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Linking cellular metabolism to hematological malignancies, volume II

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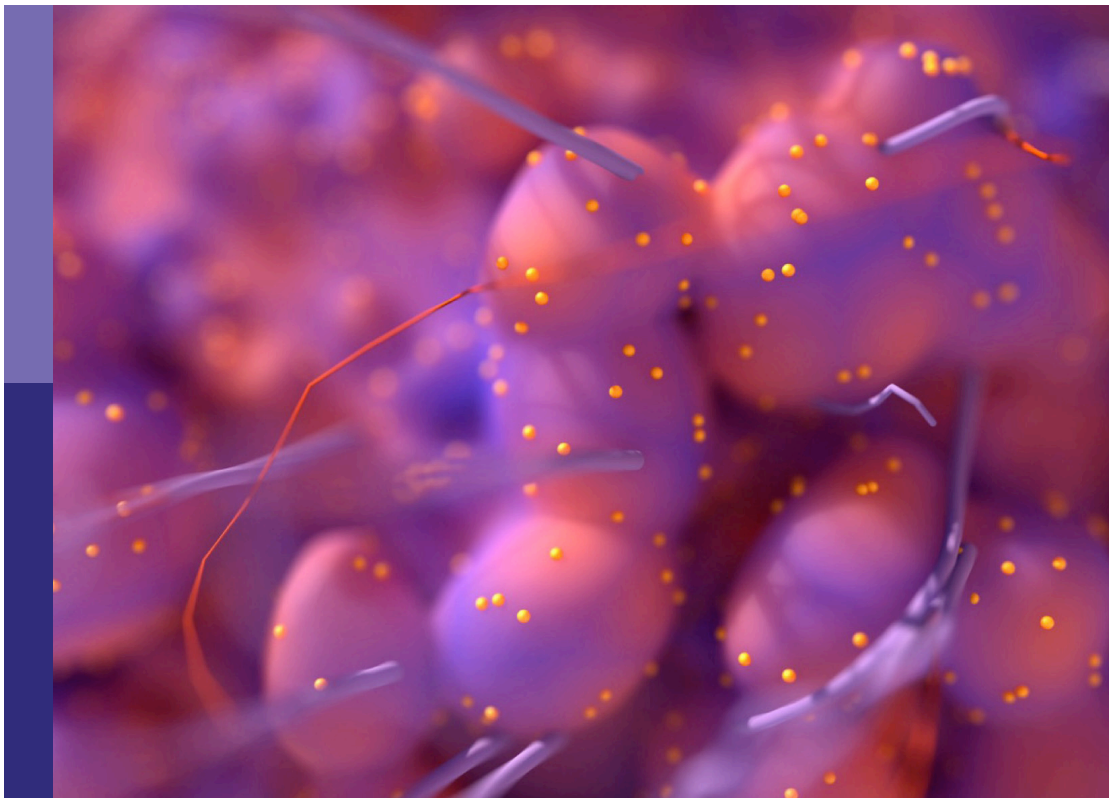

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Linking cellular metabolism to hematological malignancies, volume II

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Editorial: Linking cellular metabolism to hematological malignancies, volume II

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KEYWORDS

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Editorial on the Research Topic
Linking cellular metabolism to hematological malignancies, volume II

Cancer cells reprogram their metabolism to survive in hostile environments, which may reveal potential vulnerabilities to targeted therapies. Metabolic-based therapies can specifically target cancer metabolism, resulting in substantial antitumor effects, while sparing normal cells. This Research Topic highlights the importance of understanding tumor metabolic remodeling and immunometabolism in hematological malignancies.

Rana et al. studied cell metabolism changes in multiple myeloma. Cancer cells use aerobic glycolysis for energy, unlike healthy cells that use mitochondrial oxidative phosphorylation. This shift helps cancer cells obtain nucleotides, amino acids, and lipids for replication. Proteasomes, which regulate protein levels affecting metabolism, survival, and growth, are crucial in both healthy and cancerous cells. Proteasome inhibitors, developed over the past two decades, have improved patient survival and quality of life in multiple myeloma.

Ferroptosis is a recently discovered, iron-dependent cell death process. This leads to Oxidative stress and cell death occur due to the accumulation of lipid reactive oxygen species (ROS). Liu et al. revealed that blood tumor cells, such as leukemia and lymphoma cells, are sensitive to ferroptosis.

Lipid metabolism and hematological malignancies have a complex relationship, presenting the challenges and opportunities for therapeutic approaches. Lipid reprogramming is crucial for tumor cell physiology and influences cellular functions are necessary for cancer growth and survival. Zhang et al. highlighted the complexity arising from the interconnectedness of glucose, lipid, and amino acid metabolism within cancer cells, describing how these metabolic pathways affect and regulate each other in intricate ways, creating challenges in effectively inhibiting cancer growth by targeting a single metabolic process alone.

L-Asparagine (L-ASNase) is a hydrolytic enzyme that reduces circulating asparagine, a crucial amino acid for the survival and growth of leukemia cells, particularly lymphoblasts in acute lymphocytic leukemia (ALL). As leukemic cells lack the enzyme asparagine synthetase, they depend on external asparagine sources. By starving them of this essential amino acid, L-ASNase selectively targets and kills leukemia cells, while sparing normal cells that can synthesize asparagine. It has been widely used to treat leukemia, including childhood leukemia. Zhou et al. summarized the different mechanisms of drug resistance to L-ASNase. L-ASNase immunogenicity can trigger the production of antidrug antibodies that neutralize L-ASNase activity by binding to this preventing its action on leukemia cells. As a result, the drug is cleared from the bloodstream faster, thereby reducing its therapeutic effects. Addressing L-ASNase immunogenicity is crucial for optimizing treatment outcomes and minimizing adverse effects in leukemia patients receiving this therapy.

The interplay between RBPs, mRNA editing, pyroptosis, and the impact of these factors on AML is an intriguing area of research. Pyroptosis, a form of programmed cell death, modulates the immune response in acute myeloid leukemia (AML) cells. Identifying pyroptosis-related RBP genes and their potential prognostic value in patients with AML could provide critical insights into disease progression and therapeutic strategies. Bin et al. used the Gene expression omnibus (GEO) database to identify pyroptosis-RBP-related differentially expressed genes (PRBP-DEGs) and offer a comprehensive understanding of the interplay between pyroptosis, RNA-binding proteins, and AML prognosis. The established risk model and nomogram hold promise for improving the prognostic accuracy and can guide potential therapeutic strategies for AML.

In the tumor-microenvironment (TME), fatty acid metabolism (FAM) affects tumor cell behavior, interactions with neighboring and immune cells, and extracellular matrix. The complexities of FAM's influence of FAM on AML in TME is not well understood. Ye et al.'s investigation of scRNA-Seq and bulk transcriptome data on AML patients to explore the association between FAM, TME, and patient outcomes is a key advancement in understanding AML biology and therapeutic opportunities. Elevated FAM-related genes in leukemic stem cells suggest metabolic characteristics driving leukemia progression. PLA2G4A, a highly expressed FAM gene, is linked to poor prognosis in AML, and its targeting enhances NK Cell-mediated Immunosurveillance. This study provides insights into FAM, TME, and immune surveillance in AML, offering potential targeted therapies and personalized interventions to improved the patient outcomes.

Examination of the effects of tyrosine kinase inhibitors (TKI) on cellular metabolism is crucial for improving treatment outcomes in chronic myeloid leukemia (CML) and Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph + ALL) and reducing side effects in pediatric patients. Although TKIs have improved prognosis, challenges like drug resistance, off-target effects, and drug tolerance can impede efficacy. Li et al. highlighted the significance of alterations in glucose, lipid, and amino acid metabolism in influencing treatment responses and drug resistance during TKI therapy in children with Ph+ leukemia. These metabolic changes can affect drug sensitivity and resistance, potentially affects TKI efficacy in targeting leukemia cells.

A study by Zhou et al. on aplastic anemia in children used single-cell RNA sequencing to identify new gene expression patterns and cell subsets, revealing significant findings related to metabolic changes, including genes such as NENF, INPP4B, AKR1C3, and CHST2, which play crucial roles in neurotrophic support, phosphoinositide signaling, steroid and prostaglandin metabolism, and glycosaminoglycan biosynthesis.

FLT3 mutations, including ITD and TKD, are common in AML and affect patient prognosis. FLT3-ITD mutations have been extensively studied due to their adverse prognostic impact on AML. A meta-analysis by Li et al. on FLT3-TKD mutations in AML showed intriguing differences in prognosis between Asian and Caucasian populations. The presence of FLT3-TKD mutations may have a favorable prognosis for DFS and OS in Asian AML patients, whereas in Caucasians, these mutations are linked to an adverse prognosis for DFS. However, caution is advised when interpreting DFS results in Caucasians due to observed heterogeneity among studies.

Transcriptome analysis is crucial for understanding the molecular mechanisms of leukemia, especially mutations in SHP2 that affect signaling pathways involved in cell growth and survival. Zhao et al. conducted transcriptome profiling and identified 2443 and 2273 differentially expressed genes in HCD-57 cells expressing SHP2 mutants compared to parental cells, revealing the impact of mutant SHP2 gene expression.

Research on metabolic reprogramming in hematological malignancies has provided valuable insights, deepening our understanding of cellular metabolic mechanisms and identifying potential treatment targets. Further validation and exploration of these targets is necessary to ensure their efficacy and safety. Continued research is crucial to translate these findings into clinically relevant treatments that could improve the management and outcomes of hematological malignancies.

Author contributions

ZL: Writing – original draft, Writing – review & editing: JY: Writing – review & editing, Writing – original draft, Supervision, Software. ZZ: Writing – review & editing, Writing – original draft. HS: Writing – review & editing, Writing – original draft. MK: Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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Reprogramming lipid metabolism as potential strategy for hematological malignancy therapy

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Hematological malignancies are one of the most lethal illnesses that seriously threaten human life and health. Lipids are important constituents of various biological membranes and substances for energy storage and cell signaling. Furthermore, lipids are critical in the normal physiological activities of cells. In the process of the lethal transformation of hematological malignancies, lipid metabolism reprogramming meets the material and energy requirements of rapidly proliferating and dividing tumor cells. A large number of studies have shown that dysregulated lipid metabolism, commonly occurs in hematological malignancies, mediating the proliferation, growth, migration, invasion, apoptosis, drug resistance and immune escape of tumor cells. Targeting the lipid metabolism pathway of hematological malignancies has become an effective therapeutic approach. This article reviews the oncogenic mechanisms of lipid metabolism reprogramming in hematological malignancies, including fatty acid, cholesterol and phospholipid metabolism, thereby offering an insight into targeting lipid metabolism in the treatment of hematological malignancies.

KEYWORDS

lipid metabolism reprogramming, cholesterol, fatty acids, phospholipids, hematological malignancies

Introduction

Hematological malignancies are a collection of malignant tumors that aberrant hematological cells or immune cells are blocked in differentiation and proliferate indefinitely, leading to the dysfunction of biological organisms (1). There are three main types of hematological malignancies: leukemia, multiple myeloma (MM), and

lymphoma (2). Hematological malignancies are one of the most lethal illnesses that seriously threaten human life and health with a high mortality rate. According to the World Cancer Report 2020 released by the World Health Organization, the new number of non-Hodgkin's lymphoma, leukemia, MM and Hodgkin's lymphoma in 2020 were 544,352, 474,519, 176,404 and 83,087, respectively, accounting for 6.6% of the total number of patients. The corresponding number of deaths were 259,793, 311,594, 117,077 and 23,376 respectively, accounting for 7.1% of the total number of patients (3). Due to its particularity, hematological malignancies cannot be surgically removed like solid tumors, and its clinical first-line treatment options mainly include chemotherapy, radiotherapy and hematopoietic stem cell transplantation (4). Although the traditional first-line therapies have a certain effect, the overall efficacy is not optimistic due to the relapse/refractory caused by the occurrence of primary/secondary drug resistance (5). With the deepening of research, new cancer therapies have brought dawn to relapsed/refractory patients, including CAR-T cell therapies, ADC drugs and immune checkpoint inhibitors (6). The U.S. Food and Drug Administration approved anti-CD19 CAR-T cell therapy Tisagenlecleucel for the treatment of B-cell acute lymphoblastic leukemia (ALL) (7), ADC drug Loncastuximab Tesirine-Lpyl for the treatment of relapsed/refractory diffuse large B-cell lymphoma (DLBCL) (8) and PD-1 inhibitor Pembrolizumab for the treatment of Hodgkin lymphoma (9). However, even under novel therapies, there are still a large number of patients with poor clinical prognosis. Therefore, it is still a work with clinical application value and important scientific significance to study the oncogenic mechanism and find new therapeutic targets in order to develop new therapeutic methods (10).

It has been widely reported that cell metabolism affects tumor cell proliferation, apoptosis, migration, invasion, chemical resistance and immune escape (11–14). There is a close relationship between cellular metabolism and functional output, once the metabolic pathway is abnormal, leading to abnormal cell function and disease progression (15). Compared with normal cells, tumor cells undergo metabolic reprogramming due to their excessive proliferation, growth, migration and metastasis requiring faster and more energy, and tumor cell metabolic reprogramming has been identified as a new marker of cancer (16). Clinical observations found that lipid metabolism reprogramming often predicts poorer prognosis in cancer patients (17). A large number of lipid droplets that store lipids and cholesterol can be detected in tumor cells, high lipid droplets and high cholesterol esters are also considered indicators of cancer aggressiveness in tumor cells (18). *De novo* lipid synthesis pathway and uptake of exogenous lipids are often enhanced in rapidly dividing and energy-consuming tumor cells, such as malignant plasma cells from obese myeloma patients with high expression of acetyl-CoA synthase 2 (19). Acetyl-CoA synthase 2 is a key precursor for the *de novo* synthesis of fatty acids (FAs). Fatty acid synthase (FASN) is also upregulated in various hematological malignancies (20). The fatty acid transporter protein (FATP), which mediates cellular uptake of FAs, is expressed at high levels on both the cell surface and the intracellular space of patients with MM (21). Lipids are not only important components of organelles and energy substances, but also signaling molecules that are crucial for maintaining cellular homeostasis (22). Lymphoma-derived exosomes promote tumorigenesis by increasing lipid metabolism in recipient cells through surface phospholipase A2 (23). Lysophosphatidic acid (LPA)-mediated activation of the MEK1/2-ERK1/2 signaling pathway increases oxidative phosphorylation in the mitochondria of MM cells, which in turn produces large amounts of NAD^+ and ATP. It impairs the activity of proteasome inhibitors and enhances protein folding in the endoplasmic reticulum (ER), thereby conferring resistance to proteasome inhibitors in MM (24). In this review, we discuss the lipid metabolism reprogramming and its oncogenic mechanisms in hematological malignancies, including FA metabolism, cholesterol metabolism, phospholipid metabolism and lipid-related signaling pathways.

FA metabolism in hematological malignancies

FAs are essential molecules in the entire lipid metabolism, not only involved in the synthesis of biological membranes and secondary signaling molecules, but also substrates for mitochondrial ATP and NADH synthesis, eicosanoid production and post-translational protein–lipid modifications of signaling proteins (25, 26). As early as 1924, Warburg proposed that even under sufficient oxygen conditions, tumor cells also prefer the low-utilization form of glycolysis for energy production (27). Even if tumor cells use a large amount of carbohydrates, it is challenging to meet the needs of energy substances, so lipid metabolism is also required for energy. Tumor cells increase lipid metabolism and energy supply mainly by enhancing the *de novo* synthesis pathway of endogenous FAs, exogenous FAs uptake and lipid mobilization (28). *De novo* FA synthesis mainly depends on two key rate-limiting enzymes, acetyl-CoA carboxylase (ACC) carboxylates acetyl-CoA to malonyl-CoA, and FASN converts acetyl-CoA and malonyl-CoA Conversion of acyl-CoA to long-chain FAs (Figure 1) (29). It has been reported that the FA *de novo* synthesis pathway is generally up-regulated in tumor cells, and FASN overexpression has been an independent prognostic marker for the aggressive clinical course of tumor cells (30). MYC^+ $BCL\text{-}2^+$ DLBCL with high expression of FASN has the characteristics of high invasion and poor prognosis (31). Up-regulated FASN can promote the growth, metastasis, invasion and anti-apoptosis of DLBCL through the pERK/BCL-2 signaling pathway, and FASN inhibition can cause cell growth

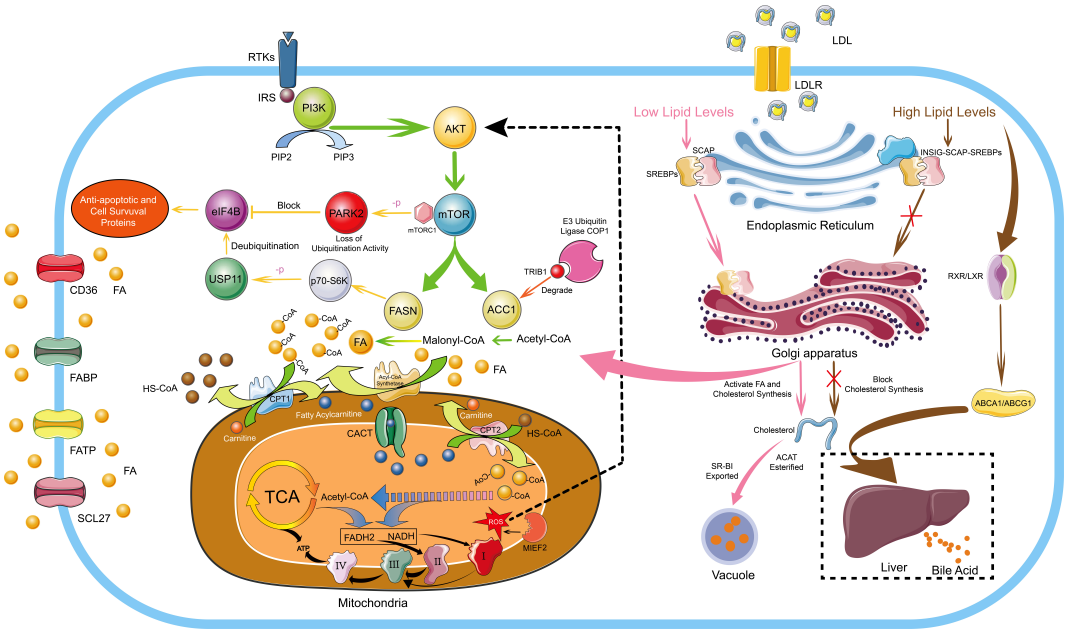

FIGURE 1
FA And Cholesterol Metabolism in Hematological Malignancies. The *de novo* synthesis of FA is regulated by the PI3K/Akt signaling pathway. *De novo* FA synthesis mainly depends on two key rate-limiting enzymes, ACC carboxylates acetyl-CoA to malonyl-CoA, and FASN converts acetyl-CoA and malonyl-CoA Conversion of acyl-CoA to long-chain FA. ACC and FASN are regulated by mTOR, which is a downstream target of the PI3K/Akt signaling pathway. Overexpressed FASN can prompt p70-S6 kinase to phosphorylate USP11 and the phosphorylated USP11 mediates eIF4B deubiquitination to increase its stability. eIF4B mediates the expression of anti-apoptotic and cell survival proteins, ultimately driving the development of lymphoma. In addition, PARK2 can also be phosphorylated by mTORC1 to lose its ubiquitination activity, thereby blocking the ubiquitination and degradation of eIF4B protein. The E3 ubiquitin ligase COP1 binds to ACC1 through Trib1 and causes ACC1 ubiquitination and degradation to inhibit FA synthesis. Cells uptake exogenous FAs mainly through CD36, FATP, FABP and SCL27. Endogenous FAs and exogenous FAs enter the mitochondria through the transmembrane mechanism, and generate a large amount of ATP through FA β-oxidation, the TCA cycle and the electron transport chains. Moreover, MIEF2, a key regulator of mitochondrial fission, can stimulate the production of mitochondrial ROS and activate the AKT/mTOR signaling pathway to further enhance FA synthesis. Cholesterol homeostasis is mainly regulated by SREBPs and LXRs. In conditions of low cholesterol levels, SCAP-SREBP2 can be smoothly transferred from the ER to the Golgi apparatus to activate the *de novo* synthesis pathway of cholesterol. In conditions of high cholesterol levels, INSIG, SCAP and SREBP2 form a stable trimolecular complex to block the export and activation of SREBP2, and finally inhibiting the *de novo* cholesterol synthetic route. Moreover, by activating LXR-RXR to express ABCA1 and ABCG1, excessive cholesterol is transported to the liver and excreted in the form of bile acids. Furthermore, excessive cholesterol is rapidly esterified and exported under the action of ACAT and SR-BI to form vacuoles containing cholesterol ester derivatives. I, II, III and IV represent complex I, complex II, complex III and complex IV in the electron transport chains, respectively.

arrest and apoptosis (32). Overexpressed FASN can also regulate the PI3K/Akt signaling pathway in DLBCL, prompting p70-S6 kinase to phosphorylate USP11. The phosphorylated USP11 mediates eIF4B deubiquitination to increase its stability, and eIF4B promotes key oncogenes biosynthesis, ultimately driving the development of lymphoma (33). PARK2 can also be phosphorylated by mTORC1 to lose its ubiquitination activity, thereby blocking the ubiquitination and degradation of eIF4B protein (Figure 1) (34). Meanwhile, up-regulation of FASN has been reported in acute myeloid leukemia (AML) (35), mantle cell lymphoma (MCL) (36), and MM (37).

There is a lack of reports about ACC1 in hematological malignancies, only one recent paper mentioned the function of ACC1 to suppress tumors. The E3 ubiquitin ligase COP1 binds to ACC1 through Trib1 and causes ACC1 ubiquitination and degradation to inactivate its biological activity, which leads metabolic reprogramming to support the energy requirements of leukemia progression. A general downregulation of ACC1 can be observed in AML. However, stabilizing the biological activity of ACC1 protein can increase intracellular ROS levels and NADPH consumption, thereby inhibiting leukemia progression, which may be caused by the material and energy competition conflict between ACC1-mediated FA synthesis and tumor cell proliferation (38). As an effective strategy for the treatment of patients with AML and endemic Burkitt lymphoma, the drug combination of bezafibrate and medroxyprogesterone acetate can effectively reduce the expression of FASN and stearoyl-CoA desaturase 1. However, ACC1, which is also a key enzyme in lipid synthesis, did not show a significant change in expression (39). The role of ACC1 remains unknown in hematological malignancies. Interestingly, ACC1 promotes tumor cell growth in some solid tumors.

MIEF2, a key regulator of mitochondrial fission, stimulates the production of mitochondrial ROS and activates the AKT/mTOR signaling pathway. The result causes the upregulation of ACC1, FASN, SREBP1/2, SCD1, HMGCS1 and HMGCR to increase lipid synthesis, ultimately promoting the growth and metastasis of ovarian cancer cells (40). Overexpression of the long non-coding RNA (lncRNA, CTD-2245E15) in lung cancer can also regulate ACC1 and pyruvate carboxylase to promote lung cancer development (41).

Tumor cells enhance the uptake of exogenous FAs, mainly through the CD36, FATP, lipid chaperone FA binding protein (FABP) and solute carrier protein family 27 (SCL27). Up-regulation of CD36 has been reported in hematological malignancies such as AML (42), chronic lymphocytic leukemia (CLL) (43), MM (44), DLBCL (30), and MCL (45). Sudjit Luanpitpong (45) used Synchrotron-Based Fourier Transform Infrared Spectroscopy of Single Cells to detect significant increases in total lipids and lipid esters in MCL resistant to Bortezomib (BZ). BZ is a protease inhibitor that leads to accumulation of misfolded and unfolded proteins, mainly by inhibiting protein degradation, ultimately causing the ER stress response. Subsequent Oil Red O staining detection revealed significant lipid droplets accumulation in BZ-resistant MCL cells. Detection of lipid metabolism-related targets revealed increased expression of CD36 protein responsible for exogenous FAs uptake, and CD36 inhibited apoptosis in MCL, which was associated with BZ-resistance. It has been reported that BTK inhibitors can inhibit lipid droplet accumulation in MCL (46). Moreover, CD36 is associated with tumor invasion and metastasis and is a prognostic biomarker for various types of cancer (47–49). Apolipoprotein C2, which is highly expressed in AML, can interact with CD36 to activate LYN-ERK signaling and enhance the metabolic activity of leukemia cells (42). CD36 was also found to promote FAs uptake by activating STAT3 in CLL (43). Up-regulation of CD36 is also one of the reasons why tumor cells develop drug resistance (50). Exogenous interleukin 6 (IL-6) mediates the up-regulation of CD36 by activating STAT3, promoting the uptake of FAs and causing chemotherapy resistance (51). CD36 also induces lipid peroxidation and ferroptosis with concomitant reduction of cytotoxic cytokines and impaired antitumor capacity (52). In addition to the drug resistance of tumor cells caused by increased lipid synthesis, decreased lipid synthesis can also lead to drug resistance. BZ exerts its anti-tumor effect through ER stress caused by the protein accumulation (53), because the ER is the site of lipid synthesis, and BZ also causes lipid accumulation (45). In BZ-resistant MM, it was found that the expression of SREBP1 and its downstream target FA elongase ELOVL6 are reduced, resulting in inhibiting lipid synthesis, thereby reducing the accumulation on the ER and ultimately causing BZ-resistance (54). In addition to CD36 mediating cellular uptake of FAs, FATP also mediates cellular uptake of FAs. FATP is expressed at high levels on the cell surface and intracellular space in MM. Furthermore, MM cells can induce lipolysis of bone marrow adipocytes, and the decomposed free fatty acids (FFA) are taken up by adjacent MM cells through FATP (21). FABP and SCL27 were also observed to be up-regulated in tumor cells, increasing the uptake of exogenous FAs of tumor cells (55–58). Glycoprotein prostaglandin D2 synthase (PTGDS) has dual roles in prostaglandin metabolism and lipid transport. More interestingly, PTGDS exhibits different functions in different tumor cells. PTGDS promotes DLBCL progression by regulating tumor cell viability, proliferation, cell cycle, apoptosis and invasion through MYH9 stimulated Wnt-β-catenin-STAT3 signaling pathway (59). However, PTGDS showed antitumor effect in testicular cancer (60), gastric cancer (61) and breast cancer (62).

Fatty acid oxidation (FAO) provides energy mainly through FA β-oxidation. In order to successfully carry out FAO, FAs first need to enter the mitochondria. Firstly, long-chain FAs need to generate fatty acyl-CoA under the action of fatty acyl-CoA synthase. Fatty acyl-CoA is converted to fatty acylcarnitine under the action of CPT1, which is then transported into the mitochondrial matrix by carnitine/acylcarnitine translocase (CACT) on the inner mitochondrial membrane, and fatty acylcarnitine entering the mitochondrial matrix are reconverted to fatty acyl-CoA by CPT2. The fatty acyl-CoA that smoothly enters the mitochondria repeats the cycle of dehydrogenation, water addition, dehydrogenation, and thiolysis, and finally decomposes the fatty acyl-CoA into acetyl-CoA, accompanied by the generation of a large amount of NADH and FADH2. These substances eventually enter the TCA cycle and the electron transport chains to be oxidized to generate a large amount of ATP for cellular physiological activities (Figure 1) (28). FAO is dysregulated in a variety of malignancies, and it mediates tumor cell proliferation, survival, drug resistance, metastatic progression, immunosuppression and tumor-promoting microenvironment (63). As an enzyme involved in FAO, HADHB is commonly overexpressed in malignant lymphomas and is a poor prognosis predictor in DLBCL, and high expression of HADHB promotes the proliferation and growth of malignant lymphomas (64). HADHA, which forms a heterodimer together with HADHB, is also widely up-regulated in malignant lymphomas, and down-regulation of HADHA can cause G0/G1 cell cycle arrest (65). Acyl-CoA oxidase 1 (ACOX1), a key rate-limiting enzyme in FAO, is overexpressed in malignant lymphomas and confers resistance to the anthracycline antibiotic doxorubicin, mainly by reducing doxorubicin-induced activation of caspase-9 and caspase-3 and reduction of mitochondrial membrane potential. Simultaneously, ACOX1 can also destabilize the tumor suppressor gene family p73 protein and inhibit its expression (66).

Lipid metabolism can affect the immune system in tumor cells, causing immune evasion and promoting tumor growth. Natural killer (NK) cells play an important role in the prevention

of hematological malignancies. However, FAs, both in lymphoma cells and in the tumor microenvironment, can reprogram lipid metabolism in NK cells and inhibit the production of cytokines such as IFN-γ, making NK cells lose their immune function to tumor cells (67). The prostaglandin PGD 2, a lipid compound of the eicosanoid family, is abundantly generated under the catalysis of cyclooxygenase overexpressed in tumor cells. PGD 2 can stimulate innate lymphocytes ILC2 to overexpress IL-5, which subsequently promotes the proliferation of Tregs cells. Tregs cells can be involved in immunosuppression, such as inhibition of T effector cell proliferation and production restriction inflammatory response factor IL-10, ultimately promoting the proliferation of hematological stem and progenitor cells (68). Tumor-associated macrophages (TAMs) up-regulate CD36 to uptake lipids resulting in lipid accumulation. Excess lipids provide a large amount of energy through FAO and lead to activation of STAT6, which is accompanied by TAMs differentiation and cancer promotion (69).

Since FA biosynthesis, uptake, and oxidation are significantly enhanced in various types of tumor cells, inhibiting FA mobilization has become a promising antitumor strategy in tumor cells. FASN, a key rate-limiting enzyme in the *de novo* synthesis pathway of endogenous FAs, is associated with multidrug resistance in tumor cells (70) and is an effective target for the treatment of malignant tumors. Clinically, glucocorticoids such as prednisone and dexamethasone can inhibit the expression of FASN and thereby inhibit the proliferation and growth of tumor cells (71). At the same time, the study found that ginger extract can inhibit the expression of FASN, and combined use with dexamethasone can enhance the drug sensitivity of ALL cells to dexamethasone (72). The combination of bezafibrate and medroxyprogesterone acetate (39), orlistat (73), N-phenylmaleimide (74) and methyl jasmonate (75) can all regulate the expression of FASN to inhibit the growth of tumor cells. In leukemia cells, FABP4 regulates DNMT1 expression through the IL-6/STAT3 axis and DNMT1 controls FABP4 through VEGF signaling, thereby forming a mutually reinforcing positive feedback regulation that ultimately promotes AML aggressiveness. The selective inhibitor BMS309403 can cause FABP4 dysfunction, which in turn promotes the downregulation of DNMT1. Subsequent induction of global DNA methylation and re-expression of tumor suppressor genes ultimately induce AML cell differentiation and inhibit AML progression (76).

Cholesterol metabolism in hematological malignancies

Cholesterol is an important substance for cell function, and cholesterol homeostasis is essential for the normal physiological activities of the body (77). Cholesterol homeostasis is mainly regulated by two transcription factor families, sterol regulator element binding proteins (SREBPs) and liver X receptors (LXRs). SREBPs mediate lipid synthesis and LXRs mediate cholesterol transport. Andrea Brendolan has made a detailed summary of cholesterol homeostasis regulation. In brief, in conditions of low cholesterol levels, SREBP2 is escorted to the Golgi apparatus by SREBP cleavage activator protein (SCAP), where a series of biological reactions activate cholesterol synthesis, and LXRs are in an inhibited state at this time. In conditions of high cholesterol levels, INSIG, SCAP and SREBP2 form a stable trimolecular complex in the ER, thereby blocking the export and activation of SREBP2 and finally blocking the *de novo* cholesterol synthetic route. Moreover, excess oxysterols or desmosterol bind and activate the LXR/RXR heterodimer, which in turn activates specific LXR target genes, such as ATP-binding cassette transporters A1 and G1 (ABCA1 and ASCG1), allowing excess cholesterol to be transported to the liver and excreted as bile acids (Figure 1) (78).

The mechanisms that maintain cholesterol homeostasis are disrupted in tumor cells due to their addiction to cholesterol. It has been reported that a large amount of cholesterol is widely present in malignant tumor cells (79). In human hepatocellular carcinoma cells, the stability of the INSIG, SCAP, and SREBP2 trimolecular complex is destabilized by cascade phosphorylation of the AKT-PCK1-INSIG axis. SCAP-SREBP complex is translated to the Golgi apparatus to activate cholesterol synthesis and ultimately promotes the proliferation and growth of tumor cells (80). Up-regulation of LDLR, SREBP2 and nuclear PBR are detected in CLL, which also explains that hypocholesterolemia in lymphocytic leukemia patients is due to over-uptake of LDL particles from plasma by high LDLR expression (81). At the same time, it has also been found that tumor cells can secrete cytokines through autocrine and paracrine mechanisms to stimulate cellular uptake of LDL in AML (82). In T-cell ALL, the Wnt-β-catenin signaling pathway mediates the oncogenic synergy of Akt and Dlx5 by enhancing cholesterol synthesis (83). Yajie Shen (84) found that SOX9 was highly expressed in the GC-DLBCL with IGH-BCL2^{+} mutation. Through whole transcriptome analysis and chromatin immunoprecipitation sequencing, it was found that SOX9 could directly bind and transcriptionally activate DHCR24, which is a terminal enzyme in cholesterol biosynthesis that catalyzes the conversion of sterol to cholesterol. Using simvastatin to inhibit cholesterol synthesis, it can effectively inhibit the growth of DLBCL and the progression of lymphoma. Moreover, peroxisome proliferator-activated receptor (PPARδ) is co-expressed with cholesterol synthesis-related genes. The expression level of the key cholesterol synthesis enzyme HMGCR increases nearly 4-fold in malignant B cells with high PPARδ gene expression, and a significant increase in membranous cholesterol was also observed in malignant B cells, indicating changes in cell signal pathways (85). In promyelocytic leukemia (APL) driven by the PML-RAPα oncoprotein, it was found that PML-RAPα can reduce the expression of PPARγ. PML-RAPα and TRIB3 cooperate to destroy the PPARγ/RXR heterodimer to inhibit

PPARγ activity, eventually causing abnormal blood lipids in APL (86). Although cholesterol is necessary for maintaining cell homeostasis and cancer cell proliferation, excess free cholesterol is harmful to cells (87). Therefore, cholesterol is rapidly esterified and exported under the action of acetyl-coenzyme A:cholesterol acetyltransferase (ACAT) and scavenger receptor class B member I (SR-BI) to form vacuoles containing cholesterol ester derivatives (Figure 1). A large number of vacuoles have been observed in highly aggressive lymphoma cells, which have been shown to contain lipids by Sudan black positive staining, and up-regulation of molecules related to cholesterol metabolism has also been detected (88).

Cholesterol metabolism reprogramming is also one of the reasons for the drug resistance of tumor cells. HMGCS1, a key enzyme in the mevalonate pathway for cholesterol synthesis, is overexpressed by the upstream regulator GATA1 in patients with relapsed/refractory AML. Activated HMGCS1 protects ER and mitochondria by upregulating the unfolded protein response (UPR) signaling pathway to avoid cell damage caused by RE stress and mitochondrial stress, ultimately endow tumor cells with drug resistance (89). In the tumor microenvironment of MM, there is a large number of oxidatively modified low-density lipoproteins (OxLDLs). These OxLDLs make proteasome inhibitors such as BZ lose their inhibitory and pro-apoptotic effects on the proteasome, and finally make MM patients acquire drug resistance (90). Chemotherapy generally causes drug resistance in tumor cells. After chemotherapy of AML, cellular cholesterol biosynthesis is significantly up-regulated and extracellular vesicles carrying a large number of cholesterol synthesis-related enzymes are secreted, which can be uptake by recipient cells to promote cholesterol synthesis and cell signal transduction, resulting in tumor formation (91).

Because cholesterol affects the occurrence and development of tumors, inhibition of cholesterol synthesis has become a new strategy for the treatment of cancer in recent years (92). In DLBCL, the BCR/SYK/PI3K/AKT signaling pathway regulates the biosynthesis of cholesterol (93). Metformin, a commonly used drug for the treatment of diabetes, can reduce the biosynthesis of cholesterol by blocking the BCR signaling pathway and inhibiting the expression of HMGCS1, thereby inhibiting the growth of DLBCL. Furthermore, limiting the biosynthesis of cholesterol affects the integrity and biological function of the cell membrane and the lipid rafts, further blocking the BCR signaling pathway and the cell activity is severely inhibited (94). Nicotinamide phosphoribosyltransferase inhibitor (KPT-9274) can selectively kill leukemia stem cells (LSC) and is an effective treatment for AML. However, due to the up-regulation of SREBP-regulated genes, LSC developed a certain resistance to KPT-9274 (95). Inhibiting the SREBP signaling pathway with dipyridamole can enhance the drug sensitivity of LSC cells to KPT-9274. In addition, simvastatin, a common statin that reduces plasma cholesterol levels, has been reported to promote apoptosis by inhibiting the miR-19a-3p/HIF-1α axis (96). Conversely, increasing cholesterol biosynthesis can also kill tumor cells. Overactivation of ERK/MAPK signaling pathway using limonoid compounds A1542 and A1543 induces excessive cholesterol biosynthesis in leukemia cells, leading to cholesterol accumulation and programmed apoptosis in leukemia cells (97). In addition, blocking cholesterol efflux by SR-BI inhibitor, resulting in intracellular cholesterol accumulation, can also stimulate ER stress response and eventually lead to apoptosis (88).

Phospholipid metabolism in hematological malignancies

Phospholipids, as a large class of lipids, are the main components of biological membranes and important signaling molecules for cell proliferation and growth (98). Sphingosine 1-phosphate (S1P), which is generated by phosphorylation of sphingosine by sphingosine kinase 1 (SK1), is an important lipid metabolite that mediates cellular signal transduction (99). There have been numerous reports that S1P mediates tumor cell proliferation, invasion, angiogenesis, and anti-apoptosis (Figure 2) (100, 101). In addition, overexpression of SK1 also induces malignant transformation and promotes tumor proliferation (102). Michael S. Lee (103) found that S1P is up-regulated in MCL, and S1P can inhibit the activation of NK cells and allow MCL cells to escape immune immunity. Once targeting S1P or inhibiting SK1, the killing effect of NK cells can be restored on MCL cells, and accompanied by the up-regulation of cardiolipin, phosphatidylcholine, phosphatidylethanolamine and sphingomyelin, especially the up-regulation of cardiolipin suggests that cardiolipin induce the activation of NK cells. Furthermore, S1P has also been reported to interact with S1PR3 to promote osteosarcoma proliferation, anti-apoptosis and aerobic glycolysis through the YAP/c-MYC/PGAM1 axis (104).

Phospholipids also play an important role in the occurrence and development of tumor cells and the generation of drug resistance. SK1 is overexpressed in DLBCL, and its downstream product S1P can induce angiogenesis and promote cell proliferation and growth (105). For the increased expression of SK2 in large granular lymphocytic leukemia, inhibiting the expression of SK2 can lead to the degradation of the downstream pro-survival protein Mcl-1, and ultimately induce cell apoptosis (106). In addition to S1P, bioactive phospholipids such as ceramide 1-phosphate (C1P), lysophosphatidylcholine (LPC) and LPA also promote tumor progression. These bioactive phospholipids can stimulate the p42/44 MAPK and AKT signaling pathways. In addition, as substances that can inhibit the migration of hematological cells, HO-1 and iNOS can be down-regulated by bioactive phospholipids in a p38 MAPK-dependent manner, thereby promoting the migration and

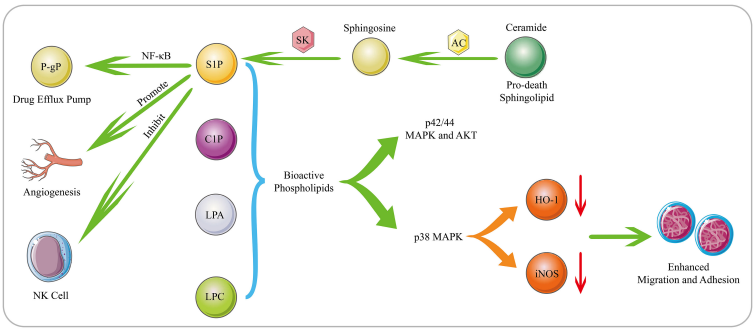

FIGURE 2
Biological Functions of Bioactive Phospholipids. S1P is generated through phosphorylation of sphingosine by SK. S1P mediates tumor cell proliferation, invasion, angiogenesis, drug resistance and immune escape. Moreover, AC can decompose the pro-death sphingolipid ceramide to generate sphingosine, which subsequently generates S1P. AC and S1P can activate the NF-κB pathway, and mediating the expression of the drug efflux pump P-gp. The bioactive phospholipids such as S1P, C1P, LPC and LPA can stimulate the p42/44 MAPK and AKT signaling pathways. Moreover, as substances that can inhibit the migration of hematological cells, HO-1 and iNOS can be down-regulated by bioactive phospholipids in a p38 MAPK-dependent manner, thereby promoting the migration and adhesion of human leukemia cells.

adhesion of human leukemia cells (107). Lysophospholipase D enzyme converts lysophospholipids into more water-soluble LPA, which activates GPCR-mediated signaling pathways and produces important lipid mediators. They are required to maintain chronic myelogenous leukaemia stem cells function *in vivo* (108). In MM patients, acid sphingomyelinase (ASM) is significantly up-regulated, which can lead to the increase of ceramide and the decrease of sphingomyelin to cause phospholipid metabolism disorders. The exosomes secreted by MM contain a large amount of ASM, which can make recipient cells resistant to melphalan and BZ (109). Overexpressed Acid ceramidase (AC) in AML can decompose pro-death sphingolipid ceramide to generate sphingosine and FFA, which are converted to S1P by SK1. AC and S1P together stimulate the activation of the NF-κB pathway, which in turn causes the expression of the ATP-binding cassette transporter P-gp. P-gp mediates the excretion of multiple drugs, ultimately conferring resistance to chemotherapeutics in AML (Figure 2) (110).

Conclusion

The rapid growth and continuous invasion of tumor cells require a large amount of energy supply, and metabolic reprogramming is commonly used to meet the material and energy requirements of tumor cells. As an important component of various biological membranes, lipids are also important substances involved in energy storage, production and cell signaling, and play an important role in cell physiological activities (111). Therefore, lipid metabolism reprogramming can be used to meet the material and energy required for rapid proliferation and growth of tumor cells, and lipid metabolism reprogramming has become one of the new markers of cancer (112). In this review, we summarize the proliferation, growth, differentiation, migration, invasion, apoptosis, drug resistance, immune escape and oncogenic mechanisms of tumor cells due to the lipid metabolism reprogramming in hematological malignancies, including FAs, cholesterol, and phospholipids. Tumor cells can increase lipid metabolism by enhancing endogenous lipid *de novo* synthesis pathway and exogenous lipid uptake, including the overexpression of FASN, ACC1, HMGCR, CD36, FABP and LDLR. Moreover, SREBPs have specific important roles in regulating lipid homeostasis, and SREBPs have three subtypes: SREBP-1a, SREBP-1c and SREBP-2. SREBP-1c mainly regulates the expression of genes required for FA synthesis, while SREBP-1a can regulate FA and cholesterol synthesis, as well as cholesterol uptake. SREBP-2 is relatively specific for the regulation of cholesterol synthesis and uptake (113). SREBPs need to be escorted by SCAP from the ER to the Golgi apparatus to perform their biological functions (114).

Lipid metabolism reprogramming plays an important role in the physiological activities of tumor cells, so targeting the lipid metabolism pathway of tumor cells has become an effective therapeutic approach. For example, inhibition of key rate-limiting enzymes in lipid biosynthesis reduces lipid synthesis, overstimulation of lipid biosynthesis causes ER stress, disruption of mitochondrial oxidative homeostasis causes mitochondrial stress, and blocking lipid-related signaling pathways causes signal pathway dysregulation. However, targeting lipid metabolism reprogramming still faces many challenges for the treatment of hematological malignancies. The main reason is that the relevant mechanisms of lipid metabolism are not fully revealed in hematological malignancies. Therefore, lipid synthesis, storage, utilization and efflux cannot be effectively regulated in hematological malignancies. Ferroptosis, which is a hot research topic in recent years, is also closely related to lipid metabolism.

Ferroptosis is a programmed cell death caused by excessive accumulation of iron-dependent lipid peroxidation and reactive oxygen species, and various hematological malignancies are sensitive to ferroptosis. Therefore, ferroptosis is also a promising therapeutic strategy for hematological malignancies. However, what we need to pay attention to is that glucose metabolism, lipid metabolism and amino acid metabolism are interconverted and affect each other, which is a complex connection. These results in that single inhibition of a certain metabolism cannot effectively inhibit the growth of tumor cells because the salvage of other metabolisms is activated. Tumor cells acquire the function of MYC through chromosomal translocations, gene amplifications and single nucleotide polymorphisms, causing a variety of metabolic dysregulations. For example, transporters and enzymes of glycolysis, fatty acid synthesis, glutaminolysis, serine metabolism and mitochondrial metabolism (115). The single inhibition of a downstream metabolic change is not effective in inhibiting cancer development. Therefore, combined inhibition of lipid metabolism, glucose metabolism and amino acid metabolism needs to be considered in clinical applications. In conclusion, understanding the oncogenic mechanism of lipid metabolism and targeting lipid metabolism reprogramming to find new therapeutic targets has important scientific significance and clinical application value. This review provides experience and direction for targeting lipid metabolism reprogramming in the treatment of hematological malignancies.

Author contributions

Conception and design: LZ, LS, and WC. Initial manuscript writing: LZ, NC, LS, and WC. Revision of the manuscript: JL, NC, ZL, and YW. Confirmation of Manuscript: LS and WC. 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.

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Fatty acid metabolism predicts prognosis and NK cell immunosurveillance of acute myeloid leukemia patients

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Background: Cell metabolic reprogramming is a hallmark of tumor prognosis, and fatty acid metabolism (FAM) plays a crucial role in the tumor microenvironment (TME). However, the relationship between FAM, TME, and prognosis of acute myeloid leukemia (AML) patients remains elusive.

Methods: We extracted the single-cell RNA sequencing (scRNA-Seq) and bulk transcriptome data of AML patients from the TCGA and GEO databases and assessed the relationship between FAM, TME, and AML patient prognosis. We also performed functional enrichment (FE) assay to evaluate the significance of FAM in anti-AML immunosurveillance.

Results: Our scRNA-Seq analysis revealed that the leukemic stem cell (LSC)-enriched population exhibited elevated levels of FAM-related genes. Using these FAM-related genes, we developed a prognostic model that accurately estimated AML patient outcome. FE analysis showed that FAM was strongly related to alterations of TME-based immunosurveillance in AML patients. More importantly, we demonstrated that FAM inhibition *via* pharmaceutical targeting of PLA2G4A, a highly expressed FAM gene in AML patients with poor prognosis, enhanced the NK cell-mediated immunosurveillance in leukemia cells.

Conclusions: Leukemic stem cell (LSC)-enriched population exhibited elevated levels of FAM-related genes. We have successfully established the FAM formula that predicts AML patient prognosis and alterations in the TME-based immunosurveillance. We also found that PLA2G4A was a highly expressed FAM gene in AML patients with poor prognoses. Pharmaceutical targeting of PLA2G4A increased the expression of NKG2DL in leukemia cells *in vitro* and thus enhanced the NK cell-mediated immunosurveillance.

KEYWORDS

acute myeloid leukemia, fatty acid metabolism, prognostic model, tumor micro environment, NK cells

Introduction

Acute myeloid leukemia (AML) is a widespread hematopoietic malignancy characterized by uncontrolled clonal expansion of primitive myeloid precursors (1). The global AML incidence has progressively increased over the years, with 75% of AML patients initially diagnosed after 60-year-old age (2). Although a diverse range of targeted therapy strategies has emerged in recent years, intensive chemotherapy remains the standard treatment for AML patients. Despite increasing complete remission (CR) rates, AML prognosis remains poor due to the high relapse rate and drug resistance (3).

