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Dehydroabietic acid chemosensitizes drug-resistant acute lymphoblastic leukemia cells by downregulating survivin expression

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ABSTRACT

Objective: To explore the mechanism of drug resistance in acute lymphoblastic leukemia and the anti-tumor effect of combination therapy of dehydroabietic acid and vincristine against acute lymphoblastic leukemia cells.

Methods: Acute lymphoblastic leukemia cells REH and CCRF-CEM were employed to detect the anti-tumor effect of vincristine and doxorubicin on proliferation and apoptosis using EdU assay, human active caspase-3 Quantikine ELISA kit, and flow cytometry. Vincristine-resistant REH cells (REH-R), survivin knockdown and overexpressing REH cells were established to verify the role of survivin in drug resistance. Additionally, *in vitro* and *in vivo* assays were performed to determine the effect of dehydroabietic acid on the cytotoxicity of vincristine.

Results: Vincristine and doxorubicin markedly suppressed proliferation and induced apoptosis of REH and CCRF-CEM cells. Survivin expression was upregulated in REH-R cells compared with REH cells. Knockdown of survivin expression obviously restored the sensitivity of REH-R cells to vincristine. Akt phosphorylation was also increased in REH-R cells compared to REH cells. In addition, LY294002, a PI3k/Akt pathway blocker, inhibited survivin expression and enhanced cytotoxicity of vincristine to REH-R cells. Dehydroabietic acid effectively reduced survivin expression in REH-R cells, thereby enhancing the therapeutic effect of vincristine on drug-resistant cells. Survivin overexpression markedly reduced the effect of dehydroabietic acid on enhancing the anti-proliferation and inducing apoptosis effect of vincristine. Moreover, the combination of dehydroabietic acid with vincristine significantly extended the survival rate in a mouse xenograft model of acute lymphoblastic leukemia, compared with vincristine treatment alone.

Conclusions: Dehydroabietic acid may be used as a potential candidate for the treatment of acute lymphoblastic leukemia in combination with vincristine.

KEYWORDS: Acute lymphoblastic leukemia; Drug resistance; Survivin; Dehydroabietic acid; REH cells; Vincristine

1. Introduction

Acute lymphoblastic leukemia is the most common pediatric malignancy accounting for 80% of all childhood leukemia cases[1]. Unlike many pediatric solid tumors, children with relapsing refractory hematologic malignancies often suffer from acute diseases due to high disease burden and rapid disease progression. Unless treated, these malignancies progress rapidly[2]. Despite significant progress in its treatment and research, hematological malignancy remains an important cause of childhood cancer deaths,

Significance

Dehydroabietic acid is a novel survivin inhibitor. Our data indicated that survivin played a critical role in drug resistance of REH cells. Dehydroabietic acid inhibited survivin expression, reversed the drug resistance of acute lymphoblastic leukemia cells, and enhanced the cytotoxicity of vincristine *in vitro* and *in vivo*. Dehydroabietic acid may be used as a potential candidate for the treatment of acute lymphoblastic leukemia in combination with vincristine.

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and survivors are at risk of life-long complications[3]. These diseases require new treatments to overcome chemotherapy resistance and reduce long-term toxicity[4].

Transcriptional factors and cytokines contribute to the pathogenesis of acute lymphoblastic leukemia. Among these transcriptional factors, survivin, a member of the inhibitor of an apoptosis protein family, regulates cell cycle and affects cancer progression, drug resistance, and patient survival[5,6]. Survivin is of prognostic significance in rectal cancer patients who received surgery and postoperative concurrent chemoradiation therapy[7,8]. Its overexpression suppresses caspase 3/7 activity and is significantly resistant to apoptotic stimuli in survivin-reconstituted HEK cells[9]. Survivin-specific cytotoxic T lymphocytes combined with *CCL17* and *GM-CSF* genes bring more suppression to mouse melanoma[10]. Meanwhile, there are a growing number of reports on the relationship between survivin and acute lymphoblastic leukemia. According to a report, survivin overexpression can be used as a parameter to determine poor prognosis in acute lymphoblastic leukemia patients[11]. Another study found that survivin knockdown improves the anti-tumor effect of chemotherapy agents in acute lymphoblastic leukemia models[12]. Therefore, survivin plays a pivotal role in the progression of acute lymphoblastic leukemia.

