Gambogic acid inhibits proliferation and induces apoptosis of human acute T-cell leukemia cells by inducing autophagy and downregulating β -catenin signaling pathway: Mechanisms underlying the effect of Gambogic acid on T-ALL cells

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Abstract. The main active compound of *Garcinia hanburyi* (referred to as gamboge) is gambogic acid (GA), which has long been a Chinese herbal medicine for treating several types of cancer. However, the potential therapeutic role and mechanisms of GA in T-cell acute lymphoblastic leukemia (T-ALL) remain unclear. In the present study, the effects of GA on proliferation, cell cycle, apoptosis, and autophagy in T-ALL cell lines were investigated. The possible mechanisms underlying GA activity were also examined. The results showed that GA inhibited proliferation, induced apoptosis, and activated autophagy in T-ALL cell lines (Jurkat and Molt-4 cells). Findings confirmed that GA has an antileukemia effect against peripheral blood lymphocyte cells in patients with ALL. GA inhibited phospho-GSK3 β S9 (p-GSK3 β S9) protein levels to inactivate Wnt signaling and suppress β -catenin protein levels. In addition, the inhibitory effect of GA on T-ALL was reversed by overexpression of β -catenin. Thus, GA can inhibit the growth and survival of T-ALL cells. GA also had antileukemic activity, at least in part, through the downregulation of the Wnt/ β -catenin signaling pathway.

Introduction

Acute lymphoblastic leukemia (ALL) is a heterogeneous hematopoietic malignancy, characterized by the expansion of lymphoid progenitor cells. Massive proliferation of cells, extensive infiltration, and inhibition of normal hematopoiesis are the main clinical manifestations of ALL. It is the most common neoplasm in pediatric patients. Approximately 85% of ALLs are of the B-cell lineage; the rest are of T-cell lineage (1). T-cell ALL (T-ALL) presents as an uncontrolled malignant accumulation of T-cell progenitors (1,2). T-ALL cases can occur at any age, while the majority of cases occur between 2 and 5 years of age (3). Current treatments focus on intensive chemotherapy, targeted therapy, and bone marrow transplantation (4). Although 80% of cases initially achieve remission (5), most patients show relapse or the cancers resist chemotherapy, resulting in a poor prognosis of patients (1). Therefore, efficient and low-toxicity antitumor compounds are crucial to ameliorate the prognosis of patients with T-ALL.

Currently, several treatment approaches methods for T-ALL are in practice; these include inducing apoptosis, inhibiting proliferation, causing cell cycle arrest and inducing differentiation (6,7). Chinese herbs have been widely used to treat cancers as they are cost-effective and have positive outcomes. More than half antitumor compounds in use today are herbaceous (8).

Gambogic acid (GA) is a natural medicinal compound that is extracted from the traditional Chinese herb *Garcinia hanburyi*. Its molecular formula is C₃₈H₄₄O₈, and it has a molecular weight of 628.75 g/mol. GA is a potential antineoplastic drug against several solid tumors and hematological malignancies (9-11), including hepatocellular (12), malignant (13), breast carcinoma (14), and chronic myeloid leukemia (15). GA has been shown to affect the physiological function and signaling pathways of several tumor cell lines (16,17). Activation of cell apoptosis has been suggested to be the main mechanism underlying the repressive effect of GA on solid tumors. The related molecular mechanisms possibly

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include inhibition of telomerase activity or obstructing transferrin receptor internalization (9,10). In addition, it has been found that GA can inhibit multiple cell signaling pathways, including the Wnt/ β -catenin pathway, and transcription factors, including nuclear factor-kappa B, activator protein 1, NRF2, PPAR- γ , tumor necrosis factor- α , sonic hedgehog, and nitric oxide synthase (18,19). However, the potential effect of GA on T-ALL is not fully understood, and the underlying molecular mechanisms require further investigation.

β -catenin, a transcriptional coactivator of the Wnt/ β -catenin signaling pathway, is degraded by proteasomes when cell function is normal. Once the pathway is activated, β -catenin constantly accumulates in the cytoplasm and then translocates to the nucleus. In the nucleus, β -catenin interfaces with high-mobility group transcription factors belonging to the T-cell factor/lymphoid enhancer factor family to activate downstream target genes, including *c-Myc*, *Axin2*, and *CyclinD1*. All of these mechanisms are attractive therapeutic targets (20-23).

