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Original Article

Colon cancer stem cells from HT29 cell line resist to arctigenin via suppressing ROS production

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Abstract: Cancer stem cells (CSCs) are a minority of the tumor cell population with stem cell properties, being considered as the major culprit for tumor initiation, relapse and metastasis. Arctigenin (AR), a natural lignan product identified in traditional Chinese herbs, has been reported to have multiple pharmacological effects such as anti-tumor, anti-inflammatory, anti-oxidant, anti-proliferative and anti-diabetic activities. In this study, we evaluated the different sensitivity of cancer stem cells versus non-stem cells in response to AR. Under both adherent and sphere forming conditions, tumor suppressive effects of AR were observed, while colon cancer stem cells may display a higher resistance to suppressive effects of AR than those non-cancer stem cells. Further experiments showed that the reduced pro-apoptotic and growth prohibitory effects in sphere culture versus monolayer under AR treatment. Mechanism studies disclosed that the levels of anti-oxidant enzymes, including uncoupling protein 2 (UCP2), nuclear transcription factor erythroid-2-related factor 2 (Nrf2) and superoxide dismutase (SOD), were dampened in adherent condition, as a contrast, the adaptive up-regulations of those enzymes in sphere condition were detected. In conclusion, cancer stem like cells appeared to be more resistant to tumor-suppressive effects of AR, possibly related to higher anti-oxidant capability and restricted reactive oxygen species (ROS) induction.

Keywords: Colon cancer, cancer stem cell, arctigenin, colosphere, reactive oxygen species

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer mortality worldwide, with more than 1.2 million new cases and over 600,000 deaths per year [1]. Current chemotherapy regimens are largely ineffective due to chemo-resistance [2]. Hence, it is urgent to explore the underlying mechanisms of the resistance to treatment of cancer.

Accumulating evidences indicate that tumor is a heterogenous group of cells, consisting of a small subpopulation of cells characterized by precursor or stem cell-like features, namely cancer stem cells (CSCs) or tumor-initiating cells (TICs). These stem cell-like subgroups of cells are identified with higher stem cell markers, such as CD44+, CD133+ staining [3, 4], and endowed with higher tumor initiating and tumor metastasis capacity [5]. Many evidences demonstrate that those CSCs are more resistant to both radio-therapy and chemotherapy, thus leading to the metastasis and relapse of cancer [6]. Therefore it's imperative to explore the mechanisms that CSCs are more resistant to cancer therapy.

Arctigenin (AR) is a novel anti-inflammatory lignan derived mainly from the seeds of the plant *Arctiumlappa* [7]. *Arctiumlappa*is widely used in traditional Chinese medicine to treat inflammation related diseases such as cough, cold and swelling of throat [7]. The anti-carcinogenic effect of AR and arctiin were also applied on several cancers, consistent with induction of apoptosis, inhibition of proliferation and modulation of multiple signaling pathways [8, 9]. It's interesting to know whether this drug have any preference on killing cancer stem cells.

In the present study, by employing with sphere forming assay, we intend to evaluate the potential usage of AR in cancer treatment by compar-

ing the different sensitivity of cancer stem cell versus non-stem cells towards AR treatment in colon cancer cell model and then define the relevant mechanisms.

Materials and methods

Cell culture and sphere formation assay

The colon cancer cell line HT-29 was obtained from China Center for Type Culture Collection and grown in 1640 medium supplemented with 10% fetal bovine serum (FBS). For sphere formation, cells were grown in serum free culture medium DMEM/F12+ GlutMAX™ (Gibco, Paisley, UK) supplemented with growth factors including 0.4% BSA, EGF (20 ng/mL), bFGF (10 ng/mL) and B27 (100×, Gibco, Paisley, UK). Cells were plated in 6-well ultra low attachment plates, at a density of 2000 cells per well and cultured for 5-7 days. To test the effect of drugs in the sphere formation ability, cells were plated in the presence of DMSO (vehicle) and AR (10 μg/mL) at the beginning of the experiment, without further addition of the drug. After 5 days, plates were analyzed for colon cancer sphere formation and the number of spheres per well was quantified using an inverted microscope (Olympus, Hamburg, Germany).

