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Evolution of myeloid leukemia in children with Down syndrome

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Abstract Children with Down syndrome (DS) have a markedly increased risk of leukemia. They are at particular risk of acute megakaryoblastic leukemia, known as myeloid leukemia associated with DS (ML–DS), the development of which is closely linked to a preceding temporary form of neonatal leukemia called transient abnormal myelopoiesis (TAM). Findings from recent clinical and laboratory studies suggest that constitutional trisomy 21 and GATA1 mutation(s) cause TAM, and that additional genetic alteration(s) including those in epigenetic regulators and signaling molecules are involved in the progression from TAM to ML–DS. Thus, this disease progression represents an important model of multi-step leukemogenesis. The present review focuses on the evolutionary process of TAM to ML–DS, and advances in the understanding of perturbed hematopoiesis in DS with respect to GATA1 mutation and recent findings, including cooperating genetic events, are discussed.

Keywords Myeloid leukemia · Down syndrome · Transient leukemia

Introduction

Down syndrome (DS) results from trisomy 21 and occurs in 1 in 700–1,000 births [1, 2]. Although the incidence of neoplasms in DS does not differ significantly from that in the general population, the distribution of malignancies is different [3]. Patients with DS show a unique spectrum of malignancies, with a 10- to 20-fold higher risk of acute leukemia and a lower incidence of solid tumors [3, 4]. Death from malignancies other than leukemia is strikingly less common in patients of all ages in DS, and death from leukemia is more likely in children younger than 10 years of age with DS than in those without [5]. The most frequent form of leukemia during childhood, both with and without DS, is acute lymphoblastic leukemia (ALL), and the incidence of ALL in children with DS is approximately 20-fold higher than in non-DS children [3]. However, the most marked increase in incidence in DS infants is with acute megakaryoblastic leukemia (AMKL), a myeloid malignancy of platelet precursors. The relative risk of developing AMKL is estimated to be 500 times higher in children with DS than in the general population [6]. Unlike AMKL in non-DS patients, most AMKL associated with DS (DS–AMKL) patients usually respond well to chemotherapy.

In most cases, DS–AMKL is preceded by a temporary form of megakaryoblastic leukemia unique to newborns with DS and known as transient abnormal myelopoiesis (TAM) or transient leukemia. TAM, a clonal myeloproliferative syndrome, presents in the fetus or a few days after birth and in most cases resolves spontaneously within 3 months [7, 8]. Typically, TAM is characterized by the presence of high numbers of circulating blast cells expressing CD33, CD38, CD117, CD34, CD7, CD56, CD36, CD71, and CD42b [9], which are indistinguishable from blasts observed in DS–AMKL. More often, the clinical presentation of TAM varies from asymptomatic alterations in the blood count to disseminated leukemic infiltration. Clinically severe TAM (liver failure, pleural/pericardial effusion, ascites, renal failure, and coagulopathy) affects 10–30 % of patients with clinically diagnosed TAM, and

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treatment could be required for the patient with these life-threatening symptoms. A small proportion of these TAM patients will die from their disease, usually due to liver failure/fibrosis [10, 11].

After spontaneous remission, 20 % of TAM patients develop myelodysplastic syndrome (MDS) and DS–AMKL within 4 years [6, 12]. These clinical findings have led many physicians to consider TAM as pre-leukemic and the progression of TAM to DS–AMKL as an attractive model to investigate multi-step leukemogenesis. MDS and DS–AMKL were given a special World Health Organization (WHO) sub-classification of "Myeloid leukemia associated with Down syndrome (ML–DS)" because of their unique clinical and biological features [13, 14]. Accordingly, in this paper, the term ML–DS will be used to refer to both entities (DS-related MDS and DS–AMKL).

