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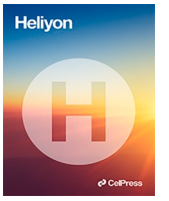

Asperuloside regulates the proliferation, apoptosis, and differentiation of chronic myeloid leukemia cell line K562 through the RAS/MEK/ERK pathway

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ABSTRACT

Context: Chronic myeloid leukemia (CML) is a malignant hematopoietic stem cell disease caused by excessive proliferation and abnormal differentiation of hematopoietic stem cells. *Asperuloside* (ASP) is considered to have good biological activity and may be a good anti-CML drug.
Objective: This study aimed to explore the effects and possible mechanisms of ASP on the biological behavior of K562 cells based on RNA-seq.
Materials and methods: The IC_{50} of ASP in K562 cells was calculated by the concentration-effect curve. Cell viability, apoptosis, and differentiation were detected by CCK8, flow cytometry, benzidine staining, and WB analysis, respectively. Further, RNA-seq was used to analyze the possible mechanism of ASP regulating K562 cells.
Results: ASP significantly inhibited the proliferation, and promoted apoptosis and differentiation of K562 cells. A total of 117 differentially expressed genes were screened by RNA-seq, mainly involved in the RAS/MEK/ERK pathway. PD98059 was used to inhibit the RAS/MEK/ERK pathway in K562 cells, and results confirmed that PD98059 could not only inhibit the RAS/MEK/ERK pathway, but also inhibit the regulation of ASP on the proliferation and differentiation of K562 cells.
Conclusion: ASP inhibited the proliferation, promoted apoptosis and differentiation of K562 cells by regulating the RAS/MEK/ERK pathway, and played a good anti-CML role.

1. Introduction

Chronic myeloid leukemia (CML) is a malignant clonal disease originating from pluripotent hematopoietic stem cells in the bone marrow, accounting for approximately 20 % of all adult leukemias [1]. Usually, CML starts with a chronic phase to an accelerated phase, then ends in a terminal phase called a blast crisis, which seriously affects the life and health of patients [2]. Epidemiological analysis showed that CML was common in adults, and the incidence gradually increased with age [3]. The main pathogenesis of CML is the translocation between the short arm of chromosome 9 and the long arm of chromosome 22 at the chromosome level, resulting in

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the generation of the BCR-ABL1 fusion gene [4]. BCR-ABL1 has strong tyrosine kinase activity, which is involved in the regulation of excessive proliferation and impaired differentiation of hematopoietic stem cells, and plays a crucial role in the occurrence of CML [5]. At present, the therapeutic drugs used in clinical practice, such as imatinib and rofitinib, are tyrosine kinase inhibitors, and have achieved good clinical therapeutic effects [6,7]. However, there are still some patients who lack or have poor responses to tyrosine kinase inhibitors [8]. Therefore, it is particularly important to develop new therapeutic modalities for CML.

Asperuloside (ASP) is an iridoid compound extracted from Rubiaceae or Eucommiaceae plants [9]. In recent years, pharmacological studies have shown that ASP has a variety of pharmacological activities, such as anti-inflammatory, analgesic, lowering blood pressure, and anti-cell chromosome mutagenesis [10]. In addition, as one of the members of the iridoid, ASP also has a good potential for anti-cancer [11]. It is cytotoxic to human breast cancer, leukemia, and oral cancer cells and can be used as a potential anticancer compound [12]. Previous studies have shown that ASP plays an anti-leukemia role by inducing endoplasmic reticulum stress-induced apoptosis in leukemia cell lines and primary human leukemia blasts [13]. However, the effects of ASP on CML remain unclear. Based on its excellent anticancer potential, we speculate that ASP may also play an important role in anti-CML.

The maintenance of undifferentiated cells and increased proliferation levels are typical characteristics of CML [14]. Inhibiting the proliferation of malignant cells and promoting their normal differentiation is the focus of the current treatment of hematological malignancies [15]. K562, a cell line isolated from CML patients, has the characteristics of stem cells and is more similar to undifferentiated early multipotent hematopoietic progenitor cells in behavior [16]. It is an ideal model for inducing cell differentiation [17]. It is an effective way to explore new therapeutic drugs for CML by analyzing the effects on K562 proliferation and differentiation.

In this study, the K562 cell line was selected as the research object to observe the effects of ASP on the proliferation, apoptosis, and differentiation of K562 cells, hypothesizing that ASP can play an anti-CML role by regulating the progression of K562 cells, and the possible mechanism was explored based on RNA-seq. The research results can provide a new idea for the treatment of CML, and provide a solid theoretical basis for the wider clinical application of ASP, which has the potential for scientific innovation and research and development application.

2. Materials and methods

2.1. Cell culture

Human chronic myeloid leukemia cells K562 was purchased from Procell Life Science & Technology Co., Ltd. (cat. no. CL-0130, China) and authenticated by STR profiling. Cells were cultured in IMDM (Procell, China) containing 10 % fetal bovine serum (FBS; cat. no. 164210–50, Procell, China) and 1 % Penicillin-Streptomycin Solution (cat. no. PB180120, Procell, China) with an incubator at 37 °C with 5 % CO_2.

ASP was obtained from Solarbio (cat. no. SA9770, China). K562 cells were treated with 0.1 % DMSO as the control and 0, 200, 400, 800, 1600 μg/mL ASP for 24 h, separately, and the IC_{50} of ASP was detected. Subsequent experiments were performed using 1/2 IC_{50}, IC_{50}, and 2 IC_{50} ASP to test the effects of ASP on K562 cells.

2.2. Cell viability analysis

K562 cells were inoculated into 96-well culture plates at a density of 5×10^3 per well and treated with different concentrations of ASP (0, 200, 400, 800, 1600 μg/mL) for 24 h. Cell viability was detected using a Cell Counting Kit-8 (CCK8; Beyotime, China) at 37 °C for 1 h [18]. The absorbance was measured at 450 nm using a microplate spectrophotometer (cat. no. 1681150; Bio-Rad, USA) and cell viability was calculated as follows: Cell viability (%) = $(OD_{experimental} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100$.

