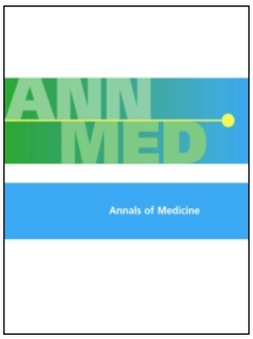

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REVIEW ARTICLE

Molecular mechanisms of the progression of myelodysplastic syndrome to secondary acute myeloid leukaemia and implication for therapy

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Myelodysplastic syndrome (MDS) includes a heterogeneous group of clonal haematological stem cell disorders characterized by dysplasia, cytopenias, ineffective haematopoiesis, and an increased risk of progression to acute myeloid leukaemia (AML), which is also called secondary AML (sAML). Approximately one-third of patients with MDS will progress to sAML within a few months to a few years, and this type of transformation is more common and rapid in patients with high-risk MDS (HR-MDS). However, the precise mechanisms underlying the evolution of MDS to sAML remain unclear. Currently, chemotherapy for sAML has minimal efficacy. The only method of curing patients with sAML is allogeneic haematopoietic stem cell transplantation (Allo-HSCT). Unfortunately, only a few patients are appropriate for transplantation because this disease primarily affects older adult patients. Additionally, compared to *de novo* AML, sAML is more difficult to cure, and the prognosis is often worse. Therefore, it is important to clarify the molecular mechanisms of the progression of MDS to sAML and to explore the potent drugs for clinical use. This review will highlight several molecular mechanisms of the progression of MDS to sAML and new therapeutic strategies of this disease.

Key words: Chromosomal abnormalities, clonal evolution, epigenetic changes, genetic mutations, immune suppression, myelodysplastic syndrome, secondary acute myeloid leukaemia

Key message

- This review primarily summarizes several possible molecular mechanisms of the progression of MDS to sAML, including clonal evolution, genetic mutations, chromosomal abnormalities, epigenetic changes, and immune suppression.

Introduction

Myelodysplastic syndrome (MDS) is one of the most common haematopoietic malignancies that arises in primitive $CD34^+$ stem cells and includes a highly heterogeneous group of myeloid disorders characterized by dysplasia, peripheral blood cytopenias, ineffective haematopoiesis, and a variable risk of transformation to acute myeloid leukaemia (AML) (1), which is also referred to as secondary origin AML (sAML). Approximately 30% of patients with MDS will develop into sAML within a few months to a few years. This type of transformation is relatively infrequent in patients with low-risk MDS (LR-MDS) but is more common and rapid in patients with high-risk MDS (HR-MDS). Nevertheless, the precise molecular mechanisms underlying the progression of MDS to sAML are poorly understood. MDS primarily affects older adult patients; thus, co-morbidities are an important consideration. For decades, the mainstay treatment for MDS was supportive care that included transfusions of blood products and growth factors. Regarding therapeutic drugs, the approvals of lenalidomide and azacitidine for MDS are major recent breakthroughs. Nonetheless, the responses to these agents are limited, and most patients progress within 2 years. Allogeneic haematopoietic stem cell transplantation (Allo-HSCT) remains the only potentially curative therapy for patients with MDS and sAML, but only a minority of patients are eligible for this treatment. Additionally, compared to *de novo* AML, sAML is more difficult to cure, and the prognosis is often worse. Therefore, it is important to clarify the molecular mechanisms of the progression of MDS to sAML and to explore new therapeutic strategies for improving the prognosis of this disease. In this review, we primarily summarize several possible molecular mechanisms of MDS with a particular focus on HR-MDS and the progression to sAML due to clonal evolution, genetic mutations, chromosomal abnormalities, epigenetic changes, and immune suppression.

Clonal evolution

Although clonality is not sufficient for defining malignant transformation, it is a cardinal manifestation of most human cancers. Recently, clonal evolution has been found to play an important role in the progression of MDS (2). Walter et al. (3) performed whole-genome sequencing of seven paired samples of skin and bone marrow (BM) from seven subjects with sAML to identify somatic mutations that were specific to sAML, and genotyped BM samples from each subject that were obtained during the antecedent MDS stage to determine the presence or absence of

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the specific somatic mutations. These authors identified recurrent mutations in coding genes and defined the clonal architecture of each pair of samples based on the MDS stage and the sAML stage using the allele burdens of hundreds of mutations. The authors revealed that the founding clones (containing 182–660 mutations) in the seven MDS samples persisted in all seven sAML samples and included at least one tier 1 mutation, although this mutation was out-competed by daughter subclones in some cases. With the acquisition of each new set of mutations, all of the pre-existing mutations were carried forward, which resulted in subclones that contained numbers of mutations that increased over the course of evolution. Notably, the majority of the mutations were randomly acquired and not causally related to the pathogenesis of MDS. These findings suggest that most cases of MDS are clonally heterogeneous and have a founding clone and multiple subclones. The transformation of MDS to sAML is characterized by multiple cycles of mutation and clonal selection, and complex clonal evolution with the acquisition of multiple additional mutations co-exists with the MDS founding clone.