Leukemic stem cell (LSC) is functionally defined by the ability to initiate and establish diseases upon transplantation (4). Similar to hematopoietic stem cells, LSCs stand at the top of the leukemia lineage by self-renewing and differentiating into proliferative leukemic cells (5). In addition, the quiescent LSCs are resistant to chemotherapeutic interventions, thus leading to their survival and disease re-establishment (6). Therefore, targeting the chemoresistance and self-renewal mechanism to eliminate LSCs is crucial for effective AML treatment.

Over time, research revealed that fatty acid metabolism (FAM) plays an essential role in AML cell survival and chemoresistance (7). Leukemic cells prefer to metabolize fatty acids to meet the augmented bioenergetic demands as fatty acid oxidation (FAO) generates more than twice as much ATP as glucose oxidation. LSCs have relatively low levels of prolyl hydroxylase 3 (PHD3), a crucial enzyme in glucose oxidation (8). Meanwhile, LSCs highly express the fatty acid transporter CD36 and the fatty acid-binding protein 4 (FABP4) that promotes fatty acid uptake and transport to fuel fatty acid lipolysis in bone marrow adipocytes (9–12), Given this evidence, targeting LSC metabolic vulnerabilities like FAM dependency may be a possible approach for eradicating chemoresistant LSCs and improving AML prognosis (13). However, the prognostic value of FAM-related genes and their relationship to the tumor microenvironment (TME) in AML are rarely reported and require further investigations (11).

Here, we identified that AML LSCs have high levels of FAM-related genes. We constructed a FAM prognostic model for better predicting AML patient outcomes and alterations in the TME-based immunosurveillance. More importantly, pharmaceutically targeting the FAM gene PLA2G4A enhanced the NK cell-mediated immunosurveillance by increasing NKG2D ligand expression in leukemia cells.

Materials and methods

Data acquisition and identification of the FAM-related genes

We gathered RNA sequencing data and corresponding clinical data from 553 AML patients on the GEO website (GSE37642, https://www.ncbi.nlm.nih.gov/geo/) and utilized them as the training cohort (TC) for analysis (Table S1). Similarly, the Cancer Genome Atlas (TCGA) dataset (n=140) with clinical information from the UCSC Xena (http://xena.ucsc.edu/) was used as the validation cohort (VC) (Table S2). FAM-related genes were obtained from the "c2.cp.kegg.v7.0.symbols". Genes were selected for further investigation only if they were listed in both the TC and VC. The genes involved in the FAM formula construction are presented in Table S3.

scRNA-Seq data processing

Raw data, with accession number GSM5400788, was downloaded from the Gene Expression Omnibus (GEO) websiteViable primary human AML cells (CD33+/CD45+/AnnexinV-) from mice bone marrow of PDX mice before chemotherapy, or accepted chemotherapy treatment were included in the analysis. The chemotherapy treatment consists in 5 days of AraC treatment (30 mg/kg for PDXs) by intra-peritoneal injection, or with 7 days of venetoclax treatment (100 mg/kg) by oral gavage We conducted normalization, dimensionality reduction, and clustering using the Seurat 3.2.3 R package. Cell filtration was done such that the system identified > 500 and < 5,000 genes and < 5% of total UMIs mapped to the mitochondrial genome. The data was normalized by dividing the UMI counts per gene into the total UMI counts in the corresponding cells, followed by log-transformation to achieve results, then scaling and centering. We next performed dimensionality reduction on the cells using Stochastic Neighbor Embedding (t-SNE). Pseudotime trajectory was then assessed *via* monocle2, depending on the Seurat clustering. Next, we retrieved the signature genes from each cluster with the Seurat function FindMarkers with the "wilcox" test. The Gene Ontology (GO) and plots were then employed *via* cluster Profiler and the ggplot2 R package. Lastly, gene summaries were pre-stratified by DEG fold change values *via* Seurat, and the gene sets were acquired *via* the GO database.

Construction and validation of the FAM formula

To establish an effective prognostic prediction model, we used the univariate Cox regression analysis of 553 GEO-LAML patients in the TC to construct the FAM formula. The clinical characteristics of these patients are presented in Table S1. The FAM formula was generated with data from the multivariate analysis with the lowest Akaike Information Criterion (AIC) value. Lastly, we computed the risk score of individual patients using the FAM formula as follows: Risk Score = e ^ sum (normalized individual FAM-associated gene levels multiplied by the corresponding regression coefficiency). The same FAM formula was used to compute the risk scores of the training cohort (TC) and validation cohort (VC) patients. Next, both TC and VC patients were stratified into a high risk (HR) or low risk (LR) cohort, based on the same threshold value. Subsequently, the Kaplan-Meier analysis was used to assess

overall survival (OS) between the HR and LR cohorts in VC. We next generated the predictive nomogram A hybrid using the "rms" R package that integrated both the FAM formula and corresponding clinical patient profile to estimate the AML patient OS at the 1-, 2-, and 3-year time points. Lastly, the calibration curve and consistency index (C-index) were employed to evaluate the predictive ability of the generated nomogram.

Gene set and functional enrichment analysis

GSEA v4.1.0 (http://software.broadinstitute.org/gsea/login.jsp) was employed to determine relevant physiological networks between the HR and LR cohorts, as evidenced by the FAM formula and c5.go.bp.v7.5.symbols gene sets. The GSEA analysis was performed in both the TC and VC. A nominal p-value < 0.05 was set as the significance threshold. The GO analysis was conducted to explore biological processes, while the Kyoto Encyclopedia of Genes and Genomes (KEGG) network analysis was conducted to determine signaling pathways.

Analysis of immune cells involved in leukemia

The XCELL algorithm was utilized to quantify various types of tumor-invading immune cells in AML patients from the TC and VC cohorts, and a p-value < 0.05 was considered significant. Next, we evaluated each category of immune cells to evaluate the differential tumor microenvironment (TME) profiles between the HR and LR cohorts.

mRNA isolation and qPCR

Total mRNA was extracted with the MagZolTM Reagent (R4801-03, Magen) following kit directions, and the transcript purity and quantification were evaluated *via* NanoDrop (Thermo Scientific) prior to qPCR. To conduct RT-qPCR, transcript samples were converted to cDNA with the TransScript All-in-One First-Strand cDNA Synthesis SuperMix (AT341, Transgen), and qPCR was carried out with the SYBR Green I Master Mix reagent (11203ES03, YEASEN) in the Bio-Rad CFX96 TouchTM Real-Time PCR Detection system. The primers used for the qPCR are listed in Table S4. The expression levels of the NKG2DL were normalized using GADPH as the internal control.

Apoptosis analysis

The three leukemia cell lines, including THP1 (CTCC-001-0044, Meisen), U937 (CTCC-001-0027, Meisen) and HL60 (CTCC-001-0025, Meisen), were grown in RPMI 1640 containing 10% FBS. After treatment with AACOCF3 (GC16115, GLPBIO) at 25μM for 48 hours, we evaluated cellular apoptosis with Annexin-V (640907, Biolegend) by flow cytometric analysis.

In vitro NK killing assay

NK92(CTCC-001-0016, Meisen) cell lines were grown in RPMI 1640 containing 10% FBS, 10% horse serum (Solarbio), and 100 ng/ml IL-2 (Peprotech).Firstly, we labelled the leukemia cells with CFSE for 30 minutes and washed off CFSE carefully. Then we treated AML cell lines with 5μM AACOCF3(GC16115, GLPBIO) for 48 hours, after which we washed off the remaining AACOCF3 and continued culturing in the presence or absence of NK92 cells for 60 hours before counting live leukemia cells by flow cytometric analysis. The killing efficiency of NK92 cells were calculated as follows: Killing efficiency= (the number of leukemia cells grown without NK92 cells - number of leukemia cells grown with the indicated frequency of NK92 cells)/number of leukemia cells grown without NK92 cells.

Statistical analyses

The Chi-squared test was used to evaluate the correlation between the FAM formula and the corresponding patient clinical profile. R (Version 4.1.0) and SPSS (Version 23.0) were employed for data analyses. Data from cell lines *in vitro* were compared with the Student's t tests, and $p < 0.05$ was set as the significance threshold.

Results

FAM-related genes are highly expressed in chemoresistant LSCs

AML patient-derived xenografted mice were treated with or without chemotherapy, and leukemia cells were collected and proceeded for scRNA-Seq (Figure 1A). We used these scRNASeq data deposited in the GEO website for t-SNE dimensionality reduction analysis and revealed 14 clusters based on their gene profile (Figure 1B). We next used the pseudotime ordering analysis to construct the cell lineage differentiation trajectory. Compared with other clusters, cluster 1 is located at the root of the trajectory (Figures 1C–E and Supplementary Figure 1A), and the cell number of cluster 1 is increased after chemotherapy (Figure 1F), suggesting that cluster 1 is the leukemic stem cell (LSC)-enriched population. We further checked whether LSC markers were enriched in cluster 1 before and after chemotherapy. We chose LILRB2 (14), VNN2 (15), and KLF4 (16) as LSC markers since they exhibited higher

expression levels in AML patients compared with normal volunteers in TCGA database and the Genotype-Tissue Expression (GTEx) project (Figure 1G). The number of LILRB2-high, VNN2-high and KLF-high cells in cluster 1 are 85, 73, and 234 respectively before chemotherapy and increased to 240, 227, and 480 after chemotherapy (Figures 1H–J). These results confirm that cluster 1 is the LSC population related to AML relapse. Subsequently, we chose the upregulated genes in cluster 1 to perform GO analysis and revealed that fatty acid metabolism (FAM) is highly enriched (Figure 1K). In summary, by analyzing the scRNA-Seq data we identified that cluster 1 is the AML LSC population and has upregulated FAM-related genes.

The FAM formula accurately predicts AML patient prognosis

We established a prognostic prediction model termed the FAM formula to elucidate the relationship between FAM and AML patient prognosis. The clinical features of our training cohorts (TC, GEO database, N=553) and validation cohorts (VC, TCGA database, N=140) are summarized in Tables S1, S2. First, we employed 201 FAM-related genes from the c2.all.v7.0.symbols (Table S3) to perform the univariate Cox regression analysis with the TC database and identified 27 genes with significant prognostic values (Figure 2A). Next, these genes were further analyzed with the LASSO Cox algorithm to construct a prognostic model (Figures 2B, C), which reduced the significant gene number to 18 (highlighted in Figure 2A). The risk score was calculated as follows: risk score = ACADS levels*(-0.0385781574616528) + ALDH2 levels*(0.115943870930126) + ACSL5 levels*(0.116279878492424) + GCDH levels*(-0.023543074746657) + ACSL3 levels*(0.0034823102467818) + SCD levels*(0.278308214066157) + HSD17B12 levels*(0.125378923880509) + SLC27A3 levels*(-0.247889819557295) + OLAH levels*0.329315900149358 + ACOT13 levels*(-0.119336558434181) + CYP4B1 levels*(-0.730901151964483) + ACOT8 levels*(-0.233925370148131) + MLYCD levels*(-0.29877179590025) + PTGS2 levels*(0.0130085245854858) + PLA2G4A levels*(0.177305593342932) + CBR1 levels*(0.273311180808465) + SLC22A5 levels*(0.00204092301483134) + LTC4S levels*(-0.00758524180721233).

We set the median value of the TC risk score calculated by the FAM formula as the threshold to distinguish between high risk (HR) and low risk (LR) cohorts. Data in Figures 2D, E illustrates the risk score distribution and survival status of the HR and LR cohorts in the TC and VC database. We Use the Kaplan-Meier analysis to demonstrate that the HR cohort experienced considerably worse overall survival (OS) than the LR cohort (Figures 2F, G, p-value<0.001). We validate the FAM formula by drawing ROC curve in which the area under ROC curve (AUC) is positively related with the prognosis accuracy. The AUC for the 1-year, 3-year, and 5-year survival rates in the TC were 0.696, 0.783, and 0.782, respectively (Figure 2H). The AUC for the 1-year, 3-year, and 5-year survival rates in the VC were 0.761, 0.795, and 0.810, respectively (Figure 2I). The AUC values in both the TC and VC are greater than 0.65, indicating that the FAM formula was highly effective in predicting AML patient prognosis.

Integrating clinicopathological characteristics into the FAM formula optimizes its predictive ability of AML patient prognosis

To optimize the predictive ability of the FAM formula, we combined the FAM formula with other clinicopathological characteristics to construct a prognostic nomogram. First, we compared the relationship between the FAM formula and the clinicopathological characteristics listed in Tables S1, S2, such as runx1 fusion protein, runx1 mutation, FAB subtype, age, platelet count, leukocyte count, blast cell count, and gender. As shown in Figures 3A, B, AML patients with younger age and M3 FAB subtype are more likely to have a FAM-based low risk score. To compare the sensitivity and specificity of the FAM formula with other clinicopathological characteristics in the prognostic model, we conducted ROC analysis and calculated the AUCs. The FAM formula risk scores were the highest in both the TC and VC, suggesting that FAM is most accurate for predicting AML prognosis (Figures 3C, D). We next used univariate and multivariate Cox regression analyses to test the independence of these signatures (Figures 3E, F). Based on our univariate analysis, the 18-gene FAM formula was strongly correlated with AML patient prognosis. In particular, in the TC database hazard ratio (HR) = 3.389, 95% confidence interval (CI) = 2.733–4.202, P< 0.001,and in the VC database HR = 3.117, 95% CI = 2.121–4.580. This 18-gene signature was determined to be an independent stand-alone risk factor by multivariate analysis for AML patient outcome in the TC: HR = 2.879, 95% CI = 2.291–3.617, P< 0.001, and the VC: HR = 3.142, 95% CI = 1.983–4.982. To optimize the clinical value and application probability of the FAM formula in predicting AML patient prognosis, we utilized the "rms" package of the R software to combine the clinicopathological characteristics with the risk score to generate a new signature named Combine. We generate a nomogram to compare these signatures for estimating the 1-, 2-, and 3-year prognosis of AML patients (Figure 3G). The consistency index (C-index) and the calibration curve of the nomogram were performed to evaluate the predictive efficiency and accuracy of the nomogram. In the Combine group, the C-index = 0.74 and represents the highest among all groups (Figure 3H), indicating that it has the best predictive ability. The calibration curve showed that the curves of 1-, 2-, and 3-year are very close to the diagonal dotted line, confirming that the Combine signature has a high predictive ability in the nomogram (Figure 3I). In summary, the Combine signature that incorporates the FAM formula and clinicopathological profiles of patients has excellent stability and

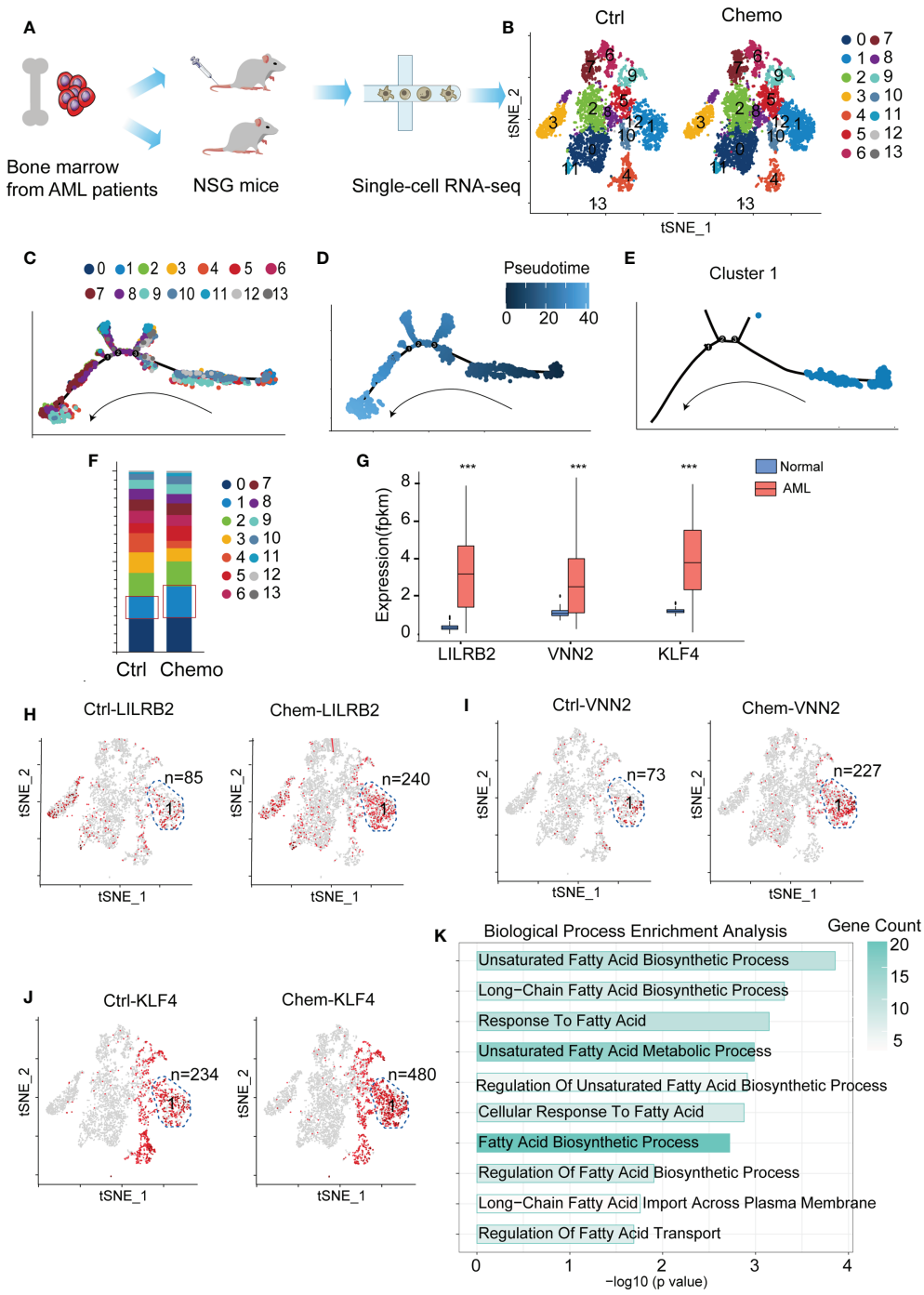

FIGURE 1
Fatty acid metabolism (FAM)-related genes are highly enriched in chemoresistant leukemic stem cells (LSCs). **(A)** A flow chart depicting the process of scRNA-Seq data acquisition. **(B)** Cluster the untreated (n=2 mice) and chemotherapy-treated leukemia cells (n=4 mice) with the t-SNE dimensionality reduction analysis. **(C–E)** The pseudotime trajectory analysis of the cells in the chemotherapy group. **(F)** Cell quantification of each cluster before and after chemotherapy. **(G)** Bar plot depicting expressions of KLF4, VNN2, and LILRB2 in AML patients and normal people. The p-value was calculated by unpaired two-tailed Student t-tests, $p < 0.05$, $p < 0.01$, $p < 0.001$. **(H–J)** LSC markers (LILRB2, VNN2 and KLF4) are enriched in the cells of cluster 1. The LILRB-, VNN2-, and KLF4-high cells are highlighted in red, and the n number depicts the red cell number in cluster 1 before and after chemotherapy. **(K)** GO enrichment of the upregulated genes in cluster 1. Data are displayed as specific values or mean ± SD.

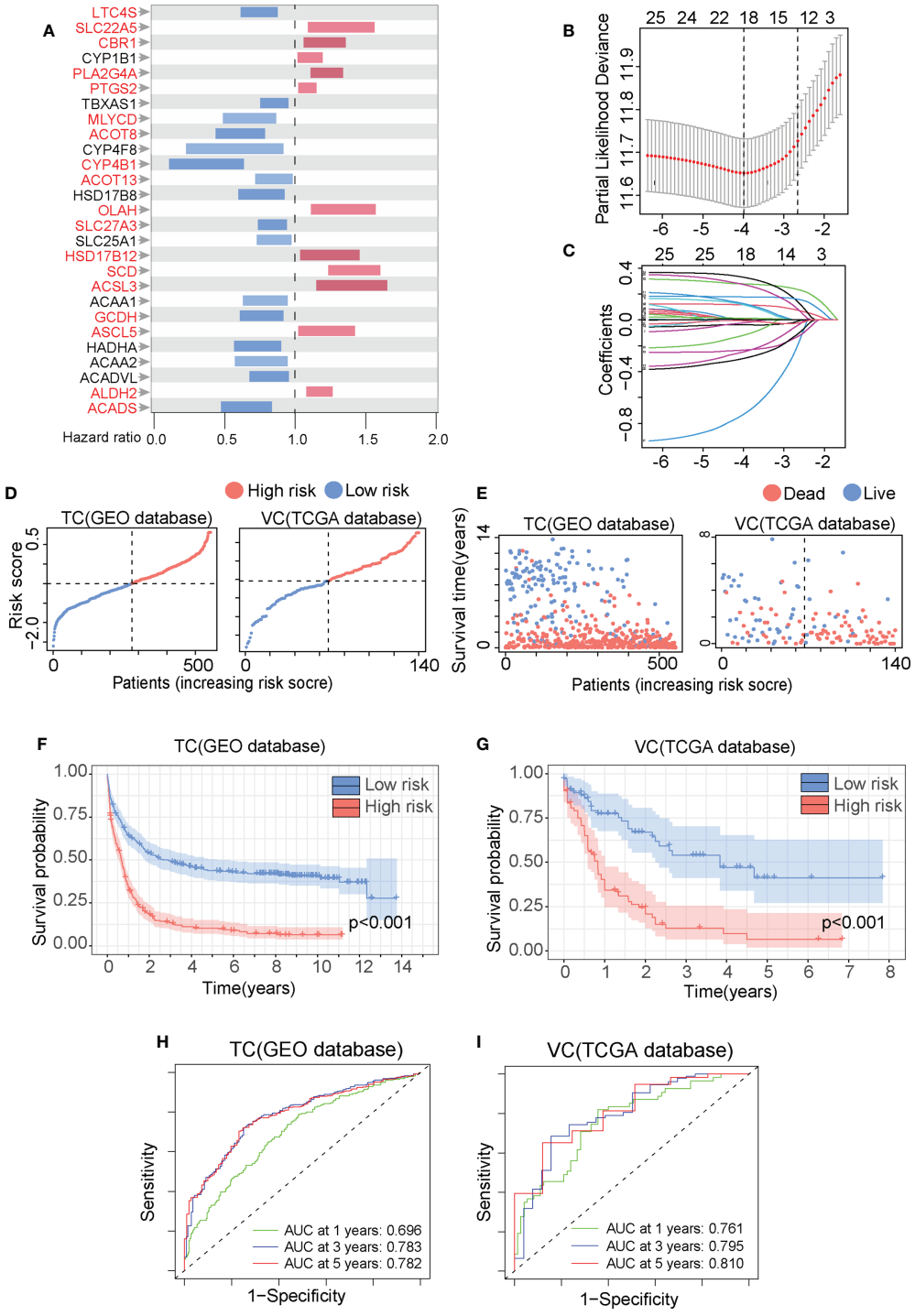

FIGURE 2
The fatty acid metabolism (FAM) formula accurately predicts AML patient prognosis. **(A)** The overall survival (OS) of 553AML patients in the GEO database was analyzed by the univariate Cox regression with the 201 FAM-related genes and summarized in Forest plots. **(B)** The FAM-related genes were analyzed by the least absolute shrinkage and selection operator (LASSO) regression model based on the minimal criteria. **(C)** The FAM-related gene in the LASSO regression analysis was calculated for coefficient. **(D, E)** The metabolic risk score distribution **(D)** and the survival outcome (SO) analysis I of the training cohorts (TC) and validation cohorts (VC). **(F, G)** The Kaplan-Meier survival curves of the HR and LR patients in the TC and VC. **(H, I)** The time-dependent ROC analyses of the FAM prognostic model to estimate the 1-, 3-, and 5-year OS of TC and VC patients.

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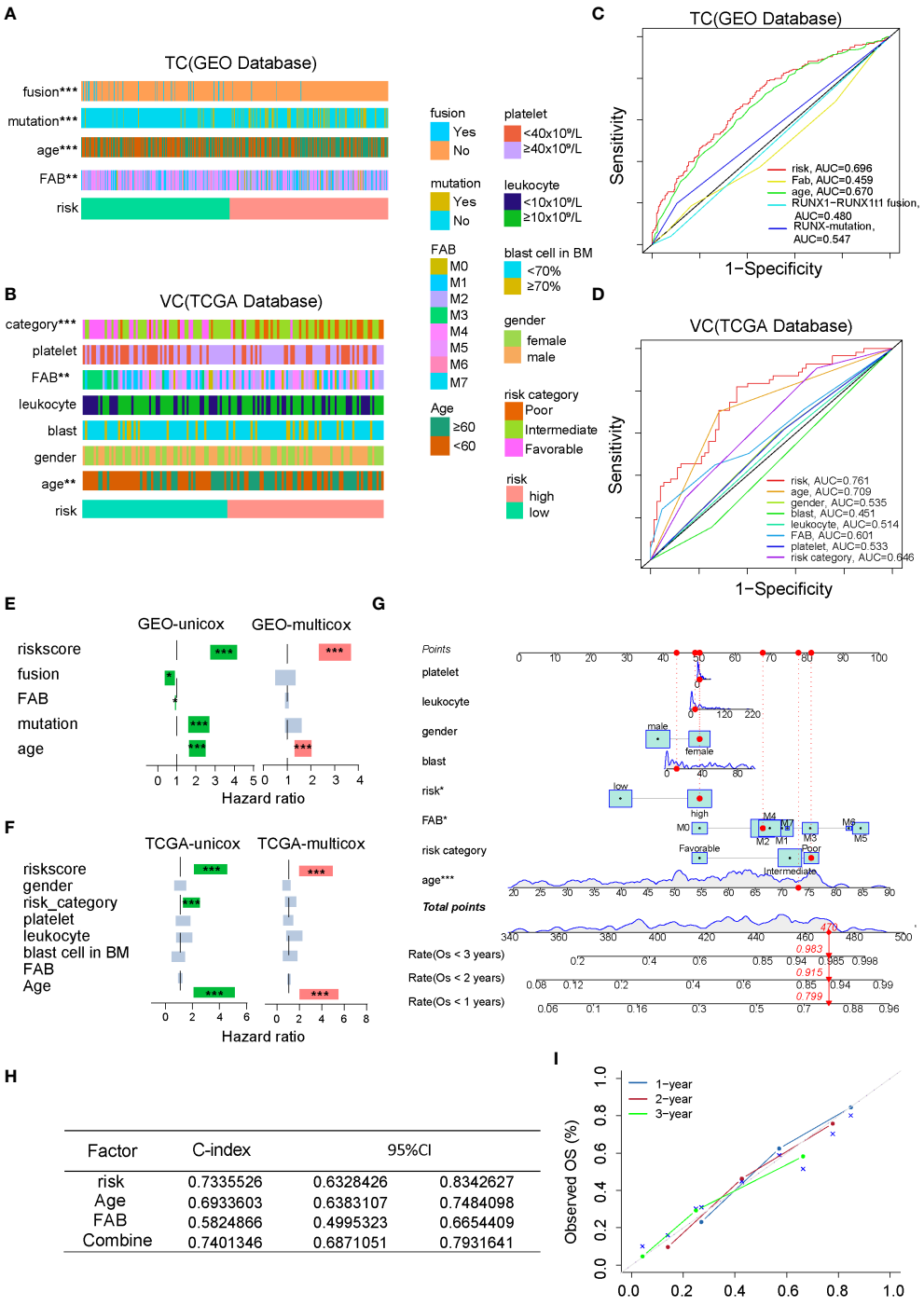

Factor	C-index	95%CI	
risk	0.7335526	0.6328426	0.8342627
Age	0.6933603	0.6383107	0.7484098
FAB	0.5824866	0.4995323	0.6654409
Combine	0.7401346	0.6871051	0.7931641

FIGURE 3
Combining the clinicopathological characteristics with the FAM formula optimizes the predictive capacity of the nomogram. **(A, B)** A strap plot summarized various clinicopathological features and FAM-related risk score, and the correlation between clinical features and the FAM risk score was analyzed by chisq.test. *p < 0.05,**p < 0.01, ***p < 0.001. **(C, D)** Evaluation of the prognostic prediction accuracy *via* the area under the time-dependent receiver operating characteristic (ROC) curve (AUC). **(E, F)** Stand-alone prognostic ability of the FAM formula or clinical features in the TC and VC. **(G)** Nomogram plot, based on the risk score and other clinicopathological patient profiles in the VC. **(H)** The consistency index (C-index) of the nomogram. **(I)** The calibration curve of the nomogram.

accuracy in predicting AML prognosis, suggesting that it has the potential for application in clinics.

The FAM formula predicts alterations in the tumor microenvironment (TME)

To determine the underlying mechanism behind the opposite prognoses in the HR and LR cohorts, we performed the functional enrichment (FE) analysis. We first compared the HR and LR cohorts to identify the differentially expressed genes (DEGs) with the p-value cut off < 0.05. We then conducted Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Set Enrichment Assay (GSEA) to establish underlying enriched processes. Based on our GO analysis, DEGs between LR and HR patients in the TC and VC databases were primarily enriched in immune-related processes (Figure 4A; Supplementary Figure 2). In addition, KEGG analysis revealed that immune-regulatory pathways, including IL-17 signaling and NF-κB signaling, were highly enriched in the HR cohort (Figure 4B). And GSEA analysis revealed that the immune-related biological processes were highly enriched in the HR cohort (Figures 4C–F; Supplementary Figures 2C–F). As tumor microenvironment (TME) plays an important role in the development, proliferation, and survival of leukemia blasts (17), we next analyzed TME of AML patients. Based on the signaling patterns of the HR and LR patients, the XCELL algorithms (18) can delineate various immune cell populations located in the TME. As shown in Figure 4G and Supplementary Figure 2G, both the VC and TC demonstrated alteration in immune cell composition, with an evident NKT cell decrease in the HR cohort. Collectively, TME is altered in HR cohorts, which may be a critical factor in determining AML patient prognosis and providing a chance to foster novel AML treatment strategies.

PLA2G4A inhibition enhances the killing efficiency of NK cells against LSCs

Based on the aforementioned FE analysis, we speculated that alteration in the immune microenvironment might be a critical factor in determining AML prognosis. To assess whether FAM regulates AML immune microenvironment, we performed overall survival (OS) analysis with the single gene of the FAM formula and found that 7 out of 18 genes have significant predictive value on AML patient prognosis with a p-value cut-off <0.05 in VC (TCGA database). Among them, enrichment of PLA2G4A most strongly represented the poor prognosis (Figures 5A, B, S3). Then, we compared the expression of eighteen FAM formula genes between AML patients and normal individuals. PLA2G4A, a member of the cytosolic phospholipase that catalyzes the hydrolysis of membrane phospholipids to release arachidonic acid, increased by 7.21 fold, representing one of the most upregulated genes in AML patients with poor prognosis (Figures 2A, 5A). These results indicate that PLA2G4A may be a crucial FAM enzyme involved in AML progress. Thus, we treated leukemia cell lines THP1, U937, and HL60 with PLA2G4A inhibitor AACOCF3, an analog of arachidonic acid that inhibits the PLA2G4A phospholipase activity by competing for the active catalytic site. AACOCF3 is the trifluoromethyl ketone derivative of arachidonic acid. This compound is a selective inhibitor of soluble PLA2 and Ca2+ independent PLA2 in human cells. (19, 20). The IC50 values of THP1, U937, and HL60 are 31.58μM, 42.38μM, and 36.72μM, respectively, indicating that AACOCF3 had a low cytotoxic effect on leukemia cells at low dosages but induced cell death at high dosages (Figures 5C–E). In parallel, Annexin-V+ apoptotic cell number was highly increased in THP1, U937, and HL60 cells when exposed to the high concentration of AACOCF3 (25μM) (Figures 5F–H), suggesting that high dose AACOCF3 directly induced leukemia cells death. As we identified an evident decrease of NKT cells in the high-risk TME, we examined the effect of PLA2G4A inhibition on NK-mediated cytotoxicity against leukemia cells. We labeled THP1, U937, and HL60 cells with CFSE and treated them with low dose AACOCF3 (5μM), in which leukemia cells should still be viable according to the survival curve in Figures 5C–E. We then co-cultured AACOCF3 pretreated leukemia cells with NK cell line NK92 before conducting FACS and quantitative PCR analyses (Figure 5I). AACOCF3 treatment significantly enhanced the cytotoxicity of NK92 against leukemia cells (Figures 5J–L). The NK group 2D (NKG2D) is a cell surface receptor to activate the NK-mediated cytotoxic effect when binding to NKG2D ligands such as MICA, MICB, and ULBP family members. We examined the expression of NKG2D ligands in leukemia cell lines treated with low concentration AACOCF3 (5μM) and found that expression levels of the MICA, MICB, and ULBP family were all significantly elevated (Figures 5M–O). Overall, our results revealed that inhibiting phospholipase PLA2G4A enhances NK-mediated immunosurveillance toward leukemia cells.

Discussion

Our findings demonstrated that the leukemic stem cell (LSC)-enriched population exhibited elevated levels of FAM genes. Using these FAM genes, we constructed a prognostic model that accurately evaluates AML patient prognosis. We also observed that FAM correlates with immunosurveillance alteration in AML patients. Pharmaceutically inhibiting the FAM enzyme PLA2G4A increased expressions of NKG2D ligands in leukemia cells, thus enhancing NK cell-mediated cytotoxicity against leukemia cells.

Although studies on metabolic reprogramming during AML progression are gradually increasing (21), the effect of metabolic drugs on life expectancy is generally limited. Fatty acid metabolism

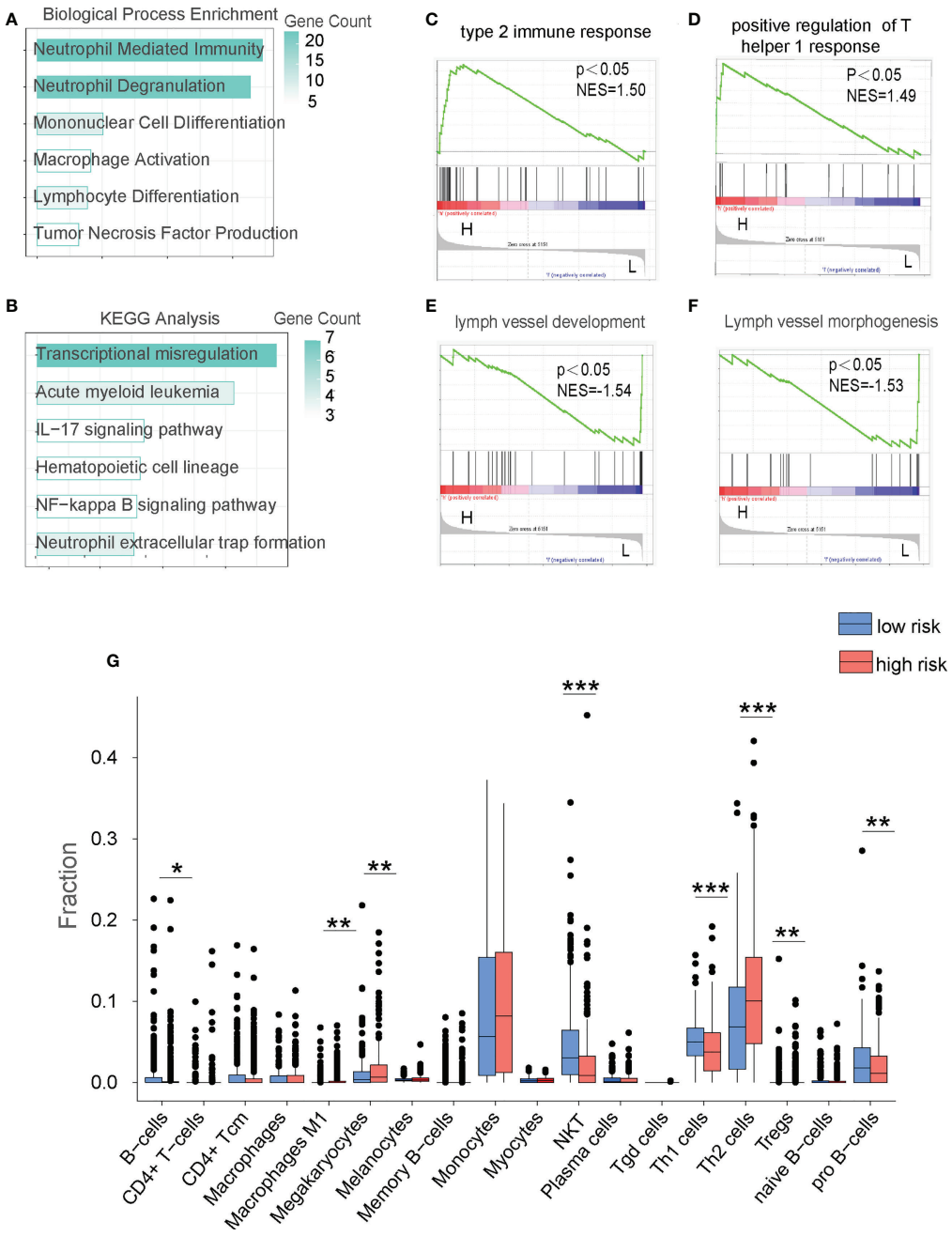

FIGURE 4
The FAM formula identifies alterations in the immune microenvironment of AML patients in the TC database. **(A)** GO analysis of the low risk (LR) and high risk (HR) cohorts in the TC. **(B)** KEGG network analysis of the LR and HR cohorts in the TC. **(C–F)** GSEA analysis of LR and HR cohorts in the TC. **(G)** Analyze the immune cell populations of the LR and HR patients using the XCELL algorithm in the TC. *p < 0.05,**p < 0.01, ***p < 0.001.

(FAM) is a well-recognized hallmark of AML prognosis (22), but the relationship and the underlying molecular mechanisms between FAM-related genes and AML prognosis are not elucidated. Herein, we analyzed the scRNA-Seq data of patient-derived xenografted AML cells and identified that the LSC-enriched population has elevated expression of FAM-related genes. Based on the hypothesis that FAM is required for AML progression, we constructed a prognostic model named the FAM formula composed of eighteen FAM-related genes to predict AML patient prognosis. We have performed the univariate cox

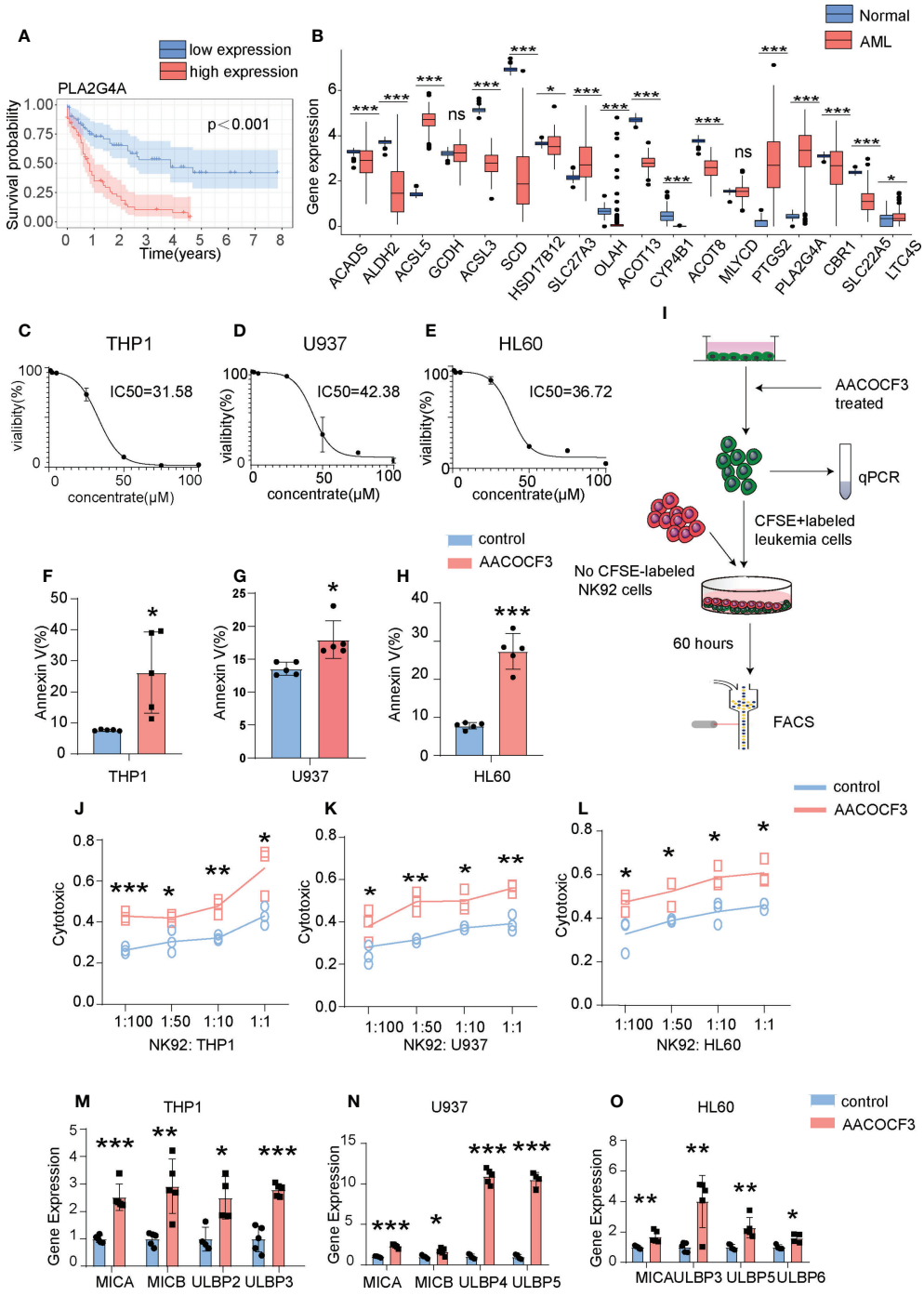

FIGURE 5
PLA2G4A inhibition enhances NK cell-mediated cytotoxicity against AML cells. **(A)** Kaplan-Meier analysis of patients in the VC based on PLA2G4A expression. **(B)** The Barplot illustrates the expression levels of individual FAM formula genes between leukemia and normal groups from the TCGA database. **(C–E)** Three leukemia cell lines (THP1, U937 and HL60) were treated with increasing dosages of PLA2G4A inhibitor AACOCF3 for recording the cell viability (n=3 independent experiments). **(F–H)** Apoptotic analysis of THP1, U937 or HL60 cells (n=5 independent experiments). **(I)** A flow chart depicting leukemia cell lines co-cultured with NK92 cells, followed by FACS analysis. **(J–L)** THP1, U937, or HL60 cells was treated with or without AACOCF3 before co-culturing with NK92 cells, and leukemic cell cytotoxicity was calculated (n=3 independent experiments). **(M–O)** Realtime PCR results show the expression of NKG2DL genes in leukemia cell lines treated with the PLA2G4A inhibitor AACOCF3 (n=3 independent experiments). All experiments were analyzed using unpaired two-tailed Student's t-tests. *P<0.05, **P<0.01, ***P<0.001. ns, no significance.

regression analysis and found that some FAM genes like ACADS, GCDH, SLC27A3, ACOT13, LTC4S were considered the protective factor with favorable prognosis. In contrast, other FAM genes like ALDH2, ASCL5, ASCL3, SCD, HSD17B12, OLAH, CYP4B1, ACOT8, MLYCD, PTGS2, PLA2G4A, CBR1 and SLC22A5 were considered as risk genes with poor prognosis. Among these genes, high expressions of ALDH2, SCD, and PLA2G4A were experimentally confirmed as the risk genes with poor prognosis *in vitro* or *in vivo* (20). ALDH2, the aldehyde dehydrogenase in the mitochondria of leukemia cells that suppresses formaldehyde accumulation, was closely related to AML relapse (23). SCD is the enzyme that converts saturated fatty acids to monounsaturated fatty acids (24). Inhibiting the activity of SCD induces leukemia cell apoptosis and may be a novel way to eradicate leukemia stem cells. Interestingly, PLA2G4A is a biomarker predicting the poor prognosis of AML patients (20), which is in line with our findings (Figure 5A). Furthermore, we compared the clinicopathological characteristics with FAM-predicted risk factors and found that the poor prognosis factors, such as runx1 mutation and old age, are highly enriched in the high-risk (HR) cohort (Figures 3A, B), confirming that FAM indeed plays a role in AML progression. We further integrated the FAM formula with clinical characteristics to construct the Combine formula that more accurately predicts AML prognosis and might have the potential for clinical application in the future.

More importantly, our mechanistic study identified that FAM in LSCs influences AML progression by suppressing the immune microenvironment. LSCs require an immunosuppressive microenvironment for survival during chemotherapy and disease re-establishment (25). Stromal cells like MSCs secrete TGFβ to reduce NKG2D expression and inactivate NK cells and other T cell subpopulations (26). Moreover, LSCs themselves downregulate NKG2D ligand expressions to escape NK-mediated immunosurveillance (27, 28). Our results in Figure 4B and Supplementary Figure 2G indicate that the HR AML patients identified by the FAM formula exhibited markedly reduced NKT cells and other immune cell populations in both the training cohort (TC) and validation cohort (VC). These results suggest that immune microenvironment alteration correlates with AML patient prognosis. More importantly, we found that FAM suppression by pharmaceutically targeting PLA2G4A enhanced NK cell immunosurveillance towards AML cells *in vitro*. Mechanistically, PLA2G4A inhibition upregulated NKG2D ligand expressions for boosting NK-mediated anti-leukemic cytotoxicity. Taken together, our study revealed that FAM suppression might be a novel strategy for optimizing AML treatment by enhancing immune surveillance.

Data availability statement

The original contributions presented in the study are included in the article and Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

Both TCGA database, GEO database, and Gtex database belong to public databases and the patients involved in these databases have obtained ethical approval. There are no ethical issues and other conflicts of interest occur in our study.

Author contributions

ZY performed most of the experiments. XT, YW, and KL collected the data from the database and wrote the manuscript. YY and YL analyzed the data. KY, ZQ, and JL cultured the cells. MZ, DL, and XX designed the present study, analyzed the data and revised 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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.1018154/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Pseudotime trajectory analysis of the cells in the chemotherapy group. The pseudotime trajectory analysis of each cluster in the chemotherapy group.

SUPPLEMENTARY FIGURE 2
The FAM formula identifies alterations in the immune microenvironment of AML patients in VC database. **(A)** GO analysis of the low risk (LR) and high risk (HR) cohorts in the VC. **(B)** KEGG network analysis of the LR and HR cohorts in the VC. **(C–F)** GSEA analysis of LR and HR cohorts in the VC. **(G)** Analyze the immune cell populations of the LR and HR patients in the VC using the XCELL algorithm.

SUPPLEMENTARY FIGURE 3
Kaplan-Meier analysis of AML patients based on the expression level of individual FAM-related genes. Kaplan-Meier analysis of AML patients in the VC based on the expression levels of SCD, SLC22A5, ACADS, MLYCD, ACSL3, and CBR1. Significance: *P<0.05, **P<0.01, ***P<0.001.

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Establishment of a risk model correlated with metabolism based on RNA-binding proteins associated with cell pyroptosis in acute myeloid leukemia

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Background: RNA-binding protein (RBP) regulates acute myeloid leukemia (AML) by participating in mRNA editing and modification. Pyroptosis also plays an immunomodulatory function in AML. Therefore, this study aimed to identify pyroptosis-related RBP genes that could predict the prognosis of AML patients.

Methods: AML related expression data were downloaded from the UCSC website and Gene Expression Omnibus (GEO) database. Pyroptosis-RPB-related differentially expressed genes (PRBP-DEGs) were conducted with a protein-protein interactions (PPI) network to screen out the key PRBP-DEGs, based on which a risk model was constructed by Cox analysis, and evaluated by plotting Receiver operating characteristic (ROC) curves and survival curves. Independent prognostic analysis was performed and a nomogram was constructed. Finally, enrichment analysis was performed for high and low risk groups.