The canonical Wnt pathway (Wnt/ β -catenin pathway) is involved in the regulation of various physiological and pathological processes during early- and late-stage embryonic development (24) and in carcinogenesis (25,26). Different types of tumors cause aberrance of the Wnt/ β -catenin pathway. Previous studies have shown that Wnt/ β -catenin pathway plays a crucial role in various leukemias (27-29). This pathway is closely associated with self-renewal, overexpression and deletion of mutations in hematopoietic stem cell (HSC). These factors all play important roles in leukemogenesis, drug resistance, and tumor relapse (30). Furthermore, it has been demonstrated that Wnt/ β -catenin signaling is involved in the development of T cells and often the deregulation of T-ALL (31,32). Thus, Wnt/ β -catenin signaling is a worthy target for improving T-ALL therapy results.

In the current study, we investigated the antitumor effects of GA using T-ALL cell lines and patient samples. The study was expanded to examine the possible mechanisms causing the observed effects of GA. GA demonstrated marked potency in inducing apoptosis, regulating cell cycle arrest, inhibiting proliferation, and inducing autophagy in T-ALL cells. Further investigations revealed that the Wnt/ β -catenin signaling pathway plays an important role in the inhibitory function of GA. Thus, GA is a potential antitumor adjuvant agent.

Materials and methods

Cell culture. The human ALL lines Jurkat and Molt-4 were obtained from the cell bank of the Chinese Academy of Science (Shanghai, China). Jurkat cells and Molt-4 cells were routinely cultured in RPMI-1640 (Gibco) medium supplemented with 10% fetal bovine serum (Gibco) with 1% glutamine-penicillin (Beyotime). Cells were cultured at 37°C with 5% CO₂.

T-ALL patients and healthy samples lymphocyte cells isolation. Following institutional guidelines, blood samples were obtained following informed consent from six patients with T-ALL and six healthy patients. Patients were recruited from the Zhejiang Provincial People's Hospital in November and December, 2018. Lymphocytes were isolated from EDTA-treated blood by density gradient centrifugation using Lymphocyte Separation Medium (TBD) as per the manufacturer's instructions. Peripheral

anti-coagulated blood (4 ml) was mixed with calcium-magnesium-free phosphate-buffered saline (PBS) (ratio 1:1). Samples were layered onto 5-ml lymphocyte separation medium and centrifuged at 300 x g for 25 min at room temperature. The interface layer was centrifuged at 50 x g for another 10 min at room temperature, and the pellet was collected. The enriched cells were cultured in RPMI-1640 medium supplemented with 20% fetal bovine serum.

The relevant ethics approval was granted by the Zhejiang Provincial People's Hospital Ethics Committee.

Gambogic acid preparation. GA with a purity of >98% was purchased from Chengdu Must Bio-Technology Co. Ltd. It was dissolved in dimethyl sulfoxide and then stored at -20°C for further analyses.

Cell viability assay using Cell Counting Kit-8. Cell viability after GA treatment was determined using a Cell Counting Kit-8 (CCK-8) assay (Beyotime). Cells were seeded in 96-well plates (1x10⁵ cells/well) and treated with different concentrations (1.2, 2.4 and 4.8 μ M) of GA, and controls were treated with 0.1% DMSO. The plates were incubated for 24, 48 and 72 h. After incubation, 20 μ l of CCK-8 solution was added to each well. The plates were further incubated at 37°C in a CO₂ incubator for 3 h. Optical density was analyzed using a microplate reader at 450 nm.

Cycle analysis. The cell cycle distribution of Jurkat cells after GA treatment (at various concentrations) were analyzed using a Cell Cycle Staining Kit (LianKe) following the manufacturer's instructions. Jurkat cells were suspended and mixed in DNA staining solution (50 μ g/ml RNase A for 20 min at room temperature, followed by 50 μ g/ml propidium iodide for 10 min in the dark). After the 30 min treatment, distribution of cell cycle stages were detected by flow cytometry (Becton Dickinson). In the present analysis, each phase of the cell cycle was recorded as a percentage.

