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ORIGINAL ARTICLE

GCS induces multidrug resistance by regulating apoptosis-related genes in K562/AO2 cell line

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Abstract We have previously shown that the expression of glucosylceramide synthase (GCS) gene in drug-resistant K562/AO2 human leukemia cell was higher than that in drug-sensitive K562 cell, and the sensitivity to adriamycin of K562/AO2 cell was enhanced by inhibiting *GCS*. It is concluded that the overexpression of GCS gene is one of the reasons which lead to multidrug resistance (MDR) of leukemia cell. Meanwhile, we also found that higher expression of Bcl-2 gene and protein were exhibited in K562/AO2 cell compared with K562 cell. Basing on this, we hypothesized that the high expression of GCS gene which results in MDR of leukemia cell is correlated with Bcl-2 signal transduction. In order to validate the hypothesis, the inhibition of GCS gene in K562/AO2 cell was observed by using chemical suppressor PPMP and siRNA targeted at *GCS*, and applying RT-PCR and flow cytometry, the expression levels of apoptosis-related gene *Bcl-2* and *Bax* were analyzed before and after inhibiting GCS gene in K562/AO2 cell. The results demonstrated that the gene and protein of Bcl-2 in K562/AO2 cell were both down-regulated significantly after GCS gene being inhibited; however, the *Bax* mRNA expression had no apparent change in different groups. This suggested that GCS gene may contributed to MDR of human leukemia cell K562/AO2 by Bcl-2 signal transduction.

Keywords Glucosylceramide synthase · Leukemia cell · Drug resistance · Bcl-2 · Bax · PPMP · siRNA

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Introduction

The generation of MDR in leukemia cell is essential reason which leads to recurrence and refractoriness in patients with leukemia [1, 2]. Chemotherapeutic agents play an important role in inducing apoptosis of tumor cell, and cell apoptosis has close relation to MDR of leukemia [3]. Ceramide, as a basic unit of the lipid sphingomyelin, is a second messenger in apoptotic signaling. It can increase the sensitivity of cell to chemotherapy by inducing apoptosis. Intracellular level of ceramide is elevated by a variety of stimuli and/or agents which include chemotherapeutic drugs, dactinomycin, tumor necrosis factor, Fas ligand engagement of CD95, ionizing radiation, ultraviolet radiation and heat shock. MDR may be enhanced or weaken by regulating level of ceramide in cell [4]. GCS locates in membrane of eukaryote widely and Golgi body. Ceramide combines with glucosyl of UDP-2-glucose under catalysis of GCS [5], and glucose ceramide (GlcCer) is generated with glycosylation of ceramide, then cell apoptosis is induced as ceramide is antagonised by GlcCer. Some researches [6–8] showed that the expression of *GCS* up-regulated in a variety of drug-resistant cells. For example, the level of *GCS* in HL-60/ADR cell of leukemia was higher than that in HL-60 cell; compared with MCF-7 cell of breast cancer, the expression of GCS in MCF-7/ADR cell was higher. Our previous

study also indicated that higher expression of *GCS* was exhibited in K562/AO2 cell compared with K562 cell [9].

The expression of Bcl-2 gene family which is closely correlative to apoptosis is a critical determinant of cell survival or death [10]. Bcl-2 gene, as pro-apoptotic gene, may inhibit cell apoptosis induced by different kinds of drugs by playing a role in regulating the common downstream of cell apoptosis pathway. *Bax*, the antagonistic gene of Bcl-2, can combine with *Bcl-2* to form dimer [11]; they both determine the regulation of cell apoptosis; so *Bcl-2/Bax* ratio can be an index of regulation of cell apoptosis. We also found that expressions of Bcl-2 gene and protein were higher in K562/AO2 cell compared with K562 cell. In order to explore the molecular mechanism of MDR induced by GCS, the study observed the change of apoptosis-related gene and protein of K562/AO2 cell after GCS gene of cell being inhibited by PPMP and GCSsiRNA.

Materials and methods

Cell lines and culture conditions

The drug-sensitive K562 and drug-resistant K562/AO2 human leukemia cell lines were kindly provided by Haematology Institute of Chinese Academy of Medical Sciences in Tianjin. The cells were maintained in RPMI 1640 medium (Sigma Co. Ltd., USA) containing 10% (v/v) FBS (Sigma Co. Ltd., USA), and 1 μg/ml adriamycin (Shanghai Hua Lian Co. Ltd., China) was added in maintaining components for K562/AO2 Cells. K562 and K562/AO2 cells were cultured in a humidified, 5% CO_2 atmosphere tissue culture incubator and subcultured a time every 3 days.

