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REVIEW

Heterogeneity of molecular markers in chronic myelomonocytic leukemia: a disease associated with several gene alterations

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Abstract The relatively homogenous clinical features and poor prognosis of chronic myelomonocytic leukemia (CMML) are associated with a molecular heterogeneity, with various mutations impacting several convergent pathways. Due to the restricted understanding of the mechanism involved in leukemogenesis, CMML still appears as a diagnostic and therapeutic undertaking, and poor prognosis of leukemia. Contrary to chronic myelogenous leukemia, *BCR–ABL1*-positive, cytogenetic, and molecular abnormalities of CMML are not specific and not pathognomonic, confirming the different levels of heterogeneity of this disease. Various mutations can be associated with a common phenotype not distinct at the clinical level, further demonstrating that molecular probings are needed for choosing individual targeted therapies.

Keywords Chronic myelomonocytic leukemia · Somatic mutations · Biomarkers · Heterogeneity · Mouse models

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Introduction

Myelodysplastic/myeloproliferative neoplasms (MDS/MPN) form an independent group in the WHO (World Health Organization) classification of malignant myeloid diseases. Since 2008, this group includes chronic myelomonocytic leukemia (CMML), juvenile myelomonocytic leukemia (JMML), atypical chronic myeloid leukemia *BCR–ABL1*-negative, unclassifiable MDS/MPN, and refractory anemia with ring sideroblasts and thrombocytosis (RARS-T). Chronic myelomonocytic leukemia, which is the more frequent of these rare diseases, is characterized by a wide heterogeneity of clinical presentation and courses. The recent identification of a variety of somatic gene mutations provides a new level of heterogeneity. The present article summarizes the present knowledge of genetic abnormalities in CMML cells and their prognostic significance.

Clinical features of CMML

Chronic myelomonocytic leukemia is a rare malignancy with an estimated incidence of <1 case per 100,000 persons per year. The median age at diagnosis is approximately 70 years, with a male predominance of 1.5–3:1. In the majority of patients, the white blood cell (WBC) count is increased at the time of diagnosis, and the disease appears as an atypical MPN. In other patients, the WBC is normal or slightly decreased with variable level of neutropenia and the disease resembles MDS. The main symptoms at the presentation of the disease correspond to fatigue, weight loss, fever, and night sweats. Although splenomegaly and hepatomegaly may be found, they are more frequent in patients with leukocytosis.

The diagnostic criteria for CMML according to WHO classification include persistent peripheral blood monocytosis $>1 \times 10^9$/l, lack of Philadelphia chromosome or *BCR–ABL1* fusion gene, lack of rearrangement of *PDGFRA* or *PDGFRB* (should be specifically excluded in cases with eosinophilia), a blood and bone marrow blast count lower than 20%, and dysplasia in one or more myeloid lineages. If dysplasia is absent or minimal, the diagnosis of CMML may still be made if the other requirements are met, and associated with an acquired, clonal cytogenetic or molecular genetic abnormality present in the hemopoietic cells, or if the monocytosis has persisted for at least 3 months and all other causes of monocytosis have been excluded.

Chronic myelomonocytic leukemia is further subdivided into two subsets, CMML-1 and CMML-2, depending on the number of blast cells plus promonocytes in the peripheral blood (PB) and bone marrow (BM).

CMML-1: blasts are less than 5% in peripheral blood and less than 10% in bone marrow. CMML-2: blasts represent 5–19% in peripheral blood and 10–19% in bone marrow, or Auer rods are present and blasts are less than 20% in peripheral blood or bone marrow.

Prognosis and predictive factors in CMML

Survival of patients with CMML is reported to vary from one to more than 100 months, but the median survival time in most series is 20–40 months. Progression to acute myeloid leukemia (AML) occurs in approximately 15–30% of cases. A number of clinical and hematological parameters, including splenomegaly, severity of anemia, and degree of leukocytosis, have been reported to be important factors in predicting the course of the disease [1]. Nevertheless, the percentage of PB and BM blasts is the most important factor in determining survival. Factors predicting the course of disease are poorly understood and, thus far, rely on clinical parameters, such as anemia, splenomegaly, or leukocytosis.

