Suppression of glucosylceramide synthase by RNA interference reverses multidrug resistance in human breast cancer cells*

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Glucosylceramide synthase (GCS), the enzyme that converts ceramide to glucosylceramide, induce multidrug resistance (MDR) in cancer cells. Recently, RNA interference (RNAi) is a powerful strategy for gene therapy by introducing double-stranded RNA and leading to the sequence-specific destruction. We have designed two different short hairpin RNAs (shRNAs) targeting GCS and introduced them into adriamycin-resistant human breast cancer cells (MCF-7/AdrR cells) to inhibit GCS expression. The results demonstrated that the shRNAs targeting GCS decreased GCS mRNA, abolished GCS protein levels and restored the sensitivity of MCF-7/AdrR cells to several antineoplastic drugs. This study revealed that this approach can reverse MDR effectively and it may be applicable to cancer patients as a specific means to restore the sensitivity to chemotherapy.

Key words: glucosylceramide synthase, multidrug resistance, RNA interference, breast cancer

Multidrug resistance (MDR) is characterized by a cross-resistant phenotype against several unrelated drugs that differ widely with respect to molecular structure and target specificity [1, 2]. It is the major cause of the failure of chemotherapy-based treatment modalities of malignant tumors [3].

Glucosylceramide synthase (GCS), the enzyme that converts ceramide to glucosylceramide, induces multidrug resistance (MDR) in cancer cells [4–6]. Ceramide, a second messenger in cellular apoptotic signaling, has shown to participate in reactions to chemotherapy and radiotherapy of cancer [7, 8]. Loss of ceramide production is one reason of cellular resistance to apoptosis induced by tumor necrosis factor α, adriamycin or ionizing radiation [9–11]. The levels of glucosylceramide (GlcCer), a simple glycosylated form of ceramide, are elevated in some multidrug-resistant cancer cells and tumor specimens from patients who demonstrate poor response to chemotherapy [12, 13]. Decreasing the potential for ceramide glycosylation has been shown to overcome resistance to several classes of anticancer drugs [14, 15]. Pharmocologically active compounds could limit GCS activity and enhance ceramide generation, such as PPPP (1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol), PDMP (1-phenyl-2-dsdecanoylamino-3-morpholino-1-propanol) and cyclosporin A [10, 16, 17]. They increased cancer cell sensitivity to antineoplastic agents [18]. In clinical trials, their commonly occurring inherent adverse pharmacokinetic side effects became an obstacle for clinical application of these MDR modulators [19]. It is necessary to develop an alternative strategy which would be more efficient and less toxic, and the approach of antisense oligonucleotides modulating GCS activity or hammered ribozymes has shown effection on reversal of multidrug resistance [20, 21].

RNA interference (RNAi) is a novel and powerful tool for the analysis of gene function in many organisms by introducing double-stranded RNA into cells and leading to the sequence-specific destruction [22]. RNAi can be induced by double stranded RNA (dsRNA), which is cleaved into 21-to 23-bp RNA fragments, known as small interfering RNA (siRNAs), by an RNase III-like enzyme, Dicer [23]. siRNAs are then incorporated into a protein complex called RNA-induced silencing complex (RISC), then recognizes and cleaves mRNA in a sequence-specific manner [24]. On the

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other hand, short hairpin RNAs (shRNAs), which resemble siRNA in their struction, are also known to trigger RNAi [25, 26]. Compared with siRNA, shRNAs have been shown to have a more sustained expression than synthetic siRNA. To explore this promising approach in order to reverse drug resistance, we applied shRNAs to examine the role of GCS in multidrug resistance by using adriamycin-resistant human breast cancer cells.

Material and methods

Cell lines and cell culture. The human breast adenocarcinoma cell line, MCF-7/AdrR, which is resistant to adriamycin, was kindly provided by National Cancer Institute in the United States. MCF-7/AdrR cells and MCF-7 cells were maintained in RPMI1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum (FBS). Cells were cultured in a humidified, 5% CO_2 atmosphere tissue culture incubator and subcultured using trypsin-EDTA solution.