Inhibiting the GCS gene of K562/AO2 cells by chemical reagent

Treating K562/AO2 cells with chemical inhibitor

DL-Threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP), which is a chemical inhibitor for GCS, was from Sigma Co. (USA). In order to determine the relationship between GCS and apoptosis-related genes in the generation of MDR of leukemia, cells were divided into three groups. The first two groups were K562 and K562/AO2 cells which would be incubated in RPMI 1640 medium without chemical agent; however, K562/AO2 cells of the third group were incubated with PPMP (20 μM). 48 h later, the target genes mRNA and proteins of various cells were tested as below.

RNA extraction and RT-PCR mRNA analysis

Total RNA of the cells from three groups were isolated using TRIzol reagent (Invitrogen GIBCO Co.). Random primers (Shanghai Sangon Co., China) was used for reverse transcript reaction at 37°C for 60 min. PCR primers sequences for *GCS*, *Bcl-2*, *Bax* and *β*-actin are as follows: *GCS* upstream 5′-CTGGAAACATTCTTTGAATTGGAT-3′ and downstream 5′-CTCATTAAACAAGACATTCCTG TC-3′; *Bcl-2* upstream 5′-GGATTGTGGCCTTCTTTGAG-3′ and downstream 5′-CCAAACTGAGCAGAGTCTTC-3′; *Bax* upstream 5′-TGCTTCAGGGTTTCATCCAGG-3′ and 5′-TGGCAAAGTAGAAAAGGGCGA-3′; *β*-actin upstream 5′-GTGGGGCGCCCCAGGCACCA-3′ and downstream 5′-CTCCTTAATGTCACGCACGATTTC-3′. DNA was amplified in a 30-cycle PCR reaction, using the following conditions: denaturation at 94°C for 60 s, annealing at 58°C for 45 s, and elongation at 72°C for 45 s. RT-PCR products were analyzed by 2% agarose gel electrophoresis stained with ethidium bromide. The fragment size of RT-PCR products was 421 bp for GCS, 233 bp for Bcl-2, 276 bp for Bax and 540 bp for *β*-actin that was used as control. The intensity of the gel spots (OD value) was determined by Auto-ultraviolet/visible Light Gel Analysis System (FR-200A, Shanghai, China).

Assay of Bcl-2 protein by flow cytometry

After pipetting 100 μl adjusted cells (equivalent to 1×10^6 cells) into each tube, add 100 μl of fixation medium (MultiSciences Biotech Co., Ltd) and incubate for 15 min at room temperature. Wash once in 3 ml PBS. Later, add 100 μl permeabilization medium (MultiSciences Biotech Co., Ltd) and the recommended volume of the FITC-conjugated intracellular Bcl-2 antibody or the corresponding isotype control Mouse IgG1 (Invitrogen GIBCO Co). Then, vortex for 1–2 s and incubate for 20 min. Soon, wash once in 3 ml PBS. The last step was to resuspend cells in sheath fluid for immediate FCM analysis.

Inhibiting the GCS gene of K562/AO2 cells by RNA interference technology

The observation of transfection efficiency

One day before transfection, plate cells in growth medium. For transfection sample, prepare oligomer-Lipofectamine 2000™ complexes as follows: First, dilute fluorescently labeled siRNA oligomer (Shanghai GenePharma Co., Ltd.) in 1640 Medium without serum. Mix gently. Second, mix Lipofectamine2000 (Invitrogen GIBCO Co.) gently before use, then dilute appropriate volume in 1640 Medium. Mix

gently and incubate for 5 min at room temperature. After the 5-min incubation, combine the diluted oligomer with the diluted Lipofectamine 2000. Mix gently and incubate for 20 min at room temperature. Finally, add the oligomer-Lipofectamine2000 complexes to well containing cells and medium. 6, 12, 24 and 48 h later, transfection efficiency was observed, respectively, by fluorescence inverted microscope.