A short time thereafter, Walter et al. (4) screened a panel of 94 candidate genes in a cohort of 157 patients with MDS or sAML (including 150 MDS cases, and 15 sAML cases, in which 8 were also analysed at the MDS stage). These authors detected at least one mutation or cytogenetic abnormality in 83% of the 150 MDS patients, and 17 genes were significantly mutated. All tumours contained a founding clone and at least one subclone that contained all of the founding clone mutations. No single gene was exclusively mutated in a founding clone or subclone, which indicated that the genes with recurrent mutations were detected in both the founding clones and the daughter subclones. Overall, these studies demonstrate that a founding clone containing hundreds of mutations is present in MDS BM, persists when the patients progress to sAML, and gives rise to daughter subclones that contain founding clone mutations. Therefore, clonal evolution combined with genetic mutation might play an important role in the progression of MDS to sAML.

Genetic mutations

With the rapid development of molecular biological techniques, many genetic mutations have been found to be closely related to the transformation of MDS to sAML. Sequencing of MDS genomes has identified genetic mutations that are implicated in RNA splicing, signal transduction, chromatin modification, transcription regulation, etc. The majority of MDS patients have two or three driver oncogenic mutations and hundreds of background mutations. Some authors argue that one genetic event is sufficient to cause sAML (5), while others believe that several genetic events are required for the transformation. In fact, reports of mutation analyses of *de novo* AML cases have indicated that the vast majority of the detected genetic alterations do not play any important causative role in leukaemogenesis (6). Based on an analysis of a large database ($n = 1{,}079$) of MDS patients, Shukron et al. (7) have also indicated that although many genetic alterations occur during sAML evolution, only a single random biological event (genetic or epigenetic) is crucial and leads to the abrupt or gradual deterioration of the patient to sAML. Several genetic mutations that are involved in the progression of MDS to sAML will be reviewed in the subsequent sections.

RNA splicing genes—SRSF2 and SF3B1

Recently, mutations in mRNA splicing genes, including SRSF2 and SF3B1, have been described in MDS, which underscore the molecular complexity that underlies the development of this heterogeneous disease. Additionally, mutations of SF3B1 and SRSF2 occur not only in myeloid lineage tumours but also in lymphoid lineage tumours (8), which suggests that such splicing gene mutations play important roles in the pathogenesis of haematologic tumours.

SF3B1 mutations were found to be more frequent in 33% of patients with MDS that were characterized by ring sideroblasts (RS); in contrast, only 2.3% of patients with the other subtypes of MDS were found to have SF3B1 mutations (9). This recurrent nature strongly suggests that SF3B1 mutations might play important roles in disease development. The close relationship between SF3B1 mutations and RS is consistent with a causal relationship and makes SF3B1 the first gene to be associated with a specific morphological feature of MDS. SF3B1 is also mutated in 70%–85% of refractory anaemia with ring sideroblasts (RARS) patients and is highly associated with the presence of RARS (9). Based on an analysis of the haematopoietic function of SF3B1 in SF3B1(+/−) mice, Wang et al. (10) found that the SF3B1(+/−) mice maintained nearly normal haematopoiesis and did not develop haematological malignancies during a long observation period. However, the SF3B1(+/−) cells exhibited a significantly impaired capacity to reconstitute haematopoiesis in a competitive setting and exhibited some enhancement of apoptosis, but they did not show any obvious defects in differentiation. Additional depletion of SF3B1 in SF3B1(+/−) haematopoietic stem cells (HSCs) with shRNA severely compromised their proliferative capacities both *in vitro* and *in vivo*. Finally, Wang et al. (10) unexpectedly found no changes in the frequencies of sideroblasts in either SF3B1(+/−) erythroblasts or cultured SF3B1(+/−) erythroblasts expressing shRNA against SF3B1. These studies suggest that the level of SF3B1 expression is critical for the proliferative capacity of HSCs, but haploinsufficiency for SF3B1 is not sufficient to induce a RARS-like phenotype. Therefore, while the level of SF3B1 expression is critical for the proliferative capacity of HSCs, and the mutation of SF3B1 plays an important role in disease development, we boldly speculate that the abnormal expression and recurrent mutation of SF3B1 might be involved in the progression of MDS to some extent.

