopenia(s), <5% blasts, no Auer rods, $<1x10^9$/L monocytes (cases with Auer rods and <5% blasts in the peripheral blood and <10% blasts in the marrow should be classified as RAEB-2)	Unilineage or multilineage dysplasia, 5%-9% blasts, no Auer rods (cases with Auer rods and <5% blasts in the peripheral blood and <10% blasts in the marrow should be classified as RAEB-2)
Refractory anemia with excess blasts-2 (RAEB-2)	Cytopenia(s), 5%-19% blasts, occasional Auer rods, $<1x10^9$/L monocytes.	Unilineage or multilineage dysplasia, 10%-19% blasts, occasional Auer rods.
Myelodysplastic syndrome, unclassified (MDS-U)	Cytopenias, no or rare blasts (β1%)°	Unequivocal dysplasia in less than 10% of cells in one or more myeloid cell lines when accompanied by a cytogenetic abnormality considered as presumptive evidence for a diagnosis of MDS, <5% blasts *Cases of RCUD with pancytopenia °Cases of RCUD and RCMD with 1% myeloblasts in peripheral blood.
Myelodysplastic syndrome associated with isolated del(5q)	Anemia, normal to increased platelet count, no or rare blasts (<1%)	Normal to increased megakaryocytes with hypolobated nuclei, <5% blasts, no Auer rods, isolated del(5q)

In addition to these intrinsic limitations of morphological parameters in capturing the biology of the disease, diagnosis and classification of MDS are compromised by the scarce reproducibility of morphological analysis of dysplasia and by the poor specificity of dysplastic changes.[27-29] Much effort has been made to standardize morphological parameters, including myeloblasts, ring sideroblasts and monocytes and their precursors, as well as to define minimal diagnostic criteria.[30-32] Nonetheless, according to previous observations, morphological abnormalities involving 10% or more of cells (mostly in erythroid lineage) were detected in a significant proportion of control patients affected with non-clonal cytopenia, and in some non-cytopenic controls.[27] In addition, various studies showed a low rate of inter-observer concordance for assessment of erythroid, megakaryocytic and granulocytic dysplasia, as well as of bone marrow blast cell count,[28,29] resulting in considerable diagnostic discordance.[33]

These limitations might be partly overcome by using more reproducible tools to assess bone marrow dysplasia, such as flow cytometry immunophenotyping, in the diagnostic workup.[34,35] However, these observations clearly suggest that the current pathology-based system is able to provide a robust and clinically useful classification of myeloid neoplasms with myelodysplasia, but that in order to overcome these biological and analytical limitations, we need to integrate molecular data.

Genomic landscape of MDS and correlations between genotype and disease phenotype

Our understanding of the molecular basis of MDS has improved dramatically in the last few years.[3-6] Recent studies made a systematic analysis of known or putative genes relevant in myelodysplasia combining massive parallel sequencing with array-based genomic hybridization, and showed that approximately 90% of MDS patients carry one or more oncogenic mutations, and two-thirds of them are found in individuals with normal karyotype.[7,8,36] Driver mutant genes include those of RNA splicing (*SF3B1, SRSF2, U2AF1, ZRSR2*),[5,6,37] DNA methylation (*TET2, DNMT3A, IDH1/2*),[3,4,38,39] histone modification (*ASXL1, EZH2*),[2,40,41] transcription regulation (*RUNX1*),[42] DNA repair (*TP53*),[43-45] signal transduction (*CBL, NRAS, KRAS*),[43,46,47] and cohesin complex (*STAG2*) (Table 2).[9] Only 4-6 genes are consistently mutated in 10% or more MDS patients, while there is a long tail of around 40 genes that are mutated less frequently.[7,8,36]

In a group of disorders classified on the basis of morphological criteria, it is essential to identify specific associations between genotype and disease phenotypes in order to recognize disease entities according to distinctive genetic profiles (Table 2). In MDS, this genotype-phenotype correlation is illustrated by the 5q- syndrome, first described as a distinct clinical entity by Van den Berghe in 1974.[48] The typical hematologic phenotype includes macrocytic anemia, normal or elevated platelet count with

Table 2. Recurrently mutated driver genes potentially relevant to the classification of patients with myeloid neoplasms with myelodysplasia.