Apoptosis analysis. GA-treated Jurkat cells (at various concentrations) were suspended in 2-ml PBS to obtain a cell density of 1-5x10⁶/ml. The cells were tested using a PI Apoptosis Detection Kit (MultiSciences Biotech Co.) according to the manufacturer's protocol. Briefly, 10- μ l PI was added to the suspension, incubated for 20 min at room temperature, and maintained in the dark. The percentage of apoptotic cells was examined by flow cytometry. The results were analyzed using Novo Express software.

Immunocytochemistry. Jurkat cells were treated with various concentrations of GA and immersed overnight. Coverslips were treated with 0.2-mg/ml poly-L-lysine for 4 h prior to placing them on coverslips. After 1.5 h of incubation, the cells were placed firmly on the coverslips. These were fixed with 4% PFA (Beyotime) for 10 min at room temperature. Samples were fixed in 0.1% Triton X-100 (Beyotime) and 5% BSA prior to incubation. The primary antibody against p-GSK3 β S9 (1:200, Cell Signaling Technology, cat. no. 5558) was added, and the specimens were incubated overnight at 4°C. The next day, the cells were washed with PBS three times and the appropriate fluorescent secondary antibody Alexa Fluor 488

Figure 1. GA suppressed the proliferation of Jurkat and Molt-4 cell lines. (A) CCK-8 assays were performed to detect the proliferation of Jurkat and Molt-4 cells after GA treatment at an increasing concentration range. IC₅₀ value was calculated by SPSS21.0. (B) Jurkat and Molt-4 cells were cultivated with GA at the concentration of 1.2, 2.4, 4.8 μM, respectively, after 24, 48 and 72 h. The viability of Jurkat cells was measured by CCK-8 assays. GA suppressed the proliferation of Jurkat cells by arresting cell cycle at G₂/M phase. (C) Flow cytometric analysis detected the cell cycle distributions of Jurkat cells after GA treatment under various concentrations (from 0 to 4.8 μM). The corresponding statistical chart is also shown. (D) Western blot detected the expression of cycle-related proteins in Jurkat cells after GA treatment, CyclinB and CDK1, compared with control, *P<0.05 and **P<0.01, controls were treated with 0.1% DMSO.

was added (1:1,000, Beyotime, cat. no. A0423). The samples were incubated at room temperature for 1 h. Cells were stained with DAPI (Beyotime) to allow visualization of the nucleus. Finally, images were obtained using a fluorescence microscope (Olympus).

Monodansylcadaverine staining. Jurkat cells were plated in 6-well plates and treated with different concentrations

(1.2, 2.4 and 4.8 μM) of GA, and controls were treated with 0.1% DMSO. After treatment, the cells were collected and suspended with PBS at a density of 1.5×10⁶ cells/ml, followed by co-staining with monodansylcadaverine (MDC) plus DAPI for another 30 min at 37°C. Approximately 10-20 μl of the cell suspension was placed onto a glass slide and covered with a cover glass. The MDC staining was examined and photographed using a fluorescence microscope.

Figure 2. GA promoted the apoptosis of Jurkat cells. (A) Flow cytometric analysis detected the apoptosis rates of Jurkat cells after GA treatment at a growing concentration range (from 0 to 4.8 μ M) with PI single-staining. The corresponding statistical chart is also shown. (B) Western blotting detected the expression of apoptosis-related proteins in Jurkat cells after GA treatment, cleaved PARP (c-PARP), Bcl-2, cleaved-Caspase3 (c-Caspase3) and Bax. GA promoted the apoptosis of Jurkat cells. (C) MDC staining was used to observe autophagic vacuoles formed in Jurkat cells treated with GA. Statistical chart is shown on the right. (D) Western blot detected the expression of autophagy-related proteins in Jurkat cells after GA treatment, Atg7, Beclin1 and LC3-II. Bar scale: 20 μ m. Compared with control, *P<0.05 and **P<0.01, controls were treated with 0.1% DMSO.

Western blot analysis. Lysed cells in RIPA lysis buffer (Beyotime) were maintained on ice for 30 min and centrifuged at 14,000 x g for 10 min at 4°C. The protein concentration in the supernatant was verified by BCA Protein Assay

Figure 3. GA treatment down-regulated Wnt/ β -catenin signaling pathways in Jurkat cells. (A) Western blot detected the expression of Wnt signaling pathway proteins in Jurkat cells after GA treatment (β -catenin, p-GSK3 β S9, GSK3 β , CyclinD1, c-Myc). Corresponding statistical chart of β -catenin, p-GSK3 β , GSK3 β , p-GSK3 β S9/GSK3 β , CyclinD1 and c-Myc is also shown. (B) Single immunocytochemistry of p-GSK3 β S9 (green) in Jurkat cells treated with GA at indicated concentrations for 24 h. * $P < 0.05$ and ** $P < 0.01$ compared with control, controls were treated with 0.1% DMSO. Bar scale: 20 μ m.

