Heliyon 10 (2024) e34717

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Exploration of the mechanism of fraxetin in treating acute myeloid leukemia based on network pharmacology and experimental verification

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ARTICLE INFO

Keywords:
Acute myeloid leukemia
Fraxetin
Network pharmacology
Molecular docking
AMPK signaling pathway

ABSTRACT

Objective: To explore the pharmacological mechanism of the effect of fraxetin in treating acute myeloid leukemia (AML) by the network pharmacology method combined with experimental validation.

Methods: The targets of fraxetin were identified through Swisstarget prediction, PhammerMap, and CTDBASE. Disease-related targets of AML were explored using GeneCards and DisGenet databases, and the intersected targets were analyzed in the String website to construct a protein-protein interaction (PPI) network. Subsequently, gene ontology (GO) functional enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment were conducted using the DAVID database. Molecular docking of core proteins with drugs was performed using Auto Dock Vina software. Finally, the effect of fraxetin on AML was evaluated by in vitro experiments. The effect of fraxetin on AML cell proliferation was assessed by CCK8, the effect of fraxetin on AML cell apoptosis was assessed by flow cytometry, and the expression of relevant protein targets was detected by Western blotting to evaluate the anti-AML effect of fraxetin.

Results: In this study, fraxetin exerts its effect against AML through 101 intersecting genes. The pathway enrichment analysis revealed that the pharmacological effects of fraxetin on AML were related to the Adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) signaling pathway, and the molecular docking results indicated that fraxetin had an excellent binding affinity to both the core target and AMPK. In vitro experiments have demonstrated that fraxetin inhibited the proliferation and induced apoptosis of THP1 and HL60 cells, and the western blotting results indicated that the *p*-AMPK of the fraxetin intervention group was significantly changed in a dose-dependent manner.

Conclusion: Fraxetin may modulate the AMPK signal pathway by interactine with the core target, thereby potentially therapeutic effect on AML.

Abbreviations: AML, acute myeloid leukemia; AMPK, Adenosine 5'-monophosphate (AMP)-activated protein kinase; CCl4, carbon tetrachloride; ERS, endoplasmic reticulum stress; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes nd Genomes; PPI, protein-protein interaction; ROS, reactive oxygen species; TCM, traditional chinese medicine; UPR, unfolded protein response.

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https://doi.org/10.1016/j.heliyon.2024.e34717
Received 11 May 2024; Received in revised form 14 July 2024; Accepted 15 July 2024
Available online 16 July 2024

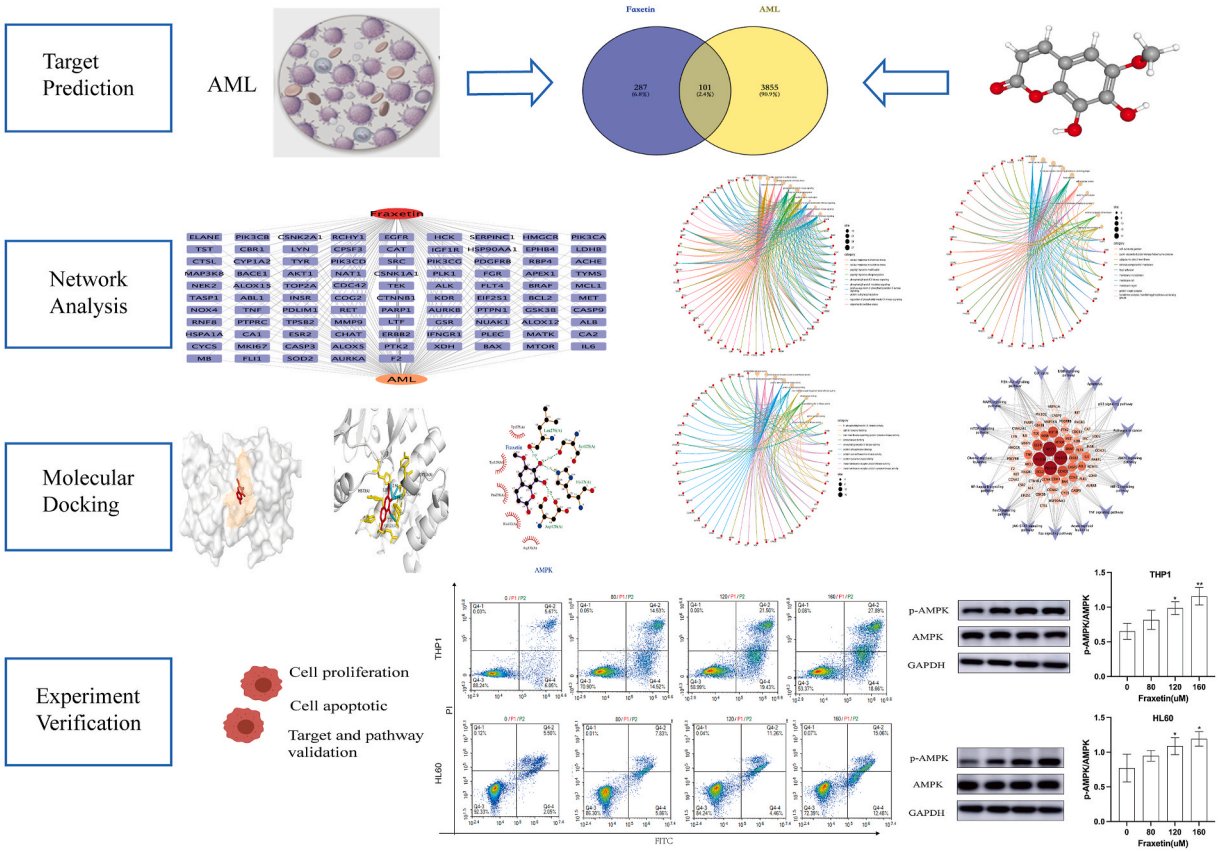

Fig. 1. The flow chart of this study.

1. Introduction

Acute myeloid leukemia (AML) is a highly heterogeneous hematologic malignancy characterized by the destruction of bone marrow differentiation and abnormal proliferation of myeloid precursor cells [1]. AML is a significant form of leukemia and is the most common adult acute leukemia [2]. There is no mention of AML in the ancient Chinese medical records. However, based on the disease's characteristics and clinical manifestations of the disease, it is categorized as"warm disease," Consumptive disease," "Hemorrhage Zheng," and "Phlegm Nucleus" [3]. The general pathogenesis is the mixing of deficiency and excess. Currently, chemotherapy drugs such as cytarabine, arsenic trioxide, and erythromycin are widely used in leukemia treatment, with a 5-year survival rate of AML in adults being only about 30 % [4]. Moreover, chemotherapeutic drugs often lack the specificity of the target, resulting in damage to the normal tissues during the treatment process, which causes a series of adverse reactions and causes great pain to the patients. Meanwhile, chemotherapy resistance is also a significant difficulty in the treatment of leukemia [5–7]. Therefore, it is crucial to evaluate the molecular mechanisms regulating AML and find new therapeutic targets for AML therapy. Therefore, the use of traditional chinese medicine (TCM), which is multi-targeted and has low side effects, has emerged as a promising research direction in treating AML.

