Original Article

Artesunate induces apoptosis and inhibits the proliferation, stemness, and tumorigenesis of leukemia

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Background: Leukemia is characterized by the presence of highly malignant tumors formed in the hematopoietic system. Artesunate (Art), a semi-synthetic derivative of artemisinin, is commonly used as an antimalarial drug and has been proven to possess anticancer potential.

Methods: In this study, the effect of Art on the proliferation and stemness of human acute promyelocyte leukemia HL-60 cells and acute myeloid leukemia KG1a cells was investigated. Flow cytometry, colony formation assay, the protein expressive levels of survivin, P21, cleaved caspase 3, Bax, Bcl-2, Ki67 were detected the effect of Art on HL-60 and KG1a cells proliferation and apoptosis. At the same time, cell sphere formation assay and the protein expressive levels of CD44, SOX2, ALDH1 and OCT4 were used to analyze the effects of Art on cancer stem cell-like property *in vitro*. The orthotopic xenograft mouse models were established by using KG1a cells in BALB/c athymic nude mice. Tumor weigh was detected. The protein levels of survivin and Ki67 were detected by immunohistochemistry assays.

Results: Art induced cell apoptosis and inhibited cell proliferation and stemness in a dose-dependent manner. In the meantime, the results exhibited that Art inhibited the growth and stemness of transplanted tumors via the suppression of the MEK/ERK and PI3K/Akt pathway.

Conclusions: Our present study provides new insights into the mechanisms of Art's anticancer potential in leukemia.

Keywords: Artesunate; leukemia; stemness; apoptosis; proliferation; tumorigenesis

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Introduction

Leukemia, which is characterized by tumors of the hematopoietic system, is one of the main malignancies endangering human health. Its incidence among all malignant tumors (liver cancer, stomach cancer, lung cancer, oesophagus cancer, rectum cancer, breast cancer, pancreas cancer, leukaemia, bran cancer and central nervous system cancer and colon cancer) ranks seventh in China (47 cases per 100,000) and sixth in Europe and America (64 cases per 100,000) (1). Chemotherapy and bone marrow transplantation are the main treatment methods for leukemia. Although they have a certain curative effect and prolong the survival of patients, due to the lack of specificity of chemotherapy drugs, they often cause severe side effects to patients, and some patients eventually develop drug-resistant recurrence (2). Therefore, more effective and less adverse therapeutic strategies need to be explored.

Artesunate (Art), a semi-synthetic derivative of artemisinin from *Artemisia annual*, is a first-line medicine for malaria (3). In the past decade, Art has been tested for its anticancer effects on various types of human malignancies

including leukemia (4-6). For instance, Art has been found to induce the apoptosis of acute myeloid leukemia THP-1 cells, myelodysplastic syndrome SKM-1 cells, and HepG2 cells (7-9), and inhibit the cell proliferation of breast cancer cells (10). Leukemia stem cells possess self-renewal capacity and differentiation potential, which play a critical role in leukemia resistance, relapse, and prognosis (11). Stem cells can also confer a unlimited proliferative capacity to cancer cells (12). Art has been shown to kill several kinds of leukemia cells (6,7,13), suggesting its antileukemia effect. However, the mechanism underling this effect is poorly understood.

Therefore, this study aimed to clarify the antileukemia mechanism of Art in regards to the proliferation, stemness, and tumorigenesis of this malignancy. To this end, human acute promyelocyte leukemia HL-60 cells and acute myeloid leukemia KG1a cells were examined *in vitro*. Then, the expression of MEK/ERK and PI3K/Akt pathway proteins were determined in both Art-treated and non-Art-treated leukemia cells. Finally, a nude mice xenograft model was established *in vivo* to detect the effects of Art on tumor growth and stemness.

We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi.org/10.21037/atm-20-4558).

Methods

Cell culture

Human acute promyelocyte leukemia HL-60 cells and acute myeloid leukemia KG1a cells were purchased from the American Type Culture Collection (ATCC)(Manassas, VA, USA) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), penicillin (100 U/mL, Gibco), and streptomycin (100 μg/mL, Gibco). These cells were maintained at 37 ℃ in an incubator with 5% CO_2. Art was purchased from Sigma-Aldrich and dissolved in phosphate-buffered saline (PBS).

Flow cytometry

HL-60 and KG1a cells were treated with Art (0, 10, 25, and 50 μM) for 24 hours. They were washed with ice-cold phosphate-buffered saline (PBS) before resuspension in Annexin V binding buffer. Then, incubation with fluorescein isothiocyanate (FITC)-conjugated Annexin V antibody (Cell Signaling Technology, Danvers, USA) and propidium iodide took place for 15 min at room temperature. Data analysis was performed using FlowJo (Tree Star, Ashland, OR, USA).