Genes and biological pathways	Relationship between mutation and clinical phenotype
RNA splicing	
SF3B1	Strictly associated with ring sideroblasts phenotype
SRSF2	Associated with RCMD, RAEB, CMML, secondary AML
U2AF1	Mainly associated with RCMD, RAEB, secondary AML
ZRSR2	Mainly associated with RCMD, RAEB, secondary AML
DNA methylation	
TET2	Associated with multilineage dysplasia, high mutation rate in CMML
DNMT3A	Associated with multilineage dysplasia
IDH1/IDH2	Associated with RCMD or RAEB
Histone modification	
ASXL1	Associated with RCMD or RAEB, CMML, secondary AML
EZH2	Associated with RCMD or RAEB, secondary AML
Transcription	
RUNX1	Associated with RCMD or RAEB
BCOR	Associated with secondary AML
DNA repair	
TP53	Associated with high blast count, complex karyotype, secondary AML
Cohesin complex	
STAG2	Associated with RCMD, RAEB, secondary AML.
RAS pathway	
NRAS/KRAS, CBL, NF1	Associated with multilineage dysplasia, JMML
Signaling	
CSF3R	Strictly associated with CNL, found in a subset of patients with aCML
JAK2, MPL, CALR	Strictly associated with RARS associated with marked thrombocytosis

hypolobated megakaryocytes, and a lower rate of progression to AML than other types of MDS. The molecular basis of this MDS subtype was then identified as the haploinsufficiency of genes that map in the common deleted region, including the *RPS14* gene, which leads to activation of the p53 pathway and the macrocytic anemia characteristic of this disorder,[49,50] miRNA-145 and miRNA-146a contributing to aberrant megakaryopoiesis,[51] and *CSNK1A1* that induces hematopoietic stem cell expansion and a competitive repopulation advantage.[52] A major step forward in genotype-phenotype correlation has been the identification of somatically acquired mutations in *SF3B1*, a gene encoding a core component of RNA splicing machinery, in MDS patients with ring sideroblasts.[5,6] In the original reports, 25%-30% of MDS patients carried a somatic mutation of *SF3B1*, but the proportion of positive patients was significantly higher in the refractory anemia with ring sideroblasts (RARS) and refractory cytopenia with multilineage dysplasia and ring sideroblasts (RCMD-RS) subgroups (about 70%) than in the remaining WHO categories.[5,6] However, in patients with MDS or MDS/MPN, the association between ring sideroblasts and *SF3B1* mutations is even stronger than that suggested by the higher prevalence of these mutations in WHO categories with ring sideroblasts. In fact, ring sideroblasts can be detected at variable percentages also in patients assigned to WHO categories that are not defined by this morphological feature. A fraction of these patients show a proportion of ring sideroblasts below the diagnostic threshold of 15% for assignment to a sideroblastic subtype, whereas others may have a proportion of ring sideroblasts equal to or higher than 15% in the presence of classification criteria that would lead to them being assigned to a different category, such as isolated del(5q) or excess blasts. The analysis of a subgroup of patients in whom an accurate quantitative enumeration of ring sideroblasts was performed irrespective of WHO categories using recently established consensus criteria[31] showed that the *SF3B1* mutation status had a positive predictive value for disease phenotype with ring sideroblasts of 97.7%, while the absence of ring sideroblasts had a negative predictive value for *SF3B1* mutation of 97.8%. The causal relationship between *SF3B1* mutation and ring sideroblasts was also supported by the significant association between *SF3B1* mutant allele burden and percentage of ring sideroblasts.[53] These observations suggest that *SF3B1* is the first mutated gene in MDS to be strongly associated with a specific disease phenotype. Taken together, these data suggest that the incorporation of genetic information may potentially improve the current classification system, and have prompted basic and translational investigations aimed at identifying genetically-defined subsets irrespective of current morphological classification criteria. The results of these studies and their clinical implications are discussed in the next sections and summarized in Table 3.