Cytogenetic alterations associated with CMML

Contrary to chronic myelogenous leukemia (CML), CMML is not associated with a specific cytogenetic or molecular abnormality, which contributes to the disease heterogeneity. Clonal cytogenetic abnormalities are found in 20–40% of patients. The most frequent recurring abnormalities include +8, −7/del(7q) and structural abnormalities of 12p. Complex caryotypes are rare and the frequency of reciprocal translocations is exceptional. A recent survey of 414 CMML patients at diagnosis found abnormal caryotype in 27% of the patients. Multivariate analysis of survival and progression to AML allowed three cytogenetic risk categories to be identified: (1) low-risk (normal karyotype or loss of Y chromosome as a single anomaly) (median survival 37 months); (2) high-risk (presence of trisomy 8 or abnormalities of chromosome 7 or complex karyotype) (median survival 11 months); and (3) intermediate risk (all other abnormalities) (median survival 18 months) [2]. Uniparental disomy (UPD) is the presence of a chromosome pair derived only from one parent present in a disomic cell line [3]. Somatic UPD were observed in 48% of CMML patients [4]. In these cases, various homozygous mutations were associated with regions of UPD [4, 5].

Molecular mutations

A number of somatic gene mutations identified in other myeloid malignancies were investigated in CMML. A recent study analyzed 81 characterized patients with CMML (45 CMML type 1; 36 CMML type 2) by applying next-generation sequencing (NGS) technology to study *CBL*, *JAK2*, *MPL*, *NRAS*, and *KRAS* at known hotspot regions. At least one molecular mutation was observed in 72.8% of patients (59 of 81 patients) [6]. However, although the occurrence of gene alterations started to be identified, their respective role in leukemogenesis or the clonal progression of the tumoral pathology remain to be elucidate, especially as most of abnormalities described are not specific of CMML.

The frequency of somatic mutations leads to a classification in three groups:

- Frequent mutations (30–50%) include somatic mutations of *TET2* (*tet* oncogene family member 2) [7], *RUNX1* [8, 9], *ASXL1* (additional sex combs like 1) [10], and *SRSF2* (serine/arginine-rich splicing factor 2, also known as *SC35*) [11].
- Aberrations with an intermediate frequency (10–30%) include mutually exclusive mutations of *RAS* and *CBL* [8, 12].
- Abnormalities with a rare frequency (<10%) are found in *JAK2* [13], *FLT3* [14], and genes involved in Notch signaling [15].

The complex combination of these various abnormalities makes difficult the distinction between the oncogenic initial mutation and secondary mutations responsible for the clone evolution. Another way to distinguish these abnormalities corresponds to gene functions: *RUNX1*, *ASXL1*, *UTX*, *EZH2*, *DNMT3A*, and *TET2* regulate transcription and chromatin conformation, while *RAS*, *CBL*, *JAK2*, and *FLT3* play a role in cytokine receptors signaling.

A few studies investigated the frequency and prognostic impact of these mutations in CMML [6, 9, 10]. These different alterations are not considered as formally specified markers of prognostic subsets or as responses to

therapies, e.g., demethylating agents. Some of these novel markers seem to be over-represented in CMML, e.g., the *TET2* and the *CBL* mutations [6] as compared to other myeloid malignancies.

Mutations in genes affecting transcription and epigenetics

TET2 *TET2* (Ten-eleven translocation 2) is one of three homologous human proteins (i.e., TET1, TET2, and TET3) catalyzing conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine, and thus impacting the epigenetic regulation of transcription. Human *TET2* is located on chromosome 4q24, a breakpoint that is also found in other AML-associated translocations, including t(3;4)(q26;q24), t(4;5)(q24;p16), t(4;7)(q24;q21), and del(4)(q23q24) [16]. Ten-eleven translocation 2 has multiple isoforms and isoform A, which includes 12 exons, is affected by most of the *TET2* mutations described so far. The higher frequencies of *TET2* mutations or deletions in myeloid malignancies have been observed in CMML [5, 7, 17–21]. Ten-eleven translocation 2 mutations have limited impact on survival and do not appear to predict the clinical outcome upon decitabine therapy [7, 22].