MTT assay

MTT assay was performed to analyze the tumor suppressive effect of arctigenin on HT-29 colon carcinoma cell line. In brief, 1×10^4 cells/well were plated in 96-well plates for 72 hours at 37°C for cell viability assay. Cells were exposed to different doses of arctigenin (0 μg/mL, 10 μg/mL and 50 μg/mL). After the incubating period, 20 μL of 3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT; Sigma) solutions were added to the treated cells and were left for incubation at 37°C for 4 hours. Cell viability was recorded by mitochondrial succinate mediated MTT formazan production. Afterwards, the supernatant was removed and the formed MTT formazan was dissolved by adding 20 μL of DMSO. The optical density of the samples at 570 nm wavelength was recorded to calculate the cell viability. For accuracy and reproducibility all the experimental sets were performed in triplicate and expressed as mean ± SD.

Flow cytometry analysis

The anti-CD44-FITC (BD Biosciences, San Diego, CA, USA) antibody was analyzed to detect CD44 on the cell surface. FITC-conjugated Annexin V and propidium iodide (PI) (Annexin-V-FLUOS, Boehringer Mannheim, Mannheim, Germany) were used to assess apoptotic cells and cell cycle. The cells were then incubated for 15 min at room temperature. Following incubation, the cells were washed once with cold PBS buffer. The labeled cells identified by fluorescence intensity were analyzed using BD FACSAria III (BD Biosciences).

Quantitative real-timepolymerase chain reaction (qRT-PCR)

Total mRNA was isolated from HT-29 monolayer cells and spheres using TRIzol reagent (Invitrogen) in accordance with the manufacturer's instruction. 2 μg sample of total RNA was reverse-transcribed into the cDNA (M-MLV Reverse Transcriptase, Promega). Quantitative real-time PCR was performed in 96-well plates with the Power SYBR Green PCR Master Mix (Takara, Dalian) on CFX96 Real-Time PCR system (Bio-Rad). The primers for amplification were: Nrf2: 5'-TCTGCCAACTACTCCCAGGT-3' (forward) and 5'-AACGTAGCCGAAGAAACCT-CA-3' (reverse); SOD: 5'-TGGACAAACCTCAGCC-CTAA-3' (forward) and 5'-TTGAAACCAAGCCAA-CCC-3' (reverse); UCP2: 5'-GATACCAAAGCACC-GTCAATG-3' (forward) and 5'-GGCAAGGGAGG-TCATCTGTC-3' (reverse); β-actin: 5'-AGCGGGA-AATCGTGCGTGAC-3' (forward) and 5'-TGGAAG-GTGGACAGCGAGGC-3' (reverse).

Western blotting analysis

HT-29 monolayer cells and spheres cultured in different conditions were washed two times with ice cold phosphate-buffered saline (PBS) and lysed in RIPA buffer. Protein concentrations were determined using the BCA assay kit (Thermo). Total protein of 50 μg were separated on 12% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membrane, Membranes were then probed with indicated primary antibodies, reacted with corresponding HRP-conjugated secondary antibodies. Membranes were exposed to ECL Western Blotting Detection Reagents (GE Healthcare Biosciences). β-actin antibody was used as an internal protein control. The experiments were repeated in triplicate.