Colony formation assay

The proliferation capacity of HL-60 and KG1a cells was measured by colony formation assay. Then, 2×10^3 cells were cultured in six-well plates with 2 mL of RPMI 1640 medium containing 10% FBS at 37 ℃ for 10–14 days, until the colonies were visible to the naked eye. The cells were fixed with methanol and stained with 1% crystal violet for 15 minutes. Then, colony numbers were counted. All experiments were performed in triplicate.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Purified RNAs were extracted from HL-60 and KG1a cells using Trizol reagent (Invitrogen, USA). First-strand cDNA synthesis was performed using a PrimeScript™ RT reagent Kit (TaKaRa). The cDNA synthesis was performed at 37 ℃ for 60 minutes after heating at 95 ℃ for 10 minutes. The cDNA was amplified using SYBR Premix Ex Taq™ II (TaKaRa). The qRT-PCR data were analyzed using $2^{-\Delta\Delta Ct}$ method to calculate the relative expression levels of Ki67, survivin and P21. Relative Ki67, survivin and P21. mRNAs were normalized to β-actin. All reactions were performed in triplicate.

Western blotting analysis

HL-60 and KG1a cells were treated with Art (0, 10, 25, and 50 μM) for 48 hours. HL-60 and KG1a cells were collected, and tumor tissues were homogenized. Protein was extracted with radioimmunoprecipitation assay (RIPA) lysis buffer plus cocktail (Sigma Aldrich) and phenylmethanesulfonyl fluoride (PMSF, Sigma Aldrich). Next, 20 μg of protein extract was separated on 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skimmed milk and then incubated with the primary antibodies(anti-survivin, ab76424; anti-P21, ab188224; anti-cleaved caspase 3, ab2302; anti-Bax, ab32503; anti-Bcl-2, ab59348; anti-Ki67, ab16667; anti-CD44, ab157107; anti-SOX2, ab93689; anti-ALDH1, ab52492; anti-OCT4, ab181557; anti-MEK1, ab96379; Anti-p-MEK1, ab214445; anti-ERK1/2, ab17942; Anti-p-ERK1/2, ab223500; anti-

PI3K,ab32089; Anti-p-PI3K, ab182651; anti-Akt, ab235958; Anti-p-AKT, ab8805) on a shaker overnight at 4 ℃. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (rabbit IgG, 1/1,000 diluted; UK). A β-action antibody (Santa Cruz Biotech) was used as a control. All bands were detected using an enhanced chemiluminescence (ECL) Western blot kit (Amersham Biosciences, UK). The bands were measured with an Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, UK).

Cell sphere formation assay

First, 0.5×10^3 cells were seeded in Ultra-Low Attachment six-well plates (Corning, Corning, NY,USA) in the serum-free RPMI 1640 medium supplemented with 20 ng/mL EGF, 20 ng/mL bFGF, N-2 and, 2% B27 (Invitrogen, Carlsbad, CA, USA). After 14 days of culture, microscope images were acquired from the plate. The number and diameters of tumor spheres (diameter >30 μm) were counted from 4 to 6 images for each well. All results were reported relative to the negative control.

Animal experiments

Each of the animal experiments in this study was conducted according to the principles of the NIH Guide for the Care and Use of Laboratory Animals and received approval from The First Affiliated Hospital of Zhengzhou University (No. 20190918). Five-week-old male athymic nude mice were purchased from the Model Animal Research Institute of Zhengzhou University. Approximately 1×10^7 KG1a cells were injected subcutaneously into the right flank of each nude mouse. The mice were randomly divided into the control and treatment group. The treatment group received 100 mg/kg Art i.p. three times a week for 3 weeks. The mice were humanely killed, and the tumors were photographed.

Immunohistochemistry

Immunohistochemistry for Ki67 and survivin was performed to detect tumor expression. Sections (2 μm) were deparaffinized and pretreated with citrate buffer using a heat-induced epitope retrieval protocol. Endogenous peroxidase was blocked with 20% hydrogen peroxide for 15 minutes at room temperature followed by incubation with anti-Ki67 and anti-survivin for 30 minutes respectively. A biotinylated goat anti-mouse immunoglobulin G secondary antibody (Dako, Denmark) was then applied for 30 minutes. Slides were incubated with peroxidase-conjugated streptavidin reagent (Dako, Denmark) and developed with 3,3'-diaminobenzidine for 5 minutes. The slides were counterstained and dehydrated.

Statistics analysis

The results are expressed as mean values ± standard deviation (SD) or standard error (SE). Statistical comparisons were analyzed by one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test. A significance level of <0.05 was used for all tests. SPSS-PASW statics software version 18.0 (SPSS Inc., Chicago, IL, USA) was used for all the statistical analyses.

Results

Art promoted apoptosis of leukemia cells

To determine the influence of Art on the apoptosis of leukemia cells, HL-60 and KG1a cells were treated with 0, 10, 25, and 50 μM of Art, and flow cytometry assay was performed. As shown in *Figure 1A*, 10, 25, and 50 μM Art stimulated cell apoptosis in a dose-dependent manner. To confirm the enhanced apoptosis, Western blotting was conducted. As depicted in *Figure 1B,C,D*, the protein level of cleaved caspase3 and the ratio of Bax/Bcl-2 were significantly up-regulated by Art in a dose-dependent manner ($P<0.05$).

