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REVIEW

Mechanisms underlying the heterogeneity of myelodysplastic syndromes

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Myelodysplastic syndromes (MDS) are hematopoietic stem cell (HSC) disorders in which recurrent chromosome abnormalities and gene mutations define a clonal hematopoiesis. The MDS-initiating cell is a rare HSC which transmits the genetic abnormalities to its myeloid and lymphoid progeny. The heterogeneity of MDS phenotypes could be linked to the diversity of genetic events involving epigenetic regulators, chromatin modifiers, splicing factors, transcription factors and signaling adaptors, the various combinations and order of mutations in cooperating genes, and the variegation of clonal hematopoietic hierarchy. Usually, epigenetic and splicing gene mutations occur first. A combination of one epigenetic event with a splicing gene alteration is frequent. The HSC compartment is invaded by a dominant and few minor clones organized linearly or with a branched architecture. The dominant clone containing the first initiating mutations produces myeloid and lymphoid lineages in transplanted immune-deficient mice. The mutations confer a selective advantage to myeloid progenitors at the expense of lymphoid progenitors. In the context of differentiation, one mutation may favor the amplification of granulomonocytic progenitor, which drives the transformation into acute myeloid leukemia. Understanding the hierarchy of mutations provides insights on the mechanism of transformation. Investigation of mutation pattern and distribution along the hematopoietic tree may influence the therapeutic decision for targeted therapy.

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Myelodysplastic syndromes (MDS) are common myeloid malignancies of the elderly. These syndromes are characterized by ineffective hematopoiesis and a propensity to develop an acute myeloid leukemia (AML) [1]. The diversity of disease subtypes is described in the 2016 World Health

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Table 1. Cytogenetic abnormalities related to myelodysplasia according to the WHO classification of 2016

Types	Chromosomal abnormalities
Complex Karyotype (three or more abnormalities)	
Unbalanced Abnormalities	–7/del(7q)
	del(5q)/t(5q)
	i(17q)/t(17p)
	–13/del(13q)
	del(11q)
	del(12p)/t(12p)
	Idic(X)(q13)
Balanced Abnormalities	t(11;16)(q23.3;p13.3)
	t(3;21)(q26.2;q22.1)
	t(1;3)(p36.3;q21.2)
	t(2;11)(p21;q23.3)
	t(5;12)(q32;p13.2)
	t(5;7)(q32;q11.2)
	t(5;17)(q32;p13.2)
	t(5;10)(q32;q21.2)
	t(3;5)(q25.3;q35.1)

Organization (WHO) classification [2]. Acquisition of genomic abnormalities drives the transformation of a normal hematopoietic cell into a clonal cell. The type of mutation and the combination of genetic events may be responsible for the heterogeneity of phenotypes and clinical outcomes. Clonal hematopoiesis is defined by the presence of genetic events such as chromosome rearrangements and gene alterations. Losses, gains, and translocations that usually affect chromosomes 3, 5, 7, 8, 11, 12, 17, 19, 20, X, and Y are not always specific for these diseases. This led the WHO committee to retain abnormalities of chromosomes 5, 7, 11, 12, 13, 17, and X as MDS-related abnormalities [2] (Table 1). Alterations such as nucleotide changes, deletions or insertions in genes involved in epigenetic modifications of DNA or histones, splicing, transcription, and signaling [3–6] have been reported extensively (Table 2). These mutations are not restricted to myeloid diseases but are also seen in lymphoid malignancies [7,8]. The mechanisms of tumor heterogeneity will be depicted with a special attention to the diversity of genetic events, the variegation of mutational landscape in the hematopoietic hierarchy, and the clonal evolution during natural course of the disease or under therapy.

Table 2. Recurrent mutations in MDS

Genes	Symbols
Epigenetic Regulators	*TET2*, *DNMT3A*, *IDH2*, *IDH1*
Splicing Factors	*SF3B1*, *SRSF2*, *U2AF1*, *ZRSR2*, *PHF6*
Chromatin Modifiers	*ASXL1*, *EZH2*, *MLL2*, *KDM6A*, *ATRX*
Transcription Factors and Cofactors	*RUNX1*, *TP53*, *BCOR*, *GATA2*, *CUX1*, *EP300*, *CREBBP*, *WT1*, *IRF1*, *ETV6*
Signaling	*CBL*, *NRAS*, *JAK2*, *KRAS, KIT*, *MPL*, *PTPN11*, *NF1*, *CDKN2A*
Cohesins	*STAG2*, *RAD21*
Other	*NPM1*

Onset of clonal hematopoiesis

The Darwinian model of evolution is convoked to explain cancer development, implicating the onset of random mutations in a cancer-initiating cell and their selection. Primitive tumors demonstrate a variable prevalence of genetic events with the lowest prevalence in hematological malignancies compared with solid tumors such as melanoma or lung carcinoma, which are mostly secondary to toxic exposure [9]. The bone marrow could be assimilated to a sanctuary relatively protected from environmental toxics. In addition, the hierarchical organization of hematopoiesis may protect the cells from a clonal evolution [10]. Mutations accumulate during decades, so the number of mutations in self-renewing tissues could be correlated directly with age [11]. A mutational signature of aging defined by the accumulation of C > T or T > C transitions is highly predominant in MDS and is a plausible consequence of DNA damage that accumulates in cycling hematopoietic stem cells (HSCs) [9,12].