Recurrent mutations of SRSF2 have been identified in a substantial proportion of patients with MDS. SRSF2 mutations might prompt poor prognosis and a higher rate of progression to sAML. In a study of 233 MDS patients, 34 patients were found to have SRSF2 mutations; such mutations occurred concurrently with at least one additional mutation in 29 patients and were closely associated with RUNX1, IDH2, and ASXL1 mutations (11). Patients with SRSF2 mutations, particularly those with LR-MDS, exhibited inferior overall survival (OS). Further exploration revealed that the prognostic impact of SRSF2 mutations might be attributed to their close association with old age. Sequential analyses of 173 samples from 66 patients revealed that all of the SRSF2-mutated patients retained their original mutations, whereas none of the SRSF2-wild patients acquired a novel mutation during disease evolution (11). Therefore, SRSF2 mutations are associated with poor clinical prognosis of MDS patients and might play a role in disease progression.

Signal transduction genes—C-CBL

The detection of 11q-acquired uniparental disomy (UPD) has led to the identification of C-CBL mutations in various myeloid neoplasms; such mutations are particularly common in chronic myelomonocytic leukaemia (CMML) with a frequency of 5% to 25% (12,13). In contrast, C-CBL mutations seem to be less frequent among patients with MDS and AML. In rare cases of human AML ($< 2\%$), C-CBL mutants have been identified, and the frequency

of such cases is higher among the core binding factor leukaemias. Kao et al. (14) analysed a large cohort of matched pair BM samples from 51 patients with *de novo* HR-MDS (13 were RCMD, 19 were RAEB-1, and 19 were RAEB-2) who progressed to sAML to determine the frequencies and characteristics of the C-CBL mutations in both phases of the disease. Of the 51 paired samples, C-CBL mutations were identified in 6 patients in the sAML phase. One patient retained an identical C-CBL mutation over sAML evolution and exhibited clonal expansion. The other five patients acquired C-CBL mutations during sAML evolution. These results suggest that although C-CBL mutations are very rare in patients with MDS, the acquisition and/or expansion of C-CBL mutant clones occurs during sAML transformation. Therefore, the high occurrence of C-CBL mutations during sAML transformation among patients with HR-MDS suggests that C-CBL mutations might play a role.

Barresi et al. (15) also described an expansion of a C-CBL mutated subclone that occurred in a case during the progression of HR-MDS to sAML. Additionally, acquisition with expansion of C-CBL mutated clones has been reported in one patient during the progression of primary myelofibrosis to sAML (16). In the literature review, except for one with refractory anemia (RA) (13), patients with MDS harbouring C-CBL mutations were mostly of RAEB or RCMD subtypes (12,15). Taken together, these results indicate that C-CBL mutations are associated with aggressive types of MDS and that C-CBL mutated subclones confer a particular growth advantage during the progression of HR-MDS progression to sAML.

Other genes—FLT3 and NPM1

FLT3 and NPM1 are among the most common mutated genes in AML (17,18), but such mutations are rare in MDS (19–21). Bains et al. (22) reviewed FLT3 and NPM1 mutation data from a large cohort ($n = 1{,}316$) of MDS patients and found that FLT3 and NPM1 mutations were rare (2.0% and 4.4%, respectively) and occurred predominantly in HR-MDS patients, particularly those with RCMD and RAEB. These authors also found that the presence of a FLT3 mutation is significantly associated with greater likelihood of progression to sAML. Furthermore, none of the patients in this cohort with only NPM1 mutations experienced disease progression. It has been proposed that NPM1 mutations alone might not be sufficient to cause progression to overt sAML, but that such mutations in conjunction with secondary events in the form of other mutations, such as FLT3 mutations, predispose progenitor/stem cells to malignant transformation. Pinheiro et al. (19) previously indicated that, although FLT3-ITD anomalies appeared only rarely in MDS patients, these anomalies played a very important role in the short-term progression of MDS to sAML and should be recognized as a marker of MDS progression. Additionally, Bains et al. (22) have indicated that MDS cases with diploid cytogenetics that progress to sAML are more likely to harbour FLT3 mutations or concomitant NPM1 and FLT3 mutations, which indicates that FLT3 mutations most likely contribute to disease progression. Recently, dynamic acquisitions of FLT3 alterations have also been found to drive a subset of patients with LR-MDS to sAML (23). Moreover, Bains et al. (22) have found that FLT3 mutations are not only associated with NPM1 mutations in cases with a diploid karyotype, but also strongly associated with complex cytogenetics, which suggests that FLT3 mutations can be induced through multiple mechanisms. Consequently, although FLT3 and NPM1 mutations are rare in HR-MDS patients, these mutations, especially FLT3 mutations, might play an important role in the progression of MDS to sAML. Therefore, routine screening of MDS patients for these mutations is potentially useful for clinical stratification and predicting the progression to sAML.

Additionally, mutations of RAS (23), SETBP1 (24), and STAG2 (3) have also been reported to be involved in the evolution of MDS to sAML. Taken together, these findings indicate that a large number of genetic mutations might play specific roles in the progression of MDS to sAML. Unfortunately, due to the limited amount of samples of these studies, several of genetic mutations were found in only a few MDS patients and could not clearly explain the mechanisms of the progression of MDS to sAML. Therefore, the roles of genetic mutations in the progression of MDS to sAML remain to be further explored.