IDH *IDH1* (Isocitrate dehydrogenase), located on chromosome 2q33.3, and *IDH2*, located on chromosome 15q26.1, encode for isocitrate dehydrogenase 1 and 2, respectively, which are enzymes catalyzing oxidative decarboxylation of isocitrate to α-ketoglutarate, generating NADPH from NADP+. As *TET2*, *IDH1/2* mutations may provoke a decrease in 5mC hydroxylation. Isocitrate dehydrogenase 1/2 mutations were identified in 1–4% of CMML patients [20, 23] and are exclusive of *TET2* mutations [24].

ASXL1 *ASXL1* (Additional sex combs like 1) is located on chromosome 20q11.1 and is part of the enhancer of trithorax and Polycomb gene family. Its function seems to correspond to transcription factor modulation through epigenetic regulation, probably through interaction with Polycomb regulatory complex 2 (PRC2). Mutations have been reported in 30–45% of CMML patients [10, 20, 25]. The worse prognosis and acute transformation associated with *ASXL1* mutations in some CMML series remains controversial [20, 26].

UTX *UTX* (Ubiquitously transcribed tetratricopeptide repeat) located on chromosome Xp11.2, which encodes for a demethylase specific for H3K27, has been found mutated in myeloid malignancies [27, 28]. Ubiquitously transcribed tetratricopeptide repeat mutations identified in CMML are mainly present in the region adjacent to the JmjC domain required for UTX activity [23]. Ubiquitously transcribed tetratricopeptide repeat mutations are less frequent than mutations in *TET2* or *ASXL1* and in some cases were simultaneously present. Ubiquitously transcribed tetratricopeptide repeat mutations were identified in more aggressive forms of CMML and AML derived from CMML [23]. Therefore, inactivation of *UTX* in hematopoietic stem cells should induce the maintenance of H3K27me3 transcription repressive marks on specific genes and should alter differentiation.

EZH2 *EZH2* located on chromosome 7q35–q36, encodes for a histone H3 lysine 27 (H3K27) methyltransferase. Enhancer of zeste 2 was recently found mutated in CMML [17, 19]. Enhancer of zeste 2 mutations were found in 11–13% of CMML [19, 20]. Enhancer of zeste 2 mutations affect DNA methylation likely because EZH2 interacts with DNA methyltransferases 1, 2, and 3 [29]. These mutations are associated with poor survival. Indeed, according to the clinical data, a poor outcome was observed for these patients [19, 20].

DNMT3A *DNMT1* (DNA (cytosine-5)-methyltransferase 1), *DNMT3A* (DNA (cytosine-5)-methyltransferase 3) located on chromosome 2p23, and *DNMT3B* encode DNA methyltransferases catalyzing the addition of a methyl group to the cytosine residue of CpG dinucleotides. Clusters of CpG dinucleotides (CpG islands) are found in regions located upstream of genes. An increased methylation of CpG islands is usually correlated to a reduced gene expression. DNA (cytosine-5)-methyltransferase 3A mutations are highly frequent in patients with de novo AML with an intermediate-risk cytogenetic profile and are independently associated with a poor outcome [30]. On the basis of the recent discovery of *DNMT3A* mutations, their presence was identified in 10% of CMML patients [23]. The occurrence of *DNMT3A* mutations was increased in patients with normal karyotype [23].

RUNX1 *RUNX1* (Runt-related transcription factor 1 gene), located on chromosome 21q22, encodes a subunit of a DNA core-binding factor that is a regulatory transcription factor essential for normal hematopoiesis [31]. Runt-related transcription factor 1 somatic mutations are highly frequent in human MDS [32]. Runt-related transcription factor 1 alterations (mutations and cryptic rearrangement) were also identified in 8–38% of CMML patients [8, 20].

Mutations affecting cell signaling

RAS The signaling kinase RAS (Rat sarcoma) oncogene has been found mutated in a high number of malignancies comprising MDS. It was established that 10–15% of MDS patients harbor *RAS* mutations, usually codon 12 *NRAS*

mutations [33]. In CMML, a mutation rate of 22 and 12% has been observed in NRAS and KRAS, respectively [19, 20] while a burden of 30.8% in N/KRAS has been measured [6]. In the future, CMML patients having RAS pathway mutations may take advantage of pharmacological molecules targeting the RAS/RAF/MAPK pathway [34, 35].