2.3. Apoptosis analysis

Cells with different treatments were collected, washed by PBS and resuspended. About 5×10^5 suspended cells were centrifuged at 300 *g* for 5 min, and the supernatant was discarded. The cells were suspended with 100 μL diluted 1 × Annexin V Binding Buffer, and 2.5 μL Annexin V-APC and 2.5 μL PI staining solution (Elabscience, China) were added to cell suspension for incubating at room temperature away from light for 15–20 min. Then, 400 μL diluted 1 × Annexin V Binding Buffer was added, and the samples were mixed and detected by flow cytometry (Thermo, USA) [19].

2.4. Benzidine staining

K562 cells were stained with peroxidase staining solution (benzidine method, cat. no. G2370, Solarbio, China) to analyze the erythroid differentiation ability of K562 cells [20]. Briefly, K562 cells under different treatments were fixed with pre-cooled BFA fixative at 4 °C for 30–60 s, and slightly washed. Then the cells were incubated with the prepared POX incubation solution for 10–15 min at room temperature under darkness, and washed with water for 2 min. After incubation with WG staining solution for 30–60 s, the same volume of WG buffer was added, and the staining was performed for 10–15 min. The staining results were observed with a microscope (Nikon Corporation, Japan).

2.5. Western blot analysis

K562 cells undergoing different treatments were collected separately. Total cellular proteins were obtained by lysing cells in RIPA buffer (Solarbio, China) and the proteins were determined by the BCA Protein Assay Kit (Solarbio, China). Protein samples were denatured, separated via SDS-PAGE, and subsequently transferred onto PVDF membranes (EMD Millipore). The membranes were blocked for 1 h at room temperature in TBST containing 5 % skimmed milk, then incubated overnight at 4 °C with anti-β-actin (1:4000; cat. no. ab8227), anti-GATA-1 (1:1000; cat. no. ab133274), anti-β-globin (1:1000; cat. no. ab214049), anti–NF–E2 (1:1000; cat. no. ab140598), anti-CD61 (1:1000; cat. no. ab179473), anti-CD41 (1:1000; cat. no. ab134131), anti-RAS (1:1000; cat. no. ab52939), anti-RAF (1:1000; cat. no. ab200653) anti-p-MEK (1:1000; cat. no. ab278723), anti-MEK (1:1000; cat. no. ab178876) anti-p-ERK (1:1000; cat. no. ab201015), anti-ERK (1:1000; cat. no. ab184699) antibodies all purchased from Abcam (Cambridge, MA, USA). After washing three times with TBST, the membranes were incubated with HRP-conjugated secondary antibody (1:4000; cat. no. ab205718; Abcam) for 2 h at room temperature. Proteins on the membranes were visualized with an enhanced chemiluminescence detection kit (Bio-Rad Laboratories, Inc.) using ChemiScope 6000 (Clinx Science, China). Densitometry was performed using ImageJ software version 1.8.0, and relative protein expression was expressed as the ratio of target protein/β-actin [19].

2.6. RNA-seq analysis

Total RNA was extracted from the cells in the control group and the IC_{50} ASP group. RNA agarose electrophoresis and Agilent 2100 Bioanalyzer were used to detect the concentration and purity of RNA. The mRNA in total RNA was purified by the polyA structure characteristic of mRNA, and the mRNA was broken down to 200–300 bp fragments by ion disruption. The cDNA was synthesized using RNA as a template. After the construction of PCR-enriched libraries, the double-end Sequencing of these libraries was performed using next-generation Sequencing technology based on the Illumina Sequencing platform [21].

2.7. Statistical analysis

All experimental data were analyzed using SPSS 20.0 software, and expressed as mean ± standard deviation (SD). The differences among groups were statistically compared by one-way analysis of variance (ANOVA) and Tukey HSD *post hoc*, and $P < 0.05$ was considered statistically significant. All experiments were independently repeated at least three times.