Reuslts: A total of 71 PRBP-DEGs were obtained and a pyroptosis-RPB-related risk model was constructed based on IFIT5, MRPL14, MRPL21, MRPL39, MVP, and PUSL1 acquired from Cox analysis. RiskScore, age, and cytogenetics risk category were identified as independent prognostic factors, and the nomogram based on these independent prognostic factors could accurately predict 1-, 3- and 5-year survival of AML patients. Gene set enrichment analysis (GSEA) showed that the high-risk and low-risk groups were mainly enriched in metabolic- and immune-related processes and pathways.

Conclusion: In this study, a risk score model correlated with metabolism based on RNA-binding proteins associated with cell pyroptosis in acute myeloid leukemia was established, which provided a theoretical basis and reference value for therapeutic studies and prognosis of AML.

KEYWORDS

acute myeloid leukemia, pyroptosis, prognostic model, RNA-binding protein, metabolism

Introduction

Acute myeloid leukemia (AML) is a clonal malignant proliferative disease of myeloid primitive cells in the hematopoietic system, and it is highly heterogeneous. AML can be transformed into malignant changes of hematopoietic progenitors at different stages of normal myeloid cells (1). Currently, the treatment of AML mainly includes chemotherapy, biologics, and hematopoietic stem cell transplantation (HSCT) (2), but about 70% of patients who achieve remission will eventually relapse or evolve into refractory leukemia, leading to treatment failure and death (3). The prognosis and survival rate of AML prognosis are unsatisfactory, and it has been reported the 5-year overall survival (OS) ratein young AML patients is less than 50%, and the 2-year OS rate in older patients after diagnosis is only 20% (4).

Pyroptosis, also known as cell inflammatory necrosis, is a programmed cell death (5), It is mainly manifested as the cell membrane rupture, leading to the release of cell contents and then activation of strong inflammatory response (6). Pyroptosis plays an important role in the fight against infection, and it is widely involved in the development of infectious diseases and nervous system-related diseases (7). Moreover, Johnson et al. found that small-molecule inhibitors of the serine dipeptidases DPP8 and DPP9 (DPP8/9) induced-pyroptosis in mouse and human monocytes and macrophages for treatment of AML, it also shown that there is a strong correlation between pyroptosis and antileukemic therapy (8).

RNA-binding proteins (RBPs) are a general term for a group of proteins that perform their functions by specifically binding to RNA. To date, the human genome-wide screen has identified 1,542 RBP genes, accounting for 7.5% of all egg and white matter-encoding genes (9). RBP plays a crucial role in processes such as RNA maturation, transport, localization and translation, and it is also vital in gene expression and maintenance of genomic integrity (10, 11). Currently, Kharas et al. found that RBPs of the musashi-2 regulates normal hematopoiesis and promotes aggressive myeloid leukemia, it may be as a new prognostic marker and target for therapy in AML. However, there are still few reports on the relationship between pyroptosis and RBPs (12).

Cell pyroptosis is closely related to RBPs. Mast cells can identify the nucleic acid fragments (DNA or RNA), bacterial cell wall components (LPS), and flagella of these pathogenic microorganisms, thus stimulating immune measures, which can lead to the pathogen elimination by immune cells (13). However, there was no report on the RBPs associated with cell pyroptosis in patients with AML in public database. In this study, the RBP genes related to cell pyroptosis in AML patients were studied by bioinformatics analysis methods, and they were constructed and verified by the prognostic feature model, providing new ideas for clinical treatment.

Materials and methods

Data source

Gene expression data, survival information, and clinical information of AML patients were obtained from the UCSC Xena website (http://xena.ucsc.edu/), which has 173 AML samples and 70 normal samples, of which 161 AML samples have survival information and clinical information. The GSE37642 dataset was downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/), which comprises 136 AML samples with survival information. In addition, 33 pyroptosis-related genes (14) and 1542 RBP-related genes (RBPGs) (9) were available in the published literature.

Identification and enrichment analysis of pyroptosis-RPB-related differentially expressed genes (PRBP-DEGs)

Differential analysis was performed on 173 AML samples and 70 normal samples in the UCSC Xena dataset using the "limma" package (15), and the threshold for DEGs was set at adj.$p< 0.05$ and $|\log_2$fold change (FC)$| > 2$. Then, Pearson's correlation between pyroptosis-related genes and RBP genes was calculated. The Benjamini & Hochberg method was used for multiple test correction, and the RBP genes related to pyroptosis

were screened according to |r| > 0.9 and q value< 0.05, and they were intersected with the above DEGs to obtain the PRBP DEGs related to pyroptosis. Finally, the Gene ontology (GO) system and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed for the PRBP DEGs using the "cluterProfiler" R package with a significance threshold of $p< 0.05$ (16).

Construction of a protein-protein interactions (PPI) network

To investigate the interactions between PRBP DEGs, a PPI network was constructed for PRBP DEGs using the STRING website. The confidence was set to 0.4, and the protein interaction pairs were obtained by removing discrete proteins, and the protein network graph was constructed by Cytoscape software.

Construction of the risk model

The 161 AML patients from the UCSC Xena datasetwere used as the training set, and a univariate Cox regression analysis was performed using the PRBP DEGs in the PPI network. Then the multivariate Cox regression analysis was performed based on the factors with $p< 0.05$ in the univariate Cox analysis to obtain prognostic biomarkers. Risk values were calculated for each patient using the formula: *Riskscore* $\sum_{i=1}^{n} coef \times id$. Subsequently, 161 AML patients in the training set were divided into high and low risk groups based on the median risk values, and risk curves were plotted for the risk model. In addition, this study used the "pheatmap" package to plot biomarker expression heat maps in high and low risk groups. Finally, survival analysis was performed for the high- and low-risk groups, and ROC curves were plotted using the "survivalROC" package.

Independent prognostic analysis and construction of a nomogram

To further investigate the prognosis of clinicopathological characteristics with the risk model, clinicopathological factors (RiskScore, cytogenetics risk category, age, platelet result count, gender, race, prior malignancy diagnoses, and prior treatment diagnoses) were included in univariate and multivariate Cox analyses to explore the independent prognosis of the risk model and clinicopathological factors. Then, the "RMS" R package was used to construct a nomogram for the risk model and other clinical factors at 1, 3, and 5 years (17). Finally, the validity of the nomogram was verified by plotting the calibration curve of the nomogram with the "survival" package.

Functional enrichment analysis

The KEGG Pathway and GO biological process gene sets were used as enrichment backgrounds, and the high and low risk groups were used as phenotype files. Enrichment analysis was performed based on the different multiples of High-risk and Low-risk to obtain the up- and down-regulated pathways or functions involved in genes that differed between high- and low- risk groups, and the significant enrichment threshold was set at NOM P< 0.05. The top 10 enrichment results for each phenotype of GO biological process and KEGG pathway were selected and presented according to the ranking of NOM P values. In addition, the "limma" package was used to identify DEGs between high- and low-risk groups (15). The threshold of DEGs was set as adj. $p< 0.05$ and $|\log_2$fold change (FC)$| > 1$. Then, DAVID was used to analyze the GO and KEGG pathways in which the DEGs were involved. $p< 0.05$ and count > 2 were considered as significant enrichment results.

Results

71 PRBP-DEGs were enriched to 107 GO and 8 KEGG pathway

There were 18045 DEGs between AML and normal samples, with 12613 up-regulated genes and 5432 down-regulated genes (Figures 1A, B). Pearson correlation analysis showed that 124 pyroptosis genes were associated with RBP genes. The DEGs were crossed with RBP genes associated with pyroptosis-RPB to obtain 71 PRBP-DEGs (Figure 1C), and they were enriched to 74 GO biological processes (GO BPs), 16 GO cell components (GO CCs), 17 GO molecular fFunctions (GO MFs), and 8 KEGG pathway, including cellular protein complex disassembly, RNA catabolic process, RNA transport, RNA destabilization, negative regulation of protein acetylation, ribonucleoprotein complex assembly, protein export from nucleus, and other protein-related pathways (Figure 1D; Supplementary Tables 1-4).

Construction of a PPI network

The PPI network included 58 nodes with 145 protein interaction pairs. The connectivity degree of MRPL40, MRPS24, MRPL21, SNRPD2, and SNRPG was high. In addition, MBNL3 was associated with ZC3H12D, ZC3HAV1L, and CELF2. MRPS12 interacts with DDX60, MRPL14, MRPL21, and MRPL39, etc. (Figure 2).

Construction of the risk model

23 PRBP-DEGs with prognostic merit were identified by univariate Cox analysis (Table 1; Figure 3A). 6 biomarkers

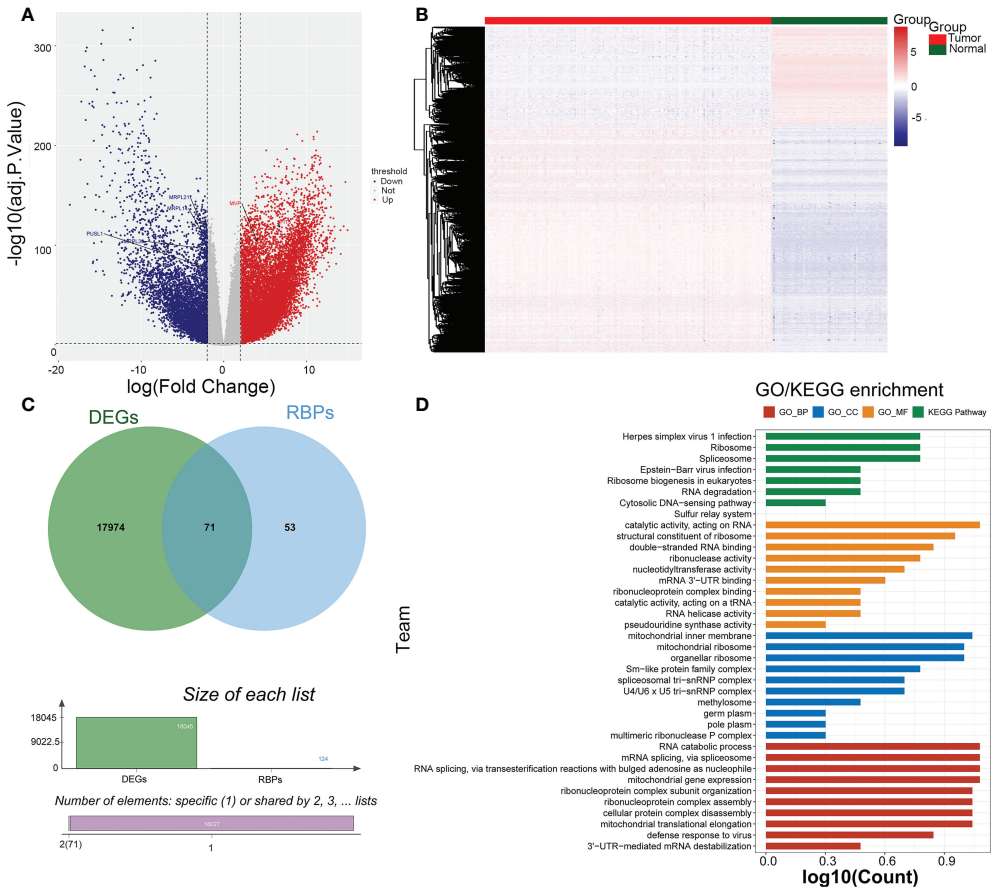

FIGURE 1
Identification of pyroptosis-RNA-binding protein (RBP)-related differentially expressed genes (PRBP-DEGs). **(A)** Volcano plot of PRBP-DEGs between AML and normal samples, the red and blue dots indicate up-regulated and down-regulated genes with adj.$p < 0.05$ and $|\log_2$fold change (FC)$| > 2$ setting as criteria. **(B)** Heatmap of PRBP-DEGs in AML and normal samples. **(C)** A Venn-gram of DEGs and pyroptosis-related RPB genes. **(D)** Bar plot of Gene Ontology (GO) enrichment analysis and Genes and Genome (KEGG) pathway analysis, $p < 0.05$ were set as criteria.

(IFIT5, MRPL14, MRPL21, MRPL39, MVP, and PUSL1) were further detected by enrolling the 23 PRBP-DEGs in multivariate Cox analysis (Tables 1, 2; Figures 3A, B). Among them, MRPL14 was a protective factor (HR< 1) and the rest of the genes were risk factors (HR > 1). The risk score of each sample was calculated as follows: Risk score = 0.287637545 × IFIT5 + (-0.40877001) × MRPL14 + 0.623197758 × MRPL21+ 0.697194958× MRPL39 + 0.39822836 × MVP + 0.545513896 × PUSL1, and 161 AML patients in the training set were divided into high and low risk groups by the median risk score (1.037), including 80 samples in the high-risk group and 81 samples in the low-risk group. Subsequently, we conducted principal component analysis (PCA) and t-distributed Stochastic Neighbor Embedding (tSNE) analysis on two subgroups, the results showed that the distribution of patients between high and low risk groups had clear pattern (Supplementary Figure 1). The risk curve was shown in Figure 3C. The expression of biomarkers in the high- and low-risk groups indicated that IFIT5, MRPL14, MRPL21, MRPL39, MVP, and PUSL1 were more expressed in the high-risk group (Figure 3D).

The survival rate of patients in the high-risk group was lower (Figure 3E). The area under the curve (AUC) values for 1, 3, and 5 years were 0.804, 0.734, and 0.741 in the ROC curves, respectively, indicating that the constructed risk model could be effectively used as a prognostic model (Figure 3F). In addition, the GSE37642 validation set verified the applicability of the risk model. (Figures 4A-D)

Independent prognostic analysis and construction of a nomogram

The results of univariate Cox analysis indicated that RiskScore, age, and cytogenetics risk category were associated

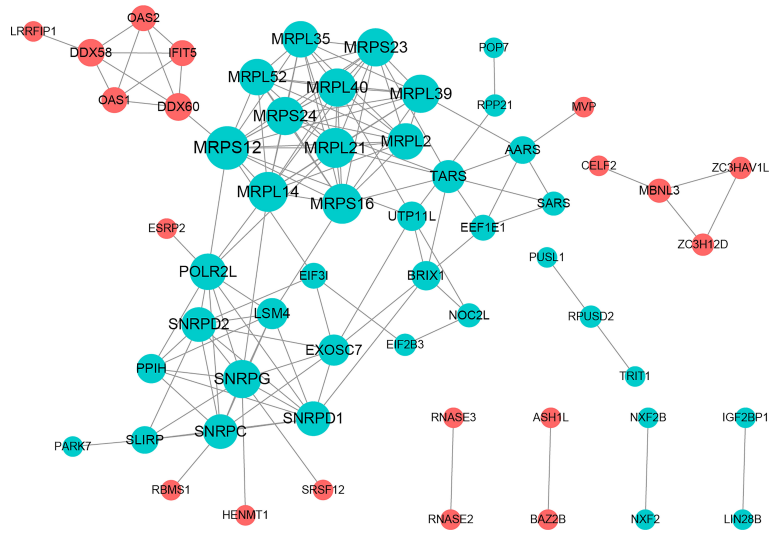

FIGURE 2
Protein-protein interactions (PPI) network constructed with PRBP-DEGs by STRING.

TABLE 1 Univariate Cox analysis to construction of the risk model.

	HR	lower.95	upper.95	p.val
SARS	3.117488934	1.712043071	5.676689692	0.00020056
PUSL1	1.838609238	1.331883653	2.538122547	0.000213762
MVP	1.461761066	1.183495432	1.80545303	0.000425672
LSM4	2.01752522	1.364048755	2.984063433	0.000440509
MRPL21	2.057589787	1.297256312	3.263561479	0.00217121
EIF3I	2.229857828	1.307191919	3.803776524	0.003249682
IFIT5	1.349731374	1.105013713	1.648644501	0.003299985
MRPS12	1.681045975	1.1839943	2.386764505	0.003680263
MRPS16	2.357846249	1.318203912	4.217434709	0.003838036
PARK7	2.166217062	1.280276367	3.665221416	0.003966997
POLR2L	1.569319071	1.134044602	2.171662686	0.006549722
SRSF12	0.793474996	0.666845724	0.944150267	0.009111989
MRPL14	1.646578327	1.123176021	2.413887171	0.010615133
MRPL40	1.990977661	1.163242997	3.407707638	0.012024053
OAS2	1.202730281	1.041115227	1.389433265	0.012168065
OAS1	1.17945007	1.034102183	1.345227282	0.013904728
MRPL39	1.623422988	1.102192997	2.391144024	0.014188686
RPUSD2	1.647408776	1.099687005	2.467934659	0.015487769
PPIH	1.808519442	1.095300701	2.98615948	0.020572574
ZC3HAV1L	0.806026539	0.65992094	0.984479719	0.034578933
SLIRP	1.555633176	1.018761732	2.375427446	0.040753344
MRPL2	1.501582438	1.016008862	2.219222591	0.041385212
NOC2L	1.532380646	1.010057548	2.324808569	0.044747457

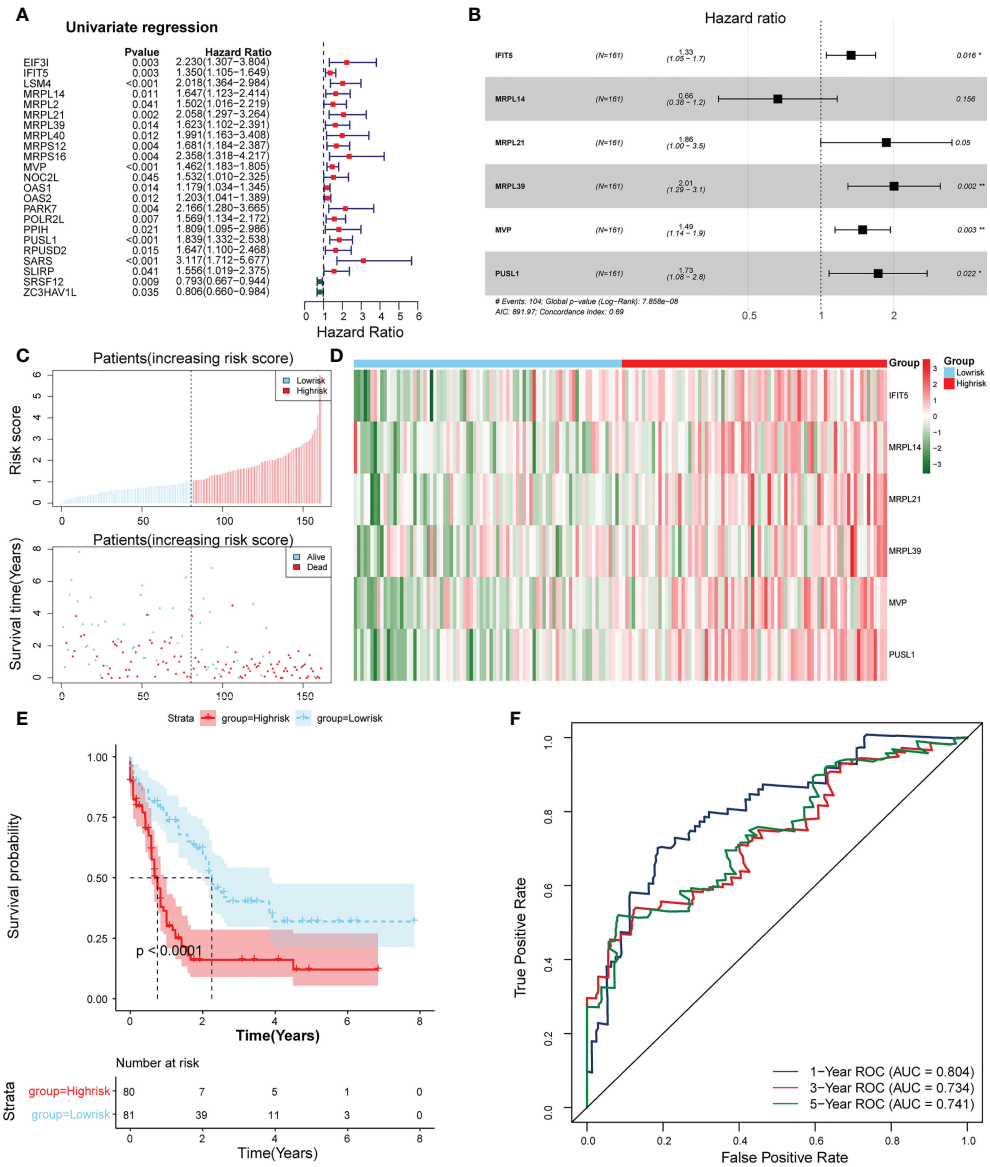

FIGURE 3
Establishment of a risk model based on 6 biomarkers. **(A)** Univariate Cox analysis of PRBP-DEGs which were selected by PPI network. **(B)** Multivariate Cox analysis to screen biomarkers. * represents p < 0.05, ** represents p < 0.01. **(C)** Distributions of risk scores and survival states between high- and low-risk groups in the training set. **(D)** Heatmap of 6 biomarkers in high- and low-risk groups. **(E)** The Kaplan-Meier survival curve for the high-and low-risk groups in the training set. **(F)** ROC curves at 1-, 3-, and 5 years in the training set.

TABLE 2 Multivariate Cox analysis to construction of the risk model.

	coef	HR	HR.95L	HR.95H	p.val
MRPL39	0.697194958	2.008111962	1.293783268	3.116838618	0.001881772
MVP	0.39822836	1.489184061	1.144212347	1.938162242	0.003056813
IFIT5	0.287637545	1.333273965	1.054794808	1.685275138	0.01611802
PUSL1	0.545513896	1.725494879	1.081634196	2.752624305	0.022063051
MRPL21	0.623197758	1.864881958	0.999043984	3.481112718	0.050352116
MRPL14	-0.40877001	0.664467036	0.377842855	1.168518698	0.155827125

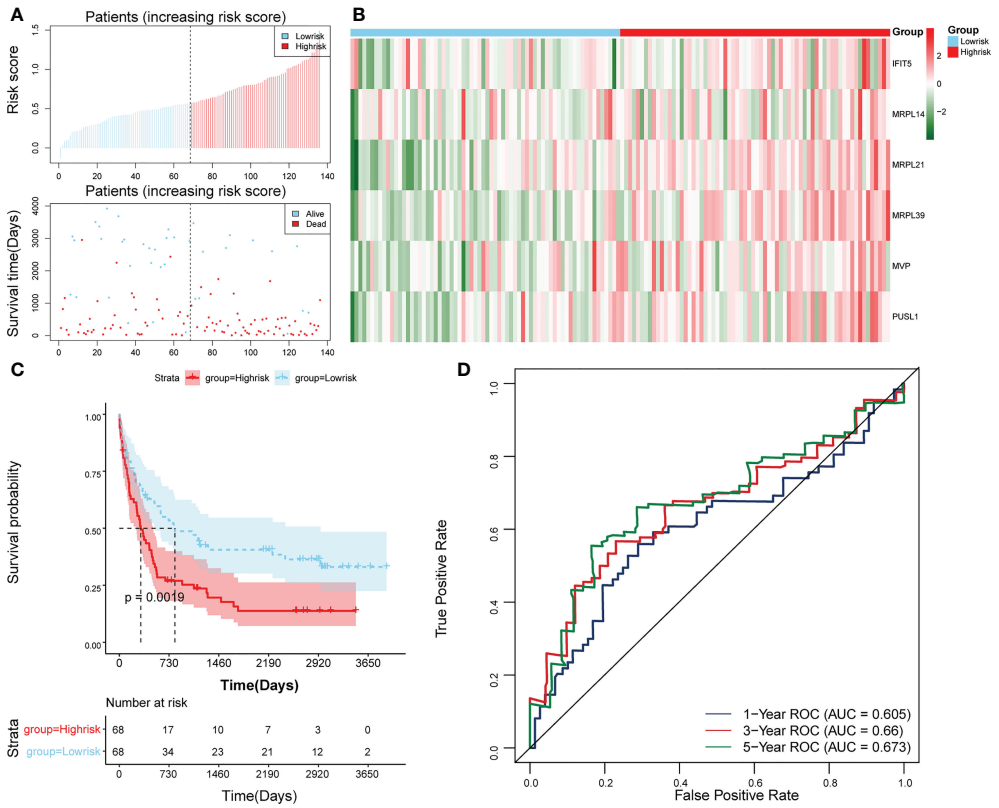

FIGURE 4
Validation of the risk model in GSE37642. **(A)** Distributions of risk scores and survival states between high- and low-risk groups in GSE37642. **(B)** Heatmap of 6 biomarkers in high- and low-risk groups in GSE37642. **(C)** The Kaplan-Meier survival curve for the high-and low-risk groups in GSE37642. **(D)** ROC curves at 1-, 3-, and 5 years in GSE37642.

with the overall survival of AML patients ($p< 0.05$), and the multivariate Cox analysis showed that RiskScore, age, and cytogenetics risk category were independent prognostic factors (Figures 5A, B; Tables 3, 4). A nomogram using these three independent prognostic factors can predict survival at 1 year, 3 years, and 5 years (Figure 5C). The slope of calibration curve at 1 year was close to 1, indicating that the constructed prediction model can be used as a valid model (Figure 5D).

Functional enrichment analysis

Gene set enrichment analysis (GSEA) enrichment analysis showed that the high-risk group was associated with immune functions such as activation of the innate immune response and innate immune response activating signal transduction, as well as metabolism-related processes, such as mitochondrial transport, ATP metabolic processes, regulation of cellular amino acid metabolism process, and it was also involved in antigen processing and presentation, apoptosis, and metabolism-related processes, such as citrate cycle TCA cycle and pentose phosphate pathway. The low risk group was mainly enriched in the pathways of cellular glucuronidation, negative regulation of execution phase of apoptosis, and regulation of execution phase of apoptosis (Figures 6A-C). Moreover, there were 997 DEGs between high and low risk groups (Figures 7A, B), and they were enriched to 87 GO BPs, 28 GO CCs, 23 GO MFs, and 11 KEGG pathways, mainly including immune response, immune response-inhibiting cell surface receptor signaling pathway, adaptive immune response, immune system processes, positive regulation of T cell activation and other immune-related pathways (Figure 7C). Thus, metabolic- and immune-related processes and pathways were closely associated with the risk model.

Discussion

Acute myeloid leukemia is one of the most common malignant hematological and systemic diseases in adults, which is mainly characterized by susceptibility to relapse, poor prognosis, and low survival rate (1, 3, 4). Pyroptosis is a

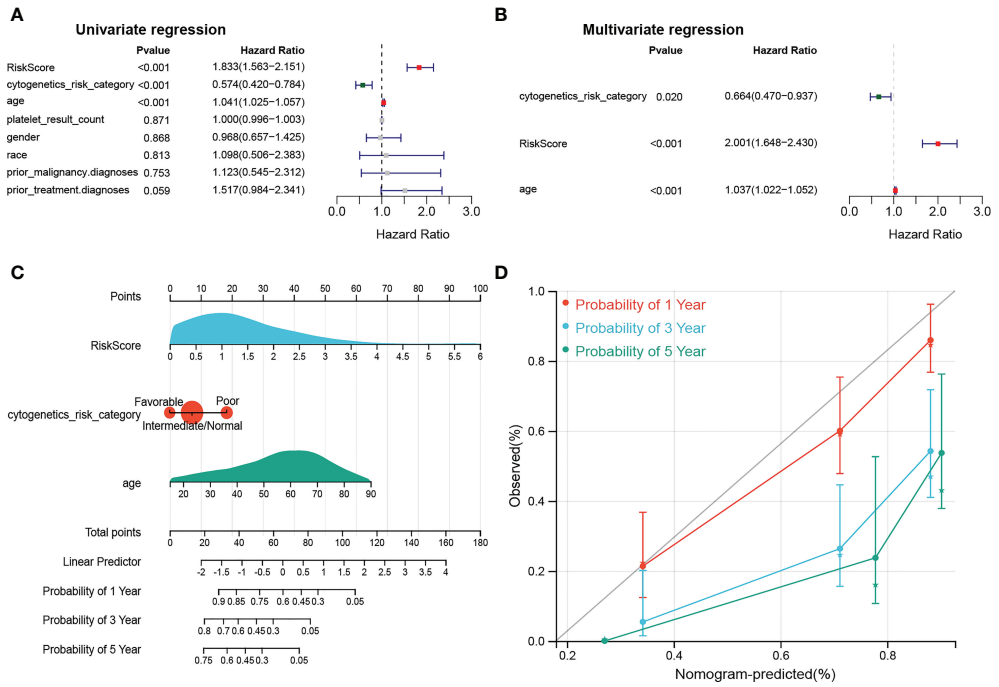

FIGURE 5
dentification of independent prognostic factors. **(A, B)** Outcomes for the univariate and multivariate Cox regression analysis of clinical parameters in AML patients ($p< 0.05$). **(C)** Nomogram of 1-, 3- and 5- year OS for AML patients. **(D)** Calibration plot of 1-, 3- and 5- year OS associated nomogram.

TABLE 3 Univariate Cox analysis in independent prognostic analysis.

	HR	lower.95	upper.95	p.val
RiskScore	1.833126262	1.562553294	2.150551859	0.000000000000103
cytogenetics_risk_category	0.574282951	0.420484124	0.78433617	0.000487834
age	1.041147542	1.025465494	1.05706941	0.000000191
platelet_result_count	0.999728697	0.996454031	1.003014125	0.871232716
gender	0.967616131	0.657072989	1.424926901	0.867601929
race	1.098041572	0.50600686	2.38276472	0.812957083
prior_malignancy.diagnoses	1.123048347	0.545488816	2.312123645	0.752783001
prior_treatment.diagnoses	1.517484175	0.983541135	2.34129325	0.059435784

TABLE 4 Multivariate Cox analysis in independent prognostic analysis.

	HR	lower.95	upper.95	p.val
cytogenetics_risk_category	0.663518376	0.470082914	0.936551027	0.019661517
RiskScore	2.0013394	1.648433066	2.429798016	0.00000000000239
age	1.036902937	1.021671191	1.052361769	0.00000159

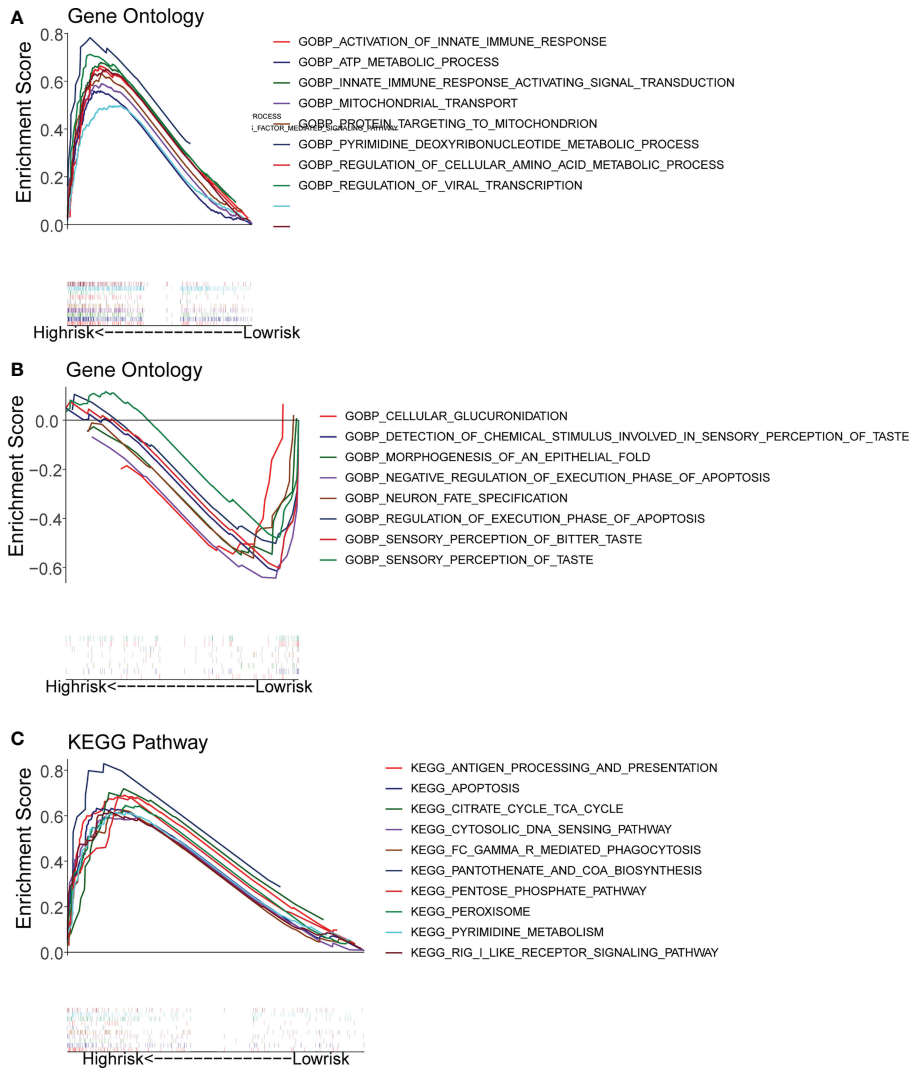

FIGURE 6
Gene Set Enrichment Analysis (GSEA) for the high- and low-risk groups. **(A, B)** GO enrichment analysis. **(C)** KEGG pathway analysis.

programmed death (5), and RBPs are essential modulators of transcription. Kebin Huang et al. found that MicroRNA-519 enhances HL60 human acute myeloid leukemia cell line proliferation and induces cell apoptosis by reducing the expression level of RBPhuman antigen (18). Additionally, B Mitton et al. reported that a small molecule inhibitor of CREB (cAMP Response-Element Binding Protein), XX-650-23, interaction mostly affects apoptotic, cell-cycle, and survival pathways, which may represent a novel approach for AML therapy (19). However, the relationship between pyroptosis-related RBPgenes and AML remains unclear, so it is important to predict the relationship between pyroptosis-related RBPgenes and AML.

In this study, 71 PRBP DEGs were enriched to cellular protein complex disassembly, RNA catabolic process, RNA transport, RNA destabilization, negative regulation of protein acetylation, ribonucleoprotein complex assembly, and protein export from protein-related pathways such as nucleus. In a study on the expression pattern and clinical value of key m6A RNA modification regulators in abdominal aortic aneurysm, it found that the modified genes were primarily enriched in RNA catabolic process, RNA transport et al. (20). Nelsonet al. demonstrated that a block to efficient splicing can occur at multiple steps in the pathway of normal splicing complex assembly, and plice site selection and ribonucleoprotein complex assembly during *in vitro* pre-mRNA splicing (21). However, the role of the above enriched pathways in AML has not been reported. Stefan Gattenloehner et al. found that the CD56 expression on AML cells correlates with an abnormal expression pattern of runt-related transcription factor 1

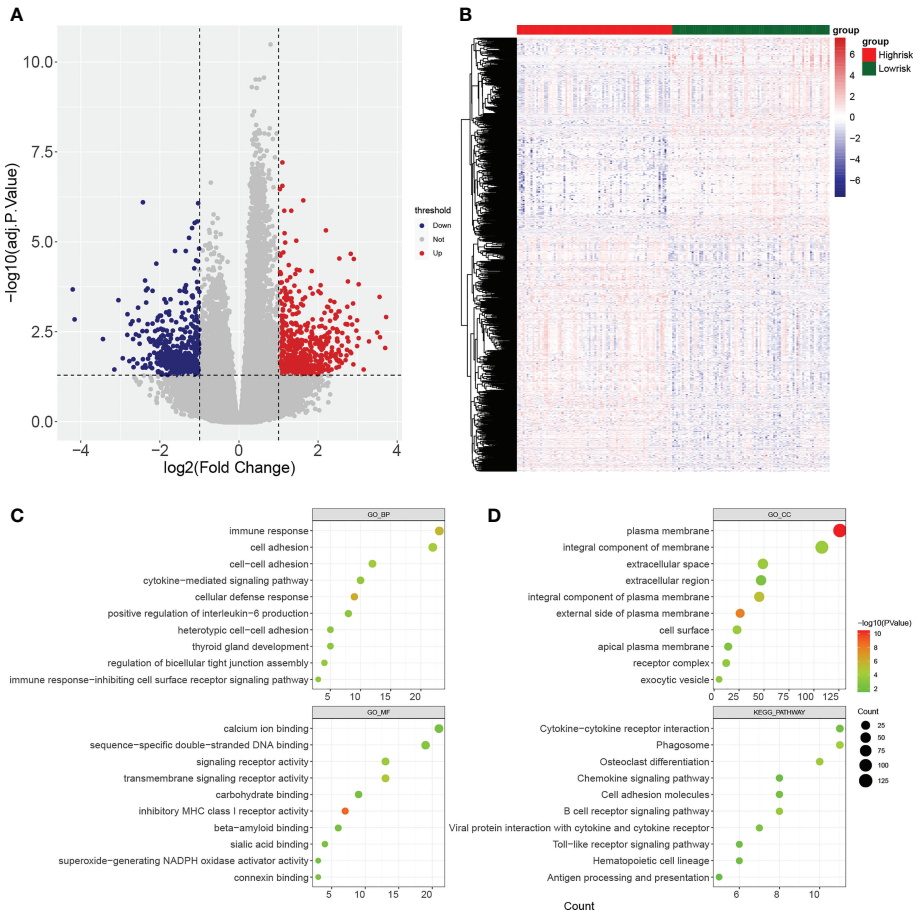

FIGURE 7
Identification of differentially expressed genes (DEGs) between high- and low-risk groups. **(A, B)** Volcano plot and heatmap of DEGs filtered with adj.p.< 0.05 and $|\log_2$fold change (FC)| > 1. **(C, D)** Bubble diagrams of GO enrichment analysis and KEGG pathway analysis (p< 0.05).

(RUNX1) isoforms and the potential for new therapy of CD56 (high) AML by suppression of the "overactive" RUNX1/CD56/NF-kappa B signaling pathway(s) (22). Therefore, we guess that certain pathways play an important role in the pathogenesis of AML, and in the future work, we need to further focus our attention on the significance of the enriched pathways such as cellualr protein complex disassembly in AML.

We constructed a risk model of the RBP genes associated with cell pyroptosis in patients with Acute Myeloid Leukaemia. Yi Zhang et al. constructed a novel prognostic scoring model for newly diagnosed FLT3-ITD-positive AML (23), but the model has some limitations such as induction and consolidation treatment regimens cannot be fully harmonized due to the retrospective nature of the study. Yun Wang et al. built an immune risk score to predict survival of patients with AML receiving chemotherapy (24), however, they lacked data on some important predictive covariates, such as mutation topography and results of MRD testing in subjects achieving a complete remission. Piyanuch Kongtim et al. constructed anovel disease risk model for patients with AML receiving allogeneic hematopoietic cell transplantation (25), while this is a retrospective study conducted in a single institution, and the limited number of patients in some subgroups may not detect relevant differences between the groups. Compared with the above models, the risk model we constructed started from the direction of the RBP genes associated with cell pyroptosis. Meanwhile, it had the advantage of fewer model genes.

In constructing this prognostic-related risk model, we obtained a total of six biomarkers. IFIT5, MRPL14, MRPL21, MRPL39, and PUSL1, and none of these genes have yet been reported in AML. MVP encodes the major component of the vault complex. The encoded protein may play a role in multiple cellular processes by regulating the MAP kinase, JAK/STAT and phosphoinositide 3-kinase/Akt signaling pathways. The encoded protein also plays a role in multidrug resistance, and expression of this gene is a prognostic marker for several types of cancer. However, H J Broxterman et al. found that it is shown that Pgp function, but not Mvp/LRP or MRP1 expression correlate with a

low steady-state DNR accumulation in *de novo* AML. The Pgp activity does, however, not predict the DNR sensitivity in AML measured as *in vitro* DNR LC50 with an MTT-based assay. The reason for that seems to be that a low DNR accumulation may not be the most important factor in determining the LC50 (26). Therefore, the drug resistance effect of MVP in AML still needs further investigation.

In this study, we performed pathway enrichment analysis between high and low risk groups using GSEA software, and finally analyzed differentially expressed genes between high and low risk groups using the R package limma, and performed functional enrichment analysis of differential genes using the enrichment tool DAVID. Our findings found that the high- and low-risk groups were associated with several immune-related and metabolic-related biological processes and pathways. The high-risk group was associated with activation of the innate immune response and innate immune response activating signal transduction. Curran et al.' laboratory has recently characterized the host innate immune system generates a T cell tolerant state in an animal AML model (27). Antigen-specific T cell tolerance is a potent immune evasion mechanism in hosts with AML that can be reversed *in vivo* after CD40 engagement (28). These results indicate that immune tolerance to AML may be initiated at the level of the innate immune system (27, 28). Long Zhang et al. discovers antigen-specific T cell tolerance is a potent immune evasion mechanism that can be reversed *in vivo* after CD40 engagement *via* a murine AML model (28). Our findings are consistent with those of the above investigators. Marko Skrtic et al. found that inhibition of mitochondrial translation as a therapeutic strategy for human AML (29). Marvin M van Luijn et al. found that the myeloid leukemic blasts with expressing HLA class II molecules, abnormalities in the processing pathways of endogenous antigens could also result in impaired HLA class II-restricted tumor-associated antigen presentation to CD4(+) T helper cells (30). Hideaki Mizuno et al. revealed that suppression of Fbp1, as well as pentose phosphate pathway enzymes by shRNA-mediated knockdown selectively decreased Evi1-driven leukemogenesis *in vitro*, Considering Evi1 upregulates Fbp1, and supports progression of AML through pentose phosphate pathway activation. Our findings also found that the high-risk group was associated with mitochondrial transport, antigen processing and presentatio and pentose phosphate pathway. Overall, the functional enrichment results for high- and low-risk groups suggested the linkages between RBPs associated with cell pyroptosis and metabolism in AML.

Some limitations of this study also exist, (a) Some functional experiments were needed to further illustrate the underlying molecular mechanisms to predict the role of the cellular pyroptosis-related differential RBP genes in AML; (b) The prognostic model should be validated by more datasets and clinical samples; (c) This study only used bioinformatics methods to conduct multiple analyses based on retrospective data from the public databases, confirmatory experiments *in vivo* and *in vitro* will be required subsequently. Therefore, we will continuously focus on the role of these genes.

In conclusion, through the above analysis, the differential RBP genes related to pyroptosis in AML were screened, and through the regression analysis of these genes, six biomarkers were obtained, and a risk model associated with metabolism was constructed, which provided a theoretical basis and reference value for the future treatment research and prognosis of AML.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ Supplementary Material.

Author contributions

TB and CL are the principal investigator and conducted statistical analysis and drafted the article. JT and BL performed data management and bioinformatics analysis. TB, CL, F-JL, YW, X-JX, D-JL, JT, and BL edited and revised the article. All authors have read and agreed to the published version of the manuscript.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.1059978/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The PCA and tSNE analyses of patients from high and low risk groups. **(A)** PCA analysis. **(B)** tSNE analysis.

SUPPLEMENTARY TABLE 1
Gene ontology (GO) analysis on Biological Process (BP).

SUPPLEMENTARY TABLE 2
Gene ontology (GO) analysis on Cell Components (CC).

SUPPLEMENTARY TABLE 3
Gene ontology (GO) analysis on Molecular Functions (BP).

SUPPLEMENTARY TABLE 4
Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis.

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Alterations in cellular metabolisms after TKI therapy for Philadelphia chromosome-positive leukemia in children: A review

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Incidence rates of chronic myeloid leukemia (CML) and Philadelphia chromosome-positive (Ph+) acute lymphoblastic leukemia (ALL) are lower but more aggressive in children than in adults due to different biological and host factors. After the clinical application of tyrosine kinase inhibitor (TKI) blocking BCR/ABL kinase activity, the prognosis of children with CML and Ph + ALL has improved dramatically. Yet, off-target effects and drug tolerance will occur during the TKI treatments, contributing to treatment failure. In addition, compared to adults, children may need a longer course of TKIs therapy, causing detrimental effects on growth and development. In recent years, accumulating evidence indicates that drug resistance and side effects during TKI treatment may result from the cellular metabolism alterations. In this review, we provide a detailed summary of the current knowledge on alterations in metabolic pathways including glucose metabolism, lipid metabolism, amino acid metabolism, and other metabolic processes. In order to obtain better TKI treatment outcomes and avoid side effects, it is essential to understand how the TKIs affect cellular metabolism. Hence, we also discuss the relevance of cellular metabolism in TKIs therapy to provide ideas for better use of TKIs in clinical practice.

KEYWORDS

cellular metabolisms, TKI therapy, Philadelphia chromosome-positive, leukemia, children

Introduction

Protein kinases (PKs), a class of enzymes, are able to transfer phosphate groups from ATP to the hydroxyl side chain of certain amino acid residues (1). PKs can be classified into tyrosine kinases (TKs) and serine/threonine kinases (STKs) based on the origin of the phosphorylated hydroxyl groups (2). TKs are essential cellular signaling enzymes regulating signal transduction pathways for metabolism, transcription, differentiation, proliferation, development, migration and apoptosis (3). TKs may be divided into two major classes: transmembrane receptors linked receptor tyrosine kinases (RTKs), like the PDGF receptors, and non-receptor tyrosine kinases (NRTKs), like c-SRC and BCR-ABL (4). Oncogenic mutations or overexpression of TK are a hallmark of cell cycle dysregulation often related to tumorigenesis (5) in hematological malignancies (6, 7), breast cancer (8), and non-small-cell lung cancer (9). Hence, TK inhibition (TKI) is regarded as a targeted treatment for cancer as it can selectively inhibit TK proteins and halt the proliferation and growth of tumor cells (3). At present, a variety of structurally different TKIs acting at singular or multiple targets like BCR-ABL, EGFR, VEGFR, PDGFR, KIT, and ALK, have been developed with minimal toxicities and good pharmacokinetics (10, 11).

A myeloproliferative tumor known as chronic myelogenous leukemia (CML) is characterized by unchecked proliferation of bone marrow myeloid progenitor cells resulted from the translocation of t (9:22) producing the hallmark BCR-ABL1, a constitutively active tyrosine kinase (12). Pediatric CML accounts for about 9% of leukemia in teenagers between the ages of 15 and 19 and around 2% of leukemia in children under the age of 15 (13, 14). However, previous studies showed that owing to the underlying biology and host characteristics, clinical presentations in children are often more aggressive than those in adults (14, 15). Similar to adult, the natural history of pediatric CML also progresses through three phases (16). The first and most prevalent stage is the chronic phase (CP), which is characterized by the absence of any subjective symptoms 3–5 years after diagnosis. The second stage is the accelerated phase (AP), during which aberrant granulocyte differentiation increases. The last stage is known as the blast crisis (BC), which is characterized by an expansion of undifferentiated blasts. Fortunately, TKIs can be used to treat patients in the CP effectively, leading to improved survival (17); however, the majority of AP and BC patients show no response to TKIs (18) because their growth is no longer influenced by BCR-ABL. Before the introduction of TKIs, pediatric CML was mainly treated with hematopoietic stem-cell transplantation (HSCT) and had an overall survival (OS) of about 64% at a median follow-up period of 6 years (19). In May 2001, the US Food and Drug Administration (FDA) approved Imatinib (IM, a small molecule TKI) for adult CML (20) and this strategy was successful in improving disease prognosis. In 2003, the US FDA authorized IM for pediatric (under 18 years of age) CML based on its effectiveness in adults (21). Since then, the OS of all children with CML treated with TKIs has improved to about 90% at the median 3-year follow-up period (19).

Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ ALL) which is characterized by the t(9;22)(q34; q11) translocation and BCR-ABL1 fusion gene accounts for 3% to 5% of children with ALL (22, 23). Children with Ph+ ALL also have a more aggressive clinical presentation because of the secondary cytogenetic abnormalities and cooperative mutations such as IKZF1 deletions (22, 24). Ph + ALL is an adverse subtype of ALL with poor prognosis. Historically, less than half of children with Ph+ ALL survived when treated with chemotherapy with or without HSCT (25–27), while survival for children with ALL exceeds 90% in the same period (28). Fortunately, due to the success of TKI treatment in CML, TKIs was introduced for Ph+ ALL treatment, leading to an improved OS and event-free survival (EFS) rates in pediatric Ph+ ALL when combined with intensive chemotherapy (29, 30).