Table 3. Currently recognized genetically-defined subtypes of myeloid neoplasms with myelodysplasia and disease entities under characterization.

Disease entity	Classification criteria	Clinical features and implications of the molecular basis
Currently recognized genetically-defined subtype		
MDS with isolated del(5q)	Normal to increased megakaryocytes with hypolobated nuclei, <5% BM blasts, no Auer rods, no or rare PB blasts (<1%), isolated del(5q)	Anemia and normal to increased platelet count, favorable prognosis. Selective sensitivity to lenalidomide induced by haploinsufficiency of *CSNK1A1*. Higher risk for progression associated with *TP53* mutations.
Genetically-defined subtype under characterization		
MDS associated with *SF3B1* mutations	≥1% BM ring sideroblasts, <5% BM blasts, no Auer rods, no or rare PB blasts (<1%), *SF3B1* mutation	Isolated erythroid dysplasia, favorable prognosis. Ineffective hematopoiesis resulting in low hepcidin levels and propensity to parenchymal iron loading. Mutations in *RUNX1* associated with worse survival and increased risk of progression. *SF3B1* modulators currently under development. Preliminary evidence of high response rate to TGF-β superfamily ligands.
MDS associated with multilineage dysplasia-type mutations	<5% BM blasts, no Auer rods, no or rare PB blasts (<1%), mutations in DNA methylation genes, splicing factors other than *SF3B1*, RAS pathway or cohesin complex	Multilineage dysplasia and significantly worse prognosis compared to other MDS without excess blasts.
MDS/MPN with t(8;9)(p22;p24)	<20% BM or PB blasts; t(8;9)(p22;p24); *PCM1-JAK2* rearrangement	Clinical picture of aCML, some cases presenting with eosinophilia. The *PCM1-JAK2*-fusion is likely to be a potential target of JAK2 inhibitors.
AML associated with secondary-type mutations	≥20% BM blasts, mutations in *SRSF2, SF3B1, U2AF1, ZRSR2, ASXL1, EZH2, BCOR, or STAG2*	Recognized antecedent MDS or leukemogenic exposures prior to AML diagnosis not required. Low rate of complete remission after induction chemotherapy, decreased event-free survival.
Myeloid neoplasm with inv(3)(q21q26.2) or t(3;3)(q21;q26.2)	< or ≥ 20% BM blasts, inv(3)(q21q26.2) or t(3;3)(q21;q26.2)	Mutations in genes activating RAS/receptor tyrosine kinase signaling pathways. Homogeneous mutational patterns and gene expression.
Myeloid neoplasm associated with mutations in splicing factors	< or ≥ 20% BM blasts, mutations in *SRSF2, SF3B1, U2AF1*	Two distinct subtypes with different morphological, molecular, and clinical features.
Myeloid neoplasm associated with *TP53* mutation	< or ≥ 20% BM blasts, mutations in *TP53*	High prevalence of monosomal and complex karyotypes, poor prognosis. Poor outcome after allogeneic stem cell transplantation. Small molecules targeting p53 mutations under development.

