

Original Article

Study on the function and mechanism of atorvastatin in regulating leukemic cell apoptosis by the PI3K/Akt pathway

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Abstract: Objective: To investigate the effects of atorvastatin on the proliferation and apoptosis of leukemic cell lines (Jurkat, K562 and HL-60), and explore the function of TLR4/MYD88/NF- κ B and PI3K/AKT signal pathway in this process. Methods: Cells in logarithmic growth phase were divided into negative control group and experimental group (cells were treated with atorvastatin with intervention concentrations of 1, 5 and 10 μ mol/L respectively) and cultured for 24 hours. Changes in apoptosis and cell cycle of leukemic cells were detected utilizing the Flow Cytometry. Changes in the expression of TLR4/MYD88/NF- κ B and PI3K/AKT signal pathway related genes were detected utilizing Real-time PCR and Western Blot method. Results: Atorvastatin inhibit proliferation and induce apoptosis in K562, HL-60 and Jurkat cells in a dose-dependent manner. K562, HL-60 and Jurkat cells in G0/G1 phase increased and that in S phase decreased after being treated with atorvastatin for 24 hours compared with that in control group, suggesting that the atorvastatin can retard the three cells in the G0/G1 phase. The study find that the basal expressions of TLR4, MYD88 and NF- κ B gene in K562, HL-60 and Jurkat cells are obviously down-regulated in a dose-dependent manner after being treated with atorvastatin with different concentrations. This down-regulation action of atorvastatin to the expression of the TLR4, MYD88 and NF- κ B gene becomes more obvious with the increase of the drug level. In addition, the PI3K, AKT and their phosphorylation levels in the above cells down-regulate obviously in a dose-dependent manner after being treated with atorvastatin. This down-regulation action of atorvastatin to the PI3K, AKT and their phosphorylation levels become more obvious with the increase of the drug level. Conclusions: Atorvastatin can inhibit proliferation and induce apoptosis in leukemia cells, which may be associated with the regulation of atorvastatin to the TLR4/MYD88/PI3K/AKT/NF- κ B signaling pathway.

Keywords: Atorvastatin, leukemia, apoptosis, PI3K/AKT signaling pathway, TLR4/MYD88/NF- κ B signaling pathway

Introduction

The statins are a class of hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase inhibitors. They can not only reduce the cholesterol levels, but also play roles in immunosuppression, inhibiting inflammation, preventing dementia and anti-tumor process. Recent studies have found that the statins can inhibit the proliferation of varied leukemic cells, induce cell differentiation and promote apoptosis. The anti-tumor mechanism of the statins mostly depends on the regulation on the expression of the apoptosis signal transduction pathway related genes [1]. The PI3K/AKT signal pathway can inhibit apoptosis, promote vasculogenesis and involve in tumorigenesis in various cells [2, 3]. In recent years, more and more evidence

are suggesting the close association between inflammation and tumor.

TLR4/MYD88/NF- κ B is an important hinge between inflammation and tumor. Previous studies have certified that the occurrence of blood neoplasm malignant is closely associated with the TLR4/MYD88/NF- κ B signal pathway. Current research aiming at the effects of atorvastatin on the proliferation and apoptosis of leukemia cells and its mechanism is still rare. To investigate the mechanism of the atorvastatin-induced apoptosis in leukemia cells, this study aim at the effects of atorvastatin on the proliferation and apoptosis of leukemia cells and the role of the PI3K/AKT and TLR4/MYD88/NF- κ B signaling pathway in this process.

Method

Leukemic cell culture

Human acute promyelocytic leukemia cell lines Jurkat, K562 and HL-60 were purchased from College of Basic Medicine, Tongji Medical College of Huazhong University of Science and Technology. The three types of leukemia cells were all conventionally cultured in 1640 culture medium (containing 10% Gibco fetal bovine serum, 100 U/ml benzylpenicillin and 100 µg/ml streptomycin) at 37°C with 5% CO₂ condition. All cells for experiment were in logarithmic phase and their viability determined by trypan blue exclusion test was above 95%.

Grouping

Four group of experiments were designed in the study, they were negative control group, 1 µmol/L, 5 µmol/L and 10 µmol/L atorvastatin group respectively.

Detection of apoptosis changes in leukemia cell with flow cytometry

Detected the leukemia cell apoptosis with the AnnexinV/PI kit. Cells in each group were treated with atorvastatin with different concentration and cultured for 48 hours. Collected HL-60 cells in each group and inoculated them to the 96-well plate with the density of 1×10^5 /well. Continued as follows according to the instruction: Added 500 µl Binding Buffer, 5 µl AnnexinV and 5 µl PI to each sample and mixed them respectively. Then kept them away from light at room temperature for 5~15 min. [established negative control (normal cells without Annexin and PI), positive control 1 (took the most effective solvent group inducing apoptosis as the positive control and treated it with only 5 µl AnnexinV) and positive control 2 (took the most effective solvent group inducing apoptosis as the positive control and treated it with only 5 µl PI)]. Finally detected them utilizing the flow cytometer.