Dehydroabietic acid is a major compound derived from rosin that has been traditionally used as herbal medicine[13,14]. The biological activity of dehydroabietic acid includes antibacterial, antifungal, and anticancer effects[15]. Kim *et al*. reported that dehydroabietic acid, a novel survivin inhibitor for gastric cancer[16], had a greater inhibitory effect than YM155, a small imidazolium-based compound, on *survivin* gene transcription and protein expression[17]. YM155 exhibits potent anti-proliferative effects in a variety of human cancer cells[18-20] and induces apoptosis in patients with T-cell acute lymphoblastic leukemia[21]. YM155 synergizes with doxorubicin to achieve high-throughput selective inhibition of survivin expression and restore apoptosis in heterogeneous breast cancer cells[22]. Inhibition of survivin by YM155 reverses multidrug resistance in osteosarcoma and castration-resistant prostate cancer[23,24]. Previous experiments also verify the role of YM155 in reducing survivin expression in pediatric acute lymphoblastic leukemia cells[25]. However, the clinical trial of YM155 was discontinued because of its high toxicity and low antitumor effects[26]. Therefore, our study aimed at exploring the important role of dehydroabietic acid in the treatment of drug-resistant acute lymphoblastic leukemia.

2. Materials and methods

2.1. Chemicals and reagents

Dehydroabietic acid was purchased from J&K Scientific Ltd. (Cat. 522318, Beijing, China). LY294002 was purchased from Beyotime (Cat. S1737, Shanghai, China). Antibodies against survivin (Cat. sc-17779), p-Akt (Cat. sc-514032), Akt (Cat. sc-81434), and β-actin (Cat. sc-8432) were purchased from Santa Cruz Biotechnology, USA. GV-lentiviral shRNA vectors targeting the human *survivin* gene, the GV control vector, and lentivirus particles that show survivin overexpression were purchased from GENE (Shanghai Genechem Co., Ltd).

2.2. Cell culture

REH and CCRF-CEM cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco, Invitrogen). Cells were maintained in a humidified atmosphere with 5% CO_2 at 37℃.

2.3. Establishment of vincristine-resistant REH cell lines

The REH cells with a logarithmic growth phase, were exposed to increasing concentrations of vincristine (Sigma-Aldrich): 1 nM and 2 nM (the density of living cells was more than 2/3 in the microscope field). When the vincristine concentration was increased to 20 nM, a steady-growing drug-resistant REH cell line (REH-R) was finally obtained by stepwise screening. After the cell line was established, it was cultured in RPMI 1640 medium containing 20 nM vincristine. Vincristine resistance index was calculated using the IC_{50} value of REH-R cells versus the IC_{50} value of REH cells.

2.4. Lentiviral shRNA vector constructs and viral infections

GV248 cloning vector containing Turbo EGFP reporter and elements were required to package into the virus. The sequence of survivin shRNA was 5-TGACGACCCCATAGAGGAA-3[12] (shSurvivin), while the scrambled oligonucleotide 5-TCTCGCTTGGGCGAGAGTAAG-3 (Ctrl-shRNA) was cloned into the same vector as the control. REH cells were transfected by lentiviral particles and screened by puromycin (1 μg/mL) for 10 d.

2.5. Lentiviral overexpression and viral infection

The human survivin (BIRC5) sequence (NM_001168.3) was inserted into the vector of overexpressed lentivirus (oeSurvivin). REH cells were transfected by lentiviral particles and screened by puromycin (1 μg/mL) for 10 d. The cells stably overexpressing survivin were maintained by a supplement of puromycin.

2.6. Cell proliferation assay

Measurement of cell proliferation was determined using the EdU kit (Cat. C0088L; Beyotime Institute of Biotechnology, Shanghai, China). Briefly, 5 000 cells were incubated with different concentrations of vincristine or doxorubicin at 37℃ under 5% CO_2 for 48 h. After washing with phosphate buffer saline (PBS), cells were incubated with 10 μM EdU for 2 h at room temperature. Then the click reaction buffer, HRP-Streptavidin solution, 3,3',5,5'-tetramethyl benzidine,

and H_2SO_4 were added to the wells. Optical density was measured at 450 nm using a microplate reader (PowerWave XS2, BioTek).

