MYELOID NEOPLASIA

The genetic basis of myelodysplasia and its clinical relevance

Mario Cazzola,1,2 Matteo G. Della Porta,1,3 and Luca Malcovati1,2

1Department of Hematology Oncology, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico Policlinico San Matteo, Pavia, Italy; and Departments of 2Molecular Medicine and 3Internal Medicine, University of Pavia, Pavia, Italy

Myelodysplasia is a diagnostic feature of myelodysplastic syndromes (MDSs) but is also found in other myeloid neoplasms. Its molecular basis has been recently elucidated by means of massive parallel sequencing studies. About 90% of MDS patients carry ≥1 oncogenic mutations, and two thirds of them are found in individuals with a normal karyotype. Driver mutant genes include those of RNA splicing (SF3B1, SRSF2, U2AF1, and ZRSR2), DNA methylation (TET2, DNMT3A, and IDH1/2), chromatin modification (ASXL1 and EZH2), transcription regulation (RUNX1), DNA repair (TP53), signal transduction (CBL, NRAS, and KRAS), and cohesin complex (STAG2). Only 4 to 6 genes are consistently mutated in ≥10% MDS patients, whereas a long tail of ~50 genes are mutated less frequently. At presentation, most patients typically have 2 or 3 driver oncogenic mutations and hundreds of background mutations. MDS driver genes are also frequently mutated in other myeloid neoplasms. Reliable genotype/phenotype relationships include the association of the SF3B1 mutation with refractory anemia with ring sideroblasts, TET2/SRSF2 comutation with chronic myelomonocytic leukemia, and activating CSF3R mutation with chronic neutrophilic leukemia. Although both founding and subclonal driver mutations have been shown to have prognostic significance, prospective clinical trials that include the molecular characterization of the patient’s genome are now needed. (Blood. 2013;122(25):4021-4034)

Introduction

Myelodysplasia is a term used in pathology for describing morphologic abnormalities, or dysplasia, in ≥1 of the major myeloid cell lines of hematopoiesis and is a typical feature of myelodysplastic syndromes (MDSs).1 In the World Health Organization (WHO) classification of tumors of hematopoietic and lymphoid tissues, MDSs are defined as clonal hematopoietic stem cell disorders characterized by cytopenia, myelodysplasia, ineffective hematopoiesis, and increased risk of progression to acute myeloid leukemia (AML).1 Representative examples of morphologic abnormalities of myelodysplasia are reported in Figure 1.

Myelodysplasia is not restricted to MDS but may be found also in other myeloid neoplasms of the WHO classification (Table 1). Although the different subtypes of myeloid neoplasms have distinctive characteristics, they may share morphologic abnormalities. The paradigmatic example is refractory anemia with ring sideroblasts associated with marked thrombocytosis (RARS-T), which has both the myelodysplastic features of RARS and the myeloproliferative characteristics of essential thrombocytosis. This suggests that the myelodysplastic features of various myeloid neoplasms may reflect common underlying genetic lesions and that these latter contribute to determining clinical phenotypes.

In this article, we will review the most recent advances in our understanding of the genetic basis of myelodysplasia and will discuss its clinical relevance. The Chronic Myeloid Disorders Working Group of the International Cancer Genome Consortium has just completed a study of targeted gene sequencing in a large cohort of patients with MDS and closely related neoplasms.5 For additional information on the genomic characterization of myeloid neoplasms, the reader is referred to recent landmark studies of genomic and epigenomic landscapes of AML,3,4 and a review article in Blood.5 A very detailed analysis of what has been learned about cancer genomes in the last few years has been published recently by Vogelstein et al.6 Definitions for basic terms used in studies of the genetic basis of myeloid neoplasms are reported in the Appendix.

Clonal expansion of myelodysplastic stem cells, ineffective hematopoiesis, and leukemic transformation: a working model of the pathophysiology of myelodysplasia

For a better understanding of the pathophysiology of myelodysplasia, we summarized the current concepts in the model reported in Figure 2. Some steps of this model are still working hypotheses, which will hopefully be verified in the near future.

An essential component of the WHO definition of MDS is the clonal nature of myelodysplastic hematopoiesis.1 Although various approaches can be used to prove the existence of a clonal population of hematopoietic cells, the more straightforward one is the use of chromosomal abnormalities or discrete gene rearrangements to show the uniform presence of these markers in purified hematopoietic cell populations.7 Using this approach, chromosomal aberrations were found to be restricted to committed myeloid progenitor cells in MDS patients, suggesting that the genetic lesion occurred in a hematopoietic cell with the capacity to differentiate into mature myeloid cells.8

Perspectives


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In a landmark study, Walter et al\textsuperscript{10} used whole genome sequencing to identify somatic mutations in bone marrow samples from patients with AML developing from MDS and then genotyped in each patient a bone marrow sample obtained during the antecedent MDS phase. About 85\% to 90\% of unfractionated bone marrow cells were found to be clonal in these patients, both in the MDS and in AML phase, irrespective of the number of blasts. This study formally proved that almost all cells of the bone marrow myeloid cell lines (ie, immature red cells, granulocytic/monocytic precursors, and megakaryocytes) are clonally derived in MDS patients at any stage of the disease and not only after AML transformation.\textsuperscript{10}

The clonal architecture of MDS has been elegantly studied by Delhommeau et al\textsuperscript{11} following the identification of somatic TET2 mutations. CD34\textsuperscript{+} cells from MDS patients were fractionated into immature CD34\textsuperscript{+}CD38\textsuperscript{−} and mature CD34\textsuperscript{−}CD38\textsuperscript{+} progenitors. Although TET2 mutations were detected in only a small fraction of CD34\textsuperscript{+}CD38\textsuperscript{−} cells, they were present in a high proportion of more mature progenitors. This suggests that the initial somatic TET2 mutation occurred in a CD34\textsuperscript{+}CD38\textsuperscript{−} cell and was then transmitted to its CD34\textsuperscript{−}CD38\textsuperscript{+} progeny. A similar clonal architecture has been more recently observed also in patients with chronic myelomonocytic leukemia (CMML).\textsuperscript{12}

The occurrence in an immature hematopoietic stem cell of a somatic mutation that provides survival and growth advantage (for instance, lower propensity to apoptosis) leads to formation of a local clone (Figure 2, step 1). For this clone to become fully dominant in the whole body, the mutated stem cells must have additional advantages. In adulthood, migration and trafficking of hematopoietic

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stem cells are of crucial importance in maintaining homeostasis of the hematopoietic system. Despite several investigations, the mechanisms by which neoplastic hematopoietic cells leave the primary site and migrate to other bone marrow districts remain largely unclear. Ultimately, however, mutated hematopoietic stem cells achieve full clonal dominance in the bone marrow, and the vast majority of circulating mature cells derive from the dominant clone (Figure 2, step 2).