(Thermo Scientific Inc). Total proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes. The membranes were blocked in 5% de-fatted milk for 1 h at room temperature and then incubated with primary antibodies at 4°C overnight. The matched secondary antibody (1:3,000, Beyotime, cat. nos. A0208, A0192) was incubated for 2 h at room temperature. Protein levels were detected using an enhanced chemiluminescence

reagent and quantified by ImageJ software. The antibodies used in the study were as follows: Anti-CyclinB (1:1,000, Abcam, cat. no. ab32053), anti-CDK1 (1:500, Beyotime, cat. no. AF0111), anti-Beclin1 (1:1,000, Thermo, cat. no. MA5-15825), and anti-LC3-II (1:1,500, Sigma, cat. no. 610832); anti-Bax (1:1,000, Abcam, cat. no. ab32503), anti-Bcl-2 (1:1,000, Abcam, ab182858), anti-cleaved caspase3 (1:1,000, Cell Signaling Technology, cat. no. 9661S),

Figure 4. Overexpression of β -catenin blocked the apoptosis of Jurkat cells induced by GA treatment. (A) Flow cytometric analysis detected the apoptotic rates of Jurkat cells after blank PV vector and the β -catenin plasmid transfection and GA treatment with PI single-staining. The corresponding statistical chart is shown below. (B) Western blotting was used to detect the expression of apoptosis-related proteins in Jurkat cells after plasmid transfection via GA treatment (2.4 μ M). Overexpression of β -catenin rescued the inhibition of downstream of Wnt/ β -catenin signaling in Jurkat cells induced by GA treatment (2.4 μ M). (C) Western blot was used to detect the expression of Wnt signaling pathway proteins in Jurkat cells after plasmids transfection by GA treatment. ** $P < 0.01$ compared with test treatment group, receiving blank plasmids plus GA.

anti-cleaved PARP (1:1,000, Abcam, cat. no. ab191217) anti-p-GSK3 β S9 (1:1,000, Cell Signaling Technology, cat. no. 5558), and anti-Atg7 (1:1,000, Cell Signaling Technology, cat. no. 8558s); anti- β -catenin (1:1,000, Cell Signaling Technology, cat. no. 19807S), anti-c-Myc (1:1,000, Cell Signaling Technology, cat. no. 2278s), and anti-CyclinD1 (1:1,000, Abcam, cat. no. ab134175); and anti- β -actin (1:3,000, Beyotime, cat. no. AF5001).

Cell transfection. Jurkat cells were seeded in 6-well plates and transfected using Lipofectamine 3000 (Invitrogen) with recombinant β -catenin and blank plasmids. The procedure was performed in accordance with the manufacturer's instructions. After 24 h, the cells were treated with GA (2.5 μ M). Observations were made after 48 h using an inverted fluorescence microscope. The expression of marker proteins in stably transfected cells were analyzed by western blot analysis.

Statistical analysis. Data are presented as mean $\pm$ standard deviation. Data were analyzed using SPSS21.0. The data

were collected from at least three independent experiments. Significance of the difference between two groups means of data sets was determined using the Student's t-test. Multiple comparisons test was analyzed by one-way ANOVA (Dunnett's correction). Results with $P < 0.05$ and $P < 0.01$ were considered statistically significant.

Results

GA inhibits the proliferation of human leukemia cell lines. To detect the anti-proliferative effects of GA *in vitro*, the human leukemia cell lines Jurkat and Molt-4 cells were exposed to increasing concentrations of GA. GA reduced the viability of Jurkat and Molt-4 cells in a dose-dependent manner (Fig. 1A-B). The IC50 values of GA for Jurkat and Molt-4 were 2.23 and 3.03 μ M, respectively. Jurkat and Molt-4 cells were treated with GA (1.2, 2.4 and 4.8 μ M) for 0, 24, 48 and 72 h. At the higher concentrations (2.4 and 4.8 μ M), GA inhibited the growth of cells significantly, and the inhibition rate increased with time. These results suggested that GA cytotoxicity is dose- and time-dependent in leukemia cells.

