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Reviews in hematologic malignancies

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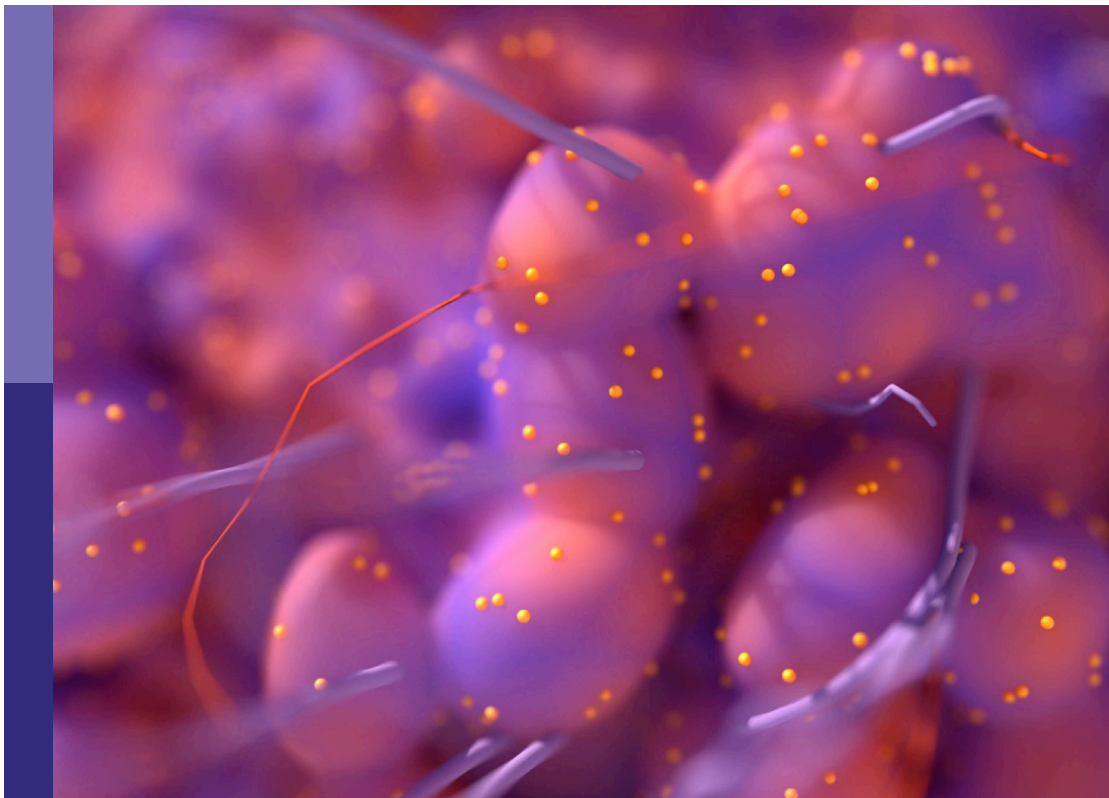

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Reviews in hematologic malignancies

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Editorial: Reviews in hematologic malignancies

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Editorial on the Research Topic
Reviews in hematologic malignancies

Hematological malignancies are a group of heterogeneous diseases that have always formed the basis of a very active research field in terms of diagnostic approaches, prognostic stratification models, and therapeutic weapons, aiming to achieve personalization of care to the individual patient. Major steps forward have been taken in terms of the study of tumor cells, but increasing attention has been paid in recent years to the tumor microenvironment.

In this collection, titled "Reviews in hematologic malignancies," three out of the nine reviews pertain to biological insights into hematological malignancies, treating such current and important themes as tumor-associated macrophages (TAMs) and alterations in calcium homeostasis, and highlighting the importance of a biological–metabolic characterization of hemopathies. In particular, Xie et al. describe the novel role of TAMs in the proliferation of malignancies, not only oncological but also hematological. The authors differentiate two phenotypes: M2 TAMs have a low antigen-presenting capability and are involved in angiogenesis, tumor cell invasion, resistance to therapy, and release of anti-inflammatory molecules; in contrast, M1 TAMs may provoke a Th-1 response and secrete pro-inflammatory molecules. The authors describe the clinical–biological implications of their findings in relation to several hemopathies, also proposing potential future therapeutic targets. Immanuel et al. focus on metabolomics, metabolic dysregulation having also emerged in recent years as a hallmark of several hematologic malignancies and a contributor to tumor initiation, progression, metastasis, and drug resistance. The authors demonstrate, using data collected from databases on lymphoid and myeloid malignancies, that calcium could be considered to be a second messenger controlling a wide range of cellular functions, highlighting for the first time the importance of alterations to calcium homeostasis in hematological malignancies and their genetic basis, and thus paving the way for future avenues of research into new metabolic therapeutic pathways.

Moreover, the reviews presented in this Research Topic address lymphoproliferative and myeloproliferative diseases separately, covering current hot topics in each case. Firstly, Mendeville et al. propose a genomic classification of diffuse large B cell

lymphomas (DLBCL), outlining a plan of work toward the construction of this genomic classification, harmonization of each classification with others, and translation of this harmonization into clinical practice in order to improve diagnosis and therapy decisions. Recognizing the role of the deregulation of F-box and WD-repeat domain-containing protein 7 (FBW7) as a key component of UPS proteins and a critical tumor suppressor in human cancers, Wan et al. separately examine its role in various hematological malignancies. In particular, they note that FBW7 mutations or inactivation are described as drivers of chemoresistance and poor prognosis, but clinical trials are needed to confirm these data and develop novel therapeutic strategies against them.

With regard to multiple myeloma (MM), there is currently an unmet medical need for improved identification of high-risk cases and the tailoring of effective treatments for these, considering that high-risk patients have been demonstrated to have poorer outcomes than the MM population as a whole. Among the clinical features of high-risk disease, renal failure is one of the most challenging in clinical practice. Xing et al. highlight the available data on the role of HCO hemodialysis, whose role is still controversial. Data from observational or randomized trials demonstrate that its use could reduce free light chains on serum plasma, but may not significantly improve outcomes. There seems to be a trend toward more positive renal outcomes, but data from randomized trials are needed to demonstrate this and to prove the existence of a tangible and significant advantage.

With regard to acute myeloid leukemia (AML), the authors of several reviews tackle specific areas of interest with the objective of improving customization of therapeutic programs for individual patients and widening the applicability of personalized medicine. Wang et al. focus on nucleophosmin (NPM1), which is the most commonly mutated gene in adult AML, presenting either alone or along with other mutations. The authors describe the results of multiple trials employing different therapies in this context, highlighting the future role of association therapy in NPM1-mutated AML and the key role of its use in MRD assessment. The observation that high CD38 expression occurs in AML blasts without an obvious correlation with cytomorphological and genetic characteristics, and that targeting CD38 has demonstrated efficacy and interesting power in modulation of immune surveillance, paves the way for ongoing trials employing anti-CD38 treatments in AML. In particular, Zhong and Ma describe the exploration of novel anti-CD38-based therapies in clinical trials for acute leukemias, especially daratumumab in T-ALL. Visani et al. clearly sum up the therapeutic landscape of optimal treatments for younger AML patients, from intensive chemotherapy to targeted therapy, attesting to major improvements in survival and steps toward a curative strategy in this setting.

Finally, there is also movement toward a genomic classification of myeloproliferative neoplasms (MPNs). Rapid advancements have been made in gene sequencing technology over the last decade. Li et al. tackle 8p11 myeloproliferative syndrome (EMS), which is an aggressive hematological neoplasm occurring with or without eosinophilia and caused by a rearrangement of the FGFR1 gene at 8p11-12. The authors describe in detail the genomic mechanisms and classifications of this rare entity, focusing their attention on the role of TKI in this hematological malignancy. However, data from clinical trials involving targeting of FGFR1 will be needed in the future to develop improved prognostication and ways to therapeutically attack EMS.

In conclusion, many findings have emerged from biological studies of hematological malignancies over recent decades, and these findings are being successfully translated into clinical practice, enabling more precise diagnosis and improved outcomes for various hemopathies.

Author contributions

FS and SM wrote the manuscript draft and reviewed the final manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Role and Mechanisms of Tumor-Associated Macrophages in Hematological Malignancies

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Mounting evidence has revealed that many nontumor cells in the tumor microenvironment, such as fibroblasts, endothelial cells, mesenchymal stem cells, and leukocytes, are strongly involved in tumor progression. In hematological malignancies, tumor-associated macrophages (TAMs) are considered to be an important component that promotes tumor growth and can be polarized into different phenotypes with protumor or antitumor roles. This Review emphasizes research related to the role and mechanisms of TAMs in hematological malignancies. TAMs lead to poor prognosis by influencing tumor progression at the molecular level, including nurturing cancer stem cells and laying the foundation for metastasis. Although detailed molecular mechanisms have not been clarified, TAMs may be a new therapeutic target in hematological disease treatment.

Keywords: macrophage, lymphoma, myeloma, leukemia, prognosis

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INTRODUCTION

Macrophages are important cellular components of the innate immune system that originate from bone marrow (BM) precursors. Plasticity and diversity are traits of the monocyte-macrophage differentiation pathway. The level of macrophage activation in different locations and at different times indicates the polarization of macrophages. Macrophages are usually polarized into the M1 or M2 type, and these types have different functional characteristics and different abilities to induce T helper cell (Th1 or Th2) responses (1, 2). M1 macrophages are found in settings dominated by Toll-like receptor (TLR) and interferon signaling. M2 macrophages arise in immunity *via* Th2 responses. Both of these macrophage types can indicate the current inflammation and trauma repair statuses.

Recent studies have shown that a group of cells derived from bone marrow called tumor-associated macrophages (TAMs) is recruited to tumors and enhance tumor hypoxia and aerobic glycolysis in solid tumors (3). In particular, some tumor-derived molecules, such as CSF-1 and IL-10, stimulate a considerable proportion of TAMs to differentiate into M2 macrophages (4, 5). Several studies have shown that most kinds of cancer linked to TAMs have poor progression and prognosis (6). M1 type, triggered by GM-CSF, IFN-γ, and LPS, could release pro-inflammatory molecules, such as TNF-α, NO, CXCL9, CXCL10, CXCL11, IL-1, IL-6, IL-12, IL-23; Conversely, M2 phenotype can be activated by M-CSF, TGF-β, IL-4, IL-10, IL-13, which leads to the high secretion of ant nflammatory molecules, such as CCL17, CCL18, CCL22, TGF-β, IL-10 (**Figure 1**) (7–10). In hematological malignancies, like myeloma, lymphoma, leukemia, and other malignancies,

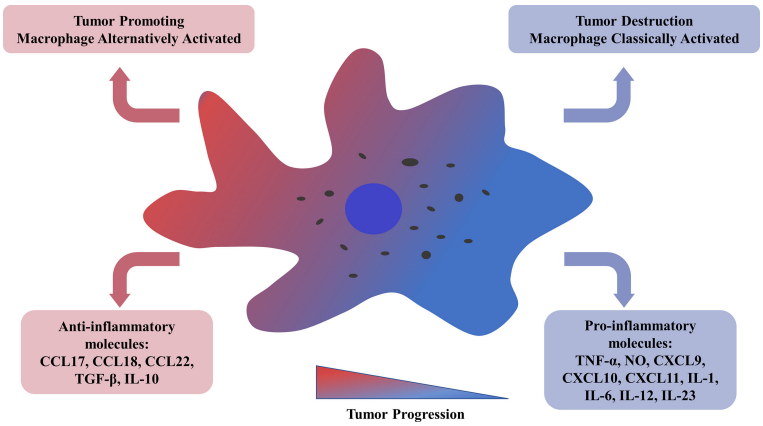

FIGURE 1 | Tumor-associated macrophages could be alternatively activated related to tumor progression and metastasis. M2 TAMs, with a low antigen-presenting capability, get involved in angiogenesis, tumor cell invasion, resistance to therapy, and release of anti-inflammatory molecules, such as CCL17/18/22, TGF-β, IL-10. M1 TAMs could provoke a Th-1 response and secrete pro-inflammatory molecules, such as TNF-α, NO, CXCL9/10/11, IL-1/6/12/23. TNF, tumor necrosis factor; NO, nitric oxide; CXCL, chemokine ligands with CX3-C motif; IL, interleukin; TGF, tumor growth factor.

macrophages invade tissues and acquire an activated phenotype to participate in disease processes. The relationship between TAMs and Hodgkin lymphoma (HL) has been studied relatively more than the relationships between TAMs and other hematological malignancies. A new study revealed that the adverse overall survival impact of TAMs in classical Hodgkin lymphoma (cHL) is dependent on checkpoint expression, especially on programmed death ligand 1 (PD-L1) and indoleamine 2,3-dioxygenase (IDO-1) expression (11). However, there are few reports on the relationship between TAMs and leukemia. In leukemia, TAMs are referred to as leukemia-associated macrophages (LAMs); they are referred to

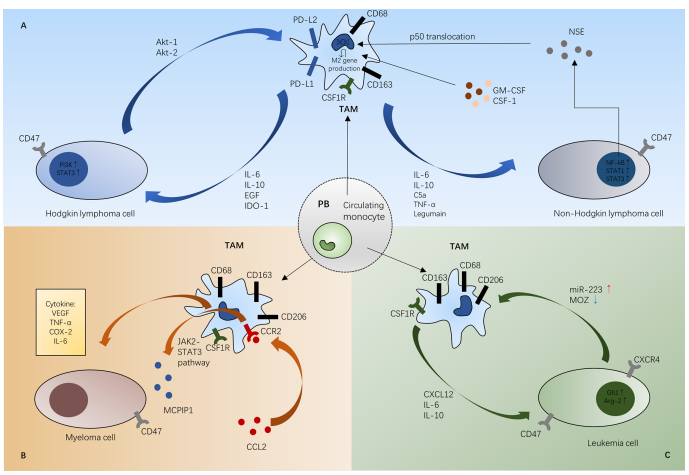

FIGURE 2 | Schematic representations of mechanisms between TAMs and tumor cells in Hodgkin lymphoma and non-Hodgkin lymphoma **(A)**, myeloma **(B)**, and leukemia **(C)**. In the different tumor microenvironment, TAMs infiltrate different tumor tissue to promote tumor growth. **(A)** In Hodgkin lymphoma, TAMs can activate HL cell proliferation through the STAT3 pathway and PI3K-Akt pathway. Besides, M1 macrophage polarization can be predominated by the NF-κB and STAT1 pathways in non-Hodgkin lymphoma. **(B)** CCL2 induces MCPIP1 expression *via* the JAK2-STAT3 signaling pathway in the MM bone marrow microenvironment. TAMs can also secrete proangiogenic cytokines like VEGF in MM microenvironment. **(C)** In the leukemia microenvironment, CSF-1R signaling paves the way for TAM recruitment. Gfi1 polarizes M1 phenotype macrophages into M2 macrophages to suppress the immune system and MOZ is a direct target of miR-223 promoting monocyte-to-macrophage development and M1 polarization. STAT3, signal transducer and activator of transcription; EGF, epidermal growth factor; IDO-1, indoleamine 2,3-dioxygenase; CSF1R, colony-stimulating factor receptor; CSF-1, colony-stimulating factor-1; GM-CSF, granulocyte macrophage-colony stimulating factor; TNF- α, tumor necrosis factor-α; NSE, neuron-specific enolase; VEGF, vascular endothelial growth factor; COX-2, cyclooxygenase-2; MOZ, monocytic leukemia zinc-finger; Gfi1, growth factor independent 1; Arg-2, arginase-1; PB, peripheral blood; TAM, tumor-associated macrophage.

as acute leukemia-associated macrophages (AAMs) in acute myeloid leukemia (AML) and nurse-like cells (NLCs) in chronic myeloid leukemia (CML). The objective of our review is to discuss the role of macrophages and their activated phenotype in different hematological malignancies.

MACROPHAGES IN LYMPHOMA

Macrophages can infiltrate malignant tumor tissues. Due to the similarity between lymphomas and solid tumors, many publications have clarified the existence of macrophages in malignant lymphoma. Here, we summarize the mechanisms by which TAMs are involved in angiogenesis, immunosuppression, and activation of tumor cells of HL, T-cell lymphoma and B-cell lymphoma and the clinical prognostic implications.

Macrophages in Hodgkin Lymphoma

cHL affects young people and is characterized by good prognosis in most cases. There are a small number of neoplastic Hodgkin and Reed-Sternberg (HRS) cells in the microenvironment of cHL with an abundant inflammatory infiltrate of immune cells.

TAMs are linked to adverse prognostic outcomes in HL in a checkpoint-dependent manner. In 1973, Coppleson LW confirmed the existence of macrophages in HL; however, poor prognosis with respect to TAMs was found later by Steidl C et al. (12, 13). The researchers demonstrated that TAM density could predict the treatment outcome by experimenting with paraffin-embedded particles. They also indicated that M2 TAMs could lead to tumor progression and immune escape. Roemer, M. G et al. confirmed that in cHL, the prognostic impact of TAMs on overall survival is checkpoint-dependent. Affected by the genetic/genomic variation of chromosome 9p24.1, PD-1 interacts with PD-L1 and PD-L2 on TAMs (14). IDO-1, which is a tryptophan-catabolizing enzyme, is also expressed by macrophages. Researchers confirmed that large amounts of PD-L1+ and IDO-1+ TAMs lead to adverse survival in patients and that biomarkers of the tumor microenvironment are checkpoint-dependent (11). As Carey et al. reported PD-1^+ CD4 T cell and $CD8^+$ T cell, together with PD-L1+ macrophages and HRS cells played an important role in cHL microenvironment (15). Tislelizumab, a humanized immunoglobulin G4 antiprogrammed cell death protein 1 antibody, binding to Fcg receptor on macrophages, demonstrated a favorable safety outcome for patients with relapsed/refractory cHL in a 3-year follow-up phase II study (16). Werner, L. et al. also confirmed in 2020 that moderate quantities of macrophages were associated with a better prognosis than very low or very high numbers using MYC-positive macrophage detection (17).

Due to the severity of HL progression, accurate prognostic models and clinically relevant biomarkers have become increasingly important. Whiteside TL et al. showed that TAMs were significantly related to primary treatment failure *via* gene expression analysis. They also demonstrated that relapse after auto-HSCT (P=0.008) and reduced progression-free survival (P=0.03) were correlated with $CD68^+$ macrophages in HL (18). Subsequently, an increasing number of studies have confirmed the relationship between macrophages and HL. Although CD68 and CD163 are recognized to be specific surface molecules of TAMs, some research has found that the prognostic significance of CD68 is not sufficiently related to clinical outcomes in cHL (19). A phase II clinical trial of CS1001 (PD-L1 inhibitor) of five relapsed or refractory (R/R) cHL elucidated that multiplex immunofluorescence staining was less intense for CD163 than CD68 (20).However, scholars from Korea found that CD163 is a better prognostic marker of macrophages in cHL (21). New reports suggested that HL patients with the highest M2 TAM count using CD163 as an M2 polarization marker had reduced disease-free survival and overall survival (11). These findings indicate that CD163 is better than CD68 as a prognostic marker for TAMs in HL.

The abovementioned findings indicate that it is likely that the effect of TAMs on outcome in HL may be related to potential changes in macrophage polarization. HRS cells can differentiated TAMs towards M2 phenotype by secreting molecules such as TGF-β and IL-13 (22). Ruella et al. cultured M2-like phenotype macrophages from monocytes, together with HDLM-2 cells and GM-CSF. The results showed that these M2 macrophages, expressed CD163, CD206, PD-L1, inhibited the growth of human CD19 chimeric antigen receptor (CAR) T cells, which suggests an unsatisfactory therapeutic effect of CAR T cell. CD123 expresses on macrophages in the microenvironment of HL, suggesting that CD123-targeted therapies might impact on the tumor microenvironment (23).It was previously shown that TAMs can activate HL cell proliferation through the STAT3 pathway (24). The STAT3 pathway also induces macrophage polarization toward the M2 phenotype (25). The STAT3 pathway can be activated by macrophage-derived factors such as epidermal growth factor (EGF), IL-6, and IL-10 (26–29). In cHL patients, IL-10 is also regarded as a marker of tumor burden and as an unfavorable host-tumor factor (30, 31). IL-10 was reported to promote the poor overall survival of cHL *via* genetic regulation of the tumor microenvironment. Single-nucleotide polymorphisms (SNPs) in IL-10 can be regarded as prognostic markers in adult cHL. Furthermore, the percentage of macrophage activating factor (MAF)-expressing cells can change, which suggests a role of these cells in determining the host genetic background that induces macrophage polarization and an indirect role in microenvironment shaping (32). The PI3K-Akt pathway is also involved in HL pathogenesis (33). PI3K inhibitor RP6530 decreases the release of lactic acid by downregulates the metabolic regulator pyruvate kinase muscle isozyme 2 (PKM2) (34). A first-in-human phase I, open-label study of Tenalisib (RP6530) enrolled 35 patients across 11 dose levels with R/R hematological malignancies correlated well with clinical outcome and further phase I/II studies are being undertaken to evaluate the efficacy (35). Later, Locatelli et al. found that downregulating lactic acid released by HRS favors M2 type macrophage. They also found that the blockade of PI3K could lead M1-type macrophages to transition into the M2 type, which suggests a new therapeutic strategy to treat patients with HL (36). However, how these mechanisms truly affect macrophage polarization remains unclear.

Macrophages in Non-Hodgkin Lymphoma

Macrophages in Diffuse Large B-Cell Lymphoma

Diffuse large B-cell lymphoma (DLBCL) accounts for 30-40% of non-HL clinical cases (5). TAMs play an active role in the progression of DLBCL. Some studies have confirmed that TAMs and specifically M2-TAMs are linked to poor prognosis in DLBCL and central nervous system DLBCL (37, 38). In *in vitro* assessment of the progression of DLBCL, M2 TAMs were found to affect the overexpression of legumain by disrupting the extracellular matrix and promoting angiogenesis (39).

In DLBCL, the relationship between $CD68^+$ TAMs and overall survival varies. Some studies have shown that there is no relationship between TAMs and prognosis, whereas others have reported a significant influence (40, 41). Many studies have shown that CD163+ TAMs and the CD163/CD68 ratio are linked to clinical outcome (37, 42, 43). Specifically, it is thought that different therapeutic options could exert different influences on TAMs. 85% to 95% of *de novo* DLBCL patients express PD-L1, correlated with macrophages and STAT3 expression (44). Pollari et al. collected tumor tissue from 74 primary testicular lymphoma patients, and examined macrophage markers, T-cell markers, B-cell marker, and checkpoint molecules, illustrating that PD-1- PD-L1 signaling have a promising role in clinical outcome (45).

In DLBCL, TAMs have been demonstrated to produce cytokines such as C5a, IL-6 and TNF-α to activate the Stat3 and NF-kB pathways (46). A new study investigated the relationship between neuron-specific enolase (NSE) levels and the prognosis of DLBCL. The researchers found that the protein expression of, which mediates nuclear p50 translocation with subsequent dysfunction of classical nuclear factor-κB (NF-κB), thereby promoting M2 polarization and shifting the role of macrophages, was increased in DLBCL (47). This mechanism is related to the IRF/STAT signaling pathway of macrophage polarization. Polarization can be skewed toward the M1 phenotype *via* STAT1 signaling and toward the M2 phenotype *via* STAT6 signaling. Two adaptors, MyD88 and TRIF, regulate signaling downstream of TLR4, which ultimately activates NF-κB, a pivotal transcription factor influencing M1 macrophage activation. M1 macrophage polarization is predominated by the NF-κB and STAT1 pathways, which play a proinflammatory role (48, 49). It is well known that the most common therapy for DLBCL is CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) or the combination of rituximab and CHOP (R-CHOP) chemotherapy (50, 51). R-CHOP combined with granulocyte-macrophage colony-stimulating factor (GM-CSF) prolongs the survival of elderly DLBCL patients (52, 53). Zhang et al. first reported the antitumor, macrophage polarization-related molecular mechanisms by which GM-CSF affects CHOP and R-CHOP therapy in 2021 (54). The researchers found that GM-CSF induced repolarization of M2 macrophages to increase M1 macrophages, providing ideas for how macrophages mediate the AKt pathway, a well-characterized pathway in DLBCL.

Macrophages in Follicular Lymphoma

Follicular lymphoma is a common indolent B-cell lymphoma characterized by a slow clinical course that is usually considered incurable. Research on macrophages in follicular lymphoma has mostly focused on predicting overall survival. Kridel R et al. found that two patient groups treated with different therapies showed an opposite correlation between M2 TAM density and prognosis (55).Some studies have suggested that the number of CD68+ TAMs is related to the prognosis of follicular lymphoma (56). Another study found that patients with an increased number of CD68+ TAMs had longer survival (57). Although the number of CD163+ macrophages can predict the prognosis of patients with follicular lymphoma, their impact depends on the treatment that the patients received from a study involved 395 samples treated with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone, and randomized to rituximab maintenance or observation (55). Furthermore, a recent meta-analysis revealed that high CD68+ LAM numbers, diffuse patterns of FOXP3+ regulatory T (Treg) cells and PD1+ cells, and high PD-L1 cell numbers are adverse factors leading to early transformation of follicular lymphoma. A study on the immune microenvironment of follicular cell lymphoma showed that immune infiltrate diversity portends good clinical efficacy in follicular lymphoma, implying that a rich immune microenvironment in follicular lymphoma is important (58).

Colony-stimulating factor-1 (CSF-1) and its receptor CSF-1R have been thoroughly studied in follicular lymphoma, and the results have indicated that macrophages can be a new therapeutic target because CSF-1 is one of the most important recruitment factors for macrophage polarization. Regarding solid tumors, glioma has been reported to be cured by treatment targeting the CSF-1R/CSF-1 axis. A recent study demonstrated that CSF-1R inhibition by PLX3397 has a higher impact on M2 macrophages than on M1 macrophages and leads to their repolarization toward an M1-like phenotype (59).

Macrophages in Marginal Zone B-Cell Lymphoma

Splenic marginal zone lymphoma (SMZL) is also an incurable indolent small B-cell lymphoma usually occurring in elderly people (average age of 65 years old) (60). Escape from immune control is the main change leading to exacerbation of the disease, characterized by abundant T-cells in the periphery but low numbers in the stroma, which surrounds large amounts of tumor cells (61). Chen and Mellman et al. suggested that SMZL is characterized by an inflamed phenotype, with the presence of intratumoral infiltration of T-cells into the tumor microenvironment in addition to myeloid-derived suppressor cells (MDSCs) and tissue-associated macrophages, which is closely related to poor overall survival (61). SMZL with an inflamed phenotype features the expression of PD-L1 as a mechanisms for immune escape; PD-L1 is colocalized with CD163, a marker of alternatively activated macrophages (62).

Macrophages in Peripheral T-Cell Lymphoma

Peripheral T-cell lymphoma (PTCL) is an aggressive form of lymphoma in Asia that usually leads to poor overall survival (63). Early research found that in acute T-cell leukemia/lymphoma, $CD68^+$ TAM infiltration exists; however, this feature is not related to poor prognosis and angiogenesis. In contrast, the number of $CD163^+$ TAMs was found to be associated with for

the prognosis of T-cell lymphoma (64, 65). Iqbal J et al. analyzed the influence of CD68 expression on the promotion of macrophage differentiation by GATA-binding protein 3 (GATA3). GATA3 and T-box family transcription factor (T-bet) are Th1 and Th2 cell differentiation markers, respectively, and T-bet-positive PTCL has a better prognosis than GATA3-positive PTCL (66, 67).

Cutaneous T-cell lymphoma (CTCL) is a heterogeneous group of T-cell lymphomas located in the skin in which macrophages behave as M2 macrophages (68, 69). In a study of CTCLs, a high number of $CD163^+$ M2 TAMs was linked to a poor clinical prognosis and was correlated with the level of soluble IL-2 (65). IL-10 is reported to be higher than average in CTCLs. IL-10 has been confirmed to increase the expression of PD-L1 to induce anti-inflammatory regulation. Xuesong W et al. proved that IL-10 is not only a biomarker but also a key cytokine in macrophage polarization that leads to tumor growth and can inhibit effective cutaneous T-cell lymphoma therapy (70). A retrospective study of 205 patients published in 2020 showed that an increased level of IL-10 is an independent factor that indicates poor overall survival, a low complete response rate and a higher early relapse rate (71).

MACROPHAGES IN MYELOMA

Multiple myeloma (MM) is a B-cell hematological tumor characterized by a large number of malignant plasma cells in the bone marrow. MM cells are highly dependent on the bone marrow microenvironment and can create an immunosuppressive microenvironment conducive to tumor growth by secreting cytokines or directly contacting surrounding immune cells. Macrophages are abundant in the bone marrow of patients with MM and can promote the growth, proliferation and drug resistance of tumor cells and participate in the formation of an immunosuppressive microenvironment.

TAMs can negatively influence MM growth and progression, leading to a poor outcome. Angiogenesis is a major feature of MM and features stimulation of angiogenic factors by plasma cell, inducing the transformation of monoclonal gammopathy of undermined significance (MGUS) into MM (72). TAMs can secrete proangiogenic cytokines like VEGF and TNF-α and express proangiogenic enzymes such as cyclooxygenase-2 (COX-2) (73, 74). There has been some research progress related to microRNAs involved in the bone marrow microenvironment. Exosome-derived miR-let-7c promotes angiogenesis by polarizing M2 macrophages in the MM microenvironment (70). Macrophages also regulate tumor growth by controlling cell metabolism. One hypothesis is that M2 macrophages can inhibit PGK1 phosphorylation by secreting IL-6 to disrupt the connection between macrophages and tumor cells (75).

Elevated microvessel density has been linked to CD163-positive TAMs and CD68/CD163 double-positive M2 TAMs. Andersen et al. suggested that in MM patients, CD163 expression was higher in bone marrow than in blood samples, and high CD163 expression correlated with a poor prognosis and a higher International Staging System (ISS) stage. Moreover, an increased number of $CD163^+$ TAMs has also been found to be a powerful predictor of poor prognosis in MM in the era of novel drugs. CD163 and inducible NO synthase (iNOS) expression have been combined with ISS stage as new prognostic factors. Furthermore, increased expression of CD206, a soluble M2 macrophage marker, indicates reduced overall survival (76–79). Sanyal et al. found a novel cell surface marker for M2 macrophages MS4A4A which includes CD20 (MS4A1), FcRβ (MS4A2) and Htm4 (MS4A3), suggesting immunotherapeutic potential in the treatment of MM (80).

CCL2 is a critical molecule that recruits monocytes and induces inflammation (81). The inactivation of CCL2-CCR2 was found to reduce tumor growth in solid neoplasms. The chemokine CCL2 was found to promote macrophage infiltration in the MM bone marrow microenvironment and to encourage proliferation (82). De Beule and colleagues revealed that AZD1480, a Janus kinase 2 (JAK2) inhibitor, was correlated with protumor effects *via* the STAT3 pathway in 5T33MM cells (78, 83). A recent study found that increased CCL2 induces MCPIP1 expression *via* the JAK2-STAT3 signaling pathway, which promotes tumor growth (79, 84). Trabectedin, a drug that kills monocytes and macrophages, triggers antiangiogenic activity by suppressing CCL2 and VEGF production. Due to this effect, the potential of trabectedin as a targeted agent in anti-MM therapeutic strategies has been proposed (85). CSF1R blockade significantly inhibits myeloma-associated macrophage polarization to the M2 type, implying that CSF1R-blocking antibodies could be a new tool for MM therapy (86).

MACROPHAGES IN LEUKEMIA

Since lymphoma is similar to solid tumors, research in lymphoma is relatively common. However, leukemia is unique. There are significant pathological differences between leukemia and solid tumors, and thus, studies of macrophage properties and actions in leukemia are lacking. Compared to solid tumors, hematological malignancies have a unique immunological microenvironment. Leukemia originates from leukemic stem cells (LSCs), and these LSCs maintain the hematopoietic microenvironment and hematopoietic stem cell (HSC) survival and function, which supports LSC proliferation through complex signals. Leukemia is propagated by LSCs, which cannot be totally eradicated and persist, ultimately leading to recurrence (87). TAMs, existing in the microenvironment of different types of leukemia are called LAMs.

Macrophages in Acute Lymphocytic Leukemia

Acute T-cell leukemia is characterized by infection with human T-cell leukemia virus. Komohara et al. showed that $CD163^+$ M2 macrophages are closely associated with the progression of T-cell acute lymphocytic leukemia (T-ALL) (88). Recently, researchers reported that an inhibitor of the M-CSF receptor may suppress

the stimulation of macrophages, which can be used as a therapeutic strategy (89). A JAK2/FLT3 inhibitor, pacritinib, was found to block CSF-1R to improve the microenvironment (89). Further research found that CSF-1R signaling paves the way for TAM recruitment and obstructs TAM proliferation in a T-ALL mouse model (90). The CXCR4/CXCL12 axis was found to inhibit TAM polarization toward the M1 phenotype. Some preclinical studies have demonstrated that the CXCR4 inhibitor plerixafor improves the clinical scores of T-ALL (91).

In B-cell acute lymphocytic leukemia (B-ALL), there are few studies about macrophages. MDSCs and Treg cells have become the focus of B-ALL research in recent years given their mutual relationship. MDSCs have emerged as a great contributor to tumor angiogenesis, drug resistance, and the promotion of tumor metastases (92). Recent studies have shown that MDSCs can continue to differentiate into TAMs in the tumor environment and can be divided into an M1 subgroup, which inhibits tumor growth, and an M2 subgroup, which promotes tumor growth. MDSCs consist of two types: monocytic MDSCs (MO-MDSCs) and polymorphonuclear MDSCs (PMN-MDSCs) (93). A recent study found that PMN-MDSCs and Treg cells play important roles in maintaining the immune-suppressive state of B-ALL, which means that they may be independent predictors of B-ALL progression. However, the relationship between peripheral Treg cells and MDSCs has not been fully recognized (94). Lineage reprogramming could be a promising future treatment in B-ALL therapy, and such a strategy was shown to eliminate the leukemogenicity of Ph-positive B-ALL cells and turn them into macrophage-like cells *in vitro* (91).

Macrophages in Acute Myeloid Leukemia

A German scholar named the macrophages in AML as AML-associated macrophages (AAMs). In AML, which factors induce macrophage polarization remains unclear. Al-Matary YS et al. proved that LAMs exert an important influence on the overall survival and drug resistance of AML patients. Their results suggest that leukemic cells and the microenvironment can induce the proliferation and infiltration of monocytes and macrophages and promote their differentiation into AAMs. The main reason for relapse in AML is LSCs, which can be supported by AAMs *via* extracellular matrix remodeling, angiogenesis, and lymphangiogenesis (95). AAMs highly express Gfi1, which polarizes M1 phenotype macrophages into M2 macrophages to suppress the immune system (96). In addition, a growth factor-independent transcriptional repressor was found to reprogram LAMs toward the antitumor state. The leukemia hematopoietic microenvironment is complex and includes fibroblasts, macrophages and other components. Variations in the hematopoietic microenvironment in leukemia have not been reported. There is mounting evidence that illustrates that the AML microenvironment can re-educate monocytes and macrophages to transition into the M2 phenotype. Mussai et al. provided the first reports showing that arginase II secreted from primary AML blasts reeducates healthy donor-derived monocytes toward an M2-like phenotype, as demonstrated by upregulation of CD206 (97).

The AML microenvironment has tissue-specific heterogeneity. In the MLL-AF9 AML mouse model, splenic LAMs more often exist in the M2 phenotype, while bone marrow LAMs more often exist in the M1 phenotype. AML creates an immunosuppressive microenvironment. By demonstrating an arginase-dependent ability of AML, Mussai et al. polarized surrounding monocytes into a suppressive M2 type macrophage. The researchers also found that repolarization of LAMs through targeting of the SAPK/JNK pathway and IRF7-SAPK/JNK pathway by interferon regulatory factor 7 (IRF7) can prolong the survival of AML mice, providing regarded a new immunotherapy strategy against leukemia (96, 98). Keech et al. showed a high degree of leukemia burden in MLL-AF9 AML mice and that nonmalignant and AML bone marrow macrophages display a decrease in M1 macrophage markers (99). It has also been found that monocytic leukemia zinc-finger (MOZ) is a direct target of miR-223 promoting monocyte-to-macrophage development and M1 polarization (100). A recent study showed that peritoneal resident macrophages in mice with AML induced by MLL-AF9 show an M2-like phenotype (101). These results strongly suggest that the leukemia microenvironment may enhance the immunotherapy effect in AML by affecting the apoptosis and killing ability of macrophages. Switching M1 to M2 is through lasting exposure to polarizing molecules or direct cell-to-cell contact between macrophages and cancer cells (102). Smirnova, T. further proved that in the presence of GM-CSF, inhibiting CSF1R could repolarization macrophage, thus improving the efficiency of AML therapy, which indicated a promising therapeutic method to modulate macrophage phenotype (103).

Macrophages in Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of $CD5^+$ B cells in blood, secondary lymphoid organs and bone marrow. Burger JA et al. found that nurse-like cells (NLCs) derived from blood could protect CLL B-cells from apoptosis through stromal cell-derived factor-1 (104). In fact, NLCs are CLL-specific TAMs characterized by expression of the markers CD68 and CD163 (105). It is unknown why NLCs increase the survival rate and drug-induced apoptosis of CLL cells. Boissard et al. reported that LFA-3 appeared to have an adverse influence on prognosis in an exploratory cohort of 60 CLL patients receiving frontline immunochemotherapy (105). IFN-γ was found to reprogram NLCs into the M1 state (106). The JAK2/FLT3 inhibitor pacritinib restrains the CSF-1R signaling pathway, thus preventing the generation and survival of NLCs (107). Edwards V DK et al. showed that significant synergy was observed when combining CSF1R inhibitors with two current CLL therapies that block the signaling pathway of the tumor cell-intrinsic B-cell receptor (108). The proinflammatory switch of NLCs plays an important role in modulating the CLL microenvironment. Trabectedin also induced an antileukemia effect in a CLL mouse model by depleting TAMs *via* the CCL2-CCR2 signaling axis (109). The CSF1 receptor also participates in antineoplastic activation by interfering with leukemic cell and NLC interactions (107).

Macrophages in Chronic Myeloid Leukemia

Previous studies have shown that M2-type macrophages are the predominant infiltrate in the bone marrow microenvironment of CML patients, with their functions being dominant, and the number of positive cells increases gradually with the progression of the disease (110). By CSF1/CSF-1/M-CSF pathway, autophagy can be induced through the differentiation from human monocytes to macrophages. Researchers found that P2RY6 agonist activated CSF-1 treated monocytes differentiation to promote autophagy induction in some CMML patients (111). Researchers found high accumulation of $CD68^+$, $CD163^+$ and $CD206^+$ macrophages in bone marrow biopsy samples. Macrophages have been reported to increase the cytotoxicity of natural killer (NK) cells against solid tumor cells. In CML bone marrow aspirates, there are higher proportions of macrophages and NK cells. Choo et al. found that mycoplasma-infected CML cells were protected from NK cytotoxicity by macrophages, which was related to macrophage-mediated maintenance of NK cells (112). Besides, the polarization of the M2-like macrophages was found to be associated with K562-derived exosomes in CML (113). This can be a new sight into leukemia-derived exosomes in the development of leukemic niches.

TAM-TARGETED THERAPEUTIC STRATEGIES

TAMs may be a therapeutic target because they are involved in cancer progression and characterized by unique transcriptional profiles (**Figure 2**) (114). It has been reported that M1-like TAMs have an antitumor effect, while M2-like TAMs have a protumor effect (115). Therefore, induction of TAM polarization from the M2 to M1 phenotype could be a therapeutic strategy to treat hematological malignancies. The different signaling pathways in TAM polarization mainly include five pathways: the JAK/STAT signaling pathway, Notch signaling pathway, PI3K/Akt signaling pathway, TLR/NF-κB signaling pathway and hypoxia-dependent signaling pathway. In addition, some natural compounds also downregulate M2 polarization (116). Our summary of the pathways shifting macrophage polarization is shown in **Table 1**.

TAM-targeted therapy towards the conjugation of antibodies and ligands to the therapeutic molecule carrier. The role and prognostic markers of TAMs, which can be also recognized as crucial receptors in hematological macrophages, is shown in **Table 2**. CD163 receptor on the surface of macrophage can recognize the complex of hemoglobin (Hb) and plasma haptoglobin (Hp). Conjugating the anti-cancer drug dichloroacetic acid (DCA) to the Hb-Hp complex targets the delivery of DCA into cancerous monocytes and scavenges cancer cells (117, 118). Wang et al. suggested that CD71 is a invariably marker and highly expressed in different subtypes of leukemia cells based on which they designed a biomimetic carrier for precision delivery of As^{III}, As@Fn nanomedicine, to bind to HL-60 AML leukemia cells characterized by CD71 (119). This finding gives us a new perspective into ferritin-based targeted therapy connected to hemoglobin targeted therapy delivered to TAMs. More anti-tumor drugs and clinical research are expected to extend our therapeutic scope.

The relationship between overexpression of CD47, a glycoprotein highly expressed in myeloid and lymphoid malignancies, and poor prognosis is under investigated (120). CD47 induces immune escape by binding to the receptor SIRPα to inhibit macrophage phagocytosis and improve T-cell cytotoxicity (121). Advani et al. confirmed that the Hu5F9-G4 antibody has a synergistic effect with rituximab in 22R/R DLBCL and follicular lymphoma patients, indicating that blocking the CD47 immune checkpoint, a so-called "don't eat me" signal, could exert antitumor effects (122). A recent study demonstrated that the addition of rituximab to CHOP chemotherapy improved the overall outcome of DLBCL patients (123). *In vitro* experiments illustrated that novel fully human anti-CD47 monoclonal antibodies increased macrophage-mediated phagocytosis and improved the prognosis of AML models (124). CC-90002 is an anti-CD47 antibody to block CD47-SIRPα interaction and enhance macrophage-mediated killing ability. However, a phase 1 study of anti-CD47 monoclonal antibody CC-90002 in patients with R/R AML and high-risk MDS still suggested insufficient evidence in clinical activity as expected in spite of well preclinical effect (125).

Trabectedin together with Zoledronic acid are two drugs used to kill tumor cell and TAMs. Trabectedin targets DNA transcription, leading to DNA double strand breaks and cell cycle blockade, which demonstrates a potent anti-tumor effect against Hodgkin Reed Sternberg cells. Tumors of trabectedin-treated mice had fewer TAMs, reducing secretion of CCL5, M-CSF, IL-6, IL-13 in HRS cells (126). Zoledronic acid is a potential therapy to change tumor microenvironment, affecting the secretion of CCL5 and IL-6. In prostate cancer, zoledronic acid repolarizes M2 macrophages to M1 type, exhibiting antitumor effect (127).

Lenalidomide has been proven to influence the tumor microenvironment by improving T-cell and NK-cell function (128). In mouse MM models, lenalidomide was proven to promote M2 macrophage depletion and affected the Th1/Th2 balance (129). The effect of adding ASCT to triplet therapy (lenalidomide, bortezomib, and dexamethasone [RVD]) in patients with multiple myeloma was associated with longer progression-free survival than RVD alone in a phase 3 clinical trial (NCT01208662) (130). Martino et al. reported a retrospective multicenter analysis of 600 RRMM patients treated with the combination of lenalidomide and dexamethasone (KRd) with a 79.9% overall response rate after a median of 11 KRd cycles (131). Pyridoxine, a specific treatment in AML, induces monocyte-macrophage death and apoptosis in THP-1 cells to play an antitumor role (132).

TAK-981 is the first-in-class small-molecular inhibitor of SUMOylation in clinical trials. Small ubiquitin-like modifier (SUMO) is a ubiquitin-like protein superfamily, promoting

TABLE 1 | Pathways shifting macrophage polarization in hematological malignancies.

Disease	Mechanism of Action	Results
HL	PI3K-Akt pathway	Leading M1 type macrophage to M2 type
DLBCL	GM-CSF synergistic enhancement effect	Enhancing M1 polarization from M2
DLBL	NSE protein mediates nuclear p50 translocation *via* IRF/STAT signaling pathway	Promoting M2 polarization and migration ability of macrophage
PTCL	GATA3-dependent mechanism	M2 macrophage differentiation
FL	CSF-1R inhibition by PLX3397	repolarization towards an M1-like phenotype
MM	Inactivation of CCL2-CCR2	Macrophage bone marrow homing, proliferation, and polarization
MM	STAT3 pathway	A Janus kinase (JAK)2 inhibitor was correlated to the pro-tumor effect
MM	Exosome-derived miR-let-7c promotes angiogenesis	Polarizing M2 macrophages in MM microenvironment
MM	CSF1R blockade	Inhibits myeloma-associated macrophage polarizing to M2 type
T-ALL	CXCR4/CXCL12 axis	Inhibiting TAM polarization towards M1 phenotype
AML	Demonstrating an arginase-dependent ability of AML	Suppressive M2-like phenotype *in vitro*
AML	Expressing Gfi1	Polarizing M1 phenotype macrophage into M2
AML	MOZ Forms an Autoregulatory Feedback Loop with miR-223	Promoting monocyte-to-macrophage development and M1 polarization
AML	Inhibiting CSF1R, in the presence of GM-CSF	Reprogramed MΦ orientation and promoted myeloblast apoptosis
CLL	IFN-γ	Reprogramming tool to polarize NLCs to M1 state
CLL	CSF-1R signaling inhibition	LAMs polarization blocking

inflammatory responses and expressing IFN-1.By blocking SUMOylation, TAK-981 allowing NK cell activation and M1 polarization to enhance antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) *via* upregulating IFN-1. Nakamura Et al. showed a preclinical research that TAK-981 and rituximab in xenograft models of human B cell lymphoma have antitumor effect (133). Assouline et al. further proved in a phase 1b/2, open-label, dose-escalation and expansion study that TAK-981 plus rituximab resulted in promising clinical activity (ORR 29%) in the R/R NHL (134). Combination of TAK-981 with anti-CD38 antibody daratumumab also resulted in protective clinical antitumor immune response (135). TAK-981 increased phagocytic activity of macrophages and natural killer cell cytotoxicity *via* IFN-1 signaling, which could be a promising treatment for patients with hematological malignancies (136).

CONCLUSION

Macrophages have attracted wide attention in solid tumor research, and their role in hematologic malignancies should also remain a focus. TAMs are referred to as LAMs, AAMs or NLCs in hematologic malignancies. Distinct microenvironments induce different molecular mechanisms of TAMs. The microenvironments of hematologic malignancy can induce the activation of macrophages into type M2 macrophages, which

TABLE 2 | The role and prognostic markers of TAMs in hematological malignancies.

Disease	Reference	Marker	Number of Patients	Survival Correlation
cHL	Karihtala et al. (11)	PD-L1, IDO-1	130	High proportions of PD-L1 and IDO-1 TAMs are associated with unfavorable outcomes
	Carey et al. (15)	PD-L1	180	Increased PD-L1 expression had superior PFS
	Kayal et al. (19)	CD68	100	CD68 TAM marker does not have prognostic value
	Suh et al. (21)	CD68, CD163	144	CD163 is a better prognostic marker of macrophages than CD68
DLBCL	Marchesi et al. (37)	CD68/CD163	61	High CD68/CD163 M2 TAM is correlated to unfavorable prognostic factors
	Wang et al. (42)	CD163	355	LMR was negatively correlated with CD163 M2 TAM
	Cencini et al. (43)	CD68/CD163	37	CD68+/CD163+ TAM have a prognostic role for IPI ≥ 2 DLBCL patients receiving R-CHOP
	McCord et al. (44)	PD-L1	777	PD-L1 did not identify high-risk in *de novo* DLBCL
	Pollari et al. (45)	PD-L1/CD68	74	High PD-L1/CD68 macrophages predict favorable survival
FL	Kridel et al. (55)	CD163	186	CD163 TAM predict outcome dependent on treatment received
	Kelley et al. (56)	CD68	94	CD68 TAMs is related to the prognosis
	Bjoürck et al. (57)	CD68	57	Patients with an increased number of CD68 TAMs had longer survival
	Kridel et al. (55)	CD163	395	CD163 macrophages can predict the prognosis depending on the treatment
SMZL	Vincent-Fabert et al. (62)	PD-L1	54	Exhibiting inflammation with the expression of PD-L1
PTCL	Sugaya et al. (65)	CD163	28	CD163 M2 TAMs was linked to a poor clinical prognosis
	Iqbal et al. (66)	CD68	372	CD68 TAM differentiation by GATA3 related to worse prognosis
ATLL	Saito et al. (64)	CD204	58	CD204 TAMs were closely associated with lymphoma cell proliferation
MM	Andersen et al. (76)	CD163	131	Soluble CD163 was found to be a prognostic marker
	Suyanı et al. (77)	CD163	68	High MVD was found to be associated with increased CD163 TAM
	Chen et al. (78)	iNOS, CD163	240	iNOS and CD163 TAMs as independent prognostic factors
	Wang et al. (79)	CD163	198	High CD163 TAM correlate with poor prognosis

PFS, progression-free survival; LMR, lymphocyte-to-monocyte ratio; MVD, microvessel density.

play an important role in angiogenesis, immunosuppression, and the activation of tumor cells. Strategies to reprogram the polarization of macrophages are new therapeutic options in hematologic malignancies.

AUTHOR CONTRIBUTIONS

The manuscript was conceptualized by LG and XZ. YX wrote the majority of the manuscript and HY cowrote the manuscript. The figures were drawn by CY and LP. HZ and LH produced the table. All authors contributed to the article and approved the submitted version.

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Targeted therapy in NPM1-mutated AML: Knowns and unknowns

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Acute myeloid leukemia (AML) is a heterogeneous disease characterized by malignant proliferation of myeloid hematopoietic stem/progenitor cells. NPM1 represents the most frequently mutated gene in AML and approximately 30% of AML cases carry NPM1 mutations. Mutated NPM1 result in the cytoplasmic localization of NPM1 (NPM1c). NPM1c interacts with other proteins to block myeloid differentiation, promote cell proliferation and impair DNA damage repair. NPM1 is a good prognostic marker, but some patients ultimately relapse or fail to respond to therapy. It is urgent for us to find optimal therapies for NPM1-mutated AML. Efficacy of multiple drugs is under investigation in NPM1-mutated AML, and several clinical trials have been registered. In this review, we summarize the present knowledge of therapy and focus on the possible therapeutic interventions for NPM1-mutated AML.

KEYWORDS

NPM1, AML, targeted therapy, venetoclax, menin inhibitors, XPO1 inhibitors

Introduction

Nucleophosmin (NPM1) is the most common mutated gene in acute myeloid leukemia (AML). AML with NPM1 mutations accounts for approximately 30% of adult AML, which is characterized by the cytoplasmic localization of NPM1 (NPM1c) (1). NPM1-mutated AML was recognized as a distinct entity in the World Health Organization classification of myeloid neoplasms.

NPM1, shuttling between the nucleus and cytoplasm, is predominantly located in the nucleus (2, 3). NPM1 protein contains three structural domains including N terminal, central and C terminal domain. Nuclear export signals (NESs), located in N terminal domain, promote the translocation of NPM1 from the nucleus to the cytoplasm (3, 4).

Nucleolar localization signals (NoLS), formed in highly conserved aromatic region of C terminal domain, is critical for the localization of NPM1 to the nucleus (5). The nuclear export of NPM1 is mediated by the interaction of two NESs and the nuclear exporter exportin 1 (XPO1) (3). NPM1 is a multifunctional protein involved in diverse cellular functions such as ribosome synthesis, genomic stability, cellular growth and stress response (6–9).

NPM1 mutations result in the generation of a new C-terminal NES and the loss of tryptophan residues 288 and 290, which endow mutated-NPM1 stronger nuclear export ability and ultimately lead to the cytoplasmic localization of NPM1 (10, 11). NPM1c mediates cytoplasmic dislocation of promyelocytic leukemia (PML) nuclear bodies (NB) (12). Researchers found that NPM1c interacts and delocalizes PU.1, FBW7γ and APE1, which block myeloid differentiation, promote cell proliferation and impair DNA damage repair, respectively (13–15).

NPM1-mutated AML is a kind of AML with favorable prognosis. The overall survival rate was about 40% and complete remission (CR) rate was about 80% (16). However, approximately 50% of patients will eventually relapse (17). The standard therapy of NPM1-mutated AML patients includes "3+7" induction chemotherapy and consolidation therapy. NPM1 often co-exists with fms-like receptor tyrosine kinase-3 internal tandem duplication (FLT3-ITD), which results in poor survival and high relapse rates. Allogeneic hematopoietic stem cell transplantation (allo-HSCT) and FLT3 inhibitors may be considered as important choices for these high-risk patients. It should be underscored that despite the favorable outcome of NPM1-mutated AML patients, disease-free survival (DFS) and overall survival (OS) of older NPM1-mutated patients remain disappointing and worse than those in younger NPM1-mutated patients (18). This may be partly due to treatment options, disease biology and age-related factors.

It has been about 15 years since NPM1-mutated AML was first discovered. However, there is no consensus over how to treat this type of AML, especially relapsed NPM1-mutated AML. Up to now, several studies targeting NPM1-mutated AML are undergoing, including inhibiting NPM1c functions, interfering with abnormal transport of NPM1c protein, promoting NPM1c degradation and immunotherapy such as monoclonal antibodies. Herein, we summarize available data (Table 1) and ongoing clinical trials (Table 2) and focus on the potential targeted therapy (Figure 1) of NPM1-mutated AML.

Venetoclax-based therapies

B-cell lymphoma 2 (Bcl-2), an anti-apoptotic protein, is overexpressed in AML. High Bcl-2 expression is associated with survival of AML cells and chemotherapy resistance (19, 20). Venetoclax is a potent and selective small molecule inhibitor of Bcl-2, has shown efficacy in preclinical and clinical practice (21, 22). Recently, safety and efficacy of venetoclax-based therapies, in combination with hypomethylating agents (HMAs) or low-dose cytarabine (LDAC), has been confirmed in several AML clinical studies. To figure out the effect of venetoclax-based therapies on NPM1-mutated AML, we summarize the data from recent prospective clinical studies, real-world reports and the latest ASH abstracts (Table 1).

Venetoclax + HMA/LDAC/IC

The phase 1 clinical trial of venetoclax with decitabine or azacitidine (NCT02203773) enrolled 145 AML patients and NPM1-mutated AML accounts for 16% (N = 23) (23). CR and CR with incomplete count recovery (CRi) (CR + CRi) was observed in 21 NPM1-mutated patients. In the phase 3 clinical trial of venetoclax plus azacitidine, 66.7% of NPM1-mutated AML patients achieved CR + CRi (NCT02993523) (24). The phase 1/2, phase 3 clinical trials of venetoclax and LDAC were successively conducted in AML patients (NCT02287233, NCT03069352). Patients with NPM1 mutations represented 11% and 9% of the study cohort and experienced CR + CRi at 89% and 78%, respectively (25, 26). In the clinical trials of venetoclax in combination with intensive chemotherapy (IC), patients with NPM1 mutations also had good responses. NPM1-mutated AML patients attained CR and CRi at 80% and 100% in the venetoclax combined with 5 + 2 (cytarabine + idarubicin) study and venetoclax combined with FLAG + IDA (fludarabine, cytarabine, granulocyte colony-stimulating factor, and idarubicin) study, respectively (ACTRN12616000445471, NCT03214562) (27, 28).

In real-world settings, venetoclax combined with HMA also gained good outcomes in NPM1-mutated AML patients. CR and CRi were achieved at 100%, 71.4% and 66.7% in three reports, respectively (29–31). We also collect venetoclax-based regimens data from the latest ASH abstracts, CR and CRi rates were high in NPM1-mutated AML patients ranging from 76.6% to 100% (32–39).

Furthermore, venetoclax was recently identified as a selective agent for NPM1-mutated AML through clinical drugs screening (40). A retrospective analysis compared outcomes of NPM1-mutated AML patients treated with 3 approaches including venetoclax plus HMA, HMA and intensive chemotherapy (IC). This analysis demonstrated that venetoclax plus HMAs significantly reduced the risk of death and achieved a higher CR rate when compared with standard IC or HMAs (41). Venetoclax plus LDAC showed encouraging activity in eradicating persistent or relapsing mutated NPM1 measurable residual disease (MRD) (42). The multicenter and prospective clinical trials of venetoclax-based regimens are required to confirm its safety and efficacy in NPM1-mutated AML. A phase 2, multicenter trial evaluating the efficacy of venetoclax plus azacitidine in molecular relapse/progression has been registered (NCT04867928).

TABLE 1 Summary of venetoclax-based therapies in NPM1-mutated AML.

Basic information	Interventions	Settings	Numbers of NPM1-mutated AML patients	Clinical outcomes (CR/CRi)
Prospective clinical studies				
NCT02203773 (phase 1)	Venetoclax + Decitabine/Azacitidine	ND AML	N = 23	CR + CRi = 21/23 = 91.5%
NCT02287233 (phase 1/2)	Venetoclax + LDAC	ND AML	N = 9	CR + CRi = 8/9 = 89%
NCT03069352 (phase 3)	Venetoclax + LDAC	ND AML	N = 18	CR + CRi = 14/18 = 78%
NCT02993523 (phase 3)	Venetoclax + Azacitidine	ND AML	N = 27	CR + CRi = 18/27 = 66.7%
ACTRN12616000445471 (phase 1b)	Venetoclax + 5 plus 2 (cytarabine + idarubicin)	ND AML	N = 10	CR + CRi = 8/10 = 80%
NCT03214562 (phase 1b/2)	Venetoclax + FLAG+IDA	ND AML and R/R AML	N = 8	CR + CRi = 8/8 = 100%
Real-world experience				
2019	Venetoclax + Azacitidine	ND AML	N = 8	CR + CRi = 8/8 = 100%
2021	Venetoclax + Decitabine	R/R AML	N = 7	CR + CRi = 5/7 = 71.4%
2021	Venetoclax + HMA	R/R AML	N = 3	CR + CRi = 2/3 = 66.7%
ASH abstracts				
2019, NCT03586609, phase 2	Venetoclax + Cladribine +LDAC/ Azacitidine	ND AML	N = 6	CR + CRi = 6/6 = 100%
2019, retrospective study	Venetoclax + Decitabine/Azacitidine/ LDAC/Mylotarg	ND AML and R/R AML	N = 2	CR + CRi = 2/2 = 100%
2020, real-world outcomes	Venetoclax + HMA/LDAC/IC	ND AML and R/R AML	N = 7	CR + CRi = 6/7 = 86%
2020, retrospective study	Venetoclax + Decitabine/Azacitidine	ND AML and R/R AML	N = 21	CR + CRi = 18/21 = 86%
2020, retrospective study	Venetoclax + HMA	ND AML and R/R AML	N = 18	CR + CRi = 16/18 = 88.9%
2021, R/R AML patients with NPM1 mutation	Venetoclax + IC (cytarabine/idarubicin ± nucleoside analog) or Venentoclax + LIC (HMA/LDAC)	R/R AML	N = 12	CR + CRi = 10/12 = 83%
2021, NCT03404193, phase 2	Venetoclax + Decitabine	ND AML and R/R AML	N = 47	CR + CRi = 36/47 = 76.6%
2021, retrospective study	Venetoclax + Azacitidine	ND AML and R/R AML	N = 18	CR + CRi = 14/18 = 77.8%
Summary				
			N =244	CR + CRi = 200/244 = 82%

ND, newly diagnosed. LDAC, low-dose cytarabine. FLAG+IDA, fludarabine, cytarabine, granulocyte colony-stimulating factor, and idarubicin. HMA, hypomethylating agents. IC, intensive chemotherapy (cytarabine/idarubicin ± nucleoside analog). LIC, low intensity chemotherapy (HMA/LDAC).