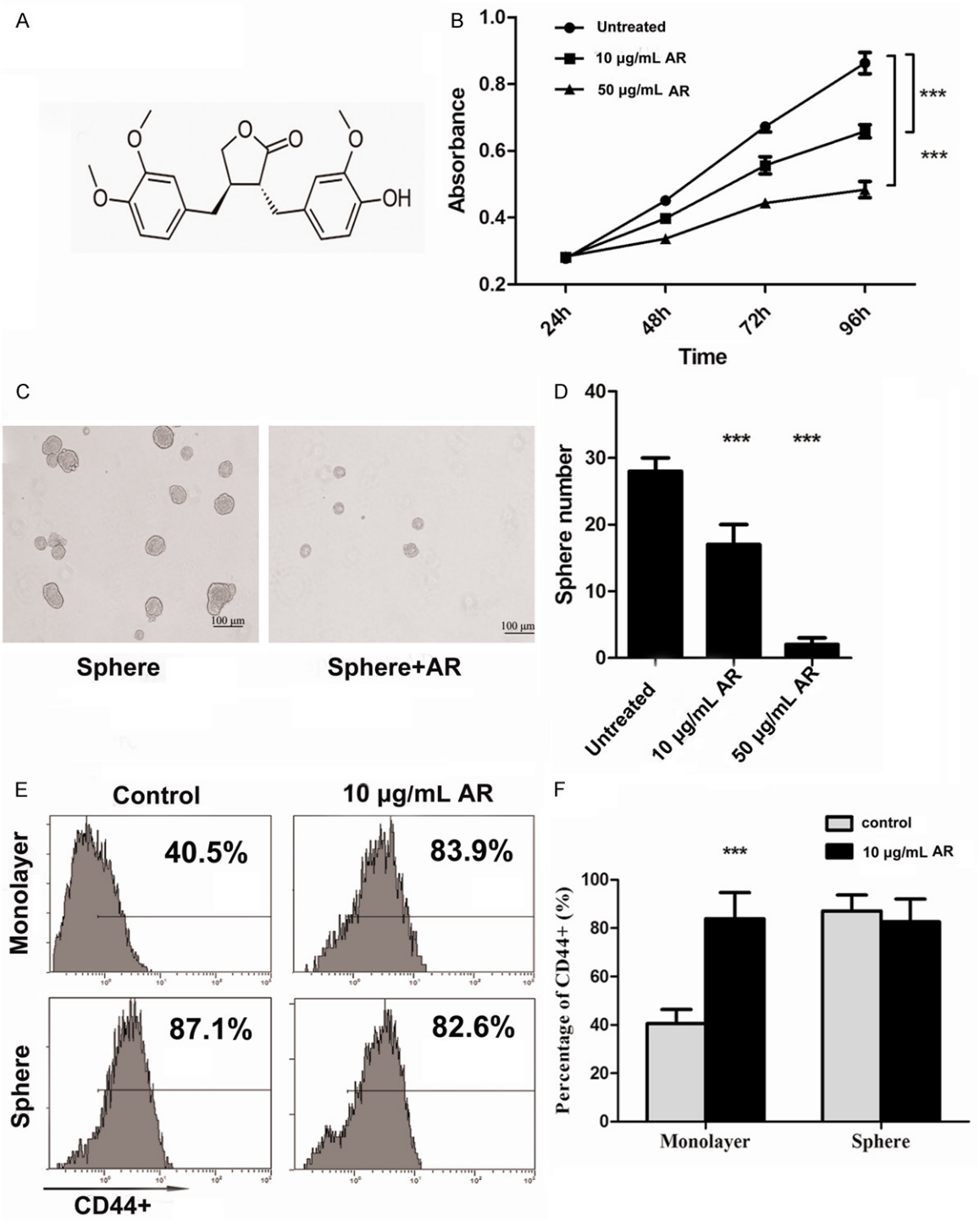


Figure 1. Tumor suppressive effects of AR under both adherent and sphere culture conditions and enrichment of CD44 positive stem like cells after treatment with AR. A. Chemical structure of AR. B. MTT assay showed remarkable tumor suppressive effect of AR on HT-29 colon carcinoma cell line in a dose dependent manner (one-way ANOVA). C. Sphere formation assay revealed decreased sphere number. D. The number of spheres per well was quantified using an inverted microscope (one-way ANOVA). E and F. Flow cytometry analysis indicated colon cancer stem cells may display a higher resistance to pro-apoptotic or anti-proliferative effects of AR than those non-cancer stem cells (Student's t-test). Data represented three repeated experiments and were shown as mean ± SD (n = 3). (***P<0.001).

