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Original Article

Artesunate affects proliferation, apoptosis, cell cycle and PTEN/FAK signaling in multiple myeloma RPMI 8226 cells

Zhiyong Cheng[1], Lijun Zhang[1], Yongsheng Bian[1], Jianzhu Fu[1], Feng Qi[1], Suyun Wang[2,3], Hongling Hao[3], Hongjie Wang[4], Wengtong Liang[1]

[1]Department of Hematology, The No.1 Hospital of Baoding, 966 Baihua Road, Baoding 071000, Hebei Province, P. R. China; [2]Department of Hematology, Shenzhen Longhua District Central Hospital, 187 Guanlan Road, Shenzhen 518000, Guangdong Province, P. R. China; [3]Department of Hematology, Hebei General Hospital, 348 Heping Xi Road, Shijiazhuang 050000, Hebei Province, P. R. China; [4]Department of Anesthesiology, Affiliated Hospital of Hebei University, 212 Yuhua Road, Baoding 071000, Hebei Province, P. R. China

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Abstract: We investigated the effect of Artesunate on cell proliferation, cell cycle, apoptosis and the PTEN/FAK signaling pathway on the RPMI8226 cell line *in vitro*. After treatment with different concentration of ART, the proliferation of RPMI8226 cells was significantly inhibited. The cell apoptotic rate was increased in a dose-dependent manner. Cell cycle analysis showed that the proportion of cells in G_0/G_1 phase decreased, while the proportion of cells in G_2/M phases increased significantly as compared to that in untreated cells. The mRNA and protein expression levels of cyclin B1, PTEN, caspase-3/7 significantly increased, while survivin, CDC2 and FAK significantly decreased when compared with the untreated control group. These results indicate that ART can inhibit RPMI8226 cells proliferation and trigger cell apoptosis with G_2/M arrests, which may occur through up-regulating caspase-3, caspase-7, cyclin B1, and PTEN expression while inhibiting survivin, CDC2, and FAK expression.

Keywords: Artesunate, multiple myeloma, cell cycle, PTEN, FAK

Introduction

Artesunate (ART), a derivative of artemisinin, is now recommended for intravenous treatment of severe falciparum malaria due to its efficacy, tolerability, and safety [1, 2]. Additionally, ART inhibits many tumor cell lines including hematopoietic malignancy [3-7], and there is no cross-drug resistance with traditional chemotherapeutic drugs [8]. The anticancer effects of ART also include cell cycle arrest [9], inhibition of angiogenesis [10], reduction of cell invasion and metastasis [11]. A salient feature of artemisinins and its derivatives is the lack of cross-resistance with conventional chemotherapy drugs. It is not only active against multidrug resistant plasmodium strains, but also active against drug-resistant tumor cells [12]. Another propitious characteristic is the good tolerability and the lack of significant adverse side effects. ART may be promising for the treatment of refractory multiple myeloma [5]. However, the exact mechanisms underlying ART-mediated anticancer activity have not been fully elucidated.

Plasma cells are terminally differentiated B-lineage cells with their unique capacity to synthesize and secrete large amounts of antigen-specific immunoglobulins. The control of B-cell terminal differentiation by cellular differentiation, apoptosis, and cell cycle is of great importance in B-cell immunity that warrants further exploration. In contrast to other hematological malignancies, invasion of the adjacent bone often occurs in patients with multiple myeloma, which can destroy skeletal structures and result in bone pain and fractures. Previous studies [13, 14] showed that expressions of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and focal adhesion kinase (FAK) mRNA and protein were significantly different between patients with MM and controls. PTEN could regulate proliferation and apoptosis

and inhibit the invasive capacity of multiple myeloma cells via regulating the expression of FAK signal pathway [13]. In this study, we assessed the impact of ART on proliferation, apoptosis and cell cycle as well as understanding the relevant molecular mechanisms mediating its effects, such as the gene and protein expression of survivin, caspase-3, caspase-7, cyclin B1 and CDC2 using RPMI8226 cells (a human multiple myeloma cell line).