Design of shRNA. Because not all siRNA target sequences are potent, we selected two different shRNAs according to recommendations (http://www.oligoengine.com and http://sirna.qiagen.com). BLAST search agaist EST libraries was performed to ensure that no other human gene was targeted. DNA templates encoding DNA were designed as follows: 19 nucleotide target sequence as sense strand followed by antisense orientations separated by a 6 nucleotide spacer sequence and flanked at either end by Bgl II and HindIII restriction enzyme sites and the five repeats of T as termination signal. The annealed oligonucleotides were cloned into pSUPER to generate psGCS1 and psGCS2 respectively. They were verified by DNA sequencing.

Transfection with shRNA expression vectors. Twelve hours before transfection, cells were seeded onto a 6-well plate with antibiotics-free growth medium, at a density of $5x10^5$ cells/well, so that MCF-7/AdrR cells reached 90% confluence before starting transfection. GCS sense and antisense oligodeoxynucleotides have the same sequence as shRNA has; they were introduced into cells using LipofectamineTM 2000 (Invitrogen). 250 µl of RPMI1640 medium without serum and 4 µg sense or antisense oligodeoxynucleotide per well were pre-incubated for 5 minutes at room temperature. During this pre-incubation period, 250 µl of RPMI1640 medium without serum was mixed with 10 µl LipofectamineTM2000. The two media were mixed and incubated for 20 minutes at room temperature for complex formation and the cells were then transfected. Similarly, transfection of psGCS1 or psGCS2 was carried out using LipofectamineTM 2000. Moreover, 72 h after transfection, the cells were selected with 0.5 µg/ml puromycin (Sigma).

Western blot analysis. MCF-7/AdrR cells were lysed in a buffer consisting of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.2% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 0.1% NP-40, and 0.5% sodium deoxycholate. Proteins in the lysates were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically blotted onto a polyvinylidene difluoride filter (Bio-Rad). To avoid unspecific binding, the filters were incubated in 5% skim milk, 0.05% Tween 20 in TBS for 2 h at room temperature. Subsequently, filters were incubated with rabbit moloclonal antibody against human GCS diluted in 5% skim milk in 1xTBST (1:1000) overnight at 4 °C and, afterwards, with peroxidase-conjugated goat anti-rabbit IgG diluted at 1:4000. As control for equivalent protein loading, the filters were simultaneously incubated with a goat mAb directed against actin (1:4000). Protein bands were visualized by an enhanced chemiluminescence method (ECL; Amershan-Pharmacia Biotech) according to the manufacturer's instruction. The intensity of each band was quantified using an image processing and analysis program.

Reverse transcription (RT) and polymerase chain reaction (PCR). Total cellular RNA was obtained from MCF-7/AdrR cells and MCF-7 cells by using RNA isolation kit. The RNA samples were reverse transcribed using avian myeloblastosis virus reverse transcriptase (TaKaRa) and a random primer, and the resultant cDNAs were subsequently amplified with Taq DNA polymerase (TaKaRa) by 30 PCR cycles, each consisting of 1 min at 95 °C, 45 sec at 55 °C, 1.5 min at 72 °C. A set of primers, GCS (sense: 5'-CCT TTC CTC TCC CCA CCT TCC TCT-3', antisense: 5'-GGT TTC AGA AGA GAG ACA CCT GGG -3') were used to amplify a portion of GCS gene. Another set of primers, β-actin (sense: 5'-ACC CCC ACT GAA AAA GAT GA-3', antisense: 5'-ATC TTC AAA CCT CCA TGA TG-3') were used for amplification of β-actin mRNA. The PCR products were electrophoresed in a 2% agarose gel containing ethidium bromide and visualized by UV illumination.

Cytotoxicity assay for cell survival. MTT assay was adopted to assess the silencing effect of GCS on chemosensitivity of MCF-7/AdrR cells to anticancer drugs. Cells were plated in a 96-well plate at a density of $5x10^3$ cells/well, 24 h later, the cells were incubated with different concentration of the anticancer drug for another 48 h, and then cells were stained with 15 µl sterile MTT dye (5 mg/ml, Sigma, USA) for 4 h at 37 °C, subsequently, culture medium was removed and 150 µl of dimethyl sulfoxide (DMSO) was added and thoroughly mixed for 10 min. Spectrometric absorbance at wavelength of 570 nm was messured against a background at 630 nm on a microplate reader (Bio-Tek, USA). The value of [A570 (anticancer drug+)/A570 (anticancer drug–)] x 100% indicated the cell survival index. A dose-response curve was plotted and IC_{50} values were calculated from multiple (at least three) independent experiments. Cytotoxicity analyses were performed 48 h after psGCS treatment of MCF-7/AdrR cells.