Chromosomal abnormalities

Chromosomal abnormalities are frequent in both *de novo* and secondary MDS and AML, particularly in HR-MDS (25) and sAML (26). Gale et al. (27) found that the most common chromosomal abnormalities were del(5/5q), del(7/7q), + 8, + 1q, del(20q), del(13), t(11q23), del(12p), and + 21, which were present in 40%–60% of MDS and AML patients. These chromosomal abnormalities are not only closely related to the leukaemic progression of MDS (28) but also related to the efficacy of chemotherapy for sAML (29). Generally, AML secondary to MDS is characterized by a low treatment response rate and a shorter OS compared to *de novo* AML (30). Larson (26) previously reported that the incidence of + 8, -7, and other detrimental cytogenetic abnormalities were higher in sAML than that in *de novo* AML, which suggested that chromosomal abnormalities might be related to the occurrence of sAML. Corrêa de Souza et al. (31) found that the chromosomal abnormalities in patients who evolved to sAML included -7, + 8, del(6q), del(7q), del(11q), i(7q), t(7;9), and i(9q) abnormalities and complex karyotypes, and some of these abnormalities might play critical roles in this process; however, the frequency of the evolution from hypocellular primary MDS to sAML was low. Currently, the causative roles of chromosomal abnormalities in disease progression are still not completely understood. Several chromosomal abnormalities associated with the progression of MDS to sAML will be discussed in the following section.

Inv(3)/t(3;3)

Inv(3)/t(3;3) is a rare (approximately 1%) recurrent cytogenetic abnormality in a subset of AML patients (32). Patients with AML associated with inv(3)/t(3;3) are frequently refractory to conventional chemotherapy regimens and have dismal clinical outcomes (33). A recent study has demonstrated that AML with inv(3)/t(3;3) is associated with unfavourable outcome and a median survival of 9.6 months (34). AML with inv(3)/t(3;3) is also recognized as a distinct subtype in the World Health Organization (WHO) classification (32) and can occur *de novo* or arise from a preceding MDS. However, the natural history of MDS with inv(3)/t(3;3) is poorly understood. Cui et al. (35) studied 17 MDS patients (11 *de novo* MDS and 6 t-MDS) with inv(3)/t(3;3) and found that nearly 65% of these patients progressed to sAML and that these patients were characterized by chemoresistance and short overall and median survival rates. Additionally, these findings were similar to those for AML associated with inv(3)/t(3;3) (34); patients with MDS with inv(3)/t(3;3) frequently have common additional cytogenetic abnormalities, including -7/7q and -5/5q, or complex karyotypes, which suggests that MDS and AML associated with inv(3)/t(3;3) might be a continuum of the same entity. These findings indicate that MDS with inv(3)/t(3;3) is an aggressive

disease that is associated with a dismal clinical outcome and a high risk of progression to sAML.

Del(6p)

Using high-resolution single nucleotide polymorphism microarrays, Puda et al. (36) detected frequent deletions in the short arm of chromosome 6 (del(6p)) and observed a strong association of del(6p) with leukaemic transformation in 61 MDS patients. The common deletion region in 6p mapped to a 1.1-Mb region that contains only the JARID2 gene, which is a member of the polycomb repressive complex 2 (PRC2). Puda et al. applied deletion mapping to 31 post-MDS AML patients and identified frequent lesions of JARID2. These authors subsequently analysed the deletion profiles of other PRC2 members and found frequent losses of genes such as EZH2, AEBP2, and SUZ12. Using next-generation exome sequencing, they identified only one somatic mutation in the PRC2 member SUZ12, indicating that these deletions were the main type of lesions that targeted PRC2 members. Otherwise, PRC2 members could also be secondary targets of deletions that had effects that were additive with those of deletions of other tumour suppressors and likely result in stronger effects on clonal progression or leukaemic transformation (36). Therefore, del(6p), which contains only the PRC2 member JARID2, might play an essential role in the leukaemic transformation of MDS. Additionally, isolated isochromosome 17q might also be associated with the leukaemic transformation of MDS (37).

Complex karyotypes

In multivariate analyses comparing clinical and genetic data, complex cytogenetic karyotypes are associated with a particularly poor prognosis. MDS with complex karyotypes belongs to the International Prognostic Scoring System (IPSS) high-risk group and has a short survival and a high risk of progression to sAML. The deletion of chromosome 5 (del(5q)) is the most common cytogenetic abnormality in MDS and portends a favourable prognosis (38). MDS with isolated del(5q) is typified by a relatively low rate (below 10%) of progression to sAML compared to other subtypes of MDS (39). However, del(5q) was recently found to be unstable and frequently involved in different types of cryptic unbalanced rearrangements (40). The involvement of del(5q) in complex karyotypes in newly diagnosed MDS was correlated with an extremely adverse prognosis (40). Using genetically engineered mice, Stoddart et al. (41) have recently demonstrated that the loss of TP53 activity in combination with haploinsufficiency for both Egr1 and Apc (two genes that are lost in del(5q)) creates an environment that is permissive for malignant transformation and the development of AML. These authors have also indicated that alterations to the function of additional genes on 5q are likely required for myeloid leukaemia development and full transformation. However, the mechanism of del(5q) in the progression of MDS has not been well studied and has rarely been reported.