Materials and methods

Main reagents

The RPMI8226 cell line was cultured in RPMI 1640 medium supplemented with 15% FCS (Hangzhou Sijiqing biotechnology Co. Ltd) at 37°C and 5% CO_2. The RPMI8226 cells have been stored for long-term and passaged in our laboratory. ART was purchased from Shanghai Fuxing Pharmaceutical Corp. Ltd. The quantity obtained was 60 mg per ampule and the approval document number was H10930195. A 1000 μg/ml stock solution was prepared by dissolving ART in dimethyl sulfoxide (DMSO), sterilized by ultrafiltration and stored at -20°C. SYBR Green Real Master Mix was purchased from Beijing Tiangen biotechnology Co. Ltd. Primary antibodies used for Western blotting were purchased from Santa Cruz Biotechnology, Inc., and secondary antibodies were purchased from Beijing Ding Guo biotechnology Co. Ltd. Reverse transcription system and caspase-3/7 apoptosis assay kits were purchased from Promega biotechnology Co, USA. Chemicals were purchased from Sigma-Aldrich and Qiangen Biotechnology (Qiangen, Beijing). This study was approved by the institutional ethics committee.

Proliferation assays

The ability of ART to inhibit the proliferation of RPMI8226 cells was measured by the MTT assay. The assay was performed in triplicate, and each experiment was repeated four times. After being treated with ART at different concentrations (0, 12.5, 25, or 50 μg/ml) for 48 h, 10 μl of MTT solution was added into each well at the end-point of cell culture and the plates were incubated for another 4 h at 37°C. Finally, 200 μl DMSO was added to dissolve formazan. Absorbance values (*A*) were measured at a wavelength of 490 nm with a microplate reader. The rate of growth inhibition was calculated as follows: growth inhibition rate = (A_{490} of control - A_{490} of experiment)/A_{490} of control × 100%.

Cell cycle assays

RPMI8226 cells were cultured in 1640 medium in the absence of FCS for 16 h, and then treated with ART at different concentrations for 48 h. At the time-point of culture as indicated, the cells were collected, washed with PBS, fixed with 70% ethanol, and placed at 4°C overnight. The cells were then treated with 10 mg/ml of RNase for 30 min, stained with propidium iodide (PI), and placed at 4°C for 15 min. The cell cycle was measured by FACS analysis. Experiments were performed in triplicate and repeated three times.

Apoptosis assays

Each 1 ml aliquot of RPMI8226 cell suspension (1×10^6 cells/ml) was plated into individual wells of a 12-well culture plate. ART, at different concentrations (0, 25, or 50 μg/ml), was added to each well in triplicate and mixed gently with the cells. After 24 h cultures, the cells were stained with both annexin V and PI. The cellular apoptosis rate and cell cycle distribution were detected by flow cytometry (FCM). Experiments were performed in triplicate and repeated three times.

Real-time quantitative PCR (FQ-PCR) analysis of mRNA expression

RPMI8226 cells were collected after treatment with varying concentrations of ART for 48 h. Total RNA was extracted from the cells of each treatment group using Trizol reagent (Promega, USA) according to the manufacturer's instructions. The concentration and the purity of the isolated RNA were determined by spectrophotometric analysis. The integrity of the RNA was verified by electrophoresis in a formaldehyde agarose gel followed by ethidium bromide staining. 1 μg of total RNA was used for the reverse transcription (RT) reaction (Promega) according to the manufacturer's instructions. FQ-PCR was performed on the resultant cDNA. PCR was carried out in a reaction volume of 25 μl with the SYBR Green I PCR Reagent kit (Tiangen Biotech.) and a Lightcycler fluorescent quantitative amplification analyzer (Bio-Rad, Hercules, CA, USA). PCR were performed for an initial dena-