Abnormal hematopoiesis in DS

Trisomy 21 can affect hematopoietic cell biology in multiple complex ways [15]. There are ~300 protein-encoding genes on human chromosome 21 (Hsa21). Some researchers investigated gene expression by human embryonic stem (ES) cells or induced pluripotent stem (iPS) cells derived from primary trisomy 21 samples, showing an intrinsic disturbance of multi-lineage myeloid hematopoiesis at specific stages [16, 17]. Some used a series of transgenic mouse models, including the Tc1 mouse, which contains a copy of most of Hsa21 [18], and other mice containing additional copies of all or part of mouse chromosomal regions corresponding to Hsa21 [19, 20]. These transgenic mouse models provided important insight into the function of a number of genes on chromosome 21 [18–20]. Tc1 mice have macrocytic anemia and increased extramedullary hematopoiesis [18]. Ts65Dn mice, which are trisomic for 104 orthologs of Hsa21 genes, display persistent macrocytosis and develop a myeloproliferative disease characterized by megakaryocyte hyperplasia and dysplasia, and myelofibrosis [19]. Several genes on Hsa21, such as *RUNX1*, *ETS2*, *ERG*, *GAPBA*, *BACH1*, and *DYRK1A*, encode proteins relating to hematopoiesis and leukemogenesis [9, 21–26]. Further analysis of the models may reveal the effect of chromosome 21 gene dosage on human hematopoiesis and hematological malignancies.

GATA1 mutation induces TAM in DS neonates

The GATA proteins are a family of transcription factors, three of which are expressed principally in hematopoietic cells (GATA1, GATA2, and GATA3) [27]. GATA1 is encoded on the X chromosome and is typically present in erythroid, megakaryocytic, mast, and eosinophilic lineage cells [28–31]. GATA2, encoded on chromosome 3, is crucial for the proliferation and survival of early hematopoietic cells, and as a partner of GATA1 is also involved in lineage-specific transcriptional regulation [32]. GATA3, encoded on chromosome 10, plays an essential role in T cell development and immune regulation [33].

GATA1 somatic mutations, first identified from a small series of ML–DS patients [34], are present in blast cells both in DS neonates with TAM and children with ML–DS [34–38]. *GATA1* mutations disappear when TAM/ML–DS enters the remission phase, indicating that these are acquired events [35]. Although it is not yet clear when in fetal development *GATA1* mutations arise, the earliest point at which mutations have been identified is 21 weeks of gestation [39].

In a recent UK prospective study, very sensitive next-generation sequencing (NGS), which allows the detection of mutant *GATA1* clones present at very low frequency (0.3 %), identified *GATA1* mutations in 30 % of all neonates with DS, at least half of which were 'silent' with no clinical features of TAM [40]. The reasons for the high frequency of *GATA1* mutations in neonates with DS are not known. Of note, none of the 70 neonates without NGS-detected *GATA1* mutations in this report developed ML–DS [40]. Accordingly, silent TAM is clinically important because it can subsequently give rise to ML–DS. The study found that 11 % of neonates with a *GATA1* mutation (including cases with clinical and silent TAM) went on to develop ML–DS [40]. Although a test for *GATA1* gene mutation has not yet been standardized in neonates with DS, genetic screening using NGS could be an important tool not only for the diagnosis of TAM, but also for detecting candidates with a potential risk of developing ML–DS.

GATA1 mutations, in the absence of trisomy 21, have not been associated with the development of leukemia. Specific hematopoietic alterations due to *GATA1* mutation alone include cytopenias [34], Diamond–Blackfan anemia [41], and, in the case of germline *GATA1* mutations, tri-lineage bone marrow dysplasia [42]. Given that *GATA1* mutations are not leukemogenic in the absence of constitutional trisomy 21 (cT21), cT21 and mutated *GATA1* are thought to be both necessary and sufficient for the generation of TAM [42]. However, clinical findings such as spontaneous remission in TAM suggest that mutation in *GATA1* is necessary, but insufficient for the development of ML–DS.

Originally, it was believed that the loss of the N-terminal transactivation domain (exon 1) reduces the activity of GATA1 in regulating the terminal differentiation of megakaryocytes, thereby leading to accumulation of poorly differentiated megakaryocytic progenitors [34]. However, most of the mutations are found in exon 2 of the *GATA1* gene, with a minority in exon 3 or at the intronic boundary

