# Functional analysis of the effect of isoimperatorin on human acute monocytic leukemia at the transcriptome level

RUNYANG YIN[1,2], RUI XU[2], HUI MING[2] and JUNTAO SONG[1,2]

[1]Department of Anesthesiology, The Affiliated Hospital of Inner Mongolia Medical University, Hohhot, Inner Mongolia Autonomous Region 010050, P.R. China; [2]Department of Oncology Hematology, Zibo 148 Hospital, Zibo, Shandong 255399, P.R. China

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**Abstract.** Acute myeloid leukemia (AML), which is characterized by the aberrant proliferation of primitive and immature myelocytes within the bone marrow, represents the most prevalent subtype of leukemia in both adults and children. Isoimperatorin, a derivative of coumarins, has been reported to possess antitumor, antioxidative and anti-inflammatory activities. In the present study, the effects of isoimperatorin on human AML cells were evaluated. Tohoku Hospital Pediatrics-1 (THP-1) and AML-193 cells were treated with or without isoimperatorin. Subsequently, induction of apoptosis was detected by flow cytometry, whereas cell proliferation was assessed using Cell Counting Kit-8. Furthermore, the potential transcriptional regulatory effects of isoimperatorin on THP-1 cells were investigated using RNA sequencing and reverse transcription-quantitative PCR (RT-qPCR). Isoimperatorin significantly induced apoptosis and inhibited cell proliferation in the AML cells. Furthermore, transcriptome sequencing analysis demonstrated 688 differentially expressed genes between isoimperatorin-treated THP-1 cells and untreated controls. Gene Ontology annotation enrichment analysis, Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis and Gene Set Enrichment Analysis indicated that isoimperatorin altered the expression genes associated with 'cell migration' and 'cell motility'. The compound primarily regulated signaling pathways, including the 'calcium signaling pathway', 'microRNA in cancer' and 'MAPK signaling pathway', while influencing biological processes related to 'cancer biology', 'metabolic pathways' and 'immune responses'. These findings were verified using RT-qPCR. Collectively, the present results elucidated the potential effects of isoimperatorin on AML and provided a theoretical foundation for the clinical treatment of AML.

*Correspondence to:* Dr Juntao Song, Department of Oncology Hematology, Zibo 148 Hospital, 20 Zhanbei Road, Zhoucun, Zibo, Shandong 255399, P.R. China
E-mail: songjuntao002@163.com

*Abbreviations:* AML, acute myeloid leukemia; DEG, differentially expressed gene; GSEA, Gene Set Enrichment Analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; PCA, principal component analysis; THP-1, Tohoku Hospital Pediatrics-1

*Key words:* isoimperatorin, acute monocytic leukemia, apoptosis, transcriptome

## Introduction

In clinical practice, leukemia can be primarily classified into four main types: Acute myeloid leukemia (AML), acute lymphoblastic leukemia, chronic lymphocytic leukemia and chronic myeloid leukemia (1,2). Among them, AML represents the most prevalent subtype in adults (2), and is characterized by the unregulated proliferation of immature mononuclear cells in the peripheral blood, bone marrow and/or other tissues (3). Annually, AML impacts ~1 million individuals globally and results in >100,000 mortalities worldwide, posing a serious threat to human health (4). The clinical manifestations of AML are often severe, with the disease progressing rapidly and the prognosis being poor. There exist substantial disparities in the cure rates among patients with AML of different ages. For instance, the cure rate is ~35% in individuals <60 years of age, while it is only ~10% in those >60 years old. The prognosis of AML is poor; the 3-year disease-free survival and overall survival rates are 26 and 31% respectively, underscoring the challenges associated with the management and treatment of this hematological malignancy (5). In the absence of treatment, a patient's life expectancy may be measured in months or, in certain cases, even weeks (6).

AML predominantly affects the elderly population, and the incidence is higher in men than in women (2). Recent studies using novel single-cell techniques have unveiled the intricate scenario of the co-existence and evolution of multiple drug-resistant subclones during the treatment of AML. This complexity significantly impedes the success of targeted therapies (7-9). Consequently, novel therapeutic agents, such as tyrosine kinase inhibitors (trial nos. NCT06734585, NCT03934372), menin inhibitors (e.g. NCT04988555, NCT06222580) and monoclonal antibodies (e.g. NCT02724163, NCT06892223), are currently being investigated in clinical trials. The underlying objective is to precisely modulate the signaling pathways and crucial molecules within tumor

cells (10-13). Numerous studies have reported that cytogenetic or molecular alterations in AML can exert a profound impact on gene expression. This perturbation leads to the generation of abnormal oncoproteins, tumor suppressors, transcription factors, signaling molecules and metabolites (14-17). In turn, these abnormal molecules are capable of promoting cancer cell proliferation and regulating cell cycle progression, apoptosis and differentiation, eventually culminating in the onset of leukemia (18). Further understanding of the molecular pathogenesis of AML is necessary to support the development of new drugs for its prevention or treatment.

Natural phytochemicals such as coumarin derivatives are regarded as a key source for the development of preventive and therapeutic strategies against various cancer types, including hematopoietic malignancies (19). Isoimperatorin serves as the principal active coumarin component in the roots of the plant species, *Angelica dahurica*. The compound exhibits a diverse range of biological and pharmacological activities, encompassing antioxidant, anti-inflammatory, neuroprotective and anticancer properties (20). It has been reported that isoimperatorin reduces the expression and binding of c-Myc and sirtuin 1, thereby inducing apoptosis in hepatocellular carcinoma cells (21). Furthermore, isoimperatorin attenuates the epithelial-mesenchymal transition process by modulating the NF-κB signaling pathway and CXCR4 expression in colorectal and hepatocellular carcinomas (22). However, to the best of our knowledge, no reports have elucidated the effects and underlying mechanisms of isoimperatorin treatment in hematopoietic malignancies.

The present study aimed to explore the potential regulatory mechanisms underlying the effects of isoimperatorin on AML cells. The primary objectives were to detect the functions of isoimperatorin and to uncover the key signaling pathways associated with isoimperatorin treatment in AML cells. To accomplish these goals, the current study adopted a multi-faceted approach involving *in vitro* apoptosis and cell proliferation assays, along with RNA sequencing (RNAseq) analysis. By integrating these methods, the present study intended to provide a comprehensive understanding of the effects of isoimperatorin on AML, and to lay a foundation for the development of novel therapeutic targets for the treatment of AML.

## Materials and methods

*Materials*. Roswell Park Memorial Institute 1640 (RPMI-1640) medium (cat. no. PM150110), Iscove's Modified Dulbecco Medium (IMDM; cat. no. PM150510) and fetal bovine serum (FBS; cat. no. 164210) were purchased from Wuhan Pricella Biotechnology Co., Ltd.Isoimperatorin (cat. no. HY-N0286) was purchased from MedChemExpress. For the experiments, isoimperatorin was dissolved in DMSO (5 mM) and further diluted to 100 $\mu$M in RPMI-1640 medium for storage. Cell Counting Kit-8 (CCK-8; cat. no. P-CA-001), TRIzol™ (cat. no. 15596018), RevertAid First Strand cDNA Synthesis Kit (cat. no. K1622) and PowerTrack™ SYBR-Green Master Mix (cat. no. A46012) were purchased from Thermo Fisher Scientific, Inc.

*Cell culture*. The human Tohoku Hospital Pediatrics-1 (THP-1; cat. no. TIB-202) and AML-193 (cat. no. CRL-9589) cell lines were purchased from the American Type Culture Collection. THP-1 cells were cultured in RPMI-1640 medium, while AML-193 cells were cultured in IMDM. Both media were supplemented with 10% FBS, 100 U/ml penicillin and 100 $\mu$g/ml streptomycin. Cell cultures were maintained under standard conditions at 37˚C with 5% $CO_2$.

*Half-maximal inhibitory concentration ($IC_{50}$) detection*. THP-1 and AML-193 cells were seeded into 96-well plates at a density of $5x10^3$ cells per well. The cells were then cultured overnight to allow for cell adherence. Subsequently, the cells were treated with 5, 10, 20, 40, 80, 160 and 320 $\mu$M isoimperatorin for 24 h. In parallel, a control group was set up, where an equivalent volume of DMSO was added. After incubation, 10 $\mu$l CCK-8 solution was added to each well, and the plates were then incubated for an additional 2 h. Subsequently, a spectrophotometer (Synergy HT; BioTek; Agilent Technologies, Inc.) was used measure the absorbance value at 450 nm, which was then used to calculate the $IC_{50}$ values using nonlinear regression analysis performed on the GraphPad Prism (version 7; Dotmatics) software.