2.7. Western blot

Cells were lysed and 10 μg of protein extracts were resolved by SDS-PAGE and electro-transferred to PVDF membrane (Invitrogen). After blocking with 5% nonfat milk for 1 h at room temperature, the membranes were incubated with primary antibodies (1∶1 000) overnight at 4℃. Then the membranes were washed and incubated with horseradish peroxidase (HRP)-labeled secondary antibody for 1 h at room temperature. Finally, the blots were visualized using a chemiluminescent detection system (ECL, Amersham Life Science). Densitometry for immunoblotting was performed by using AlphaEaseFC-v4.0.0 software.

2.8. Immunofluorescence

For γH2AX immunostaining, cells grown on a round 12 mm microscope chamber were permeabilized with 0.2% Triton X-100 for 5 min on ice, washed 3 times with PBS, blocked with 0.25% bovine serum albumin, and incubated for 1 h at room temperature with mouse monoclonal antibody against γH2AX [1∶500, H2A.X (phospho S139) (EP854(2)Y), ab81299, Abcam]. After washing 5 times with PBS, cells were incubated with rabbit Alexa-Fluor-488-conjugated antibody (1∶1 000) (Life Technologies) for 1 h at room temperature. Then cells were washed 3 times with PBS again, and 1 μg/mL DAPI was used to stain nuclei. Images were captured using the Leica TCS SP8 confocal microscope.

2.9. Measurement of caspase-3 activity

Caspase-3 activity was determined using a human active caspase-3 Quantikine ELISA kit (R&D Systems). Briefly, cells were incubated with 10 μM Biotin-ZVKD-FMK inhibitor for 1 h at 37℃. Then, cells were harvested, washed, and resuspended in an extraction buffer containing protease inhibitors. The microplate was coated with caspase-3 antibody and added with standards and sample extracts containing covalently linked active caspase-3-ZVKD-biotin. Finally, streptavidin-conjugated HRP and substrate were added to the wells. The amount of active caspase-3 was measured using a microplate reader at 450 nm.

2.10. Apoptosis analysis

Cells were seeded in a 6-well plate. After 24 h treatment, cells were harvested and stained using an annexin V-FITC/propidium iodide kit (Best Biotech, China). Briefly, cells were incubated in dark with annexin V-FITC and propidium iodide at room temperature for 15 min. Then samples were analyzed using a flow cytometer with WinMDI version 2.9 software.

2.11. Animal experiments

In this study, 7-week-old female NOD/SCID mice were purchased from Shanghai SLRC Laboratory Animal Co., Ltd. Mice were randomly housed in cages (five mice per cage) under a 12-h light/dark photoperiod, and at room temperature of (25±2)℃ and a relative humidity of (50±15)%. Food and water were free to access. REH cells (3×10^6/100 μL PBS per mouse) were injected intravenously into mice *via* tail vein (n=10 mice per group). Mice received vehicle, vincristine (once a week, *i.p.*, 0.15 mg/kg) alone or combined with dehydroabietic acid (*i.p.*, 20 mg/kg/d) until being sacrificed when showing physical signs of leukemia (>5% weight loss, abnormal posture, decreased activity, labored breathing, and/or hind limb paralysis). Survival time was monitored daily. The dosage of dehydroabietic acid was determined according to the results of previous experiments[27].

2.12. Statistical analysis

All results are expressed as mean±SEM of three independent experiments. Data were analyzed by Student's t-test or one-way ANOVA. Multiple comparisons between the groups were performed using Student-Newman-Keuls method. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software for Windows (SPSS, Inc., Chicago, Illinois).

2.13. Ethical statement

All procedures complied with national and institutional guidelines for the care and use of animals. This study was evaluated and approved by the Guidelines of the Animal Care and Use Committee of Jiangsu University (Approval No. 2021/043).

3. Results

3.1. Chemotherapeutic drugs suppress the proliferation of acute lymphoblastic leukemia cells

Vincristine and doxorubicin were employed in the treatment of acute lymphoblastic leukemia. Therefore, these two chemotherapeutic drugs were chosen to evaluate the inhibitory effect on the proliferation of acute lymphoblastic leukemia cells. EdU assay indicated that vincristine and doxorubicin inhibited the proliferation of REH and CCRF-CEM cells in a concentration-dependent manner (Figures 1A-D). Meanwhile, we detected cell apoptosis by testing active caspase-3 activity. Vincristine and doxorubicin markedly induced caspase-3 activity (Figures 1E-F) and cell apoptosis (Figure 1G) in acute lymphoblastic leukemia cells (5 nM of vincristine and doxorubicin in REH cells and 20 nM in CCRF-CEM cells, respectively) (P<0.05). The above results indicated that chemotherapeutic drugs suppress proliferation and induce apoptosis of acute lymphoblastic leukemia cells.

