Research Article

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Casticin inhibits the hedgehog signaling and leads to apoptosis in AML stem-like KG1a and mature KG1 cells

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Abstract

Objectives: Acute myeloid leukemia (AML) is a severe blood cancer with less than 50 % long-term survival. Despite advancements in treatment options, relapse is still the major obstacle. The main reason of this problem is ineffective targeting of leukemic stem cells (LSCs), which play an important role in tumor development and relapse. In our previous studies, we found that casticin, the major polyphenolic component of *Vitex trifolia*'s fruit, targets both leukemic cells and LSCs without affecting healthy tissues. Therefore, in this study, we aimed to investigate the effect of casticin-mediated cell death in relation to the LSCs-favored survival pathways at gene and protein expression levels using *in vitro* LSC-like and parental leukemic cell models.
Methods: We validated the LSC character of KG1a and KG1 cells (84.55 % CD34+, CD38- and 93.55 % CD34+, CD38+, respectively) by flow cytometry. For the investigation of casticin's mechanism of action, we employed real time-PCR, western blotting and bioinformatics analyses.
Results: Our results showed an increase in cleaved PARP/β-actin ratio but no change in LC3BI/II and SQSTM/β-actin ratios. Our gene expression, bioinformatics and immunoblotting analyses represented significant decrease in Shh, Gli and Wnt levels. We also elucidated a possible crosstalk between Hedgehog and other oncogenic cascades via the Gli, Notch, YAP, p38, Mcl-1, and Myc proteins in casticin mediated anti-leukemic effect.
Conclusions: In conclusion, we found that casticin induces apoptosis in both LSC-like and parental leukemia cells mainly by suppressing Shh signaling, which is crucial for LSC survival and AML relapse.

Keywords: acute myeloid leukemia; apoptosis; cancer stem cells; casticin; cell signaling

Introduction

Acute myeloid leukemia (AML) is the most common form of acute leukemia in children. Standard treatment for AML dates back to the 1970s, a regimen that disrupts DNA and RNA synthesis in all rapidly dividing cells of the body [1]. Conventional chemotherapies have some limitations in AML treatment due to their cytotoxic effects on healthy tissues and inability to target leukemia stem cells [2–4]. Although the U.S. Food and Drug Administration (FDA) has approved new therapeutic agents for AML treatment, their effectiveness is still limited with less than 50 % 5-year survival rates [5–6].

Leukemic stem cells (LSCs) represent a small fraction (only 1–4 %) of the total AML cells, which can self-regenerate and have unlimited re-population capacity. Therefore, LSCs have the ability to initiate leukemia, and insufficient eradication of these cells leads to drug resistance and relapse [7–8]. Hence, it is important to incorporate LSC-targeted agents into therapeutic strategies.

Natural products have been a promising source for anticancer drug discovery for decades [9]. Phytochemicals have the ability to induce specifically cancer cell death by affecting multiple oncogenic signaling pathways [10]. Since survival

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signaling pathways like Notch, Wnt, Hedgehog, and Hippo cascades are critical for targeting LSCs [11–15], phytochemicals may be a promising source to disrupt these signals, leading to programmed cell death in both LSCs and leukemic blasts for ultimate AML treatment [16, 17].

Casticin (3′, 5-dihydroxy-3, 4′, 6, 7-tetramethoxyflavone) is a predominant polyphenolic constituent of the fruit of Vitex trifola L [18]. and studies reported that casticin induces apoptosis in AML cells [19–21]. In particular, Righeschi et al. reported that casticin has the most potent anti-proliferative effect on lymphoblastic leukemia cells among the well-known polyphenols [22]. In our previous study, we showed that 2 μM casticin induces apoptosis in both leukemic cells and LSCs without affecting healthy cells [23]. Although the anti-proliferative effect of casticin on leukemia cell lines is known, the mechanism of action of casticin is still unelucidated. Hence, this study aimed to uncover the underlying molecular mechanism focusing on signaling pathways associated with casticin-induced cell death in LSC-like KG1a and AML blast model KG1 cell lines at gene and protein expression levels.

Materials and methods

Cell culture

KG1 and KG1a cells were obtained from ATCC (ATCC, Rockville, MD, USA). KG1a and KG1 cells cultured in 20 % fetal bovine serum (Biochrom, Germany) involved Iscove's Modified Dulbecco's Medium (IMDM) (ATCC, 30–2005) at 37 °C in 5 % CO_2 incubator. KG1a cell line consists of stem-like AML cells that lack of differentiation capacity. However, KG1 cell line, which involves a more mature AML cells, exhibits differentiation capability into macrophages [24].

Cell viability assays

Casticin (≥98 % purity, Sigma, MO, USA) was dissolved in dimethyl sulfoxide (DMSO; AppliChem, Germany) with a stock concentration of 50 mM. Further dilutions were made with fresh culture medium, maintaining DMSO concentration<0.1 %. Cell viability and IC50 values of casticin were assessed using MTT colorimetric assay. In brief, 50 μL of cell suspension was seeded into a 96-well plate, after overnight starvation, cells were treated with casticin for 24 or 48 h, then incubated with 10 μL/well MTT solution (5 mg/mL). After solubilization, absorbance at 562 nm was measured by using microplate reader Biotek (ELX800, USA).

Phenotypes of the cell lines

To validate our *in vitro* stem cell model, flow cytometry was used to assess CD34+ and CD38-expression in KG1a and KG1cell lines. Cells (5×10^5–1×10^6) were washed and treated with CD34- FTC (Invitrogen) and CD38-APC (Invitrogen) antibodies, then analyzed using Navios Flow Cytometer (Beckman Coulter, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

To explore the mechanism of action of casticin treatment, RT-qPCR analyses were performed using gene panel (IDT, 14246249) comprising 47 genes, including 42 genes involved in cell survival and apoptotic signaling along with 5 housekeeping (Table 1). Briefly, after the treatment the total RNA was extracted using High Pure RNA Isolation Kit (Roche) followed by cDNA synthesis using EvoScript Universal cDNA Master (Roche). PCR amplification conditions included predenaturation (95 °C) for 10 s, denaturation (95 °C) for 10 min, annealing and extension for 30 s at 60 °C, over 45 cycles. The RT-qPCR was performed with Roche Light Cycler 480 Real-time PCR system by using FastStart Essential DNA Green Master Mix (Roche) in 96-well plates. For data normalization, the mean ΔCT value of five housekeeping genes was used and the data were analyzed with the $2^{-\Delta\Delta Ct}$ method [25]. Fold change cut off was set at -fold and higher to identify upregulated and downregulated genes. For the visualization of all

Table 1: Genes employed in the panel.

Pathway/phenotype	Genes
Drug resistance	*ABCB1, ABCC1, ABCG2*
Seramid formation pathway	*CERS, NSMAF, UGCG*
PI3K/Akt/mTOR pathway	*AKT1, mTORC1, PIK3CB, PIK3CG, PTEN, PTPN11*
Ras/MEK/ERK	*ERK1, JNK1, KRAS, NRAS, MEKK1, P38 (MAPK14)*
NFKB	*P65, HSP90*
Notch	*NOTCH1*
Hedgehog	*GLI1, SHH*
Hippo	*ALDH1A1, ALDH3A1, YAP/TAZ*
Apoptosis	*BAD, BAX, BCL-2, BCL-XL, MCL-1, MYC, PARP1, P53*
Autophagy	*BECN1 (Beclin), LC3B, SQSTM1*
Wnt/Beta-catenin	*CTNNB1 (Beta-catenin), LEF, WNT1*
Jak/Stat	*STAT3, STAT5*
Housekeeping	*ACTB (Beta-actin), B2M, RPL37A, SRP14, UBE2D2*

gene expression changes, heat maps were created by using package 'heatmap' version 1.0.12 in R (v.4.2.2).

