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Experimental study of reversal of multidrug resistance in human leukemia K562/DOX cells by toad venom

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Abstract. Acute leukemia is a malignant tumor originating from the hematopoietic system with the highest incidence and mortality. At present, the main clinical treatment of leukemia is still chemotherapy, during the course of which the multidrug resistance (MDR) will significantly reduce remission rate and disease-free survival rate of patients. MDR is the most important factor affecting refractory/recurrent acute leukemia. Therefore, reversing leukemia MDR is one of the best ways to improve the complete remission rate of refractory/recurrent acute leukemia, and the study of drugs and methods to overcome leukemia MDR has received extensive attention in the leukemia research field. This study was to primarily investigate the effects of Liushen pills on leukemia drug-resistant cell line K562/DOX in inhibiting growth, reversing resistance and inducing apoptosis in anticipation of providing useful cytological and molecular biological basis for the treatment of refractory/recurrent acute leukemia. The serum containing toad venom was prepared by means of Chinese drug serum pharmacology. MTT assay was used to detect the inhibitory rates of human leukemia cell line K562/DOX after being treated with the serum containing toad venom as well as daunorubicin, or with the serum containing toad venom alone at different time points. Real-time fluorescent quantitative analysis (RT-PCR) was performed to determine the effects of serum containing toad venom on the expression of BCL-2 mRNA in human leukemia cell line K562/DOX. Compared to the control group, toad venom showed inhibitory effects on K562/DOX cells; the expression level of BCL-2 mRNA in toad venom group were decreased, indicating that toad venom may reverse the resistance of K562/DOX cells by down-regulating the expression level of MDR1.

Keywords: K562/DOX cells; mechanism; multidrug resistance; toad venom

1. Introduction

Acute Leukemia (AL) is a clonal disease originating from hematopoietic stem cells and is a malignant tumor of the blood system (Hou *et al*. 2021, Huang *et al*. 2021a, Jiao *et al*. 2021, Moradi *et al*. 2021). In the field of leukemia research, an increasing number of new drugs and chemotherapy regimens have emerged, suggesting a great progress in the treatment of leukemia (Pang *et al*. 2019, Wu *et al*. 2021, Xu *et al*. 2021a, Zhao *et al*. 2021a). However, multidrug resistance (MDR) of tumor cells is a major cause of chemotherapeutic failure (Habibi *et al*. 2017, 2019a, c, f, 2020). Currently, the majority of in vitro resistance-reversal agents are not effective enough, unable to overcome MDR (Habibi *et al*. 2016, 2018a, b, 2019b, d). In recent years, the role of traditional Chinese medicine (TCM) in reversing the drug resistance of tumor cells has attracted much attention (Hu *et al*. 2021, Huang *et al*. 2021b, Liu *et al*. 2021b, Ma *et al*. 2021, Zhao *et al*. 2021b). Clinical trials have shown that Liushen pills, a kind of traditional Chinese medicine, can reverse the multidrug resistance of leukemia cells by inducing cell apoptosis and reducing the expression of drug-resistant genes (Shi *et al*. 2007, Jiang *et al*. 2020, Pan *et al*. 2020, Wang *et al*. 2020). However, the effective ingredients playing a key role in Liushenpills have not been clear, and the ways to reverse the multidrug resistance of leukemia cells remain to be further explored (Zhang *et al*. 2019b, Zou *et al*. 2019, Liu *et al*. 2021a, Xu *et al*. 2021b, c). In this study, the effects of toad venom, a component in TCM Liushen pills, on the proliferation of drug-resistant leukemia cells and the expression of BCL-2 mRNA were observed to explore its mechanism of reversing K562/DOX cells, providing a clinical basis for the future application.

2. Meterials

2.1 Cell lines

Amycin-resistant human erythroleukemia cell lines K562/DOX were purchased from Guangzhou Da'an Science and Technology Biology Co., Ltd.

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2.2 Drugs and reagents

Liushen pills and toad venom were purchased from Guangzhou Baozhilin Pharmacy (Liushen pills were produced by Shanghai Lei Yun Shang Pharmaceutical Co., Ltd.. Specification: 3.125 g/1000 pills); daunorubicin was purchased from Guangzhou New Special Pharmacy (Dalian Meilun Biotech Co., Ltd. 10mg/tube); DNA polymerases and Trizol were purchased from TaKaRa Bio Inc.; BCA-100 Protein Quantitative Assay Kit was purchased from Biocolor Company (Shah *et al*. 2015, 2016a, Nosrati *et al*. 2018, Toghroli *et al*. 2018, Ziaei-Nia *et al*. 2018).