Art impeded proliferation of leukemia cells

To determine the role of Art on proliferation of leukemia cells, HL-60 and KG1a cells were treated as previously described, and colony formation assay was performed. As shown in *Figure 2A*, 10, 25, and 50 μM of Art suppressed the colony forming rate in both HL-60 and KG1a cells in a dose-dependent manner ($P<0.05$). Quantitative real-time polymerase chain (qRT-PCR) and Western blotting were applied. The Ki67 expression in both the mRNA level and protein level was significantly reduced by Art in a dose-dependent manner (*Figure 2B,C*, $P<0.05$). As depicted in *Figure 2D,E*, the mRNA level and protein level of survivin were both significantly reduced by Art in a dose-dependent manner ($P<0.05$). *Figure 2F,G* shows that mRNA level and protein level of P21 were both significantly reduced by Art in a dose-dependent manner ($P<0.05$).

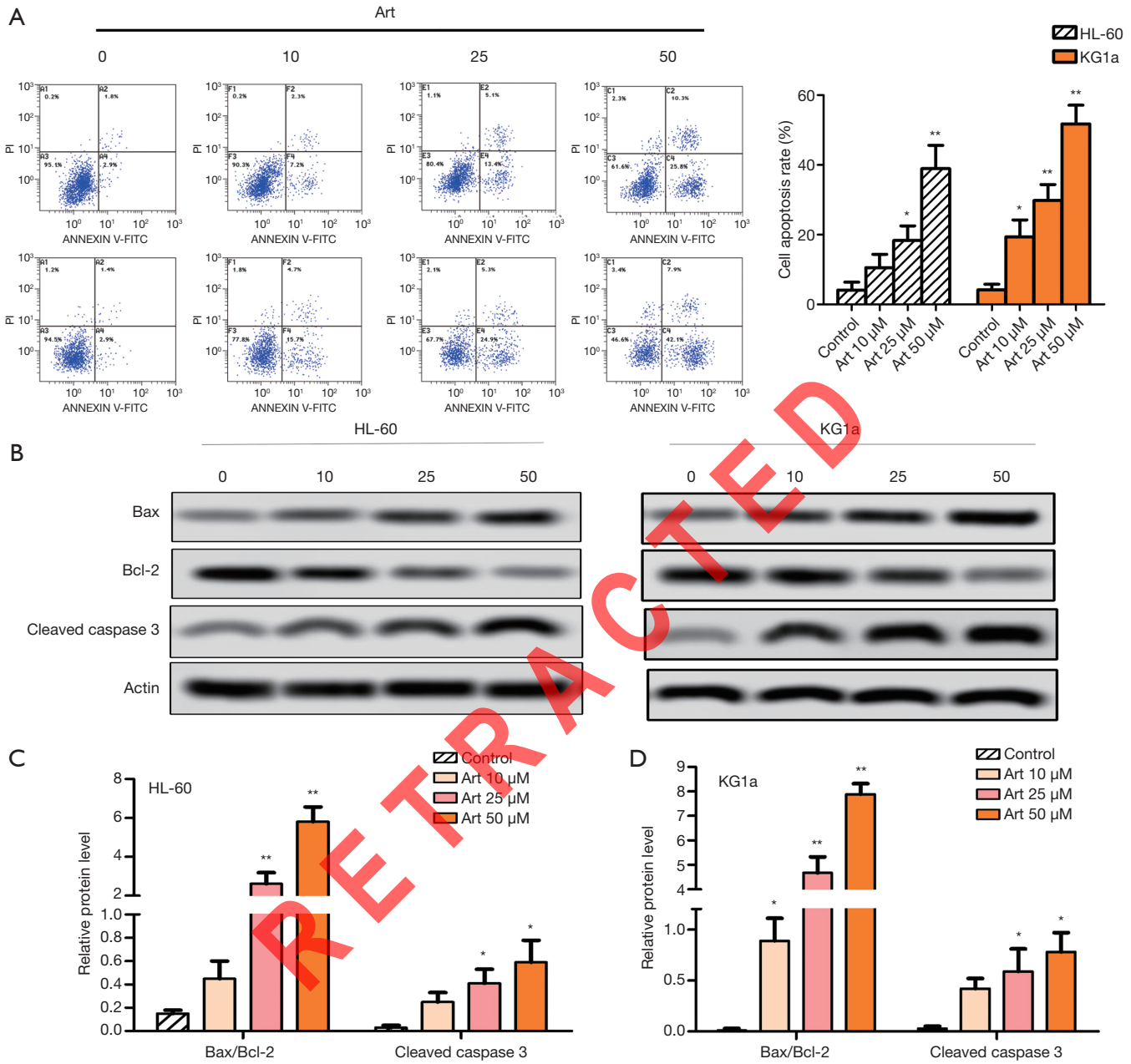

Figure 1 Art promoted apoptosis of leukemia cells. (A) Apoptosis of HL-60 and KG1a cells was detected by flow cytometry. The representative column diagrams show results of the apoptosis rate of HL-60 and KG1a cells. (B) The protein expression of cleaved caspase 3, Bax, and Bcl-2 was detected by western blot in both HL-60 and KG1a cells, respectively. (C,D) The representative column diagrams show results of relative protein expressions in both HL-60 and KG1a cells. β-actin was used as the loading control. *, P<0.05, compared with Art 0 μM group. **, P<0.01, compared with Art 0 μM group. Data are mean ± SD for the results of three replicates.