Venetoclax + ATO

In addition to the most common combinations between venetoclax and HMA, LDAC or IC, there are several new combinations under investigation, such as the ones with arsenic trioxide (ATO). ATO, as an ancient drug, has exerted its function in several malignancies. Both ATO and venetoclax can downregulate Bcl-2 expression to induce apoptosis (43). Myeloid cell leukemia sequence 1 (MCL-1) is critical for the survival of AML cells and plays an essential role in venetoclax resistance (44, 45). ATO was reported to attenuate MCL-1 upregulation induce by venetoclax (46). The synergistic antileukemic activity of ATO and venetoclax was also confirmed in primary leukemia stem cells from AML patients (46). Therefore, this combination might represent an alternative option for NPM1-mutated AML. ATO and venetoclax synergistically induces the apoptosis of NPM1-mutated OCI-AML3 cells *in vitro* and showed anti-leukemia activity in two relapsed and/or refractory (R/R) NPM1-mutated AML patients (47).

The aforesaid results highlight the promising efficacy of venetoclax-based regimens, providing a rationale for further trials in NPM1-mutated AML. Current venetoclax-based regimens are mainly applied in elderly patients who are unfit for chemotherapy or young patients who are ineligible for standard induction therapy. More studies are required to

TABLE 2 Summary of ongoing or completed clinical trials in NPM1-mutated AML.

Clinical trials identifier	Trial phase	Status	Intervention	Comments
NCT00893399	3	Completed	Gemtuzumab Ozogamicin (Mylotarg) Standard chemotherapy (Idarubicin, Etoposide, Cytarabine, ATRA, Pegfilgrastim)	Evaluating efficacy
NCT01237808	3	Completed	Cytarabine, Etoposide, All-trans retinoic acid	Evaluating efficacy
NCT03031249	1/2	Recruiting	Cytarabine, All-trans retinoic acid, Arsenic Trioxide	Evaluating safety and efficacy
NCT03769532	2	Recruiting	Pembrolizumab, Azacitidine	Evaluating safety and efficacy
NCT04689815	2	Recruiting	Oral Arsenic Trioxide Formulation	Evaluating efficacy
NCT04867928	2	Recruiting	Venetoclax, Azacitidine	Evaluating efficacy
NCT05020665	3	Recruiting	Entospletinib, Placebo, Cytarabine, Anthracycline	Evaluating efficacy
NCT04067336	1/2	Recruiting	KO-539	Two NPM1-mutated AML patients obtained CR
NCT04065399	1/2	Recruiting	SNDX-5613, Cobicistat	The overall response rate of NPM1-mutated AML: 38% (5/13)
NCT04811560	1	Recruiting	JNJ-75276617	Evaluating safety and tolerability of JNJ-75276617
NCT04988555	1/2	Recruiting	DSP-5336	Evaluating safety, tolerability and clinical activity of DSP-5336
NCT04752163	1/2	Recruiting	DS-1594b, Azacitidine, Venetoclax	Evaluating safety, toxicity and efficacy of DS-1594b
2014-000693-18	2	Completed	Dactinomycin	Evaluating anti-tumor activity and safety
2014-003490-41	2	Recruiting	Dactinomycin	Evaluating anti-tumor activity and safety

expand the application of this approach, for example, to achieve greater overall survival in young patients. Furthermore, future researches should concentrate on optimizing the venetoclax-based therapies and overcoming venetoclax resistance.

Menin inhibitors

NPM1-mutated AML cells are characterized by high expression of HOXA and HOXB clusters, which are necessary

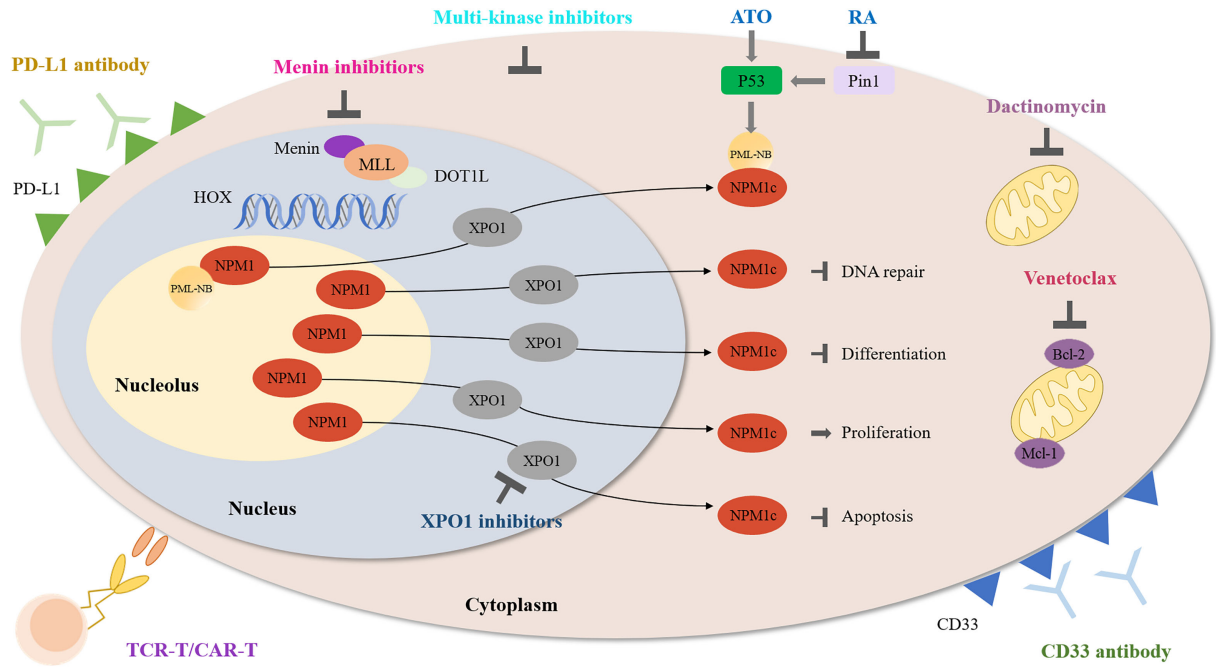

FIGURE 1
Schematic diagram describing the function of multiple agents in NPM1-mutated AML.

for the maintenance of the leukemic state (48). Histone modifiers MLL1 and DOT1L control HOX and FLT3 expression and differentiation in NPM1-mutated AML (49). Combinatorial inhibition of menin-MLL1 and DOT1L showed synergistic activity against primary AML cells in this study. Another preclinical result also indicated that inhibition of menin-MLL1 reversed leukemic development of NPM1-mutated AML mice models (50). It was reported that menin-MLL1 inhibition combined with venetoclax demonstrated anti-leukemia activity in primary NPM1-mutated AML samples (51). It seems that targeting menin could be a therapeutic strategy in NPM1-mutated AML.

FLT3-ITD often co-exists with mutated NPM1, accounting for approximately 40% of NPM1-mutated AML. Combining menin inhibitors with FLT3 inhibitors induced synergistic inhibition of proliferation and enhanced apoptosis in AML blasts (52). The combination of menin and FLT3 inhibitors significantly reduced leukemia burden and induced the long-term remissions in a PDX model with both NPM1 and FLT3-ITD mutations (53). Since XPO1 inhibition potently downregulate HOX expression in NPM1-mutated AML, the combination of menin and XPO1 inhibitors appeals as a rational therapeutic option in NPM1-mutated AML (48).

Several clinical studies are recruiting to assess the safety and efficacy of menin inhibitors such as SNDX-5613 and KO-539 on leukemia with MLL-rearrangement or NPM1 mutation (NCT04067336, NCT04065399, NCT04811560, NCT04752163, Table 2). Early results demonstrated tolerance and biologic activity of KO-539 (54). This phase 1/2A study evaluated clinical activity in 6 R/R AML patients and KO-539 induced CR in two patients. One patient achieved MRD-negative CR, who had AML with NPM1, DNMT3A, and KMT2D mutations and received KO-539 at 200 mg daily as the eighth line of treatment. SNDX-5613 exhibited safety and promising antileukemic activity in R/R MLL-rearrangement and NPM1-mutated AML in preliminary results (55). As of data cutoff on October 18, 2021, the overall response rate in 13 NPM1-mutated AML patients was 38%. The most common side effects included prolonged QTc, nausea, vomiting and differentiation syndrome.

XPO1 inhibitors

Exportin 1 (XPO1) is a nuclear exporter implicated in the export of proteins and RNAs (56). NPM1 mutation results in the increased nuclear export ability of mutated NPM1 (10, 11). XPO1 inhibitors can relocate mutated NPM1 to the nucleus. However, XPO1 inhibitors are not NPM1-specific and also inhibit nuclear export of other proteins such as TP53 and P21.

Considering the relationship between XPO1 and NPM1, XPO1 inhibitors might be a promising approach for NPM1-mutated AML. The combination of selinexor and venetoclax showed a synergistic effect on the anti-leukemic activity of AML cells (57). Current studies mainly focus on the effects of XPO1 inhibitors in AML, not specifically in NPM1-mutated AML. To date, the combinations of selinexor and traditional chemotherapy, such as decitabine, cytarabine, mitoxantrone and idarubicin, are under study (58–60). However, systemic toxicities of selinexor, such as nausea and anorexia, limit its clinical usage to twice per week. Eltanexor, a second-generation XPO1 inhibitor, exhibits lower blood-brain penetration, improved tolerability and better anti-leukemic efficacy when compared with selinexor (61, 62). The combination of eltanexor and venetoclax reduce cell viability and induce apoptosis of AML cell lines (63). This combination therapy also enhanced anti-leukemia effect in AML cell-derived and patient-derived xenograft models. Eltanexor seems to be a prospective drug and further investigations are needed to validate the clinical activity in NPM1-mutated AML.

XPO1 is widely expressed in normal cells and interacts with hundreds of proteins, inhibition of XPO1 might generate some side-effects such as hematologic adverse events. Future efforts should focus on combining XPO1 inhibitors with either traditional chemotherapy or novel agents to enhance efficacy and safety.

ATO plus ATRA

Arsenic trioxide (ATO) plus all-trans retinoic acid (ATRA) had been proved a successful strategy in acute promyelocytic leukemia (APL), a unique AML subtype characterized by the fusion protein of promyelocytic leukemia (PML)–retinoic acid receptor α (RARα). The combination has been proved to induce the degradation of PML-RARα fusion protein to cure APL (64).

NPM1-mutated AML cells are more sensitive to ATO because the presence of C-terminal cysteine 288 of NPM1c protein makes cells sensitize to oxidative stress induced by ATO (65). The anti-leukemia ability of ATO and ATRA support the further application in NPM1-mutated AML. Thus, two groups simultaneously demonstrated that the combination of ATO and ATRA induced the degradation of mutated NPM1 protein and apoptosis in both NPM1-mutated AML cell lines and primary cells (66, 67). Furthermore, ATO plus ATRA activated p53 signaling and restored nuclear organization of PML bodies. The combined treatment also significantly reduced bone marrow blasts in 3 NPM1-mutated AML patients and recovered the abnormal localization of both NPM1 and PML (67). ATRA was reported to induce mutated NPM1 degradation through the Pin1/PML/P53 axis, thereby promoting the response of blasts to chemotherapy or ATO (68). It is reported that ATRA improved survival of elderly NPM1-mutated AML patients without FLT3-ITD mutations when added to traditional chemotherapy (69). These findings provide convincing evidence for further clinical application of ATO and ATRA in NPM1-mutated AML. Relevant research are undergoing in NPM1-mutated AML (NCT03031249, NCT04689815, Table 1).

Dactinomycin

Dactinomycin, a famous antibiotic, exhibits potent antibacterial and anticancer activity by inhibiting topoisomerases and RNA polymerases (70). Investigators found that low dose dactinomycin can efficiently generate stress response in NPM1-mutated cells, illustrating NPM1-mutated AML might be sensitive to nucleolar stress (71). Dactinomycin targets mitochondria particularly primed by mutant NPM1, induces ROS production and restore PML NBs formation. Dactinomycin was initially shown its efficacy on a NPM1-mutated AML patient without FLT3-ITD mutations. The patient achieved morphologic and immunohistochemical CR after two cycles of therapy and showed a molecular CR after the fourth cycle (72). The clinical safety and efficacy of dactinomycin in AML patients with NPM1 mutations was further established (71, 73). Dual targeting of mitochondria with dactinomycin and venetoclax exerts strong anti-leukemic activity in NPM1-mutated AML (74). Dactinomycin seems to be a potential clinical choice for NPM1-mutated AML. Two clinical trials have been registered to evaluate anti-leukemic activity and safety of dactinomycin in NPM1-mutated AML (Table 1).

Immunotherapy

CD33 antibody

CD33 is a myeloid differentiation antigen expressed at the very early stages of myeloid cell development and is absent outside the hematopoietic system or on pluripotent hematopoietic stem cells (75). CD33 expression was found in leukemic blasts in almost all AML patients and associated with adverse disease features (76, 77). Gemtuzumab ozogamicin (GO) is CD33-directed immunoconjugate by delivering a DNA-damaging calicheamicin derivative to exert its function.

CD33 expression was significantly higher in the NPM1-mutated AML cases compared with the NPM1-unmutated cases (78). The results support the therapeutic application of CD33 antibodies in NPM1-mutated AML. A study showed that the addition of GO to standard chemotherapy improves the event-free survival (EFS) and OS in *de novo* AML patients aged 50–70 years (79). Among this cohort, NPM1-mutated AML patients accounted for 33% of all cases.

One clinical study was registered to evaluate the efficacy of GO in NPM1-mutated AML (NCT00893399). The study failed to show significant benefits on EFS when GO was added to intensive therapy, which might be due to a higher early mortality in the GO arm. However, patients who achieved CR + CRi after induction therapy significantly had fewer relapses in the GO arm than in the standard arm (80). In the following attempts, the combinations of GO and other treatments are required to be optimized in NPM1-mutated AML.

PD-1 and PD-L1 antibody

Programmed cell-death protein PD-1 and its ligands PD-L1 are immune checkpoint molecules that are involved in T-cell activation and dampen T-cell anti-tumor response. PD-1/PD-L1 pathway plays an essential role in tumor immune evasion, thus promoting the progression of tumor (81).

NPM1-mutated AML patients have a stronger cytotoxic T-lymphocyte response against mutated NPM1-derived peptides compared with healthy volunteers (82). Immune responses might be a contributing factor for the better prognosis of NPM1-mutated patients (83). High PD-L1 expression was detected in NPM1-mutated AML patients and predicted worse overall survival (84, 85). It should be noted that NPM1 was identified as a transcriptional regulator of PD-L1 and is associated with poor prognosis in triple-negative breast cancer (86). The aforementioned results indicated that PD-L1 might be a potential therapeutic target in NPM1-mutated AML. Unfortunately, current study suggested clinical activity of PD-L1 antibody in AML is limited (87, 88). Thus, more fundamental research and clinical studies are needed to investigate the exact role of PD-L1 in NPM1-mutated AML.

Hypomethylating agents, such as azactidine, have a dual effect against tumor immunity. In addition to enhancing anti-tumor immune response, HMAs can restrain immune response by upregulating PD-1 and PD-L1 expression, which can promote the exhaustion of tumor-specific T cells (89). It seems necessary to combine HMAs with immune checkpoint inhibitors such as PD-1 or PD-L1 antibody (90). Recently, a clinical study to evaluate the safety and efficacy of pembrolizumab when administered in combination with azacitidine in NPM1-mutated AML patients with molecular relapse was recruiting (NCT03769532, Table 1).

CAR-T/TCR-T cell therapy

The adoptive immunotherapy, such as T cell receptor (TCR) and chimeric antigen receptor (CAR) T cell therapy, is an important milestone in the development of genetically modified cell therapies for leukemia. Due to low antigen expression in healthy tissues, TCR-T and CAR-T targeting tumor-associated antigens could be accompanied by severe toxicity. Neoantigens are derived from tumor-specific gene mutations but most neoantigens are encoded by patient-specific passenger mutations, which can be lost due to immunoediting and ultimately result in immune evasion (91). Nevertheless, neoantigens from driver gene mutations are unlikely to induce immune evasion because leukemic cells need to express the driver gene to maintain their malignant phenotype (92). Therefore, neoantigens derived from driver gene mutations are ideal targets for immunotherapy.

Mutated NPM1 is an essential driver gene and occurs in approximately 30% of AML. Besides the primary genetic lesion, NPM1 mutations also cooperate with other mutations to contribute to leukemogenesis (93). Moreover, NPM1-mutated protein does not exist in normal tissues, so it is an ideal leukemic-specific antigen and a potential target for NPM1-mutated AML. Recently, TCR-T and CAR-T directed against NPM1-mutated peptides obtained preliminary success in NPM1-mutated AML.

Van der Lee et al. transduced CD8+ and CD4+ T cells with the TCR for NPM1-mutated peptide, which demonstrated efficient specificity against NPM1-mutated and HLA-A2-restricted primary leukemic blasts (94). T cells transduced with TCR for NPM1-mutated protein could efficiently kill AML cells and prolonged OS of NSG mice engrafted with HLA-A*02:01-positive NPM1-mutated OCI-AML3 human cells. NPM1-mutated CAR-T cells showed efficient and specific anti-leukemia activity against NPM1c+HLA-A2+ leukemia cells and primary AML blasts (95). CAR-T cells could significantly reduce leukemia burden and prolonged survival of NSG mice engrafted with OCI-AML3 cells. Both TCR-T and CAR-T exhibit strong specificity and cytotoxicity against NPM1-muated AML without evident side effects. Further studies are warranted to investigate in clinical application and overcome potential drawbacks.

Conclusion

NPM1-mutated AML is a clinically heterogeneous group because it almost always exists in the context of other mutations. NPM1 mutations often co-occur with FLT3, DNMT3A or other mutations to contribute to leukemogenesis (96, 97). The latest report classified NPM1-mutated AML into two novel subtypes, primitive and committed subtype, based on a stem cell signature through RNA-seq (98). Interestingly, they found that leukemic cells in the primitive subtype are more sensitive to certain kinase inhibitors. The addition of kinase inhibitors to the treatment might achieve therapeutic benefits in this specific subtype of NPM1-mutated AML. These results may prompt us to make a more accurate risk stratification of NPM1-mutated AML based on multidisciplinary technology, thereby giving a guidance for clinical treatment. Furthermore, some controversial issues in diagnosis and treatments of NPM1-mutated AML still exists. Falini et al. recently summarized how he diagnose and treat NPM1-mutated AML and he constructively proposed that NPM1 mutational status, the timing of HSCT, MRD monitoring and ELN genetic-based risk stratification should be considered during the therapy (99).

NPM1 mutations are ideal targets for MRD monitoring because they are AML-specific, frequent, stable at relapse and do not drive clonal hematopoiesis of indeterminate potential. Investigators found that MRD, as determined by real-time quantitative PCR (RT-qPCR) of NPM1-mutated transcripts, provides important prognostic information for AML (100). Patients with persistence of NPM1-mutated transcripts in blood after the second cycle of chemotherapy was associated with a greater risk of relapse and a lower rate of survival compared with those without such transcripts. In multivariate analysis, the presence of MRD was the only significant prognostic factor for relapse and death. RT-qPCR remains the standard method for MRD monitoring in NPM1-mutated AML, the application of highly sensitive digital droplet PCR and NGS will be expanded in the future.

Considering the above findings, the combination of multiple agents is the dominant trend in the future treatment of NPM1-mutated AML, such as venetoclax-based regimens and XPO1 inhibitors combinations. The pathogenesis of NPM1-mutated AML and diverse drugs combinations need to be further studied. Joint efforts should be made to overcome the limitation of currently promising drugs, such as resistance for venetoclax and toxicity for XPO1 inhibitors. Novel targeted drugs for NPM1-mutated AML are also urgently developed. We are looking forward to acquiring the consensus on treatment of NPM1-mutated AML.

Author contributions

H-HZ and S-ZZ conceived the idea of the paper. RW, PX, and L-LC contributed equally to data collection and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher’s note

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Treatment options for acute myeloid leukemia patients aged <60 years

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Treatment of acute myeloid leukemia (AML) has changed over the last few years, after the discovery of new drugs selectively targeting AML blasts. Although 3/7 remains the standard of care for most AML patients, several new targeted agents (such as FLT3 inhibitors, CPX-351, gemtuzumab ozogamicin, BCL-2 inhibitor, and oral azacitidine), either as single agents or combined with standard chemotherapy, are approaching clinical practice, starting a new era in AML management. Moreover, emerging evidence has demonstrated that high-risk AML patients might benefit from both allogeneic stem cell transplant and maintenance therapy, providing new opportunities, as well as new challenges, for treating clinicians. In this review, we summarize available data on first-line therapy in young AML patients focusing on targeted therapies, integrating established practice with new evidence, in the effort to outline the contours of a new therapeutic paradigm, that of a "total therapy", which goes beyond obtaining complete remission.

KEYWORDS

AML - acute myeloid leukemia, target therapy, induction, allogeneic stem cell transplantation, young

Introduction

Acute myeloid leukemia (AML) is a malignant disorder of the hematopoietic system mostly diagnosed in the elderly population. However, it can occur at any age. Previously incurable, only 35% to 40% of younger patients (aged <60 years) and 5% to 15% of older patients are alive and disease-free at 5 years (1). Historically, long-term survival without allogeneic hematopoietic stem cell transplantation (allo-HSCT) has been extremely poor (2). Results from 2,551 adults with AML who received intensive chemotherapy on Cancer and Leukemia Group B (CALGB) trials before the targeted therapy era, without undergoing allo-HSCT in first remission, showed 10-year disease-free survival (DFS) in only 15% of younger patients and <2% of older patients (3). This poor outcome is in part due to a myriad of chromosomal alterations and gene mutations, which are

frequently found in AML blasts, thereby promoting a clinically heterogeneous group of diseases, which share the common features of drug resistance and high relapse rate (2). Despite steady progress in the understanding of AML biology and novel technologies to better characterize the biology of the disease, such as multicolor flow cytometry, droplet digital polymerase chain reaction (ddPCR), and next-generation sequencing (NGS), the treatment paradigm has not drastically changed during the last 40 years. Therefore, there are still relevant unmet needs to improve survival and quality of life for most AML patients by optimizing the combination of novel therapies with conventional agents, including innovative approaches to allo-HSCT. Here, we will discuss current therapeutic approaches in young AML patients and future prospects with a focus on promising drugs in development.

General considerations

Treatment of AML depends on several prognostic factors including age, overall health status, and presence of genetic or chromosomal abnormalities. Thus, there is a common trend to better characterize AML subtypes at diagnosis, to stratify tailored therapies earlier in the treatment course. AML has often been assumed to require urgent treatment; however, data from a cohort of 599 patients reported that time from diagnosis to initiation of intense treatment (TDT) had no effect on survival even in patients presenting with white blood cells (WBCs) > 50,000/μl or age > 60 years (4). Röllig et al. recently confirmed these results analyzing data from a registry of 2,200 patients, suggesting that TDT is not related to response or survival, neither in younger nor in older patients (5).

The choice between conventional and investigational therapy is guided by ELN 2017 recommendations (2), in which patients are divided into "favorable", "intermediate", and "adverse" groups. Recently, the German Group has validated this stratification for remission, survival, and relapse-free survival (6). In particular, patients with TP53 mutations and/or complex cytogenetics, which are highly associated with each other, showed a particularly dismal outcome and should be considered a distinct "very adverse" group, which may benefit most by enrolment in a clinical trial. Using this refined classification, complete remission (CR) rates for the very favorable [patients with inv(16)/t(16;16) or biallelic CEBPA mutations], favorable, intermediate, adverse, and very adverse groups were 77%, 71%, 66%, 44%, and 27%, respectively, and estimated OS rates at 5 years were 70%, 50%, 31%, 14%, and 0%, respectively (6). It is important to keep in mind that risk classification systems must always be interpreted in conjunction with treatment regimens, which may change over time. In this view, neither ELN2017 nor its modifications incorporate data from patients treated with new drugs recently approved. Finally, prediction of long-term outcome based on pre-treatment disease-related factors alone has a major bias, since patient-related factors are not included in the ELN categories. Criteria for unfitness are, in most trials, age either ≥ 65/70/75 years, performance status, or comorbidities, all precluding the use of intensive induction. As known, "geriatric assessment" has been shown to refine the prognostic effect of age, which should not be the only determinant of fitness for intensive induction, as reported in ELN 2017 (2). A panel of experts from the Italian Society of Hematology (SIE) has proposed a new definition of unfitness to intensive and non-intensive chemotherapy (7), using an analytic hierarchy process-based consensus process. The definition of unfitness to intensive therapy should require the fulfillment of at least one of nine criteria (Table 1).

Moreover, analyses of measurable residual disease (MRD) during and after treatment by flow cytometry [with a threshold set at 0.1%, the amount of residual leukemic cells (2)], quantitative PCR, or NGS have emerged as novel tools to assess response to therapy and to guide the post-remission strategy. Different methods have specific indications and require highly specialized expertise. The ELN Working Party consensus document on MRD in AML (2) indicates that molecular assessment for NPM1 mutations, RUNX1-RUNX1T1, CBFB-MYH11, and PML-RARA fusion transcripts should be performed at diagnosis, at least after two cycles of induction/consolidation therapy, and every 3 months, for 24 months after the end of treatment. Monitoring of NPM1-mutated transcripts may be more informative when performed in PB as compared to BM. In general, obtaining an MRD-negative CR is associated with longer remissions, DFS, and OS, independently from the intensity of chemotherapy and allo-HSCT. Accordingly, as recognized by the ELN2017 panel (1), the goal of induction therapy should be CR with MRD negativity in all patients, independently from the type of treatment. In addition to MRD assessment, the detection of small subclones by NGS is important to evaluate clonal evolution during the disease course (even though its current error rates set the sensitivity level at about 1%). Regarding therapeutic intervention in patients with an MRD positivity after allo-HCT, different strategies might be pursued. Fast tapering of immunosuppressive treatment, donor lymphocyte infusion, hypomethylating agents, or FLT-3 inhibitors are valid options, and might be used alone or in combination with each other (8).

First-line treatment

The mainstay treatment for AML in the fit population is based on an induction chemotherapy with cytarabine plus an anthracycline, with or without a purine analogue, followed by two to four cycles of consolidation chemotherapy and/or allo-HSCT, to eliminate residual leukemic cells (2). This approach achieves CR in 60%–70% of patients aged <60 years, with a lower

TABLE 1 Conceptual and operation criteria to define AML patients' unfitness to intensive chemotherapy.

Conceptual criteria	Operation criteria
Advanced age (over 75 years)	An age older than 75 years
Severe cardiac comorbidity	Congestive heart failure or documented cardiomyopathy with an EF ≤ 50%
Severe pulmonary comorbidity	Documented pulmonary disease with DLCO ≤ 65% or FEV1 ≤ 65%, or dyspnea at rest or requiring oxygen, or any pleural neoplasm or uncontrolled lung neoplasm
Severe renal comorbidity	On dialysis and age older than 60 years or uncontrolled renal carcinoma
Severe hepatic comorbidity	Liver cirrhosis Child B or C, or documented liver disease with marked elevation of transaminases (>3 times normal values) and an age older than 60 years, or any biliary tree carcinoma or uncontrolled liver carcinoma or acute viral hepatitis
Active infection resistant to anti-infective therapy	Active infection resistant to anti-infective therapy
Cognitive impairment	Current mental illness requiring psychiatric hospitalization, institutionalization or intensive outpatient management, or current cognitive status that produces dependence (as confirmed by the specialist) not controlled by the caregiver
Low performance status (ECOG functional scale)	ECOG performance status ≥3 not related to leukemia
Any other comorbidity that the physician judges to be incompatible with conventional intensive chemotherapy	Any other comorbidity that the physician judges to be incompatible with conventional intensive chemotherapy

AML, acute myeloid leukemia; DLCO, diffusing capacity of the lungs for carbon monoxide; ECOG, Eastern Cooperative Oncology Group; EF, ejection fraction; FEV1, forced expiratory volume in 1 s.

remission rate and a dismal outcome in patients with adverse risk disease.

Intensive induction therapy is based on 7 days cytarabine with 3 days anthracycline (i.e., 7 + 3). Several trials have been done to improve the outcome of "7 + 3": adding a third drug, and increasing the dose of cytarabine and daunorubicin.

The SWOG trial 1203 randomized 754 adults aged < 60 years among 7 + 3 (with a daunorubicin dose of 90 mg/m^2), idarubicin + cytarabine 1.5 g/m^2 daily by continuous infusion × 4 days (IA) and IA + vorinostat (9). CR rates were 75%–79%, and there were no differences in EFS, RFS, or survival among the three arms, neither in patients with NPM1, FLT3, or CEBPA mutations or intermediate or adverse cytogenetics. In patients with favorable cytogenetics, outcomes were significantly better with 7 + 3 than with IA or IA + V; however, different doses of cytarabine were administered during post-remission therapy, and this may have led to confusing results (9).

The NCRI/MRC group (AML15 trial) randomized 3,106 AML patients in two comparison arms, with the aim to compare cytarabine, daunorubicin, etoposide (ADE) with daunorubicin and cytarabine (DA), fludarabine, cytarabine, granulocyte colony-stimulating factor (G-CSF), and idarubicin (FLAG-Ida). They observed reduced relapse rates using FLAG-Ida instead of ADE and DA, but no survival benefits due to higher treatment-related mortality after obtaining CR, in the former group (10).

The randomized phase 2 trial on alvocidib, cytarabine, and mitoxantrone hydrochloride (FLAM) compared to cytarabine and daunorubicin hydrochloride (3 + 7) was conducted on 165 treating patients with newly diagnosed AML. The study showed no significant differences in overall survival (OS) between FLAM and 3 + 7, despite significantly higher CR rates with FLAM. The OS appeared to be better in patients <50 years old, compared with those ≥50 years old, without significant differences (11, 12).

A phase 1/2 trial of G-CSF, cladribine, cytarabine, and dose-escalated mitoxantrone (G-CLAM), conducted on 199 patients with newly diagnosed or relapsed/refractory AML or high-risk myelodysplastic syndromes, showed higher rates of CR/CRi and higher MRD-negative CR (measured by multiparameter flow cytometry [MFC]) than standard "7 + 3" therapy in fit patients with newly diagnosed AML or other high-grade myeloid neoplasm with ≥10% blasts (HG-MN) (13).

Results of E1900 reported in 2009 (14) showed a benefit of anthracycline intensification [45 (standard dose) versus 90 (high dose) mg/m^2 daunorubicin for 3 days in combination with cytarabine] in younger patients, but not in patients with adverse cytogenetics, FLT3-ITD, or aged 50 years or older. In 2016, the updated results of the same trial reported broader benefit in high-risk patients, both in unfavorable cytogenetics and in FLT3-ITD mutant AML, with a 10% advantage; the FLT3-ITD positive patients received a higher dose of daunorubicin (15).

Another study that evaluated different doses of daunorubicin, in the same period, was the HOVON trial (16), although this study enrolled only patients aged ≥60 years, eligible for intensive chemotherapy. DFS and OS for AML patients treated with high-dose daunorubicin were not superior to standard dose in the whole population. However, there was a benefit of survival in patients aged 60–65 years.

Another randomized trial, which compared standard versus high-dose daunorubicin induction in 383 young adults with AML, was performed in Korea and published in 2011 (17). High-dose daunorubicin produced a statistically significant higher DFS and OS after a median follow-up of 52.6 months.

The survival benefits of high-dose daunorubicin therapy were more prominent in patients with intermediate-risk cytogenetics, and toxicities were similar in the two arms.

Finally, patients with FLT3 ITD (n = 200) appear to have fewer relapses leading to longer survival when treated with the 90 mg/m^2 dose of daunorubicin (18). In this setting, Luskin et al. (15) showed a benefit in a significant subgroup of older patients aged 50 to 60 years with FLT3-ITD or NPM1 mutations. In particular, NPM1 mutant patients receiving intensified daunorubicin had a remarkable increase in median OS (75.9 vs. 16.9 months) and a >20% increase in 4-year OS (52% vs. 29%) (15).

Current evidence suggests that the dose of daunorubicin should not be less than 60 mg/m^2 (2).

Although 3/7 remains the standard of care for most AML patients, several new targeted agents, already approved or under investigation, either as single agents or combined with standard chemotherapy, are approaching clinical practice, starting a new era in AML management. Since 2017, the US Food and Drug Administration (FDA, Table 2) have approved nine new drugs, and some of these have already been incorporated in clinical practice.

Finally, it is broadly accepted that post-remission therapy is needed to prevent relapse. Allo-HSCT reduces the risk of relapse, with its graft vs. leukemia (GVL) effect. Nonetheless, allo-HSCT is affected by a significant non-relapse mortality and morbidity, due to acute and chronic graft vs. host disease (GVHD). The general recommendation is to offer allo-HSCT to all patients with an ELN2017 risk either intermediate or adverse. In contrast, the risk of relapse in the ELN 2017 favorable subgroup is low, and does not justify the use of allo-HSCT in this setting. However, patients with MRD-positive CR should be candidates for frontline allo-HSCT, regardless of the initial risk group, in order to improve OS and DFS. In fact, MRD positivity after the second course of chemotherapy significantly affects OS in patients with favorable and standard risk AML, as reported by Freeman et al. (19), much more than in high-risk patients. Moreover, the presence of NPM1-mutated transcripts after the second cycle of chemotherapy in the peripheral blood of patients in CR was the only significant adverse prognostic factor in the UK NCRI AML 17 trial (20). In summary, allo-HSCT remains the best post-remission treatment option for the vast majority of AML patients. It is still debated whether maintenance after allo-HCT is warranted and if it should be reserved only to high-risk patients, or to the entire population submitted to allo-HCT.

FLT3 inhibitors

FMS-like tyrosine kinase 3 (FLT3) gene mutations are present in approximately 30%–35% of all AML patients. Within this subgroup, 25% of AML patients show internal tandem duplications (ITDs), whereas 5%–8% present a mutation in the tyrosine kinase domain (TKD) (21). The presence of a high FLT3-ITD allelic ratio of the TKD mutation confers a poor prognosis (2). FLT3-mutated AML is the paradigm for therapies combining molecularly targeted agents with standard intensive chemotherapy.

First-generation FLT3 inhibitors were not specifically designed to target the FLT3 receptor. These drugs are nonspecific, multikinase inhibitors, with additional activity against other targets such as c-Kit, platelet-derived growth factor receptor (PDGFR), and vascular endothelial growth factor receptor (VEGFR). This class of drugs include lestaurtinib, sunitinib, sorafenib, ponatinib, and midostaurin. In 2017, based on the randomized "RATIFY" trial, the FDA approved 3 + 7 plus midostaurin for adults aged <60 years with FLT3-ITD or TKD mutation, regardless of the allelic ratio. In this global, randomized, placebo-controlled phase 3 trial of 717 patients, the addition of midostaurin to induction and consolidation chemotherapy resulted in superior survival rates compared to chemotherapy alone (7.2% difference in 4-year OS)

TABLE 2 Recent FDA approvals in newly diagnosed AML.

Drug or regimen	FDA approval indication
Midostaurin	FLT3^{MUT} AML
CPX-351 (liposomal daunorubicin HCl and cytarabine)	tAML, AML MRC
Gemtuzumab Ozogamicin	Newly diagnosed adults with CD33+ AML with IC
Glasdegib + LDAC	Newly diagnosed AML >75 years or unfit for IC
Venetoclax +HMA	Newly diagnosed AML >75 years or unfit*
Venetoclax + LDAC	Newly diagnosed >75 years or unfit*
Ivosidenib	Newly diagnosed >75 years or unfit* with IDH1^{MUT} RR AML with IDH1^{MUT}
Enasidenib mesylate	RR AML IDH2^{MUT}
Oral azacytidine	Post-IC Maintenance

AML MRC, and AML with myelodysplasia-related changes; HMA, hypomethylating agent; IC, intensive chemotherapy; LDAC, low-dose cytarabine; t-AML, therapy-related AML; RR, relapsed/refractory. *Adult patients with newly diagnosed AML who are >75 years old or who have comorbidities that preclude use of intensive induction chemotherapy.

(22). The superiority of midostaurin was independent of allo-HCT, with best results seen in patients receiving allo-HCT in CR1. Moreover, there was a trend toward better OS in all FLT3-mutation subtypes (TKD, ITD with low allelic ratio, and ITD with high allelic ratio) in the midostaurin arm (17). Another endpoint of the trial was to test the efficacy of 12-month maintenance with midostaurin, but only in patients not submitted to allo-HCT. Among 717 enrolled patients, only 174 began maintenance therapy, and only 104 completed the 12-month schedule. In the unplanned *post-hoc* subset analysis of the CALGB 10603/RATIFY trial reported by Larson et al. (23), there was no difference in DFS between the two arms (HR = 1.4 [95% CI, 0.63–3.3]; $p = 0.38$) from the end of maintenance. Moreover, midostaurin did not produce an improvement in OS from the start of maintenance (HR = 0.96 for M [95% CI, 0.58–1.59]; $p = 0.86$). Accordingly, midostaurin was licensed for the treatment of adult patients with newly diagnosed AML who are FLT3+ in combination with standard cytarabine and daunorubicin induction and cytarabine consolidation, but not as a single agent for maintenance therapy.

In order to answer some of the unanswered questions left by the RATIFY trial, the German–Austrian AML Study Group (AMLSG) designed the 16-10 trial. This was a phase 2, open-label, single-arm study that enrolled patients aged 18–70, designed to test the role of midostaurin during induction and consolidation, in association with chemotherapy, and as a maintenance (12 months) after allo-HCT (24). The trial enrolled 284 patients with FLT3-ITD only; 134 patients received allo-HCT during the first complete remission (CR1), 75 of whom received midostaurin maintenance. Patients started midostaurin after a median time of 71 days post-allo-HCT. The median duration of maintenance was 9 months, and toxicities were mainly gastrointestinal (70%) and infections (51%), but were low in grade and manageable. The 16-10 trial showed a statistically significant advantage for midostaurin maintenance over no maintenance for both EFS ($p = 0.01$, HR 2.51) and OS ($p = 0.02$, HR 2.64) (24).

Recently, the RADIUS trial tested, in a randomized fashion, the efficacy of 12-month maintenance with midostaurin after allo-HCT in 60 FLT3-ITD+ AML patients. Thirty patients received midostaurin, and 30 received standard-of-care therapy. However, only 16 patients in the midostaurin arm and 14 in the placebo arm completed the 12 months of treatment. The primary outcome, RFS, was comparable in the two arms. There were no differences in 24-month OS (25). A positive note regarded toxicity, intended as serious adverse events and GVHD (any grade) during maintenance, because there was no significant difference between midostaurin and placebo. Given the small sample size of the trial, it is not possible to draw a definitive conclusion on the role of midostaurin after allo-HCT.

Despite being not licensed for the treatment of AML, sorafenib has been one of the first and most widely used FLT3 inhibitors. It is an orally administered multikinase inhibitor with potent inhibitory activity against the Raf kinase and MAPK signaling pathway and FLT3, c-Kit, VEGFR, RET, and PDGFR. This drug was evaluated in many clinical trials in AML patients, both in combination with induction chemotherapy and for maintenance. Ravandi et al. reported a high response rate in patients with previously untreated AML who received a combination of sorafenib, cytarabine, and idarubicin (CR with incomplete recovery rates were 95%) (26).

In the SORAML randomized trial, sorafenib combined with standard induction chemotherapy significantly prolonged EFS (26 months) and RFS (63 months) as compared with placebo plus chemotherapy (9 and 22 months, respectively) in younger patients (≤60 years). Notably, only 17% of these patients had FLT3-mutated AML (27), and no significant difference was observed for patients with FLT3-ITD. The SORAML trial indicated, for the first time, the role of a maintenance therapy in young AML patients, independently from molecular aberrations, in addition to standard chemotherapy.

Additionally, in the context of post-transplant maintenance, results of the randomized phase 2 SORMAIN study (28) indicated that sorafenib reduces the risk of relapse (2-year RFS was 85% in the sorafenib group compared with 53.3% in the placebo group) and death. However, although well tolerated, sorafenib maintenance was associated with higher incidence of acute and chronic GvHD (≥3 grade: 76.8% in the sorafenib arm vs. 59.8% in the placebo group). More recently, Bazarbachi et al. reported the results of the European Group for Blood and Marrow Transplantation (EBMT) registry-based study of 62/462 patients with FLT3-mutated AML (FLT3-ITD-95%) who received posttransplant sorafenib either as a prophylactic ($n = 19$), as pre-emptive therapy ($n = 9$), or as treatment for relapse ($n = 34$) (29). On multivariate analysis, maintenance with sorafenib significantly improved leukemia-free survival (LFS) (hazard ratio [HR] = 0.35), OS (HR = 0.36), and graft-versus-host disease-free RFS (HR = 0.44).

On the other hand, second-generation inhibitors, such as gilteritinib, quizartinib, and crenolanib, were designed to selectively and potently inhibit the FLT3 receptor, and presumably have an improved tolerability at the concentrations necessary to fully inhibit FLT3 *in vivo*. Many early-phase trials combining the more potent next-generation FLT3-TKIs with 7 + 3 induction chemotherapy in the frontline setting have been reported recently with meaningfully high response rates. Gilteritinib has demonstrated the potent and selective inhibitory activity of FLT3-ITD and FLT3-TKD mutations, with additional inhibitory activity against EML4-ALK and Axl. In accordance with the final results of the phase 3 ADMIRAL study (30), in 2018, the FDA approved gilteritinib for FLT3-mutated, relapsed/refractory disease. In 2018, Pratz et al. reported the updated results of a phase 1/2 study of gilteritinib combined with 7 + 3 and HIDAC consolidation in 62 unselected AML patients. The CRc rate was 100% in FLT3-mutated patients, with a median DFS of 297 days (31).

Currently, numerous clinical trials of gilteritinib are underway to evaluate its role in various settings such as first-line, rescue, consolidation, or maintenance. In this last setting, the BMT-CTN 1506 study is ongoing to establish whether there is a benefit of FLT3 inhibition in the post-HCT setting and, if so, in which patients. This study is a randomized, double-blind, placebo-controlled, phase 3 trial conducted on 346 AML patients with FLT3-ITD mutation, randomly assigned in a double-blinded fashion to placebo or gilteritinib (32).

Crenolanib is another potent FLT3 inhibitor with activity against both FLT3-ITD and FLT3-TKD with additional inhibitory activity of PDGFR and c-Kit. Additionally, crenolanib is a potent inhibitor of the mutant FLT3-D835, which is one of the main mechanisms of resistance to FLT3 inhibitors. The addition of crenolanib to standard chemotherapy has been assessed in 29 young patients (<60 years) with newly diagnosed FLT3-mutated AML included in a phase 2 trial. After 20.8 months of median follow-up, CR was achieved in 72% after one cycle of induction, 81% of patients were alive, and median overall survival, event-free survival, and cumulative incidence of relapse had not been reached (33, 34). The randomized phase 3 trial is currently evaluating the addition of crenolanib vs. midostaurin to standard chemotherapy in newly diagnosed FLT3 mutated AML patients, and has been designed to match the same inclusion criteria as those of the RATIFY trial.

In summary, midostaurin is the only approved FLT3 inhibitor in combination with induction chemotherapy in newly diagnosed FLT3-mutated AML and it can be used regardless of FLT3 mutation settings (ITD, TKD, ITD with NPM1 mutation, FLT3-ITD low, and ITD with poor prognostic driver mutations). However, based on the very high response rate achieved with next-generation FLT3 inhibitors (80%–90%), two phase 3 randomized trials are ongoing and investigating frontline quizartinib/crenolanib + 7 + 3 chemotherapy (NCT02668653 and NCT03258931 respectively) (Table 3). Recent data confirmed that allo-HCT remains the best consolidation therapy for AML patients with FLT3-ITD pertaining to the high-risk ELN category, and should be performed as soon as possible in CR1 regardless of the use of FLT3 inhibitors. It is important to remember that patients with NPM1mut/FLT3-ITD low (<0.50 AR) included in the intermediate-risk AML category should be carefully considered for allo-HCT as first-line treatment, given their more favorable outcome with respect to high-risk ELN (36–38).

BCL2 inhibitors/venetoclax

Recently published phase 3 studies have established a new standard-of-care therapy in patients not eligible for intensive chemotherapy in light of survival benefit for patients treated with azacitidine plus venetoclax or LDAC plus venetoclax (39, 40). Where and when targetable mutations are found, it is expected that therapy may be optimized with the molecular-targeted doublet (i.e., substitution of HMA or LDAC for a molecularly targeted therapy) or triplet (the addition of a molecular-targeted therapy to a venetoclax backbone) with appropriate schedule modifications. These studies have also paved the way for other uses of venetoclax. In particular, in the setting of fit, younger patients, the combination of venetoclax with intensive chemotherapy and the role in the maintenance phase of therapy for patients in CR should be investigated. Early-phase trials have evaluated the combination of venetoclax with intensive chemotherapy and set the safety dose of venetoclax at 200 mg daily for 4 days in the younger population (41).

The MD Anderson group recently reported the interim results of a phase 1b/2 trial with the combination FLAG-IDA plus venetoclax in a mixed AML population (treatment naïve and R/R patients) (42). Twenty-nine newly diagnosed AML

TABLE 3 FLT3 inhibitors combined with intensive chemotherapy in frontline AML therapy: clinical trials.

FLT3 inhibitor + chemotherapy	Trial design	Enrolled/ goal	Primary results to date
Midostaurin vs. placebo plus 7 + 3 (cytarabine + daunorubicin) (22)	Phase 3	717 patients	CR/CRi: Midostaurin 54% vs. placebo 59% (p = NS) 4-year OS: Mido 51.4% vs. 44.3% (7.1% difference)
Midostaurin plus 7 + 3 (cytarabine + daunorubicin or idarubicin) and maintenance NCT03379727	Phase 3b	300 patients	Completed accrual; Results pending
Quizartinib plus 7 + 3 (35)	Phase 1	19 patients	CR/CRi = 84%; well tolerated
Quizartinib vs. placebo plus 7 + 3 (cytarabine + daunorubicin) NCT02668653	Phase 3	539 patients	Completed accrual; Results pending
Crenolanib plus 7 + 3 (cytarabine + daunorubicin/idarubicin) (33)	Phase 2	29 patients	CR/CRi = 72%; 2-year OS not reached
Crenolanib vs. midostaurin plus 7 + 3 (cytarabine + daunorubicin) NCT03258931	Phase 3	510 patients	Currently accruing
Gilteritinib plus 7 + 3 (cytarabine + idarubicin or daunorubicin) (31)	Phase 1	70 patients	CR/CRi = 93%

patients were enrolled. As expected, the most important grade III–IV toxicities were infections (febrile neutropenia 50%, bacteremia 35%, pneumonia 28%, and sepsis 12%). This combination induced a very high rate or MRD-negative composite CR (96%) in the newly diagnosed setting. Allo-HCT was performed in 69%, and 94% of patients were alive 1 year after the transplant. Even if the results are appealing, especially if we consider the high rate of MRD-negative remissions, numbers are very small and the median follow-up is too short to draw any significant conclusion. Patients with TP53 mutations continued to have poor outcomes despite treatment with this combination, and survival in TP53-mutated was significantly inferior to that of wild-type patients (43). Furthermore, a recent trial confirmed the safety and efficacy of the combination of venetoclax (400 mg *per os* on days 2–8) with the intensive CLIA regimen (cladribine, cytarabine, and idarubicin) in newly diagnosed AML. Fifty patients aged 18–65 years were enrolled; 47/50 (94%) achieved a remission, and only 1 patient died of toxicity during induction. Again, the combination induced a high rate of MRD-negative CR (82%), and infections were the most common grade III–IV adverse event reported. Notably, two patients with FLT-3 positive AML died due to infectious complications while on treatment with CLIA-VEN and FLT-3 inhibitors. Drug–drug interaction between venetoclax, FLT-3 inhibitors, and azoles, which are all metabolized *via* CYP3A, should be deeply investigated in order to avoid possible unexpected toxicities in this population of AML patients. Twelve-month EFS and OS are promising, but also in this case, we need to wait for more data (and more mature) in order to correctly weigh the impact of the combo in newly diagnosed AML (44).

Notably, the US investigators recently started a prospective, multicenter, double-blind, randomized, placebo-controlled phase 3 clinical study (NCT04628026) exploring the role venetoclax plus intensive chemotherapy in newly diagnosed patients with AML. Different dosages of venetoclax (100, 200, and 300) will also be tested in the trial, in order to clarify the role of bcl-2 inhibition in combination with intensive chemotherapy (Table 4).

Currently, too many questions remain unanswered: the dosage of venetoclax, the duration of therapy, the characteristics of the patients, both clinical and biological, and the lack of a randomized trial still do not allow us to recommend its use outside from clinical trials, in the younger population fit for intensive chemotherapy.

Monoclonal antibodies

Since the early 1980s, attempts to use antigen-targeted immunotherapy to selectively kill AML cells resulted in the development of monoclonal antibodies (mAbs). However, none of them have demonstrated a significant efficacy to be incorporated into standard of care. Nonetheless, recent advancements in antibody engineering have attracted a lot of interest in antibody-based therapies in AML. Reinforcing mAbs by investigating agents with novel targets or mechanisms, as well as combination strategies, hold a promise to make a great progress in this field.

CD33 (SIGLEC-3) is a member of the sialic acid-binding immunoglobulin-like lectin (Siglec) family, which has been the most exploited target in AML treatment due to its expression on at least one subset of leukemic blasts in almost all patients. CD33 is highly expressed in acute promyelocytic leukemia (APL), NPM1-mutated AML, and FLT3/ITD mutated AML, whereas expression is usually low in leukemias with core-binding factor translocations (45).

Gemtuzumab ozogamicin (GO) is a highly potent antitumor antibiotic disulfide derivative of calicheamicin-γ1 conjugated to a recombinant humanized antibody (IgG4) against CD33. GO targets CD33, allowing the fast release of the toxic part in the tumor cell lysosomes (46). Calicheamicin binds to the minor groove of DNA and triggers single- and double-stranded breaks,

TABLE 4 Bcl2 inhibitors/Venetoclax + intensive chemotherapy in AML young and fit patients.

Venetoclax + chemotherapy	Trial design	Enrolled/ goal	Primary results to date
Venetoclax plus FLAG-IDA (NCT03214562)[36]	Phase 1b/2	116 patients	Recruiting. Results on 29 patients enrolled: CR MRD negative = 96%; allo-HCT = 69%; alive 1 year after transplant = 94%
Venetoclax plus CLIA (NCT02115295)[38]	Phase 2	458 patients	Recruiting. Results on 50 patients enrolled: CR MRD negative = 82%
Venetoclax plus 7 + 3 (NCT05356169)	Phase 2/3	300 patients	Not yet recruiting
Venetoclax and azacitidine for non-elderly adult patients (NCT03573024)	Phase 2	36 patients	Recruiting
Venetoclax plus intensive chemotherapy (7 + 3) (NCT04628026)	Phase 3	650 patients	Recruiting

inducing cell death through mitochondrial pathways and caspase activation. Single-drug activity in three uncontrolled phase 2 studies resulted in accelerated regulatory approval of GO in 2000 by FDA for patients with CD33-positive AML in first relapse (47). The preliminary data from phase 3 trial SWOG S0106 were associated with a significantly higher risk of fatal adverse events during the induction phase, which led to early trial termination and the decision to withdraw the drug in 2010 (48). Four other randomized trials investigated whether adding GO to the first cycle of intensive chemotherapy of adults with newly diagnosed AML improved outcomes: MRC/NCRI AML-15 (49), AML16 (50), ALFA-0701 (51), and GOELAMS AML 2006 IR (52). Despite the heterogeneity between the studies, adding GO resulted in significantly improved survival in all studies except S0106 (48), which, unlike the other studies, used a lower daunorubicin dose (45 mg/m^2) in the experimental vs. control arm (60 mg/m^2). A recent meta-analysis on 3,325 subjects reported that adding GO had no impact on remission rate but reduced 5-year cumulative incidence of relapse [OR = 0.81 (p = 0.0001)] and improved survival [OR for death = 0.90 (0.82, 0.98), p = 0.01] (53). These benefits were confined to those with favorable risk disease (n = 246), in whom survival probability at 6 years was 55% without, vs. 76% with GO (odds ratio 0.47, 95% confidence interval 0.31–0.73; p = 0.0006) and with intermediate cytogenetics (34% vs. 39%, odds ratio 0.84, 95% CI 0.75–0.95, p = 0.005). One of the major adverse effects of GO is that it increases the risk of fatal hepatic injury and sinusoidal obstruction syndrome [formerly known as a veno-occlusive disease (VOD)], especially when administered before HSCT. The AML 17 trial confirmed that single doses higher than 3 mg/m^2 should not be employed because of increased incidence of VOD and early mortality (54). GO should routinely be combined with 7 + 3 or FLAG-IDA in those with ELN favorable or intermediate risk assuming an acceptable risk of TRM. Fournier et al. also found the addition of GO in patients with mutations in NPM1, CEBPA, FLT3 ITD or TKD, NRAS, KRAS, and SRSF2 to be beneficial (55).

Expression levels of CD33 and the correlation with clinical response have been evaluated from multiple phase 2 and phase 3 clinical trials. The results from two published meta-analyses did not reveal any significant association between leukemic blast CD33 expression and GO clinical efficacy (56, 57). Recently, six nonsynonymous single-nucleotide polymorphisms (SNPs) were reported to have clinical relevance in pediatric patients treated with GO (58). The most interesting of these polymorphisms is CD33 SNP rs12459419 (C<T; Ala14Val), resulting in a shorter isoform of CD33 that lacks exon 2 (loss of the IgV domain) within the CD33 protein (55). Loss of the V-set domain directly impact the binding, internalization, and clinical efficacy of GO is associated with differential response in GO versus no-GO treatment arms. Specifically, patients with at least one copy of the variant T allele (CT/TT genotypes) derived no benefit from the addition of GO (59). Validation studies for the role of CD33 SNP in mediating response to CD33-directed therapy are forthcoming.

Cpx-351

One-fourth of AML cases is secondary to previous hematological disorders (sAML) or developing after chemotherapy or radiotherapy (tAML) (60). sAMLs and tAMLs are more frequent in older patients, and their prognosis is often worsened by the presence of adverse cytogenetic, high-risk molecular aberrations, and impaired performance status. Allo-HSCT is the only curative therapeutic option in this unfavorable setting, where conventional treatment is usually able to induce less than 40% short-term CRs. In 2017, FDA approved CPX-351 (VYXEOS®, Jazz Pharmaceuticals), a liposomal encapsulation of cytarabine and daunorubicin, with a molar ratio of 5:1 for patients with t-AML or with AML and "myelodysplasia related changes" (AML-MRC). The approval followed the phase 2 clinical trial (NCT00788892) of CPX-351 versus 7 + 3 in older patients (60–75 years) with newly diagnosed AML. The study was conducted on 127 patients randomly assigned to receive CPX-351 or 7 + 3. CPX-351 produced a higher CR/Cri rate than 7 + 3 (66.7% vs. 51.2%), without statistical difference in EFS and OS. In a subgroup analysis of patients with secondary AML, the CR/CRi rate was higher (57.6% vs. 31.6%) with an improved EFS (HR = 0.59, p = 0.08) and OS (HR = 0.46, p = 0.01) (61).

In the setting of newly diagnosed secondary AML, a randomized study conducted on 309 patients, aged 60 to 75 and treated with CPX or 7 + 3 (daunorubicin at the dose of 60 mg/m^2), showed C/CRi rates of 48% vs. 33% (p = 0.02) and event-free survival longer with CPX (p = 0.02, medians 2.5 vs. 1.3 months) (62). Recently published data with a median follow-up of 5 years confirm a reduction in the risk of death by 30% (HR 0.7, 95% CI 0.6–0.9, and thus $p < 0.05$, medians 9.3 months CPX, 6.0 months 7 + 3) with the CPX administration (63). The best outcome was observed in patients who promptly underwent transplant after achieving CR.

Given its efficacy, with a favorable safety profile, CPX-351 is now under investigation, alone or in combination, also in the younger population. Few data are available up to now, in a limited number of patients, and thus they should be taken with caution. First, data from a multi-institutional retrospective analysis on 30 younger patients (mean age, 53 years; range, 23–59) with confirmed s-AML showed a worst outcome for CPX-351, with respect to the elderly population. Response rate was unsatisfactory (CR/CRi 27.6%), and median overall survival was shorter (7 months) than reported in the recently published phase 3 trial in patients aged 60–75 years old (64). However, 10/30 patients had TP53 mutation, 19/30 had complex karyotype, and 6 had received prior HMAs, thus representing a very high-risk population with extremely dismal outcome. A phase 2 trial is

now prospectively enrolling AML patients with secondary AML aged less than 60 years (NCT04269213), in order to clarify the role of CPX-351 in this setting.

More interest, however, is coming from the possible use of CPX-351 in combination with other drugs, since preclinical data suggest a synergistic activity in combination with venetoclax or FLT3 inhibitors (65). The ongoing V-FAST study (65) is evaluating the safety and establishing the recommended phase 2 dose (RP2D) of CPX-351 combined with other agents for patients with newly diagnosed AML aged 18–75 years. Preliminary data on 26 patients were presented at the EHA 2021 (65). RP2D was established for all the combinations. Even if the results are preliminary, it seems that the combination of venetoclax+CPX-351 is quite toxic (three deaths due to AE, one due to sepsis), with CR rate approximately 50%. On the contrary, the combination of CPX-351 with midostaurin and enasidenib was very well tolerated and able to induce 100% of CR, paving the way for further exploration. The phase 2 of the study is enrolling patients (NCT04075747).

Azacitidine maintenance

A randomized phase 3 trial (HOVON97) assessed the value of azacitidine as post-remission therapy, in older patients (≥60 years) with AML or myelodysplastic syndrome, in CR/CRi after at least two cycles of intensive chemotherapy. A total of 116 eligible patients were randomly (1:1) assigned to either observation (N = 60) or azacitidine maintenance (N = 56; 50 mg/m^2, subcutaneously, days 1–5, every 4 weeks) until relapse, for a maximum of 12 cycles. Fifty-five patients received at least 1 cycle of azacitidine, 46 at least 4 cycles, and 35 at least 12 cycles. DFS was significantly better for the azacitidine treatment group (logrank; p = 0.04), and the 12-month DFS was estimated at 64% for the azacitidine group and 42% for the control group. OS did not differ between treatment groups (66).

Also, single treatment with the oral azacitidine formulation CC-486 has been investigated in different clinical settings. In the phase 3, randomized QUAZAR AML-001 trial, CC-486 (300 mg once daily on days 1–14 every 28 days) significantly improved OS and RFS in older AML patients who were in first remission after intensive chemotherapy and not candidates for allogeneic HSCT (67). In a phase 1/2 study in 28 AML/MDS patients in CR after HSCT, CC-486 maintenance therapy showed preliminary efficacy, with an estimated 1-year survival rate of 86% and 81%, respectively (68); the phase 3 trial is ongoing (NCT04173533). In the context of young patients with AML, oral azacitidine should probably find its place in patients unfit for allo-HCT. CC-486 could also represent an excellent bridging therapy to allo-HCT in CR patients waiting for the donor selection; it can be used after consolidation cycles, without the need for hospitalization, thus reducing the risk of relapse.

To date, maintenance therapy with oral azacitidine is licensed only for patients aged ≥55 years with AML and intermediate- or poor-risk cytogenetics who achieved CR/CRi after intensive chemotherapy ± consolidation and are transplant-ineligible.

HSCT

Upfront allo-HSCT for adult AML patients in CR1 is the gold standard, if cure is the final goal of treatment. Its usefulness was firstly demonstrated in 2005, with a meta-analysis of five studies in which allo-HCT was performed in CR1 AML patients from an HLA-identical sibling donor. This meta-analysis revealed that the efficacy of allo-HSCT for patients with AML in CR1 depends on cytogenetic risk: the beneficial effect of allo-HSCT was evident for the poor-risk group patients, and probably for the intermediate-risk groups, but was absent for the favorable-risk group (69).