Treating K562/AO2 cells with siRNA

RNA interference technology is a powerful in gene knockout; by using this technology, we explored the mechanisms of GCS inducing MDR of leukemia again. The culture cells were divided into six groups, e.g. K562 cells, K562/AO2 cells, K562/AO2 cells with GCSsiRNA, K562/AO2 cells with GAPDHsiRNA (positive control), K562/AO2 cells with siRNA (negative control) and K562/AO2 cells with Lipofectamine2000. The groups, which need to be transfected, were treated according to procedure "The observation of transfection efficiency". The cells of other groups were incubated in 1640 medium. 48 h later, the following tests were performed.

RNA extraction and real-time PCR analysis

Total RNA of K562/AO2 cells with GCSsiRNA, K562/AO2 cells with GAPDHsiRNA, K562/AO2 cells with siRNA (negative control) and K562/AO2 cells with Lipofectamine2000™ (Mock group) were isolated using TRIzol reagent. Reverse transcript reaction was performed at 37°C for 60 min. Real-time PCR primers sequences for *GCS* as follows: *GCS* upstream 5′-TGCTCAGTACATTGCCGAAGA-3′, downstream 5′-TGGACATTGCAAACCTCCAA-3′, and TaqMan probe sequence for *GCS*: 5′-TTATGGCCAAAGCGATAGCTGACCGAG-3′. DNA was amplified in a 35-cycle PCR reaction, using the following conditions: denaturation at 94°C for 5 s, annealing and elongation at 60°C for 30 s. Real-time PCR products were analysed by LightCycler PCR Instrument (Roche Co.).

RT-PCR mRNA analysis

The RT-PCR mRNA detection of the cells in various groups was as same as "RNA extraction and RT-PCR mRNA analysis"

Assay of Bcl-2 protein by flow cytometry

The expressions of Bcl-2 protein in K562, K562/AO2 and K562/AO2 with GCSsiRNA cells were measured, respectively, by the procedure "Assay of Bcl-2 protein by flow cytometry"

Table 1 Influence of PPMP on expression of GCS mRNA in K562/A02 cells

PPMP (μmol/L)	OD_{GCS}	$OD_{GCS}/OD_{\beta\text{-actin}}$	Inhibited ratio (%)*
0	79.9 ± 4. 12	0.98 ± 0.19	1.00
2.5	76.6 ± 4.89	0.92 ± 0.22	0.94
5	63.2 ± 5.86	0.85 ± 0.32	0.86
10	38.0 ± 3.21	0.51 ± 0.26	0.52
25	36.4 ± 5.32	0.49 ± 0.34	0.50

K562/AO2 cells was incubated with gradually increasing concentrations of PPMP (0, 2.5, 5, 10 and 25 μM) for 48 h. RNA was extracted, and GCS mRNA was detected by RT-PCR

* The expression of *GCS* in K562/AO2 cells incubated with 10 or 25 μM PPMP was inhibited obviously, compared with 0 μM PPMP ($n = 2$)

Results

Results of inhibiting the GCS mRNA in drug-resistant K562/AO2 cells by chemical reagent PPMP

Influence of PPMP on expression of GCS mRNA in K562/A02 cells

In order to observe the effect of PPMP on GCS mRNA in K562/A02 cells, we treated cells with different concentrations of PPMP. As shown in Table 1, when K562/A02 cells were treated in 2.5 or 5 μM PPMP, the expression of GCS gene ($OD_{GCS}/OD_{\beta\text{-actin}}$) had no apparent change. However, GCS mRNA expression ($OD_{GCS}/OD_{\beta\text{-actin}}$) in K562/A02 cells with 10 and 25 μM PPMP after 48 h was reduced to 52 and 50%, respectively, compared with untreated K562/A02 cells.

Expression of GCS, Bcl-2 and Bax genes in K562 cells and K562/AO2 cells without or with PPMP

To explore the role of GCS inducing MDR of leukemia cells through Bcl-2 family-mediated inhibiting apoptosis pathway, GCS, Bcl-2 and Bax mRNA in human leukemia K562 cells and K562/AO2 cells, which treated or untreated with PPMP, were examined by RT-PCR (shown in Fig. 1). First, *GCS* was assessed in the three groups. The level of GCS mRNA ($OD_{GCS}/OD_{\beta\text{-actin}}$) exhibited higher expression in K562/AO2 cells compared with K562 cells ($P < 0.05$). GCS mRNA expression in K562/AO2 cells treated with 20 μM PPMP after 48 h was reduced significantly, compared with untreated K562/AO2 cells ($P < 0.05$). We also examined the level of Bcl-2 and Bax genes, both of which are involved in cellular apoptosis. The results showed that the expression of Bcl-2 mRNA ($OD_{Bcl\text{-}2}/OD_{\beta\text{-actin}}$) in K562/AO2 cells is higher than that in K562 cells ($P < 0.05$). After