Flow cytometry. Cell apoptosis profiles were analyzed by flow cytometry. $1x10^5$ cells were collected and washed with phosphate-buffered saline (PBS), fixed in ice-cold ethanol and stored at 4 °C. After resuspension in PBS containing 10 µg/ml RNase A and 20 µg/ml propidium iodide (PI) for

30 min, samples were analyzed by flow cytometry (Becton-Dickinson,USA). The apoptotic cells were expressed as a subdiploid peak.

Statistical analysis. Experiments were repeated three times. The statistical analyses were performed using SPSS10.0 for Windows. Student's t-test was used to determine the statistical significance of the data obtained. $P<0.05$ was considered as significant difference.

Results

Construction and identification of shRNA expressing vectors. The two shRNAs selected, which target the GCS mRNA at bases 1069–1087 and 497–515, were cloned into the expression vector pSUPER (Fig. 1A). The predicted forms and sequences of these shRNAs are shown in (Fig. 1B). No other human gene was targeted after BLAST search against EST libraries was performed. Because the Bgl II restriction enzyme sites were lost in recombinant plasmids, and thus they could not be digested by Bgl II and could still be digested by Hind III. Therefore, we used double enzyme digestion analysis to identify if the plasmids were successfully constructed. In addition, DNA sequencing confirmed that all vectors were correct.

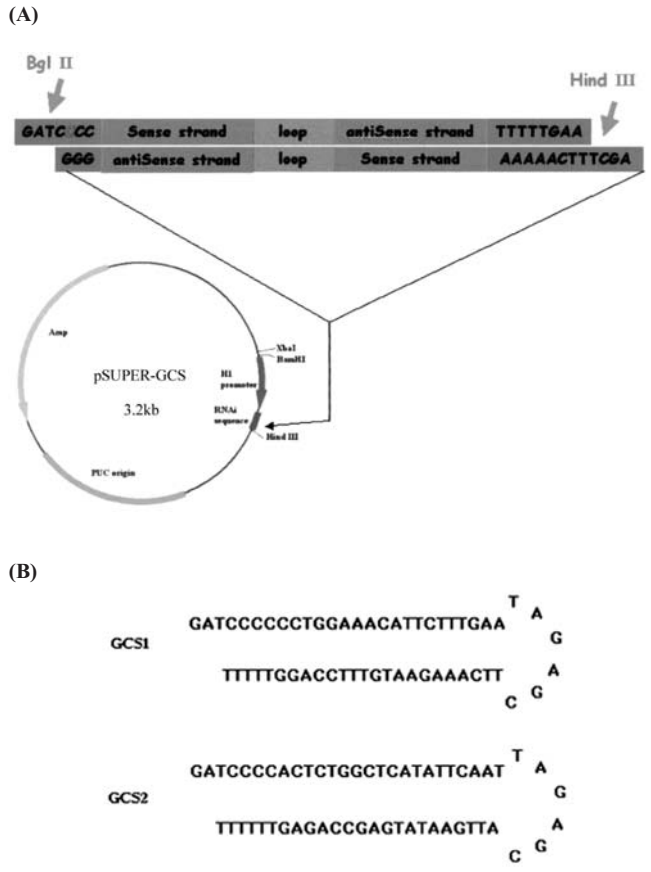

Figure 1. (A) Schematic diagram of pSUPER-GCS. (B) Sequences of small hairpin RNAs (shRNAs).