*Apoptosis analysis*. THP-1 and AML-193 cells were seeded into 12-well plates at a density of $1x10^5$ cells per well. The cells were then incubated overnight to allow for cell adherence. Based on the $IC_{50}$ values for the two cell lines, THP-1 cells were treated with 12.5, 25 and 50 $\mu$M isoimperatorin, while AML-193 cells were exposed to 15, 30 and 60 $\mu$M isoimperatorin. All treatments were performed at 37˚C in a humidified incubator with 5% $CO_2$ for 24 h. In parallel, a control group was set up, where an equivalent volume of DMSO was added. After the treatment, the cells were carefully collected and then subjected to centrifugation at 300 x g for 5 min at 4˚C to pellet the cells. Subsequently, apoptosis detection was performed using a fluorescein isothiocyanate annexin V/PI kit (cat. no. 556547, Becton, Dickinson and Company) according to the manufacturer's instructions. Within 1 h of staining, the samples were analyzed using a flow cytometer (BD FACSCelesta™; BD Biosciences). The acquired data were processed and analyzed using FlowJo™ (version 10.8.1; BD Biosciences) software. The percentages of early apoptotic cells (characterized by being annexin $V^+$ and $PI^-$) and late apoptotic cells (characterized by being annexin $V^+$ and $PI^+$) were next calculated.

*Cell proliferation assay*. Cell proliferation assay was conducted using the CCK-8 assay according to the manufacturer's protocol. Briefly, THP-1 and AML-193 cells were seeded into 96-well plates at a density of $5x10^3$ cells per well. The cells were cultured overnight to allow for cell adherence at 37˚C with 5% $CO_2$. Subsequently, THP-1 cells were treated with 6.25, 12.5 and 25 $\mu$M isoimperatorin, while AML-193 cells were exposed to 7.5, 15 and 30 $\mu$M isoimperatorin. Cells were incubated at 37˚C for 0, 24, 48, 72 and 96 h to observe the time-dependent effects of isoimperatorin on cell proliferation. Subsequently, 10 $\mu$l CCK-8 solution was added to each well. The plates were then incubated at 37˚C with 5% $CO_2$ for an additional 2 h. Finally, a spectrophotometer (Synergy HT; BioTek; Agilent Technologies, Inc.) was utilized to measure the absorbance values at 450 nm, which was then used to assess the cell proliferation status, since such absorbance values are directly proportional to the number of viable cells.

*Total RNA extraction, library construction and sequencing.* A total of $1x10^7$ THP-1 cells were initially seeded in a 6-cm cell culture dish and cultured overnight. Taking into account the apoptosis experiment results, to investigate signaling pathways without eliciting excessive apoptosis, 12.5 $\mu$M isoimperatorin was added to each culture plate and the cells were further incubated for 24 h. Meanwhile, the control cells were treated with an equivalent volume of DMSO. Subsequently, total RNA was isolated from both the isoimperatorin-treated and non-treated THP-1 cells using TRIzol reagent (Thermo Fisher Scientific, Inc.), following the manufacturer's instructions. Next, a NanoDrop 2000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.) was utilized to determine the quality and concentration of the extracted total RNA. Purified total RNA from three replicate samples of both solvent-controlled and isoimperatorin-treated THP-1 cells was then used to prepare an mRNAseq library using the Illumina Stranded Total RNA Prep in combination with Ribo-Zero Plus rRNA Depletion kit (cat. no. 20040529, Illumina, Inc.). Ribosomal RNAs were eliminated through a poly-(A)-containing mRNA selection process, in order to reduce their presence in the subsequent sequencing results. Following ribosomal RNA removal, the remaining mRNAs underwent a series of procedures, including cDNA strand synthesis, purification, end-repair, A-tailing adaptor ligation and PCR amplification. The quality of the constructed libraries was analyzed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc.) and the library concentrations were determined through qPCR analysis as described below. Subsequently, each library was adjusted to a concentration of 20 pM and then sequenced using an Illumina NextSeq500 instrument (Illumina, Inc.) equipped with NextSeq System Suite software (version 2.2.0; Illumina, Inc.). Finally, paired-end reads with a length of 150 bp were acquired using the NextSeq 550 System High-Output Sequencing Kit (Illumina, Inc.).

*Functional and signaling pathway enrichment analysis of differentially expressed genes (DEGs).* Differential gene expression analysis and Principal Component Analysis (PCA) were conducted using the DESeq package (https://bioconductor.org/packages//2.10/bioc/html/DESeq.html) within the R environment (version 4.4.2; https://www.r-project.org/; Posit Software, PBC). The resultant data were then visualized using the FactoMineR package (https://cran.r-project.org/web/packages/FactoMineR/index.html). Genes were classified as differentially expressed when |log2 fold-change| >1 and P<0.05 (adjusted P-value following false discovery rate correction). Subsequently, a two-way cluster analysis on all the identified DEGs was performed using the pheatmap package (https://cran.r-project.org/web/packages/pheatmap/index.html). Furthermore, volcano plots were generated using the ggplot2 package (https://cran.r-project.org/web/packages/ggplot2/index.html) to visually display the differential gene expression data. For gene expression data analysis, the Database for Annotation, Visualization and Integrated Discovery (https://davidbioinformatics.nih.gov/) software was employed. The DEGs underwent Gene Ontology (GO) enrichment analysis (http://www.geneontology.org/) to identify significantly enriched biological processes, cellular components and molecular functions. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.genome.jp/kegg/) was utilized to explore the associated signaling pathways. Additionally, Gene Set Enrichment Analysis (GSEA) was carried out with GSEA software (https://www.broadinstitute.org/gsea/).

*RT-qPCR.* A total of $1x10^7$ THP-1 and AML-193 cells were seeded into 6-cm cell culture dishes and cultured overnight to allow for cell adherence. Subsequently, 12.5 and 15 $\mu$M isoimperatorin was added to THP-1 and AML-193 cells, respectively, and further cultured for 24 h. Control cells were treated with an equal volume of DMSO. Total RNA was extracted from both isoimperatorin-treated and non-treated cells using TRIzol reagent, according to the manufacturer's instructions. Specifically, 200 $\mu$l chloroform was added to each sample to fully dissociate the nucleoprotein complex. Subsequently, the samples were centrifuged at 12,000 x g for 15 min at 4˚C. The upper aqueous phase was then carefully transferred to a new microcentrifuge tube and mixed with 500 $\mu$l isopropyl alcohol to precipitate the RNA. After centrifugation at 12,000 x g for 10 min at 4˚C, the resulting RNA pellet was washed twice with 1 ml 75% ethanol. Once air-dried, the RNA samples were resuspended in 100 $\mu$l diethyl pyrocarbonate-treated deionized water. The concentrations of the resuspended RNA samples were measured using a microspectrophotometer (NanoDrop 2000; NanoDrop Technologies; Thermo Fisher Scientific, Inc.). For RT, first-strand cDNA was synthesized following the manufacturer's instructions of the RevertAid First Strand cDNA Synthesis Kit (cat. no. K1622; Thermo Fisher Scientific, Inc.). Subsequently, qPCR was performed on a Bio-Rad CFX-Touch Real-Time PCR System (Bio-Rad Laboratories, Inc.) using the PowerTrack™ SYBR-Green Master Mix (cat. no. A46012; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The thermocycling conditions were as follows: cDNA pre-denaturation, 95˚C for 30 sec; cDNA denaturation, 95˚C for 10 sec; and primer annealing and new strand extension, 60˚C for 30 sec. The denaturation, annealing and extension steps were repeated for a total of 40 cycles. For each pathway, two most significantly up- and downregulated genes were selected to verify the reliability of RNAseq results. *GAPDH* was selected as a reference gene and quantification of gene expression was performed using the $2^{-\Delta\Delta Cq}$ method (23). The primer sequences are shown in Table SI.

*Statistical analysis.* Data are presented as the mean ± SD of three independent experiments. Statistical analysis was performed using GraphPad Prism (version 7; Dotmatics) software. Statistical significance was evaluated using a one- or two-way analysis of variance with Tukey's post hoc test for multiple comparisons, or unpaired two-tailed Student's t-test for comparisons between two groups. P<0.05 was considered to indicate a statistically significant difference.