Once the myelodysplastic clone has become fully dominant in the bone marrow, the disease may or may not become clinically apparent. For instance, a somatic SF3B1 mutation appears to be able to cause a clinical phenotype per se, whereas a driver TET2 mutation can determine clonal hematopoeisis without hematologic manifestations, suggesting that cooperating mutant genes might be required for phenotypic expression. Myelodysplastic hematopoeisis is characterized by excessive apoptosis of hematopoietic precursors, at least in patients with low-risk disease. Ineffective hematopoeisis, ie, the premature intramedullary death of erythroblasts, immature granulocytes/monocytes, and megakaryocytes, is primarily responsible for the defective production of mature blood cells and peripheral blood cytopenia. We must therefore assume that the somatic mutation responsible for gain of function at the stem cell level involves loss-of-function at the hematopoietic precursor level (Figure 2, step 3).

Recurrent chromosomal abnormalities are mostly secondary genetic events with established clinical relevance in MDS

Recurrent chromosomal abnormalities have been very important thus far for diagnosis and prognostication of MDS. Regarding
diagnosis, the detection of a cytogenetic aberration in a patient with peripheral cytopenia and bone marrow dysplasia provides an important marker of clonal proliferation. By contrast, the diagnosis of MDS may be difficult in patients with a normal karyotype or noninformative cytogenetics.19

Recurrent chromosomal abnormalities are detected in about half of patients with MDS,20 and the most common single cytogenetic aberrations include del(5q), trisomy 8, del(20q), and monosomy 7 or del(7q).21-22 These are likely secondary genetic events, deriving from the genome instability caused by the founding genetic mutation.3 The only exception to the rule known thus far is isolated del(5q), which characterizes the 5q- syndrome: in fact, haploinsufficiency for RPS14 and miR-145, mapping to the common deleted region, represents the pathophysiological basis of this MDS subtype.23-25

With respect to the prognostic relevance of recurrent chromosomal abnormalities, in a recent collaborative study aimed to develop the revised International Prognostic Scoring System (IPSS-R) for MDS, patient databases from international institutions were coalesced to assemble a combined database including 7012 patients.26 Cytogenetic abnormalities were categorized into 5 prognostic subgroups that were shown to have significant prognostic relevance with different median survival and risk of evolution into AML. (Table 2).

The 5-group cytogenetic risk classification reported in Table 2 was recently found to predict the outcome of allogeneic hematopoietic stem cell transplantation in MDS patients.27 In particular, patients with a complex karyotype (very poor cytogenetic subgroup) had a very poor outcome after transplantation. This was also true for monosomal karyotype, defined as the karyotype of patients who had ≥2 autosomal monosomies or 1 monosomy in combination with other structural abnormalities.27

Thus, chromosomal aberrations will likely continue to have clinical relevance in MDS even in the era of genomic medicine. Because they basically consist in copy number changes, their detection will likely be improved by array-based karyotyping28 and/or by massive parallel sequencing itself.2

### Spliceosome mutations: their unexpected promotion of clonal proliferation and the concept of genetic predestination

Pre-mRNA splicing is catalyzed by the spliceosome, a macromolecule composed of 5 small nuclear RNAs associated with proteins to form particles termed small nuclear ribonucleoproteins (snRNPs).16 More than 50% of patients with myelodysplasia carry somatic mutations in spliceosome genes encoding proteins involved in the 3′ splice site recognition and U2 snRNP function.2 Spliceosome mutations are rarely found in childhood myeloid neoplasms, suggesting that they are typically acquired in the elderly.36

Mutations of the RNA splicing machinery are largely mutually exclusive and are most often founding events. In fact, the mutant allele burden is typically between 40% and 50%, indicating a dominant bone marrow clone that is heterozygous for the mutation.37,38 Mutation hotspots have been shown in the 3 most frequently mutated genes, ie, SF3B1, SRSF2, and U2AF1, and almost all described mutations are missense, with no evidence of nonsense or frameshift changes.15,31,32 Different patterns of missplicing associated with the above mutant genes have been described.32,39,40 Altogether, the available evidence suggests that spliceosome mutations affecting the 3′ splice site recognition and U2 snRNP function are likely to create novel protein isoforms that can drive clonal dominance of mutated hematopoietic stem cells.5 This conclusion was totally unpredictable since, as emphasized by Vogelstein et al,6 a spliceosome mutation was

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**Table 2. MDS cytogenetic scoring system: prognostic relevance of cytogenetic abnormalities in 7012 patients included in the International Working Group for Prognosis in MDS (IWG-PM) database**

<table>
<thead>
<tr>
<th>Prognostic cytogenetic subgroup</th>
<th>Cytogenetic abnormalities</th>
<th>Proportion of MDS patients, %</th>
<th>Median overall survival, years</th>
<th>Median time to 25% AML evolution, years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very good</td>
<td>–Y, del(11q)</td>
<td>4</td>
<td>5.4</td>
<td>Not reached</td>
</tr>
<tr>
<td>Good</td>
<td>Normal karyotype, del(5q), del(12p), del(20q), double abnormality including del(5q)</td>
<td>72</td>
<td>4.8</td>
<td>9.4</td>
</tr>
<tr>
<td>Intermediate</td>
<td>del(7q), +8, +19, i(17q), any other single or double independent clones</td>
<td>13</td>
<td>2.7</td>
<td>2.5</td>
</tr>
<tr>
<td>High</td>
<td>–7, inv(3)(t3q)/del(3q), double including –7del(7q), complex: 3 abnormalities</td>
<td>4</td>
<td>1.5</td>
<td>1.7</td>
</tr>
<tr>
<td>Very high</td>
<td>Complex karyotype: ≥3 abnormalities</td>
<td>7</td>
<td>0.7</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Reproduced from Greenberg et al.26

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**Somatic gene mutations in myelodysplasia: from studies of candidate genes to whole genome sequencing**