2.3 Animals

12 New Zealand male rabbits weighing 2-3kg (purebred adult New Zealand rabbits are all qualified conventional animals. License Number: SCK (Guangdong) 2009-0002, Guangdong monitoring certificate 2008A023) were provided by Animal Experimental Center of Guangzhou University of Chinese Medicine.

2.4 Main instruments

PCR instrument: SENSO (Germany); gel imaging system: Shanghai Peiqing Technology Co., Ltd.; ELISA plates and culture flasks: Coring (United States); CO2incubators, water bath (HH-W21-600S), cyclotron oscillator (HY-5): Shanghai Yuefeng Instrument Co., Ltd.; centrifugal precipitation machine: Beijing Medical Centrifuge Factory; inverted phase contrast microscope (COIC): China Guangzhou Da'an Gene Company.

3. Methods

3.1 Preparation of drug-containing sera

Liushenpills and toad venom, the experimental drugs to be tested on New Zealand rabbits, were converted from human equivalent dose (0.933mg/kg) to animal dose(13.125mg/kg) (Chen 2006) and blended with normal saline to be prepared into corresponding concentrations. 12 rabbits were randomly divided into control group, Liushen pill group, toad venom group, and daunorubicin group, three in each group (Zhan *et al*. 2014, Iqbal *et al*. 2018, Qi *et al*. 2019, 2020, Zhang *et al*. 2019a). The rabbits in the control group were administratedby IG of 20ml of normal saline once every day for continuous 3 days (Habibi *et al*. 2019e, 2021, Jahandari *et al*. 2021). On the last day, drug administration was executed at an interval of 2 hours for continuous 3 times (Jiang *et al*. 2021, Li *et al*. 2021, Lou *et al*. 2021b, Razzaghi *et al*. 2021). One hour after the last administration, blood samples were collected from the hearts under sterile conditions, centrifuged, inactivated, filtered through 0.22um millipore filters on thesuperoclean bench, and then stored at -20℃ refrigerator for future use (Lou *et al*. 2021a, Lv *et al*. 2021a, c, Yu *et al*. 2021).

3.2 K562/DOX cell culture and experimental grouping

Amycin-resistant human erythroleukemia cell lines (K562/DOX cells) were cultured in the presence of 1 mg/L of amycin to maintain drug resistance (Zhou *et al*. 2019, 2020, 2021, Lv *et al*. 2021b). They were inoculated in RPMI1640 medium supplemented with 15% fetal calf serum (FCS) at 37°C and in 5% CO_2 atmosphere in sterile T75 culture flasks (Shah *et al*. 2016b, Zandi *et al*. 2018, Naghipour *et al*. 2020a, Yazdani *et al*. 2020, Rajaei *et al*. 2021). Cellsfrom log phase which had a doubling time of 2-3 days were cultured for one week until further use (Khorramian *et al*. 2016, Khorami *et al*. 2017, Hosseinpour *et al*. 2018, Safa *et al*. 2019, Afshar *et al*. 2020, Naghipour *et al*. 2020b). They were cultured in media supplemented by sera containing normal saline, 5ug/ml of daunorubicin, toad venom and Liushenpills respectively, the volume of which making up 15% of the total culture system (Sinaei *et al*. 2012, Khorramian *et al*. 2017, Toghroli *et al*. 2017, Milovancevic *et al*. 2019, Sajedi *et al*. 2019).

3.3 MTT assay to determine cell inhibitory rate

The K562/DOX culture media were adjusted to contain 1x105cells/mL. 100μL of adjusted cell suspension was seeded into 96-well plates and treated with 100μL of normal saline, Liushenpills, toad venom, and daunorubicin for 24h, 48h and 72h respectively. Then 20 μl of MTT solution (5 g/L) was added into each well and cells were incubated for another 4h. The supernatantswere removed and 100 μl of DMSO was added into each well. The plate was rotated for 10 min at room temperature and the absorbance (A) was detected at 490nm wavelengths by an enzyme-linked immunosorbent assay reader. The inhibitory rate was calculated using the following formula: cell inhibitory rate = (Ablank group-Acontrol group)/Ablank group×100%. Cells were plated in 5 duplicate wells and exposed to the sameconcentration each time. All the experiments were repeated three times.