## Results

*Isoimperatorin treatment significantly promotes apoptosis and suppresses proliferation in AML cells.* Fig. S1A depicts the chemical structure of isoimperatorin. The $IC_{50}$ values for isoimperatorin-treated THP-1 and AML-193 cells were 51.93 $\mu$M (Fig. S1B) and 67.55 $\mu$M (Fig. S1C), respectively. To explore the impact of isoimperatorin on tumor biological progression

in AML, THP-1 cells were incubated with 12.5, 25 and 50 $\mu$M isoimperatorin for 24 h. Subsequently, induction of apoptosis was analyzed using flow cytometry. A concentration of 12.5 $\mu$M isoimperatorin induced a statistically significant increase in both early-stage and late-stage apoptosis within THP-1 cells when compared with control cells (Fig. 1A). Within the 25 and 50 $\mu$M groups, the proportions of cells undergoing early and late apoptosis were also significantly increased compared with the control group, which demonstrated that isoimperatorin induced apoptosis in THP-1 cells in a dose-dependent manner (Fig. 1A-C). Given that 50 $\mu$M isoimperatorin induced substantial apoptosis within 24 h, lower concentrations of isoimperatorin, specifically 6.25, 12.5 and 25 $\mu$M, were selected for cell proliferation assays on THP-1 cells. Treatment with isoimperatorin significantly inhibited cell proliferation in a dose-dependent manner; 25 $\mu$M isoimperatorin led to a significant reduction in the proliferation of THP-1 cells following 24, 48, 72 and 96 h of incubation compared with control group (Fig. 1D). Furthermore, in AML-193 cells, isoimperatorin induced significant apoptosis at both early and late apoptotic stages at concentrations of 15, 30 and 60 $\mu$M (Fig. 1E-G). In cell proliferation assays with further reduced concentrations, isoimperatorin significantly inhibited AML-193 cell proliferation at a concentration of 7.5 $\mu$M, whereas 15 and 30 $\mu$M isoimperatorin induced a more pronounced and significant loss of cell proliferation (Fig. 1H). These results suggested that isoimperatorin has the capacity to induce apoptosis and impede cell proliferation within the THP-1 and AML-193 cell lines, and thereby exhibited potential antitumor activity.

*Isoimperatorin treatment affects gene expression profiles in AML cells*. RNAseq analysis was conducted for THP-1 cells treated with isoimperatorin and untreated control cells. As a 24-h treatment with 25 and 50 $\mu$M isoimperatorin led to a prominent increase in the apoptosis of THP-1 cells, while 12.5 $\mu$M isoimperatorin induced a slight apoptosis in THP-1 cell, 12.5 $\mu$M isoimperatorin was selected to explore its regulatory impacts on gene expression in THP-1 cells. PCA demonstrated a distinct separation between isoimperatorin-treated and untreated THP-1 cells (indicating treatment-induced transcriptional shifts) and tight intragroup clustering (validating experimental reliability), supporting the robustness of analyses (Fig. 2A). In total, 688 DEGs were identified in THP-1 cells subsequent to the administration of isoimperatorin. Among these, the expression of 466 genes was significantly downregulated, while 222 genes were significantly upregulated compared with those in the control cells (Fig. 2B and C). Administration of isoimperatorin significantly altered the gene expression profiles in THP-1 cells. To determine the GO terms and KEGG signaling pathways into which the DEGs were clustered post-isoimperatorin treatment, analyses were performed based on the rich factor and FDR values. The top 20 most significantly altered (based on the FDR value) GO term classifications and KEGG enrichment results are shown. A substantial number of DEGs were involved in processes related to 'cell communication' and 'signaling' and certain DEGs were clustered into categories such as 'cell migration', 'cell projection' and 'cell motility' in GO enrichment (Fig. 2D). Furthermore, KEGG enrichment analysis demonstrated that numerous significantly enriched pathways in all treated isolates were associated with the nervous system, including 'cholinergic synapse', 'axon guidance' and 'GABAergic synapse'. Additionally, numerous DEGs were also significantly enriched in the 'MAPK signaling pathway', 'calcium signaling pathway' and 'Rap1 signaling pathway' (Fig. 2E). Collectively, these results indicated that isoimperatorin exerts a marked regulatory effect on tumor biology-associated signal transduction in THP-1 cells.

*Isoimperatorin treatment affects gene expression profiles associated with tumor biology processes in AML cells*. DEGs that were significantly enriched and implicated in tumor biology processes were analyzed based on the rich factor and FDR value (Fig. 3). The influence of isoimperatorin on tumor biology was exerted through several key signaling pathways, namely 'microRNAs in cancer' (Fig. 3A), 'prostate cancer' (Fig. 3B), 'small cell lung cancer' (Fig. 3C) and 'proteoglycans in cancer' (Fig. 3D). Among these pathways, the DEGs affected by isoimperatorin treatment were predominantly clustered within the 'microRNAs in cancer' signaling pathway. In this pathway, nine genes were upregulated, while nine genes were markedly downregulated (Fig. 3A). Regarding the 'prostate cancer' signaling pathway, upon the administration of isoimperatorin, four genes were significantly upregulated, while six genes were downregulated (Fig. 3B). In the context of 'small cell lung cancer' signaling, four genes were downregulated and five genes were significantly upregulated (Fig. 3C). In the context of 'proteoglycans in cancer' signaling, isoimperatorin treatment led to the upregulation of five genes and the downregulation of nine genes (Fig. 3D). To validate the RNAseq results, two most significantly up- and downregulated genes were selected from each of the four biological processes identified in the analysis. These genes were then subjected to RT-qPCR evaluation in the THP-1 (Fig. 3E-H) and AML-193 (Fig. S2A-D) cell lines. RT-qPCR results corroborated the RNA-seq data: the expression trends of selected genes were consistent between the two methods, confirming the reliability of the RNA-seq findings. For key genes in tumor biology-such as MYC and BCL2, which were significantly downregulated by isoimperatorin-*in vitro* validation revealed that their expression changes following isoimperatorin treatment were associated with altered cell proliferation and apoptosis and supports the conclusion that isoimperatorin regulates tumor-related biological processes through these genes.

*Isoimperatorin treatment affects gene expression profiles associated with metabolic processes in AML cells*. DEGs that were enriched and involved in metabolic processes were analyzed based on the rich factor and FDR value (Fig. 4). The influence of isoimperatorin on metabolism was manifested through several key metabolic pathways, namely 'glycosaminoglycan biosynthesis-heparan sulfate/heparin' (Fig. 4A), 'mucin type O-glycan biosynthesis' (Fig. 4B), 'steroid hormone biosynthesis' (Fig. 4C) and 'glycosphingolipid biosynthesis-ganglio series' (Fig. 4D). Among these pathways, the metabolic pathways associated with 'glycosaminoglycan biosynthesis-heparan sulfate/heparin' and 'glycosphingolipid biosynthesis-ganglio series' were inhibited by isoimperatorin. This was further substantiated as four genes within the 'glycosaminoglycan biosynthesis-heparan sulfate/heparin' pathway