Art suppressed stemness of leukemia cells

To determine the role of Art on stemness of HL-60 and KG1a cells, cell sphere formation assay was performed, and the result was observed under a phase contrast microscope. As shown in *Figure 3A*, 10, 25, and 50 μM of Art suppressed the cell sphere formation ability in a dose-dependent manner as evidenced by the reduced diameters

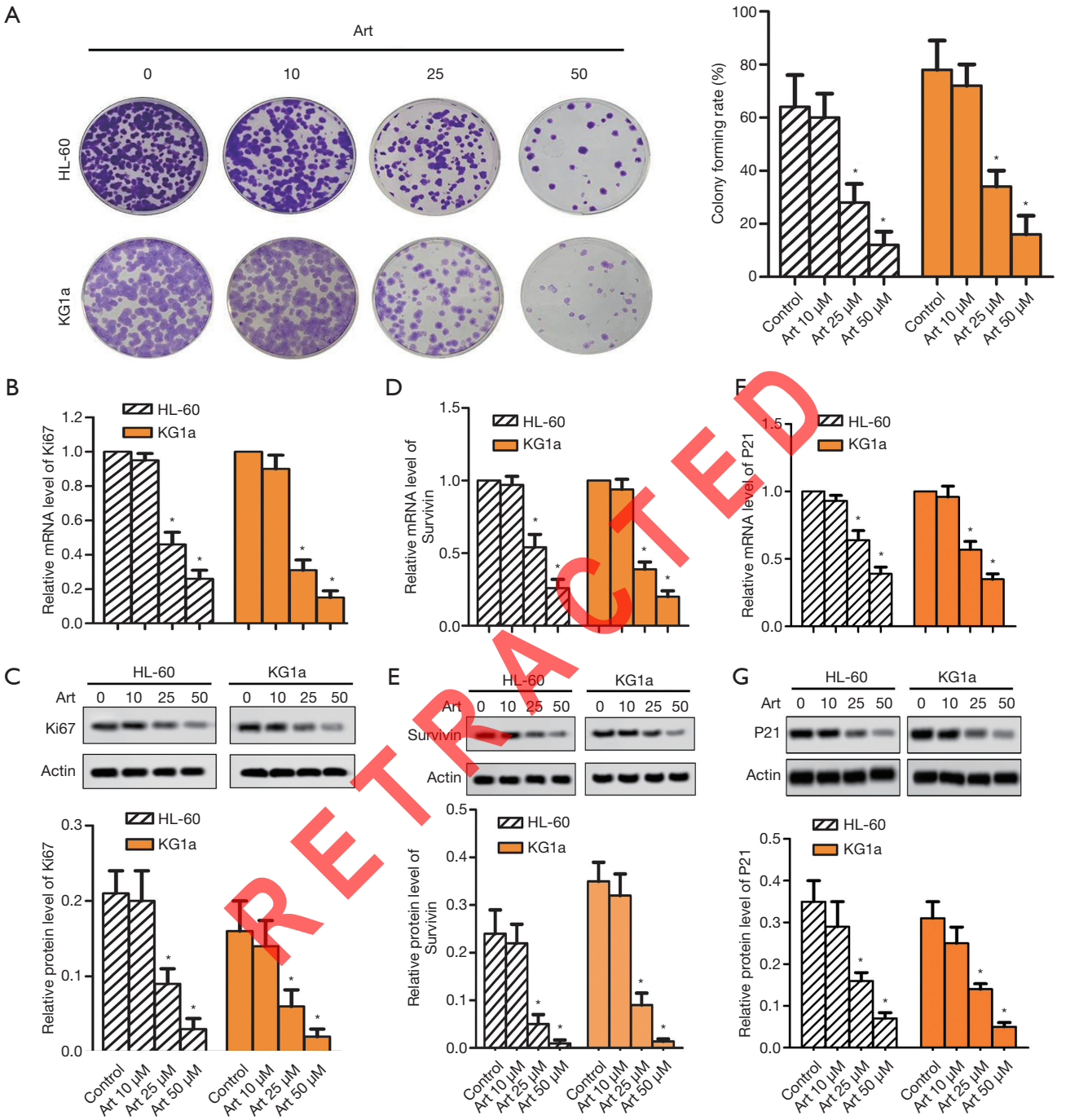

Figure 2 Art impeded proliferation of leukemia cells. (A) HL-60 and KG1a cells proliferation was analyzed by colony formation assay, and was stained with 1% crystal violet. The colony formation rate is shown as a column diagram (magnification, ×40). (B) The mRNA level of Ki67 was detected by qRT-PCR. (C) The protein expression of Ki67 was detected by Western blot. The representative column diagrams show results of relative protein expressions in both HL-60 and KG1a cells. (D) The mRNA level of survivin was detected by qRT-PCR. (E) The protein expression of survivin was detected by Western blot. The representative column diagrams show results of relative protein expressions in both HL-60 and KG1a cells. (F) The mRNA level of P21 was detected by qRT-PCR. (G) The protein expression of P21 was detected by Western blot. The representative column diagrams show results of relative protein expressions in both HL-60 and KG1a cells. β-actin was used as the loading control. *, P<0.05, compared with Art 0 μM group. Data are mean ± SD for the results of three replicates.