The presence of mutations in leukemia and/or lymphoma-associated genes can arise without evidence of hematological disorder. A pioneer study from Busque et al. reported mutations in the *TET2* gene in women older than 60 years [13]. Whole-exome sequencing of peripheral blood samples in large cohorts of individuals confirmed that mutation frequency increases with age and affects more than 20% of individuals older than 90 years [14–17]. The most common recurrently mutated genes are *DNMT3A*, *TET2*, *ASXL1*, *TP53*, and *JAK2*. A new entity called clonal hematopoiesis of indeterminate potential (CHIP) has been defined based on the presence of mutations (at least 2% variant allele frequency in peripheral blood) in the absence of dysplasia or cytopenia [18]. A low level of mutations can also be found in a fraction of patients with moderate dysplasia or idiopathic cytopenias of undetermined significance (ICUS), and this may represent a transition to MDS, a positive diagnosis of which is based on morphological WHO criteria, clinical evolution, and variant allele frequency over 10% [18]. Preclinical clonal hematopoiesis seems to indicate a high risk of hematological malignancy development and disease progression. The subjects have a decreased overall survival compared with age-matched controls [15,16,19]. Prospective studies of patients devoid of disease-modifying treatments are required to evaluate the evolution of CHIP. Related to the evolution from CHIP to MDS and secondary AML after MDS is the occurrence of mutations in cooperating genes leading to functional defects of affected cells and their deleterious interaction with the bone marrow microenvironment.

Unexpectedly regarding the incomplete penetrance of dysplastic features, whole-genome sequencing of MDS bone marrow samples has revealed the presence of a dominant clone in which mutations are expressed in more than 90% of the cells coexisting with few minor clones. Those mutations seg-

regate in a mean of two clones in early MDS and in more than three clones at the time of AML transformation [20]. Ten to 20 mutations are detected in individual samples by whole-exome sequencing, confirming the oligoclonal nature of the bone marrow [6,21,22]. A median of four to six mutations affect genes that are mutated recurrently in hematological malignancies; that is, epigenetic regulators, splicing factors, chromatin modifiers, transcription factors, cohesins, and signaling adaptors. Analysis of sequential samples allows backtracking the initiating events [21,22]. In most cases, the founder genetic lesions target recurrently epigenetic genes involved in the regulation of DNA methylation, such as *TET2* or *DNMT3A*; genes involved in the remodeling of chromatin, such as *ASXL1* or *EZH2*; and genes involved in RNA splicing, such as *SF3B1*, *SRSF2*, *U2AF1*, or *ZRSR2* [3–6]. Mutations affecting cell differentiation or proliferation pathways (*RUNX1*, *GATA2*, *BCOR*, *N/KRAS*, *CBL*) or cohesins (*STAG2*, *RAD21*) more likely occur during the progression from MDS to AML [23,24]. These secondary mutations usually accumulate in cells that exhibit a genetic background constituted by mutations present at the onset of the disease [23–25]. Secondary AML post-MDS and de novo AML share mutations in *DNMT3A*, *TET2*, *IDH1/2*, and *TP53* genes. In contrast, mutations in *NPM1*, *FLT3*, and *MLL/KTM2A* and chromosomal rearrangements of the core binding factor are usually absent in MDS.