CBL *CBL* (Casitas B-lineage lymphoma), located at 11q23.3, encodes for a cytosolic protein harboring two roles, i.e., a negative regulator of kinase signaling mediated by E3 ubiquitin ligase activity, and an adaptor protein with a positive effect on downstream signaling [36]. Casitas B-lineage lymphoma catalyzes the ubiquitination of FLT3, KIT, and MPL [37, 38]. Casitas B-lineage lymphoma mutations in myeloid malignancies are mainly associated with 11q acquired uniparental disomy [39]. Further studies have demonstrated that *CBL* mutations were most often associated with JMML and CMML (5–22%) [20, 39–43]. Casitas B-lineage lymphoma mutations associated with JMML and CMML usually correspond to missense substitutions or in-frame deletions. In addition, mutant CBL is co-expressed with *TP53*, *JAK2*, *FLT3*, and *RUNX1* mutants [39, 41].

JAK2 *JAK2* (Janus kinase 2) located on chromosome 9p24, belongs to the JAK family (Janus family non-receptor protein tyrosine kinases), which comprises four kinases (JAK1, 2, and 3 and TYK2) that attach to cytokine receptor cytosolic domains. Janus kinase 2 has a crucial function in the signaling pathways coming from "myeloid" cytokine receptors [44]. The *JAK2*V617F mutation impacts the non-catalytic "pseudo-kinase" domain and disrupts its kinase-regulatory function. In addition, *JAK2*V617F acts on epigenetic regulation through nuclear translocation by directing histone H3 phosphorylation [45]. This mutation has been found in the majority of BCR–ABL-negative MPNs (95% of patients with PV, 50–70% with ET, and 40–50% with PMF), as well as in some cases of atypical MPN (30–50% splanchnic vein thrombosis and sideroblastic anemia associated with a thrombocytosis) [46]. Finally, it was also identified *JAK2*V617F in 1–10% of CMML patients [20, 23].

FLT3 *FLT3* (Fms-like tyrosine kinase 3), located on chromosome 13q12, is often mutated in AML, and contributes to a damaging prognosis, most likely by triggering a proliferative advantage to the leukemic cells [47]. Fms-like tyrosine kinase 3 mutations have also been rarely described in MDS. The majority of *FLT3* mutations identified in MDS corresponds to internal tandem duplication (ITD) events in high-risk MDS patients, and could be one of the genetic alterations leading to their transformation into AML [48]. Furthermore, FLT3-ITD knock-in generates a CMML-like phenotype in mice and 3.1% of CMML patients have been found positive for FLT3-ITD [14].

NOTCH Notch signaling is an essential modulator of differentiation in tissue and cell types. Its activity is regulated by the multi-subunit γ-secretase (γSE) complex [49]. This signaling was already shown to have both oncogenic and tumor-suppressor functions in solid tumors and in T cell acute lymphoblastic leukemia (T-ALL), a leukemia characterized by Notch1 activating mutations [50]. Recently, 12% of CMML patients have been found to harbor somatic heterozygous mutations in multiple notch pathway genes including *NCSTN*, *APH1*, *MAML1*, and *NOTCH2* [15]. Interestingly, CMML patients with *notch* mutations also had somatic alterations in *JAK2*, *KRAS*, *TET2*, and *ASXL1*, suggesting molecular cooperation between notch signaling and other oncogenic pathways in CMML. Activation of Notch signaling using peptides or specific antibodies will be certainly investigated in the near future.

Other gene mutations

NPM1 *NPM1* (Nucleophosmin 1), located on chromosome 5q35.1, encodes for a nucleolar shuttling protein. This protein is localized primarily in the nucleolus, but shuttles rapidly between the nucleus and cytoplasm. Nucleophosmin has been shown to have a crucial role in a high number of cellular processes. C-terminal somatic mutations in *NPM1* were described in 35% of karyotypically normal AML [51]. Nucleophosmin 1 mutations are rare in chronic myelogenous diseases. In CMML, 1–5% of patients have been found positive for mutated *NPM1* [20, 52]. When present, they may forewarn about rapid progression to AML and likely a poorer prognosis.