Also in 2009, another meta-analysis of clinical trials was performed using a "donor versus no donor" methodology, which showed statistically significant RFS and OS for allo-HCT in young patients with intermediate- and poor-risk AML (70).

The pivotal question in deciding whether to recommend HSCT in CR1 is if the reduction in relapse risk reached with the transplant outweighs NRM. Since the NRM of fit AML patients transplanted with a matched sibling or unrelated donor is approximately 15%, patients with a relapse risk >50% are likely to benefit from allo-HSCT. In recent years, the prognostic classification of AML patients has been refined, and several factors influence the success of intensive chemotherapy. Among these factors, hyperleukocytosis (>100,000/mmc) at diagnosis, secondary AML, adverse karyotype or adverse genetic risk, and a resistant disease have been identified as bad prognostic factors (2). Finally, yet importantly, persistence of MRD positivity after consolidation therapy is also an important independent predictor of relapse risk in AML, and can be used to refine the decision-making process (19). Nonetheless, two scoring systems are able to predict transplant outcome in AML, specifically NRM. The EBMT score comprises age, donor type, HLA disparity, and disease status, and is able to stratify NRM risk between 15% and 45% (71). The HCTCI, derived using a weighted assessment of 17 comorbidities, and the updated comorbidity-age index (age cutoff, 40 years), has also been shown to predict NRM and OS after allo-HCT, and has been validated in AML (72, 73). Recently, a simplified comorbidity index (SCI) has been developed in a single-center cohort of 573 adult patients (217 AML patients) who underwent CD34-selected allo-HCT following myeloablative conditioning. The SCI includes comorbidities associated with a significant increase in NRM: cardiac comorbidity, pulmonary disease, hepatic injury, and renal disfunction. Age with a cutoff of 60

years was also included because it is related to NRM risk (HR 1.64, 95% CI, 1.23–2.19; $p = 0.001$) (74).

Another matter of debate is the optimal conditioning regimen for young, fit patients with AML. In fact, despite optimization of transplant procedures, leukemia recurrence remains the main cause of transplant failure. Recent lines of evidence, coming from randomized clinical trials, demonstrated that reducing the conditioning intensity might not be the right strategy in AML. Recently, a phase 3 clinical trial compared outcomes by conditioning intensity [myeloablative conditioning (MAC) or a reduced-intensity conditioning (RIC)] in adult patients with myeloid malignancy undergoing an allo-HCT while in morphologic CR. Conditioning intensity made no difference in MRD-negative patients who underwent transplantation. In patients with a detectable mutation by NGS, relapse (3-year cumulative incidence, 19% MAC vs. 67% RIC; $p < 0.001$) and survival (3-year OS, 61% MAC vs. 43% RIC; $p = 0.02$) were significantly different between the two arms. Multivariable analysis for NGS-positive patients, adjusting for disease risk and donor group, confirmed the inferior OS for patients receiving RIC compared with MAC (HR, 1.97; 95% CI, 1.17 to 3.30; $p = 0.01$) (75).

The combination of thiotepa, busulfan, and fludarabine (TBF) could represent a valid alternative, as a myeloablative regimen, to busulfan and cyclophosphamide in AML patients. In a registry-based retrospective study reported by the EBMT group (76) and in a multicenter trial (77), TBF regimen confirmed its antileukemic potential with an impressive low number of relapses in patients transplanted in CR1, which translated in a trend towards better LFS in AML patients.

Conclusions

Intensive chemotherapy, with or without targeted agents, remains the backbone of treatment in fit patients with AML. Treatment of patients with favorable ELN risk does not necessitate an allo-HCT to cure those patients. On the contrary, allo-HCT is the most effective strategy to achieve MRD-negative CR, which is the ideal condition to pursue a long-lasting OS in patients with intermediate or adverse ELN risk. In these patients, it is necessary to further investigate venetoclax with or without intensive chemotherapy or other targeted agents, to evaluate its potential role in increasing MRD-negative CR after induction/consolidation and prior to allo-HCT. Furthermore, maintenance therapy after allo-HCT should be considered for patients with either FLT-3-positive AML or adverse-risk features. Maintenance with sorafenib or midostaurin, even if not formally approved in all countries, demonstrated a survival advantage for patients with FLT-3-positive AML, when administered after allo-HCT, even if burdened with a certain degree of toxicity. Small phase 2 clinical trials suggest that HMAs, with or without drugs targeting bcl-2 or specific molecular markers, could be used as a safe and effective maintenance in patients with adverse ELN risk, with high relapse risk. MRD monitoring, in order to early identify MRD fluctuations or relapse, should be performed in all patients, in order to support an early intervention in those adverse risk patients not receiving any maintenance after allo-HCT. In conclusion, even if the landscape of AML treatment is changing, there is still room for improvement for survival of the fit and young population. Further development of precision medicine, together with the improvement of the knowledge of the biological mechanisms of the interactions between immune cells and AML blasts, will probably lead us to the next level, in order to significantly increase the number of AML patients for whom a curative intent is possible.

Author contributions

GV, MC, and AI wrote, commented and approved the manuscript. SP and FL wrote the revised version of the manuscript, commented and approved the final version of the manuscript. GV and AI equally contributed to the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Targeting CD38 for acute leukemia

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Acute leukemia (AL) is a hematological malignancy, and the prognosis of most AL patients hasn't improved significantly, particularly for relapsed or refractory (R/R) AL. Therefore, new treatments for R/R adult acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) are urgently necessary. Novel developments have been made in AL treatment, including target and immune therapies. CD38 is one of the targets due to its high expression in many hematological malignancies, including multiple myeloma, ALL and a subset of AML. Consequently, targeting CD38 therapies, including CD38 monoclonal antibodies (mAbs), bispecific antibodies, and CAR-T cell therapy, exhibit promising efficacy in treating multiple myeloma without significant toxicity and are being explored in other hematological malignancies and nonhematological diseases. Herein, this review focuses on targeting CD38 therapies in ALL and AML, which demonstrate sound antileukemic effects in acute leukemia and are expected to become effective treatment methods.

KEYWORDS

CD38mAb, bispecific antibodies, anti-CD38 CAR T, acute myeloid leukemia, acute lymphoblastic leukemia

Introduction

Acute leukemia (AL), including acute myeloid leukemia (AML) and acute lymphocytic leukemia (ALL), is a hematological malignancy originating from the abnormal cloning of hematopoietic stem cells. It is characterized by aberrant proliferation of immature hematopoietic cells, thereby inhibiting normal bone marrow hematopoietic function. The prognosis of most AML patients has not improved significantly owing to its heterogeneity. Indeed, its five-year overall survival rate is less than 40% in patients under 60 years old, with a worse prognosis in patients older than 60 years old as well as in relapsed or refractory (R/R) AML (1, 2). Similarly, the prognosis of adult ALL is poor, particularly for R/R ALL patients, with an overall survival rate of less than 7% (3, 4). Therefore, new treatments for R/R adult AML and ALL are urgently needed. Based on a better understanding of the pathogenesis and pathophysiology of AL in recent years, novel advancements have been made in AL treatment, including target and immune therapies. CD38 is one of the targets in view of its high expression in several

hematological malignancies, particularly in multiple myeloma (MM), with minimal or no expression in normal tissues (5). CD38-targeting therapies, including CD38 monoclonal antibodies (mAbs), bispecific antibodies, and CAR-T cell therapy, have exhibited outstanding efficacy in treating MM without significant toxicity. They are also being explored for other hematological malignancies and have demonstrated promising outcomes. This review focuses on the utilization of targeting CD38 therapies in ALL and AML, including preclinical and ongoing clinical trials of these agents, with the goal of providing novel insights for the treatment of R/R AL.

Structure and functions of CD38

Reinherz EL et al. discovered CD38 in 1980 while searching for T cell receptors *via* murine monoclonal antibodies. They initially thought it was a molecule that activates thymus T cells (6). Later, CD38 was revealed to be a class II transmembrane glycoprotein on the cell surface that contains a C-terminal extracellular domain, a transmembrane domain, and an N-terminal intracellular domain (7–9), acting as a tetramer on the cell surface (9).

The CD38 gene is located on chromosome 4p15 and consists of eight exons and seven introns (10, 11). The CD38 promoter region comprises a CpG island, several immunological transcription factor binding sites located upstream of the CpG island (11), and a retinoic acid response element in the first intron (12). CD38 transcription and expression are enhanced by binding to the aforementioned sites.

The human CD38 antigen acts as a receptor (5, 8, 13, 14) and a catalytic enzyme (5, 15–18). As a receptor, it is primarily involved in cell adhesion and migration through binding with CD31 and is also related to cell activation, proliferation, and differentiation. Apart from receptor functions, CD38 exerts extracellular enzyme activities with multiple functions of cyclase and hydrolase. It uses nicotinamide adenine dinucleotide (NAD+) as a substrate to generate cyclic ADP ribose (cADPR) and ADPR and catalyzes nicotinic adenine dinucleotide phosphate (NAADP+) production from nicotinamide adenine dinucleotide phosphate (NADP+) (5, 15–18), thereby mediating Ca2+ influx (19, 20), activating various signaling pathways (20, 21), and participating in effector cell-mediated immunosuppression (22).

CD38 expression in healthy tissues and diseases

CD38 is predominantly expressed in hematopoietic cells such as lymphocytes and myeloid cells, with a high expression in early and activated cells and a low expression in mature cells (5). Previous studies have validated that CD38 is highly expressed in plasma cells (378.4 nTPM) but moderately expressed in activated T cells (64.8 nTPM) and NK cells (79.3 nTPM) (5). In addition, it is expressed in germinal center lymphocytes, dendritic cells (33.8 nTPM), red blood cells (78.6 nTPM), and platelets (5, 23–25). Additionally, nonhematopoietic tissue cells, including Purkinje cells, neurofibrils, prostate endothelial cells (8.1 nTPM), islet β cells, retinal cells, and muscle fibroblasts of smooth and striated muscles (41.5 nTPM), express CD38 (5).

Various studies have corroborated that CD38 is expressed at different levels in various neoplastic and nonneoplastic diseases, particularly in hematological malignancies such as multiple myeloma (100% CD38+) (26), chronic lymphocytic leukemia (30-50% CD38+) (27), Waldenstrom macroglobulinemia (40% CD38+) (28), primary systemic amyloidosis (53% CD38+) (29), mantle cell lymphoma (60% CD38+) (30), T cell lymphoma (50-80% CD38+) (31), NK/T cell lymphoma (90% CD38+) (32, 33), etc.

CD38 is expressed in AML and ALL. Marinov J was the first to evaluate CD38 expression in 72 leukemia patients and noted that the positive rate of CD38 was up to 75% in myeloid tumors and acute non-T lymphoblastic leukemia (34). Afsaneh K et al. reported that 58.2% of 304 AML cases expressed CD38, whereas only 5% of acute promyelocytic leukemia (APL) cases did (35). Wang et al. conducted an immunophenotypic analysis on 109 AML patients and noted that the positive rate of CD38 was as high as 91.7% (100/109) (36). Although the positive rates of CD38 in AML reported by various studies are not low, its expression level on cell surfaces varies significantly. Naik et al. examined CD38 expression in leukemia cells from 37 AML patients and found significant differences in expression levels in these samples, ranging from 300+ to 6000+ of CD38 antigen density on leukemic blast (37). They further measured CD38 levels in AML patients upon diagnosis and after treatment with MRD; no significant difference was found. Similarly, Dos Santos et al. examined CD38 expression levels in AML cell lines (n=9) and AML patient cells (n=10) (38), and confirmed that CD38 expression significantly differed between AML cell lines (12,827 ± 19,320 molecules/cell) and AML primary cells (11,560 ± 8,175 molecules/cell). In another study of AML cell lines, CD38 expression levels significantly differed among AML cell lines (39). Indeed, the cell surface CD38 density of seven AML cell lines (HL-60, U-937, THP-1, MOLM-13, UOC-M1, Oci-AML2, and KasUMI-1) was detected, with CD38 levels ranging from very high (UOC-M1, 46.5 ± 4.9), high (OCI-AML2, 16.6 ± 1.5), low (THP-1, 7.0 ± 0.2), very low (molm-13, 2.1 ± 1.0 and Kasumi-1, 1.1 ± 1.2), to CD38-negative (HL-60, U-937).

For ALL, Afsaneh K et al. determined that the positive rate of CD38 in 138 ALL patients accounted for 78.2% of all cases (35). Immunophenotypic analysis of 282 ALL patients conducted in Iraq by Sana D. Jalal et al. revealed that 80.5% (194/241) of B-ALL patient samples and 95.1% (39/41) of T-ALL patient samples expressed CD38 (40). Jutta Deckert et al. investigated CD38 expression levels in different CD38-positive cell lines and identified that CD38 levels were significantly lower in B-ALL

cells than in T-ALL cells (41). A study examining 50 B-ALL and 50 T-ALL primary cells showed that the median density of CD38 in T-ALL and B-ALL samples was 41,026 copies/cell and 28,137 copies/cell, respectively (42). According to Naik et al., CD38 expression levels were more consistent in 12 adult T-ALL samples than in 37 samples from AML patients at the same time (37). Collectively, these studies suggest that the CD38 expression level in T-ALL is more consistent than that in AML, with higher expression than that in B-ALL. Moreover, Bride et al. identified that CD38 was stably expressed in tumor cells of T-ALL patients, regardless of diagnosis, after induction chemotherapy or relapse (43). Researchers from India determined comparable results when examining CD38 expression levels in patients with T-ALL at diagnosis, after chemotherapy, in relapses, and in refractory status (44).

CD38 mAbs

CD38 is abundantly expressed in multiple myeloma (MM) (26) and some non-Hodgkin's lymphomas (NHLs) (27, 30, 33, 34), and is emerging as a new therapeutic target for these diseases. In addition, CD38 mAbs, including daratumumab (DARA), isatuximab, MOR202, and TAK079, have exhibited remarkable efficacy in MM.

Akin to other mAbs, CD38 antibodies inhibit tumor cell growth through complement-dependent cytotoxicity (CDC) (41, 45–47), antibody-dependent cytotoxicity (ADCC) (41, 45, 46), and antibody-dependent macrophage phagocytosis (ADCP) (48, 49) and induce apoptosis (45, 50). In addition, CD38 mAbs can prevent calcium influx and inhibit cell signal transduction by suppressing the catalytic activity of CD38 cyclase and hydrolase (51). It can further inhibit tumor cell growth by modulating the bone marrow microenvironment (52). (Figure 1) However, different monoclonal antibodies have varied action focuses. DARA is a humanized IgG1κ monoclonal antibody(mAb) that specifically binds to the epitopes of the cell surface of CD38, directly killing tumor cells through immune-mediated cytotoxicity, such as ADCC, CDC, and ADCP, and inducing apoptosis *via* FCγ receptor-mediated cross-linking (45–50). It can further inhibit the enzyme activity of CD38 and influence tumor cell metabolism (51). In addition, DARA can regulate the bone marrow microenvironment (52), activate immune cells, kill tumors, or directly govern the growth of tumor cells by inhibiting mitochondria (53). FDA approved it in 2015 for refractory or relapsed MM.

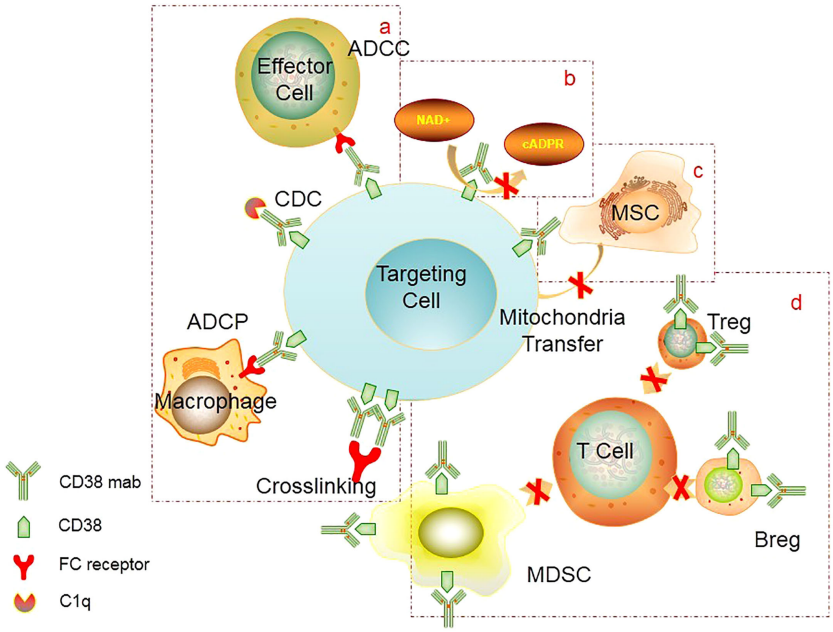

FIGURE 1
Mechanism of CD38 mAb: **(A)** FC-mediated ADCC, ADCP, cross-linking to induce apoptosis, CDC. **(B)** Inhibition of CD38 activity. CD38 mAb can block the catalytic activity of CD38 cyclase and hydrolase, thereby preventing calcium influx and inhibiting cell signal transduction. **(C)** Inhibition of mitochondrial transfer. CD38 mAb can inhibit mitochondrial transfer from mesenchymal stem cells to AL cells, thereby reducing metabolic capacity of tumor cells and ultimately inhibiting tumor proliferation. **(D)** Inhibition of immunosuppressive cells. CD38 mAb can eliminate CD38+ regulatory cells (Treg, Breg, MDSC), which can promote the expansion of effector T cells and enhance the immune killing effect. ADCC, Antibody-dependent cell-mediated cytotoxicity; ADCP, Antibody-dependent cellular phagocytosis; CDC, complement-dependent cytotoxicity; MSC, mescenchymal stem cell; MDSC, myeloid-derived suppressor cells; Treg, regulatory T cells; Breg, regulatory B cells.

Isatuximab, another humanized IgG1κ mAb with a different action site from DARA, has a significant pro-apoptotic effect on tumor cells. It can effectively inhibit CD38 cyclase action, activate NK cells, and inhibit CD38-positive T regulatory cells (41). FDA approved it in March 2020 for the treatment of R/R MM. It is currently being evaluated in CD38-positive hematological malignancies for its efficacy and safety.

MOR202, a humanized CD38 antibody developed by Morphosys, is being studied as a monotherapy and combined with lenalidomide/dexamethasone or pomalidomide/dexamethasone in phase I/II trials for the treatment of refractory or relapsed MM.

TAK079, another humanized CD38 antibody(IgG1λ mAb) and TAK053, an IgG4mAb, are both in the preliminary stage

Based on the high expression of CD38 on the surface of acute leukemia cells, CD38 mAb has also gradually been applied for the treatment of acute leukemia.

Application of CD38 mAbs in ALL

Preclinical studies in ALL

Studies on CD38 mAbs in B-ALL are scarce. Jutta Deckert et al. confirmed that isatuximab inhibited B-ALL cell line growth through ADCC *in vitro* (41). In a B-ALL xenograft model, isatuximab also exerted an antitumor effect, effectively prolonging mouse survival. Anlai Wang et al. reported that isatuximab exerts ADCC and ADCP effects on ALL cell lines (16 T-ALL and 11 B-ALL) and can induce robust antitumor activity *in vivo* (42).

Numerous studies have explored the efficacy of CD38 mAbs in T-ALL. It has been reported that Daratumub, Isatuximub, and other CD38 mAbs can inhibit the growth of T-All cell lines *in vitro* and exert their effects through ADCC, CDC, ADCP, and proapoptotic mechanisms (37, 41–43). In a mouse xenograft model, Naik et al. determined that DARA significantly lowered tumor load in mice compared to the control group (37). However, a patient sample (T-ALL2) mouse model manifested early death compared to the other T-ALL mouse models. The researchers hypothesized that this might be due to the high aggressiveness of the T-ALL2 patient sample. They used luciferase to label samples from this patient and then implanted the labelled samples into mice following the addition of DARA. They found no early death in mice and detected a significant reduction in fluorescence intensity. Bride et al. successfully constructed a PDX model in nonobese diabetic/severe combined immunodeficient (NOD/SCID/Il2rgtm1wjl/SzJ) mice from 15 pediatric T-ALL patients (seven ETP and eight non-ETP) (43). Daratumumab significantly reduced the leukemic burden in the bone marrow and spleen in six of the seven ETP samples compared to the control group (IgG1 mAb). Mice that received five of the eight non-ETP samples perished after the initial administration of DARA, presumably due to tumor lysis. When the tumor burden was reduced in the model, that is, following the administration of DARA, and the model was similar to the MRD-positive state, DARA exhibited significant therapeutic benefits in all eight non-ETP patient sample models compared to the control group. Both studies conducted by Naik and Bride concluded that DARA has an antitumor effect in T-ALL and can effectively eliminate MRD. Studies on its mechanism of action have identified that DARA has immune cell-mediated effects on ADCC and CDC in immune-normal mice and may promote apoptosis and inhibit CD38 enzyme activity in immune-deficient mice through nonimmune cell-mediated activities.

Regarding studies on DARA combinations, Fotini et al. demonstrated that DARA monotherapy or combined with chemotherapy can effectively improve xenograft mouse survival (54). All the mice had long-term asymptomatic survival in the DARA plus chemotherapy group. This suggests that DARA possesses remarkable antileukemia properties and may enhance the effect of chemotherapy. The researchers further assessed MRD in the bone marrow of living mice *via* PCR; seven out of eight mice were MRD-negative. These results signify that DARA also plays a decisive role in eliminating T-ALL with persistent positive MRD. P Doshi et al. described that DARA effectively inhibited the proliferation of T-ALL ($P < 0.05$) and B-ALL ($P < 0.001$) in animal models compared to the control group. DARA alone or combined with vincristine significantly prolonged mouse survival (> 88 days) compared to 22 days in the control group and 43 days in the vincristine monotherapy group (55).

Correlation between CD38 density in ALL and efficacy of CD38 mAb

The correlation between CD38 density in ALL and the efficacy of CD38 mAbs remains controversial. Jutta Deckert et al. assessed the antitumor effect of isatuximab in 15 cell lines with different CD38 expression levels (including four T-ALL and one B-ALL) and found that isatuximab induced an ADCC effect in all cell lines (41). However, its proapoptotic and CDC effects were more evident in cell lines with high CD38 expression. Similarly, Anlai Wang et al. speculated that the effects of isatuximab-mediated ADCC and ADCP were positively correlated with CD38 expression levels (42). However, Fotini did not find a correlation between CD38 expression level and DARA efficacy (54). P Doshi et al. likewise reported that DARA-mediated effects of ADCC and CDC were not directly related to CD38 expression levels (55). As previously mentioned, the antitumor effect mediated by CD38 mAb is related to the affinity between the antibody, tumor antigen, antibody, and Fc receptor. In ALL studies, distinct types of CD38mAbs were correlated with CD38 expression levels, potentially due to antibody-antigen affinity. More studies are necessary to elucidate this possibility.

Clinical application in ALL

Several studies concerning the efficacy and safety of DARA in ALL have been reported in recent years. Bonda et al. reported for the first time a patient with ETP-ALL who relapsed after two allogeneic hematopoietic stem cell transplantations (allo-SCTs) and multiline chemotherapy (56). Following reinduction chemotherapy, the bone marrow morphology revealed 4% blasts, while MRD reached 15.6%. Owing to the high expression of CD38 in the patient's leukemic cells, DARA was administered. MRD was negative after four doses (16 mg/kg weekly on days 1, 8, 15, and 22). Then, the patient received DARA every two weeks for 16 weeks, along with 6-mercaptopurine and methotrexate. MRD negativity lasted for more than five months. Sumeet Mirgh et al. reported that patients with ETP were MRD-positive after chemotherapy and allo-SCT (57). Although the expression of CD38 was downregulated in residual blasts, the patient still received DARA treatment (16 mg/kg weekly for two weeks) and became MRD-negative. Yisai et al. also confirmed that in two patients with non-ETP ALL who relapsed after allo-SCT, MRD was positive after nelarabine treatment but became negative after DARA therapy (16 mg/kg dose weekly on days 1, 8, 15, and 22) (58).

T-ALL patients ineligible for standard chemotherapy, including ETP patients, can achieve MRD negativity with DARA monotherapy, followed by allo-SCT (Sumeet Mirgh et al. utilized a DARA regimen of 16 mg/kg dose weekly for eight weeks, then every two weeks for eight weeks, followed by monthly doses for two months) (57) or to reduce tumor burden before receiving standard chemotherapy (Sandra D. R used a DARA regimen of 16 mg/kg dose on days 1 and 15 for two weeks) (59). Regrettably, these two patients eventually died as a result of infection and disease relapse. These reports are also consistent with the results of preclinical studies demonstrating that CD38 mAb (DARA) has an excellent antileukemia effect in T-ALL, especially MRD-positive T-ALL. Yisai et al. reported a case of a B-ALL patient who relapsed after BITE (anti-CD3/CD19) therapy (58). Due to the high expression of CD38 in leukemia cells, DARA was added (16 mg/kg dose weekly for three weeks), and CR was achieved. Eventually, allo-SCT was performed, but the patient relapsed three months after transplantation. (Table 1).

A phase II trial of daratumumab combined with standard chemotherapy for relapsed and refractory B-ALL and T-ALL in children and young adults is currently underway (ClinicalTrials.gov identifier: NCT03384654). The study included B-ALL patients with two or more relapses or who had been refractory to at least two chemotherapy regimens, as were T-ALL patients relapsing for the first time or who had been refractory to one chemotherapy regimen. All patients were treated with a combination of daratumumab, prednisone, and vincristine. In the T-ALL group, doxorubicin, PEG-asparagase, cyclophosphamide, cytarabine, 6-mercaptopurine,

TABLE 1 Case reports of CD38 targeted therapies in AL.

Author	Age	Sex	Disease	Previous treatment	Disease status before CD38 targeted therapy	Treatments	response to therapy	last follow-up
Bonda A et al.	32yrs	female	ETP ALL	Chemotherapy twice allo-SCT re-chemotherapy	MRD+	Daratumumab	MRD-	Sustained remission
Sumeet Mirgh et al.	57yrs	male	ETP ALL、Hypertension, diabetesInfection	–	Unfit for chemotherapy	Daratumumab Allo-SCT	MRD-	Die of infections
Sumeet Mirgh et al.	26yrs	male	ETP ALL	Chemotherapy allo-SCT	MRD+	Daratumumab	MRD-	Sustained remission for 326 days
Yishai Ofran et al.	44yrs	female	T-ALL	Chemotherapies Allo-SCT Re-chemotherapy	MRD+	Daratumumab	MRD-	Sustained remission for 10.5 months
Yishai Ofran et al.	24yrs	female	T-ALL	Chemotherapies Allo-SCT Re-chemotherapy	MRD+	Daratumumab	MRD-	Sustained remission for 10 months
Yishai Ofran et al.	40yrs	male	B-ALL	Chemotherapies BITEChemotherapies Allo-SCT	MRD+	Daratumumab	MRD-	Daratumumab was discontinued due to severe GVHD reaction
Marco Cerrano	44yrs	male	ETP ALL	Chemotherapies Allo-SCT	MRD+	Daratumumab	MRD-	Sustained remission for 3 months
Sandra D	2yrs	male	T-ALL	Chemotherapies	Relapsed, unfit for chemotherapy	Daratumumab	NCR	Die of T-ALL
Guo YL et al.	24yrs	female	B-ALL	Chemotherapy CD19/CD22 CART	Relapsed	Anti-CD38 CART	NA	died of severe CRS

and methotrexate were added to enhance further efficacy. Although the response rate in B-ALL patients was low, the efficacy in T-ALL patients was highly promising, with 5/8 patients achieving CR. Therefore, the study was expanded to include 20 additional T-ALL patients. It is unknown whether the poor response of CD38 mAb in B-ALL patients was attributable to the chemotherapeutic regimen or the lower expression level of CD38 in B-ALL patients compared to T-ALL patients. Another clinical trial enrolling 14 patients to evaluate the efficacy and safety of isatuximab in T-ALL (ClinicalTrials.gov identifier: NCT02999633) was prematurely terminated on November 8, 2017, due to an unsatisfactory benefit/risk ratio (Table 2).

Application of CD38 mAbs in AML

Preclinical studies in AML

Various studies have corroborated that DARA exerts antitumor effects in AML both *in vivo* and *in vitro*. Dos et al. examined the antileukemic effect of CD38 mAb in AML cell lines and primary AML cells (38). They reported that DARA inhibited the proliferation of AML cells (MOLM-13, MOLM-16, MV-4-11, and NB4 cell lines) *in vitro*. Meike Farber et al. also found that DARA could significantly reduce AML cell growth in 5/8 AML cell lines (35). Further mechanistic studies determined that DARA induced apoptosis of AML cell lines *via* cross-linking and could induce a 5~20% ADCC effect and a 2~5% CDC effect on six of the nine AML cell lines (38). Moreover, compared with homotypic IgG1 monoclonal antibodies, DARA significantly enhanced the ADCP effect of macrophages (49, 60). Other studies have found that DARA exerts a significant antileukemic effect in coculture systems of AML cells and mesenchymal cells but only has a weak cell-autonomous effect in monoculture, suggesting that DARA is more likely to act through the bone marrow microenvironment (49). Recent studies have discovered another mechanism by which DARA limits mitochondrial transfer from mesenchymal stem cells to AML cells, thereby reducing the metabolic capacity of AML tumor cells and ultimately inhibiting tumor proliferation (53). Moreover, in a patient-derived xenograft (PDX) mouse model, DARA significantly reduced tumor burden in peripheral blood and spleen but did not affect the bone marrow (38). The authors observed that the addition of DARA did not reduce bone marrow tumor burden; nevertheless, it significantly reduced CD38 expression on the surface of bone marrow AML cells. They theorized that the bone marrow microenvironment suppressed the antileukemic effect of DARA. Another study revealed that DARA significantly reduced leukemic burden in peripheral blood compared to the control group (42%, P=0.0354), but there was no significant difference in the bone marrow (60). Last, Naik et al. demonstrated that DARA reduced tumor burden in mice compared to the control group (37).

Correlation between CD38 density and efficacy of DARA in AML

Earlier DARA studies in MM patients have established a positive correlation between CD38 surface density and DARA efficacy (41, 45, 46, 61). In AML studies, the relationship between the CD38 expression level and the efficacy of DARA monotherapy is complex. Meike Farber et al. noted that CD38 density seemed to be related to the efficacy of CD38 mAb (60).In contrast, Dos et al. did not observe a direct correlation between the expression level of CD38 and the effect of CD38 mAb (38). Enguerran Mouly et al. (39) determined that in THP-1 cells (low CD38 expression), the maximum ADCC effect induced by DARA was approximately 7%, whereas the maximum ADCC effect was approximately 28% in UOC-M1 cells (high CD38 expression). Furthermore, there was no significant difference in the effect of DARA-induced ADCC between the two groups of AML cell lines with low and high CD38 expression levels (Spearman's rank correlation r=0.6134, P=0.09). After incubation with inecalcitol (a vitamin D receptor agonist enhancing CD38 expression in 10 out of 11 AML cell lines), CD38 expression levels were upregulated in both CD38-negative cell lines (HL-60, U-937) and CD38-high cell lines in varying degrees. The corresponding DARA-induced maximum ADCC effect was increased to 20% and 42%, respectively. Researchers further evinced that DARA-induced ADCC was correlated with

TABLE 2 Clinical trials of CD38 mAbs in ALL.

Study	Phase	Disease	Setting	Treatment	Status
NCT03860844	I/II	Pediatric AML/ALL	refractory/ relapsed	Isatuximab+Chemotherapy	Recruiting
NCT02999633	II	T-ALL/T-LBL	refractory/ relapsed	Isatuximab	Terminated
NCT03384654	II	B/T Precursor Cell Lymphoblastic Leukemia/Lymphoma	refractory/ relapsed	Daratumumab +Chemotherapy	Active, not recruiting
NCT04972942	II	T-ALL/T-LBL	high risk	Daratumumab+TBI+allo-SCT	Not yet recruiting
NCT01084252	I/II	CD38-positive hematologic malignancies including NHL, MM, AML, ALL, and CLL	relapsed/ refractory	Isatuximab	Recruiting

the expression level of CD38 after incubation with inecalcitol (Spearman's rank correlation r=0.8333, P<0.01). Furthermore, the expression level of CD38 was correlated with the ADCC effect of DARA but not with CDC. Similarly, the combination of DARA and ATRA led to a significant improvement in anti-leukemic effects, which was positively correlated with the upregulation of CD38 expression in AML cell lines (60). Collectively, these results signify that there was no significant correlation between the CD38 expression level and the effect of the CD38 mAb in leukemia. However, with an improvement in CD38 expression after stimulation by other drugs, the action of the CD38 mAb is further amplified. The antitumor effect mediated by CD38 mAb is linked to various factors, including the affinity between the antibody and tumor antigen, the affinity between the antibody and Fc receptor, the density of tumor antigen, the characteristics of the tumor target cells, and the characteristics of the immune effector cell. The expression level of CD38 in AML cells did not correlate with the efficacy of CD38 mAb alone but rather with the efficacy of combination therapy. We infer that this may be attributed to the lower expression level of CD38 in AML cells than in MM cells, which is insufficient to observe the differences in antitumor effects of CD38 mAb in AML cells with varying CD38 expression levels. However, the combination with other drugs significantly increases the CD38 expression level in AML cells, reaching the threshold to observe the efficacy of CD38 mAb. Nonetheless, whether this observation is also related to the features of AML cells remains to be determined.

DARA-based combination for AML

The above studies provide a theoretical foundation for a DARA-based combination that may enhance antitumor activity.

Prior studies have identified that ATRA can upregulate CD38 expression in various tumor cells, including APL (62) and MM (46). Yoshida et al. reported that ATRA upregulated CD38 expression in KG-1, U937, other AML cell lines, and primary cells of patients (61). Correspondingly, Buteyn et al. demonstrated that ATRA upregulated CD38 expression in AML cell lines (MV4-11, OCIAML3, MOLM-13, and U937) and primary cells of AML patients (63). Thus, there is a binding site of the ATRA receptor RAR (retinoic acid receptor) in the first intron of the CD38 gene, directly activating RAR and initiating CD38 transcription after ATRA binding (12). The increased expression of CD38 can improve the antibody-mediated cytotoxicity of DARA and the anti-leukemic effect. Additionally, Buteyn et al. reported that ATRA combined with DARA improved conjugate formation and the antibody-mediated cytotoxicity of primary AML cells through FCR induction *in vitro* compared to the control and DARA monotherapy groups. The survival of AML cell lines and primary cells was significantly reduced at 24, 48, and 72 h. *In vivo*, ATRA with DARA inhibited tumor proliferation in mice, significantly decreased tumor size, and prolonged survival compared to DARA monotherapy (63).

Venetoclax, a Bcl-2 inhibitor, also showed a synergetic effect with DARA. It is FDA-approved for AML combined with low-dose chemotherapy or demethylation. However, its extensive use eventually leads to resistance, making its combination with other drugs essential. According to some reports, mitochondria can be transferred from mesenchymal cells to primary AML cells to promote AML growth. DARA has been reported to inhibit mitochondrial be transferred from mesenchymal cells to primary AML cells (53). Mistry et al. (64) determined that venetoclax monotherapy inhibited AML cell growth and promoted apoptosis in primary AML cells and mesenchymal cell cocultures, whereas DARA monotherapy did not. Compared to venetoclax and DARA monotherapies, combination therapy inhibited tumor cell growth and promoted apoptosis. *In vivo*, they also demonstrated that venetoclax combined with DARA effectively reduced tumor burden in mice.

In addition, Fatehchand et al. (65). reported that IFN-γ promoted the differentiation of primary AML cells and upregulated CD38 and FcγRI expression. *In vitro*, IFN-γ enhanced the antibody-mediated cytotoxicity of DARA, while IFN-γ combined with DARA inhibited tumor growth in animals.

Clinical trials of CD38 mAbs in AML

Preclinical studies have corroborated the efficacy of CD38 mAbs in AML, and DARA-related clinical trials in AML are underway. Researchers at the MD Anderson Cancer Center evaluated the efficacy of daratumumab in patients with refractory relapsed AML and high-risk MDS (ClinicalTrials.gov identifier: NCT03067571). A phase I/II trial of daratumumab and DLI (donor lymphocyte infusions) in patients with relapsed AML after hematopoietic stem cell transplantation (ClinicalTrials.gov identifier: NCT03537599) is ongoing at Ohio State University. Another CD38 mAb, isatuximab, is also being tested in phase I/II clinical trials combined with chemotherapy for refractory relapsed AML and ALL in children (ClinicalTrials.gov identifier: NCT03860844). The outcomes of these clinical trials are not yet available (Table 3).

Anti-CD38 bispecific antibody

Anti-CD38/CD3 bispecific antibodies, including AMG 424, GBR1342, and Bi38-3, have been shown to be effective in MM both *in vitro* and *in vivo* (66, 67). CD38 bispecific antibodies can bind to CD38 on tumor cells and cytotoxic immune effector cells (T cells, a small number of NK cells), playing the antitumor role of the antibody itself and activating effector cells to attack tumor cells. However, these antibodies have not been explored in AL.

XmAb18968, a novel anti-CD38/CD3 bispecific antibody, binds to CD38 on the cell surface *via* the FC segment and

TABLE 3 Clinical trials of CD38 mAbs in AML.

Study	Phase	Disease	Setting	Treatment	Status
NCT03067571	I/II	AML and high-risk MDS	refractory/ relapsed	daratumumab	Unknown
NCT03537599	I/II	AML with allo-SCT	relapsed	Daratumumab+DLI	Recruiting
NCT03860844	I/II	Pediatric AML/ALL	refractory/ relapsed	Isatuximab +Chemotherapy	Recruiting
NCT01084252	I/II	CD38-positive hematologic malignancies including NHL, MM, AML, ALL, and CLL	refractory/ relapsed	Isatuximab	

nonselectively activates effector T cells (68). The researchers optimized XmAb18968's affinity for CD38 and CD3 to lower cytokine release. A phase I clinical trial of XmAb18968 (NCT05038644) will be conducted to evaluate its safety and tolerability in patients with relapsed and refractory T-ALL, T-LBL, and AML. The inclusion criteria are as follows: patients older than 18 years, having CD38 expression levels greater than 20%, and having not undergone allo-SCT within the past six months. The study's primary endpoint is to determine the recommended dose of the phase II study and the toxicity profile, while the secondary endpoints include response rate, response duration, and survival. The study will begin to enroll patients in January 2022.

Anti-CD38 CAR-T cells therapy

Adoptive cell therapy has achieved favorable success in B-ALL, but complications such as late B cell failure still exist, and its application in AML and T-ALL requires further exploration. Therefore, it is critical to identify new therapeutic targets for AML and ALL cell immunotherapy. As pointed out earlier, CD38 can serve as a new target for adoptive cell therapy of AML and ALL.

Preclinical studies of CD38 CAR-T cells in AL

Esther Drent et al. designed anti-CD38 CAR-T cells using different CD38 antibody sequences and assessed their efficacy and safety (69). They found that anti-CD38 CAR-T cells were effective in lysing primary cells from AML patients, regardless of whether these cells had a high or low expression of CD38. Yoshida et al. reported that anti-CD38 CAR-T cells displayed time- and quantity-dependent cytotoxicity to AML cell lines with high expression of CD38 and had a selective killing effect on AML cell lines with partial or low expression of CD38 but had no effect on CD38-negative cells (70). However, these cytotoxic effects of anti-CD38 CAR-T cells alone on AML cell lines and primary AML cells were limited. Anti-CD38 CAR-T cells exhibited significantly enhanced antitumor activity when AML cells were cocultured with ATRA.

Clinical studies of CD38 CAR-T cells in AL

The preclinical studies provided a theoretical foundation for the clinical application of anti-CD38 CAR-T cells in AL. Guo et al. reported a case of relapsed B-All treated with anti-CD38 CAR-T cells (71). It was a 24-year-old relapsed and refractory B-ALL patient who had received bispecific CD19/CD22 CAR-T therapy and achieved CR but relapsed five months later. At that time, CD19 was negative, and CD22 was marginally positive, but the positive rate of CD38 was 63%. Therefore, the patient was treated with anti-CD38 CAR-T cells (1×10^6/kg). After treatment, the tumor burden in the patient's bone marrow and peripheral blood was significantly reduced (14.52% vs. 0.8%, and 5% vs. 0%, respectively). However, the patient suffered from a severe CRS reaction combined with liver and lung toxicity (Table 4). Moreover, CD38 was expressed in CAR-T cells, leading to CAR-T cell death and loss of function. Finally, the patient discontinued all treatments. This case shows that anti-CD38 CAR-T cell therapy may be effective in patients with relapsed ALL even after anti-CD19 CAR-T cell therapy. Can the combination of CAR-T cell therapy with different targets improve therapeutic effects? Relevant clinical studies are currently underway.

Han et al. conducted a phase I/II study (NCT03754764) to assess the safety and feasibility of anti-CD38 CAR-T cell therapy in B-ALL patients who had previously received CD19 CAR-T therapy. There is also a phase I/II study to evaluate the safety and efficacy of CD38 CAR-T therapy in CD19-negative ALL patients, particularly in ALL patients treated with CD19 CAR-T cells (NCT04016129). Chang et al. performed a phase I/II study of multitarget CAR-T cells, including CD38, in refractory relapsed AML patients to evaluate the feasibility, safety, and efficacy of treatment (NCT03222674). Another similar study is ongoing (NCT03473457) (Table 4). In another study, Cui et al. evaluated the efficacy and safety of anti-CD38 CAR-T cells in patients with relapsed AML following allogeneic hematopoietic stem cell transplantation (NCT04351022) (72). The study included six patients with relapsed posttransplant AML with a median pre-treatment CD38 positive rate of 95% (92–99%). All patients received FC regimens (fludarabine and cyclophosphamide) prior to CD38 CART cell infusion ($6.1\text{-}10\times10^6$/kg). Four weeks after anti-CD38 CAR-T cell infusion, 4/6 patients (66.7%) achieved

TABLE 4 Clinical trials of CD38 CAR-T cell therapies in AL.

Study	Phase	Disease	Setting	Treatment	Status
NCT03754764	I/II	B-ALL	refractory/relapsed, after CD19 CART	Anti-CD38 CART	Recruiting
NCT04016129	I/II	CD19 negative ALL	refractory/relapsed	Anti-CD38 CART	Recruiting
NCT03222674	I/II	AML	refractory/relapsed	Muc1/CLL1/CD33/CD38/CD56/CD123 CART	Unknown
NCT03473457	I/II	AML	refractory/relapsed	CD38/CD33/CD56/CD123/CD117CD133/CD34/Mucl CART	Terminated
NCT04351022	I/II	AML	relapsed/refractory	Anti-CD38 CART	Recruiting

CR or CRi, with a median time to CR or CRi of 191 days, a median OS of 7.9 months, and a median leukemia-free survival (LFS) of 6.4 months. Five patients experienced grade 1-2 CRS, and one developed grade 3 hepatic toxicities.

These findings provide evidence of the effectiveness of anti-CD38 CAR-T cells in the treatment of AL. However, owing to various adverse reactions, it is vital to explore new approaches for mitigating adverse reactions and prolonging the time of action *in vivo*. Presently, some researchers are attempting to employ proteins or antibodies to block CD38 on CAR-T cells, knock out the CD38 gene in effector cells, and use the caspase-9 suicide gene to mediate the autolysis of CAR-T cells.

Conclusions

Targeting CD38 antibodies and cellular therapies have demonstrated a unique and encouraging therapeutic effect in MM. They have also exhibited promising results in AL, especially in T-ALL. Combining CD38 mAb with ATRA, venetoclax, or IFN-γ for AML are being explored in clinical trials and real-world studies to validate the efficacy. The correlation between CD38 density and the efficacy of CD38 mAb in AL remains controversial and warrants further study. In the future, screening eligible predictors will assist in stratifying AL patients who can benefit from CD38-targeted therapies. Additional studies are needed to elucidate the efficacy and safety of CD38-targeted therapies, such as bispecific antibodies, trispecific antibodies, ADCs, multitarget CARTs, and CAR-NK cell therapy, in acute leukemia.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Deregulated calcium signaling in blood cancer: Underlying mechanisms and therapeutic potential

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Intracellular calcium signaling regulates diverse physiological and pathological processes. In solid tumors, changes to calcium channels and effectors *via* mutations or changes in expression affect all cancer hallmarks. Such changes often disrupt transport of calcium ions (Ca^{2+}) in the endoplasmic reticulum (ER) or mitochondria, impacting apoptosis. Evidence rapidly accumulates that this is similar in blood cancer. Principles of intracellular Ca^{2+} signaling are outlined in the introduction. We describe different Ca^{2+}-toolkit components and summarize the unique relationship between extracellular Ca^{2+} in the endosteal niche and hematopoietic stem cells. The foundational data on Ca^{2+} homeostasis in red blood cells is discussed, with the demonstration of changes in red blood cell disorders. This leads to the role of Ca^{2+} in neoplastic erythropoiesis. Then we expand onto the neoplastic impact of deregulated plasma membrane Ca^{2+} channels, ER Ca^{2+} channels, Ca^{2+} pumps and exchangers, as well as Ca^{2+} sensor and effector proteins across all types of hematologic neoplasms. This includes an overview of genetic variants in the Ca^{2+}-toolkit encoding genes in lymphoid and myeloid cancers as recorded in publically available cancer databases. The data we compiled demonstrate that multiple Ca^{2+} homeostatic mechanisms and Ca^{2+} responsive pathways are altered in hematologic cancers. Some of these alterations may have genetic basis but this requires further investigation. Most changes in the Ca^{2+}-toolkit do not appear to define/associate with specific disease entities but may influence disease grade, prognosis, treatment response, and certain complications. Further elucidation of the underlying mechanisms may lead to novel treatments, with the aim to tailor drugs to different patterns of deregulation.

To our knowledge this is the first review of its type in the published literature. We hope that the evidence we compiled increases awareness of the calcium signaling deregulation in hematologic neoplasms and triggers more clinical studies to help advance this field.

KEYWORDS

Calcium signaling, calcium homeostasis, blood cells, lymphoma, myeloproliferative neoplasms, red cell abnormalities, leukaemia, cancer biological pathways

1 Introduction

The deregulation of signaling by calcium ions (Ca^{2+}) has been extensively studied in solid tumors (1, 2). Changes to Ca^{2+} channels and effectors *via* mutations or changes in expression affect many functional capabilities responsible for cancer growth, invasion, and metastasis (2–5). The function of the endoplasmic reticulum (ER), the main site of Ca^{2+} storage in a cell, and Ca^{2+} transfer from the ER to mitochondria, the main regulation point for apoptotic cell death, are often deregulated in solid tumors (6, 7). Our review presents the rapidly accumulating data that this deregulation appears similar in many types of blood cancer. Therapeutic opportunities targeting Ca^{2+} signaling are emerging for disorders such as leukemia, lymphoma, and myeloproliferative neoplasms (MPN) (8–11), but this information is not yet widely known. Therefore, to increase awareness, we provide an outline of core findings that demonstrate deregulation of Ca^{2+} signaling in blood cancer. Research in this field has accelerated enormously in recent years, therefore, despite our great efforts, this review is unlikely to be complete. Nevertheless, we hope our compilation of data makes the subject of abnormal Ca^{2+} signaling in blood cancer more widely known. To our knowledge, this is the first review of this type in the published literature.

1.1 Unique relationship between extracellular Ca^{2+} and hematopoietic stem cells

Ca^{2+} signaling regulates many cellular processes, including gene expression, cell proliferation, motility, apoptosis, enzyme activity, and cytoskeletal dynamics, all of which are crucial to supporting normal cell differentiation including of hematopoietic stem cells (HSCs) (12–14). Specific effects of Ca^{2+} signaling are achieved through a tight control of intracellular Ca^{2+} homeostasis. At the resting state, cytosolic Ca^{2+} concentrations are maintained at very low levels: ~50–100 nM in most cells and reported to be as low as 20–30 nM in HSCs (15). This contrasts with high extracellular Ca^{2+} concentrations of ~1.5 mM in most fluids, including in blood plasma and bone marrow interstitial space (16). On the background of this high extracellular-intracellular Ca^{2+} gradient, precisely regulated spatio-temporal increases in cytosolic Ca^{2+} levels trigger signaling events (17).

The bone marrow environment provides a unique extracellular context for Ca^{2+} signaling. High Ca^{2+} levels in the endosteal niche have been shown to assist homing of HSCs through their calcium-sensing receptor (18). Nevertheless, it remains unclear if low or high Ca^{2+} concentrations are required to support HSC quiescence, both were shown to apply (14, 15, 19, 20). A recent study demonstrates that there is heterogeneity in Ca^{2+} levels between bone marrow cavities, depending on the level of bone resorption, but unexpectedly, no sharp gradient towards the endosteal niche was observed (16). HSCs reside in locations with higher extracellular Ca^{2+} levels compared to the serum and to the overall Ca^{2+} levels in the bone marrow. With aging, there is a significant increase in extracellular Ca^{2+} levels in the bone marrow associated with clonal expansion of activated HSCs. It has been proposed that deregulated Ca^{2+} homeostasis may be involved in leukemic transformation of HSCs, but experimental validation is required (21). In support, changes in Ca^{2+} homeostasis influence cancer stem cell properties in other cancer types (22).

1.2 Principles of intracellular calcium signaling

Cytoplasmic free Ca^{2+} levels are maintained by Ca^{2+} buffer systems (23) and modulated by a system of molecules re-distributing Ca^{2+} between the intracellular stores (the ER, mitochondria, Golgi apparatus and lysosomes), taking Ca^{2+} in from the extracellular space, or extruding it from the cell (12). Various channels, exchangers and pumps regulate Ca^{2+} levels in cells, including in blood cells. The collective involvement of these molecules, often referred to as a Ca^{2+}-signaling toolkit (13, 24), is shown in Figure 1 (with molecular details described in the figure legend).

In this review, we wish to highlight the role of ER as the main site of Ca^{2+} storage in almost any cell, as this functionality is

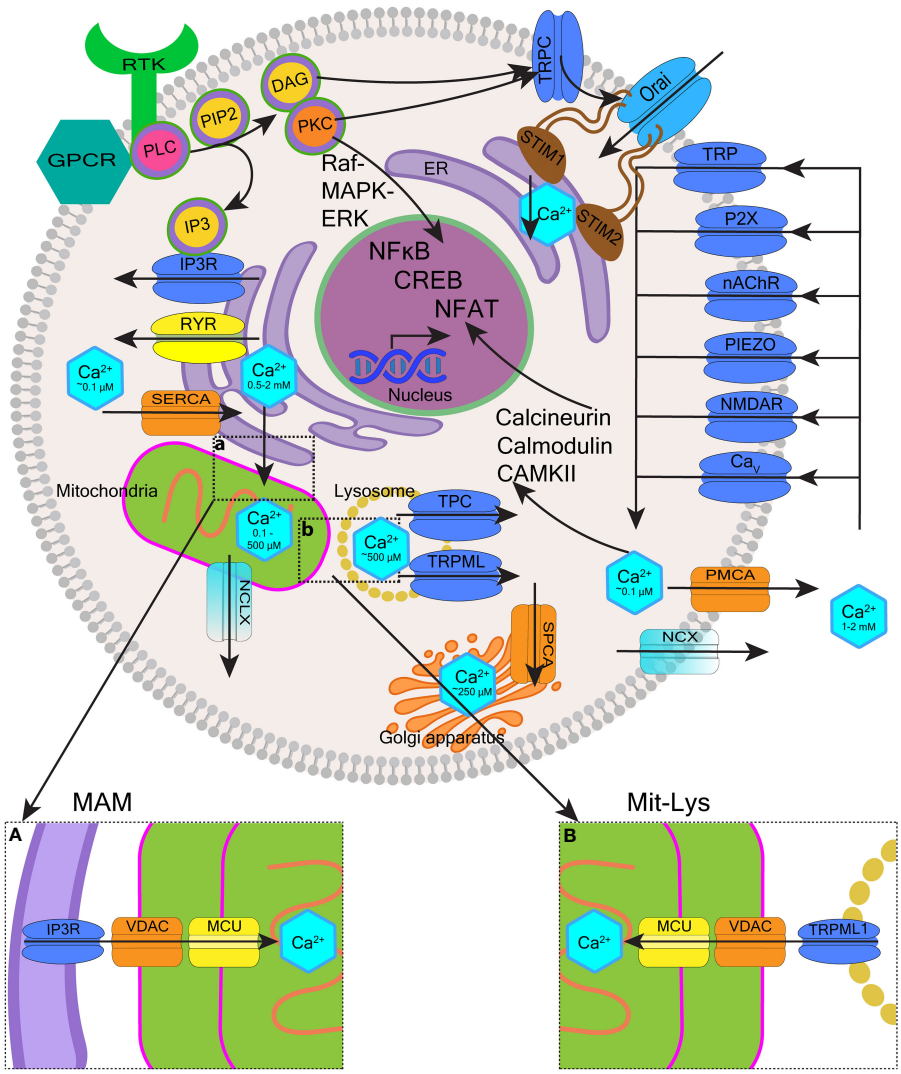

FIGURE 1
Overview of intracellular calcium homeostasis. Calcium homeostasis is maintained by the influx and efflux of Ca^{2+} through calcium channels and pumps located on the plasma membrane, as well as membranes of organelles such as the endoplasmic reticulum (ER), the Golgi apparatus, mitochondria, and endo-lysosomes. The cytoplasm, extracellular space, and each organelle have unique resting Ca^{2+} concentrations that have been indicated. Extracellular Ca^{2+} is transported into the cytosol through different channels such as transient receptor potential (TRP) channels, purinergic receptor (P2X) channels, nicotinic acetylcholine receptor (nAChR) channels, Piezo mechanosensitive channels, ionotropic glutamate receptor channels (e.g. N-methyl-D-aspartate receptors, NMDARs), and voltage-gated calcium (Ca_v) channels. Ca^{2+} is removed from the cytosol to the extracellular space by plasma membrane calcium ATPase (PMCA) efflux pumps and sodium-calcium exchangers (NCX). Activation of cell surface transmembrane receptors with tyrosine-based activation motifs (RTK e.g. B-cell and T-cell receptors) or G-protein coupled receptors (GPCR e.g. neurokinin-1 receptor) activate phospholipase C (PLC). PLC hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP2) located in the plasma membrane which generates two second messengers inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG). IP3 binds to IP3 receptors (IP3Rs) located on the ER membrane leading to the release of Ca^{2+} from the ER and DAG activates protein kinase C (PKC). Depletion of ER Ca^{2+} activates stromal interaction molecules STIM1-STIM2 (located in the ER membrane), which then activates Orai1-Orai3 channels (located in the plasma membrane) to induce Ca^{2+} influx into the cytosol. This mechanism is called store-operated calcium entry (SOCE). Ryanodine receptors (RYRs) represent an alternative pathway for Ca^{2+} release from the ER regulated by Ca^{2+}, Mg^{2+} and other molecules including ATP, calmodulin and CaMKII. The Ca^{2+} concentration in the ER is replenished *via* sarco-endoplasmic reticulum calcium ATPase 2b (SERCA2b) pump. The influx of Ca^{2+} from the ER to mitochondria occurs through voltage-dependent anion channels (VDAC) and mitochondrial calcium uniport (MCU) located in high numbers within mitochondria-associated ER membranes (MAMs) (**insert a**). Ca^{2+} leaves mitochondria mostly through $Na^{+}/Ca^{2+}/Li^{+}$ exchanger (NCLX). Ca^{2+} stored in the endo-lysosomes is mobilized mostly by two-pore channels (TPC) and transient receptor potential mucolipin (TRPML) channels in response to nicotinic acid adenine dinucleotide phosphate (NAADP.) TRPML1 is involved in the mitochondrial-lysosomal contact sites (Mit-Lys), facilitating Ca^{2+} transfer to mitochondria through VDAC and MCU (**insert b**). Multiple effector molecules mediate effects of Ca^{2+} signaling including PKC, Raf-MAPK (mitogen-activated protein kinase)-ERK (extracellular signal-regulated kinase), calmodulin, calcium/calmodulin-dependent protein kinases (e.g. CaMKII), and calcineurin. These signaling molecules influence gene expression through transcription factors such as nuclear factor kappa B (NF-κB), cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB), and nuclear factor of activated T-cells (NFAT).

often deregulated in cancer including blood cancer (25). The concentration of free Ca^{2+} in the ER is ~500 μM and ~2 mM for total ER Ca^{2+}, most of which is bound to Ca^{2+}-binding proteins such as calreticulin (CALR) (26). Many pathways of cell activation converge on the efflux of Ca^{2+} from the ER that occurs through channels called inositol 1,4,5-trisphosphate (IP3) receptors (IP3Rs) (27) (Figure 1). IP3Rs induce the release of Ca^{2+} from the ER upon binding of IP3 generated by phospholipase C (PLC) (28). PLC operates downstream of G-protein coupled receptors (GPCRs) and tyrosine kinase receptors located in the plasma membrane (29, 30). When ER Ca^{2+} becomes depleted, extracellular Ca^{2+} influx is initiated to maintain signaling. In non-neuronal cells most extracellular Ca^{2+} enters the cell through the mechanism called store-operated calcium entry (SOCE) (31, 32). SOCE is triggered by stromal interaction molecules (STIM1-STIM2) located in the ER membrane. Upon sensing ER Ca^{2+} depletion, STIM proteins oligomerize and redistribute to the plasma membrane where they interact with Orai1-Orai3 channels to activate Ca^{2+} influx into the cytosol (26, 32) STIM2 has low affinity for Ca^{2+} and activates when ER Ca^{2+} stores are <500 μM. In contrast, STIM1 has high affinity for Ca^{2+} and only activates when Ca^{2+} stores are <300 μM (33). Loss of STIM2 occuring in certain cancers is thought to reduce ER Ca^{2+} content (7). PLC also generates 1,2-diacylglycerol (DAG) that performs its signaling functions by binding and activating other proteins, including protein kinases C (PKC) and certain transient receptor potential (TRP) canonical (TRPC) channels, in particular TRPC1, that can interact with Orai1 and STIM1 to support SOCE (29, 34) (Figure 1).

Ca^{2+} transfer from the ER to mitochondria is another important mechanism often hijacked in solid tumors and of emerging importance in blood cancer (21, 35). ER and mitochondria interact through specialized ER-mitochondrial contact sites called mitochondria-associated ER membranes (MAMs) (36) (Figure 1, insert a). Within MAMs, IP3Rs on the ER interact with voltage-dependent anion channels (VDACs) located in the outer mitochondrial membrane allowing unrestricted Ca^{2+} entry into the inter-membrane space (37). The passage of Ca^{2+} through the inner mitochondrial membrane is restricted by the mitochondrial calcium uniport (MCU) and the membrane potential ($\Delta\Psi m \sim -150$ mV) (38). Small amounts of mitochondrial Ca^{2+} support mitochondrial metabolism, providing a mechanism that couples cellular activity with the generation of adenosine triphosphate (ATP). Ca^{2+} uptake into mitochondria activates pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and isocitrate dehydrogenase, thereby stimulating the tricyclic acid cycle and energy generation (39, 40). In contrast, high levels of Ca^{2+} in the mitochondria induce apoptosis (41, 42). Prolonged accumulation of Ca^{2+} in the mitochondria leads to the opening of the mitochondrial permeability transition pore (mPTP) formed when VDAC1 clusters with adenine nucleotide translocase (on the inner mitochondrial membrane) and cyclophilin D (in the mitochondrial matrix). The mPTP opening causes depolarization of the inner mitochondrial membrane, which uncouples the respiratory chain leading to increased mitochondrial membrane permeability and the release of cytochrome c (21, 35, 36).