Despite the recent advancement of TKI therapies, drug resistance remains a problem in clinical anticancer treatment. In recent years, mechanisms of acquired resistance have been identified. TKI resistance in the treatment of CML can result from both BCR-ABL dependent and independent pathways (31). The majority of the BCR-ABL-dependent resistance (32–36) is mediated by the T315I “gatekeeper” mutation, BCR-ABL overexpression, MDR1 upregulation, and ABL kinase domain mutation, respectively. Currently, it is not so clear about BCR-ABL-independent resistance. Recent findings indicate that such TKI resistance may be influenced by the insensitivity of leukemia stem cells (LSCs) (37) and abnormal activation of the PI3K (38) and RAS/MAPK (39, 40) signaling pathways. To overcome IM resistance, second-generation TKIs, such as dasatinib (DAS) and nilotinib (NIL), were developed and they have activity against most IM-resistant BCR-ABL1 mutants (41). But, they are not able to overcome ABL-T315I-induced resistance. Then, ponatinib, the third-generation TKI, has been developed and is effective against T315I-mutated Ph+ leukemias (42), but the risk of life-threatening cardiovascular side effects limits its clinical application (43). On the other hand, due to the need of growth and development, the long-term clinical safety of TKIs has to be considered. In recent years, several long-term side effects have been reported, including growth deceleration (44), dysregulation of bone (45, 46), and decreased fertility (47, 48).

To achieve better treatment outcomes while avoiding drug resistance and side effects, a deeper comprehension of TKIs’ mechanism is necessary. Therefore, this review discusses the metabolic pathways alterations after TKIs therapy in children, including the following five aspects: glucose metabolism, lipid metabolism, amino acid metabolism, nucleotide metabolism, and immunometabolism.

Glucose metabolism

Glucose metabolism, which serves as a significant source of energy for cell development, includes glycolysis pathway, pentose phosphate pathway (PPP), oxidative phosphorylation and serine synthesis pathway (SSP) (49). It is known that in aerobic settings, intracytoplasmic glycolysis provides energy first, followed by mitochondrial oxidative phosphorylation. When oxygen is scarce, cells depend on glycolysis instead of the oxygen-consuming TCA cycle to produce energy (49). However, Otto Warburg found that even in the presence of enough oxygen, cancer cells prefer to engage in aerobic glycolysis, generally referred to as "Warburg effect" or "aerobic glycolysis", to produce ATP and metabolic intermediates (50). Studies showed that although glycolysis produces ATP per glucose molecule considerably less effectively than oxidative phosphorylation, the production rate increases significantly (51). Besides, this reprogramming of glucose metabolism provides additional macromolecular precursors such as acetyl-CoA, glycolytic intermediates and ribose which meet the needs of fast growth and proliferation of cancer cells (52). Like other malignancies, the aberrant cellular metabolism also occurs in CML cells. The BCR-ABL oncoprotein can boost glucose uptake and glycolysis and overexpress glucose transporter-1 (GLUT-1) to influence metabolism (53). Furthermore, the PI3K/Akt/mTOR pathway is considered to be responsible (54). Since the introduction of the first BCR-ABL TKI, IM (Gleevec, previously STI571), the effects of BCR-ABL TKIs on glucose metabolism in tumor cells have been explored.

Using [1,2-13C2] glucose as the single tracer with biological mass spectrometry, J Boren et al. demonstrated that (55) by lowering hexokinase and glucose-6-phosphate 1-dehydrogenase activity and changing pathway carbon flux of the pentose cycle in K562 human myeloid blast cells, IM reduced the use of glucose carbons for *de novo* nucleic acid and fatty acid synthesis. In 2004, Gottschalk S et al. used magnetic resonance spectroscopy to examine changes in endogenous metabolites, energy status, and glucose metabolism of human BCR-ABL+ cells (CML-T1 and K562) and BCR-ABL- cells (HC-1) following IM therapy (56). They found that the "Warburg effect" was reversed in BCR-ABL+ cells at the appropriate therapeutic doses of IM (0.1-1.0 μmol/L) by switching glucose metabolism from anaerobic glycolysis to the mitochondrial Krebs cycle. In this situation, BCR-ABL+ cells decreased the glucose uptake from the media but the glucose metabolism in mitochondrial increased, leading to elevated absolute concentrations of the high energy phosphate nucleoside triphosphate (NTP). Subsequently, Barnes K et al. investigated the function of BCR-ABL-induced glucose transport regulation anomalies in CML (9). This work showed that by upregulating the GLUT-1 glucose transporter's expression on the cell surface, BCR-ABL-expressing cells may accelerate the absorption of hexose. Interestingly, IM treatment leads to a 90% internalization of the cell-surface GLUT-1 transporters, substantially decreasing hexose uptake in BCR-ABL-expressing cells (57). These findings suggested that reversing the aerobic glycolysis and inhibiting glucose transport significantly contributes to IM's anti-tumor effects.

Pyruvate-Kinase (PK), as an enzyme, catalyzes the last stage of glycolysis, converting phosphoenolpyruvate and ADP into pyruvate and ATP (58). According to previous literature, the Warburg effect is accomplished by regulating expression of the embryonic M2 isozyme of PK (PKM2), rather than the M1 isozyme (PKM1) expressed in normal cells, through an alternative splicing repressor polypyrimidine tract-binding protein1 (PTBP1) (59, 60). A study suggested that IM inhibits glycolysis through the inhibition of phosphorylation of BCR-ABL and the down-regulation of miR-124/PTBP1/PKM2 signaling (61). Through downregulation of PTBP1, IM changes PK isoforms from PKM2 to PKM1, leading to reversal of the Warburg effect (61). Apart from this, Damaraju VL et al. reported how TKIs reduce glucose uptake by evaluating the interaction of TKIs with GLUT-1 in the human nasopharyngeal carcinoma cell line (FaDu) and GIST-1 cells. They discovered that [3H]2-deoxy-d-glucose ([3H]2-DG) and [3H]Fluoro-2-deoxy-D-glucose (FDG) uptake were competitively suppressed by IM and NIL, and that IM had reversible [3H] FDG uptake inhibition whereas NIL did not (62). Additionally, molecular modeling demonstrated that TKIs impair GLUT-1's ability to take up glucose by interacting with the glucose binding site *via* hydrogen bonds and van der Waals interactions (62).

Intrinsic metabolic differences between IM sensitive and resistant cell lines were also previously characterized. A study showed that IM treatment in sensitive BCR-ABL positive cells (K562-S, LAMA84-S) leaded to the reduction of glucose absorption and lactate generation and the enhancement of oxidative TCA cycling (53). On the other hand, the drug-induced IM resistant cells (K562-r and LAMA84-r) displayed a highly glycolytic metabolism with increased glucose absorption and lactate generation. Additionally, in IM-resistant cells, oxidative synthesis of RNA ribose from 13 C-glucose using glucose-6-phosphate dehydrogenase was decreased, while the non-oxidative transketolase pathway was boosted (53). In line with the literature mentioned above (57), in IM-treated sensitive cells, GLUT-1 moved from the plasma membrane to the intracellular fraction leading to reduced glucose uptake, while GLUT-1 remained at the plasma membrane in IM-resistant cells (53). However, different from the finding in the drug-induced IM resistant cells, Ko BW et al. reported that the KBM5-T315I cells which acquired drug resistance resulting from the T315I mutation have metabolically suppressive status compared to KBM5 cells (IM-sensitive) (63). KBM5-T315I cells showed low glycolytic activity, decreased fatty acid synthesis and reactive oxygen species (ROS) generation potentially participating to the reduced proliferative activity of KBM5-T315I cells. The researchers came to the conclusion that the decreased

expression of glycolysis-related genes and ROS levels might be responsible for reduced growth ability of KBM5-T315I CML (63). These biological and metabolic characteristics of CML cells with different resistance mechanisms should be take into account in future studies overcoming the IM resistance.

As indicated previously, glycolysis was extremely important for B-ALL cells. T Liu et al. reported that 2-deoxyglucose (2-DG) suppressed aerobic glycolysis, leading to the inhabitation of B-ALL cell growth, the increasing of the pro-apoptotic protein Bim and re-sensitization of B-ALL cells to the tyrosine kinase inhibitor DAS *in vivo* (64). Apart from this, deletion of GLUT-1 partially inhibits glucose uptake (64). Through metabolic reprogramming, the decreased glucose transport capacity was sufficient to decrease anabolism and promote catabolism in B-ALL cells. As a result, GLUT1-deficient B-ALL cells were unable to accumulate *in vivo*, and GLUT-1 depletion inhibited leukemia progression. These data suggested that inhibition of aerobic glycolysis and glucose uptake by GLUT-1 could be plausible adjuvant approaches for B-ALL therapies. In another study, researchers found that TKI treatment creates a new metabolic state in leukemic cells that is highly sensitive to specific mitochondrial perturbations. As a result, patients with BCR-ABL+ leukemia may respond better to TKI when receiving adjuvant therapy with targeting mitochondrial metabolism (65). Since TKI treatment changed glucose metabolism in BCR -ABL+ cells from anaerobic glycolysis to the mitochondrial tricarboxylic acid cycle, they indicated that oligomycin A, a mitochondrial ATP synthase inhibitor, greatly promotes TKI sensitivity in leukemia cells at very low concentrations *in vitro*. In a mouse model, oligomycin A enhanced the ability of TKI to eliminate BCR-ABL+ leukemia cells (65). In spite of strong suppression of glycolysis, Shinohara H et al. found that by upregulating carnitine O-palmitoyltransferase 1 (CPT1C), the rate-limiting fatty-acid oxidation (FAO) enzyme, IM triggers compensatory FAO, which enabled glucose-independent cell viability. AIC-47 suppresses CPT1C expression and directly inhibits the metabolism of fatty acids and. Combined with AIC-47, IM enhanced the attack on cancer energy metabolism, leading to an increased cytotoxicity (61). Overall, these studies illustrate that metabolic reprogramming after TKIs treatment could be a potential therapeutic target.

In addition to BCR-ABL cells, the effects of BCR-ABL TKIs on other cells were also investigated. Recent studies reported the influences of IM and DAS on skeletal muscle cell metabolism (66, 67). Damaraju VL et al. showed 2-deoxy-D-glucose absorption was suppressed by to almost 50% in C2C12 murine skeletal muscle cells pre-incubated for 15 min with IM. Moreover, in a skeletal muscle cell model, IM lowered energy generation and mitochondrial function by inhibiting mitochondrial complex V activity and nucleoside absorption. This may contribute to tiredness, one of the most prevalent side effects of TKIs (66). In addition to inhibiting glucose transport proteins and metabolic enzymes, IM also exerts antidiabetic effects by protecting against β-cell death and ultimately increasing insulin production in a mouse model (68). In CML patients, Fitter S. et al. found a 3-fold increase in plasma adiponectin concentrations after three months of IM treatment; adiponectin elevation enhanced glucolipid metabolism, which explains why diabetes improved after IM treatment (69).

Lipid metabolism

There are two mechanisms by which mammals acquire lipids: *de novo* synthesis and direct exogenous uptake. The *de novo* lipogenesis pathway is restricted to hepatocytes and adipocytes in normal tissue; however, cancer cells can also reactivate this pathway even with exogenous lipids (70). An elevated of lipid uptake, storage and lipogenesis was reported in a variety of cancers, contributing to rapid tumor growth (71, 72). Research suggested that lipid metabolism in cancer cells is regulated by PI3K/Akt/mTOR pathway (24). Sterol regulatory element-binding protein 1c (SREBP-1c), a transcription factor that promotes lipid synthesis *de novo*, is controlled by mTORC1 (70, 73). In spite of lipids being widely used as cancer biomarkers, little is known about TKIs' impact on lipid metabolism and pathways.

Previous studies have suggested that exposure to the first-generation TKI (IM) may lead to a reduction in cholesterol and triglycerides in people and animal models, as well as a better serum lipid profile, while the second-generation TKIs may cause a worse metabolic profile (74–77). A cohort study researched how first- and second-generation TKIs affected the patients with CML's glucose and lipid metabolism. They discovered that compared to the IM and DAS groups, the NIL group had substantially higher fasting plasma glucose, insulin, C-peptide, insulin resistance, total cholesterol, and low-density lipoprotein (LDL) cholesterol levels (76). In a translational mouse model, plasma cholesterol and atherosclerosis areas were reduced by IM and ponatinib, while they were not affected by NIL. On the other hand, IM showed a beneficial cardiovascular risk profile compared to NIL and ponatinib (74).

It is not entirely clear how IM reduces lipid levels. The PDGF receptor (PDGFR) inhibitory action of IM has been proposed as a potential reason. The phosphorylation of LDL receptor-related protein (76) may be facilitated by excessive PDGFR expression, leading to atherosclerosis brought on by cholesterol. However, NIL, which also inhibits PDGFR, was found to significantly increase lipid levels in patients, rendering this explanation unsatisfactory (76). Ellis M et al. explored the possible biological mechanisms behind the lipid-lowering effects of IM in CML. Results indicated that two genes, apobec1 that inhibit lipid synthesis and LDL-R that promote clearance of circulating LDL, are significantly induced by IM. In addition, IM induced HMG-coAR expression, which regulates hepatic cholesterol

synthesis (78). To elucidate the effects and mechanisms of TKIs on lipid metabolism, additional studies will be needed.

Studies on BCR-ABL TKIs and lipid metabolism are limited so far. The aforementioned study by Gottschalk et al. reported that phosphocholine concentrations, which are known to be raised in all rapidly proliferating malignant cells, were significantly reduced in IM-treated BCR-ABL+ cells (56). Similarly, subsequent study, which assessed a global metabolic profile including lipid metabolism of human leukemia cell after incubation with IM, showed that IM-treated K562 cells had a decreased concentrations of phosphocholine (PC) and phosphatidylcholine (PtdCho) (79). Following the first week of IM treatment, this reduction was significant and even increased after 2 -4 weeks. Polyunsaturated fatty acids (PUFAs) signal intensity increased after 2 and 4 weeks of treatment with increasing apoptosis rate. Furthermore, the amount of methylene/methyl (CH2/CH3) resonances of fatty-acid chains also enhanced (31). Previous studies have reported the decrease in PtdCho concentration with advancing apoptosis stages (80). Additionally, the accumulation of PUFAs as well as increased CH2 and CH3 resonances of free fatty acids is two other characteristics associated with cell death (81). Collectively, these lipid metabolic events following TKI treatment in BCR-ABL+ cells showed pathways linked to a continuous process of cell death.

Amino acid metabolism

Amino acids are basic units for protein synthesis in the organism and can divide into two groups: essential amino acids and non-essential ones. Essential amino acids cannot be synthesized by humans and can only be provided by food sources (82), including phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine and histidine (can be synthesized in adults). Non-essential amino acids include arginine, cysteine, glycine, glutamine, proline, tyrosine, alanine, aspartic acid, asparagine, glutamate and serine which can be synthesized in the body. After malignant transformation, tumor cells have an increased demand for amino acids, which can be utilized as intermediates in many metabolic pathways (83). According to studies in recent years, under genotoxic, oxidative, and nutritional stress, amino acids can act as metabolic regulators to promote cancer cell proliferation and survival (84, 85). Among them, studies on glutamine, serine, glycine and branched-chain amino acids (BCAAs) have drawn more attention.

Glutamine

As mentioned above, TKIs can efficiently hamper glucose metabolism in Ph+ leukemia. But, due to compensatory metabolic pathway activation, glycolysis inhibition alone frequently falls short of eliminating cells (86, 87). Glutamine is the most prevalent amino acid in human plasma and the second only to glucose in the metabolism of tumor cells (88). This metabolic alteration is frequently observed in cancer (89). Glutamine may provide its nitrogen and carbon to a variety of mechanisms in cancer cells, including energy production, macromolecular synthesis, and signal transmission (90).

Through the transporters (e.g., SLC1A5 or ASCT2), glutamine is brought into the cytoplasm where it is converted to glutamate by the enzyme glutaminase(GLS) (91). An earlier work shown that human B cell Burkitt lymphoma cell line P493 cells may use glutamine to carry out glucose-independent mitochondrial oxidative phosphorylation in the presence of low oxygen levels (92). Combined transcriptome and metabolome profiling, Pallavi Sontakke et al. found that (93), despite the prominent glycolysis, BCR-ABL positive cells also undertake glutaminolysis, which was demonstrated by elevated intracellular glutamine levels both in normoxia and hypoxia. In agreement with these findings, they also discovered that both protein and RNA levels of the glutamine importer SLC1A5 increased in BCR-ABL-expressing cells. Given this circumstance, glutamine may play a significant function as an additional source of carbon in the replenishment of tricarboxylic acid cycle (TCA) metabolite. Other researches have demonstrated that this is indeed the case. Anne Trinh et al. found that, after the treatment of IM, survived CML cells can continue to consume glutamine to create alphaKetoGlutarate, a TCA intermediate, that keeps the state of high mitochondrial oxidative metabolism (94). After that, they found that the combination of Kidrolase (an FDA-approved drug of L-asparaginase) and IM can deplete extracellular glutamine and therefore restrict mitochondrial metabolism. Finally, they discovered that this combination stimulates the intrinsic apoptotic pathway, effectively killing CML cells. Furthermore, TKIs are unable to eradicate the leukemia stem cells (LSCs) and/or progenitor cells that could cause relapses (95). In order to kill LSCs, Anne Trinh et al. also provided evidence that both glycolysis and glutamine-dependent mitochondrial metabolism required to be impaired (94). To prevent recurrence and achieve a longer OS of children, it may be an interesting therapeutic approach to eradicate LSCs by combining TKI and mitochondrial inhibitors.

Serine and glycine metabolism

Serine is well known as the one-carbon source in the methionine cycle and folate cycle and contributes to nucleotide synthesis, methylation reactions, and production of NADPH, an antioxidant defense mechanism (96, 97). Serine and glycine can be imported from the extracellular environment produced by the *de novo* serine synthesis pathway (SSP) (97). The SSP, starting from the glycolytic metabolite 3-phosphoglycerate (3-PG), is composed of three steps. First, phosphoglycerate dehydrogenase (PHGDH) converts 3-PG into 3-phosphohydroxypyruvate; then,

phosphoserine-amino transferase (PSAT-1) converts 3-phosphohydroxypyruvate into phosphoserine; last, phosphoserine phosphatase (PSPH) eventually catalyzing the dephosphorylation of phosphoserine into serine (98). As mentioned above, as a key metabolite to support cell proliferation, increasing serine supply is required to sustain cancer progression (99). In various cancers, to meet the large serine demand for survival, the three SSP enzymes (PHGDH, PSAT, and PSPH) are all highly expressed (100). In addition, recent work on cancer metabolomics has shown that unexpected increased reliance on glycine metabolism was discovered in rapid proliferation cancer cells and this phenotype that was not observed in rapidly proliferating nontransformed cells (101). Interestingly, an animal experiment found that restriction of serine and glycine intake can inhibit tumor growth and extend the survival time of tumor-bearing mice (102). Hilal Taymaz-Nikerel et al. reported some new multi-omics findings in yeast on the mechanism of IM, using the model organism Saccharomyces cerevisiae (103). They performed the whole-genome analysis of the transcriptional response of yeast cells *via* flux-balance analysis (FBA) and modular analysis of protein/protein interaction network which consist of proteins encoded by differentially expressed genes (DEGs). FBA indicated that IM alters multiple metabolic pathways by decreasing and increasing the fluxes of reactions and the fluxes related to metabolic pathway of glycine and serine were increased. However, through proteomics and metabolomics profiling of IM-resistant CML cells (ImaR), a previous study showed that serine-glycine-one-carbon metabolism and proline synthesis were enhanced in KU812 ImaR cells (104). In summary, IM can indeed suppress the proliferation of CML cells and induce the increasing of metabolic pathway of glycine and serine in parallel. Based on these, could we then assume that over-represented glycine and serine might counteract the inhibitory effect of IM and promote CML cell survival and chemoresistance? If so, suppressing the metabolism of glycine and serine could possibly serve as a novel therapeutic target.

BCAAs

For mammals, the BCAAs are necessary amino acids, including leucine, isoleucine, and valine. In mammalian proteins, about 63% of the hydrophobic amino acids are BCAAs (105) and may only be attained from food intake and recycled scavenged protein (106). After being catabolized by enzymes, intracellular BCAAs can provide nitrogen and carbon groups to take part in the synthesis of biomass, energy production, nutritional signaling, and epigenetic regulation (107). In humans, branched-chain amino transferases (BCATs) comprise two compartment-specific BCAA transaminases (BCAT1 and BCAT2) and can produce glutamate and the corresponding branched-chain α-ketoacids (BCKAs) (108, 109). When the transamination and transfer of nitrogen to α-ketoglutarate (α-KG) by BCATs initiate, catabolism of BCAAs begins. BCAAs and BCKAs often coexist in a balanced state, however this is not the case with malignancies. By fluorescent markers unique to each amino acid, Hattori A et al. measured the amino acid contents in CML-initiating cells isolated from a BC-CML mouse model. They discovered that BCAA levels were significantly higher in these cells (110). They later found that increased BCAT1 (or cBCAT), which may catalyze the conversion of a BCKA plus glutamic acid (Glu) into a BCAA and a -KG, may be a factor in this heightened BCAA metabolism. Additionally, they discovered that BC-CML-initiating cells had higher levels of BCAT1 mRNA, and that transduction of shRNA targeting BCAT1 mRNA reduced intracellular BCAA levels, which impeded the ability to form colonies *in vitro*. Through multi-omic investigations in yeast, Hilal Taymaz-Nikerel et al. found that (103) after IM treatment, the fluxes of the processes involved in the production of various amino acids, such as isoleucine, lysine, histidine, threonine, and valine, were drastically downregulated. In addition, Miriam G. Contreras Mostazo et al. indicated that KU812 ImaR cells might consume more BCAAs than parental cells in normoxia (104). According to these findings, inhibiting BCAA metabolism may be a promising therapeutic strategy for reducing ImaR cells.

Others

Methionine and homocysteine, two sulfur-containing amino acids, are the primary precursors of glutathione, a tripeptide that lowers reactive oxygen species (ROS) and upholds redox equilibrium (111). Additionally, Hilal Taymaz-Nikerel et al. discovered that (103) following IM therapy, the reaction fluxes through the production of methionine and cysteine, as well as the absorption of sulfate, were determined to be drastically decreased.

Nucleotide metabolism

In all areas of life, nucleotide metabolism is a critical activity. In order to enable cell proliferation (112), nucleotides, a type of biological information macromolecule, are primarily used as the raw materials for the synthesis of nucleic acids. Nucleotides comprise of both purine (adenine and guanine) and pyrimidine (cytidine, uridine and thymidine). Therefore, inhibitors of purine or pyrimidine synthesis have also been applied in hematological malignancies (113).

Purines and pyrimidines get synthesized separately but they have one same thing: 5-phosphoribose-1-pyrophosphate (PRPP), which is a donor of phosphate and ribose sugar and is an active form of ribose generated from ribose 5-phosphate (Figure 1). In purine biosynthesis, the primary source of

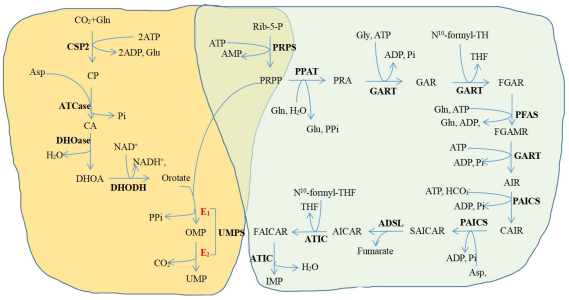

FIGURE 1
The de novo pyrimidine and purine synthesis pathways. Yellow background, the de novo pyrimidine synthesis pathway; green background, the de novo purine synthesis pathway Purines and pyrimidines get synthesized separately but they have one same thing. PRPP Gl. Glutamine, CSP2 Carbamoyl-phosphate synthetase 2: Glu, Glutamate: CP. Carbamoyl phosphate; ATCase, Aspartate transcarbamylase; CA, N-carbamoyl-L-aspartate DHOase. Dihydroorotase. DHOA. Ddihydroorotate, DHODH. Dihydroorotate dehydrogenase; OMP Orotidine 5'-monophosphate: UMPS. Undine monophosphate synthetase: UMP. Uridine monophosphate: Rib-5-p. Ribose-5-phosphate: PRPS. Phosphoribosylpyrophosphate synthetase; PRPP 5-phosphonbosyl-1-pyrophosphate: PPAT. Phosphoribosylpyrophosphate amidotransferase; PRA, Phosphoribosylamine, Gly. Glysine, GART Glycinamide ribonucleotide formyltransferase; GAR, Glycinamide ribonucleotide, THF. Tetrahydrofolate; FGAR, N-fotuvlelycinamide boucleotide, PEAS, Phosphoribosylformylglycinamidine synthase; FGAMR, N-formylglycinamidme ribonucleotide, AIR, Annnoimidazole ribonucleotide: PAICS, Phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase: CAIR. Carboxyaminoimidazole nbonucleotide; Asp. Aspartate: SAICAR, Nsuccinocarboxamide-5-aminoimidazole ribonucleotide, ADSL. Adenylosuccinate lyase: AICAR 5-aminoimidazole-4-carboxamide ribonuckotide ATIC 5-aminoimidazole-4-carboxamide rbouucleotide transfonuylase FAICAR 5-formamido-4-imidazolecarboxamide ribonucleotide, IMP. Inosine-5-monophosphate: PP. Pyrophosphate: ATP. Adenosine-S-triphosphate: ADP Adenosine 5'-diphosphate; AMP Adenosine 5'-monophosphate; Pi. Phosphate, HCO_3^-. Hydrogen carbonic acid, CO., Carbon dioxide.

nucleotides *in vivo* is *de novo* synthesis, and negative feedback mostly controls the rate of nucleotide synthesis (114). In the process of making purines, PRPP is transformed into inosine monophosphate (IMP), which needs 6 ATP, glutamine, glycine, and aspartate. Then, IMP can be converted into guanosine monophosphate (GMP) or adenosine monophosphate (AMP) through different enzymes. The purine nucleotide salvage mechanism is easier and uses less energy than the *de novo* synthesis method (115, 116). However, since the lack of the enzyme system which can synthesize purine nucleotides from scratch, the brain and bone marrow can only conduct the salvage approach to generate purines (117, 118). In addition, previous studies have shown that the *de novo* nucleotide synthesis pathway is also usually found in proliferating cells, including immune cells and cancer cells (119, 120). An earlier study (121) revealed that a higher level of *de novo* purine synthesis was identified in the leukocytes and plasma of newly diagnosed CML patient due to the enhancement of 5-aminoimidazole-4-carboxilic acid ribonucleoside (CAIR). They discovered that the majority of the purine levels had stabilized toward the control values after IM and NIL therapy. However, adenosine 5'-monophosphate, guanine, guanosine, guanosine 5'-monophosphate, and inosine 5'-monophosphate did not change toward the control values in the patients receiving DAS.

Pyrimidine synthesis is also split into *de novo* synthesis and salvage pathways, just like purine biosynthesis (122). In addition to PRPP, pyrimidine biosynthesis also needs aspartate, glutamine, bicarbonate, and 2 ATP (123). Leukocytes from newly diagnosed patients had elevated levels of pyrimidine metabolism, particularly cytosine, cytidine 5'-monophosphate, cytidine 2',3'-cyclic phosphate, and uridine 5'-monophosphate (121). Most of these metabolites returned to control levels after TKI therapy. But, some pyrimidine metabolites (cytidine, cytidine 5'-triphosphate, and uridine) were still present at similar levels in the patients receiving DAS.

By multi-omics, Taymaz-Nikerel et al. revealed that, after IM treatment, the fluxes of the processes involved in the production of purine and pyrimidine nucleotides were dramatically downregulated in yeast (103).

Immunometabolism

Historically, abnormal energy metabolism has been known as a hallmark of cancer (124). Recent research has revealed that immune cells undergo metabolic reprogramming during the activation and differentiation processes, giving rise to the notion of "immunometabolism" (125). The field of immunometabolism is about how metabolic processes affect immune cell functions in physiological and pathological situations (126). In cancer cells, complex and dynamic metabolic reprogramming can make themselves to accommodate tumor microenvironment (TME), which can restrict the biosynthetic and bioenergetic demands for growth (127). Additionally, cancer cell metabolism not only aggressively competes for essential resources but also produces metabolic byproducts that affect immune cell activation, fitness, and effector function in a direct or indirect ways (128–131). Instead of inhibiting or killing cancer cells, these defective immune cells even may become tumor-supporting cells to speed up the spread and invasion of cancer (125).

In recent years, the way to manage tumor patients has significantly changed as a result of tumor immunotherapy (132). During the treatment, TKIs do not only target BCR-ABL1 but also inhibit additional targets such c-KIT, TEC, SRC, FLT3, Lck, and mitogen-activated kinases (MAPK) (133). This "off-target" effect can alter immune responses, both harmful and beneficial. Lisa Christiansson et al. found that (134) IM and DAS can both lower immune escape mechanisms by reducing the

number of myeloid suppressor cells and the inhibitory factors arginase 1 (Arg1), Myeloperoxidase (MPO), and IL-10. According to researches, the proportion of Myeloid-derived suppressor cells (MDSC) and the blood concentrations of Arg1 and inducible nitric oxide synthase (iNOS) were both considerably higher in CML patients at diagnosis and significantly lower after TKI therapy (135). ZIYUAN LU et al. found that (136) following treatment with a TKI (IM, DAS or NIL), Total T cells, Tregs (whose decline became more pronounced over time), CD4+ T cells, and CD8+ T cells all reduced to varying degrees in CML patients. They also revealed that IM and DAS may be more effective than NIL on decreasing the number and function of Tregs. Silke Appel et al. (137) reveals that IM inhibits dendritic cell (DC) differentiation and function *via* Akt and nuclear factor-κB signal transduction. After then, Daniela Dorfel et al. (138) discovered that both IM and NIL considerably and similarly hampered monocyte differentiation into DCs, with only a partial recovery after TKI discontinuation.

Taking into account the long-term side effect and the cumulative cost of TKI therapy in children, it is clear that it is important to avoid lifelong treatment with TKIs. As mentioned above, LSCs have high correlations with tumor recurrence and it is crucial to eliminate LSCs. One potential solution is to stop after a certain period of deep molecular remission and restore normal immune functions, especially the NK cells (139–142). Previous studies indicated that patients with stable NK cell counts accompanied by higher cytotoxicity and increased killing capacity are more inclined to get sustained treatment-free survival (142, 143). Previous study showed that following TKI treatment, the proportion of effector NK cells were increased (135). The results indicate that the number and killing capacity of NK cells may be utilized to further assess the risk of TKIs discontinuation. Geoffrey D. Clapp et al. suggested that (144) carefully timed vaccines may stimulate the patient's immune system to drive the residue LSCs to extinction. In recent trials of Ph+ ALL, Schultz KR et al. (145) used limited duration, more intensive chemotherapy in combination with TKIs for children and adolescents and had an initial observation of substantially good outcomes. It may be another option to eliminate the CML LSCs.

TKI adverse effects in children

Due to off-target effects, IM can cause substantial growth abnormalities in children with CML (146–148) by a direct effect on the growth plate (149), acquired growth hormone deficiency and disturbing the GH : IGF-1 axis (150). Second- and third-generation TKIs in children have less clinical data, but DAS appears to have a similar impact on growth (151).

TKIs' teratogenic potential makes them potentially harmful to pregnant women as well (152). Associated studies showed that (153) female partners of male patients are not at risk for pregnancy-related complications and TKI should be continued. For female patients, contraception should be planned during TKI, so when in major molecular remission for more than two years, pregnancy can be planned. In addition, NIL appears to be the safest (153).

As recommendations for monitoring and supportive care in children with CML receiving TKI therapy, height, weight, gonadotropins, sex steroids, thyroid function (TSH, free T4), echocardiogram and electrocardiogram be examined routinely (14).

Summary

Overall, TKI is obviously regarded to be the most effective kinase inhibitor for CML treatment nowadays and the role of TKI in Ph+ ALL treatment has also attracted increased attention. Recently, an increasing number of studies have demonstrated that TKIs can affect the normal metabolism of Ph+ leukemia cells to achieve therapeutic purposes. But in children, there are still many difficulties to surmount, such as the off-target effects, drug tolerance, disease recurrence, adverse effects, cumulative cost. In this study, we perform a more detailed analysis about cellular metabolism alterations after TKI therapy, but further research is needed because so many interested targets can be combined with TKI therapy to provide great benefits to Ph+ leukemia children.

Author contributions

JH, JY, TWL, LL, TQL, and ZX performed the collection and interpretations of all relevant literature. CL and LW write the manuscript. CC and JD critically read and revised 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.

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Transcriptome analysis reveals effects of leukemogenic SHP2 mutations in biosynthesis of amino acids signaling

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Gain-of-function mutations of SHP2, especially D61Y and E76K, lead to the development of neoplasms in hematopoietic cells. Previously, we found that SHP2-D61Y and -E76K confer HCD-57 cells cytokine-independent survival and proliferation *via* activation of MAPK pathway. Metabolic reprogramming is likely to be involved in leukemogenesis led by mutant SHP2. However, detailed pathways or key genes of altered metabolisms are unknown in leukemia cells expressing mutant SHP2. In this study, we performed transcriptome analysis to identify dysregulated metabolic pathways and key genes using HCD-57 transformed by mutant SHP2. A total of 2443 and 2273 significant differentially expressed genes (DEGs) were identified in HCD-57 expressing SHP2-D61Y and -E76K compared with parental cells as the control, respectively. Gene ontology (GO) and Reactome enrichment analysis showed that a large proportion of DEGs were involved in the metabolism process. Kyoto Encyclopedia of Gene and Genome (KEGG) pathway enrichment analysis showed that DEGs were the mostly enriched in glutathione metabolism and biosynthesis of amino acids in metabolic pathways. Gene Set Enrichment Analysis (GSEA) revealed that the expression of mutant SHP2 led to a significant activation of biosynthesis of amino acids pathway in HCD-57 expressing mutant SHP2 compared with the control. Particularly, we found that *ASNS*, *PHGDH*, *PSAT1*, and *SHMT2* involved in the biosynthesis of asparagine, serine, and glycine were remarkably up-regulated. Together, these transcriptome profiling data provided new insights into the metabolic mechanisms underlying mutant SHP2-driven leukemogenesis.

KEYWORDS

leukemia, SHP2 mutations, metabolism, transcriptome, biosynthesis of amino acids

Introduction

Src Homology 2 domain-containing protein tyrosine Phosphatase-2 (SHP2), encoded by *PTPN11* gene, is a classical non-receptor protein tyrosine phosphatase (PTP) (1). It is the first PTP recognized as an oncogene. SHP2 plays key roles in regulating RAS-ERK, PI3K-AKT, JAK-STAT and other signaling pathways, which are mainly downstream signals of growth factor, cytokine, and integrin receptors (2, 3). In general, SHP2 mutations are rare in solid tumors (3). Germline mutations in SHP2 present in ~50% of Noonan Syndrome and ~90% of LEOPARD syndrome, both congenital developmental disorders and mainly characterized by growth retardation, short stature, facial features, and heart defects (4). Somatic SHP2 mutations mainly occur in several types of hematologic malignancies, including ~10% myelodysplastic syndromes, ~5% juvenile acute myeloid leukemia, ~7% B-cell acute lymphoblastic leukemia, and particularly ~35% juvenile myelomonocytic leukemia (JMML) (3, 5–7). However, the molecular mechanisms of leukemogenesis driven by mutant SHP2 are not fully understood. Previous studies about SHP2 mutants mainly focus on the activation of tumor proliferation signaling pathways and the tumor microenvironment (1, 3). However, the effects of SHP2 mutants on cancer-cell metabolism have not been investigated. Characterizing the alterations in cellular biosynthesis can provide insights into the mechanism of mutant SHP2-driven leukemogenesis.

Various biological hallmarks of tumor cells are closely related to cell metabolism, including rapid proliferation, immune escape and drug resistance (8). The use of cellular nutrient generally requires the binding of growth factors to their receptors to activate a series of signaling pathways that initiate cell metabolism (9). However, gain-of-function mutations occur in growth factor receptors or downstream pathway genes in most tumor cells (10), leading to constantly activated signals that overcome the growth factor dependency (11). As the result, cancer cells acquire the ability to autonomously uptake nutrients, providing a material basis for the uncontrolled division and proliferation (9). Meanwhile, abnormal metabolic pathways often induce cancer cell-specific vulnerabilities, which provided potential therapeutic targets.

Metabolic reprogramming is believed to result from oncogene activation or metabolic enzymes alterations (12). Previous studies have shown that some key proteins in cell proliferation-related signaling pathways are involved in metabolic reprogramming (13). The serine/threonine kinase AKT, for instance, does not only activate cell division signals, but also regulates glucose uptake to provide energy to cancer cells. The activation of AKT has been found to support the growth factor-independent survival *via* multi-step regulation of glucose metabolism, including promotion of glucose uptake by up-regulation of glucose transporter 1 (GLUT1) and activation of hexokinase (HK) (14, 15). In most cases, a variety of oncogenes lead to metabolic reprogramming by inducing broad changes in gene expressions (16). For instance, MYC enhances aerobic glycolysis by up-regulating GLUT1, PKM, LDH and MCT1, which also reprograms the glutathione biosynthesis (13, 17). Besides, cancer cells with specific oncogenic activation exhibit a defined metabolic preference (16). For example, EGFR activation promotes the serine synthesis pathway whereas FGFR activation enhances aerobic glycolysis and recycles lactate (16).

Our previous studies have shown that the expression of mutant SHP2 led to growth factor-independency of HCD-57, an erythropoietin (EPO)-dependent erythroid leukemia cell line (18), suggesting the possibility of mutant SHP2-reprogrammed cell metabolism in HCD-57. For this reason, we investigated altered metabolism pathways of parental and SHP2-mutant HCD-57 based on transcriptome analysis. Analysis of Kyoto Encyclopedia of Gene and Genome (KEGG) metabolism-related pathways revealed that differentially expressed genes (DEGs) were mainly enriched in glutathione metabolism and biosynthesis of amino acids pathways. Gene Set Enrichment Analysis (GSEA) showed the biosynthesis of amino acids pathway was significantly activated by the expression of mutant SHP2. In addition, we found that mRNA expression of *ASNS* involved in asparagine synthesis, *PHGDH* and *PSAT1* involved in serine biosynthesis, and *SHMT2* involved in glycine synthesis were significantly increased in HCD-57 expressing mutant SHP2, compared with parental cells. Taken together, we identified aberrant metabolic pathways in mutant SHP2-driven leukemia cells, which may provide potential metabolism-targeted therapies for leukemia with SHP2 mutations.

Materials and methods

Cell culture

HCD-57 was a kind gift from Dr. Zhizhuang Joe Zhao, the University of Oklahoma, Health Science Center. HCD-57 was cultured in IMDM (Gibco, MA, USA) supplemented with 20% FBS (Hyclone, UT, USA) and 20 ng/mL EPO (Peprotech, NJ, USA). Parental HCD-57 cells were starved for 8 h without EPO before total RNA isolation. HCD-57/SHP2-D61Y and HCD-57/SHP2-E76K, as the mutant SHP2-expressing cells, have acquired EPO-independent survival and proliferation. They were cultured in IMDM supplemented with 20% FBS and in absence of EPO. All cells were cultured in a humidified atmosphere at 37°C with 5% CO_2.

Generation of mutant SHP2-transformed HCD-57

Retroviruses carrying mutant SHP2 were generated by using pMSCV-IRES-GFP as described previously (19). Briefly, the full-length SHP2-D61Y and SHP2-E76K were cloned to pMSCV-IRES-GFP, respectively. Plasmids containing mutant SHP2 were used to transfect GP2-293 cells together with pVSV-G helper plasmid using Lipofectamine 3000 reagent (Thermo Fisher Scientific, MA, USA). Subsequently, the medium was collected and centrifuged at 20, 000 g for 2 h at 4°C to enrich retroviruses. HCD-57 cells were infected by retroviruses with 5 μg/mL polybrene (Sigma-Aldrich, MO, USA) with centrifugation at 1, 800 g for 2 h at room temperature. The infected cells were cultured in IMDM in absence of EPO. Single colonies were picked after 8-10 days of culture and further expanded in EPO-free IMDM supplemented with 20% FBS.

Total RNA isolation

Total RNA from cells was isolated using Trizol (Invitrogen, CA, USA) per the manufacturer's instructions. The isolated total RNA was qualified and quantified by using a Nano Drop and Agilent 2100 bioanalyzer (Thermo Fisher Scientific, MA, USA).

mRNA library construction

RNA-seq library construction and RNA high-throughput sequencing were entrusted to Beijing Genomics Institute (Beijing, China). In brief, mRNA for each sample was purified using Oligo (dT)-attached magnetic beads and then fragmented into small pieces with fragment buffer. First-strand cDNA was generated using random hexamer-primed reverse transcription, followed by a second-strand cDNA synthesis and end repair using A-Tailing Mix and RNA Index Adapters. The cDNA fragments were then amplified by PCR, and purified by Ampure XP Beads. The product was validated on the Agilent Technologies 2100 bioanalyzer. The double-stranded PCR products from previous step were heated denatured and circularized by the splint oligo sequence to get the final library, which was amplified with phi29 to make DNA nanoballs (DNBs) containing more than 300 copies of one molecular. DNBs were loaded into the patterned nanoarray and single end 50 bases reads were generated on BGIseq500 platform.

Bioinformatics analysis

Clean reads were filtered by FASTQ (version 0.18.0). Reads containing sequencing adapters, unknown nucleotides ('N' base) and low-quality bases were removed. Clean reads were obtained and stored in FASTQ format. The clean reads were mapped to the reference genome using HISAT2 (v2.2.4). StringTie (V1.3.1) was applied to assemble the map reads and gotten fragments per kilobase of transcript per million mapped reads (FPKM) to calculated gene expression. Bioinformatic analyses were performed using the Omicsmart online platform (http://www.omicsmart.com). Principal component analysis (PCA) was performed with R package gmodels (http://www.r-project.org/). DEGs analysis was performed by edgeR. The parameter of false discovery rate (FDR) below 0.05 and absolute fold change ≥ 2 were considered DEGs. Analysis of Gene Ontology (GO), KEGG, and Reactome were based on database (http://www.geneontology.org/), (https://www.genome.jp/kegg/), and (https://reactome.org/). Enrichment analysis identified significantly enriched in DEGs comparing with the whole genome background. The calculated *P* value was gone through FDR correction, taking FDR ≤ 0.05 as a threshold. GSEA was performed using software GSEA to identify whether a set of genes in specific KEGG pathways shows significant differences in two groups.

Results

Transcriptomic profiling analyses of HCD-57 expressing SHP2-D61Y and -E76K

To investigate the effect of mutant SHP2 on HCD-57 cells, RNA-seq was performed among HCD-57 cells expression SHP2-D61Y, SHP2-E76K, and parental HCD-57 cells. Owing to the expression of mutant SHP2 led to growth factor-independency of HCD-57, we cultured parental HCD-57 cells in medium deprived EPO for 8 h to remove the stimulation of growth factor. PCA analysis clearly separated the parental HCD-57 cells from HCD-57 expressing SHP2-D61Y and -E76K based on PC1, with PC1 contributing 69.6% variation, making it the dominant component (Figure 1A). The number of DEGs was 2443 and 2273 for HCD-57 cells expressing mutant SHP2-D61Y and -E76K compared to the control (Figure 1B), respectively. Hierarchical clustering of differential gene expression patterns was performed, and a heatmap was used to present the results. The analysis revealed comparable patterns among the HCD-57 cells expressing SHP2-D61Y and SHP2-E76K, while the transcriptome profiles of these mutant cells were much different from parental HCD-57 cells deprived of EPO (Figure 1C). A Venn diagram was performed and 1436 mutual DEGs were identified among the two compared groups (Figure 1D).

Mutant SHP2 dysregulated cellular metabolic biological processes

We performed multiple enrichment analyses to investigate biological functions and altered pathways related to these DEGs. We found that a mass of dysregulation genes was related to metabolic process using GO classification in SHP2-D61Y and SHP2-E76K transformed cells compared to the control (Figure 2A). In cells expressing SHP2-D61Y, genes involved in metabolism accounted for ~60% in the total up-regulated DEGs, and ~58% in down-regulated DEGs, respectively. Cells transformed by SHP2-E76K showed ~59% for up-regulated proportion and ~57% for down-regulated proportion involved in metabolism. In addition, KEGG analysis in whole pathway maps identified metabolic pathways as the most significantly enriched pathways in both two comparison groups (Figure 2B). As expected, Reactome analysis also showed significant enrichment of metabolism reaction (Figure 2C). These results indicated that the expression of SHP2 mutants leads to a remodeling of cellular metabolism.

Altered metabolism pathways in HCD-57 expressing mutant SHP2

We performed KEGG pathway analysis of all DEGs to identify significantly enriched metabolic pathways. We found that the most enriched pathways in cells expressing SHP2-D61Y and SHP2-E76K compared with the control were glutathione metabolism and biosynthesis of amino acids pathways (Figure 3A). Subsequently, we found that the expression of mutant SHP2-D61Y and SHP2-E76K significantly activated the biosynthesis of amino acids pathway based on GSEA analysis (Figure 3C). The schematic diagrams of alterations in KEGG pathways regarding biosynthesis of amino acids was revealed in Supplementary Figures 1 and 2. There was a down-regulation in the glutathione metabolism pathway whereas the nominal p-value and FDR q-value (false discovery rate) did not reach a statistical significance (Figure 3B). The remaining metabolic pathways that were significantly enriched ($P<0.05$) both in SHP2-

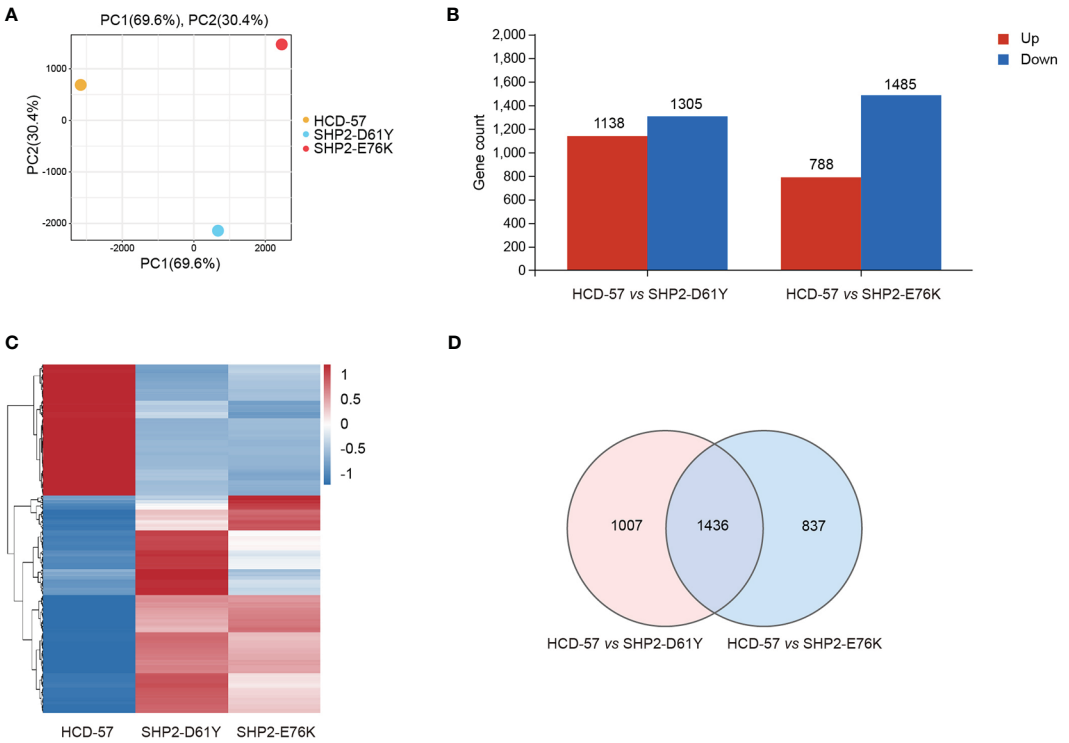

FIGURE 1
Basic transcriptomic analysis profile among mutant-SHP2 transfected cells and parental HCD-57 cells deprived EPO. **(A)** PCA plots of DEGs identified in HCD-57 cells expressing SHP2-D61Y and -E76K compared to parental HCD-57 cells. **(B)** The number of DEGs (up-regulated and down-regulated) in two compared groups. FDR<0.05, |log2FC|>1. **(C)** Heatmap of hierarchical clustering results for all identified DEGs at SHP2-D61Y, SHP2-E76K and parental HCD-57 cells (red, up-regulated; blue, down-regulated). **(D)** Venn diagram of the numbers of DEGs in HCD-57 vs SHP2-D61Y and HCD-57 vs SHP2-E76K.