Gene overrepresentation analysis

In order to determine the biological processes and signaling pathways related to cell death, the Consensuspathdb tool were used in gene set overrepresentation analyses [26]. Gene Ontology (GO) annotations [27] were utilized to provide information on molecular functions and biological processes, while the Kyoto Encyclopedia of Genes and Genomes (KEGG) [28] and Reactome [29] databases served as valuable resources for signaling and metabolic pathway information. Statistical significance was determined using Fisher's exact test, and the Benjamini-Hochberg correction was applied to adjust the p-values. Results with adjusted p-value ≤ 0.05 were considered statistically significant.

Protein-protein interaction analysis

A protein-protein interaction network was constructed to depict the connections among the proteins encoded by the significantly altered genes, as well as other proteins involved in relevant biological processes. Establishment of this network was achieved by using human physical protein-protein interaction data (version 4.4.206) from the Biological General Repository for Interaction Datasets (BioGRID) and Cytoscape software facilitated network visualization [29, 30].

Assessment of protein expressions

Significantly altered gene expressions, as identified by RT-qPCR and bioinformatics, were also assessed at protein expression level by using western blotting. Briefly, whole-cell lysates were prepared using RIPA buffer (Thermo, MA, USA). Subcellular fractionation was performed using the NEPER kit (Thermo, MA, USA). Protein concentrations were detected by BCA assay (Pierce Chemical, USA) with approximately 40 ug of total protein was loaded per well. Following SDS-PAGE, proteins were transferred to PVDF membranes by semi-dry or wet-transfer methods. Membranes were blocked with 5 % BSA (Biochrom, Germany) in Tris-buffered saline containing 0.1 % Tween 20 (Sigma, St. Louis, MO, USA) and immunoblotted overnight at 4 °C with primary antibody (PARP, LC3BI/II, SQSTM1/p62, Sonic hedgehog (Shh), Gli, Wnt) followed by 1 h incubation with the horseradish peroxidase-linked secondary antibody (Goat anti-rabbit IgG-HRP; Goat anti-mouse IgG-HRP, Cell signaling). Protein detection was carried out using the West Pico chemiluminescent substrate kit (Thermo, MA, USA).

Statistical analysis

All experiments were conducted three times and the results are expressed as mean±SEM. Statistical significance was determined using one-way-ANOVA in SPSS version 22.

Results

Phenotypes of the cell lines

According to our results, approximately 84.55 % of KG1a cells exhibited the CD34+, CD38-phenotype, while 93.55 % of KG1 cells demonstrated the CD34+, CD38+ phenotype (Figure 1). Since CD34+ and CD38-phenotype is accepted as the characteristic feature of AML LSCs [7], we used the KG1a cell line as an *in vitro* model for LSCs and the KG1 cell line as their parental cell line to represent mature leukemic cells.

mRNA expressions of signaling pathways

All genes in the designed panel were categorized according to relevant biological processes, molecular pathways, and significant expression changes for KG1a, KG1 cells (Supplementary Figures S1 and S2).

The changes in gene expression were visualized by a heat map, which allowed us to comparatively evaluate the effects of casticin treatment on target genes in the KG1a and KG1 cell lines (Figure 2). RT-qPCR results showed that treatment of KG1a cells with casticin resulted in a significant decrease in the expression of *SHH, GLI*, and *WNT* mRNA levels, genes known to play an important role in LSC survival [4]. An increase in the expression of *P53* and *P38* genes, which are closely associated with apoptosis, was detected. In addition, a significant increase was found in the expression of genes that support the survival of leukemic cells and LSCs, such as *ABCC1, ABCG2, MCL-1, NOTCH1, NRAS,* and *ERK*. In addition, treatment with casticin resulted in a significant increase in the expression of *ALDH1A1*, which has high expression and enzymatic activity in LSCs (Figure S1 and Figure 2A). In KG1 cells, casticin treatment significantly decreased the expressions of *SHH, GLI,* and *WNT* genes. In addition, there was also a significant decrease in the gene expression of *ABCB1*, which encodes p-glycoprotein that plays a role in drug resistance (Figure S2 and Figure 2A).

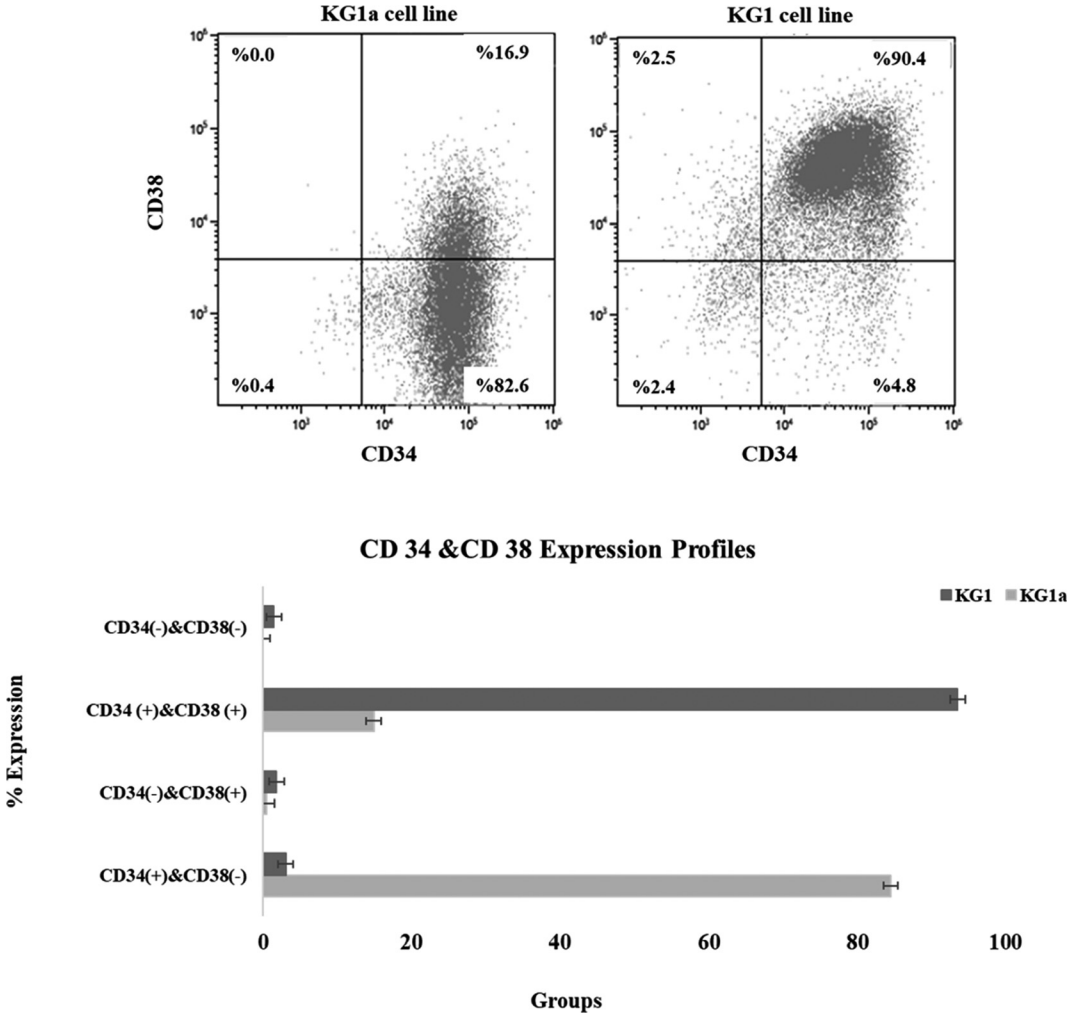

Figure 1: Flow cytometry diagrams and graph of CD34 and CD38 expression in KG1a and KG1cell lines.