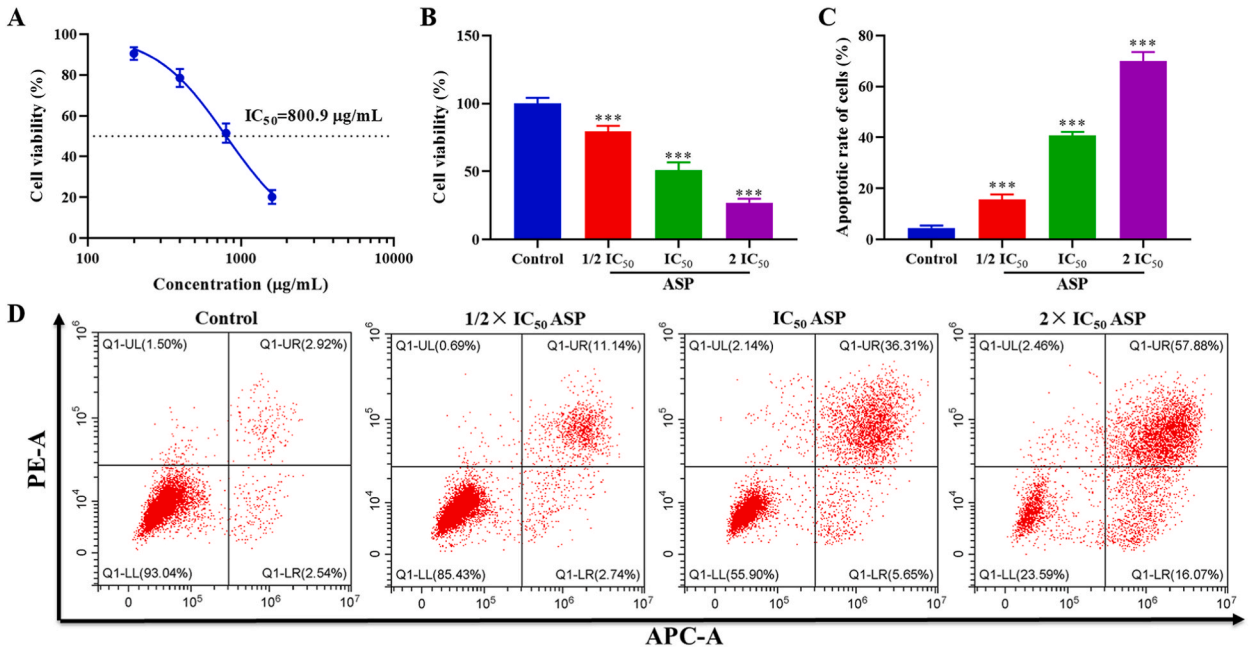

Fig. 1. ASP inhibited cell proliferation and promoted apoptosis of K562 cells. (A) The concentration-effect curve of ASP in K562 cells. IC_{50}: 800.9 μg/mL **(B)** K562 cell viability after ASP treatment was detected by CCK-8 assay. **(C–D)** Cell apoptosis was verified by flow cytometry in K562 cells. Data were shown as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with the control group. ASP, Asperuloside.

3. Results

3.1. Effects of ASP on proliferation, apoptosis, and differentiation of K562 cells

To explore the effects of ASP on CML, K562 cells were treated with different doses of ASP (0, 200, 400, 800, 1600 μg/mL), and it was found that ASP could inhibit the proliferation of K562 cells in a dose-dependent manner, and its IC_{50} was 800.9 μg/mL (Fig. 1A). Then, K562 cells were treated with ½ × IC_{50}, IC_{50} and 2 × IC_{50} doses of ASP respectively to analyze the effects of ASP on proliferation, apoptosis and differentiation of K562 cells. The CCK-8 results indicated that ASP significantly inhibited the proliferation of K562 cells compared with the control group in a dose-dependent manner (Fig. 1B, $P < 0.001$). And flow cytometry results showed that ASP significantly promoted apoptosis of K562 cells in a dose-dependent manner compared with the control group (Fig. 1C and D, $P < 0.001$). The effects of ASP on K562 cell differentiation were further analyzed, and the results of benzidine staining showed that the rate of positive cells significantly increased after ASP treatment (Fig. 2A and B, $P < 0.001$), and the WB results showed that the protein expressions of GATA-1, β-globin and NF-E2 in the ASP group were significantly up-regulated compared with the control group (Fig. 2C-F, $P < 0.05$), indicating that ASP promoted the erythroid differentiation of K562 cells. In addition, compared with the control group, the protein expressions of megakaryocyte-specific markers CD61 and CD41 were also significantly increased in the ASP group (Fig. 2G-I, $P < 0.001$), suggesting that ASP could also promote the megakaryocyte differentiation of K562 cells. The differentiation promotion effect of ASP was also dose-dependent. These results suggested that ASP could inhibit proliferation, and promote apoptosis and differentiation of K562 cells in a dose-dependent manner.

3.2. ASP activates RAS/MEK/ERK pathway activity in K562 cells

To further analyze the possible mechanism of ASP regulation in K562 cells, RNA-seq sequencing analysis were performed in the