D61Y and -E76K cells were presented in Table 1. These data suggest that biosynthesis of amino acids may play an important role in leukemogenesis induced by mutant SHP2.

Genes associated with serine and glycine synthesis were significantly up-regulated in SHP2-mutant HCD-57

To further investigate genes with significantly altered expression in the biosynthesis of amino acids caused by the expression of SHP2-D61Y and SHP2-E76K, we obtained 13 mutually dysregulated genes using the Venn diagram (Figure 4A). The heatmap showed significantly up-regulated genes in cells expressing mutant SHP2, including *ASNS*, *PSAT1*, *PHGDH*, *SHMT2*, and *ALDH18A1* (Figure 4B). The FPKM values of 13 mutually dysregulated genes are shown in Table 2. Phosphoglycerate dehydrogenase (PHGDH) catalyzes the reversible oxidation of 3-phosphoglycerate to 3-phosphohydroxypyruvate, the first step of the *de novo* serine biosynthesis pathway. Subsequently, 3- phosphohydroxypyruvate is converted to phosphoserine by phosphoserine aminotransferase 1 (PSAT1) and then to serine by phosphoserine phosphatase. Serine hydroxymethyltransferase (SHMT2) catalyzes the reversible transition from serine to glycine and promotes the production of one-carbon units. Asparagine synthetase (ASNS) converts aspartate and glutamine to asparagine. *ALDH18A1* is a member of the aldehyde dehydrogenase family, and its encoded protein catalyzes the reduction of glutamate to delta1-pyrroline-5-carboxylate, a critical step in the *de novo* biosynthesis of proline, ornithine, and arginine. In addition, our analysis found carbon metabolism, as well as glycine, serine, and threonine metabolism pathways also dysregulated in SHP2-mutant cells, and the differentially expressed genes were also mainly *PSAT1*, *PHGDH*, and *SHMT2* (Supplementary Figure 3). These data suggest that the gain-of-function SHP2 mutants could promote serine and glycine synthesis *via* up-regulating the mRNA expression of *PSAT1*, *PHGDH*, and *SHMT2*.

Discussion

In this study, we performed RNA-seq transcriptome sequencing analysis to identify dysregulated metabolic pathways and key genes based on HCD-57 cells transformed by SHP2-D61Y or -E76K. We found that DEGs caused by the expression of mutant SHP2 were mainly enriched in metabolic pathways, especially glutathione metabolism and biosynthesis of amino acids pathways. Importantly, we found that the biosynthesis of amino acids pathway was significantly activated in HCD-57 cells expressing SHP2-D61Y and SHP2-E76K. In addition, our data showed that the mRNA expression of *ASNS*, *PHGDH*, *PSAT1*, and *SHMT2* involved in asparagine, serine, and glycine biosynthesis were significantly increased in cells expressing mutant SHP2. Furthermore, our analysis found that *PSAT1*, *PHGDH*, and *SHMT2* were also key genes leading to the upregulation of carbon metabolism, as well as glycine, serine, and

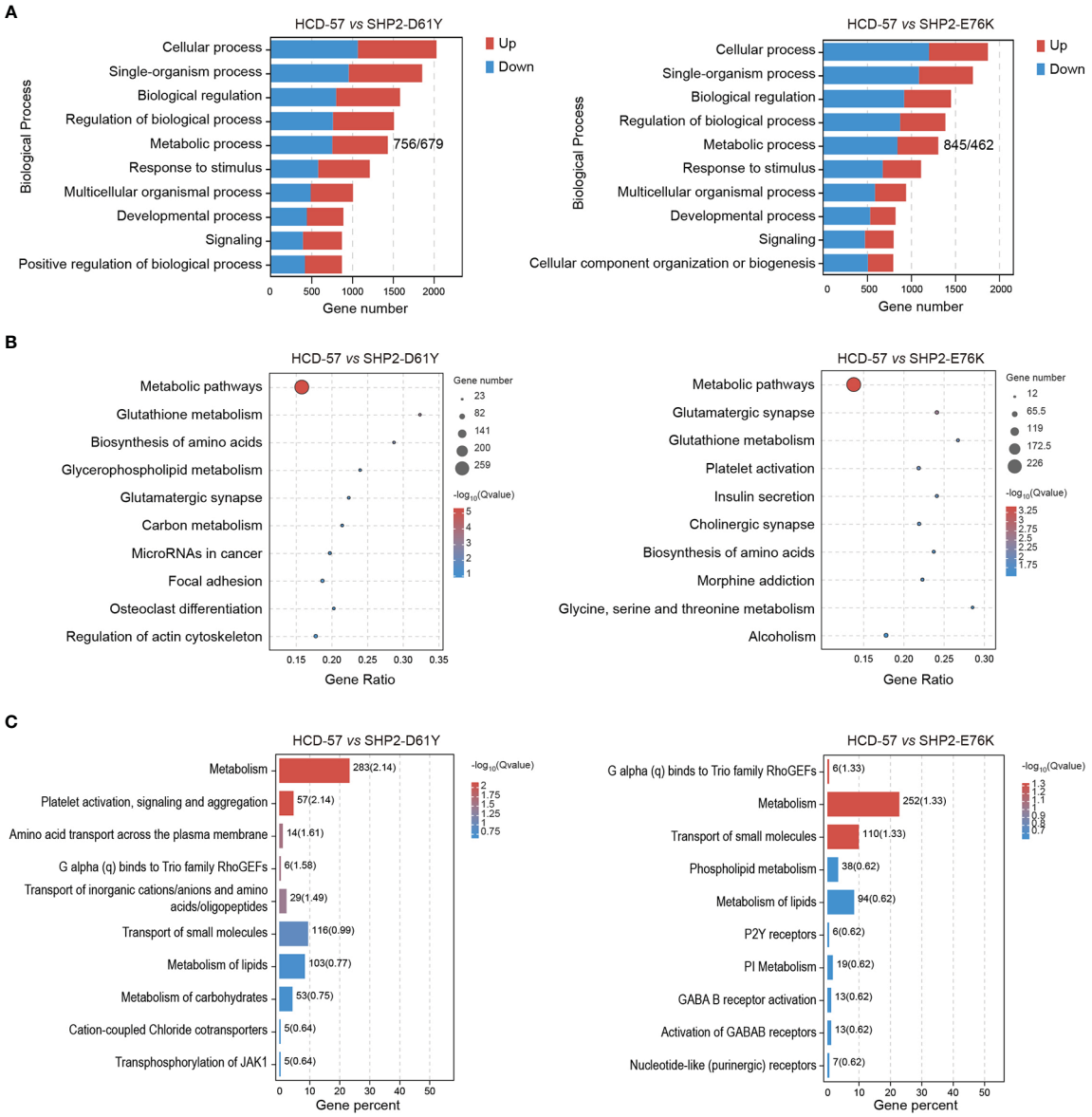

FIGURE 2
Enrichment analyses of all DEGs in HCD-57 vs SHP2-D61Y and HCD-57 vs SHP2-E76K. **(A)** Top 10 biological processes based on descending order by number of DEGs through GO analysis. **(B)** Top 10 pathways enriched by KEGG enrichment analysis. **(C)** Top 10 biological pathways and processes enriched by Reactome analysis.

threonine metabolism pathways. These findings suggest that gain-of-function mutants of SHP2 might promote serine synthesis by activating the expression of *PSAT1* and *PHGDH*, and promote glycine biosynthesis by activating the expression of *SHMT2* for leukemia initiation and progression.

Reprogramming of metabolic pathways ensures the survival and proliferation of cancer cells in a nutrient-deficient environment (20). Besides, immune cell metabolic reprogramming alters immune cell function by interfering with critical transcriptional and post-transcriptional activation mechanisms, to keep growing tumors from being attacked by the immune system (20, 21). Alterations in carbohydrate metabolism in tumor cells have been reported. Tumor cells take up and use more glucose than they need, which is known as the Warburg effect (22). Recently, the amino acid dependence of tumor cells has received more and more attention (23). Amino acids have been demonstrated to be the dominant nitrogen source for hexosamines, nucleotides, and other nitrogenous compounds in rapidly proliferating cells (24, 25). Indeed, like glucose, there are major differences in the uptake and secretion of several amino acids in tumors relative to normal tissues. Compared to normal tissues, tumors require a large number of amino acids for bioenergetic, biosynthetic, and redox balance support (26, 27). This high demand is not limited to essential amino acids, but also for nonessential amino acids (NEAA) (24, 27). NEAA are not only components of proteins but also intermediate metabolites fueling multiple biosynthetic pathways. For example, glycine is synthesized from serine, threonine, choline, and hydroxyproline, and is degraded through the glycine cleavage system, serine hydroxymethyltransferase, and conversion to glyoxylate (28). In addition, glycine is utilized for the biosynthesis of glutathione, heme, creatine, nucleic acids, and uric acid (28).

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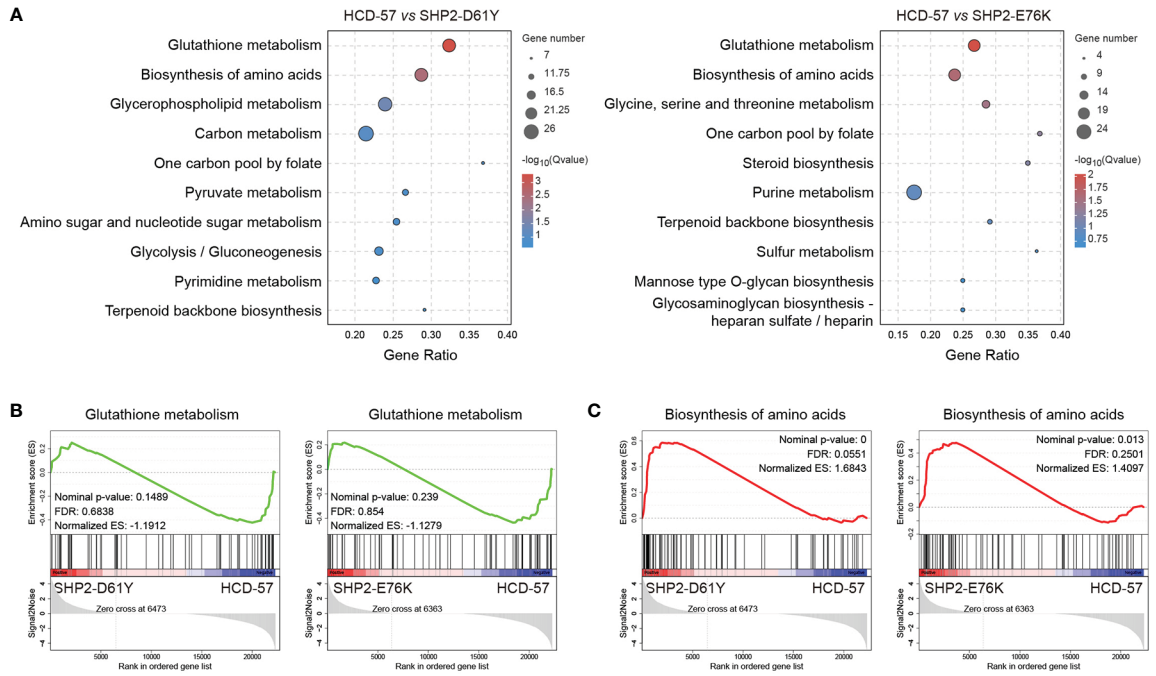

FIGURE 3
Pathways for metabolism-specific dysregulation caused by mutant SHP2 expression. **(A)** Top 10 enriched pathways related to metabolism based on KEGG enrichment analysis for all DEGs. GSEA plots of **(B)** Glutathione metabolism and **(C)** Biosynthesis of amino acids target genes on HCD-57 expressing SHP2-D61Y or -E76K vs parental cells. Normalized enrichment score (NES), nominal *P*-value and FDR Q-values are indicated.

The serine synthesis pathway (SSP) has been widely reported as a critical pathway enabling cancer cell proliferation and metastasis. Serine is a central precursor of biosynthetic metabolism, including being charged onto transfer RNAs for protein synthesis, providing head groups for sphingolipid and phospholipid synthesis, and serving as a precursor for cellular glycine and one-carbon unit (29). PHGDH is a rate-limiting enzyme for *de novo* serine biosynthesis and is mainly up-regulated to active serine biosynthesis. A high PHGDH expression has been extensively reported in several tumors, particularly breast and melanoma, and its high expression in these tumors is associated with poor prognosis (27). Importantly, its knockdown and silence exhibit obvious anti-tumor responses both *in vitro* and *in vivo* (30). PSAT1 is the transaminase for serine. It catalyzed the phosphohydroxypyruvate oxidized by PHGDH to produce phosphoserine, which is then dephosphorylated by 1-3-phosphoserine phosphatase (PSPH) to form serine. *PSAT1* expression is elevated in colon cancer and lung adenocarcinoma, and has been shown to enhance cell proliferation, metastasis, and chemoresistance (31, 32).

TABLE 1 List of metabolic pathways significantly enriched both in HCD-57 cells expression SHP2-D61Y and -E76K (*P*<0.05).

Pathways		HCD-57 *vs* SHP2-D61Y				HCD-57 *vs* SHP2-E76K			
Name	KEGG-B-Class	DEGs	*P*-value	Q-value	NES (GSEA)	DEGs	*P*-value	Q-value	NES
Metabolic pathways	Global and overview maps	259	0.0000	0.0000	NA	226	0.0000	0.0004	NA
Glutathione metabolism	Metabolism of other amino acids	23	0.0000	0.0005	-1.1912	19	0.0001	0.0090	-1.1279
Biosynthesis of amino acids	Global and overview maps	23	0.0000	0.0030	1.6843	19	0.0004	0.0217	1.4097
Carbon metabolism	Global and overview maps	26	0.0014	0.0748	1.5362	19	0.0450	0.2511	1.2666
One carbon pool by folate	Metabolism of cofactors and vitamins	7	0.0041	0.1219	0.8887	7	0.0021	0.0486	-0.7613
Pyrimidine metabolism	Nucleotide metabolism	13	0.0124	0.2027	-0.8285	7	0.0093	0.1126	-0.8684
Glycine, serine and threonine metabolism	Amino acid metabolism	10	0.0200	0.2634	1.2259	12	0.0009	0.0302	1.0145
Sulfur metabolism	Energy metabolism	4	0.0309	0.3111	NA	4	0.0213	0.1769	NA
Purine metabolism	Nucleotide metabolism	23	0.0451	0.3758	0.8631	24	0.0074	0.1061	-0.9653

The threshold of significant differentially expressed genes (DEGs) was set as FDR<0.05, |log2FC|>1. *P*-value and Q-value were calculated from KEGG analysis. Normalized Enrichment Score (NES) were obtained from Gene Set Enrichment Analysis (GSEA).

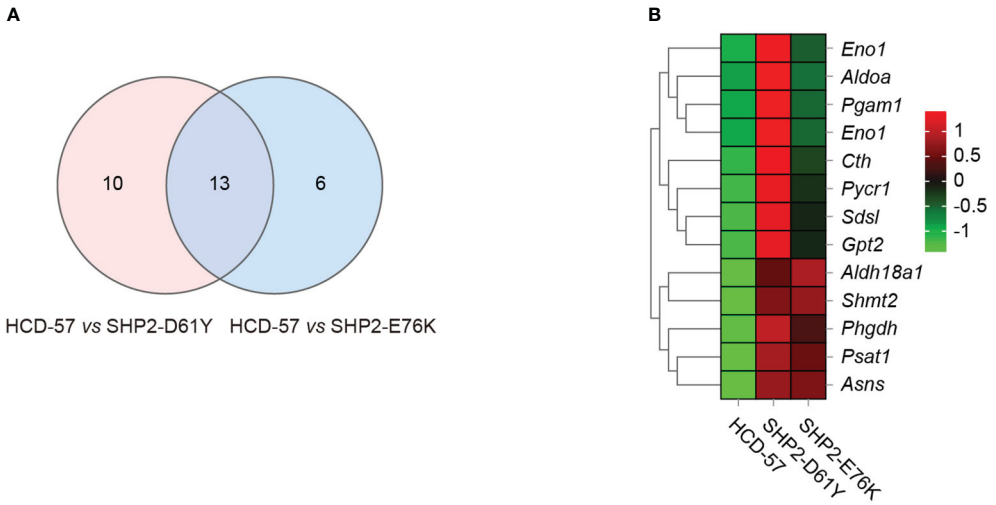

FIGURE 4
Differential gene analysis involved in the biosynthesis of amino acids pathway caused by mutant SHP2 expression. **(A)** Venn diagram indicating the overlap dysregulated genes in the biosynthesis of amino acids pathway in HCD-57 vs SHP2-D61Y and HCD-57 vs SHP2-E76K. **(B)** Heatmap of 13 DEGs shared in both comparisons in the biosynthesis of amino acids metabolites pathway.

Serine and glycine metabolism are closely linked, as glycine is directly generated from serine *via* the serine hydroxymethyltransferase enzymes SHMT1 and SHMT2 (24). Importantly, the conversion of serine to glycine provides one-carbon units, which provide the necessary proteins, nucleic acids, lipids, and other biological macromolecules to support tumor growth (27). Serine, glycine, and their relation to one-carbon metabolism are highly relevant aspects of tumor metabolism (33). The directionality of serine/glycine conversion is a significant factor for cancer cell metabolism and evidence indicates that mitochondrial SHMT2 is the main serine-glycine converting enzyme (34). SHMT2 is upregulated in various cancer cells, and its depletion could trigger ROS-dependent mitochondria-mediated apoptosis (35).

ASNS converts aspartate and glutamine to asparagine and glutamate through an ATP-dependent amidotransferase reaction (36). Asparagine plays a crucial regulatory role in conditions of glutamine depletion (37). The precise role of asparagine in modulating tumor growth is unknown (38). ASNS is frequently up-regulated in tumors and is associated with poor prognosis (37, 39). In acute lymphoblastic leukemia (ALL), primary cells and many ALL cell lines exhibit a low expression level of ASNS (40). Su et al. found that different cells and patients expressed different amounts of ASNS mRNA and suggested it should pay attention to the differentiation of mRNA, protein content, and kinase activity in ASNS (41). Besides, Hutson et al. demonstrated that ASNS mRNA content increased in cells deprived of free amino acids (42). Later studies have also shown that endoplasmic reticulum stress increases ASNS transcription *via* the unfolded protein response (43). Ye et al. concluded that activation of ASNS by ATF4 with amino acid limitation may serve a vital biological process for tumor initiation and growth (44).

Exploring the metabolic adaptation mechanisms of uncontrolled tumor proliferation led by driver mutations has become a hot topic in cancer research. Preclinical research and clinical practice have shown therapeutic benefits by targeting tumor amino acid metabolism. One example is asparaginase that depletes both asparagine and glutamine in serum, which has been widely used to treat childhood acute lymphoblastic leukemia (45). A detailed understanding of the metabolic adaptation mechanisms of tumor cells may help the discovery of novel therapeutic targets, especially for relapsed and refractory neoplasms including SHP2-mutant JMML. The identification of specific driver mutation-dependent metabolic vulnerabilities is the bottleneck for the precise tumor treatment, which requires further investigation in the further.

TABLE 2 FPKM values of 13 genes commonly dysregulated in biosynthesis of amino acids pathway in HCD-57 cells expression SHP2-D61Y and -E76K.

Symbol	*ENO1*[1]	*ALDOA*	*PGAM1*	*ENO1*[2]	*CTH*	*PYCR1*	*SDSL*
HCD-57	75.26	122.95	18.87	55.07	0.80	1.86	7.60
SHP2-D61Y	633.94	1349.77	239.24	527.25	15.18	8.98	36.05
SHP2-E76K	195.85	281.77	56.60	136.91	5.30	4.64	20.00
Symbol	*GPT2*	*ALDH18A1*	*SHMT2*	*PHGDH*	*PSAT1*	*ASNS*	
HCD-57	2.72	36.96	65.34	61.91	48.00	104.82	
SHP2-D61Y	13.12	88.33	159.39	231.75	192.18	318.86	
SHP2-E76K	7.11	100.42	165.38	183.02	169.66	305.13	

ENO1[1], Enolase 1, alpha non-neuron; ENO1[2], Enolase 1B, retrotransposed.

Data availability statement

The data presented in the study are deposited in the SRA repository, accession number PRJNA821339.

Author contributions

YZ, ZP, CC, and YC conceived the project. YZ, ZC, CH, and YG performed the experiments. YZ, QZ, and DZ analyzed data. YZ, ZC, BH, ZP, CC, and YC 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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1090542/full#supplementary-material

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Possible mechanism of metabolic and drug resistance with L-asparaginase therapy in childhood leukaemia

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L-asparaginase, which hydrolyzes asparagine into aspartic acid and ammonia, is frequently used to treat acute lymphoblastic leukaemia in children. When combined with other chemotherapy drugs, the event-free survival rate is 90%. Due to immunogenicity and drug resistance, however, not all patients benefit from it, restricting the use of L-asparaginase therapy in other haematological cancers. To solve the problem of immunogenicity, several L-ASNase variants have emerged, such as *Erwinia*-ASNase and PEG-ASNase. However, even when *Erwinia*-ASNase is used as a substitute for *E. coli*-ASNase or PEG-ASNase, allergic reactions occur in 3%-33% of patients. All of these factors contributed to the development of novel L-ASNases. Additionally, L-ASNase resistance mechanisms, such as the methylation status of ASNS promoters and activation of autophagy, have further emphasized the importance of personalized treatment for paediatric haematological neoplasms. In this review, we discussed the metabolic effects of L-ASNase, mechanisms of drug resistance, applications in non-ALL leukaemia, and the development of novel L-ASNase.

KEYWORDS

L-asparaginase, asparagine synthetase, metabolic, drug resistance, childhood leukaemia

Introduction

L-asparaginase (L-ASNase), an enzyme that hydrolyzes asparagine, is one of the most successful drugs for metabolic targeting to date and one of the most important chemotherapeutic drugs in standardized regimens for childhood ALL. L-ASNase is essential for improving the complete remission rate and long-term survival in children with ALL. In the moderate/low-risk group mainly according to the treatment response of 15-19 days and the level of minimal residual disease in 29-45days, event-free survival and overall survival rates can reach 90% when combined with other chemotherapeutic drugs (1–3). L-ASNase (L-ASNase) has been shown to have anticancer activity that depends on its ability to hydrolyse asparagine since it was discovered in guinea pig serum in 1953 (4–7). In 1966, Dolowy et al. first reported complete remission in a case of refractory childhood acute

lymphoblastic leukaemia (ALL) treated with guinea pig-derived L-ASNase (8). In 1970, Clarkson et al. first reported the treatment of ALL with purified *E. coli*-derived L-ASNase (*E. coli*-ASNase) and the induction of remission (9). In the following decades, L-ASNase was widely used in the treatment of ALL. Currently, the clinically used L-ASNases include *E. coli*-ASNase, *Erwinia*-ASNase, and PEG-ASNase, among which PEG-ASNase has the longest half-life and lowest immunogenicity (5, 10). Nevertheless, allergic reactions to L-ASNase still occur in 30%-70% of patients, which limits its efficacy (11). To provide new insights into using L-ASNase in treating paediatric leukaemia, we discussed the metabolic effects of L-ASNase, mechanisms of drug resistance, applications in non-ALL leukaemia, and the development of novel L-ASNase in this review.

Metabolic effects of L-ASNase on leukaemic cells

Tumour cells have different metabolic patterns compared to normal cells. This metabolic pattern is manifested by increased glycolysis, glucose uptake, and uptake and catabolism of amino acids (12–14). Metabolic reprogramming allows tumour cells to show resilience in hypoxic and nutrient-deficient environments. At the same time, however, such metabolic alterations also make tumour cells exhibit specific vulnerabilities, such as an increase in certain specific metabolic demands (15). The increased metabolic demands determine the importance of glucose and amino acids in tumour metabolism. Unlike normal cells, amino acids that are not essential to normal cells may be essential to tumour cells because tumour cells usually lose the ability to synthesize these amino acids *de novo*, enabling the amino acid deprivation therapy.

Asparagine is a nonessential amino acid involved in protein synthesis for normal cells, which can be obtained from food or produced by the combination of aspartate acid with ammonia catalysed by asparagine synthase (ASNS) (16, 17).Different from normal cells, due to the lack of ASNS, leukaemia cells frequently fail to synthesize asparagine and therefore must rely on the host to supply asparagine for their protein synthesis requirements. By catabolizing asparagine in serum, L-ASNase can expose leukaemia cells to an asparagine-deficient environment, and thus affecting protein synthesis in leukaemic cells and leading to their growth inhibition or death (5, 16–20). In addition, Hermanova et al. further demonstrated the molecular mechanism by which L-ASNase inhibits protein synthesis in leukaemic cells (21). The mammalian target of rapamycin protein complex 1 (mTORC1) plays a central role in the amino acid response. RagA/RagB switches from a GDP-bound state to a GTP-bound state as amino acid levels rise, which activates mTORC1, and in turn stimulates a series of downstream reactions, including protein synthesis (22). However, it was recently discovered that RagB-expressing cells can still activate mTORC1 even in an amino acid-deficient environment (23). By treating wild-type RagB cells and RagB-mutant cells (in a permanent GTP-bound state) separately with L-ASNase and assaying the levels of the mTORC1 downstream molecule p-S6 protein, Hermanova et al. found that wild-type RagB cells had significantly lower p-S6 protein levels, while RagB-mutant cells did not show significant changes in p-S6 protein levels. That is, L-ASNase can inhibit protein synthesis by inhibiting RagB-mTORC1 (21) (Figure 1).

L-ASNase also has glutaminase (GLNase) activity that can hydrolyze glutamine. In the presence of ASNS, glutamine can act as an amino donor to facilitate the production of aspartic acid into asparagine. Therefore, the hydrolysis of glutamine by L-ASNase also contributes to the reduction of asparagine levels, improving the efficacy (16, 19, 24) (Figure 1). However, whether the anti-leukaemic effect of L-ASNase depends on GLNase activity is controversial (25–32). First, Offman and Parmentier et al. demonstrated that the killing effect of L-ASNase on leukaemic cells was reliant on GLNase activity and that the cytotoxicity of L-ASNase on leukaemic cells increased with increasing GLNase activity (25, 29). Chan et al. also demonstrated in a recent study that L-ASNase with GLNase activity was more cytotoxic to leukaemic cells and could better prolong the survival of mice in an ASNS-negative SUP- B15 xenograft model (32). In contrast, a previous study showed that L-ASNase without GLNase activity could achieve the same level of antitumour effects as wild-type L-ASNase in ASNS-negative leukaemia cell lines (30). Nguyen et al. also demonstrated that L-ASNase mutants with low GLNase activity had the same level of antitumour activity as L-ASNase mutants with high GLNase activity in an ASNS-negative SUP-B15 leukaemia cell xenograft model (31). However, the L-ASNase in their research is not completely devoid of GLNase activity. Therefore, we believe that GLNase activity of L-ASNase is required for the killing effect of L-ASNase in ASNS-negative tumour cells, but at what level of GLNase activity needs to be maintained is a question that require confirmation through more experiments.

In addition to protein synthesis and amino acid metabolism, Hermanova et al. found that L-ASNase can also affect the energy metabolism of leukaemic cells, including increased fatty acid oxidation and inhibition of glycolysis. They suggested that the inhibition of mTORC1 by L-ASNase was responsible for inducing fatty acid oxidation. Moreover, they found that fatty acid oxidation inhibitors and L-ASNase can act synergistically to kill cells (21). Therefore, the combination regimen of fatty acid oxidation inhibitors with L-ASNase may provide a brand-new option for the treatment of ALL. Furthermore, Takahashi et al. demonstrated in their study that L-ASNase can inhibit glycolysis in leukaemic cells (18), but the precise molecular mechanism of this is unknown. In general, L-ASNase can affect the energy metabolism of leukaemia cells, which in turn may hurt the efficacy of L-ASNase. Clarifying the specific mechanism will provide new selections to ALL treatment, and more research on combined treatment regimens targeting energy metabolism such as fatty acid oxidation will bring new hope to ALL treatment.

Mechanism of drug resistance

Asparagine synthase

Studies on the mechanism of L-ASNase resistance have been widely conducted. Numerous studies have found elevated expression of ASNS in L-ASNase-resistant tumour cells. It has been confirmed that L-ASNase-resistant cells express higher levels of ASNS than L-ASNase-sensitive cells (33–36). Scherf and Holleman et al. reported that L-ASNase-sensitive cells express lower levels of ASNS mRNA *in vitro* (37, 38). In contrast, however, the results of Fine et al. did not

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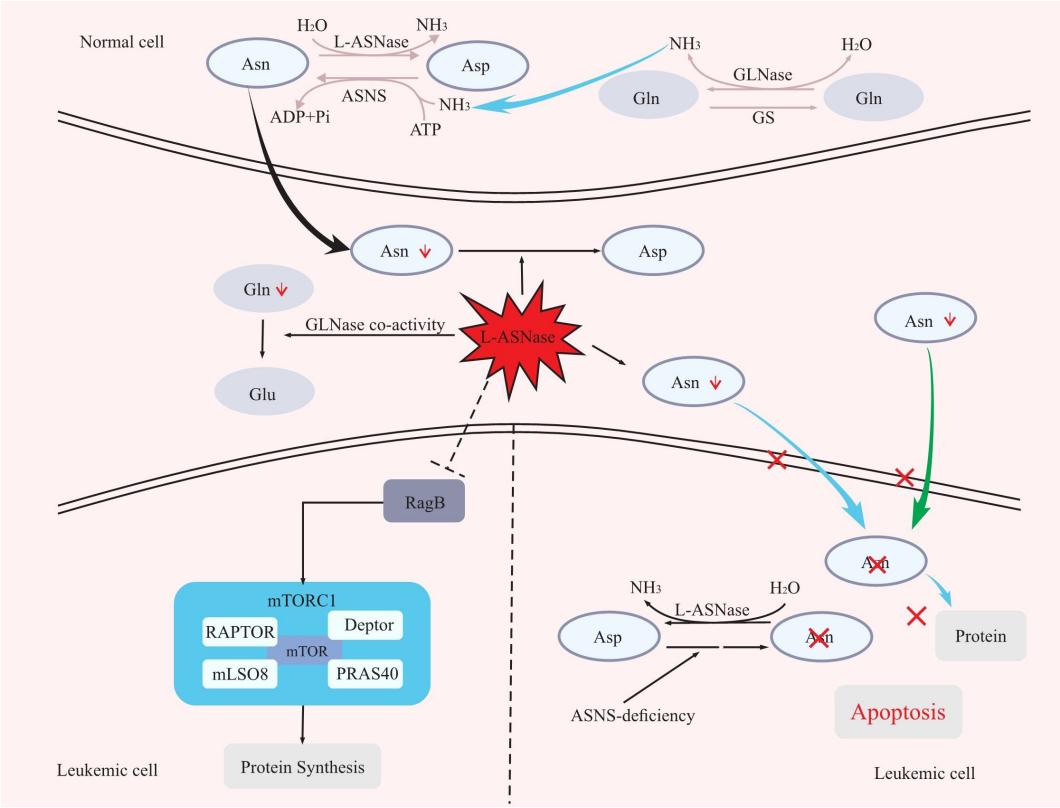

FIGURE 1
The mechanism of L-ASNase. L-ASNase depletes Asn, and GLNase coactivity hydrolyzes Gln, which further reduces Asn, leading to apoptosis of leukaemia cells. L-ASNase can inhibit RagB-mTORC1 and thus inhibit protein synthesis. Asp, aspartic acid; Asn, asparagine; Gln, glutamine; Glu, glutamic acid; ASNS, asparagine synthetase; L-ASNase, L-asparaginase; GS, glutamine synthetase; GLNase, glutaminase.

find a correlation between the expression level of ASNS mRNA and sensitivity to L-ASNase (39). In B-lineage lymphocytic leukaemia cells carrying the TEL-AML 1 translocation, Stams et al. also obtained the same results as Fine et al. (40). Even in other studies, higher expression levels of ASNS mRNA were found in L-ASNase-sensitive TEL-AML1-positive cells compared with TEL-AML1-negative cells that were resistant to L-ASNase (41), but they did not further elucidate the relationship between TEL-AML1 fusion genes and ASNS gene expression. In addition, Su et al. stated that high ASNS expression did associate with resistance to L-ASNase. But they suggested that it should be the ASNS protein, rather than the mRNA, to be tested as indicators of L-ASNase resistance as there was no significant correlation between the levels of ASNS mRNA and ASNS protein (42). In any case, these studies illustrate the point that ASNS expression confers L-ASNase resistance in leukaemic cells.

Other studies have revealed that the methylation status of the *ASNS* promoter region can affect the transcription of *ASNS* and thus affect the sensitivity of L-ASNase. *ASNS* is part of the amino acid response pathway that is activated by amino acid deficiency (43, 44). When asparagine is deprived, tumour cells can respond *via* the GCN2-ATF4 pathway. ATF4 binds to the *ASNS* promoter in a hypomethylated state and induces its expression (45). Jiang et al. found that the hypermethylated state of the *ASNS* promoter restricted the binding of the transcription factor ATF4 upon amino acid depletion, and thereby inhibiting *ASNS* expression (46). Overall, amino acid deficiency-induced *ASNS* expression requires both GCN2 activation and hypomethylation of the *ASNS* promoter region, which enable ATF4 binding to drive *ASNS* expression. A cohort study by Akahane et al. further confirmed that the hypomethylation status of the *ASNS* promoter region is associated with L-ASNase resistance. Their analysis of 75 Japanese children with T-ALL revealed an intermediate (33.3% < methylation <66.7%) or low (<33.3%) methylation status of the *ASNS* promoter region in 92% of refractory/relapsed cases (47). In addition, Touzart et al. found ASNS to be expressed at low levels in TLX1$^+$ T-ALL cells (high *ASNS* methylation levels). TLX1$^+$ T-ALL was more sensitive to L-ASNase than the TLX$^-$CCRF-CEM cell line (low *ASNS* methylation level) (48). Recently, the important role of amino acid stress response genes in L-ASNase sensitivity was further confirmed by Ferguson et al., who identified a novel L-ASNase resistance gene, *SLC7A11*, whose high expression leads to L-ASNase sensitivity in cancer cells (49). In conclusion, these studies all suggested that the hypomethylation status of the *ASNS* promoter region contributes to the expression of ASNS induced by L-ASNase treatment, and thus conferring L-ASNase resistance to leukaemic cells. However, there are limited cohort studies to refer to at present, therefore, additional larger cohort studies are needed to further confirm the possibility of *ASNS* promoter region methylation as a predictor of treatment response.

Meanwhile, the factors affecting the methylation status of the ASNS promoter region have been reported. Worton et al. reported that L-ASNase induces *ASNS* promoter demethylation, which confers drug resistance to leukaemic cells (50). However, the mechanism by

which L-ASNase induces demethylation has not been further confirmed. The study by Akahane et al. focused on the significance of *SPI1* fusion in the methylation status of *ASNS*. In their cohort study, all seven *SPI1* fusion cases had an *ASNS* promoter hypomethylation status, and the *ASNS* gene expression levels were significantly higher than those of *SPI1* fusion-negative cases (47). This suggests that genetic modifications may play an important role in the methylation status of the *ASNS* promoter region. Yet, it is critical to confirm the relationship between poor prognosis-associated fusion genes and *ASNS* gene methylation status and the molecular mechanism of L-ASNase-induced demethylation, providing information for treatment option and improving the prognosis for ALL patients.

Energy metabolism and autophagy

Several recent studies showed that L-ASNase resistance is related to phosphatase and tensin homologue (PTEN) deficiency and phosphatidylinositol-3 kinase (PI3K)/Akt/mTOR signalling pathway. PTEN is a major negative regulator of the PI3K/Akt/mTOR signalling pathway. Deletion of PTEN can occur in 20% of children with T-ALL and plays an important role in the development and prognosis of T-ALL in children (51–53). Hlozkova et al. proposed that the metabolic pattern of leukaemic cells is associated with L-ASNase resistance after investigating the effect of L-ASNase treatment on the extensive metabolic reprogramming of leukaemic cells. They found that cells with a high glycolytic response are resistant to L-ASNase (54). They subsequently confirmed the relationship between glycolytic levels and L-ASNase sensitivity by investigating the effects of PTEN deficiency on the metabolism of leukaemic cells and changes in L-ASNase sensitivity. Furthermore, a recent study by Hlozkova et al. found that, compared to PTEN wild-type cells, PTEN-deficient T-ALL cells have a higher glycolytic function and overactivated Akt, and these changes made T-ALL cells resistant to L-ASNase. Meanwhile, the resistance of PTEN-deficient cells to L-ASNase could be improved by inhibiting Akt signalling (53). These results suggest that Akt inhibitors may contribute to the treatment of T-ALL patients with PTEN mutations, but further experiments are still needed for verification.

Amino acid deprivation has been shown to induce the activation of autophagy, which is considered a self-protective mechanism in tumour cells (55–57). Hermanova et al. showed that L-ASNase can induce the activation of protective autophagy in leukaemia cells by inhibiting mTORC1 (21). As in previous studies (58), they suggested that autophagy could counteract nutrient imbalance by recycling amino acids, thus resisting the cytotoxicity of L-ASNase (21). Takahashi et al. also reported that L-ASNase treatment reduced glycolysis in leukaemia cells while causing mitochondrial damage and activating autophagy. However, they concluded that the function of L-ASNase-induced autophagy was to eliminate mitochondrial damage and thus reducing ROS production rather than amino acid recycling. Notably, in this study, they demonstrated that by inhibiting autophagy, the cytotoxicity of L-ASNase could be enhanced and such synergistic effect works through the ROS-p53 positive feedback loop (18). In addition, Takahashi et al. and Polak et al. further confirmed that autophagy inhibitors and L-ASNase have synergistic anti-leukaemic effects (59, 60). The activation of autophagy is one of the mechanisms leading to L-ASNase resistance, but the role of autophagy in this mechanism still needs to be further refined. The abovementioned studies suggest that timely detection of autophagy activation during L-ASNase treatment would be more helpful in the selection of treatment regimens, and the combination of L-ASNase with autophagy inhibitors may provide better clinical outcomes.

Host factor

The role of the bone marrow haematopoietic microenvironment

The tumour microenvironment affects the cytotoxicity of L-ASNase (61). A study by Iwamoto et al. revealed the interaction between leukaemic cells and their surrounding microenvironment. The expression of ASNS is much higher in normal bone marrow mesenchymal stem cells (MSCs) than in leukaemic lymphoblastoid cells. *In vitro*, leukaemic cells can acquire resistance to L-ASNase by receiving asparagine from MSCs (62). Glutamine synthetase expression is increased in bone marrow adipocytes after induction of chemotherapy with L-ASNase, producing more glutamine and thus protecting leukaemic cells from L-ASNase (63). Future studies focusing revealing the molecular mechanism of the interaction between leukaemia cells and the haematopoietic microenvironment in the bone marrow will further elucidate the anti-leukaemic effect of L-ASNase and hence improving the L-ASNase therapy.

Neutralizing antibodies and silent inactivation

Due to its immunogenicity, an L-ASNase treatment can cause an immune response, which is associated with the production of neutralizing antibodies. Neutralizing antibodies can inactivate L-ASNase, and thereby reducing efficacy. The production of neutralizing antibodies in patients without clinical symptoms is known as silent inactivation, which is usually not clinically evident and thus difficult for early detection (11, 64, 65). Although it has been suggested that patients with allergic reactions to *E. coli*-ASNase and PEG-ASNase should be switched to *Erwinia*-ASNase (66), 3-33% of patients can develop an immune response against *Erwinia*-ASNase, resulting in neutralizing antibodies against L-ASNase and thus resistance to L-ASNase (67, 68).

Other pathways

It has been shown that leukaemic cells can acquire L-ASNase resistance through the OPRM1-cAMP-caspase pathway. Kang et al. identified the opioid receptor μ1(OPRM1) as a key factor for L-ASNase resistance in paediatric ALL using unbiased genome-wide RNAi. They analysed OPRM1 expression levels in primary leukaemic cells from five children with ALL in relation to L-ASNase sensitivity and found that cells with low levels of OPRM1 were more resistant to L-ASNase (69). In addition, Lee et al. identified the Huntington-associated protein 1 gene (*HAP1*) as an L-ASNase resistance gene, and by examining the relationship between HAP1 levels and L-ASNase sensitivity in the cells of six ALL patients, they found that the lower HAP1 level, the more resistant they were. Furthermore, they found that HAP1 deletion prevented Ca^{2+} release from the endoplasmic reticulum and downregulated the Calpain-1-Bid-caspase-3/12 pathway, conferring L-ASNase resistance in leukaemic

cells (70). Additionally, a recent study demonstrated that if the Wnt pathway is blocked, cells may degrade proteins *via* GSK3-dependent protein ubiquitination and proteasome degradation pathways to synthesize asparagine to counteract the cytotoxicity of L-ASNase (71).

In conclusion, ASNS expression remains a pivotal factor in the resistance of L-ASNase in leukaemic cells, and ASNS expression is closely related to the methylation status of its promoter region. In addition, activation of autophagy, high glycolysis levels, or inhibition of apoptotic signalling pathways can all promote L-ASNase resistance. The gradual uncovering of L-ASNase resistance mechanisms further emphasizes the significance of individualized therapy and continues to provide new ideas for the further development of individualized combination therapy regimens.

Application of L-ASNase in other childhood leukaemia

Although L-ASNase is currently used primarily for the treatment of ALL and some NK/T-cell lymphomas, there is growing evidence that L-ASNase can play a critical role in the treatment of other childhood leukaemias (26).

Dübbers et al. found that leukaemic cells from M1, M4, and M5 subtypes had negative ASNS staining among all FAB subtypes of AML and that AML-M5 had the lowest ASNS activity (72). This is in agreement with the results of Okada et al., who found L-ASNase to be effective against specific subtypes of AML (M1, M4, M5) *in vitro* (73). Additionally, according to Buaboonnam et al., patients with refractory/relapsed AML who received treatment with L-ASNase in combination with MTX had 1- and 2-year survival rates of 35.6% and 17.8%, respectively (74). Whether this regimen can be used as a treatment for patients with refractory/relapsed AML after intensive therapy still needs further study. More recently, Chen et al. reported that the combination of L-ASNase with MIT and Ara-C for AML could enhance the inhibition of tumour cell proliferation (75). It is the current belief that the toxic effect of L-ASNase on AML may be related to GLNase activity. Glutamine is a nutrient that AML cells require. L-ASNase can remove glutamine and thus inhibit the growth of AML cells. However, L-ASNase simultaneously promotes the production of glutamine synthetase, leading to L-ASNase resistance (76, 77). Thus, further research is still needed to clarify the role of the GLNase activity of L-ASNase. Furthermore, as in ALL, it has been shown that the bone marrow haematopoietic microenvironment protects AML cells. Cells in the bone marrow microenvironment can either release ASNS to counteract L-ASNase action or release lysosomal cysteine protease B (CTSB) to inactivate L-ASNase, which confers L-ASNase resistance (78)

The potential of L-ASNase in CML treatment has been uncovered. Song et al. found that L-ASNase inhibited growth and induced apoptosis in the human CML cell Lines K562 and KU812, among which the apoptosis induction of K562 cells by L-ASNase was dependent on caspase3 (79). This discovery makes it possible to use L-ASNase in the treatment of CML. Trinh et al. also demonstrated that L-ASNase could inhibit the growth of CML cells, and the combination of L-ASNase and imatinib can significantly induce CML cell death by downregulating antiapoptotic factors such as Bcl-2 and upregulating proapoptotic factors such as Bim, and thereby eradicating CML stem cells (80). A recent study by Konhauser et al. also demonstrated the synergistic effect of L-ASNase in combination with etoposide on killing K562 cells (81).

With the continuous development of studies on the metabolic and nonmetabolic effects of L-ASNase on paediatric leukaemia, studies on the therapeutic effects of L-ASNase on other non-ALL leukaemia are proliferating. These studies suggest that L-ASNase may provide a new option for the treatment of other paediatric leukaemias. These results are based on the enzymatic activity of L-ASNase, which depletes asparagine and glutamine in the blood and inhibits mTOR, which in turn affects protein synthesis and induces apoptosis. Meanwhile, these studies found that L-ASNase caused the activation of protective autophagy in tumour cells, so the combination of L-ASNase and autophagy inhibitors will benefit both ALL patients and non-ALL patients.

Novel L-asparaginase

As mentioned above, L-ASNase is a xenogeneic protein agent that is highly immunogenic. Efficacy is compromised during L-ASNase treatment because of immunological or nonimmunological side effects. *Erwinia*-ASNase is often chosen as an alternative treatment for patients with *E.coli*-ASNase allergy (82), and PEG-ASNase has been introduced into the clinic for its longer half-life and lower immunogenicity. However, none of these variants can completely solve the problem. Neutralizing antibodies can still be produced and therefore inactivating L-ASNases (83, 84). To address these issues, several approaches have been used to develop novel L-ASNase preparations, such as reduced GLNase coactivity of L-ASNase, enzyme engineering modifications, and vector packaging.

Since most of the nonimmunological side effects of L-ASNase are attributed to GLNase activity, reducing the GLNase coactivity of L-ASNase may effectively ameliorate the side effects of L-ASNase. Consequently, L-ASNase variants with or without negligible GLNase activity were generated. Wolinella succinogenes-derived L-ASNase (*WOA*) was the first reported L-ASNase variant with low GLNase activity that did not suppress immune responses in mice (85–87). Reinert et al. showed no significant changes in glutamine in the liver and spleen of mice treated with the *WOA* variant compared to L-ASNase (88). Recent studies have also identified a guinea pig-derived humanized variant of L-ASNase that is completely devoid of GLNase activity. This variant has reduced immunogenicity while maintaining anti-leukaemic activity (89, 90).

Enzyme engineering has been widely employed to change the characteristics of L-ASNase in search of L-ASNase with low immunogenicity, a longer half-life, and lower GLNase activity. Since L-ASNase can be cleaved by CTSB and aspartate endopeptidase (78, 91), Offman et al. used site-directed mutagenesis to design an L-ASNase variant that is resistant to CTSB cleavage and has lower immunogenicity. They also designed a variant with low GLNase activity, N24A/R159S, which reduced the toxicity of L-ASNase (25). Furthermore, in a recent study, Maggi et al. designed an N24S mutant with improved protease resistance and thermostability in response to the instability and brief half-life of *E. coli*-ASNase (92).

In addition to the above methods, carrier packaging can also be used to reduce the immunogenicity of L-ASNase and make it more stable *in vivo*. Common carriers include erythrocytes, liposomes, nanocapsules, and microcapsules. The performance of these L-ASNases has also been demonstrated *in vivo* and *in vitro* (93–95). For instance, because it is encapsulated within cells, Eryaspase, a product that encapsulates *E. coli*-ASNase into erythrocytes, has a long half-life similar to that of erythrocytes and has low immunogenicity (96–98). Last year, Eryaspase was approved by the FDA for the treatment of ALL patients who are allergic to PEG-ASNase (99).