After casticin treatment, a significant decrease in the mRNA levels of *SHH*, *GLI*, and *WNT* was detected in both cell lines. While there was a significant decrease in *ABCB1* mRNA expression in KG1 cells following casticin treatment, there was no significant alteration in KG1a cells. In addition, although casticin caused very significant increase in *ALDH1A1* mRNA levels in KG1a cells, the treatment did not affect *ALDH1A1* mRNA levels in KG1cells (Figure 2B).

We also analyzed cell signaling pathways-associated gene alterations and pathway-pathway interactions considering differentially expressed genes (by p-value as ≤0.05 and fold change as ≥2) in response to the casticin treatment (Figure 3).

In evaluating the effects of casticin on cell survival signaling pathways, it was appropriate to focus on Shh and Wnt/β-catenin signaling pathways in both cell lines. To reveal the functional relationships between the proteins and their potential roles in casticin-mediated cell death, a protein-protein interaction network (PPI) was constructed (Figure 4). According to the PPI network and RT-qPCR results, the most interactive proteins were found to be Gli, Shh, and Wnt.

Protein expression levels

Confirmation of apoptotic cell death was achieved through the analysis of PARP protein levels. The ratio of cleaved PARP/β-actin was significantly increased in casticin-treated KG1a and KG1 cells compared with the corresponding control groups ($p<0.01$ for both KG1a and KG1) (Figure 5A and B). The findings were consistent with the flow cytometry results [23] and it was confirmed that 2 μM casticin treatment induced apoptotic cell death in both cell lines.

The involvement of autophagy in casticin-mediated cell death was investigated by detecting the expression of

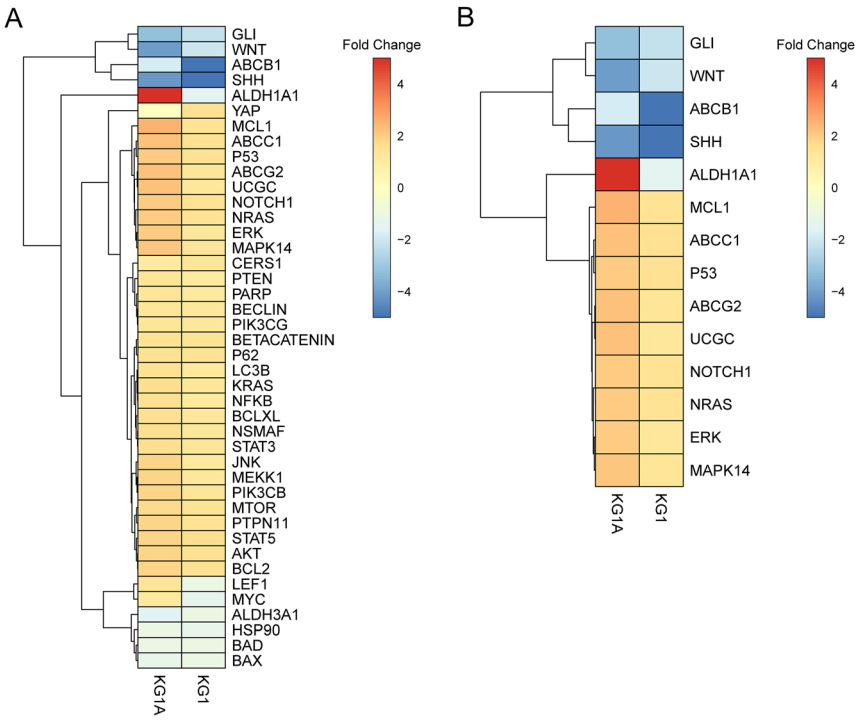

Figure 2: Heat map representing the mRNA expression changes in casticin-treated KG1a and KG1 cell lines. (A) Fold changes in 42 genes in response to casticin treatment (compared to their respective non-treated control groups). (B) Differentially expressed genes with at least 2-fold changes in response to casticin treatment (compared to their respective non-treated control groups). The blue color indicates a decrease in expression, while the red color indicates an increase in expression.

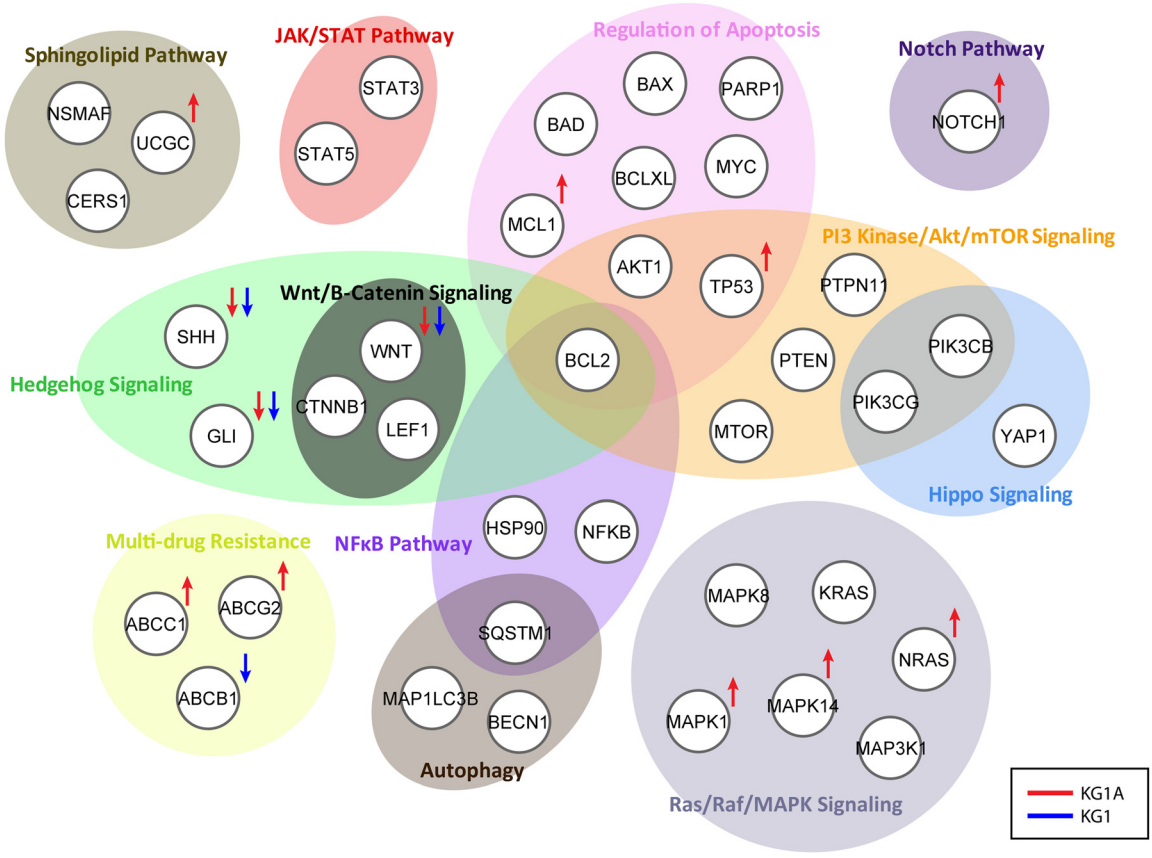

Figure 3: Cell signaling pathways-associated gene alterations and pathway-pathway interactions.