Table 1. Primer sequence for FQ-PCR

Primer	Sequence	Product Size	Accession Number
Survivin	F: 5'-GACCACCGCATCTCTACATTC-3'	194 bp	BC065497
	R: 5'-TGCTTTTTATGTTCCTCTATGGG-3'		
Caspase-3	F: 5'-TTCAGAGGGGATCGTTGTAGAAGTC-3'	264 bp	NM_004346
	R: 5'-CAAGCTTGTCGGCATACTGTTTCAG-3'		
Caspase-7	F: 5'-TGACCTATCCTGCCCTCA-3'	107 bp	NM_001227
	R: 5'-TCTCCTGCCTCACTGTCC-3'		
Cyclin B1	F: 5'-AAATACCTACTGGGTCGGGA-3'	160 bp	NM_031966.3
	R: 5'-CATCTTCTTGGGCACACAAT-3'		
CDC2	F: 5'-TCCCAATAATGAAGTGTGGC-3'	130 bp	NM_001786.4
	R: 5'-CGAGAGCAAATCCAAGCC-3'		
PTEN	F: 5'-ATACCAGGACCAGAGGAAACC-3'	101 bp	000314
	R: 5'-TTGTCATTATCCGCACGCTC-3'		
FAK	F: 5'-GCGGCCCAGGTTTACTGAA-3'	103 bp	L05186
	R: 5'-GGCCTGTCTTCTGGACTCCAT-3'		
β-actin	F: 5'-CTGGCACCACACCTTCTACAAT-3'	382 bp	NM_001101
	R: 5'-AATGTCACGCACGATTTCCCGC-3'		

turation at 94°C for 5 min, followed by 30 cycles of two-step reactions: 94°C for 45 s and 60°C for 1 min. Fluorescence signals were measured at the step of 60°C for 1 min during each cycle. Relative quantification of gene expression was calculated by the following method: for each reaction to amplify the target gene and β-actin gene, the C_t value was determined. The ratio of each target mRNA to β-actin mRNA was calculated by the formula $2^{-\Delta Ct}$. Specific primer sequences of each target gene are shown in **Table 1**.

Western blot analysis of protein expression

RPMI8226 cells were collected following 48 h treatment with ART at varying concentrations. The protein concentration of the cell lysate was measured by coomassie brilliant blue kit (Kang Chen Biotechnology, Beijing, China). The cell extract was equally loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and run at 100 V for 2 h. Proteins were transferred to nitrocellulose (NC) membranes. Membranes were blocked with 5% skim milk in Tris-buffered saline (TBS with 0.1% Tween 20) for 1 h, then washed and incubated with the primary antibody at 4°C overnight. The membranes were then washed and incubated with the secondary antibody, and imaged with a chemiluminescent substrate, ECL Plus. Protein levels were measured using a Fluors scanner and Bio-Rad software for quantitative analysis.

Measurement of caspase-3 and -7 activities

Caspase-3/7 activity was measured using a caspase-3/7 activity assay kit according to the manufacturer's protocol. To evaluate the effect of ART on regulating caspase-3 and -7 activity, each 100 μl aliquot of RPMI8226 cell suspension (1×10^5 cells/ml) was plated into individual wells of a 96-well culture plate. ART, at different concentrations (0, 25, or 50 μg/ml), was added to each well in triplicate and mixed gently with the cells. Each experiment was repeated three times. After being treated with ART for 24 h, 100 μl mixed caspase 3/7 reagent was added into each well and the plates were incubated for another 3 h at 37°C. Absorbance values (A) were measured at a wavelength of 405 nm with a microplate reader. A blank sample was used as a control. Caspase-3/7 activity was determined by A490 of experiment/A490 of control.

Statistical analysis

Statistical analyses were performed using the SPSS software version 11.5. All data were normal distribution, and the measurement data were expressed as means ± standard deviations (SD). The independent samples t-test was used to compare 2 variables. Multiple variables (Such as the expression levels of mRNA and proteins treated with different concentrations of ART) were analyzed by one-way analysis of variance (ANOVA) and q test was used with the comparative analysis between two groups. Data were statistically significant with a *P* value of less than 0.05.