Ash Bark comes from the dried branch bark or dried bark of *Fraxinus rhynchophylla Hance, Fraxinus chinensis Roxb, Fraxinus szaboana Lingelsh, or Fraxinus stylosa Lingelsh*. It has the effects of clearing heat and drying dampness, astringency and relieving dysentery, arresting leucorrhea, and improving eyesight. Modern pharmacological studies have proved that Ash Bark has many pharmacological effects, such as anti-inflammatory, anti-oxidative stress, antitumor, and so on [8–13]. *Ash Bark* is mainly composed of esculin, esculetin, fraxetin, fraxin, and so on, which have many biological activities such as anti-inflammation, anti-oxidation, anti-apoptosis, anti-bacteria, and organ protection [8]. Fraxetin, a hydroxycoumarin compound, has a major pharmacological mechanism as an antioxidant, anti-inflammatory, anti-apoptotic, anti-fibrotic, and antimicrobial agent [14–18]. Furthermore, fraxetin has a wide range of biological activities that make it a potential candidate for treating specific disease indications, such as cancer, organ protection, diabetes, and its complications [19–22]. Fraxetin exerts anti-breast cancer pharmacological effects by modulating the expression of the apoptotic protein family and anti-fibrotic pharmacological effects by modulating inflammatory pathways in carbon tetrachloride (CCl4)-induced liver fibrosis [13,16]. In addition, TCM has been used to treat AML in China for more than 2000 years [23]. For example, arsenic trioxide has shown therapeutic effects on AML [24]. Recent studies have shown that resveratrol, salidroside, Shen Qi Sha Bai Decoction, and other TCM have therapeutic effects on AML [25–27]. Based on the potential anti-tumor pharmacological effects

of fraxetin, its potential mechanisms for treating hematological tumors deserve to be studied.

A network pharmacology approach based on bioinformatics, systems biology, and polypharmacology has recently been developed to analyze the complex relationships between biological systems, drugs, and diseases through a network perspective [28]. This new research field is based on the theory of systems biology, the direction of pharmacology, and the research method of constructing the network of "drug-disease-target-pathway," which provides clear ideas and perspectives for the complex research of TCM. The "multi-component, multi-target" research method can meet the demand for comprehensive treatment of multiple complex diseases. Therefore, the network pharmacological method is suitable for studying TCM treatment of AML [29]. In this study, we explore the anti-AML effect of fraxetin and deeply analyze its possible mechanism of action on AML through network pharmacology. The research thinking diagram is shown in Fig. 1.

2. Materials and methods

2.1. Network pharmacology analysis

2.1.1. Access to intersection genes of fraxetin and AML

To gather the targets of fraxetin, we searched the Swisstarget prediction (http://www.swisstargetprediction.ch/index.php), PhammerMap (http://lilab-ecust.cn/pharmmapper/submitfile.html) and CTDBASE (The Comparative Toxicogenomics Database | CTD (ctdbase.org)). AML targets were retrieved by entering "acute myeloid leukemia" in the GeneCard and DisGenet databases. UniProt (https://www.genecards.org/) was used to convert to standardized gene names. The intersecting target genes of fraxetin and AML were obtained through the online venny platform.

2.1.2. PPI network construction and analysis

We entered the intersecting targets obtained above into the STRING database to establish the protein-protein interaction (PPI) network. By setting the target type as "Homo sapiens" and removing the nodes with irrelevant links to obtain the network of interaction, we downloaded the TSV format file. We imported it into Cytoscape 3.8.2 software to visualize and analyze the PPI network.

2.1.3. Go and KEGG analysis

We imported the intersecting genes into the DAVID database (DAVID Functional Annotation Bioinformatics Microarray Analysis (ncifcrf.gov)) [30]. We selected the genus "Homo sapiens" for gene ontology (GO) functional enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis aiming to find the critical signaling pathways. We visualized them using an online platform (https://www.bioinformatics.com.cn)for data analysis and visualization [31].

2.1.4. Construction of the drug-target-autophagy network

The "drug-target-autophagy" network was constructed by Cytoscape.

2.1.5. Module analysis

The MCODE plugin in Cytoscape 3.8.2 was used to find several closely connected proteomes in a large and complex target network and to analyze the biological functions of proteomes.

2.1.6. molecular docking

We downloaded the SDF format of fraxetin (PubChem CID 5273569) from PubChem (https://pubchem.ncbi.nlm.nih.gov) website [32]. We optimized the mechanical structure in ChemBio3D exported the mol2 format file. Download the PDB file of the corresponding target protein from the Protein Data Bank (https://www.rcsb.org/) [33]. Import the target protein into pymol 2.5.0 and run the process of removing ligand molecules and water molecules through the software program, and finally save the protein file as a new PDB format file, then import it into autodock software for hydrogenation and searching for active pockets and export the pdbqt format file of the target, and finally use Autodock_vina to perform the molecular docking with the following grid coordinates: the binding localization points were defined using a grid of 40,40,40 with a spacing of 1.000 Å per grid, and default values were used for other parameters. The lower the score, the higher the affinity. Finally, visualize the results using Ligplot and Pymol.

2.1.7. External validation of key genes

GEPIA (http://gepia2.cancer-pku.cn/) was used to verify the differential expression of key genes between AML patients and healthy individuals [34]. The Kaplan-Meier Plotter (http://kmplot.com/analysis/index.php?p=service) website was used to explore the effect of central targets on AML overall survival (AML) [35]. AML patients were divided into two groups: high expression group and low expression group, and Kaplan-Meier survival plots were used to compare the two groups to evaluate the prognostic significance of key genes.

3. Experimental Verification in vitro

3.1. cell lines, reagents, and antibodies

AML cell lines HL60 and THP1 were obtained from the Central Laboratory of the First Hospital of Lanzhou University. All cell lines

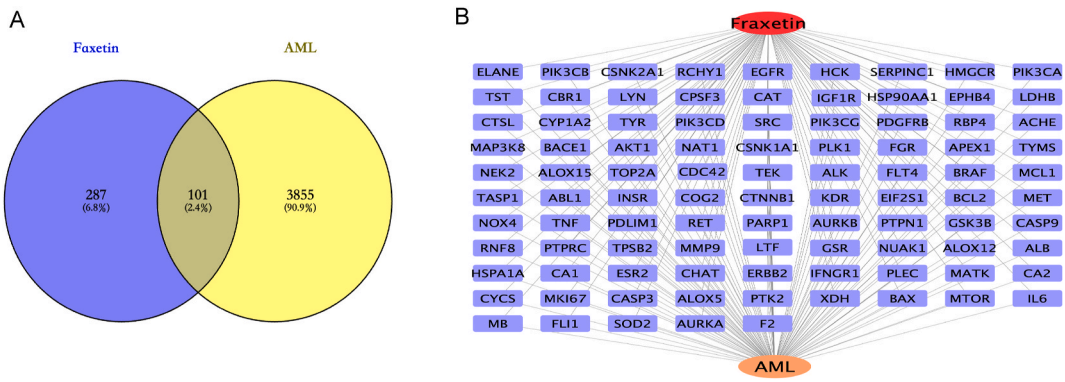

Fig. 2. Venny diagram and CTD network (Nodes represent shared target genes, fraxetin, and AML, and edges represent their interactions).

were grown in RPMI-1640 supplied with 10 % FBS at 37 °C in a humid atmosphere containing 5 % CO2. Fraxetin (MedChemExpress, Product No. HY-N0580, purity >99.96 %, molecular weight 208.17) was ordered commercially. GAPDH (60004-1-Ig, Proteintech, 1:10000) , Rabbit anti-*p*-AMPK (Thr 172) (#2535, Cell Signaling Technology [CST], 1:1000), rabbit anti-AMPK (#5831, CST, 1:1000), Bax (50599-2-Ig, Proteintech, 1:1000) and Bcl2 (68103-1-Ig, Proteintech, 1:1000).

3.2. cell viability assay

To analyze the effect of fraxetin on AML cell proliferation, AML cells were treated with fraxetin (40, 80, 120, 160, 200 μM) and seeded into 96-well plates, placed in an incubator at 37 °C for 24h. Cell viability was measured by the CCK8 cell counting kit (Biosharp, Nanjing, China) according to the manufacturer's instructions. Each sample was allocated in 96-well plates, and CCK8 was added. The absorbance at 450 nm was measured using a multifunctional enzyme marker (Thermo Fisher Scientific).

3.3. Apoptosis assay

THP1 and HL60 cells were treated with fraxetin at concerntrations 80, 120, and 160 μM) for 24 h. Cell apoptosis was assessed using the Annexin V-FITC/PI apoptosis detection kit (Multisciences, Hangzhou, China) according to the manufacturer's instructions. The cells were resuspended and mixed in 500 μl of binding buffer along with 5 μl of annexin V-FITC and 10 μl of PI. After incubation for 5 min, cell apoptosis was detected by flow cytometry (Agilent NovoCyte).