Discussion

In summary, L-ASNase is still the cornerstone drug for the treatment of paediatric ALL. In addition to affecting the protein synthesis and amino acid metabolism of ALL cells, L-ASNase can affect energy metabolism. Also, changes in energy metabolism and autophagy in ALL cells may affect the efficacy of L-ASNase. The focus of current research on the mechanism of L-ASNase resistance is gradually shifting from the protein level to the gene expression regulation level. Meanwhile, there are studies that elucidate the relationship between leukaemia metabolic profiles and autophagy and L-ASNase resistance. Although the mechanism of L-ASNase resistance has not been fully elucidated to date, these studies suggested that the combination of fatty acid oxidation inhibitors or autophagy inhibitors and L-ASNase can provide better anti-leukaemic effects, which provide brand-new options for the future treatment of childhood leukaemia.

The immunogenicity of L-ASNase is a reason for its drug resistance. Using carrier packaging L-ASNase such as erythrocytes and nanocapsules can effectively reduce its immunogenicity and therefore L-ASNase can work better. The performance of these L-ASNases has also been demonstrated *in vivo*. Moreover, the necessity of GLNase activity for the anticancer effect of L-ASNase is still highly controversial. Although the development of L-ASNase variants with low GLNase activity continues, the necessity of GLNase activity and the level of GLNase activity that should be maintained for L-ASNase still needs to be further investigated. Moreover, some glutamine-dependent haematological tumours may not benefit from L-ASNase variants without GLNase activity.

Finally, addressing the above issues will not only help to solve the problem of ALL resistance to L-ASNase but also help to explain the potential application of L-ASNase in other tumours.

Author contributions

TLia and TLi performed the collection and interpretations of all relevant literature. RZ wrote the manuscript. CC and JH critically read and revised 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.

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FLT3-TKD in the prognosis of patients with acute myeloid leukemia: A meta-analysis

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Background: Fms-like tyrosine kinase 3 (FLT3) gene mutations occur in approximately 30% of all patients with acute myeloid leukemia (AML). Internal tandem duplication (ITD) in the juxtamembrane domain and point mutations within the tyrosine kinase domain (TKD) are two distinct types of FLT3 mutations. FLT3-ITD has been determined as an independent poor prognostic factor, but the prognostic impact of potentially metabolically related FLT3-TKD remains controversial. Hence, we performed a meta-analysis to investigate the prognostic significance of FLT3-TKD in patients with AML.

Methods: A systematic retrieval of studies on FLT3-TKD in patients with AML was performed in PubMed, Embase, and Chinese National Knowledge Infrastructure databases on 30 September 2020. Hazard ratio (HR) and its 95% confidence intervals (95% CIs) were used to determine the effect size. Meta-regression model and subgroup analysis were used for heterogeneity analysis. Begg's and Egger's tests were performed to detect potential publication bias. The sensitivity analysis was performed to evaluate the stability of findings in meta-analysis.

Results: Twenty prospective cohort studies (n = 10,970) on the prognostic effect of FLT3-TKD in AML were included: 9,744 subjects with FLT3-WT and 1,226 subjects with FLT3-TKD. We found that FLT3-TKD revealed no significant effect on disease-free survival (DFS) (HR = 1.12, 95% CI: 0.90–1.41) and overall survival (OS) (HR = 0.98, 95% CI: 0.76–1.27) in general. However, meta-regressions demonstrated that patient source contributed to the high heterogeneity observed in the prognosis of FLT3-TKD in AML. To be specific, FLT3-TKD represented a beneficial prognosis of DFS (HR = 0.56, 95% CI: 0.37–0.85) and OS (HR = 0.63, 95% CI: 0.42–0.95) for Asians, whereas it represented an adverse prognosis of DFS for Caucasians with AML (HR = 1.34, 95% CI: 1.07–1.67).

Conclusion: FLT3-TKD revealed no significant effects on DFS and OS of patients with AML, which is consistent with the controversial status nowadays. Patient source (Asians or Caucasians) can be partially explained the different effects of FLT3-TKD in the prognosis of patients with AML.

KEYWORDS

AML, FLT3-TKD, meta-analysis, metabolism, prognosis

Introduction

Acute myeloid leukemia (AML) is the most common acute leukemia in adults with the features that poorly differentiated cells from hematopoietic system infiltrate in bone marrow, blood, and other tissues (1). Nowadays, it tends to be assumed that valuable and accurate prognostic assessments benefit patients with AML by providing optimized treatments for their survivals. Hence, more and more recurrent genetic mutations, such as FLT3-ITD, Nucleophosmin (NPM1), and CCAAT enhancer-binding protein alpha (CEBPA), have been used to guide disease management and refine individual prognosis.

Fms-like tyrosine kinase 3 (FLT3) is a potential prognostic genetic marker, which encodes a class 3 receptor tyrosine kinase to plays a crucial role in hematopoiesis. FLT3 gene mutations occur in approximately 30% of all patients with AML (2). There are two distinct forms of FLT3 mutations: internal tandem duplication (ITD) in the juxtamembrane domain and point mutations within the activation loop of the tyrosine kinase domain (TKD), affecting D835 in most cases (3). These gain-of-function mutations lead to ligand-independent activation of FLT3, which contributes to uncontrolled proliferation of AML blasts (2, 4, 5). Numerous studies have found that FLT3-ITD is an independent factor for adverse prognosis (6). However, the prognostic value of FLT3-TKD remains controversial due to the relatively low incidence and limitations of single center studies (2). The relationship between FLT3-TKD and cytoplasmic Src family tyrosine kinases has been confirmed recently (7) while we have known about the associations between Src family members and multiple nutrient metabolism, including glucose (8), lipid (9), and glutamine (10). From our perspectives, FLT3-TKD is regarded as a potentially metabolically related mutation in tumorigenesis and progression of AML. For this reason, we performed a meta-analysis within the published studies before 30 September 2020 to investigate the prognostic significance of FLT3-TKD in patients with AML.

Methods

Search strategy

Two independent investigators implemented a systematic search in PubMed, Embase, and Chinese National Knowledge Infrastructure (CNKI) databases systematically, with the last search updated on 30 September 2020. The following terms "(acute myeloid leukemia) or (acute myeloblastic leukemia) or (acute myelocytic leukemia) or (acute myelogenous leukemia) or (acute nonlymphoblastic leukemia) or AML", "FLT3 or CD135 or (fms-like tyrosine kinase 3) or (fetal liver kinase-2) or (fetal liver kinase-3) or (human stem cell tyrosine kinase-1)", "(tyrosine kinase domain mutation) or (TKD mutation) or D835 or I836", and "prognosis or prognoses or (prognostic factors) or (prognostic implication) or (prognostic element)" were retrieved in PubMed entries in the National Institutes of Health and European Embase databases without any limitation applied. The key words "急性髓系白血病or急性髓性白血病or急性非淋巴细胞性白血病", "FLT3", and "TKD突变" were retrieved in CNKI. The reference lists in retrieved studies and relevant reviews were also manually searched for more eligible studies.

Selection criteria

All literature studies involved in AML and FLT3-TKD were electronically retrieved for the next filter. Afterward, prospective cohort studies were identified according to their titles and abstracts. The full texts of the literature studies that fulfilled the inclusion criteria were perused to validate their eligibility. Inclusion criteria were as follows: (a) the evaluation of association between prognosis of AML and FLT3-TKD; (b) untreated patients with AML were included in study; (c) complete original materials with specific explanation of sample size; (d) they provided data of all enrolled subjects on either or both of overall survival (OS) and disease-free survival (DFS) after a period of follow-up in the study; (e) with survival information based on the FLT3 status: FLT3-TKD and wild type; and (f) prospective cohort study focusing on human being. Exclusion criteria were as follows: (a) not conforming to inclusion criteria; (b) abstract, review article, letter, comment, and editorial; (c) duplicate publication of previous publications; (d) family-based studies of pedigrees; (e) without detailed FLT3 status data (FLT3-TKD and wild type); (f) with incomplete specific explanation or without specific explanation of sample size; and (g) studies were excluded if they focused exclusively on acute promyelocytic leukemia (APL) (M3). For multiple publications from a same population, the largest study was included only to exclude duplicate studies or overlapping data. According to the inclusion and exclusion criteria, the two independent investigators accomplished study selection independently by screening title, abstract, and full text. Any dissent was solved by discussion. If agreement could not be reached, then a third researcher was consulted. Four studies were discussed and excluded after discussion (11–14).

Data extraction

The data of the eligible studies were extracted in duplicate by two independent researchers. The data extracted comprised first author's name, publication year, diagnostic criteria for AML, the resource of the subjects, genotyping methods, the number of subjects, the FLT3 status (FLT3-TKD or wild type) of subjects, the number of OS and/or DFS from all subjects (if any), hazard ratio (HR) with 95% confidence interval (CI) of OS and/or DFS, and the baseline data of all subjects in all included prospective cohort studies [age, sex, patient source, other mutations (if any), the usage of chemotherapeutics (if any), and so on] (Table S1). HR and its 95% CI were extracted directly or calculated the observed minus expected (O-E) according to the ratio of event or were extracted by using Engauge Digitizer according to the Cox curve (15). Various patient source descents were classified as Caucasian and Asian. Two investigators would check the extracted data and reach to consensus on all collected data. If a dispute existed, then original data of the included studies would be rechecked and be discussed again to reach consensus. If the dispute still existed, then the third investigators would be appointed as the decider to adjudicate the disagreements.

Quality assessment

The Newcastle-Ottawa (NOS) scale was used to score the strength of all included studies by the three independent investigators (16). The scale has nine items classified into three major categories: selection (four items), comparability (two items), and outcome (three items) (Supplement 1. Method). In this scoring system, selection, comparability, and outcome categories could be awarded a maximum of four, two, and three points, respectively. High quality was considered as six or more points that each cohort study scored. Any discrepancies were resolved among authors. The results of quality assessment are displayed in Table S2.

Publication bias and sensitivity analysis

Potential publication bias was checked by Begg's funnel plots (17) and Egger's test (18). An asymmetric plot with p-value less than 0.05 was considered a significant publication bias. Moreover, sensitivity analysis was performed on the pooled HRs to evaluate the effect of each study, in which the results of the meta-analysis were recalculated after removal of each study in a turn.

Data analysis

Among entire conduction of the meta-analysis, we strictly abided by the PRISMA checklists as a guideline (19). All statistical analyses were performed with Stata 16.0 software (StataCorp, College Station, TX, USA). A two-tailed $p < 0.05$ was considered significant except for specified conditions, where a certain p-value was declared. HR and corresponding 95% CI were applied to assess the prognostic impact of FLT3-TKD in patients with AML. Furthermore, HR > 1 was considered as poorer prognosis in patients with FLT3-TKD than patients with FLT3-WT, whereas HR < 1 was considered as beneficial prognosis in patients with FLT3-TKD than patients with FLT3-WT. The heterogeneity of the studies was assessed by I^2 statistic (I^2 = 0%–25%, no heterogeneity; I^2 = 25%–50%, moderate heterogeneity; I^2 = 50%–75%, large heterogeneity; and I^2 = 75%–100%, extreme heterogeneity) (20). When the heterogeneity was statistically significant ($I^2 > 50\%$), the random-effects model was used for assessing information; otherwise, the fixed-effects was conducted (21). Heterogeneity was analyzed by meta-regression model including age and patient source, and subgroup analysis was stratified by age and patient source. In addition, Begg's and Egger's tests were performed to detect potential publication bias. Furthermore, sensitivity analysis was conducted to determine the stability of findings in meta-analysis.

Results

Study retrieval

The study selection process for the meta-analysis about the prognosis of FLT3-TKD in AML is shown in Figure 1. Following the initial retrieval of 917 publications through database search (253 from PubMed, 638 from Embase, and 26 from CNKI), 696 relevant publications were selected after the removal of duplicates. Moreover, after a careful review of the title and abstract, 152 publications were rejected because of their irrelevance to this meta-analysis. The remaining 544 publications were full-text–reviewed; of these, 524 were excluded. The reasons for excluding are shown in Figure 1. Finally, 20 prospective cohort study studies (22–41) consisting of 10,970 participants (FLT3-WT = 9,744; FLT3-TKD = 1,226) were included in our meta-analysis. The general characteristics of the 20 studies are shown in Table 1, and additional information is shown in Table S1.

The DFS and OS between FLT3-WT and FLT3-TKD in all patients with AML

Before analysis, we determined two outcomes: one of them is relapse of AML or death from AML relapse as the outcome of DFS and the other one is death from all causes as the outcome of OS to represent the prognosis of AML in this meta-analysis. In all the patients with AML from included studies, we analyzed two outcomes between the group with FLT3-WT and the group with only FLT3-TKD in FLT3 gene in random-effects model. The pooled HR of DFS is 1.12 (95% CI: 0.90–1.41; I^2 = 70.7%, p = 0.000) (Figure 2A). In same model, the pooled HR of OS is 0.98 (95% CI: 0.76–1.27; I^2 = 79.9%, p = 0.000) (Figure 2B).

Meta-regression was used for analyzing heterogeneity

Age and patient source were included in meta-regression model for heterogeneity analysis. The coefficient of age is −0.005 (p = 0.497) and 0.008 (p = 0.299) in patients of AML for DFS and OS, respectively, and the coefficient of patient source is −1.004 (p = 0.015) and −0.358 (p = 0.324) in patients of AML for DFS and OS, respectively (Table 2, Figure 3).

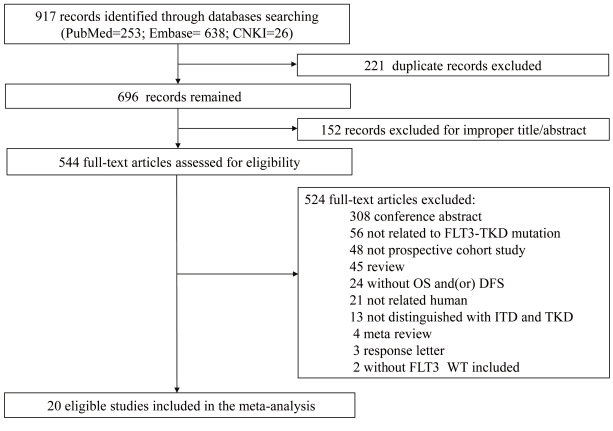

FIGURE 1
Article search and selection.

TABLE 1 Characteristics of studies included in the meta-analysis.

Code	First Author	Country/Region	Year	Patient number		DFS			OS		
				FLT3-WT	FLT3-TKD	HR	95% CI	p value	HR	95% CI	p value
1	Jeong Yeal Ahn	USA	2013	49	4	1.820	0.64-5.23	0.3	1.430	0.19-10.78	0.727
2	C Allen	UK	2013	319	35	NA	NA	NA	0.480	0.22-1.03	0.06
3	Costa Bachas	Netherlands	2014	123	4	0.560	0.08-4.04	0.56	0.670	0.16-2.71	0.57
4	Ulrike Bacher	Germany	2008	2935	147	1.380*	1.17-1.64	NA	2.010*	1.66-2.42	
5	Claudia Bănescu	Romania	2019	214	12	NA	NA	NA	0.900	0.47-1.70^{U}	0.739
6	Prajwal Boddu	USA	2017	117	21	0.245	0.058-0.980	0.048	0.678	0.260-1.768	0.427
7	Hyoung Jin Kang	Korea	2005	55	2	0.510$^{\#}$	0.12-2.14	NA	NA	NA	NA
8	D-C Liang	Taiwan, China	2003	74	3	0.510$^{\#}$	0.16-1.66	NA	NA	NA	NA
9	Adam J Mead	UK	2007	980	127	0.710	0.52-0.97	0.03	0.710	0.52-0.96	0.03
10	Soheil Meshinchi	USA	2006	515	38	1.930$^{\#}$	1.17-3.17	NA	0.910$^{\#}$	0.57-1.45	NA
11	Isabel Moreno	Spain	2003	156	12	2.580$^{\#}$	0.63-10.52	NA	NA	NA	NA
12	Man Qiao	Mainland, China	2011	49	7	NA	NA	NA	0.429	0.204-0.653	0.779
13	Patricia Rubio	Argentina	2016	39	5	NA	NA	NA	1.410$^{\#}$	0.18-10.92	NA
14	Hirozumi Sano	Japan	2013	135	8	0.470	0.06-3.45	0.45	NA	NA	NA
15	Susanne Schnittger	Germany	2012	2676	689	1.280*	1.17-1.4	NA	1.350*	1.23-1.48	NA
16	Akira Shimada	Japan	2008	110	11	0.740$^{\#}$	0.38-1.43	NA	0.850$^{\#}$	0.45-1.58	NA
17	Christian Thiede	Germany	2002	904	75	1.750*	1.38-2.22	NA	1.690*	1.33-2.14	NA
18	Susan P Whitman	the USA	2008	123	16	2.300	1.1-4.7	0.02	NA	NA	NA
19	Y Yamamoto	Japan	2001	17	8	0.380$^{\#}$	0.18-0.8	NA	1.000$^{\#}$	0.31-3.19	NA
20	G Yoshimoto	Japan	2005	24	2	1.330$^{\#}$	0.18-10.05	NA	0.670$^{\#}$	0.15-2.9	NA

*: HR and 95% CI was by using Engauge Digitizer according to the cox curve; #: HR and 95% CI was calculated the O-E according to the ratio of event; U, univariable analysis; DFS, disease-free survival; OS, overall survival; NA, not available.

The DFS and OS of patients with AML between FLT3-WT and FLT3-TKD in the Asian subgroup and in the Caucasian subgroup

To further analyze heterogeneity, considering with the background knowledge of FLT3-TKD, we conducted meta-analysis in subgroups according to the patient source (ethnicity) of all participants included (Table S1). The pooled HR of DFS is 1.34 (95% CI: 1.07–1.67; I^2 = = 72.9%, p = 0.000) in the Caucasian subgroup, and the pooled HR of DFS is 0.56 (95% CI: 0.37–0.85; I^2 = 0.0%, p = 0.777) in the Asian subgroup (Figure 4A). The pooled HR of OS is 1.11 (95% CI: 0.85–1.44; I^2 = 80.5%, p = 0.000) in the Caucasian subgroup, and the pooled HR of OS is 0.63 (95% CI: 0.42–0.95; I^2 = 5.1%, p = 0.368) in the Asian subgroup (Figure 4B).

Publication bias and sensitivity analysis

Begg's and Egger's tests have been used to detect any publication bias that indicated that there was no significant bias between studies of FLT3-TKD in prediction of DFS (p = 0.499 of Begg's and p = 0.233 of Egger's) and also of OS (p = 0.488 of Begg's and p = 0.061 of Egger's). Symmetrical Begg's funnel plot was obtained (Figure 5). We also conducted sensitivity analysis to examine the stability of the meta-analysis to determine the influence of each study on pooled HRs in patients with AML by deleting a single study in every model. It showed no individual study affected the pooled HR of DFS (Figure 6A) and OS (Figure 6B) in all patients with AML.

Discussion

In this meta-analysis, 20 prospective cohort studies (n = 10,970) on FLT3-TKD in AML were included: 9,744 subjects with FLT3-WT and 1,226 subjects with FLT3-TKD (22–41). The incidence of FLT3-TKD in this meta-analysis was 11.2%, which was nearly consistent with previous studies (approximately 7%–10% of all AML cases) (2). Our results indicated that, within 20 cohort studies (n = 10,970) included, the pooled HR of DFS was 1.12 (95% CI: 0.90–1.41; I^2 = 70.7%, p = 0.000) and OS was 0.98 (95% CI: 0.76–1.27; I^2 = 79.9%, p = 0.000), which revealed no significant effect of FLT3-TKD on DFS and OS of patients with AML by random effect models. However, meta-regressions demonstrated that patient source associated with the prognosis effect of FLT3-TKD in patients with AML. To be specific, FLT3-TKD represented a beneficial prognosis of DFS (HR = 0.56,

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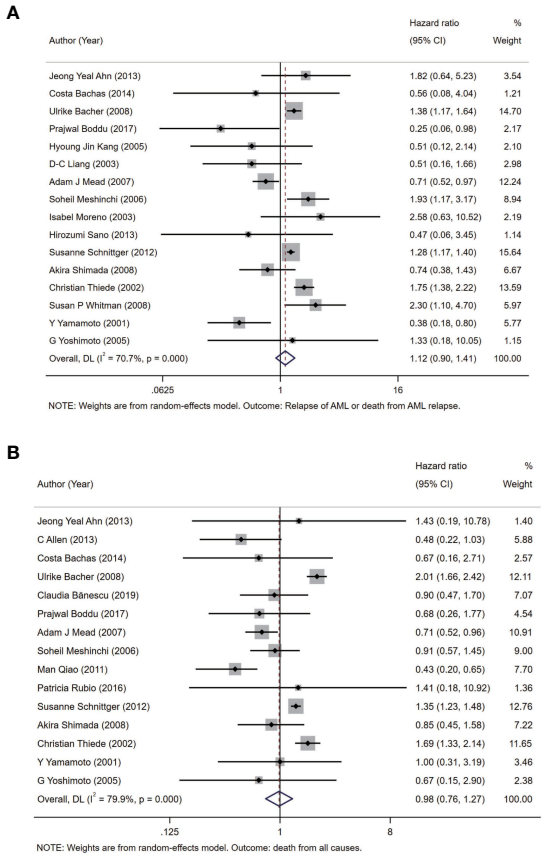

FIGURE 2
Forest plots of HRs and 95% CI for DFS **(A)** and OS **(B)** in patients with AML. The size of blocks or diamonds represents the weight in this meta-analysis, and the length of straight line segment represents the width of 95% CI. HR, hazard ratio; CI, confidence intervals; DFS, disease-free survival; OS, overall survival; AML, acute myeloid leukemia.

95% CI: 0.37–0.85) and OS (HR = 0.63, 95% CI: 0.42–0.95) for Asians with AML, whereas FLT3-TKD represented an adverse prognosis of DFS for Caucasians with AML (HR = 1.34, 95% CI: 1.07–1.67). However, the results of DFS from Caucasians ought to be interpreted with caution due to the heterogeneity (I^2 = 72.9%, p = 0.000).

In general, FLT3-TKD reveals no significant effects on DFS and OS of patients with AML, which is consistent with the controversial statue nowadays. The controversies of prognostic impacts on FLT3-TKD in patients with AML were supported by two-sided laboratory evidence of FLT3-TKD. On one hand, many studies indicated that FLT3-TKD associated a beneficial prognosis of AML. In some cases, patients with FLT3-TKD companying with other mutations, such as NPM1 mutation, showed a favorable prognosis (27) with the reasons that the localization and signaling of FLT3-TKD was changed by NPM1c in AML (42). On the other hand, other research studies manifested that FLT3-TKD was considered as a harmful mutation in prognosis of AML. Since FLT3-ITD was first recognized as a frequently mutated gene in AML in 1996 (43), growing studies indicated that a lot of FLT3-ITD–positive patients with AML relapse with the appearance of FLT3-TKD after initial response to FLT3 inhibitor treatments. Moreover, several FLT3 inhibitors including Sorafenib and Quizartinib potently had effects on inhibiting FLT3-ITD but were not effective toward FLT3-TKD (2, 44, 45). The phenomenon was plausibly interpreted as the coexistence of two kinds of FLT3 mutations and the presence of FLT3-TKD in a very low level at initial stage of disease, which subsequently became prevalent after FLT3-ITD–positive leukemic cells, are eliminated (46).

Our results indicated that FLT3-TKD represented a beneficial prognosis for Asians with AML, whereas it represented an adverse prognosis of DFS for Caucasians with AML, but with heterogeneity. First, in the Caucasian subgroup, the pooled HR of DFS was 1.34 (95% CI: 1.07–1.67; I^2 = 72.9%, p = 0.000), and the pooled HR of OS was 1.11 (95% CI: 0.85–1.44; I^2 = 80.5%, p = 0.000) (Figure 4). There were 635 Asians from four different countries and 10,335 Caucasians from eight different countries in this meta-analysis, which revealed that Caucasians outnumbered Asians. Hence, we speculated that confounding factors from large sample capacity of the Caucasian subgroup accounted for the heterogeneity in Caucasians (I^2 = 72.9%, p = 0.000). In this situation, if related confounding factors were well controlled, then the conclusion might be more convinced. However, it was indicated that Caucasians reveals a distinct genetic alteration profiles of AML than Eastern Asian population (47), but what we focused on is the ratio of FLT3-TKD in AML, which would not be influence by population base too much at this meta-analysis. Second, according to different therapeutic guidelines of AML, the chemotherapy regimens for patients with AML vary internationally. On one hand, included studies from Japan accounted for a large population in the Asian subgroup in this meta-analysis. The conventional "3 + 7" induction is regarded as basic regimens for complete remission (CR) with anthracyclines for 3 days and standard dose of cytarabine for 7 days. We noticed that the dose of cytarabine and anthracyclines in Japan was less than the dose in Caucasian countries according to practical guideline for AML (48, 49). Cytarabine of 100 mg/m^2 was recommended for 7 days in Japan, whereas cytarabine of 100–200 mg/m^2 was recommended for 7 days in Caucasian countries; and daunorubicin of 50 mg/m^2 was recommended for 3 days in Japan, whereas daunorubicin of 60–90 mg/m^2 for 3 days was recommended in Caucasian countries (48, 49). Hence, we speculated prognosis of AML in different countries or regions might attribute to the dose of cytarabine and daunorubicin,

TABLE 2 Multivariate meta-regression analysis for FLT3-TKD in patients of AML.

Outcome	DFS		OS	
	Coefficient	p-value	Coefficient	p-value
Age	−0.005	0.497	0.008	0.299
Patient source	−1.004	0.015*	−0.358	0.324

DFS, disease-free survival; OS, overall survival; *p < 0.05.

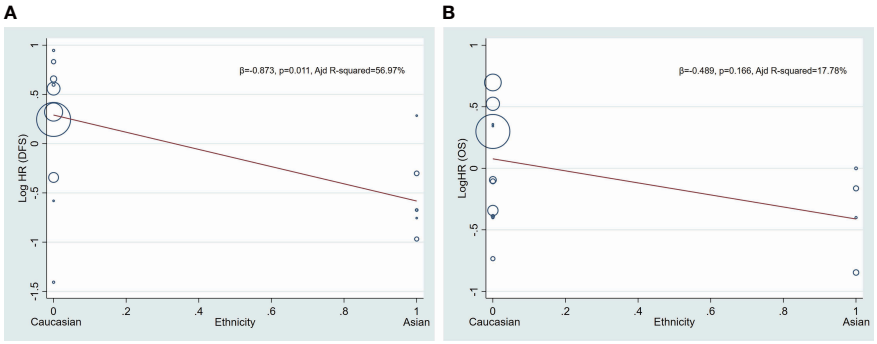

FIGURE 3
Univariate meta–regression analysis. Log HR of DFS and OS according patient source (ethnicity). A circle represents a study, and the diameter of circle represents sample capacity. Various ethnicity (patient source) descents were classified as Caucasian and Asian. HR, hazard ratio; DFS, disease-free survival; OS, overall survival.

which consist of the conventional "3 + 7" induction. On another hand, anthracyclines have been used extensively with standard dose of cytarabine to induce remission of patients with AML worldwide (48, 50). However, clinicians in China tend to attach great importance to homoharringtonine (HHT) and apply HHT-based induction regimens to induce the CR of patients with AML (not APL), which was considered as another discrepancy between Asian countries and Caucasian countries in the treatments of AML (48, 50). Concretely, clinical hematologists and oncologists in China are apt to replace anthracyclines with HHT in CR induction of AML or added HHT upon prime chemotherapy regimens for higher CR rate of AML. HHT is a kind of alkaloid deriving from genus Cephalotaxus and exerts selective antileukemia effects on patients with AML, especially on these carrying FLT3-ITD and elderly patients with AML (51–53). What is noteworthy is that the FLT3-TKD status associates with chemotherapy regimens, especially in relapse (2), whereas some salvage therapies for relapsed AML include HHT as the basic members of chemotherapy regimens, such as HAA regimen (54).

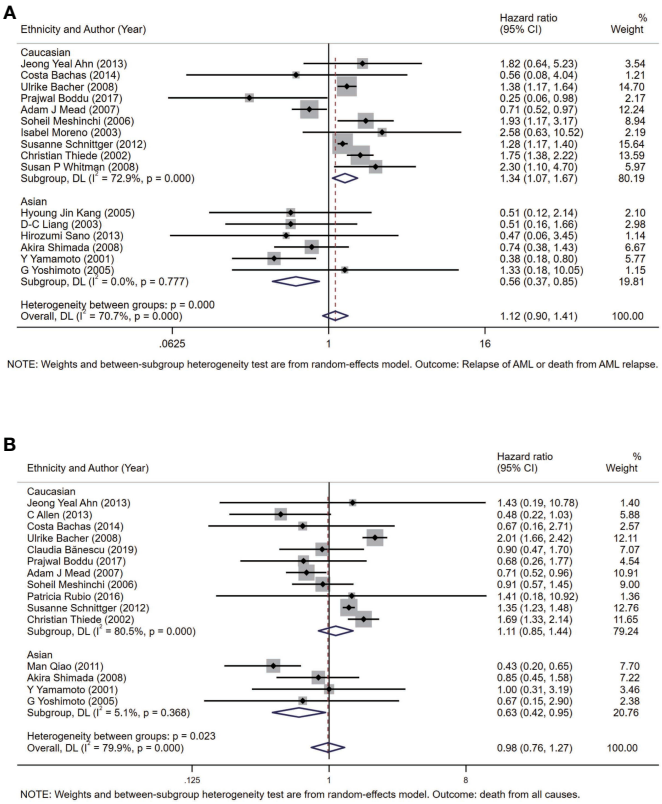

FIGURE 4
Forest plots of HRs and 95% CI for DFS **(A)** and OS **(B)** in patients with AML in the Asian subgroup and the Caucasian subgroup. The size of blocks or diamonds represents the weight in this meta-analysis, and the length of straight line segment represents the width of 95% CI. HR, hazard ratio; CI, confidence intervals; DFS, disease-free survival; OS, overall survival; AML, acute myeloid leukemia.

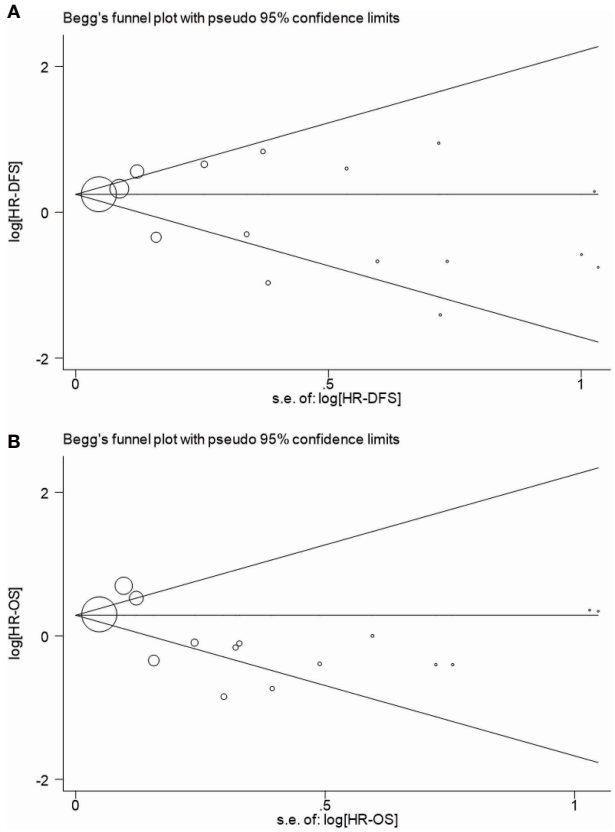

FIGURE 5
Begg's funnel plot for analyzing publication bias of DFS **(A)** and OS **(B)** in patients with AML. A circle represents a study, and the diameter of circle represents sample capacity. DFS, disease-free survival; OS, overall survival; AML, acute myeloid leukemia.

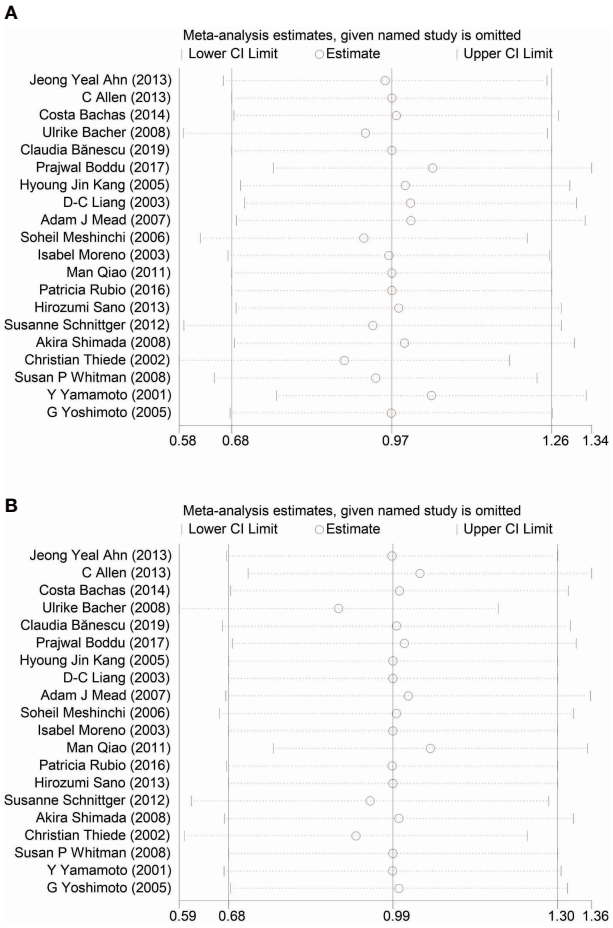

FIGURE 6
Sensitivity analysis of DFS **(A)** and OS **(B)** in patients with AML. DFS, disease-free survival; OS, overall survival; AML, acute myeloid leukemia.

Hence, we speculated that prognosis of AML in different ethnicities may also due to the clinical usage of HHT partially. Third, race diversity is the result of different genetic backgrounds, not only the gene encoding FLT3. Patients with AML usually were with genomic anomaly or sporadic gene mutations (42). Currently, the contribution of different genetic backgrounds to the occurrence and progression of AML remains unclear. In conclusion, FLT3-TKD exerts impacts on contrasting prognosis of AML in different ethnicities due to multiple reasons, which deserve further explorations.

FLT3-TKD is considered as a potentially metabolically related mutation in AML. FLT3-TKD associates with cytoplasmic Src family tyrosine kinases by increasing the phosphorylation of activating tyrosines, such as FGR and HCK (7). In addition, Src family members are involve in multiple nutrients metabolisms, including glucose (8), lipid (9), and glutamine (10), indicating that FLT3-TKD is a potentially metabolically related mutation. As for glucose metabolism, Src is able to regulate cyclin B1, N-cadherin, and E-cadherin under high glucose as a response to hyperglycemia in colorectal cancer (8). For glutamine metabolism, epidermal growth factor receptor (EGFR)-promoted tumor progression is considered as being Src signaling pathway related by influencing glutamine metabolism in multiple malignancies (10). For lipid metabolism, Src is able to being recruited by CDCP1 into lipid rafts, which affect HGF receptor Met *via* the activation of STAT3 (9). Overall, FLT3-TKD is considered as being metabolically related based on the functions of Src family tyrosine kinases, which can be used to explain FLT3-TKD status in AML.

Our meta-analysis has several strengths. First, we selected 20 eligible prospective cohort studies, which was considered as an ideal epidemiological method to predict prognosis of AML. In addition, selection bias was well controlled by two independent investigator and an unlimited literature search. Furthermore, most included studies were of high quality with regard to quality assessment of the NOS scale (16). Moreover, no evident publication bias was identified by either Begg's funnel plot or Egger's test. Finally, we conducted sensitivity analysis by deleting a single study in every model, and we did not find obvious abnormal studies contributing to the pooled HR.

Begg's funnel plot was used to detect publication bias in this meta-analysis (Figure 5): As is shown in Figure 5, publication bias is not evident when DFS was regarded as an evaluated end point (Figure 5A); however, it indicated a publication bias when OS was regarded as an evaluated end point (Figure 5B). Indeed, OS reflects the multiple influences to individuals, not just AML. The OS of AML is able to attribute to multiple factors, which make the manuscript

easy to publish. Moreover, OS can be concluded according to the status of the patients with AML (live/dead), whereas DFS is usually diagnosed on the basis of medical examination, such as bone marrow biopsy. The difference contributed to the difficulty of obtaining data from AML, which also may lead to the publication bias. All in all, DFS of AML is considered as a better evaluated index in the prognosis AML, and DFS of AML in this meta-analysis did not indicate a publication bias.

There are several limitations of this study that should be acknowledged. First, there was a heterogeneity in Caucasians (I^2 = 72.9%, p = 0.000), which may attribute to the fact that Caucasians outnumbered Asians: 635 Asians from four different countries and 10,335 Caucasians from eight different countries in this meta-analysis. In our opinion, the heterogeneity from Caucasian group was from some unknown confounding factors in the united prospective cohorts of Caucasians. In this situation, if related confounding factors were well controlled, then the conclusion might be more convinced. In addition, we failed to get the specific information about chemotherapy from 10,970 participants including chemotherapy regimens, chemotherapy dose, and chemotherapy time, because different types of treatment may exert distinct impacts on the prognosis of the patients with AML. However, FLT3-TKD does not result from general chemotherapies, so the FLT3-TKD in AML in this study is regarded as being from individuals' genetic backgrounds. Furthermore, because of the limitation from the extracted data, we were unable to perform more stratification analysis according to other confounding factors. Although confounding factors work on the ending events, it is considered as a common problem in clinical research studies because we cannot predict everything before the start.

In conclusion, our results showed that FLT3-TKD revealed no significant effect on DFS and OS of patients with AML. However, meta-regressions demonstrated that patient source associated with the prognosis effect of FLT3-TKD in patients with AML. To be specific, FLT3-TKD represented a beneficial prognosis of DFS and OS for Asians with AML, whereas FLT3-TKD represented an adverse prognosis of DFS for Caucasians with AML. However, the results of DFS from Caucasians ought to be interpreted with caution due to the heterogeneity. This meta-analysis provided new information about the distinct prognosis of patients with AML between Asians and Caucasians. From our perspectives, the Caucasians with FLT3-TKD at the initial diagnostic stage of AML could be recommended the Asians dose of cytarabine and daunorubicin (cytarabine of 100 mg/m^2 + daunorubicin of 50 mg/m^2) in conventional "3 + 7" induction, so that they could receive a better prognosis of AML for survivals. An adequately designed prospective study including a large population with AML with clear FLT3 gene statue is needed to confirm our results.

Role of the funding source

The funder of the study supported the study design, data analysis, and publication of the manuscript.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

SL was involved in project conceiving, data collection, data curation, literature assessment, investigation, formal analysis, software performance, visualization, and original manuscript writing. NL was involved in data collection, data curation, and literature assessment. YC was involved in project conceiving, study supervision, and manuscript editing. ZZ was involved in study supervision and funding acquisition. YG was involved in project conceiving, literature assessment, study supervision, investigation, funding acquisition, and manuscript editing. All of authors read and approved the final manuscript.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1086846/full#supplementary-material

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Single-cell RNA sequencing depicts metabolic changes in children with aplastic anemia

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Introduction: Aplastic anemia (AA) is a bone marrow hematopoietic failure syndrome mediated by immune cells. The mechanism of this immune disorder is not well understood and therapeutic strategies still need to be improved.

Methods: Studies have found that abnormalities in metabolisms promote the survival of AA cells. In recent years, an increasing number of studies have reported the immunosuppressive therapy for the treatment of AA. In this study, we analyzed the transcriptome of AA from peripheral blood compared with healthy donors by single-cell sequencing and identified the affected metabolic pathways including lysine degradation. We demonstrated that the metabolic abnormalities of T lymphocytes mainly focus on glycolysis/gluconeogenesis. In addition, the metabolic abnormalities of natural killer cells concentrated in oxidative phosphorylation.

Results: The key genes involved in abnormal metabolic processes were Neustein neurotrophic factor (*NENF*), inositol polyphosphate-4-phosphatase type II B (*INPP4B*), aldo-keto reductase family 1, member C3 (*AKR1C3*), and carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 2 (*CHST2*) by differential gene expression analysis.

Discussion: Molecule interaction analysis showed that tumor necrosis factor superfamily, member 12 (TNFSF12) in tumor necrosis factor (TNF) signaling was broadly activated in AA. In conclusion, we suppose that the treatment of the immune cells' abnormal metabolic pathway may contribute to the development of novel strategies to treat AA.

KEYWORDS

aplastic anemia (AA), cell metabolism, scRNA seq, T lymphocytes, NK cells, NENF

Introduction

Aplastic anemia (AA) is a bone marrow hematopoietic failure syndrome caused by various etiologies. It is characterized by a decreased proliferation of bone marrow hematopoietic cells and peripheral blood pancytopenia (1). The main clinical manifestations are anemia, hemorrhage, and infection (2). The pathogenesis of AA is complicated, including the abnormality of the hematopoietic microenvironment (3), deficiency of hematopoietic stem cells/progenitor cells (4), and disorders of the immune system (5). AA occurs at any age. However, compared with adults, a large proportion of children with AA have a relatively high incidence rate of the bone marrow failure syndrome (6). The incidence of AA varies geographically, which is two-to-three times higher in Asia than in the western world (7).

Most acquired AA is considered to be the destruction of bone marrow hematopoietic cells mediated by T cells (8). Early studies showed that removing lymphocytes from the bone marrow with AA could increase the number of cell colonies in tissue culture, while adding the same lymphocytes to a normal bone marrow would inhibit *in vitro* hematopoiesis (9). In clinic, human leukocyte antigen (HLA)-matched sibling bone marrow transplantation is the first-line treatment for AA patients under 40 years old (10). The combined immunosuppressive therapy of eltrombopag, thymoglobulin, and cyclosporine A is the initial treatment of refractory AA, and patients' survival rate is approximately 90% (11).

Cell metabolism promotes the absorption of nutrients and various components required for cell synthesis (12), enabling organisms to grow and reproduce, maintain their structure, and react to the external environment (13). In terms of obtaining energy and biosynthesis, aerobic glycolysis is very important for cell proliferation (14). The transformation of intracellular metabolic pathways in immune cells alters the function of immune cells (15). In immune cells, six metabolic pathways were intensively discussed: glycolysis, the tricarboxylic acid cycle, the pentose phosphate pathway, fatty acid oxidation, fatty acid synthesis, and amino acid metabolism (15). Amino acid metabolism plays an important role in regulating the function of innate and adaptive immune systems. Previous investigations indicate that the deletion of the transporter Alanine/Serine/Cysteine Transporter 2 (*ASCT2*) (responsible for glutamine and leucine uptake) gene in T cells will damage the function of helper T cell 1 (TH1) and helper T cell 17 (TH17) cells (16). The transition of the metabolic pathway from oxidative phosphorylation to aerobic glycolysis is a sign of T-cell activation and a key step to satisfy the metabolic requirements in the process of cell proliferation (17). Studies have shown that the glucose analog 2-deoxyglucose, an inhibitor of the glycolysis pathway, inhibits T cells from developing into TH17 cells (18). Therefore, metabolic reprogramming is critical for T-cell activation and functional execution (19). In order to promote drug uptake and enhance the delivery ability to target T-cell populations, we coupled them with glucose transporters (20). Therefore, regulating the metabolism of T cells may be a therapeutic means to treat AA.

Single-cell messenger RNA (mRNA) sequencing (scRNA-seq) is a technology for an unbiased, high-throughput, and high-resolution transcriptome analysis of cell heterogeneity in populations (21). It aggregates cells and identifies new subsets, as well as gene expression in various tissues (22). We created a thorough transcriptional map of immune cells from healthy controls and patients with AA by single-cell RNA sequencing. Then, we explored the changes of the cellular transcriptome in patients and identified the key metabolic pathways that may affect the occurrence of AA.

Materials and methods

Clinical samples

The transcriptomic profile was obtained from peripheral blood from five children with AA (AA: LJX-AA, LZL-AA, SLT-AA, WJL-AA, and XF-AA) and three healthy donors (Ctrl: CBC-Ctrl, CRC-Ctrl, and LJJ-Ctrl) (Figure S1). All samples were recruited *via* The Seventh Affiliated Hospital of Sun Yat-sen University. All participants provided written informed consent before inclusion in the study.

Single-cell mRNA library preparation and sequencing

The complementary DNA (cDNA)/DNA/small RNA libraries were sequenced on the Illumina sequencing platform by Genedenovo Biotechnology Co., Ltd (Guangzhou, China). Cellranger was used to remove the reads with low sequencing quality and then compare them with the reference genome to annotate as a specific gene. After unique molecular identifiers (UMI) correction and statistics, the unfiltered feature barcode matrix was obtained. The cells in the data were identified and distinguished according to the unfiltered feature barcode array. To filtrate multicellular samples, Doublet-Finder was applied to calculate the gel beads in emulsion (GEM) probability [pattern analysis and neural networks (pANN) value]. In addition, we used the following indicators to perform cell filtration: the number of genes identified in a single cell (200.0–3,600.0), the total number of UMI in a single cell (<17,000.0), and the proportion of mitochondrial gene expression in a single cell (<25.0%).

Data analysis and visualization

We conducted Harmony for data consolidation and batch effect correction. Dimension reduction, cell clustering, and differential gene expression were performed using the Seurat package. Based on the subset information of cells, we set $|\log2FC| \geq 0.36$ and the proportion of cells expressing target genes in each group ≥ 0.1 as the threshold. Then, we used the modeling and simulation team (MAST) obstacle model to test the significance of differences. We screened the pathway with the enrichment degree of top 20 in kyoto

encylopaedia of genes and genomes (KEGG) A class Metabolism from the KEGG enrichment of clusters (Ctrl *vs.* AA). We calculated the number of differential genes in each term in the Gene Ontology (GO) database (http://www.geneontology.org/) and applied a hypergeometric test to find the GO entries that are significantly enriched. The Kyoto Encyclopedia of Genes and Genomes pathway was performed by Omicshare tools (http://www.omicshare.com/tools/). Gene Set Enrichment Analysis (GSEA) was performed by using the software GSEA and MSigDB (23). Disease Ontology Analysis was performed by the disease ontology (DO) database (http://disease-ontology.org/). The CellPhoneDB package was used to estimate cell–cell communication.

Statistical analysis

Data visualizing and statistical analysis were performed using GraphPad Prism 8.0 (GraphPad Software Inc, CA, USA). Differences between experimental groups were analyzed using unpaired Student's t-test. P value < 0.05 was considered significant.