TP53 mutations are frequent in MDS and AML with complex karyotypes that include del(5q) and are often associated with shorter survival (42). Studies have suggested a role for TP53 mutations and complex karyotypes in disease progression and poor outcomes. Multivariate analysis has confirmed that TP53 mutations are an independent adverse prognostic factor for OS in sAML (43). Bejar et al. (44) have indicated that TP53 mutations are consistently associated with complex karyotypes with -7/7q- and -5/5q- and predict poor outcomes for patients with MDS. Others have also found that TP53 mutations tend not to occur with other gene mutations in MDS but frequently co-occur with complex karyotypes, such as del(5q) or del(7q), and are correlated with IPSS intermediate-2 (Int-2) or high-risk IPSS scores and leukaemic progression (45,46). Similarly, patients with a chromosomal abnormality of chromosome 5 or 7 often acquire additional mutations, such as TP53 mutations, that result in disease progression (46). TP53 alterations (either mutations or deletions) have also been detected in nearly 70% of AML cases with complex karyotypes and are correlated with dismal outcomes (47). Taken together, these findings indicate that TP53 mutations are an independent prognostic indicator of MDS. Such mutations frequently co-occur with complex karyotypes, particularly del(5q) or del(7q), and contribute to disease progression. Therefore, both TP53 mutations and chromosomal abnormalities play important roles in the progression and prognosis of MDS.

Epigenetic changes

Recently, epigenetic research has come to play an important role in the malignant tumour genotyping and clinical treatment. Epigenetic changes, i.e. heritable changes in gene expression that do not represent changes in DNA sequence, have been recognized as critical factors for physiological phenomena such as embryogenesis and the differentiation of normal cells. DNA methylation is a well-established epigenetic mechanism that has been shown to play significant roles in tumour initiation and progression. DNA methylation often occurs on the cytosine of CpG islands located in gene promoter regions and is thought to be closely correlated with tumourigenesis. DNA methylation is particularly frequent in MDS and AML (48). This affects genetic mutation and the stability of the genome, which can result in epigenetic inactivation of tumour suppressors and open the way for evolution towards extended life-spans for (potential) cancer stem cells. Accordingly, subtypes of MDS can be largely explained by disordered stem cell epigenetics. Epigenetic modifications of key target antigens have been indicated to play important roles in the pathogenesis and evolution of MDS and AML (49). Mahmud et al. (50) believe that the progression of MDS and AML is associated with increased methylation levels of key promoter regions. In HR-MDS, hypermethylations of several tumour suppressor genes adversely affect survival and increase risk of leukaemic evolution (51).

Genetic methylations

CDKN2B and CDKN2A are two frequently methylated genes. The methylations of these genes were studied in 63 patients with MDS and in 13 patients with AML by Cechova et al. (52). Aberrant methylations were present in the CDKN2A and CDKN2B genes in 38% and 77%, respectively, of the patients in the MDS group. The level of methylation was higher in the group of AML patients; 77% and 100% of the CDKN2A and CDKN2B genes, respectively, were methylated. In MDS patients, aberrant methylation was associated with a tendency for disease progression towards more advanced forms according to the WHO classification and the IPSS risk stratification. Significant differences in methylation levels have been observed between the early and advanced forms of MDS in the CDKN2B gene but not in the CDKN2A gene. The methylation of the CDKN2B gene in patients treated with azacytidine was analysed and found to be correlated with the course of the disease. Increased methylation was connected with disease progression. Cechova et al. (52) concluded that hypermethylation is an important event in the progression of MDS to sAML and that methylation level of the CDKN2B gene might be used as a marker of leukaemic transformation in MDS.

Mutations of DNMT3A have been shown to play important roles in the regulation of epigenetic patterning and might be used as molecular predictors of pathogenesis and clinical outcome of patients with MDS (53). DNMT3A mutations often indicate worse OS and a more rapid progression to sAML. Recently, DNMT3A mutations were identified in 4%–22% of patients with *de novo* AML (54), and predicted poor prognosis. These mutations were closely associated with advanced age (over 60 years), the M(4) and M(5) subtypes, and intermediate-risk cytogenetics. Walter et al. (55) found DNMT3A mutations in 8% (12/150) of MDS patients and reported that the DNMT3A mutations were associated with worse OS in MDS. These alterations also presented in MDS and primary myelofibrosis prior to the development of obvious leukaemia, which indicated that DNMT3A mutations might contribute to leukaemogenesis.