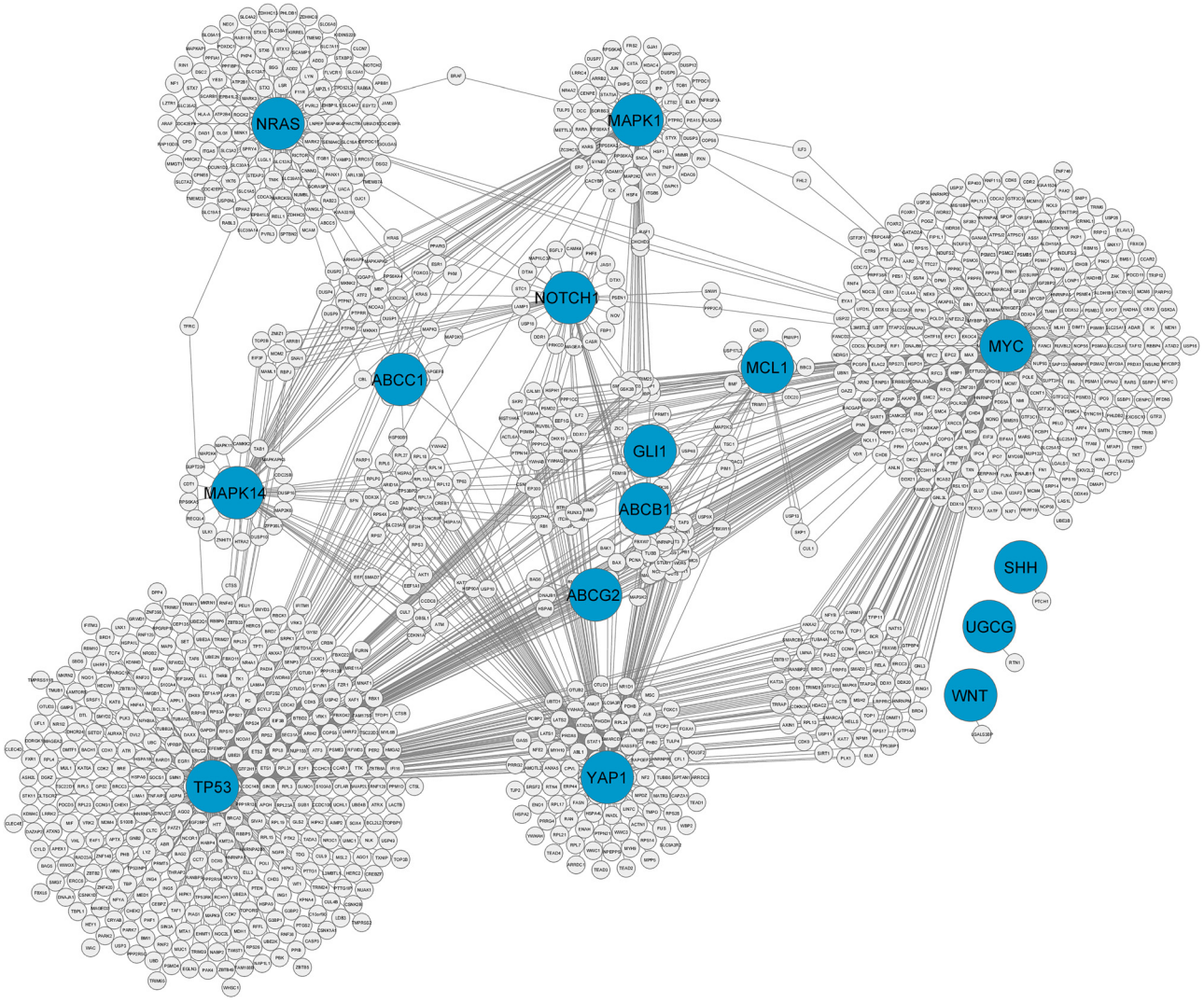

Figure 4: Protein-protein interaction (PPI) network depicting the interactions between proteins encoded by genes that show significant changes (fold change of 2 or higher) in RT-qPCR analysis. The blue nodes represent the proteins included in our RT-qPCR gene panel, while the others represent other proteins that could potentially interact with them.

autophagic proteins LC3B I/II and SQSTM. However, the results showed that casticin treatment did not significantly alter the ratio of LC3BI/II and SQSTM/β-actin in KG1a and KG1 cell lines compared to the control groups ($p>0.05$) (Figure 5C–F).

Western blotting analyses of the most interactive proteins (Shh, Gli and Wnt) showed that 2 µM casticin treatment caused a decrease in Gli/β-actin ratio in KG1a, KG1 cell lines after 2 µM casticin treatment compared to their control groups (KG1a and KG1 $p<0.01$) (Figure 6A and B). When we assessed the transcriptor factor of the Hedgehog pathway, we also found a decrease in Shh/β-actin ratio in KG1a and KG1 cell lines compared to their control groups (KG1a $p<0.05$; KG1 $p<0.01$) (Figure 6C and D). However, different results were obtained in the two cell lines for Wnt protein expressions. Following casticin treatment a decrease in Wnt protein expression was found for KG1a cells ($p<0.05$), but no significant changes was found in KG1cells ($p>0.05$) (Figure 6E and F).

Discussion

In our present study, we investigated the underlying molecular mechanism of casticin-induced cell death by focusing on signaling pathways in *in vitro* LSC-like cell and AML blast models at both gene and protein expression levels.

