

Research Article

Open Access

Jinshen Wang, Lixia Wei, Yueqin Han, Daogang Qin, Qiaozhi Yang, Xiuli Ju*

The impact of *Tegillarca granosa* extract haishengsu on HL-60 cell

DOI 10.1515/med-2015-0048

received April 6, 2015; accepted May 25, 2015

Abstract: Haishengsu (Hss) is a purified protein from *Tegillarca granosa* that has been used as a traditional Chinese medicine to treat cancer for more than a century. In this study, we observed the impact of Haishengsu (Hss) on the proliferation and differentiation of HL-60 cells in the leukemic cell line by taking tretinoin and AS_2O_3 as a positive control and making a comparative analysis between the effect of Hss and tretinoin and AS_2O_3 . We found that Hss could significantly inhibit the proliferation of HL-60 cells and caused most of the cells to stay in the G0/G1 phase. Its effect was much stronger than that of tretinoin and AS_2O_3 , and the ability of Hss to induce differentiation was close to tretinoin. Hss functions probably by inhibiting the expression of the Bcl-2 and MPO genes and further promoting the expression of the Bax gene. Hss has a significant effect on both inhibiting the proliferation and inducing the differentiation of HL-60 cells. It is possible that Hss may be a new kind of clinical differentiation inducer.

Keywords: *Tegillarca granosa*; Cell differentiation; Leukemia

*Corresponding author: Xiuli Ju, Department of Pediatric Hematology, Qilu Hospital, Shandong University, 107 West Wenhua Rd, Jinan, Shandong 250012, China; Tel:+86 13184177168; E-mail: juxiuli2015@163.com

Cryomedicine Laboratory of Qilu Hospital of Shandong University, Jinan, Shandong, 250000, P.R.China

Jinshen Wang, Department of Pediatric Hematology, Qilu Hospital, Shandong University, Jinan, Shandong, 250000, P.R.China,

Department of Pediatric, Liaocheng People's Hospital, Liaocheng, Shandong, 252000, P.R.China

Lixia Wei, Yueqin Han, Daogang Qin, Qiaozhi Yang, Department of Pediatric, Liaocheng People's Hospital, Liaocheng, Shandong, 252000, P.R.China

1 Introduction

Marine creatures, in the process of their growth and metabolism, produce certain unique bioactive substances that accumulate in them. In vitro study has shown that some of these bioactive substances have anti-tumor effects. Ascidiemin, a marine alkaloid in the Mediterranean, could significantly induce the apoptosis of leukemic cells HL-60 and P388 [1]. Bryostatin, a macrolides compound in *bugula neritina*, could help induce human leukemic cells [2]. *Tegillarca granosa* is a sea creature that has been used as a traditional Chinese medicine to treat cancer for more than a century. Haishengsu (Hss) is a purified protein from *Tegillarca granosa*, and recent research has confirmed that Hss has a distinct effect in both inhibiting the proliferation of renal carcinoma cells and leukemic cells and inducing the apoptosis of cells [3,4]. This study focuses on HL-60 cells in the human leukemic cell line to observe the impact of Hss on the proliferation and differentiation of HL-60 cells and make a comparative analysis between the effect of Hss and tretinoin and AS_2O_3 , which are currently the most commonly applied clinical differentiation inducers.

2 Material and Method

2.1 Cells and reagents

HL-60 cells were introduced from Shandong Medical Academy of Science. Hss was obtained from Qingdao Haisheng Oncology Hospital (Shandong, China, batch number 990211). Medium powder RPMI-1640 was purchased from Gibco, further compounded with tri-distilled water and combined with a final concentration of 100 u/ml penicillin and 100 ug/ml of streptomycin. Calf serum

was produced by Sijiqing Company, and tretinoin and AS_2O_3 were purchased from Sigma. The water-insoluble drugs, tretinoin and AS_2O_3 were dissolved by dimethylsulfoxide (DMSO from Sigma), filtered, sterilized and diluted into different concentrations via culture solution RPMI-1640 before the experiment. The final concentration of DMSO was less than 0.1%. FTCT labeled mAb CD11b, PE labeled McAb CD15 and its corresponding isotype control were purchased from Sigma. Methyl thiazolyl tetrazolium (MTT) was purchased from Sigma and prepared as 5g/L solution with PBS of pH 7.4 before use. RT-PCR, dNTP, TaqDNA polymerase and RNA enzyme inhibitor were purchased from Qiagen. The objective primer, according to the reported reference, was synthesized by the DNA synthesis department of Shanghai Biotechnology Company.