Results

Single-cell analysis and cell type identification

To interrogate the metabolism of immune cells in patients with AA, clinical peripheral blood specimens were analyzed by 10x Genomics based on scRNA-seq [single-cell tagged reverse transcription sequencing (STRT-seq)]. The cells were labeled and differential genes were analyzed by Seurat to complete the statistics and distribution mapping. Then, we used the GO database and KEGG database to analyze the enrichment of divergence genes (Figure 1A). We distinguished cell subsets by immune cells' specific surface markers. Among the 19 subsets in the bubble plot, except for

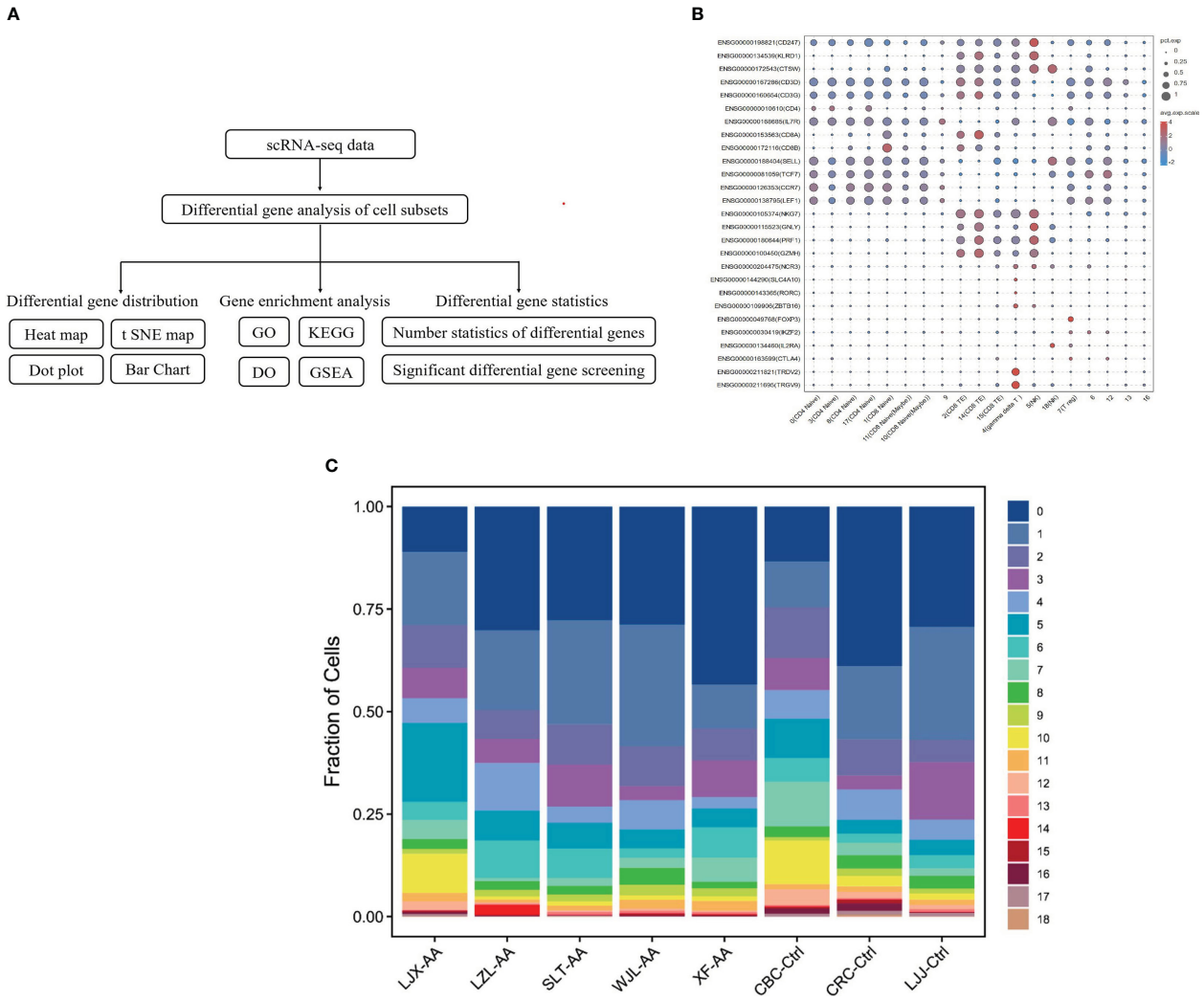

FIGURE 1
Identify cells and classify subsets with single-cell analysis. Schematic representation of the experimental design and single-cell mRNA sequencing (scRNA-seq) sequencing procedure **(A)**. The surface marker of immune cells was used to identify cells and classify subsets. Dot sizes represent percent expressed and dot colors represent the average-expressed scale **(B)**. Fraction of cell subsets' differences between groups. Colors indicate cell clusters with numbered labels **(C)**.

clusters 5 and 18, which are NK cells, the rest are T cells, including CD4 T cells, CD8 T cells, and Treg cells (Figure 1B). The percentage of different clusters showed that in AA, cluster 1 and cluster 2 are significantly distinct with the control group (Figure 1C).

The initial dimension reduction and unsupervised clustering of single-cell transcriptomes classified cells into 19 groups (Figures 2A, B). Compared with the control group, the difference of cluster 3 and cluster 5 is rather obvious in AA. At the same time, we conducted GO enrichment analysis from three aspects (biological process, cell component, and molecular function). The research found that, in the biological process part, genes upregulated in the AA group were significantly more than those downregulated (Figure 2C). Further study on GO enrichment analysis showed that, compared with the control group, most of the first 20 GO terms in the AA group were related to the regulation of cell metabolism (Figure 2D). Therefore, the abnormal regulation of cell metabolism and abnormal expression of related genes may be associated with the occurrence of AA.

Single-cell mRNA sequencing revealed metabolic differences in aplastic anemia

In order to further understand which metabolic processes are unusually regulated and metabolism-related genes are abnormally expressed in patients with AA, we conducted the KEGG pathway and GSEA. So as to display the distribution characteristics of metabolic pathways in different cell subsets, we manufactured the t-distributedstochastic neighbor embedding (t-SNE) map through the R package. The soft k-means clustering algorithm is used to cluster the dimension-reduced data, and cells were clustered into 19 cell types (Figure 3A). In the t-SNE difference distribution map of metabolism pathways, it was intuitively seen that lysine degradation was more common in AA (Figure 3B). We manufactured the homogenized gene expression into a t-SNE map. The results showed that the expression of the Neustein neurotrophic factor (*NENF*) gene in AA was significantly higher than that in the control group (Figure 3C).

Compared with the healthy donors, analysis found that the dysregulation of lysine degradation in patients with AA had marked statistical significance (AA *vs.* Ctrl, q = 0.0051) (Figure 4A). Interestingly, in the GSEA, we found that valine, leucine, and isoleucine degradation–related genes were more abundant in healthy donors (Figure 4B), which may indicate that valine, leucine, and isoleucine accumulated multiple times in the AA patients. To sum up, we concluded that the pathobolism of amino acids may play a key important role in the occurrence of AA. Next, we further analyzed from the aspect of metabolizing gene expression. In the heat map, there are significantly more abnormal regulated genes in cluster 3 and cluster 5 in AA compared with the control group (Figure 4C). Seurat software was performed to analyze the divisions between the subset of cells. The results were similar to the said GO analysis. The genes upregulated in AA were significantly more than those downregulated. At the same time, the metabolized gene expression in the third and fifth subsets was more obvious than that in other subsets (Figure 4D). These findings provided clues for our follow-up research on the mechanism of AA.

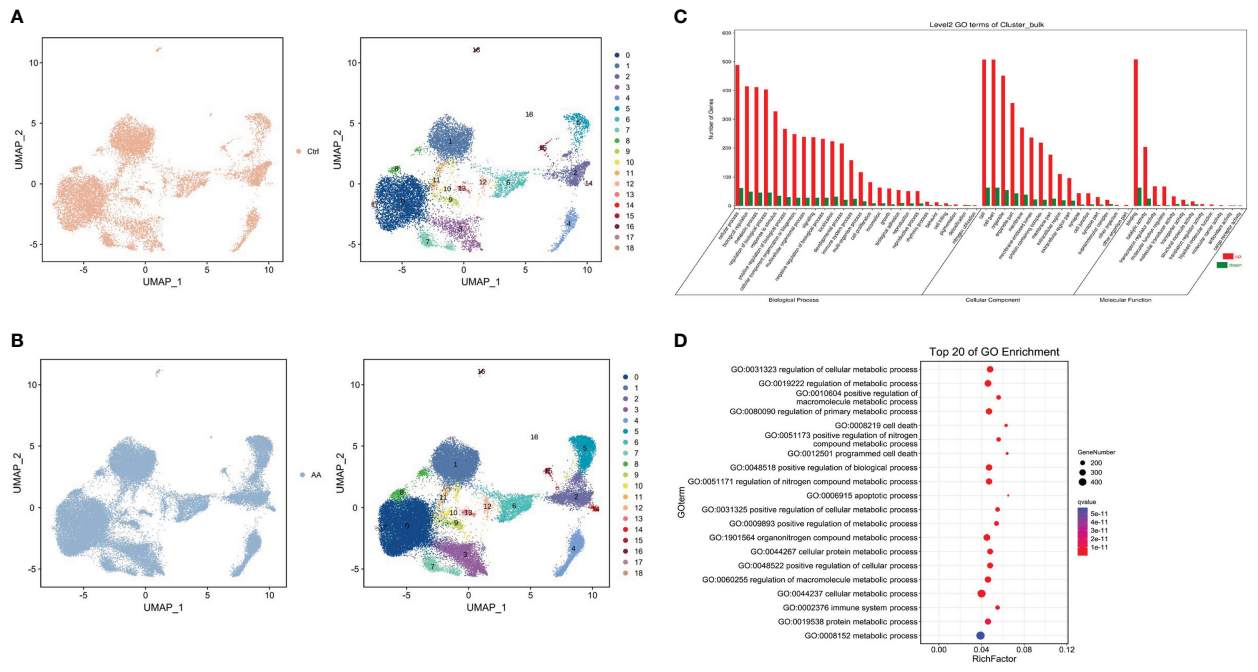

FIGURE 2
Single cell type identification and enrichment analysis. Uniform manifold approximation and project visualization of the control group **(A)** and aplastic anemia (AA) **(B)** based on single-cell transcriptomes (left). Gene Ontology (GO) enrichment classification histogram depicting the number of up- or downregulated genes in the biological process, cell component, and molecular function ontologies **(C)**. Dot plot representing top 20 enriched GO terms (ranked by Q values) based on bulk RNA-seq **(D)**.

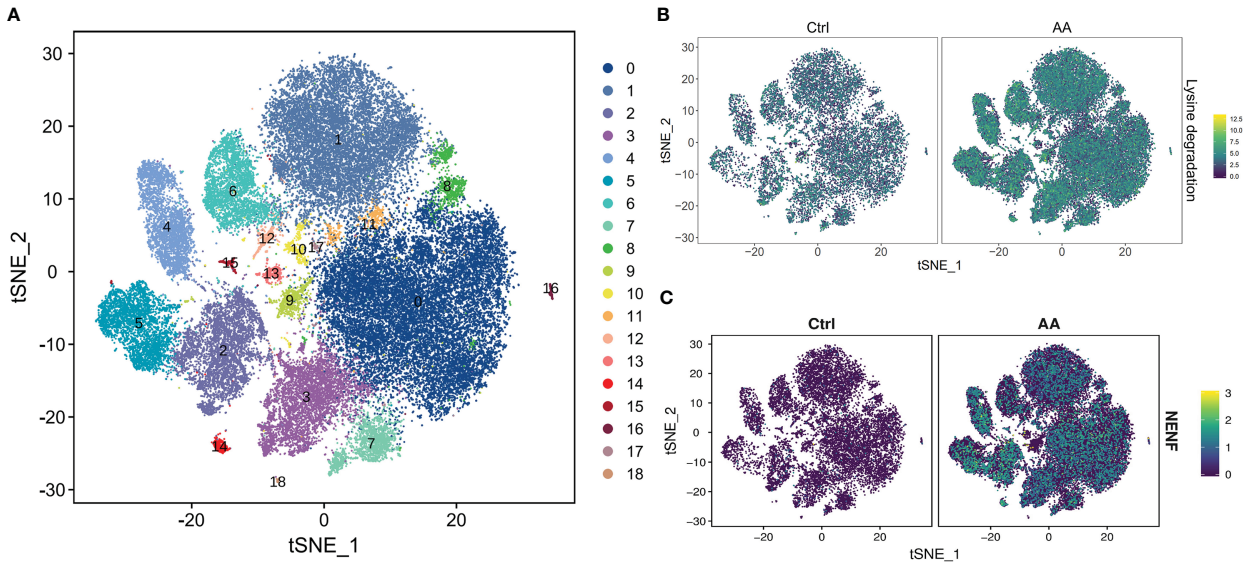

FIGURE 3
scRNA-seq revealed metabolic differences in AA. t-SNE plot of all cells representing the cell clusters analyzed by scRNA-seq. Each dot represents a single cell; each color corresponds to one cluster **(A)**. t-SNE plot showing the metabolism differences of lysine degradation between AA patients and healthy donors. Colors indicate logarithmic-transformed P values **(B)**. t-SNE plot showing the significantly expressed gene *NENF* in AA patients compared with those in healthy donors **(C)**.

T-lymphocyte metabolism analysis

As previously mentioned, compared with the control group, the number of abnormally expressed genes in the fifth subset of the AA group is the highest among all subsets. Then, we analyzed the changes of metabolic pathways in the fifth subgroup through the KEGG pathway, listed the metabolic pathways of top 20, and drew dot plots. It was found that the glycolysis/gluconeogenesis metabolic pathways were the most significant (Figure 5A). Because most cases of AA are an immune system disorder disease mediated by T cells, this article mainly studies the divergences of immune cell metabolism between the AA group and the control group. Then, we drew a t-SNE map according to the expressed genes through the R package and found that the *INPP4B* gene was specifically expressed in T cells but almost not in NK cells (Figure 5B). To check out which diseases may be caused by abnormal metabolic T cells in the process of AA progression, we conducted Disease Ontology analysis, and the results showed that AA was most likely to be converted to leukemia (Figure 5C). The said results provide an important basis for our follow-up research on the treatment and prevention of AA.

Natural killer cell metabolism analysis

It was reported that the dysfunction of natural killer (NK) cells may also be related to the occurrence of AA. Then, we analyzed the changes of metabolic pathways in NK cells through the KEGG pathway and found that the oxidative phosphorylation in NK cells in AA was abnormal, which had statistical significance (Figure 6A). We continued to explore which metabolism-related genes are abnormally expressed in NK cells and drew the t-SNE map. Interestingly, the expression amount of the *AKR1C3* gene and *CHST2* gene in NK cells is significantly higher than that in other cell subsets (Figures 6B, C). *CHST2* (carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 2) gene encodes a sulfotransferase protein, which catalyzes the sulfation of non-reducing n-acetylglucosamine residues and participates in the metabolism of lymphocytes at the inflammatory sites (24). Similar to what was mentioned earlier, we also did the Disease Ontology analysis of NK cells, listed the metabolic pathways of top 20, and drew bar charts. It was found that the metabolic abnormalities of NK cells were the most likely to cause AA to revert to cancer and the disease of cell promotion (Figure 6D). The intercellular interaction network between 18 subsets showed that cluster 5 had the strongest correlation among all cell subsets in the AA group (Figure 6F), while cluster 4 had the strongest correlation among all cell subsets in the control group (Figure 6E), indicating that the communication relationship of subgroup 5 in the AA group is enhanced. Among molecule interactions, the expression of the TNF signaling pathway, mechanistic target of rapamycin (mTOR) signaling pathway, and PI3K-Akt signaling pathway in AA (Figure 6H) and control (Figure 6G) had the most obvious difference. We further observed that TNFSF12 in TNF signaling was broadly activated in AA, which might contribute to the high reduction of normal blood cells. The metabolic correlation analysis of NK cells in AA provides a new perspective for us to study the mechanism of AA.

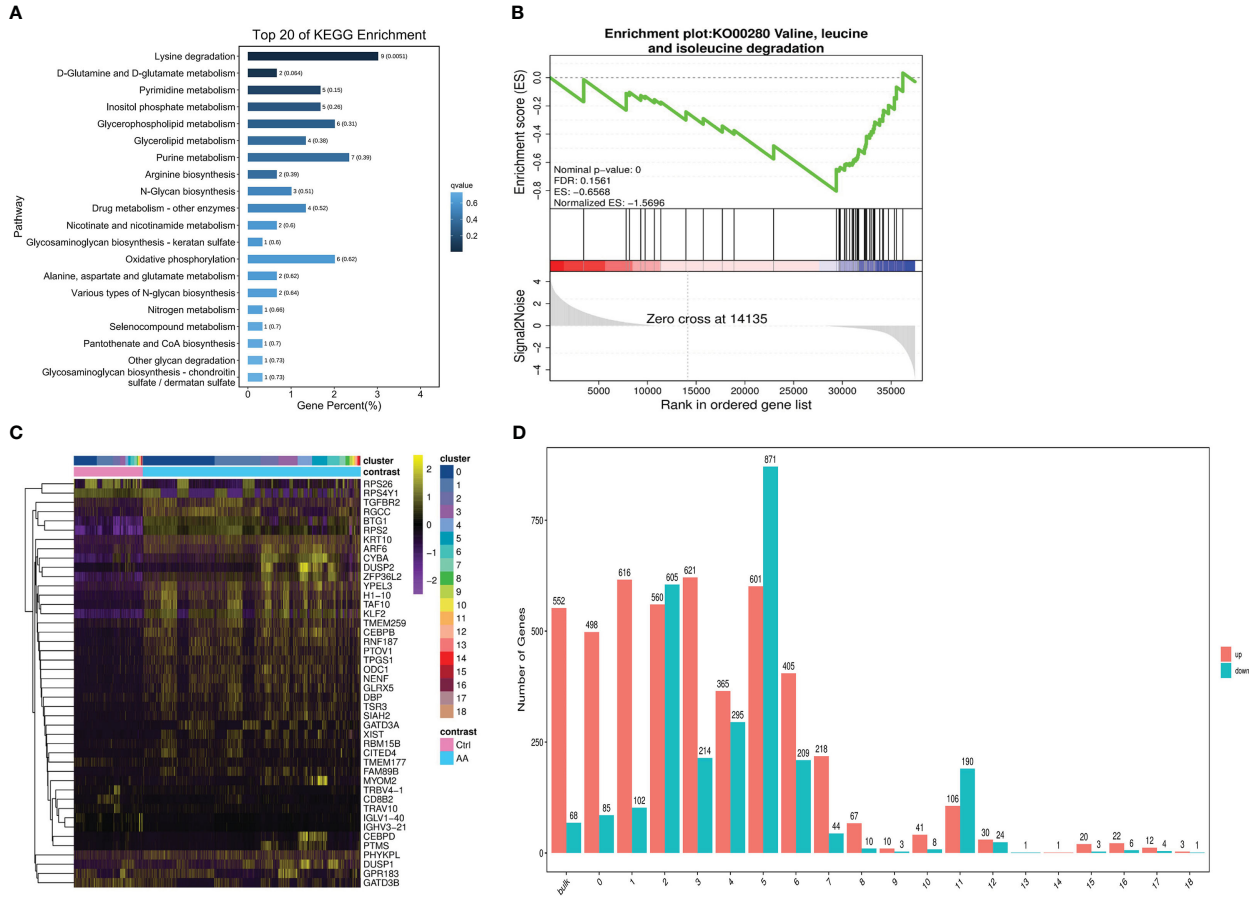

FIGURE 4
scRNA-seq revealed metabolic differences in AA. Bar chart showing top 20 of the KEGG pathway enriched in cell metabolism compared to those in the control group **(A)**. Gene set enrichment analysis (GSEA) plots of valine, leucine, and isoleucine degradation differences in AA patients compared with those in healthy donors **(B)**. Heat map of differential genetic expression handled with the z-score to normalize gene expression **(C)**. Bar chart showing the number of up- or downregulated genes in bulk and 19 subsets. Red indicates upregulation, and green indicates downregulation **(D)**.

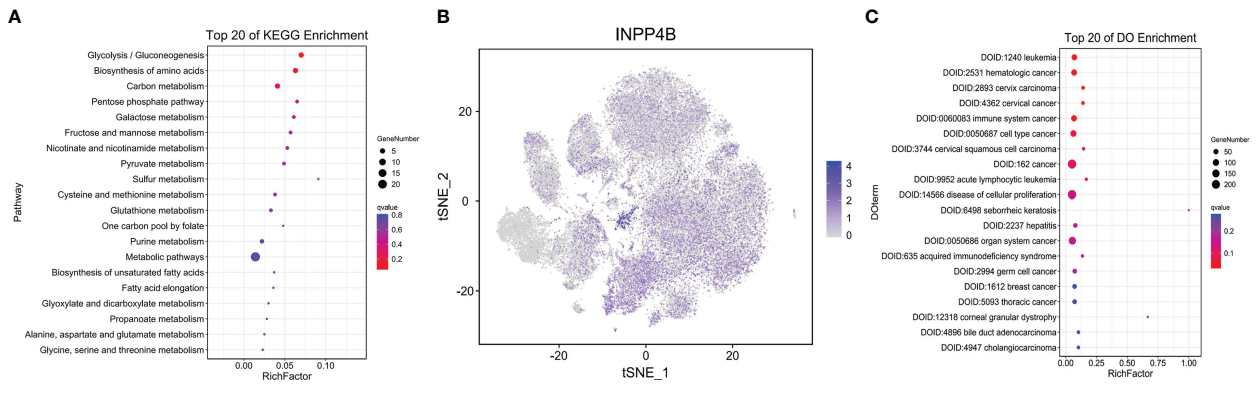

FIGURE 5
T-lymphocyte metabolism analysis. Dot plot depicting the top 20 KEGG pathway in T lymphocytes. Dot sizes represent the gene number, and dot colors represent Q values **(A)**. t-SNE plot showing the differently expressed gene *INPP4B* in T lymphocytes. All but clusters 5 and 18 are lymphocytes. Each dot represents a single cell. Colors indicate logarithmic-transformed P values **(B)**. Dot plot showing top 20 of disease ontology enrichment in T lymphocytes **(C)**.

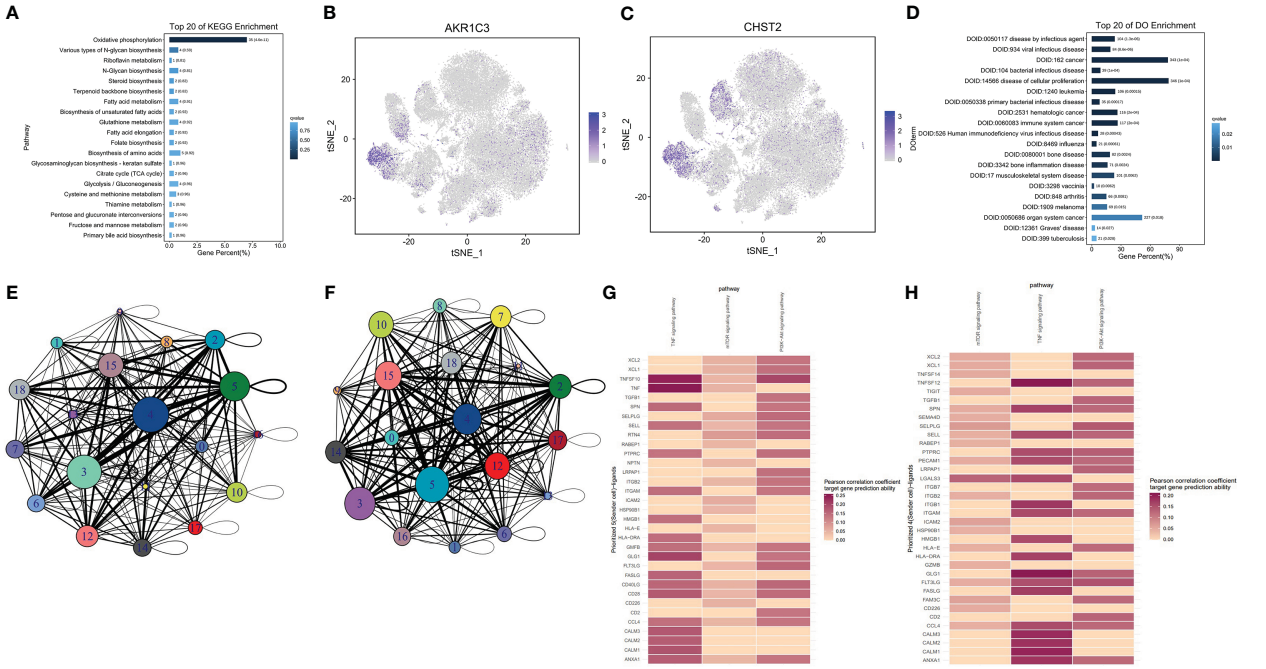

FIGURE 6
Natural killer (NK) cell metabolism analysis. Bar chart showing top 20 of the KEGG pathway in NK cells. Column length represents the percentage of differential genes, and column colors represent Q values **(A)**. t-SNE plot showing differently expressed genes *AKR1C3* **(B)** and *CHST2* **(C)** in NK cells. Cluster 5 is an NK cell. Each dot represents a single cell; Colors indicate logarithmic transformed P values. Bar chart depicting top 20 of disease ontology enrichment in NK cells **(D)**. Intercellular interaction network between 18 subsets of control **(E)** and AA **(F)**. Molecular interaction states of 36 ligand–receptors between clusters 4 and 5 in the control **(G)** and AA **(H)** groups. Molecules in red indicate that the Pearson correlation coefficient target gene prediction ability is higher.

Discussion

T cells were divided into cytotoxic T cells, helper T cells, regulatory T cells, and memory T cells according to their functions and surface markers. After being activated by antigens in the peripheral circulation, naive CD4 T cells proliferate and differentiate into various subsets of T helper cells, including Th1, Th2, and Th17 cells (25). The metabolic reprogramming of T cells enables them to shift from oxidative metabolism to biosynthetic metabolism to support rapid cell growth (26). Activated CD4 T cells require efficient glucose uptake, glycolysis, glutamate decomposition, and lipid synthesis to maintain cell proliferation (27). The enhancement of aerobic glycolysis in cells makes glucose and other nutrients not oxidized in mitochondria to produce ATP but used for the biosynthesis of nucleic acids, lipids, and amino acids (28). The boost of the glycolytic metabolic pathway occurs mostly in activated NK cells (29), T lymphocytes (30), and B lymphocytes (31). Previous studies have shown that pyruvate dehydrogenase is a key enzyme in T-cell glycolysis and oxidative metabolism (32). In our study, we found that the lysine degradation pathway of immune cells in AA is significantly higher than that in normal samples through the single-cell sequencing analysis of peripheral blood clinical samples. Lysine degradation is caused by ϵ-deamination or α-deamination reaction and produces two acetyl coenzyme A and several reductants (33). It has been found that the selective modification of lysine sites in proteins by aminophiles disrupts the interaction between proteins and RNA in the immune response (34). Our results showed that the disturbance of the immune system in AA was also related to the degradation of valine, leucine, and isoleucine. Previous studies indicated that L-amino acid transporters that are composed of Slc7a5 and CD98 induce leucine uptake, activate the mTOR pathway, and affect T-cell metabolism (35). During the activation of T cells and B cells, the transcription of intracellular glutamine transporters SNAT1 and SNAT2 is enhanced (36). It is covered that the NK cell count is decreased and the activity is impaired in patients with Fanconi anemia (37). NK cells mainly rely on oxidative phosphorylation to generate energy and activate downstream to produce interferon-γ (IFN-γ) (38). Oxidative phosphorylation and glycolysis are two major metabolic pathways for energy production and cell function maintenance. In immune cells, oxidative phosphorylation can regulate the formation of memory cells and related inflammatory reactions (39). Several recent studies in the US have shown that Cox10 (a gene encoding the composition of mitochondrial electron transfer chain complex IV) plays an important role in NK-cell antigen-specific amplification and murine cytomegalovirus (MCMV) infection (40).

At the transcriptional level, our research found that the NENF gene was upregulated in the immune cells of AA. Neudesin was initially identified as a secreted protein with neurotrophic activity. It has a conservative cytochrome 5-like heme/steroid binding domain and can activate intracellular signal pathways by binding to G protein–coupled receptors (41). NENF is essential in a variety of

biological processes, including neural function, fat metabolism, and tumorigenesis (42). It has been found that neudesin inhibits adipogenesis in mouse embryonic fibroblasts cells 3T3-L1 (3T3-L1) cells through mitogen-activated protein kinase (MAPK) cascade reaction (43). We also detected that *INPP48B* gene was upregulated in the T cells of patients with AA, and *AKR1C3* and *CHST2* were specifically upregulated in NK cells. INPP4B was initially identified as an enzyme that preferentially hydrolyzes the 4-phosphate of phosphatidylinositol-3,4-bisphosphate (PI(3,4)P2), to generate phosphatidylinositol-3-phosphate (PI(3)P) (44). In recent studies, the overexpression of INPP4B in AML cells enhancescolony-forming potential and induces chemotherapy resistance in acute myelocytic leukemia (AML) patients (45). As a soluble enzyme of the aldehyde ketone reductase family, AKR1C3 plays an important role in regulating prostaglandin, the steroid hormone, and retinoic acid metabolism (46). Chst2 encodes a carbohydrate sulfotransferase that catalyzes the sulfation of the C6 position of GlcNAc during keratan sulfate biosynthesis (47).

Through disease ontology enrichment analysis, the abnormal metabolism of T cells is likely to cause AA to develop into leukemia, and the metabolic changes of NK cells are likely to lead to abnormal cell proliferation diseases and tumors. Relevant results have also confirmed that the secondary myelodysplastic syndrome and acute leukemia usually develop from severe AA after immunosuppressive therapy (48). These findings support the broad potential of targeting functional lysine in the human proteome. It has been discovered that drugs acting on the surface receptors of the CTLA-4 and PD-1 can limit the uptake of glucose and amino acids, so as to negatively regulate the activation of T cells (49). Detailed investigations on specific mechanisms of metabolic abnormalities in the immune cells of AA are needed in the future.

Data availability statement

The data presented in the study are deposited in the CNCB repository, submission of HRA: HRA003544, project: PRJCA012914.

Ethics statement

The studies involving human participants were reviewed and approved by Shenzhen Science and Technology Innovation Commission. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

Author contributions

LH, JH, and YL conceived the project. LH and LW performed the experiments. QZ, JH, and JY analyzed data. QZ 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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1075408/full#supplementary-material

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Ferroptosis in hematological malignant tumors

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Ferroptosis is a kind of iron-dependent programmed cell death discovered in recent years. Its main feature is the accumulation of lipid reactive oxygen species in cells, eventually leading to oxidative stress and cell death. It plays a pivotal role in normal physical conditions and the occurrence and development of various diseases. Studies have shown that tumor cells of the blood system, such as leukemia cells and lymphoma cells, are sensitive to the response to ferroptosis. Regulators that modulate the Ferroptosis pathway can accelerate or inhibit tumor disease progression. This article reviews the mechanism of ferroptosis and its research status in hematological malignancies. Understanding the mechanisms of ferroptosis could provide practical guidance for treating and preventing these dreaded diseases.

KEYWORDS

ferroptosis, ROS, GPx4, leukemia, lymphomas

Introduction

Ferroptosis is a novel iron-dependent mode of death induced by elastin and Ras selective lethal 3 (RSL3) (1). Different from apoptosis, classic necrosis, autophagy, and other forms of cell death. The morphological features of ferroptosis are reduced cell size, increased mitochondrial membrane density, and decreased cristae. It is characterized by the induction of lipid peroxides (LPO) accumulation dependent on iron ions and reactive

Abbreviations: RSL3, Ras-selective lethal 3; ROS, reactive oxygen species; GSH, glutathione; GPX4, glutathione peroxidase 4; RPL8, ribosomal protein L8; IREB2, iron response element binding protein 2; PEBP1, phosphatidylethanolamine-binding protein 1; Nrf2, nuclear factor E2-related factor 2; PTGS2, cyclooxygenase-2; Fer-1, ferrostatin-1; DFO, deferoxamine; NCOA4, Nuclear receptor coactivator 4; ATP5G3, ATP synthase F0 complex subunit C3; NTBI, non-transferrin bound iron; BMDMs, bone marrow-derived macrophages; FAC, ferric ammonium citrate; HSC, hematopoietic stem cell; NOX, NADPH oxidase; AML, acute myeloid leukemia; PUFAs, polyunsaturated fatty acids; DHA, dihydroartemisinin; LOX, lipoxygenase; DLBCLs, diffuse large B lymphomas; TYP, Typhaneoside; AMPK AMP-activated protein kinase; CLL, chronic lymphocytic leukemia; TBH, tert-butanol; FINO2, 1, 2-dioxolane.

oxygen species (ROS). Polyunsaturated fatty acid (PUFA) in phospholipids, REDOX active iron, and repair defects of LPO are three characteristics of ferroptosis, which determine the sensitivity of tumor cells to ferroptosis (2, 3). Targeted small molecule substances reduce the antioxidant glutathione (GSH) levels or glutathione peroxidase 4 (GPX4) by acting on specific targets in the cell, which leads to the accumulation of intracellular ROS and lipids. In the synergistic action of iron, peroxidation induces ferroptosis (4, 5). Cystine/glutamate antiporter (system Xc-) exists on the cell surface to maintain REDOX homeostasis, promote the entry of cystine into the cell, and then reduce it to cysteine, which removes excess glutamate from the cell and provides the raw material for intracellular GSH synthesis. Meanwhile, GSH is an essential substrate for the degradation of LPO by glutathione peroxidase 4 (GPX4). When the GSH synthesis pathway is inhibited, LPO accumulates, and ferroptosis occurs (6, 7). Ferroptosis requires GSH depletion, decreased glutathione peroxidase 4 (GPX4) activity, and the inability to metabolize lipid peroxides through GPX4-catalyzed glutathione reductase reaction, thereby destroying the integrity of the cell membrane (8). Initially, Dixon et al. suggested that ferroptosis is mainly regulated by ribosomal protein L8 (RPL8), iron response element binding protein 2 (IREB2) at the gene level. ATP synthase F0 complex subunit C3 (ATP5G3), tetratricopeptide repeat domain 35 (TTC35), Regulation of citrate synthase (CS) and acyl-CoAsynthetase family member 2 (ACSF2) (1). Later, many studies have shown that many genes/proteins and molecular are involved in this particular cell death process, such as cyclooxygenase 2(PTGS2) (9), p53 (10), nuclear factor E2-related factor 2(Nrf2) (11), phosphatidylethanolamine binding protein 1 (PEBP1) (12) and miRNA (13). In addition to these key regulators, the onset of ferroptosis is associated with excess glutamate, an increase in the intracellular iron concentration, or the handling of small-molecule substances such as elastin, RSL3, and others in Table 1.

In recent years, with the in-depth study of the mechanism of ferroptosis, ferroptosis has been found in a variety of tumor cells, including breast cancer (20), lung cancer (1), liver cancer (21), kidney cancer (22) and brain tumors (23). In 2015, Jiang et al. found that p53 is essential in regulating ferroptosis, and this study found that ferroptosis contributes to embryonic development (10). Leukemia and lymphoma are the most common hematological malignancies. The main treatment methods are chemotherapy and stem cell transplantation. Although the treatment level of stem cell transplantation has been dramatically improved in recent years, its application has certain limitations (24). At the same time, the remission rate of chemotherapy is low, and there has been no substantial progress in recent years. Therefore, exploring treatment options that will benefit patients is still necessary. As one of the cell death modes, ferroptosis is a popular research direction in tumor research and treatment (25, 26). The known ferroptosis inducers can be divided into the following two categories. The first category includes Erastin, sulfasalazine, glutamate. Which can act through system xc-. The second class includes RSL3 and DP17, which can directly inhibit glutathione peroxidase activity (GPX). Unlike other ferroptosis inducers that usually mediate a single pathway, Erastin can mediate multiple molecules with efficient, rapid, and long-lasting effects (5, 27). In addition, depending on the mechanism of ferroptosis, many molecules have been identified as ferroptosis inhibitors, including ferrostatin-1(Fer-1, inhibiting lipid ROS) (1), deferoxamine (DFO, chelating iron) (1). Examples include deferoxamine (DFO, chelating iron) (1), zileuton (inhibiting 5-

TABLE 1 Main inducers, inhibitors, and regulators in hematological malignant tumors relevant to ferroptosis.

Target	Compound name/ protein	Effect	Hematological Malignant Tumors with Relevance to Ferroptosis
Inducers			
VDACs and system x_c^-	Erastin Piperazine erastin	Induce ferroptosis	Chronic Myelogenous Leukemia [14] Acute myeloid leukemia [9] Diffuse large B cell ymphomas[9]
GPX4	RSL3	Induce ferroptosis	Acute myeloid leukemia [9] Diffuse large B cell lymphomas [9]
System x_c^-	Sulfasalazine	Induce ferroptosis	Non-Hodgkin's lymphoma [15]
Lipid peroxidation	FINO2	Induce ferroptosis	B-lymphoblastic cell leukemia[16] B-cell lymphoma[16]
GSH depletion	Buthioninesulfoximine	Induce ferroptosis	Acute myeloid leukemia [9] Diffuse large B cell lymphomas [9]
Inhibitors			
P38	SB202190	Inhibit ferroptosis	Acute myeloid leukemia[17]
ROS from lipid peroxidation	Ferrostatin-1	Inhibit ferroptosis	Acute lymphoblastic leukemia [18]
Intracellular iron	Ciclopirox olamine	Inhibit ferroptosis	Acute lymphocytic leukemia [19]

lipoxygenase) (28), and the newly discovered FINO2(iron oxide) (14). This article reviews the research progress of ferroptosis in hematological malignancies, and the main inducers, regulators, and inhibitors of ferroptosis in hematological malignancies, as shown in Table 1.

Iron metabolism and hematological tumors

Iron is a nutrient that promotes cell metabolism, proliferation, and growth. Iron ions can gain and lose electrons. They are the active sites of many iron- and heme-containing enzymes in the body, such as mitochondrial enzymes related to respiratory complexes, enzymes related to DNA synthesis and cell cycle, and enzymes related to detoxification functions (such as peroxidase and catalase); the ability to freely gain and lose electrons allows iron to participate in redox reactions and form free radicals (15). Through the Fenton reaction, hydrogen peroxide reacts under the catalysis of ferrous iron to generate reactive oxygen species (16) Reactive oxygen species can causelipid and protein damage and oxidative DNA damage, including DNA base modification and DNA strand breaks, inducing mutations and promoting the occurrence and progression of tumors (17). In recent years, evidence has shown that ROS are essential for health. Under physiological conditions, generating low levels of ROS is considered a signaling molecule. On the other hand, abnormal iron accumulation, ROS overproduction, and ROS buffer system dysfunction can cause many chronic diseases. Therefore, the chronic accumulation of ROS is involved in many diseases. Moreover, excessive ROS production induces oxidative damage of biomolecules, leading to aging, cancer, and many other diseases (18). Many studies have shown that iron metabolism disorders are related to the occurrence of a variety of diseases, including hereditary hemochromatosis, myocardial ischemia-reperfusion injury, neurodegenerative diseases, and even cancer (19, 29, 30). Specifically, excessive intracellular iron levels can mediate carcinogenesis due to its pro-oxidative properties and DNA-damaging effects. At the same time, tumor cells acquire large amounts of iron to maintain rapid growth and proliferation (31). Under normal homeostasis, transferrin binds to free iron in the circulation, and metabolic iron in the body is maintained at a steady state. When iron overload occurs in the body, serum transferrin binding is close to saturation, and there is non-transferrin-bound iron (NTBI) in circulation (6, 32, 33). However, excessive iron can increase intracellular ROS content through the Fenton reaction, thus promoting ferroptosis (16). *In vitro* treatment of mouse primary hepatocytes and bone marrow-derived macrophages (BMDMs) with ferric ammonium citrate (FAC) significantly increased lipid peroxidation, decreased NADPH, and decreased cellular viability. These changes were reversed by ferroptosis inhibitors and iron chelators (34). In addition, the team also found that the Slc7a11-/- mouse model did not cause ferroptosis under basal iron conditions. The high-iron diet caused increased non-transferrin-bound iron in mice, decreased GSH levels, and increased ROS levels, indicating that iron-induced ferroptosis differs from that induced by erastin. Nuclear receptor coactivator 4 (NCOA4) is a selective receptor for the selective autophagy flip of ferritin in ferroptosis Hou et al. knocked out NCOA4 in PANC1 cells, which reduced the level of intracellular divalent iron. Ferroptosis is induced by erastin, and the level of iron is increased in the cells overexpressing NCOA4 by transfection, and the level of ferroptosis is increased. Therefore, the increase of intracellular iron caused by the degradation of ferritin mediated by NCOA4 participates in ferroptosis (35). The exosomes secreted by GPX4 inhibitor-treated cells contain a large amount of ferritin (36).

Compared with normal hematopoietic cells, leukemic cells have altered iron uptake, storage, and efflux and an altered iron transporter-hepcidin regulatory axis (Figure 1) (17). Although iron and the reactive oxygen species generated by its catalysis are crucial to maintaining the balance of the hematopoietic system, the accumulation of iron and the subsequent abnormal increase in reactive oxygen species can disrupt various biological functions of hematopoietic stem cells (HSCs) (37), including quiescence, self-renewal, and multilineage differentiation potential. Excessive ROS promotes cell senescence and apoptosis and impairs self-renewal ability and tumor formation, similarly, too much iron can lead to changes in the tumor microenvironment that promote cancer cell ferroptosis (38, 39). In addition, iron is crucial to the development of leukemia because iron-dependent ribonucleotide reductase is required for DNA synthesis to maintain the rapid proliferation of leukemia cells (40–42). In addition, iron overload induces apoptosis of neighboring NK cells, CD4+ T cells, and CD8+ T cells while simultaneously increasing the ratio of regulatory T cells, allowing leukemia cells to evade immune system attack (43, 44). In addition, unlike malignant tumors of other systems, patients with hematological malignancies require repeated blood transfusions due to normal erythropoiesis and chemotherapy, resulting in increased iron load in the body. Excessive iron and ROS can promote the malignant transformation of hematopoietic stem cells by consuming smoke NADPH oxidase (NOX) and GSH (39). In myelodysplastic syndromes, ROS-induced DNA damage may increase patients' risk of leukemia (38). These results indicate that excessive iron promotes ferroptosis through the ROS pathway, and changes in intracellular iron levels mediated by the ferritin metabolism pathway are also closely related to ferroptosis. Regulating the homeostasis of iron metabolism alters the susceptibility of AML cells to ferroptosis, and disease progression in leukemia also increases iron accumulation in patients.

The mechanism of ferroptosis and its research in hematological tumors

ROS

Reactive oxygen species (ROS), produced during normal metabolic processes, play essential roles in cell signaling and tissue homeostasis. However, ROS overproduced during metabolic processes are key promoters of the ferroptosis cascade

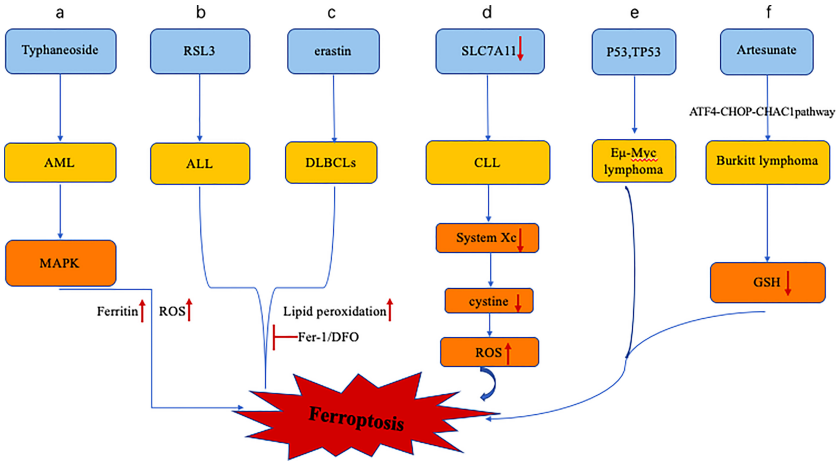

FIGURE 1
Altered iron metabolism in leukemia includes dysregulation of the ferroportin-hepcidin regulatory axis. **(A)** Typhaneoside treatment of AML cells resulted in ferroptosis in AML cells by activating AMPK signaling, accompanied by ferritin degradation and ROS accumulation. **(B, C)** RSL3 and erastin treatment of ALL and DLBCLs resulted in ferroptosis, accompanied by increased lipid peroxidation, which was inhibited by antioxidant and DFO. **(D)**, Expression of SLC7A11 in CLL cells was down-regulated, and the system xc- transporter cystine ability was decreased, leading to the increase of intracellular ROS and the promotion of cell ferroptosis. **(E)**, p53 inhibits System xc- and promotes ferroptosis in Eμ-Myc lymphoma cells, while the deletion of TP53 gene accelerates the formation of Eμ-Myc lymphoma model. **(F)**, Artesunate can induce ferroptosis in Burkitt lymphoma cells by activating ATF4-CHOP-CHAC1 pathway and degrading GSH.

and can lead to unfavorable modifications of various intracellular molecules such as lipids, proteins, and DNA damage (45, 46). This is called "lipid peroxidation." It is generally believed that lipid peroxides, especially the peroxidation of lipid hydrogen, can lead to damage reactions in the lipid bilayer of the cell membrane, which is a signal of cell death and can induce programmed cell death is an essential mediator of ferroptosis (47). Besides, lipid peroxides can produce additional toxicity due to their degradation products by altering the structure and function of nucleic acids and proteins such as Michael receptors and aldehydes (48). The high-throughput screening results showed that ferstatin-1 (fer-1) and lipoxstatin-1 (lip-1) could prevent erastin-induced ROS accumulation, specifically inhibiting RSL-induced ferroptosis. This also demonstrates the role of ROS accumulation in ferroptosis (49).

GPX4

GPX4 is one of the 25 human genome proteins containing selenocysteine and belongs to the GPxs family (24). It uses GSH as a cofactor to reduce lipid peroxide to lipid alcohol, preventing ROS synthesis (9, 50). GPX4 is the direct target of RSL3. GPX4 can avoid the toxicity of lipid peroxides and maintain the stability of the membrane lipid bilayer through its enzymatic activity (51). RSL3 can specifically inhibit the activity of GPX4, which leads to ROS accumulation in cells and induce ferroptosis (52). Furthermore, overexpression of GPX4 can reduce ferroptosis (27). Pedro et al. Demonstrated that the knockout of the GPX4 gene resulted in ferroptosis in cells (53). GPX4 - deficient mouse embryonic fibroblasts showed resistance to erastin-induced cell death, suggesting that ferroptosis depends on ROS (54). Selenium can effectively inhibit GPX4-dependent ferroptosis by activating tfap2c and Sp1 to enhance genes in this transcription program, such as GPX4. Selenium deficiency can inactivate GPX4, thus making cells more sensitive to oxidative damage (55, 56). In addition, fino2 and fin56 induced ferroptosis by indirectly inhibiting GPX4 levels and activity without interfering with glutathione metabolism (57, 58).

System Xc–

Glutamate-cystine antiport system x c – (system x c –) is a component of cell membrane transporter and is a heterodimer composed of SLC7A11 and SLC3A2, and responsible for the exchange of extracellular cystine and intracellular glutamate (58). Under physiological conditions, extracellular cystine is transported into the cell through system Xc-, the substrate for synthesizing the antioxidant glutathione, and glutathione is the main component for removing reactive oxygen species (59). Blocking system xc - can inhibit the synthesis of cysteine-dependent glutathione (GSH) and then damage cells' antioxidant defense ability, thereby promoting ROS accumulation and inducing ferroptosis in cells. For example, sulfasalazine can inhibit system xc - and cause ferroptosis, while β-mercaptoethanol increases cystine uptake, thus inhibiting elastin-induced ferroptosis in HT1080 cells (5). Erastin and sulfasalazine prevented cultured cancer cells from absorbing radiolabeled cystine (60).

Using traditional biochemical methods and more advanced metabonomics analysis, erastin treatment can lead to large consumption of intracellular GSH (61). How erastin or SAS inhibits SLC7A11-mediated Cys2 import remains unclear. Initial studies suggested that erastin could bind to the related transporter SLC7A5 and further inhibit SLC7A11 in trans2. However, recent findings deny this possibility and suggest that erastin can directly

inhibit SLC7A11 (60). Previous studies have revealed more potent drug analogs of erastin, and these findings are helpful for future studies on the targets and effects of erastin *in vitro* and *in vivo* (62). Wang et al. found that the source of cystine/cysteine in SLC7A11 knockout mice was decreased, which limited the subsequent GSH synthesis and increased the sensitivity of cells to ferroptosis induced by iron overload (34). Similarly, studies have shown that Nrf2 plays a key role in leukemia cell resistance to DOX and in triptolide induced iron death, suggesting a potential strategy for using triptolide and DOX in combination therapy in leukemia treatment (63). Moreover, Sorafenib induces ferroptosis in hepatocellular carcinoma through SHP-1/Stat3-mediated MCL1 downregulation and subsequent inhibition of SLC7A11 by increased BECN1 binding (64).