Decrease of GCS mRNA expression using shRNA expression vectors. The pSUPER system we used in this study was able to sturdily suppress the expression of GCS gene. The cells transfected with psGCS1 or psGCS2 were selected with 0.5 μg/ml puromycin and were cultured under such conditions for about 1 month. Then we measured the amount of GCS mRNA in the cells by semi-quantitative RT-PCR. As shown in Figure 2A and 2B, in adriamycin-resistant MCF-7/AdrR cells, GCS gene was clearly detectable after 30 cycles of PCR amplication. On the other hand, in cells transfected with plasmids expressing shRNAs targeting GCS, only a faint band was detectable after 30 cycles of amplification. As shown in Figure 2C, multidrug-resistant MCF-7/AdrR cells exhibited a 129-fold overexpression of GCS mRNA in comparison to the drug-sensitive MCF-7 cells. Treatment with psGCS1 and psGCS2 reduced GCS mRNA level to 14% and 12% of the initial GCS mRNA expression value respectively, i.e. 86% and 88% gene-inhibiting activity in breast cancer cells. In contrast, the single-stranded GCS antisense oligodeoxynucleotides (ASODNs) demonstrated less GCS mRNA expression-inhibiting activity which decreased to 52% and 46%, respectively. In other words, 48% and 54% gene-silencing activity could be showed in MCF-7/AdrR cells (Fig. 2A, B). GCS mRNA expression in the cells transfected with the controls had no change compared to the MCF-7/AdrR cells.

Knockdown of GCS on protein level by shRNA expression plasmids. We used pSUPER vector to express shRNAs. The shRNAs expression plasmids and a control vector were transfected into MCF-7/AdrR cells and GCS protein expression was monitored by western blotting. psGCS1 and psGCS2 suppressed GCS protein expression strongly, however the control vector did not inhibit GCS protein expression. (Fig. 3A, B). As shown in Figure 3C, the multidrug-resistant breast cancer cell line MCF-7/AdrR revealed a 88-fold GCS protein expression, whereas the non-resistant counterpart MCF-7 showed only a 7-fold GCS protein value. The shRNAs expression plasmids decreased the GCS protein level to 20% and 18% respectively, in other words, the GCS protein expression inhibition rates were 80% and 82%, respectively. As demonstrated at the mRNA level, single-stranded GCS ASODNs also showed less inhibition rates of GCS protein, which were 43% and 47%, respectively (Fig. 3C). In summary, shRNA-mediated silencing was much more pronounced than ASODNs, and no effect could be observed by any other of the controls or GCS sense.

Reversal of the drug-resistant phenotype by shRNA expression vectors. The reversal of the drug-resistant phenotype was assessed by comparison of the IC_{50} values determined by a cell proliferation assay and performed on cells