3.4 Expression of BCL-2 mRNA in K562/DOX Cells

The K562/DOX cells treated with drugs for 48h were taken to detect the expression levels of BCL-2 genes in differently-treated cells by Sybrgreen, β-actin as an internal standard. Total RNA was extracted (following the Invitrogen's instruction of Trizol Kit) and RT-PCR was used. After primer was dissolved, ordinary PCR reaction was used for exploration and optimization. When the reaction systemwas set up according to the reagent directions, RealTime PCR could be performed for amplification and detection. During the whole process, the fluorescence signals were collected to draw melting curve. The experiment was repeated three times.

4. Statistical analysis

All data were expressed as mean± standard deviation (x±s) and SPSS 17.0 software was used for statistical analysis.

5. Results

5.1 Effects of Sera containing Liushen pills and toad venom on inhibitory rate of K562/DOX cells

Daunorubicin had no significant inhibitory effects on

Table 1 Absorbance and inhibitory rate of K562/DOX cells treated with sera containing Liushen pills and toad venom

Doses (mg/kg)	Duration (h)	n	Absorbance	Inhibitory Rate (%)
Control Group	24	3	0.1773±0.02364	
	48	3	0.4762±0.02474	
	72	3	0.8567±0.02089	
Liushen Pill Group	24	3	0.088±0.02168 ⊿	50.4
	48	3	0.3280±0.03564⊿*	31.1
	72	3	0.6540±0.0.2793⊿@	23.6
Toad Venom Group	24	3	0.08232±0.01734 *#	53.07
	48	3	0.3325±0.02588 *@	30.10
	72	3	0.6733±0.3847 *$	21.40
Daunorubicin Group	24	3	0.1933±0.02988 #	
	48	3	0.3519±0.02266#⊿	26.20
	72	3	0.6333±0.0.2793 #@	26.07

Table 2 Effects of Expression of BCL-2 mRNA Detected by RT-PCR (24h)

Group	Copy Number of BCL-2 mRNA
Control Group	1.3839±0.005312
Daunorubicin Group	1.0737±0.001236*
Liushen Pill Group	⊿0.8211±0.01344*
Toad Venom Group	⊿0.5912±0.01399

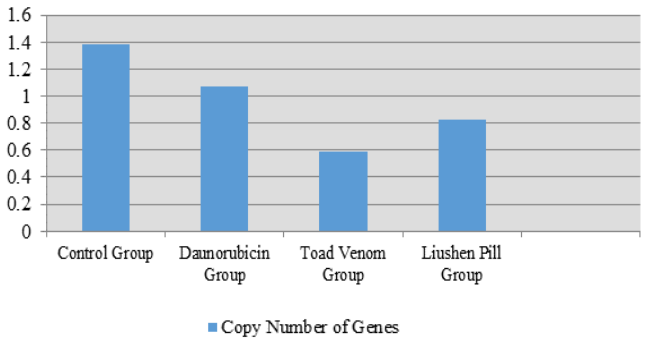

Fig. 1 A configuration of a MEEAD

K562/DOX cells, while seracontaining toad venom and Liushenpills obviously inhibited cells. See Table 1.

Note: Absorbance and inhibitory ratewere shown in Table 1. At different time points, the control group was significantly different from the groups treated with drug-containing sera (⊿P < 0.01, *P < 0.05, #P < 0.05), revealing that all drug-containing sera had certain inhibitory effects on leukemia K562 cells, among whichLiushenpill group andtoad venom group exhibited more obvious inhibition. Within 24h, there was no significant difference of inhibitory rates between Liushenpill group (⊿) and toad venom group (*#) (P > 0.01); within 48h, Liushenpill group (⊿*) and toad venom group (*@) had no obvious difference of inhibitory effects (P > 0.01); whereas within 72h, inhibitory rateswere strongly differentiated between Liushenpill group (⊿@) and toad venom group (*$) (P < 0.01), indicating that with the passage of time, toad venom group demonstrated more obvious inhibitory effects on leukemia K562 cells, and the effects of toad venom and Liushenpills on K562 cells were time-dependent.

5.2 Effects on the expression of BCL-2 mRNA in K562/DOX cells treated with sera containing Liushen pills and toad venom

The results showed that compared with the control group, the groups treated with drug-containing sera displayed significant differences in the expression of BCL-2 mRNA. The expression levels of BCL-2 mRNA were significantly decreased in toad venom group and Liushen pill group, of which toad venom group showed more obvious decrease (P < 0.01), suggesting that toad venom with inhibitory effects on BCL-2 mRNA in K562/DOX cells may be the main functional component of Liushen pills (see Table 2, Fig. 1).