The order of mutation could contribute to the phenotypic presentation of the disease. This has been emphasized in myeloproliferative neoplasms, in which the initiating event may be either a *TET2* or a *JAK2* mutation. Patients presenting with a *TET2* mutation as a first event are older than patients with a *JAK2* mutation as a first event. The bone marrow hierarchy is different, with more common myeloid progenitors (CMPs) in "*TET2*-first" and more erythroid and megakaryocytic progenitors in "*JAK2*-first" cases. Patients with a *JAK2* mutation at the beginning present with polycythemia vera more likely than essential thrombocythemia and have an increased risk of thrombosis [26]. In MDS, the influence of mutation order on the patient phenotype is less clear. However, even if the two mutations are clonal, meaning that they affect more or less 50% of bone marrow cells, the clone that drives the disease in immunodeficient mice is the clone that contains the earliest events. In two-thirds of patients with an *SF3B1* mutation, this event occurs before mutations in *DNMT3A* or *TET2*. In this case, the dominant *SF3B1*-mutated clone of the hematopoietic stem/progenitor cell (HSPC) compartment gives an *SF3B1*-mutated progeny of myeloid and lymphoid cells in xenograft experiments. In contrast, when the mutation *SF3B1* occurs after the epigenetic event, and even if *SF3B1* mutation belongs to a dominant clone, it appears as a minor myeloid clone in mouse bone marrow [27]. The companion mutations in the different clones may influence engraftment capacities and cooperate with the initiating events to propagate the disease. The type of founder mutation may also influence the phenotype. The diagnosis of sideroblastic anemia is considered as soon as the *SF3B1* mutation is detected, even if the percentage of bone marrow ring sideroblasts is below 15%. There is a significant correlation between *SF3B1* variant allele burden and the percentage of ring sideroblasts [25]. Indeed, the knock-in of *SF3B1*^{K700E} in mice induces a macrocytic anemia and an increased expansion of long-term HSCs (LT-HSCs) [28]. Furthermore, *SF3B1*-mutated HSCs isolated from patients with a sideroblastic anemia and injected to immunodeficient mice undergo abnormal erythroid differentiation assessed by the onset of ring sideroblasts at the polychromatophilic stage [29]. *SF3B1* mutations are never detected in CHIP. This suggests that the onset of *SF3B1* mutation drives the entry into a symptomatic disease. *SF3B1* mutation is rarely isolated, but rather is usually associated with a *DNMT3A* or *TET2* mutation. However, the co-occurrence of an epigenetic event does not prevent the formation of ring sideroblasts. This also suggests that *SF3B1* and *TET2* or *DNMT3A* do not act as epistatic genes. Targeted resequencing using next-generation sequencing often fails to ascertain the order of mutations in the dominant clone. Single-cell genotyping is required to identify the first initiating event among the diversity of combinations. Therefore, the question of the impact of mutation order on the phenotype of MDS remains an open question.

Normal hematopoietic hierarchy

The recent contributions to the understanding of normal hematopoiesis help in deciphering the mechanisms of initiation and propagation of myeloid malignancies, which mainly result from the insult of the HSCs. At the top of hematopoietic hierarchy, the HSC demonstrates self-renewal activity and differentiation capacities toward both myeloid and lymphoid lineages. The capability of HSCs to reconstitute human hematopoiesis beyond 12 weeks with mature cells of different types into irradiated immunodeficient mice defines the LT-HSC. Its immediate progeny, the short-term HSCs (ST-HSCs), support a transient multilineage engraftment in mice. Phenotypically, $CD34^+CD38^{-/lo}CD45RA^-$ LT-HSCs or ST-HSCs are distinguished by the expression of CD90/Thy1, which is lost by ST-HSCs [30,31]. Membrane expression of integrin α6, CD49f, also discriminates $CD49f^+$ LT-HSCs from $CD49f^-$ ST-HSCs. The self-renewing compartment of $CD34^+CD38^-CD45RA^-CD90/Thy1^-$ cells extends to the multipotent progenitors (MPPs).

Single HSC transplantation experiments have demonstrated that the maintenance of the graft and the reconstitution of myeloid and lymphoid lineages is variable, suggesting that the HSC compartment is highly heterogeneous [32,33]. In particular, these experiments identified a myeloid-restricted progenitor with long-term repopulating activity that produces either a megakaryocytic and megakaryocytic-erythroid progeny or a common myeloid progeny [33]. The heterogeneity of the HSC compartment might be explained by the sequential loss of lineage potential by differentiating HSCs

or the coexistence of distinct subsets of HSCs with multilineage or unilineage potential. MPPs with various lineage potentials have been identified downstream of the HSC and are separated in several classes (MPP1, MPP2, MMP3, and MPP4) on the basis of phenotypic, epigenomic, transcriptomic, proteomic, and functional characteristics [34–37]. The most abundant MPPs are the lymphoid-primed MPPs (LMPPs or MPP4). This is a lineage-biased population with low myeloid potential and high lymphoid potential controlling the fate of T, B, and natural killer lymphocytes and monocytes [38–40]. However, this cell retains both lymphoid and myeloid transcriptomic programs, whereas its epigenome is oriented toward the lymphoid lineage by inactivating DNA methylation of myeloid enhancer regions and not lymphoid enhancers [41]. MPP1 cells have a multilineage myelo-erythro-megakaryocytic (My-Er-Mk) potential and a lymphoid potential in primary transplantation that is lost by secondary transplantation into mice [34]. MPP2 and MPP3 have a quite exclusive myeloid orientation with an Er-Mk potential for MPP2 and a granulomonocytic potential for MPP3 [35,37,42]. According to Notta et al., MPPs with multilineage potential $CD34^+CD38^-CD45RA^-CD90/Thy1^-CD71^-MPL^-$ can be distinguished from MPPs with Er-Mk or Er potential on the basis of CD71 expression or CD71 and MPL expression, respectively [37]. The proposed model in mice is that two distinct populations of MPPs—MPP2 and MPP3—are myeloid specified and one LMPP/MPP4 supplies the lymphoid lineage [42].