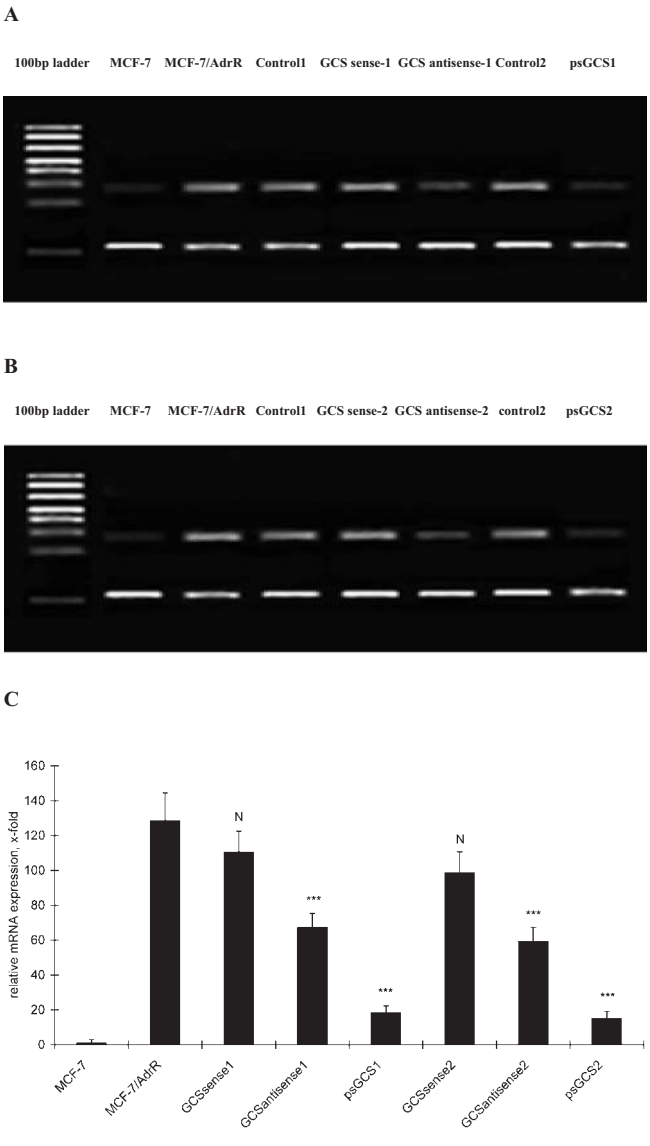

Figure 2. Analysis of shRNA-mediated silencing of GCS mRNA expression in MCF-7/AdrR cells. A: Semi-quantitative RT-PCR analysis demonstrating psGCS1-mediated decrease of GCS mRNA expression in MCF-7/AdrR cells. B: Semi-quantitative RT-PCR following psGCS2 treatment in MCF-7/AdrR cells. RT-PCR for β-actin was performed in parallel to show an equal amount of total RNA in sample. C: Relative GCS mRNA expression level normalized against β-actin expression in MCF-7/AdrR cells. The GCS/β-actin ratio in drug-sensitive MCF-7 cells was set at 1. MCF-7, drug-sensitive breast cancer cells; MCF-7/AdrR, drug-resistant breast cancer cells; control1, medium with transfection reagent; GCS antisense-1, single-stranded GCS1 oligodeoxynucleotide in antisense orientation; GCSanti sense-1, single-stranded GCS1 oligodeoxynucleotide in antisense orientation; control2, medium with empty plasmid; psGCS1, recombinant plasmid expressing shRNA against GCS; GCS sense-2, single-stranded GCS2 oligodeoxynucleotide in sense orientation; GCS antisense-2, single-stranded GCS2 oligodeoxynucleotide in antisense orientation; psGCS2, recombinant plasmid expressing shRNA against GCS. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