2.2 Cell culture

The HL-60 cells were put into the culture medium, which contained a 10% volume inactivated newborn calf serum RPMI-1640, and it was placed in an incubator at 37°C, 5% CO_2 and saturated humidity to culture and passage. The solution was replaced once every 2 days.

2.3 HL-60 cell activity detection via MTT method

The HL-60 cells were removed in the exponential period and vaccinated into the 96-hole culture plate, with each hole having 199ul (including 1×10^5 /ml cells). They were put into an incubator at 37°C, 5% CO_2 and saturated humidity and culture for 24 hours. Tretinoin, AS_2O_3 and Hss were added separately into each experimental group. The final concentration was tretinoin 5umol/L, AS_2O_3 5umol/L and Hss 40mg/L. (Previous research has shown that the survival rate is lower with an increased concentration of Hss; IC50 (half maximal inhibitory concentration) is 40mg/L.) A certain volume of DMSO solution was added to the control group until the final volume of each hole reached 200 ul. Also, blank contrast groups, each with 6 parallel holes, were set up. After culturing for 24, 48 and 72 hours, 20ul MTT were added to each of these holes. Then the mixture was put into an incubator for 4 hours before a horizontal centrifuge was made and the supernatant extracted. Then 150ul DMSO was added into each well and blended fully. Finally, the ELISA was used to test the absorption value of optical density of each well and calculate the inhibition rate according to the following formula:

Inhibition rate % = $\frac{\text{average OD value of control group} - \text{average OD value of drug group}}{\text{average OD value of control group}} \times 100\%$.

2.4 Morphological observation under light microscope

HL-60 cells were removed in the exponential period and put into RPMI-1640 culture solution, which contained 10% fresh new calf serum and dilute. After they were diluted into 1×10^5 /ml cell suspension, the cell suspension was vaccinated into the 24-well culture plate, each with 995ul, which was set with aseptic cover glass in advance. Tretinoin, AS_2O_3 and Hss were added separately to each experimental group. The final concentration was tretinoin 5umol/L, AS_2O_3 5umol/L, Hss 40mg/L. A corresponding volume of DMSO solution was added to the control group until the final volume of each well reached 1000 ul. Then three parallel wells were set up in each of these groups and put into an incubator at 37°C, 5% CO_2 and saturated humidity for culturing. After 24, 48 and 72 hours, the cover glass from each experimental group was removed and they were dried naturally. Then methanol was used to fix and Giemsa to stain the cells, followed by observation with photos.

2.5 Measure the expression of CD11b and CD15 by means of FCM and analyze the change of cell cycle

HL-60 cells in the exponential period were removed and put into RPMI-1640 culture solution, which contained 10% fresh new calf serum and dilute. After they were diluted into 1×10^5 /ml cell suspension, the cell suspension was vaccinated into the culture bottles, each with 19.9ml. Tretinoin was added to each experimental group, and AS_2O_3 was added separately. The final concentration was tretinoin 5umol/L, AS_2O_3 5umol/L, Hss 40mg/L. A corresponding volume of DMSO solution was added to the control group until the final volume of each bottle reached 20ml. They were then incubated at 37°C, 5% CO_2 and saturated humidity and culture for 72 hours. Seventy-two hours later, the cells were centrifuged and collected. Then the following process was started: 1: the cells were washed with PBS twice and then suspended into 200ul Binding Buffer, 30ul Annexin V-FITC and 5ul propidium iodide (PI). They were blended together, and the mixture was kept in darkness. After 15 minutes' reaction under room temperature, 300ul buffer solution was poured into