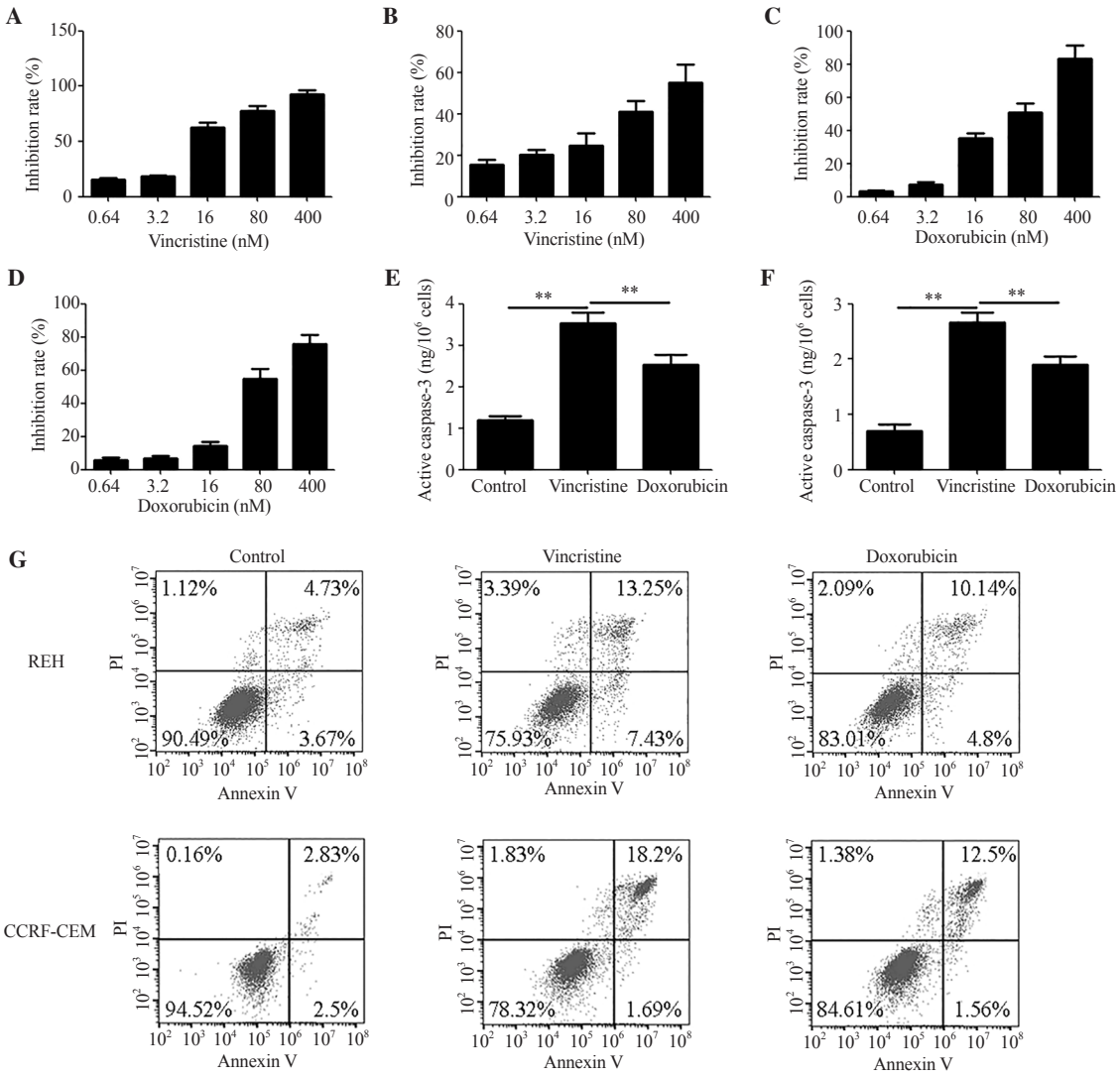

Figure 1. Vincristine and doxorubicin suppress proliferation and induce apoptosis of acute lymphoblastic leukemia cells. REH (A and C) and CCRF-CEM (B and D) cells were treated with different concentrations of vincristine or doxorubicin for 48 h. EdU assay was used to detect cell proliferation. (E) REH cells were treated with 5 nM vincristine and doxorubicin. (F) CCRF-CEM cells were treated with 20 nM vincristine and doxorubicin. Caspase-3 activity was determined using Quantikine human active caspase-3 kit. (G) Flow cytometric analysis. Data represent mean±SEM (n=3). $^{**}P$<0.01.