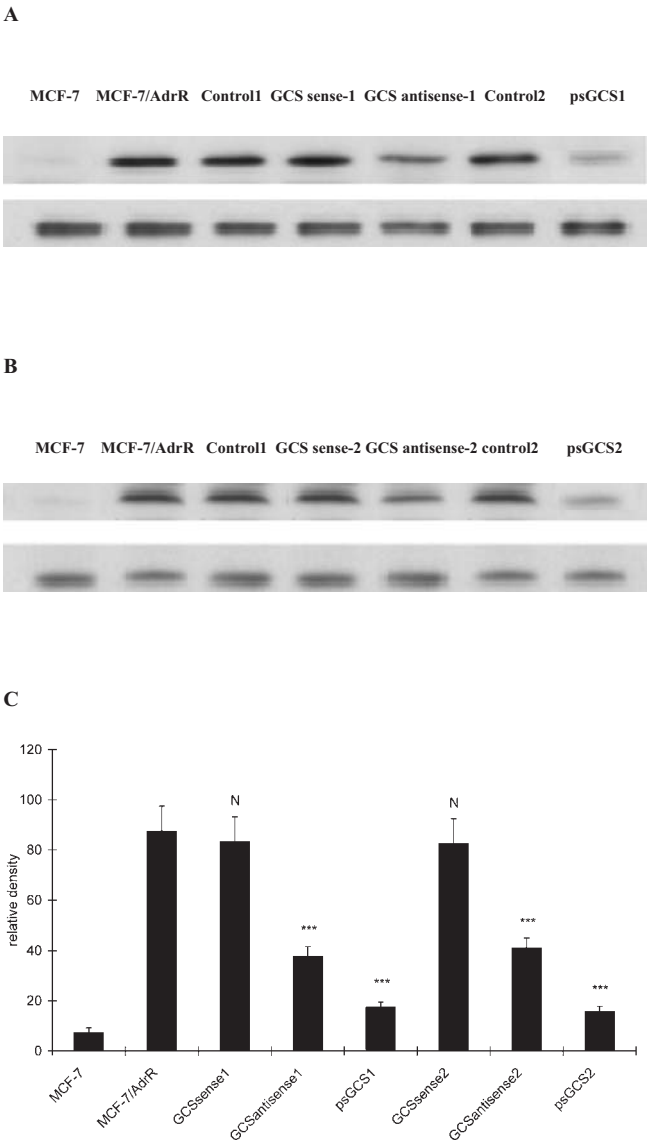

Figure 3. Charaterization of shRNA-mediated decrease of GCS protein content in MCF-7/AdrR cells.
A: Western blot analysis depicting GCS protein expression after psGCS1 treatment in MCF-7/AdrR cells. B: Western blot following psGCS2 treatment in MCF-7/AdrR cells. C: Relative GCS protein levels normalized against actin protein level in MCF-7/AdrR cells. The levels of actin expression were also determined as control for equivalent protein loading. MCF-7, drug-sensitive breast cancer cells; MCF-7/AdrR, drug-resistant breast cancer cells; control1, medium with transfection reagent; GCS sense-1, single-stranded GCS1 oligodeoxynucleotide in sense orientation; GCS antisense-1, single-stranded GCS1 oligodeoxynucleotide in antisense orientation; control2, medium with empty plasmid; psGCS1, recombinant plasmid expressing shRNA against GCS; GCS sense-2, single-stranded GCS2 oligodeoxynucleotide in sense orientation; GCS antisense-2, single-stranded GCS2 oligodeoxynucleotide in antisense orientation; psGCS2, recombinant plasmid expressing shRNA against GCS. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