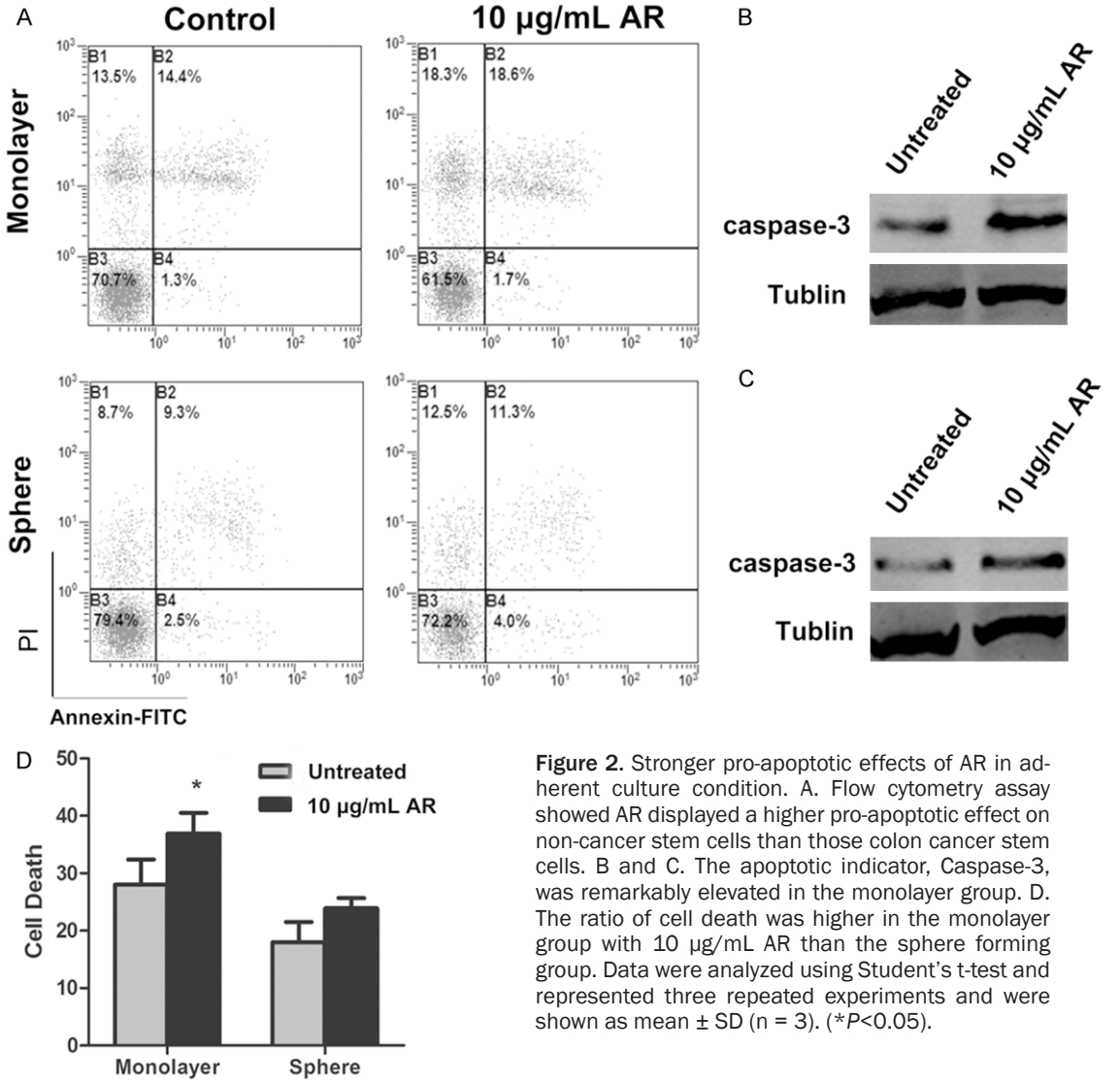


Figure 2. Stronger pro-apoptotic effects of AR in adherent culture condition. A. Flow cytometry assay showed AR displayed a higher pro-apoptotic effect on non-cancer stem cells than those colon cancer stem cells. B and C. The apoptotic indicator, Caspase-3, was remarkably elevated in the monolayer group. D. The ratio of cell death was higher in the monolayer group with 10 μg/mL AR than the sphere forming group. Data were analyzed using Student's t-test and represented three repeated experiments and were shown as mean ± SD (n = 3). (**P*<0.05).

Measurement of ROS

ROS level in HT-29 cells was detected with a Reactive Oxygen Species Assay Kit by employing the cell-permeable fluorogenic probe 2',7'-dichlorodihydrofluorescin diacetate (DCFH-DA). In brief, DCFH-DA was diffused into the cells and deacetylated by cellular esterases to the non-fluorescent compound 2',7'-dichlorodihydrofluorescin (DCFH), which was rapidly oxidized to the highly fluorescent compound 2',7'-dichlorodihydrofluorescein (DCF) by ROS. The fluorescence intensity is proportional to the ROS level within the cell cytosol. In the assays, HT-29 cells and spheres were incubated with AR at a dose of 10 μg/mL or DMSO for 24 h in 6-well plate. Then, cells were treated with 1 mmol/L DCFH-DA for 1 hour at 37°C. Fluorescence was measured with a fluorescence plate reader (Molecular Devices, SpectraMax M5) at 480 nm excitation/530 nm emission.