Results

ART inhibited the proliferation of RPMI8226 cells in a dose- and time-dependent manner

After ART treatment, at a final concentration of 12.5 μg/ml to 50 μg/ml, the proliferation of

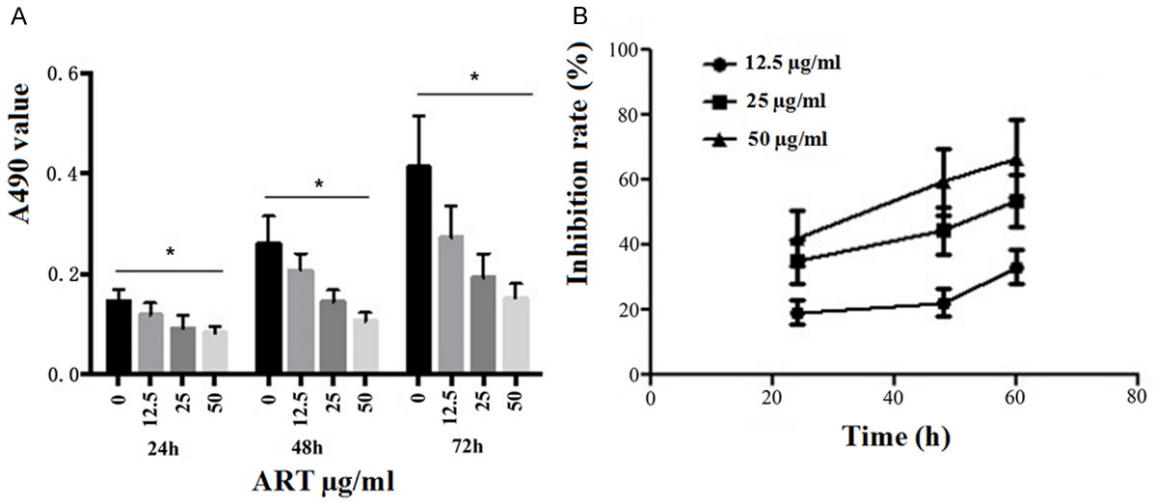

Figure 1. The inhibitory effect of different concentrations of ART on RPMI8226 cell proliferation. A: The growth of RPMI8226 cell treated with ART. B: The growth inhibition rate of RPMI8226 cell treated with ART. *P<0.05 compared with control group.