Detection of cell cycle changes in each group with flow cytometry

Collected HL-60 cells in logarithmic phase, inoculated them to the 6-well plate with the density of 1×10^5 /well and cultured them for 24 hours. The cultured cells in each group were

collected by centrifugation (1000 r/min, 5 min), rinsed twice with PBS. After thoroughly mixed with 1 ml 70% alcohol at 4°C overnight, the cells were centrifugated (1000 r/min, 5 min), rinsed twice with PBS and added with 150 µl RNA enzyme inhibitors and 150 µl PI (20 µg/ml). The mixture were kept away from light at room temperature for 30 min and finally sent to the flow cytometer to analyze the cell cycle.

Detection of the transcription level changes of TLR4, MYD88 and NF-κB genes with Real-time PCR method

RNA extraction: Added 1 ml Trizol and 200 µl trichloromethane to the treated cells, mixed them lightly and kept them at room temperature for 5 min before they were centrifugated (12000 rpm, 15 min, 4°C). Transferred the upper water phase (about 400 µl) to a new 1.5 ml eppendorf tube, added 400 µl isopropanol, mixed them and kept them at room temperature for 10 min before they were centrifugated (12000 rpm, 10 min, 4°C). Threw away the supernatant and rinsed the precipitation three times with the precooled 70% dehydrated alcohol. The extract were dissolved into 20 µl DEPC water after air seasoning for 5-10 min and the RNA concentration was measured by spectrophotometer.

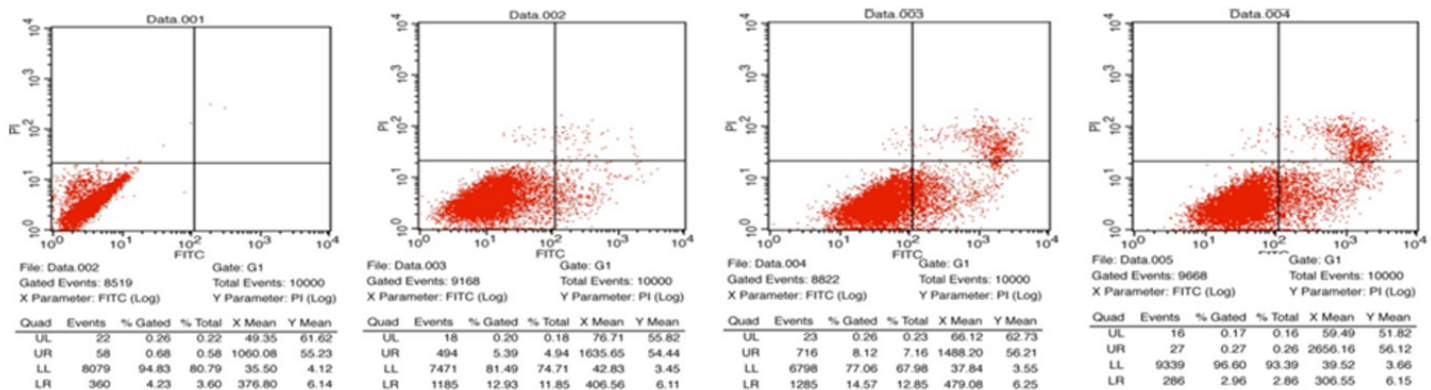
Reverse transcription: The reaction system contained 4 µl 5 × RT buffer, 1 µl RNase Inhibitor, 2 µl dNTP and 1 µl M-MLV was first kept at 42°C for 60 min to react thoroughly, and then kept at 72°C for 5 min to inactivate the M-MuLV.

Real time PCR: Brief introduction as follows: 1). prepared the positive standard substances and their concentration gradients; 2). The reaction systems for both the test samples and the positive standard substances were 50 µl mixture containing 10 µl 5 × SYBR Green I PCR buffer solution, 1 µl sense primer F (10 pmol/µl), 1 µl anti-sense primer R (10 pmol/µl), 1 µl Taq enzyme (3 U/µl), 5 µl cDNA template solution and 31 µl double-distilled water. 3). The above mixture were predegenerated at 93°C for 3 min and then followed by 40 cycles of 93°C for 30 s, 55°C for 45 s and 72°C for 45 s.