In summary, the accumulation of ROS and the increase of lipid peroxidation are associated with ferroptosis. The level and activity of GPX4 can affect the level of ferroptosis. In addition, system xc - regulates ROS balance by affecting GSH metabolism, an essential part of ferroptosis.

Jin et al. found that dihydroartemisinin (DHA) can strongly inhibit the activity of AML cell lines and can further effectively induce the death of AML cells, which is often considered ferroptosis because of its apparent iron dependence and accompanied by mitochondrial dysfunction. Mechanistically, DHA induces autophagy by regulating the AMPK/mTOR/p70S6k signaling pathway, which can accelerate ferritin degradation and increase the labile iron pool, further promoting the accumulation of ROS in cells and eventually leading to ferroptosis (65). Probst et al. Used acute lymphoblastic leukemia (all) cell lines as a cell model. Increased lipid peroxidation levels accompanied cell death after rsl3 treatment. The cell death was inhibited by adding a lipid peroxidation inhibitor fer-1 or lipoxygenase (LOX), and the iron chelator DFO could reverse RSL3-triggered cell death (66). Data analysis showed that diffuse large B lymphomas (DLBCLs) were particularly sensitive. Besides, lipid peroxides are produced in the DLBCL cell line treated with erastin. Lipophilic antioxidants can save cell death, indicating that the cell death in this cell line has ferroptosis characteristics. The sensitivity of DLBCL cell lines and other hematopoietic cell lines to 203 different lethal compounds was further analyzed, and it was found that the average resistance of DLBCL cell lines to all compounds was weak, indicating that the enhanced sensitivity of DLBCLs to erastin-induced ferroptosis was not due to Universal sensitivity to all compounds (9).

These results suggest that the susceptibility of leukemia and lymphoma cells to ferroptosis is consistent with the response to classical ferroptosis, which is characterized by excessive accumulation of ROS and increased lipid peroxidation. Typhaneoside (TYP) is a major flavonoid in the extract of Pollen Typhae, showing significant biological and pharmacological effects. Typ activates the AMP-activated protein kinase (AMPK) signaling pathway, which leads to ferritin degradation, ROS accumulation, and ferroptosis, and further significantly triggers autophagy in AML cells (67). Similarly, artesunate can induce an ER stress response and activate the ATF4-CHOPCHAC1 pathway to degrade intracellular GSH, thereby inducing ferroptosis in Burkitt lymphoma cells. The protection of the cells evidences this by Lip-1, Fer-1, and DFO (68). These studies provide new ideas for promoting ferroptosis in treating hematological malignancies. Human lymphoma and leukemia cells cannot convert methionine to cystine by their metabolic functions. Therefore, its growth and proliferation must be mediated by the extracellular uptake of cysteine. Interestingly, SLC7A11 was down-regulated in chronic lymphocytic leukemia (CLL) compared with other systemic solid tumors. The systemic xc transporting cystine capacity was reduced, which could increase the intracellular ROS level. This suggests that CLL is strongly associated with ferroptosis (69). Similarly, clinical studies have also shown that in patients with DLBCLs, GPX4 expression accounted for 35.5%(33/93). The survival time of the gpx4 positive group was significantly higher than that of the gpx4 negative group (70). GPX4 overexpression is an independent prognostic indicator of diffuse large B-cell lymphoma and AML (71, 72). This may be related to the role of GPX4 in reducing intracellular lipid oxidation and making cells insensitive to ferroptosis. In conclusion, ROS accumulation and lipid peroxidation play essential roles in the mechanism of ferroptosis. Studies on ferroptosis sensitivity show that leukemia and lymphoma cells can be more sensitive to ferroptosis by increasing the accumulation of intracellular ROS through multi-channel regulation. This opens up a new research direction for selecting drugs for the treating hematological tumors.

P53 and hematological tumors

p53 controls the cell cycle, DNA replication, and uncontrolled cell division during tumor growth. When p53 is mutated, it leads to tumor initiation and progression (71). By inhibiting the transcription of SLC7A11, p53cystine uptake, reduces and intracellular GSH, increases intracellular ROS accumulation, and increases the susceptibility to cell ferroptosis. They used an acetylation-deficient mutant p53 (3KR) that cannot induce other forms of apoptosis but retains the ability to regulate the expression of SLC7A11 and found that SLC7A11 is overexpressed in many types of human cancers. High expression levels of SLC7A11 can significantly reduce the tumor growth inhibitory activity induced by p53(3KR), indicating that this inhibitory activity has nothing to do with the disturbance of cell cycle, cell apoptosis, and cell senescence. At the same time, high levels of reactive oxygen species can trigger p53-mediated ferroptosis. The regulation of ROS levels by p53 is a complex biological process. When ROS levels are at basal or relatively low levels, p53 can prevent cellular cells from continuing to accumulate ROS. However, when ROS levels are abnormally high, p53 may promote cell death through the ferroptosis pathway. Therefore, ferroptosis can be regulated by p53 affecting intracellular ROS levels. The Alox12 gene is located on human chromosome 17p13.1 in a very close position to the TP53 gene. Some scholars have suggested that many human tumors lose one alox12 allele (73). Chu et al. used p53(3KR) H1299 cells lacking six lipoxygenase isoforms to test ROS-induced ferroptosis levels after butyl alcohol (TBH) treatment and found that p53-mediated ferroptosis could be specifically blocked by loss of ALOX12 function. SLC7A11 specifically binds to alox12, thereby reducing

its enzymatic activity, confirming that p53 can indirectly activate ALOX12 lipoxygenase activity by inhibiting SLC7A11 transcriptional repression system Xc, leading to Alox12-dependent ferroptosis induced by ROS death. The pathway of ferroptosis is independent of the GPX4 pathway (74).

Thus, p53 regulates ferroptosis levels by regulating SLC7A11 transcript levels and activity. Further studies showed that alox12 deletion inhibited p53-mediated iron mineralization. In the Eμ-myc mouse model, the loss of one Trp53 allele significantly accelerated the formation of the Myc-induced classical eμ-myc lymphoma model. In contrast, the loss of an alox12 allele shortened the median survival of these mice (74). In conclusion, p53 increases the sensitivity of cell death with iron by regulating ROS level, and the loss of p53 function plays an essential role in the occurrence and prognosis of Eμ-myc lymphoma.

Conclusion

Ferroptosis is a cell death mediated by various small molecules, such as erastin and RSL3. It is affected by GPX4, GSH metabolism, iron metabolism, and other pathways. Its occurrence is often accompanied by the accumulation of ROS and other substances, which can further lead to the peroxidation of cell membrane lipids. The specific mechanism of ferroptosis needs further study. The study of cell death mode is still an essential link in overcoming the tumor treatment problem. In recent years, ferroptosis has been more popular in tumor research. Many studies have shown that the sensitivity of leukemia and lymphoma cells to ferroptosis can be regulated by changing the concentration of ferroptosis-inducing molecules, the balance between intracellular ROS level and cell death, and the level of intracellular iron metabolism, to achieve the goal of eliminating leukemia and lymphoma cells. Studies have also found that many compounds are closely related to the ferroptosis of tumor cells, and the level of ferroptosis inducers is related to the prognosis of the disease. In addition to the existing iron chelators and targeted iron metabolism-related proteins, treating redox imbalance disorders targeting high intracellular iron levels also has broad application prospects in treating leukemia. The application of ferroptosis and ferromacrophages as a new ferroptosis therapy in leukemia has just begun. With the rapid development of nanotechnology, efforts have been made to exploit the therapeutic advantages of iron-based nanoparticles. The Magnetic field can not only concentrate nanoparticles but also promote the production of intracellular ROS, thus exerting an anti-leukemia effect. The progression of blood system tumor diseases and the changes brought about by treatment will also affect the ferroptosis process. There will be expected to be more related studies to provide new ideas for treating leukemia and lymphoma.

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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Shutting off the fuel supply to target metabolic vulnerabilities in multiple myeloma

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Pathways that govern cellular bioenergetics are deregulated in tumor cells and represent a hallmark of cancer. Tumor cells have the capacity to reprogram pathways that control nutrient acquisition, anabolism and catabolism to enhance their growth and survival. Tumorigenesis requires the autonomous reprogramming of key metabolic pathways that obtain, generate and produce metabolites from a nutrient-deprived tumor microenvironment to meet the increased bioenergetic demands of cancer cells. Intra- and extracellular factors also have a profound effect on gene expression to drive metabolic pathway reprogramming in not only cancer cells but also surrounding cell types that contribute to anti-tumor immunity. Despite a vast amount of genetic and histologic heterogeneity within and between cancer types, a finite set of pathways are commonly deregulated to support anabolism, catabolism and redox balance. Multiple myeloma (MM) is the second most common hematologic malignancy in adults and remains incurable in the vast majority of patients. Genetic events and the hypoxic bone marrow milieu deregulate glycolysis, glutaminolysis and fatty acid synthesis in MM cells to promote their proliferation, survival, metastasis, drug resistance and evasion of immunosurveillance. Here, we discuss mechanisms that disrupt metabolic pathways in MM cells to support the development of therapeutic resistance and thwart the effects of anti-myeloma immunity. A better understanding of the events that reprogram metabolism in myeloma and immune cells may reveal unforeseen vulnerabilities and advance the rational design of drug cocktails that improve patient survival.

KEYWORDS

metabolism, multiple myeloma, proteasome inhibitor, oxidative phosphorylation, glycolysis, fatty acid synthesis

1 Introduction

The hallmarks of cancer constitute an organizing principle to rationalize the complexities of neoplastic disease (1–4). Six biological capabilities - sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis acquired during the multistep development of human tumors - were initially identified as the hallmarks of human cancers (1). Genomic instability underlies these features that promote genetic diversity and intratumoral heterogeneity. Recently, reprogramming of energy metabolism and evading immune destruction have also been recognized as cancer hallmarks (2–4). Cancer cells sustain prodigious anabolic requirements that exceed those of neighboring somatic cells. Metabolic pathways in cancer cells are reprogrammed to achieve the required bioenergetic, biosynthetic and redox demands. Reprogramming of energy metabolism is also required to support continuous cell growth and proliferation, replacing the metabolic program that operates in most healthy tissues and fuels physiological operations within cancer cells (4–6). Tumorigenesis stems from the direct and indirect consequences of oncogenic mutations to reprogram key metabolic pathways (4, 7–12). Cancer-associated metabolic reprogramming also alters the level of key intracellular and extracellular metabolites (2, 11, 12).

Tumors display an added dimension of complexity since they contain a repertoire of recruited, ostensibly normal cells that contribute to the acquisition of hallmark traits by creating the tumor microenvironment (TME) (7–15). The TME is comprised of heterogeneous and interactive cell types including cancer cells and cancer stem cells surrounded by a multitude of recruited stromal cell and immune cell types. Cellular metabolism is reprogrammed in cancer cells by tumor-intrinsic and extrinsic factors. Cancer cells proliferate within the tumor permissive bone marrow (BM) and are surrounded by a complex environment that consists of cellular and acellular components, e.g., blood and lymph vessels, fibroblasts, endothelial cells, numerous immune cell types, osteoblasts, osteoclasts, pericytes, platelets, hematopoietic stem cells and other cell types. In addition, cancer cells are also influenced by surrounding cytokines, extracellular vesicles, cartilage, fat, bone and the extracellular matrix these reside within the BM milieu (10–15).

Oncometabolites are metabolites that aberrantly accumulate from distorted metabolism and are considered novel pathognomonic hallmarks in certain human cancers, e.g., glioma, leukemia, neuroendocrine tumors, and renal cancer (16–19). Oncometabolites have been shown to play a pivotal role in neoplastic transformation, cancer metabolism, and the development of therapeutic resistance. As a consequence of gain-of-function mutations and loss of tumor suppressors, oncometabolites accumulate within cancer cells and within the TME. For example, mutations in isocitrate dehydrogenase 1 and 2 (*IDH1/2*) occur in a subset of acute myeloid leukemia (AML) patients and *IDH2* mutant leukemic cells produce elevated levels of the oncometabolite D-2-hydroxyglutarate (D2-HG) (17, 18). D2-HG is a structural homolog and antagonist of the Krebs cycle intermediate α-ketoglutarate (α-KG) that disrupts the Krebs cycle leading to metabolic and epigenetic derangements. D2-HG changes the catalytic activity of α-ketoglutarate–dependent dioxygenases leading to genome-wide histone and DNA methylation alterations (16–19).

2 Linking altered cellular metabolism to multiple myeloma

Multiple myeloma (MM) is a cancer of terminally-differentiated plasma cells (PCs) that accumulate and proliferate predominantly within the tumor permissive BM microenvironment (20–25). PCs are primary effectors of humoral immunity and function as antibody-producing factories that secrete vast amount of immunoglobulins. PC proliferation within the BM leads to increased production and circulation of the monoclonal (M-spike) protein in serum and/or urine (6, 26). Cardinal clinical features of MM include anemia, hypercalcemia, renal impairment and myeloma-related bone lesions (6, 20–22). The clinical course of nearly all MM patients is characterized by cycles of continuously shortening periods of remission followed by relapse. The prevalence of obesity, cardiovascular disease and diabetes increases with age and elderly patients diagnosed with MM generally present with these concurrent co-morbidities (6, 27–32). The prognosis of MM patients has significantly improved over the past two decades, primarily due to the incorporation of novel agents developed based upon the biology of disease (20, 22, 33). MM cells synthesize and secrete vast amounts of protein, especially immunoglobulins, and have adapted to withstand an enhanced capacity for unfolded polypeptides. Hence, MM cells are exquisitely sensitive to drugs that disrupt protein homeostasis, e.g., proteasome inhibitors (PIs). Although PIs represent a highly effective anti-myeloma therapy and transformed the management of MM, drug resistance inevitably emerges through compensatory protein clearance mechanisms, e.g., the aggresome+autophagy pathway (34). Genome-wide profiling identified individual microRNAs (miRs), e.g., miR-29b, that were differentially expressed in bortezomib-resistant MM cells compared to drug-naive cells. The highly distinct function and specialized habitat of MM cells shapes the circuitry of intracellular pathways that contribute to drug resistance (35).

Genomic, proteomic and metabolic changes in myeloma cells stimulates their clonal evolution and expansion that eventually leads to the emergence of drug resistant clones that are responsible for disease relapse (36–38). Altered cellular metabolism also reduces the anti-myeloma effect of standard-of-care agents, e.g., PIs and immunomodulatory drugs (IMiDs). Metabolic changes within the TME further decreases the beneficial anti-myeloma effects of PIs and IMiDs, monoclonal antibodies and cellular immunotherapies (14, 15, 23, 36). Despite the development of novel anti-myeloma drugs over the past two decades, disease heterogeneity, high-risk disease, early relapse and treatment resistance remain challenges (14, 20, 22, 24, 33).

Moreover, subclonal heterogeneity of PCs evolves alongside disease progression through the selection of genetically and metabolically adapted subclones (37, 38). Importantly, the incidence of MM is associated with metabolic syndrome and inflammatory cytokines, while the anti-diabetic agent metformin that lowers blood glucose levels and statins, which lower the level of low-density lipoprotein (LDL) cholesterol, are positive prognostic factors in patients diagnosed with MM (39–42).

3 Metabolic pathways altered in multiple myeloma

MM cells employ specialized metabolic programs that differ from neighboring, untransformed somatic cells to sustain their extraordinary anabolic and catabolic needs (6, 42–45). Features of altered metabolism in MM include deregulated uptake and metabolism of glucose and amino acids especially glutamine, capacity to acquire scarce nutrients, enhanced glycolytic and tricarboxylic acid (TCA) cycle intermediates, elevated nicotinamide adenine dinucleotide phosphate (NADPH) production and elevated level of fatty acid (FA) synthesis.

3.1 Glucose metabolism

The glycolytic enzyme hexokinase II (*HKII*) is overexpressed in MM cells relative to PCs from healthy donors (46). Ikeda et al. found that hypoxia-inducible HKII impaired glycolysis and contributed to autophagy activation as well as the acquisition of an anti-apoptotic phenotype in myeloma cells. To detect candidate genes crucial for the acquisition of hypoxia-inducible autophagy, a comprehensive expression analysis was performed using MM patient samples incubated under normoxia or hypoxia. Hypoxic stress upregulated glycolytic genes (*PFKFB4*, *ENO2*, *ALDOC*, *PFKFB3*, *HK2*, *PFKP, GPI, PGK1*, *LDHA*, *ALDOA*, *ENO1*, *PKM*, and *GAPDH*) including *HKII* in samples obtained from MM patients (46). These results suggest that hypoxia-drive event may permit myeloma cells to metabolize glucose in an energetically favorable multi-step process. Antisense oligonucleotide (ASO) directed against *HKII (HII-ASO1*) suppressed *HKII* expression in MM cell cultures and in MM patient tumor cells xenografted into murine models (47). *HKII-ASO1* shows selective *HKII* inhibition to support the clinical development of this approach. Aerobic glycolysis also activates the TCA cycle to produce NADPH and glutathione (GSH) which reduces oxidative stress. Since oxidative damage is essential for bortezomib-mediated cytotoxicity, drug resistance may be accompanied by increased tolerance towards oxidative insults. Soriano et al. showed that PI-adapted myeloma cells tolerate subtotal proteasome inhibition owing to metabolic adaptations that favor the generation of NADPH reducing equivalents, supported by oxidative glycolysis (48).

Lactate dehydrogenase A (*LDHA*) expression is increased in relapsed MM patients to suggest that glucose metabolism is enhanced (49). Proliferator-activator receptor-γ coactivator-1β (*PGC-1β)* and *LDHA* are highly expressed in MM cells and *LDHA* is upregulated by *PGC-1β* through the *PGC-1β/RXRβ* axis by acting on the *LDHA* promoter. Overexpression of *PGC-1β* or *LDHA* potentiated glycolysis metabolism and increased cell proliferation and tumor growth. Conversely, knockdown of either *PGC-1β* or *LDHA* suppressed glycolysis, increased reactive oxygen species (ROS) formation and apoptosis, suppressed tumor growth and enhanced mouse survival. Liu et al. investigated whether excess glucose induced hypoxia-inducible factor-1α (HIF-1α) and stimulated glucose metabolism and cell migration in pancreatic cancer cells (50). The authors studied wild-type (WT) MiaPaCa2 pancreatic cancer cells and a MiaPaCa2 subline transfected with an HIF-1α-specific small interfering (siRNA). Excess glucose stimulated the migration of WT and siRNA-treated MiaPaCa2 cells grown under normoxia and hypoxia, while glucose stimulated cell migration independent of HIF-1α. These studies indicated that excess glucose increases HIF-1α and ATP in hypoxic WT-MiaPaCa2 cells. Extracellular glucose levels and hypoxia regulate glucose metabolism independent of HIF-1α while glucose stimulates cell migration through HIF-1α-dependent and independent mechanisms.

The Warburg effect describes an increase in the rate of glucose uptake and preferential production of lactate, even in the presence of oxygen (51–53). Further evidence that Warburg's

experiments on tumor tissue *in vitro* were valid *in vivo* was demonstrated in experiments on surviving tumor tissue and replicated in tumor-bearing animals (54, 55). The effect is clinically utilized in ^{18}F-fluorodeoxyglucose (^{18}F-FDG) positron emission tomography (PET) scans as sensitive diagnostic and prognostic tools (56, 57). Glucose is transported across the cell membrane through glucose transporters (GLUTs) through a facilitated diffusion mechanism (58–61). Owing to its elevated glycolytic gene profile, MM cells have been shown to be dependent on glycolysis and, therefore, susceptible to glycolysis inhibitors, e.g., GLUT inhibitors (58). Of the 14 GLUT subtypes, *GLUT1* overexpression is most associated with poor clinical outcomes in cancer cell lines and cancer patients (44, 47, 58, 61). In MM cells, *GLUT1* upregulation increases glucose uptake and enhances susceptible to GLUT1 inhibitors (61). MM cells are also dependent on *GLUT4* for glucose uptake, survival, and elevated expression of the anti-apoptotic protein *Mcl-1*, that has been associated with tumorigenesis, poor prognosis, and drug resistance (58).

Upregulation of the GLUT membrane transporters, e.g., GLUT1 GLUT4, GLUT8 and GLUT11, increases the level of glycolytic metabolites in MM cells. The Federal Drug Administration (FDA)-approved HIV protease inhibitor ritonavir demonstrates an off-target inhibitory effect on GLUT4 as well as a dose-dependent inhibitory effect on glucose uptake and proliferation in L363 and KMS11 cells (62). However, a subset of MM cells survive glucose deprivation or ritonavir treatment, possibly through mitochondrial oxidative phosphorylation (OXPHOS). Targeting the mitochondrial complex I using the FDA-approved anti-diabetes drug metformin combined with ritonavir induced apoptosis in primary MM cells. The PI3K/AKT pathway, through mTOR-dependent activity, is linked to increased

glucose metabolism and may explain the elevated levels of glycolytic intermediates seen in MM cells (63–66). The combination also suppressed AKT and mTORC1 phosphorylation and downregulated *Mcl-1* expression (62).

LDH, which converts pyruvate and NADH to lactate and NAD^+, is elevated in ~10% of patients with newly-diagnosed, symptomatic MM (67). *HIF-1α* is upregulated in drug resistant MM cells and leads to enhanced lactate production and the accumulation of glycolytic metabolites (68). *HIF-1α* upregulation is associated with metastasis, unfavorable prognosis, and reduced OS in cancer patients (68, 69). Since bortezomib decreases HKII activity in MM cells grown under hypoxic conditions and loss of HKII decreases LDHA activity, targeting LDHA could enhance effects of bortezomib (45). Indeed, inhibition of *HIF-1α* and *LDHA* have been shown to restore sensitivity to bortezomib and melphalan in MM cells (45). FX11 is a selective and potent LDHA inhibitor which reduces ATP levels by inducing oxidative stress and ROS production (70) (Table 1). PX-478 selectively inhibits HIF-1α to suppress cell migration, angiogenesis and drug resistance (71). Pyruvate kinase M2 (PKM2) regulates glycolysis and promotes tumor cell survival and proliferation (86). Never in mitosis gene A (NIMA)-related kinase 2 [*NEK2*] increases the PKM2/PKM1 ratio by splicing PKM to promote enhanced glycolysis that drives oncogenesis (54).

TABLE 1 Pharmacologically targeting metabolic vulnerabilities in hematologic malignancies.

Metabolic Pathway	Target	Drug	Mechanism of Action
Glycolysis	HIF-1α	PX-478 (Phase I, NCT00522652)	Decreases nuclear HIF-1α protein levels to reduce HIF-1α (71).
	LDHA and HK2	FX11 (Preclinical)	Inhibits aerobic glycolysis (70).
	GLUT4	Ritonavir (Phase I, NCT02948283)	Cytostatic and/or cytotoxic effects by chemosensitizing tumor cells both *in vitro* and *in vivo* (62, 72).
	GLUT1	Vincristine (Phase II, NCT00003493)	Inhibits microtubule formation in mitotic spindle, resulting in an arrest of dividing cells at the metaphase stage (73).
		Bortezomib (Phase IV, NCT00257114)	Binds reversibly to the chymotrypsin-like subunit of the 26S proteasome, resulting in its inhibition and preventing the degradation of various pro-apoptotic factors (48).
		WZb117 (Preclinical)	Inhibits passive sugar transport in human erythrocytes and cancer cell lines and, by limiting glycolysis (74).
		Phloretin (Preclinical)	Blocks cyclins and cyclin-dependent kinases and activates mitochondria-mediated cell death to promote cell death (61, 75).
	Hexokinase	Vincristine	See above.
		Bortezomib	See above.
OXPHOS	Glycerophosphate dehydrogenase	Metformin (Phase II, NCT04850846)	Inhibits MM proliferation by inducing cell cycle arrest and apoptosis (39, 40, 76).
Amino acid metabolism	Glutaminase	Benzophenanthridinone 968 (Preclinical)	Promotes apoptosis in both human MMCL and patient primary cells (77, 78).
		CB-839 (Telaglenastat) (Phase I, NCT03798678)	Allosteric, noncompetitive inhibitor of both splice variants of the broadly expressed glutaminase-1. Enhanced CFZ-induced ER stress and apoptosis, characterized by a robust induction of ATF4 and CHOP and the activation of caspases (79).
	Guanine and Guanosine GSH	Melphalan (Phase II, NCT02669615)	Alkylates guanine and causes linkages between strands of DNA leading to cytotoxicity in dividing and non-dividing cells (80).
	SNAT1	α-Methylamino-isobutyric acid (Preclinical)	Competitive inhibitor of the neutral amino acid transport A system which decreases glutamine uptake and reduces cell growth (81, 82).
	ASCT2	V-9302 (Preclinical)	Blocks ASCT2 to attenuate cancer cell growth and proliferation, increase cell death, increase oxidative stress, to contribute to anti-tumor responses *in vitro* and in murine models *in vivo* (83).
	LAT1	Nanvuranlat (JPH203) (Phase I, in solid tumors, PMID: 32198649)	Inhibits essential amino acids uptake in tumor cells to activate apoptosis (84).
Fatty acid metabolism	Carnitine palmitoyltransferase-1 (CPT1)	Etomoxir	Inhibits β-oxidation and *de novo* fatty acid synthesis in MM cells (43, 85).
	Fatty acid synthase (FASN)	Orlistat	Inhibits lipases and induces apoptosis in myeloma cells (43, 85).

The HK isoform HKII is the rate-limiting step in aerobic glycolysis and is overexpressed in many cancers including MM (87, 88). Vincristine and bortezomib suppressed GLUT-1 and HK expression to induce apoptosis in MM cells (73), while WZb117 and phloretin inhibited GLUT-1 activity to decrease glucose uptake with synergistic anti-tumor effects in leukemia, lung, colon and breast cancers (74, 89, 90). Under hypoxic conditions, phloretin enhanced the effects of daunorubicin and overcame hypoxia-conferred drug resistance (91). Targeting glucose consumption through enzymatic regulators and transporters could serve as an effective anti-myeloma therapy.

3.2 Amino acid metabolism

Glutamine is an abundant amino acid crucial for cell proliferation, differentiation, apoptosis, and cytokine production (92). Glutamine is needed in MM cells for nucleic acid biosynthesis, to generate energy in the TCA cycle and to support increased amino acid and FA synthesis. MM cells are particularly dependent on extracellular glutamine since they exhibit high glutaminase levels and low glutamine synthetase expression. Glutamine depletion prevents MM growth and enhances sensitivity to anti-myeloma agents (77, 79, 84, 93–95). The histidine/large neutral amino acid transporter LAT1 (SLC7A5) glutamine transporter is overexpressed in MM cells and is associated with reduced overall survival (OS) (84). MM cells primarily rely upon the alanine, serine, cysteine transporter 2 (ASCT2/SLC1A5) and glutamine transporters for glutamine uptake. Targeting glutamine transporters, specifically ASCT2 inhibitors combined with the PI carfilzomib induced proteotoxic stress and ROS generation (81, 83). The need for extracellular glutamine makes glutamine transporters interesting targets for MM therapy.

Glutamine serves as an important energy source for cancer cells and glutamine deficiency or the glutaminase inhibitor benzophenanthridinone 968 induces apoptosis in MM cells (13, 78, 82). Benzophenanthridinone 968 effectively inhibits glutaminase and this inhibition induces apoptosis in MM cell lines (MMCLs) and patient primary tumor cells. Elevated expression of the glutamine transporters SNAT1, ASCT2 and LAT1, makes these an attractive target for anti-myeloma therapy (6). The prognostic significance of LAT1 in MM was investigated by immunohistochemistry to monitor the expression of LAT1 and its functional subunit, 4Fc heavy chain (CD98), on tumor cells in 100 newly diagnosed MM (NDMM) patients (84). *LAT1* overexpression was associated with high proliferation and poor prognosis in NDMM patients. LAT1 may be a promising pathological marker to identify high-risk MM.

3.3 Fatty acid metabolism

A lipid profiling study uncovered large differences in lipid composition as well as amino acid and energy profiles from NDMM, relapsed and/or refractory (RRMM), monoclonal gammopathy of unknown significance (MGUS) and healthy controls (96). The metabolomic profile was quite different between that observed with samples from healthy controls compared to that of samples from patients with either MGUS, NDMM or RRMM. Significant alterations in amino acid, lipid, and energy metabolism were observed between the different patient groups. Eight metabolites, i.e., free carnitine, acetylcarnitine, glutamate, asymmetric dimethylarginine and phosphatidylcholine species, differed between MGUS and NDMM patients, supporting the notion that metabolic changes occur during myelomagenesis. A second lipidomics study revealed upregulation of ceramides and phosphatidylethanolamines (PEs) and downregulation of phosphatidylcholines, sphingomyelin and one species of PE in MM patients (97). Increased sphingomyelinase expression in primary patient samples was found and inhibition of sphingomyelinase by GW4869 further increased bortezomib and melphalan-mediated cell death (80). Treatment of MM cells with ixazomib led to the accumulation of lipids. Pre-treatment of MM cells with docosahexaenoic acid (DHA) or eicosapentaenoic acid (EHA) also increased the sensitivity to bortezomib by altering the GSH metabolic pathway (98). Tirado-Velez et al. tested the hypothesis that inhibition of β-oxidation and *de novo* FA synthesis would reduce cell proliferation in myeloma cells (85). The authors found that the RPMI-8226, NCI-H929 and U-266B1 cells displayed increased FA oxidation (FAO) and elevated expression of FA synthase (FAS). Inhibition of FAO by etomoxir and FAS by orlistat inhibited β-oxidation and *de novo* FA synthesis without significantly altering glucose metabolism. These effects were associated with cell cycle arrest in G0/G1 and reduced cell proliferation (43, 85). Etomoxir-mediated inhibition of FAO modestly increased the amount of lactate generated without altering glucose metabolism, to suggest that the inhibition of FAO in myeloma cells did not result in an adaptive mechanism to sustain energy homeostasis. FAS was elevated in ~70% of MM patients compared to healthy volunteers and inhibition of FAS by cerulenin promoted apoptosis (99). MMCLs and primary MM cells overexpress FAS to promote their survival and proliferation. MM patients have been reported to exhibit greater levels of saturated FAs and n-6 polyunsaturated FAs (PUFA), compared to healthy controls. *Acetyl-CoA synthetase 2 (ACSS2)* is overexpressed in MM cells derived from obese patients and contributes to myeloma progression (100). ACSS2 interacts with the oncoprotein interferon-regulated factor 4 (IRF4), and enhances IRF4 stability and IRF4-mediated gene transcription through activation of acetylation. The importance of *ACSS2* overexpression in myeloma was confirmed by finding that an ACSS2 inhibitor reduced myeloma growth *in vitro* and in a diet-induced obese mouse model. The findings demonstrated a key impact for obesity-induced ACSS2 on myeloma progression and could be important for other obesity-related malignancies. Glioma cells were incubated with tetradecylthioacetic acid (T11111141), which cannot be β-oxidized, and the oxidizable FA palmitic acid (PA), in the presence of L-carnitine and the carnitine palmitoyltransferase inhibitors etomoxir and aminocarnitine. L-carnitine partially abolished PA-mediated growth reduction of glioma cells, whereas etomoxir and aminocarnitine enhanced the anti-proliferative effect of PA (101). Similarly, Samudio et al.

demonstrated that inhibition of FAO with etomoxir or ranolazine reduced the proliferation and sensitized human leukemia cells to ABT-737-induced apoptosis (102). The conventional view has been that cancer cells predominately produce ATP by glycolysis, rather than by oxidation of energy-providing substrates. Mitochondrial uncoupling, i.e., continued reduction of oxygen without ATP synthesis, may obviate the ability of oxygen to inhibit glycolysis and promote the preference for glycolysis by shifting from pyruvate oxidation to FAO.

4 Oncogenic *MYC* and myeloma metabolism

Transcription factors of the *MYC* family are deregulated in up to 70% of all human cancers and *MYC* deregulation is a determinant of myeloma progression (103–105). Oncogenic levels of *MYC* regulate almost every aspect of cellular metabolism. *MYC* plays a key role in the regulation of aerobic glycolysis and activates glycolytic genes not only by transcription, but also through alternative splicing. In addition, enhanced *MYC* expression upregulates the level of glutamine transporters and suppresses inhibition of glutaminolysis (77, 94, 106). Glutamine depletion led to the rapid loss of the MYC protein, independent of *MYC* transcription and post-translational modifications. However, *MYC* loss was dependent on proteasomal activity and this loss was paralleled by an equally rapid induction of apoptosis (106). *MYC* transcription is upregulated in certain MM cells, especially during later stages of disease. The estimated 24-month progression-free survival was found to be significantly shorter in patients with intermediate to high *MYC* expression compared with patients with low *MYC* expression (107). However, this did not translate into a significant difference in OS. Somewhat different results were presented by Chng et al. which indicated that patients with *MYC*-expressing tumors had a significantly shorter OS (105). Chng et al. further reported that nearly all tumors with *RAS* mutations expressed a *MYC* activation signature. *MYC* activation, assessed by gene expression signature or immunohistochemistry was associated with hyperdiploid MM, and shorter survival even in tumors non-proliferative.

5 Impact of the hypoxic microenvironment on metabolism in myeloma cells

MM cells are exposed to different levels of oxygen and nutrients leading to metabolically heterogeneous phenotypes that differentially respond to therapeutic intervention (108–112). Hypoxia-inducible factors (HIFs), e.g., HIF-1α, are stabilized (108, 111, 113) within the TME and HIF-1α activation intensifies conversion of pyruvate into lactate instead of the oxidation of pyruvate in mitochondria. HIF-1α is also essential in regulating vascular endothelial growth factor (VEGF) which is associated with a poor prognosis in MM (114). HIF-1α was reported to be increased in MM as compared to controls (115, 116). The expression of *HIF-1α* was also correlated with serum levels of VEGF, basic fibroblast growth factor (bFGF) and angiopoietin-2 (Ang-2) (117–125). Gene expression datasets indicated that *HIF-1α* and *HIF-2α* were enriched in cells from NDMM patients compared to those from healthy donors (45, 126, 127). IMiDs treatment has been shown to decrease *HIF-1α* expression within the BM indicating that HIF-1α could also serve as a target in MM (128).

The TME consumes vast amounts of oxygen that is required for aerobic glycolysis within tumor cells (129) (Figure 1). Hypoxia increases anaerobic glycolysis by activating HIF (130) and hypoxia-induced *LDHA* and *HKII* promote PI-resistance in MM cells (45). It was also shown that activation of miR-210 due to hypoxia significantly reduced tumor susceptibility to $CD8^+$ cytotoxic T-lymphocytes (CTLs) by downregulating *PTPN1, HOXA1*, and *TP53I11* in melanoma and lung cancer cells (131). Hypoxia inducible miR-210 significantly downregulated *PTPN1* and *TP53I11* in MMCLs (132). Moreover, the HIF-inducible factor adrenomedullin is released from MM cells and stimulates vascular endothelial cells to express the angiogenic receptors CRLR and RAMP2 to promote angiogenesis (133). HIF-1α regulates interleukin (IL)-32 release from myeloma cells that is taken up by osteoclasts (134). The hypoxia-inducible p38-cyclic adenosine monophosphate (AMP) response element-binding protein (CREB)-Dickkopf-related protein 1 (DKK1) axis and upregulation of the HIF-1α-inducible MM SET domain-containing histone methyltransferase (MMSET) suppress osteoblastic bone formation (135). Taken together, hypoxic stress creates a favorable environment for myeloma survival by regulating chemotaxis, stimulating osteoclasts and endothelial cells, and inhibiting osteoblasts (Figure 1).

Bortezomib inhibits HIF-1α at the transcriptional level which in turn impairs recruitment of the coactivator CBP/p300 (136). The effects of PIs are attenuated within the hypoxic TME possibly due to reduced endoplasmic reticulum (ER) stress. In addition, the degradation of unfolded proteins normally mediated by proteasomes may be alternatively removed by autophagy.

HIF-inducible HKII activates autophagy by inhibiting mammalian target of rapamycin (mTOR) signaling (137). HKII is a promising therapeutic target and HKII inhibitors may increase the efficacy of anti-myeloma agents.

6 Metabolic alterations that alter anti-myeloma immunity

MM cells remodel the BM milieu to reshape the TME and negatively impact effectors of anti-tumor immunity (138–140). Deregulated tumor metabolism impairs the functional capacity of neighboring immune cells and compromises their differentiation (141–147). Adaptations within the TME create a competition for nutrients required by myeloma cells with their neighboring non-tumor cells. MM cells outcompete neighboring cells for nutrients to enhance tumor growth and impair anti-tumor immunity. Further dissecting the metabolic requirements of tumor and non-tumor

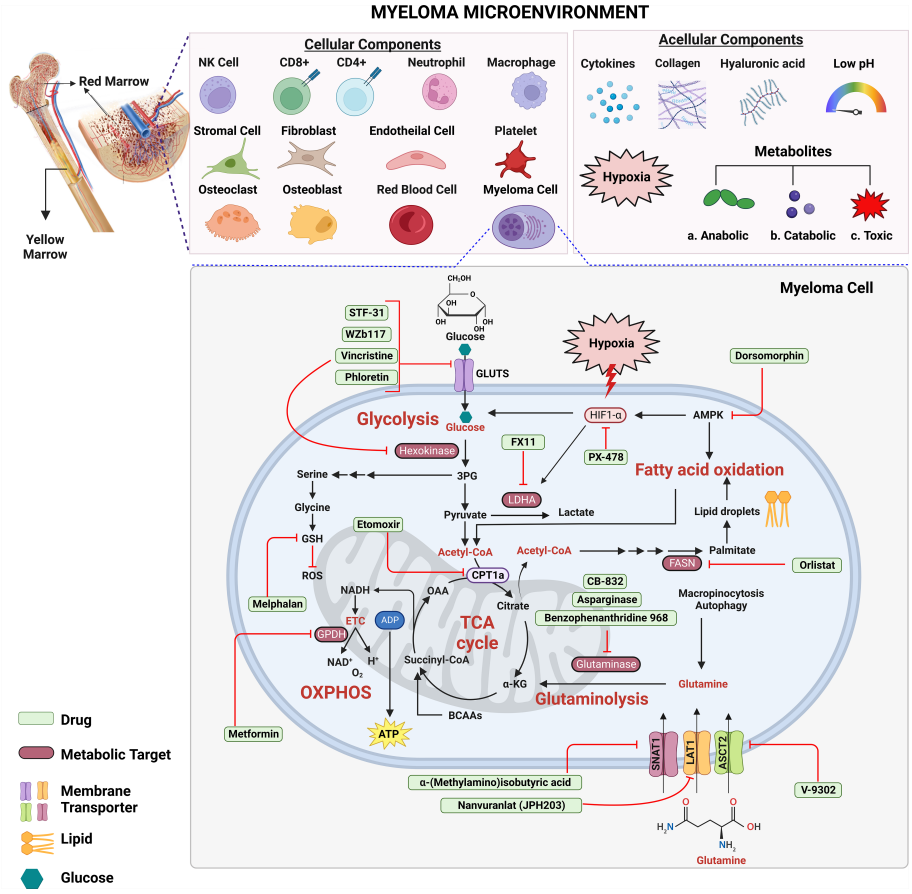

FIGURE 1
Targeting metabolic energy supply chains to enhance anti-myeloma therapy. Multiple myeloma cells use glucose as a primary source of energy followed by glutamine and fatty acids. Within cytosol, glucose is metabolized *via* glycolysis into two molecules of pyruvate and adenosine triphosphate (ATP) each. Next, pyruvate is transported across the mitochondrial matrix and is oxidized *via* TCA cycle to acetyl-CoA. Glutamine is transported across the membrane *via* transporters where it is metabolized into α-ketoglutarate (α-KG) *via* glutaminolysis. Oxidation of fatty acids results in breakdown of fatty acids into acetyl-CoA units. Which supplies energy to other tissues when glycogen stores are depleted. Each metabolic step releases energy in the form of electrons which are accepted by the electron transport chain to generate even more ATP through oxidative phosphorylation (OXPHOS). Metabolism targeting drugs (green) inhibit the key metabolic steps in glycolysis, TCA cycle, fatty acid synthesis, OXPHOS and glutaminolysis. Figure 1 is an original image created with Biorender.com, Toronto, Canada.

cells in the TME may enhance immunotherapeutic responses. In addition, a disrupted vasculature deprives the TME of adequate blood supply and enhances competition between tumors and infiltrating immune cells (148). In MM, $CD4^+$ and $CD8^+$ T-cells form the primary immune defense, however, tumor-induced remodeling of the TME is unfavorable to T-cells due to nutrient deprivation, acidosis, and the accumulation of toxic metabolites (146) (Figure 1). The hypoxic microenvironment also upregulates PD-L1 expression on tumor cells through HIF-1α and a hypoxia response element (HRE) (149, 150). In MM, PD-L1 expression has been shown to be upregulated on malignant PCs (151, 152). NK cells from MM patients express PD-1, in contrast to NK cells from healthy individuals, which suppresses NK cell cytotoxicity (153). Immune cells take up and utilize amino acids, e.g., L-arginine, a non-essential amino acid present in macrophages and DCs, and lipids that are necessary for functional activity (154–157). As a product of aerobic or anaerobic glycolysis in tumors, lactic acid induces *VEGF* expression and M2-like polarization of tumor-associated macrophages (158). Tumor secretion of lactate also promotes overexpression of arginase I isoform in macrophages and is associated with immunosuppression. Lactate is not only a secondary product of cancer metabolism, but also promotes immune evasion through various mechanisms (159–161). Adenosine, and other products of cancer cell metabolism, interfere with the antitumor effect of infiltrating T-cells (162, 163). Tryptophan metabolites, especially kynurenine generated through indoleamine 2,3-dioxygenase (IDO1), have been shown to modulate T-cell activity (141–144). Kynurenine, produced by both *IDO-1* and tryptophan-2,3-dioxygenase-2 (TDO-2), upregulated the PD-1 co-inhibitory pathway on activated $CD8^+$ T-cells *in vitro* compared with vehicle-treated cells (140). Since tryptophan catabolites suppress immunity, blocking tryptophan catabolism with IDO inhibitors is a potential anticancer strategy (164). Targeting the tryptophan catabolic kynurenine pathway using immune-based approaches has been shown to enhance antitumor immunity and cytotoxicity in MM (165).

7 Conclusions

A century after Warburg discovered that tumor cells switch from mitochondrial respiration to glycolysis to generate energy, even under aerobic conditions, cancer metabolism remains perplexing. Myeloma cells exhibit a metabolic phenotype characterized by enhanced glycolytic flux for ATP production, glucose to lactate conversion and reduced mitochondrial OXPHOS (166–168). In contrast to healthy, differentiated cells, which rely on mitochondrial OXPHOS to generate energy, cancer cells rely on aerobic glycolysis. The switch to aerobic glycolysis may represent an adaptation to facilitate the uptake of nucleotides, amino acids, and lipids required for replication (169–171). Reprogramming of the metabolic pathways that contribute to tumor growth has exposed molecular vulnerabilities and actionable targets that can be exploited (Table 1). Warburg described aerobic, *not anaerobic*, glycolysis and therefore there must exist factors other than HIF1-α which elicit the Warburg effect. In addition, HIF1-α is not expressed in MM cells unless grown under hypoxic conditions. Recent work by Abdollahi et al. (172, 173) demonstrated a role for PRL-3 in the induction of glucose uptake and enhanced glycolysis. Importantly, this effect was not mediated through HIF1-α, c-Myc or AMPK, but rather through STAT1 and STAT2. In hypoxia there was synergy between HIF1-α and PRL-3 in promoting glycolysis. Contrary to HIF1-α, PRL-3 does not seem to reduce OXPHOS, and recent research has shown that many hematological cancers do not downregulate OXPHOS activity (174).

Proteasomes are central to the protein degradation machinery of eukaryotes (175, 176). Healthy and transformed cells depend on proteasomes to control the level of proteins linked to metabolism, survival and proliferation (177). Based upon these findings, over the past two decades PIs have emerged as a transformative anti-myeloma therapy that has improved patient OS and quality-of-life. Proteasome abundance and catalytic activity is controlled at the level of assembly and is finely tuned by adaptations in cellular metabolism (177). Sequencing of PCs from NDMM patients has shown that MM is frequently dominated by *RAS* (43% of patients) and nuclear factor kappa B (NF-κB) pathway (17%) mutations (178). Malignant PCs undergo extensive metabolic reprogramming during myelomagenesis that is enhanced by *KRAS*, *NRAS*, and *BRAF*-activating mutations to elevate proteasomal capacity and reduce ER stress (179). *Ras* and related proteins are mutated or deregulated in many solid tumors, but PIs are ineffective against these cancers (180). Future studies are needed to decipher how solid tumors reprogram cell metabolism to evade the cytotoxic effect of PIs. Novel agents and drug delivery systems that target cancer metabolism may broaden the therapeutic impact of PIs in rationally-designed drug cocktails that improve patient survival.

Author contributions

JD, PR, and KG developed the concept, wrote, edited, made substantial contributions, and approved the final version of the manuscript.

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Conflict of interest

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Glossary

α-KG	α-ketoglutarate
ACSS2	Acetyl-CoA synthetase 2
AKT	A serine/threonine kinase from the ***t***hymoma cell line AKT-8, derived from the Stock ***A*** strain ***k*** AKR mouse. Also known as Protein kinase B (PKB)
AML	Acute Myeloid Leukemia
AMP	Adenosine monophosphate
Ang2	Angiopoietin-2
ASO	Antisense oligonucleotide
ASCT2	Alanine, serine, cysteine transporter 2
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
BiTE	Bispecific T cell engager
BM	Bone marrow
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CTL	Cytotoxic T-lymphocyte
CREB	Cyclic AMP response element-binding protein
D2-HG	{{sc}}d{{/sc}}-2-hydroxyglutarate
DKK1	Dickkopf-related protein 1
DLBCL	Diffuse large B-cell lymphoma
DNA	Deoxyribonucleic acid
DHA	Docosahexaenoic acid
EHA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
FA	Fatty acid
FAO	Fatty acid oxidation
FAS	Fatty acid synthase
FDG	Fluorodeoxyglucose
GLUT	Glucose transporter protein type
GSH	Glutathione
HKII	Hexokinase II
HIF	Hypoxia-inducible factor
HRE	Hypoxia response element
HIV	Human immunodeficiency virus
IDO	Indoleamine 2,3-dioxygenase
IL	Interleukin
IRF4	Interferon-regulated factor 4
LAT1	L-Type Amino Acid Transporter 1

(Continued)

Continued

LDHA	Lactate dehydrogenase A
LSC	Leukemic stem cell
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
miR	MicroRNA
M-spike	Monoclonal or Myeloma protein spike or paraprotein
MGUS	Monoclonal gammopathy of unknown significance
MM	Multiple myeloma
MMSET	MM SET domain-containing histone methyltransferase
NADPH	Nicotinamide adenine dinucleotide phosphate
NDMM	Newly diagnosed MM
NEK2	NIMA-related kinase 2
NIMA	Never in mitosis gene A
NHL	Non-Hodgkin's lymphoma
NK	Natural killer
NF-κB	Nuclear factor kappa B
OXPHOS	Oxidative phosphorylation
PE	Phosphatidylethanolamine
PET	Positron emission tomography
coactivator-1βPFK	Phosphofructokinase
PKM1	Pyruvate kinase M1
PKM2	Pyruvate kinase M2
PI3K	Phosphatidylinositol 3-kinase
PI	Proteasome inhibitor
PUFA	Polyunsaturated fatty acid
R-CHOP	Rituximab, cyclophosphamide, hydroxydaunorubicin HCl, vincristine (Oncovin) and prednisone used to treat both indolent and aggressive forms of NHL
ROS	Reactive oxygen species
RRMM	Relapsed and/or refractory MM
siRNA	Small interfering RNA
SLC	Solute carrier
STAT-3	Signal transducer and activator of transcription 3
SGLT	Sodium-dependent glucose transport
TCA	Tricarboxylic acid
TCR	T-cell receptors
TDO-2	tryptophan-2,3-dioxygenase-2
TP53	Tumor protein 53
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
WT	Wildtype.

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