Our understanding of the molecular basis of MDS has improved dramatically in the last 4 years. The first major breakthrough was the identification of somatic mutations of TET2 in patients with rearrangements of chromosome 4q24, where the gene maps.11,29 Subsequently, Bejar et al30 used next-generation sequencing and mass spectrometry—based genotyping to screen mutational hotspots in 111 genes in 439 patients with MDS. In the last 2 years, studies of whole exome or whole genome sequencing led to the discovery of mutations in the RNA splicing machinery,15,31,32 of SETBP1 mutations in aCML,33 and of CSF3R mutations in CNL.34

Recent studies have performed a systematic analysis of panels of known or putative genes relevant in myelodysplasia by coupling high-throughput sample handling with massive parallel sequencing2,35 or by combining deep sequencing with array-based genomic hybridization (Seishi Ogawa, Kyoto University, personal communication, August 9, 2013). The panels used included from 94 to 111 candidate or noninformative cytogenetics,19

Thus, chromosomal aberrations will likely continue to have clinical relevance in MDS even in the era of genomic medicine. Because they basically consist in copy number changes, their detection will likely be improved by array-based karyotyping28 and/or by massive parallel sequencing itself.2
expected to lead to a plethora of nonspecific cellular abnormalities rather than to promote clonal proliferation.

The different spliceosome mutations are associated with different phenotypes and different clinical outcomes (Table 3). Somatic SF3B1 mutations are found almost exclusively in patients with refractory anemia with ring sideroblasts without or with thrombocytosis (RARS and RARS-T, respectively), and this clearly suggests a causal relationship between mutation and ring sideroblast formation. In addition, the vast majority of patients with SF3B1 mutation have a good clinical outcome with a low propensity to AML transformation. SRSF2 mutations are found mainly in patients with multilineage dysplasia and/or excess blasts and, at variance with SF3B1 mutations, predict for high risk of leukemic evolution and poor survival. SRSF2 mutations have been detected in about one fifth of cases of AML transformed from MPN and, in particular, have been reported in ~40% to 50% of patients with CMML, where they are frequently associated with TET2 mutations. Somatic mutations of U2AF1 have been reported in various MDS subtypes and found to be predictive of a high risk of leukemic evolution and shorter survival.

Table 3. Most common driver genes in patients with MDS and MDS/MPN

<table>
<thead>
<tr>
<th>Biological pathways and genes</th>
<th>Frequency, %*</th>
<th>Timing of mutation acquisition†</th>
<th>Relationship between mutant gene and clinical phenotype</th>
<th>Prognostic or predictive relevance of mutant gene</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RNA splicing</strong></td>
<td></td>
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</tr>
<tr>
<td>SF3B1</td>
<td>15-30%</td>
<td>More often a founding mutation</td>
<td>Strictly associated with ring sideroblasts phenotype (RARS, RARS-T)</td>
<td>Associated with good overall survival and low risk of leukemic evolution</td>
</tr>
<tr>
<td>SRSF2</td>
<td>10-20%</td>
<td>More often a founding mutation</td>
<td>Associated with RCMD or RAEB, co-mutated with TET2 in CMML</td>
<td>Associated with poor overall survival and high risk of leukemic evolution</td>
</tr>
<tr>
<td>U2AF1</td>
<td>&lt;10%</td>
<td>More often a founding mutation</td>
<td>Mainly associated with RCMD or RAEB</td>
<td>Associated with high risk of leukemic evolution</td>
</tr>
<tr>
<td>ZRSR2</td>
<td>&lt;10%</td>
<td>More often a founding mutation</td>
<td>Not defined</td>
<td>Not defined</td>
</tr>
<tr>
<td><strong>DNA methylation</strong></td>
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<tr>
<td>TET2</td>
<td>20-30%</td>
<td>More often a founding mutation</td>
<td>Found in all MDS subtypes, high mutation frequency (50-60%) in CMML</td>
<td>No impact on overall survival, may predict response to hypomethylating agents</td>
</tr>
<tr>
<td>DNMT3A</td>
<td>~10%</td>
<td>More often a founding mutation</td>
<td>Found in all MDS subtypes, co-mutated with SF3B1 in RARS</td>
<td>Associated with unfavorable clinical outcome (negative prognostic relevance mitigated by SF3B1 co-mutation in RARS)</td>
</tr>
<tr>
<td>IDH1/IDH2</td>
<td>~5%</td>
<td>More often a founding mutation</td>
<td>Associated with RCMD or RAEB</td>
<td>Associated with unfavorable clinical outcome in all myeloid neoplasms (MDS, MDS/MPN, MPN)</td>
</tr>
<tr>
<td><strong>Chromatin modification</strong></td>
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<tr>
<td>ASXL1</td>
<td>15-20%</td>
<td>More often a subclonal mutation</td>
<td>Associated with RCMD or RAEB, high mutation frequency (40%) in CMML</td>
<td>Associated with unfavorable clinical outcome in all myeloid neoplasms (MDS, MDS/MPN, MPN)</td>
</tr>
<tr>
<td>EZH2</td>
<td>~5%</td>
<td>More often a subclonal mutation</td>
<td>Associated with RCMD or RAEB</td>
<td>Associated with unfavorable clinical outcome in all myeloid neoplasms</td>
</tr>
<tr>
<td><strong>Transcription</strong></td>
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<tr>
<td>RUNX1</td>
<td>~10%</td>
<td>Typical subclonal mutation</td>
<td>Associated with RCMD or RAEB</td>
<td>Associated with unfavorable clinical outcome in all myeloid neoplasms (MDS, MDS/MPN, MPN)</td>
</tr>
<tr>
<td>BOR</td>
<td>&lt;5%</td>
<td>Typical subclonal mutation</td>
<td>Associated with RCMD or RAEB</td>
<td>Associated with unfavorable clinical outcome in all myeloid neoplasms</td>
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<tr>
<td><strong>DNA repair control</strong></td>
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<tr>
<td>TP53</td>
<td>~5%</td>
<td>Typical subclonal mutation</td>
<td>Associated with advanced disease and complex karyotype, mutated in 20% of patients with MDS with del(5q)</td>
<td>Associated with poor overall survival and high risk of leukemic evolution, predicts poor response to lenalidomide in MDS with del(5q)</td>
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<tr>
<td><strong>Cohesin</strong></td>
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<tr>
<td>STAG2</td>
<td>&lt;10%</td>
<td>More often a subclonal mutation</td>
<td>Associated with RCMD or RAEB. Mutated in about 10% of patients with AML</td>
<td>Associated with unfavorable clinical outcome</td>
</tr>
<tr>
<td><strong>RAS pathway</strong></td>
<td></td>
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<tr>
<td>CBL</td>
<td>&lt;5%</td>
<td>More often a subclonal mutation</td>
<td>Found in different MDS subtypes, associated with JMML in children</td>
<td>Not defined in MDS</td>
</tr>
<tr>
<td>NRAS/KRAS</td>
<td>&lt;5%</td>
<td>More often a subclonal mutation</td>
<td>Found in different MDS subtypes, associated with JMML in children</td>
<td>Not defined in MDS</td>
</tr>
<tr>
<td>NF1</td>
<td>&lt;5%</td>
<td>More often a subclonal mutation</td>
<td>Found in different MDS subtypes, associated with JMML in children</td>
<td>Not defined in MDS</td>
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<tr>
<td><strong>DNA replication</strong></td>
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<tr>
<td>SETBP1</td>
<td>&lt;5%</td>
<td>More often a subclonal mutation</td>
<td>Found in 25% of patients with aCML and in subsets of patients with advanced MDS or CMML</td>
<td>Associated with poor overall survival and high risk of leukemic evolution</td>
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<tr>
<td><strong>Receptors</strong></td>
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</tr>
<tr>
<td>CSF3R</td>
<td>&lt;1%</td>
<td>Founding driver mutation in CNL</td>
<td>Strictly associated with CNL, found in a subset of patients with aCML</td>
<td>Mutation type may predict response to specific inhibitors</td>
</tr>
</tbody>
</table>