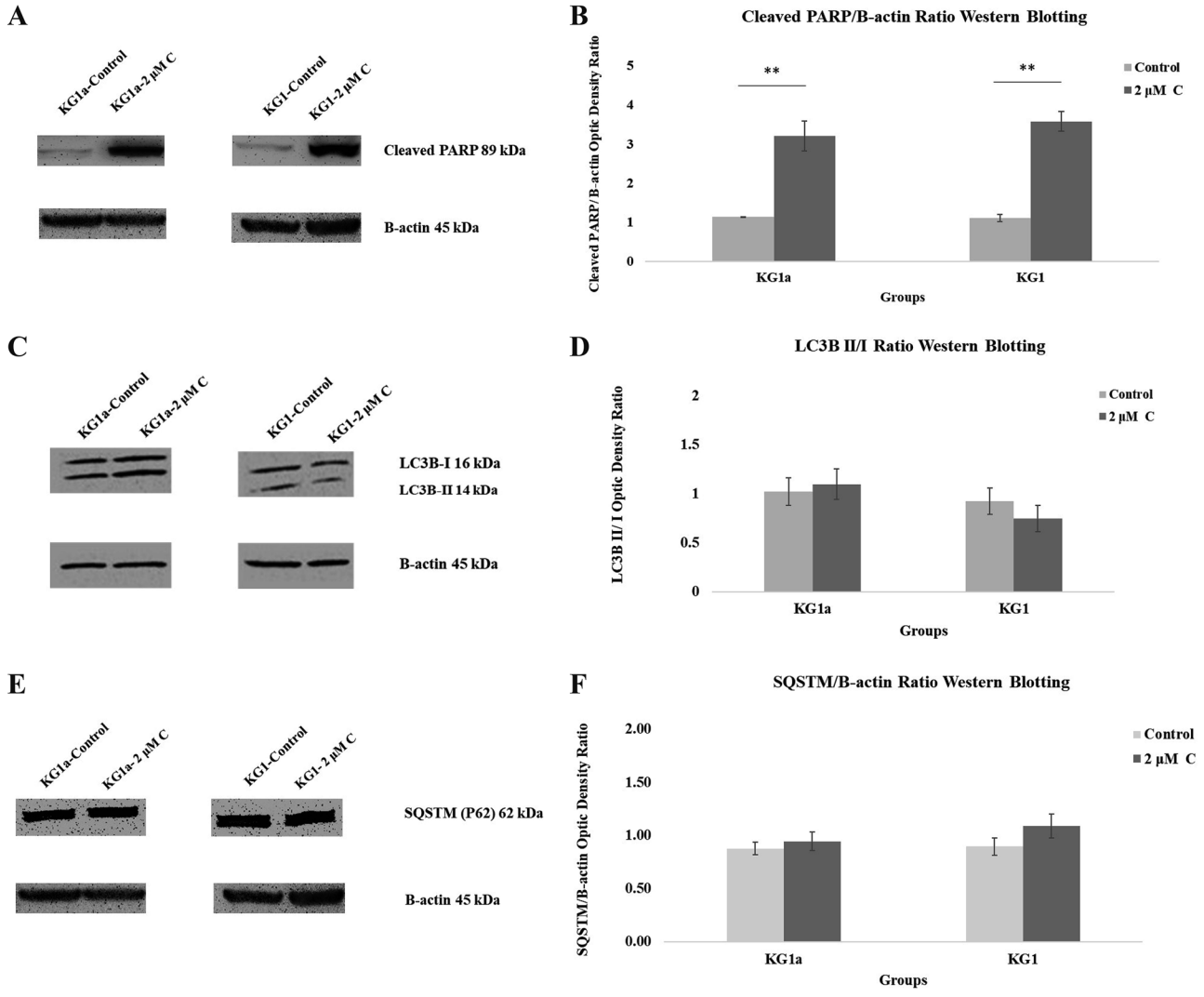

Figure 5: Western blotting results of apoptosis and autophagy. (A) Western blot images of cleaved PARP and β-actin levels of casticin-treated KG1a and KG1 cell lines and their non-treated control groups, respectively. (B) Comparison of the cleaved PARP/β-actin optic density ratio (mean ± SEM) with corresponding control groups (** $p<0.01$). (C) Western blot images of LC3B-I, LC3B-II, and β-actin protein levels of casticin-treated KG1a and KG1 cell lines and their non-treated control groups. (D) Comparison of the LC3B I/LC3B II optic density ratio (mean ± SEM) with corresponding control groups. (E) Western blot images of SQSTM (p62) and β-actin levels of casticin-treated KG1a and KG1 cell lines and their non-treated control groups. (F) Comparison of the SQSTM/β-actin optic density ratio (mean ± SEM) with corresponding control groups.

The first aspect investigated in this study was the suitability of the *in vitro* models. A major feature of the LSCs is to re-initiate AML and a study found that CD34+CD38− subpopulations were consistently successful in engrafting in *in vivo* models of AML [31]. Therefore, to confirm our cell lines as an *in vitro* stem cell-like and blast model, we evaluated the CD34+ CD38- expression features of KG1a and KG1 cell lines. Our analyses of revealed that approximately 85 % of KG1a cells have the CD34+, CD38-phenotype, while 94 % of KG1 cells have the CD34+, CD38+ phenotype. In addition, it is known that KG1 cell line is the parental cell line of KG1a and unlike KG1, KG1a cell line is insensitive to the differentiation [24–32]. Furthermore, the French-American-British (FAB) classification system, which classifies cells according to their differentiation degree, categorizes KG1a and KG1 cell lines as M0 and M2, respectively [33]. In the light of the literature and our results, we used KG1a cell line as an *in vitro* stem cell-like model. Although KG1 cell line has also been used as a stem cell model in some studies [34, 35], based on our result, we used KG1 cell line as AML blast model to evaluate the effects of casticin on AML cells with different degrees of differentiation.

Aldehyde dehydrogenase 1A1 (ALDH1A1) is the predominant isoform of ALDH in mammals, protecting both