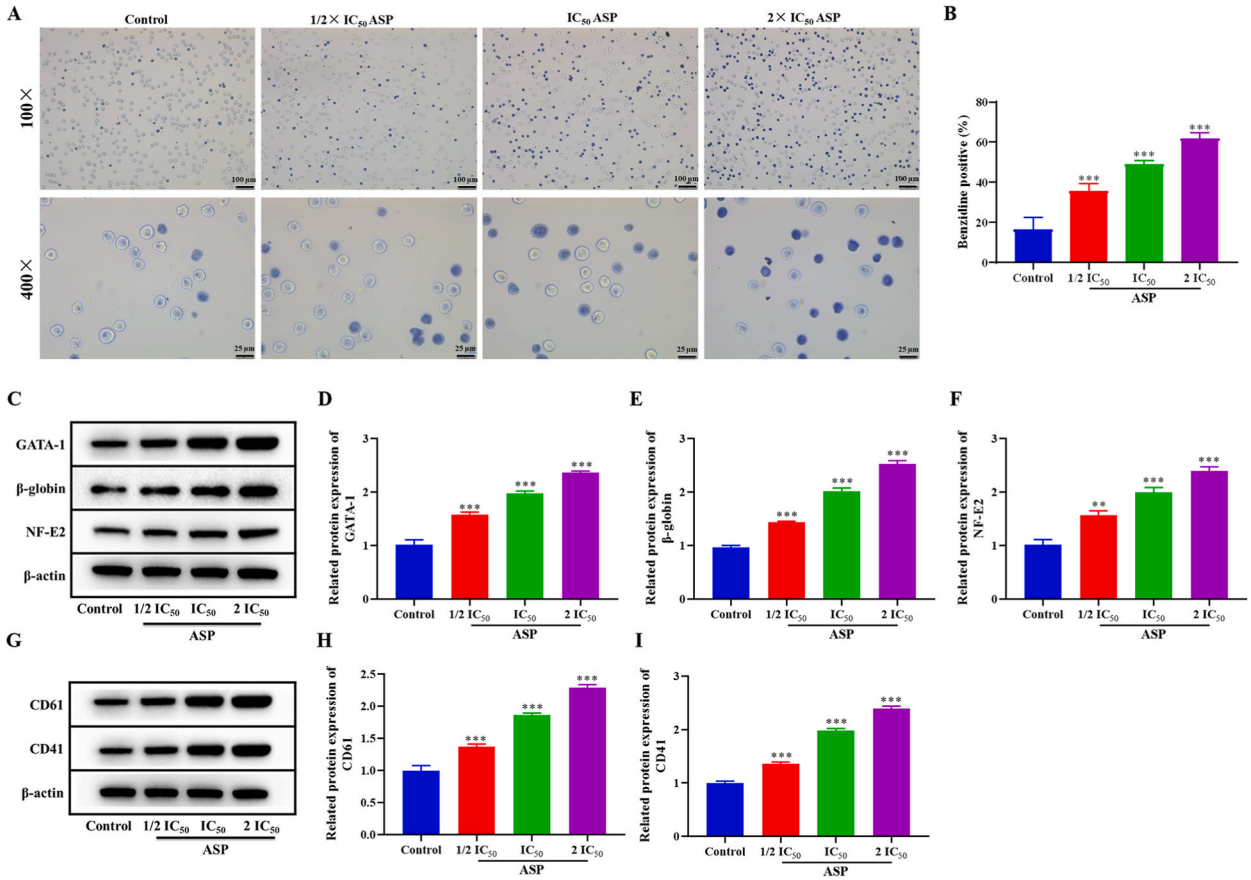

Fig. 2. ASP promoted the differentiation of K562 cells. (A) K562 cells were treated with ASP and then analyzed with benzidine staining assay. Magnification 100 × and 400 × . **(B)** Percentage of positive cells in a benzidine staining. **(C)** Protein expressions of GATA-1, β-globin and NF-E2 in K562 cells were detected by Western blot. **(D)** Related protein expression of GATA-1. **(E)** Related protein expression of β-globin. **(F)** Related protein expression of NF-E2. **(G)** Protein expressions of CD61 and CD41 in K562 cells were detected by Western blot. **(H)** Related protein expression of CD61. **(I)** Related protein expression of CD41. Data were shown as mean ± SD. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, compared with the control group. ASP, Asperuloside.

control and IC_{50} ASP treated groups. The statistical results of gene expression characteristics in each sample are shown in Fig. 3A. Before differential expression analysis, the correlation of gene expression levels among samples was checked first. As shown in Fig. 3B, the correlation coefficient between samples with biological replicates was close to 1, indicating a high correlation between samples. Further principal component analysis (PCA) was performed on each sample according to the expression level, and the results showed that there were significant differences between the control group and the IC_{50} group (Fig. 3C). The DESeq was used for differential analysis of gene expression, and the union of differential genes and samples of all comparison groups were analyzed by bidirectional cluster analysis using the Pheatmap software package in R language, and the results are shown in Fig. 3D. And the ggplots2 software package of R language was used to draw the volcano map of differentially expressed genes (DEGs), as shown in Fig. 3E. Genes down-regulated by IC_{50} compared with control were shown on the left, and genes up-regulated by IC_{50} compared with control were shown on