Oncogenic effects have also been shown for certain endo-lysosomal Ca^{2+} storage and release mechanisms (43). Endo-lysosomes are heterogenous and dynamic acidic organelles that in addition to other roles, act as intracellular Ca^{2+} stores (44, 45). Endo-lysosomes sequester and release Ca^{2+} to the cytosol mainly through two-pore channels (TPC1-TPC2) and TRP mucolipin channels (TRPML1-TRPML3) activated by second messengers such as nicotinic acid adenine dinucleotide phosphate (NAADP), the most potent Ca^{2+}-mobilizing second messenger known (46, 47). Effects of endo-lysosomal Ca^{2+} release may be both local and global. The latter occur when endo-lysosomal mechanisms act in conjuction with the ER to induce or inhibit ER Ca^{2+} release (48). Endo-lysosomal Ca^{2+} signaling regulates processes such as membrane trafficking, vesicle fusion and secretion which impacts a range of cellular behavious e.g. immune responses, autophagy, cell proliferation, and migration (43, 49). In analogy to MAMs, mitochondrial membrane contact sites have also been shown to involve lysosomes (50, 51) (Figure 1, insert b). The release of lysosomal Ca^{2+} through TRPML1 supports Ca^{2+} transfer to mitochondria, providing an additional mechanism through which intracellular Ca^{2+} signaling, mitochondrial bioenergetics and lysosomal effects can be regulated (51).

This review emphasizes importance of abnormal Ca^{2+} signaling in hematologic cancers. We begin by presenting the long-standing foundational data on Ca^{2+} homeostasis in red blood cells (RBCs) as historically, this work provided guidance for research into Ca^{2+} signaling in selected blood cancers. We then focus on the neoplastic impact of deregulated Ca^{2+} influx through the plasma membrane and the ER, Ca^{2+} efflux *via* Ca^{2+} pumps and exchangers, and the impact of deregulated Ca^{2+} sensor and effector proteins in blood cancer. Throughout the review we highlight potential therapeutic strategies being developed to abrogate this deregulation.

2 Foundational research into calcium signaling in red cells with an outline of the toolkit components

Research into Ca^{2+} homeostasis in RBCs has a long history and has been regularly reviewed (52–54). While reticulocytes and immature RBCs of patients with sickle cell disease retain some of the mitochondria (55), normal mammalian RBCs do not have true Ca^{2+} storage organelles. However, RBCs often contain inside-out vesicles that are formed in response to

increased Ca^{2+} uptake. These vesicles contain plasma membrane calcium ATPases (PMCAs) that pump Ca^{2+} from the cytosol into vesicles and thus protect the cytosolic and membrane proteins from Ca^{2+}-induced damage (oxidation, proteolysis, irreversible dehydration) (see Figure 2A and the corresponding legend for molecular details). Some of these Ca^{2+}-filled vesicles are extruded, other inside-out endosomes are retained inside the cells (56–58). The resting concentration of cytosolic Ca^{2+} in RBCs are similar to nucleated cells, ranging from 30–60 nM in normal RBCs to the pathological 300 nM levels in patients with certain hereditary anemias (59). This compares with 1.2-1.8 mM in blood plasma (52, 53). Cytosolic Ca^{2+} concentrations in RBCs affect many aspects of red cell physiology including cell hydration, metabolic activity, redox state, and proteolysis. Regulation of Ca^{2+} concentrations translates into the control over the remodeling of the cytoskeletal elements and concomitant changes in cell shape, cell volume, rheological properties and ultimately, RBC longevity and clearance (52) (Figure 2A).

Multiple types of channels permeable for Ca^{2+} are present in the RBC membrane supporting versatility and plasticity of intracellular Ca^{2+} signaling (53) (Figure 2A). These channels are present in RBCs in very low copy numbers to keep the basal Ca^{2+} permeability of the plasma membrane low. Each channel type responds to its own stimulus (e.g. mechanical, electrical or chemical) to induce Ca^{2+} oscillations under specific conditions. Due to the broad variance in channel copy number per cell, there is variation in RBC responses to stimulation, and the numbers of "responding cells" typically range from 10% to 30% (60–62).

One of the first Ca^{2+} signaling processes identified in RBCs was the function of the Gárdos channel (potassium calcium-activated channel subfamily N member 4, KCNN4) (63) (Figure 2A). KCNN4 is activated by Ca^{2+} that enters through any of the non-selective cation channels [e.g. piezo type mechanosensitive ion channel component 1, Piezo1 (64)]. Piezo channels are the largest plasma membrane Ca^{2+} channels known containing a three-bladed propeller-shaped structure that spans the lipid bilayer sensing membrane stretch (65–67). The activation of Piezo links mechanical forces applied to RBCs with the control of cell volume and lifespan (64, 68). The KCNN4 activation leads to K^+ efflux and water loss (69), which reduces RBC volume and facilitates cell shape change. Activation of KCNN4 in RBCs of healthy people most likely enables better passage of RBCs through narrow capillaries (70), while its overactivation causes Ca^{2+}-overload and RBC dehydration (71) (Figure 2A). Hereditary stomatocytosis/xerocytosis are caused by gain of function mutations in genes encoding either KCNN4 (58, 72, 73) or Piezo1 channels (74–76). Different mutations cause distinctive clinical phenotypes, including some with syndromic features (72). The increasing use of next-generation-sequencing will help characterize the scope of genetic variants that are clinically relevant.

Other Ca^{2+} channels in RBCs include selected TRP and voltage-gated Ca^{2+} channels (Ca_v), *N*-methyl-D-aspartate (NMDA) receptors, and VDACs (52, 53). The TRP channels are a large family of approximately 30 structurally related but diverse members, the majority of which function as non-selective cation channels with variable Ca^{2+} permeability (77, 78). TRP channels can be activated by multiple external ligands including inflammatory and pain mediators, certain spices (e.g. garlic, mint, camphor and chili), metabolites, or physical stimuli such as temperature and stretch. TRP channels act as environmental sensors and transduction channels that regulate intracellular Ca^{2+} levels in response to the depletion of internal Ca^{2+} stores with or without simultaneous activation by PLC (79–81). The importance of TRP and Piezo channels in human physiology and pathology is underscored by the award of the Nobel Prize in Physiology or Medicine in 2021 to David Julius and Ardem Patapoutian "for their discoveries of receptors for temperature and touch" (82–84). Based on the amino acid sequence homology, activation mode and function, TRP channels are divided into six subfamilies: TRPC (canonical, TRPC1-TRPC7), TRPV (vanilloid, TRPV1-TRPV6), TRPM (melastatin, TRPM1-TRPM8), TRPA (ankyrin, TRPA1), TRPML (mucolipin, TRPML1-TRPML3), and TRPP (polycystin, TRPP1-TRPP2) (77). All TRP channel types are tetrameric assemblies of subunits containing six transmembrane domains arranged around a central ion permeation pore (79).

All TRP channels mediate receptor-operated Ca^{2+} entry but some also function as components or regulators of SOCE (85, 86). The latter applies mostly to TRPC1 and TRPC4 as they can interact with and be activated by STIM1 upon depletion of the ER Ca^{2+} stores; in turn, TRPC1, TRPC3 and TRPC6 can interact and activate Orai1 channels to support ER Ca^{2+} store refilling (87). Other TRPC channels do not interact with STIM1 directly, however heteromeric assemblies combining TRPC1 with TRPC4/5 or TRPC3 with TRPC6/7 contribute to SOCE, implying single TRPC components can provide SOCE regulation (88). Other TRP channel types (e.g. TRPV4 and TRPV6) and other proteins also interact with TRPC channels, which influences diversity of their functioning (89).

TRPC6 is abundant in human RBCs and contributes to stress-stimulated Ca^{2+} entry but its specific function in RBCs remains elusive (90). The discovery of TRPV2 in RBCs is relatively recent (91). Similar to Piezo1, TRPV2 mediates Ca^{2+} influx into RBCs in response to mechanical activation, which modulates RBC osmotic fragility and may contribute to the RBC storage lesion (92).

NMDA receptors are ligand-gated non-specific cation channels with high Ca^{2+} permeability activated by glutamate and glycine (93). NMDA receptors play critical functions in the brain but are also expressed in non-neuronal cells, including all types of blood cells: red cells (60, 94, 95), platelets (96–98), neutrophils (99), monocytes (60), and lymphocytes (100, 101). In RBCs, NMDA receptor regulates hemoglobin oxygen affinity, nitric oxide production, cell hydration status, and proliferation of

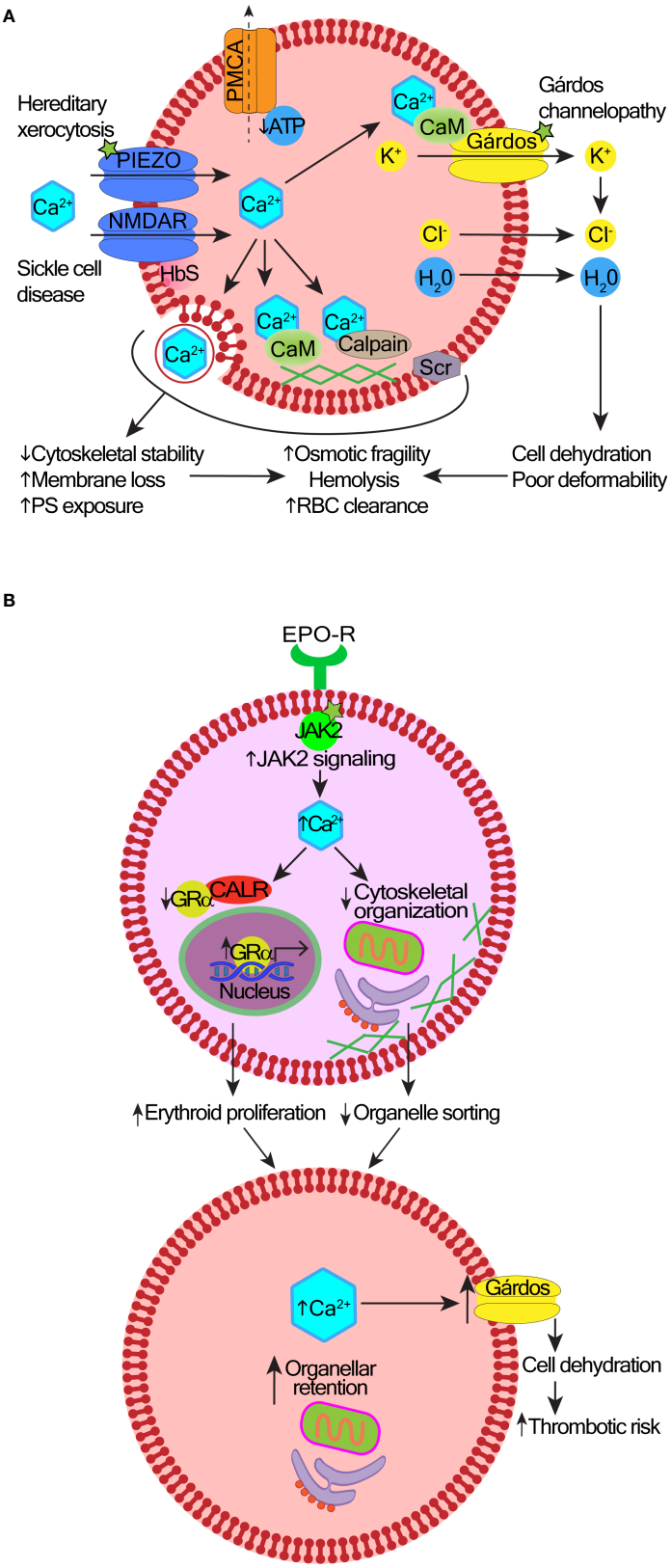

FIGURE 2 (Continued)

FIGURE 2 (Continued)
Mechanisms and consequences of deregulated calcium signaling in red cells and erythroid precursors. **(A)** In hereditary stomatocytosis/xerocytosis, heterogenous gain-of-function mutations in the Piezo1 or Gárdos channels cause excessive Ca^{2+} entry into red blood cells (RBCs). In sickle cell disease, there is an abnormally high abundance and activity of NMDA receptor channels, and probably other Ca^{2+}-transporting ion channels contributing to the increased permeability of the RBC membrane to Ca^{2+}. A layer of aggregated hemoglobin S (HbS) interferes with the shedding of NMDA receptor channels from the cell surface. Reduced levels of adenosine triphosphate (ATP) impair the function of the plasma membrane calcium pump (PMCA); as a result, Ca^{2+} uptake exceeds its efflux. Excess intracellular Ca^{2+} can be sequestered into vesicles and extruded, protecting the cytosolic and membrane proteins from Ca^{2+}-induced damage. However, over time, increased cytosolic Ca^{2+} overactivates the Gárdos channel leading to cell dehydration. Membrane and cytoskeletal instability are induced by the overactive calcium/calmodulin (CaM) complexes, calpain, or scramblase (Scr). This leads to premature RBC clearance, hemolysis, and anemia. The exact contribution of these mechanisms to different types of anemia remains under investigation. **(B)** Effects of high intracellular Ca^{2+} in polycythemia vera (PV). Hyperactive JAK2 V617F mutation increases Ca^{2+} levels in erythroid precursors. Ca^{2+} overload impairs the nuclear export function of calreticulin (CALR), which results in nuclear retention of the glucocorticoid receptor α (GRα) responsible for stress response and erythroid proliferation. Defective organelle sorting and extrusion from erythroblasts leaves organellar remnants in reticulocytes.

erythroid precursors (95). RBCs from patients with sickle cell disease carry higher numbers of NMDA receptors than in healthy donors (Figure 2A). NMDA receptor overactivity leads to Ca^{2+} overload, K^+ loss, cell dehydration, and oxidative stress, which may contribute to sickle cell crises (94). The efficacy of NMDA receptor inhibitor memantine for symptomatic treatment of sickle cell disease is currently being explored (102, 103).

VDACs are the major components of the outer mitochondrial membrane but they are also present in the plasma membrane including in RBCs (104, 105). VDACs conductance and selectivity are voltage-dependent. In the plasma membrane, VDACs may be involved in the transmembrane electron transport (37, 104). VDACs permeability for Ca^{2+} is low but considering the large intracellular-extracellular Ca^{2+} gradient, their activation may still contribute significant Ca^{2+} influx (53).

Finally, voltage-gated Ca^{2+} (Ca_V) channels transduce changes in the plasma membrane potential to intracellular Ca^{2+} transients that initiate many crucial physiological processes (106). In neurons and muscle cells Ca_V channels primarily regulate synaptic transmission and contraction respectively but these channels also regulate secretion and biochemical processes such as enzyme activity, protein phosphorylation/dephosphorylation, and gene expression in other cell types. Ca_V channels are subdivided into Ca_V1, Ca_V2, and Ca_V3 (107). $Ca_V2.1$ is epressed in RBCs but its function is poorly defined (108).

Overall, Ca^{2+} channels play important roles in RBC membrane transport, metabolism, volume, shape and lifespan regulation, although many specific functions remain unknown (53). It has been proposed that increased Ca^{2+} levels in RBCs due to abnormal function of Ca^{2+} channels represents a common mechanism underlying an accelerated clearance of RBCs from the bloodstream and pathological hemolysis in a range of anemias, which is a new area for investigation (59).

3 Calcium signaling in normal and neoplastic erythropoiesis

The role of intracellular Ca^{2+} signaling during erythropoiesis has been recently reviewed (54). Ca^{2+} signaling regulates erythroid progenitor proliferation, differentiation, survival, and terminal enucleation. Changes in Ca^{2+} homeostasis are seen in reactive ineffective erythropoiesis (e.g. in β-thalassemia) (109) or in neoplastic erythropoiesis driven by Janus kinase 2 (JAK2) V617F mutation in polycythemia vera (PV) (110, 111). CALR is an ER-resident protein that regulates functions of other proteins by chaperoning them to their active sites in response to changing intracellular Ca^{2+} levels (112). In normal erythroid precursors, CALR promotes the nuclear export of glucocorticoid receptor α, which resets precursor proliferation to differentiation (110). In contrast, hyperactive JAK2 signaling in PV increases free intracellular Ca^{2+} levels, which impairs the nuclear export function of CALR (Figure 2B). Glucocorticoid receptor α is retained in the nucleus maintaining the expression of stress genes that increase proliferation of erythroblasts (110). Elevated levels of Ca^{2+} may also impair actin reorganization required to extrude organelles during enucleation (111). Consequently, PV reticulocytes have a high content of organellar remnants e.g. mitochondria, ER and ER-associated proteins including CALR. In mature RBCs from PV patients, high Ca^{2+} levels increase the activity of the Gárdos channel leading to cell dehydration (111) (Figure 2B).

Increased levels of cytoplasmic Ca^{2+}, cell dehydration and the presence of organelle remnants in RBCs have the potential to promote thrombosis in PV (111). Dehydrated RBCs are more rigid, thus less amenable to shape changes required to pass through narrow capillaries, and also more susceptible to hemolysis under high-shear rates that occur in arterial circulation (113, 114). Higher cytoplasmic Ca^{2+} levels are known to increase adhesion between RBCs (115), and of RBCs to the endothelium (116, 117). Most previous work into PV-associated thrombosis focused on the role of a high hematocrit, white cell and platelet activation, coagulation factors and inflammation (118). However, a recent study used a laser-assisted optical rotational red cell analyzer to demonstrate abnormal RBC morphodynamics in 48 patients with PV (119). The deformability and stability of RBCs were reduced and RBC aggregation was increased. These alterations correlated with the incidence of ischemic stroke in 13 of these patients, suggesting a link between abnormal RBC morphodynamics and the increased risk of arterial thrombosis in PV, although this requires confirmation in larger studies (119).

Collectively, emerging data highlight a possible connection between the JAK2 V617F mutation and deregulated Ca^{2+} signaling in PV RBCs and precursors, with the potential to contribute to autonomous erythropoiesis and thrombosis. Therefore, strategies to modulate Ca^{2+} signaling may be useful for PV treatment.

4 Calcium signaling deregulation in blood cancer

Similar to solid tumors (3, 120), many blood cancers remodel Ca^{2+} signaling to promote their cancerous properties. Altered expression or activity of Ca^{2+} channels, pumps, and effectors can lead to the activation of transcription factors involved in the control of cell survival and proliferation.

4.1 Plasma membrane calcium-permeable channels

A number of Ca^{2+} influx channels located on the plasma membrane have been reported to impact on leukemic cells. These include the non-selective cation channels such as the TRP family, purinoreceptors (P2X7), nicotinic acetylcholine receptor (nAChR), Piezo1, NMDA receptor, and the Ca^{2+} selective Orai1 channels (Figure 1). Table 1 provides a summary of such changes in different blood cancers, and an explanation of their functional effects follows.

4.1.1 Transient receptor potential channels

It is thought that the primary physiological roles of TRP channels are perception of various sensations ranging from pain, pressure, temperature, taste and vision. However, evidence accumulates that TRP channels also regulate proliferation, differentiation, invasion, metastasis, autophagy and apoptosis of malignant cells (80, 81, 166–170). TRP channels have been shown to contribute crucial oncogenic functions in a number of hematologic malignancies (166, 167). Leukemia, lymphoma, myeloma and Waldenström macroglobulinaemia patient cells and cell lines have altered expression of TRP channels that has been linked with changes in cell proliferation, cell death and cell migration (165–167) (Table 1).

TRPM2 is overexpressed in cells from patients with acute myeloid leukemia (AML) and in AML cell lines (e.g. Kasumi-1, U937, KG-1, MV-4-11, SKNO1, THP-1, MonoMac-6, AML-193, MOLM13 and SHSY5Y) (129). TRPM2 depletion in AML cells and xenograft mouse models has anti-leukemic effects. TRPV2, TRPM7 and TRPC1 have been studied in chronic myeloid leukemia (CML) cell lines (K-562, KU812, MOLM-6 and 32D-p210) (127, 146, 147, 171). The silencing of TRPV2 induces significant apoptosis in K-562 cells (127), while inhibition of TRPM7 reduces cell proliferation and increases

TABLE 1 The differential expression of plasma membrane calcium channels and their relative contribution to the malignant phenotype in different blood cancers.

Cancer type	Molecule	Change in disease	Functional effects	References
AML	P2X7	↑ expression	↑ Ca^{2+} influx $^{(C,P,M)}$, ↑ proliferation $^{(C,M)}$, ↓ proliferation $^{(C,P)}$, ↓ remission rate $^{(P)}$, ↓ overall survival $^{(P,M)}$, altered sensitivity to chemotherapy $^{(P,M)}$, ↑ migration $^{(M)}$	(121–126)
	TRPV2	↑ expression	↑ proliferation $^{(C)}$, ↓ apoptosis $^{(C)}$	(127)
		↓ expression $^{(P)}$		(128)
	TRPM2	↑ expression	↑ proliferation $^{(C)}$, ↑ autophagy $^{(C)}$, ↑ mitochondrial Ca^{2+} influx $^{(C)}$, ↓ ROS production $^{(C)}$	(129, 130)
	TRPM4	↑ expression	↑ proliferation $^{(C)}$, cell cycle progression $^{(C)}$	(131)
	Orai1	↑ expression	↑ proliferation $^{(C)}$, ↑ migration $^{(C)}$, ↑ cell cycle progression $^{(C)}$	(132–134)
	IP3R2	↑ expression	↓ overall survival $^{(P)}$, ↓ event-free survival $^{(P)}$	(135)
	$Ca_V1.1$ (CACNA1S) $Ca_V1.2$ (CACNA1C)	↑ expression $^{(P)}$		(136, 137)
	$Ca_V1.2$ (CACNA1C)	↓ expression in AML-MSCs	↑ AML proliferation in 2D and 3D co-culture models $^{(P)}$	(138)
ALL	P2X7	↑ expression	↑ Ca^{2+} influx $^{(C)}$,↑relapse $^{(P)}$	(121, 123)
		↓ expression $^{(P)}$		(124)
	TRPC4/C5	↑ expression $^{(C)}$		(139)
	TRPV5/V6	↑ expression	cell cycle progression $^{(C)}$, endocytosis $^{(C)}$, cell migration $^{(C)}$	(140–142)
	TRPM2	↑ expression	↑ Ca^{2+} influx $^{(C)}$, ↓ ROS production $^{(C)}$	(130, 143)

(Continued)

TABLE 1 Continued

Cancer type	Molecule	Change in disease	Functional effects	References
	TRPM4		abnormal Ca^{2+} oscillation pattern (C), ↓ cytokine secretion (C)	(144)
	Orai1	↑ expression (P)		(134)
	NMDAR	mutations in *GRIN2C*	↑ relapse in high-risk pediatric patients (P); effects on expression and functional consequences are unknown	(145)
	$Ca_V1.1$ (CACNA1S) $Ca_V1.2$ (CACNA1C)	↑ expression (P)		(136, 137)
CML	P2X7	↑ expression	↓ remission rate (P)	(121)
		↓ expression (C)		(125)
	TRPV2	↑ expression	↑ proliferation (C), ↓ apoptosis (C)	(127, 146, 147)
	TRPM2	↑ expression (C)		(130)
	TRPM7	↑ expression	↑ Ca^{2+} influx (C), ↑ proliferation (C), differentiation (C)	(146)
	Piezo1	↑ expression (C)		(148)
	Orai1	↑ expression (C)		(134)
CLL	P2X7	↑ expression	↑ Ca^{2+} influx (P), ↓ proliferation (P)	(149)
	TRPC1	↑ expression	↑ cytokine secretion (P)	(150)
	α7-nAChR	↑ expression	↑ proliferation (C), ↑ migration (P,C)	(151)
	Orai1	↑ expression	↑ Ca^{2+} influx (P), ↓ event-free survival (P), ↓ progression-free survival (P)	(11, 134)
	CACNA1A	↑ expression (P)		(136, 137)
DLBCL	TRPM4	↑ expression	↓ overall survival (P), ↓ progression-free survival (P)	(152)
	IP3R2	↑ expression	↑ sensitivity to BIRD-2-mediated cell killing (C)	(153–155)
	$Ca_V1.1$ (CACNA1S) $Ca_V1.2$ (CACNA1C) $Ca_V1.3$ (CACNA1D) $Ca_V1.4$ (CACNA1F)	↑ expression of CACNA1D in ABC-DLBCL and of CACNA 1S, 1D and 1F in GCB-DLBCL (P)		(156)
	Orai1	↑ expression (C,P)		(134, 156)
Plasma cell myeloma	TRPV1	↑ expression	↑ proliferation (C,P), ↑ cell survival (C,P), ↑ drug resistance (C)	(157, 158)
	TRPV2	↑ expression	↓ overall survival (P), ↓ event-free survival (P), ↑ bone lesions (P), ↑ cytokine secretion (P)	(159, 160)
	TRPM7	↑ expression	↑ migration (C)	(161)
	TRPM8	↑ expression (P)		(162)
	TRPML2	↑ expression	↑ sensitivity to ibrutinib and/or bortezomib (C)	(163)
	α7-nAChR	↑ expression	↑ proliferation (C), ↑ migration (C)	(151)
	Orai1	↑ expression	↑ migration (C), ↓ progression-free survival (P)	(161, 164)
Waldenström macroglobulinaemia	TRPC1	↓ expression (P)		(165)

(P) = Patient cells, (C) = Cell lines, (M) = Mouse model. ↑ = increased, ↓ = decreased. Expression changes are often found in particular cell lines or leukemic subtypes and not in others. Empty cells indicate there is no data. AML, acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; CLL, chronic lymphocytic leukaemia; CML, chronic myeloid leukaemia; DLBCL, diffuse large B-cell lymphoma; MSCs, mesenchymal stromal cells.

differentiation (146). In BCR::ABL1-expressing murine myeloid progenitor cells (32D-p210), TRPC1 expression is reduced and may be one of the factors associated with SOCE reduction in these cells (171).

TRPV1, TRPV6 and TRPM2 contribute to the growth of cells derived from acute lymphoblastic leukemia (ALL) (142, 172). TRPV1 activation by resiniferatoxin (an analog of capsaicin, a vanilloid agonist) induces apoptosis, interferes with cell cycle progression and decreases proliferation in both Jurkat T-cells and patient-derived T-ALL lymphoblasts; however, the affect of resiniferatoxin on non-leukemic cells was not tested (172). TRPV6 is one of the necessary elements for migration and oncogenic signaling in Jurkat T-cells (142). TRPM2 is crucial for cell cycle arrest and decreases apoptosis of

irradiated Jurkat T-cells and Bcl-2-overexpressing T-lymphoblasts (143).

In chronic lymphocytic leukemia (CLL) cells, patient-derived and the Jok-1 cell line, TRPC1 plays a role in promoting cell survival. It does so by contributing to the production of anti-inflammatory cytokines and the activation of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathways triggered by CD5 activation (150, 168). TRPML2 is associated with the sensitivity of plasma cell myeloma cell lines to ibrutinib and/or bortezomib treatment. TRPML2 expression is low in ibrutinib-resistant U266 cells but high in ibrutinib-sensitive RPMI8226 cells (163). Upon TRPML2 RNA-silencing, RPMI8226 cells show worse response to ibrutinib than controls (163). These data raise the possibility that TRPML2 expression levels may help predict ibrutinib sensitivity in patients with myeloma (163).

Most recently, somatic mutations and copy number variations in *TRP* genes have been reported in 33 cancer types including hematologic malignancies, in particular diffuse large B-cell lymphoma (DLBCL) and AML cells (173). TRP mutations in the transmembrane regions were concluded to be likely deleterious and these genetic alterations were possibly linked to transcripitional deregulation of *TRP* genes and the consequent change in expression of TRP channels (173). The frequency of mutations in TRP channels was higher in DLBCL than in AML cells, with TRPM2, TRPM3 and TRPM6 showing the greatest mutation frequency (173). However, it is not clear what significance these genetic alterations have in the pathogenesis of cancer. Further work is required to uncover how these mutations contribute to cancer initiation and progression, and whether they can serve as markers for diagnosis, prognosis, or as treatment targets (173). *Ex vivo* studies with patient-derived cells demonstrate that targeting of TRP channels offers potential to inhibit malignant cell proliferation and improve chemotherapy effects (129, 172).

4.1.2 Purinoreceptor channels

P2X receptors are a family of ATP-dependent cation channels that have seven members (P2X1–7). An increase in extracellular ATP, often due to damage to the plasma membrane or exocytosis of ATP-containing granules, is the principal physiological stimulus for P2X receptor activation (174). Altered expression or function of P2X7 has been reported in a number of hematologic cancers (175). P2X7 is upregulated in cells from patients with AML and CLL and downregulated in B-ALL (124). Reports have differed on whether P2X7 in CML cells is up- or down-regulated (121, 124). When P2X7 activation is prolonged, and the receptor is exposed to high ATP levels, P2X7 opens an unselective membrane macropore and can trigger cell death (175, 176). P2X7RB is a splice variant that is unable to form this macropore (176). Both full-length P2X7RA and truncated variant P2X7RB are overexpressed in AML cells; whereas in relapsed AML patients, P2X7RB is increased and P2X7RA is decreased (126). AML blasts with high levels of P2X7RB have higher viability and much lower Ca^{2+} uptake than those expressing high levels of P2X7RA (126). AML development is slower and overall survival is extended in mice transplanted with P2X7-null AML cells compared to mice transplanted with control AML cells (125). Ca^{2+} influx is decreased in murine P2X7-null leukemia-initiating cells (LICs) and bulk AML cells compared to wild-type. The transcription factor cAMP-response element binding protein (CREB), which is involved in calcium signaling, is decreased in P2X7-null LICs and upregulated in AML patients. When CREB is overexpressed in P2X7-null AML cells, the development of leukemia is similar to wild-type AML cells (125). These results suggest that CREB-mediated Ca^{2+} signaling is required for the leukemogenic activities of P2X7.

4.1.3 Nicotinic acetylcholine receptor

Upon binding acetylcholine, nAChR channels assist the movement of cations into the cell, which causes membrane depolarization (177) and triggers the opening of voltage-gated Ca_v channels leading to Ca^{2+} influx (151). Homomeric α7-nAChRs are more permeable to Ca^{2+} and desensitize faster than heteromeric nAChRs (177). Primary CLL cells express α7-nAChR at a higher level than normal B-cells, and inhibiting α7-nAChRs in a range of leukemic cell lines reduces cell migration (151). Conversely, protein expression levels of α7-nAChRs in AML, CML and ALL patient peripheral blood or bone marrow-derived mononuclear cells was lower than in healthy subjects (178). Acetylcholine causes an increase in intracellular Ca^{2+} levels in CML-derived K-562 cells, and the α7-nAChR antagonist methyllycaconitine citrate inhibits K-562 cell proliferation as well as reduces the intracellular Ca^{2+} levels (177). The opposite was observed in Jurkat T-ALL cells, with methyllycaconitine causing intracellular Ca^{2+} levels to rise but this did not require extracellular Ca^{2+} (179).

4.1.4 *N*-methyl-D-aspartate receptor

Typical neuronal NMDA receptors are ligand-gated non-specific cation channels with high Ca^{2+} permeability activated by glutamate and glycine (93). In non-neuronal cells, including in megakaryocytes, NMDA receptors may also function in a metabotropic-like (i.e. flux independent) manner (97, 98, 180, 181) (see Figure 3A and the corresponding legend for molecular details). In leukemic cell lines with megakaryocytic features Meg-01, K-562, and Set-2, NMDA receptor supports cell proliferation (182). Deletion of NMDA receptor in Meg-01 cells shifts cell differentiation toward the erythroid lineage, suggesting NMDA receptor function at the level of a bipotential megakaryocyte-erythroid progenitor (183). NMDA receptor inhibitor memantine enhances cytotoxic effects of cytarabine in Meg-01 cells, thus this drug combination warrants testing on patient cells (183). In non-leukemic mice, the NMDA receptor regulates proplatelet formation through a

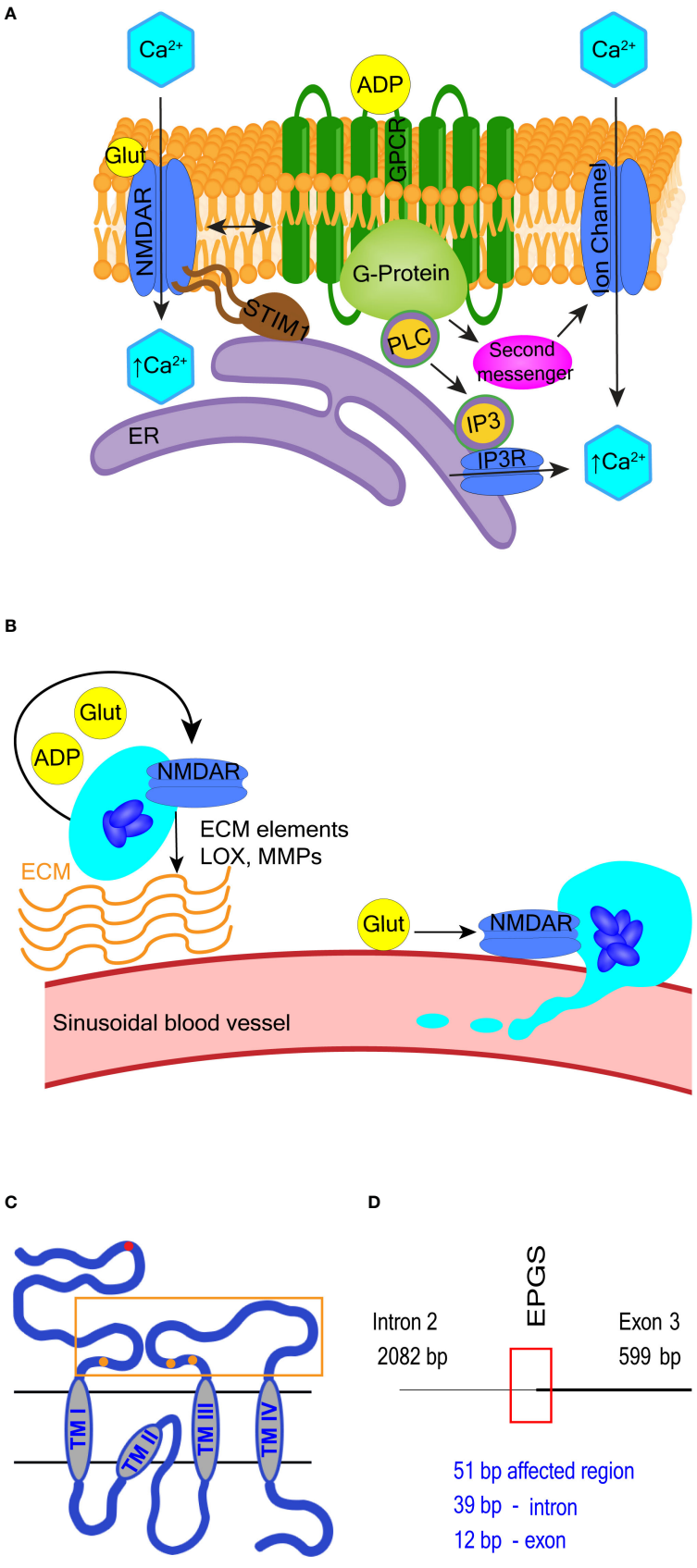

FIGURE 3 (Continued)

FIGURE 3 (Continued)
Selected NMDA receptor effects in hematopoietic cells. **(A)** Overview of NMDA receptor-induced calcium signaling. NMDA receptor directly facilitates Ca^{2+} entry into cells but may also operate in a metabotropic manner to induce Ca^{2+} release from the ER or *via* secondary messenger activation of ion channels such as transient receptor potential (TRP) channels. Adenosine diphosphate (ADP) and glutamate are both released from maturing megakaryocytes. ADP binds G protein-coupled receptors (GPCR) and activates PLC-β to increase intracellular Ca^{2+} levels. NMDA receptor modulates GPCR function in neuronal cells, so potentially may do so in hematopoietic cells. **(B)** Overview of NMDA receptor-associated effects in megakaryocytes. NMDA receptor assists proplatelet formation by regulating the expression of extracellular matrix (ECM) elements (e.g. collagen) and ECM remodeling enzymes (e.g. lysyl oxidase, LOX and matrix metalloproteinases, MMPs). **(C, D)** Schematics of NMDA receptor subunit GluN2C and the *GRIN2C* gene variants discovered in B-ALL. In **(C)**, the glutamate-binding domain (400-539; 659-800 aa) is enclosed in an orange rectangle, and glutamate binding sites are represented by orange dots (at 509-511, 516, 687-688, and 729 aa respectively). Location of *GRIN2C* variants found in B-ALL is marked by a red dot in **(C)** and red rectangle in **(D)** The affected region is 51 base pairs long; the EPGS sequence is translated (134-137 aa).

mechanism that involves megakaryocyte interaction with the extracellular matrix and cytoskeletal reorganization (180). NMDA receptor exerts these effects by influencing Ca^{2+} and adenosine diphosphate (ADP) signaling, and the expression of transcripts involved in extracellular matrix remodeling (180) (Figure 3B). These mechanisms are relevant to the pathophysiology of primary myelofibrosis (PMF); therefore, NMDA receptor inhibitors should be tested in PMF models.

In a random survival forest model, variants in the *GRIN2C* gene encoding the GluN2C subunit of NMDA receptor were part of a group of 7 variant genes found to predict shorter event-free survival in high-risk pediatric patients with B-ALL (145). The mutated *GRIN2C* region in ALL covers an intron/exon boundary located in the GluN2C protein's N-terminal domain (see Figures 3C, D for further details). The presence of *GRIN2C* mutations was associated with accelerated relapse in children with high-risk B-ALL, but their functional impact is not known. These findings call for experimental studies to determine the NMDA receptor role in normal and leukemic B-cell precursors.

4.1.5 Voltage-dependent anion channels

VDAC has three isoforms in mammals with VDAC1 being the most abundant (37, 104). VDAC1 is a key regulator of metabolite transfer between the mitochondria and cytosol including of ATP, ADP, and of small ions such as Ca^{2+} and Na^{+}. These functions are crucial for normal mitochondrial bioenergetics (37, 184). In its open state, VDAC1 facilitates metabolite exchange but is lowly permeable to Ca^{2+}. In contrast, in the "closed" state VDAC1 is highly permeable to Ca^{2+} providing a proapoptotic signal (37, 185).

VDAC1 is overexpressed in U266 myeloma cells, which together with CD45 expression enhances the cells sensitivity to apoptosis *via* mitochondrial pathways (186). VDAC1 is also overexpressed in CLL patient cells compared to healthy controls (187). VDAC1-derived decoy sequences (Antp-LP4 and N-terminal-Antp) induce cell death in peripheral blood mononuclear cells from patients with CLL but not from healthy donors (187). Similarly, in a large panel of leukemic cell lines including from CLL (MEC-1, MO1043, and CLL), T-ALL (MOLT4, Jurkat), and AML (U-937, THP-1, K-562), VDAC1-targeting peptides induce cell death (188).

VDAC1 associating with Bcl-2, Bcl-xL or hexokinase prevents apoptosis in cancer cells. VDAC1 peptides disrupt this association leading to VDAC1 oligomerization, mitochondrial Ca^{2+} overload, cytochrome c release and apoptosis (187, 188). Combined treatment of acute promyelocytic leukemia (APL) cell line HL-60 with melatonin and retinoic acid decreases VDAC1 expression, suggesting its leukemia-promoting role (189). In B-ALL cell lines, VDAC1 was upregulated after prednisolone treatment in three steroid-sensitive cell lines (697, Sup-B15, RS4;11) but unchanged in the steroid-resistant cell line (REH), suggesting that VDAC1 has a role in steroid-induced apoptosis (190). Similar was seen in T-ALL cells. Cell death occurred in Jurkat T-cells when either rice or human VDAC proteins were overexpressed, and the effect was blocked by ectopically expressed Bcl-2 (191). Overall, VDAC1 interactions with pro-survival proteins support anabolic metabolism and inhibit apoptosis thus maintaining leukemia growth. Strategies that target these interactions are being explored for treatment of leukemia, with T-ALL cells emerging as the most vulnerable to this form of manipulation (192, 193).

4.1.6 Voltage-gated ion channels (Ca_V channels)

Voltage-gated Ca^{2+} channels are coded by *CACNA* genes (calcium voltage-gated channel subunit alpha) and are subdivided into three families Ca_V1, Ca_V2 and Ca_V3 (194). Ca_V1 and Ca_V3 channels are expressed in many cell types while Ca_V2 are mostly expressed in neurons (106). Ca_V channels mediate Ca^{2+} influx in response to plasma membrane depolarization, influencing muscle contraction and neurotransmission, as well as secretion and gene expression in may cell types (137). Ca_V1 channels are activated by high voltage (> −40 mV with a peak at 0 mV) and mediate long-lasting (L-type) currents with slow inactivation. In contrast, Ca_V3 channels are activated by low voltage (around −60 mV with a peak at −20 mV) and mediate transient currents (T-type) with faster kinetics than the L-type currents. Ca_V2 channels are activated by high voltage and mediate P/Q-type, N-type and R-type Ca^{2+} currents (106).

Bioinformatic analysis of Oncomine, a web-based cancer microarray database of patient tissue revealed increased expression of *CACNA* transcripts in diverse cancer types

including of *CACNA1S* and *CACNA1C* (coding for $Ca_V1.1$ and $Ca_V1.2$ channels respectively) in AML and B-ALL samples and of *CACNA1A* (coding for $Ca_V2.1$) in samples from patients with CLL, marginal zone lymphoma and monoclonal gammopathy of unknown significance (136, 137). On the other hand, downregulation of *CACNA1C* transcripts (coding for $Ca_V1.2$) was seen in centroblastic lymphoma, *CACNA1F* (coding for $Ca_V1.4$) in anaplastic large cell lymphoma, and *CACNA1G* (coding for $Ca_V3.1$) in mantle cell lymphoma (195). A recent study confirmed distinct expression of Ca_V channels in a range of lymphoma cell lines and patient-derived samples (156). $Ca_V1.2$ (*CACNA1C*) expression was increased in classical Hodgkin lymphoma cell lines when compared to other B-cell lymphoma cell lines. $Ca_V1.3$ (*CACNA1D*) showed higher expression in samples from patients with activated B-cell-like DLBCL (ABC-DLBCL), whereas expression of $Ca_V1.1$ (*CACNA1S*), $Ca_V1.2$ (*CACNA1C*), and $Ca_V1.4$ (*CACNA1F*) were higher in germinal centre B-cell like DLBCL (GCB-DLBCL) (156). Therapeutic potential of inhibiting $Ca_V1.2$ in AML was revealed in an elegant 3D-culture model that mimicked the human bone marrow niche and utilized AML-derived mesenchymal stromal cells (AML-MSCs) from pediatric patients (138). Inhibition of $Ca_V1.2$ channel in AML-MSCs using lercanidipine (an anti-hypertensive drug) interfered with leukemia growth *ex vivo* and *in vivo*, with no toxic effects on normal MSCs or healthy CD34-positive HSCs (138). Further work is required to determine the mechanism through which Ca_V channels influence blood cancer growth.

4.1.7 Orai1 channels

Multimers of Orai1 proteins form an ion pore in the plasma membrane that is highly selective for Ca^{2+}. SOCE is triggered when Orai1 and STIM1/STIM2 proteins interact in response to ER Ca^{2+} store depletion. Increased expression of Orai1 or STIM1/STIM2 has been recorded in cell lines derived from AML (132, 133), T-ALL (134), CLL (11) and various lymphoma cell lines (134, 156). Mantle cell lymphoma Rec-1 cell line does not have high expression of Orai1 and STIM1 but Rec-1 and patient cells have significantly higher cytoplasmic Ca^{2+} concentrations under physiological conditions compared to normal cells suggesting "leaky SOCE" (196). High expression of Orai1 and STIM1 in CLL patients is associated with worse treatment- and progression-free survival (11). In mice models of T-ALL, deletion of STIM1 and STIM2 abolishes SOCE and results in prolonged survival (134). The underlying mechanism is intriguing, as the absence of SOCE does not change leukemic cells proliferation; instead, prolonged survival is associated with reduced inflammation in organs infiltrated by leukemia (134). In the HL-60 APL cell line, silencing of Orai1 and Orai2 reduces cell migration and proliferation (132). In the KG-1 and U937 AML cell lines, Orai1 contributes to cell proliferation and cell cycle progression (133). *ORAI1* gene expression was increased in peripheral blood mononuclear cells from 9 patients with AML compared with normal cells and correlated with adverse risk in the cohort of 439 AML patients (133).

Orai1 and STIM1 function is also linked with the CD20 molecule in B-cells and required for the efficacy of anti-CD20 antibody therapy in B-cell cancers. CD20 (MS4A1) is part of the membrane-spanning 4-domain family, subfamily A (MS4A) (197). The exact biological role of CD20 is unknown but it may act as an amplifier of Ca^{2+} signals transmitted through the B-cell receptor (BCR) in immature and mature B-cells to modulate cell proliferation and differentiation (198). CD20 has been reported to be physically coupled to or affect the phosphorylation of BCR and BCR-associated kinases; which are upstream regulators of the signaling cascade that activates SOCE (199–201).

Monoclonal anti-CD20 antibodies such as rituximab and obinutuzumab activate Ca^{2+} influx in patient CLL cells and cell lines such as SUDHL-4, BL2, Ramos, BL60, Raji, Daudi, and normal B-cells (202–206). Using genetically encoded Ca^{2+} indicator GCaMP-CD20 as a precise method to measure Ca^{2+} concentration changes around CD20, it was determined that anti-CD20 antibodies do not cause Ca^{2+} influx through or near CD20 (207). Instead, obinutuzumab activates intracellular Ca^{2+} efflux from either lysosomes or the ER into the cytosol (206) (see Figure 4A and the corresponding legend for molecular details). Inhibition of this intracellular Ca^{2+} movement reduces obinutuzumab-induced cell death (206, 207). Binding of rituximab to CD20 induces co-clustering of CD20 with Orai1 and STIM1 in SUDHL-4 cells, leading to extracellular Ca^{2+} influx and internal Ca^{2+} store release (205). CD20 overexpression in HEK293 cells increases Ca^{2+} influx, which is abolished when Orai1 and STIM1 are knocked down (207). CD20 strongly interacts with STIM1 but only when Orai1 is present (207). Influx of Ca^{2+} induced by rituximab or obinutuzumab is significantly reduced in Orai1 knockdown cells (205, 206). In B-CLL cells expressing high levels of STIM1, a combination of an anti-STIM1 monoclonal antibody and rituximab significantly reduces cell viability compared to rituximab alone (11). Thus, CD20 interactions with Orai1/STIM1 are important for the therapeutic efficacy of anti-CD20 antibodies. Manipulation of these interactions may help develop more effective therapeutic combinations for B-cell malignancies (Figure 4A).

4.2 IP3 signaling cascade and Ca^{2+} release from the ER

IP3 is a major regulator of Ca^{2+} signaling; it binds to IP3Rs on the ER to release Ca^{2+} into the cytosol (27, 28) (Figure 1). IP3 and another second messenger DAG are generated when PLC, activated downstream of G-protein coupled or tyrosine kinase receptors, hydrolyses PIP2 located in the plasma membrane (26).

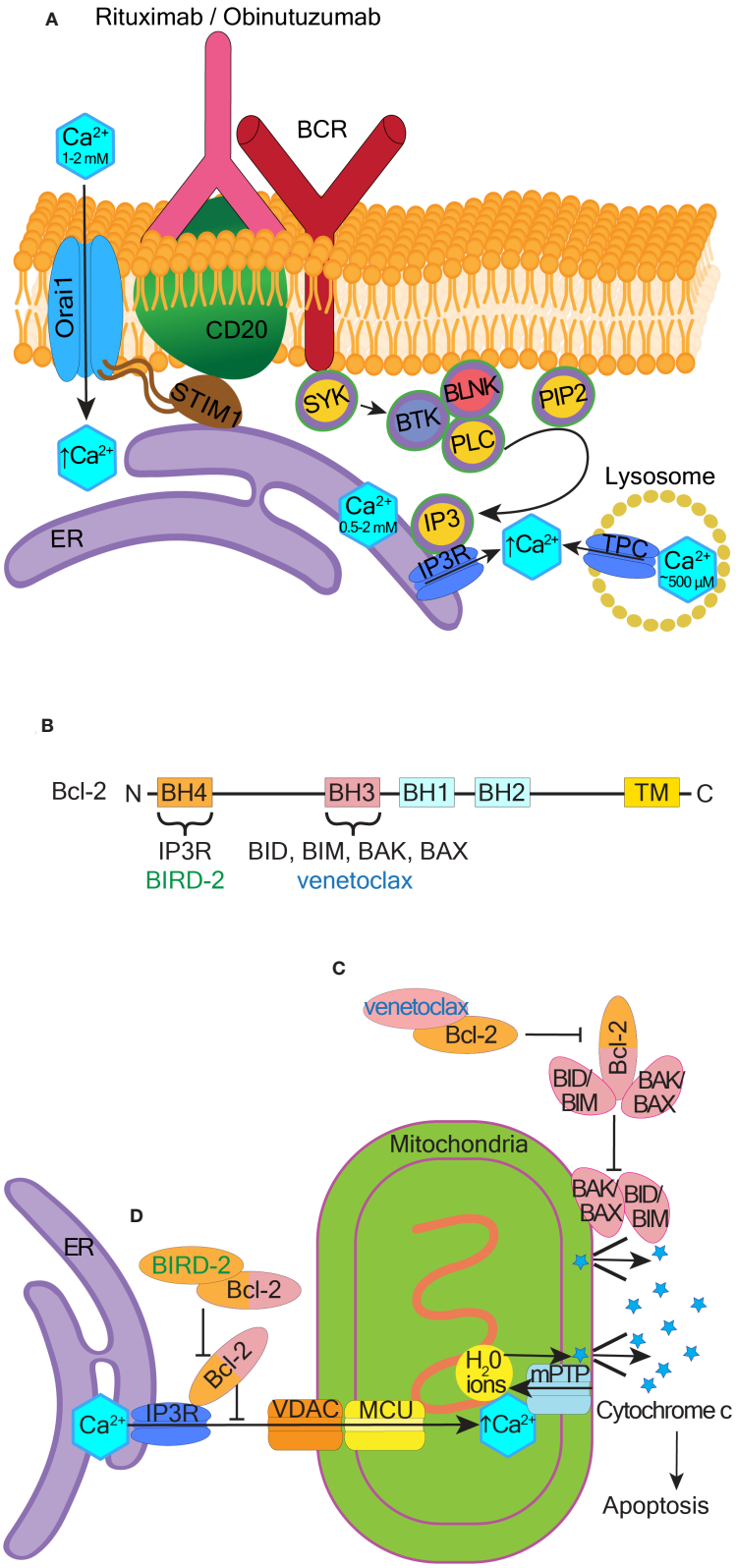

FIGURE 4 (Continued)

FIGURE 4 (Continued)
Therapeutic potential of pro-apoptotic calcium signaling at the ER and mitochondria. **(A)** Mechanism of BCR activated Ca^{2+} influx in response to rituximab and obinutuzumab. The membrane-spanning 4-domain protein CD20 is physically-coupled to the BCR. Rituximab and obinutuzumab induce phosphorylation of several proteins involved in BCR signaling, including BLNK (B-cell linker kinase), BTK (Bruton's tyrosine kinase), and PLC-γ (phospholipase C-γ). CD20 binds STIM1 and this binding is dependent on the presence of Orai1. Upon binding of rituximab/obinutuzumab, Ca^{2+} is released from lysosomes, the ER, and/or extracellularly *via* activation of store-operated calcium entry, which assists cell killing. **(B)** Schematic showing the four Bcl-2 homology (BH) domains. Venetoclax binds to the hydrophobic cleft located in the BH3 domain, and BIRD-2 binds to the BH4 domain. Transmembrane domain (TM), N- and C- termini are indicated. **(C)** Canonical BAX and BAK dependent pathway of apoptosis and the mechanism through which venetoclax inhibits this pathway. **(D)** Non-canonical ER Ca^{2+}-dependent pathway of apoptosis and the mechanism through which BIRD-2 inhibits this pathway.

4.2.1 Phospholipase C

PLC has six isoforms; β, γ, δ, ϵ, ζ, and η. PLC-β operates downstream of GPCRs and PLC-γ downstream of tyrosine kinase receptors (29, 30). Important activators of PLC-γ in hematologic cells are BCR and T-cell receptors (TCR), which are transmembrane tyrosine kinase receptors (208). T-cells mainly express the PLC-γ1 isoform, and B-cells mainly express the PLC-γ2 isoform. PLC-γ1 is essential for IP3 production and Ca^{2+} release in normal T-cells, whereas PLC-β3 is the main regulator of these responses in Jurkat T-cells and patient-derived T-ALL blasts (209).

Leukemic stem cells (LSCs) are multipotent, proliferative, and self-renewing cells that propagate leukemia (210). In AML LSCs, oxysterol-binding protein-related protein 4L (ORP4L) acts as a scaffold protein that facilitates PIP2 presentation to PLC-β3 for cleavage into IP3 (211). ORP4L is expressed in LSCs, but not in normal HSCs (211). Knocking down or inhibiting ORP4L decreases the survival of T-ALL cells and AML LSCs and reduces spontaneous cytosolic and mitochondrial Ca^{2+} oscillations (209, 211, 212). In T-ALL cells, ORP4L also interacts with PLC-β3 and IP3R1, which enhances Ca^{2+} release from the ER by facilitating the binding of IP3 to IP3R1 (212). Overall, the ORP4L regulated Ca^{2+} release into the mitochondria helps sustain mitochondrial oxidative respiration required for survival of T-ALL cells and AML LSCs (209, 211, 212)

BCR and TCR recruit kinases such as SYK (spleen tyrosine kinase), BTK (Bruton's tyrosine kinase), BLNK (B-cell linker kinase), and ZAP70 (zeta chain of T-cell receptor-associated protein kinase 7) to phosphorylate and activate PLC-γ. PLC-γ2 plays a role in CLL, DLBCL, Hodgkin lymphoma, endemic Burkitt lymphoma, MALT-associated gastric lymphoma, and plasma cell myeloma (208). For example, B-CLL cells showing high responsiveness after BCR engagement have higher PLC-γ2 activity and calcium signaling compared to non-responding cells (213). Patients with such hyperresponsive B-cells have a poorer prognosis than non-responders (213). Ibrutinib, which inhibits BTK and thus blocks PLC-γ2 signaling, has become an important and effective treatment for CLL and other B-cell lymphomas (208).

4.2.2 Inositol 1,4,5-trisphosphate receptors

Three isoforms of IP3Rs exist and most cell types express more than one isoform (214). Mice with all three IP3R isoforms deleted develop T-cell malignancies throughout the body that resemble T-ALL (215). Cytogenetically normal AML cells have higher expression of IP3R2 than healthy progenitors and patients with high IP3R2 expression have shorter overall and event-free survival (135). DLBCL SU-DHL-4 cells also have high levels of IP3R2 and constitutive IP3 signaling, which leads to elevated basal levels of cytoplasmic Ca^{2+} and increased cell survival (154). Inhibition of IP3 production *via* inhibiting PLC reverses the prosurvival effect and increases cell death in SU-DHL-4 cells (154).

Several oncogenes and tumor suppressors directly interact with IP3Rs and regulate their activities to control Ca^{2+} influx into the mitochondria. Among such IP3R regulators are the Bcl-2 family proteins, which consist of different anti-apoptotic members (e.g. Bcl-2, Bcl-xL, Mcl-1, and Bcl-10) and pro-apoptotic members (e.g. BIM, BID, BAX, and BAK) (216) (Figure 4B). Bcl-2 overexpression is common in blood cancer, including in DLBCL, AML and CLL (154, 217, 218). Bcl-2 binds to and prevents the activation of pro-apoptotic proteins through their BH3 domains (219, 220). To overcome Bcl-2 effects in cancer cells, BH3 mimetics like venetoclax were developed that target the hydrophobic BH3-binding groove of Bcl-2 (Figure 4B). Venetoclax binding to Bcl-2 liberates BIM, which activates BAX or BAK, leading to apoptosis (221) (Figure 4C).

In addition to the canonical BAX/BAK-dependent pathway of apoptosis, Bcl-2 also directly binds to IP3Rs on the ER through its BH4 domain. The binding of Bcl-2 to IP3R inhibits Ca^{2+} release from the ER and prevents cell apoptosis triggered by mitochondrial Ca^{2+} overload (222). Venetoclax does not interfere with this BH4-dependent mechanism of cell death (223). In contrast, a designer peptide Bcl-2 IP3R Disruptor-2 (BIRD-2) targets the BH4 domain of Bcl-2 (224) (Figure 4B). BIRD-2 binding to Bcl-2 unleashes IP3R activation and cytotoxic Ca^{2+} levels are released from the ER (155, 225) (Figure 4D).

BIRD-2 induces apoptosis in multiple blood cancer cell lines and/or patient-derived cells, including from DLBCL, CLL, plasma cell myeloma, and follicular lymphoma (153, 226, 227). DLBCL cells with high levels of IP3R2 and constitutive IP3 signaling are particularly sensitive to BIRD-2 (154). In DLBCL cell lines, BIRD-2 induces cell death in a caspase-dependent manner, however in contrast to venetoclax, BIRD-2-induced cell

death is independent of BAX/BAK (228). In both DLBCL cell lines and primary CLL patient samples, BIRD-2 triggers mitochondrial Ca^{2+} overload to induce caspase-dependent cell death. Cyclosporine A, which desensitizes mPTP to excessive Ca^{2+}-induced opening, and ruthenium265 (Ru265), which inhibits mitochondrial Ca^{2+} uptake, both counteract BIRD-2-induced cell death (228). Combining venetoclax with BIRD-2 enhances cell death of DLBCL cell lines, however DLBCL cells with acquired resistance to venetoclax were not sensitized to BIRD-2 (155, 229). BIRD-2 may be useful in combination with venetoclax as a therapeutic strategy in DLBCL, however the clinical relevance of these approaches remains to be determined.

Another therapeutic target that can induce mitochondrial Ca^{2+} overload is the GPCR neurokinin-1 receptor (NK-R1), expression of which is elevated in patient-derived AML cells and cell lines (230). Targeting NK-R1 with the antagonists SR140333 or aprepitant in AML and CML cell lines increases cytosolic and mitochondrial Ca^{2+} concentrations, resulting in increased production of reactive oxygen specied (ROS) and apoptosis (230). When IP3R or VDAC1 are inhibited, ROS production and apoptosis are decreased (230), but neither antagonist inhibits proliferation of normal CD34-positive HSCs. Aprepitant has been approved by the US Food and Drug Administration for the treatment of chemotherapy-induced nausea and vomiting (230). Therefore, this and other NK-R1 antagonists could be repurposed for testing efficacy in AML (230).