Mutations of splicing components

It was recently reported that genetic alterations of the major splicing components could be involved in myeloid neoplasms with features of myelodysplasia [11]. The splicing machinery components were mutated in 16 out of 29 cases (55.2 %) of MDS in a mutually exclusive manner. This novel pathway of mutations involves multiple components of the RNA splicing machinery, i.e., SF3B1, SRSF2, U2AF35, and ZRSR2, and to a lesser extent, SF3A1, SF1, U2AF65, and PRPF40B. Mutations of the splicing machinery were highly specific to MDS either with (84.9%) or without (43.9%) increased ring sideroblasts, CMML (54.5%), and therapy-related AML or AML

with myelodysplasia-related changes (25.8%), but were rare in de novo AML (6.6%) and MPN (9.4%). *SRSF2* (serine/arginine-rich splicing factor 2, also known as *SC35*) mutations were more frequent in CMML cases [11]. Serine/arginine-rich splicing factor 2 is also involved in the regulation of DNA stability and depletion of SRSF2 can lead to genomic instability [53]. This very frequent new mutation (47%) is characterized by higher age, higher hemoglobin levels, and a high coincidence with *TET2* and *RUNX1* mutations (Schnittger S, ASH congress, 2011). Moreover, it is mutually exclusive of *EZH2* mutations. Finally, in the subset of *RUNX1*-mutated CMML, *SRSF2* mutations showed a favorable impact on outcome (Schnittger S, ASH congress, 2011).

Gene downregulation through promoter hypermethylation

TIF1γ

TIF1γ (Transcription intermediary factor 1 gamma, also called *TRIM33*) located at 1p13.1, encodes for an E3 ubiquitin ligase, as CBL, which belongs to the TRIM (tripartite motif) family. Four TIF1 members (α–δ) have been identified in mammals, and orthologs are present in organisms such as *Drosophila* [54–64].

Mutations in the zebrafish *mon* (*tif1γ*) gene cause a disruption in both primitive embryonic and definitive adult hematopoiesis, resulting in a severe loss of erythroid cells [65]. In zebrafish and human stem/progenitor CD34+ cells, TIF1γ functionally links positive elongation factors to blood-specific transcription complexes to regulate the erythroid commitment [66]. Recently, we and others have identified *TIF1γ* as a tumor suppressor in murine hematopoietic cells [67, 68], mimicking the essential features of human CMML [67]. This finding prompted us to investigate TIF1γ expression in CMML patients. Transcriptional Intermediary Factor 1γ level was very low and almost undetectable in leukemic cells of 35% of patients. We have shown that TIF1γ decreased expression is not due to gene mutation but to the gene promoter hypermethylation [67].

The demethylating agent decitabine induces a clinical and a biological response in about 30% of high-grade CMML [69]. We demonstrated that the gene expression increases in peripheral blood monocytes of patients who respond to decitabine, which was confirmed in leukemic cells cultured ex vivo in presence of decitabine. Hence, our data identify *TIF1γ* as an epigenetically regulated tumor suppressor gene in hematopoietic cells, and suggest that changes in *TIF1γ* expression may be a biomarker of response to demethylating agents in CMML.

Mouse models of CMML

Tif1γ

As mentioned above, two mouse models for *Tif1γ* were generated so far. Loss of *Tif1γ* leads to severe defects in hematopoiesis from the HSC compartment to myelomonocytic lineages. Indeed, the effects of hematopoietic tissue-targeted deletion of *Tif1γ* in mice (Mx-Cre and *cFES*-Cre mouse models) were examined [67, 68]. Transcription intermediary factor 1 gamma deletion affects the transition from very primitive progenitors (i.e., LT-HSCs population) to common myeloid progenitors, and leads to a selective expansion of granulo-monocytic progenitors [67, 68]. At older age (>6 months), the phenotype recapitulates the human CMML [67].