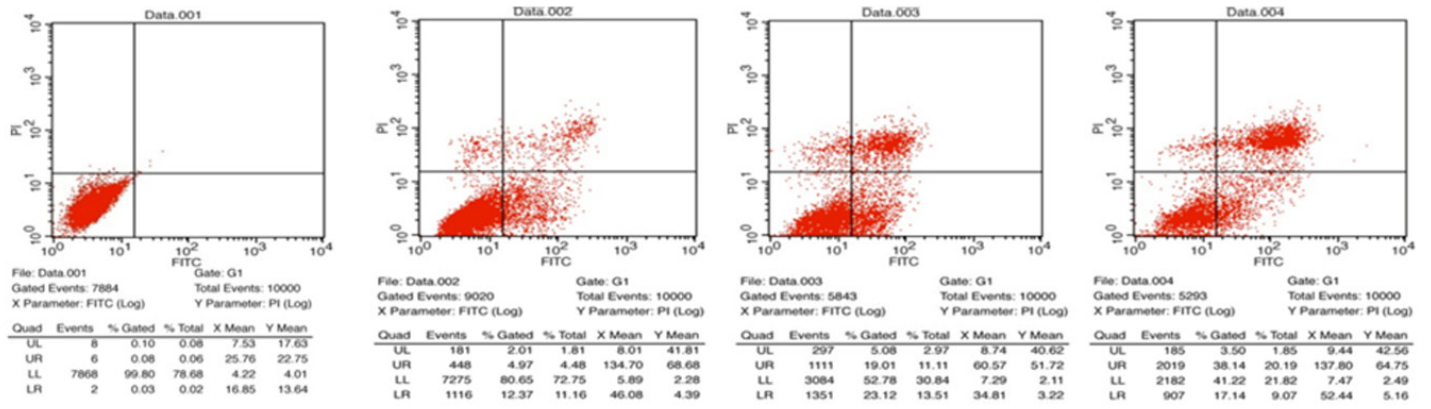
1.2.6 Detection of the expression level changes of TLR4, MYD88, PI3K, AKT, NF-κB, p-PI3K and p-AKT with western blot.

Atorvastatin and leukemic cell apoptosis

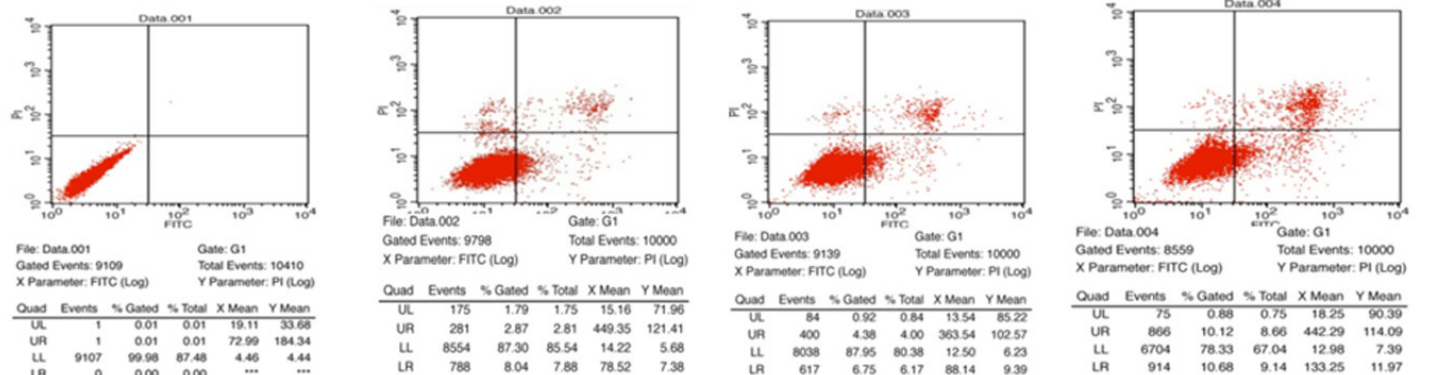
A: Jurkat



B:K562



C:HI-60



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Atorvastatin and leukemic cell apoptosis

Figure 1. Atorvastatin-induced apoptosis of leukemia cells. 1: Control; 2: 1 µg/ml of atorvastatin; 3: 5 µg/ml of atorvastatin; 4: 10 µg/ml of atorvastatin.

Collected HL-60 cells in each group treated with atorvastatin in different concentration and cultured for 48 hours for the followed experiments:

Protein extraction and quantification: Cells in each group were added with 200 µl protein lysate, mixed and schizolysed thoroughly to extract the total proteins. The protein levels were determined by the BCA method (determining the wavelength at A590 and calculating the protein levels with the standard curve) before they were subpackaged.

Detection of the interest protein with immunoblotting: Prepared the spacer gel (3.9%) and the separation gel (8% and 10%) and kept them at room temperature for 1 hours to agglomerate. Added 40 µl loading buffer and 10 µl β-mercaptoethanol to 160 µl samples and boiled the mixture for 6 min. Set up the voltage at 60 V for the first step electrophoresis and turned it to 120 V when the samples reached the separation gel. Stop the electrophoresis when the bromphenol blue reached the bottom. Electrotransferred the protein to the nitrocellulose filter for the immunodetection. After blocking the protein with 5% dried skim milk for 2 hours, diluted the rabbit anti-PI3K (1:1000), the rabbit anti- AKT (1:2000) and the Mtor (1:1000), added them to the nitrocellulose filters and kept them in 4°C freezer overnight. The nitrocellulose filters were rinsed three times (10 min in each time) with the TBST solution, added the corresponding secondary antibodies and shaken for 1 hours in shaking table. Rinsed the nitrocellulose filters three times (10 min in each time) with the TBST solution again before developing with the ECL reagent and film exposure with X-ray in darkroom. Taking the rabbit anti-GAPDH (1:1000) as control (the same method with primary antibodies), calculated the absorbance value of the protein band ($IA = \text{mean absorbance} \times \text{area}$) and denoted the relative level of protein with the ratios of $IA_{\text{target protein}}/IA_{\text{GAPDH}}$

Statistical analyses

All data were treated with SPSS12.0 software. The results were present as Mean ± SD. Utilized

the one-factor analysis of variance for the multiple comparisons and the T-test for the two group comparisons and considered $P < 0.05$ as statistical significance.