4.3 Endo-lysosomal Ca^{2+} channels

The role of endo-lysosomal Ca^{2+} signaling in blood cancer has not been systematically studied but there are a number of observations pointing towards its importance. TRPML3, TPC1 and TPC2 endo-lysosomal Ca^{2+} efflux channels are expressed in the megakaryoblastic leukemia cell line Meg-01 (231). NAADP releases Ca^{2+} from the lysosomal-like Ca^{2+} stores in Meg-01 cells and TPC knockdown reduces this response (231). TPC2 is localized to platelet dense granules that are lysosome-related organelles and is involved in their maturation and function (232). TPC2 mediates Ca^{2+} release and formation of perigranular Ca^{2+} nanodomains in Meg-01 cells that mark "kiss-and-run" events mediating material transfer between different granules (232). Upon genetic deletion of NMDA receptors in Meg-01 cells, accumulation of lysosomal organelles and upregulation of *MCOLN3* transcripts (coding for TRPML3) were observed. This suggests a link between lysosomal biogenesis, NMDA receptor function and Meg-01 cell proliferation (183). TPC1 and TPC2 inhibitor tetrandrine suppresses growth and increases cell death in several AML cell lines (U937, NB4, K-562, HL-60 and THP-1) (233–236). A recent study also demonstrates that TPC2 inhibition and its genetic deletion sensitizes ALL cells (cell lines and patient-derived) to cytotoxic drugs (237). Upon TPC2 deletion, leukemic cells are not able to sequester cytotoxic drugs within lysosomes, which increases drug concentration in the cytoplasm and enhances its cytotoxic effectiveness. Therefore, targeting lysosomal TPC2 may help overcome chemoresistance in ALL cells (237). Similar may apply in AML, although different mechanisms may contribute to lysosomal deregulation in different types of leukemia (238–240).

4.4 Calcium ATPases and secondary-active Ca^{2+} transporters

Several types of Ca^{2+} ATPases are involved in the maintenance of transmembrane Ca^{2+} gradients between the cytosol and the blood plasma as well as between the cytosol and the inner compartments of the organelles. Plasma membranes are equipped with several isoforms of the plasma membrane calcium ATPases (PMCAs) (241, 242) (Figure 1). Human monocytes and macrophages express plasma membrane Na^+/Ca^{2+} exchanger NCX that actively extrudes Ca^{2+} while taking in Na^+ transported passively using the energy of transmembrane Na^+ gradient generated by the Na^+/K^+ ATPase (243). Mitochondrial membrane contains its own $Na^+(Li^+)/Ca^{2+}$ exchanger (NCLX) that controls Ca^{2+} levels in the mitochondria (244). Cells also pump Ca^{2+} out of the cytoplasm into the Golgi apparatus through secretory pathway Ca^{2+} ATPases (SPCA), and to the ER through SERCA (245) (Figure 1). The activity of SERCA reflects the activation state of T-cells (246), B-cells (247), Th1 and Th2 lymphocytes (248). Of these molecules, SERCA has been reported to be dysregulated in a number of hematologic malignancies.

4.4.1 Sarco-endoplasmic reticulum calcium ATPase

There are three SERCA genes in humans and alternative splicing can give rise to many isoforms (10). In response to differentiation, SERCA3 expression changes in leukemic megakaryocytic cell line Meg-01, precursor B-ALL cell lines (Kasumi-2 and RCH-ACV), and APL cell lines (NB4 and HL-60) (249–251). All-trans retinoic acid-induced differentiation of APL cells results in increasing SERCA3 expression and SERCA3-dependent Ca^{2+} accumulation (249). When SERCA activity is inhibited, lower concentrations of retinoic acid can induce differentiation of NB4 and HL-60 cell lines (252).

In a human T-ALL xenograft mouse model, SERCA inhibition with thapsigargin reduces tumor growth (253). Thapsigargin prevents the activation of the transmembrane receptor, NOTCH1, which often contains activating mutations in T-ALL, CLL, mantle cell lymphoma, and a subset of DLBCL (10, 253). The reduction of Ca^{2+} entering the ER upon SERCA inhibition changes the folding and trafficking of NOTCH1 (10, 253). As reviewed by

Pagliaro et al, other SERCA inhibitors have been developed that induce apoptosis in a range of leukemic cell lines and xenograft models including T-ALL, B-ALL, mantle cell lymphoma, and AML (10). In contrast, when SERCA expression is reduced or its activity is inhibited by oncogenes such as Bcl-2 and Ras, the decrease in ER Ca^{2+} concentrations reduces the potential for a Ca^{2+} overload and initiation of apoptosis in response to ER stress (219, 254).

4.5 Calcium sensor and effector proteins

Some of the downstream Ca^{2+} sensor proteins implicated in blood cancer include the S100 family, calcium/calmodulin-stimulated protein kinases (CaMKs), calcineurin, CALR, and PKC. Dysregulation of these Ca^{2+} sensors alters gene regulation associated with cell apoptosis, proliferation, and migration (Table 2).

TABLE 2 The differential expression of calcium sensor and effector proteins and their relative contribution to the malignant phenotype of different blood cancers.

Cancer type	Molecule	Change in disease	Functional effects	References
AML	S100-A4	↑ expression	↓ overall survival $^{(P)}$, ↑ cell proliferation $^{(C)}$, ↑ migration $^{(C)}$, ↑d rug resistance $^{(C)}$	(255–259)
	S100-A8 or -A9	↑ expression	↓ overall survival $^{(P,M)}$, ↓ event free survival $^{(P)}$, ↑ drug resistance $^{(C)}$, ↑ autophagy $^{(C)}$, ↓ apoptosis $^{(C)}$, differentiation $^{(C,P,M)}$	(255, 260–265)
	S100-P	↑ expression	↑ overall survival $^{(P)}$, differentiation $^{(C)}$	(259, 266–268)
	CAMKI	↑ expression	↑ cell proliferation $^{(C,M)}$, ↓ overall survival $^{(P)}$	(269)
	CAMKII	↑ activation	↓ differentiation $^{(C)}$, ↑ cell proliferation $^{(C)}$	(270)
	CAMKIV	↑ expression	↑ cell proliferation $^{(C,M)}$, ↓ overall survival $^{(P)}$	(269)
	PKCα	↑ expression and activation	↑ Bcl-2 phosphorylation $^{(P)}$, ↓ overall survival $^{(P)}$	(271–273)
ALL	S100-A4	↑ expression $^{(C,P)}$		(258, 274)
	S100-A6	↑ expression	↓ overall survival $^{(M)}$, ↑ cell proliferation $^{(M)}$, ↓ apoptosis $^{(C)}$	(274–278)
	S100-A8 or A9	↑ expression	↓ event free survival $^{(P)}$, ↓ Ca^{2+} influx $^{(C)}$, ↑ drug resistance $^{(C,P)}$, ↑ relapse $^{(P)}$	(274, 279)
	Calcineurin	↑ activation	↑ cell proliferation $^{(C,M)}$, ↓ apoptosis $^{(C,M)}$, ↑ disease progression $^{(M)}$	(280, 281)
CML	S100-A4	↑ expression	↑ drug resistance $^{(C)}$	(282)
	S100-A8 or A9	↑ expression	↑ drug resistance $^{(C)}$, ↑autophagy $^{(C)}$, ↓ Ca^{2+} influx $^{(C)}$	(260, 283)
	CAMKII	↑ activation	↑ cell proliferation $^{(C)}$	(270)
	CAMKII	↑ expression	↑ drug resistance $^{(P)}$	(284)
	PKCα	↓ expression	↑ association with cell membrane $^{(P)}$	(285)
	PKCβ2	↑ expression $^{(P)}$		(286)
MPN	S100-A4	↑ expression	↑ inflammation $^{(P)}$	(287)
	S100-A8 or A9	↑ expression	↑ inflammation $^{(P)}$, ↓ cell proliferation $^{(P)}$	(287)
	FKBP5	↑ expression in megakaryocytes	↑ cell survival $^{(P)}$, ↓ calcineurin activity $^{(P)}$, ↑ STAT5 activation $^{(C,P)}$	(288, 289)
	CALR	mutations, predicted loss of Ca^{2+} binding	↑ Ca^{2+} influx $^{(P)}$, ↑ proliferation of megakaryocytes $^{(P)}$	(9, 290, 291)
CLL	S100-A8	↑ expression	↑ disease progression $^{(P)}$, ↑ need for early treatment $^{(P)}$	(292)
	PKCα	↓ expression $^{(P)}$		(286)
	PKCβ2	↑ expression, ↑ activation	↓ Ca^{2+} influx $^{(P)}$, ↑ cell survival $^{(P,M)}$, ↓ apoptosis $^{(P)}$	(286, 293, 294)
DLBCL	S100-A4	↑ expression	↑ drug resistance $^{(P)}$	(295)
	S100-A8 or A9	↑ expression	↑ drug resistance $^{(P)}$	(295)
	Calcineurin	↑ activation	↑ cell proliferation $^{(C)}$, ↓ apoptosis $^{(C)}$	(280, 296)
T-cell lymphoma	S100-A9	↑ expression	↑ drug resistance $^{(P)}$, ↓ overall survival $^{(P)}$, ↓ progression free survival $^{(P)}$, ↑early reoccurrence rate $^{(P)}$	(297, 298)
Plasma cell myeloma	CAMKII	↑ expression	↑ disease progression $^{(P)}$, ↓ overall survival $^{(P)}$, ↑ cell proliferation $^{(C)}$, ↓ apoptosis $^{(C)}$	(299)

(P) = Patient cells, (C) = Cell lines, (M) = Mouse model. ↑ = increased, ↓ = decreased. Expression changes are often found in particular cell lines or leukemic subtypes and not in others. Empty cells indicate there is no data. AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; DLBCL, diffuse large B-cell lymphoma; MPN, myeloproliferative neoplasms (classical Philadelphia chromosome-negative).

4.5.1 S100 family

The S100 family are Ca^{2+} binding proteins of which many have been reported to be dysregulated in AML, ALL, CLL, MPN, and lymphomas (292, 295, 300, 301). S100- A8 and A9 are the most well-studied members of the S100 family in leukemia. Dysregulation of their expression, and changes in plasma levels, or secretion levels in the bone marrow microenvironment have been reported in AML (300, 301). S100- A8 and A9 are dose-dependent regulators of myeloid differentiation and leukemic cell proliferation and can play contradictory roles, depending on their expression levels as monomers, homodimers, or heterodimers (300, 301). S100- A8 and A9 are expressed constitutively in the cytoplasm of myeloid cells, including myeloid precursors, but are absent from lymphocytes (301). Increased activity of multiple S100 family members is associated with increased drug resistance in hematologic malignancies including AML, CML, ALL, and B-cell lymphomas (302, 303). For example, S100-A8/A9 contribute to gilteritinib resistance in FLT3- internal tandem duplications- (FLT3-ITD)-positive AML primary cells and cell lines (304). Particularly, S100-A9 expression has been found to be more consistently and remarkably altered than S100-A8 in human FLT3-ITD-positive AML cell lines (MOLM13 and MOLM13-RES) after gilteritinib treatment. The potential mechanism is gilteritinib promotes Bcl-6 dissociation from the S100-A9 promoter, which leads to upregulation of S100-A9 (304).

4.5.2 Calcium/calmodulin-stimulated protein kinase family

Calmodulin is a Ca^{2+}-binding protein that regulates a wide variety of cellular processes *via* interaction with multiple target proteins (305). The CaMK family members are activated when bound to Ca^{2+}-saturated calmodulin (306). CaMK family members are overexpressed or aberrantly activated in CML, AML, and plasma cell myeloma (269, 270, 284, 299, 307). Inhibition or knockdown of CaMKI, CaMKII, or CaMKIV reduces proliferation in different myeloid leukemia cells and multiple CAMK or calmodulin antagonists have been used to inhibit leukemic cell growth and proliferation (269, 270, 305–307). High expression of CaMKs is associated with a poor overall survival probability in patients with plasma cell myeloma or AML (269, 299). Deletion of CaMKII suppresses T-cell lymphomagenesis in mice, and T-cell lymphoma cell line growth (comprising H9, JB6, Jurkat, and SU-DHL-1) is suppressed when CaMKII activity is inhibited (308). CaMKII is also activated by the constitutively active tyrosine kinase BCR::ABL1 in CML cells. The tyrosine kinase inhibitor (TKI) imatinib, inhibits proliferation of BCR::ABL1 expressing cells and is accompanied by a rapid decrease in activated (autophosphorylated) CaMKII (270). In an inducible BCR::ABL1 cell line (TonB210.1), decreased BCR::ABL1 expression is also accompanied by a reduction of autophosphorylated CaMKII, and inducing BCR::ABL1 expression restores CaMKII autophosphorylation (270). CAMKII-γ is highly activated in CML LSCs and its aberrant activation accelerates CML blast crisis (309). In a mouse xenograft model of patient-derived CML cells, LSCs were eliminated by an ATP-competitive CAMKII-γ inhibitor berbamine (310).

4.5.3 Calcineurin

Calcineurin is a calcium-calmodulin-dependent phosphatase, that when activated by Ca^{2+} and calmodulin, dephosphorylates its substrates including nuclear factor of activated T-cells (NFAT) (311). Dephosphorylated NFAT proteins translocate into the nucleus to regulate the transcription of genes important for cell proliferation, growth, migration, differentiation, and survival (311). The calcineurin-NFAT pathway negatively regulates megakaryopoiesis (312). Inappropriate inhibition of this pathway may contribute to the pathological expansion of megakaryocytes and their precursors, in particular in the context of Down syndrome (313, 314) (see Figure 5A and the corresponding legend for molecular details). The calcineurin inhibitor FKBP5 (FK506 binding protein) is overexpressed in megakaryocytes from patients with PMF. FKBP5 overexpression in UT-7 cells (a factor-dependent human cell line with a megakaryocytic phenotype) promotes cell survival after cytokine deprivation, suggesting a pathway to disease development through the inhibition of calcineurin (288).

Calcineurin and NFAT have also been implicated in other hematologic malignancies, including Burkitt lymphoma, T-cell lymphoma, T-ALL, DLBCL, CML, CLL, and AML (311, 315, 316). The calcineurin inhibitors, cyclosporin A, and tacrolimus (FK506), have anti-leukemic effects in mice T-ALL models, and deletion of calcineurin specifically in T-ALL leukemic cells results in impaired leukemia progression (280, 281). Inhibition of calcineurin by cyclosporin A or FK506 is also selectively cytotoxic against the ABC-DLBCL (296). This response to calcineurin inhibitors is associated with reduced NFAT-mediated expression of critical genes, including c-Jun, signal transducer and activator of transcription 3 (STAT3), interleukin-6 and interleukin-10 that are crucial for survival of ABC-DLBCL cells (296).

4.5.4 Calreticulin

CALR, amongst its other functions, is an ER-resident Ca^{2+}-buffering protein that helps maintain intracellular Ca^{2+} homeostasis and assists the folding of proteins destined for secretion or insertion into the plasma membrane (317). CALR is mutated in approximately one-quarter of the Philadelphia chromosome-negative MPNs, PMF and essential thrombocythemia (290, 318). The mutations in *CALR*, as well as *JAK2* and *MPL* (Figure 5B, left), converge to constitutively activate JAK2-STAT signaling, which drives deregulated expansion of HSCs and megakaryocytes (317, 318). *CALR* mutations have two main variants, type 1 and type 2. Type 1 or type 1-like mutations are mostly large deletions of which a 52-bp deletion is the most common; while type 2 or type 2-like are mostly small insertions of which a 5-bp insertion is the

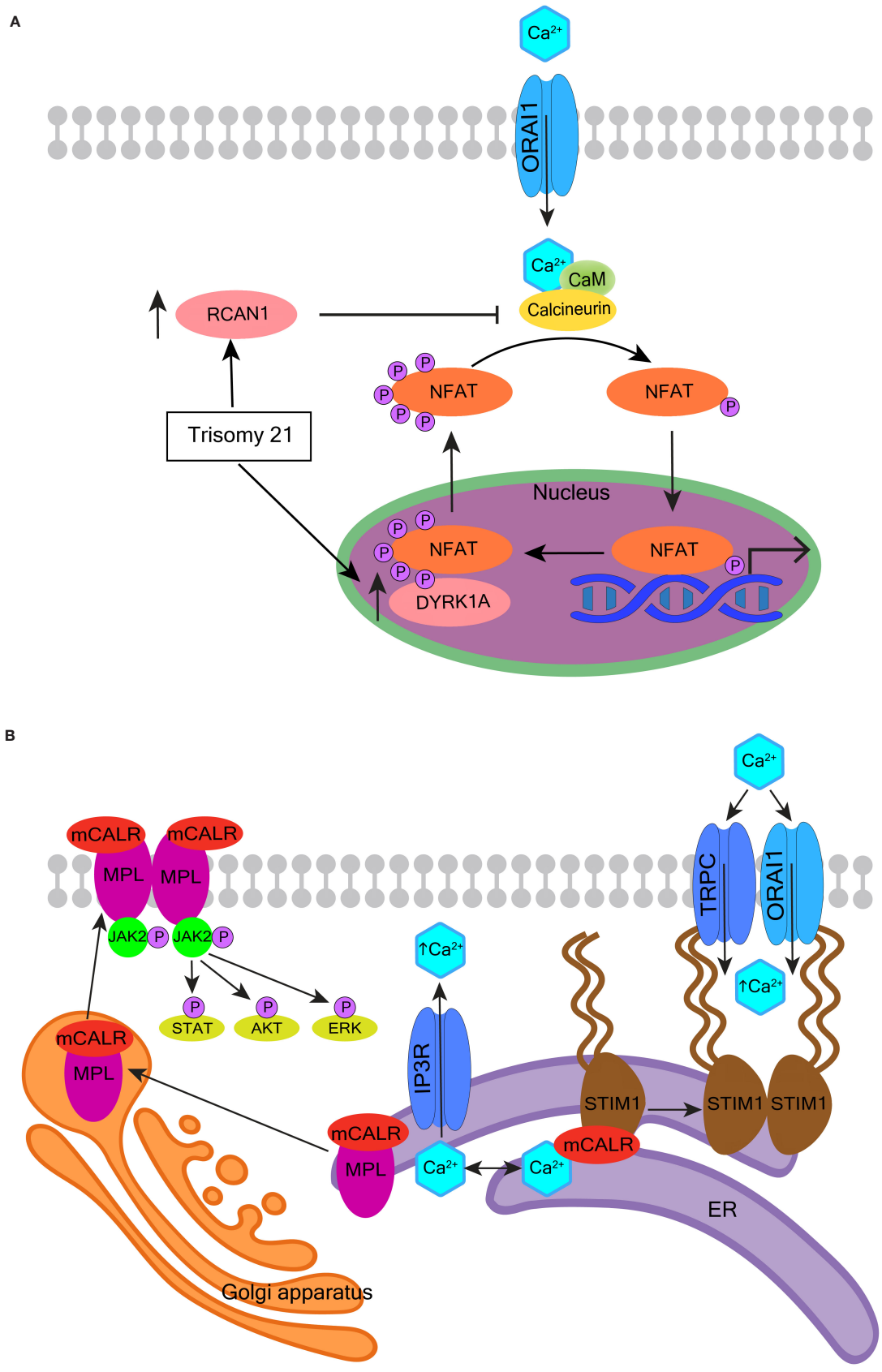

FIGURE 5 (Continued)

FIGURE 5 (Continued)
Oncogenic effects of calcineurin and calreticulin. **(A)** The role of calcineurin-NFAT signaling in the pathogenesis of myeloid proliferation associated with Down syndrome. Human chromosome 21 encodes two important regulators of nuclear factor of activated T-cells (NFAT) - regulator of calcineurin 1 (RCAN1) and dual specificity tyrosine phosphorylation regulated kinase 1A (DYRK1A). When calcineurin is activated by Ca^{2+} and calmodulin (CaM), it dephosphorylates NFAT. Dephosphorylated NFAT translocates to the nucleus and transcriptionally regulates numerous genes involved in cell proliferation, growth, migration, differentiation, and survival. NFAT is re-phosphorylated by DYRK1A and is exported back to the cytoplasm. RCAN1 inhibits calcineurin, and so also inhibits the dephosphorylation and translocation of NFAT. RCAN1 and DYRK1A are overexpressed in Down syndrome and are suspected to contribute to the development of transient abnormal myeloproliferation and megakaryoblastic leukemia in Down syndrome children. **(B)** The role of mutated calreticulin (CALR) in myeloproliferative neoplasms. More than 50 mutations have been reported in exon 9 of the *CALR* gene; most generate a +1-frameshift causing the mutated CALR protein (mCALR) to stably associate with the thrombopoietin receptor MPL protein in the ER. The mCALR-MPL complex is transported from the ER through the Golgi apparatus and secretory system to the plasma membrane. The binding of mCALR to MPL constitutively activates signaling through JAK2 and its downstream targets such as STAT, AKT, and ERK (left). The most common mCALR variants are type 1 that also impair the Ca^{2+} binding activity of mCALR more than type 2. The type 1 mCALR with reduced Ca^{2+} binding dissociates from STIM1 in the ER. This allows STIM1 to dimerize and bind Orai1 and TRPC, which leads to constitutive activation of SOCE (right).

most common. Type 1 mutations are predicted to impair the Ca^{2+}-binding activity of CALR more than type 2 (319). Congruently, type 1 mutations associate with higher ER Ca^{2+} release, higher SOCE, and spontaneous cytosolic Ca^{2+} oscillations in cultured megakaryocytes (9, 290).

CALR assists the folding of STIM1, and whilst they are bound, STIM1 is in an inactive configuration on the ER membrane inhibiting SOCE (9). Megakaryocytes with mutated CALR have a decreased association between CALR and STIM1 (Figure 5B, right), and the CALR type 1 variant has the highest level of dissociation from STIM1. Defective interaction between mutant CALR and STIM1 activates SOCE and generates spontaneous cytosolic Ca^{2+} influx. This, in turn, increases megakaryocyte proliferation, which can be reversed using a specific SOCE inhibitor (9). Thus, the impact of mutated CALR on Ca^{2+} homeostasis may be influencing the course of MPN in combination with its aberrant activation of JAK2-STAT signaling. Further elucidation of these mechanisms may inform the development of new drugs to improve the effects of JAK2 inhibition.

4.5.5 Protein kinase C

PKC is activated by the second messenger DAG, which is hydrolyzed from PIP2 following receptor engagement and PLC activation (320, 321). The PKC family has many isoforms that can be categorized into three groups: conventional PKC isoforms, novel PKC isoforms, and atypical PKC isoforms (320, 321). A range of PKC isoforms are up- or down-regulated which can affect cell growth and survival in AML, CML, CLL, and plasma cell myeloma (286, 322, 323). Only the conventional PKC isoforms (α, β, and γ) are activated by Ca^{2+} as well as DAG (320, 321). In CML, the BCR::ABL1 phosphorylates a range of PKC isoforms leading to altered activity (323). ER Ca^{2+} release and SOCE are reduced in cell lines that express BCR::ABL1 (171, 324). These abnormal Ca^{2+} responses are Bcl-2 independent but PKC dependent. PKC-β overexpression is significantly associated with resistance to TKIs such as imatinib (323, 325). Suppressing PKC-β activity or expression in TKI-resistant CML patient cells and cell lines increases the sensitivity to imatinib (325). Inhibition of PKC-β increases the effect of imatinib on reducing leukemic cell proliferation in a CML mouse model and also prolongs survival (325). Outside of CML, PKC-β was found to be essential for CLL development in a mouse model and promotes cell growth and survival of CLL cells (294, 322, 326).

5 Mutational landscape in the calcium-toolkit encoding genes recorded in publically accessible blood cancers databases

We reviewed publically available genetic cancer databases for mutations affecting molecules described in this review across the main blood cancer types. Figure 6 demonstrates the mutational landscape in lymphoid cancers and Figure 7 in myeloid cancers. We obtained this data using cBioPortal for Cancer Genomics platform (https://www.cbioportal.org/) (354, 355). The Ca^{2+}-toolkit encoding genes were queried as gene sets grouped according to function (see Supplementary Table S1). Results are observational only and require validation but are useful to generate hypotheses for future research and to assist discussion. The datasets available for interrogation and the samples reviewed are listed in Supplementary Tables S2 and S3. The lymphoid neoplasms reviewed included B-ALL (n = 234-269 patients depending on the gene), DLBCL (n = 1288), low-grade B-cell neoplasms (n= 1542) including CLL (n = 1254), monoclonal B-cell lymphocytosis (n = 54), mantle cell lymphoma (n = 29), plasma cell myeloma (n= 205) and low-grade T-cell neoplasms (n = 43) including Sezary syndrome (n = 26), primary cutaneous CD8/CD30 positive lymphomas (n = 14) and mycosis fungoides (n = 3) (Figure 6, Table 3 and Supplementary Table 2). Of these lymphoid cancers, patients with B-ALL had the lowest frequency of variants in the Ca^{2+}-toolkit encoding genes (5.9%) and patients with low-grade T-cell neoplasms had the highest frequency (48.8%), most carrying

FIGURE 6
Calcium-toolkit mutations in lymphoid neoplasms. Oncoprints are shown generated using cBioportal for Cancer Genomics platform (https://www.cbioportal.org/). Events were analyzed per patient, frequencies are listed. Unaltered columns and whitespaces between columns are not shown. The Ca^{2+}-toolkit genes were grouped according to function (see Supplemental Table 1). The molecular profiles queried included mutations but excluded copy number variations and structural variants as these were not available for most patients. The databases analyzed are indicated in the figure and referenced below: 1) For B-ALL: Acute Lymphoblastic Leukemia databases St Jude Nat Genet 2015 (327), St Jude Nat Genet 2016 (328) and Pediatric Acute Lymphoid Leukemia Phase II TARGET 2018. TARGET data was generated by the Therapeutically Applicable Research to Generate Effective Treatments initiative and is available at https://portal.gdc.cancer.gov/projects. The St Jude database also contained 8 T-ALL, 10 AML, and 5 unspecified leukemias - none had relevant mutations and these cases were excluded from the total. There were no other T-ALL cases with mutational data available for analysis so this cancer type could not be analyzed further. 2) For DLBCL: Diffuse Large B cell Lymphoma databases DFCI Nat Med 2018 (329), Duke Cell 2017 (330), Broad PNAS 2012 (331), TCGA PanCancer Atlas (332–340), and BCGSC Blood 2013 (341). 3) For low-grade B-cell neoplasms: Chronic Lymphocytic Leukemia databases Broad Cell 2013 (342), Broad Nature 2015 (343), IUOPA Nature 2015 (344), ICGC Nature Genetics 2011 (345), Mantle Cell Lymphoma database IDIBIPS PNAS 2013 (346), and Multiple Myeloma database Broad Cancer Cell 2014 (347). 4) For low-grade T-cell neoplasms: Cutaneous T Cell Lymphoma database Columbia U Nat Genet 2015 (348). Only patients with the appropriate diagnoses were selected. Specific cases analyzed are listed in Supplemental Table 2. The cBioPortal queries can be retrieved at the following links: B-ALL https://bit.ly/3Q9ZGvv; DLBCL https://bit.ly/3CZJpq8; low-grade B-cell neoplasms https://bit.ly/3AP87Xx; low-grade T-cell neoplasms https://bit.ly/3TvDZsT. Specific genetic variants can be found through these links, all were of unknown significance. *Numbers of patients analyzed and disease groups are clarified in Table 3.

multiple variants (Figure 6 and Table 3). The *GRIN* genes encoding NMDA receptor subunits and the *ITPR* genes encoding IP3Rs were mutated in 1.3% of B-ALL patients each, other variants were present in <1% of B-ALL patients. The cBioPortal data did not yet contain results of Bohannan et al. published earlier this year that reported the presence of *GRIN2C* mutations in high-risk B-ALL patients (145) (Figure 3C). It would be interesting to review these data when it becomes publically available, and pursue similar analysis in larger studies in the future.

In mature T-cell neoplasms, the most affected genes were those coding for PLC (16.3%), $Ca^{2+}/Na^{+}/K^{+}$ exchangers (14.0%), TRPC and TRPM channels (11.6% each), Ca_V and NMDA receptor channels (9.3% each), ryanodine receptors (RYR) (7.0%), and a few others at 4.7% each (TRPV, P2RX, and SERCA) (Figure 6 and Table 3). This particularly high frequency of variants in cutaneous T-cell neoplasms requires confirmation in larger cohorts. Unfortunately, no other mature or precursor T-cell malignancies could be reliably reviewed. Less than 10 T-ALL samples with mutational data were identified and

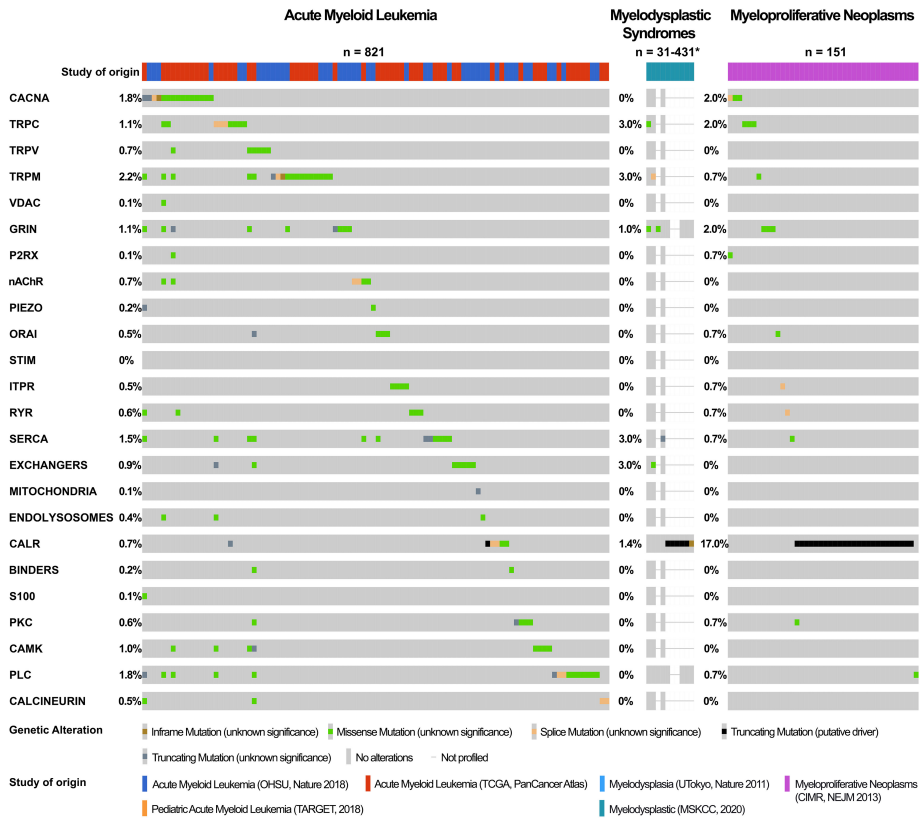

FIGURE 7
Calcium-toolkit mutations in myeloid neoplasms. Oncoprints are shown generated using cBioportal for Cancer Genomics platform (https://www.cbioportal.org/). Events were analyzed per patient, frequencies are listed. Unaltered columns and whitespaces between columns are not shown. The Ca^{2+}-toolkit genes were grouped according to function (see Supplemental Table 1). The molecular profiles queried included mutations but excluded copy number variations and structural variants as these were not available for most patients. The databases analyzed are indicated in the figure and referenced below: 1) Acute Myeloid Leukemia databases OHSU Nature 2018 (349); TCGA PanCancer Atlas (332–340), and Pediatric Acute Myeloid Leukemia TARGET 2018. TARGET data was generated by the Therapeutically Applicable Research to Generate Effective Treatments initiative and is available at https://portal.gdc.cancer.gov/projects. 2) Myelodysplasia databases UTokyo Nature 2011 (350)), MSKCC 2020 (349, 351, 352). 466 MDS patients were excluded from the analysis as they were not profiled for any queried genes. 3) Myeloproliferative Neoplasms database CIMR NEJM 2013 (353). Only patients with the appropriate diagnoses were selected. Specific cases analyzed are listed in Supplemental Table 3. The cBioPortal queries can be retrieved at the following links: AML https://bit.ly/3Ts6NlX; MDS https://bit.ly/3AuvJkc; MPN https://bit.ly/3q1TZ8C. Specific genetic variants can be found through these links, apart from *CALR* mutations all were of unknown significance. *Numbers of patients analyzed are clarified in Table 3.

none had relevant mutations. Ca^{2+} signaling is critical for T-cell activation downstream of TCR and linked closely with the regulation of T-cell metabolism (356). Multiple studies highlighted the role of Orai, Ca_V, TRP, NMDA receptors and other Ca^{2+} regulators in normal and malignant T-cells (134, 192, 357–361). Further studies are required to examine potential contribution of these changes in T-cell cancers.

In mature B-cell neoplasms, mutated genes were broadly similar between the low-grade and high-grade cancers (Figure 6 and Table 3). The frequency of variants was higher in patients with low-grade B-cell neoplasms, 23.0% compared with 13.9% in DLBCL, but many patients with DLBCL had multiple variants. The types of variants were similar between patients with CLL, monoclonal B-cell lymphocytosis, mantle cell lymphoma and plasma cell myeloma. In low-grade B-cell lymphoproliferative disorders, variants in *RYR1-3* were the most common (4.5%), followed by *PLC* (3.2%), *CACNA* (2.8%), *TRPM* (2.6%) and *TRPC* genes (2.1%), with others present in <2% of patients. Some of these mutations appeared exclusive between each other. In DLBCL, *RYR* mutations were less common (2.5%) but *CACNA* variants were more common (3.8%) (Figure 6 and Table 3). One could hypothesize that the acquisition of multiple mutations associates with a higher grade. Further bioinformatics analysis and laboratory studies to determine the role of gene variants in lymphoid cancers appears warranted. For example, experimental studies to examine the contribution of mutations in plasma membrane Ca^{2+} channels and RYRs would be of interest. The existing literature in this area is limited. RYRs facilitate the

TABLE 3 Frequencies of genetic variants in the calcium-toolkit encoding genes documented in lymphoid and myeloid cancer databases.

Cancer type	Lymphoid								Myeloid		
	B-cell							T-cell	AML	MDS	MPN
	B-ALL	DLBCL	Low-grade B-cell neoplasms					Low-grade T-cell neoplasms			
			Total	CLL	MBL	MCL	PCM				
Total number of patients per cancer type	**234 except [1]269**	**1288**	**1542**	**1254**	**54**	**29**	**205**	**43**	**821**	**31 except [2]209 [3]431 [4]33**	**151**
Gene sets analyzed	% of patients with gene variants in each cancer type										
CACNA	0.0	3.8	2.8	2.2	5.6	0.0	6.3	9.3	1.8	0.0	2.0
TRPC	0.4	2.6	2.1	2.3	1.9	0.0	1.5	11.6	1.1	3.2	2.0
TRPV	0.4	0.3	0.8	0.6	0.0	3.4	2.0	4.7	0.7	0.0	0.0
TRPM	0.4	2.7	2.6	1.9	3.7	10.3	5.4	11.6	2.2	3.2	0.7
VDAC	0.0	0.0	0.1	0.0	0.0	0.0	1.0	0.0	0.1	0.0	0.0
GRIN	1.3	1.6	0.9	0.8	0.0	3.4	1.5	9.3	1.1	1.0[2]	2.0
P2RX	0.0	0.7	0.3	0.2	0.0	0.0	1.0	4.7	0.1	0.0	0.7
nAChR	0.4	0.9	1.2	0.8	1.9	3.4	3.4	4.7	0.7	0.0	0.0
PIEZO	0.0	0.4	0.5	0.5	0.0	0.0	0.5	0.0	0.2	0.0	0.0
ORAI	0.0	0.4	0.3	0.1	0.0	0.0	2.0	0.0	0.5	0.0	0.7
STIM	0.4	0.2	0.1	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0
ITPR	1.3	1.4	0.8	0.6	1.9	0.0	2.0	2.3	0.5	0.0	0.7
RYR	0.4	2.5	4.5	3.8	0.0	0.0	10.2	7.0	0.6	0.0	0.7
SERCA	0.0[1]	1.2	1.6	1.4	3.7	0.0	2.0	4.7	1.5	3.2	0.7
Calcium exchangers	0.0	1.2	1.4	1.1	1.9	0.0	2.9	14.0	0.9	3.2	0.0
Mitochondrial calcium regulators	0.0	0.5	0.6	0.8	0.0	0.0	0.0	0.0	0.1	0.0	0.0
Endolysosomal calcium channels	0.4	0.7	0.5	0.3	0.0	3.4	1.0	2.3	0.4	0.0	0.0
CALR	0.0	0.2	0.1	0.1	0.0	0.0	0.0	0.0	0.7	1.4[3]	16.6
Calcium binders	0.0[1]	1.1	0.1	0.1	0.0	0.0	0.5	0.0	0.2	0.0	0.0
S100 family	0.4	0.2	0.1	0.2	0.0	0.0	0.0	0.0	0.1	0.0	0.0
PKC	0.0	1.9	1.4	1.2	0.0	0.0	3.4	2.3	0.6	0.0[4]	0.7
CAMK	0.0	0.8	1.0	0.9	0.0	0.0	2.4	0.0	1.0	0.0	0.0
PLC	0.9	3.4	3.2	3.1	1.9	0.0	4.9	16.3	1.8	0.0[2]	0.7
Calcineurin	0.0	0.5	0.1	0.1	0.0	0.0	0.5	0.0	0.5	0.0	0.0
Total % of patients with genetic variants[5]	5.9[1]	13.9	23.0	20.3	22.2	24.1	39.0	48.8	11.9	2.3[3]	26.5

This table provides a summary of data displayed in Figures 6, 7. The gene sets are listed in Supplemental Table 1 and the full list of cases reviewed is provided in Supplemental Table 2 (lymphoid cases) and Supplemental Table 3 (myeloid cases). [1,2,3,4] Patient numbers varied for these groups from the overall total as shown. [5]Many patients had multiple genetic variants. AML, acute myeloid leukemia; B-ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; MCL, mantle cell lymphoma; MBL, monoclonal B-cell lymphocytosis; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasms; PCM, plasma cell myeloma.

release of Ca^{2+} from the ER in addition to IP3Rs (362) (Figure 1). They are the largest ion channels known regulated by $Ca_V1.1$/$Ca_V1.2$-mediated Ca^{2+} entry, as well as other small molecules including ATP, calmodulin, calsequestrin and CaMKII (363). RYR1 is primarily expressed in skeletal muscles but is also present in B-lymphocytes (364, 365) and Burkitt lymphoma-derived Namalwa cells (366). The RYR role in B-cell function remains unclear but its activity downregulates CD38 expression (366). High levels of CD38 associate with poor risk CLL (367). Studies to determine the mechanism of the RYR contribution to the regulation of CD38 and the impact of RYR mutations on CD38 expression and B-cell activation may be helpful.

In the group of myeloid neoplasms, we reviewed 821 patients with AML, 151 with MPN and 31-431 patients with myelodysplastic syndrome (MDS) (depending on the gene) (Figure 7, Table 3 and Supplementary Table S3). Compared with B-ALL, AML patients carried more variants (11.9% versus 5.5% in B-ALL patients). Variants in *CACNA* (1.8%) and *TRP* genes (2.2% in *TRPM*, 1.1% in *TRPC*, 0.7% in *TRPV*) were the most common in AML but no variants exceeded 3% frequency. MDS patients had a

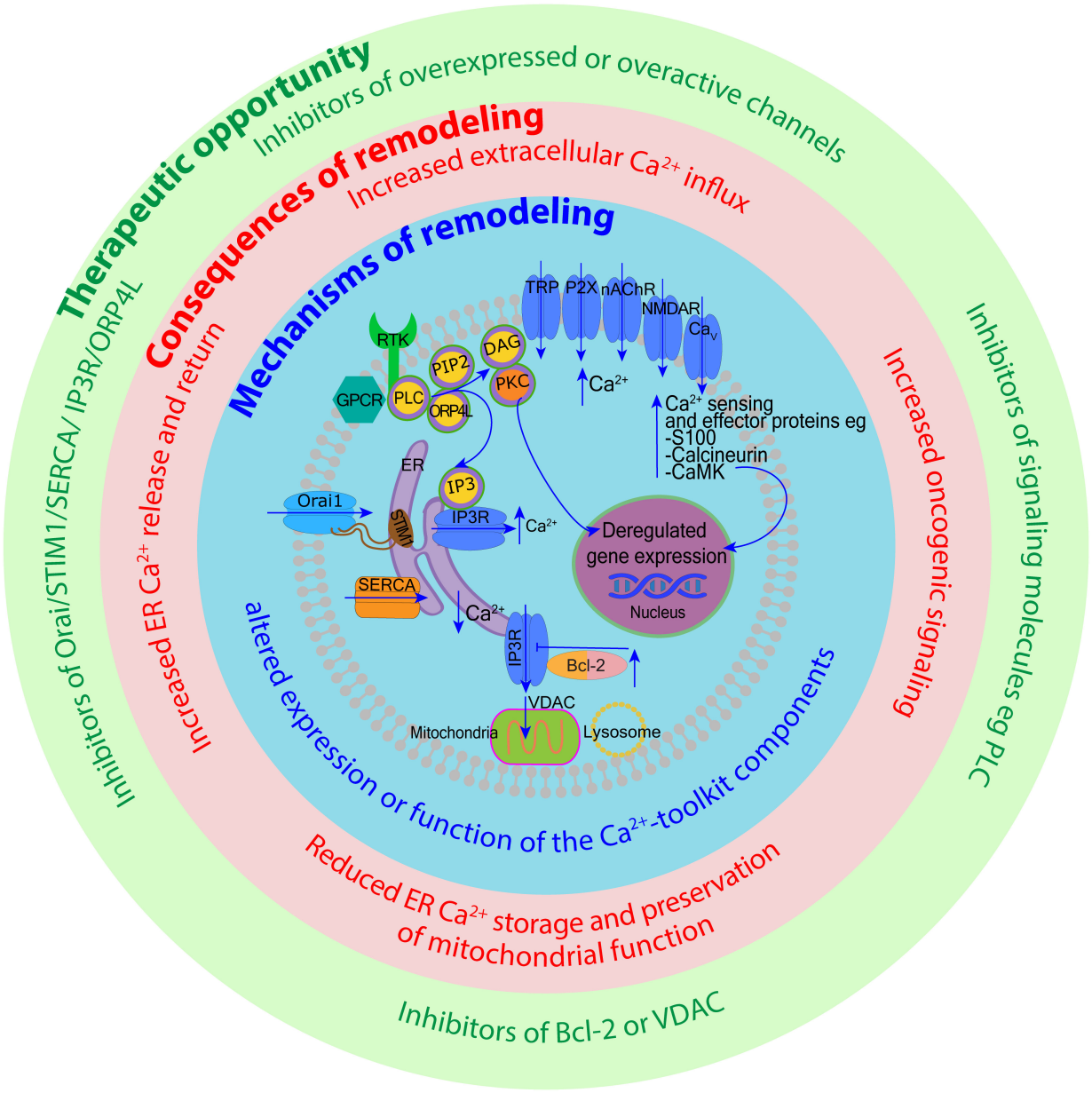

FIGURE 8
Remodeling of calcium signaling in hematologic cancers and therapeutic opportunities this presents. Examples of mechanisms responsible for the remodeling of Ca^{2+} signaling in blood cancer are depicted within the central blue circle. Their direct consequences are listed in the middle red circle, and therapeutic opportunities arising from these changes are highlighted in the outer green circle. For example, the increased expression of the plasma membrane Ca^{2+} channels (a mechanism of remodeling) leads to increased extracellular Ca^{2+} influx supporting oncogenic signaling (a consequence). Such changes could be counteracted by specific Ca^{2+} channel inhibitors (a therapeutic opportunity). In another example, mechanisms that spare cancer cells from mitochondrial Ca^{2+} overload (e.g. through the overexpression of Bcl-2 or VDAC) could be counteracted by inhibitors of these molecules. The design of novel therapies heavily relies on our understanding of the Ca^{2+}-toolkit remodeling in different blood cancers.

lower frequency of variants at 2.3% overall, although data for these patients was limited. In the MPN group, 17% had *CALR* mutations, consistent with it being a known MPN driver (353, 368). In addition, a further 9.5% of MPN patients had variants in other Ca^{2+}-toolkit genes (including 2.0% in *CACNA*, *TRPC* and *GRIN* genes each). It was intriguing that these variants were exclusive with *CALR* mutations and also appeared exclusive with each other, raising the possibility of their independent effects in MPN (Figure 7 and Table 3). Evidence slowly accumulates that Ca^{2+} signaling is aberrant in *CALR*-mutated MPN (9, 290, 291, 319). The

TABLE 4 Demonstrative examples of calcium-related compounds/drugs (specific inhibitors or activators of calcium channels and receptors) that have been found to exert an impact on the functional outcome *in vivo* or *in vitro* in diverse blood cancer types.

Target	Compound	Functional effects	Cancer type	References
Plasma membrane Ca^{2+} channel activators				
TRPV2	Cannabidiol	↑ cytoplasmic [Ca^{2+}], ↑ ROS production, induces mitophagy, ↓ cell viability, ↓ cell proliferation, ↓ cell cycle progression	CML (C)	(147)
			Plasma cell myeloma (C)	(372)
NMDAR	Glutamate	↑ cytoplasmic [Ca^{2+}], ↑ cell proliferation	AML (C)	(182, 183, 373)
	NMDA	↑ cytoplasmic [Ca^{2+}], ↑ cell proliferation	AML (C)	(182, 183, 373)
Plasma membrane Ca^{2+} channel inhibitors				
P2X7	A740003	↓ cell proliferation, ↓ self-renewal of leukemia-initiating cells, ↑ survival time	AML (C,M)	(124, 125)
	AZ10606120	↓ cytoplasmic [Ca^{2+}] influx, ↓ leukemic growth	AML (P,M)	(126)
TRPC3	Pyr3	Pyr3 and Dex - co-treatment: ↓ Dex-mediated Ca^{2+}signaling, ↑ cell death, ↑ cell cycle arrest, ↑ apoptosis, ↑ mitochondrial membrane potential depolarization, ↑ ROS production	ALL (P,C)	(374)
TRPV2	Tranilast	↓ cytoplasmic [Ca^{2+}], ↓ cell growth, ↑ apoptosis, ↑ cell cycle arrest	AML (C) CML (C) Non-Hodgkin lymphoma (C)	(127)
α7-nAChR	Methyllycaconitine citrate	↓ cytoplasmic [Ca^{2+}], ↓ proliferation	CML (C)	(177)
	NBP-14	↓ migration, ↓ α7-nAChR expression	AML(C) CLL (C,P) Plasma cell myeloma (C)	(151)
NMDA receptor	Memantine	↓ cytoplasmic [Ca^{2+}], ↓ proliferation, ↓ cell viability, facilitates differentiation, inhibits proplatelet formation, alteres expression of Ca^{2+} channels and pumps, ↑ cytarabine-mediated cell killing	AML (C)	(182, 183, 373)
	MK-801	↓ cytoplasmic [Ca^{2+}], ↓ cell proliferation, ↓ cell viability, facilitates differentiation, inhibits proplatelet formation	AML (C)	(182, 373)
VDAC	VDAC-based (decoy) peptides	mitochondrial dysfunction, ↓ ATP production, mitochondrial Ca^{2+} overload, cytochrome c release and apoptosis	ALL (C)	(188, 192)
	Avicin	as above	ALL (C)	(192, 375)
$Ca_V1.2$	Lercanidipine	↓ Ca $^{2+}$ influx into AML-MSCs, ↓ proliferation of AML-MSCs and of AML blasts, sensitizes leukemia cells to other drugs	AML (P,M)	(138)
Ca_V3	mibefradil and NNC-55-0396	↓ cytoplasmic [Ca^{2+}], ↓ proliferation, ↑ apoptosis	ALL	(376)
ER/SOCE Ca^{2+} channel and effector modulators				
Broad inhibitor of SOCE*	BTP-2	↓ megakaryocyte proliferation	CALR-mutated MPN	(9)
Bcl2 inhibitor	BIRD-2	Disrupts the Bcl2-IP3R interaction, ↑cytoplasmic [Ca^{2+}] through IP3R, ↑ apoptosis	CLL (C,P)	(154, 226–228)
			DLBCL (C)	(153–155, 225, 228)
			Follicular lymphoma (C)	(227)
			Plasma cell myeloma (C)	(227)
IP3R	Wogonoside – IP3R1 activator	↑ cytoplasmic [Ca^{2+}], increases differentiation, induces cell cycle arrest, ↓ cell viability, ↓ STAT3 activation	AML (C,M,P)	(377, 378)
			ALL (C,M)	(379)
	Xestospongin – IP3R inhibitor	Inhibits Ca^{2+} release into cytoplasm, ↑ cell death, synergy with Dex to further ↑cell death	ALL (C)	(380)

(Continued)

TABLE 4 Continued

Target	Compound	Functional effects	Cancer type	References
Orai1 inhibitor	RP4010	↓ cell proliferation	AML (C,P,M)	(381)
Orai1 inhibitor	Synta66	↓ cell proliferation	CLL (C,P)	(11)
Orai3 activator	Tipifarnib	↑ cytoplasmic [Ca^{2+}], loss of membrane integrity, ↑ cell death	AML (C)	(382)
			Plasma cell myeloma (C)	(382)
ORP4L inhibitor	LYZ-81	↓ Ca^{2+} oscillations, ↑ cell death of LSCs, ↓ PIP2 hydrolysis	AML (C,P,M)	(211)
		↑ cell death, ↓leukemic engraftment, ↑ survival	Adult T-cell leukemia (P,M)	(383)
PLC inhibitor	U73122	↓ cytoplasmic [Ca^{2+}], ↑ cell death,	CLL (P) DLBCL (C)	(154)
CaMKII inhibitor	berbamine	Eliminates CML LSCs	CML (P,M)	(310)
SERCA inhibitor	CAD204520	↑ cytoplasmic [Ca^{2+}], ↓ cell viability, ↑ cell cycle arrest, targets mutated NOTCH1, SERCA inhibition achieved *in vivo* without cardiac toxicity	Mantle cell lymphoma (C)	(384)
			ALL (C,P,M)	(384)
	Casearin J	↑ cytoplasmic [Ca^{2+}] *via* ER and SOCE activation, ↑ ROS production, ↓ cell viability, inhibits NOTCH1 signaling	ALL (C)	(385, 386)
	CXL017	↓ cell viability	AML (C)	(387)
	HA14-1 (also binds hydrophobic cleft of Bcl-2)	↑ cytoplasmic [Ca^{2+}], has adverse effects on platelet survival (388)	ALL (C)	(389, 390)
			CLL (P)	(391)
			DLBCL (C)	(391)
	JQ-FT	↓ cell viability, ↑cell cycle arrest, ↓ proliferation, targets mutated NOTCH1	ALL (C,M)	(392)
	Thapsigargin	↑ cytoplasmic [Ca^{2+}], ↓ cell viability, ↓ cell size, ↑ cell cycle arrest, targets mutated NOTCH1 cell lines, impairs cardiac cell mechanics	ALL (C,M)	(253, 384–386)
Endo-lysosomal Ca^{2+} modulators				
TPC1/2 inhibitor	Tetrandrine	↓ cell proliferation, ↑ cell death	AML (C,P)	(233–236)
		Inhibits cytotoxic drug sequestration in the lysosomes which helps overcome chemoresistance	ALL (C,P)	(237)
Co-localizes with lysosomes	Imipramine blue + pimozide (STAT5 inhibitor)	↑ cytoplasmic [Ca^{2+}], loss of mitochondrial membrane potential, liberation of ROS, ↑ apoptosis	AML (C,P)	(240, 393)

References were prioritized that include data on the effects on calcium signaling. ↑ = increased, ↓ = decreased. *Broad inhibitors of SOCE were used in many studies. Their effects are not listed but this particular example has been included to reflect the emerging therapeutic potential for the modulation of SOCE in CALR-mutated MPN. (P) = Patient cells, (C) = Cell lines, (M) = Mouse model. ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; Dex, dexamethasone; DLBCL, diffuse large B-cell lymphoma; LSCs, leukemia stem cells. MPN, myeloproliferative neoplasms.

pattern of the mutational landscape revealed by our review argues that further work into Ca^{2+} signaling in MPN is warranted, including in patients without *CALR* mutations.

6 Concluding remarks

In all cell types, including hematopoietic, Ca^{2+} is an essential second messenger controlling a wide range of cellular functions, including activation of gene transcription, protein kinase signaling, cell cycle, cell survival, proliferation, differentiation, migration, and apoptosis (2–4). Others have reviewed deregulation of Ca^{2+} signaling in specific cancer subtypes such as AML, CLL and plasma cell myeloma, and summarized the influence of Ca^{2+} remodeling on cell proliferation and differentiation (369–371). Our review is the first one, to our knowledge, that highlights the extent and intricacies of alterations in the Ca^{2+}-toolkit in a wide range of hematologic cancers. We also provide a review of blood cancer databases for genetic variants in the Ca^{2+}-toolkit encoding genes.

It has been well documented that in solid tumors, cancer cells remodel Ca^{2+} signaling to enhance cancer hallmarks (1, 2). Our review emphasizes that similar alterations occur in blood cancer, driven by changes in expression, function and possibly mutations in the Ca^{2+}-toolkit components. Figure 8 provides a schematic summary of the underlying mechanisms and their consequences in blood cancer cells. Normal cells in response to activation mostly use Ca^{2+} released from the ER to support signaling. However, prolonged IP3R-mediated Ca^{2+} release in chronically activated cancer cells (e.g. due to oncogenic mutations) may lead to mitochondrial Ca^{2+} overload triggering apoptosis. Therefore, to

escape apoptosis, cancer cells shift to preferentially utilize extracellular Ca^{2+} transferred into cells by overexpressed or overactive plasma membrane Ca^{2+} channels. On the other hand, mechanisms that utilize ER-derived Ca^{2+} may be reduced, and overactive SERCA2 pumps Ca^{2+} back into the ER. This remodeling of Ca^{2+} signaling helps cells maintain heightened oncogenic signaling while shielding mitochondria from Ca^{2+} overload. The examples of mechanisms providing anti-apoptotic effects include Bcl-2-mediated inhibition of IP3R in DLBCL and reduced ER Ca^{2+} binding by mutated CALR in MPN. In addition, intracellular mediators of Ca^{2+} signaling may be overexpressed or overactive e.g. PLC, PKC, ORP4L, calcineurin and CaMK. Understanding how Ca^{2+} signaling remodels in cancer cells creates therapeutic opportunities, with the potential to spare normal cells and tailor therapies according to the underlying mechanism in different cancers/patients (Figure 8).

Research into Ca^{2+} signaling in blood cancer has become very active in recent years. Many critical discoveries have been made but multiple challenges remain. We highlighted some areas for future investigation throughout this review, including the need to characterize diverse mechanisms of Ca^{2+} remodeling and determine the significance of the mutational landscape affecting the Ca^{2+}-toolkit genes in different cancer types. Such work is not easy. Ca^{2+} signaling is a complex network of intertwining pathways and ubiquitous for cellular functioning. It can be difficult to identify the causes and consequences of the changes found in blood cancer. Most of the previous studies focused on a specific mechanism of Ca^{2+} signaling in isolation and used cell lines to characterize it. Moving forward, we should consider changes to the entire Ca^{2+} toolkit and use multiple disease models *ex vivo* and *in vivo* to study effects of multiple gene networks in cancer cells and stromal cells. This will require innovative approaches and collaboration between experts in Ca^{2+} signaling, hematological sciences and clinical hematologists.

The ultimate aim of pursuing research in this area is to improve the treatment of patients. Calcium pathways are amenable to modulation and may offer novel points for therapeutic intervention. The main targets/pathways were recently summarized for AML (371). Our Table 4 provides a range of examples of compounds/drugs that have been found to exert an impact on the functional outcome *in vitro* or *in vivo* in diverse blood cancer types. Some of these compounds are in clinical use for other applications or undergo pre-clinical/clinical testing in solid tumors (394, 395). If their targets are found to be pathogenic in blood cancer, these drugs could be rapidly transitioned to hematologic applications.

In conclusion, multiple Ca^{2+} homeostatic mechanisms and Ca^{2+} responsive pathways are altered in hematologic cancers. Some of these alterations may have genetic basis, including in MPN, B-cell and T-cell lymphoproliferative disorders, but studies are limited. Most changes in the Ca^{2+}-toolkit do not appear to define or associate with specific cancer types but may influence variables such as grade (e.g. in mature B-cell neoplasms), prognosis including responsiveness to chemotherapy (e.g. in ALL, AML and CLL), and complications (e.g. thrombosis and bone marow fibrosis in MPN). Deregulation of Ca^{2+}signaling provides an opportunity to design novel therapeutic interventions. Some options are currently investigated mostly at the pre-clinical level in various cancer models (e.g. of AML, ALL and DLBCL). Similar opportunities are being considered in solid tumours, which may facilitate faster clinical translation to blood cancer. Future research to define the role of specific Ca^{2+} regulatory mechanisms in different blood cancer types will be challenging but such work is likely to advance therapies.

Author contributions

MLK-Z conceived and designed the work. TI and MLK-Z wrote most of the paper. JL and TNG helped with the literature review, figure preparation, and writing of specific sections; JL wrote the TRP section, TNG wrote about VDACs and helped analyze the cBioPortal data. AB provided advice and wrote about calcium homeostasis in mature red cells. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

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High-cutoff hemodialysis in multiple myeloma patients with acute kidney injury

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Multiple myeloma (MM), an incurable hematological malignancy with clonal proliferation of plasma cells, is mainly characterized by excessive production of monoclonal immunoglobulins and free light chains (FLCs). Kidney injury is one of the main clinical manifestations and is also a significant predictor of the prognosis of symptomatic MM patients, especially those who require dialysis-supported treatment. Overproduction of FLCs is the trigger for kidney injury, as they can induce the transcription of inflammatory and profibrotic cytokines in the proximal tubule and bind to Tamm–Horsfall protein in the distal tubules to form casts that obstruct the tubules, leading to kidney injury and even renal fibrosis. In addition to traditional antimyeloma treatment, high-cutoff hemodialysis (HCO-HD), which can effectively remove FLCs *in vitro*, has attracted much attention in recent years. Due to its greater membrane pore size, it has significant advantages in removing larger molecules and can be applied in rhabdomyolysis, sepsis, and even myeloma cast nephropathy. However, mounting questions have recently been raised regarding whether HCO-HD can truly provide clinical benefits in MM patients with acute kidney injury (AKI). Therefore, in this study, we discussed the pathological causes of AKI secondary to MM and summarized the current situation of HCO-HD in MM patients compared with other available extracorporeal techniques. In addition, pivotal clinical trials that reflect the ability of the clearance of FLCs and the side effects of HCO-HD are highlighted, and the relevant protocol of HCO-HD is also provided to assist clinicians in decision-making.

KEYWORDS

multiple myeloma, acute kidney injury, tubular nephropathy, high-cutoff hemodialysis, high-cutoff membrane

Introduction

Multiple myeloma (MM) is a malignant proliferative tumor of plasma cells, and is the second most common hematological malignancy (1), accounting for 13% of malignant hematological diseases and 1% of all malignancies (2, 3). MM affects mostly elderly men aged approximately 65 years (4), with an age-standardized incidence rate of 2.1/100,000 in 2016 worldwide (5). MM is two to three times more common among people of African descent than among Caucasian people, while it is less common among Asian and Hispanic people (6, 7). With improved diagnostic procedures and increased clinical knowledge regarding this disease, the number of people suffering from MM is steadily increasing. Hypercalcemia, kidney injury, anemia, and bone lesions are the most common symptoms of symptomatic MM (3, 8). Kidney injury has previously been proven to be an important risk factor that has a direct impact on patient survival (9–11). According to studies on symptomatic MM, approximately 20%–50% of patients who present with symptoms will develop kidney injury (2, 12–15), 12%–20% of patients will develop acute kidney injury (AKI), and approximately 10% of patients with AKI will eventually require dialysis treatment (16, 17). Previous studies have shown that 70%–90% of dialysis-supported patients with AKI secondary to MM develop myeloma cast nephropathy (MCN) due to the significant production of monoclonal free light chains (FLCs) by malignant plasma cells (18, 19). Therefore, effective clearance of FLCs is particularly imperative in these patients. With the advent of chemotherapy drugs such as proteasome inhibitors, immunomodulatory imide drugs and monoclonal antibodies, the production of FLCs has been suppressed, and the prognosis for MM has steadily improved. Although chemotherapy, which can inhibit the formation of FLCs, is the cornerstone, extracorporeal techniques that can facilitate the removal of FLCs are likewise receiving much interest.