Genetically-defined MDS subtypes

In 2001, the WHO classification recognized MDS with isolated del(5q) as a distinct category, representing the first subtype of MDS to be defined by a genetic abnormality (Table 1).[19] This definition captures the previously identified 5q- syndrome.[48] However, the morphological features of cases classified in this subtype are diverse, and most patients do not have the specific constellation of signs that constitute the 5q- syndrome, suggesting that additional molecular abnormalities may also contribute to the phenotype of these patients. Nonetheless, recognition of MDS with isolated del(5q) has important clinical implications, providing the frame for targeted therapeutic interventions and identification of specific mechanisms of resistance. In fact, lenalidomide was proven to induce high rates of transfusion independency and cytogenetic response in patients with International Prognostic Scoring System (IPSS) low or intermediate-1 MDS with del(5q) and red blood cell transfusion-dependency,[54-56] and it was recently demonstrated that this agent induces the ubiquitination and consequent degradation of *CSNK1A1* by the CRBN-CRL4 E3 ubiquitin ligase, deriving its therapeutic window from specifically targeting a haploinsufficient gene.[57] In addition, mutations in *TP53* were found to be associated with higher risk of disease progression during treatment with lenalidomide,[44] and screening of these mutations is currently recommended as part of clinical decision making.[20] In a recent study based on a comprehensive mutation analysis in a large and well clinically characterized cohort of MDS patients,[7] our group analyzed significant associations between genotype and disease phenotype, and adopted unsupervised hierarchical clustering analyses to identify genetically defined MDS subtypes.[58] The results of this study showed that *SF3B1* mutation is a major classification criterion *per se*, able to identify a distinct subset of MDS patients with homogeneous genotypic and phenotypic features and favorable prognosis, irrespective of current classification criteria. In fact, in this group, neither the threshold of 15% or more ring sideroblasts nor the presence of uni- or multilineage dysplasia were able to recognize separate subsets. Patients with MDS carrying *SF3B1* mutation showed homogeneous disease phenotype with high prevalence of isolated erythroid dysplasia, and cases with multilineage dysplasia according to current WHO morphological criteria had only mild dysplasia in myeloid or megakaryocytic lineage.[58]

More recently, in a large and well-characterized cohort of myeloid neoplasms with 1% or more ring sideroblasts, we found that patients with *SF3B1* mutation showed significantly better overall survival and lower risk of disease progression compared with *SF3B1*-unmutated cases.[59] The independent prognostic value of *SF3B1* mutations was retained when the analysis was limited to sideroblastic categories, suggesting that these mutations are indeed able to recognize a distinct subset within MDS with ring sideroblasts. Within MDS associated with *SF3B1,* mutations in DNA methylation genes (*TET2* and *DNMT3A*) were significantly associated with multilineage dysplasia. When comparing patients with uni- or multilineage dysplasia, no significant effect of multilineage dysplasia was found on survival or risk of progression. In addition, mutations in *RUNX1* were significantly associated with worse survival and increased risk of progression.[59]

Taken together, these results suggest that MDS associated with *SF3B1* mutation is indeed a homogeneous subset of disease and should be recognized as a distinct disease entity within MDS, irrespective of current WHO criteria. Conversely, MDS with ring sideroblasts negative for *SF3B1* mutation, mainly classified as refractory cytopenia with multilineage dysplasia, show a significantly worse prognosis and and segregate into a different cluster with other MDS subtypes.[58,59]

The recognition of a disease subtype with a unique molecular basis may have potential clinical implications. We previously showed that patients with *SF3B1* mutation have a high degree of ineffective hematopoiesis resulting in inappropriately low hepcidin levels and propensity to parenchymal iron loading.[60] Recently, a transforming growth factor-β superfamily ligand was found to correct ineffective erythropoiesis and promote late-stage erythroid differentiation in mice,[61,62] and preliminary results from a phase II study in patients with MDS showed a higher response rate in patients with ring sideroblasts and *SF3B1* mutation.[63] In addition, several compounds were previously reported to bind to the SF3b complex and to inhibit mRNA splicing,[64,65] and preliminary results showed that *SF3B1* modulators may induce tumor regression and increase survival in *SF3B1*-mutant xenografts.[66]