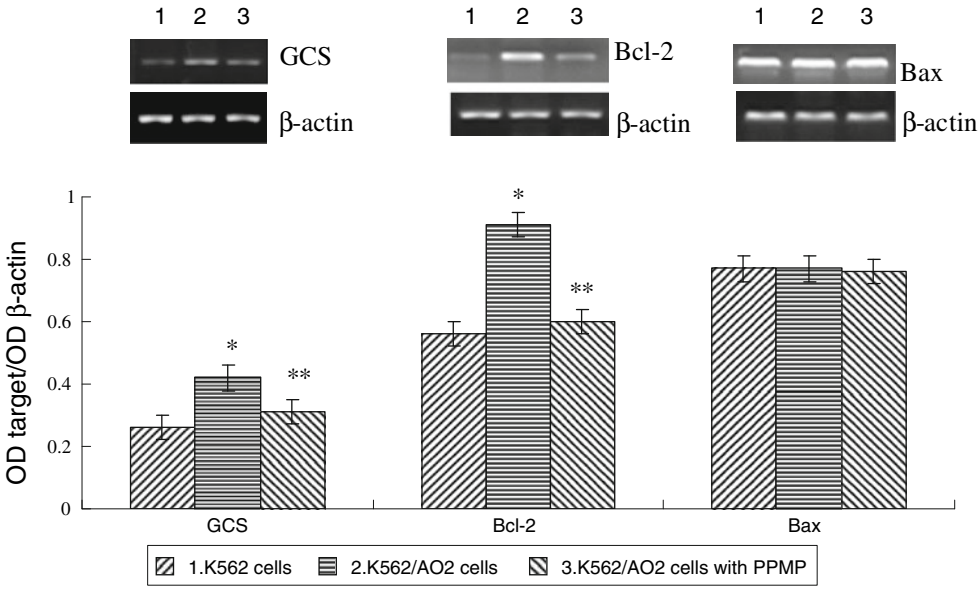

Fig. 1 Expression of GCS, Bcl-2 and Bax genes in three groups. Isolated mRNA was amplified by RT-PCR. The reverse PCR products, 421 bp for *GCS*, 233 bp for *Bcl-2*, 276 bp for *Bax* and 540 bp for *β*-actin, were resolved on 2% agarose gel electrophoresis stained with ethidium bromide. Housekeeper gene *β*-actin was used as a control for even loading. The intensity of the gel spots (OD value) was also determined ($n = 4$). *$P < 0.05$ compared with K562; **$P < 0.05$ compared with K562/AO2

being treated with PPMP, Bcl-2 mRNA in K562/AO2 cells decreased markedly ($P < 0.05$), whereas the level of Bax mRNA ($OD_{Bax}/OD_{\beta\text{-actin}}$) in the three groups has no apparent change ($P > 0.05$).

The expression level of Bcl-2 protein in K562 cells and K562/AO2 cells without or with PPMP

With the purpose of indicating the level of Bcl-2 protein in different cells, we applied FCM. As shown in Fig. 2, the expression of Bcl-2 protein in drug-resistant K562/AO2 cells was higher than that in drug-sensitive K562 cells ($P < 0.05$), but Bcl-2 protein of K562/AO2 cells treated with PPMP decreased significantly ($P < 0.05$). The result indicated that the expression of Bcl-2 protein in K562/AO2 cells was down-regulated after *GCS* being inhibited by PPMP.

Results of inhibiting the GCS mRNA of drug-resistant K562/AO2 cells by RNA interference technology

Observation of siRNA transfection efficiency

Treated cells with fluorescently labeled siRNA oligomer, 6, 12, 24 and 48 h later, transfection efficiency was observed, respectively (shown in Fig. 3), by fluorescence inverted microscope (Olympus IX71, Japan). The result indicated the transfection efficiency was highest after 48-h transfection, which was 65%.

Detection of GCS mRNA inhibition by real-time PCR

By using the method of real-time quantitative PCR, the level of gene knockdown by siRNA was measured. As shown in Fig. 4, compared with negative control and Mock group, the expression of GCS gene in K562/AO2 cells which were transfected GCSsiRNA was silenced significantly. The inhibition ratio was 73.38%.