3.4. Western blot analysis

Collected cells were lysed using RIPA buffer (Beyotime) for 30 min at 4 °C to extract the total protein. Then, the protein concentration was determined using the BCA assay kit (EpiZyme, Shanghai, China) . In each Western blot analysis, equal amounts of proteins (20ug) from different groups were separated by 10 % SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, U.S.A.). After being blocking with 5 % non-fat milk for 1 h, the membranes were washed with TBST (tris-buffered saline with 0.05 % Tween 20, pH 7.4) and incubated with the primary antibodies for BAX, Bcl-2, P-AMPK, AMPK, and GAPDH at 4 °C overnight. After three washes with TBST, the membranes were incubated with corresponding secondary antibodies for 1 h at room temperature. Finally, an enhanced chemiluminescence (ECL) solution (Vazyme , Nanjing, China) was applied to detect the protein bands through a chemical imaging system (Amersham Imager 680). Image J software was used for density analysis to determine the relative abundance of protein expression.

3.5. Statistical analysis

The experimental data from this study were presented as mean ± standard deviation (mean ± SD); the *t*-test for independent samples was employed to compare between two groups, while one-way ANOVA was used to compare between multiple groups; all the experimental data were statistically and graphically analyzed by GraphPad Prism 10.0, A significance level of $P < 0.05$ was considered statistically significant.

4. Results

4.1. Screening and collection of fraxetin and AML targets

We obtained a total of 388 fraxetin targets from the Swisstarget prediction, PhammerMap, and CTDBASE databases. Additionally, we acquired 3956 AML targets from the GeneCards and DisGenet databases. By using the venny diagram (Fig. 2A), we identified 101

Table 1
molecular docking binding energies.

Target gene	PDB ID	affinity (kcal/mol)
AKT1	1UNQ	−5.2
BCL2	6GL8	−6.2
CASP3	2DKO	−5.5
CCND1/CDK4	2W96	−6.7
TNF	5UUI	−5.0
PI3K	8EXL	−6.9
mTOR	4DRI	−6.9
IL6	1ALU	−5.1
ERBB2	7PCD	−6.3
EGFR	8A27	−7.5

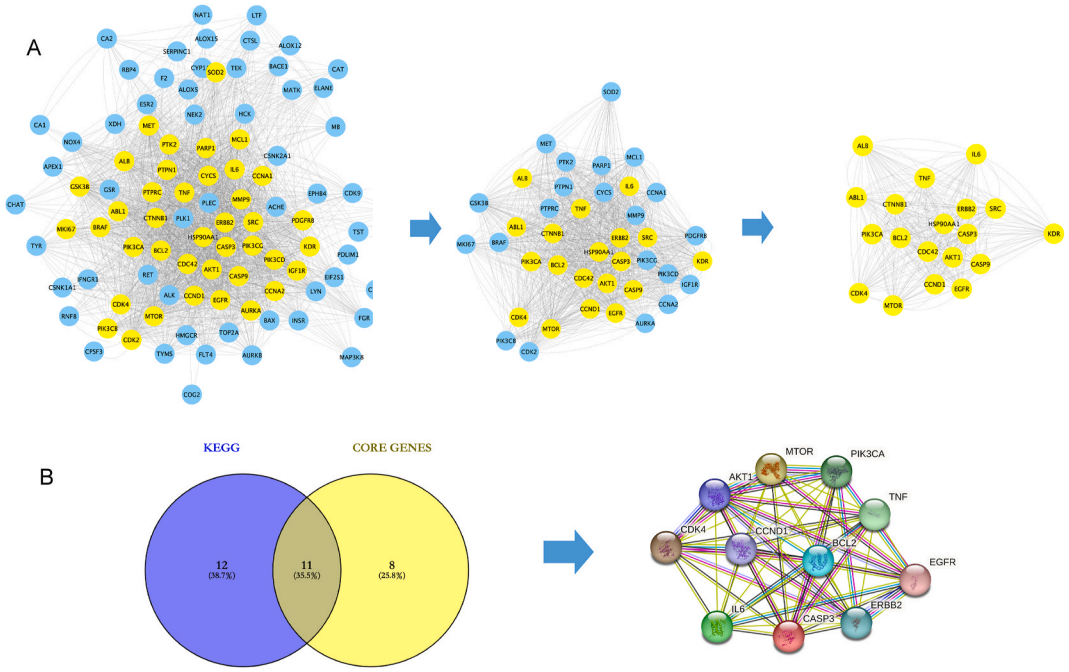

Fig. 3. PPI network (A) PPI network of 101 intersecting genes (B) the core genes (C) the intersecting genes of core genes and KEGG pathway-related genes.

targets in fraxetin that have the potential therapeutic targets for intestinal AML. We constructed a fraxetin-target gene- AML network to visualize and elucidate the pharmacological potential of fraxetin for AML. A total of 101 nodes (purple) represent common target genes, and 2 additional nodes represent fraxetin (represented in red) and AML (represented in orange), as shown in Fig. 2B.

4.2. PPI network analysis of AML intersecting targets

We visualized and analyzed the PPIs of AML intersecting genes using Cytoscape 3.8.2 software. This analysis resulted in a PPI network comprising of 96 nodes and 2284 edges, where the nodes represent the proteins and the lines represent the interactions between them (supplementary material). Then, we filtered out 19 core targets based on the median values of Degree, Betweenness, and Closeness (Table 1). These core target genes play essential roles in the treatment of AML by fraxetin (Fig. 3A).

4.3. GO and KEGG enrichment analysis

To elucidate the multiple biological functions and mechanisms of fraxetin in related to AML, the 101 intersecting genes were imported into the DAVID database for GO and KEGG enrichment analysis. We selected the top 10 BP, CC, and MF for visualization (Fig. 4A). The results showed that the intersecting targets of fraxetin for AML: GO-Biological Process (GO-BP) was mainly enriched in protein autophosphorylation, protein phosphorylation, and, negative regulation of apoptotic processpositive regulation of cell proliferation, GO-Cellular Component (GO-CC) was mainly enriched in the cytoplasm, receptor complex and nucleus, GO-Molecular Function (GO-MF) was enriched primarily on protein serine/threonine/tyrosine kinase activity, protein tyrosine kinase activity and