Results

Atorvastatin induce the leukemic cell apoptosis

To identify the effects of atorvastatin to the apoptosis of the K562, HL-60 and Jurkat cells, we detected the apoptosis levels of the three types of cells treated with atorvastatin with different concentration for 24 hours utilizing the flow cytometry. The results showed that the apoptosis effects induced by atorvastatin to K562, HL-6 and Jurkat cells are dose-dependent. The apoptosis rate increased with the increase of drug concentration. The apoptosis rate of K562, HL-6 and Jurkat cells treated with 1 µmol/L of atorvastatin for 24 hours were 11.5%, 11.5% and 11.5% respectively while that treated with 10 µmol/L of atorvastatin for 24 hours were raised to 20.4%, 72.6% and 89.2%, respectively. (the P value were 0.02 and 0.043 versus the controls, respectively, **Figure 1**).

The effects of atorvastatin to the leukemic cell cycle

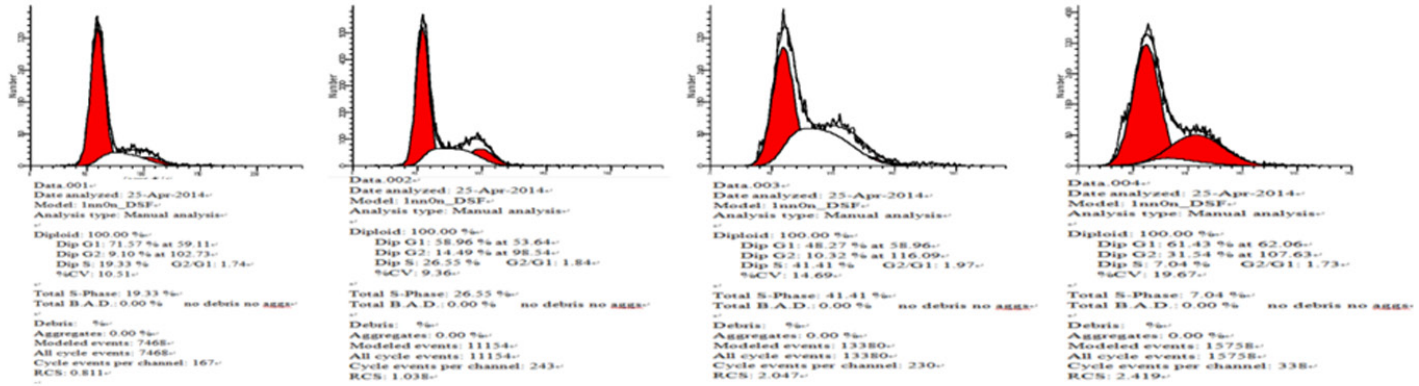
To investigate the relationship between atorvastatin-induced apoptosis and cell cycle in the K562, HL-60 and Jurkat cells, the study detect the cell cycle of the three types of cells treated with atorvastatin with different concentration for 24 hours utilizing the flow cytometry. The results show that K562, HL-60 and Jurkat cells in G0/G1 phase increase and that in S phase decrease after being treated with atorvastatin for 24 hours compared with that in control group, suggesting that the atorvastatin can retard the three cells in the G0/G1 phase (**Figure 2**).

Atorvastatin induces the leukemic cell apoptosis by the TLR4/MYD88/NF-κB signal pathway

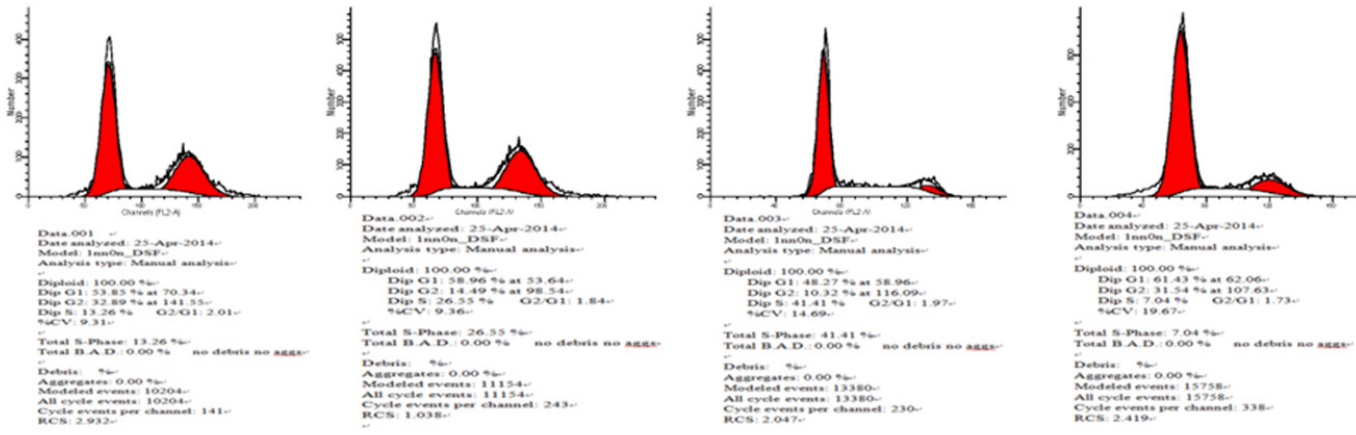
The signal pathway inducing tumor cell apoptosis have been widely studied. This study aims to investigate whether the atorvastatin can induce