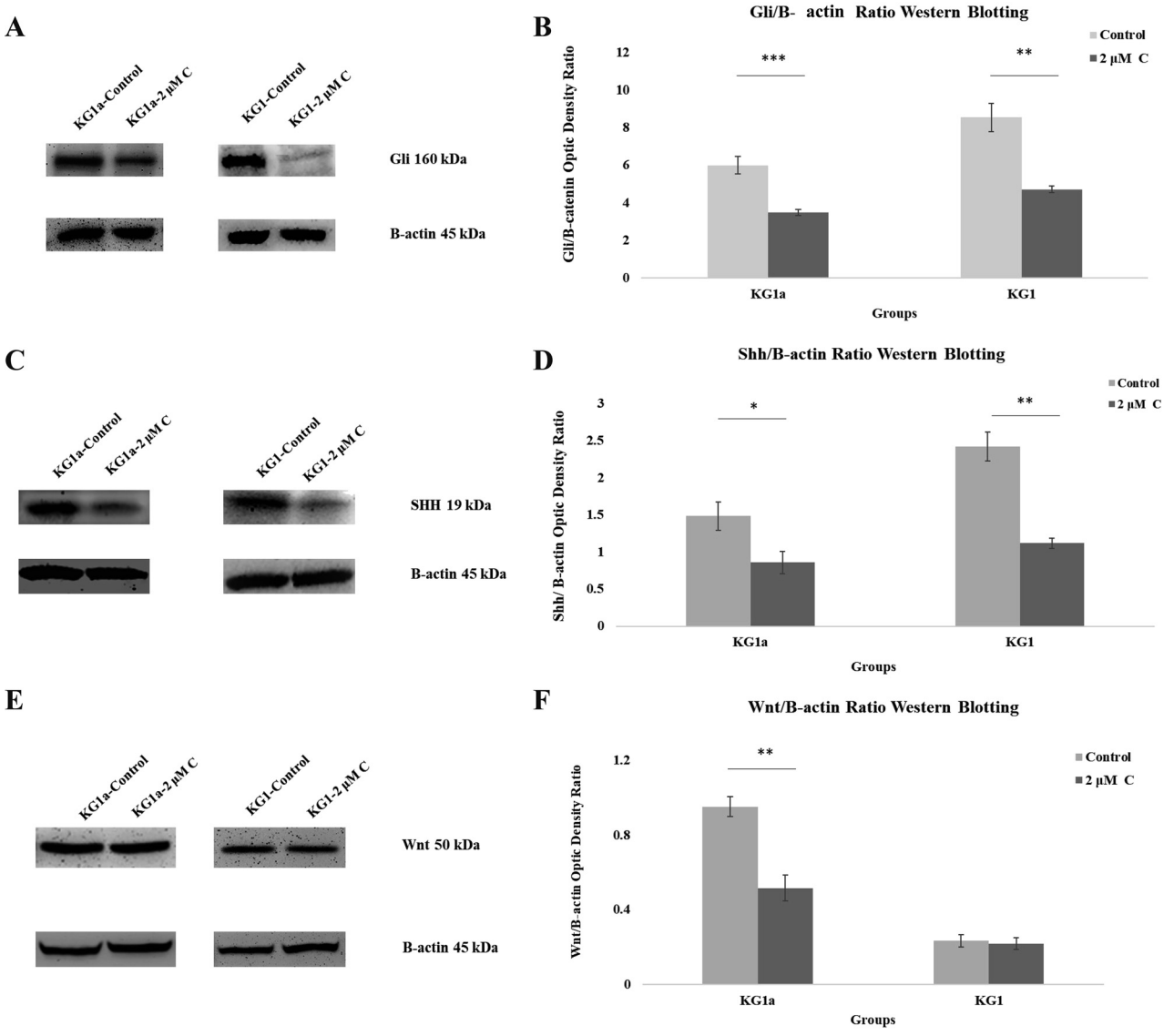

Figure 6: Western blotting results of signaling pathways. (A) Western blot images of gli and β-actin levels of casticin-treated KG1a and KG1 cell lines and their non-treated control groups. (B) Comparison of the gli/β-actin optic density ratio (mean ± SEM) with corresponding control groups (**:p<0.01; ***:p<0.001). (C) Western blot images of Shh and β-actin levels of casticin-treated KG1a and KG1 cell lines and their non-treated control groups. (D) Comparison of the Shh/β-actin optic density ratio (mean ± SEM) with corresponding control groups (*:p<0.05; **:p<0.01). (E) Western blot images of Wnt and β-actin levels of casticin-treated KG1a and KG1 cell lines and their non-treated control groups. (F) Comparison of the Wnt/β-actin optic density ratio (mean ± SEM) with corresponding control groups (**:p<0.01).

hematopoietic stem cells and leukemia stem cells against reactive aldehyde metabolites [36]. ALDH1A1 is accepted as a metabolic marker of leukemia stem cells [37] and CD34+CD38− leukemic stem cells isolated from AML patients show high ALDH activity [38]. In our study, we found a significant increase in ALDH1A1 mRNA expression LSC-like KG1a cells following casticin treatment. We think that the increase in ALDH1A1 gene expression in KG1a cells may be a response to casticin-mediated anti-cancer effect. However, to fully understand the role of ALDH1A1 in casticin-mediated anti-leukemic effect, further research is needed.

Furthermore, we confirmed apoptosis-triggering effect of casticin in AML LSC-like cells and blasts through increased levels of cleaved PARP protein and flow cytometry analysis. We also assessed the role of autophagy in casticin's anti-leukemic effect. Although many studies reported the anti-leukemic effects of some polyphenols occurred via induction of autophagy [39–41], casticin treatment did not induced

autophagic flux, evaluated by assessing LC3B II/LC3B I and SQSTM (p62) levels in KG1a and KG1 cell lines. Therefore, we speculate that casticin may not induce autophagy in LSC-like cells and blasts in AML, but it has the capacity to trigger apoptosis in both cells.

Studies have shown that maintenance of LSC survival and drug resistance are ensured by survival signaling pathways, particularly the Hedgehog and Wnt-β-catenin pathways in AML [11–43]. Shh, a Hedgehog pathway ligand, binds to the transmembrane patched (PTC) receptor, which regulates the translocation of Smoothened (SMO) into the cilium to alter the function of Gli transcription factors for survival effect [44]. Studies reported that *GLI1* gene is over-expressed in relapsed or drug-resistant AML patients [12–45]. Zhang et al. reported that casticin targets the Hedgehog signaling pathway and reduces key epithelial-mesenchymal transition factors, potentially decreasing cancer cell migration in the ovarian cancer SKOV3 cell line [46]. In our study, casticin treatment led to decrease in *SHH* and *GLI* mRNA expression, which was also confirmed at protein level. To suppress hedgehog pathway, Glasdegib, a SMO inhibitor, has been used in AML treatment for several years, but research showed that inhibition of SMO is not sufficient to trigger cell death due to the major survival effect of Gli, especially in LSCs [47–50]. Therefore, we think that casticin-mediated apoptosis through inhibition of Shh ligand and Gli transcription factor in LSC-like cells and blasts might be a promising outcome for drug development.

In addition to Shh pathway, studies also showed that Wnt/β-catenin pathway also supports LSC maintenance directly or through interaction with oncogenic pathways such as Hedgehog [14, 15] and inhibition of Wnt leads to LSC death [51, 52]. In parallel with the literature, we detected decreased WNT mRNA expression after casticin treatment in the cell lines. However, in protein level, the decrease of Wnt was only confirmed for KG1a cells. Therefore, we speculated that it might be related to higher dependence of LSCs on Wnt/β-catenin signaling for survival. Moreover, Su et al. reported that Shh activation promotes cell survival via interaction with Wnt/β-catenin signaling in CD34+ chronic myeloid leukemia cells [53] and our pathway interaction analysis revealed that there is a possible crosstalk between Hedgehog signaling and Wnt. Hence, further studies are needed to uncover possible crosstalk between Hedgehog signaling and Wnt in casticin-induced cell death in AML.

In this study, we aimed to understand the effects of casticin on AML LSC-like cell line and its mature form. However, we believe that further studies, including an *in vivo* AML model that enables us to consider AML micro-environment and drug metabolism, might overcome the limitations of the study. Moreover, our bioinformatics analyses indicated a possible crosstalk between Hedgehog and the proteins of the MAPK, PI3K/Akt/mTOR, Notch, Hippo signaling cascades after treatment with casticin. Therefore, we think that investigating oncogenic pathway interactions may enhance long-term treatment efficacy in AML, but further studies are necessary to explore these interactions.