3.2. Role of survivin expression in drug resistance of acute lymphoblastic leukemia cells

REH cells were more sensitive to vincristine and doxorubicin compared to CCRF-CEM cells. Therefore, we cultured REH cells by continuously adding increasing concentrations of vincristine, and a steady-growing REH-R strain was finally obtained by stepwise screening. Then, we determined the IC_{50} value of vincristine and doxorubicin against REH-R cells. The IC_{50} value against drug-resistant cells (REH-R) was more than 7 times that of REH cells when the cells were treated with vincristine (Figure 2A). This shows a significant decrease in the sensitivity of a resistant strain to vincristine (P<0.05). As survivin plays a pivotal role in the development of acute lymphoblastic leukemia, we detected survivin expression in both REH and REH-R cells. Western blot assay showed that survivin expression was significantly upregulated in drug-resistant REH cells (P<0.05) (Figure 2B).

To more clearly verify the effect of survivin expression on the resistance of acute lymphoblastic leukemia cells, we employed GV-lentiviral shRNA targeting human *survivin* gene and the scramble GV vector. Western blot assay demonstrated that survivin expression in REH cells was significantly knocked down (Figure 2C). EdU result indicated the survivin knockdown group significantly elevated inhibition rate compared to Ctrl-shRNA group cells (Figure 2D). The cytotoxic effect of vincristine was significantly enhanced after silencing survivin expression. Moreover, the apoptotic rate was significantly increased in the survivin knockdown group (Figure 2E).

3.3. PI3k/Akt pathway contributes to survivin expression in REH-R cells

To verify the role of survivin expression in drug resistance and

the underlying mechanism, we detected the signaling pathway in REH-R cells. Whether vincristine activates PI3k/Akt pathway in REH cells was evaluated first. The results showed that vincristine and doxorubicin upregulated p-Akt level and survivin expression (Figure 3A). Moreover, p-Akt and survivin expressions were also increased in REH-R cells compared to REH cells (Figure 3B). The findings indicated that PI3k/Akt pathway and survivin expression may contribute to drug resistance. To verify this hypothesis, LY294002, a PI3k/Akt signaling pathway blocker, was employed to treat the REH-R cells. According to the results, LY294002 markedly suppressed p-Akt level and survivin expression in REH-R cells (P<0.05) (Figure 3C). Meanwhile, LY294002 enhanced the cytotoxicity of vincristine to drug-resistant cells in a concentration-dependent manner (Figure 3D).

3.4. Dehydroabietic acid downregulates survivin expression and re-sensitizes REH-R cells to vincristine

Based on the important role of survivin in the proliferation of REH and REH-R cells, we tried to combine vincristine with dehydroabietic acid, a small-molecule inhibitor of survivin, to more effectively treat drug-resistant REH-R cells. EdU results showed combined treatment with dehydroabietic acid (Figure 4A) and vincristine significantly inhibited the proliferation of REH-R cells, compared to vincristine treatment alone (P<0.05) (Figure 4B). We also measured DNA damage and apoptosis by detecting γH2AX and caspase-3 expression (Figures 4C-E). Dehydroabietic acid enhanced the cytotoxic effect of vincristine, as evidenced by increased γH2AX and caspase-3 expression. In further experiments, we found that dehydroabietic acid significantly decreased survivin expression in REH-R cells (P<0.05) (Figure 4F). To further verify the role of survivin in drug resistance, survivin was overexpressed using lentivirus. Compared with the vector control group, survivin was markedly overexpressed in the oeSurvivin group (Figure 4G). Survivin overexpression reduced the effect of dehydroabietic acid in inhibiting proliferation (Figure 4H) and inducing apoptosis (Figure 4I). These results demonstrated that dehydroabietic acid re-sensitizes resistant cells to vincristine through suppressing survivin expression.