Atorvastatin and leukemic cell apoptosis

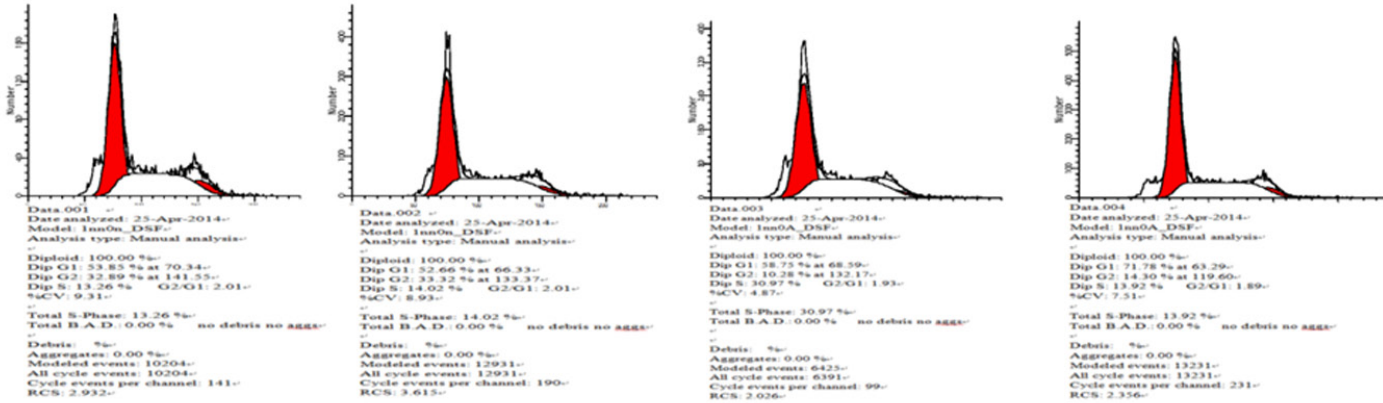
A: Jurkat



B: K562



C: HI-60



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Atorvastatin and leukemic cell apoptosis

Figure 2. Atorvastatin-induced leukemia cell apoptosis and cell cycle. 1: Control; 2: 1 $\mu\text{g}/\text{ml}$ of atorvastatin; 3: 5 $\mu\text{g}/\text{ml}$ of atorvastatin; 4: 10 $\mu\text{g}/\text{ml}$ of atorvastatin.