Additionally, abnormal methylations of the TET2, IDH, ASXL1, FANCF, and FZD9 genes have also been reported to be associated with a short progression time and poor prognosis in MDS (56,57). Demethylation treatment is effective for a portion of MDS and AML patients, which further suggests that abnormal genetic methylation is one mechanism of the progression of MDS and AML (57,30). The use of demethylation agents in MDS treatment will be mentioned in detail subsequently. Taken together, these findings indicate that abnormal epigenetic methylation, particularly DNA methylation, plays a significant role in the initiation and progression of MDS.

Immune suppression

Significant deregulation of the immune system has been suggested to occur in the complex pathogenesis of MDS (58,59). Mounting evidences indicate that the heterogeneities of the progression of MDS and leukaemia are driven by inflammation and immune suppression (60). It is clear that key immunologic molecules function as extrinsic tumour suppressors in some MDS patients (59,61,62). These molecules are not only toxic to developing leukaemic blasts but also destroy normal haematopoietic progenitors via antigen cross-reactivity or indirect mechanisms of cytokine-mediated suppression (63). The features of an 'effector' disease state in MDS are pancytopenia, low blast counts, dysplasia, and T-cell responses to leukaemia-associated antigens (63). Years of clinical evidences have shown that immunosuppressive therapy based on agents such as cyclosporine or anti-thymocyte globulin can effectively improve haematopoiesis in this highly selected subgroup of patients (61), which suggests the important roles of immunosuppression in the pathogenesis, development, and progression of MDS.

Tregs

Regulatory T cells (Tregs) play a crucial role in the immune surveillance for many malignancies (64) and exert their immunosuppressive functions via the secretion of immunosuppressive cytokines, such as interleukin-10 (IL-10) and transforming growth factor-β (TGF-β). High numbers of Tregs inhibit immune responses against the dysplastic clones, which facilitates clonal expansion and disease progression. Tregs are known to play a prominent immunosuppressive role in patients with *de novo* AML (65). Studies have confirmed the presence of increased numbers of Tregs in patients with HR-MDS (58). Compared to relatively low numbers of Tregs in LR-MDS, HR-MDS is associated with significantly high numbers of Tregs (66,67), which suggests the expansion of Tregs numbers during disease progression. Kordasti et al. (58) have suggested that the increasing numbers of Tregs, which is correlated with lower Th1/Th2 ratios, might be related with the increasing of the number of BM blasts and result in disease progression in HR-MDS.

Mailloux et al. (63) have recently investigated the phenotypic features of $Treg^{EM}$ ($CD3^+CD4^+FOXP3^+CD25^+CD127^{dim}CD27^-CD45RA^-$) in association with the progression of MDS in a retrospective study and found that the subset of MDS patients with increased percentages and absolute numbers of $Treg^{EM}$ cells had higher percentages of abnormal BM myeloblasts compared to the patients with normal Treg profiles or high numbers of other Treg subtypes, such as $Treg^{CM}$ ($CD3^+CD4^+FOXP3^+CD25^+CD127^{dim}CD27^+CD45RA^-$). These authors also found that the presence of high numbers of $Treg^{EM}$ cells was associated with significantly reduced OS in these patients. Isolated $Treg^{EM}$ cells have been shown to be significantly more suppressive than other Treg subtypes *in vitro*, and the presence of such highly suppressive $Treg^{EM}$ cells has been shown to be independent of the presence of other Treg subtypes and the established MDS risk factors in multivariate models (68), which suggests that the presence of $Treg^{EM}$ cells might serve as a prognostic indicator. Furthermore, some angiogenic factors, including vascular endothelial growth factor, are immunosuppressive and therefore probably contribute to the immunosuppressive environment in HR-MDS. These immune-suppressive effects might aid in the alleviation of the auto-immune symptoms observed in some MDS patients (69,70). Consequently, the aberrant immune responses are the result of MDS, and the disturbed immune response contributes in turn to the symptoms and progression of this disease.

Overall, the above-mentioned biological abnormalities provide up-to-date understanding of the molecular mechanisms of the progression of MDS to sAML and might shed light on the development of new treatment strategies. The following section will briefly introduce the current therapeutic approaches for MDS.

Current therapies for MDS

The last decade has seen many scientific and therapeutic advances that have culminated in the United States (US) Food and Drug Administration's (FDA) approval of three drugs for the treatment of haematopoietic malignancies: lenalidomide, azacitidine, and decitabine. Lenalidomide and azacitidine have been approved for patients with del(5q) and HR-MDS, respectively. However, each of the available treatment modalities has shortcomings, and the lack or loss of response after standard therapies is associated with dismal outcomes. Currently, both combination strategies and novel agents are under investigation in clinical trials to improve the outcomes.