Statistical analysis

All data were reported as the mean ± standard deviation (SD). Data were analyzed using either a one-way ANOVA with Bonferroni's correction for comparison of multiple groups or an unpaired Student's t-test for comparison of two groups, as described in the figure legends (GraphPad Prism 6.0 software). *P*<0.05 was considered to be statistically significant (**P*< 0.05; ***P*<0.01; or ****P*<0.001).

Results

Tumor suppressive effects of AR under both adherent and sphere culture conditions

AR has been reported to treat various types of cancer [10-12]. However, its efficiency on colon cancer stem cells was still unknown. Firstly, under conventional adherent culture condition in the presence of serum, the proliferation and cell viability of adherent HT-29 cells were significantly inhibited by AR in a dose dependent manner (0, 10 and 50 μg/mL) (**Figure 1B**).

Cancer stem cells are supposed to be responsible for cancer progression, regression and metastasis [13, 14]. In order to test the effects of AR on CSCs, sphere forming in *in-vitro* serum-free medium under non-adhesive condition is a way of enrichment of cancer stem cells. After treatment with AR at doses of 10 and 50 μg/mL in serum-free medium for 5 days, the numbers of sphere were almost abolished at the higher dose of AR (50 μg/mL), or obviously diminished at a lower dose of AR (10 μg/mL, as shown in **Figure 1C** and **1D**), Thus, our results showed that both colon cancer stem cells and non-stem cells are sensitive to AR administering.

In order to evaluate cancer stem cells sensitivity towards the AR treatments, CD44 positive staining on cell surface was regarded as a specific stem cell marker, as shown **Figure 1E** and **1F**, under sphere culture condition, the ratio of CD44 positive was about 87.1% compared with that of 40.5% under conventional culture. Unexpectedly, After treatment with AR, the CD 44 positive percentage in monolayer culture was raised to up to 83.9% at a dose of 10 μg/mL and 72.5% at 50 μg/mL (data not shown), similarly in sphere culture, in the presence of AR at a dose of 10 μg/mL, the ratio was mildly reduced to 82.6% compared with the control 87.1% (**Figure 1E**, **1F**). These data suggested that colon cancer stem cells may display a higher resistance to suppressive effects of AR than those non-cancer stem cells.