The multipotent cell populations are in a poised state from which they exit for lineage commitment driven by exogenous signals. This state could be governed by low levels of transcription factors under a threshold required for lineage commitment. By tracking fluorochrome-tagged GATA-1 and PU-1 transcription factors at the single-cell level, Hoppe et al. demonstrated that no cell, even putatively multipotent cells, coexpress these transcription factors [43]. Progenitors express one transcription factor that drives the lineage commitment. In the classical model of hematopoiesis, the first branching point arising at the level of committed $CD34^+CD38^+$ progenitors splits lymphoid and myeloid lineages into common lymphoid progenitors (CLPs) and CMPs, which then separate into megakaryocytic–erythroid progenitors (MEPs) and granulomonocytic progenitors (GMPs). Fed by the upstream multilineage or unilineage-specified MPPs, the $CD34^+CD38^+CD45RA^-Flt3^+$ CMPs and the $CD34^+CD38^+CD45RA^-Flt3^-$ MEPs have a predominant unilineage outcome: myeloid and erythroid, respectively [37].

In basal conditions, blood homeostasis is maintained by long-survivor myeloid progenitors without the contribution of HSCs, which are mostly quiescent [44]. The feeding role is devoted to MPPs. HSCs stay out of the cell cycle, which means that they are not prone to acquiring mutations in case of DNA misrepair during mitosis. In regenerating conditions, HSCs self-renew to feed the MPP compartment transiently. LMPP/MPP4 cells can be reprogrammed toward the myeloid lineage to participate in the bone marrow reconstitution [42]. HSCs differentiate into MPP2 and MPP3 and then into GMPs that organize in clusters upon stimulation by cytokines stem cell factor (SCF) and granulocyte colony-stimulating factor (G-CSF). This process is arrested by the release of inhibitory factors by neighboring megakaryocytes, which restore HSC quiescence [45].

HSC is targeted by genetic events

Infection, inflammation, or chronic blood loss are physiological stresses that may instruct HSCs to exit from quiescence. When cycling for self-renewal or asymmetrical division, HSCs and progenitors are more sensitive to genetic alterations than more differentiated cells. In these conditions, HSCs are submitted to collapse of mitochondrial membrane potential, production of reactive oxygen species, and DNA damage. Repeated stresses may provoke their attrition [12,46]. The functional potential of HSCs also changes during aging. Old HSCs exhibit a reduced capacity to generate their progeny and an altered lymphoid differentiation. In addition, lymphoid-biased HSCs should have a longer lifetime and lower capacities to proliferate than myeloid-biased HSCs [39,40,42]. The positive selection of myeloid-biased HSCs and the alterations in lineage specification programs is beneficial to myeloid differentiation [47,48]. In old mice, HSCs are less functional compared with young mice in the conditions of bone marrow transplantation. Importantly, old HSCs accumulate DNA damage due to limited DNA repair capacities compared with proliferating progenitor cells [49–51]. They experiment a replicative stress leading to double-strand DNA breaks in the progeny [51]. This genotoxic stress may contribute to the onset of mutations and could originate from a deleterious interaction with an inflammatory microenvironment.

In a mouse model of Shwachman–Diamond syndrome, which mimics a preleukemic syndrome, $CD271^+$ mesenchymal stromal cells produce inflammatory molecules named damage-associated molecular pattern (DAMP) S100A8/9. Recombinant S100A8 induces mitochondrial dysfunction, oxidative stress, and a DNA damage response in HSPCs [52]. Interestingly, the expression of S100A8/9 markers is increased in several MDS subtypes, including the 5q– syndrome and therefore could contribute to the induction of a genotoxic stress at the level of MDS HSPCs [52,53].