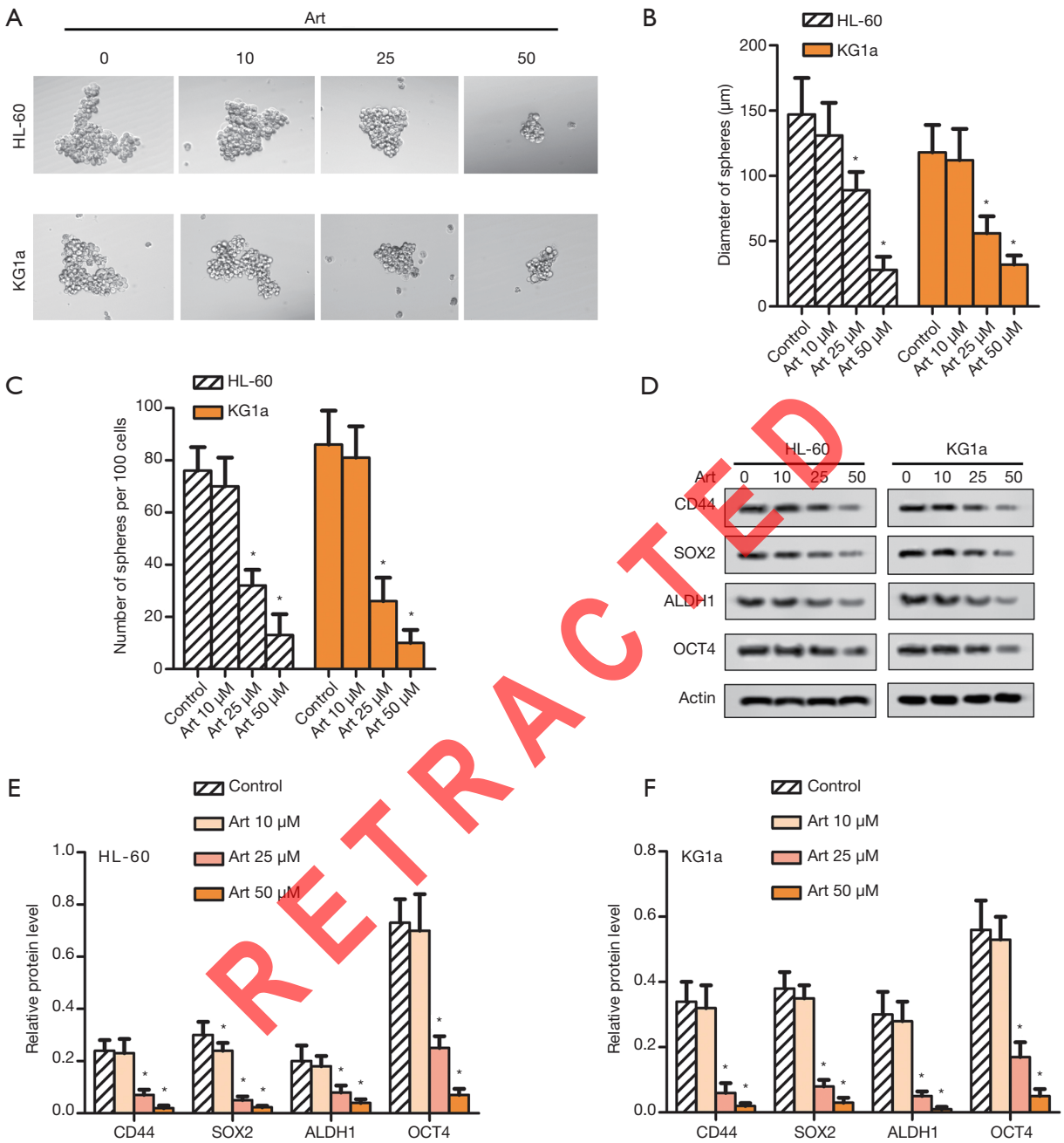

Figure 3 Art suppressed stemness of leukemia cells.(A) Cell stemness of HL-60 and KG1a cells was analyzed by sphere formation assay (magnification, ×100). (B) The diameters of the spheres were quantified. (C) The number of spheres was counted. (D) The expressions of proteins CD44, SOX2, ALDH1 and OCT4 were determined by Western blot in both HL-60 and KG1a cells. (E-F) The representative column diagrams show results of relative protein expressions in both HL-60 and KG1a cells. *, P<0.05, compared with Art 0 μM group. Data are mean ± SD for the result of three replicates.

of the spheres (*Figure 3B*) and sphere numbers (*Figure 3C*). Detection of expression of stemness marker proteins was then performed. The expressions of CD44, SOX2, ALDH1 and OCT4 were all inhibited by Art in a dose-dependent manner (*Figure 3D,E,F*, P<0.05). These results indicated that Art significantly reduced stemness of HL-60 and KG1a

leukemia cells.

Art inhibited MEK/ERK and PI3K/Akt signaling pathways

MEK/ERK and PI3K/Akt signaling pathways have been shown to play essential roles in leukemia. We thus used Western blot to detect the pathway protein. As shown in *Figure 4A,B*, the expressions of p-PI3K, p-AKT, p-MEK1, and p-ERK1/2 were all inhibited by Art in a dose-dependent manner in both HL60 and KG1a cells. After an inhibitor was added, the HL60 and KG1a cells was divided into control group, inhibitor group, Art (25 μM) group and Art (25 μM) + inhibitor group (*Figure 4C,D*). The expressions of p-PI3K, p-AKT, p-MEK1, and p-ERK1/2 were inhibited in the Art + inhibitor group compared with the Art (25 μM) group. The results indicated that Art inhibited MEK/ERK and PI3K/Akt signaling pathways.