The above mentioned study on genotype-phenotype correlations in MDS also focused on MDS categories without ring sideroblasts, and found that mutations in genes implicated in DNA methylation (*DNMT3A, TET2, IDH1, IDH2*), splicing factors other than *SF3B1 (SRSF2, U2AF1, ZRSR2)*, and those of the RAS pathway (*KRAS, NRAS, CBL, NF1*) and cohesin complex (*STAG2, RAD21*) independently predicted disease phenotype with multilineage dysplasia. Unsupervised clustering analysis among MDS categories without excess blasts suggested that these mutations were able to discriminate a homogeneous group of patients, invariably characterized by multilineage dysplasia, and with a significantly worse prognosis compared to cases with different mutation patterns. The results of unsupervised hierarchical clustering analyses including somatic mutations and currently classification features according to WHO criteria are schematically represented in Figure 1.[58]

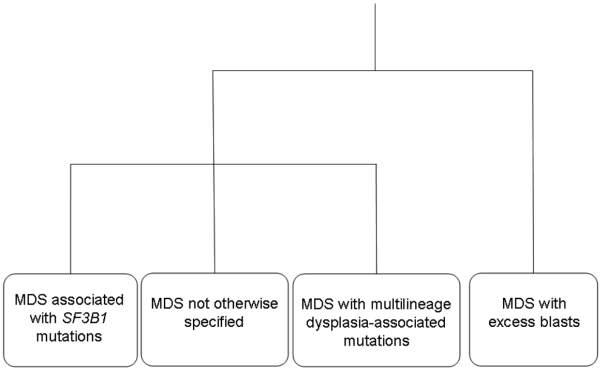

Figure 1. Schematic representation of clusters of MDS resulting from unsupervised hierarchical clustering analyses including somatic mutations and current classification features according to WHO criteria.[58] Multilineage dysplasia-associated mutations include mutations in the following gene categories: DNA methylation, splicing factors other than *SF3B1*, RAS pathway, cohesin complex.

Myeloid neoplasms at the boundaries of MDS and AML

Acute myeloid leukemia with inv(3)(q21q26.2) or t(3;3)(q21;q26.2) is currently a distinct disease entity in the WHO classification. Previous studies had showed that MDS with inv(3)(q21q26.2) or t(3;3)(q21;q26.2) are characterized by advanced disease phenotype with multilineage dysplasia or excess blasts, and a high risk of progression to acute myeloid leukemia.[67,68] More recently, a molecular characterization of myeloid malignancies with inv(3)/t(3;3), including both AML and MDS cases, showed that 98% of inv(3)/t(3;3) myeloid malignancies harbor mutations in genes activating RAS/receptor tyrosine kinase signaling pathways, and showed that neither mutational patterns nor gene expression profiles differ across inv(3)/t(3;3) AML and MDS cases, supporting the recognition of myeloid neoplasms with inv(3)/t(3;3) as a single disease entity irrespective of blast count.[69]

A recent study by Delwel and co-workers focusing on MDS with excess blasts and AML adopted unsupervised clustering approaches and provided evidence that RAEB and AML carrying mutations in splicing factors (*SF3B1*, *U2AF1*, or *SRSF2*) are clinically, cytologically, and molecularly very similar, and concluded that RAEB/AML with these mutations constitute a related disorder overriding the artificial separation between AML and MDS, and should be considered as myeloid malignancies that transcend the boundaries of AML and MDS.[70]

A more recent study by Lindsley and co-workers defined the mutational profile in cases of AML that develop following an antecedent MDS or CMML, and found eight genes that were mutated with more than 95% specificity in secondary AML compared to *de novo* AML, including *SRSF2, SF3B1, U2AF1, ZRSR2, ASXL1, EZH2, BCOR*, and *STAG2* (named secondary-type mutations).[71] In addition, three alterations were identified that were significantly under-represented in secondary AML compared to *de novo* AML, including *NPM1* mutations, *MLL*/11q23 rearrangements, and CBF rearrangements (termed *de novo*-type alterations). Finally, mutations in the *TP53* gene were associated with a distinct clinical phenotype, and reduced overall survival. All other mutations identified were not specific to either AML subtype and were labeled pan-AML mutations.