Figure 5. GA reduced the viability and promoted the apoptosis of lymphocyte cells from T-ALL patients. (A) CCK8 assays were performed to detect the viability of lymphocyte cell extract from healthy individuals (n=6) or T-ALL patient peripheral blood (n=6) treated by GA for 4 h. (B) Flow cytometric analysis was performed on lymphocyte cells from healthy individuals and T-ALL patients after 4 h GA treatment at 2.4 μ M with PI single staining. The corresponding statistical chart is shown on the right. **P<0.01 compared with samples from healthy individuals, receiving 0.1% DMSO.

GA induces G2/M phase cell cycle arrest in Jurkat cells. To test whether GA could induce Jurkat cell cycle arrest, cell cycle distribution was performed. The results demonstrated that the exposure of cells to GA at concentrations of 2.4 and 4.8 μ M led to a remarkable increase in G2/M phase, with a corresponding decrease in the S phase compared with exposure of cells to control or 1.2- μ M GA (Fig. 1C). To determine the underlying mechanisms causing G2/M phase arrest in Jurkat cells, we examined the effect of GA on the expression of CyclinB and CDK1 (key regulators of G2/M transition). Western blot results revealed that the protein levels of CyclinB and CDK1 were significantly decreased by GA (Fig. 1D). Taken together, these results indicate that GA may inhibit the proliferation of Jurkat cells by inducing cell cycle arrest at the G2/M phase.

GA induces apoptosis of Jurkat cells. It is known that apoptosis is programmed cell death. Thus, we examined whether GA induces apoptosis in cancer cells. Using a flow cytometric analysis the presence of apoptosis in GA-treated cells was identified. GA treatment induced significant apoptosis in Jurkat cells in a dose-dependent manner (Fig. 2A). Apoptosis was detected by western blot analysis (Fig. 2B). It was noted that GA treatment upregulated the expression of c-PARP, c-Caspase-3, and Bax but downregulated the expression of Bcl-2 compared with control treatment. Overall, these results demonstrated that GA upregulated the expression of pro-apoptotic proteins, thus causing apoptosis in Jurkat cells.

GA induced autophagy in Jurkat cells. We observed whether autophagic vacuoles were formed when Jurkat

cells were treated with GA by MDC staining (Fig. 2C). The number of MDC-positive fluorescent points in GA-treated cells was markedly greater than that in the control group. These results demonstrated that the inhibitory effect of GA on cell viability is related to the induction of autophagy. To investigate whether GA regulated the process of autophagy, the protein expression of autophagy-related markers were assessed by western blot analysis (Fig. 2D). Significant upregulation of Atg7, Beclin-1, and LC3-II occurred in GA-treated groups. These results suggest that GA may encourage autophagy of Jurkat cells.

GA regulates Wnt/ β -catenin signaling in Jurkat cells. To identify other mechanisms involved in the inhibitory effect of GA, we focused on the Wnt pathway (Fig. 3A). The protein levels of β -catenin, as well as downstream proteins CyclinD1 and c-Myc, were significantly decreased in a dose-dependent manner after GA treatment. GSK3 β , a highly effective kinase within the β -catenin destruction complex, phosphorylated β -catenin and promoted its destruction by proteasomes. Therefore, GA suppressed the expression of p-GSK3 β S9 and subsequently promoted β -catenin degradation to reduce β -catenin nuclear import. Immunostaining results showed that after GA treatment (Fig. 3B), the expression of p-GSK3 β S9 decreased. Our findings indicated that GA inhibits the growth of Jurkat cells by regulating the Wnt/ β -catenin pathway.