of exon 1 and 2, and lead to expression of N-terminally truncated GATA1 (GATA1s) protein [34, 43]. GATA1s lacks the N-terminal transactivation domain, but retains both DNA-binding zinc fingers [34]. The presence of GATA1s is thought to impair GATA1-mediated regulation of other transcription factors, including GATA2, MYB, MYC, and IKAROS family zinc finger 1 (IKZF1), in fetal megakaryocytes [44]. GATA1 knockdown mice accumulate immature erythroid progenitors [45], and when transgenically 'rescued' with GATA1s these mice have an abnormal accumulation of CD41+ megakaryocytic progenitors [46]. Furthermore, forced expression of GATA1s in fetal hematopoietic progenitors from mice bearing wild-type GATA1 causes marked expansion of megakaryoblastic progenitors [25]. Toki et al. [47] also revealed that GATA1s promotes megakaryocyte proliferation more profoundly than that induced by GATA1 deficiency, supporting a gain-of-function mechanism for GATA1s. Collectively, although both GATA1 dysfunction and GATA1s expression are important in the development of TAM, GATA1 dysfunction alone does not appear to be sufficient, and it may be overexpression of GATA1s that contributes to the development of DS-related myeloid disorders. Interestingly, although how GATA1s contributes to the TAM phenotype remains unclear, *GATA1* mutation type and GATA1s expression levels are significantly associated with a risk of progression to ML–DS [45, 48].

Genetic heterogeneity in TAM

Cancer arises through an evolutionary process of somatic mutation and selection [49]. This evolutionary process, clonal evolution, is associated with significant intratumoral heterogeneity [50]. Recently, new genomic technologies have led to a better understanding of the complex clonal architecture of leukemia [51–54].

TAM xenograft model

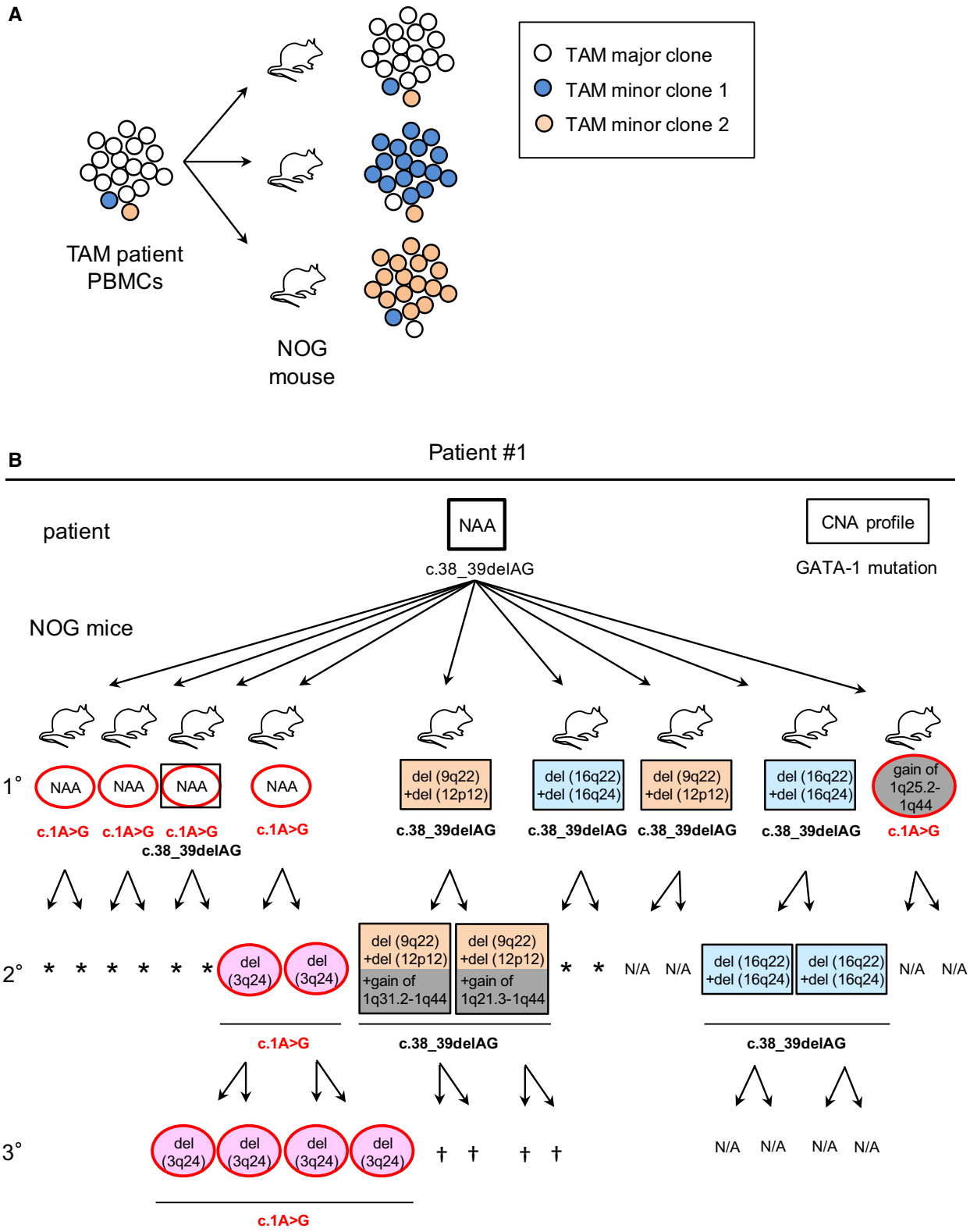
Mouse models in which primary human leukemic cells were transplanted into immunodeficient hosts provide significant clues to advance our understanding of the pathogenesis of human leukemia [51–53, 55]. However, xenograft models using primary patient samples from the pre-leukemic phase have been rarely reported. We hypothesize that TAM xenograft models would be an attractive method for investigating the evolutionary process of leukemia. In contrast to a previous study in which TAM cells showed a limited ability to expand in immunodeficient mice [56], we established a xenograft model of TAM using NOD/Shi-scid, IL-2Rγnull (NOG) mice, which mimicked the progression of TAM to DS–AMKL [57]. The presence of the same *GATA1* mutation was confirmed in the primary TAM cells and the engrafted cells in the NOG mice. These TAM-derived cells were morphologically similar to the primary TAM cells obtained from the patients. Flow cytometric analysis of surface antigens detected an expression pattern consistent with that observed in primary cells of TAM patients. Interestingly, in this model, the engrafted cells predominantly comprised a single genetically defined subclone of the TAM patient cells (Fig. 1a).