*Approximate proportion of patients with MDS carrying the mutant gene reported in studies published so far.
†Based on values for mutant allele burden or variant allele frequency.
has led Papaemmanuil et al.\textsuperscript{2} to hypothesize that they give rise to initiating clones with different genetic predestination. More specifically, the initial driver mutation would shape the future trajectory of clonal evolution through constraints on the repertoire of cooperating genetic lesions. The molecular mechanisms underlying genetic predestination remain to be defined.

\subsection*{Somatic mutations in genes encoding epigenetic regulators}

In a recent review article in \textit{Blood}, Issa\textsuperscript{45} described cellular differentiation as an epigenetic process that requires specific and highly ordered DNA methylation and chromatin modification programs. The disordered differentiation of MDS is often associated with somatic mutations in genes that control DNA methylation (\textit{TET2, DNMT3A, and IDH1/IDH2}) or regulate chromatin modification (\textit{ASXL1} and \textit{EZH2}).\textsuperscript{5,45}

Somatic \textit{TET2} mutations were first described in patients with myeloid neoplasms in 2009.\textsuperscript{11,29} \textit{TET2} is mutated in 20\% to 25\% of patients with MDS\textsuperscript{2,30} and in 50\% to 60\% of patients with CMML.\textsuperscript{46} In an elegant study, Busque et al.\textsuperscript{47} found recurrent somatic \textit{TET2} mutations in elderly females who had clonal hematopoesis demonstrated by X-chromosome inactivation skewing but no hematologic phenotype. This and other observations support the notion that \textit{TET2} mutation can lead per se to increased hematopoietic stem cell self-renewal and clonal myeloid proliferation.\textsuperscript{48,49} \textit{TET2} mutations are frequently found in patients with normal karyotype, and therefore represent a useful marker of clonality in these subjects.\textsuperscript{50} In addition, co-occurrence of \textit{TET2} and \textit{SRSF2} mutations is typically found in CMML.\textsuperscript{2} Thus far, no prognostic relevance in terms of overall survival has been clearly defined.\textsuperscript{50,51} but recent studies suggest that \textit{TET2} mutation may predict response to hypomethylating agents.\textsuperscript{51,52} Interestingly, \textit{TET2} mutations were found to be associated with reduced overall survival among patients with intermediate-risk AML.\textsuperscript{53}

In a study based on massively parallel DNA sequencing, Ley et al.\textsuperscript{54} found that \textit{DNMT3A} mutations are highly recurrent in patients with de novo AML and are associated with a poor outcome. Somatic \textit{DNMT3A} mutations have later been detected in \textasciitilde{}10\% to 15\% of patients with different subtypes of MDSs.\textsuperscript{35,55,56} They are associated with unfavorable clinical outcome and more rapid progression to AML in patients with RCMD or RAEB\textsuperscript{55} but not in those with RARS, likely because the co-occurrence of the \textit{SF3B1} mutation mitigates the negative effect of \textit{DNMT3A} mutation.\textsuperscript{56} As suggested by Papaemmanuil et al.,\textsuperscript{2} this observation may indicate that some genes may only be transforming in specific genomic contexts.

Recurrent mutations in the isocitrate dehydrogenase genes \textit{IDH1} and \textit{IDH2} are found in AML and MDS.\textsuperscript{5,57} In AML, co-occurrence of \textit{NPM1} and \textit{IDH1} or \textit{IDH2} mutations is associated with a good clinical outcome.\textsuperscript{53} By contrast, \textit{IDH1} mutation has been found to be associated with a short leukemia-free survival in MDS.\textsuperscript{58}

Two genes involved in chromatin modification and regulation are recurrently mutated in MDS: \textit{ASXL1}, which interacts with the polycomb-group repressive complex 1 and 2 (PRC1, PRC2),\textsuperscript{39,60} and \textit{EZH2}, which belongs to PRC2.\textsuperscript{2,3,61-65} In cellular and animal models, \textit{ASXL1} mutations have been shown to promote myeloid transformation through loss of PRC2-mediated gene repression.\textsuperscript{60} \textit{ASXL1} mutations are common not only in MDS, but also in AML, CMML, and PMF, and are generally associated with poor clinical outcome in all myeloid neoplasms.\textsuperscript{46,61,62,66} Of note, \textit{ASXL1} mutation has been recently incorporated into a prognostic scoring system for CMML as a negative prognostic factor.\textsuperscript{46} Similarly, \textit{EZH2} mutations were found to be significantly associated with a shorter overall in lower risk MDS, and their incorporation in a prognostic model improved risk stratification in these patients.\textsuperscript{56}

Several studies, recently reviewed in \textit{Blood} by Issa,\textsuperscript{45} have shown that MDS patients not only carry epigenetic effector mutations but also an abnormal epigenome. Some of these patients respond to epigenetic therapy, including hypomethylating drugs (azacitidine and decitabine) and drugs that inhibit multiple histone deacetylases.\textsuperscript{45} However, although the efficacy of some of these treatments has been demonstrated in prospective clinical trials,\textsuperscript{67} we still lack reliable predictors of response to epigenetic therapy.