Art inhibited growth and stemness in KG1axenograft models

KG1a xenograft mouse models were established to confirm the anticancer role of Art *in vivo*. Some mice were treated with Art and some were not (100 mg/kg). Tumor size (*Figure 5A*) and tumor weight (*Figure 5B*) were significantly decreased by Art. Immunohistochemistry analysis (*Figure 5C,D*) showed that Art significantly reduced the expression of Ki67 and survivin, indicating that cell proliferation was restricted. Art also impeded the cell sphere formation ability as evidenced by reduced diameters of spheres and sphere numbers (*Figure 5E,F*) *in vivo*. These results suggest that Art inhibited growth and stemness in KG1a xenograft models.

Discussion

Due to advances in chemotherapy regimens and supportive therapy, the application of hematopoietic stem cell transplantation, and a greater understanding of its pathogenesis, the prognosis of leukemia has improved (14). However, many obstacles, such as multi-drug chemoresistance, high cost, severe side effects, and limited bone marrow bank resources, have greatly restricted the treatment of leukemia in modern medicine (15). Therefore, there has been an intensification of research into developing effective and low toxic anti-tumor cell agents from natural drugs.

Pharmacological studies have confirmed that artemisinin and its derivatives not only have antimalaria and antitumor effects but can also inhibit the proliferation of white blood cells (16). Pharmacokinetic studies have proven that artemisinin can be absorbed quickly, distributed widely, and excreted quickly from the body (17), while toxicological studies have confirmed that its side effects are mild (18). Interestingly, the main target organ of artemisinin toxicity is bone marrow (19). Artemisinin can inhibit bone marrow hematopoiesis and has a strong targeting effect on leukemia cells, which is one of the characteristics of antileukemia drugs. Art, as a derivative of artemisinin, has long been used clinically as an antimalarial drug (20). *In vivo* and *in vitro* experiments have shown that Art can selectively kill tumor cells and reverse the multidrug resistance of tumor cells, without cross-resistance to traditional chemotherapy drugs (21). Therefore, Art may serve as a new type of antileukemia drug in clinical application. Many researchers have studied the antitumor effect and mechanism of Art, and the main anti-tumor mechanisms primarily involve proliferation inhibition and apoptosis promotion.

Survivin is expressed in most human tumors, and can inhibit key members of the caspase protease family (such as caspase-3 and -7) and exert an apoptosis-inhibiting effect. P21, the first CKI gene found, mainly acts by modulating the activity of CDK. P21 blocks the activity of all cyclin-CDK complexes, such as cyclin E-CDK2, cyclin D-CDK4, and cyclin A-CDK2 (22). Overactivation of the P21 gene can result in cell cycle disorders and block cell proliferation (23). Previous studies have shown that, in conjunction with surviving, p21 and p27 may also inhibit cell apoptosis (24,25). Results of this study suggest that Art enhances tumor cells apoptosis and inhibits cell proliferation.

Increasing evidence shows that leukemia stem cells have an important role in chemoresistance and relapse of leukemia (26). In this study, we first explored the role of Art on stemness of leukemia cells, which showed that Art inhibited stemness of leukemia cells, as evidenced by confined sphere formation and reduced expressions of stem cell marker proteins including CD44, SOX2, ALDH1, and OCT4.

PI3K/Akt and MEK/ERK signaling pathways play important roles in mediating extracellular stimulation to intracellular response, regulating gene expression, and inhibiting apoptosis (27). Inhibition of Akt activity with PI3K inhibitor LY294002 or the protein synthesis inhibitor actinomycin can increase TRAIL-induced apoptosis, while overexpression of active Akt makes TRAIL-sensitive cell line SUN-668 resistant to induced apoptosis (28).