This ontogeny-based classification was then applied to resolve unrecognized clinical heterogeneity therapy-related AML, a category defined only on the basis of clinical exposure to leukemogenic therapy. Together, the results indicated that prior exposure to leukemogenic therapy does not define a genetically conforming therapy-related AML ontogeny. Rather, therapy-related AML could be separated into three groups more similar to AML with the same genetic alterations and no leukemogenic exposure.

Finally, this genetic classifier was applied to an unselected cohort of AML patients. Among older *de novo* AML patients, 45% had *de novo*/pan-AML mutations, whereas 33% had secondary-type mutations, and 20% had *TP53* mutations. Older *de novo* AML patients with secondary-type or *TP53* mutations showed shorter event-free survival than those with *de novo*/pan-AML mutations, suggesting that genetic ontogeny is able to capture a subset of patients with secondary-type mutations who may have had an unrecognized period of antecedent myelodysplasia prior to AML diagnosis, and that in elderly AML, genetic ontogeny more than clinical ontogeny may account for relative differences in sensitivity to chemotherapy.[71]

In the same study, patients with AML and mutations in the *TP53* gene were associated with a distinct clinical phenotype, including more complex karyotypes and reduced overall survival. Notably, in an analysis of paired MDS and secondary AML samples, no additional mutations were detected in MDS patients with *TP53* mutations at the time of disease progression, suggesting that *TP53* mutations might be able to drive leukemic evolution without the occurrence of co-operating mutations.[71]

High percentage of bone marrow blasts and high prevalence of monosomal and complex karyotypes are also typical features of patients with MDS carrying mutations in *TP53*.[43,72,73] A recent study of combined datasets from the International Working Group for MDS confirmed that patients with *TP53* mutations had a significantly shorter median overall survival (7.7 months) compared to patients with unmutated *TP53*, and a multivariate analysis identified *TP53* mutation status as the most significant prognostic marker for overall survival.[74] Taken together, these data suggest that *TP53* mutations might be considered to define a distinct class of myeloid neoplasms, irrespective of the proportion of bone marrow blasts and current WHO criteria. However, it must be acknowledged that, in contrast to current and other candidate genetically-defined subtypes of myeloid neoplasms, such as MDS associated with del(5q) or *SF3B1* mutations,[7,75] in most MDS cases *TP53* mutations are secondary genetic events driving the emergence of subclones on a dysplastic background,[7,74] and this does not fit with the concept of a unifying ontogeny as key prerequisite for the identification of unique disease entities.[17]

Integration of molecular criteria in the classification of MDS/MPN

According to the WHO classification, MDS/MPN are clonal myeloid neoplasms that at the time of initial presentation have some clinical, laboratory or morphological findings that support a diagnosis of MDS, and other findings more consistent with myeloproliferative neoplasm (MPN). These disorders comprise chronic myelomonocytic leukemia (CMML), atypical chronic myeloid leukemia (aCML), juvenile myelomonocytic leukemia (JMML), and myelodysplastic/myeloproliferative neoplasms, unclassifiable (MDS/MPN, U). The best characterized of these latter conditions is the provisional entity defined as RARS associated with marked thrombocytosis (RARS-T).[17]

Chronic myelomonocytic leukemia

Chronic myelomonocytic leukemia is currently defined as the presence of a persistent peripheral blood monocytosis over $1x10^9$/L, and at least one of the following: i) dysplasia in one or more cell lines; ii) an acquired clonal cytogenetic or molecular abnormality in hematopoietic cells; or iii) persistence of monocytosis for at least three months and no evidence of other causes of monocytosis. The diagnosis of CMML is 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 the

diagnosis of CMML uncertain. Comprehensive genetic studies reported cytogenetic aberrations in approximately 20% of patients with CMML, whereas at least one molecular mutation was observed in over 80% of patients.[76,77] However, most of these mutated genes are not specific for CMML and can be detected in different myeloid neoplasms,[7] as well as in elderly individuals with clonal hematopoiesis.[78-80]

A high prevalence of mutations in *SRSF2* was previously reported in patients with CMML, and co-operation between *SRSF2* and *TET2* mutations in this disorder has been suggested. In a large cohort of myeloid neoplasms with dysplasia, the association of *SRSF2* and *TET2* mutations was found to be highly specific for CMML disease phenotype. Notably, most of the double-mutated patients with a diagnosis of MDS according to WHO criteria had relative monocytosis at the time of mutation analysis and developed an overt CMML during follow up.[58] These data suggest that the association between these two gene mutations is highly predictive of a myeloid neoplasm characterized by myelodysplasia and monocytosis, supporting its recognition as co-criterion for the diagnosis of CMML.