Overexpression of β -catenin inhibited the apoptosis of Jurkat cells. To further confirm the role of β -catenin in GA-induced regulation of Jurkat cell proliferation and apoptosis, we transfected the cells with plasmid-carrying β -catenin. We established that the overexpression of β -catenin protects Jurkat cells from apoptosis. Flow cytometry results showed that after β -catenin plasmid transfection, a proportion of the apoptosis (induced by GA) recovered (Fig. 4A). Western blot results revealed that the transfection of β -catenin plasmid reversed the increase of Bax and c-caspase-3 and decreased Bcl-2 induced by GA (2.4 μ M) (Fig. 4B). These results confirmed the hypothesis that the overexpression of β -catenin significantly inhibited the apoptosis of Jurkat cells. After transfection of β -catenin plasmid into GA-treated Jurkat cells, the protein levels of β -catenin, CyclinD1, GSK-3 β , p-GSK3 β S9, and c-Myc were measured using western blot analysis (Fig. 4C). The results showed that the expression of GSK3 β significantly decreased in GA-treated Jurkat cells transfected with β -catenin compared with that in cells transfected with the negative control. In the negative control group, the expression of β -catenin, p-GSK3 β S9, CyclinD1, and c-Myc are significantly increased.

Effects of GA on healthy people and T-ALL patients. GA toxicity was then examined. The results showed that GA is toxic to Jurkat cells. To investigate the potential of GA for cancer therapy, we evaluated the effects of GA on lymphocyte cells from healthy individuals and patients with T-ALL. Apoptotic assays revealed that cell viability of healthy individuals decreased slightly when treated with GA (Fig. 5). Conversely, GA treatment induced significant apoptosis in lymphocyte cells from patients with T-ALL.

Discussion

T-ALL is one of the most common types of aggressive hematological malignancies in children, and has high heterogeneity (33). The current treatment options for ALL include chemotherapy (such as VDLP or VILP regimen) and allogeneic bone marrow transplantation. However, the side effects of myelosuppression and central nervous system issues are still severe (34) leading to a poor prognosis (35). Drug resistance and inevitable recurrence also contribute to the poor prognosis of patients (36). Therefore, there is a need to explore new therapeutic targets for T-ALL treatment. GA, obtained from *G. hanburryi*, exhibits cytotoxic abilities in several solid tumors both *in vitro* and *in vivo*. The assumed mechanism of GA in treating solid tumors primarily involves activating apoptotic pathways (9,10). However, the exact role and possible molecular mechanisms underlying GA activity against T-ALL are still poorly understood and require extensive studies. In the current study, we evaluated the potential antileukemic effect of GA on T-ALL cells. We found that GA suppressed cell proliferation, affected cell cycle distribution, induced apoptosis, and activated autophagy in T-ALL cells. We identified that the antileukemic effect of GA on T-ALL cells may involve regulating the Wnt/ β -catenin signaling pathway by inactivating p-GSK3 β S9 and activating GSK3 β . These combined activities promote the degradation of the β -catenin protein.

Apoptosis induction plays an essential role in the antitumor strategies of various therapies. Previous findings have emphasized the fact that traditional Chinese medicines can strengthen the activation of caspase-independent cell apoptosis pathways (37). Flow cytometry analysis revealed that GA induces apoptosis in T-ALL cells. c-Caspase3 is known to activate other caspases, playing a critical role in the execution of the mitochondrial apoptotic pathway. In the present research, GA increased the expression levels of c-caspase 3 in T-ALL cells, indicating that the mitochondrial apoptosis pathway is affected by GA. Bax and Bcl2 are critical regulators of apoptosis, however, Bax is a proapoptotic protein, and Bcl2 is anti-apoptotic protein (38). GA reduced the expression of Bcl2 and increased that of Bax. Therefore, GA's ability to regulate the balance between Bax and Bcl2 provides evidence that GA induces apoptosis in T-ALL cells. The ability of GA to induce apoptosis is one of the mechanisms contributing to the anticancer effects of GA in T-ALL.

The existing literature shows that T-ALL stem cells are highly dependent on the Wnt/ β -catenin signaling pathway (39). Downstream target proteins of Wnt/ β -catenin signaling play major roles in neoplastic transformation, including cell cycling (c-Myc and CyclinD1), tumor growth, cell invasion, and tumor metastasis (40). Our flow cytometry results show that GA disturbs T-ALL cell cycle and induces G2/M arrest. Cell cycle arrest is closely related to cycle-regulating proteins (41). CyclinB1 and CDK1 are critical regulators of cell cycle progression, specifically in the G2/M transition (42). In our experiment, GA regulated the expression levels of CyclinD1, CyclinB1, and CDK1 proteins. The ability of GA to induce cell cycle arrest highlights its antineoplastic role. This demonstrates another mechanism that GA uses to induce anticancer effects on T-ALL.