\subsection*{Somatic mutations in other cellular pathways}

Acquired mutations of transcription factors have been described not only in AML but also in MDS.\textsuperscript{3,30} Somatic mutations of \textit{RUNX1} are found in \textasciitilde{}7\% to 8\% of all patients with MDS and are generally associated with advanced disease, severe thrombocytopenia, and poor clinical outcome.\textsuperscript{2,3,30,56}

The gene \textit{TP53}, located on chromosome 17p13.1, encodes p53, which coordinates transcription programs contributing to tumor suppression, and mutant p53 proteins have been identified in various cancers.\textsuperscript{6,68} \textit{TP53} mutations are found in about 5\% of patients with MDS, mainly in subjects with advanced disease, complex karyotype, abnormalities of chromosome 17, or deletions of chromosome 5 and 7.\textsuperscript{3,35,55} MDS patients carrying \textit{TP53} mutation have an unfavorable clinical outcome and a high risk of leukemic evolution.\textsuperscript{2,3,30} and the same is true for patients with MPN.\textsuperscript{69} In particular, \textit{TP53} mutated subclones may occur at an early disease stage in MDS with del(5q), where they are associated with a lower response to lenalidomide and an increased risk of progression to AML.\textsuperscript{70}

The Ras superfamily includes small GTP-binding proteins involved in intracellular signal transduction. Several genes of this superfamily have been found to be mutated in patients with myelodysplasia, including \textit{NRAS}, \textit{KRA}, \textit{NFI}, \textit{PTPN11}, and \textit{CBL}.\textsuperscript{7} Somatic or germ-line mutations of the Ras pathway gene are present in \textasciitilde{}90\% of patients with juvenile CMLM,\textsuperscript{71} an MDS/MPN in which secondary mutations of \textit{SETBP1} and \textit{JAK3} may cause disease progression.\textsuperscript{72}

\textit{SETBP1} encodes a protein that binds the \textit{SET} nuclear oncogene involved in DNA replication. Although heterozygous de novo germ-line mutations in \textit{SETBP1} have been shown to be associated with the Schinzel-Giedion midface retraction syndrome,\textsuperscript{73} somatic mutations have been recently detected in patients with myeloid malignancies.\textsuperscript{3,34} In particular, \textit{SETBP1} mutations are found in \textasciitilde{}25\% to 30\% of patients with aCML.\textsuperscript{3,35} \textit{SETBP1} has a direct role in the transcriptional regulation of other genes,\textsuperscript{70} and \textit{SETBP1} mutations are more often genetic events associated with disease progression in MDS.\textsuperscript{7,4,57}

\textit{CSF3R} encodes the receptor for colony stimulating factor 3. The acquisition of nonsense mutations in this gene, resulting in the expression of truncated CSF3R protein, has been previously found to be associated with progression to MDS/AML in patients with severe congenital neutropenia.\textsuperscript{7} Activating somatic mutations in \textit{CSF3R} have been recently detected in \textasciitilde{}90\% of patients with CNL and in \textasciitilde{}40\% of those with aCML.\textsuperscript{74} This study also showed that distinguishing between these 2 disorders may be difficult using
the WHO criteria, whereas follow-up investigations demonstrated that **CSF3R** and **SETBP1** mutations are not mutually exclusive. As pointed out by Gotlib et al, CNL and aCML are likely overlapping neoplasms; although the pathogenesis of the former is mainly characterized by **CSF3R** mutation, that of aCML is likely more multifactorial.

Cohesin is a highly conserved 4-subunit ring structure that encircles sister chromatids during metaphase, allowing their cohesion, and also plays critical roles in transcriptional regulation and post-replicative DNA repair. Somatic mutations in **STAG2**, a gene involved in the cohesion complex, have been found in ~6% of MDS patients. In a recent work, Kon et al detected mutations and deletions involving various genes of the cohesion complex (**STAG2**, **RAD21**, **SMC1A**, and **SMC3**) in 8% of patients with MDS, 10% of those with CMML, and 12% of those with AML. A similar frequency was previously reported in AML patients, suggesting that altered cohesin function plays a role in myeloid leukemogenesis.

The gene **BCOR**, located on chromosome Xp11.4, encodes a coressor of BCL6, a POZ/zinc finger transcription repressor that is required for germinal center formation and may influence apoptosis. Germ-line mutations of this gene are associated with oculofaciocar-dioledent, and Lenz microphthalmia syndromes. Inactivating somatic mutations of **BCOR** have been described in AML with normal karyotype and more recently in a subset of patients with MDS. These are typical subclonal driver mutations, associated with a poor clinical outcome.

### Familial MDSs and genetic predisposition to acquisition of somatic mutations associated with myelodysplasia: germ-line **GATA2** mutations

For a deeper analysis of this subject, the reader is referred to a comprehensive review article by Liew and Owen. Familial syndromes predisposing to MDS or AML include bone marrow failure inherited disorders (Diamond-Blackfan, dyskeratosis congenita, severe congenital neutropenia), DNA repair deficiency syndromes, Noonan syndrome and neurofibromatosis I, Down syndrome, and familial platelet disorder with propensity to myeloid malignancy (associated with germ-line mutations of **RUNX1** or **CEBPA**).

More recently, germ-line mutations of **GATA2** have been described in familial syndromes characterized by predisposition to MDS and AML. In 3 families, subjects carrying the **GATA2** (C1061T) mutation had macrocytic anemia and developed MDS/AML between the second and fifth decade of life. Various germ-line mutations of **GATA2** have been detected in patients with the Emberger syndrome, characterized by primary lymphedema associated with a predisposition to AML. Finally, several germ-line mutations in **GATA2** have been reported to be associated with the autosomal dominant and sporadic monocytopenia and mycobacterial infection (MonoMAC) syndrome, which predisposes to myeloid malignancy. Typically these patients have a combination of severe monocytopenia with mild neutropenia and marginally reduced hemoglobin level, and progression to MDS/AML typically occurs in the second or third decade of life. It should be noted that, although germ-line mutations of both **RUNX1** or **GATA2** may predispose to MDS, somatic mutations of the same genes may represent mechanisms of disease progression in myeloid neoplasms.