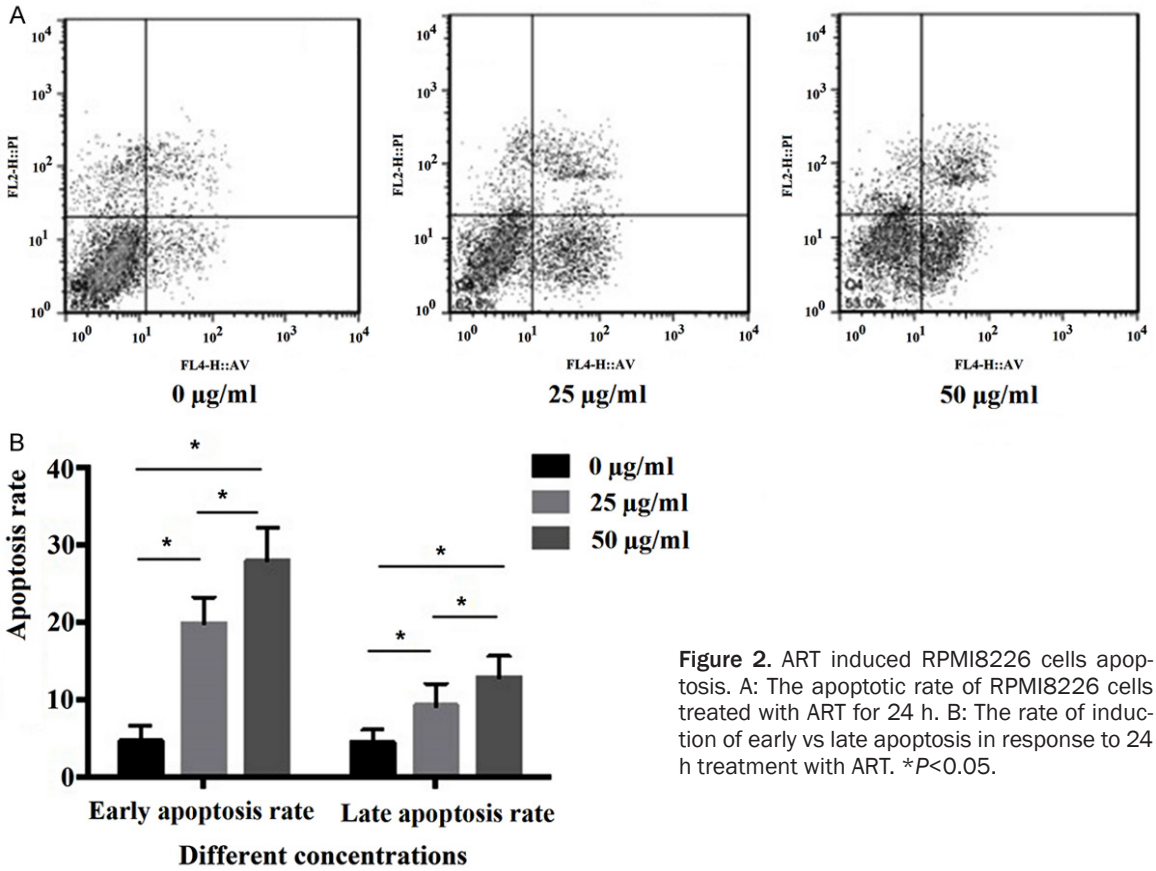

Figure 2. ART induced RPMI8226 cells apoptosis. A: The apoptotic rate of RPMI8226 cells treated with ART for 24 h. B: The rate of induction of early vs late apoptosis in response to 24 h treatment with ART. *P<0.05.

RPMI8226 cells was significantly inhibited as compared with that in untreated group in the range of 24 h to 72 h. The inhibition rate curve is shown in **Figure 1**. The inhibitory effect of ART on cell proliferation was enhanced in a dose dependent manner. Additionally, the inhibitory effect of ART at each concentration was enhanced by the extension of treatment