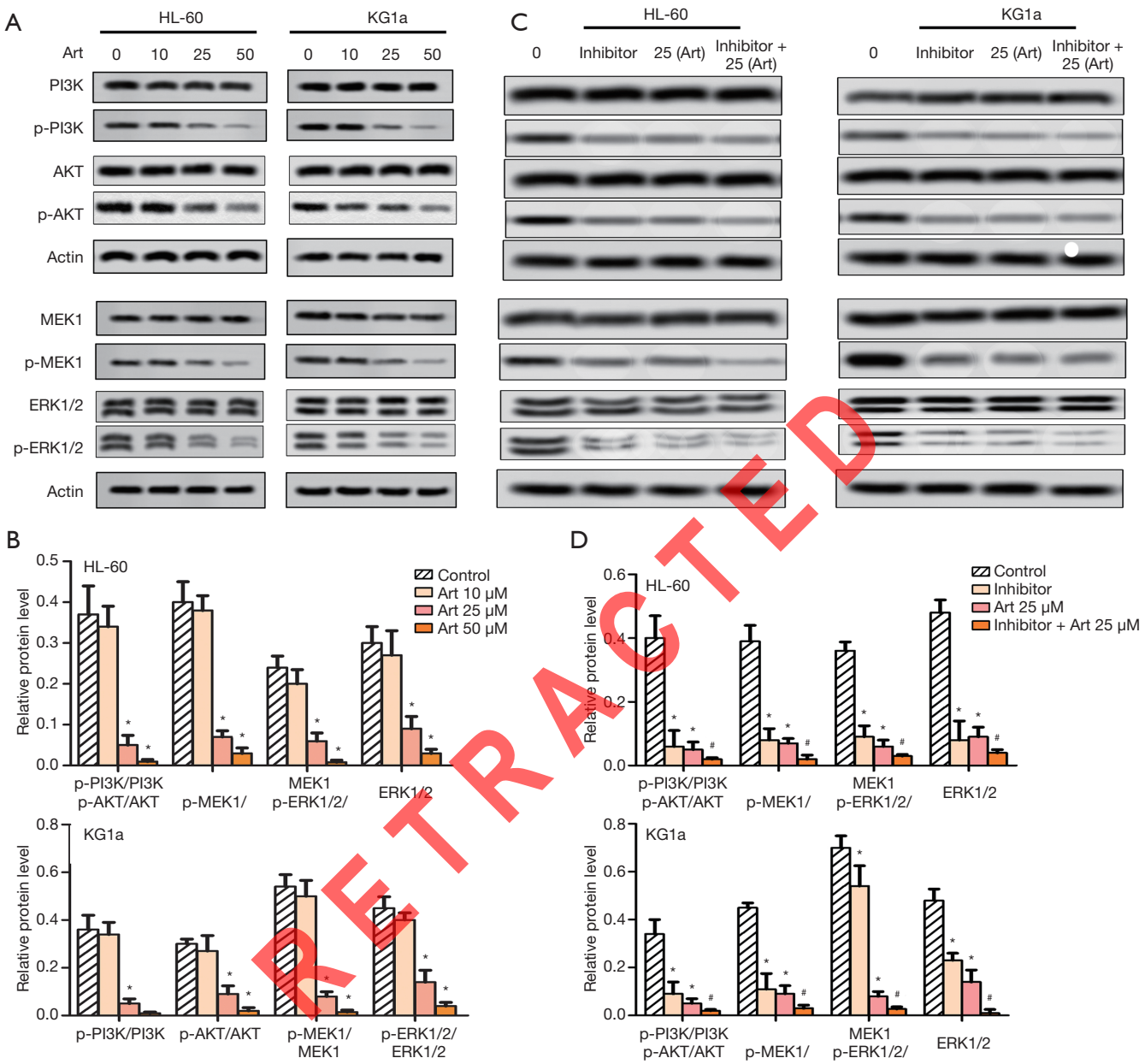

Figure 4 Art inhibited MEK/ERK and PI3K/Akt signaling pathways. (A) The expressions of proteins MEK1, p-MEK1, ERK1/2, p-ERK1/2, PI3K, p-PI3K, AKT, and p-AKT were determined by Western blot in both HL-60 and KG1a cells. (B) The representative column diagrams show results of relative protein expressions. (C) The expressions of proteins MEK1, p-MEK1, ERK1/2, p-ERK1/2, PI3K, p-PI3K, AKT, and p-AKT were determined by Western blot in both HL-60 and KG1a cells. (D) The representative column diagrams show results of relative protein expressions.*, P<0.05, compared with Art 0 μM group; #, P<0.05, compared with Art 25 μM group. Data are mean ± SD for the results from three replicates.

The addition of MEK1/2 inhibitor in Hela cells also significantly increase the rate of apoptosis induced by TRAIL, indicating the protective effect of cells against apoptosis (29). Recent studies have shown that the MEK/ERK cascade phosphorylates Thr125 on caspase-9, thereby inactivating caspase-9 (30). In this study, PI3K/Akt and MEK/ERK signaling pathways were inhibited by Art. This result suggests that Art may promote apoptosis of leukemia