Extracorporeal techniques

Solute and water can be removed by using different mass separation mechanisms, which are diffusion, convection, and adsorption (20, 21). Plasma exchange (PE) is another purification method in which a patient's plasma and blood cells are separated and disease-causing plasma or hazardous substances are filtered out (22). Conventional hemodialysis is diffusion-based and removes small molecules such as urea nitrogen and serum creatinine. For middle molecules like β2-microglobulin, high-flux hemodialysis (HF-HD) has more power to remove them than conventional hemodialysis. However, due to the size of the membrane pores, HF-HD can only remove molecules of approximately 10–20 kDa, while the molecular weights for the κ and λ chains of FLCs are 22.5 and 45 kDa, respectively. Therefore, HF-HD is theoretically ineffective in removing FLCs from the blood, but some FLCs can be removed due to the nonuniformity in pore size (23). With advancements in dialysis mode and membrane technology, hemodiafiltration with ultrafiltrate regeneration by adsorption in resin (SUPRA-HFR) based on diffusion, convection, and adsorption (24); high-cutoff (HCO) membranes and medium-cutoff (MCO) membranes with larger membrane pores (25); and polymethylmethacrylate (PMMA) membranes with powerful adsorption have been developed (26). An HCO membrane with pores ranging from 0.008 to 0.01 μm and a 50- to 60-kDa cutoff in blood, which can be used in rhabdomyolysis and sepsis, is also effective in removing FLCs from the blood and can thus be used in MM. The size of the membrane area is the fundamental difference between the two frequently adopted types of HCO filters, HCO1100 and Theralite®, which is 1.1 m^2 for HCO1100 and 2.1 m^2 for Theralite®. HCO1100 can be used in series to yield a better effect as Theralite®. As high-cutoff hemodialysis (HCO-HD) combined with chemotherapy has progressed over the last decade, questions have arisen regarding whether it can yield better clinical benefits than HF-HD and other conventional hemodialysis techniques for patients with AKI caused by MM and whether there is a target threshold of serum FLC reduction in these patients.

Pathological features of AKI secondary to MM

The major cause of AKI in patients with MM is an increased concentration of serum monoclonal FLCs, which are reabsorbed in the proximal tubules and degraded in the lysosomes of proximal tubular cells after passing through the glomerulus (27). The body can produce approximately 500 mg of polyclonal FLCs each day (4, 28); however, less than 10 mg of polyclonal FLCs can be excreted in the urine because of reabsorption (29). In MM and other diseases in which clonal proliferation of plasma cells leads to an increase in monoclonal FLCs, mass-produced FLCs eventually exceed the renal tubular reabsorption capacity (4), as shown in Figure 1. Massive reabsorption of FLCs also reduces the catabolic capacity of proximal tubular cells (30) and induces the generation of hydrogen peroxide and redox signaling (27), ultimately promoting the activation of multiple inflammatory response pathways, such as the nuclear factor kappa-B (NF-κB) pathway (31), which is essential for MM progression (32, 33). In the distal tubule, FLCs can bind to Tamm–Horsfall protein to form casts that obstruct the tubules, which leads to tubular rupture and extravasation of Tamm–Horsfall protein, resulting in tubulointerstitial nephritis (34) and even AKI in some severe cases (35). The combination of these effects eventually leads to impairment of renal function and even irreversible fibrosis.

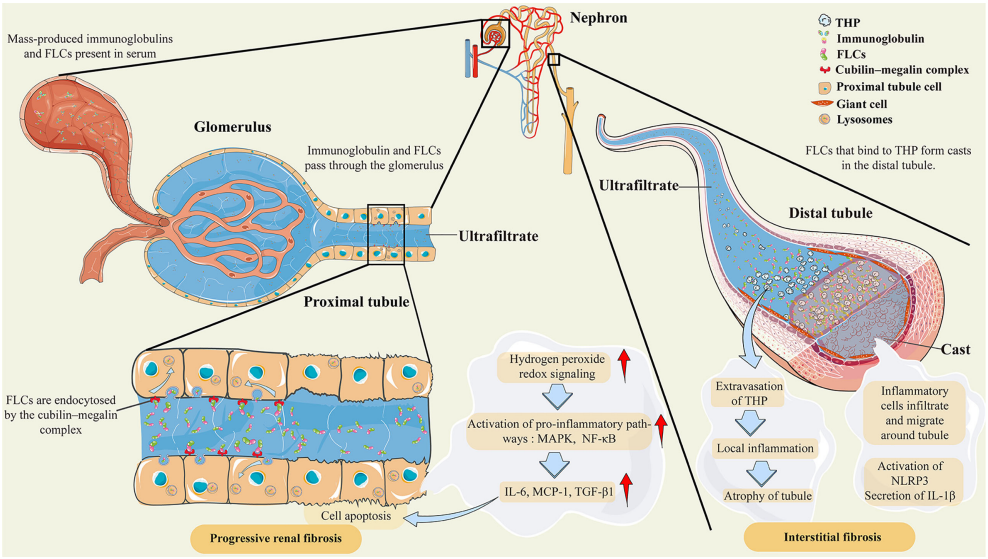

FIGURE 1
Pathological features of AKI secondary to multiple myeloma. Abbreviations: THP, Tamm–Horsfall protein; FLCs, free light chains; MAPK, mitogen-activated protein kinases; NF-κB, nuclear factor-κB; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; TGF-β1, transforming growth factor β1; NLRP3, NOD-like receptor family protein 3; IL-1β, interleukin-1β. Under normal conditions, FLCs are endocytosed by the cubilin–megalin complex in the proximal tubule cell. Almost all FLCs will ultimately degrade in lysosomes. However, mass-produced immunoglobulins and FLCs are present in the serum as a result of clonal proliferation of plasma cells, exceeding the renal tubular reabsorption capacity. The massive amount of FLCs will induce hydrogen peroxide and redox signaling, leading to the activation of proinflammatory pathways, such as MAPK and NF-κB. Then, the transcription of inflammatory and profibrotic cytokines, such as IL-6 and MCP-1, is initiated. In the distal tubule, FLCs can bind to Tamm–Horsfall protein to form casts that obstruct the tubules, which leads to tubular rupture and extravasation of Tamm–Horsfall protein. These effects lead to local inflammation, inflammatory cell infiltration, and migration around tubules, and even to atrophy of tubules. There are also postulations that the crystalline organization of casts participates in inflammation and giant cell reactions by activating NLRP3 and secreting IL-1β.

HCO-HD research and the removal of FLCs

The significant production of FLCs in serum leads to renal impairment, which, in turn, leads to a reduction in FLCs that were cleared by the kidney and an increase in serum FLC concentration. Attempts to remove serum FLCs through appropriate renal replacement therapies are therefore urgently needed. In recent years, there have been many debates regarding the use of appropriate extracorporeal techniques to accelerate serum FLC removal on the basis of effective chemotherapy. PE is the first method to directly remove serum FLCs. Serum FLCs constitute approximately 15%–20% of the total FLC volume, and PE with 3.5 L of plasma removes approximately 65% of serum FLCs. A model of FLCs in MM suggests that only 25% of the total FLC volume can be removed by PE within 3 weeks (36). However, due to the similar concentration of FLCs in the serum, extravascular compartment, and tissue edema fluid (37) and the short duration of each session of PE (23), the reduction of FLCs in plasma enabled their easy diffusion back into blood vessels from the outside. Therefore, the substantial rebound of FLCs concentration, resulted in the ineffectual removal of FLCs by PE. Moreover, PE also removes many essential proteins, such as intact immunoglobulins and blood coagulation factors (23). A randomized controlled trial also revealed that using PE has no significant clinical benefits (38), which indicated that new alternative treatments need to be explored.

As the κ and λ chains of FLCs weigh 22.5 and 45 kDa, respectively, it is challenging for routine HF-HD to remove them. Before the introduction of novel chemotherapeutic agents, less than 25% of patients with AKI secondary to MM requiring dialysis treatment could be dialysis-independent (39). In the era of new chemotherapeutic agents, the dialysis-independent rate among patients treated with routine intermittent hemodialysis was approximately 30% (40). With the development of dialysis membranes and dialysis modalities, the advent of the HCO membrane has made it possible to efficiently remove serum FLCs through extracorporeal techniques.

Hutchison et al. performed *in vivo* and *in vitro* studies to demonstrate that the HCO1100 dialyzer with an extended dialysis time could produce more effective FLC clearance than PE, with an FLC clearance rate of 10–40 ml/min (36). A model of FLCs in MM indicates that 90% of the total FLC volume can be removed by HCO-HD within 3 weeks, and renal recovery was

observed in three out of five patients with AKI secondary to MM (36). In subsequent research, it was discovered that raising the ultrafiltration rate increased the κ chain removal rate and that changing dialyzers in extended dialysis procedures to reduce membrane passivation could increase the λ chain removal rate (41). A 60% reduction in FLCs within 21 days restores renal function in 80% of patients with AKI secondary to MM (42).

Many studies have reported the removal rate of FLCs by HCO-HD. Specifically, Zannetti et al. observed that three 4-h weekly sessions of HCO-HD prior to a bortezomib-based regimen resulted in a 27% reduction in the difference between involved and uninvolved serum FLC (dFLC) concentrations and a significant increase in that difference after drug administration (43). Peters et al. performed a 5-h dialysis session combined with chemotherapy and observed that the use of a session of HCO-HD removed approximately 61 ± 20% of FLCs (44). In MYRE with a 5-h dialysis session, the median reduction rate of FLCs after the first HCO-HD was 68% (40). Berni et al. performed a 6-h session of HCO-HD in combination with chemotherapy, which resulted in a 40.2%–75.4% decrease in FLCs per session (45). Steiner et al. observed an average of 66.5 ± 20.88% FLC elimination with a session of HCO-HD in a real-world study but also a 35% rebound in FLC concentration following HCO-HD (46). In EuLITE (39), the median reduction rates of the κ and λ chains after an 8-h dialysis session combined with chemotherapy were 77% and 72%, respectively. Those abovementioned studies preliminarily indicated that the clearance of FLCs will increase with the extension of dialysis time and the addition of effective chemotherapy.

Several studies revealed that 41.7%–85.7% of patients recovered renal function and became hemodialysis-independent of HCO-HD, as summarized in Table 1 (43–51, 53, 54). However, due to the inherent nature of retrospective studies, confounding factors, such as whether patients had a history of chronic kidney disease, hypercalcemia, and other precipitating factors of cast nephropathy, the percentage of atypical plasma cells, small sample sizes, different estimated glomerular filtration rates (eGFR) as end points for hemodialysis independence, and inconsistent follow-up times, were present across studies. Furthermore, not all patients in those studies had undergone a biopsy. Low urinary albumin with high serum FLC levels (>500 mg/L) is a warning sign for MCN in MM patients (4). Table 1 also demonstrates that a large percentage of individuals who had a biopsy had MCN. Therefore, even without biopsy information, it appears reasonable that MCN is the most likely cause of AKI secondary to MM. However, we remain convinced that biopsy is a reliable way to assess renal prognosis and guide treatment because other monoclonal immunoglobulin-related kidney lesions, such as light-chain amyloidosis and light-chain deposition disease, are also potential reasons for AKI in MM patients. For patients with suspected MCN and serum FLC levels less than 500 mg/L, renal biopsy should be conducted without any contraindications (4, 55, 56).

To date, only two randomized controlled trials (RCTs), the EuLITE (39) and MYRE (40) experiments, have been performed to confirm the differences between HCO-HD and routine HF-HD. The major results are listed in Table 2. The results showed that HCO-HD does have a hemodialysis-independent rate of approximately 60% at 12 months, which was an improvement over the previous 30% renal recovery rate. However, neither of the two RCTs showed a statistically significant dialysis-independent rate in the first 3 months compared with the control group, although MYRE had a higher hemodialysis independence rate at 6 and 12 months than the control group. This may indicate that the recovery of kidney function takes a longer time in these patients. However, in EuLITE, there was no higher hemodialysis-independent rate and no better trend in the HCO-HD group. At 3 months, the MYRE showed a good rate of hematological response (partial response, very good partial response, and complete response) in the HCO-HD group because of the different methods of hemodialysis ($p < 0.01$). However, at 6 months, the hematological response was not statistically significant in either RCT. At 12 months, the EuLITE even showed that the hematological response of the HF-HD group was superior to that of the HCO-HD group ($p < 0.05$). EuLITE finally concluded that HCO-HD did not yield any practical benefits over HF-HD and was even associated with lower hematological remission at 12 months and higher mortality at the end point of follow-up than the HF-HD group.

There were also certain differences between the two RCTs, such as whether medication to correct unfavorable variables was administered before randomization and differences in filters, chemotherapy regimens, and dialysis protocols. Because of the above differences, it has also been postulated that intensive HCO-HD is harmful in patients who can achieve renal response by steroids and symptomatic measures (57). However, determining the accurate cause of such opposing outcomes is challenging, as hemodialysis or chemotherapy cannot be individually administered to these patients. Thus, more trials based on uniform rules are needed and expected in the future, such as consistent chemotherapy regimens, to clarify the accurate reasons for this difference. The results of the only two RCTs placed serious doubt on the use of HCO-HD, yet both trials revealed that the HCO membrane combined with chemotherapy treatment exhibited strong clearance qualities for serum FLCs. The high clearance properties of HCO-HD versus HF-HD for FLCs and the higher hematological response at 3 months were also confirmed in a meta-analysis incorporating the results of these two RCTs and three cohort studies (44, 53, 54). Nevertheless, no significant difference was found between HCO-HD and HF-HD in terms of improving renal recovery

TABLE 1 The renal recovery rate at the end point of previous studies.

Reference	Design	Sample size	Age (years)	Male (%)	Follow-up (months)	*De novo* MM (%)	FLC concentration at baseline (mg/L)	Serum creatinine at baseline (μmol/L)	MCN in biopsy patients [b]	Renal recovery rate (%)
Hutchison et al., 2009 (47)	Prospective/ Pilot study/ Single-center	19	60	74	NA [a]	84.2	2,600	714	19/19	73.7
Hutchison et al., 2012 (48)	Multi-center	67	65.1	62.7	NA	74.6	5,770	662	33/38	62.6
Heyne et al., 2012 (49)	Retrospective/ Single-center	19	69	36.8	NA	52.6	8,580	601.13	6/6	73.7
Sinisalo et al., 2012 (50)	Single-center	7	64	28.6	NA	100	13,300	589	6/6	85.7
Tan et al., 2014 (51)	Retrospective/ Multi-center	6	66	NA	7.5	83.3	12,500	651	6/6	50
Zannetti et al., 2015 (43)	Prospective/ Single-center	21	62	57.1	17.2	100	6,040	569.3	15/15	76
Berni et al., 2016 (45)	Single-center	13	63	76.9	NA [a]	NA	7,110	565.8	5/8	76.9
Steiner et al., 2021 (46)	Retrospective/ Multi-center	61	66	68.9	NA [a]	100	7,883	530.4	NA	42.6 at 3 months 49.2 at 6 months
Marn et al., 2016 (52)	Retrospective/ Single-center	28	68	53.6	NA	75	κ 14,433 λ 10,307	552	NA	60.7 [c]
Peters et al., 2011 (44)	Prospective/ Case–control/ Single-center	HCO-HD 5 HD 5	HCO-HD 67.8 HD 74.2	HCO-HD 80 HD 20	HCO-HD 12.9 HD 13.3	NA	HCO-HD 2,528 HD NA	HCO-HD 660 HD 457.8	10/10	HCO-HD 60 HD 0
Gerth et al., 2016 (53)	Retrospective/ Case–control/ Single-center	HCO-HD 42 HD 17	HCO-HD 64.4 HD 58.4	HCO-HD 47.6 HD 58.8	NA [a]	54.2	HCO-HD κ 8,545 λ 5250 HD κ 5,230 λ 12,400	HCO-HD 388.97 HD 442.01	16/17	HCO-HD 64.3 HD 29.4
Curti et al., 2016 (54)	Retrospective/ Cohort/ Multi-center	HCO-HD 12 HDF 7	HCO-HD 62.5 HDF 63.9	HCO-HD 83 HDF 57	HCO-HD 27.2 HDF 26.5	73.7	HCO-HD 11,924 HDF 8,043	NA	10/10	HCO-HD 41.7/58.3 at 3 months/end point HDF 16.7/33.3 at 3 months/end point

NA, not available or could not obtain all of the participants' detailed information; FLCs, free light chains; MCN, myeloma cast nephropathy; HCO-HD, high-cutoff hemodialysis; HDF, hemodiafiltration.
Age, follow-up, FLC concentration, and serum creatinine are presented as the mean or median.
Renal recovery is defined as hemodialysis independence with no other extracorporeal techniques needed.
[a]Data represent the follow-up period, which lasted more than 3 months.
[b]The denominator represents the number of patients who underwent a biopsy, and the numerator represents the number of MCN patients.
[c]Hemodiafiltration was applied to the treatment.

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TABLE 2 The major results of the two randomized controlled trials.

Groups	EuLITE (39)		MYRE (40)	
	HCO-HD	HF-HD	HCO-HD	HF-HD
Patients	43	47	46 [a]	48
Study period	2008–2013		2011–2016	
Area	UK and Germany		France	
Age (years)	66	65	68	69
Serum creatinine (μmol/L)	623	499	566	645
Previous kidney disease (%)	7	2	6 [b]	17 [b]
Serum FLCs concentration (mg/L)	κ 9,300 λ 7,200	κ 11,600 λ 7,200	6590	5230
Bone marrow plasma cells (%)	NA	NA	38	31
Albumin (g/L)	37	38	32	34
First-line chemotherapy	BAD		BD	
Follow-up (months)	24		17.5 [c]	
Hemodialysis independence rate at 3 months (%)	56	51	41	33
Myeloma response rate at 3 months (%)	NA	NA	89	63
Hemodialysis independence rate at 6 months (%)	58	66	57	35
Myeloma response rate at 6 months (%)	63	72	78	60
Hemodialysis independence rate at 12 months (%)	58	66	61	38
Myeloma response rate at 12 months (%)	42	68	NA	NA
Death rate at 12 months (%)	NA	NA	20	21
Death rate at end point (%)	37	19	28	33

NA, not available or could not obtain all of the participants' detailed information; HCO-HD, high-cutoff hemodialysis; HF-HD, high-flux hemodialysis; FLCs, free light chains; BD, bortezomib and dexamethasone; BAD, bortezomib, doxorubicin, and dexamethasone.
Age, serum creatinine, serum FLC concentration, bone marrow plasma cells, and albumin are presented as the mean or median.
Hemodialysis independence is defined as sustained renal recovery without extracorporeal techniques after treatment.
[a]Data show that 46 patients were included in the primary analysis.
[b]Data represent patients with previous kidney diseases with an estimated glomerular filtration rate greater than 30 ml/min/1.73 m^2.
[c]Data represent a median follow-up of 17.5 months (interquartile range, 12.0–30.0 months).

outcomes (58). In another study of 83 patients with AKI secondary to MM, in which 31 patients needed dialysis, bortezomib-based triplets were associated with a high potential for kidney response (59). Among the 31 patients with routine hemodialysis and 70% who received a triplet, 15 patients ultimately became hemodialysis-independent. In EuLITE, where a triplet regimen based on bortezomib was used as the first-line treatment, but MYRE used a doublet, hemodialysis independence rates were also shown to be approximately 50% in the HF-HD group at 3 months and even 66% at 12 months. This may indicate that a combination of multiple chemotherapy drugs can make a significant difference in treatment. A phase II trial combining quadruple medication therapy with routine hemodialysis is now ongoing (46), which is expected to provide an alternative treatment for patients with AKI secondary to MM. As we mentioned above, the prevention of FLC formation is the primary goal. However, combining multiple medications may make critically ill patients more vulnerable to complications and even death. Trials with novel drugs such as monoclonal antibodies without the addition of more drugs are also needed for patients in the future.

Adverse effects associated with HCO-HD

No serious complications associated with dialysis occurred during HCO-HD (36, 45–47, 50, 51, 60), and major reported adverse effects were almost always attributable to chemotherapy. The side effects related to hemodialysis included hypotension, fever, infections, muscle weakness, and thrombosis in the catheter. However, in two RCTs (39, 40), there were some dialysis-related adverse effects. The MYRE revealed no statistically significant dialysis-related complications in HCO-HD compared with HF-HD, demonstrating that HCO-HD is as safe as HF-HD, while there is a trend toward more adverse effects in HCO-HD (40). In EuLITE (39), 364 adverse events were recorded, with 52% in HCO-HD, and 54% of the serious adverse effects with grades 3–5 occurred in HCO-HD. The EuLITE trial finding that 8-h hemodialysis combined with triplet therapy resulted in a greater incidence of infection and mortality may demonstrate that HCO-HD should be adopted in a relatively mild manner rather than using a dialysis duration that is too long once combined with a triplet regimen.

Because of the high-molecular-weight cutoff of the HCO membrane, HCO-HD can cause considerable albumin loss. The amount of albumin loss decreased rapidly in the dialysis procedure, probably due to the blockage of the filter membrane (61). The loss of albumin can be avoided by proactive albumin supplementation during the last hour of dialysis. Hutchison et al. demonstrated that HCO-HD will result in the loss of 1.5 g and 5.7 g of albumin per hour in a single HCO1100 and a series of HCO1100, and at least 12 g and 45 g of albumin will be needed for 8-h HCO-HD, respectively (41). Twenty grams of albumin was frequently supplied after HCO-HD with Theralite® (40, 45). During HCO-HD, the loss of electrolytes, such as calcium and magnesium, is common (47, 62).

Protocols for HCO-HD

Since there is no consensus regarding the HCO-HD protocol, it is often left to the physician's discretion. The protocols of the previous HCO-HD are provided in Table 3. The timing of HCO-HD initiation was not mentioned in previous studies. However, it is critical that HCO-HD should be implemented as soon as possible once AKI patients who need dialysis are diagnosed. A session is usually conducted intermittently for durations longer than 4 h. A serum FLC concentration of 500 mg/L has been set as the threshold for myeloma cast nephropathy (63). Thus, most studies terminated HCO-HD when the serum FLC concentration dropped below 500 mg/L (45, 49, 50, 54) and kept using HF-HD if dialysis was still needed until the eGFR was more than 15 ml/min/1.73 m^2. Blood and dialysate flow were set to 250–350 and 500 ml/min, respectively, with ultrafiltration performed according to clinical needs. There is also a study that applied HCO membranes in post-dilution hemodiafiltration in MM patients with AKI (52).

Other extracorporeal techniques can be available to achieve a reduction in FLCs

Although the reduction of FLCs is not as significant as HCO-HD, researchers also reported other extracorporeal techniques that may have beneficial effects on patients with AKI secondary to MM. The removal efficacy of FLCs by different extracorporeal techniques is demonstrated in Table 4.

TABLE 3 The protocols of previous high-cutoff hemodialysis or high-cutoff hemodiafiltration.

Reference	Design	eGFR (ml/min/1.73 m^2)	Dialysis Membrane area (m^2)	Hemodialysis procedure	Hemodialysis end point	Albumin supplement	Dialysis-related adverse effect	Hemodialysis sessions/days in patients became independent of dialysis
Hutchison et al., 2009 (47)	Prospective/ Pilot study/ Single-center	7	1.1 × 2	8 h/session daily for 5 days and on alternate days for next 12 days, then three 6 h/session per week	Until the patients became independent of dialysis	40 g after 8 h of dialysis	0	28 d [h]
Hutchison et al., 2012 (48)	Multi-center	NA	1.1/1.1 × 2	2–4 h/4-6 h/>6 h [c]	NA	NA	6% patients happened relevant side effect [f]	11.5 [i]
Heyne et al., 2012 (49)	Retrospective/ Single-center	7	1.1	6 h/session, five sessions in the first week, followed by every other day	sFLC concentrations < 500 mg/L	NA	NA	15 d [h] 6 [i]
Sinisalo et al., 2012 (50)	Single-center	8	2.1	3 h low-flux hemodialysis at first session, then daily 6 h/session	sFLC concentrations < 500 mg/L	NA	0	17.5 [i]
Tan et al., 2014 (51)	Retrospective/ Multi-center	8	1.1/1.1 × 2/2.1	NA	NA	NA	0	6 [i]
Zannetti et al., 2015 (43)	Prospective/ Single-center	8 [a]	1.1	4 h/session, thrice weekly	The reduction of sFLC concentrations > 60%	NA	NA	32 d [h]

(Continued)

TABLE 3 Continued

Reference	Design	eGFR (ml/min/1.73 m^2)	Dialysis Membrane area (m^2)	Hemodialysis procedure	Hemodialysis end point	Albumin supplement	Dialysis-related adverse effect	Hemodialysis sessions/days in patients became independent of dialysis
Berni et al., 2016 (45)	Single-center	9	2.1	6 h/session daily for 6 days and then 6 h/session on alternate days	sFLC concentrations < 500 mg/L or renal recovery	20 g in the last hour of dialysis	0	NA
Steiner et al., 2021 (46)	Retrospective/ Multi-center	7 [a]	NA	NA	NA	NA	0	11 [i]
Marn et al., 2016 (52)	Retrospective/ Single-center	NA	2.1	8 h/session daily or every other day [d]	sFLC concentrations < 500 mg/L or renal recovery	40 g in the last 2 h of dialysis	0	NA
Peters et al., 2011 (44)	Prospective/ Case–control/ Single-center	NA	1.1	5 h/session, 6 days/week for 6 weeks	It depended on patients' tolerance or creatinine clearance > 15 ml/min	20 g in the last hour of dialysis	NA	41 [i]
Gerth et al., 2016 (53)	Retrospective/ Case–control/ Single-center	NA [a]	1.1 × N [b]/2.1	About 6 h/session and at least 5 sessions per week	sFLC concentrations < 1,000 mg/L	NA	NA	NA
Curti et al., 2016 (54)	Retrospective/ Cohort/ Multi-center	7.7	1.1/2.1	8 h/session daily for 5 days and on alternate days for next 12 days, then 6 h/session thrice weekly	sFLC concentrations < 500 mg/L	NA	NA	NA
Bridoux et al., 2017 (40)	Randomized controlled trial	NA	2.1	Eight 5 h/session for the first 10 days. If needed, 3 additional weekly hemodialysis sessions until completion of 3 cycles of chemotherapy	Individual investigators determined hemodialysis withdrawal	20 g if albumin less than 25 g/L prior to dialysis	Incidence of dialysis-related effect is 43% [g]	NA
Hutchison et al., 2019 (39)	Randomized controlled trial	7	1.1 x 2	6 h/session on day 0, then 8 h/session on day 2, 3, 5–7, 9, 10. After day 12, 8 h/session on alternate days and from day 21, 6 h/session thrice weekly up to 90 days	Nephrologist determined hemodialysis withdrawal [e]	60 g in the last hour of dialysis	NA	NA

NA, not available or could not obtain all of the participants' detailed information; eGFR, estimated glomerular filtration rate, presented as the mean or median; sFLC, serum free light chain.
[a]Data are calculated by the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) equation.
[b]Data indicate the 1.1-m^2 dialysis membrane used in single or series.
[c]Data indicate the duration of hemodialysis (2–4 h, 4–6 h, or>6 h) without detailed protocol information.
[d]Hemodiafiltration was applied to the treatment.
[e]Patients with a predialysis eGFR of more than 20 ml/min/1.73 m^2 and an adequate urine output were advised to stop dialysis.
[f]Dialysis-related adverse effects, including hypotension, fever with negative culture, reversible muscle weakness, and thrombosis of the central venous catheter, occurred in 6% of patients.
[g]Rate of hemodialysis-related adverse events of any grade was 43% in HCO-HD.
[h]Data presented as the median number of days until dialysis independence was achieved.
[i]Data presented as the median number of sessions in patients who became independent of dialysis.

Hemodialysis with an adsorbent polymethylmethacrylate membrane

The PMMA membrane is highly biocompatible and can be used in standard HD. If the PMMA membrane is replaced halfway through the session, the reduction in FLCs will be increased. Santoro et al. (64) observed a 27.7% reduction for the κ chain and 15.2% for the λ chain by using a PMMA membrane in 4-h routine hemodialysis sessions from the beginning to the end of treatment. However, the reduction increased to 33.1% and 53.1% for the κ and λ chains, respectively, when two PMMA membranes were adopted. In another study using PMMA membranes for 4-h routine hemodialysis sessions in dialysis-dependent patients with serum FLCs above 500 mg/L, the reduction rates of κ and λ chains were only 22.3% and 21%. respectively, but increased to

TABLE 4 Other extracorporeal techniques can be available to reduce FLCs.

Reference	Design	Sample size	Age (years)	Male (%)	Extracorporeal techniques	FLC concentration at baseline (mg/L)	Serum creatinine at baseline (μmol/L)	Hemodialysis procedure	Reduction of κ FLC	Reduction of λ FLC
Santoro et al., 2013 (64)	Pilot study/ Single-center	7	77	14.3	PMMA	3,648	NA	4 h/session	1 PMMA [a] 27.7% 2 PMMA [b] 33.1%	1 PMMA [a] 15.2% 2 PMMA [b]53.1%
Fabbrini et al., 2013 (26)	Retrospective/ cohort/ Multi-center	10	NA	NA	PMMA	1 PMMA 6,538 2 PMMA 7,925	NA	4 h/session	1 PMMA [a] 22.3% 2 PMMA [b] 31.0%	1 PMMA [a] 21.0% 2 PMMA [b] 53.1%
Sens et al., 2017 (65)	Retrospective/ cohort/ Single-center	17	62	47.1	PMMA	4,220	485	Six 6-h sessions a week for a maximum of 3 weeks	NA	NA
Pendón-Ruiz et al., 2013 (66)	Single-center	3	63	33.3	Supra-HFR	κ 1,873.6 λ 160.4	NA	2 cases with three 4-h sessions a week and 1 case initially with 3-h session a day then a progressive reduction of sessions	53.8%–63.4% per session [c]	38% per session [c]
Pasquali et al., 2015 (24)	Single-center	4	63	25	Supra-HFR	10,145	715.7	4 h/session for 8 consecutive days and then every 2 days	4.9–15.3ml/ min [c]	3.2–11.5 ml/ min [c]
Menè et al., 2018 (67)	Pilot study/ Single-center	6	61	50	Supra-HFR	6,910	972.4	5 consecutive sessions on alternate days, the first 2 sessions lasted 3 h and 4 h for the rest of 3 sessions	84.01%	69.3%
Li et al., 2018 (68)	Single-center	3	NA	NA	Supra-HFR	1,130	NA	Three 3.5-h sessions a week	NA	32.2%–49.5%
Pendón-Ruiz et al., 2020 (69)	Observational/ Single-center	9	72	44.4	Supra-HFR	κ 11,200 λ 1,313	750	The first two sessions last 2.5 h and 3 h, respectively; the remaining sessions last 4 h three times a week	57.6%	33.5%
Cazorla et al., 2020 (70)	Single-center	3	72	66.7	MCO-HD	14,300	415.5	6 h/session	44.8% per session [c]	NA

Abbreviations: NA, not available or could not obtain all of the participants' detailed information; FLCs, free light chains; PMMA, polymethylmethacrylate; Supra-HFR, hemodiafiltration with ultrafiltrate regeneration by adsorption in resin; MCO-HD, medium-cutoff hemodialysis.

Age, FLC concentration, and serum creatinine are presented as the mean or median.

[a]Data show that only one PMMA membrane was adopted in hemodialysis.

[b]Data show replacement of the PMMA membrane at the midpoint of hemodialysis.

[c]Data present the trend of FLC reduction.

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31% and 53.1% after changing the PMMA membrane halfway through the session (26). Briefly, two PMMA membranes may be an option for patients with a high-concentration λ chain. In a series of 17 patients with AKI secondary to MM requiring dialysis (65), 88% of patients received a bortezomib-based chemotherapy regimen combined with six 6-h hemodialysis sessions a week with two PMMA membranes until the serum FLCs dropped below 200 mg/L. Twelve (70.6%) patients recovered renal function in 60 days. Among the 14 patients who could be evaluated for hematological response, 7 patients achieved a very good hematological response or better at 3 months and did not require albumin supplementation during treatment. The dialyzer was replaced midway through the study due to the adsorption saturation properties of the PMMA membrane, which indicated the effectiveness of the PMMA membrane.

Hemodiafiltration with ultrafiltrate regeneration by adsorption in resin

SUPRA-HFR utilizes convection, adsorption, and diffusion by connecting a high-permeability filter to a low-permeability filter and making the ultrafiltrate from a high-permeability filter pass through a resin cartridge followed by reinfusing it into a low permeability filter. The process is shown in Figure 2. The resin cartridge plays a major role in reducing FLCs. SUPRA-HFR achieves effective removal of FLCs by resin and avoids the loss of albumin, which was verified in some small-sample observation studies (24, 66–69). Pasquali et al. (24) observed a significant decrease in FLCs, and three out of four patients with AKI secondary to MM became dialysis-independent in 6 weeks through SUPRA-HFR combined with bortezomib-based chemotherapy. They also found a reduction rate of 4.9–15.3 ml/min and 3.2–11.5 ml/min for the κ chain and λ chain, respectively. However, it was also found in another study that only 3 out of 9 MM patients with AKI became dialysis-independent after 2.75 ± 0.43 months of treatment (69). These two studies all show that the efficiency of the resin cartridge decreases over time, especially for the λ chain. The efficiency of the κ chain decrease is greater than that of the λ chain in SUPRA-HFR, which has also been observed in other studies (24, 66, 71). The total reduction of the κ chain was also better than that of the λ chain (67, 69). Therefore, it is recommended that SUPRA-HFR should be applied in patients with a high concentration of the κ chain (66).

Medium-cutoff hemodialysis

The MCO membrane has a relatively smaller pore size than the HCO membrane, which also induces a moderate loss of albumin and is more commonly used in patients requiring maintenance hemodialysis. Medium-cutoff hemodialysis (MCO-HD) has been confirmed to be superior to HF-HD and hemodiafiltration (72, 73). A crossover multicenter clinical trial also confirmed that MCO-HD in patients with end-stage kidney disease for 6 months is safe, with no substantial drop in albumin and no influence on quality of life, functional status, or nutrition (74). However, research on the use of MCO-HD for AKI secondary to MM is limited. In a series of three patients with AKI secondary to MM requiring dialysis treatment due to an elevated concentration of κ chain, MCO-HD combined with chemotherapy induced a sustained decrease in the κ chain with an average decrease of 44.8 ± 19.5% per session. Eventually, all three patients became dialysis-independent (70).

As mentioned above, two PMMA membranes can reduce the λ chain by more than 50% and the κ chain by 30%. SUPRA-HFR has the ability to reduce more κ chains than λ chains. The MCO-HD for AKI secondary to MM was limited. However, at least an average 44.8% reduction for the κ chain was available. Their adverse effects are less than those of HCO-HD, but the efficiency of FLC reduction is also inferior to that of HCO-HD.

What we should know

In recent years, extracorporeal techniques have made great progress in the removal of larger middle molecules. Nevertheless, it is not sufficient to simply speed up the clearance of FLCs in patients with AKI secondary to MM. It is crucial to use effective chemotherapeutic medications to control the production of FLCs. Proteasome inhibitors such as bortezomib have been proven to be associated with a better and faster renal response due to their effect on rapidly reducing FLCs through the NF-κB pathway (75). More importantly, bortezomib can be used without dose adaptation in patients with severe renal impairment, especially those requiring dialysis treatment (76, 77).

For patients with AKI secondary to MM who require dialysis support, hemodialysis and chemotherapy cannot be used separately. The duration of AKI prior to treatment initiation has been found to be an independent predictor of renal recovery (48, 49). However, the key predictor of renal recovery is the initiation time of efficient treatment to reduce the amount of FLCs (47, 48, 59). Therefore, there is an urgent need for a strong working relationship between nephrologists and hematologists. There is no doubt that the timely adoption of HCO-HD in conjunction with antimyeloma treatment in patients who require dialysis support is important. However, starting HCO-HD without effective chemotherapy would have limited benefits in MM patients with AKI. The use of HCO-HD alone does not effectively reduce the total amount of FLCs because the generation of FLCs is still ongoing. Additionally, FLCs easily diffuse back into blood vessels from the extravascular compartment or tissue edema fluid. It is critical to reduce the

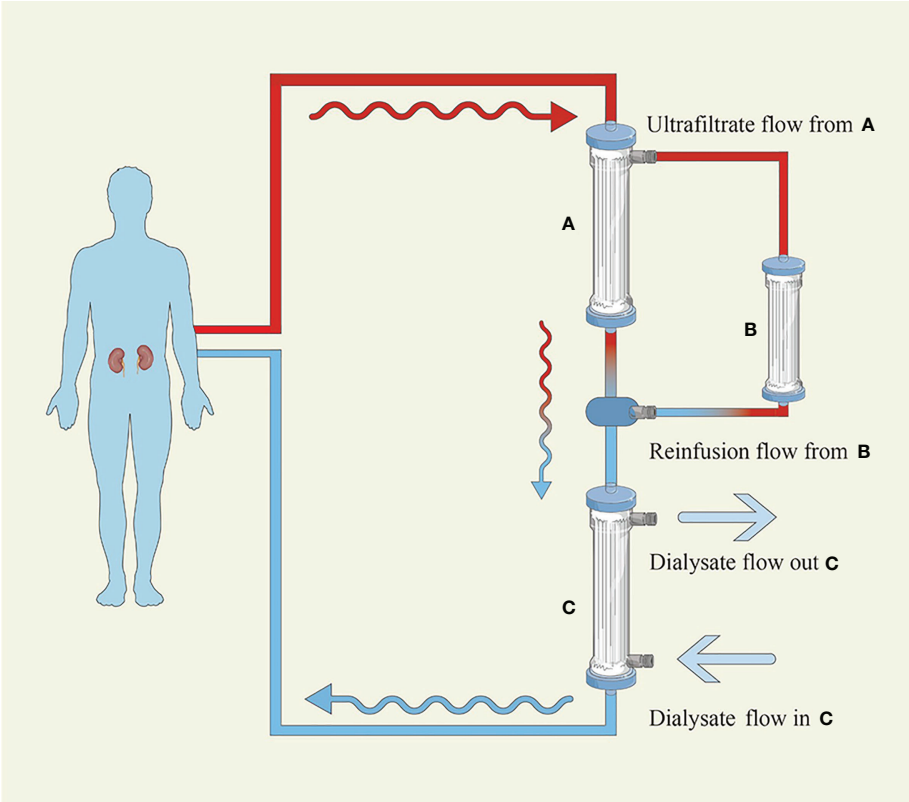

FIGURE 2
Hemodiafiltration with ultrafiltrate regeneration by adsorption in resin. **(A)** High-permeability filter with a polyphenylene membrane surface of 0.7 m^2 **(B)** Resin cartridge with a high affinity for κ and λ **(C)** Low-permeability filter with a polyphenylene membrane surface of 1.7 m^2. The blood flows into the A filter initially, where convection is performed, and the produced ultrafiltrate transits the B cartridge, where adsorption takes place, before passing through the C filter, where diffusion is carried out.

formation of serum FLCs through effective chemotherapy. Bortezomib-based chemotherapy is currently recommended for MM patients with AKI. Triplet chemotherapy regimens should be considered in fit patients (4); however, the best drug combination is still unclear, and there are tolerance concerns (4, 39). In critical patients, bortezomib and dexamethasone doublets should be considered first (4). It should also be noted that monoclonal anti-CD38 antibody, which has proven its rapidity and depth of hematological responses in MM patients (78, 79), is becoming increasingly more considerable in MM patients with AKI who need dialysis support (80). However, more trials are needed in the future.

Finally, there is no adequate evidence that HCO-HD should be the standard of care for all MM patients with AKI. MM patients with AKI who do not require dialysis support may recover renal function if the reversible factors can be corrected by volume expansion, urine alkalinization, blood calcium reduction, avoidance of nephrotoxic drugs, and initiation of chemotherapy. Hemodialysis should be performed as soon as possible when someone has indications for dialysis, such as severe acute kidney injury (AKI stage 3 [KDIGO]), electrolyte disorder (blood potassium elevated more than 6.5 mmol/L), or severe volume overload (18). The absence of standards for HCO-HD also results in a variety of dialysis durations. The target threshold of serum FLC reduction and the timing of HCO-HD initiation are still unclear. Whether HCO-HD is the most cost-effective option is also still under debate due to the increased cost of HCO filters, albumin supplementation, and careful monitoring of electrolytes. However, some studies concluded that the total cost of HCO dialyzers was comparable to those of standard dialyzers (54), and some even demonstrated significant cost savings (45). This difference may be due to the different reimbursement policies in different countries. Therefore, the promotion of HCO-HD also needs to take the actual situation into account in each country. Although HCO-HD and other extracorporeal removal techniques are successful in removing pathogenic materials, current clinical trials have not yet confirmed the obvious advantage in recovering kidney function in AKI secondary to MM.

Conclusions

In summary, there are no ideal extracorporeal techniques that have emerged that can achieve large reductions for FLCs other than HCO-HD. HCO-HD and bortezomib-based chemotherapy have a tendency to improve renal outcomes, but more RCTs based on novel drugs are warranted. Perhaps HCO-HD should be used in MM patients with AKI who are unable to achieve restored renal function by steroids, symptomatic measures, and effective chemotherapy. For patients with high concentrations of κ or λ chains, there are other, less expensive extracorporeal techniques available even though the reduction of FLCs is inferior to HCO-HD, which also requires further investigation and validation.

Author contributions

Conceptualization: YX, JY and SS. Validation: ZY and JZ. Supervision: YW, XL and YQ. Visualization: YX and JY. Writing—original draft: YX. Writing—review and editing: YX, JY, ZY and SS. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The path towards consensus genome classification of diffuse large B-cell lymphoma for use in clinical practice

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Diffuse large B-cell lymphoma (DLBCL) is a widely heterogeneous disease in presentation, treatment response and outcome that results from a broad biological heterogeneity. Various stratification approaches have been proposed over time but failed to sufficiently capture the heterogeneous biology and behavior of the disease in a clinically relevant manner. The most recent DNA-based genomic subtyping studies are a major step forward by offering a level of refinement that could serve as a basis for exploration of personalized and targeted treatment for the years to come. To enable consistent trial designs and allow meaningful comparisons between studies, harmonization of the currently available knowledge into a single genomic classification widely applicable in daily practice is pivotal. In this review, we investigate potential avenues for harmonization of the presently available genomic subtypes of DLBCL inspired by consensus molecular classifications achieved for other malignancies. Finally, suggestions for laboratory techniques and infrastructure required for successful clinical implementation are described.

KEYWORDS

diffuse large B-cell lymphoma (DLBCL), next generation sequencing (NGS), consensus classification, genomics, bioinformatics

Introduction

Molecular diagnostics of cancer has entered a new era, propelled by advances in omics- and bioinformatic technologies that provide a new layer of characteristics for tumor classification. In general, current state-of-the-art diagnostic pathology categorizes tumors using phenotypic macro- and microscopic and immunohistochemical (IHC)

characteristics, combined with molecular assays for single or limited numbers of markers like PCR, and fluorescent *in situ* hybridization (FISH). Analyses of highly complex omics data by bioinformatic technologies have identified molecular patterns and pathways that underly biologically distinct, and thereby newly recognized categories. *Vice versa*, accepted diagnostics distinct categories may proof to be molecularly so closely related they may even be combined into a single entity.

Diffuse large B-cell lymphoma (DLBCL), the most prevalent type of non-Hodgkin lymphoma and the focus of this review, is characterized by a complex, heterogeneous tumor biology that is reflected in clinical heterogeneity (1). This is evident from a wide outcome spectrum with cure for 60% of patients treated with standard immune-chemotherapy (R-CHOP) and disease progression for the other 40% of which the far majority eventually succumbs due to relapsing and/or refractory disease (2, 3). Since 2000, omics information started to contribute layers of comprehensive biological information to the diagnosis of DLBCL (4). At that time, RNA expression profiling by means of microarray analysis followed by unsupervised clustering revealed a relatively simple dichotomous distinction based on cell-of-origin (COO) (5). For universal application in daily clinical practice, this distinction was translated into various algorithms that relied on classic immunohistochemistry (IHC) assay data rather than complex RNA analytics. This undoubtedly aided to have DLBCL COO classification to be included in the updated 4th edition of the World Health Organization (WHO) Classification for Hematolymphoid Malignancies in 2016 (6). Nonetheless, it was never widely applied outside clinical trials, largely since the clinical implications ultimately proved to be limited (7–9). Almost 20 years after the RNA-based COO classification concept, several independent studies proposed DNA-based subtyping by next-generation sequencing (NGS) as an alternative means to capture the biological heterogeneity of DLBCL and to supersede or complement COO classification (10–13). The different DNA-subtyping studies bear significant similarities, but also differ in some *a priori* concepts, applied technologies, bioinformatical approaches and ultimately in part in recognized genomic subtypes (14, 15). These differences preclude uniform classification, which is a quintessential step towards clinical implementation and essential to perform meaningful clinical trials (16–18).

Molecular classifications of DLBCL

Classifications based on RNA-expression

The more than 20-year-old RNA-based COO classification recognizes 2 major molecularly distinct classes considered to reflect different stages of B-cell differentiation; activated B-cell (ABC) and germinal center B-cell (GCB) while a small group of patients remains 'unclassified'. Both in the primary discovery studies and various subsequent validation studies, patients with a GCB-type DLBCL consistently showed a better prognosis under guideline therapy than patients with an ABC-type DLBCL (4). The differential clinical outcomes coupled with distinctive underlying biology served as a justification for differential treatment. In the years that followed it became clear however that the complex and heterogeneous biology of DLBCL was not fully captured by this simple dichotomous classification (5). In particular, phase 2 and phase 3 clinical trials that either used COO as an inclusion parameter, or were *post-hoc* analyzed based on COO class, failed to demonstrate differential improvement of outcome for patients receiving experimental, targeted treatment alternatives (7, 8, 19).

This does however not imply that RNA-based information would not provide essential information to dissect DLBCL biology, as specific host-immune response signatures could already be identified in the early 2000s (20). Most recently, deconvolution algorithms using known cell type specific RNA signatures to computationally infer cellular components from bulk RNA data have allowed to further dissect information on tumor features as well as non-malignant tumor immune microenvironment (TME) features. Thereby, the original GCB class was further divided into three to four differentiation phases (germinal center, dark zone, precursor memory B-cell, light zone) and ABC into two phases (pre-plasmablast, plasmablast/plasmacell). Hence, TME analysis from RNA expression data provided complementary signatures that could further and largely independently describe DLBCL biology in a clinically meaningful manner (21).

DLBCL defining DNA-alterations and subtyping approaches

The first larger DNA-based next-generation sequencing (NGS) studies for DLBCL that were undertaken revealed a spectrum of mutations, numerical chromosomal copy number aberrations (CNAs) and translocations that were largely characteristic for either of the RNA expression-based COO classes (22–27). For example, mutations in the chromatin modifying genes *CREBBP, KMT2D* and *EZH2*, were described as characteristic of GCB-type DLBCL and chromosome *18q* gain and *MYD88* mutations characteristic of ABC-type DLBCL. Apart from these few COO-characteristic DNA alterations, the majority was shown to be only limitedly overrepresented in either class, explanatory for the extensive genetic heterogeneity of DLBCL.

In 2018, research groups from the National Cancer Institute (NCI) and the Dana Farber Cancer Institute (DFCI) independently and practically simultaneously proposed DNA-based subtyping approaches based on whole exome sequencing

(WES) (1, 10, 11). The NCI group made a first step towards harmonization of the two approaches by, like DFCI, also including CNAs to their classification which resulted in the LymphGen algorithm (12). The DFCI- and NCI studies included retrospectively collected patient cohorts and identified 5- and 7 genomic subtypes, respectively. Encouraging is that despite the different cohorts and bioinformatical approaches, both defining features and the resulting subtypes are largely overlapping (Figure 1 and Box 1). Other groups, with other cohorts using overlapping bioinformatical approaches have been able to reproduce these subtypes by and large (13, 31–33), including unpublished results by the authors of this review. This all provides confidence that a DNA-based characterization of DLBCL has the potential to disentangle the biological heterogeneity that underlies DLBCL's clinical heterogeneity.

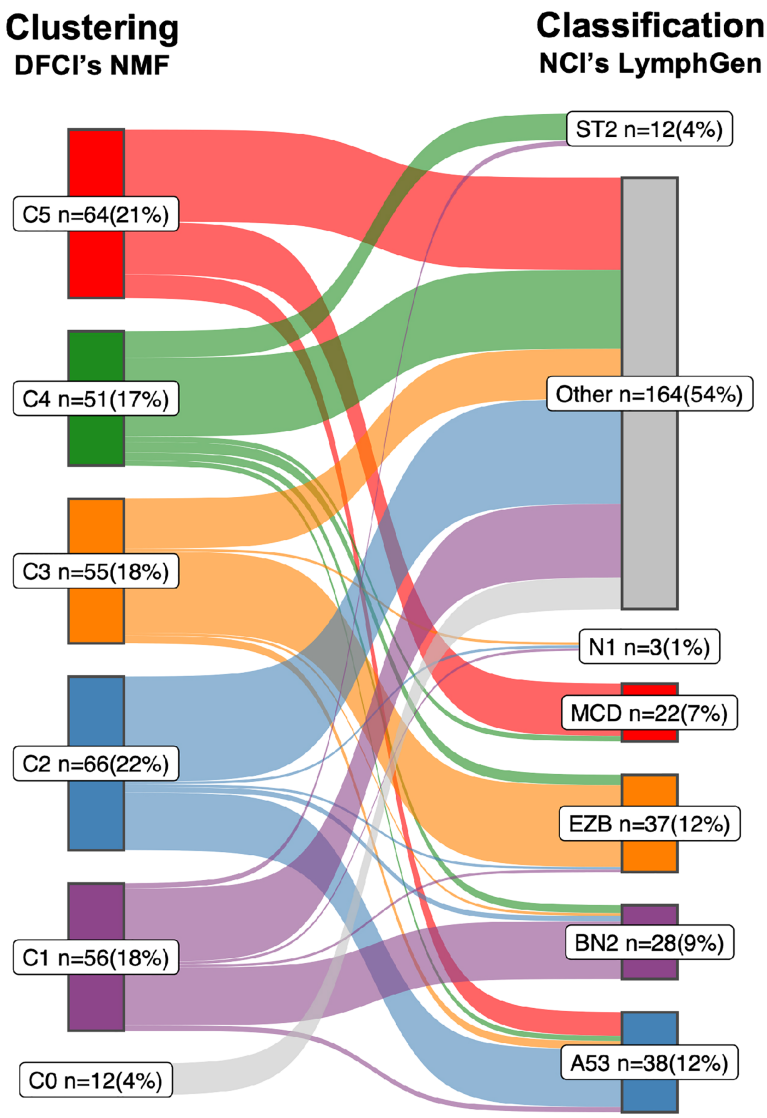

FIGURE 1
Sankey diagram comparing the two DLBCL subtypes. A Sankey diagram was constructed to illustrate how the LymphGen (12) and NMF (10) subtyping systems compare, as described in Box 1. Therefore the NGS data of 304 diffuse large B-cell lymphoma (DLBCL) cases published by the Dana Farber Cancer Institute (DFCI) (10) was used as input. Left stage: Clustering by means of non-negative matrix factorization (NMF). Right stage: Classification by means of LymphGen algorithm. Flows between the subtypes resemble DLBCL cases and are labelled according to their molecular counterpart; C1/BN2, purple; C2/A53, blue; C3/EZB, orange; C4/ST2, green; C5/MCD, red; samples not assigned to a cluster (NMF, C0) or unclassified (LymphGen, Other) are in gray. Each subtype with numbers of samples (n) and percentage of total (=304).

Box 1 Comparison of the LymphGen and NMF subtyping systems.

The correspondence between the NCI's LymphGen and DFCI's NMF subtypes is 75% based on the 63.1% of patients classified by the LymphGen algorithm (12). If also the LymphGen unclassified samples are considered, the overall agreement between the two subtyping systems is around 50%. Approaches: Both studies performed comprehensive genomic profiling to detect somatic mutations, CNAs and translocations. Because of the lack of matched normal tissue for most samples, both studies applied custom computational pre-processing techniques to eliminate sequencing artifacts and distinguish somatic and germline variations. The DFCI group performed WES on a series of tissue biopsies of 304 patients with primary DLBCL. Samples were from 4 different trials and cohorts, of which 55% were derived from FFPE tissue, and 44% had matched-normal tissue availability (10). The NCI group performed WES on a series of fresh-frozen DLBCL tissue biopsies of 574 patients for which 96.5% were primary DLBCL tissues and the other 3.5% from relapsed or refractory, without matched-normal tissue (12). Below is a short summary of the most defining features which the NCI and DFCI proposed subtypes have in common. For a more comprehensive overview on details of their differences and commonalities we refer to a recent review by Crombie et al. (28).

i. The C1 subtype recognized by DFCIs NMF algorithm finds its analogue in the BN2 subtype recognized by NCIs LymphGen algorithm. Combined, the two algorithms determined 21 defining genetic alterations, of which eight overlap. Overlapping genes include *BCL6* translocations, alterations in *NOTCH2* signaling genes and mutations targeting the NF-kB pathway. Furthermore, the C1/BN2 subtype is enriched for, but not restricted to ABC-type, and shows a favorable outcome. The C1/BN2 alterations form a genetic basis of immune evasion corresponding to mutations seen in marginal zone lymphoma. Non-overlapping genes include mutations of *B2M*, *FAS*, *HLA-B* and translocations of *PD-1* ligands.

ii. The NMF-C2 subtype is analogues to the LymphGen-A53 subtype. Both have characteristic *TP53* inactivation, and a high degree of genome instability as reflected by the prominence of genome-wide CNAs. This subtype is not significantly enriched for either of the two COO types, which underpins that the original COO dichotomy was indeed an oversimplification of DLBCL biology. Overall survival of this C2/A53 subtype under R-CHOP treatment is unfavorable. A notable difference between the two subtypes is the high number of discordant subtype-defining features (36 from 41), including driver alterations such as chromosomal deletion of the *CDKN2A* locus (9p.21).

iii. The NMF-C3 subtype is analogues to the LymphGen-EZB subtype, with a relatively high concordance of subtype-defining alterations (10 out of 18); including translocations of *BCL2*, and mutations in chromatin modifying genes. Discordant features include amplification of the *REL* locus (2p16.1) and mutations of *FAS*. The C3/EZB subtype represents classic GCB-type DLBCLs, and the genetic features are to a large extent alike follicular lymphoma (FL), which suggest that these DLBCLs represent transformed FL (29). Clinically, C3/EZB subtype tumors are considered of most high risk within the GCB-type of DLBCLs. Notably, also the RNA-based DHITSig is enriched in this subtype and used to further subdivide EZB.

iv. The NMF-C4 subtype is analogous to the LymphGen-ST2 subtype. C4/ST2 subtype defining alterations affect BCR/PI3K signaling, the JAK/STAT pathway, and histone genes. Most of these DLBCLs belong to the GCB-type with favorable outcome. Few alterations linked to this subtype are concordant between the two classification systems (6 out of 24). The less defined nature of this subtype is further underpinned by a recent study suggesting that this subtype may be further subdivided into two subtypes with divergent biology: a *TET2/SGK1* and a *SOCS1/SGK1* subtype (13).

v. The NMF-C5 subtype is analogues to the LymphGen-MCD subtype. Nine of the 24 characteristic alterations overlap which include mutations in genes associated with extranodal involvement (*MYD88*, *CD79B, TBL1XR1*). This C5/MCD subtype is highly enriched for ABC-type DLBCLs and is the subtype with the least favorable survival under R-CHOP treatment. Discordant alterations include other markers of immune evasion (mutations of *HLA-B* and translocations of *PD-1* ligands) and copy number gains of chromosomal arms 3q and 18q.

vi. Finally, the LymphGen classification describes the N1 subtype which is characterized by *NOTCH1* mutations. This subtype occurs in less than 2% of DLBCLs Figure 1 and has the worst survival among the LymphGen subtypes. This subtype is not recognized by the NMF algorithm with the DFCI cohort. Also, when we extend the DFCI cohort with another 500 DLBCLs treated with R-CHOP, the NMF algorithm still does not recognize this class (authors unpublished results).

The bioinformatic approaches of the current DNA-based subtyping systems for DLBCL

The DFCI group used unsupervised clustering combined with alteration-centric features (Box 2). Driver alterations were discriminated from passengers, thereby reducing the genetic dataset to 158 features. Non-negative matrix factorization (NMF), an unsupervised clustering algorithm that detects patterns of co-occurring features and assigns a subtype to each included tumor, was used. The number of clusters to be identified was predefined between 4 and 10, which is actually an arbitrary choice. The NMF algorithm identified the optimal stability of clusters to be represented by 5 DLBCL groups of similar sizes, which the authors labelled as C1 to C5.

The NCI group used semi-supervised clustering combined with gene-centric features (Box 2). The prior knowledge given were four predefined classes, each composed of 1 or 2 specific DNA "seed" alterations: MCD (seed is co-mutation of *CD79B* and *MYD88*^{L265P}), BN2 (seed is *NOTCH2* mutation or *BCL6* translocation), N1 (seed is *NOTCH1* mutation) and EZB (seed is *EZH2* mutation or *BCL2* translocation). Finally, the algorithm selected the additional genomic features that had the strongest association with the four classes through an iterative approach. All patient samples were included for classification with this 4-class algorithm, yet of the entire

Box 2 Genome feature definition and subtyping algorithms.
The two proposed DNA-based subtyping systems differ in their bioinformatic approaches for i) genomic feature definition, and ii) subtype identification (10, 12):

i. To define genomic features a gene-centric approach can be applied that combines all DNA alterations that impact the same gene into 1 feature, independent of whether they are a mutation, translocation or CNA. For example, a point-mutation of *CDKN2A* and a deletion of the *CDKN2A*-locus 9p.21 would be recognized as 1 feature. Alternatively, an alteration-centric approach regards each DNA alteration type separately, independent of their location in the genome. In the example of *CDKN2A*, the mutation and 9p.21 deletion are regarded as two separate features.

ii. Also machine learning algorithms for patient subtyping can generally be divided in 2 main approaches, supervised or unsupervised (30). The supervised approach uses predefined classes to construct a classification rule from the features. An unsupervised approach leaves it to the algorithm to identify a number of subtypes that are composed of feature characteristics prioritized by the algorithm. Semi-supervised learning would be where some prior knowledge on classes and or features is given.

cohort, only 46% of cases could be assigned (11). In the remaining 54% of cases in the NCI cohort recurrent alterations of *TP53* (25%), *TET2* (10%) and *SGK1* (6.9%) were identified. This prompted the NCI group to refine and extend the four classes with two additional classes: A53 (seed is mutation and/or CNA of *TP53*) and ST2 (seed is mutations of *SGK1* and *TET2*), resulting in six seed classes (12). Subsequently, a Bayesian predictor model titled "LymphGen" was developed, which calculates for each individual tumor the subtype probabilities for each of the six classes based on its genetic alterations. Tumors designated as "core" tumors were defined as being attributed to one class with a probability score of >90%. Consequently, the Bayesian predictor allows tumors to be assigned to multiple classes. Those with a probability score greater than 90% for more than one class are the so called "genetically composite" tumors. Tumors with a probability score of 50%-90% for one single class were termed "extended" class members. Tumors with few subtype-specific genetic alterations were left unclassified. Thereby, the then 6-class LymphGen algorithm assigned 63.1% of cases of the NCI cohort (12). Later, the RNA expression-based *MYC* double-hit signature (DHITSig), previously developed by others (34), was added as a surrogate for *MYC* translocation status to split the EZB class in *MYC* positive and *MYC* negative cases.