Stronger pro-apoptotic effects of AR in adherent culture condition

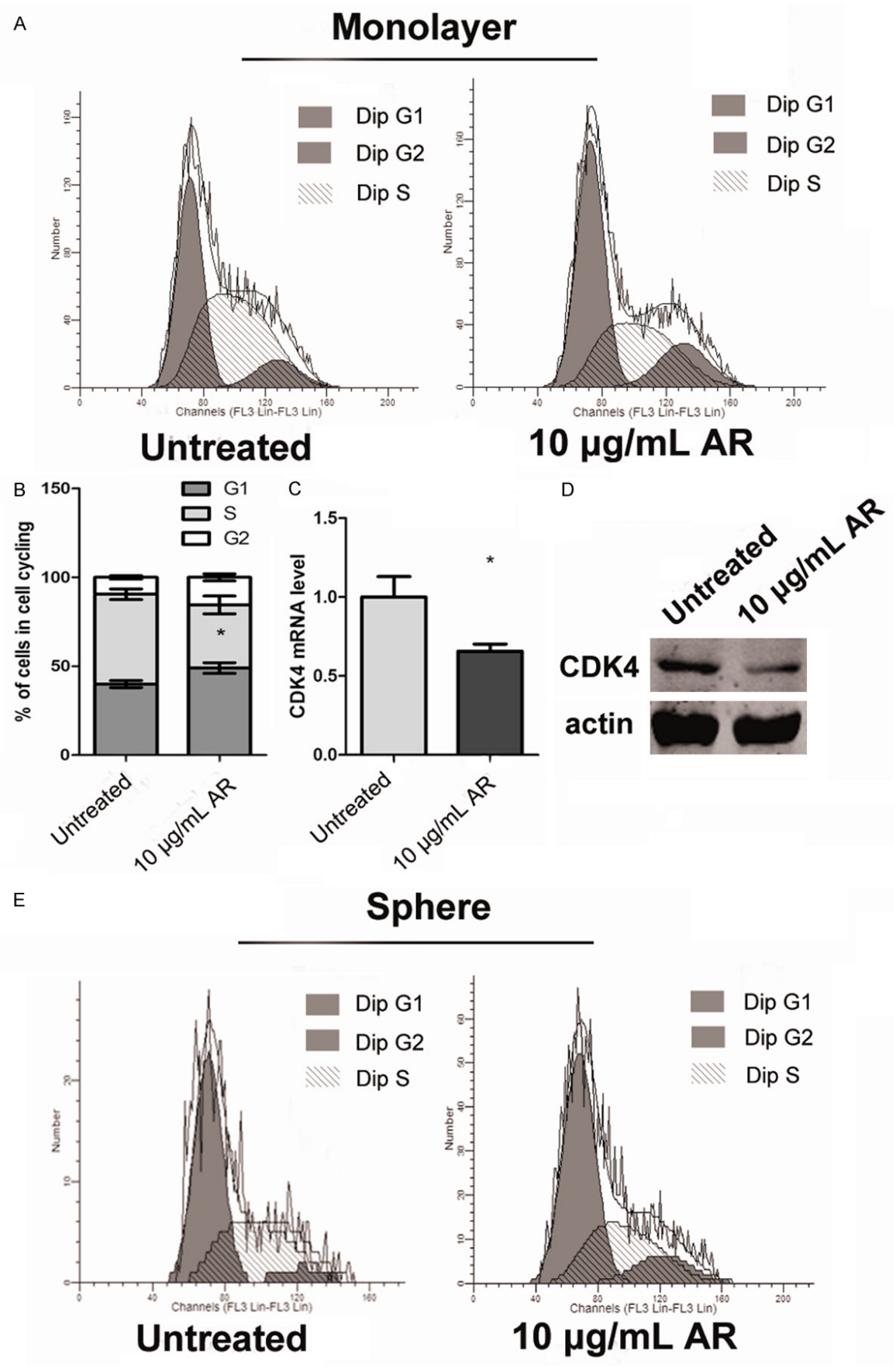
Tumor suppressive effects of AR on colon cancer cells may be due to either pro-apoptotic or growth inhibition. The positive rate of cells with double staining of Annexin V and propidium iodide (PI) is a way to detect the apoptosis. The results showed positive rates of PI and Annexin V staining in AR-treated monolayer groups were robustly raised to 18.3+18.6% at a dose of 10 μg/mL and 39+40% at 50 μg/mL (data not shown), compared with control ratio of 13.5+14.4% (**Figure 2A**, **2D**). For the sphere forming group, the ratio mildly increased to 12.5+11.3% at a dose of 10 μg/mL compared with the ratio of 8.7+9.3% in the control group. Another apoptotic indicator, Caspase-3, a key enzyme in the apoptosis pathway, its expression was also enhanced in the treatment groups for both culture cells but was higher in AR-treated monolayer group (**Figure 2B**, **2C**).

Lessened cell cycle arrest effects of AR in the sphere culture

Following analysis of pro-apoptotic effects, cell cycle patterns were subsequently detected. After AR treatment at a dose of 10 μg/mL, the cell counts in G1 phase increased, while those in S and G2/M phases decreased, suggesting cell cycle arrest at G1/S checkpoint. Apparently under the monolayer condition, the S phase reduction (from 50% to 36%) was more obvious than those of sphere one (38% to 32%) (**Figure 3A**, **3B**, **3E**, **3F**). In addition, the expression of CDK4, a G1/S cell cycle phase checkpoint, both mRNA and protein levels were alleviated, in agreement with the results in FACS (**Figure 3C**, **3D**, **3G**, **3H**). These data suggested that AR effectively promoted cell cycle arrest in the non-stem HT-29 cells, meanwhile, leaving those low-cycling cancer stem populations unaffected.