Cbl

Casitas B-lineage Lymphoma knockout mice present an expanded hematopoietic stem cell population, splenomegaly, and increased cytokine sensitivity of hematopoietic progenitor cells [39]. In addition, primary murine bone marrow retrovirally transduced with c-Cbl mutants and transplanted into mice led to a generalized mastocytosis, a myeloproliferative disease, and myeloid leukemia [37].

Notch

To investigate hematopoiesis in the absence of any notch-derived signal, Nicastrin (*Ncstn*), a member of the γSE complex and one of the few non-redundant members of the pathway has been targeted. Nicastrin$^{f/f}$ mice were crossed to both an inducible (Mx1-cre) and a hematopoietic-specific (Vav-cre) recombinase strain [15]. Both models developed a myeloproliferative/myelodysplastic disease resembling human CMML.

Ras

By using an improved mouse bone marrow transduction and transplantation model, it was demonstrated that oncogenic Nras induced CMML- or AML-like disease in mice [70]. Interestingly, palmitoylation as well as farnesylation are essential for leukemogenesis by oncogenic Nras in this model [71]. A mouse bone marrow transplantation model harboring an oncogenic G12D mutation in the *Nras* locus was also generated [72]. Around 95% of recipient mice developed a myeloproliferative disease resembling the myeloproliferative variant of CMML, with a prolonged latency and acquisition of multiple genetic alterations, including uniparental disomy of oncogenic Nras allele.

Flt3

A mouse model harboring an ITD in the murine *Flt3* locus has been generated [14]. These mutant mice displayed a myeloproliferative disease mimicking CMML. These mice harbored an increase number of multipotent stem and progenitor cells in an ITD dose-dependent manner and exhibited alterations within their myeloid progenitor compartments and a block in normal B cell development.

Development of novel therapies in CMML

Age and co-morbidity participate to the therapeutic decision. Before the area of the epigenetic therapy, hydroxyurea was the therapy most used and other chemotherapeutic approaches, including cytarabine and etoposide, were not better [73]. Allogenic stem cell transplantation, which is the only curative therapy, can be considered only in younger patients (<55 years) with a matched donor as transplant-related mortality increases with age. The first studies of efficiency of demethylating agents (i.e., azacitidine and decitabine) in CMML therapies came from investigations of MDS patients [74, 75]. Other clinical investigations confirmed these results [22, 76–78]. Azacitidine is a US Food and Drug Administration (FDA)- and European Medicines Agency-approved agent for the treatment of CMML-2, whereas decitabine is only FDA-approved. A phase 2 trial of decitabine in CMML patients with characteristics of advanced disease was performed [22]. Biological parameters predicting drug efficacy were examined. Overall response rate was 38%. With a median follow-up of 23 months, overall survival was 48% at 2 years. Mutations in *ASXL1*, *TET2*, *RUNX1*, *NRAS*, *KRAS*, *CBL*, *FLT3*, and *JAK2* genes, and hypermethylation of the promoter of *TIF1γ*, did not presage effect or survival on decitabine therapy. In contrast, low expression levels of cJUN and cMYB predicted improved overall survival.

The association of DNA methyltransferase and histone deacetylase inhibition seems to be promising in the treatment of myelodysplastic syndrome or acute myeloid leukemia. Indeed, molecular mechanisms responsible for responses to DNA methyltransferase/histone deacetylase inhibitor combinations may include reversal of aberrant epigenetic gene silencing [79]. Other interesting therapies to be studied consist of take in azacitidine and thalidomide as shown in MDS and AML [80], or azacitidine and farnesyl transferase inhibitors [81].

Contrary to CML, in which a single molecular defect was observed (BCR–ABL), leading to the targeting of one type of small molecule such as tyrosine kinase inhibitors, the various gene alterations found in CMML seem to not be correlated with a homogeneous phenotype at the clinical level, further prompting to develop molecular diagnostics for decision-making of targeted therapeutics for each patient.

Conclusions

Although clonal cytogenetic abnormalities have been usually associated so far with CMML, molecular alterations correlating with these cytogenetic abnormalities were recently evidenced. The role of each gene deficiency in disease occurrence and progression is not characterized yet. The main challenge for the next years is to determine how these molecular alterations (mutations or gene promoter hypermethylation) may be directly responsible either in the development, progression of CMML, or in the evolution of CMML to AML.

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