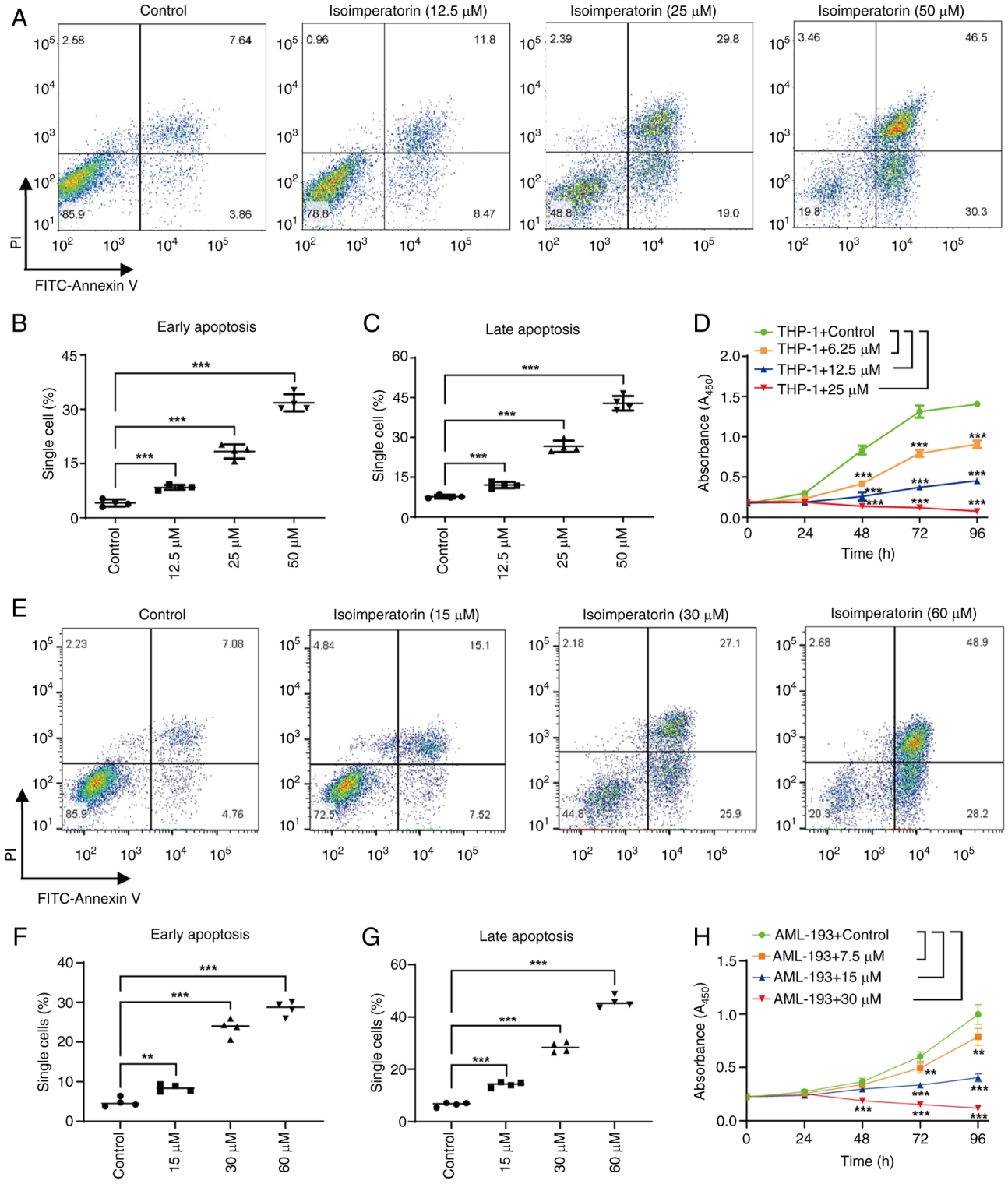


Figure 1. Isoimperatorin treatment significantly promotes apoptosis and suppresses proliferation in AML cells. (A) Isoimperatorin treatment significantly increased the apoptosis of THP-1 cells. THP-1 cells were treated with or without 12.5, 25 or 50 $\mu$M isoimperatorin for 24 h, stained with annexin V and PI, and subjected to apoptosis detection using flow cytometry. (B) Percentage of annexin V$^+$ and PI$^-$ early apoptotic cells. (C) Percentage of annexin V$^+$ and PI$^+$ late apoptotic cells. (D) Isoimperatorin significantly inhibited the proliferation of THP-1 cells. (E) Isoimperatorin treatment significantly increased the apoptosis of AML-193 cells. AML-193 cells were treated with or without 15, 30 or 60 $\mu$M isoimperatorin for 24 h, stained with annexin V and PI, and subjected to apoptosis analysis via flow cytometry. (F) Percentage of annexin V$^+$ and PI$^-$ early apoptotic AML-193 cells. (G) Percentage of annexin V$^+$ and PI$^+$ late apoptotic AML-193 cells. (H) Isoimperatorin significantly inhibited the proliferation of AML-193 cells Data are presented as the mean ± SD. Significant differences were examined using one-way analysis of variance with Tukey's post hoc test in panels (B), (C), (F) and (G) and two-way analysis of variance with Tukey's post hoc test in panels (D) and (H). $^{**}$P<0.01 and $^{***}$P<0.001. AML, acute myeloid leukemia; CCK-8, Cell Counting Kit-8; THP-1, Tohoku Hospital Pediatrics-1.

and two genes in the 'glycosphingolipid biosynthesis-ganglio series' pathway were all significantly downregulated upon treatment with isoimperatorin (Fig. 4A and D). In 'mucin type O-glycan biosynthesis' signaling, isoimperatorin treatment

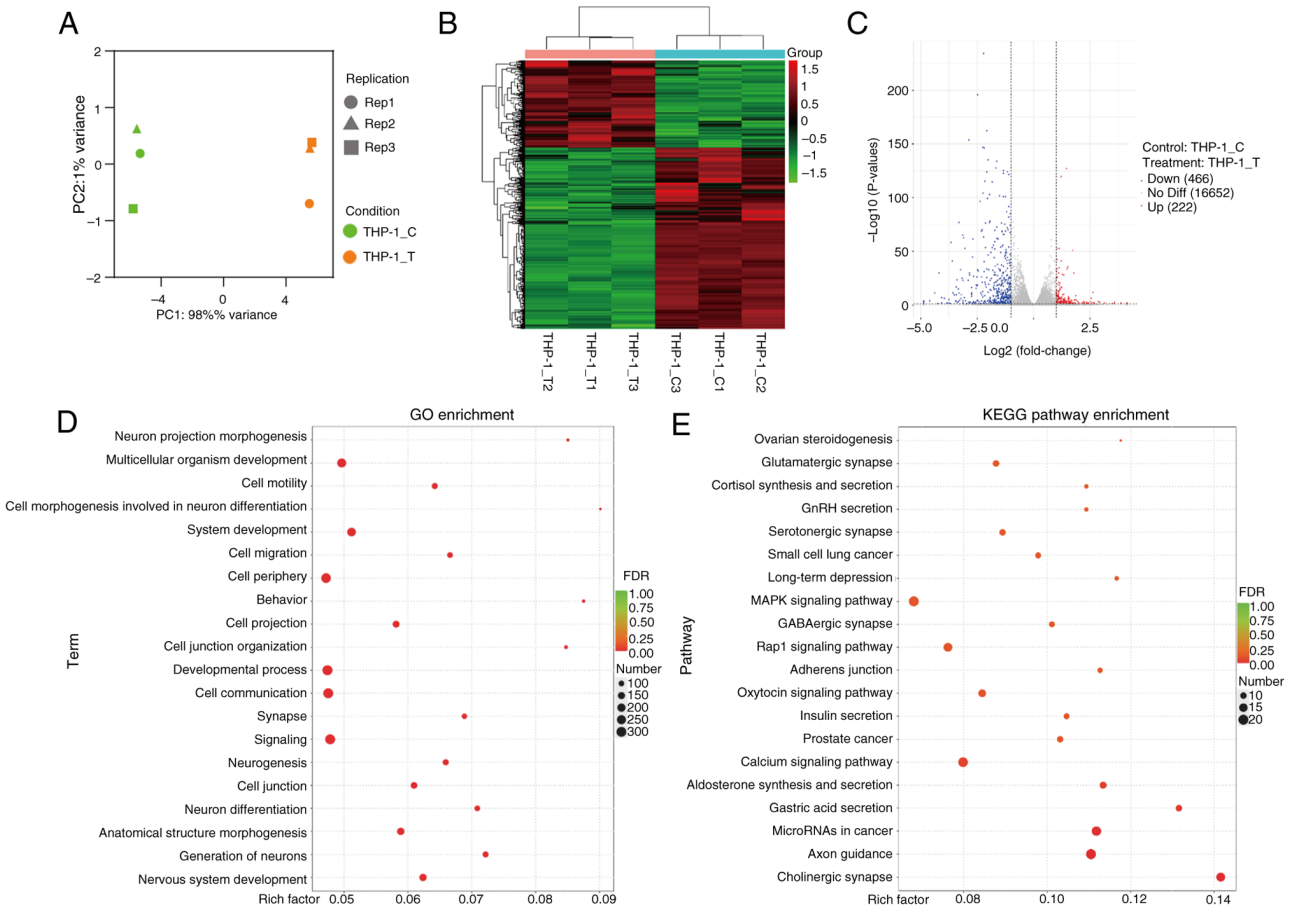


Figure 2. Isoimperatorin treatment affects gene expression profiles in AML cells. (A) Principal component analysis was performed to show inter-group differences and intra-group consistency. (B) Two-way cluster analysis of DEGs between isoimperatorin-treated and untreated THP-1 cells was performed using the pheatmap package. (C) Volcano plot indicating DEGs between isoimperatorin-treated and untreated THP-1 cells. (D) Scatter plot of the top 20 most significantly enriched GO terms (biological processes, cellular components and molecular functions) for DEGs in THP-1 cells after treatment with 12.5 $\mu$M isoimperatorin for 24 h. (E) Scatter plot of the top 20 most significantly enriched KEGG signaling pathways obtained from the RNA sequencing data of THP-1 cells after treatment with 12.5 $\mu$M isoimperatorin for 24 h. The degree of enrichment was indicated by the rich factor, FDR and number of genes. AML, acute myeloid leukemia; DEGs, differentially expressed genes; THP-1, Tohoku Hospital Pediatrics-1; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; FDR, false discovery rate; C represents the control; T represents THP-1 cells treated.

significantly suppressed the expression of three genes and promoted the expression level of one gene (Fig. 4B). In the 'steroid hormone biosynthesis' pathway, isoimperatorin treatment enhanced the expression of three genes while inhibiting that of two genes (Fig. 4C). To validate the RNAseq results, two most significantly upregulated and two most significantly downregulated genes were selected from each of the four biological processes identified in the analysis. These genes were then subjected to RT-qPCR evaluation in THP-1 (Fig. 4E-H) and AML-193 (Fig. S2E-H) cells. RT-qPCR results corroborated the RNAseq data for the expression trends of selected metabolic process-related genes, validating the reliability of RNA-seq findings. These results suggested that isoimperatorin could significantly regulate the expression of genes associated with metabolic processes in THP-1 cells.