Atypical chronic myeloid leukemia

Atypical chronic myeloid leukemia (aCML) is characterized by a neutrophilic leukocytosis with dysgranulopoiesis and circulating immature granulocytes. Its diagnosis currently relies on poorly specific criteria, and differentiating between aCML and other myeloid neoplasms, such as chronic neutrophilic leukemia (CNL), is difficult. In aCML, recurrent somatic mutations were reported, but according to the available evidence are not specific for this entity.[81,82] A high prevalence of mutations in *CSF3R* has also been reported in atypical CML, but the mutation seems to be more significantly associated with CNL.[83] At present, these mutations may represent valuable co-criteria for the diagnosis, although further efforts need to be made to identify the genetic determinants of disease phenotype in these overlapping syndromes.

An interesting variant of aCML is the MDS/MPN associated with the t(8;9)(p22; p24) translocation that fuses *JAK2* to *PCM1*. Although some cases present with eosinophilia and may be classified as chronic eosinophilic leukemia, approximately half of the patients have a clinical picture of aCML.[84] Interestingly, the *PCM1-JAK2*-fusion is likely to be a potential target of JAK2 inhibitors.[85,86] Overall, the unique molecular basis and the potential clinical implications support the recognition of this entity within MDS/MPN.

Refractory anemia with ring sideroblasts associated with marked thrombocytosis

Refractory anemia with ring sideroblasts associated with marked thrombocytosis is currently defined by the WHO according to the presence of refractory anemia associated with erythroid dysplasia and ring sideroblasts 15% or over, and platelet count $450x10^9$/L or over associated with the presence of large atypical megakaryocytes similar to those observed in *BCR/ABL1*-negative MPN. The available evidence suggests that RARS-T may result from a combination of *SF3B1*, responsible for myelodysplastic features (i.e. ring sideroblasts), and *JAK2, MPL* or *CALR* mutations, conferring the myeloproliferative phenotype (i.e. thrombocytosis).[53,87-90] This evidence suggests that RARS-T is indeed a myelodysplastic/myeloproliferative neoplasm at the clinical, morphological and molecular level, and supports its recognition as a distinct entity. Although the association of *SF3B1* and *JAK2*, *MPL* or *CALR* mutations seems specific for myeloid neoplasms with ring sideroblasts and thrombocytosis, it can be detected only in a fraction of patients, suggesting that additional as yet unknown lesions may be present.[53,88] Nonetheless, at present this mutation pattern may represent a valuable co-criterion to substantiate the morphological evidence of myelodysplastic and myeloproliferative features.

Conclusions

The current classification adopted by the World Health Organization, combining morphology, immunophenotype, genetic and clinical features to define distinct clinicopathological disease entities provides the best approach to classify myeloid neoplasms with myelodysplasia. Recent evidence in chronic myeloproliferative neoplasms suggests that biologically homogeneous entities represent the ideal framework within which innovative targeted therapies can be developed and therapeutic strategies optimized by identifying reliable indicators of response, providing markers for monitoring of minimal residual disease, and efficiently identifying specific mechanisms of resistance.[91-95] Recent progress, and the resultant wider understanding of the genetic basis of MDS and other myeloid neoplasms with myelodysplasia, offer a unique opportunity to improve the diagnosis and classification processes, overcome the limitations of the current morphology-based approach, and open new avenues of research to develop novel diagnostic and prognostic tools and therapeutic options.

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