**Several driver genes may cause myelodysplasia, and genotype/phenotype relationships have been defined**

In their recent article, Vogelstein et al concluded that there are ~140 genes whose intragenic mutations contribute cancer. Based on currently available data, the number of potential myelodys-plasia driver genes is likely somewhat lower (50-60). However, reliable estimates can be provided only by whole genome sequencing studies. Whole exome sequencing and candidate gene sequencing are clearly less informative and may not allow the identification of more rarely mutated genes.

The patient cohorts studied thus far are heterogeneous, and all studies are basically retrospective. Taking into account these limitations, only 4 to 6 genes (**SF3B1**, **TET2**, **SRSF2**, **ASXL1**, **DNMT3A**, and **RUNX1**) have been found to be consistently mutated in ~10% of MDS patients, whereas a long tail of about 40 to 50 additional genes are mutated in smaller subsets. In the study by Walter et al, the most frequently mutated genes were **TP53** and **U2AF1**: this likely reflects the fact that their patient cohort included a low proportion of low risk and a high proportion of high-risk MDS subtypes.

At presentation, most MDS patients have 2 or 3 oncogenic driver mutations and hundreds of background or passenger mutations. Considering the variant allele frequency, some mutant genes, typically those of RNA splicing and DNA methylation, appear to be mainly associated with the initial clonal proliferation, whereas others are mainly involved in subclonal evolution (Table 3). However, the temporal order of acquisition of driver mutations is not fixed and varies from subject to subject. Thus, the same mutant gene, eg, **TET2**, may be an early driver in some patients and a subclonal driver in others. Walter et al observed that mutations in driver genes belonging to the same biologic pathway tended not to co-occur, suggesting that a second mutation in the same pathway provides no additional growth advantage or is not even tolerated.

According to Vogelstein et al, most human cancers are caused by 2 to 8 sequential genetic lesions that develop over the course of 20 to 30 years. In their studies of whole genome sequencing, Welch et al estimated that as few as 2 key somatic mutations are needed to cause the malignant founding clone and clinically manifest AML. The available evidence suggests that this may apply also to MDS: at variance with AML, however, the genetic lesions responsible for MDS likely occur sequentially over years, rather than over months or weeks, at least in low-risk subtypes with long natural history of disease, as is typically RARS.

Unfortunately only few functional studies linking the various mutant genes to a cellular or disease phenotype have been performed. In an animal model of conditional Tet2 loss, Tet2 haploinsufficiency was shown to lead to a disorder resembling human CMML. Another study reported findings suggesting that SF3B1 or SF3b1 haploinsufficiency leads to ring sideroblast formation in human cells or heterozygous knockout mice, respectively. The **CSF3R** (T618I) mutation has been shown to drive a lethal myeloproliferative disorder in a murine model.

Genotype/phenotype relationships have been defined in MDS, MDS/MPN, and related myeloid neoplasms. A tentative schematic representation of our current knowledge of these relationships is reported in Figure 3. Although reliable conclusions will be made possible only by prospective studies, this scheme provides a proof of concept of the potential feasibility of a molecular classification of MDS and related myeloid neoplasms. More specifically, **SF3B1** mutation appears to be strictly associated with refractory anemia.
with ring sideroblasts with or without marked thrombocytosis, the combination of $SRSF2$ and $TET2$ mutation with CMML, and an activating $CSF3R$ mutation with CNL. Refractory anemia has no peculiar genotype thus far, raising the question as to whether it should be considered as a separate entity. RCMD and refractory anemia with blast excess can be associated with different combinations of founding mutations, primarily involving genes of RNA splicing (with the only exclusion of $SF3B1$) or epigenetic regulation and subclonal driver mutations.

Interestingly, as shown in Table 4, a molecular classification appears to be already feasible in MDS/MPN, a group of myeloid neoplasms thus far defined on the basis of cumbersome clinical, hematologic, and morphologic criteria. It is also apparent that specific driver genes are responsible for the myeloproliferative component of the different MDS/MPN, like $JAK2$ or $MPL$ in RARS-T, $SETBP1$ in aCML, and $CSF3R$ in CNL. We have previously shown that RARS-T develops from RARS through the occurrence of a subclonal driver mutation in $JAK2$ or $MPL$ in the initial $SF3B1$ mutated clone. A similar evolutionary process likely operates in CMML and aCML, which may develop from a preexisting MDS through the acquisition of subclonal driver mutations that cause monocytosis and granulocytic leukocytosis, respectively.

Myelodysplasia driver genes are recurrently mutated also in other myeloid neoplasms

The driver genes whose mutations are responsible for MDS (Table 3) are frequently mutated also in other myeloid neoplasms listed in Table 1. This is primarily true for AML, although, as underlined by Walter et al, some genes are overrepresented in MDS compared with AML and vice versa. Indeed, in the recent study by the Cancer Genome Atlas Research Network, the 20 most recurrently mutated genes in AML were $FLT3$, $NPM1$, $DNMT3A$, $IDH1/2$, $TET2$, $RUNX1$, $TP53$, $N/KRAS$, $CEPBPA$, $WT1$, $PTPN11$, $KIT$, $U2AF1$, $SMC1A$, $SMC3$, $PHF6$, $STAG2$, and $RAD21$. Only half of these genes are within the 20 most recurrently mutated ones in MDS. Although the molecular pathophysiology of MDS is different from that of AML, some AML driver genes might behave as subclonal drivers in MDS and thereby drive leukemic transformation.

Somatic mutations of $CBL$, $TET2$, $ASXL1$, and $IDH1/IDH2$ have been detected in the advanced phase of chronic myeloid leukemia. Several of the genes reported in Table 3 can also be mutated in PMF, in combination with $JAK2$ (V617F) or $MPL$ exon 10 mutations, and comutation has been shown to have a negative impact on the clinical course of this MPN. Recently, somatic mutations of $TET2$, $SRSF2$, $ASXL1$, $CBL$, and $RUNX1$ have been detected in ~90% of patients with advanced mastocytosis, and overall survival was found to be significantly shorter in patients with additional mutations than in those carrying $KIT$ (D816V) only.