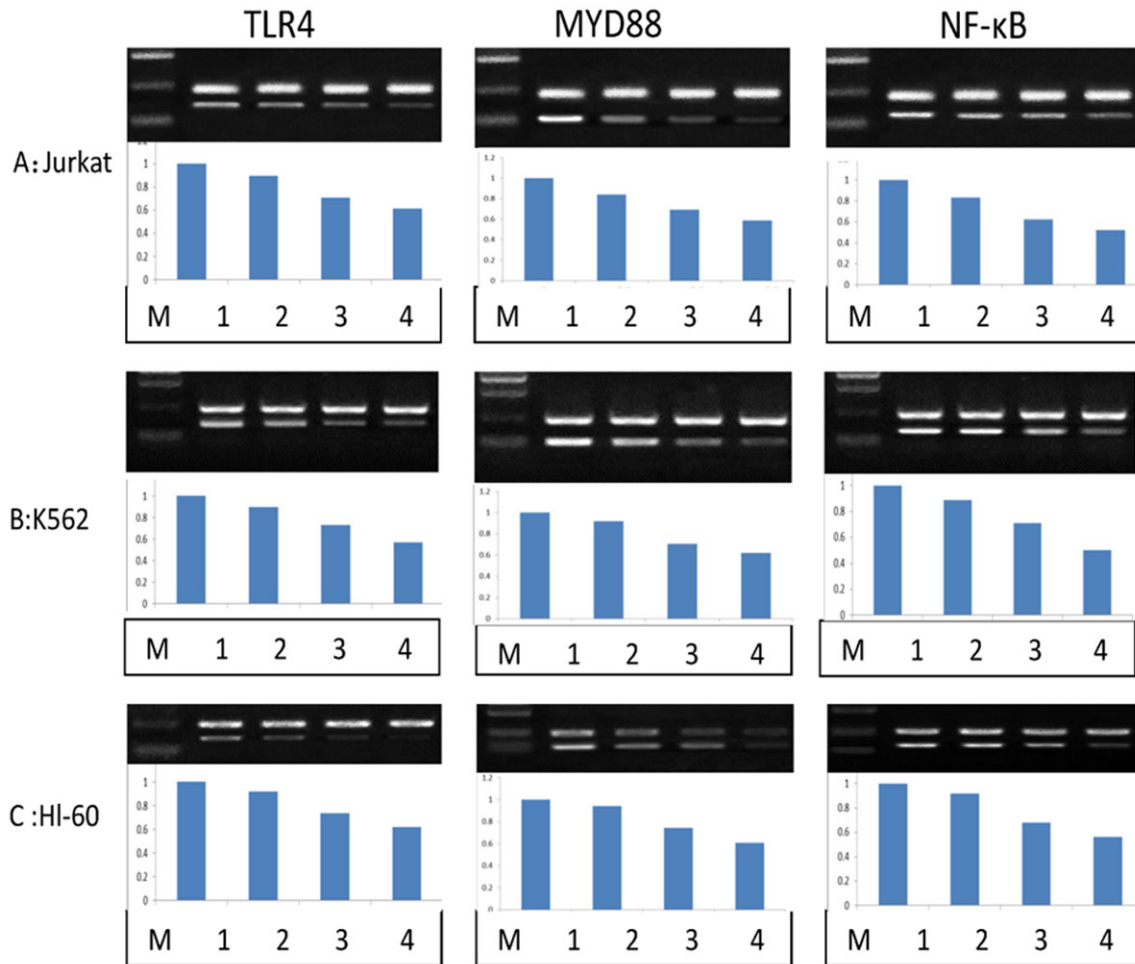


Figure 3. Atorvastatin via TLR4/MYD88/NF- κB signaling pathway induces apoptosis of leukemia cells (RT-PCR). 1: Control; 2: 1 $\mu\text{g}/\text{ml}$ of atorvastatin; 3: 5 $\mu\text{g}/\text{ml}$ of atorvastatin; 4: 10 $\mu\text{g}/\text{ml}$ of atorvastatin.

the leukemic cell apoptosis by regulating the TLR4/MYD88/NF- κB signal pathway. The study utilize the realtime RT-PCR and Westernblot method to detect the effects of atorvastatin on the TLR4/MYD88/NF- κB signal pathway in K562, HL-60 and Jurkat cells treated with atorvastatin with different concentration for 24 hours. The results showed that the TLR4, MYD88 and NF- κB genes keep basal expression in the negative control group but their expressions obviously decrease after being treated by atorvastatin with different concentration. This process is dose dependent, for the down-regulation effects of atorvastatin to the TLR4, MYD88 and NF- κB genes become more

obvious with the drug concentration increasing (Figures 3, 4).

Effects of atorvastatin on the PI3K/AKT signal pathway

The PI3K/AKT is one of the most important signal transduction signal pathway *in vivo*. Activity abnormality in this signal pathway will cause inhibition of apoptosis. To investigate whether the signal pathway participate the atorvastatin induced apoptosis in leukemic cells, we utilized the Western blot method to detect the effects of atorvastatin on the expression of PI3K/AKT signal pathway related genes in K562, HL-60