*Isoimperatorin treatment affects gene expression profiles associated with the immune response system in AML cells.* Based on the KEGG enrichment analysis, analysis of the DEGs implicated in immune response-associated signaling pathways was performed. According to the rich factor and FDR value, the top four most altered immune response-associated signaling pathways in THP-1 cells treated with isoimperatorin were as follows: 'Chemokine signaling pathway' (Fig. 5A), 'Th1 and Th2 cell differentiation' (Fig. 5B), 'NOD-like receptor signaling pathway' (Fig. 5C) and 'leukocyte transendothelial migration' (Fig. 5D). In the 'chemokine signaling pathway', treatment with isoimperatorin significantly suppressed the expression of nine genes and increased the expression levels of four genes (Fig. 5A). Additionally, in the context of the 'Th1 and Th2 cell differentiation' pathway, isoimperatorin treatment increased the expression of three genes while downregulating four genes (Fig. 5B). Furthermore, in the 'NOD-like receptor signaling pathway', isoimperatorin treatment significantly downregulated the expression of three genes and upregulated the expression levels of eight genes (Fig. 5C). The immune response process associated with 'leukocyte transendothelial migration' was inhibited by isoimperatorin. This was further corroborated as all seven genes within this pathway were significantly downregulated upon treatment with isoimperatorin (Fig. 5D). To validate the RNAseq results, two most significantly up- and downregulated genes were selected from each of the four biological processes identified in the analysis. These genes were then subjected to RT-qPCR

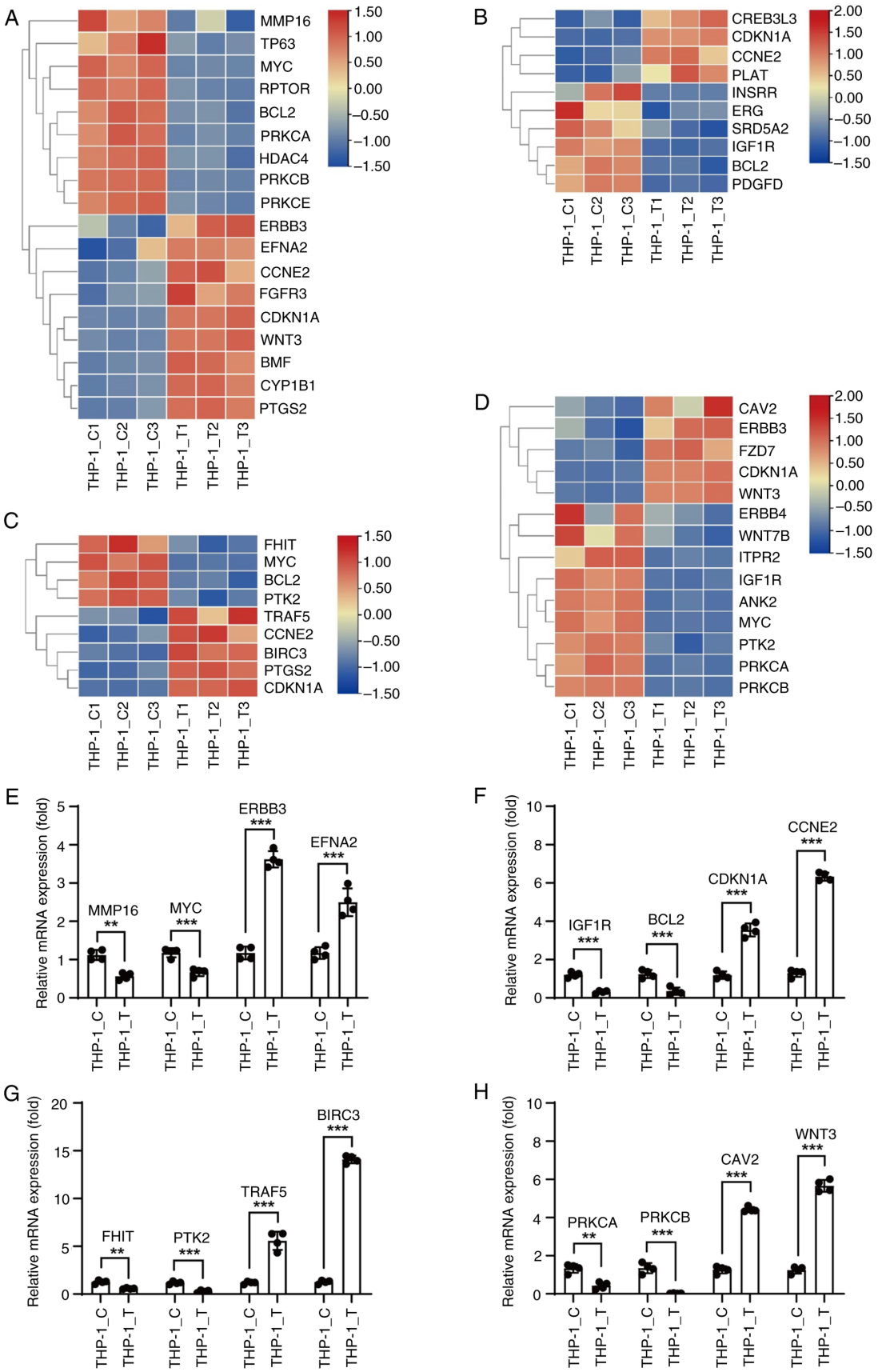


Figure 3. Isoimperatorin treatment affects gene expression profiles associated with tumor biology processes in AML cells. Heatmaps indicated that differentially expressed genes in THP-1 cells treated with isoimperatorin were involved in (A) 'microRNAs in cancer', (B) 'prostate cancer', (C) 'small cell lung cancer' and (D) 'proteoglycans in cancer'. From each pathway, two upregulated and downregulated genes in (E) 'microRNAs in cancer', (F) 'prostate cancer', (G) 'small cell lung cancer' and (H) 'proteoglycans in cancer' processes were selected and detected by reverse transcription-quantitative PCR analysis in THP-1 cells. Data are presented as the mean ± SD. Significant differences were examined using unpaired two-tailed Student's t-test in panels (E)-(H). $^{**}$P<0.01 and $^{***}$P<0.001. THP-1, Tohoku Hospital Pediatrics-1. C represents the control samples, with THP-1_C1, THP-1_C2, and THP-1_C3 as biological replicates. THP-1_T represents THP-1 cells treated with 12.5 $\mu$M isoimperatorin, including THP-1_T1, THP-1_T2, and THP-1_T3 as biological replicates.