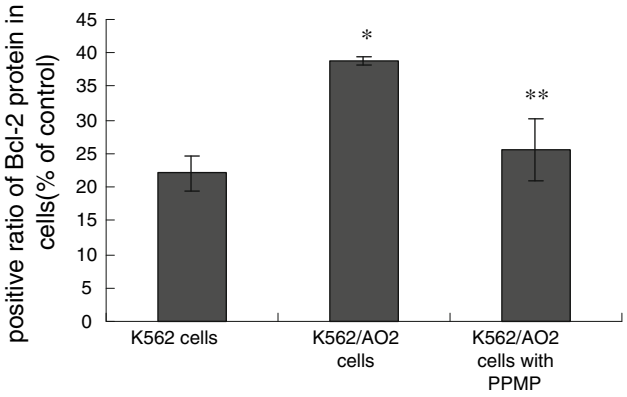

Fig. 2 Expression of Bcl-2 protein in drug-sensitive K562, drug-resistant K562/AO2 cells and drug-resistant K562/AO2 cells treated with PPMP (20 μM, 48 h). Expression levels of Bcl-2 protein in three groups were detected by FCM ($n = 3$). *$P < 0.05$ compared with K562 cells; **$P < 0.05$ compared with K562/AO2 cells

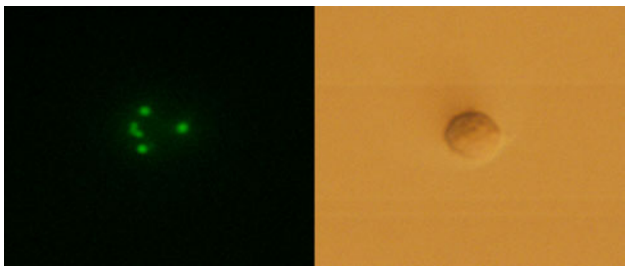

Fig. 3 Display of siRNA transfection in fluorescence inverted microscope. **a** Green fluorescence was seen in cell which was transfected successfully. **b** The same cell was shown in achromatic light

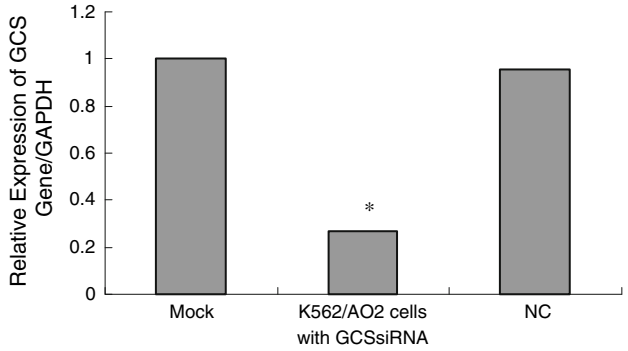

Fig. 4 The inhibition effect of siRNA on GCS mRNA in K562/AO2 cell. Using real-time quantitative PCR, target gene *GCS* and housekeeper gene *GAPDH* were detected in the cells of different groups. The *graph* indicated relative expression of GCS/GAPDH gene in K562/AO2 cells after being transfected GCSsiRNA, siRNA (negative control) and only Lipofectamine2000 (Mock group), respectively. Data are presented as means ($n = 3$). *$P < 0.05$ versus Mock group

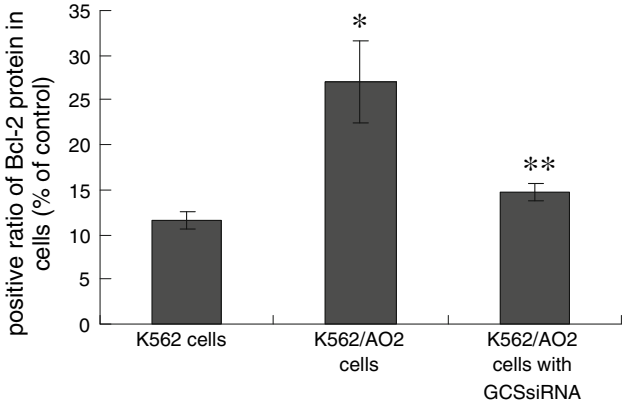

Fig. 6 Expression of Bcl-2 protein in K562 and K562/AO2 cells untreated or treated with GCSsiRNA. Leukemia cells were incubated, and transfected or untransfected GCSsiRNA. Measurements of Bcl-2 protein were made by FCM ($n = 3$). *$P < 0.05$ compared with K562 cells; **$P < 0.05$ compared with K562/AO2 cells