Genetically heterogeneous subclones with varying repopulating capacity expanded in the TAM xenograft model

Of engrafted TAM cells derived from three patients (#1, #2, and #9), only cells from patient #1 successfully engrafted into secondary as well as tertiary recipients, indicating that some TAM clones have long-term self-renewal capacity, a characteristic of leukemia, at least in xenografts. Interestingly, TAM patient #1 went on to develop ML–DS at the age of 1 year, 10 months after the TAM cell sample was taken, whereas the other patients did not. Compared with TAM, additional chromosomal alterations and copy number alterations (CNAs) are frequently observed in ML–DS [8, 58–60], and primary samples from TAM patient #1 had no CNAs other than the gain of chromosome 21 (Fig. 1b). However, serial transplantations demonstrated that TAM-derived cells from patient #1 acquired various CNAs, a hallmark of ML–DS, and showed diverse repopulating capacity in xenografts (Fig. 1b). Breakpoint-specific PCR analysis demonstrated that TAM cells with these CNAs were already present as a minor population in the original sample from patient #1. In addition, engrafted cells derived from this primary patient sample revealed the presence of another minor *GATA1* mutation that was not detected by conventional sequencing in the original cell sample (Fig. 1b). These findings suggest that pre-leukemic TAM is far more genetically heterogeneous than first suspected.

Clinical findings

Ahmed et al. [35] reported that multiple separate *GATA1* mutant clones could occur in an individual TAM patient. In addition, Xu et al. [61] reported that ML–DS can arise from a minor TAM clone with a *GATA1* mutation that is distinct from that of the patient's major TAM clone. Recently, whole exome/genome sequencing of paired TAM and ML–DS samples also indicated that ML–DS may develop not only from a major *GATA1* mutant subclone present in the TAM phase, but also from a minor mutant *GATA1* clone [62]. Furthermore, whole exome sequencing results



also suggest the presence of intratumoral heterogeneity in the majority of DS–AMKL cases [62]. Thus, recent new genomic and NGS technologies are together enabling the genetic heterogeneity of TAM and ML–DS to be unraveled.