In myeloid malignancies, the clones are thought to emerge from a unique cell that acquired novel mutations sequentially over time, suggesting that the genetic complexity may increase with evolution. Nilsson et al. reported the first evidence for an MDS-initiating cell by identifying a chromosome 5q deletion by fluorescence in situ hybridization in sorted $CD34^+CD38^-$ cells [54]. The cell that is initially targeted by genetic events (either chromosomal deletion or mutation) is a rare HSC [55,56]. The del(5q) $lin^{low}CD34^+CD38^-CD90/Thy1^+CD45RA^-$ cells exhibit long-term culture-initiating cell (LTC-IC) activity and capacities to engraft and to expand a

myeloid and a lymphoid progeny in immunodeficient NSG mice [56,57]. HSC-deriving cells isolated from patients with an *SF3B1*-mutated sideroblastic anemia are also capable of long-term engraftment [29,58]. By backtracking the mutations at the single-cell level in the $CD34^+CD38^-$ HSPC compartment, we have shown that individual $CD34^+CD38^-$ cells contains 0, 1, 2, 3, or all of the mutations detected in bulk mononuclear cells; are organized in clones that coexist; and are maintained in this compartment. A dominant clone accumulates mutations in a linear succession or a branched architecture downstream of the founding clone or in a separate clone [27,56]. Among HSPCs, LT-HSCs harboring LTC-IC activity contain the mutations of the dominant clone. In xenotransplantation experiments, engrafted cells belonged to the dominant clone and revealed their stem cell nature by yielding both $CD15^+$ or $CD14^+$ myeloid cells and $CD19^+$ lymphoid cells [21,27,56].

Alterations of hematopoietic hierarchy in myeloid malignancies

In several myeloid malignancies, the hematopoietic tree is highly modified. In aplastic anemia, the $CD34^+CD38^-$ HSC and MPP compartments are depleted and the $CD34^+CD38^+$ compartment is preserved. Among $CD34^+CD38^+$ cells, erythroid progenitors are lost and myeloid progenitors are represented normally [37]. It has been demonstrated that the transmission of mutations to the progeny disturbs the steps of development. In BCR-ABL-driven chronic myeloid leukemia (CML), the initiating cell derives from the HSC compartment and drives the relapse [59,60]. During the development of CML, HSCs differentiate into myeloid-biased MPPs. MPP2 and MPP3 feed the GMP compartment rapidly organized in clusters of GMPs that are maintained by the activation of self-renewal pathway and the default of inhibitory cytokines [45].

MDS clonal hematopoiesis is characterized by a myeloid differentiation bias of HSCs, which could be due either to the positive selection of a myeloid-specified MPP or the involution of B-cell progenitors in MDS bone marrow. Sternberg et al. have demonstrated B-cell progenitor depletion in MDS [61]. Mutations could accumulate with a higher probability in actively dividing myeloid progenitors that supply the daily production of blood cells than in lymphoid cells.

It is remarkable that the size of the HSC compartment where the disease initiates remains small. This encourages searching for disease-propagating cells at the level of MPPs. As first evidence for the involvement of MPPs, the engraftment of a clonal $lin^-CD34^+CD38^-CD90/Thy1^-CD45RA^-$ MPP into NSG mice has been obtained [62]. In this case, the MPP harbored an *ASXL1* mutation not detected in HSCs that may confer a proliferative advantage to MPP in vivo [62]. In another MDS with excess of blasts >10%, we detected seven mutations in the *STAG2*, *ETV6*, *U2AF1*, *ASXL1*, and *NRAS* genes at the level of bulk mononuclear cells, including one *STAG2* p.798fs (insC) represented with a variant allele frequency (VAF) over 50% in all compartments and two *STAG2* p.A916fs (ins TAAG) and *STAG2* p.R1045X detected at 5% and 10% of cells, respectively. These latter mutations were absent in HSCs, detected in MPPs and transmitted with the same VAF to CMPs, GMPs, and MEPs (Fig. 1). This indicates that MDS bone marrow can be made of a variegation of mutations scattered between distinct stem or progenitor subsets. We observed the emergence of three *STAG2*-mutated subclones on a common genetic background among MPPs. Whether these clones could initiate the disease in NSG mice is not known. In this case, the HSC remains the disease-initiating cell. The concept of several coexisting cancer stem cells developed from the analysis of clear cell renal cancer architecture cannot be applied to MDS [63].

Considering the diversity of phenotypes, it is not surprising that the hierarchical trees are highly variable in MDS. Will et al. studied the repartition of committed progenitors within the $CD34^+CD38^+$ compartment and showed that CMPs accumulate at the expense of MEPs and GMPs in early MDS [64]. In these latter cases, CMPs or GMPs may derive from a rapidly proliferating myeloid-specified MPP devoid of erythro-megakaryocytic differentiation capacities. Alternatively, mutations may increase the competitiveness of CMPs or GMPs (Fig. 1). We have shown that a *BCOR* mutation identified as being dominant in CMPs may become undetectable in GMPs or MEPs. This suggests that the capacity of the *BCOR*-mutated subclone to differentiate toward the granulocytic and erythroid lineages is abrogated [27]. Invalidation of *BCOR* using an shRNA strategy in normal progenitors confirmed this hypothesis.