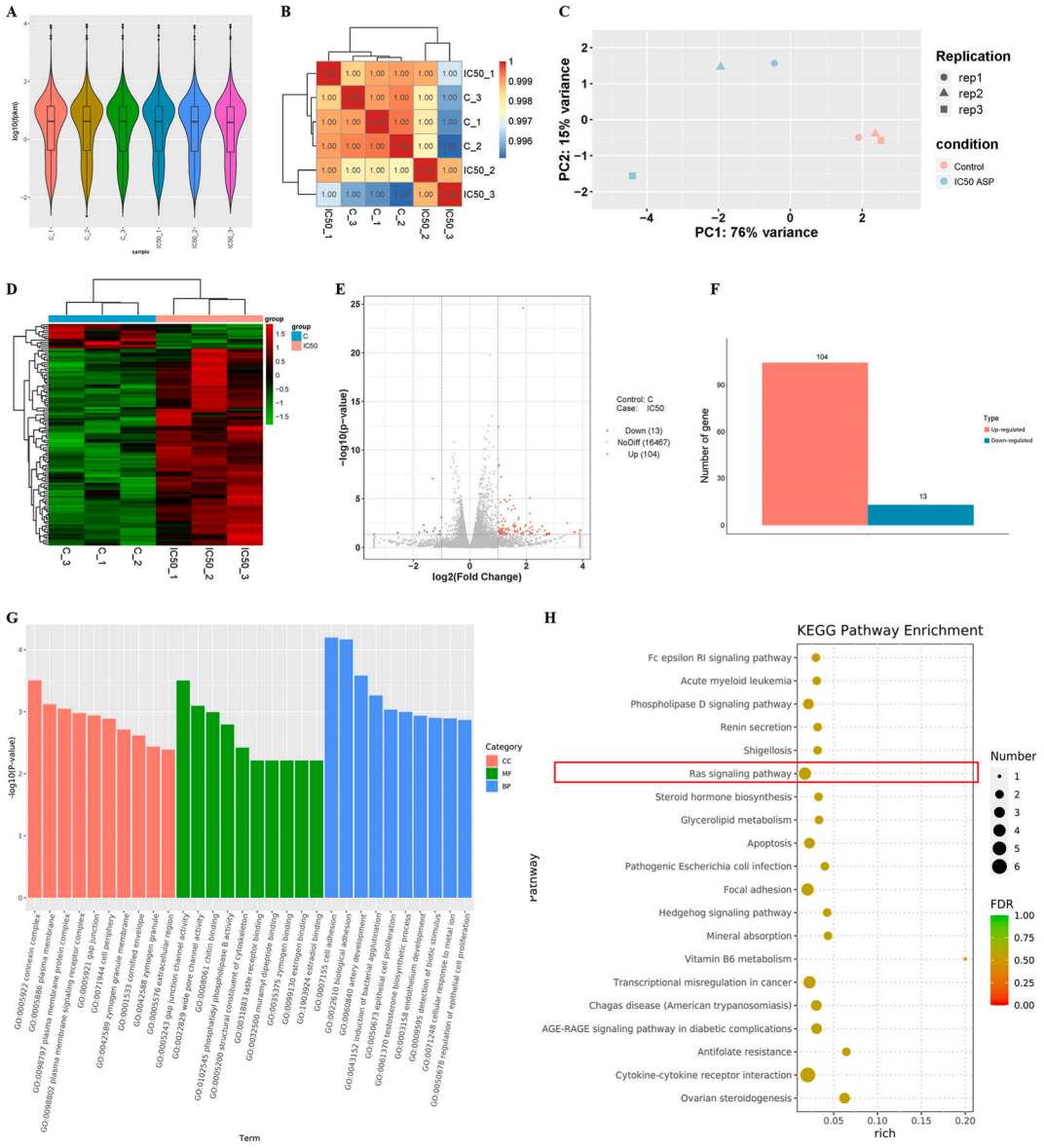

Fig. 3. The possible mechanism of ASP affecting K562 cells was analyzed by RNA-seq. (A) Violin diagram of FPKM density distribution. **(B)** Pearson's correlation coefficient was used to test sample correlation. **(C)** Principal components analysis (PCA) diagram. Different shapes indicate different samples, and different colors indicate different groups. **(D)** Cluster diagram of differentially expressed genes. Genes are shown horizontally, each column is a sample, red is high expression genes, and green is low expression genes. **(E)** Volcanic map of differentially expressed genes. The abscissa is log2(Fold Change), and the ordinate is -log 10 (p-value). Red dots indicate up-regulated genes, blue dots indicate down-regulated genes, and gray dots indicate non-significantly differentially expressed genes. **(F)** Statistical analysis of expression difference results. IC_{50} group compared with the control group. **(G)** Bar chart of GO enrichment analysis of differentially expressed genes. **(H)** KEGG enrichment analysis of differentially expressed genes. C: control group; IC_{50}: IC_{50} ASP treatment group.

the right. A total of 117 significant DEGs (104 up-regulated and 13 down-regulated compared with the control group) were screened between the control and IC50 groups according to the criteria of $|\log2FC|>1$ and $P < 0.05$ (Fig. 3F).

Further analysis of the functional enrichment of DEGs was performed, and GO results showed that in the cellular component group, DEGs were mainly enriched in the "connexin complex", "plasma membrane" and "plasma membrane protein complex", in the molecular function group, DEGs were mainly enriched in "gap junction channel activity", "wide pore channel activity" and "chitin-binding", and in the biological processes group, DEGs were mainly enriched in "cell adhesion", "biological adhesion" and "artery development" (Fig. 3G). In addition, the KEGG results showed that the main differential pathways included the "Ras signaling pathway", "focal adhesion" and "cytokine-cytokine receptor interaction" (Fig. 3H). The Ras signaling pathway is involved in many important cellular processes, such as cell proliferation, differentiation, apoptosis, cytoskeleton movement, protein transport, and secretion. Studies have shown that activation of the ERK pathway through overexpression of RAS can promote erythroid differentiation of K562 cells [22]. The effects of ASP on the expression of RAS/MEK/ERK pathway molecules were further verified by Western blot. The results showed that compared with the control group, the expressions of RAS, RAF, *p*-MEK, and *p*-ERK in the ASP treatment group were significantly increased (Fig. 4A–E, $P < 0.001$), suggesting that ASP could enhance the activity of RAS/MEK/ERK pathway.

3.3. RAS/MEK/ERK pathway was involved in the regulation of ASP on K562 cells

To further elucidate the role of the RAS/MEK/ERK pathway in the ASP regulation of K562 cells, we treated K562 cells with IC_{50} ASP and/or inhibitor PD98059 (20 μM; cat. no. HY-12028, MCE, USA) and performed subsequent assays. Western blot results showed that the inhibitor PD98059 significantly inhibited the expression of RAS/MEK/ERK pathway proteins, and inhibited the activation of the pathway caused by ASP (Fig. 5A–E, $P < 0.001$). In addition, cell proliferation, apoptosis, and differentiation of K562 were analyzed, and CCK8 and flow cytometry results showed that compared with the control group, PD98059 could promote the proliferation and inhibit apoptosis of K562 cells (Fig. 6A-C, $P < 0.001$). Similarly, compared with the IC_{50} ASP treatment group, the IC_{50} ASP + PD98059 could also significantly promote cell proliferation and inhibit apoptosis (Fig. 6A-C, $P < 0.001$). In addition, the results of benzidine staining showed that PD98059 significantly reduced the erythroid differentiation of cells in the control group and the IC_{50} ASP treatment group (Fig. 6D, $P < 0.001$). Western blot results also showed that PD98059 significantly reduced the protein expression of GATA-1, β-globin, and NF-E2 in the control group and IC_{50} ASP treatment group (Fig. 6E and F, $P < 0.001$), and also significantly reduced the protein expression of CD61, CD41 and CD42a (Fig. 6G and H, $P < 0.001$), suggesting that inhibition RAS/MEK/ERK pathway could significantly inhibit the pro-differentiation effect of ASP on K562 cells. These results demonstrated that the RAS/MEK/ERK pathway was involved in the regulation of ASP on the proliferation, apoptosis, and differentiation of K562 cells.

4. Discussion

The pathological manifestations of CML are the abnormal proliferation of hematopoietic stem cells, increased white blood cell