the cell mixture and the apoptosis rate of cells was immediately detected. 2. The cells were washed with PBS once, centrifuged and removed PBS., 1ml PBS, was added to the cells, blended fully and then transferred to a microcentrifuge tube; 5ulFTCT labeled mAb CD11b and PE labeled McAb CD15 was added to the tube. It was placed in darkness at 4°C and incubated for 20mins. After 20mins. the fluorescent intensity of the marked cells out of a total of 10000 cells was detected. 3. The cells were washed with PBS once, centrifuged, and blended. Seventy percent absolute alcohol was blended fully with the cells. The mixture was placed at 4°C and kept overnight. It was washed with PBS twice, 0.5ml PBS was added to the cells and blended fully. RNaseA was added to the mixture, and it was placed at 37°C for 1h. Then, PI was added, and it was placed in darkness at 4°C, staining for 1h. Then it was filtered through 300 mesh nylon mesh and fluorescence intensity was detected. The total cell number was 10000, and the cell cycle and DNA content were analyzed.

2.6 Adopting RT-PCR to test the expression change of bcl-2, bax and mpo gene

HL-60 cells were removed in the exponential period and added to RPMI-1640 culture solution, which contained 10% fresh new calf serum and dilute. After they were diluted into 1×10^5 /ml cell suspension, the cell suspension was vaccinated into the culture bottles, each bottle with 19.9ml. Tretinoin was added to each experimental group, and AS_2O_3 was added separately. The final concentration was tretinoin 5umol/L, AS_2O_3 5umol/L, Hss 40mg/L. A corresponding volume of DMSO solution was added to the control group until the final volume of each bottle reached 20ml. Then they were put into an incubator at 37°C, 5% CO_2 and saturated humidity and culture for 72 hours. Then, the cells were centrifuged, collected and washed with PBS twice, according to the instructions in the RT-PCR kit. The final product was placed in 1% agarose gel electrophoresis. The gel image analyzer (Gel Doc1tyoe, Bio-Rad) was used to observe and take photographs. The primer sequence of each gene is listed in Table 1.

2.7 Statistical process by means of SPSS statistical software for analysis

Data obtained from the experiment were presented with the $X \pm s$, t test, which was adopted to compare the experimental and control groups, and the inhibition rate at

different time points was measured by multiple-factor repetitive measurement and analysis.

Ethical approval: The conducted research is not related to either human or animals use.

3 Result

3.1 The impact of Hss, tretinoin and AS_2O_3 on cell proliferation

Hss, tretinoin and AS_2O_3 all had a significant effect on inhibiting HL-60 cells, which followed a time effect manner. With the passage of time, their inhibition rate gradually increased. Each time point had statistical significance. By comparison, we found that the inhibition rate of Hss was clearly superior to that of tretinoin and AS_2O_3 , as shown in Table 2.

Table 1: Primers for the RT-PCR experiments performed in this study

Gene		Sequence of primers	Product
Bax	up	5'gatgctccaccaagaag 3'	
	Down	5 gatcagttccggcacctt3'	243bp
Bcl-2	up	5'tgtttccttcttgagtt cg3'	
	Down	5'tcacttggtgctcagatagg 3'	280bp
Mpo	up	5'cctactacatgcaaggcactgt3'	
	Down	5'cccagttcctcttcaggagaacttc3'	249 bp
β -actin 1	up	5'gtggggcgc ccc agg cacca 3'	
	up	5'ctcctt aatgtcacgcacgatttc 3'	539 bp
β -actin 2	up	5'gggtcagaaggattcctgtg 3'	
	up	5'gggtctcaaacatgatctggg 3'	317 bp

Table 2: The inhibition rate, of Hss, tretinoin and AS_2O_3 on proliferation of HL-60 cell $X \pm s$ (%).