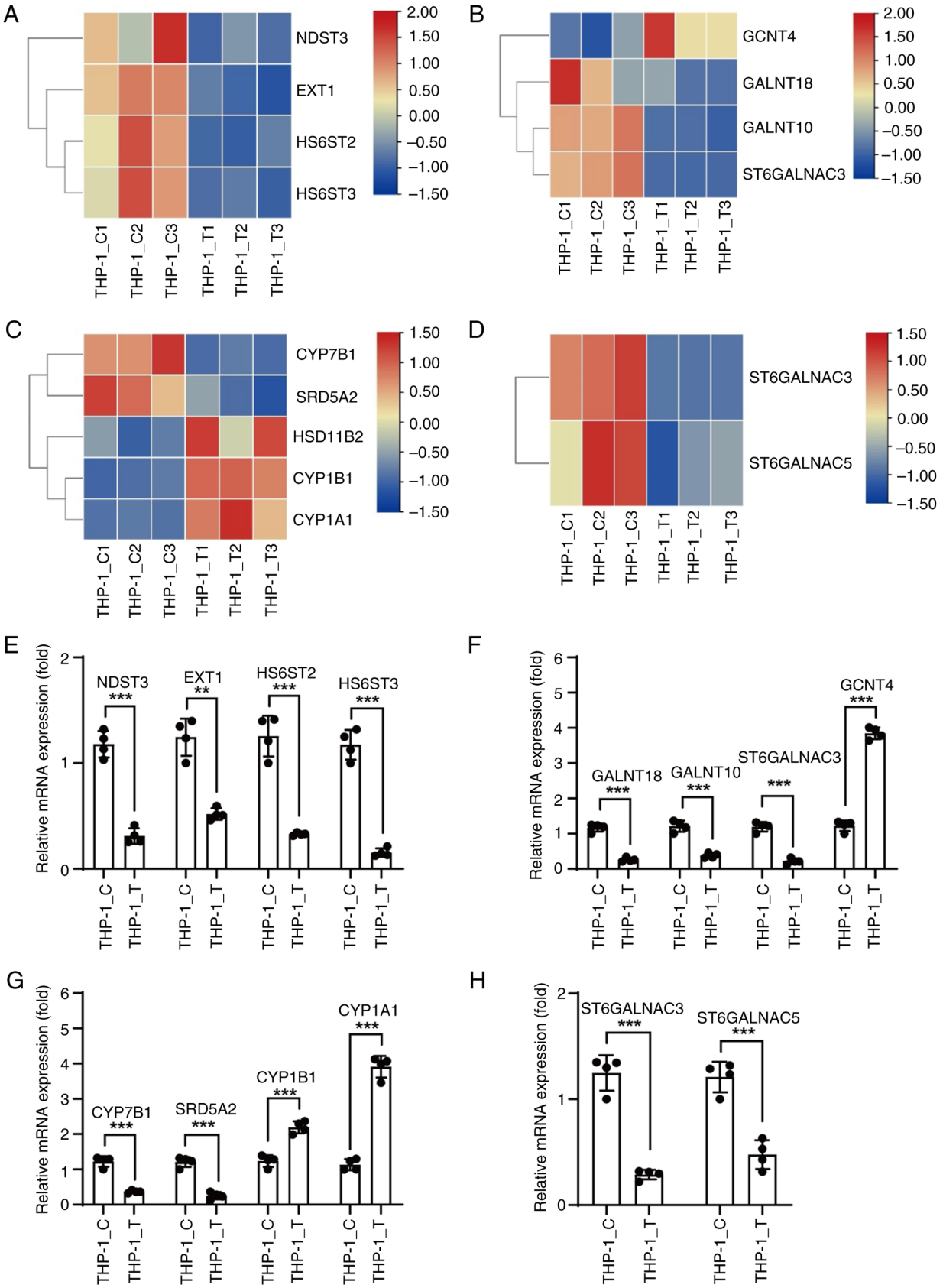


Figure 4. Isoimperatorin treatment affects gene expression profiles associated with the metabolic processes in AML cells. Heatmaps indicated that differentially expressed genes in THP-1 cells treated with isoimperatorin were involved in the (A) 'glycosaminoglycan biosynthesis', (B) 'mucin type O-glycan biosynthesis', (C) 'steroid hormone biosynthesis' and (D) 'glycosphingolipid biosynthesis' pathways. From each pathway, two upregulated and downregulated genes in (E) 'glycosaminoglycan biosynthesis', (F) 'mucin type O-glycan biosynthesis', (G) 'steroid hormone biosynthesis' and (H) 'glycosphingolipid biosynthesis' pathways were selected and detected by reverse transcription-quantitative PCR analysis in THP-1 cells. Data are presented as the mean ± SD. Significant differences were examined using unpaired two-tailed Student's t-test in panels (E)-(H). $^{**}$P<0.01 and $^{***}$P<0.001. THP-1, Tohoku Hospital Pediatrics-1. C represents the control samples, with THP-1_C1, THP-1_C2, and THP-1_C3 as biological replicates. THP-1_T represents THP-1 cells treated with 12.5 $\mu$M isoimperatorin, including THP-1_T1, THP-1_T2, and THP-1_T3 as biological replicates.

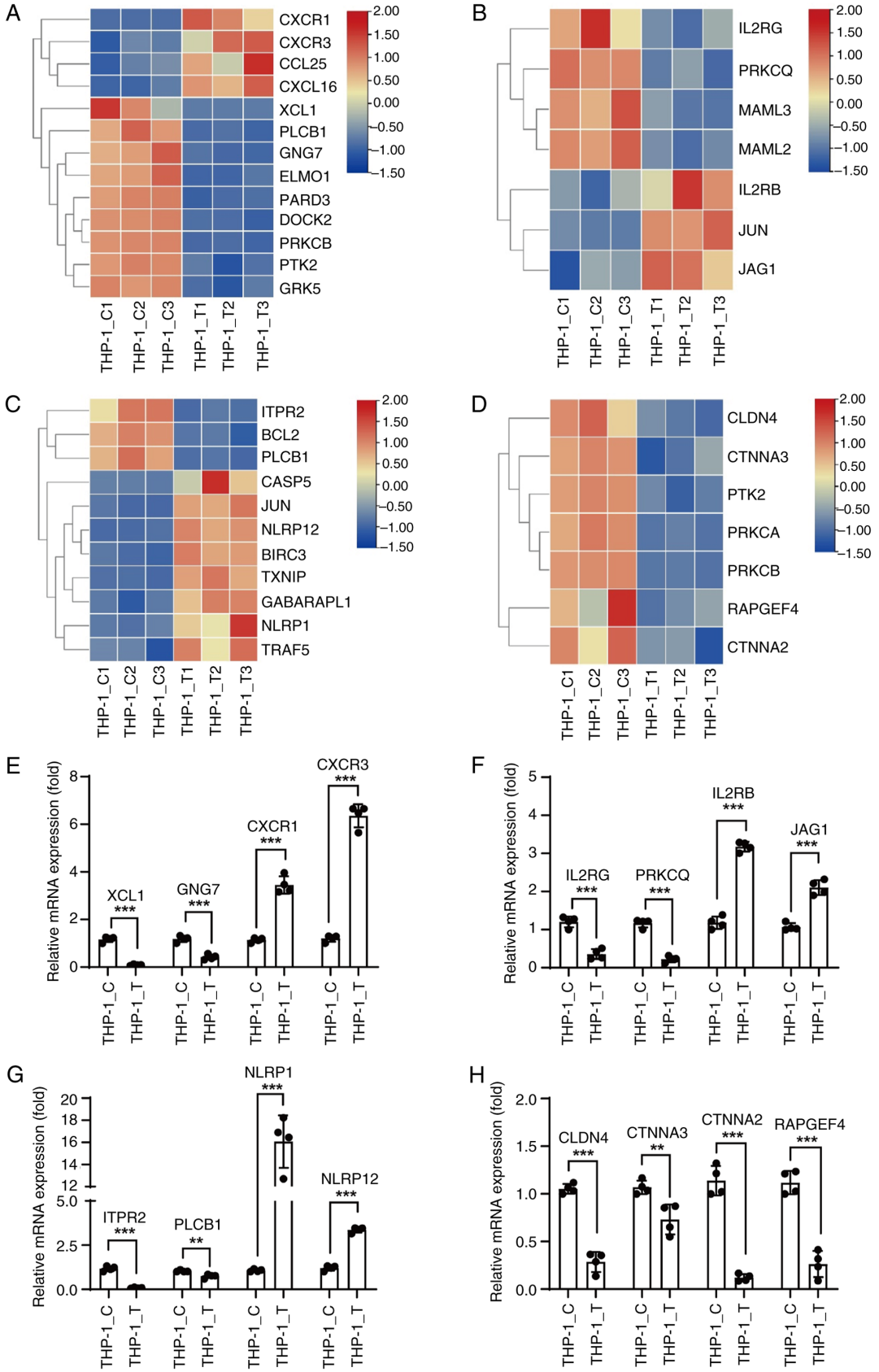


Figure 5. Isoimperatorin treatment affects gene expression profiles associated with the immune response system in AML cells. Heatmaps indicated that differentially expressed genes in THP-1 cells treated with isoimperatorin were involved in (A) 'chemokine signaling pathway', (B) 'Th1 and Th2 cell differentiation', (C) 'NOD-like receptor signaling pathway' and (D) 'leukocyte transendothelial migration'. From each pathway, two upregulated and downregulated genes in (E) 'chemokine signaling pathway', (F) 'Th1 and Th2 cell differentiation', (G) 'NOD-like receptor signaling pathway' and (H) 'leukocyte transendothelial migration' processes were selected and evaluated by reverse transcription-quantitative PCR analysis in THP-1 cells. Data are presented as the mean ± SD. Significant differences were examined using unpaired two-tailed Student's t-test in panels (E)-(H). $^{**}$P<0.01 and $^{***}$P<0.001. THP-1, Tohoku Hospital Pediatrics-1. C represents the control samples, with THP-1_C1, THP-1_C2, and THP-1_C3 as biological replicates. THP-1_T represents THP-1 cells treated with 12.5 $\mu$M isoimperatorin, including THP-1_T1, THP-1_T2, and THP-1_T3 as biological replicates.