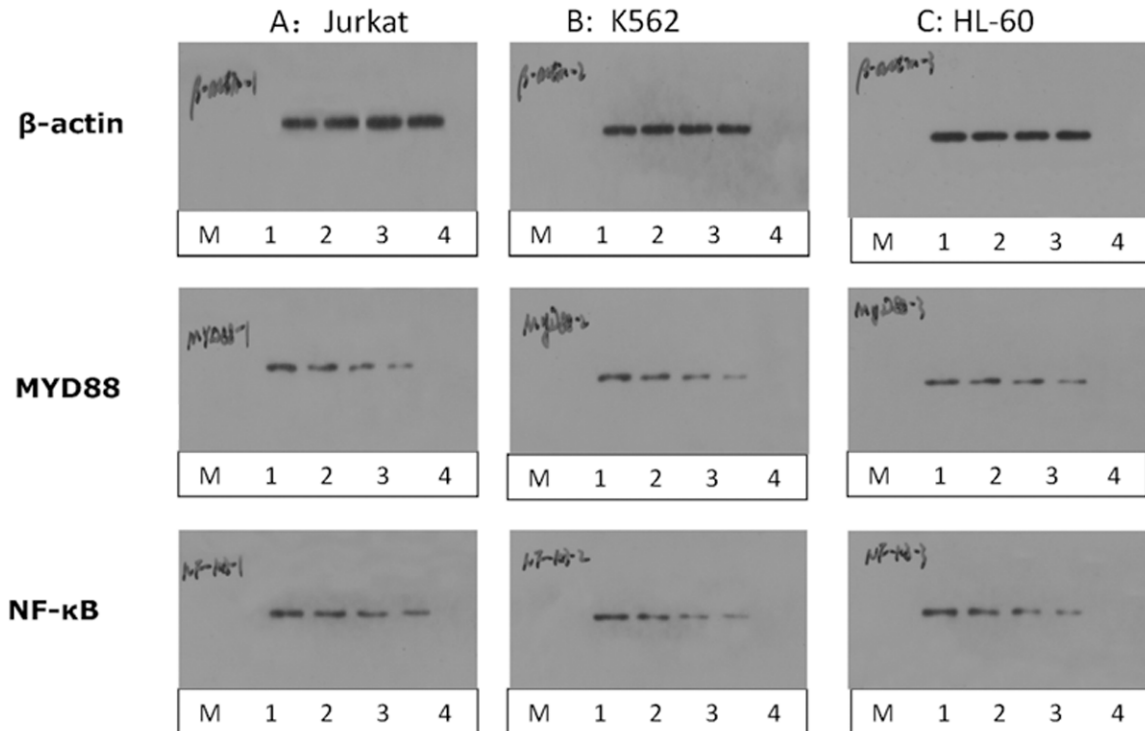


Figure 4. Atorvastatin via TLR4/MYD88/NF-κB signaling pathway induces apoptosis of leukemia cells (West blot). 1: Control; 2: 1 μg/ml of atorvastatin; 3: 5 μg/ml of atorvastatin; 4: 10 μg/ml of atorvastatin.

and Jurkat cells, which was treated with different concentration of atorvastatin for 24 hours. The results showed that the PI3K and AKT and their phosphorylation levels decrease obviously and the process are dose dependent. The down-regulation effects of atorvastatin on the PI3K and AKT and their phosphorylation levels become obvious with the drug concentration increasing (Figure 5).

Discussion

The statins are a class of hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase inhibitors. They are the main medicine for the prophylaxis and therapy of the coronary heart disease since they can effectively decrease the cholesterol and the low density lipoprotein levels. The function of the statins includes 1) decreasing the cholesterol levels; 2) inhibiting vasculitis, improving the endothelium function and enhancing the cardiac function and exercise tolerance; 3) the immunosuppressive action And 4) anti-platelet aggregation [4]. Recently studies have confirmed that the statins play an obvious role in inhibiting various

malignant tumor such as breast carcinoma, melanoma and leukemia [5, 6]. The statins can inhibit proliferation and induce apoptosis in various malignant tumor cells. The anti-tumor mechanism of the statins mostly depends on the regulation on the expression of the apoptosis signal transduction pathway related genes.

This study explored the effects of atorvastatin on the apoptosis and cell cycle of leukemic cells and investigated the role of PI3K/AKT and NF-κB signal pathway in this process. Studies *in vitro* have confirmed the statins can inhibit proliferation and induce apoptosis in tumor cells. Dimitroulakos et al [7] have found that atorvastatin can induce apoptosis in most acute myelocytic leukemia; acute myelogenous leukemia (AML) cell lines and the primary AML cell. Sassano et al [8] have confirmed that the fluvastatin and atorvastatin can induce differentiation and apoptosis in NB4 cells. In addition, it has been confirmed that the atorvastatin plays an important role in cell cycle. The atorvastatin inhibit the MVA pathway by decreasing the cholesterol synthesis and inhib-