The Wnt/ β -catenin signaling pathway plays an important role in the self-renewal of HSCs, leukemia progression, and BCR-ABL

kinase inhibitor resistance (43-46). In the Wnt signaling pathway, GSK-3 β binds to several proteins and undergoes phosphorylation, thereby inducing β -catenin degradation. GSK-3 β has been regarded as a tumor suppressor as it negatively regulates the Wnt/ β -catenin signaling pathway (47). It can regulate catalytic kinase activity through phosphorylation of different serine/threonine residue sites. Phosphorylation at tyrosine 216 significantly increases the enzymatic activity of GSK3 β , whereas phosphorylation at serine 9 decreases enzyme activity (48). Phosphorylation at S9 is the main mechanism causing GSK3 β inactivation (49). In the present study, we revealed that the p-GSK3 β S9 level decreases but the catalytic activity of GSK3 β increases by GA treatment. Preliminary results demonstrated that decreased β -catenin protein levels in T-ALL cells may result from GSK3 β activation. β -catenin overexpression is associated with neoplasm progression, cell proliferation, and cell survival (50). To evaluate our theories, we overexpressed β -catenin and verified the results using western blot analysis. The degree of proliferation and apoptosis in T-ALL cells after GA treatment showed that the inhibitory effect of GA on T-ALL cells was reversed. Overall, our results demonstrated that GA can suppress growth of T-ALL cells by inactivating the Wnt/ β -catenin signaling pathway.

It has been reported that the Wnt/ β -catenin signaling pathway is aberrantly upregulated during the occurrence and development of cancers. Thus, mature blood cells lack Wnt signaling (30). However, leukemia cells are highly proliferative with high Wnt/ β -catenin signaling. The deficiency of Wnt ligands is associated with low levels of β -catenin as destruction complexes target β -catenin for degradation. Our research found that GA could exert inhibitory effects on T-ALL cell lines and lymphocyte cells from patients with ALL. These results suggest that GA is more sensitive to lymphocyte cells from patients with T-ALL.

Autophagy is a highly conservative and protective process of cell recycling and degeneration of damaged functional proteins and organelles through lysosomal degradation and digestion (51,52). Autophagy plays an essential role in normal cell stabilization and tumorigenesis, drug resistance, and other pathophysiological processes (53-55). Currently, there is no uniform classification criterion for autophagy. Autophagy is regulated by autophagy-related genes. LC3 acts as an autophagosomal marker for monitoring autophagy (56). Beclin1 and Atg7 also belong to autophagy markers as they participate in the formation and maturation of autophagosomes (57). MDC is localized and mainly restricted to the lysosomal membrane (it is also used to assess autophagy activity). Recent research has shown that autophagy and the Wnt/ β -catenin pathway are closely related. Autophagy negatively regulates Wnt signaling (58). The tumor inhibitor GABARAPL1 suppresses the Wnt/ β -catenin pathway by activating autophagy (59). In our study, autophagy-related proteins (LC3-II, Beclin1, and Atg7) were upregulated, and MDC fluorescence intensity increased significantly after GA treatment. This suggests that GA induces autophagy in T-ALL cells. Activation of autophagy can inhibit the Wnt/ β -catenin pathway, further encouraging cell damage. However, the specific mechanisms require further research.

In summary, our study demonstrates that GA exhibits effective antileukemia characteristics in T-ALL cells. The proliferation, apoptosis, and autophagy regulation effects of GA on T-ALL cells seem to result from downregulating Wnt/ β -catenin signaling and upregulating GSK3 β activity,

thereby inducing β -catenin degradation. Collectively, our experimental results indicate that GA is a promising antileukemia agent for the treatment of T-ALL.

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Availability of data and materials

All relevant raw data in our trial will be made freely available to any researchers who wish to use them for non-commercial purposes.

Authors' contributions

YL, YW, TTW designed the study. JD, DK, GY, QZ performed the clinic trials. DK and GY analyzed the data. TTW and JD wrote the paper, and were responsible for the images. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Ethics approval was granted by the Zhejiang Provincial People's Hospital Ethics Committee. Informed consent was obtained from the subjects included in the present study.

Consent for publication

Not applicable.

Competing interests

The authors have no conflicts of interest to declare.

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