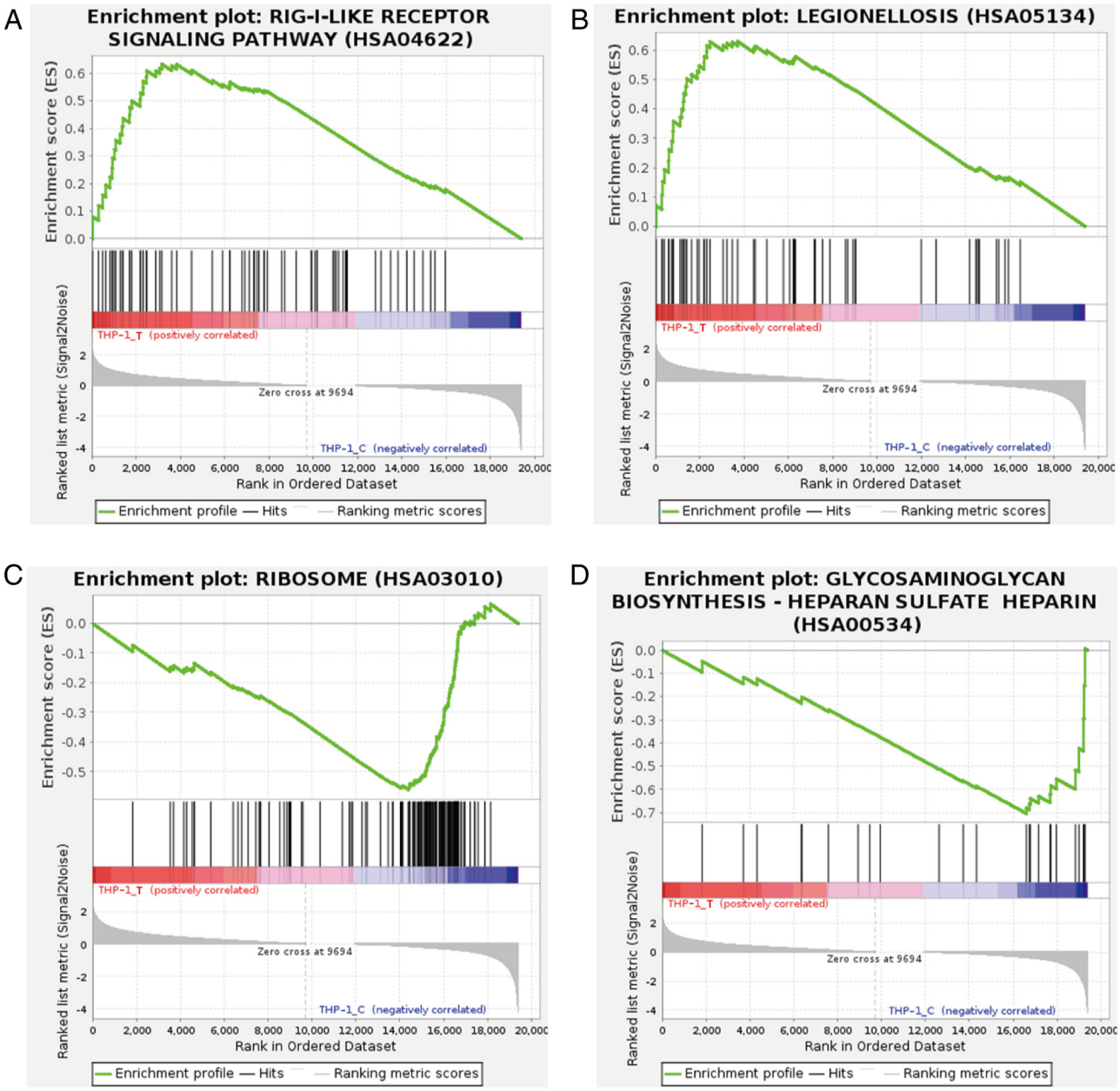


Figure 6. Signaling pathways involved in the sensitivity of THP-1 cells to isoimperatorin. Gene Set Enrichment Analysis plots indicated that differentially expressed genes in THP-1 cells treated with isoimperatorin were involved in (A) the 'Rig-I-like receptor signaling pathway', (B) 'legionellosis', (C) 'ribosome' and (D) 'glacoseminoglycan biosynthesis-heparan sulfate heparin'. ES, enrichment score; THP-1, Tohoku Hospital Pediatrics-1.

evaluation in THP-1 (Fig. 5E-H) and AML-193 (Fig. S2I-L) cells. The RT-qPCR results corroborated the RNA-seq data for the expression trends of the selected immune-response process-related genes were consistent between the two methods, confirming the reliability of the RNA-seq findings. These findings suggested that isoimperatorin could significantly regulate the expression of genes associated with the immune-response process in THP-1 cells.

*Signaling pathways involved in the sensitivity of AML cells to isoimperatorin*. To further elucidate the molecular functions implicated in tumor biology, immune response and metabolism, considering the findings derived from the GO and KEGG analyses in isoimperatorin-treated THP-1 cells, GSEA was performed on the RNAseq data (Fig. 6). The positively enriched gene sets were intricately involved in 'Rig-I-like receptor signaling pathway' (Fig. 6A) and 'legionellosis' (Fig. 6B). By contrast, the negatively enriched gene sets were associated with 'ribosome' (Fig. 6C) and 'glucosaminoglycan biosynthesis-heparan sulfate heparin' (Fig. 6D). These gene sets were constituted by genes that are closely related to specific immune signaling and metabolites, which indicates that isoimperatorin potentially exerts its functions, at least in part, through the activation or inhibition of these signaling pathways.

## Discussion

AML is a malignant disorder of myeloid hematopoietic stem/progenitor cells, which is primarily characterized by the aberrant hyperplasia of primitive and immature myeloid

cells in the bone marrow and peripheral blood. As one of the most pernicious malignant tumors, AML poses a threat to human health globally, with an estimated 187,000 new cases and 156,000 deaths annually worldwide, and a 5-year overall survival rate <30% in many regions (24). Currently, the standard treatment paradigm for newly diagnosed AML primarily consists of induction chemotherapy, followed by additional chemotherapy during the consolidation phase and/or allogeneic stem cell transplantation (2). However, a significant proportion of patients fail to achieve remission or experience relapse and disease progression post-remission (25). Immune checkpoints, such as programmed cell death protein 1/programmed cell death 1 ligand 1, serve a critical role in leukemia pathogenesis and progression. However, addressing immune-related adverse events to improve clinical outcomes in leukemia remains a challenge (26,27). The combination of targeted drugs (IDH, FLT3 and BCL2 inhibitors) with hypomethylation drugs in treating relapsed/refractory or elderly AML patients has shown some positive outcomes like certain remission rates and prolonged survival, but is limited by low response rates, short response duration, inevitable relapse, and resistance mechanisms (28-30). Natural active products in traditional Chinese medicine exhibit diverse structures and functions, rendering them key sources for innovative drug discovery (31). Presently, ~50% of the currently utilized antitumor drugs are either directly or indirectly derived from natural products, encompassing a wide array of compound types such as coumarins, alkaloids, polysaccharides, polyphenols, diterpenes and unsaturated fatty acids (32). Isoimperatorin, a coumarin compound, has been reported to possess diverse biological activities, including acetylcholinesterase inhibition, nematicidal and insecticidal effects, analgesic properties, anti-inflammatory potential and anti-tumor activity by inducing carcinoma cell apoptosis via the MAPK/ERK1/2 signaling pathway (20-22,33). The present study aimed to elucidate the function and underlying potential mechanism of isoimperatorin in the context of AML.

The capacity of cells to evade apoptosis constitutes a major contributing factor to the pathogenesis of different types of cancer, such as leukemia and multiple myeloma (34). It has been reported that isoimperatorin can induce the apoptosis of nasopharyngeal carcinoma cells and hepatocellular carcinoma by regulating the MAPK/ERK1/2, and c-Myc and SIRT1 signaling pathways (21,35). The present experimental findings demonstrate that isoimperatorin promotes both early- and late-stage apoptosis in AML cells in a dose-dependent manner. Additionally, isoimperatorin significantly suppressed cell proliferation, suggesting that isoimperatorin exerts antitumor activity. Furthermore, the results of GO analysis based on sequencing data demonstrated that isoimperatorin influences the expression of genes associated with 'cell migration', 'cell motility' and 'cell projection'. It has been reported that leukemia is a metastatic disease. Specifically, leukemia cells invade distant organs through the utilization of the same cell-surface adhesion molecules, chemokines, stromal molecules and intracellular motor signaling pathways as those employed by solid tumors (36). Whiteley *et al* (1) reported that leukemia is a prototypical metastatic disease. The unique interactions between leukemia cells and the microenvironment surrounding different organs may contribute to the heterogeneity of treatment responses observed in certain patients (1). Furthermore, similar to solid tumor cells, leukemia cells utilize a diverse range of migration strategies, including amoeboid, invadopodia-driven and lamellipodia-driven movements, each of which depends on distinct molecular signaling pathways (1,2,36). Based on these prior studies, the present results further demonstrated that isoimperatorin may serve a pivotal role in the inhibition of leukemia progression by modulating the expression of key genes involved in the migration and motility of AML cells.