Moreover, in an *in vivo* study, vincristine prolonged the survival rate of mice (Figure 5). As expected, a combination of dehydroabietic

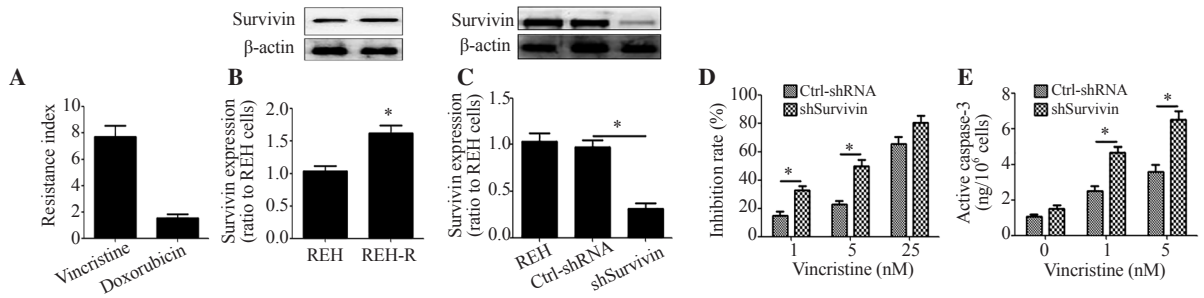

Figure 2. Survivin expression is upregulated in vincristine-resistant REH (REH-R) cells. (A) IC_{50} values after 48 h treatment of vincristine or doxorubicin. Resistance index was calculated using the IC_{50} value of vincristine against REH-R cells versus the IC_{50} value against REH cells. (B) Survivin expression was detected by Western blot analysis in REH-R and REH cells. (C) REH cells were transfected by lentiviral particles containing scrambled oligonucleotide (Ctrl-shRNA) or survivin shRNA (shSurvivin). Western blot assay was performed to detect survivin expression. (D) Inhibition rate of cells after 48 h treatment of vincristine using EdU assay. (E) Caspase-3 activity was measured in REH cells. Data represent mean±SEM (n=3). $^*P$<0.05.

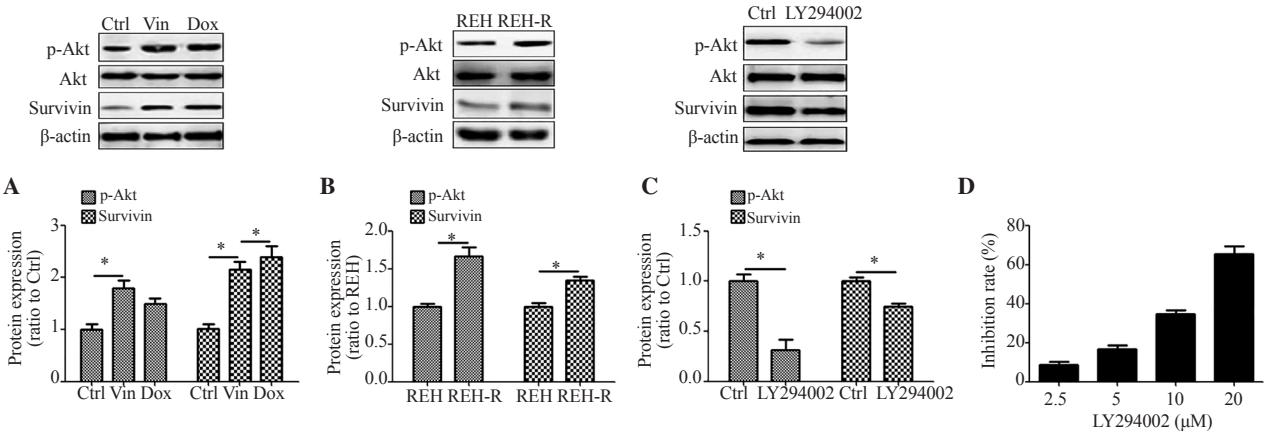

Figure 3. PI3k/Akt pathway contributes to survivin expression in resistant strains. Western blot analysis was used to detect the expressions of p-Akt, Akt, and survivin in REH (A) cells treated with vincristine and doxorubicin and in REH-R (B) cells. LY294002, a PI3k/Akt pathway blocker was employed to detect the expressions of p-Akt, Akt, and survivin (C), and cell proliferation (D) in resistant cells. Protein expression of survivin and p-Akt was normalized against actin and Akt, respectively in (A) to (C) and the expression of the examined protein in the control group was set at 1. Data represent mean±SEM (n=3). $^*P$<0.05. Vin: vincristine; Dox: doxorubicin.