By using classical biomarkers for CMP $Lin^-CD34^+CD38^+CD123^+CD45RA^-$, GMP $Lin^-CD34^+CD38^+CD123^+CD45RA^+$, and MEP $Lin^-CD34^+CD38^+CD123^-CD45RA^-$, we showed that the MEPs are well preserved or increased in sideroblastic anemias, whereas this compartment is diminished in other subtypes [27]. MEPs could be fed by selected erythroid-biased MPPs.

As a hallmark of the myeloid and lymphoid potential of disease-initiating cells, B lymphocytes and, to a lesser extent, T lymphocytes contain MDS-recurrent mutations and participate in clonal hematopoiesis [56,65,66]. However, the mutational repertoire of B and T lymphocytes is limited. Mutations of the founding clone, mainly affecting epigenetic regulators, splicing factors, and chromatin modifiers are detectable in B lymphocytes [21,27]. B lymphocytes are usually less mutated than myeloid cells. Several hypotheses can be put forward: (1) the onset of secondary mutations in the context of initial genetic background may impair the transition of HSCs to lymphoid progenitors and decrease the fitness of B cells; (2) secondary mutations may appear in an MPP with low lymphoid potential [42,67]; and (3) mature B cells may originate from the HSPC compartment downstream of a long-lived lymphoid progenitor affected by early genetic lesions but with slow proliferative capacity that precludes the acquisition of additional events [44,68,69]. Differences in

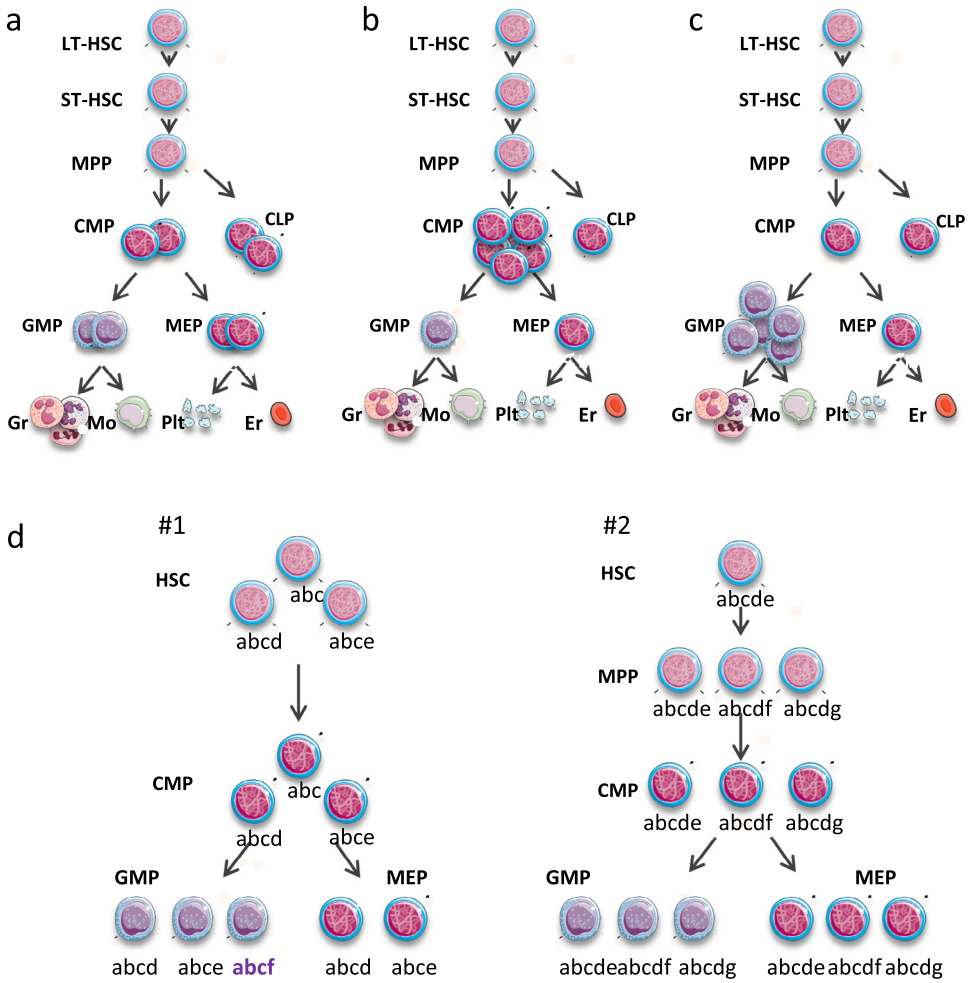

Figure 1. Hierarchy of clonal hematopoiesis. **(a)** Schematic representation of normal hematopoiesis. **(b)** Low-risk MDS showing amplification of CMPs and diminution of CLPs. **(c)** High-risk MDS showing amplification of GMPs. **(d)** Hierarchy of clonal hematopoiesis in two examples of low-risk MDS by genotype analysis of sorted bone marrow subpopulations. #1: Onset of an "f" mutation in GMP; #2: acquisition of two "f" and "g" mutations in two separate clones in MPPs transmitted to the progeny. Each letter represents one mutation. Gr = granulocyte, Er = erythrocyte, Mo = monocyte, Plt = platelet.