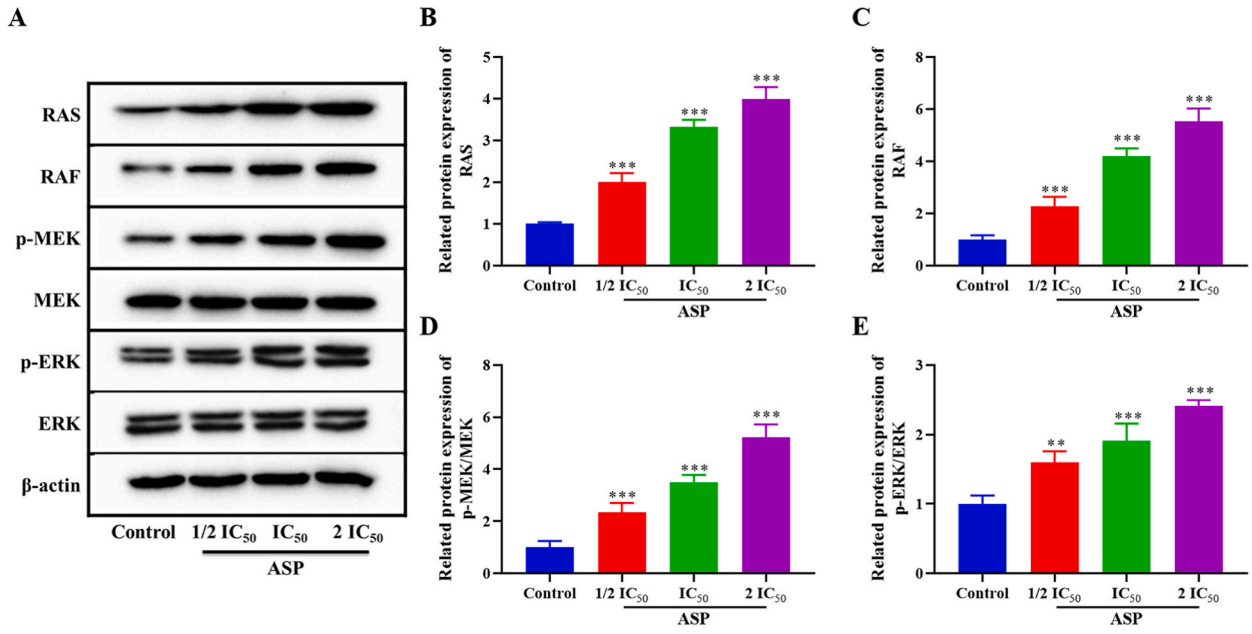

Fig. 4. Effects of ASP on RAS/MEK/ERK signaling pathway in K562 cells. (A) Protein expressions of RAS, RAF, *p*-MEK, MEK, *p*-ERK and ERK in K562 cells were detected by Western blot. **(B)** Densitometry analysis of protein expression. Data were shown as mean ± SD. *P < 0.05, **P < 0.01 and ***P < 0.001, compared with the control group. ASP, Asperuloside.

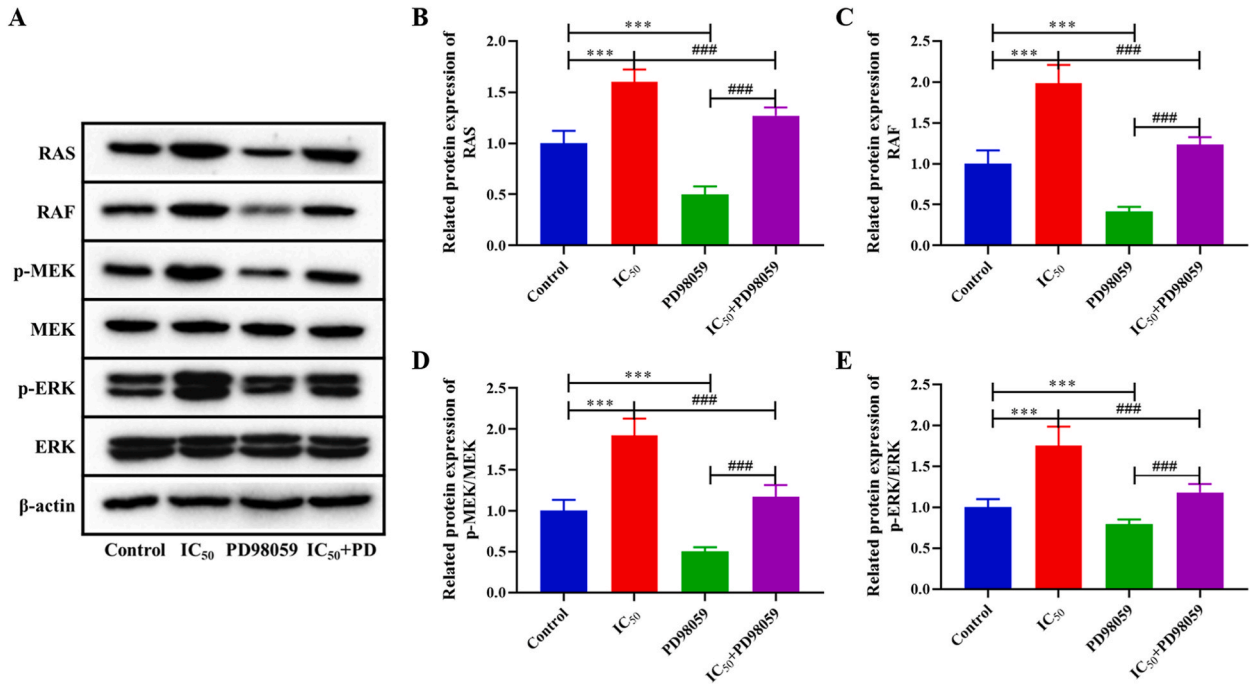

Fig. 5. The protein expressions of RAS/MEK/ERK signaling pathway in K562 cells. (A) Protein expressions of RAS, RAF, *p*-MEK, MEK, *p*-ERK and ERK in K562 cells were detected by Western blot. **(B)** Densitometry analysis of protein expression. Data were shown as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, compared with the IC_{50} + PD98059 group. PD98059, RAS/MEK/ERK pathway inhibitor; IC_{50}, IC_{50} ASP treatment group.

counts and extreme bone marrow hyperplasia are often seen in clinical examinations, and there are many moderately immature myeloid cells in blood smears [23]. Disorders of differentiation and maturation are considered to be common features of leukemia cells [24]. Inhibiting abnormal proliferation and promoting normal differentiation of leukemia cells may be novel therapeutic strategies for various hematological malignancies [25]. ASP, as a common secondary metabolite of iridoid glycosides, has a wide range of pharmacological effects [9]. Studies have confirmed that ASP has strong anti-cancer activity and may be a potential therapeutic agent for CML [11]. This study confirmed that ASP could significantly inhibit the proliferation and promote apoptosis and differentiation of K562 cells, and RNA-seq and in vitro experiments confirmed that its promoting effect on differentiation may be achieved by regulating the RAS signaling pathway.