Lenalidomide for Low- and Int-1 risk MDS

Lenalidomide, a second-generation immunomodulatory drug (IMiD), is known to have multiple biologic activities including the promotion of erythropoiesis, modulation of cytokine production, anti-angiogenic, anti-inflammatory, immunomodulating, a direct toxic effect on BM myelodysplastic clones (71,72,73), etc. Lenalidomide has been approved by the US FDA for the treatment of red blood cell transfusion-dependent (RBC-TD) LR-MDS patients with del(5q) either in isolation or in combination with other cytogenetic abnormalities. Lenalidomide results in high rates of erythroid transfusion independence (TI) in Low- and Int-1 risk del(5q) MDS, and has been shown to inhibit the growth of differentiating del(5q) erythroblasts but does not cytogenetically affect normal cells (74). Therefore, it is highly effective for patients with MDS with isolated del(5q) and is the mainstay therapy for this disorder (75,76). Lenalidomide has dramatic effects on restoring effective erythropoiesis

and inducing cytogenetic remission, which is accompanied by reductions in inflammatory cytokine generation and marrow microvessel density and improvements in primitive haematopoietic progenitor recovery. Multivariate analysis has revealed that a high baseline platelet count and a low karyotype complexity are associated with the achievement of a haematological response; however, complete cytogenetic responses are not observed in any mutated TP53 cases (77), which indicates that TP53 mutations are possibly resistant to lenalidomide. While lenalidomide has revolutionized the treatment of patients with MDS and del(5q), sequencing of the TP53 gene should be included in the study of these patients to identify single abnormalities or a complex karyotype before lenalidomide treatment (42).

Sánchez-García et al. (78) used a time-dependent multivariate methodology to analyse the influence of lenalidomide therapy on OS and AML progression in 215 patients with Low- or Int-1 risk and del(5q) MDS. The data of these authors have clearly shown that the response to lenalidomide (including achievement of RBC-TI or cytogenetic response) results in substantial clinical benefit for LR-MDS patients with del(5q) and that lenalidomide treatment does not appear to increase the AML risk in this population of patients. Kuendgen A et al. (79) have also reported that lenalidomide treatment does not increase AML progression risk but rather confers a possible survival benefit to RBC-TI patients with del(5q) Low- or Int-1 risk MDS. The time to AML progression has been found to be longer in patients who achieve RBC-TI after more than 8 weeks or exhibit any cytogenetic response versus the non-responders (80). A recent subset analysis from the MDS-004 study also supports the clinical benefits and acceptable safety profile of lenalidomide for RBC-TD patients with Low- or Int-1 risk MDS with isolated del(5q) (81). Therefore, lenalidomide is associated with improved OS and a reduced risk of AML progression in Low- or Int-1 risk MDS patients with del(5q), and it is not leukaemogenic per se. Lenalidomide also exhibits efficacy in sAML, multiple myeloma, chronic lymphocytic leukaemia, non-Hodgkin's lymphoma, and myelofibrosis (82). However, in HR-MDS and AML, the results of lenalidomide monotherapy are modest and thus mandate the use of combination therapy.

Azacitidine for Int-2 and HR-MDS

HR-MDS with del(5q) is characterized by rapid progression and poor survival. The majority of these patients are elderly and have co-morbidities, which therefore limits the use of intensive therapies. HR-MDS frequently progresses to sAML within months even in the presence of continuous therapy. Azacitidine, a DNA hypomethylating agent (HMA) with anti-neoplastic activity, was initially approved by the US FDA in 2004 for the treatment of all subtypes of MDS and was granted expanded approval in 2009 for the treatment of patients with HR-MDS. Azacitidine was approved in Japan in March 2011 and has become a standard drug of choice in the treatment of HR-MDS. In randomized clinical trials, azacitidine has been shown to reduce the risk of transformation to sAML, improve peripheral blood values, improve the quality of life, and provide a definite survival advantage compared to conventional care regimens for patients with IPSS of Int-2 or HR-MDS (83,84). The agent maintains a relatively safe toxicity profile even in older patients. A meta-analysis with 1,392 participants has indicated that, due to higher OS rates and better survival benefits, azacitidine is recommended as the first-line hypomethylating agent for MDS, particularly for elderly and high-risk patients (85). Currently, both intravenous and subcutaneous forms of azacitidine are approved for use in the US, and the oral form has been granted fast-track status by the FDA.