6. Discussion

The MDR of acute leukemia is the leading cause of chemotherapeutic failure. In recent years, the study of MDR has focused on the classical MDR pathways mediated by Permeability glycoprotein (P-gp), which hold that P-gp encoded by MDR1 can utilize the energy released by hydrolysis of ATP to pump hydrophobic lipophilic drugs out of cells, resulting in decreased intracellular drug concentration to be drug resistant. Therefore, many researchers are committed to the development of drugs that can inhibit P-gp activity so as to reverse MDR. It has been found that a variety of substances can effectively inhibit P-gpfunctioning as drug transporter in vitro, but the drugs show strong dose-dependence and toxicity in clinical trials. It is difficult for some drugs in vivoto achieve the plasma concentration required to reverse MDR so that their clinical application is constrained. Therefore, domestic and foreign scholars are actively studying drug-resistant reversal agents with high efficiency, low toxicity and extensive targets (Thomas *et al*. 2003, Bustamante *et al*. 2004).

In addition to P-gp-mediated classical MDR pathways, non-P-gp-mediated MDR pathways and pathways that prevent tumor cell apoptosis are closely related to clinical MDR of leukemia cells. Currently, there are three mechanisms concerning MDR: (1) overexpression of mRNA of P-gp-mediated MDR1; (2) overexpression of mRNA of non-P-gp-mediated MDR1 and BCL-2; (3) block oftumor cell apoptosis (Lei 2005).

Liushen pills, the traditional Chinese medicine, contain such six ingredients as realgar, toad venom, calculus bovis, moschus, borneolumsyntheticum and margarita, antipyretic, antidotal, antiphlogistic and analgesic, mainly used for sore throat, tonsillitis, scarlet fever, scarlatina, aphonia, ulcer, sore and furuncle, etc. In recent years, Liushenpills havereceived wide attention in the treatment of cancer, especially leukemia. TCM theories hold that leukemia arises from evil attackon nutrient blood, with evil struggling againstand consuming energy and blood, depletingbody fluid, and blocking internal organs and meridians to be the

crux of thermal potential. Evil is indeed the main problem, without the elimination of which, health cannot be ensured. Taking advantage of Liushen pills' antipyretic, antidotal, antiphlogistic and analgesic effects, Dai *et al*. (2003) achieved 90.48%. of total effective rate of treating chronic myeloid leukemia. Experimental studies showed that Liushen pills could significantly promote the apoptosis of leukemiaHL-60 cells, down-regulate the expression levels of such apoptosis-related genes as BCL-2 and C-myc mRNA and up-regulate the expression levels of Bax mRNA. At higher concentrations, Liushen pills can directly kill leukemia cellswhile at lower concentrations, they can activate body's immunity system, which is conducive to the treatment of leukemia. Based on these studies, it is feasible to use heat-clearing and detoxifyingtraditional Chinese medicine to reverse the multidrug resistance of leukemia. However, the main functional elements of Liushen pills have not been elucidatedyet, and the ways and targets to improve resistance have not been clear. The above-mentioned theoretical, clinical and research basis referred to as specific mechanism, in line with the Chinese philosophy "fight fire with fire", we intend to use toad venom as the subject in order to overcome MDR of leukemia and to study its role in reducing the expression of leukemia MDR genes, promoting apoptosis of leukemia drug-resistant cellsand enhancing the ability to kill leukemia drug-resistant cells.

The results showed that the inhibitory rate of K562/DOX cells did not change significantly after being treated with daunorubicin, whereas the inhibitory rates of were significantly increased after being treated with toad venom and Liushenpills. The results of RT-PCR indicated that the expression of BCL-2 in control group was significantly different from that of the other three groups treated with daunorubicin, toad venom and Liushen pills, and the difference was more significant in toad venom group than in Liushen pill group ($P < 0.01$), revealing that toad venom had inhibitory effects on BCL-2 mRNA in K562/DOX cells.

Based on the above experimental results, we believe that toad venom is an appropriate target to produce a marked anti-MDR effect on leukemia cells. The MDR reversal effect of Liushenpills may be achieved by toad venom's direct or indirect reduction of BCL-2 gene expression of K562/DOX cells, further increasing the concentration of intracellular chemotherapeutic drugs to promote the cell apoptosis. The main ingredient of Liushen pills may be toad venom, but their full mechanism remains to be further studied.

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