Erythroid differentiation is an important part of hematopoietic differentiation and an important way for the body to produce mature erythrocytes [26]. Erythrocytes are an important part of blood, and play an important physiological function in transporting oxygen to various tissues and organs of the body [27]. The production of erythrocytes is a complex process, and once the development of erythroid cells stops at a certain stage and eventually fails to differentiate into mature erythrocytes, will lead to a variety of diseases, among which leukemia is the most serious and common one [28,29]. K562 cell line was first isolated from the pleural effusion of patients with CML in blast crisis [16]. The P210 protein produced by the BCR-ABL fusion gene in K562 cells significantly increased the activity of tyrosine kinase, resulting in enhanced malignant behavior of cells [30,31]. In addition, K562 is highly undifferentiated and has the characteristics of stem cells. This cell line contains most of the *trans*-acting factors required to regulate globin gene expression, and can restart or continue to differentiate into erythroid, mononuclear, or megakaryocytes in response to a variety of inducers [32]. It was found that the degree of malignancy could be reduced by inducing K562 cell differentiation, promoting apoptosis, and inhibiting abnormal cell proliferation [33,34]. RNAi knockdown of SRPK1 inhibited the proliferation and induced apoptosis of K562 cells [35]. Cabozantinib induced erythroid differentiation of K562 cells by upregulating heme biosynthesis, globin synthesis and erythrocyte-related reactions [36]. Ormeloxifene led to increased levels of markers of megakaryocyte differentiation CD41, and CD61 as well as key transcription factors GATA1 and AML1 in K562 cells [37]. This study also found that ASP could significantly inhibit the proliferation and promote apoptosis of K562 cells, and increase the expressions of GTAT-1, β-globin, NF-E2, CD61 and CD41. As an important transcription factor, GATA-1 can activate the expression of red blood cell-specific genes, such as globin, and inhibit the expression of genes related to cell cycle and cell proliferation, which are not conducive to red blood cell development and differentiation [38]. Similarly, NF-E2, as another key transcription factor involved in erythroid differentiation and the regulation of globin gene expression, also plays a key role in erythroid differentiation [39]. In addition, CD41 and CD61 are highly expressed membrane surface glycoproteins for the directional differentiation of hematopoietic stem cells into megakaryocytes [40]. The results of this study suggested that ASP promoted the erythroid and megakaryocyte differentiation of K562 cells, showing a potential therapeutic effect on CML.