Expression of GCS, Bcl-2 and Bax genes in K562 and K562/AO2 cells untransfected or transfected GCSsiRNA

For revealing the role of GCS inhibiting apoptosis through Bcl-2 gene family-mediated pathway once again, GCS, Bcl-2 and Bax genes in human leukemia K562 cells and K562/AO2 cells untransfected or transfected GCSsiRNA were investigated by RT-PCR. As shown in Fig. 5, the level of GCS and Bcl-2 mRNA exhibited higher expression in K562/AO2 cells compared with K562 cells ($P < 0.05$); GCS mRNA expression in K562/AO2 cells treated with GCSsiRNA after 48 h was silenced significantly, compared with untreated K562/AO2 cells ($P < 0.05$). Interestingly, Bcl-2 antiapoptotic gene in K562/AO2 cells decreased markedly after being treated with GCSsiRNA ($P < 0.05$), whereas the expression level of proapoptotic gene *Bax* in cells of the three groups has no apparent change ($P > 0.05$).

The expression level of Bcl-2 protein in K562 and K562/AO2 cells untreated or treated with GCSsiRNA

In order to indicate the level of Bcl-2 protein in different cells, FCM technique was applied. As shown in Fig. 6, the expression of Bcl-2 protein in K562/AO2 cells was higher than that in K562 cells ($P < 0.05$). It is also interesting that Bcl-2 protein of K562/AO2 cells treated with GCSsiRNA decreased significantly ($P < 0.05$).

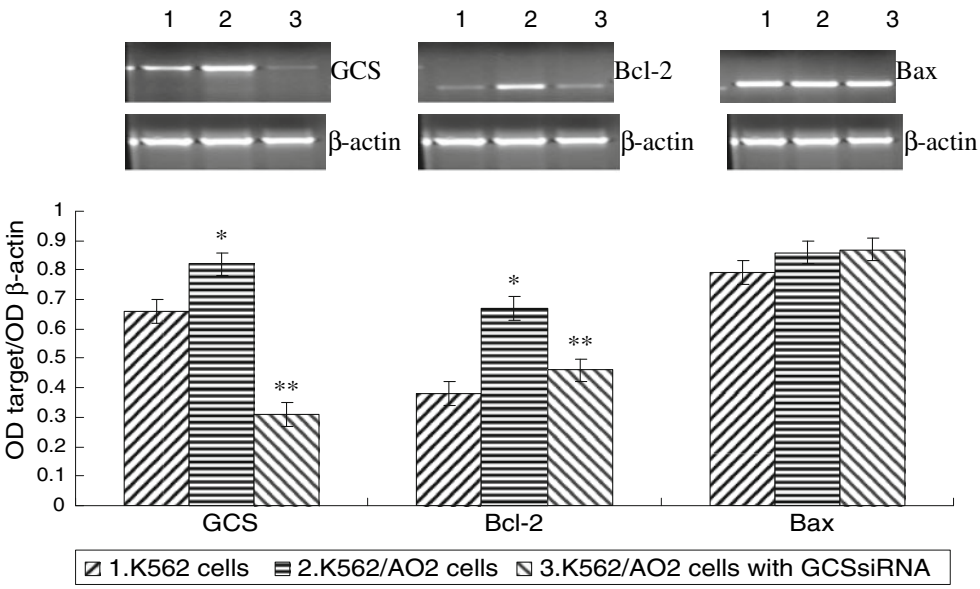

Fig. 5 Expression of GCS, Bcl-2 and Bax genes in K562, K562/AO2 cells and K562/AO2 cells transfected GCSsiRNA. RNA in three groups was extracted; isolated mRNA was amplified by RT-PCR. The reverse PCR products, 421 bp for *GCS*, 233 bp for *Bcl-2*, 276 bp for *Bax* and 540 bp for *β*-actin, were resolved on 2% agarose gel electrophoresis stained with ethidium bromide. Housekeeper gene *β*-actin was used as a control for even loading. The intensity of the gel spots (OD value) was also determined. *$P < 0.05$ compared with K562 cells, **$P < 0.05$ compared with K562/AO2 cells ($n = 5$)