groups	24h	48h	72h
Hss	36.12±4.43	75.23±3.14	96.23±0.78
tretinoin	4.49±4.21	24.20±3.14	46.18±7.55
AS_2O_3	4.04±2.94	22.19±1.53	71.51±5.61

3.2 The impact of Hss, tretinoin and AS₂O₃ on differentiation of HL-60 cell

3.2.1 The change of cell morphology under a light microscope

After a Giemsa stain was applied, we microscopically observed the control group under oil and found that HL-60 cells were relatively large, the cell nucleus was big and round, and chromosomes were dispersed. The cell contained 2-4 nucleoli, the cytoplasm was basophilic and hyperchromatic, and the nucleo-cytoplasmic ratio was large (Figure 1D). On the tretinoin side, after 72 hours' culturing, the cell morphology was significantly changed. The cell differentiated into a mature granulocyte, the cell body shrank, the cell nucleus was depressed, and the chromatin became dense and thick. The cell nucleus decreased and disappeared, the nucleo-cytoplasmic ratio was reduced, and some cells began to die (Figure 1B). On the AS₂O₃ side, after 72 hours' culturing, most of the cells began to become apoptotic, the cell body shrank, karyopyknosis occurred, and the acidophilia of chromatin was enhanced. Part of the cell nucleus broke into several round granules of different sizes. Apoptotic bodies were observed in some cells (Figure 1C). On the Hss side, after 72 hours' culturing, both differentiation and apoptosis could be observed. The cell body shrank, the cell nucleus was depressed, and the chromatin became dense and thick. The cell nucleus decreased and disappeared, the nucleo-cytoplasmic ratio was reduced, and also karyopyknosis and apoptotic body appeared (Figure 1A).

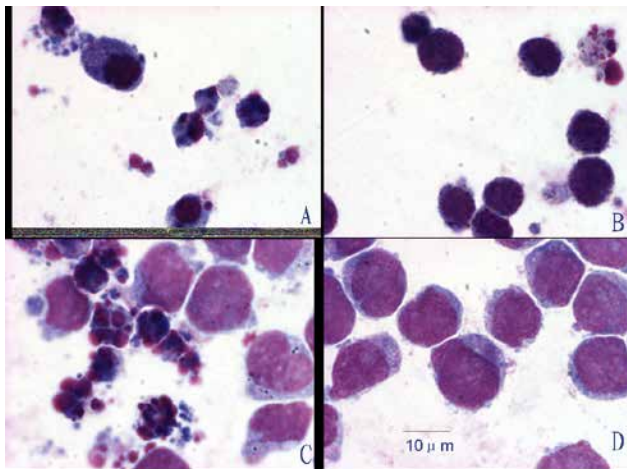


Figure 1: The morphological change ($\times 1000$) of cell under light microscope after 72 hours' culture. A: Hss; B: tretinoin; C: AS₂O₃; D: control

3.2.2 The change of differentiation antigen on cell surface

After 72 hours' culturing of Hss, tretinoin, AS₂O₃ on HL-60 cells, the expression of CD11b significantly increased. There was a statistical difference in comparison with the control group. A difference was observed between Hss and tretinoin. No difference was observed between Hss and AS₂O₃. CD15 shows a high expression in both the control group and the experimental group, without statistical difference, as shown in Table 3.

3.2.3 The change of cell cycle

The HL-60 cells in the exponential phase were mainly located in s phase and G2/M phase. After 72 hours' culturing of Hss, tretinoin, and AS₂O₃, most of the cells remained in the G1 and s phases. The number of cells in G2/M significantly decreased. There was a statistical difference in comparison with the control group. Differences were observed both between Hss and tretinoin and between Hss and AS₂O₃, as shown in Table 4.

3.3 The impact of Hss, tretinoin, and AS₂O₃ on mRNA

After 72 hours' culturing of Hss, tretinoin, and AS₂O₃, the expression of Bcl-2 in the control group was the strongest, while that in the experimental groups was relatively weak.