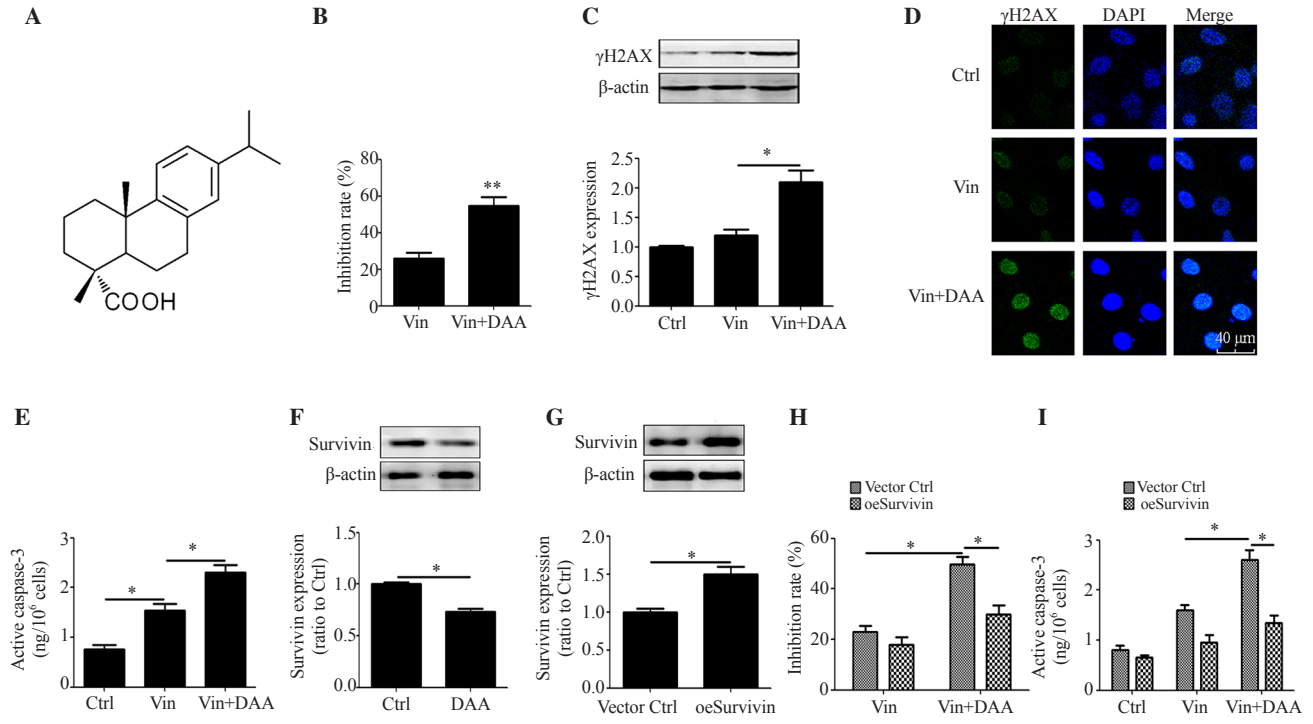

Figure 4. DAA enhances the anti-tumor effect of vincristine on a drug-resistant strain by inhibiting survivin expression. (A) Chemical structure of DAA. REH-R cells were treated with 30 nM vincristine alone or with a combination of vincristine and 100 μM DAA. (B) Cell proliferation was assessed by EdU assay. (C-D) γH2AX expression was detected using Western blot and immunofluorescence assays (200×). Scale bar: 40 μm. (E) Caspase-3 activity was determined. (F) Survivin expression was detected. Lentivirus was employed to overexpress survivin. REH cells were transfected by lentiviral particles containing human *survivin* gene to overexpress survivin (oeSurvivin). Survivin expression (G), cell proliferation (H), and caspase-3 activity (I) were detected. Data represent mean±SEM (n=3). $^{*}P$<0.05, $^{**}P$<0.01. DAA: dehydroabietic acid.

acid and vincristine significantly extended the survival rate, compared to vincristine alone (P<0.05). Altogether, these data indicated that combined treatment with dehydroabietic acid and vincristine markedly delayed the progression of leukemia *in vivo*.