Higher anti-oxidant capacity of CSCs in sphere culture under AR treatment

The above data suggested that tumor suppressive effects of AR were caused by both pro-apoptotic and proliferation inhibition. Since ROS level is a key inducer for either cell growth or apoptosis, influenced by anti-oxidant status. In order to evaluate the altered redox status after AR treatment, firstly the intracellular ROS levels stained with DCF-DA was examined. As shown in **Figure 4A**, a much higher ROS level was determined in the monolayer cells (80%) in comparing with sphere cultures (55%). After treatment with AR, under both culture conditions, ROS was further elevated. The anti-oxi-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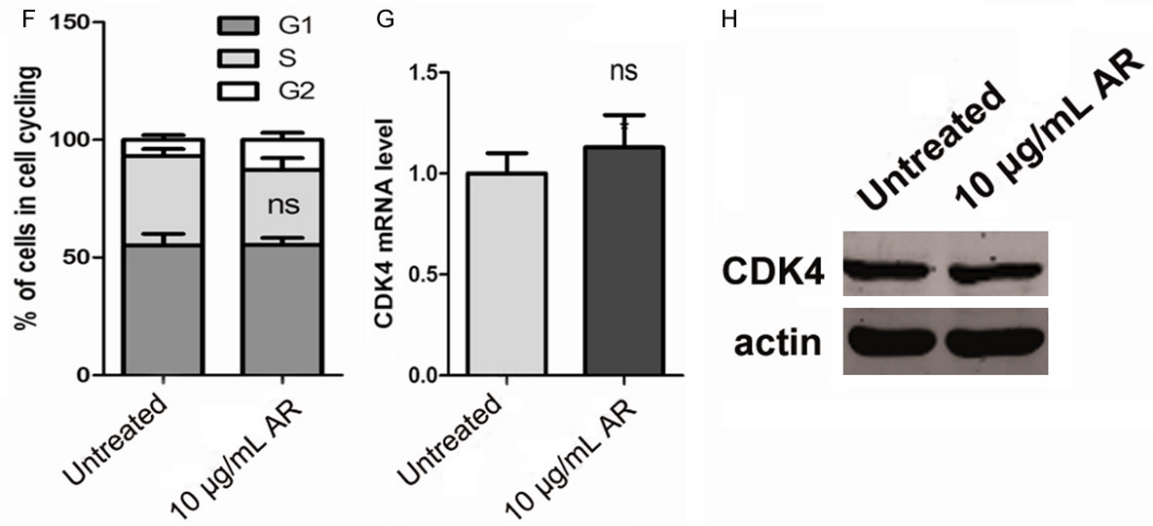

Figure 3. Lessened cell cycle arrest effects of AR in the sphere culture. A and B. Cell cycle was arrested at G1/S checkpoint in the monolayer group after 10 μg/mLAR treatment. C and D. Expression levels of CDK4 were alleviated in the monolayer group. E and F. Cell cycle of the sphere forming group was not obviously changed by 10 μg/mL ARtreatment(no statistical significance). G and H. Expression levels of CDK4 were not obviously changed (no statistical significance). Data were analyzed using Student's t-test and represented three repeated experiments and were shown as mean ± SD (n = 3). (**P*<0.05).

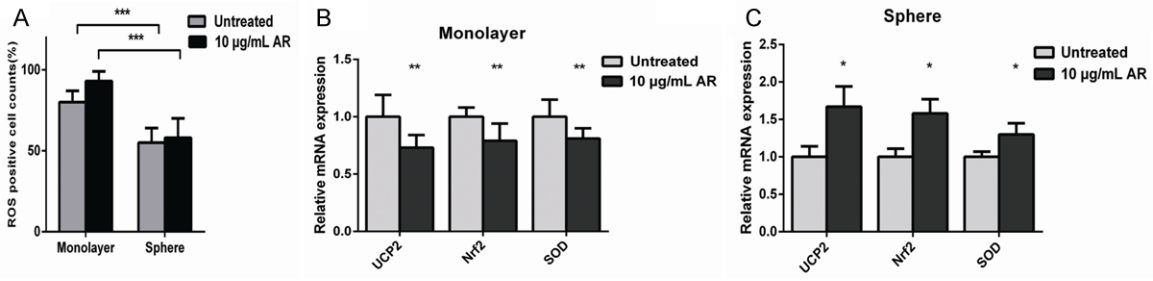

Figure 4. Higher anti-oxidant capacity of CSCs in sphere culture with AR treatment. A. ROS level was increased in the monolayer group treated with AR at a dose of 10 μg/mL. B and C. Quantitative real-time PCR results showed the expression of anti-oxidant genes: UCP2, Nrf2, SOD, were decreased in the monolayer group, while were increased in the sphere forming group. Data were analyzed using Student's t-test andrepresented three repeated experiments and were shown as mean ± SD (n = 3). (**P*<0.05; ***P*<0.01; or ****P*<0.001).

dant related proteins, such as UCP2, Nrf2 and SOD were declined or mildly reduced in monolayer cells (**Figure 4B**), while those CSCs adaptively up-regulated the above three antioxidant proteins at higher level after adding AR (**Figure 4C**). The opposite alterations of anti-oxidant proteins in CSCs versus non-CSCs indicated that CSCs were a group of cells harboring a higher anti-oxidant ability by keeping a lower level of ROS prior to or after AR treatment.