Table 3: The change of differentiation antigen on HL-60 cell surface after 72 hours' culture. $\bar{X} \pm s$ (%)

groups	CD11b	CD15
tretinoin	76.13 \pm 8.09	94.02 \pm 5.78
AS ₂ O ₃	13.44 \pm 10.58	90.00 \pm 6.59
Hss	94.38 \pm 7.14	94.74 \pm 7.09
Control	2.35 \pm 3.06	95.50 \pm 8.06

Table 4: The change of HL-60 cell cycle after 72 hours' culturing (%)

groups	G0/G1	G2/M	S
tretinoin	38.44	16.49	45.07
AS ₂ O ₃	59.73	4.84	35.44
Hss	75.08	3.26	21.66
Control	21.11	20.12	58.77

The expression change of Bax showed the opposite result. After the effect of Hss, tretinoin and As_2O_3 , the expression of the Bax gene strengthened. The Hss and the tretinoin groups showed the strongest expression, while the Bax in the control group showed the weakest. After 72 hours' culturing of Hss, tretinoin, and As_2O_3 , the MPO gene, in comparison with the control group, was weakened. The Hss group was weakened the most in expression of the MPO gene, and then the tretinoin group. The expression of MPO gene in the control group was the strongest, as shown in Figure 2.

4 Discussion

Acute promyelocytic leukemia (APL) accounts for about 8% to 15% of cases of acute nonlymphocytic leukemia, with an annual incidence of 2-3/million [5]. APL is characterized by specific chromosome translocation t(15; 17)(q22; q21) and results in a characteristic fusion gene PML/RAR α [6-8]. Traditional treatment is focused on chemotherapy. In recent years, tretinoin and As_2O_3 , through the effect on the PML/RAR α fusion gene, have induced differentiation and apoptosis of tumor cells and thus greatly improved the long-term survival rate of patients with APL [9]. However, with the expansion of clinical application to cases, we gradually discovered that both tretinoin and As_2O_3 have distinct individual differences on curative effect, drug resistance, recurrence rate and other

side effects to varying degrees [10,11]. So, finding a new differentiation inducer and apoptosis agent has become a pressing matter in clinical treatment.

The result of our study shows that Hss has an apparent effect in inhibiting the proliferation of HL-60 cells. Hss could make the most of the cell stay in G0/G1 period, in that cell synthesis is blocked and unable to enter into the division cycle, and it finally succeeds in inducing the maturation and differentiation of the cell. After 72 hours' influence of Hss on HL-60 cells, the expression of CD11b apparently becomes stronger. Normally, its expression on HL-60 cells is negative, mainly on mature monocytes, natural killer cell and neutrophil granulocytes [12,13]. mRNA in myeloperoxidase (MPO) has the highest expression rate in original granulocytes and early young granulocytes. As the granulocyte differentiates into maturity, the expression level of the MPO gene dramatically declines. An MPO mRNA test is a kind of marker for myeloid cells with a high sensitivity, specificity and a strong stage [14,15]. The result of RT-PCR shows that after 72 hours' influence by Hss, the MPO gene, in contrast with the control group, has weakened. This also confirms that Hss has an effect in inducing the maturation and differentiation of HL cells.

Hss, as a kind of glycoprotein mixture, has an anti-tumor effect. But its mechanism is still unclear. This study shows that Hss may through its impact on Bcl-2/Bax, further influence the apoptosis and differentiation of cells. The Bcl-2 gene family is one of the most significant apoptosis regulatory genes. Bcl-2 and Bax's effect in controlling