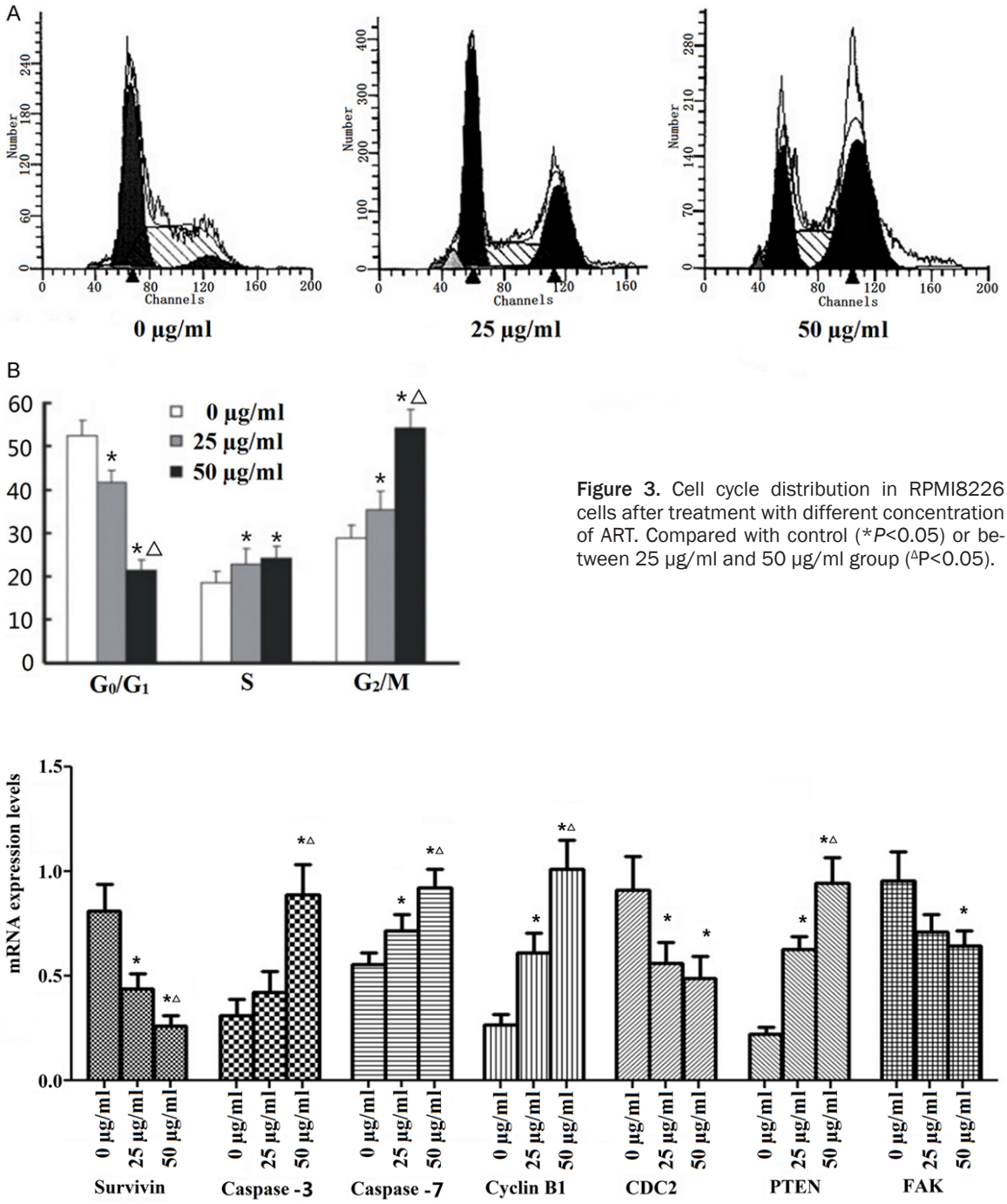

Figure 3. Cell cycle distribution in RPMI8226 cells after treatment with different concentration of ART. Compared with control (*P<0.05) or between 25 μg/ml and 50 μg/ml group ($^{\Delta}P$<0.05).

Figure 4. The mRNA expression levels in RPMI8226 cells after treatment with ART at varying concentrations for 48 h. *P<0.05, compared with ART 0 μg/ml group; $^{\Delta}P$<0.05, ART 25 μg/ml group vs ART 50 μg/ml group.

time. After 72 h of treatment with 50 μg/ml of ART, the growth inhibition rate was (66.56 ± 12.22)%. As shown in **Figure 1**, the growth inhibition effect of ART was both time- (P<0.05) and dose-dependent (P<0.05). The IC_{50} of ART was 36.47 μg/ml by using Logit method.

Apoptosis of RPMI8226 cells induced by ART treatment

As shown in **Figure 2**, ART exerted a cytotoxic effect on RPMI8226 cells at 25 μg/ml and 50 μg/ml concentrations at 24 h treatment as