Atorvastatin and leukemic cell apoptosis

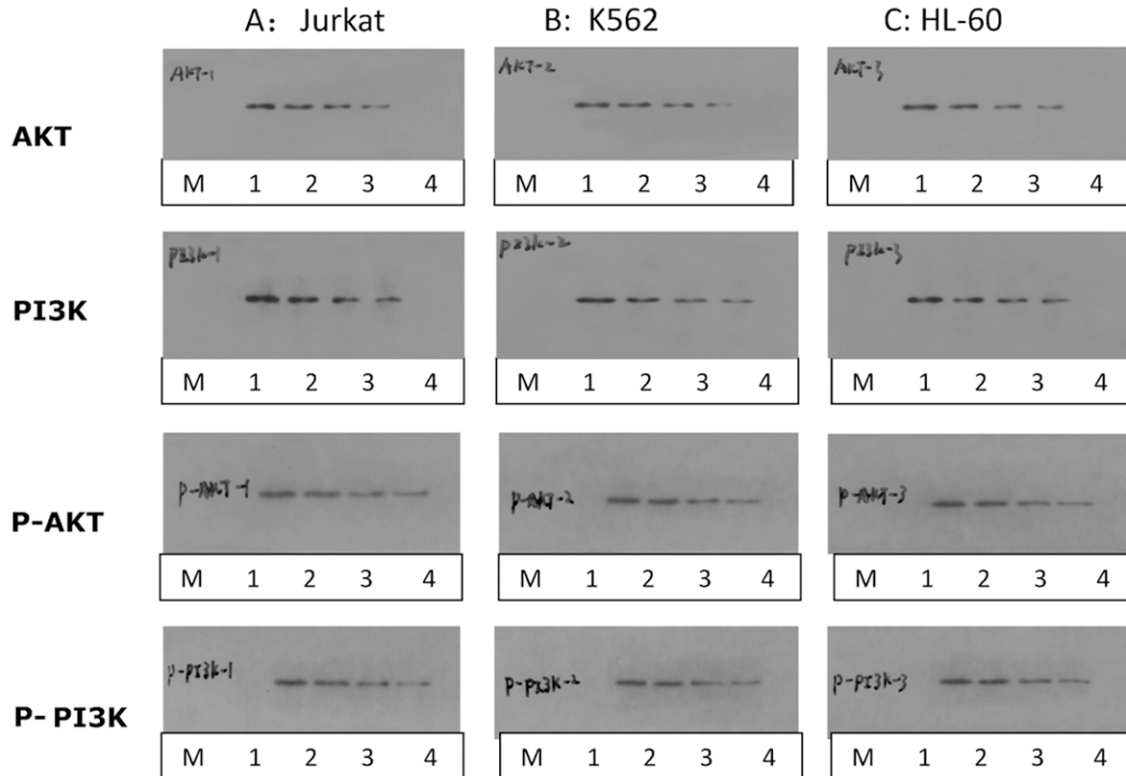


Figure 5. Impact of atorvastatin on PI3K/AKT signaling pathway. 1: Control; 2: 1 µg/ml of atorvastatin; 3: 5 µg/ml of atorvastatin; 4: 10 µg/ml of atorvastatin. P-AKT and P-PI3K indicated Phosphorylation of Akt and PI3K.

it the tumor cell proliferation by inducing various tumor cells to stagnate in the G phase.

This study has confirmed that the atorvastatin can inhibit the leukemic cell apoptotic and retard the three cells in the G₀/G₁ phase. The study has further confirmed that the mechanism of atorvastatin-induced apoptosis in leukemic cells may be closely related to the TLR4/MYD88/NF-κB and PI3K/AKT signal pathway. Toll-like receptor (TLR) signal pathway plays an important role in immunoregulation. MYD88 is an important adaptins in the TLR signal pathway. The TLR activates the IRAK and TRAF6 by to activate the MYD88. The phosphorylated TRAF6 induces the TAK1 and MKK6 to activate the downstream signal pathway such as NF-κB, JNK and P38 and induces high expression of proinflammatory cytokine such as IL-1, IL-6, IL-12 and TNF-α. Therefore, the TLR/NF-κB signal pathway can induce the high expression of the inflammatory factor related genes and further inhibit apoptosis by activating the NF-κB. In this study, the mRNA and protein levels of

TLR4, MYD88 and NF-κB genes down-regulate in the leukemic cells treated with atorvastatin, suggesting that the atorvastatin may induce apoptosis by inhibiting the TLR4/MYD88/NF-κB signal pathway- induced transcription.

The PI3K/AKT is one of the most important signal transduction pathways *in vivo* and plays important roles in the growth, proliferate, differentiation and metabolism of the normal cells. PI3K/AKT signal pathway abnormality will accelerate the cell cycle process, cause apoptosis abnormality and facilitate the differentiation from normal cells to tumor cells [10, 11]. Many studies have showed persistent activation of this signal pathway in some leukemic cells instead of normal cells. Insulin-Like Growth Factor 1 (IGF-1) is an upstream activator of PI3K. The IGF-1 receptor autophosphorylates after combining with the IGF-1 and then activates the insulin receptor substrate 1 (IRS-1). The IRS-1 recruits PI3K in the phosphorylation process and the activated PI3K causes phosphorylation of its downstream target protein

AKT, the AKT then activates the mTOR by phosphorylating it and facilitates the protein synthesis. Now the PI3K/AKT is considered as the main signal regulation pathway for protein synthesis and play important roles in the tumorigenesis process. The PI3K, AKT and their phosphorylation levels in the atorvastatin-treated leukemic cells down-regulate obviously in a dose-dependent manner. Therefore, the atorvastatin may inhibit cell proliferation and induce apoptosis by inhibiting the PI3K/AKT signal pathway (especially the phosphorylated protein of PI3K and AKT). Ogunwobi et al [12] have found that the statins can inhibit the cell proliferation and induce apoptosis by inhibiting the ERK and AKT signal pathway, which is coincidence with the conclusion in this study.

Studies on the existence of the relationship between the TLR4/MYD88/NF- κ B and PI3K/AKT signal pathway are seldom reported by now. Ghosh-Choudhury et al [15] have found that the simvastatin can inhibit the tumor cell proliferation by inhibiting the AKT activity, weakening the transcription activity of NF- κ B and decreasing the expression of the anti-apoptosis protein Bcl-XL in the breast carcinoma cells *in vitro*. This study confirmed the TLR4/MYD88/NF- κ B and PI3K/AKT signal pathway participate together the atorvastatin-induced apoptosis in leukemic cells. We presume boldly the atorvastatin may inhibit the tumor cell proliferation and induce apoptosis by the TLR4/MYD88/PI3K/AKT/NF- κ B pathway.

In conclusion, the atorvastatin can inhibit the leukemic cell proliferation and induce apoptosis and the mechanisms are complex. One of the mechanisms may be associated with the atorvastatin-regulated expression of TLR4/MYD88/PI3K/AKT/NF- κ B related genes.

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Disclosure of conflict of interest

None.

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