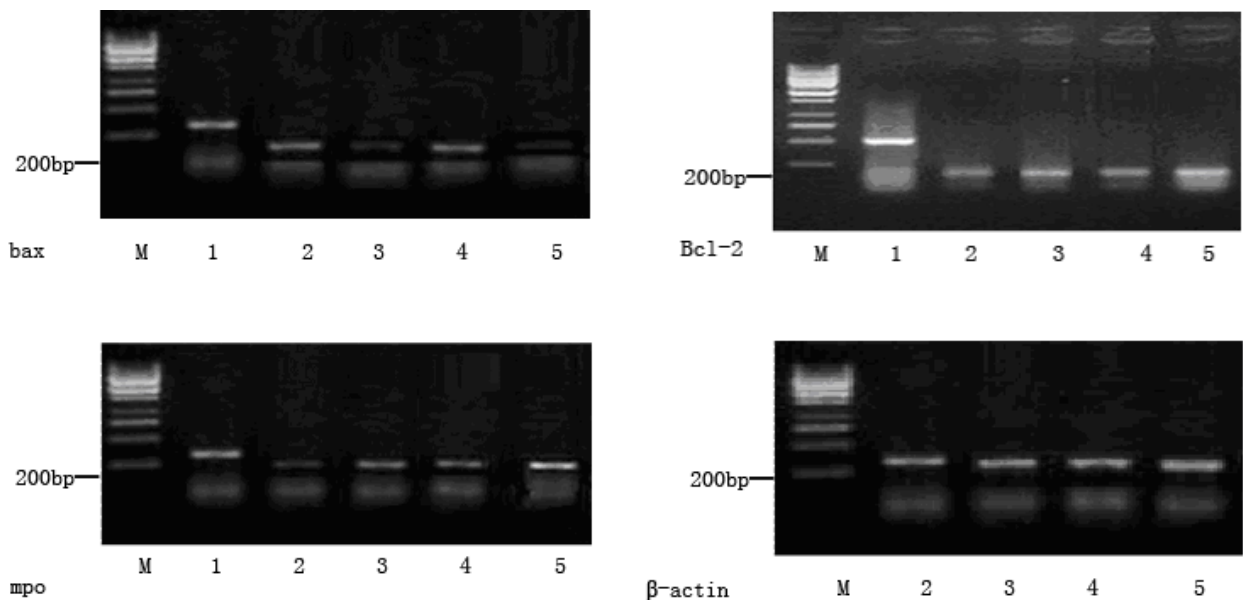


Figure 2: The change of cell gene expression after 72 hours' culture. M: marker 1- β -actin 2: Hss group 3: As_2O_3 group 4: tretinoin group 5: control group

the release of cytochrome C is the key incident that results in apoptosis and also the major means in the mitochondria-mediated apoptosis pathway [16,17]. RT-PCR confirms that Hss significantly inhibits the expression of the Bcl-2 gene and promotes the high expression of the Bax gene. When the cells show a proliferation state and the Bax gene transits into high level expression, the proliferation is inhibited and differentiation occurs. Through the comparison of Hss and tretinoin and AS_2O_3 , we found that Hss takes effect earlier than tretinoin and AS_2O_3 , and it inhibits cell proliferation more effectively than tretinoin and AS_2O_3 . Its ability to induce cell differentiation is superior to 5 μ mol/L AS_2O_3 and is close to 5 μ mol/L tretinoin. So, overall, we concluded that Hss, as a leukemia cell differentiation inducer, has a stronger effect than tretinoin and AS_2O_3 .

Our preliminary conclusion is that Hss has a significant effect on both inhibiting the proliferation and inducing the differentiation of HL-60 cells. It takes effect probably through inhibiting the expression of the Bcl-2 and MPO genes, thus further promoting the expression of the Bax gene. There is a strong possibility that Hss could be a new kind of differentiation inducer for clinical treatment. Of course, it is still requires further study, especially in vivo.