mutation composition between myeloid and lymphoid compartments contribute to tumor heterogeneity.

In the context of differentiation, the various combinations of mutations take part in the selection of myeloid compartments. In other words, the function of targeted genes participates in the selection and the functional consequences of altering one gene depends on the cell type in which the alteration occurs and on associated events [70]. The genetic progression and phenotype is linked to the tissue of origin, the context-dependent selection of pro-tumorigenic alterations, and the functional interactions of cooperating genes [71,72].

Leukemia-propagating cells

In AML, clonal hematopoiesis is driven by a leukemic stem cell (LSC) [73,74]. This cell retains the self-renewal capability of its normal counterpart in NSG mice. The clone that usually engrafts may be a subclone distinct from the one that emerges at relapse and distinct from the putative founding clone [75,76]. In $CD34^+$ AML, the hierarchical organization of hematopoiesis is highly perturbed. LSC activity has been identified in two hierarchically organized populations distinct from HSC, one being similar to LMPPs and the other resembling normal GMPs. These two populations retain a self-renewal potential [77].

In advanced MDS entering the process of AML transformation, the propagating population could be the GMPs because the percentage of GMPs increased [64]. We have reported that a *STAG2* gene mutation that was faintly detectable in HSCs ($Lin^-CD34^+CD38^-CD90^+$) can amplify in a GMP. Among three distinct clones coexisting in the GMP compartment at the time of diagnosis, the clone containing this mutation amplified as the disease progressed from a MDS with excess of blasts type 1 to a MDS with excess of blasts type 2 and AML at the expense of the two other clones [27]. In the context of lineage commitment, some mutations may increase the fitness of

clones to their microenvironment and help the leukemic transformation. As shown in the case of de novo AML, the expansion of a population of cells with putative self-renewal potential downstream of the stem cell compartment should be a key step in the transition from MDS to AML. All initiating mutations are still present as the disease evolves into AML; however, the variant allele frequencies may be different due to the amplification of the leukemic clone.

Deciphering clonal hematopoiesis during AML initiation and evolution has consolidated the concept of preleukemic events. A few genetic events can fit the definition on the basis of the repopulating advantage in xenograft experiments of a HSC containing such a mutation, its persistence in remission samples, and its involvement in the clone driving the relapse. *TET2*, *DNMT3A*, and *ASXL1* mutations and *MLL* rearrangements that may lead to leukemic and nonleukemic lymphoid repopulation are preleukemic lesions that can reinitiate the leukemic process at various stages until relapse [78,79].

Implications for treatment

A dynamic evolution that comes with a risk of clonal selection and treatment resistance or escape is the heterogeneity seen in MDS and other myeloid malignancies. We have reported a 10 year follow-up of a refractory anemia with excess blasts 1 (RAEB1) patient that was remarkably stable in terms of bone marrow blast percentage for 9 years and entered RAEB2 after that. This patient did not received disease-modifying therapy for 9 years and successively acquired new mutations in the HSPC compartment. At the time of clinical evolution, the architecture became branched, with two separate clones each containing a new mutation in two distinct signaling adapters (Fig. 2). It is clear that, under treatment with growth factors such as an erythropoiesis-stimulating agent, a recombinant thrombopoietic mimetic, or G-CSF, new mutations still accumulate. Lenalidomide clearly exerts a pressure leading to the disruption of the del(5q) clone and a balanced amplification of non-del(5q) clones. Azacytidine does not prevent the onset of new mutations such as *EZH2* [21,22].