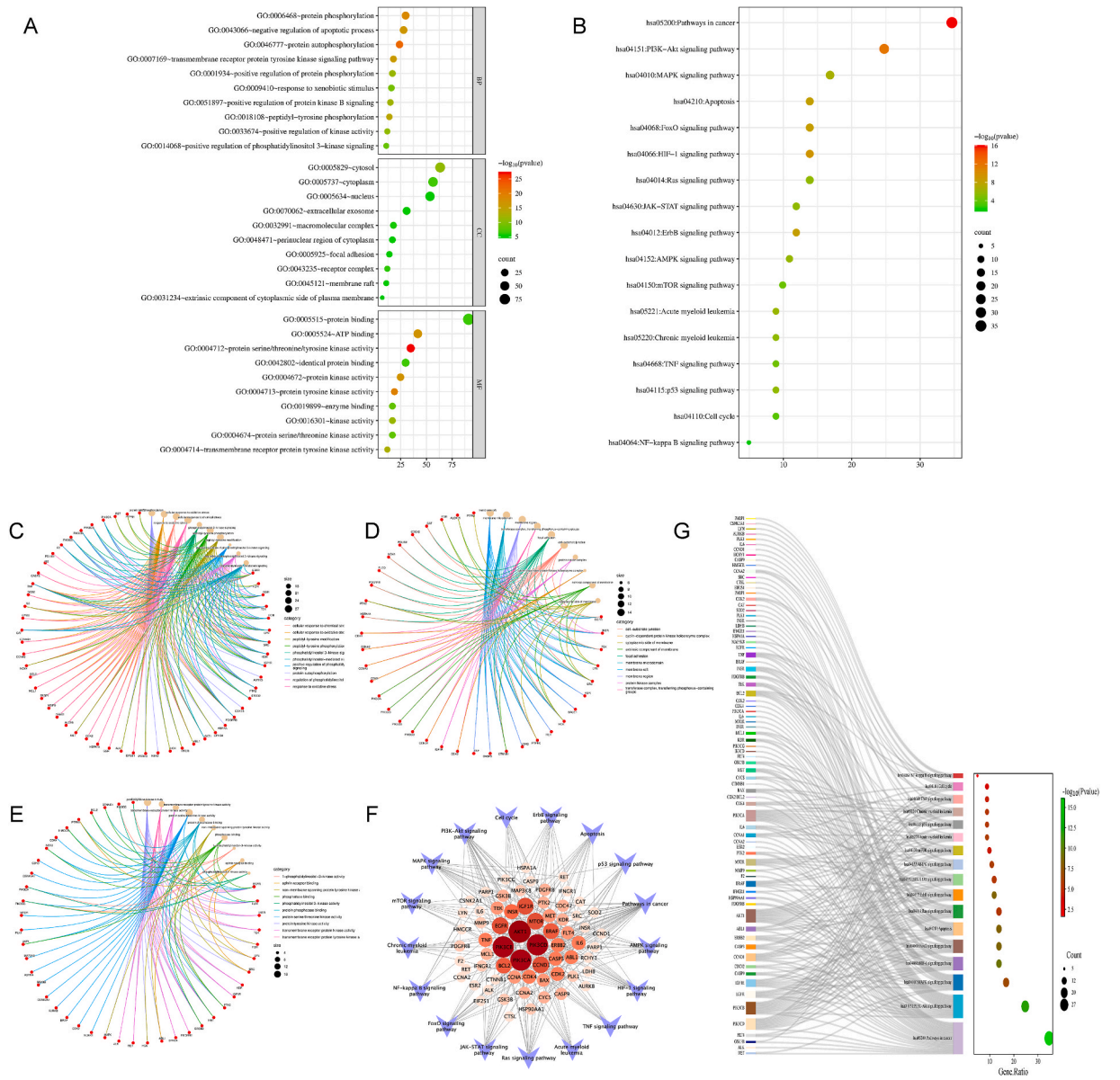

Fig. 4. GO and KEGG analysis (A) GO biological function analysis (B) KEGG pathway enrichment analysis (C) the first ten significantly enriched terms in BP and related genes (D) the first ten significantly enriched terms in the CC pathway and related genes (E) the first ten significantly enriched terms MF pathway and related genes (F) The KEGG pathway and related genes related to AML (G) the Sankey-dots of the KEGG pathway.

protein kinase activity. Meanwhile, as shown in Fig. 4C–E, we further visualized the corresponding relationship between genes and GO analysis. According to KEGG pathway analysis, based on the KEGG pathway analysis, we identified a total of there were 133 KEGG-enriched signaling pathways that were associated with 101 intersecting targets. From these, we further narrowed sown our focus to 17 KEGG signaling pathways that were specifically related to AML (Fig. 4B). Subsequently, we constructed a KEGG target gene network based on these pathways (Fig. 4F–G). The study further analyzed the network relationship between the critical signaling pathways and targets of fraxetin to improve AML. The results demonstrated that there were interconnections between different pathways, and the active components of fraxetin were more likely to exert synergistic effects by interfering with different signaling pathways to achieve the therapeutic effect of AML.

In the AML signaling pathway, we selected 23 relevant targets based on the DC value. We further compared the key targets of fraxetin with the targets related to the AML signaling pathway and obtained 11 core targets: tumor necrosis factor (TNF), interleukin-6 (IL6), (serine/threonine protein kinase B (AKT1), epidermal growth factor receptor (EGFR), recombinant Caspase （CASP3）, B-cell lymphoma-2(BCL2), mammalian target of rapamycin （mTOR）, ERBB2, recombinant Cyclin D1(CCND1), Cyclin-dependent-kinases 4(CDK4), and phosphoinositide 3-kinase (PIK3CA). These targets may play a key role in treating AML with fraxetin, as they occupy a

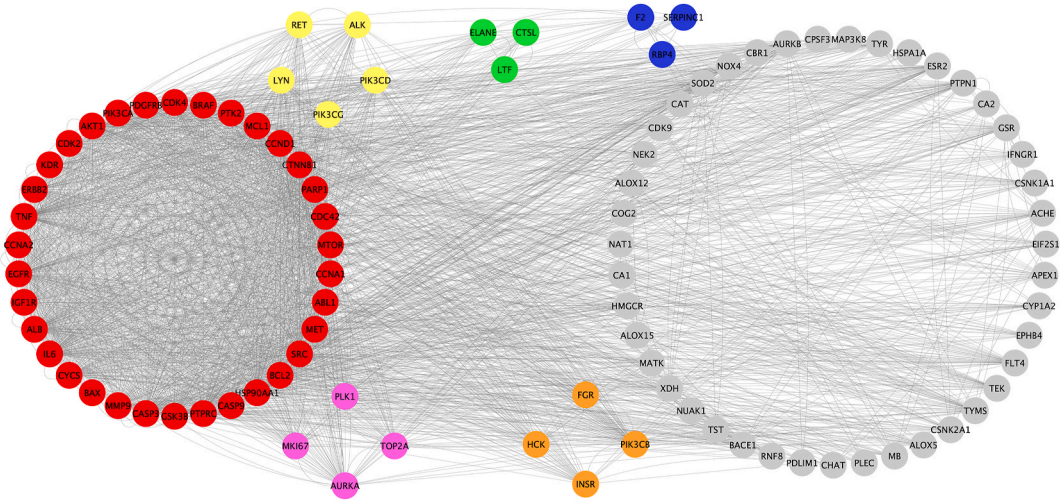

Fig. 5. Module-based network analysis of potential targets for fraxetin (red for Cluster 1, purple for Cluster 2, orange for Cluster 3, yellow for Cluster 4, green for Cluster 5, and blue for Cluster 6).

Table 2
The core target gene of fraxetin in the treatment of AML.

name	Degree	Betweenness	Closeness
AKT1	52	2.84820775	1
IL6	52	2.84820775	1
BCL2	52	2.84820775	1
CTNNB1	52	2.84820775	1
CCND1	52	2.84820775	1
CASP3	52	2.84820775	1
MTOR	52	2.84820775	1
TNF	50	2.46436937	0.96296296
SRC	50	2.31804902	0.96296296
ERBB2	50	2.31804902	0.96296296
EGFR	50	2.4270355	0.96296296
ALB	50	2.46436937	0.96296296
MMP9	50	2.46436937	0.96296296
HSP90AA1	50	1.88137769	0.96296296
GSK3B	48	1.58520544	0.92857143
PARP1	48	1.48411939	0.92857143
CASP9	46	1.05663401	0.89655172
CDK4	44	1.06681097	0.86666667
PIK3CA	44	2.00355065	0.86666667

critical part of the PPI network and play an essential role in the KEGG signaling pathway (Fig. 3B).

4.4. Module analysis

To further explore the potential pharmacological effects of fraxetin in the treatment of AML, we performed a module analysis using the MCODE plug-in in Cytoscape, scoring the proteins in the module according to the degree of association and mapping the cascade of the modules by Cluster Viz in Cytoscape 3.8.2 (Fig. 5). Only cluster 1 had a score of greater than 20 in the MCODE module score, and we performed GO and KEGG analyses on targets in cluster 1 and found that these proteins were significantly correlated with the AMPK signaling pathway, PI3K-Akt signaling pathway, and MTOR signaling pathway.