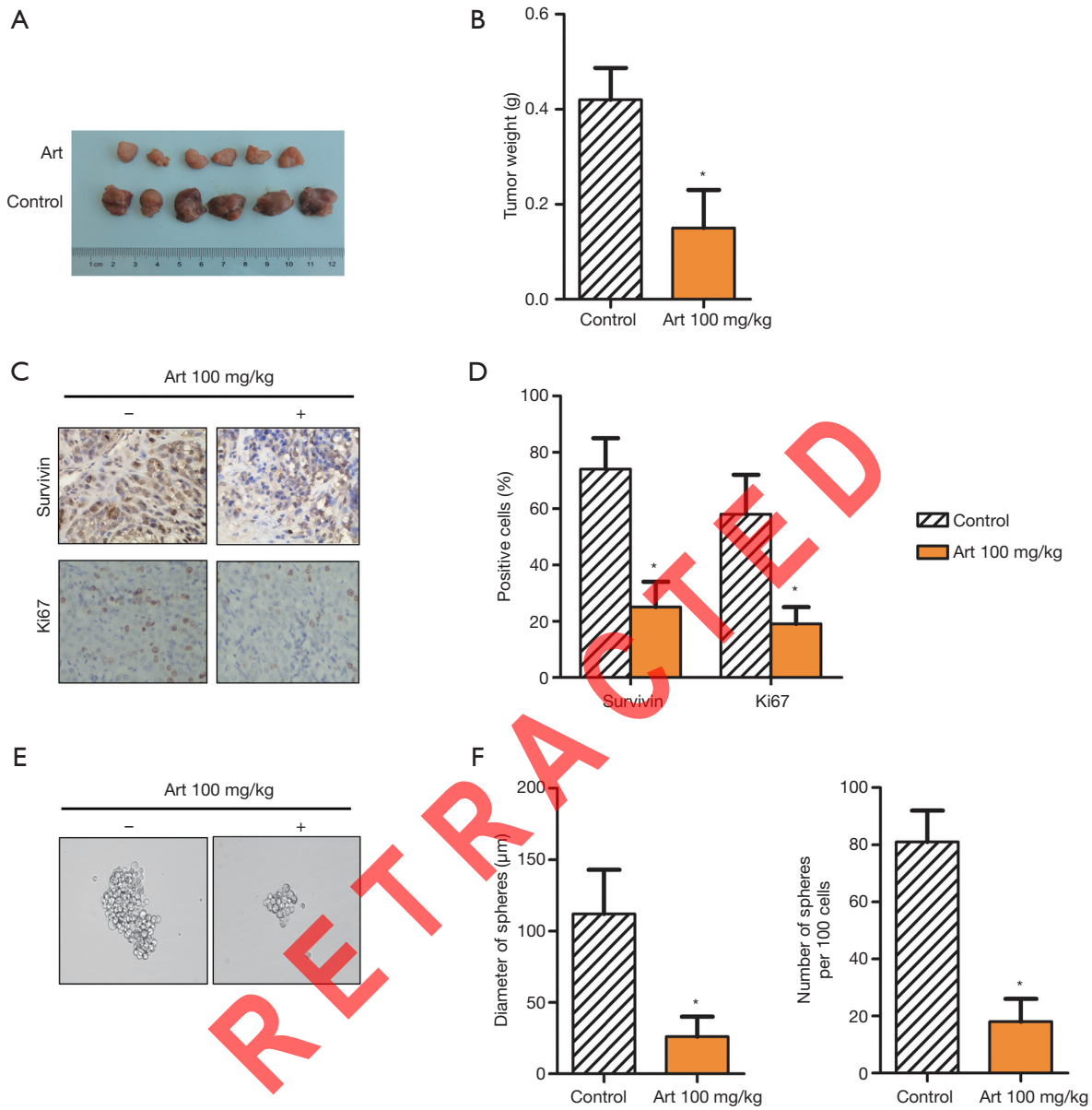

Figure 5 Art inhibited growth and stemness in KG1a xenograft models. (A) Some nude mice bearing subcutaneous xenograft leukemia were treated with100 mg/kg Art and some were not. (B) The representative column diagram shows tumor weight. (C) Immunohistochemistry analysis of expression of Ki67 and survivin (magnification, ×200). (D) The representative column diagram shows the results of positive cells (brown). (E) Cell stemness of KG1a cells was analyzed by sphere formation assay (magnification, ×100). (F) The diameters of spheres were quantified. *, P<0.05, compared with control. Data are mean ± SD for the results from three replicates.

cells via inactivating PI3K/Akt and MEK/ERK signaling pathways.

Cancer stem cells, which are defined by self-renewal, differentiation potential and tumorigenicity, are proposed to be responsible for cancer initiation and maintain the tumor mass. Typically, growth factors are known to stimulate MEK/ERK pathways, which is responsible for mitogenic activity, while the PI3K appears more related with the maintenance of stemness (31). The ING family of epigenetic regulators (ING5) also enhances PI3K/AKT

and MEK/ERK activity to sustain self-renewal of Stem cell-like brain tumor initiating cells (BTICs) over serial passage of stem cell-like spheres (32). CD44 is an important oral cancer stem cell marker, CD44v4 expression is more linked with ERK1/2 activation and promote cisplatin resistance, whereas CD44v6 expression is associated primarily with PI3K/Akt/GSK3β activation and driving tumor invasion/migration (33). Bufalin inhibited the activation of MEK/ERK and PI3K/AKT signaling pathways by inhibiting the expression of p-c-Met, thus inhibiting proliferation of GBC-SD cells and reducing the self-renewal ability of gallbladder cancer stem cells (34). Activation of PI3K/Akt and MEK/ERK signaling pathways has been proven to promote stemness of cancer cells (35,36). Consistent with the previous studies, Art inhibited stemness of leukemia cells, and interestingly, this mechanism might be regulated by PI3K/Akt and MEK/ERK signaling pathways.

In conclusion, this study showed that Art inhibited proliferation and stemness of leukemia cells *in vitro* and *in vivo*, and promoted their apoptosis. The mechanism of Art regulation of cell apoptosis, proliferation, and stemness consist of the modulation of PI3K/Akt and MEK/ERK signaling pathways.

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Footnote

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Data Sharing Statement: Available at http://dx.doi.org/10.21037/atm-20-4558

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/atm-20-4558). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Each of the animal experiments in this study was conducted according to the principles of the NIH Guide for the Care and Use of Laboratory Animals and received approval from The First Affiliated Hospital of Zhengzhou University (No. 20190918).

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