Discussion

Ceramide, a second messenger in apoptotic signaling, plays a principal role in the nature of cellular response to anticancer therapies [12, 13]. GCS is the transmembrane protein with C-terminal catalytic structure; as an enzyme, it can transform ceramide into glucosylceramide [14]. Accumulation of glucosylceramide is a characteristic of some MDR cancer cells of breast, ovarian, colon and epithelioid carcinomas [15–17]. Modification of ceramide metabolism by blocking the glycosylation pathway has been shown to increase cancer cell sensitivity to cytotoxics [18–20]. In adriamycin-resistant variant, the ceramide was converted into non-toxic form (ceramide glycosylation) by GCS; the sensitivity of cell to adriamycin can restore by inhibiting GCS [21]. The toxicity of drugs such as (*N*-(4-hydroxyphenyl)retinamide) on neuroblastoma, lung cancer and breast cancer can be enhanced by chemical inhibitor PPMP [22]. Our previous study showed that the K562/AO2 cell line was 115-fold more resistant to adriamycin and 36-fold more resistant to vincristine compared with K562 cell line. The expression of *GCS* was higher in K562/AO2 cell. MDR of K562/AO2 cell was inverted significantly after cell being treated with PPMP or GCSsiRNA. PPMP and GCSsiRNA enhanced adriamycin sensitivity by 4.4-fold [9] and 5.6-fold [23], respectively. From this, we concluded that GCS is a reason which leads to the MDR of leukemia cell. The present study investigates the mechanism that how MDR of leukemia cell is induced by GCS.

Bcl-2 gene protein family is over-expressed in tumor tissues; it has close relation to generation, development and MDR of tumor [24]. Bcl-2 gene protein family plays a key role in controlling mitochondrial function associated with cell-death pathway. Bcl-2 protein localizes in the membrane of cell; it plays anti-apoptotic role by preventing release of cytochrome *c*. Bax, as pro-apoptotic protein, has effect on inducing apoptosis of cell. In recent years, more and more evidence demonstrated that Bcl-2/Bax ratios determined cell apoptosis or cell survival [25–27].

The mechanism that ceramide regulates cell apoptosis may have relation to transcription activation of JNK pathway, which means that ceramide regulates process of cell apoptosis by activating downstream c-JUN N-terminal kinase [28]. One way that JNK mediates cell apoptosis is to phosphorylate Bcl-2 and Bcl-xl; phosphorylation of Bcl-2 and Bcl-xl leads to cell apoptosis by promoting release of cytochrome *c* in mitochondrion and activating Cascade reaction of caspase [29]. Bhalla et al. [30] showed that the expression of Bcl-2 was higher in drug resistant cell lines HL-60/VCR than that in drug sensitive cell lines HL-60. When cells were treated with ceramide, cell apoptosis was induced and level of Bcl-2 mRNA in cell was decreased [31]. Another study demonstrated the relationship between Bcl-2/Bax ratio and MDR in tumor cell. Yue et al. [32] detected Bcl-2/Bax ratio of 40 cases with AML; the results indicated that the drug resistance of the group with higher Bcl-2/Bax ratio was higher. Pepper et al. [33] found that Bcl-2/Bax ratio had significant effect on chemotherapy sensitivity of chronic lymphocytic leukemia; patients were resistant to chemotherapy drugs when Bcl-2/Bax ratio was heightened; however, accompanied by decline of Bcl-2/Bax ratio, chemosensitivity of CML was recovered.

Our study showed that the expressions of GCS and Bcl-2 in K562/AO2 cells were higher than that in K562 cells significantly. PPMP can obviously inhibit the expressions of GCS mRNA in K562/AO2 cells, whereafter the genes and proteins of Bcl-2 in K562/AO2 cells were also both down-regulated (Figs. 1, 2). The GCSsiRNA can silence the expressions of *GCS* in K562/AO2 cells significantly, and the genes and proteins levels of Bcl-2 in K562/AO2 cells were decreased after GCS gene knockdown (Figs. 5, 6). The Bax mRNA expressions had no apparent change in different groups. It is concluded that MDR gene GCS has positive correlation to Bcl-2 gene and Bcl-2/Bax ratio, but it has no relation to Bax gene. GCS prevents tumor cell apoptosis by glycosylating ceramide to reduce accumulation of ceramide in cell and up-regulate Bcl-2 gene. So GCS gene may lead to MDR of human leukemia cells by Bcl-2 signal transduction. We may develop a chemical drug targeting down-regulating GCS to reverse or partially reverse MDR of leukemia cell, which may open up a new way to cure leukemia.

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