Discussion

Colon cancer is one of the most devastating diseases in the world. Surgical removal, plus with Chemo-therapy and radio-therapy remained to be the major therapeutic modalities. Chemo-resistance and metastasis are still the hurdles threatening the patients. Accumulating evidences support that cancer is made up of a heterogenous group of cells, among which the smaller portion of cancer stem cells are responsible for tumor initiation, chemo-resistance and metastasis. Therefore specifically killing these cancer stem cells is a better way to eradicate cancer in the long run. In order to obtain the cancer stem cells, sphere-forming experiment is employed under the low attachment culture dishes plus with serum-free medium. Regarding to the cancer stem cells determination, various

surface markers are commonly applied with, such as CD44, CD133. In order to be more accurately denoted, sometimes two or more markers are combined in usage. In this study, we disclosed that sphere forming culture condition could enrich more than double numbers of CD44+ cancer stem like cells than monolayer culture did. Moreover, other stem marker gene expressions, such as CD133, Oct4, EpCAM, ABCG2, Nanog were also highly expressed (data not shown). Therefore this method could be regarded as an easy way to test the drug sensitivity towards CSCs.

From the importance of CSCs in carcinogenesis, it's better to find the drugs that preferentially target it. Arctigenin, a functional ingredient of several traditional Chinese herbs, has been reported to exert the antitumor effects. Therefore we intend to evaluate whether AR possesses such advantages or not.

The evidences showed that AR displayed the tumor suppressive effects at both culture conditions, surprisingly, further analysis of CSCs markers demonstrated that the ratio of CSCs positively labeled with CD44+ staining were not declined but rather enriched after AR applications. Moreover pro-apoptotic and growth inhibition effects supplied with AR were also more apparent in the monolayer condition. These data highly suggested that non-CSCs are more prone to be prohibited by AR treatment. As a contrast the cancer stem cells showed more resistance to it.

Regarding to the mechanism related with AR antitumor effects, previous studies reported that AR could suppress UPR response under the glucose deprivations [15]. It could also promote glucose starved A549 tumor cells to undergo necrosis elicited by higher ROS and ATP depletion [16]. Moreover, AR suppressed cell proliferation and potentiated cell apoptosis in a dose dependent manner and enhanced chemo-sensitivity to cisplatin via survivin and STAT3 down-regulations [17, 18].

As known before, CSCs showed less sensitivity towards traditional chemo and radiation therapy and contributed to chemo-resistance and cancer relapse. Underlying mechanisms are related to the stronger DNA repair ability and other anti-stress adaptability in CSCs.

ROS, as an indicator of cellular stress intensity, is responsible for mediating either cell proliferation or pro-apoptotic effects, depending on the intensity levels. Our experiments discovered that the higher ROS level and cell proliferation rate were found in monolayer culture compared with sphere culture. After AR treatments, ROS level was further enhanced at various intensity range for both conditions. Notably, the ROS scavenging and anti-oxidant proteins, such as UCP2 and SOD, are aberrantly down-regulated in the AR sensitive non-cancer stem cells, unequivocally, Nrf2, UCP2 and SOD are all up-regulated at higher intensities in cancer stem cell subgroup. On the whole, these evidences at least partially explained the underlying mechanisms related with AR resistance of the CSC.

In conclusion, AR was able to suppress HT-29 cells probably via ROS overproduction, accompanying with pro-apoptosis and cell cycle arrest. However, the sparse CSCs in the HT-29 cells are less sensitive to AR treatment by dampening the excessive production of ROS via modulating ROS scavenging related genes like UCP2, Nrf2 and SOD.

This study indicated that AR may not serve as a good supplement drug in line with traditional chemo-therapeutic regimen. An optimal cancer combined therapy should be directed towards discovering the reagent that preferentially targets the slow cycling cancer stem cells in future.

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Disclosure of conflict of interest

None.

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