Critical evaluation of the current subtyping approaches for DLBCL

Despite the different choices in feature identification and machine learning algorithms (Box 2), the NCI and DFCI groups recognize a similar and extensive underlying biological heterogeneity of DLBCL. Some subtypes are already more similar than others. For example LymphGens MCD/NMF C5, LymphGen A53/NMF C2 and LymphGen EZB/NMF C3 are already relatively consistently defined. An important difference is that the LymphGen algorithm does only assign 63.1% of patients to any of their predefined subtypes, whereas the DFCIs NMF algorithm defines a number of subtypes to which 100% of the samples in the cohort are assigned. The N1 subtype is the rarest subtype and is only recognized by the NCI with the *NOTCH1* mutation seed given to the LymphGen algorithm (Box 1).

A small fraction of DLBCL patients (<2%) carry *NOTCH1* mutations which infers potential specific sensitivity to Ibrutinib, a Bruton's tyrosine kinase (BTK) inhibitor. Due to its low frequency, the N1 subtype is not recognized using unsupervised techniques in relatively small series. The size of the currently studied cohorts has been too small, hence underpowered, to detect such rare genomic subtypes by unsupervised analysis. Unknown small genomic subtypes can only get recognized once the sample size is sufficiently large, as exemplified by Curtis et al. for breast cancer (35). Rare subtypes like N1 may be characterized by very specific biological characteristics that make them uniquely targetable with specific potent inhibitors and thereby highly relevant to be recognized. As an example from another cancer entity, in about 1% of metastatic colorectal cancers the *ERBB2* oncogene on chromosome *17q* is amplified, which can be effectively targeted by trastuzumab and neratinib and results in high response rates in these tumors (36–38). Likewise, 4-5% of non-small-cell lung cancers have a translocation of the *ALK* gene, which can be effectively targeted by the ALK inhibitor crizotinib (39).

Not recognized by either NCI or DFCI are the actual high-grade B-cell lymphoma (HGBCL), B-cell lymphomas with *MYC* translocation together with either *BCL2* and/or *BCL6* translocation (double hit/triple hit). Unsupervised NMF clustering theoretically might be able to recognize this group as a subtype but, like the N1 subtype, it may have remained undetected as a result of the limited number of *MYC*-translocation positive DLBCLs in the DFCI

Box 3 Summary of the data-driven bioinformatic path to the four consensus molecular subtypes for colorectal cancer.
Three generic methodological steps are involved in the path taken for consensus classification of colorectal cancer.

i. Independent expert team subtyping prediction on normalized raw data sets: Eighteen RNA-based CRC gene expression data sets, derived from different continents and research groups were assembled from public resources (Gene Expression Omnibus and The Cancer Genome Atlas). The data sets were compiled from various genome-wide expression analysis techniques (arrays and RNA-sequencing), different sample types (formalin-fixed paraffin embedded and fresh-frozen tissue materials) and different study designs (retrospective and prospective series, including clinical trials). The first bioinformatics step concerned central pre-processing and normalization aimed to obtain expression profiles for each of the patients of the 18 gene-expression datasets, independent of cohort or technique. Next, each of the six initial participating research teams applied their original classification algorithm to each of the 18 data sets. Thus, resulting in six classifications, with a total of 27 different subtypes for all 3,962 patients.

ii. Network analysis for consensus subtype identification: Using the six classification systems of the 3,962 patients, a network-based approach was applied to study the association between all the 27 subtypes. To detect robust clusters of recurrent molecular subtypes, an unsupervised Markov clustering approach was performed, resulting in the identification of four consensus molecular subtypes (CMSs). Of the 3962 samples, 3104 (78%) were identified as highly representative of a particular subtype and labelled as core consensus samples and the remaining n=858 as non-consensus samples. The core consensus samples were used to train the novel CMS classifier in the subsequent step.

iii. CMS classifier construction and application: To allow classifications of individual cases, which is mandatory for diagnostic routine, a classification algorithm is required. Since the data sets were created using different RNA gene expression profiling techniques across the different studies, not all genes were included in all data sets. The CRCSC first converted all 18 separate data sets into a single data set. The genes that were commonly profiled by all separate data sets were selected to allow aggregation of all 18 data sets into a single data matrix. To construct the CMS classifier, the single data matrix, CMS classes and consensus sample set were used. The consensus samples were randomly split using two-third as training and one-third as validation set, and a random forest classifier was generated to calculate a prediction value for subtype assignment for each sample, by means of bootstrapping with 500 iterations. Application of the CMS classifier on the validation set demonstrated an overall accuracy of 90%. The CMS classifier was robust enough to allow assignment of 40% of the non-consensus samples, while the rest showed heterogeneous patterns of CMS subtypes and contained biological information of more than one class.

dataset. The DHITSig signature used by the NCI is a surrogate marker to recognize a MYC subtype and troublesome for various reasons. First it is not DNA alteration derived and requires a different assay, namely RNA expression analysis. Second, the name of this signature is deceiving since it implies a genetic context of HGBCL, whereas only 64% of DHITSig-positive GCB-type DLBCLs actually carry a *MYC* translocation and 52% are actual double hit/triple hit DLBCLs (34). Third, also other lymphoma classes besides *HGBCL* double hit/triple hit such as Burkitt lymphoma score positive for DHITSig. This RNA DHIT signature is thus not specific for either *MYC* translocation or HGBCL (40, 41).

Besides the choice of subtyping algorithms, the NCI gene-centric versus DFCI alteration-centric choices for genetic features deserve attention (Box 2). The easiest solved are the focal chromosomal CNAs, aberrations smaller than 3Mb (42) which only encompass one or few genes, and can therefore be combined in a gene-centric fashion (43). The choice between alteration- or gene-centric is not obvious for the larger-scale chromosomal CNAs since they harbor hundreds of genes. Rather than rationalizing a choice between a gene-or alteration-centric approach, the machine learning algorithms can be offered data processed in either manner and side-by-side evaluated for best subtyping performance.

Although the unsupervised clustering choice is an elegant data-driven approach to identify subtypes (17, 44, 45), in the end a classifier, like LymphGen, will need to be built to diagnose individual patients in daily clinical practice, which dictates another step towards harmonization.

Towards a unifying classification for DLBCL; Lessons learned from other tumor types

Two steps towards clinical implementation of a DNA-based classification of DLBCL

The currently proposed DNA-based subtypes will be the basis for a unified biological classification that may require a two-step strategy (28). Step 1 would involve harmonization of the current DNA-subtyping systems into a single unified classification, Step 2 would be the development of a reproducible and widely applicable molecular diagnostic assay; certified, as well as cost- and time-effective to enable clinical implementation. This exposes various challenges, from the choice of laboratory technique, subtype-defining DNA alteration features and interpretation to classification algorithms and bioinformatic procedures.

A universally accepted classification is a prerequisite to improve patient management

Harmonization into a single classification is a first requirement for implementation in diagnostic routine.

Objective, reproducible, and conclusive subtype definition for each patient sample, combined with a detailed understanding of the tumor biology of each defined DNA-class, will enable to explore clinical consequences of such classification, preferably in clinical trials (46). For various organ-specific malignancies molecular classifications for tumor families have now been standardized and integrated in the 5th edition series of the WHO Classifications and are starting to be implemented in the diagnostic workflow for those settings that have access to the technology (47–49). The road towards this level of applicability has been achieved with several research groups proposing their individual molecular classification as a starting point, at different moments in time and with different laboratory and bioinformatical techniques, as is exemplified by the classification of breast cancer, central nervous system (CNS) tumors and colorectal cancer (48, 49).

Lessons learned from classifications that are universally agreed upon for other solid malignancies

Probably breast cancer classification is one of the most successful early examples. An RNA-based classification for breast cancer found its way already into the 4th edition of the WHO Classifications of Breast Tumours in 2012, which was further expanded upon in 5th edition (47). it recognizes 5 molecular classes; each with different prognosis but also different treatment recommendations. The existing close transatlantic collaborations undoubtedly facilitated consensus formation, characterized as "organic" allowing different biological and bioinformatical perspectives to converge (46, 50, 51). Once a consensus classification was established and reproducible assays were developed, exploration of personalized and targeted treatment approaches could be effectively explored to identify bespoke treatment modalities, amongst others in the multi-armed I-SPY clinical trials (52).

From the point of view of development of a molecular-based consensus classification, the present WHO classification for CNS tumors is an impressive result of intensive collaboration leading to a highly refined molecular classification. In 2014 a group of neuro-oncological pathologists, physically converged in 2014 in Haarlem (NLD) and prepared a clinically relevant histo-molecular diagnostic consensus classification, whilst reducing interobserver variability (53), which soon was implemented in the 4th edition of the WHO Classification of CNS Tumors (54). Subsequently, a largely novel approach was taken by means of genome-wide DNA methylation analysis where the large spectrum of CNS tumors were recognized by methylation profiles combined with a form of dimension reduction called t-distributed stochastic neighbor embedding (t-SNE) (55). The t-SNE methylation test alone allows for diagnoses of the large majority of CNS tumors, not seldomly more detailed and/or reliable compared to the histo-molecular diagnosis, resulting in redefinition of these entities. The collaborative effort with inclusion of samples and intellectual input from many research groups across the world as well as extensive discussions in the Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy (cIMPACT-NOW) (56) has helped a broad acceptance and indeed this molecular classification is now also included in the 5th edition of the WHO Classification of Central Nervous System Tumours (48, 48).

To harmonize colorectal cancer (CRC) classification, the Colorectal Cancer Subtyping Consortium (CRCSC) was formed to integrate six independently published RNA-based classifications (49). As opposed to the CNS assembly consensus, a predefined mathematical harmonization path was taken with the aim to resolve inconsistencies between the various CRC classification systems. This approach culminated in four consensus molecular subtypes (CMSs) (49) to which each CRC sample adheres to a higher (core samples) or lesser (non-core) extend. Since the context in CRC classification is so very similar to the current status in DLBCL, we here provide a summary of this CMS approach where three generic methodological steps were involved (Box 3).

The process to come to a single, harmonized molecular classification for DLBCL may likely be the one taken for the development of colorectal cancer CMS. For DLBCL also, a similar issue in the underlying biology result in single class (core) tumors, unclassified samples and genetically composite tumors (12, 57). What should alleviate the consensus process is that for DLBCL two, rather than the six for colorectal cancer, existing DNA-classifications as a starting point while still various independent published and unpublished (authors of this review) datasets are available.

From DLBCL genome classification to clinical implementation

DNA alterations required for DLBCL genome classification

Any consensus classification for DLBCL will include a combination of mutations and structural chromosomal variations (CNAs and translocations) (Box 1). Therefore, inclusion of this information into a single genome subtyping assay would be highly attractive. Various common laboratory and bioinformatics applications are available for mutation and CNA detection by NGS. Also NGS-based translocation detection is starting to become a cost-effective alternative for routinely used Fluorescent *in situ* hybridization (FISH) to determine translocations. (Figure 2). FISH benefits from a choice of worldwide commercially available probes and assays but is labor-

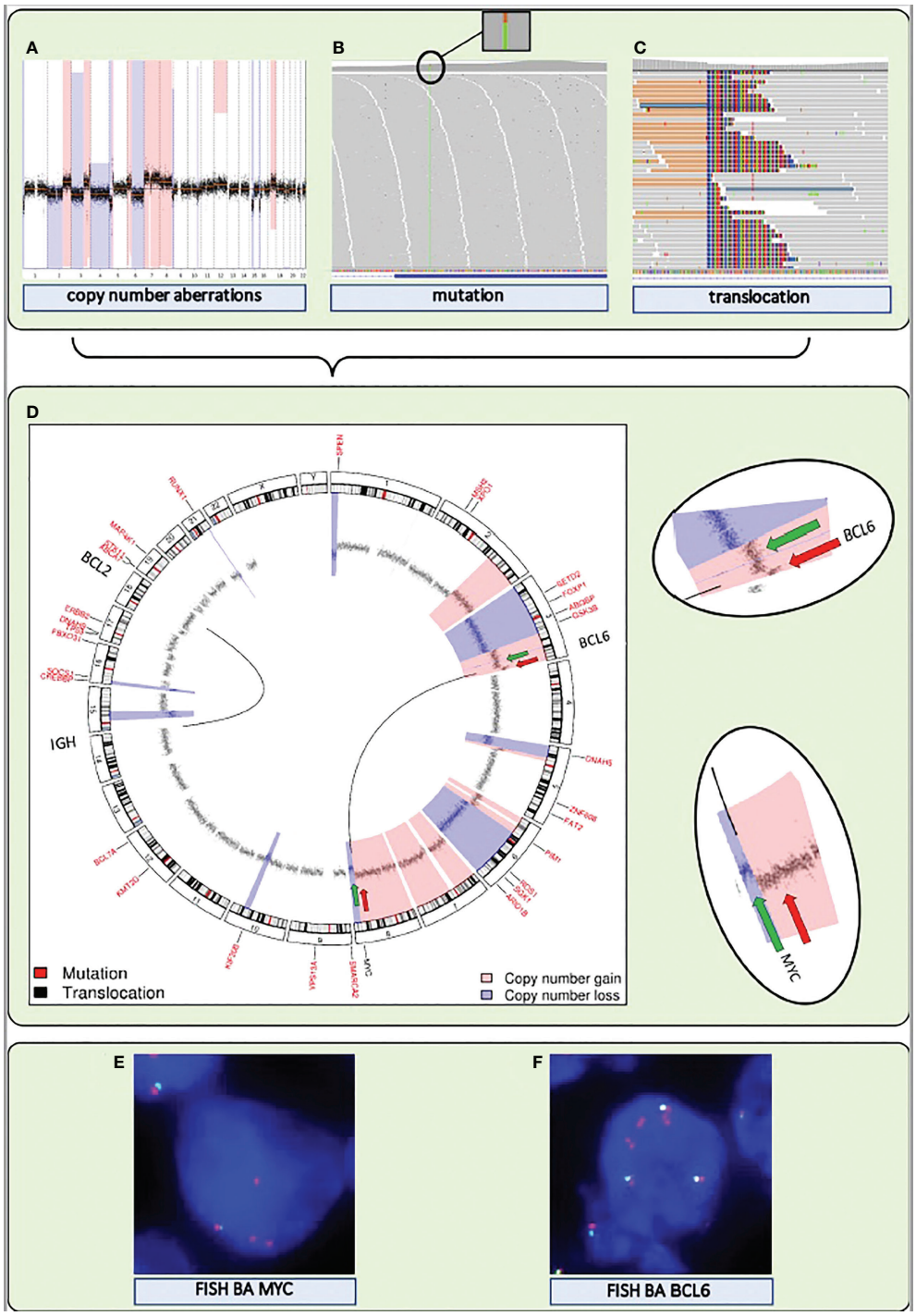

FIGURE 2
A single NGS assay to detect somatic mutations and structural variations, including translocations and CNAs, from DNA extracted from FFPE tissue. **(A–C)** Visualization of the detection by NGS of CNAs, mutations, and translocations for a DLBCL sample. **(A)** Genome-wide chromosomal CNAs. x-axis shows chromosomes 1 to 22, from left to right, y-axis shows copy number gains (light red) and copy number losses (blue). **(B)** Screenshot of high coverage (200X) NGS sequence reads aligned to the reference genome highlighting a somatic mutation in *KMT2D*. **(C)** Screenshot of high coverage (200X) NGS sequence reads aligned to the reference genome highlighting a translocation breakpoint in *MYC*. **(D)** A circular representation of the genome depicting mutations (genes denoted in small red letters), translocations (genes denoted with large black letters connected by black lines) and CNAs (inner circle: black dots are measurement bins and called losses are colored in blue and gains in light red). Green and red arrows point to the position of break apart (BA) FISH probes that were used as a control for the translocations detected by NGS. **(E)** FISH BA MYC. **(F)** FISH BA BCL6. Integrated NGS analysis explains aberrant FISH pattern: a loss (green arrow) and a gain (red arrow) at the *MYC* locus coincide with a single (green arrow) and double gain (red arrow) at the translocation partner *BCL6* locus.

intensive with a certain level of technical variability and subjectivity in interpretation. Thereby, NGS outperforms FISH in several ways: it avoids interobserver variability, it can be performed with small and histologically compromised materials, and it is able to identify exact translocation breakpoints on nucleotide level. An additional advantage of some of the NGS approaches is that unknown translocation partners may be identified, that may be of clinical relevance for the biological and clinical interpretation of DLBCL patients with a *MYC* translocation (40). Various combinations of NGS and bioinformatics platforms have been successfully developed in this direction (58–61).

Assays for clinical implementation

World-wide clinical implementation of any diagnostic routine requires relatively simple assays that are applicable to routine diagnostic tissue material, such as formalin-fixed paraffin embedded (FFPE) specimens. The elaborate laboratory- and informatics infrastructure needed for current NGS or array analysis may only be available in selected settings of large medical centers or commercial providers as exemplified for CNS tumors. Favorable aspects of commercial involvement are the wide availability, extensive standardization, quality control and rapid turnover time due to high case volumes. Downsides are amongst others worldwide availability, financial dependency and commercial goals, market dominance of individual commercial providers, lack of technical transparency and development, lack of flexibility to include most recent research developments and generally lack of integrated interpretation with other pathology parameters. Another option to bring a genome subtyping assay to implementation in daily practice is to "reduce" complex molecular information to simpler and widely applicable techniques. The DLBCL-COO classification alternative is a good example; genome-wide molecular classification with elaborate bioinformatics was translated into several simple immunohistochemistry (IHC) markers, of which the Hans classification is most widely used (62). All IHC-based COO assays show limited concordance with the gold standard of RNA expression-based assays (63). This prompted the development of a digital gene expression assay based on 20 key genes that can be applied on FFPE material (64). This Lymph2Cx assay, restricted to equipment from the company Nanostring (Seattle, USA), showed high concordance with the original RNA expression-based COO classification with a 2% error rate in COO assignment (65). These characteristics, together with a short turnaround time of less than 36 hours, allowed for rapid molecular characterization of patients, making this assay a suitable middle-ground alternative for employment in research and clinical trials (19). Similar assays have been commercialized by others (66). In view of the expected high-dimensional nature of a consensus molecular classifier for DLBCL, simple translation to an IHC is not likely. Current NGS techniques are already reliably applicable for FFPE biopsy samples offered by commercial providers. It may be expected that these companies will readily offer products for consensus molecular DLBCL classification once this would be developed.

Assay and turnaround time

A single genome subtyping assay that detects CNAs, mutations, and translocations in parallel would conceivably be most efficient in terms of labor, cost and tissue material. But is this also efficient in terms of turnaround time? A recent study showed that real-time molecular profiling of RNA-based COO determination of DLBCL is realistic to stratify patients in a timely manner, with a median turnaround time of 8 days (8). This would be a desirable timeframe for DNA-based DLBCL classification, such that based on tumor vulnerabilities, patients can be diverted after 1 or 2 cycles standard R-CHOP treatment, which is a successful approach facilitating rapid trial inclusion (67). A recent feasibility study in the Netherlands, which involves a WGS specialized non-profit organization, was performed to evaluate implementation of WGS into routine diagnostics (68). Meanwhile, they were able to optimize the turnaround time from biopsy to DNA report to 7 working days, demonstrating the potential of clinical implementation of NGS methods for these purposes.

Application in daily clinical practice and promising future developments

Bespoke treatment of DLBCL patients

Once validated, uniform and widely applicable, consensus molecular subtypes of DLBCL will be a sound basis to explore more effective, targeted treatment methods (1). The potential of DNA-based classification for precision medicine of DLBCL has been demonstrated in a recent retrospective analysis of a randomized phase-III trial (69). In this study, patients under 60 with two specific DNA subtypes (LymphGen's MCD and N1) that received R-CHOP with Ibrutinib had significantly better survival (both subtypes 100% 3-year event-free survival) than patients that received R-CHOP alone (42.9% and 50%, respectively), clearly indicating the potential predictive value of the novel genomic subtypes. Next, prospective clinical trials may further explore associations with genomic subtypes and associations with targeted compounds, such as NFkB-inhibitors, PI3K inhibitors, P53-modulators and apoptosis modulators, as well as immunotherapy such as immune checkpoint inhibitors and CAR-T cell therapy. For this purpose, various dedicated next-generation designs are now proposed (70).

It is obvious to further investigate to what extent the integration of the current DNA-based and RNA/microenvironmental-based subtyping methods for DLBCL would be of added value. Adding a layer of epigenetic information as for CNS (55) or even germline genetic characteristics might be considered (71). Also liquid biopsy strategies measuring circulating tumor DNA (ctDNA), will provide other lines of opportunities in diagnosis and disease monitoring of DLBCL patients (72–74) Future studies are required to investigate the potential integration of these approaches for the management of DLBCL patients.

Consensus classification serves the DLBCL patient

The step forward to allow evaluation of new treatment modalities based on DLBCL genetics is now impeded by a discordancy between the 2 independently suggested genomic subtyping approaches, which dictates the challenge that lies ahead of us. Based on various other tumor entities we suggest a blueprint for harmonization of the proposed DNA subtypes, which may allow more widespread clinical implementation. Once this hurdle is taken, a diagnostic work up, applicable in a clinically relevant timeframe, will enable the design of next-generation prospective biomarker-based clinical trials. If successful, the precision medicine with targeted therapies that match dependencies of the molecular subtypes of DLBCL may be brought forward.

Author contributions

MM, MR, DJ, and BY contributed to conception and design of the review and wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The 8p11 myeloproliferative syndrome: Genotypic and phenotypic classification and targeted therapy

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EMS (8p11 myeloproliferative syndrome, EMS) is an aggressive hematological neoplasm with/without eosinophilia caused by a rearrangement of the FGFR1 gene at 8p11-12. It was found that all cases carry chromosome abnormalities at the molecular level, not only the previously reported chromosome translocation and insertion but also a chromosome inversion. These abnormalities produced 17 FGFR1 fusion genes, of which the most common partner genes are ZNF198 on 13q11-12 and BCR of 22q11.2. The clinical manifestations can develop into AML (acute myeloid leukemia), T-LBL (T-cell lymphoblastic lymphoma), CML (chronic myeloid leukemia), CMML (chronic monomyelocytic leukemia), or mixed phenotype acute leukemia (MPAL). Most patients are resistant to traditional chemotherapy, and a minority of patients achieve long-term clinical remission after stem cell transplantation. Recently, the therapeutic effect of targeted tyrosine kinase inhibitors (such as pemigatinib and infigratinib) in 8p11 has been confirmed *in vitro* and clinical trials. The TKIs may become an 8p11 treatment option as an alternative to hematopoietic stem cell transplantation, which is worthy of further study.

KEYWORDS

EMS, FGFR1 rearrangement, targeted therapy, tyrosine kinase inhibitors, pemigatinib, infigratinib

1 Introduction

8p11 myeloproliferative syndrome (EMS) or stem cell leukemia/lymphoma (SCLL), which is a very rare but aggressive neoplasm with the fibroblast growth factor receptor 1 (FGFR1) rearrangement on chromosome 8p11-12, is recognized as a distinct entity in 2016 World Health Organization (WHO) classification (1). In 2022 WHO classification, EMS belongs to myeloid or lymphoid neoplasms with eosinophilia and tyrosine kinase

gene fusions (MLN-TK) (2). To date, fewer than 110 EMS patients have been reported worldwide. Most of them were male, occurring at any age. The disease progresses rapidly, usually into acute leukemia within one year. The molecular characteristics of EMS often involve chromosome 8 abnormalities, such as chromosome translocation, insertion, and inversion, which lead to the fusion of FGFR1 with partner genes to form any of 17 different fusion genes, resulting in the constitutive activation of tyrosine kinase (3). Because of its complex and diverse manifestations, EMS is often ignored or misdiagnosed as other hematological neoplasms such as aCML (atypical chronic myeloid leukemia) or CML. In the WHO classification, CML and aCML belong to myeloproliferative neoplasms, the distinction is that CML is defined as BCR-ABL1 fusion-positive resulting from t (9, 22) (q34; q11), while aCML is rare and characterized molecularly with BCR-ABL1 fusion-negative, and it is emphasized that accurate histological diagnosis has been to be the key to predict the prognosis of the disease (1, 4). To make the differentiation, it is necessary to detect BCR-ABL1 fusion-positive or FGFR1 rearrangements in peripheral blood (PB) or bone marrow (BM) by a combination of karyotype analysis, fluorescence *in situ* hybridization (FISH) and next-generation sequencing of molecular genetic techniques. In addition, the prognosis of EMS is very unfavorable. At present, only allogeneic stem cell transplantation (allo-SCT) improves the survival of these patients (5), but less than 50% of patients with EMS can undergo allo-SCT (6). Due to its resistance to traditional treatment, the drugs targeting tyrosine kinase inhibitors show the most promising results and have some advantages. This review will summarize the phenotypic and genotypic classification and the application of targeted therapy or EMS in the last 25 years, providing the latest data about the characteristics of this rare entity.

2 Genotypic and phenotypic classification

FGFR1 belongs to the fibroblast growth factor receptor (FGFR) family and is a member of the receptor tyrosine kinase (RTK) superfamily (7). At least four FGFRs have been found in the FGFR family, namely, FGFR1, FGFR2, FGFR3, and FGFR4. Their common structural feature is that they all contain extracellular immunoglobulin-like domains and cytoplasmic tyrosine kinase domains (8). The FGFR1 gene has a total length of 65 kb and contains 19 exons, which are located on the short arm of chromosome 8. The product encoded by the FGFR1 gene is a transmembrane protein located in the cytoplasmic membrane, which is divided into intracellular and extracellular regions. The extracellular domain is a signal peptide composed of an immunoglobulin-like domain I, an acidic box, a heparin-binding domain, and cell adhesion factor homologous domain, an immunoglobulin-like domain II, and an immunoglobulin-like domain III. The intracellular region consists of a near membrane domain and a tyrosine kinase domain (9). The acidic box in FGFR1 plays an important role in stabilizing its protein structure and ligand-receptor interactions (10). The molecular pathogenesis of EMS is characterized by FGFR1 rearrangement, which forms a fusion gene originating from translocation, insertion, inversion, or deletion (11), to genome variation, affecting FGFR1 mRNA transcription, and promoting the oncogenicity and genetic variation of the FGFR1 protein (12). FGFR1 fusion genes can be divided into two types: type I and type II. Type I refers to the FGFR1 gene located at the 3' end of the fusion gene, and the FGFR1 tyrosine kinase domain is fused to the N-terminal oligomerization domain of the partner protein. The N-terminal oligomerization domain of the partner protein generates a fusion type protein that cannot bind to the FGF ligand and causes a conformational change in the FGFR1 tyrosine kinase domain (13). This stimulates the function of FGFR1 oncogene and constitutively activates its tyrosine kinase function, changes its localization, and subsequently activates PI3K-AKT, RAS/MAPK, STAT, and PLCγ/PKC in the downstream cell pathways to transmit abnormal signals (7, 14). The fusion genes of FGFR1 and its partners in EMS arise from type I. Type II is the opposite to type I, and the main difference is the fusion proteins retain the extracellular domain of FGFR1, which binds to FGF ligands, and is common in solid tumors (13). Even though the domains in the fusion proteins retained by FGFR rearrangement are different, in all cases the protein retains a complete kinase domain, suggesting that the kinase domain plays a vital role in the function of the fusion protein.

Currently, it has been reported that 17 FGFR1 gene rearrangements exist in EMS, including 15 translocations, 1 insertion, and 1 inversion (Figure 1). Herein, we will further clarify the clinical characteristics of EMS with corresponding cases, and analyze the characteristics and functions of different FGFR1 rearrangements and partner genes (Tables 1 and 2).

2.1 BCR-FGFR1/t (8, 22) (p11.2; q11.2)

FGFR1 is the second-most common partner gene of BCR (breakpoint cluster region) after the ABL (abelson leukemia virus) gene (26). Sequence analysis revealed that BCR exon 4 was fused in frame with FGFR1 exon 9, and genomic breakpoints occurred in intron 4 of BCR and intron 8 of FGFR1 (25). The BCR gene locus spans 130 kb and contains 23 total exons, with alternative exon 1 and exon 2; it will eventually encode a protein of approximately 1271 amino acids (50). Exon 1 encodes one serine/threonine kinase oligomerization domain, a growth factor receptor-binding protein 2 binding site (Grb2), the BCR-associated protein 1 interaction site (BAP-1), and two SH2 domains. Exons 3-8 encode a central ornithine exchange factor domain (GEF), and exons 19-23 contain the Racgap domain, as

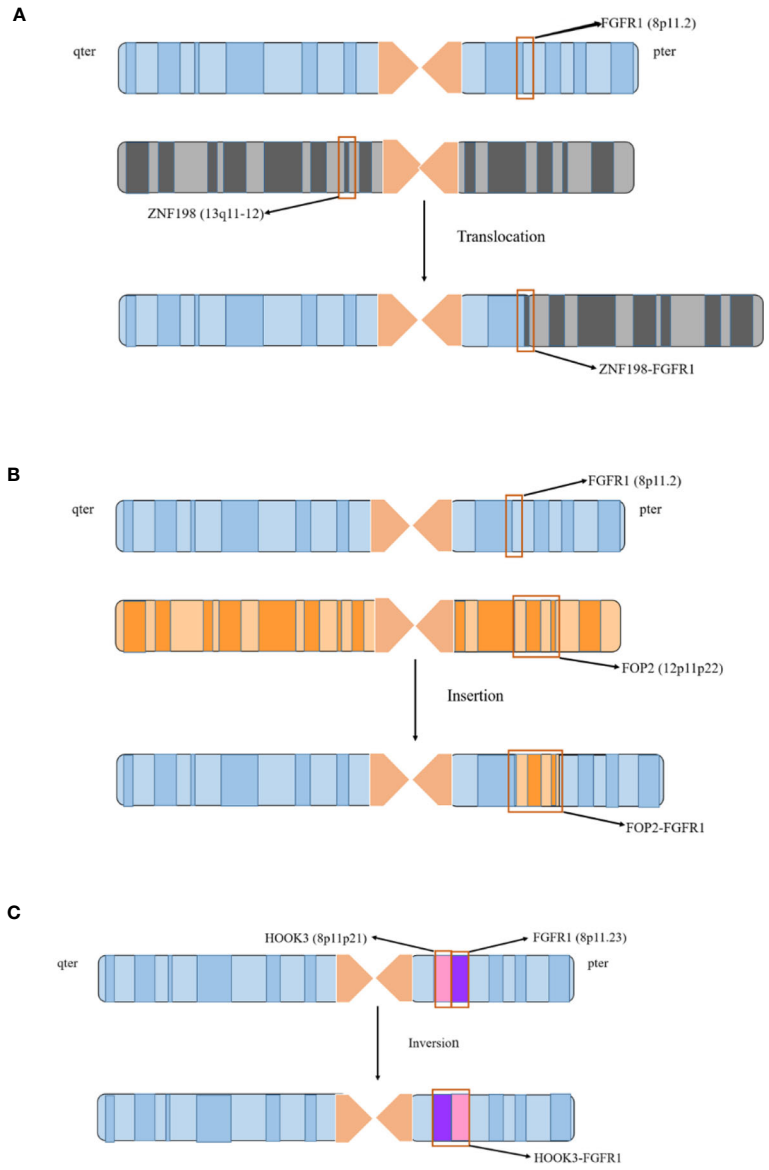

FIGURE 1
FGFR1 rearrangement involves three chromosomal abnormalities. 17 FGFR1 gene rearrangements existed in EMS, including 15 translocations, 1 insertion, and 1 inversion. **(A)** The 15 fusion genes are generated from chromosome translocation. It is illustrated by the case of ZNF198-FGFR1 to describe with which chromosome 8 and other chromosomes are formed by translocation. **(B)** FOP2-FGFR1 is the only rearrangement due to chromosome insertion. The FGFR1 gene on chromosome 8 and the FOP2 gene on the 12p11-p22 are breaking, and the dissociative FOP2 gene is reinserted into the FGFR1 gene fracture to form a fusion. **(C)** HOOK3-FGFR1 is a recently identified FGFR1 rearrangement in EMS, which is derived from chromosome inversion. The fragment between FGFR1 and HOOK3 genes located on the short arm of chromosome 8 is breaking, and the broken fragment is rotated 180 degrees, resulting in the fusion of HOOK3 and FGFR1.

well as other PSD95, D1g1, and ZO-1 (PDZ) domain binding motifs (26, 51).

There is evidence that several domains of BCR play a crucial role in the pathogenesis of EMS. The fusion protein contains part of the RhoGEF (Rho ornithine exchange factor) domain of BCR, and it has kinase activity. The BCR-FGFR1 fusion causes a kinase-kinase fusion, it is where the tyrosine kinase domain of FGFR1 is fused to the serine-threonine kinase domain of BCR (13). A recent study pointed out that the GEF domain in BCR is related not only to the rapid onset of EMS with BCR-FGFR1 positivity but also to the phenotype of EMS disease progression. The deletion of the GEF domain leads to an increase in AKT

TABLE 1 The characteristics and functions of different FGFR1 rearrangements and partner genes.

Genotypic	Chromosome abnormality	Year	Fusion site (partner; FGFR1)	Breakpoint (partner; FGFR1)	Oligomeric/dimeric domain of the fusion	Function of partner genes
ZNF198-FGFR1(15)	t (8;13)(p11.2;q11-12)	1998	exon 17;exon 9	exon 17;exon 9	Five zinc fingers or proline-rich domain (16)	DNA repair (17)
FOP1-FGFR1 (18)	t(6;8)(q27;p11.2)	1999	exon 5/6/7;exon 9	intron 6;intron 8	The LisH motif (18)	Anchoring of centrosome (19)
CEP110-FGFR1 (20)	t(8;9)(p11-12;q32-34)	2000	exon 15;exon 9	Intron x;exon 8	Leucine zipper (20)	Regulation of mitosis and cell cycle (21)
HERVK-FGFR1 (22)	t(8;19)(p11.2;q13)	2000	Not clear	Not clear	Not clear	Proliferation, transformation and tumorigenesis of normal cells (23, 24)
BCR-FGFR1 (25)	t(8;22)(p11.2;q11.2)	2001	exon 4;exon 9	intron 4;intron 8	Serine/threonine kinase domain (26)	Critical regulators of brain development (26)
NUP98-FGFR1 (27)	t(8;11)(p11.2;p15)	2001	Not clear	Not clear	The coiled-coil domain (not clear)	Nucleo-cytoplasmic transport (28)
FOP2-FGFR1 (29)	ins(8;12)(p11.2;p11p22)	2004	exon 4;exon 9	intron 4;intron 8	First two coiled-coil domains (30)	Not clear
TIF1-FGFR1 (31)	t(7;8)(q34;p11.2)	2005	Not clear	intron 11;intron 9	TRIM domain (31)	Transcription factor (32)
MYO18A-FGFR1 (33)	t(8;17)(p11.2;q25)	2005	exon 32;exon 9	Controversial	Presumably PDZ domain (34)	Unconventional myosin (34)
CPSF6-FGFR1 (35)	t(8;12)(p11.2;q15)	2008	exon 8;exon 9	intron 8;Not clear	RNA recognition motif (35)	3' cleavage and polyadenylation of pre-mRNA (36, 37)
LRRFIP1-FGFR1 (38)	t(2;8)(q37;p11.2)	2009	exon 9;exon 9	Not clear	The coiled-coil domain (38)	Organism immune response
CUX1-FGFR1 (39)	t(7;8)(q22;p11.2)	2011	exon 11;exon 10	Not clear	Not clear	Tumor suppressor (40)
TPR-FGFR1 (41)	t(1;8)(q25;p11.2)	2012	exon 22/23;exon 13	intron 22;intron 12	TprMet, NPC relavant domain (41, 42)	Nuclear pore protein (43)
NUP358-FGFR1 (44)	t(2;8)(q12;p11.2)	2013	exon 20;exon 9	Not clear	Leucine zipper part (44)	Nucleo-cytoplasmic transport (45)
SQSTM1-FGFR1 (46)	t(5;8)(q35;p11.2)	2014	exon 9;exon 9	intron 8;intron 8	PB1 domain (46)	Regulating the activation of NF-κβ (46, 47)
TFG-FGFR1 (48)	t(3;8)(q12;p11.2)	2020	exon 8;exon 10	Not clear	Not clear	Not clear
HOOK3-FGFR1 (49)	inv(8;8)(p11.23;p11.21)	2022	Not clear	exon 11;exon 10	Not clear	Not clear

The basic information about the FGFR1 rearrangements are based on studies currently present in the literature.

activation by inhibiting the activation of RHOA and PTEN, accelerating the occurrence of leukemia, strengthening the survival and proliferation of cells, and promoting the proliferation of stem cells and lymph node metastasis (52). At the same time, BCR-FGFR1 also retains the coiled-coil domain of BCR (26).

To be worthy of our attention, Hu's *in vivo* transplantation study demonstrated that microRNAs-17/92 are downstream effectors of FGFR1 in BCR-FGFR1-driven B-cell lymphoblastic leukemia (53). Moreover, the BCR-FGFR1 fusion protein also depends on the heat shock protein 90 (Hsp90) complex to escape the dissolution of the proteasome because BCR-FGFR1 acts as a client of chaperone Hsp90 (54).

Patients with BCR-FGFR1 fusion can have a similar presentation to BCR-ABL1 positive CML (55). In some case reports, it also tends to present AML-like and ALL phenotypes (56). Patients progressed rapidly into AML/ALL within one or two years of the diagnosis of EMS, but the relevant pathological mechanism is still unclear. Khodadoust and Morishige also reported three-line mixed phenotype acute leukemia with BCR-FGFR1 (57, 58). It is observed for phenotypic change in the course of the same patient. In previous research, it was proposed that the therapeutic drug blinatumomab may enhance the transformation of acute lymphoblastic leukemia into myeloid leukemia (59). Moreover, a meta-analysis of data from 20 patients indicated that BCR-FGFR1-positive cells might be derived from myeloid/B progenitor cells, but the mechanism determining the differentiation of B-myeloid cells is unclear (6).

TABLE 2 Number and common phenotypes of reported cases for EMS and the reported response for chemotherapy and TKIs.

Fusion	Number of cases	Common phenotypes	Physical and laboratory examination	Sensitivity to chemotherap	Numbers and results of allo-SCT	Sensitivity to TKIs
ZNF198-FGFR1	>30	T-LBL/T-lymphoma	Lymphadenopathy, hepatosplenomegaly, eosinophilia or monocytosis or both	Insensitive[1]	7,Remission;2,Recurrence	Sensitive (imatinib, MIDOSTAURIN)
FOP1-FGFR1	5	MPD, AML, B-ALL	Polycythemia without eosinophilia	Sensitive	No	Not tested
CEP110-FGFR1	>20	AML, T-LBL	Lymphadenopathy, purpura, skin lesions, eosinophilia and monocytosis	Insensitive	7,Remission;1,Recurrence	Sensitive (imatinib, dasatinib, pemigatinib)
HERVK-FGFR1	2	AML, SM-AHNMD	Polycythemia, poikilocyte, granulocytosis, abnormal megakaryocytes	Insensitive	1;Remission	Not tested
BCR-FGFR1	>30	CML, aCML, AML, B-ALL	Splenomegaly, eosinophilia	Insensitive	4,Remission;3,Recurrence	Insensitive (imatinib, dasatinib), Sensitive (ponatinib, pemigatinib)
NUP98-FGFR1	2	therapeutic AMML	Granulocyte hyperplasia with mononucleosis	Not tests	No	Not tested
FOP2-FGFR1	2	T-LBL, AML	Lymphadenopathy, eosinophilia	Sensitive[2]	No	Not tested
TIF1-FGFR1	5	CEL, AMML	Eosinophilia	Resistant[3]	No	Not tested
MYO18A-FGFR1	2	CML	Thrombocytopenia, monocyte, eosinophilic and basophil increased	Resistant	No	Not tested
CPSF6-FGFR1	1	Not reported	Lymphadenopathy and splenomegaly, neutrophils without eosinophilia	Resistant	No	Not tested
LRRFIP1-FGFR1	1	MDS, AML	Pancytopenia, eosinophilia	Not tests	No	Not tested
CUX1-FGFR1	1	pre-T-LBL	Neutrophils, lymphocytes and monocytes increased without eosinophils	Resistant	No	Not tested
TPR-FGFR1	4	AMML, AML-M5	Lymphadenopathy, increasing monocytes	Insensitive	1;Remission	Not tested
NUP358-FGFR1	2	MDS	Splenomegaly, a little eosinophilia	Sensitive	No	Not tested
SQSTM1-FGFR1	1	AMML	Neutrophils and monocytes increased, megakaryocytes	Not tests	No	Not tested
TFG-FGFR1	1	AML	Skin ecchymosis and splenomegaly, eosinophilia	Insensitive	No	Resistant (ponatinib)
HOOK3-FGFR1	1	MDS	Leukocytosis and thrombocytopenia.	Insensitive	No	Resistant (ponatinib)

The responsiveness to the TKIs and chemotherapy are based on the very few studies that have been reported so far; thus, the data included are not definitive. Addtionally, in many cases, the TKIs were used in conjunction with other chemotherapy or allo-SCT agents.

2.2 ZNF198/ZMYM2-FGFR1/t (8, 13) (p11.2; q11-12)

ZNF198 was also described in previous reports as ZMYM2, FIM, and RAMP. ZNF198 is located at 13q11-12, and its orientation is from the telomere to the centromere. The ZNF198 gene is organized into 26 exons with an initiation codon located in exon 4, which is predicted to encode a 1377 amino acid nucleoprotein with five zinc fingers as the MYM domain (60), and it might participate in DNA repair by other proteins to form a complex (17).

ZNF198-FGFR1 fusion is the most frequent fusion type in EMS other than BCR-FGFR1. The 17 exons of ZNF198 are fused with 9 exons of FGFR1, as well as a breakpoint located at the same position in most cases. However, new studies have shown that the fusion is not only produced by a balanced translocation but also involves the insertion and internal inversion of the 13q11-12 chromosome (60–62); therefore, the breakpoint of the fusion is still not clear. The fusion gene produces a 152 bp transcript that is located in the cytoplasm, according to *in vitro* studies (15, 63). It is speculated that the fusion also encodes a protein with a length of 146 kDa containing approximately 1309 amino acids that is located in the cytoplasm (64). The fusion protein consists of five zinc fingers, a proline-rich domain, and FGFR1 the entire tyrosine kinase domain of FGFR1 (16). Exon 9 of FGFR1 encodes the tyrosine kinase domain; in this context, the zinc finger domain of ZNF198 is fused to the tyrosine kinase domain of FGFR1 (15, 64). There is an argument as to whether

the carcinogenicity of the fusion comes from the oligomerization of the proline-rich domains or the dimerization of the zinc finger domains (16, 65). In short, the studies discussed above are basically *in vitro* studies, and more *in vivo* research is needed to confirm these ideas in the future.

There are at least 30 cases reported to be ZNF198-FGFR1 positive with EMS. At the time of onset, the diversity of clinical phenotypes and laboratory tests makes the diagnosis of the disease more difficult. Lymphadenopathy and hepatosplenomegaly are often the first symptoms. Most ZNF198-FGFR1-positive EMS patients are diagnosed with T-LBL/T-lymphoma (66). Cases involving B-ALL alone are relatively rare and are more common in cases of T/B double line involvement or acute mixed leukemia (67). The fusion suggests that the disease may originate from hematopoietic progenitor cells or stem cells and has the potential to differentiate along various lines. In a mouse model of ZNF198-FGFR1, T-cell receptors on the surface of tumor cells were found to have an α-deletion hindering the recruitment of CD3, preventing the maturation of CD4 (+)/CD8 (+) double-positive T cells, and upregulating BCL2, IL-7 receptor-α and IL-2 receptor-α in tumor precursor cells, allowing them to escape apoptosis in the thymus, which may be one of the reasons why this fusion more easily induces T-lymphocytic leukemia/lymphoma phenotype (68). The expression of ZNF198-FGFR1 is related to specific PAI-2 (plasminogen activator inhibitor-2/SERPINB2)-mediated anti-apoptosis, which is possibly one of the reasons for the high malignancy of leukemia cells (69).

2.3 CEP110-FGFR1/t (8, 9) (p11-12; q32-34)

CEP110, also known as CTNL, is located at 9q32-34. CEP110 comprises 19 exons spanning approximately 26 kb and is inferred to encode an acidic protein with 994 amino acids and a molecular weight of 110 kDa (20). The CEP110 protein binds to the centrosome *via* five repeated leucine zippers (L-X (6)- L-X (6)- L-X (6)-L), which are at amino acid positions 28-49, 98-118, 496-517 and 689-710, respectively (20). The position of CEP110 in the centrosome of mother and daughter is different (21), which might be related to the different roles of CEP110 in different stages of cell mitosis. CEP110 and nine other peptides could change the structure of the mitotic interphase centrosome, which is very important for the function of the microtubule-organizing center (21).

Because of a balanced translocation, CEP110-FGFR1 is a chimeric gene consisting of exon 15 of CEP110 fusing with exon 9 of FGFR1 (27), and its breakpoint is located in exon 8 of FGFR1 and an intron of CEP110. The N-terminal of CEP110-FGFR1 retains the leucine zipper of CEP110, the C-terminal contains the tyrosine kinase domain of FGFR1 in the cytoplasm, and the protein is approximately 150 kDa (20). The leucine zipper has dimerization potential in CEP110-FGFR1 and mediates the activation of constitutive tyrosine kinase activity. CEP110 is located in the centrosome but the fusion protein is in the cytoplasm, which may be relevant to the occurrence of EMS. CEP110-FGFR1 with EMS is the third most common phenotype, with nearly 20 cases. Although most patients present with lymphadenopathy, purpura and skin lesions are also common with such fusion genes (70). The bone marrow aspiration and peripheral hemogram of patients are often accompanied by eosinophilia and monocytosis. There is also a complex clinical phenotype, which is often characterized by myeloid leukemia. T-LBL is more common in enlarged lymph node biopsies.

2.4 FOP/FGFROP-FGFR1/t (6, 8) (q27; p11.2)

The FGFR1 oncogene partner, referred to as FGFROP and FOP, is located at locus 6q27. The whole FOP gene is 1630 bp and comprises 13 exons (71). This protein may encode a protein of approximately 44.3kDa, including 399 amino acids, with an α-helically folded conformation (71). The α-helical region contains (L-X_2-L-X_{3-5}-L-X_{3-5}-L) leucine-rich repeats, including a Lish motif, which can dimerize (71). FOP is also in the centrosome and participates in forming an MT-anchored centrosome complex (19).

The in-frame fusion results from the 5/6/7 exons of FOP fusing to the 9th exon of FGFR1, and the breakpoint of the fusion is in intron 8 of FGFR1 and intron 6 of FOP, but it is changeable (18, 27). The FOP-FGFR1 fusion protein contains the N-terminal of the leucine-rich sequence of FOP (retaining the LisH motif) (18), and while it is in the centrosome, the fusion interferes with the normal function of FOP. In addition, the fusion could also protect cells from apoptosis by regulating BCL2 (B-cell lymphoma-2) overexpression and Caspase 9 inactivation (72, 73). Furthermore, the fusion proteins target the centrosome, activate the signaling pathway of this organelle by promoting centrosome phosphorylation, and continuously participate in the regulation of the cell cycle so that cells may overcome G1 blockade and obtain the ability to proliferate and survive (74). There is a possibility that the poor proliferation signal transduction of the fusion protein may depend on its abnormal localization and dimerization, and FOP is likely to be the cause of both (75).

Cases of FOP-FGFR1 fusion are rare, and only 5 cases have been reported. Among them, three cases are incomplete due to the early stage and inconsistent diagnostic criteria at that time. It is noteworthy that three of the cases were accompanied by polycythemia vera, without eosinophilia in the bone marrow and hemogram, which might also be due to the lack of records (18, 76). This finding supports the hypothesis that the FOP gene

plays an important role in the proliferation and differentiation of erythroid cells (18). MPD (myeloproliferative diseases), AML, and B-ALL were included in their clinical phenotypes, but the complete characterization of the fusion was limited by the scarcity of cases.

2.5 NUP98-FGFR1/t (8, 11) (p11; p15)

NUP98 is situated on 11p15.4, 3.9 Mb from the telomere, and it consists of 33 exons, producing a transcript 122 kb in size that codes for 1729 amino acid residues of the NUP98-NUP96 precursor (77). The alternative transcript generates a precursor NUP186 protein, which is then proteolytically cleaved into NUP98 and NUP96 (77). NUP98 is encoded by the first 18 exons of the NUP98 gene and is composed of 860 amino acids, and the other exons are involved in coding the NUP96 protein (28). It is a nuclear pore protein with a molecular weight of 98 kDa, forming an important part of the nuclear pore complex (NPC) (28). Several scholars have found a dimeric or oligomeric domain in NUP98, including a coiled-coil structure (27). NUP98 plays a huge role in nucleocytoplasmic transport, allowing nucleolar proteins and RNA transporters to shuttle protein and RNA between the nucleus and cytoplasm (28).

The NUP98-FGFR1 fusion is formed from a balanced translocation, the fusion site and breakpoint of the fusion are not clear. Based on the other fusion genes mentioned above, we infer that the NUP98-FGFR1 fusion should contain a dimeric or oligomeric coiled-coil domain of NUP98. The fusion of NUP98 and other partner genes often contains the N-terminal GLFG domain of NUP98 (77), and we hypothesize that the GLFG domain contains a coiled-coil. In addition to being fused to FGFR1 on EMS, NUP98 also forms a fusion with the NSD gene on 8p11-12 due to translocation (78). Fusion transcripts of NUP98 usually have different characteristics, such as FG (phenylalanine motif), which provides a binding site for the homologous domain of karyopherins and chromatin interactions with its partner genes (44). One case presented with abdominal pain and fever, which was diagnosed as breast cancer with metastasis 11 years ago and the patient received radiotherapy and chemotherapy (79). The peripheral hemogram is mainly composed of blast cells, while the bone marrow is mostly composed of granulocyte hyperplasia with mononucleosis and a therapeutic AMML (acute monomyelocytic leukemia) phenotype (79).

2.6 FGFR1OP2-FGFR1/ins (8, 12) (p11.2; p11p22)

The full name of the FGFR1OP2 gene is FGFR1 oncogene partner 2, or FOP2. FOP2 is in a 12p11-12 locus with 7 exons, it encodes a protein containing 253 amino acids, approximately 29 kDa (30). As a result of a chromosome insertion, the chimeric cDNA shows an in-frame fusion of exon 4 of FOP2 to exon 9 of FGFR1, and its breakpoint is located in intron 4 of FOP2 and intron 8 of FGFR1 (30). The structure of the FOP2 protein contains four coiled-coil domains, and the first two exist in the fusion, suggesting that the fusion protein consists of 526 amino acids and is approximately 60 kDa (30). In a mouse model of the FOP2-FGFR1 fusion, the fusion protein combined with Notch1 promoted stem cells to differentiate into T cells and trigger lymphoma (80). Due to the constitutive activation of deletion mutations, the abnormal increase in Notch1 transcription in fusion T-lymphoma mice may be due to the use of an alternative Notch1 promoter (80). Notably, Hsp90 and Hsp90-CDC37 formed with the partner CDC37 could maintain the stability and activity of the FOP2-FGFR1 fusion, and Hsp90-CDC37 forms a permanent complex with FOP2-FGFR1 to protect it against hydrolysis (81). In two patients with FOP2-FGFR1, lymphadenopathy was their common clinical manifestation, their laboratory tests showed eosinophilia, and the lymph node biopsy indicated T-LBL (29, 30). It is unclear whether there is a mutation of Notch1 in patients with the T-LBL phenotype of the FOP2-FGFR1 fusion, but a mutation of Notch1 could be useful for the diagnosis and prognostication of patients in the future.

2.7 TIF1/TRIM24-FGFR1/t (7, 8) (q34; p11.2)

TIF1 is in 7q34 and is responsible for encoding TIF1α (transcription factor 1α). The N-terminal of TIF1α displays an RBBC motif composed of a RING finger, B-BOX, and a coiled-coil domain, also known as the tripartite motif (TRIM), and the C-terminal contains a PHD and a bromo domain (31). TIF-FGFR1 and reciprocal TIF1-FGFR1 were found in one patient. The breakpoint of TIF1-FGFR1 was in intron 9 of FGFR1 and intron 11 of TIF1 (31). The TRIM domain and tyrosine kinase domain were retained in the TIF1-FGFR1 protein, but the FGFR1-TIF1 protein was oriented to the plasma membrane by the extracellular domain and transmembrane domain of FGFR1, and the PHD and bromo domains of TIF1 and the pathogenicity of TIF1-FGFR1 were stronger than the latter (31). Nuclear receptors are ligand-induced transcription factors; currently, TIF1 is widely considered to be a protein that specifically interacts with the ligand-binding domains of several nuclear receptors (32). There are 5 known cases, three of which were found in Korea. One of the patients was not accompanied by eosinophilia at the time of onset and had increased but it subsided spontaneously (82). After nearly 5 years, eosinophilia appeared in the patient's peripheral blood and bone marrow, and the clinical diagnosis was chronic eosinophilic leukemia. Another case had AML-M4 with eosinophilia, and one had B-lymphocytic leukemia (31, 83).

2.8 MY018A-FGFR1/t (8, 17) (p11.2; q25)

Myosin XVIIIA, or MYO18A, is in 17q25 and it encodes an unconventional myosin. Its N-terminal contains a PDZ domain, followed by a conserved myosin head motor domain, next to several binding sites consistent with calcitonin and calcitonin-related light chains, alias IQ motifs, and there is a coiled-coil domain included in the C-terminal (34). The PDZ domain is mainly involved in protein-protein interactions and often binds to proteins with C-terminal PDZ motifs (84). The conserved myosin motor domain plays a principal role in the interaction with ATP (34). Owing to the opposite directions of MYO18A and FGFR1 at the centromere, the MYO18A-FGFR1 fusion is not only formed by a simple chromosome balanced translocation but also involves an inversion. It is a complex FGFR1 rearrangement in which t (8, 50) is derived from a three-way translocation and accompanied by a breakpoint of 8p11 (33). The 32nd exon of MYO18A is fused to the 9th exon of FGFR1; however, it is strange that although MYO18A is in 17q11, the breakpoint of the fusion is in q23 (33). There is a reason why the distal regions of 17q23 and 8p11 are translocated to reciprocal derivative chromosomes, and then the 17q chromosome region between 17q11 and 17q23 is reversed, followed by a combination with FGFR1 on 8p11 (33).

The MYO18A-FGFR1 fusion presumably encodes a protein containing 2085 amino acids (33). The activation of the oncogenicity of the FGFR1 fusion may be closely related to the cellular localization of its partner protein. It has been reported that CSF-1(colony stimulating factor-1) could phosphorylate MYO18A, and this may change its cellular localization or affect its binding to the target protein (85). Both known cases were female patients with the CML phenotype. In one case, the peripheral blood and bone marrow showed CML-like characteristics (33). Another case developed from severe urticaria to systemic malignant mast cell disease (MCD) and was diagnosed as CML (27).

2.9 CPSF6-FGFR1/t (8, 12) (p11.2; q15)

Cleavage and polyadenylation specificity factor 6, also called CPSF6, is a member of the CFIm (Cleavage Factor Im complex), which plays a key role in the 3'cleavage and polyadenylation of pre-mRNA (36, 37). It is also involved in the selection of poly-A sites for multiple genes and in the regulation of the 3'UTR (36, 37). The CPSF6-FGFR1 fusion consists of exon 8 of CPSF6 fused to exon 9 of FGFR1 in-frame, and its breakpoint is in intron 8 of CPSF6 (35). The CPSF6-FGFR1 fusion mRNA is presumed to encode a protein with 895 amino acids, approximately 97 kDa, which retains the N-terminal domain of CPSF6 and contains an RRM (RNA recognition motif) (35). Nevertheless, CPSF6 has not been confirmed to have a dimerization domain and, only the RRM domain of CPSF6 is retained in the fusion, which may mediate homodimerization (35). Currently, only one case has been reported. The first episode of this patient involved lymphadenopathy and splenomegaly, and neutrophils in the peripheral blood were increased without eosinophilia, but eosinophilia was present in the bone marrow (35). Monocyte infiltration was found on lymph node biopsy.

2.10 LRRFIP1-FGFR1/t (2, 8) (q37; p11.2)

LRRFIP1 is a leucine-rich repeat flightless-interacting protein 1, and the LRRFIP1 protein is widely expressed in the nucleus and cytoplasm, mainly in the cytoplasm (86). In reality, LRRFIP and its function have not yet been clarified, but it participates in the regulation of the immune response. For instance, the long chain of noncoding RNA upstream of TNF binds to the inhibitor LRRFIP1, which negatively regulates the expression of TNF by forming an inhibition complex (87). LRRFIP1 and ETs-1 (ETs protein-1) interact with the TNF-α-308 site. LRRFIP1 is a TNF-α repressor that does not produce TNF-α in cells and occupies 308 sites, thus reducing TNF-α, yet the combination of ETs and the 308 site produces the opposite effect (88). Exon 9 of the two genes are fused in-frame, and the transcripts of the LRRFIP1 fusion tend to encode highly differentiated proteins that contain 668 amino acids (38). Similarly, the fusion contains the N-terminal coiled-coil domain of LRRFIP1 (38). At present, there is only one known patient, aged 82, who presented with pancytopenia (38). Five years prior, BM presented with MDS (myelodysplastic syndrome) and PB had obvious eosinophilia, and the disease turned into AML after 5 years (38).

2.11 CUX1-FGFR1/t (7, 8) (q22; p11.2)

CUX1 (Cut-like homeobox 1) is in 7q22, which encodes a protein that binds to DNA, and it is one of the members of the homologous domain family (homeobox transcription factors) (39). The homeobox domain and three repeated CUT domains of DNA binding form the homeobox transcription factor and the N-terminal of the protein involves a coiled-coil domain (39). The balanced translocation of chromosomes causes an in-frame fusion between exon 11 of CUX1 and exon 10 of FGFR1 (39). CUX1 is a tumor suppressor that stabilizes the PI3K signaling pathway and decreases the number of normal cells transforming into tumor cells. Previous studies using a Drosophila cancer model and a CUX1 insertion mutation mediated by a mouse transposon found that when CUX1 is deleted, it will abnormally activate the PI3K signaling pathway, thus promoting tumor growth and sensitivity to PI3K/AKT inhibitors (40). CUX1 is frequently inactivated in myeloid tumors. Knockout of the CUX1 gene promotes PI3K signal transduction, which activates quiescent hematopoietic stem cells and causes them

to proliferate, leading to hematopoietic stem cell failure, causing MDS in mice (89). Otherwise, CUX1 participates in DNA repair, and CUX1 deletion leads to abnormal DNA repair, which also seems to be one of the pathogenic mechanisms of myeloid tumors. The PB of patients with CUX1-FGFR1 shows an increase in neutrophils and lymphocytes and a mild increase in monocytes without eosinophils (39). The blast cells in the peripheral blood are mainly pre-T-LBL, and the known patients died after one round of chemotherapy (39).