4.5. molecular docking

To further explore the binding affinity of fraxetin to potential targets, molecular docking of related molecules was performed in this study. According to the previous results, the affinity of the ligand molecule is lower than −5.0 kcal/mol, which indicates that the ligand molecule has a good binding force. The molecular docking results showed that fraxetin had a good binding affinity to the core targets of AML, and all binding free energies were < -5.0 kcal/mol (Table 2). The visual details of the molecular docking are shown in Fig. 6.

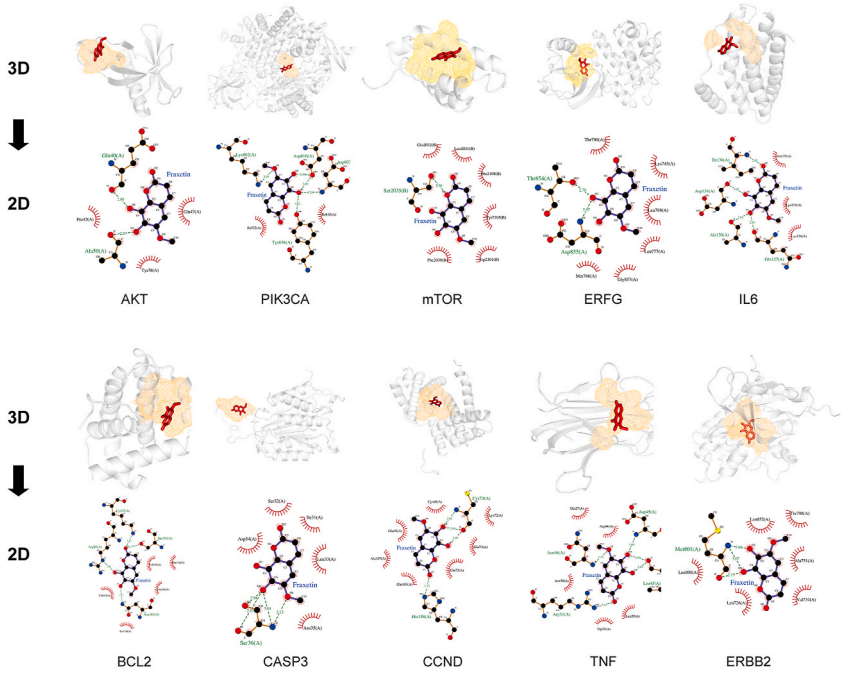

Fig. 6. Molecular docking (The 2D structure diagram shows the semicircular eyelashes as hydrophobic interactions and the green dashed line as hydrogen bonding).

5. Experimental verification

5.1. mRNA expression levels of core genes

The differential expression of key genes between AML patients and healthy individuals was analyzed using the GEPIA database. The mRNA levels of Bcl2 and TNF in AML patients were significantly higher than those in healthy individuals, while the expression level of CCND1/CDK4 and IL6 mRNA was significantly lower than that in healthy individuals ($P < 0.01$) (the results are shown in Fig. 7).

5.2. Survival analysis of core genes

We performed survival analyses for 11 hub genes. The results showed that the poor prognosis of 1608 AML patients in the TCGA database was associated with key genes ($P < 0.05$, Fig. 8).

5.3. Fraxetin inhibited the proliferation of AML cell lines

To examine whether fraxetin could affect the growth of leukemia cells, we treated THP1 and HL60 with fraxetin (40, 80, 120, 160, 200 μM) for 24h, and then cell viability was measured by CCK8 assay. As shown in Fig. 9A, fraxetin exhibited a significant dose-dependent reduction in the viability of AML cell lines. The IC50 values of fraxetin for THP1 and HL60 were 100.6 μM and 108.8 μM, respectively (Fig. 9B). Taken together, these results suggested that fraxetin reduced the viability of AML cell lines.

5.4. Fraxetin induced the apoptosis of AML cell lines

To further investigate whether fraxetin affects the survival of AML cell lines, we first performed a flow cytometry assay. AML cell lines were treated with 80, 120, or 160 μM fraxetin for 24 h, and then cells were stained with AnnexinV- FITC/PI and analyzed using flow cytometry. As shown in Fig. 10A, treatment with fraxetin at concentrations of 80, 120, and 160 μM resulted in significant increase in the apoptosis rate of THP1 and HL60 cells when compared to the contro group.

To further confirm that fraxetin induced apoptosis in THP1 and HL60 cells, we performed Western blotting of apoptosis-related proteins in fraxetin-treated cells. We found that the protein levels of the anti-apoptotic protein Bcl-2 in THP1 and HL60 cells were significantly decreased after fraxetin treatment, respectively, compared with the control group (Fig. 10B), whereas the levels of the pro-apoptotic protein Bax were significantly increased after fraxetin treatment, respectively (Fig. 10B). Taken together, these results strongly suggest that fraxetin promotes apoptosis in AML cell lines.

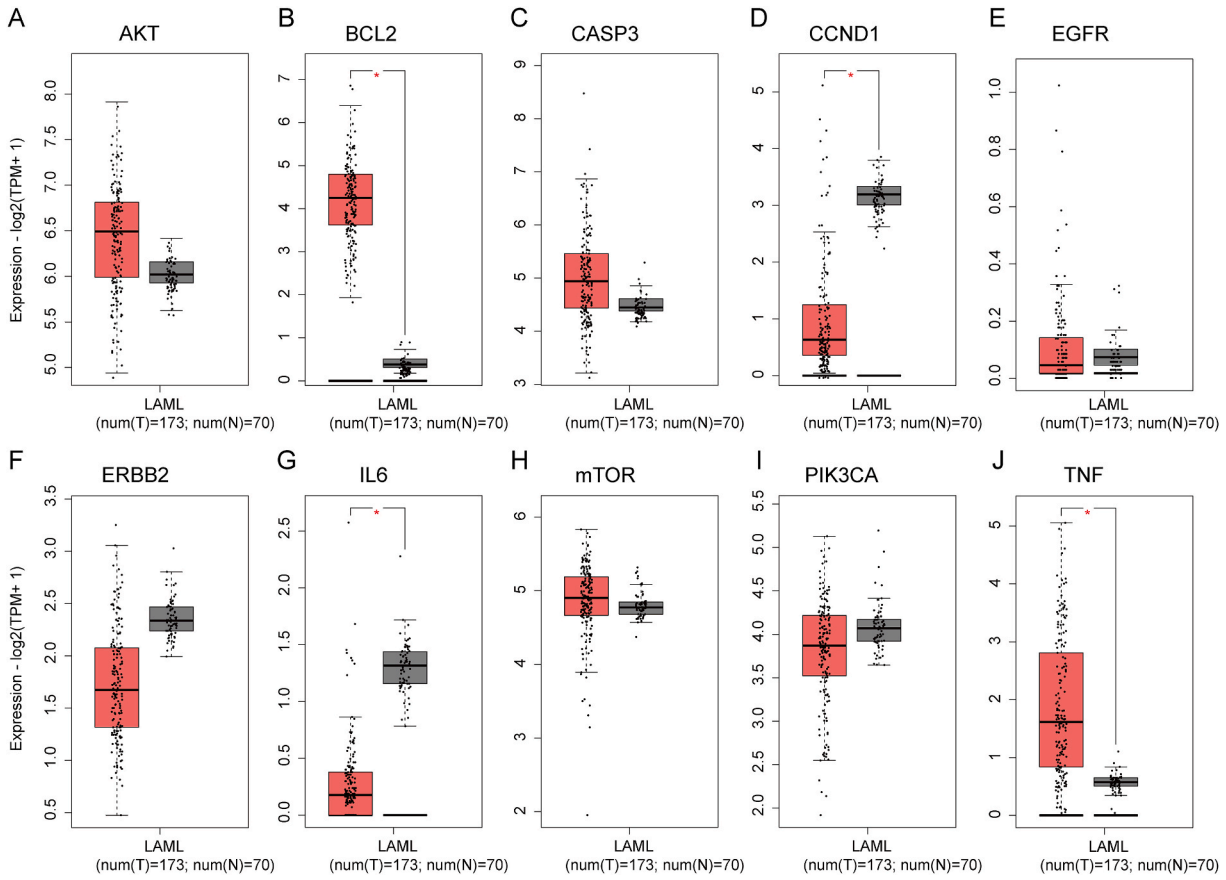

Fig. 7. mRNA expression levels of core genes in The Cancer Genome Atlas (TCGA) and Gene Tissue Expression (GTEx) databases (A-J indicate the expression of AKT, BCL2, CASP3, CCND1, EGFR, ERBB, IL6, mTOR, PIK3CA, TNF in AML, respectively. Gray indicates the normal group and red indicates the AML group).