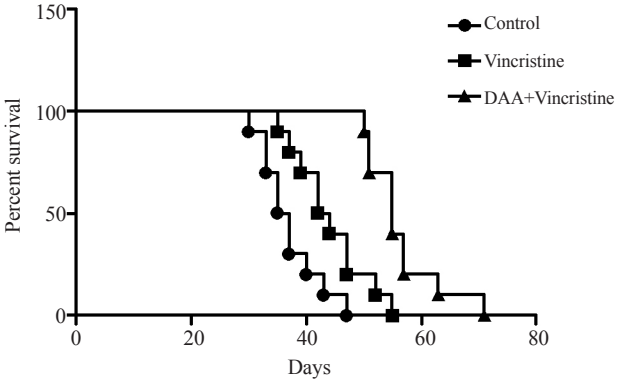

Figure 5. DAA enhances the anti-tumor effect of vincristine in a mouse xenograft model of acute lymphoblastic leukemia. Survival of female NOD/SCID mice intravenously injected with 3×10^6 REH cells (n=10 per group) was monitored.

4. Discussion

Acute lymphoblastic leukemia is the most common pediatric hematologic malignancy[28]. Although the survival rate after treatment can exceed half, relapse occurs and the prognosis of patients after relapse is poor[12]. Moreover, myeloablative therapy and hematopoietic stem cell transplantation are no longer effective after relapse[25]. Therefore, drug-resistant acute lymphoblastic leukemia has become a major problem in clinical treatment.

A previous study reported that *survivin* gene transcription is significantly increased in patients with relapsed acute lymphoblastic leukemia compared with that at initial diagnosis[29]. Given that survivin is mainly expressed during the development and progression of malignant tumors, little or no expression is present in the terminally differentiated tissues[30]. Moreover, another study confirmed the positive correlation between survivin expression and drug resistance, making it an important target for the treatment of drug-resistant pediatric acute lymphoblastic leukemia[11]. We established a model of drug-resistant acute lymphoblastic leukemia strain *in vitro* and confirmed that the protein expression of survivin in a drug-resistant strain was significantly increased.

Based on previous research and relevant clinical data, we

conducted a series of *in vitro* and *in vivo* experiments to verify the role of dehydroabietic acid in inhibiting survivin expression and reversing drug resistance. By effectively inhibiting the expression of survivin, dehydroabietic acid increased cytotoxicity of vincristine and reactivated apoptosis of drug-resistant cells. In this study, we selected the REH cell line with susceptibility to drug resistance and successfully induced the drug-resistant REH cells. The survivin expression was upregulated in REH-R cells compared with REH cells, which is similar to other work where a chemotherapeutic agent upregulated survivin expression in thyroid cancer cells[31]. Moreover, after combining dehydroabietic acid with vincristine in the treatment of drug-resistant cells, we found that dehydroabietic acid significantly increased the ability of vincristine to suppress cell proliferation, induce DNA damage and apoptosis *in vitro*, and showed significant an anti-tumor effect *in vivo* compared with vincristine treatment alone. Survivin overexpression reduced the effect of vincristine in inhibiting proliferation and inducing apoptosis. These results further verified the role of survivin in drug resistance and the effect of dehydroabietic acid in enhancing cytotoxicity of vincristine.

Previous studies reported that Akt plays an important role in vincristine resistance in RPMI8226/VCR cells[32,33]. Inhibiting survivin expression through a PI3k/Akt pathway blocker LY294002 or shRNA sensitizes resistant cells to vincristine. Silencing of survivin also inhibits invasion and proliferation of glioma cells[34]. In our study, LY294002 suppressed survivin expression and inhibited proliferation of drug-resistant cells. Our results are consistent with the previous studies where dehydroabietic acid derivatives display selective inhibitory activity on PI3Kα and decrease p-Akt expression in HCT-116 cells[35,36]. Therefore, dehydroabietic acid may reverse vincristine-induced drug resistance by inhibiting the PI3K/Akt signaling pathway, which needs to be studied in our further research. Moreover, the target of dehydroabietic acid and the transcriptional factor of *survivin* also need to be elucidated.

In summary, our results showed that dehydroabietic acid can effectively improve the sensitivity of drug-resistant REH cells to vincristine. These data will provide a research basis for the clinical treatment of drug-resistant acute lymphoblastic leukemia.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Authors' contributions

LLS and WHH experimented. LLS, WHH, and HJZ drafted the manuscript. XWY designed the project. All authors discussed the results, and they read and approved the manuscript.

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