2.12 TPR-FGFR1/t (1, 8) (q25; p11,2)

TPR is also known as the translocated promoter region, it is in 1q25 and consists of 51 exons. The mammalian TPR encodes a nuclear pore protein including 2349 amino acids of approximately 267 kDa (43). The N-terminal residues include the TprMet domain, NPC relevant domain, and multiple coiled-coil domains, followed by several leucine zipper domains and phosphorylated sequences of various kinases. The C-terminal is composed of a highly acidic spherical domain (90, 91). TPR is located in the nucleoplasmic fibrils of NPCs, which are located on the cytoplasmic surface of the nuclear membrane (91). Comparison between the cDNA sequence and the genomic DNA sequence revealed that the chimeric cDNA of TPR–FGFR1 is the result of an in-frame fusion of exon 22/23 of the TPR gene to exon 13 of FGFR1, and the breakpoint is in intron 22 of TPR and intron 12 of FGFR1 (41, 92). The chimeric protein includes 1426 amino acids of approximately 154 kDa, and it exists in the cytoplasm and contains the TprMet domain, NPC relevant domain, multiple coiled-coil domains, tyrosine kinase domain, and partial transmembrane domain of FGFR1. There are two different fusion ways for other fusion proteins and TPR-FGFR1 fusion protein (Figure 2).The TPR part has one or more dimerization domains (41, 42). In addition, dimerization may be provided by the TPR to guide the fusion to the NPC, and there is a dramatic influence on the regulation of nucleocytoplasmic transport and the molecules entering or leaving the nucleus due to the localization of the fusion protein kinase (90). There are four known cases with TPR-FGFR1. Lymphadenopathy was the common manifestation at onset, and lymphoid biopsy found was T-cell lymphoma. PB and BM had increasing monocytes, with or without eosinophilia, and the bone marrow was MPN (myeloproliferative neoplasms)-like (92). Finally, the clinical phenotype was diagnosed as AMML and AML-M5 (41).

2.13 NUP358/RANBP2-FGFR1/t (2, 8) (q12; p11.2)

NUP358 is at 2q12, and it encodes a polypeptide chain by containing 3224 amino acids of 358 kDa (45), also called

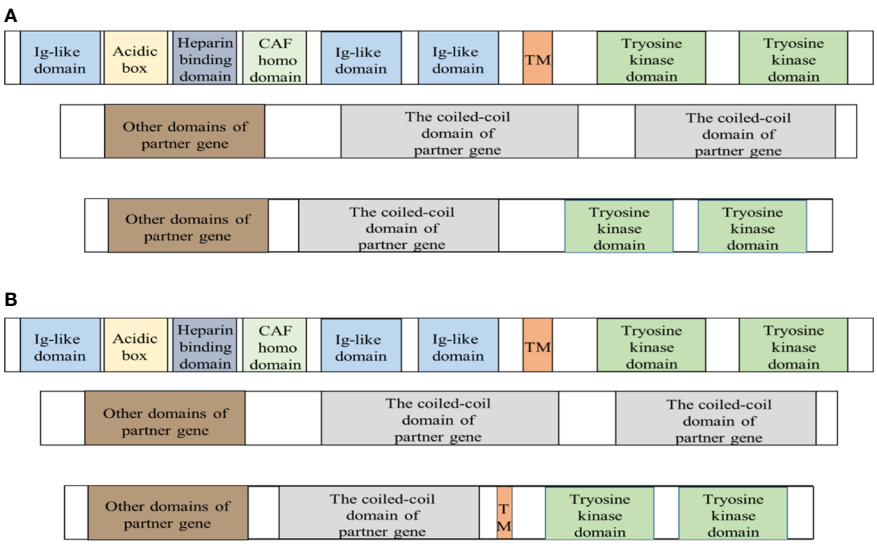

FIGURE 2
Fusion proteins are produced in two different ways. FGFR1 is a transmembrane protein and the extracellular domain of FGFR1 is a signal peptide composed of I immunoglobulin-like domain, acidic box, heparin-binding domain, and cell adhesion factor homologous domain, II immunoglobulin-like domain, and III immunoglobulin-like domain. The intracellular region consists of the tyrosine kinase domain. The proteins transcribed by the fusion gene mainly have two different protein structures. The breakpoint is indicated by the red dashed line. CAF = Cell Adhesion Factor; TM = transmembrane domain. (**A**) The fusion protein only retains the tyrosine kinase domain of FGFR1 and the coiled-coil domain of partner protein. Except for the fusion protein with unclear structure and TPR-FGFR1, others are basically of it. (**B**) TPR-FGFR1 not only contains the tyrosine kinase domain of FGFR1 and a variety of the coiled-coil domain of TPR but also retains the partial transmembrane domain of FGFR1.

RANBP2. NUP358 could approximately be divided into several distinct regions: The N-terminal of NUP358 contains an α-helical region (which has three nonstandard tetratricopeptide repeats (TPRs) with the property of right-handed torsion), followed by four RanGTP binding domains, eight consecutive zinc finger motifs, an E3 ligase domain, and a C-terminal cyclophilin A homology domain (they are presumed to be connected to an unstructured region containing phenylalanine glycine (FG) repeats, forming the docking site of the mobile transport receptor) (45). NUP358, like NUP98, is an important component of the NPC and it plays a major role in transport between the nucleus and cytoplasm. Located on the cytoplasmic surface of NPC, NUP358 has a high-intensity positive charge and it binds to single-stranded RNA and exports RNA as a major part of the nuclear-cytoplasmic transport process (45). With a balanced translocation, exon 20 of NUP358 fused to exon 9 of FGFR1, and the fusion is predicted to encode a chimeric protein containing RANBP2 as the N-terminal, as well as a leucine zipper part that mediates protein-protein interactions (44). The only known patient was a 63-year-old woman with splenomegaly and a little eosinophilia (44). Granulocytes were the main features of peripheral blood and bone marrow biopsy, mainly manifesting as MDS (myelodysplastic syndrome) (44).

2.14 HERVK-FGFR1/t (8, 19) (p11.2; q13)

Human endogenous retrovirus-K (HERVK) is a residue from being infected by a retrovirus and integrating into the human genome, and it can be divided into three families: class I, class II and class III. Among them, HERVK belongs to class IIβ retroviral-like elements and is also called the HERVK superfamily (93). The expression of HERVK family proteins can trigger the proliferation and transformation of normal cells, especially into leukemia cells. Unfortunately, due to the lack of research on its rearrangement with FGFR1, more molecular information cannot be provided. An EMS patient with HERVK-FGFR1 fusion had systemic mastocytosis, and KIT and D815V mutations could be detected (similar to case 8p11 complicated with mastocytosis, MYO18A-FGFR1, and ZNF198-FGFR1). There was a common characteristic that both had erythroid abnormalities: one had polycythemia and poikilocytes in the PB (94), and the other had erythroid maturation disorder (22). Furthermore, two cases also had granulocytosis and abnormal megakaryocytes; one case was diagnosed as AML, and the other was diagnosed as systemic mastocytosis with clonal hematopoietic nonmast cell disease (SM-AHNMD) (22, 94). The fusion protein of HERVK-FGFR1 may the development and maturation of erythroid and megakaryocytes, which requires further research to provide more evidence.

2.15 SQSTM1-FGFR1/t (5, 8) (q35; p11.2)

SQSTMI at 5q35 is composed of 8 exons and encodes a multifunctional protein with 440 amino acids and a molecular weight of 62 kDa, so it was also previously called p62. This protein binds to ubiquitin and regulates the activation of the NF-κβ signaling pathway, which is closely related to oxidative stress and autophagy (46, 47). The structural skeleton of SQSTM1 is the N-terminal of the PB1 (Phox and Bem1p) domain, ZZ type zinc finger domain, LIR (LC3 interaction domain) motif, and the C-terminal of UBA (ubiquitin associated) domain, which mediates the interaction with single or multiple ubiquitins (95, 96). Mutation of the UBA domain is associated with Page's disease of the bone (PDB) (95, 96). The PB1 domain of SQSTM1 mediates the homodimerization of SQSTM1 through the electrostatic force interaction between alkaline and acidic charge clusters at appropriate positions, in which the alkaline charge cluster plays a key role (96). Sequencing of the PCR product revealed that FGFR1 was fused to exon 9 of SQSTM1 at chromosome 5q35, showing that SQSTM1 was juxtaposed with FGFR1 as a result of chromosomal translocation and that the breakpoint was intron 8 of SQSTM1 and intron 8 of FGFR1 (46). The transcript of the fusion presumably encodes a protein containing 718 amino acids, and the N-terminal retains the PB1 domain of SQSTM1, thus enabling cell transformation (46). The activation of the constitutive tyrosine kinase SQSTM1-FGFR1 may be mainly due to its homodimerization mediated by acid and alkaline charge cluster interactions. In this case, neutrophils and monocytes were increased in the peripheral blood, but the bone marrow was dominated by monocytes and megakaryocytes. There was only one case of SQSTM1-FGFR1 without eosinophilia that was diagnosed as AMML (46).

2.16 TFG-FGFR1/t (3, 8) (q12; p11.2)

TFG is at 3q12, also known as the tropomyosin-receptor kinase fused gene or TRK fusion gene, and it was first discovered as a fusion partner of NTRK1 in human papillary thyroid carcinoma (97). The coiled-coil domain of TFG is made up of four leucine motifs with a heptapeptide repeat region, which might be the reason why it is shorter than the typical leucine zipper (98). The human TFG protein sequence is highly homologous to that in pigs and mice, and the structure of human TFG contains a coiled-coil domain of the N-terminal trimer, glycosylation, myristylation and phosphorylation region, and the SH2- and SH3-binding motifs (97). The specificity of TFG dimerization may be due to changes in the Val and Leu residues at core position A (98). The TFG-FGFR1 fusion site is not clear, but the breakpoint is in exon 8 of TFG and exon 10 of FGFR1 (48). The fusion encodes a protein located within the cytoplasm, and the results of *in vitro* coimmunoprecipitation

showed that the fusion could self-bind to form a homodimer (48). The TFG-FGFR1 fusion activates the downstream cascade signaling pathway in cells and regulates the FGFR1 downstream genes, which promotes the upregulation of BCL2, MYC, and KLF4 expression and the downregulation of SPI1 and CSFIR expression, and the fusion regulates downstream signaling pathways, mainly *via* SPI1 (48). Furthermore, the upregulation of MYC by the fusion leads to the phosphorylation of STAT3, STAT5, ERK, FLT3, and JNK, promoting the continuous proliferation of cells (48). The one known patient suffered from skin ecchymosis and splenomegaly, and eosinophilia was not found in PB and BM examination (48). The patient was diagnosed with AML with maturation; later, as with most patients with EMS of other subtypes, he died because of conventional chemotherapy (48).

2.17 HOOK3-FGFR1/inv (8, 8) (p11.23; p11.21)

HOOK3-FGFR1 positivity with EMS is the latest FGFR1 rearrangement discovered, more importantly, the reason for its formation is different from that of its other partner genes. It is an inversion forming a ring chromosome 8 but the existence of the ring chromosome is related to genomic instability (49). The breakpoints of the fusion are in exon 11 of HOOK3 and exon 10 of FGFR1, and it is speculated that the chimeric protein contains 768 amino acids (49). As expected, the N-terminus of the fusion contains a partial coiled-coil domain encoded by exons 31-11 of HOOK3, and the C-terminus contains a complete tyrosine kinase domain encoded by exons 10-18 of FGFR1, excluding the transmembrane domain (49). According to recent studies, the activation of the NF-κβ pathway is an important factor in the treatment of multiple myeloma and is associated with low sensitivity to bortezomib and ixazomib (99). Only one case of HOOK3-FGFR1 has been reported. The patient was admitted to the hospital with nonspecific clinical symptoms, the PB showed leukocytosis and thrombocytopenia, and BM revealed myelodysplasia and B-lymphoid and granulocytic infiltrative hyperplasia without eosinophilia (49). Ultimately, the patient was clinically diagnosed with MDS with abnormal monoclonal B-cell proliferation (49).

3 Targeted therapy with tyrosine kinase inhibitors

To date, the prognosis of EMS is very unfavorable. Few cases have achieved remission, and most of them were treated by allogeneic hematopoietic stem cell transplantation (HSCT). Notwithstanding that a variety of traditional chemotherapy regimens were also involved in the treatment, the outcomes were not satisfactory, and serious side effects and drug resistance resulted in undesirable events for the patients. Additionally, because of the scarcity of stem cell donors, difficulties in matching, infection, economic costs, and a long waiting time, the majority would die of disease progression before HSCT. It was exciting that in recent years, the first TKI, imatinib, was developed and applied to the treatment of CML (Philadelphia chromosome-positive), and an excellent effect was achieved. Then, a variety of TKIs was developed and tested in clinical trials, which brought hope to patients. FGFR1 rearrangement leads to the constitutive activation of a tyrosine kinase, which triggers cascade signal transduction in cells and causes abnormal proliferation, survival, differentiation, and antiapoptotic effects. Therefore, inhibitors targeting tyrosine kinase have the potential for the treatment of EMS. There are the number and the reported response for chemotherapy or TKIs (Table 2).

3.1 Mechanism

There are various types of tyrosine kinase inhibitors; in general, their mechanisms are mainly the following:

1. Small molecule TKIs target the ATP-binding cleft in growth factor receptor kinases (100);

2. Peptide inhibitors of pseudosubstrates that bind to catalytic domain peptide/protein substrate sites (101);

3. Monoclonal antibodies against receptor tyrosine kinase and ligand traps (101, 102).

Small molecule inhibitors account for the majority of TKIs, which are commonly used to treat lung cancer, breast cancer, and other tumors; in particular, the efficacy of inhibitors targeting VFGFRs has been quite apparent during treatment. Small molecule inhibitors are mostly ATP-competitive inhibitors that compete with ATP and combine with the TK receptor kinase cleft to inhibit kinase activity and its downstream intracellular signal cascade reactions, thus inhibiting the proliferation and transformation potential of oncogenes (100). All protein kinases share the same ATP binding site, and the binding of ATP to a kinase is due to the hydrogen bond between the adenine ring of ATP and the ATP binding cleft of the kinase. The inhibitors target the ATP binding site of the kinase and vicinity and the selectivity of inhibitors is controlled by simulating different parts of the ATP structure (103).

ATP small molecule inhibitors are divided into three types, I, II, and III. Type I inhibitors recognize the active conformation of kinases and are direct ATP competitive inhibitors. Inhibitors form one to three hydrogen bonds with the binding sites of kinases, simulating the formation of hydrogen bonds between normal ATP and binding sites, competing with ATP for ATP binding sites (104). Conversely, the type II inhibitor work by binding to the inactive conformation of the kinase, and it is an ATP indirect competition inhibitor that allosterically regulates the kinase activity and occupies the hydrophobic sac adjacent to the ATP binding site, and then indirectly competes with ATP (105).

Different from the first two, type III inhibitors are covalent inhibitors that covalently bind to a cysteine on particular parts of the kinase. The sulfur atom in the cysteine residue is rich in electrons and reacts with the electrophilic group of the inhibitor, sharing electrons and irreversibly binding, blocking the kinase from binding to ATP (106). Covalent inhibitor binding can occur at any variable position of cysteine residues in the kinase domain, so that position is not fixed (103). FGFs/FGFR1 signal pathway and the mechanisms of FGFR1 inhibitors (Figure 3).

It has been pointed out in the available literature that TKIs used in EMS treatment are small molecule inhibitors of ATP-binding clefts, but they are classified as nonselective or selective depending on their targeting selectivity to FGFR. The former mentions targeting a variety of growth factor receptors, including FGFRs, which are multitargeted; the latter refers to targeting only FGFRs or targeting a variety of growth factors with the highest affinity for FGFRs.

3.2 Nonselective tyrosine kinase inhibitors

PTKs (protein tyrosine kinases) play an important role in cell regulation, such as mitosis, development and differentiation, tumorigenesis, angiogenesis, cell survival and apoptosis, and cell cycle control. Therefore, abnormal PTKs interfere with normal physiological functions, thus promoting the occurrence and development of diseases and even tumors (100, 107). FGFRs, VFGFRs, and PDGFRs are members of the PTK superfamily, and their protein structures are similar to those containing the extracellular ligand-binding domain, the transmembrane domain, and the intracellular tyrosine kinase domain. Their tyrosine kinase domains are highly similar, especially their ATP binding sites (103). Therefore, small molecule ATP inhibitors target a variety of growth factors with similar kinase domains and are also known as multitarget TK inhibitors, nonselective.

3.2.1 Imatinib

Imatinib was approved by the FDA to treat Philadelphia chromosome-positive CML in 2001, and encouraging data were obtained in subsequent clinical trials (108). It is a competitive inhibitor of ATP type II and is also approved for treating some solid tumors (109, 110). Although the application of imatinib can reduce the blood cell count, no reliable data have been obtained *in vitro*, and its actual therapeutic effect on EMS is unclear. One case presented with 8p11 and ZMYM2-FGFR1 fusion positivity, and the patient then began taking imatinib on the 15th day after diagnosis (111). Although the leucocyte count

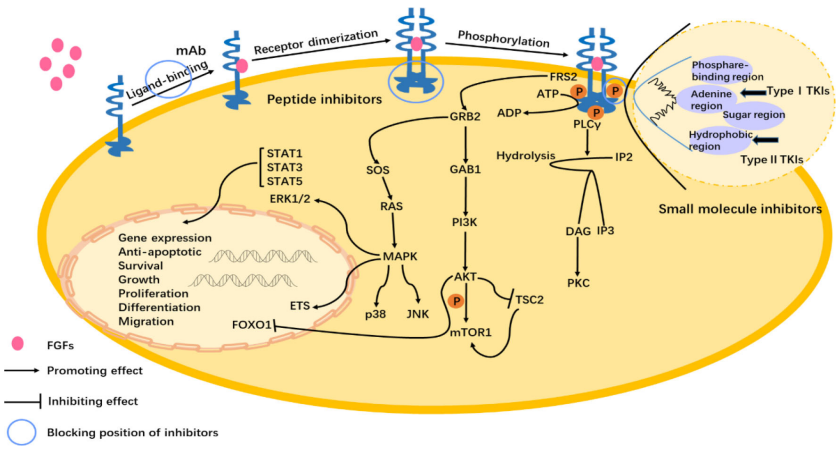

FIGURE 3
FGFs/FGFR1 signal pathway and the mechanisms of FGFR1 inhibitors. FGFs binding to the FGFR1 induces dimerization and the subsequent phosphorylation of the intracellular tyrosine kinase domain. Activation of downstream signaling occurs via FGFR substrate 2α (FRS2α), which is constitutively associated with the juxtamembrane region of FGFR. Phosphorylated FRS2 recruits the adaptor protein, growth factor receptor-bound protein 2 (GRB2), which then recruits the guanine nucleotide exchange factor SOS. The recruited SOS activates the RAS GTPase, which then activates the MAPK pathway. MAPK activates members of the ETS transcription factor family, ERK1/2, p38, and Jun N-terminal kinase pathways (JNK). The recruited GRB2 as well as recruits the adaptor protein GAB1, which then activates the PI3K, and after that phosphorylates the AKT. Next, then AKT has various activities including activation of the mTOR complex 1 by inhibition of the cytosolic tuberous sclerosis complex 2 (TSC2) and phosphorylation. And AKT pathway inhibits the activity of the forkhead box class transcription factor (FOXO1) bringing about it exiting the nucleus. Phospholipase C (PLC) binds to a phosphotyrosine and hydrolyzes phosphatidylinositol 4,5-bisphosphate (IP2) to phosphatidylinositol 3,4,5-triphosphate (IP3) and diacylglycerol (DAG), which then activates protein kinase C (PKC). The target gene expression is regulated by the activity of the signal transducer and activator of transcription STAT1 (signal transducer and activator of transcription), STAT3, and STAT5. The abnormal activation of the FGFs/FGFR pathway has an influence on physiological activity, such as anti-apoptotic, survival, and growth of cells (1–3). The mechanisms have been shown in the figure of small molecule inhibitors, peptide inhibitors, and monoclonal antibodies.

decreased briefly and returned to normal, the patient unfortunately died due to disease progression (111). Disappointingly, the results obtained with imatinib *in vitro* were unsatisfactory. Imatinib could not inhibit FGFR1 rearrangement well in cell lines compared to other small molecule inhibitors (112, 113). Imatinib might have a therapeutic effect on the chronic phase of the CML phenotype in EMS, for which the main treatment is to reduce the leucocyte count in the patients' PB, but it may have little benefit for the treatment of EMS in the acute phase.

3.2.2 Ponatinib

Ponatinib (AP24534) is an oral multitarget inhibitor that mainly targets ABL, FGFR1, FLT3, KIT, and PDGFRA (114). The application of ponatinib in EMS has also been supported by many researchers and clinicians. Ponatinib showed agreeable direction on a variety of EMS cell lines *in vitro*, mainly by inhibiting the downstream signaling pathways of ERK and STAT5 and inducing cell apoptosis (115). A 47-year-old male patient with BCR-FGFR1 positivity was planned to receive single-agent ponatinib due to the ineffectiveness of MEC chemotherapy at the beginning (57). The patient had good tolerance to the treatment and achieved satisfactory results, with the resolution of his swollen lymph nodes and neck pain, and the morphology of his BM was completely restored, providing an opportunity to implement hematopoietic stem cell transplantation (57). Attention to the NF-κβ pathway in FGFR1 rearrangement and further enrichment may be one of the reasons for the poor response to ponatinib (49). There is a presumption that ponatinib treatment can achieve more survival opportunities for patients who are resistant to EMS traditional chemotherapy and imatinib with lymphadenopathy, establish clinical remission, and improve the quality of life of the patients.

3.2.3 Midostaurin

Midostaurin is a small molecule tyrosine kinase inhibitor that is mostly involved in the treatment of AML and advanced systemic mastocytosis. Midostaurin demonstrated favorable results in ZNF198-FGFR1 mouse models and cells. The Midostaurin-treated group survived significantly longer and the spleen weight, and white blood cell count was significantly lower than the placebo-treated group (116). Based on the above data, a patient with ZNF198-FGFR1 enrolled in a randomized phase II clinical trial and obtained a nice treatment response. His lymphadenopathy and splenomegaly significantly subsided, and the patient achieved clinical stability for 6 months (116). This evidence suggests that Midostaurin can be effective for patients with a progressive myeloproliferative disorder with organ enlargement.

3.2.4 Dovitinib

Dovitinib (TKI258) is a multitarget receptor tyrosine kinase inhibitor targeting FGFRs, VEGFR, PDGFR, FLT3, and KIT. TKI258 inhibited the phosphorylation levels of ERK and STAT5 in cells transformed by the fusion in a dose-dependent manner (117). There was a significant difference in the efficacy of dovitinib between the CFU-GM (CFU-granulocyte-macrophage) and BFU-Es (erythrocyte burst-forming unit). CFU-GM was not inhibited by dovitinib, but BFU-Es were strongly inhibited at all below 100nM (117). In addition, the *in vitro* data of dovitinib compared with other TKIs are not ideal, and larger doses are required to achieve the same degree of inhibitory effect as other TKIs (118). The difference in efficacy between dovitinib and other TKIs may be related to the non-selective expression level of this kinase and the inhibition of the number of other potential kinases and downstream biological efficacy. This suggests that colony FISH analysis is very important before the treatment of actual EMS patients, which could determine whether dovitinib could reduce the number of FGFR1 rearrangement subtype cells.

3.2.5 Dasatinib

Dasatinib is an oral small molecule inhibitor that inhibits nonmutated BCR-ABL and most known BCR-ABL mutants; its targeting includes PDGFR, cKIT, SFK (SRC family kinase), FGFR1, and EGFR (114). Several clinical examples suggest that dasatinib is more suitable for EMS patients with cardiovascular disease than ponatinib, and the patients benefited for more than 9 months and significantly improved the patient's quality of life (118, 119). Perhaps dasatinib might be more suitable for the treatment of elderly and frail EMS patients with cardiovascular disease, and it improves the PB, prolongs the survival time of patients, and provides another treatment option in addition to hematopoietic stem cell transplantation.

3.3 Selective tyrosine kinase inhibitors

Even though nonselective TKIs have shown their possible efficacy in EMS, their side effects should not be ignored due to their multiple targets. To enhance the efficacy and reduce the side effects, it is necessary to selectively block the FGFR tyrosine kinase. Recently, with the development of selective FGFR inhibitors such as pemigatinib and infigratinib, phase I/II clinical trials have been carried out for treating advanced cholangiocarcinoma, and they may also have potential therapeutic effects for EMS.

3.3.1 Pemigatinib

Pemigatinib (INCB054828) is a reversible ATP competitive FGFR inhibitor, and the US FDA has accelerated the approval of pemigatinib for the treatment of previously treated and unresectable locally advanced or metastatic cholangiocarcinoma with FGFR2 fusion or other rearrangements (120). In August 2019, pemigatinib was recognized as an orphan drug in the US to treat myeloid/

lymphoid tumors with eosinophilia, PDGFRA, PDGFRB, FGFR1 rearrangement, or PCM1-JAK2. In enzymatic assays with recombinant human FGFR kinases, the $IC_{50\ values}$ of INCB054828 against FGFR1, 2, and 3 were 0.4, 0.5, and 1.0 nM, respectively, but the inhibitory effect on FGFR4 was weak (121). It is speculated that its high affinity and selectivity for FGFR1, FGFR2, and FGFR3 are due to its filling with complementary hydrophobic vesicles near the FGFR gatekeeper region (120).

Pemigatinib has been studied in several EMS registered clinical trials and *in vitro* trials, with good tolerance and excellent efficacy. *In vitro*, pemigatinib could effectively inhibit FGFR phosphorylation, pERK, and pSTAT5 levels, and restore FGFR phosphorylation to basal levels (121, 122). In addition, humanized mice subcutaneously transplanted with KG1 could significantly inhibit the growth of tumors by oral administration of pemigatinib, and phase II clinical trials have been carried out in FGFR1 rearranged myeloid/lymphoid tumors (FIGHT, NCT03011372) (121). Thirty-four MLN patients were included in the trial, and the results showed that among 31 patients with BM and/or involvement of extramedullary disease (EMD), the CR (complete response) rate and CRC (clinical research coordinator) assessment were 64.5%, and 77.4%, respectively (123). Among the 33 patients evaluable for CyR (cytogenetic responses), the CCyR (complete cytogenetic response) rates were 72.7% and 75.8, respectively, but the median CR duration had not been reached (123). Recently, these results suggest that pemigatinib may offer a long-term treatment option for EMS ineligible for HSCT or may facilitate bridging to HSCT in eligible patients.

3.3.2 Infigratinib

Infigratinib (NVP-BGJ398) is a selective and oral small molecule FGFR inhibitor. Different from non-selective TKIs, BGJ398 could significantly inhibit the expression of the FOP2-FGFR1 fusion protein, apoptosis-related protein BCL-2, and phosphorylation levels of AKT and S6K1, and upregulate activated caspase-3 (117). Apoptosis is a complex process regulated by many genes, and the regulation of apoptosis-related proteins by BGJ398 may be one of the mechanisms by which it exerts its pharmacological actions (117). What's more, Other studies have confirmed that both ponatinib and infigratinib can inhibit the proliferation of TPR-FGFR1 fusion protein, but the effect of infigratinib is stronger than ponatinib, and infigratinib induces the death of transformed cells at a very low concentration (42).

3.3.3 Other potential inhibitors

In addition to pemigatinib and infigratinib, the following FGFR inhibitors also have the potential to treat EMS. Futibatinib is an oral, potent, selective, covalently irreversible small molecule inhibitor that targets the P-loop ATP binding pocket of the tyrosine kinase domain in FGFR1-4 *in vivo* (124–126). Futibatinib effectively inhibits FGFR1-4 with an IC_{50} of a single digit of nanomolesper liter (124–126). Erdafitinib is a new pan-FGFR small molecule inhibitor. It has recently been approved for patients with advanced urothelial cancer with specific FGFR gene changes (127). At present, there is no corresponding clinical trial or *in vitro* experiment to confirm its efficacy in rare diseases such as EMS. It is hoped that additional trials will be conducted in the future to enrich the treatment options, promote the development of FGFR small molecule inhibitors for EMS treatment and prolong the overall survival rate of these patients without requiring hematopoietic stem cell transplantation.

4 Conclusion

From 1998 to 2022, 17 genotypes of EMS phenotypes with FGFR1 rearrangement were reported. In recent years, because of the progress of molecular detection technology and the expansion of the detection range, the rearrangement of FGFR1 has received attention, and new mutations have been detected. Moreover, some of these fusions are not common and are only reported in a few cases, which limits the possibility of making conclusions about these new fusions. Although FGFR1 has different rearrangements; and its fusion proteins have different structures, the main functional abnormality is always caused by the FGFR1 tyrosine kinase domain in the fusion. In the future, it could be possible to conduct in-depth research on these rare fusions, including their pathological and cellular biochemical characteristics, to provide a more extensive research base to support better treatment, such as a combination of traditional chemotherapy and targeted drugs, a combination of targeted drugs and hematopoietic stem cell transplantation, or the application of targeted drugs alone. In addition, many researchers believe that 8p11 is a leukemia stem cell cancer with the potential to differentiate into various lineages, which provides strong evidence for which hematopoietic stem cells from healthy humans are the source of leukemia and the clinical relevance of the identification of pre-leukemia HSCs (128). Because of the late diagnosis and rapid deterioration of the condition, most patients with EMS have a poor prognosis and even face recurrence after transplantation, and the treatment of these patients is challenging. More effective experiments *in vitro* and clinical trials registered in centers or multiple institutions are required in the future, but they might have to be limited to rare cases.

In summary, EMS is very rare, and its clinical features are not yet very clear. Such diseases are often resistant to traditional chemotherapy, and the application of TKIs is promising for EMS treatment. In particular, attention should be given to clinical trials of FGFR inhibitors in the future.

Author contributions

QL: Conceptualization, supervision. TL: Data curation, writing- original draft preparation. GZ: Data curation,

visualization. HL: Data curation, investigation. XZ: Investigation, methodology. QL: Writing- review and editing. All authors have read and agreed to the published version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Dysregulation of FBW7 in malignant lymphoproliferative disorders

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The ubiquitin-proteasome system (UPS) is involved in various aspects of cell processes, including cell proliferation, differentiation, and cell cycle progression. F-box and WD repeat domain-containing protein 7 (FBW7), as a key component of UPS proteins and a critical tumor suppressor in human cancers, controls proteasome-mediated degradation by ubiquitinating oncoproteins such as c-Myc, Mcl-1, cyclin E, and Notch. It also plays a role in the development of various cancers, including solid and hematological malignancies, such as T-cell acute lymphoblastic leukemia, diffuse large B-cell lymphoma, and multiple myeloma. This comprehensive review emphasizes the functions, substrates, and expression of FBW7 in malignant lymphoproliferative disorders.

KEYWORDS

FBW7, lymphoproliferative disorders, ubiquitin, Notch, c-Myc

Introduction

Malignant lymphoproliferative disorders are a heterogeneous group of disorders characterized by the abnormal proliferation of lymphocytes, ranging from indolent to highly aggressive neoplasms, including chronic lymphocytic leukemia (CLL), diffuse large B-cell lymphoma (DLBCL), multiple myeloma (MM), and acute lymphoblastic leukemia (ALL). Although targeted drugs have significantly improved the prognosis of patients with malignant lymphoproliferative disorders, a subset of patients have a remarkably poor outcome. Therefore, exploring the underlying mechanisms of malignant lymphoproliferative disorders is critical (1).

F-box and WD repeat domain-containing protein 7 (FBW7), also known as FBXW7, hCDC4, and AGO, is located at chromosome 4q31, which is a genomic region deleted in more than 30% of cancers (2). Oberg and colleagues determined that FBW7's role in substrate recognition is a key component of the Skp1–Cul1–F–box (SCF)–type E3 ubiquitin ligases (3). FBW7 has three isoforms: FBW7α, FBW7β, and FBW7γ; all of them contain conserved interaction domains of the F-box and WD repeats in the C-terminus. However, their 5'-UTR and N-terminal coding regions are different, forming the

specificity of the expression and function of these three protein isoforms. These three isoforms also have distinct cellular localizations. FBW7α is localized in the nucleoplasm, FBW7β is cytoplasmic, and FBW7γ is nucleolar. FBW7α is regulated by protein kinase C and is mainly expressed in mice. FBW7β is beneficial to fight oxidative stress in cells and more expressed in brain tissue. FBW7γ exclusively expressed in skeletal muscle (4, 5).

Substrates of FBW7 in cancer

FBW7 is an important tumor suppressor in cancer development, and *FBW7* mutations have been detected in a wide range of human malignancies, including lung cancer, ovarian cancer, colorectal cancer, T acute lymphoblastic leukemia (T-ALL), and MM. The percentage of *FBW7* mutations varies across cancers, being up to 20% in T-ALL (2). FBW7 recognizes c-Myc, Mcl-1, and other oncogenic proteins for ubiquitination and subsequent proteasomal degradation, which is closely related to the proliferation, apoptosis, invasion, and metastasis of cancer cells (6–10).

The Myc protein is a transcription factor that has crucial roles in regulating gene expression for cell proliferation, metastasis, and metabolism, and deregulation of the proto-oncogene c-Myc leads to the development of many human cancers, including hematological malignancy such as DLBCL and solid tumors such as gastric carcinoma. The c-Myc protein is ubiquitylated and degraded by the ubiquitin–proteasome system (11–13). FBW7 overexpression results in decreased c-Myc levels, inhibited cell proliferation, and induced apoptosis, while *FBW7* mutations can cause c-Myc accumulation, enhanced cell proliferation, and reduced apoptosis. Thus, FBW7 may mediate apoptosis and growth arrest in leukemia-initiating cells through the ubiquitin–proteasome system and degradation of c-Myc (14).

Another oncoprotein, cyclin E, is a crucial component of the cell cycle and regulates the G1 to S-phase transition and promotes DNA replication. However, cyclin E is frequently dysregulated in cancers, and excess cyclin E activity impairs S-phase progression and causes genomic instability. Significantly, cancers with *FBW7* mutations have increased levels of cyclin E. Furthermore, protein phosphatases, such as PP2A-B56, dephosphorylate Ser384 during the interphase of the cell cycle, resulting in a decrease in FBW7 levels and increase in cyclin E levels (15).

Moreover, the Notch pathway regulates numerous cellular functions, including differentiation, proliferation, and apoptosis. FBW7 plays a crucial role in regulating the Notch pathway, including Notch upstream regulators and downstream substrates. The oncoprotein Notch, which is also as a target for FBW7-mediated ubiquitylation, participates in the development of many human cancers, such as T-ALL and DLBCL (16, 17). Moreover, FBW7 can inhibit metastasis and invasion of CLL cells by regulating signaling pathways, such as Notch and its downstream target molecules (18).

In addition, Mcl-1, as the pro-survival Bcl-2 family member, inhibits apoptosis by blocking cell death in numerous cancers. For example, loss of FBW7 in T-ALL cells increases Mcl-1 expression and promotes chemoresistance (19). The transcription factors nuclear factor kappa B (NF-κB) and c-Jun are also important in the development of lymphoproliferative disorders. NF–κB2/p100 can interact with FBW7 thereby promoting its degradation in a GSK3β phosphorylation-dependent manner (20). In addition to these proteins, FBW7 can also ubiquitylate several essential proteins, such as BRAF (Table 1).

Regulation of FBW7

Mutations or deletions of *FBW7* have been implicated poor prognosis, indicating that aberrant regulation of FBW7 is one of the factors for cancer progression. Some of the regulators of FBW7 include p53, Numb, microRNAs (miRNAs), and CCAAT/enhancer binding protein-δ (C/EBP δ) (35). P53 is a well-known tumor suppressor protein that conserves genome stability after DNA damage. Moreover, dysregulation of p53 is

TABLE 1 FBW7 substrates and major biological functions in lymphoproliferative disorders.

Gene	Phosphorylationsites	Kinases	Cancers	Biological functions of substrates	References
c-Myc	T58, S62	GSK3	T-ALL, ATL, DLBCL, CLL, MM	Tumor proliferation, metastasis, glycolysis	(11, 12, 18, 21–23)
Notch1	T2512/E2516	CDK8	T-ALL, ATL, DLBCL, CLL,	Notch pathway, proliferation, migration and invasion	(17, 18, 24, 25)
cyclin E	T380/S384, T62/E66	CDK2, GSK3	T-ALL, MM	Cell cycle	(26, 27)
c-Jun	T239/S242	GSK3	MM	Cell proliferation	(23, 28)
Mcl-1	S159/T163 S121	GSK3	T-ALL, DLBCL, PEL, MM	Inhibit apoptosis	(19, 22, 29–31)
NF-κB	ND	GSK3	T-ALL, MM	Cell proliferation, inhibit apoptosis	(20, 32, 33)
BRAF	ND	ND	ATL	BET inhibitors resistance	(34)

ATL, adult T cell leukemia/lymphoma; T-ALL, T cell acute lymphoblastic leukemia; DLBCL, diffuse large B-cell lymphoma; CLL, chronic lymphocytic leukemia; MM, multiple myeloma; PEL, primary effusion lymphoma.

associated with the development of various cancers. Inactivation of FBW7 could lead to premature loss of hematopoietic stem cells and impaired regulation of cell apoptosis by p53 (36). Another transcription factor, C/EBP δ, decreases Notch intracellular domain (NICD) degradation by ubiquitination through inhibition of FBW7 expression and promotes Notch1 mRNA expression in cancer cells (37). Furthermore, miRNAs, such as miR-223, can regulate T-ALL proliferation by reducing FBW7 expression and contributing to drug resistance (32). In addition, miR-27 and miR-214 could also decrease FBW7 expression to regulate cell proliferation in other lymphoproliferative disorders. However, the exact function of FBW7 in malignant lymphoproliferative disorders remains unclear. Understanding the regulation network surrounding FBW7 is crucial for providing insights into the mechanisms of FBW7-mediated malignant lymphoproliferative disorders. In this review, we focus on the role of FBW7 in malignant lymphoproliferative disorders to identify a novel target for future therapies.

Proliferation of T lymphocytes

T lymphoblastic lymphoma/acute lymphoblastic leukemia

T lymphoblastic lymphoma/acute lymphoblastic leukemia (T-LBL/ALL) is a highly aggressive hematologic malignancy caused by the malignant transformation of T−cell progenitors. T−ALL accounts for 15% and 25% of the total number of childhood and adult cases of ALL, respectively. Adults with T−ALL have poor long-term survival. Approximately 50% of them develop recurrence within 1 year, and the remission rate of second-line chemotherapy is only 30%–45%, which ultimately develops refractory leukemia (38, 39). The 5-year survival rate is 10%; however, in patients with recurrent or refractory T-ALL, overall survival (OS) is less than 6 months (40). Therefore, understanding the underlying mechanisms of T−ALL development is critical.

Notch1 is the most common oncogene for T-ALL, and mutations in *Notch1* have been reported in more than 60% of T-ALL. Besides, the mutation rate of *Notch1 in* adults is higher than children. Mutations of *Notch1* occur mainly in the heterodimeric domain (HD) region and the proline, glutamine, serine, and threonine domain (PEST). Mutations in the HD domain can result in Notch1 activation by reducing the interaction of the Notch1 subunit. The PEST region regulates protein turnover by targeting proteins to ubiquitin–proteasome complex for subsequent degradation (16, 41). The cross-talk between Notch and NF-κB pathways is vital in T-ALL development, indicating NF-κB signaling is one of the major mediators of Notch-induced oncogenic transformation (42). The activation of the Notch pathway can upregulate NF-κB activity, resulting in abnormal cell proliferation and apoptosis inhibition (42). In addition, the occurrence of T-ALL is also related to the abnormal expression of noncoding regulatory elements and miRNAs, such as miR-19, miR-155, and miR-233 (43).

As early as more than 10 years ago, studies have shown that inactivation of FBW7 could lead to premature loss of hematopoietic stem cells caused by an active cell cycle and impaired regulation of cell apoptosis by p53 (36). FBW7 can regulate the cell cycle in a differentiation-dependent manner and loss leads to the accumulation of c-Myc, which results in the excessive proliferation of immature T cells, ultimately leading to the development of T-ALL or lymphoma (44). The mutation rate of *FBW7* in T-ALL is between 8%–20% (45–48). And the deletion or mutation in *FBW7* increases the protein level of c−Myc, which in turn increases the number of leukemia initiating cells. Furthermore, inhibition of the expression of c−Myc by small molecule inhibitors can inhibit the proliferation of mouse and human T-ALL cells, suggesting that *c−Myc* may be the proto-oncogene that drives T-ALL and is regulated by FBW7 (14). Besides, in T-ALL, *FBW7* mutation usually cooccurs with *Notch1* mutation, which has been widely proven to be an oncogene in T-ALL (49). Mutation of *FBW7* can reduce the cellular level of NICD protein degradation, which not only leads to abnormal activation of the Notch pathway but also reduces ubiquitination degradation of proto-oncogenes such as cyclin E and c-Myc (50). Kumar et al. demonstrated that Notch and NF-κB signaling can increase miR-223 gene expression, which in turn downregulates the expression of the onco-suppressor FBW7, known to negatively regulate Notch signaling, thus suggesting that the Notch/miR-223/FBW7 axis may reinforce Notch signaling effect in T-ALL. Furthermore, miR-223 may be involved in the mechanism of g-secretase inhibitor (GSI) treatment of patients with T-ALL; inhibiting miR-223 expression may reduce GSIs resistance (32). Moreover, *FBW7* mutations, region of R465C and R479Q, can also resist GSIs treatment by stabilizing NICD and its principle downstream target c-Myc, which may be the mechanism of GSIs resistance in patients with T-ALL (24). Moharram et al. showed the R465C mutation in *FBW7* played an important role in T-ALL progression when combined with Notch1 mutation and the R505C mutation in *FBW7* impaired Notch1 binding (46). In addition, approximately 60% of patients with T-ALL express the oncogenic transcription factor T-cell acute lymphocytic leukemia 1 (TAL1). Studies have shown that TAL1 can upregulate miR-223 expression by binding to the miR-223 promoter, reducing FBW7 expression, and increasing the expression of downstream substrates c-Myc, Notch1, and cyclin E, which ultimately induce cell proliferation (21, 26). Moreover, other studies also have shown that miR-223 can regulate T-ALL proliferation by reduce FBW7 expression; this indicates that miR-223 could regulate FBW7 activation in T-ALL (51, 52). Mcl-1 is an antiapoptotic protein of the BCL-2 family, which promotes tumor progression by inhibiting apoptosis (53). In T-ALL cell lines, the deletion of FBW7 increases in the expression level of Mcl-1 in a GSK3 phosphorylation-dependent manner and promotes T-ALL progression. Mcl-1 also upregulates resistance in the treatment of

BCL-2 inhibitor (ABT737). However, when the FBW7 function was restored or Mcl-1 lost, the sensitivity to ABT737 was restored (19) (Figure 1).

However, other studies have shown that patients with *Notch1/FBW7* mutations have a better prognosis, with 5-year event-free survival of 95.5% and 5-year OS 100% (54). FBW7 deletion can upregulate glucocorticoid receptors in primary T-ALL cells, thereby enhancing their sensitivity to glucocorticoids and improving prognosis (55). In addition, *FBW7* mutations do not affect the prognosis of children with T-ALL. Maybe other oncogenes are involved or affected by treatment regimens. Therefore, more studies are still required to confirm the mechanisms related to the role of FBW7 in T-ALL.

Adult T-cell leukemia/lymphoma

Adult T-cell leukemia/lymphoma (ATL) is a rare malignant T-cell monoclonal proliferative disease caused by human T-cell leukemia virus type 1 (HTLV1). Southwestern Japan is one of the most endemic areas for malignancy, along with the Caribbean basin, Central and South America. The Japan Clinical Oncology Group-Lymphoma Study Group proposed four clinical subtypes of ATL: acute, lymphoma, chronic, and smoldering. Nonetheless, the course of ATL is highly variable. Patients with acute, lymphoma, and chronic type with unfavorable prognostic factors, defined by levels of blood urea nitrogen or lactate dehydrogenase at higher than normal or having albumin levels lower than normal, are categorized as having aggressive clinical course; however, chronic type without unfavorable prognostic factors and smoldering type are indolent ATL (56, 57). *FBW7* is an important tumor suppressor, but some *FBW7* mutations can function as an oncogene. Yeh et al. showed that 25% (8/32) of patients with ATL had mutations in the *FBW7* WD40 domain, region of D510E and D527G, which had ability to target and degrade cyclin E, Mcl-1, and c-Myc. Mutation in *FBW7* failed to degrade NICD, activating the Notch signaling pathway and ultimately promoting ATL cell proliferation. However, FBW7 expression in wild-type, proliferation of cells was inhibited (25). In addition, c-Myc is a prognostic factor for patients with ATL, and FBW7 downregulation can lead to c-Myc accumulation and ATL cell proliferation (12). Patients with low FBW7 levels and high c-Myc levels experience poor prognosis, with 3-year OS less than 50% (12). Moreover, FBW7 mutations in ATL cells inhibit the degradation of BRAF and provide resistance to BET inhibitors through the RAF-MEK-ERK pathway, which also indicated ERK inhibitor may be the new therapeutic target in ATL (34). Therefore, FBW7 may be a potential target for the treatment of ATL (Figure 1).

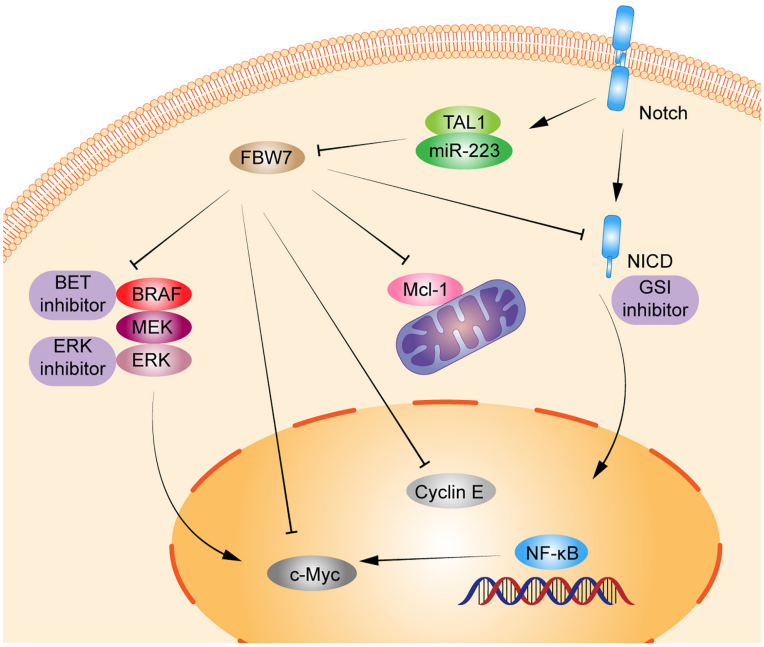

FIGURE 1
Schematic diagram of FBW7-mediated oncogene and signaling pathway in T lymphoproliferative malignancies. 1) Dysregulation of FBW7 can reduce cellular levels of Notch intracellular Domain (NICD) degradation, which leads to abnormal activation of Notch pathway, NF-κB pathway, and affect the expression of its downstream substrates c-Myc, cyclin E. 2) T-cell acute lymphocytic leukemia 1 (TAL1) can upregulate miR-223 expression, thus reducing the expression of FBW7 and increasing the expression of downstream substrates c-Myc, Notch1, cyclin E, and Mcl-1 to induce cell proliferation. 3) FBW7 mutations activate the RAF-MEK-ERK pathway, inhibiting BRAF degradation and providing resistance to BET inhibitors.

Proliferation of mature B lymphocytes

Diffuse large B-cell lymphoma

DLBCL is the most common type of non-Hodgkin lymphoma in adults, accounting for 30%–40% of new diagnoses. DLBCL is classified as germinal center B-cell (GCB) subtype and activated B-cell (ABC) subtype by genetic profiling, and patients with the ABC subtype have significantly poorer outcomes (58). Approximately 60% of patients with DLBCL can cure with regimens such as rituximab, cyclophosphamide, adriamycin, vincristine, and prednisone (R-CHOP). However, 20%–50% of patients will be refractory or will relapse after achieving complete response (59). Therefore, ongoing efforts in the understanding of the mechanism of DLBCL have identified subsets of patients with poor prognosis for immunochemotherapy.

FBW7 expression in patients with GCB-DLBCL was higher than in the ABC-DLBCL subtype, which had an inferior prognosis. The median survival time of patients in DLBCL with low FBW7 expression was 44 months, which was significantly shorter than that of those with high FBW7 expression (81 months) (22). FBW7 overexpression decreased cell viability and increased apoptosis rates in ABC-DLBCL cell lines. In terms of mechanism, stability of Stat3 and phospho-Stat3^{Tyr705} which are associated with poor survival in ABC-DLBCL, were reduced following FBW7 overexpression in ABC-DLBCL cell lines. The downstream antiapoptotic target genes of activated Stat3, including c-Myc, Mcl-1, Bcl-2, and Bcl-xl, showed decreased mRNA expression following exogenous FBW7 overexpression. Similarly, the negative relationship between FBW7 and Stat3 levels was also confirmed in DLBCL patient samples (22) (Figure 2).

In addition, epigenetic alterations play a vital role in the tumor progression of DLBCL. One study that adopted whole-genome/exome sequencing (WGS/WES) of 619 patients with DLBCL revealed that somatic mutations in *KMT2D* (19.5%) were most frequently observed, followed by mutations in *ARID1A* (8.7%), *CREBBP* (8.4%), *KMT2C* (8.2%), *TET2* (7.8%), *EP300* (6.8%), and *EZH2* (2.9%) (17). Somatic mutations in the *CREBBP* and *EP300* genes mainly occur in B-cell lymphomas, especially in DLBCL, and often relate to disease relapse and inferior prognosis. The 3-year progression-free survival and OS were 52.6% and 67.8%,

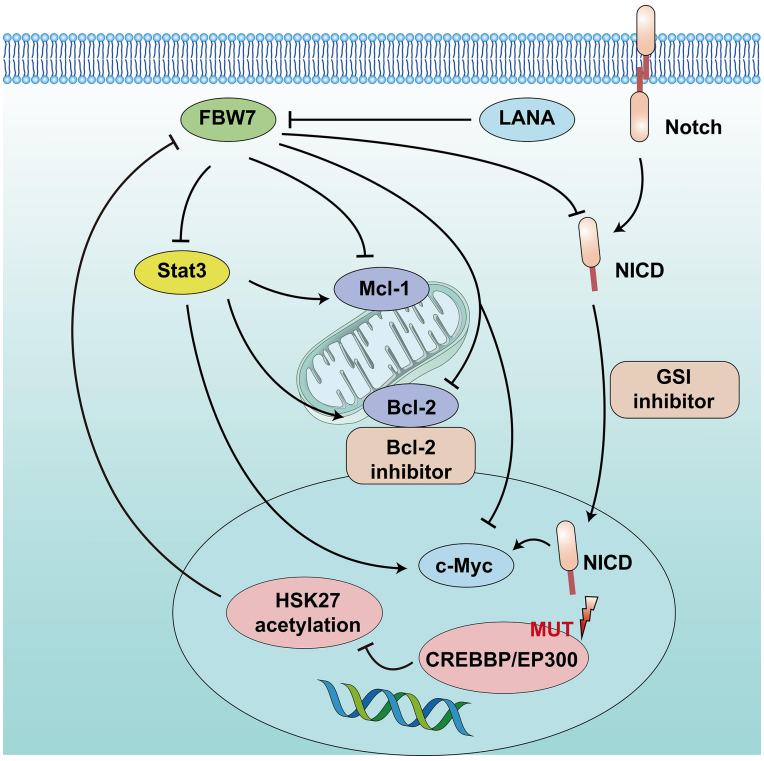

FIGURE 2
Schematic diagram of FBW7-mediated oncogene and signaling pathway in B lymphoproliferative malignancies. 1) CREBBP/EP300 mutations inhibit H3K27 acetylation, downregulate FBW7 expression, and activate the Notch pathway to promote cell proliferation. 2) Dysregulation of FBW7 could upregulate Stat3 expression and increase downstream antiapoptotic target genes, including c-Myc, Mcl-1, and Bcl-2, to induce cell proliferation and inhibit apoptosis.

respectively, lower than that of patients without mutation (17, 60). The mechanism suggested that *CREBBP/EP300* mutation inhibited H3K27 acetylation, downregulated FBW7 expression, and activated the Notch pathway, ultimately promoting DLBCL proliferation. This indicates that *CREBBP/EP300* mutation in DLBCL may regulate cell proliferation *via* the FBW7-Notch pathway (17) (Figure 2). However, another study reported that FBW7 was a tumor pro-survival factor (61). *KMT2D* is a tumor suppressor gene of DLBCL. FBW7 degrades KMT2D by ubiquitylation and promotes DLBCL cell growth. Therefore, more clinical studies are required to confirm the mechanisms related to the role of FBW7 in DLBCL.

Chronic lymphocytic leukemia

CLL is a clonal proliferative disease of mature B lymphocytes, characterized by the aggregation of small mature lymphocytes in the peripheral blood, bone marrow, spleen, and lymph nodes. In addition, CLL is a heterogeneous disease with variable clinical presentation and evolution. CLL generally has a chronic and indolent course with slow progression. The treatment of watching and waiting is required at early stages, but CLL remains an incurable disease with heterogeneous prognosis. Patients with nonmutated IGHV, Del(17p), and Del(11q) chromosomal aberrations and *TP53* gene deletions or mutations usually have an aggressive course and poor prognosis and often do not achieve sustained remission (62, 63). Approximately 12% patients with CLL may have *Notch1* mutation, which is associated with poor prognosis (64). Patients of CLL with *Notch1* or *FBW7* mutations have a higher risk of Richter's syndrome transformation (57%) (65). In addition, approximately 2%–8% of patients with CLL have detected *FBW7* mutations, mainly missense mutations, affecting the WD40 domain required for substrate binding, and the mutation sites detected at R465L, R465H and G423V (65–67). FBW7 negatively regulates Notch1. Close et al. used CRISPR/Cas9 technology to edit the WD40 domain in the CLL cell line to cause *FBW7* mutations, which could increase the transcription activity and protein level of Notch1 and c-Myc by reducing degradation (18). Moreover, *FBW7* mutations in CLL not only increase NICD levels but also increase the expression level of the Notch1 target gene, impeding its degradation through ubiquitination (18). These findings suggest that *FBW7* mutations play a role in activating the leukemia-causing Notch1 pathway (Figure 2).

Primary effusion lymphoma

Primary effusion lymphoma (PEL) is a highly aggressive B-cell lymphoma with a poor prognosis and median survival of 6.2 months (68). The etiology is associated with Kaposi's sarcoma associated herpesvirus (KSHV) (69). Latency-associated nuclear antigen (LANA) is a key gene of KSHV, which can interact with FBW7, resulting in inhibiting PEL cell apoptosis by reducing the degradation of Mcl-1 and caspase-3 through ubiquitination (29, 70) (Figure 2).

Proliferation of bone marrow plasmacytes

Multiple myeloma

MM is a hematologic malignancy characterized by the presence of abnormal clonal plasma cells in the bone marrow, accounting for 15% of adult hematologic malignancies. It is characterized by anemia, hypercalcemia, bone lesions, and kidney dysfunction. It remains an incurable disease, with a 5-year OS of approximately 45% (71–73). Therefore, understanding the underlying mechanisms of MM development is critical.

The NF-κB pathway is important for cell growth, differentiation, and survival. It also plays a vital role in the development of MM (74). The p100 protein, belonging to the NF-family, is the main inhibitor of the noncanonical NF-κB pathway. Clearance of p100 from the nucleus is required for NF-κB pathway activation and MM cell survival. FBW7α can target the nuclear phosphorylation of p100 by GSK3 for degradation through ubiquitination both *in vivo* and *in vitro*. Thus, FBW7α and GSK3 function as pro-survival factors by controlling p100 degradation in MM (20, 75). Besides, miRNAs, such as miR-32, miR-27, miR-214, and miR-21, are upregulated in MM (76). Overexpression of miR-27b and miR-214 in MM can mediate the FBW7 and PTEN/AKT/GSK3 pathways blocking degradation by ubiquitinating Mcl-1 and inducing cell proliferation and apoptosis resistance (30). In addition, patients with MM with a lower FBW7 expression and higher miR-32 expression in cancer tissues than in normal tissues, had poorer prognosis, with a median OS <3 years. Overexpression of miR-32 *in vitro* decreased FBW7 expression and increased the expression of cancer-related proteins, c-Jun and c-Myc (23). These results indicate that miRNAs can regulate FBW7 expression in MM and then affect the expression of its downstream substrates, thus promoting MM proliferation. In all, *FBW7* can be both a tumor suppressor gene and a pro-survival gene in MM. Further studies should confirm the mechanisms related to the role of FBW7 in MM (Figure 3).

Other malignant lymphoproliferative disorders

Mutations of the *FBW7* gene were also detected in B-ALL and mantle cell lymphoma (77–79). MiR-27a is upregulated in

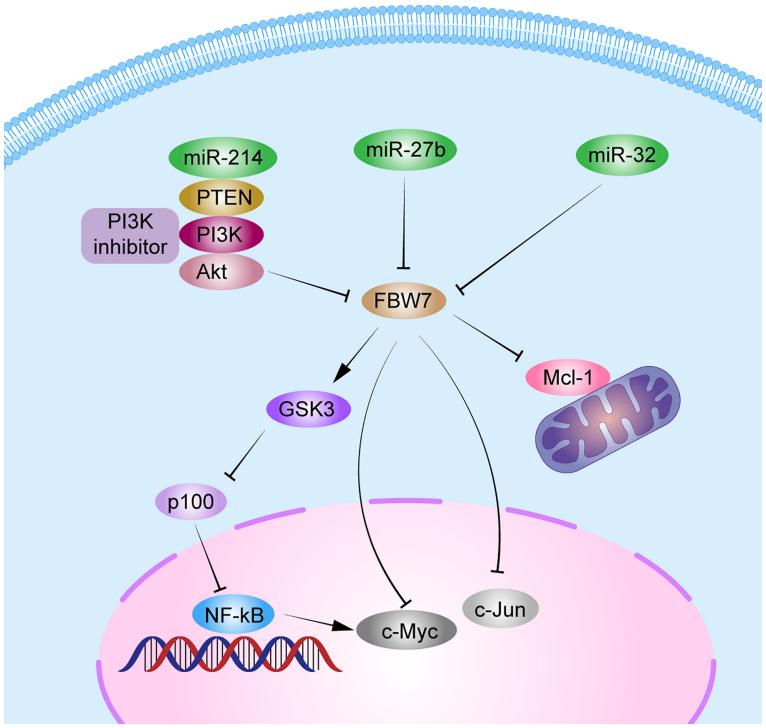

FIGURE 3
Schematic diagram of FBW7-mediated oncogene and signaling pathway in multiple myeloma. 1) FBW7 could target nuclear phosphorylation of p100 by GSK3β for degradation through ubiquitination, and subsequently activate the NF-κB pathway to promote MM cell proliferation. 2) miRNAs, such as miR-32, miR-27b, and miR-214, block degradation of Mcl-1, c-Myc, and c-Jun by FBW7 directly or indirectly by PTEN/AKT/GSK3 pathways to induce cell proliferation and apoptosis resistance.

pediatric B-ALL, and its expression is inversely correlated with FBW7 expression and disease progression (80). Further studies are required to clarify this mechanism. In addition, FBW7 overexpression in cutaneous T-cell lymphoma could increase the sensitivity of histone deacetylase inhibitor (HDACi) (81).

Conclusion

FBW7 as a critical tumor suppressor, can recognize various oncogenic proteins and degrade them through ubiquitination to maintain cell growth. However, FBW7 dysregulation is associated with the development of malignant lymphoproliferative disorders. In addition, *FBW7* mutations or inactivation is associated with chemoresistance and poor prognosis. Thus, the underlying mechanisms can potentially be targeted for treating malignant lymphoproliferative disorders. Future studies should attempt to elucidate the complex mechanisms underlying the role of FBW7 and its substrates and to identify novel targets for effective treatment of malignant lymphoproliferative disorders. Clinical studies are needed to confirm the significance of FBW7 in tumor development, progression, and resistance to therapies as well as opportunities for targeted therapies.

Author contributions

XW, WG, ZZ and OB designed the study and contributed vital data and analytical tools and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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