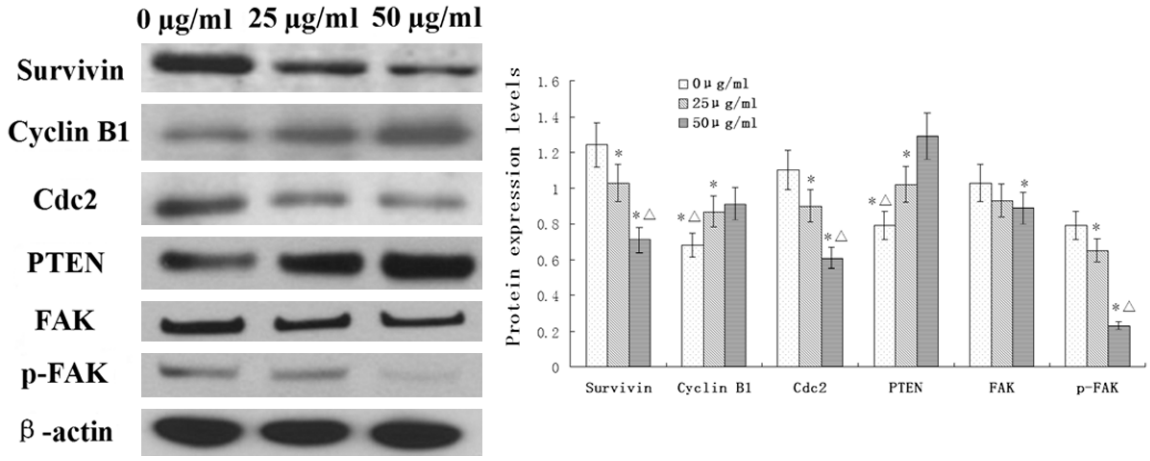

Figure 5. Protein expression in RPMI8226 cells after ART treatment at different concentrations for 48 h. **P*<0.05, compared with ART 0 μg/ml group; $^{\Delta}$*P*<0.05, ART 25 μg/ml group vs ART 50 μg/ml group. The relative protein expression levels under each treatment were determined from the average of three independent experiments.

compared with untreated cells. Measurement of apoptosis by flow cytometry revealed that ART induces apoptosis in a dose-dependent manner. When RPMI8226 cells were treated with ART at 50 μg/ml for 24 h, the apoptosis level was (43.7% ± 5.4)%.

ART alters the cell cycle distribution of RPMI8226 cells

After being cultured with 25 μg/ml and 50 μg/ml concentrations of ART for 48 h, the cell cycle distribution of RPMI8226 cells was detected by flow cytometry. The results show that the proportion of cells in G_0/G_1 phase decreased, while the proportion of cells in G_2/M phase significantly increased as compared to that in untreated RPMI8226 cells (**Figure 3**).

ART treatment modulates mRNA expression profiles in RPMI8226 cells

To determine the effect of ART treatment on the expression of survivin, caspase-3, caspase-7, cyclin B1, CDC2, PTEN and FAK mRNA in RPMI-8226 cells, the expression level of these genes were analyzed by FQ-PCR following treatment with ART at varying concentrations. As shown in **Figure 4**, the expression level of caspase-3, caspase-7, cyclin B1, PTEN mRNA significantly increased, while the expression level of survivin, CDC2 and FAK mRNA decreased significantly in ART treated RPMI8226 cells at 48 h when compared with that in the untreated control group (*P*<0.05).

ART treatment alters protein expression in RPMI8226 cells

As shown in **Figure 5**, the expression level of cyclin B1, and PTEN protein significantly increased, while the expression level of survivin, CDC2 and p-FAK protein decreased significantly in ART treated RPMI8226 cells at 48 h when compared with that in the untreated control group (*P*<0.05). β-actin was used as a loading control.

ART treatment enhances caspase-3 and -7 activities in RPMI8226 cells

RPMI8226 cells were exposed to ART for 24 h, the A490 value in RPMI8226 cells without treatment was 0.336 ± 0.041. The A490 values in 25 μg/ml and 50 μg/ml groups of RPMI8226 cells were 0.451 ± 0.042 and 0.612 ± 0.051, respectively. The caspase-3 and -7 activities were significantly increased when compared with that in the untreated control group (*P*<0.01). This indicates that ART can increase the activity of caspase-3/7, thereby inducing apoptosis in a dose dependent manner.

Discussion

Artesunate is an antimalarial agent with broad anti-cancer activity not only in solid tumor but in hematopoietic malignancy *in vitro* and animal experiments [5-7]. Clinical trials showed that ART has anti-proliferative properties in colorectal cancer and is generally well tolerated. Also ART treatment effect reduces Ki67

and increases CD31 expression. In ART treatment group, it has longer survival and less recurrence in colorectal cancer [15].

Studies showed that ART has been demonstrated to exhibit anti-myeloma effects [5, 10]. Our results demonstrated that ART effectively inhibited RPMI8226 cell proliferation in a dose- and time-dependent manner. The growth inhibition rate was (66.56 ± 12.22)% after 72 h of treatment with 50 μg/ml of ART, which is comparable to the results from previous studies [5, 10]. We also find that the number of apoptotic cells of RPMI8226 increased in a dose dependent manner induced by ART.