In 5q– syndrome or MDS with isolated del(5q), lenalidomide is a powerful drug that rescues patients from anemia, improves quality of life, and prolongs survival [80]. However, this drug targets 5q– erythroid cells but fails to eradicate the 5q– HSCs responsible for disease maintenance [55]. Several scenarios are possible [21,22]. In most cases, lenalidomide abrogates the expansion of the founding clone

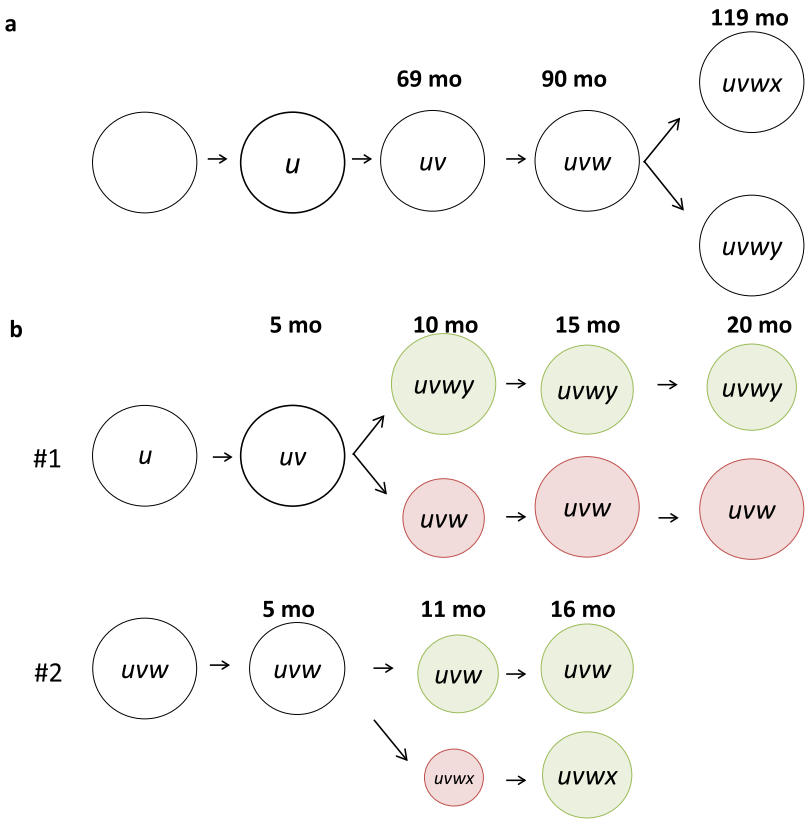

Figure 2. Clonal evolution over time. **(a)** Natural course of clonal evolution: linear acquisition until 90 months (mo) and branched architecture after 119 months of evolution. **(b)** Clonal evolution under treatment: shown are two examples of clonal evolution under lenalidomide. Upper panel: modification of clone size in one responding patient. Lower panel: acquisition of a new mutation and amplification of the new clone in one nonresponding patient. Each letter represents a mutation.

when it contains the del(5q) abnormality, leading or not to the expansion of a separate clone. In non-del(5q) MDS, lenalidomide is less efficient but may act by targeting the hematopoietic cell directly, interfering with stromal cell interaction, or inducing an immune response. We have shown that lenalidomide targets HSPCs transiently by decreasing the size of the clones in responding patients. The loss of response is associated with a re-increase of clone size and sometimes with emergence of new mutations (Fig. 2) [81]. Lenalidomide also modulates the immune populations of the bone marrow microenvironment by increasing the abundance of the cytotoxic T-cell population, suggesting that it may also induce an indirect immune-mediated antitumor effect [82].

Targeting the HSCs is elusive because these cells are partly dormant and less responsive to drugs than more differentiated cells [27,55]. Furthermore, in MDS patients, who are mostly older than 70 years, it is unrealistic to target HSCs because the residual normal HSC is weak and plausibly functionally inefficient to repopulate the bone marrow after treatment. Sparing normal HSCs is therefore challenging, particularly in cases of advanced MDS. The precise identification of disease-propagating cells could be contributive to the design of a therapy specifically directed against them to slow down the progression. Single-cell genetic profiling will provide the best resolution to decipher the clonal heterogeneity and identify the self-renewing compartment in which the disease amplifies. Single-cell DNA screening using a microfluidic platform coupled to multiplex fluorescent quantitative polymerase chain reaction allowing unbiased single-cell selection will provide a comprehensive analysis of genetic abnormalities at diagnosis and during evolution and will also serve in the detection of residual disease. This approach could reveal the potential sources of treatment resistance or failure to guide the choice of alternative therapies.

Conclusion

MDS initiates with mutations in epigenetic regulators and/or splice factors at the level of HSCs. Clonal evolution depends on the acquisition of a secondary event usually in the compartment of HSCs, but also in MPPs or GMPs, showing that the context of differentiation may be favorable to the selection of a clone that drives the transformation. Further work is needed to identify specific biomarkers of the disease-propagating compartment that could be expressed in the context of particular patterns of mutated genes. This could help to redirect to this compartment targeted therapies against signaling molecules or deregulated metabolism pathways that appear as frequent secondary transforming events.

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Conflict of interest disclosure

The authors declare no competing financial interests.

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