The enrichment outcomes derived from the KEGG analysis indicated that isoimperatorin predominantly regulated the 'calcium signaling pathway', 'MAPK signaling pathway' and 'cholinergic synapse'. Currently, one of the efficacious mechanisms for the treatment of leukemia is to induce the apoptosis and differentiation of leukemia cells. While the 'apoptosis pathway' was not ranked among the top 20 significantly altered signaling pathways, this appears inconsistent with the aforementioned findings that isoimperatorin induced AML apoptosis. This apparent contradiction may be explained by the different concentrations of isoimperatorin used. Specifically, 12.5 $\mu$M isoimperatorin was employed for transcriptome sequencing, as this dosage induced only weak cell apoptosis, whereas 25 and 50 $\mu$M were used to induce notable apoptosis. The present experimental design aimed to characterize core signaling mechanisms rather than downstream apoptotic responses under sublethal conditions. Previous studies have demonstrated that the induction of apoptosis and differentiation of leukemia cells is concomitant with alterations in the intracellular concentration of calcium ion ($Ca^{2+}$), suggesting that intracellular $Ca^{2+}$ exerts a significant influence on leukemia cells (37-39). Other studies have shown that elevated $Ca^{2+}$ can participate in apoptosis through multiple mechanisms. For instance, the activation of $Ca^{2+}$-dependent protein kinase and/or phosphorylase signals can lead to the activation of numerous apoptosis-related genes, ultimately culminating in apoptosis (40). Previous research has demonstrated that the disruption of $Ca^{2+}$ homeostasis represents a common terminal event in drug-induced cell death. Under the influence of various factors in the internal and external environments, the balance of intracellular $Ca^{2+}$ is maintained by precisely regulating intracellular $Ca^{2+}$, thereby enabling the maintenance of normal intracellular activities (41). Together with the present experimental results, it can be hypothesized that isoimperatorin may promote apoptosis in AML cells via its impact on the calcium signaling pathway. A significant number of genes were enriched in the MAPK signaling pathway following isoimperatorin treatment. Notably, in nasopharyngeal carcinoma cells, isoimperatorin has been shown to induce apoptosis via the MAPK/ERK1/2 signaling pathway (35). Furthermore, the present findings demonstrated that the protein kinase C isoform PRKCA was inhibited in multiple signaling pathways by isoimperatorin. These pathways include those related to several signaling transduction-related pathways, including the 'calcium signaling pathway', 'Rap1 signaling pathway', 'MAPK signaling pathway' and 'PI3K-AKT signaling pathway'. Previous research has indicated that increased expression of PRKCA is correlated with poor survival outcomes in patients with AML (42). Isoimperatorin significantly suppressed the expression of PRKCA, indicating a key association with its antitumor activity.

Metabolic rewiring and cellular reprogramming represent cardinal hallmarks of AML initiation and progression (17). Metabolic alterations in leukemic cells are inherently

gene-specific, with associated epigenetic and functional factor changes synergistically driving oncogenic pathways. This interplay offers extensive opportunities for the development of targeted therapies against abnormal metabolism in AML (17). In the present study, metabolism-associated pathways such as 'glycosaminoglycan biosynthesis-heparan sulfate/heparin', 'mucin type O-glycan biosynthesis', 'steroid hormone biosynthesis' and 'glycosphingolipid biosynthesis of the ganglio series' emerged as key pathways in isoimperatorin-treated AML cells. Heparan sulfate/heparin, which are key components of the extracellular matrix, interact with growth factors and cytokines, modulating signaling pathways that promote leukemic cell survival, proliferation and adhesion to the bone marrow niche (43). These interactions enhance the self-renewal capacity of leukemia stem cells, contributing to disease initiation and relapse (44). In addition, mucin type O-glycan biosynthesis modifies cell surface proteins, altering cell-cell communication and immune recognition. Aberrant O-glycosylation can shield AML cells from immune surveillance, facilitating tumor progression and resistance to immunotherapies (45). Steroid hormone biosynthesis pathways, traditionally linked to endocrine functions, exert autocrine and paracrine effects in AML. Dysregulation of these pathways disrupts normal cell differentiation and apoptosis, driving leukemic transformation (46). For instance, altered steroid metabolism can activate oncogenic signaling cascades, fueling AML cell proliferation (47). Furthermore, glycosphingolipids of the ganglio series, integral to lipid rafts, are crucial for receptor clustering and signal transduction. Changes in their biosynthesis can dysregulate key pathways such as PI3K-AKT, promoting cell survival and proliferation in AML (48). These metabolic pathways offer promising therapeutic targets. However, understanding the context-dependent roles of these pathways and their complex crosstalk is essential for developing effective and targeted AML treatments.

Immune dysregulation is crucial in the pathogenesis and progression of AML (49). In the present study, chemokine signaling, Th1/Th2 cell differentiation, NOD-like receptor signaling and leukocyte transendothelial migration were identified as key pathways involved in the isoimperatorin-mediated regulation of AML cells (50). The chemokine signaling pathway regulates immune cell migration in the bone marrow microenvironment. and AML cells hijack it by secreting chemokines to recruit immunosuppressive cells (such as regulatory T cells), forming a niche that aids leukemic survival and immune evasion (50). In our study, key chemokine genes in this pathway-including CXCRL, CCL25, and CXCR3-were identified as being upregulated by isoimperatorin. As these genes are involved in recruiting anti-tumor immune cells or antagonizing the recruitment of immunosuppressive subsets (51), their upregulation suggests isoimperatorin may counteract pathway hijacking by shifting the chemokine balance, limiting the recruitment of suppressive cells, promoting infiltration of protective immune populations, and potentially restoring immune surveillance-key anti-leukemic mechanisms (50). Th1/Th2 cell differentiation is fundamental to immune homeostasis, and in AML, an abnormal shift toward Th2 dominance-characterized by elevated anti-inflammatory cytokines like IL-4 and IL-10-suppresses Th1-mediated cytotoxic T cell activity and promotes leukemic cell survival (52,53). Our study identified key genes regulated by isoimperatorin that may counteract this pathogenic shift. For instance, it inhibits PRKCQ, a kinase critical for Th2 differentiation, thereby potentially limiting Th2 polarization (54). Additionally, it upregulates JUN, a transcription factor that modulates the production of pro-inflammatory/Th1-associated cytokines, which may enhance anti-tumor immune responses (55). Collectively, these regulatory effects indicate that isoimperatorin may alleviate the Th2-biased imbalance in AML, reinforcing cytotoxic immunity and suppressing leukemic progression as key anti-AML mechanisms. In addition, the NOD-like receptor signaling pathway functions as a sentinel for pathogen- and damage-associated molecular patterns. In AML, dysregulation of this pathway can trigger chronic inflammation, which is a key driver of leukemogenesis (2). Aberrant activation of NOD-like receptors often results in the release of pro-inflammatory cytokines, which promote angiogenesis and support the proliferation of AML cells (56,57). The present study identified key genes regulated by isoimperatorin that may counter these pathogenic processes. For instance, it suppressed BCL2, an anti-apoptotic gene supporting AML cell survival (42), and upregulated NLRP12, a negative regulator of NOD-like receptor-mediated inflammation (58). The downregulation of BCL2 by isoimperatorin likely impairs leukemic cell survival, while the upregulation of NLRP12 may dampen excessive inflammatory signaling-collectively mitigating chronic inflammation and restraining leukemic progression as potential anti-AML mechanisms. Leukocyte transendothelial migration is essential for immune cells to reach sites of inflammation or malignancy. In AML, alterations in this process hamper the effective infiltration of immune effector cells into the bone marrow, thereby restricting their antitumor function. Additionally, AML cells can exploit the migration machinery to metastasize to other tissues, contributing to disease progression (59). The present study identified key genes regulated by isoimperatorin that may counter these processes, such as suppressing PTK2, a kinase driving cell migration and adhesion (60) and inhibits PRKCA, a kinase involved in migration signaling (61). By downregulating these genes, isoimperatorin likely impairs the metastatic potential of AML cells while potentially enhancing immune cell infiltration-collectively restraining disease progression as a potential anti-AML mechanism. In conclusion, the potential of isoimperatorin to target these immune pathways presents novel therapeutic opportunities for patients with AML.

In summary, the present experimental findings demonstrated that isoimperatorin could significantly induce apoptosis and inhibit cell proliferation, predominantly by regulating 'cell migration' and 'cell motility', along with the 'calcium signaling pathway' and 'MAPK signaling pathway'. These results suggest that isoimperatorin has potential as a novel therapeutic strategy for AML. However, the present study was limited by its sole investigation on gene transcription within cell lines and the omission of potential regulatory mechanisms associated with protein expression levels. The present work lays a foundation for understanding the antitumor effects of isoimperatorin, future studies should focus on key DEGs like MYC, BCL2, IGF1R, JUN, NLRP1, and PRKCA. It will use gene editing and transgenic models to clarify their roles

in isoimperatorin-induced antitumor effects, validate associated pathways (MAPK signaling pathway and GABAergic pathway), test synergies with inhibitors (midostaurin, palbociclib), and conduct translational studies in preclinical models to support clinical potential. In conclusion, the present study, through integrating these methods, provides a comprehensive understanding of isoimperatorin effects on AML, and its findings hold notable clinical significance by laying a solid foundation for identifying and developing novel therapeutic targets, which may contribute to advancing more effective treatments for AML and improving patient outcomes.

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**Availability of data and materials**

The RNA sequencing data generated in the present study may be found in the NCBI BioProject database under accession number PRJNA1021357 or at the following URL: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1021357. The other data generated in the present study may be requested from the corresponding author.

**Authors' contributions**

RY, RX and JS conceptualized the study. RY, HM and JS designed the study, and collected and analyzed the data. RY, RX, HM and JS wrote and edited the manuscript. JS supervised the research. RY, HM and JS confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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