Additional genomic events induce ML–DS

Estimates of the risk of ML–DS following TAM vary from ~5 % in the recent prospective study to 30 % in

◀ **Fig. 1** **a** Schema of experimental design for tracking primary TAM clones in xenografts. Peripheral blood mononuclear cells (PBMCs) were obtained from TAM patients and transplanted into irradiated NOG mice via the tail vein. TAM major clone *white circle*; TAM minor clone 1 *blue circle*; TAM minor clone 2 *orange circle*. **b** Summary of the serial transplantation of TAM cells of patient #1 who developed ML–DS at 1 year of age, and the results of CNA profiling and *GATA1* mutation analysis. The original patient sample had a single *GATA1* mutation, c.38G_39delAG, and no additional CNAs. Diverse subpopulations with or without additional CNAs expanded in each recipient. *GATA1* mutation analysis showed two distinct mutations in recipients, one identical to that of the original patient (c.38_39delAG) and a different mutation (c.1A>G). The mice harboring cells with the original mutation (c.38_39delAG) are depicted as *black rectangles*, and the mice with cells harboring the other mutation (c.1A>G) are depicted as *red ovals*; the CNA profile is given within the graphic. The *GATA1* mutation is indicated *below the symbol*. *NAA* no additional alteration, *N/A* not assessed due to low blast cell count, †death of recipient before analysis. *Asterisk* represents no engraftment. This research was originally published in Ref. [57]

retrospective studies of clinically diagnosed TAM [8, 11, 40], and analysis of paired samples from the same patient found the identical *GATA1* mutation in both pre-leukemic (TAM) and leukemic (ML–DS) stages [11, 37, 43]. Because only 5–30 % of TAM cases progress to ML–DS, additional genetic events besides *GATA1* mutation are suspected to be involved in the progression [63]. For example, recurrent additional cytogenetic abnormalities are commonly observed during disease progression [8, 43, 58, 59]. Classically reported, the development of ML–DS is significantly correlated with karyotypic abnormalities such as dup(1q), del(6q), del(7p), dup(7q), +8, +11, and del(16q), which are rarely observed in the TAM phase [8, 58, 59]. However, evidence for a role of chromosomal instability in DS malignancies is conflicting, and whether these chromosomal abnormalities cause the leukemic phenotype is unclear [64, 65].

On the other hand, many studies have elucidated the details of genomic changes during ML–DS progression. Initially, a few candidate tumor-associated genes were identified in the progression to ML–DS [66–68]. NGS studies of TAM/ML–DS now reveal a high frequency of mutations in cohesin, CCCTC-binding factor (CTCF), and other epigenetic regulators [62, 69]. While the normal function of these proteins is incompletely understood, roles for cohesin and CTCF are emerging as coordinators of long-range