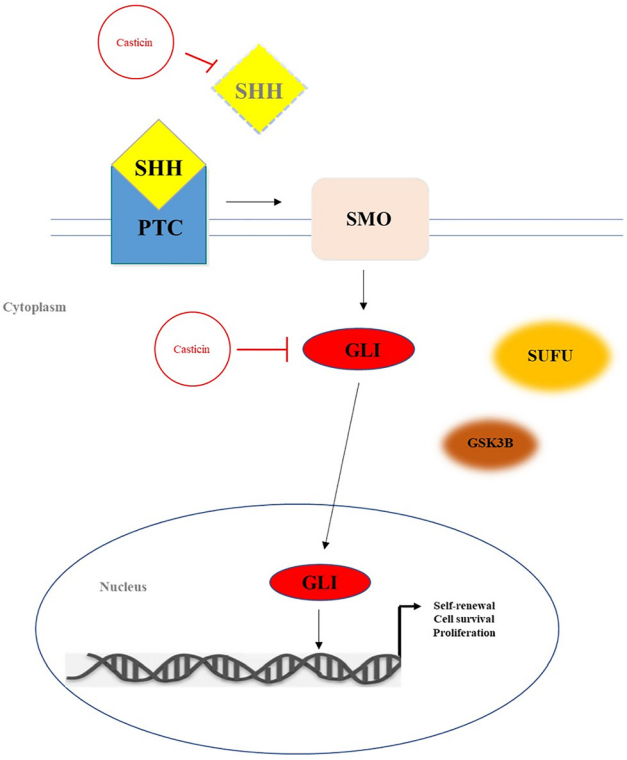

Figure 7: The possible effect of casticin on LSCs and leukemic cells.

In conclusion, our study demonstrated for the first time that casticin induces apoptosis in AML LSC-like cells and blasts primarily by suppressing the Hedgehog signaling pathway (Figure 7). Based on these results, we propose that casticin holds a potential as a candidate for AML drug development.

Research ethics: The local Institutional Review Board deemed the study exempt from review.
Informed consent: Not applicable.
Author contributions: concept – S.K., T.E.; design – S.K., K.Y.A., T.E., G.G.; supervision – S.K., K.Y.A.; resources – S.K., K.Y.A., H.A.; materials – S.K., T.E., H.A.; data collection and/or processing – T.E., K.Y.A., G.G., H.A.; analysis and/or interpretation – T.E., S.K., K.Y.A., G.G., H.A.; literature search –T.E., S.K., K.Y.A., G.G.; writing –T.E., S.K., K.Y.A., G.G.; critical reviews – S.K., K.Y.A., T.E., G.G., H.A.

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Data availability: The data supporting the findings of this study can be accessed by contacting the corresponding author, S.K., upon a reasonable request.

References

1. Döhner H, Estey EH, Amadori S, Appelbaum FR, Büchner T, Bloomfield CD, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood 2010;115: 453–74.
2. Crossnohere NL, Richardson DR, Reinhart C, O'Donoghue B, Love SM, Smith BD, et al. Side effects from acute myeloid leukemia treatment: results from a national survey. Curr Med Res Opin 2019; 35:1965–70.
3. Neuendorff NR, Loh KP, Mims AS, Christofyllakis K, Soo WK, Bölükbasi B, et al. Anthracycline-related cardiotoxicity in older patients with acute myeloid leukemia: a young siog review paper. Blood Advances 2020;4:762–75.
4. Pabon CM, Abbas HA, Konopleva M. Acute myeloid leukemia: therapeutic targeting of stem cells. Expert Opin Ther Targets 2022;26: 547–56.
5. Medeiros BC, Chan SM, Daver NG, Jonas BA, Pollyea DA. Optimizing survival outcomes with post-remission therapy in acute myeloid leukemia. Am J Hematol 2019;94:803–11.
6. Kantarjian H, Kadia T, DiNardo C, Daver N, Borthakur G, Jabbour E, et al. Acute myeloid leukemia: current progress and future directions. Blood Cancer J 2021;11:41.
7. Stelmach P, Trumpp A. Leukemic stem cells and therapy resistance in acute myeloid leukemia. Haematologica 2023;108:353.
8. Zeijlemaker W, Grob T, Meijer R, Hanekamp D, Kelder A, Carbaat-Ham JC, et al. CD34+CD38− leukemic stem cell frequency to predict outcome in acute myeloid leukemia. Leukemia 2019;33:1102–12.
9. Goel H, Kumar R, Tanwar P, Upadhyay TK, Khan F, Pandey P, et al. Unraveling the therapeutic potential of natural products in the prevention and treatment of leukemia. Biomed Pharmacother 2023; 160. https://doi.org/10.1016/j.biopha.2023.114351.
10. Maleki Dana P, Sadoughi F, Asemi Z, Yousefi B. The role of polyphenols in overcoming cancer drug resistance: a comprehensive review. Cell Mol Biol Lett 2022;27:1–26.
11. Fukushima N, Minami Y, Kakiuchi S, Kuwatsuka Y, Hayakawa F, Jamieson C, et al. Small-molecule hedgehog inhibitor attenuates the leukemia-initiation potential of acute myeloid leukemia cells. Cancer Sci 2016;107:1422–9.
12. Huang K, Sun Z, Ding B, Jiang X, Wang Z, Zhu Y, et al. Suppressing hedgehog signaling reverses drug resistance of refractory acute myeloid leukemia. OncoTargets Ther 2019;12:217.
13. Lainez-González D, Serrano-López J, Alonso-Domínguez JM. Understanding the hedgehog signaling pathway in acute myeloid leukemia stem cells: a necessary step toward a cure. Biology 2021;10: 255.
14. Pelullo M, Zema S, Nardozza F, Checquolo S, Screpanti I, Bellavia D. Wnt, Notch, and TGF-β pathways impinge on hedgehog signaling complexity: an open window on cancer. Front Genet 2019;10:711.
15. Rodrigues ACBC, Costa RGA, Silva SLR, Dias IRSB, Dias RB, Bezerra DP. Cell signaling pathways as molecular targets to eliminate AML stem cells. Crit Rev Oncol Hematol 2021;160. https://doi.org/10.1016/j.critrevonc.2021.103277.
16. Hwang D, Kim M, Park H, Jeong MI, Jung W, Kim B. Natural products and acute myeloid leukemia: a review highlighting mechanisms of action. Nutrients 2019;11:1010.
17. Khatoon E, Banik K, Harsha C, Sailo BL, Thakur KK, Khwairakpam AD, et al. Phytochemicals in cancer cell chemosensitization: current knowledge and future perspectives. Semin Cancer Biol 2022:306–39. https://doi.org/10.1016/j.semcancer.2020.06.014.
18. Yan CX, Wei YW, Li H, Xu K, Zhai RX, Meng DC, et al. A review on their traditional medicine, phytochemistry, Pharmacology. J Ethnopharmacol 2023. https://doi.org/10.1016/j.jep.2023.116273.
19. Jin J, Shen J-K, Du H, Yang M, Wang Y-G. Apoptosis was induced by casticin in acute myelocytic leukemia cells. Blood 2008;112. https://doi.org/10.1182/blood.v112.11.3991.3991.
20. Shen JK, Du HP, Yang M, Wang YG, Jin J. Casticin induces leukemic cell death through apoptosis and mitotic catastrophe. Ann Hematol 2009; 88:743–52.
21. Carbone K, Gervasi F, Kozhamzharova L, Altybaeva N, Sönmez Gürer E, Sharifi-Rad J, et al. Casticin as potential anticancer agent: recent advancements in multi-mechanistic approaches. Front Mol Biosci 2023; 10. https://doi.org/10.3389/fmolb.2023.1157558.
22. Righeschi C, Eichhorn T, Karioti A, Bilia AR, Efferth T. Microarray-based MRNA expression profiling of leukemia cells treated with the flavonoid, casticin. Cancer Genomics Proteomics 2012;9.
23. Erkmen T, Sert Serdar B, Ates H, Keskinoglu P, Kocturk S. Casticin: a promising candidate to develop a stem cell targeted strategy in AML treatment. The J Basic and Clin Hea Sci 2020;4:297–304.
24. Koeffler HP, Billing R, Lusis AJ, Sparkes R, Golde DW. An undifferentiated variant derived from the human acute myelogenous leukemia cell line (KG-1). Blood 1980;56. https://doi.org/10.1182/blood.v56.2.265.265.
25. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods 2011;25: 402–8.
26. Kamburov A, Stelzl U, Lehrach H, Herwig R. The ConsensusPathDB interaction database: 2013 update. Nucleic Acids Res 2013;41: D793–800.
27. Blake JA, Christie KR, Dolan ME, Drabkin HJ, Hill DP, Ni L, et al. Gene ontology consortium: going forward. Nucleic Acids Res 2015;43: D1049–56.
28. Kanehisa M, Goto S, Sato Y, Kawashima M, Furumichi M, Tanabe M. Data, information, knowledge and principle: back to metabolism in KEGG. Nucleic Acids Res 2014;42:D199–205.
29. Jassal B, Matthews L, Viteri G, Gong C, Lorente P, Fabregat A, et al. The reactome pathway knowledgebase. Nucleic Acids Res 2020;48: D649–55.
30. Oughtred R, Stark C, Breitkreutz BJ, Rust J, Boucher L, Chang C, et al. The BioGRID interaction database: 2019 update. Nucleic Acids Res 2019;47: D529–41.
31. Ng SWK, Mitchell A, Kennedy JA, Chen WC, McLeod J, Ibrahimova N, et al. A 17-gene stemness score for rapid determination of risk in acute leukaemia. Nature 2016;540:433–7.
32. Fardel O, Payen L, Courtois A, Drenou B, Fauchet R, Rault B. Differential expression and activity of P-glycoprotein and multidrug resistance-