Azacitidine-lenalidomide combination

However, only half of the patients with HR-MDS can benefit from azacitidine, and azacitidine resistance eventually develops. While azacitidine prolongs survival in patients with HR-MDS by a median of 9.5 months, responses only occur in fewer than half of the patients, and azacitidine therapy is not curative; most patients relapse within 2 years. Therefore, strategies to improve the outcomes of these patients are needed. Clinical trials of histone deacetylase inhibitors, lenalidomide, thrombopoietin agonists, or anticancer drugs in combination with HMAs are ongoing. Azacitidine has been combined with lenalidomide in the hope of achieving improved outcomes. Early-phase trials of HR-MDS without del(5q) have suggested increased activity with a concurrent azacitidine-lenalidomide combination. Based on a review of the results of a phase I trial of a sequential azacitidine-lenalidomide combination approach in patients with HR-MDS and AML with del(5q), Zeidan et al. (86) indicated that the combination therapy improved outcomes in these patients. Two case reports suggested that azacitidine might be effective in lenalidomide-resistant or discontinued MDS patients with del(5q) and might also be effective in progressed patients (e.g. the progression of isolated del(5q) MDS to RAEB-2) after lenalidomide therapy, although these patients eventually progressed to sAML and died (87,88). However, Ueda et al. (89) remind us that azacitidine is indeed an important drug for the treatment of MDS, but the premature withdrawal of treatment might cause rapid disease progression.

Azacitidine for AML

The role of azacitidine has also been explored in the treatment of AML and studied in the peritransplant setting. AML patients over the age of 60 years tolerate standard induction chemotherapy poorly. Therapy with azacitidine at a dose of 75 mg/m^2/day for 7 days appears to be better tolerated and is approved by the US FDA for the treatment of elderly AML patients with BM blast counts of 20%–30%. Sadashiv et al. (90) have recently suggested that the administration of subcutaneous azacitidine 100 mg/m^2/day for 5 days every 28 days is a feasible, well-tolerated, and effective alternative to standard induction chemotherapy in elderly patients with AML. Additionally, azacitidine has been shown to be effective in a 71-year-old patient with CMML who harbours del(20q) (91).

A major limitation to improving the outcomes in AML is relapse resulting from leukaemic cells that persist at clinical remission. Tregs, which are increased in AML patients, can contribute to immune evasion by residual leukaemic cells. Tumour necrosis factor (TNF) can induce TNF receptor-2 (TNFR2) expression by Tregs. It has been hypothesized that because TNFR2 is required for Treg stabilization, and TNFR2$^+$ Tregs are potent suppressors, the targeting of TNFR2$^+$ Tregs might restore the effectiveness of immune-surveillance mechanisms. In a pilot study, Govindaraj et al. (92) have found that AML patients in clinical remission have substantially increased levels of TNFR2$^+$ T cells, including TNFR2$^+$ Tregs, and impaired effector CD4$^+$ T-cell function with reduced IL-2 and IFNγ production. These authors indicated that although treatment with lenalidomide and azacitidine increased cytokine production by effector T cells in all patients, durable clinical remissions might be observed in patients with concomitant reductions in TNFR2$^+$ T cells and TNFR2$^+$ Tregs. *In vitro* studies have further demonstrated that lenalidomide can reduce TNFR2 expression and augment effector cytokine production by T cells, which can be further enhanced by azacitidine (92). These results indicate that the reduction of TNFR2$^+$ T cells in the AML post-remission phase might result from combination dazaciti-

dine and lenalidomide therapy and might contribute to improved clinical outcomes. Aggarwal et al. (46) have already observed a moderate increase in FOXP3-expressing Tregs accompanied by a modest reduction in IFNc-producing cells in HR-MDS patients treated with azacitidine, while their *in vitro* studies have revealed an increase in IFNc production. Furthermore, IL-17 production by $CD4^+$ T-cells is significantly reduced in these patients (Bontkes HJ, Alhan C, Eeltink C, et al., unpublished observations). These results indicate that azacitidine treatment has inhibitory effects on the $CD4^+$ T-cell-mediated immune response, which might be detrimental in HR-MDS.

While azacitidine and lenalidomide both have meaningful single-agent clinical activity in MDS and are moderately successful in the treatment of AML patients, the combined use of HMAs with IMiDs and even with other novel agents is inevitable. Additionally, HMAs are being used as a bridging therapy prior to Allo-HSCT and as a salvage therapy for relapsed disease after Allo-HSCT, which suggests that HMAs will continue to be key drugs for the management of MDS. Accordingly, further efforts are necessary to achieve more sustained control of HR-MDS and AML.

Summary

The diversity of abnormalities, such as clonal evolution, genetic mutations, chromosomal abnormalities, epigenetic changes, and immune suppression, that are associated with leukaemogenesis in MDS suggests that many pathways are involved in the process of this disease and underscores the molecular complexity that underlies the development of this heterogeneous disease. Early detection and identification of these abnormalities are the prerequisites for the identification of novel therapeutic strategies that are capable of tackling the aggressive nature of leukaemia that arises from MDS. Therefore, future efforts are necessary to clarify the molecular mechanisms underlying MDS progression. It is hoped that improved understanding of the complex mechanisms will be translated into novel therapeutic approaches and better prognostic tools that will facilitate accurate risk-adaptive therapy.

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