Acknowledgments: The authors thank Dr Guangyao Li and Cryomedicine Laboratory of Qilu Hospital of Shandong University for technical assistance and advice

Conflict of interest: Authors state no conflict of interest

References

- [1] Dassonneville L, Wattez N, Baldeyrou B, Mahieu C, Lansiaux A, Banaigs B, et al. Inhibition of topoisomerase II by the marine alkaloid ascididemin and induction of apoptosis in leukemia cells. *Biochem Pharmacol* 2000, 60: 527-537
- [2] Wang S, Wang Z, Grant S. Bryostatin 1 and UCN-01 potentiate 1-beta-D-arabinofuranosylcytosine-induced apoptosis in human myeloid leukemia cells through disparate mechanisms. *Mol Pharmacol* 2003, 63 : 232-242
- [3] Li GY, Liu JZ, Zhang B, Yang M, Chen SG, Hou M, et al. Tegillarca granosa extract Haishengsu (HSS) suppresses expression of mdr1, BCR/ABL and sorcin in drug-resistant K562/ADM tumors in mice. *Adv Med Sci.* 2013;58:112-117
- [4] Xu W, Chang Z, Liu X, Jiang C, Wang C, Xu L. Antitumor effects of a polypeptide isolated from *Tegillarca granosa* linnaeus and the related molecular mechanism. *Pak J Pharm Sci.* 2014;27:565-570
- [5] Chen ZX, Xue YQ, Zhang R, Tao RF, Xia XM, Li C, et al. A clinical and experimental study on all-trans retinoic acid-treated acute promyelocytic leukemia patients. *Blood* 1991;78:1413-1419
- [6] Chen SJ, Chen Z, Chen A, Tong JH, Dong S, Wang ZY, et al. Occurrence of distinct PML-RAR α fusion gene isoforms in patients with acute promyelocytic leukemia detected by reverse transcriptase/polymerase chain reaction. *Oncogene* 1992;7:1223-1232
- [7] Pandolfi PP, Alcalay M, Fagioli M, Zangrilli D, Mencarelli A, Diverio D, et al. Genomic variability and alternative splicing generate multiple PML/RAR α transcripts that encode aberrant PML proteins and PML/RAR α isoforms in acute promyelocytic leukemia. *EMBO* 1992,11:1397-1407
- [8] Wang ZY. Mechanism of action of all-trans retinoic acid and arsenic trioxide in the treatment of acute promyelocytic leukemia. *Gan To Kagaku Ryoho*, 2002, Suppl 1: 214-218
- [9] Halftermeyer J, Le Bras M, De Thé H. RXR, a key member of the oncogenic complex in acute promyelocytic leukemia. *Med Sci(Paris)*,2011, 27(11):973-978
- [10] Chamarin N, Smith GB, Green A, Andrews MI. Retinoic acid syndrome. *Lancet* 1993; 341:1289-1290
- [11] Wang ZY, Chen Z. Acute promyelocytic leukemia: from highly fatal to highly curable. *Blood*,2008,111,2505-2515
- [12] Tan SM, Hyland RH, Al-Shamkhani A, Douglass WA, Shaw JM, Law SK. Effect of integrin beta 2 subunit truncations on LFA-1 (CD11 a/CD18) and Mac-1 (CD11b /CD18) assembly, surface expression, and function. *Immuno*, 2000; 165: 2574-2581
- [13] Paietta E, Goloubeva O, Neuberg D, Bennett JM, Gallagher R, Racevskis J, et al. A surrogate marker profile for PML/RAR alpha expressing acute promyelocytic leukemia and the association of immunophenotypic markers with morphologic and molecular subtypes. *Cytometry B Clin Cytom*,2004 59:1-9
- [14] Alvarado CS, Austin GE, Borowitz MJ, Shuster JJ, Carroll AJ, Austin ED, et al. Myeloperoxidase gene expression in infant leukemia: a Pediatric oncology Group Study. *Leku LymPhoma*,1998,29:145-160
- [15] Serrano J, Román J, Sánchez J, Torres A. Myeloperoxidase gene expression in acute lymphoblastic leukemia. *Br J Haematol*, 1997; 97:841-843
- [16] Eskes R, Desagher S, Antonsson B, Martinou JC. Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. *Mol Cell Biol*, 2000, 20:929-935
- [17] Miramar MD, Costantini P, Ravagnan L, Saraiva LM, Haouzi D, Brothers G, et al. NADH oxidase activity of mitochondrial apoptosis-inducing factor. *J Biol Chem*, 2001 ,276 : 16391-163981