associated protein in CD34-positive KG1a leukemic cells. Int J Oncol 1998;12:315–24.
33. Sarde A, Eckard S, Mei L, Ruegg C, Chun P, Smith V. Selectivity of T Cell engager AMV564 against different leukemic blast populations and potential application for patient selection. Blood 2020;136:25–6.
34. Wang F, Huang X, Sun Y, Li Z, Sun R, Zhao T, et al. Sulforaphane regulates the proliferation of leukemia stem-like cells via sonic hedgehog signaling pathway. Eur J Pharmacol 2022;919. https://doi.org/10.1016/j.ejphar.2022.174824.
35. Yu W, Lutz C, Krämer A, Schmidt-Zachmann MS. The JmjC-domain protein NO66/RIOX-1 affects the balance between proliferation and maturation in acute myeloid leukemia. Exp Cell Res 2021;402. https://doi.org/10.1016/j.yexcr.2021.112566.
36. Venton G, Pérez-Alea M, Baier C, Fournet G, Quash G, Labiad Y, et al. Aldehyde dehydrogenases inhibition eradicates leukemia stem cells while sparing normal progenitors. Blood Cancer J 2016;6:e469–9.
37. Gerber JM, Smith BD, Ngwang B, Zhang H, Vala MS, Morsberger L, et al. A clinically relevant population of leukemic CD34-CD38 - cells in acute myeloid leukemia. Blood 2012;119:3571–7.
38. Dancik GM, Voutsas IF, Vlahopoulos S. Lower RNA expression of ALDH1A1 distinguishes the favorable risk group in acute myeloid leukemia. Mol Biol Rep 2022;49:3321–31.
39. Pan Y, Chen L, Li R, Liu Y, Nan M, Hou L. Tanshinone IIA induces autophagy and apoptosis via PI3K/Akt/mTOR axis in acute promyelocytic leukemia NB4 cells. Evid Based Compl Alternat Med 2021;2021:3372403–9.
40. Shi C, Lan W, Wang Z, Yang D, Wei J, Liu Z, et al. Alantolactone inhibits cell autophagy and promotes apoptosis via AP2M1 in acute lymphoblastic leukemia. Cancer Cell Int 2020;20:442.
41. Xiao J, Zhang B, Yin S, Xie S, Huang K, Wang J, et al. Quercetin induces autophagy-associated death in HL-60 cells through CaMKKβ/AMPK/mTOR signal pathway. ABBS 2022;54:1244–56.
42. Saygin C, Matei D, Majeti R, Reizes O, Lathia JD. Targeting cancer stemness in the clinic: from hype to hope. Cell Stem Cell 2019;24:25–40.
43. Garg S, Shanmukhaiah C, Marathe S, Mishra P, Babu Rao V, Ghosh K, et al. Differential antigen expression and aberrant signaling via PI3/AKT, MAP/ERK, JAK/STAT, and wnt/β catenin pathways in lin-/CD38-/cd34+ cells in acute myeloid leukemia. Eur J Haematol 2016;96:309–17.
44. Carballo GB, Honorato JR, De Lopes GPF, Spohr TCLDSE. A highlight on sonic hedgehog pathway. Cell Commun Signal 2018;16:1–15.
45. Li X, Chen F, Zhu Q, Ding B, Zhong Q, Huang K, et al. Gli-1/PI3K/AKT/NF-KB pathway mediates resistance to radiation and is a target for reversion of responses in refractory acute myeloid leukemia cells. Oncotarget 2016;7:33004.
46. Zhang J, Cui Y, Sun S, Cao J, Fang X. Casticin inhibits the epithelial-mesenchymal transition in ovarian carcinoma via the hedgehog signaling pathway. Oncol Lett 2018;15:4495–502.
47. Jamieson C, Martinelli G, Papayannidis C, Cortes JE. Hedgehog pathway inhibitors: a new therapeutic class for the treatment of acute myeloid leukemia. Blood Cancer Discov 2020;1:134–45.
48. Long B, Wang LX, Zheng FM, Lai SP, Xu DR, Hu Y, et al. Targeting GLI1 suppresses cell growth and enhances chemosensitivity in CD34 + enriched acute myeloid leukemia progenitor cells. Cell Physiol Biochem 2016;38:1288–302.
49. Desch P, Asslaber D, Kern D, Schnidar H, Mangelberger D, Alinger B, et al. Inhibition of GLI, but not smoothened, induces apoptosis in chronic lymphocytic leukemia cells. Oncogene 2010;29:4885–95.
50. Wellbrock J, Behrmann L, Muschhammer J, Modemann F, Khoury K, Brauneck F, et al. The BET bromodomain inhibitor ZEN-3365 targets the hedgehog signaling pathway in acute myeloid leukemia. Ann Hematol 2021;100:2933–41.
51. Sakoda T, Kikushige Y, Miyamoto T, Irifune H, Harada T, Hatakeyama K, et al. TIM-3 signaling hijacks the canonical wnt/β-catenin pathway to maintain cancer stemness in acute myeloid leukemia. Blood Adv 2023:2053–65. https://doi.org/10.1182/bloodadvances.2022008405.
52. Pepe F, Bill M, Papaioannou D, Karunasiri M, Walker A, Naumann E, et al. Targeting Wnt signaling in acute myeloid leukemia stem cells. Haematologica 2022;107:307.
53. Su W, Meng F, Huang L, Zheng M, Liu W, Sun H. Sonic hedgehog maintains survival and growth of chronic myeloid leukemia progenitor cells through β-catenin signaling. Exp Hematol 2012;40:418–27.

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