5.5. Fraxetin activated the AMPK signaling pathway in AML cell lines

The core targets are mainly involved in the cell cycle (CCND1, CDK4), apoptosis (CASP3, Bcl2), inflammatory factors (TNF, IL6), and tumor-related signaling pathways (PIK3CA, AKT1, MTOR). Significantly, the Adenosine 5'-monophosphate (AMP)-activated protein kinas (AMPK) signaling pathway correlates with various aspects involved in the core targets [30–34,61]. We are highly interested in the AMPK signaling pathway; therefore, we will further explore whether fraxetin exerts its therapeutic effect on AML through the AMPK signaling pathway. To confirm the role of the AMPK signal pathway in the treatment of AML, we initially explored the binding energy of fraxetin and AMPK (PDB ID: 2H6D) by molecular docking, and the results showed that the fraxetin had a good binding energy of −5.8 kcal/mol (Fig. 11A). Then, we further confirmed the effects of the AMPK signaling pathway in the therapy of AML by in vitro experiments in which THP1 and HL60 cells were treated without and with fraxetin (80, 120, 160uM) for 24h and then performed a Western blotting experiment. We found that THP1 and HL60 cells treated with fraxetin significantly increased expression levels of *p*-AMPK (Fig. 11B).

6. Discussion

Leukemia is an extremely malignant disease of the hematopoietic system, and its treatment clinically is mostly by chemotherapy. Although most young patients may experience some relief from their symptoms, the overall prognosis is generally poor. Additionally, the chemotherapeutic drugs widely used nowadays are often accompanied by adverse reactions such as vomiting, bleeding, and infections, which bring double harm to the patient's physical and mental health. To overcome drug resistance, new therapeutic methods are needed to improve the effectiveness of established therapeutic agents. Therefore, it is urgently necessary to find new natural products with anticancer properties as a new therapeutic approach to reduce the side effects of chemotherapy [36]. TCM is a traditional medicine in China, which advocates dialectical treatment and overall concepts. It has been found that TCM has a good application in cancer treatment, on the one hand, because of its obvious inhibitory effect on tumor cells. On the other hand, as a natural product with the advantage of low toxicity and side effects, TCM has tremendous research value in the treatment of cancer.

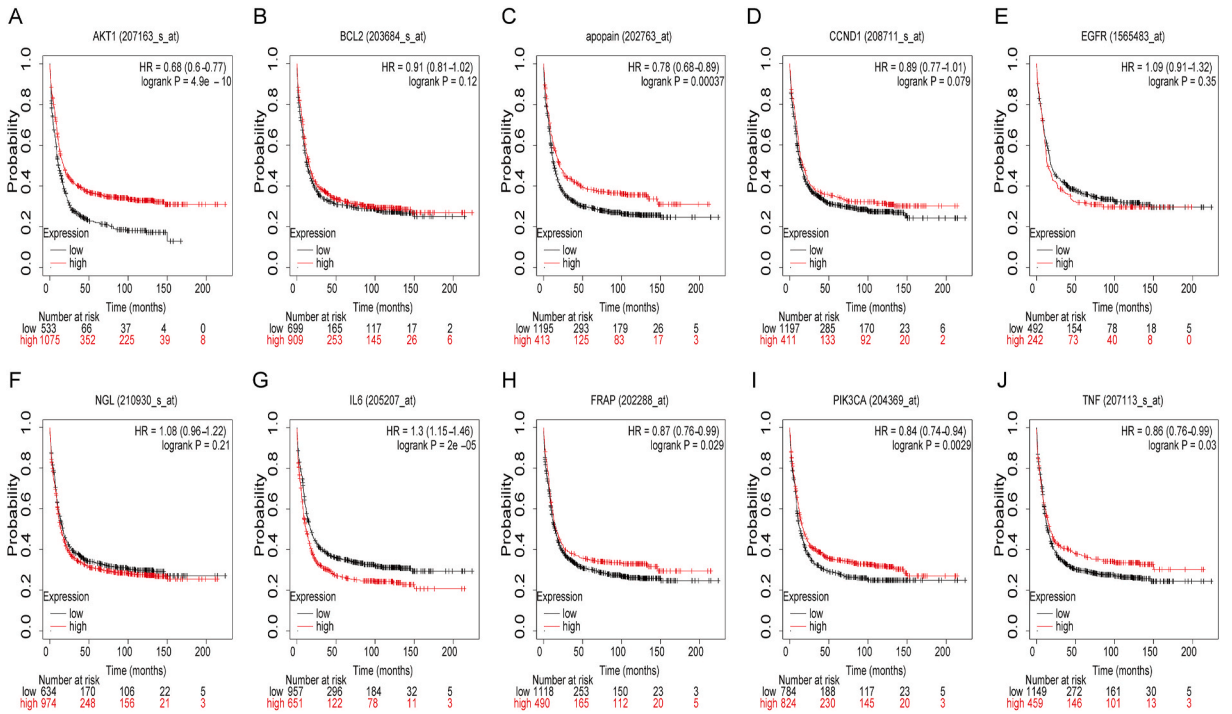

Fig. 8. Kaplan-Meier overall survival analysis (A-J indicate the relationships between Recurrence free survival (RFS) and the expression of AKT, BCL2, CASP3, CCND1, EGFR, ERBB, IL6, mTOR, PIK3CA, TNF in AML patients, respectively.).

Fraxetin has various pharmacological functions, such as antibacterial, antioxidant, neuroprotective, and anti-fibrotic. In non-small cell lung cancer cells, fraxetin plays an anticancer role by inhibiting cell proliferation and blocking the cell cycle [37]. Furthermore, the anticancer effect of Fraxetin has been demonstrated in breast cancer cells by modulating intracellular signaling pathways, including the Bcl2 family [13]. Fraxetin inhibits the proliferation and induces apoptosis in colon cancer, which may be related to the induction of reactive oxygen species (ROS) levels and calcium influx, as well as increased mitochondrial membrane permeability and destruction of endoplasmic reticulum stress (ERS) [38]. Fraxetin also synergizes with 5-FU and irinotecan to inhibit the proliferation of colon cancer cells by regulating the PI3K//serine/threonine protein kinase B (AKT) and mitogen-activated protein kinase (MAPK) signaling pathways. In addition, it enhances the sensitivity of 5-FU-resistant cancer cells [38]. Despite the many studies that have demonstrated the anti-cancer effects of fraxetin, it is not rare to find a study involving leukemia. However, it is difficult to understand the specific mechanism of Chinese herbal medicine's efficacy due to the diversity of its components. Therefore, in this study, we systematically investigated the therapeutic mechanism of fraxetin on AML by combining network pharmacology and in vitro experimental validation, which provided novel and unique insights into the treatment of complex diseases such as AML with TCM and offered new perspectives for potential targeted drug therapy for AML.