Myelodysplasia driver genes may also interact with somatic mutations involving lymphoid cell lines, thus giving rise to peculiar phenotypes. T-cell large granular lymphocytic leukemia is characterized by clonal expansion of CD3$^+$ cytotoxic T lymphocytes and may be associated with autoimmune disorders.
and immune-mediated cytopenias. The expansion of clonal T cells has been shown to be caused by somatic mutations of STAT3 or STAT5b. Autoimmune processes have been shown to contribute to cytopenia in a subset of MDS patients, and these patients may benefit from immunosuppressive treatment. Interestingly, a recent study describes the occurrence of STAT3 mutations in a subset of MDS patients and autoimmune processes have been shown to contribute to cytopenia in a subset of MDS patients, and these patients may benefit from immunosuppressive treatment.

Table 4. Somatic mutations that characterize the different types of MDS/MPN

<table>
<thead>
<tr>
<th>Myeloid neoplasm according to the 2008 WHO classification</th>
<th>Main diagnostic/clinical feature(s)</th>
<th>Most common mutant gene(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMML (classified as MDS/MPN)</td>
<td>Persistent peripheral blood monocytosis (&gt;1 × 10^9/L).</td>
<td>Co-occurrence of TET2 and SRSF2 mutations, or combinations of ASXL1 mutations with other mutant driver genes. ASXL1 mutation is associated with poor overall survival and high risk of progression to AML.</td>
</tr>
<tr>
<td>aCML (classified as MDS/MPN)</td>
<td>Peripheral leukocytosis (&gt;13 × 10^9/L) with dysgranulopoiesis and 10% or more circulating immature granulocytes.</td>
<td>Combinations of founding mutations in various genes and subclonal mutations of SETBP1 or ASXL1.</td>
</tr>
<tr>
<td>CNL (classified as MPN)</td>
<td>Neutrophilic leukocytosis (&gt;25 × 10^9/L) with less than 10% circulating immature granulocytes.</td>
<td>Activating somatic mutations of CSF3R (encoding the receptor for G-CSF) in the vast majority of patients. Oncogenic lesions can be classified as truncation or membrane proximal mutations, involving different preferential downstream kinase signaling.</td>
</tr>
<tr>
<td>JMML (classified as MDS/MPN)</td>
<td>Persistent peripheral blood monocytosis (&gt;1 × 10^9/L) in children.</td>
<td>Somatic mutations of the RAS pathway (NRAS, KRAS, NF1, PTPN11, and CBL). Heterozygous germ-line CBL mutations may predispose to JMML. Subclonal driver mutations of SETBP1 and JAK3 may cause disease progression.</td>
</tr>
<tr>
<td>RARS-T (classified as a provisional entity within MDS/MPN)</td>
<td>Macrocytic anemia, ring sideroblasts, and thrombocytosis.</td>
<td>Combinations of a founding somatic mutation of SF3B1 and subclonal driver mutations of JAK2 or MPL (and likely of other as-yet-unknown genes).</td>
</tr>
</tbody>
</table>

CNL has been included because of its overlapping features with aCML.

Figure 4. Current approach to diagnosis and prognostication of MDS and MDS/MPN and a hypothetical future approach based on massive parallel sequencing.
cytogenetics to identify nonrandom chromosomal abnormalities as single assay. Although whole genome sequencing is clearly more reliable detection of copy number changes from sequencing data.

The current diagnostic approach to MDS includes peripheral blood and bone marrow morphology to evaluate abnormalities of peripheral blood cells and hematopoietic precursors (Figure 1), bone marrow biopsy to assess marrow cellularity, fibrosis, and topography, and cytogenetics to identify nonrandom chromosomal abnormalities (Table 2). Massive parallel sequencing has the potential of dramatically improving our approach to diagnosis of MDS as illustrated in Figure 4. The price of whole genome sequencing is expected to drop below $1000 in the next years, whereas that of targeted gene sequencing should be definitely lower. Deep sequencing may allow the simultaneous detection of both somatic gene mutations and copy number changes, the cytogenetic abnormalities typical of MDS, in a single assay. Although whole genome sequencing is clearly more informative, massive parallel sequencing of a panel of myeloid genes is more feasible in a clinical laboratory. The genes to be sequenced may include ~50 to 60 myelodysplasia driver genes, genes associated with inherited disorders that predispose to MDS, and a reasonable number of germ-line single nucleotide polymorphisms that allow reliable detection of copy number changes from sequencing data.

Relevance of gene mutations in the diagnostic approach to myelodysplasia: toward a molecular classification of myeloid neoplasms

The current diagnostic approach to MDS includes peripheral blood and bone marrow morphology to evaluate abnormalities of peripheral blood cells and hematopoietic precursors (Figure 1), bone marrow biopsy to assess marrow cellularity, fibrosis, and topography, and cytogenetics to identify nonrandom chromosomal abnormalities (Table 2). Massive parallel sequencing has the potential of dramatically improving our approach to diagnosis of MDS as illustrated in Figure 4. The price of whole genome sequencing is expected to drop below $1000 in the next years, whereas that of targeted gene sequencing should be definitely lower. Deep sequencing may allow the simultaneous detection of both somatic gene mutations and copy number changes, the cytogenetic abnormalities typical of MDS, in a single assay. Although whole genome sequencing is clearly more informative, massive parallel sequencing of a panel of myeloid genes is more feasible in a clinical laboratory. The genes to be sequenced may include ~50 to 60 myelodysplasia driver genes, genes associated with inherited disorders that predispose to MDS, and a reasonable number of germ-line single nucleotide polymorphisms that allow reliable detection of copy number changes from sequencing data.

Prognostic and predictive significance of driver mutations and development of molecular models for clinical decision making

As shown in Figure 5, the current WHO classification of MDS has valuable prognostic relevance, with blast percentage and multilineage dysplasia representing the most relevant parameters from this point of view. However, the reproducibility of these latter parameters is far from optimal, and there is a need for more robust prognostic factors. The IPSS-R clearly represents a step forward but does take into account only cytogenetic abnormalities, which are secondary genetic events and not the driver lesions.

The definition of founding and subclonal driver mutations might considerably improve prognostication of MDS and more generally clinical decision-making in this field. For instance, the identification of the mutant gene responsible for the initial clone is relevant to clinical outcome. For instance, ring sideroblasts may be found not only in patients with a founding mutation in SRSF2, but also in those with an initiating oncogenic lesion in SF3B1. However, the median leukemia-free survival is >10 years in the former vs <2 years in the latter. Second, the detection of subclonal driver mutations associated with small clones may allow early diagnosis of disease progression, including evolution into AML. In chronic lymphocytic leukemia, the presence of subclonal driver mutations adversely impacts clinical outcome. Third, the number of driver mutations per patient represents an important prognostic factor per se. In the recent study by Papaemmanuil et al, the median leukemia-free survival was >3 years in patients with 1 or 2 driver mutations vs <2 years in patients with ≥3 driver mutations.