Apoptosis can be triggered by a variety of stimuli. In particular, caspase activation is known to play a pivotal role in the execution of apoptosis [16, 17]. On the other hand, the survivin-encoded product survivin is a new member of the inhibitor of apoptosis family and has multiple functions. It inhibits cell apoptosis, promotes cell transformation, and participates in cell division, vascularization, and the generation of tumor cell drug resistance among other functions. The primary function of survivin expression in tumor cells is to antagonize cell apoptosis. The anti-cell apoptosis mechanism of survivin is realized by inhibiting the activation of caspase-3 and caspase-7 [18]. Our results revealed that the expression level of survivin decreased while caspase-3 and caspase-7 increased significantly after RPMI8226 cells were treated with ART for 48 h compared with the untreated control group. Here we show that ART induced apoptosis in RPMI8226 cells potentially through the mechanism that ART attenuates survivin's inhibition on caspase-3/7 by suppressing its expression. Collectively, these data indicate that ART induced apoptosis by the activation of caspases, and survivin can increase the resistance of tumor cells to apoptosis signals through caspase-dependent channels, thereby blocking apoptosis. But in some studies showed that it has non-caspase pathway with ART [19]. So the molecular mechanism needs further investigation.

Dysregulation of the cell cycle is a hallmark of cancer. Like all cancer, self-renewing and non-cycling myeloma cells are both found in the bone marrow [20]. Cell cycle arrest and autophagy are closely linked biological processes [21]. Here we show that ART arrested the cell cycle at the G_2/M phase in RPMI8226 cells. The results are, however, not conclusive. While some authors have reported G_0/G_1 arrest, others have found G_2/M block upon artesunate treatment [5, 22-25]. In a mouse myeloma cell line SP2/0 cells, ART increased the proportion of cells in G0/G_1 phase, while decreasing the proportion of cells in G_2/M or S phase [5]. These discrepant results may be due to the heterogeneity of MM as a disease, whereby each MM cell line possesses unique oncogenic driver mutations, resulting in varying response to cell cycle arrest induced by ART. Cyclin B1 and CDC2 form a complex, which is a key regulator for the mitotic and meiotic cell cycle, and is considered a master regulator that integrates signals from several cell cycle controlling pathways [26]. Our results showed that ART affects the cyclin B1/cdc2 complex by up-regulating the expression of cyclin B1 and down-regulation of CDC2, thereby leading to inappropriate accumulation of CDC2 and cyclin B1.

As with most cancers, genetic mutations are considered to be one of the important pathogenic factors in the genesis of MM [27]. PTEN gene deletions and/or mutations were associated with high morbidity of myeloid and lymphoid neoplasms in rat models, and in human hematological neoplasms including human MM [13]. Several studies indicate that PTEN can influence cell cycle, apoptosis, migration and invasion of cells via phosphorylation of focal adhesion kinase (FAK), which results in the phosphorylation of subsequent tyrosine residues [28]. Overexpression of FAK was observed in various human cancers [14, 29]. FAK overexpression in cancer tissue has been shown to inhibit the function of PTEN and stimulate the growth of cancer cells [30]. Previous study indicated that decreased PTEN expression was accompanied by an increased FAK expression level [14]. Therefore, in the present study our data showed that the mRNA and protein expression level of PTEN increased while FAK decreased significantly in RPMI8226 cells after treated with ART, indicating that ART might influence the cell growth, cell cycle, and apoptosis by regulating the activity of PTEN/FAK signaling pathway.

In this study, we first examined the effects of ART on the survival, apoptosis, and cell cycle of the human MM RPMI8226 cell line. Secondly, we demonstrate that ART might influence the

cell growth, apoptosis and cell cycle by regulating the activity of survivin, caspase 3/7, cyclin B1, CDC2 and the PTEN/FAK signaling pathway. Therefore, ART might be a new candidate drug for the treatment of multiple myeloma.

Disclosure of conflict of interest

None.

Address correspondence to: Suyun Wang, Department of Hematology, Shenzhen Longhua District Central Hospital, 187 Guanlan Road, Shenzhen 518000, Guangdong Province, P. R. China. Tel: +86-75521003169; E-mail: suyunwcn@163.com

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