The network pharmacology approach is a new model for the research of TCM, which aims to predict drug targets and mechanisms of action by constructing a "Drug-disease-target" network. In this study, we searched AML-related genes by integrating several disease databases through the research method of network pharmacology. Finally, we obtained 101 potential biological targets of fraxetin acting on AML. The top 11 core targets were obtained by dual network topology analysis of intersecting targets and the KEGG signaling pathway. Fraxetin may affect the development of AML by targeting the above molecular targets. GO biofunction enrichment analysis and KEGG signal pathway enrichment analysis were performed on the intersecting targets further to clarify the relevant mechanism of fraxetin regulation of leukemia. The results indicated that 28 out of 101 core genes in GO analysis were associated with the regulation of apoptosis. Additionally, KEGG analysis revealed that the cancer pathway, PI3K/AKT signaling pathway, and AMPK signaling pathway were closely linked to the therapeutic effects of fraxetin on AML. The PI3K/AKT signaling pathway is involved in the whole process of leukemia, including cell proliferation, cell transformation, and extramedullary infiltration, which indicates that the PI3K/AKT signaling pathway is critical in the treatment of leukemia [39–41]. The role of AMPK in cancer is also an active research area. AMPK, a cellular energy sensor ， is a critical factor in regulating cellular energy and metabolism, promotes apoptosis by regulating multiple downstream signaling molecules, and plays a vital role in promoting differentiation in myeloid malignancies [42,43]. Several studies have shown that activation of AMPK can inhibit the proliferation and promote apoptosis of cancer cells, thereby having anticancer potential [44]. In addition, AMPK can regulate energy and lipid metabolism in cancer cells, affecting tumor growth and metastasis [45,46].

Apoptosis is considered to be a "programmed" mode of cell death, which is regulated by different molecular mechanisms; the BCL family plays a crucial role in the process of apoptosis. One of the basic characteristics of human cancer cells is to escape from apoptosis,

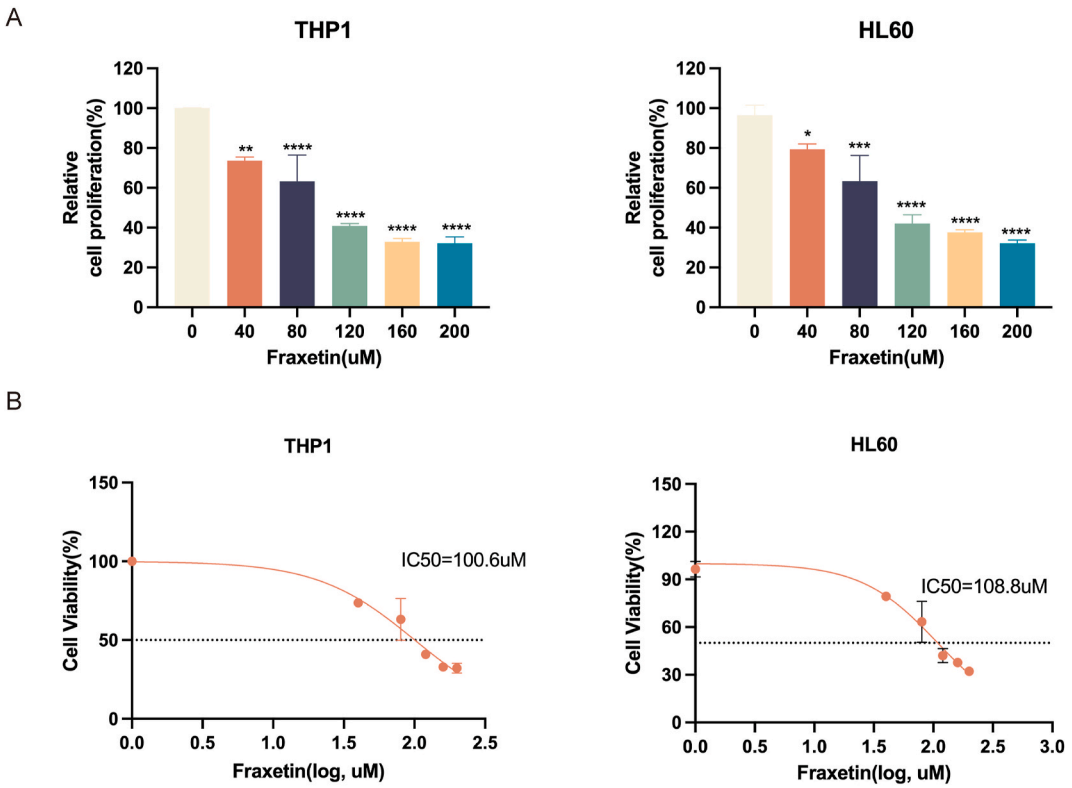

Fig. 9. Fraxetin inhibited the proliferation of THP1 and HL60 cells (A.viability of THP1 and HL60 cells after 24h of fraxetin treatment (n = 3). B. IC50 value was calculated with the GraphPad Prism. Data are presented as the means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. compared with control.).

and the anticancer effect of fraxetin is related to its powerful pro-apoptotic effect. In previous studies on fraxetin, it has been demonstrated that fraxetin plays a pro-apoptotic role by regulating the expression of BCL2 family proteins [14]. The BCL protein family includes pro-apoptotic proteins Bad, Bid, and Bax and anti-apoptotic proteins Bcl-2, Bcl-x, and mcl-1. Bcl2 play a role in apoptosis regulation by mediating the release of cytochrome C from the mitochondria to the cytoplasm, which activates downstream inactive caspase 9 and activates caspase 3 and polyadenine diphosphate ribose polymerase, which subsequently induces apoptosis [47]. Lee et al. showed that the concentration-dependent inhibition of fraxetin on colon cancer cell lines (HCT116 and HT29) significantly increased the expression of the pro-apoptotic protein Bax, suggesting that fraxetin can induce mitochondrial-mediated apoptosis in colon cancer [38]. In addition, fraxetin exerts an anti-glioma effect by regulating the expression of Bcl2 family proteins and increasing the expression of caspase 3 [48]. However, the anti-tumor effect of fraxetin is mainly focused on solid tumors and has not been found in leukemia. The literature has shown that the Bcl2 level in AML patients is significantly higher than that in the control group and is closely related to the prognosis of patients [49]. In addition, Bcl2 is a critical regulatory protein in the process of apoptosis, differentiation, and autophagy of AML cells. In this study, fraxetin treatment significantly increased the apoptosis rate of THP1 and HL60 cells compared to the control group. Furthermore, Western blotting analysis showed that fraxetin treatment resulted in decreased expression of Bcl2 and increased expression of Bax in THP1 and HL60 cells. Therefore, these results suggested that fraxetin treatment enhanced THP1 and HL60 cells' apoptosis due to the activation of mitochondrial apoptotic pathways.

Apoptosis is regulated by intracellular energy metabolism, and AMPK serves as the cellular energy receptor that regulates cellular energy metabolism. AMPK is composed of three subunits (αβγ), and the N-terminal of α-subunit contains a conserved Ser/Thr kinase region. Phosphorylation of the threonine (Thr-172) site is required for its kinase activity. A state of cellular hypoxia may lead to the activation of AMPK. Tumors are often accompanied by disturbed energy metabolism and inhibition of AMPK activation. Therefore, AMPK is considered as a potential target for cancer treatment [50]. In cancer cells, resveratrol-induced AMPK activation is closely associated with apoptosis, and the mechanism may be due to the inhibition of glucose uptake by AMPK activation in cancer cells [51] with resveratrol (200 μM) treatment of ovarian cancer cells A2780 and SKOV3, which promoted apoptosis and significantly up-regulated phosphorylation AMPK and Caspase 3 expression levels. Further blocking of AMPK activity with an AMPK inhibitor significantly inhibited resveratrol-induced apoptosis and reversed *p*-AMPK levels [51]. It has been shown that small molecule drug-induced apoptosis in AML cells is associated with sustained activation of AMPK, leading to mitochondrial respiratory dysfunction, and may also be related to the pathways of unfolded protein response (UPR) and the protein kinase R-like ER kinase (PERK) [52]. Solasonine inhibited the progression of acute monocytic leukemia in vitro and in vivo by upregulating the AMPK/FOXO2A pathway [53]. In vitro and in vivo studies have found that activation of *p*-AMPK and inhibition of MTOR activity resulted in dephosphorylation