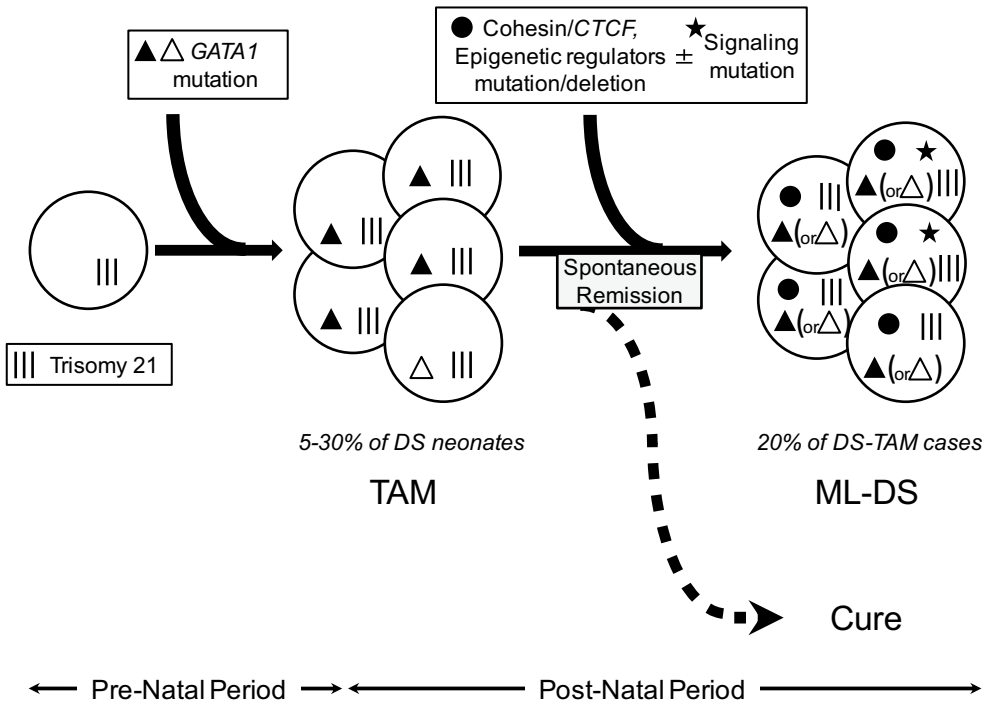


Fig. 2 The proposed model of ML–DS pathogenesis. In the setting of trisomy 21, an in utero truncating mutation in *GATA1* induces TAM in the neonatal period. At birth, multiple clones with different *GATA1* mutations (indicated by *filled triangles* and *open triangles*) might be present, whereas one *GATA1* mutant clone is predominant in most cases of overt TAM. Although TAM resolves spontaneously in most cases, residual cells acquire additional cooperating/driver mutations (cohesin, CTCF, other epigenetic regulators, and kinase-signaling molecules) leading to overt ML–DS within 4 years. ML–DS originates from one of the multiple *GATA1* mutant clones present in the TAM phase, usually representing the progeny of the largest (sometimes from the minor) subpopulation. Trisomy 21 *bars*; *GATA1* mutation *filled triangles* and *open triangles*; cohesin, CTCF, and other epigenetic regulator mutation *filled circles*; kinase-signaling molecule mutation *filled stars*. Figure adapted from Ref. [62]

interactions between genomic regulatory elements [70], and for epigenetic regulators, such as EZH2, in regulating various epigenetic events [71].

Mutations are also observed in members of signaling pathways, such as the JAK family of kinases, MPL, SH2B3, and multiple RAS pathway genes [62, 69]. The variant allele frequency (VAF) of mutations in cohesin components, CTCF, and EZH2 implies that their role is in the early stage of ML–DS development [62]. By contrast, the RAS pathway and other signaling mutations were more likely to represent subclonal mutations, which were typically preceded by mutations in cohesin components, CTCF, and EZH2 and were involved in the evolution of multiple DS–AMKL subclones [62]. Importantly, these genomic analyses of ML–DS confirm that it evolves from the cells responsible for TAM.

Conclusions and future directions

NGS-based studies unraveled the genomic landscape of DS-related myeloid disorders [72, 73]. ML–DS is shaped by multiple rounds of acquisition of new mutations and clonal selection (Fig. 2). In the setting of trisomy 21, *GATA1* mutation(s) causes TAM. Although most TAM resolves spontaneously, further mutation in cohesin, CTCF, EZH2, or other epigenetic regulators in residual TAM cells occurs with or without mutation of signal-transducing molecules, leading to ML–DS (Fig. 2). Recent studies reveal that genetic intratumoral heterogeneity was evident not only in the ML–DS, but also in the TAM phase [57, 62], and that ML–DS originated from one of the multiple subclones present in the TAM phase of the disease. NGS methodology also showed the precise frequency of *GATA1* mutation in DS [40] and may be used in the future to make diagnostic and treatment decisions in DS-related myeloid disorders.

The recent findings of mutations in epigenetic regulators in ML–DS suggest that epigenetics also play a role in the development of ML–DS. Indeed, Malinge et al. [74] showed that TAM and ML–DS are associated with sequential epigenetic changes. They showed that trisomy 21 led to global hypomethylation and that TAM samples featured new, focal gains of DNA methylation. However, the transcriptome and epigenome of TAM samples compared with ML–DS in their report were very similar [74]. A crucial role for mutations in epigenetic regulators in the evolutionary process of TAM to ML–DS remains unclear.

Furthermore, a number of key questions remain unanswered. Why are mutations in cohesin and CTCF especially common in ML–DS? How do these mutations contribute to the etiology of ML–DS and the relationship with *GATA1* mutation? Why does TAM resolve spontaneously? How do residual TAM cells survive? How can we predict and prevent the incidence of ML–DS in TAM patients? These questions will continue to drive investigations into this curious disorder.

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Compliance with ethical standards

Conflict of interest The author has no conflict of interest to declare.

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