A few studies have already suggested that incorporation of somatic mutations into prognostic scoring systems can improve prognostication of MDS. The International Working Group for Prognosis in MDS has started a research project aimed to develop a prognostic scoring system (IPSS-Mol) that includes clinical, hematologic, and molecular parameters. Our preliminary evidence suggests that parameters such as hemoglobin level, blast count, and high-risk cytogenetic abnormalities will continue to retain strong independent prognostic value (M.C., L.M., and M.G.D.P., unpublished data, August 8, 2013). Additional parameters that may significantly contribute to a refined risk assessment of MDS include gene expression profiling-based signatures.

Finally, characterization of the patient’s genome may guide therapeutic programs, and its inclusion in prospective clinical trials is therefore of crucial importance. TET2 mutations might be associated with response to hypomethylating agents, whereas U2AF1 mutations would independently predict for poor outcome after allogeneic stem cell transplantation. There is considerable therapeutic potential for epigenetic-targeted therapies in AML, and this may be also true in MDS. Several drugs that target the spliceosome are being investigated for potential use in various malignancies, whereas drugs targeting oncogenic Ras signaling might be useful in many myeloid neoplasms. Case reports suggest that ruxolitinib may be effective in CML associated with CSF3R mutation and in chronic eosinophilic leukemia associated with PCM1-JAK2 fusion gene. More generally, the identification of the biologic pathways that are activated by mutation might allow personalized treatment in the individual patient with myelodysplasia. It should also be considered
that characterization of the patient’s genome before and after treatment may allow a correct assessment of response, in particular, the impact of treatment on clonal pattern of hematopoiesis.

For the above expectations to be realized, functional studies of the mutant genes and prospective clinical trials that include the molecular characterization of the patient’s genome are now needed.

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**Authorship**

Contribution: M.C. conceived this review article, analyzed the literature, wrote the manuscript, and prepared the illustrations; and M.G.D.P. and L.M. analyzed the literature and contributed to the manuscript preparation.

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Correspondence: Mario Cazzola, Department of Hematology Oncology, Fondazione IRCCS Policlinico San Matteo, 27100 Pavia, Italy; e-mail: mario.cazzola@unipv.it.

**Appendix: Definitions for basic terms used in studies of the genetic basis of myeloid neoplasms**

**Clone:** A group of cells that derive from a common parent cell and share its genome.

- **Malignant clone:** A clone generated by the occurrence in the parent cell of a somatic mutation that alters the cell biology and function.
- **Founding or initiating clone:** A malignant clone generated by a founding somatic mutation.
- **Subclone:** A clone generated by the occurrence of an additional driver mutation in a cell of an already established clone.
- **Clonal architecture:** Clonal composition of a neoplasm based on its clonal origin and subclonal evolution.

- **Clonal dominance:** A condition in which most cells of a tissue belong to a clone. In myelodysplastic syndromes, about 85-90% of bone marrow cells are clonally derived.
- **Subclonal evolution or expansion:** The process by which the founding malignant clone generates subclones through the acquisition of additional driver mutations. In some patients, the order of genetic changes, i.e., the temporal order of acquisition of driver mutations, can be inferred by means of massive parallel sequencing though calculation of the mutant allele burden or variant allele frequency (VAF).
- **Intraclonal (intratumoral) heterogeneity:** A founding malignant clone that has undergone subclonal evolution is no longer monoclonal in the strict sense, but is instead a mosaic of several clones/genomes with different sets of somatic mutations.

**Mutation:** A change of the nucleotide sequence of the genome.

- **Germline mutation:** A mutation that is inherited though a germ cell (oocyte or spermatozoon) at the time of conception, and is therefore present in all cells of a developed body.
- **Somatic mutation:** A mutation that occurs in a non-germ cell of a body after conception (the ancient Greek somatikos means “of the body”).
- **Nonsynonymous mutation:** A nucleotide mutation that alters the amino acid sequence of a protein.
- **Driver mutation:** A mutation that causes a selective advantage in a cell with capacity for self-renewal, leading to formation of a clone of mutated cells.
  - **Founding or initiating driver mutation:** A driver mutation that gives rise to the initial clone of a malignancy.
  - **Subclonal or cooperating driver mutation:** A driver mutation that occurs in a cell of an already established clone and generates a subclone carrying both the founding and the newly acquired mutation.
- **Background or passenger mutation:** A mutation that occurs in a tissue before neoplastic transformation and has no pathophysiological significance. In most tissues, including hematopoietic stem cells, the number of passenger mutations is a function of age. Whole genome sequencing studies have shown that in hematopoietic stem cells, their number may range from about 100 to 1000, depending on age. When an initiating driver mutation gives rise to a malignant clone (neoplastic transformation), all background or passenger mutations present at that time are captured and carried forward. Additional passengers mutations can be captured during subclonal evolution.

**Mutant allele burden or VAF:** The relative proportion of a mutant or variant allele (i.e., the allele carrying a somatic mutation) in a tissue or tumor sample. The mutant allele burden can be estimated using various approaches: i) by means of allele-specific quantitative PCR [e.g., the procedure employed for estimating the proportion of JAK2 (V617F)-mutant alleles in granulocytes from a patient with polycythemia vera]; or ii) more directly by assessing variant and wild-type reads using next-generation sequencing. A mutant allele burden or VAF of about 50% in regions of diploid DNA content in a homogeneous cell population (e.g., circulating granulocytes in myeloid neoplasms) suggest a fully clonal population of cells that are heterozygous for the mutation. In myeloid neoplasms, the mutant allele burden in a bone marrow sample is most commonly between 40 and 50% due to the presence of non-mylloid cells.

**Variant-allele clusters:** Group of mutation with similar VAF, potentially belonging to the same clone or subclone.

**Whole exome sequencing:** A procedure that allows to sequence all the coding regions (exomes) of a genome.

**Whole genome sequencing:** A procedure that allows to sequence the coding and non coding regions of a genome.

For deeper insight into these concepts, the reader is referred to recent articles by Ley and colleagues⁴,⁵ and Vogelstein et al.⁶.
References


The genetic basis of myelodysplasia and its clinical relevance

Mario Cazzola, Matteo G. Della Porta and Luca Malcovati

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