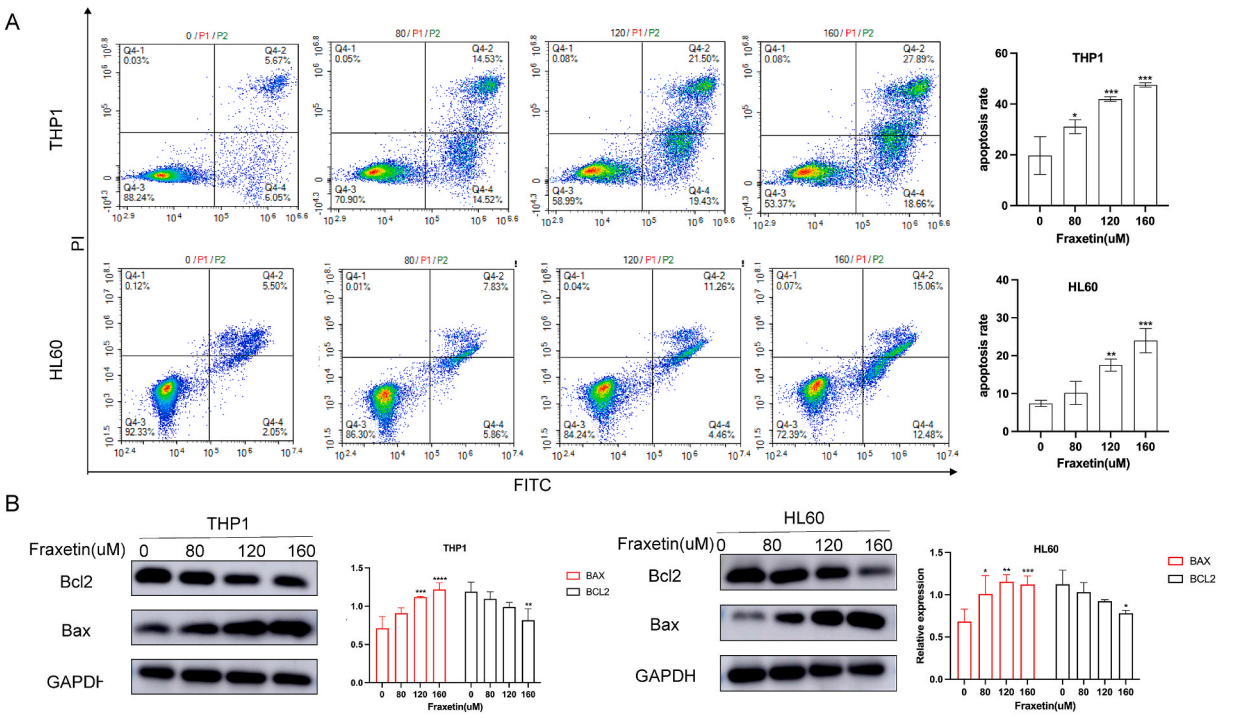

Fig. 10. Fraxetin induced the apoptosis of AML cells (A) THP1 and HL60 cells were treated with fraxetin (0, 80, 120, 160 μM) for 24 h, apoptosis rates were analyzed using Annexin V-FITC/PI flow cytometry. (B) Cells were treated with fraxetin (0, 80, 120, 160 μM) for 24 h, and the expression levels of apoptosis-related proteins Bax and Bcl-2 were analyzed by western blotting (n = 3). GAPDH was considered as an internal control. Data are presented as the mean ± SD. *p < 0.05, **p < 0.01 and ***p < 0.001.

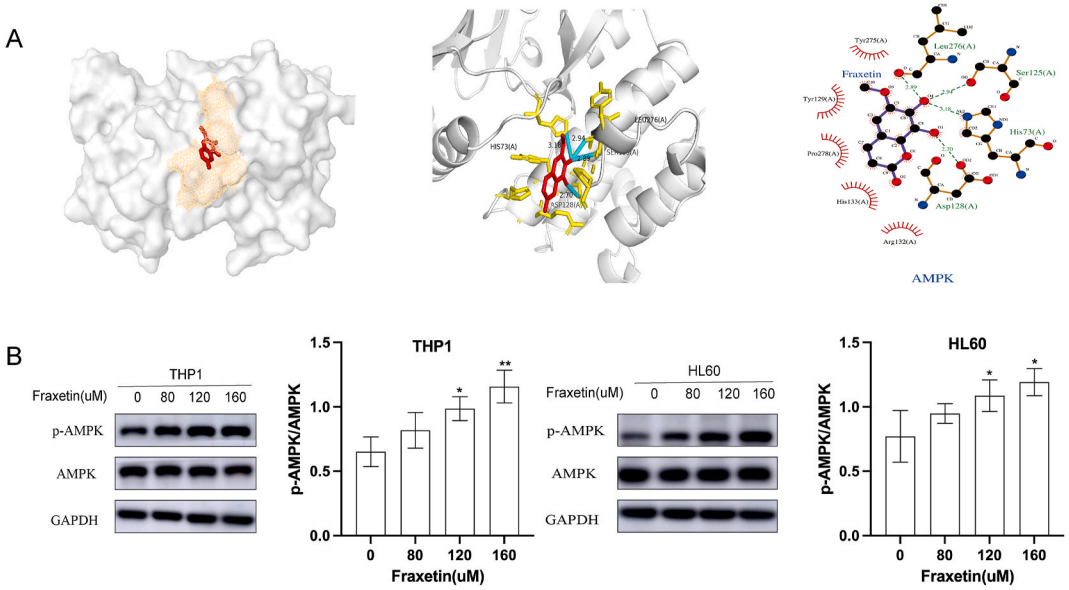

Fig. 11. Fraxetin induces AMPK signaling pathway. (A) Molecular docking of fraxetin and AMPK. (B) THP1 and HL60 cells were treated with different concentrations of fraxetin (0, 80, 120, 160 μM) for 24 h. The phosphorylation and total protein levels of *p*-AMPK and AMPK were analyzed by western blotting (n = 3). GAPDH was considered as an internal control. Data are presented as the mean ± SD. *p < 0.05 and **p < 0.01.

of the translation initiation factor 4E-BP1 to produce effects against AML but had no effect on normal myeloid hematopoietic stem cell function [54]. In our study, we found that intervention with fraxetin promoted *p*-AMPK expression with a dose-dependent pattern in THP1 and HL60 cell lines. This suggeststing that fraxetin effectively inhibitsed proliferation and promotesd apoptosis in AML cell lines.

Furthermore, it was found and that this mechanism is associated with the AMPK signaling pathway. In addition, the AMPK signaling pathway can affect glycolysis and proliferation of lymphocytic leukemia cells. In this way, activation of AMPK downregulates mTORC1 to inhibit glycolysis and induce mitochondria oxidative metabolism, thereby limiting the progression of leukemia [55]. We will follow up with further in-depth exploration of other mechanisms of fraxetin in treating AML by regulating the AMPK signaling pathway.

In summary, the present study combined network pharmacology and in vitro experimental validation to predict the effective mechanism and main targets of fraxetin in the treatment of AML and verified the predicted results by molecular docking and in vitro experiments, suggesting that fraxetin can act on multiple targets and signaling pathways at the same time to produce synergistic effects. It is an effective means of treating AML. Our study suggests that fraxetin may exert its anti-AML effect by regulating the expression of AMPK, which plays a vital role in cell proliferation and apoptosis. Therefore, we can conclude that fraxetin may be an effective therapeutic agent for AML, but further experiments are still needed to explore its mechanism.

Data availability statement

All required data are available from the corresponding author.

Funding

None.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

CRediT authorship contribution statement

Yihong Chai: Writing – original draft, Formal analysis. **Xiaohong Sun:** Writing – original draft, Software, Data curation. **Qi Zhou:** Visualization. **Hongxing Li:** Validation. **Yaming Xi:** Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors are especially grateful to the Center for Medical Frontier Innovation Research Center, The First Hospital of Lanzhou University for providing us with the experimental platform and guidance on experimental techniques.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e34717.

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