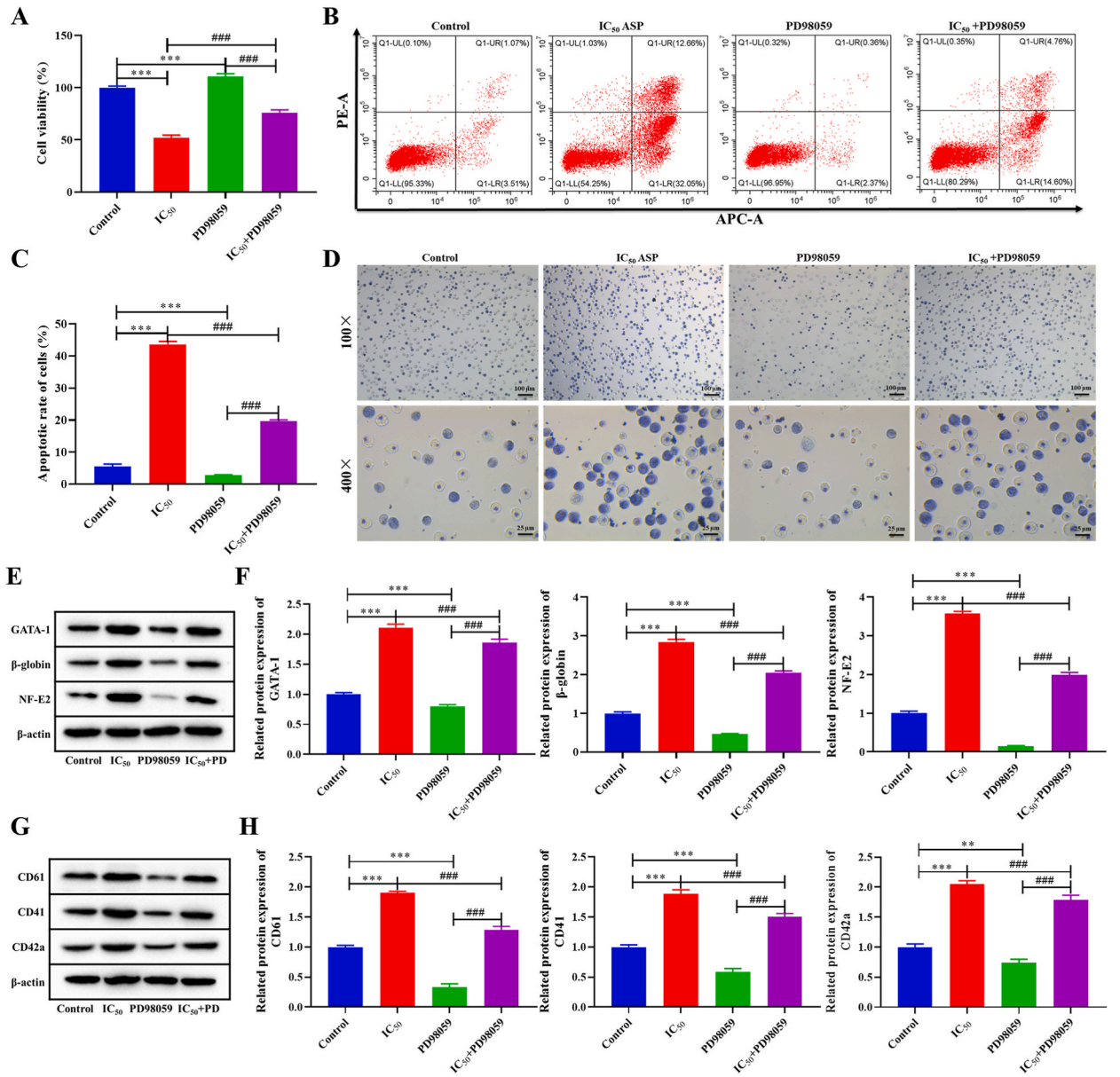

Fig. 6. Inhibition of the RAS pathway could significantly inhibit the regulatory effects of ASP on K562 cells. (A) K562 cell viability after IC50 ASP and/or PD98059 treatment was detected by CCK-8 assay. **(B–C)** Cell apoptosis was verified by flow cytometry in K562 cells. **(D)** K562 cells were analyzed with a benzidine staining assay. Magnification 100 × and 400 × . **(E)** Protein expressions of GATA-1, β-globin and NF-E2 in K562 cells were detected by Western blot. **(F)** Related protein expression of GATA-1, β-globin and NF-E2. **(G)** Protein expressions of CD61, CD41 and CD42a in K562 cells were detected by Western blot. **(H)** Related protein expression of CD61, CD41 and CD42a. Data were shown as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, compared with the IC_{50} + PD98059 group. PD98059, RAS/MEK/ERK pathway inhibitor; IC_{50}, IC_{50} ASP treatment group.

Further study on the mechanism of K562 differentiation induced by ASP and the discovery of new targets for differentiation therapy can provide a new theoretical basis for targeted therapy of CML. In this study, a total of 117 differentially expressed genes between the IC_{50} ASP treatment group and the control group were screened out by RNA-seq, which enriched several pathways, including the RAS signaling pathway. RAS/MEK/ERK pathway is a major signal-level pathway that connects extracellular signals and the regulation of intracellular transcription factors, and can be involved in the regulation of cell proliferation, migration, invasion, and other cellular functions [41,42]. The classical activation pathway is initiated by the mutual binding of ligand and cell surface receptor, that is, RAF, MEK, and ERK are activated by phosphorylation in turn [43]. Subsequently, the activated ERK can enter the nucleus and regulate the activity of transcriptional regulators to affect gene expression [44]. In addition to its role in cell proliferation and metastasis, RAS/-MEK/ERK also plays an important role in regulating erythroid differentiation physiologic hematopoiesis and leukemia development

[45]. It has been shown that RAS/MEK/ERK pathway activation can be used therapeutically and may guide new therapies for relapsed lymphoblastic leukemia [46]. Activation of the ERK pathway through overexpression of RAS can promote erythroid differentiation of K562 cells [22], and can also stimulate primary human erythroid progenitor cells with erythropoietin to leading to activation of ERK, thereby promoting erythroid differentiation [47]. In addition, it has also been reported that K562 possesses a constitutively active RAS-MAP kinase pathway due to the presence of the BCR-ABL oncogene [48], and blocking the association between BCR-ABL and Ras, Raf, and MEK in K562 cells inhibits cell proliferation and induces spontaneous erythroid differentiation [49,50]. Data obtained in a chicken erythropoiesis model suggest that ERK activity may need to be inhibited to initiate erythroid differentiation [51]. There are conflicting reports on the ERK pathway during erythroid differentiation. Studies have shown that the ERK signaling pathway has a time-dependent effect on erythroid differentiation of K562 cells and is different among different inducing drugs. Generally, ERK is activated when induced by hemin and cisplatin, while the phosphorylation activity of ERK is reduced when induced by butyrate and ArA-C [52]. In this study, ASP treatment could significantly promote the activation of the RAS/MEK/ERK pathway in K562 cells, and the use of inhibitor PD98059 further confirmed that the RAS/MEK/ERK pathway was involved in the regulation of K562 cells by ASP. These suggested that ASP may promote K562 cell differentiation by activating the ERK pathway, which is similar to hemin and cisplatin.

In conclusion, ASP could significantly inhibit the proliferation and promote apoptosis and differentiation of K562 cells. The results of RNA-seq and in vitro experiments suggested that the regulatory effects of ASP on K562 cells may be achieved by regulating the RAS/MEK/ERK pathway. The use of inhibitor PD98059 further confirmed that the RAS/MEK/ERK pathway plays an important role in the ASP regulation of K562 cells. All the results of this study suggest that ASP promoted the apoptosis and differentiation of K562 cells by regulating the RAS/MEK/ERK pathway, which has a potential therapeutic potential in CML. This study can provide a theoretical basis for the new treatment of CML and provide new evidence for the clinical application of ASP. Admittedly, there are still some limitations in this study, for example, the study mainly focused on K562 cells in vitro. Although the analysis of the influence on the proliferation and differentiation of CML cells K562 is an effective way to explore new drugs for CML treatment, in vivo studies are important to validate in vitro results. Follow-up studies will consider exploring the role of ASP in CML in animals to validate our idea. In addition, this study was conducted in only one cell line, and multiple cell lines can strengthen the robustness and generality of our findings. To further apply the research results to the clinic, a large number of scientific experiments are still needed.

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Bingjie Zhao: Writing – original draft, Conceptualization. **Hong Che:** Writing – original draft, Conceptualization. **Linlin Li:** Investigation. **Lian Hu:** Data curation. **Wenjing Yi:** Data curation. **Li Xiao:** Data curation. **Songshan Liu:** Investigation, Data curation. **Zhufa Hou:** Writing – review & editing, Writing – original draft, Conceptualization.

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.

Appendix A. Supplementary data

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