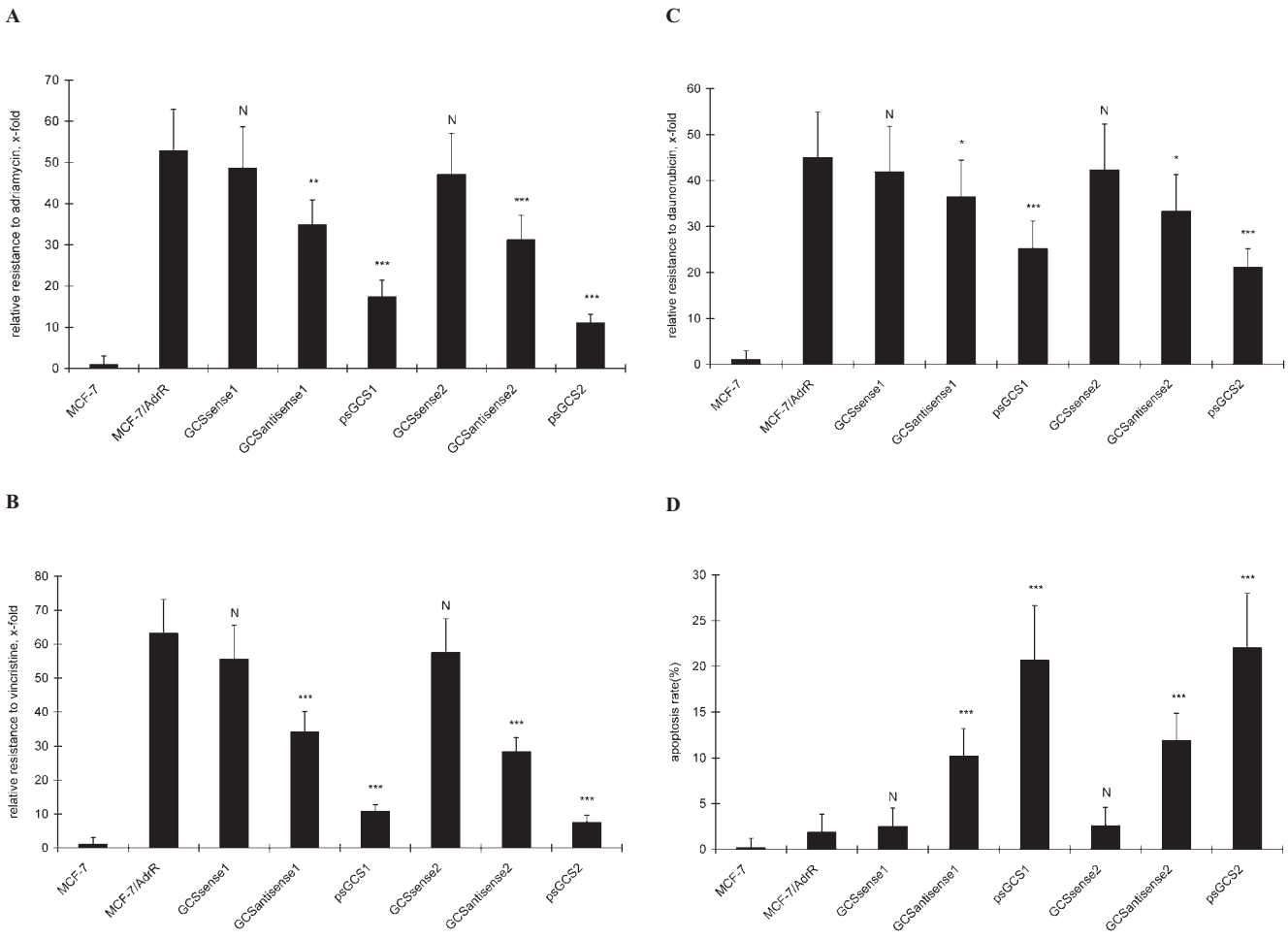

Figure 4. Effect of expression of shRNAs targeting GCS on resistance to cytotoxic drugs in MCF-7/AdrR cells.
A: Relative resistance against adriamycin in MCF-7/AdrR cells, resistance value in drug-sensitive MCF-7 cells was set at 1. B: Relative resistance against vincristine in MCF-7/AdrR cells, resistance value in drug-sensitive MCF-7 cells was set at 1. C: Relative resistance against daunorubicin cells, resistance value in drug-sensitive MCF-7 cells was set at 1. D: The apoptosis rates of MCF-7/AdrR cells induced by treatment with psGCS1 and psGCS2. MCF-7, drug-sensitive breast cancer cells; MCF-7/AdrR, drug-resistant breast carcer cells; GCS sense-1, single-stranded GCS1 oligodeoxynucleotide in sense orientation; GCS antisense-1, single-stranded GCS1 oligodeoxynucleotide in antisense orientation; psGCS1, recombinant plasmid expressing shRNA against GCS; GCS sense-2, single-stranded GCS2 oligodeoxynucleotide in sense orientation; GCS antisense-2, single-stranded GCS2 oligodeoxynucleotide in antisense orientation; psGCS2, recombinant plasmid expressing shRNA against GCS.
Drug resistance was evaluated by IC_{50} assay. The relative expression values are means of at least three independent experiments. N – non-statistical significance to multidrug-resistance breast cancer cells; $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

after the selection with puromycin. Both psGCS1 and psGCS2 reversed the drug resistance to several anticancer drugs more effectively than GCS ASODNs (Fig. 4). psGCS1 decreased the resistance factor to different drugs (adriamycin, vincristine, daunorubicin) from 53-fold to 17-fold, 63-fold to 11-fold, 45-fold to 25-fold respectively (decrease to 33%, 17%, 56% of the original value, i.e. 67%, 83%, 44% reversal; $p<0.001$). psGCS2 reversed the drug resistance to 11-fold, 8-fold, 21-fold respectively (decrease to 21%, 12%, 47% of the original value, i.e. 79%, 88%, 53% reversal; $p<0.001$). GCS ASODN1 decreased drug resistance to 66%, 54%, 81% of the original value, i.e. 34%, 46%, 19% reversal ($p<0.05$). GCS ASODN2 decreased drug resistance to 59%, 45%, 74% of the original value, i.e. 41%, 55%, 26% reversal ($p<0.05$).

Additionally, flow cytometry showed that the rates of cell apoptosis were 20.65±0.52%, 22.01±0.73% respectively after transfection with psGCS1 and psGCS2, however, the rates of MCF-7/AdrR cells transfected with GCS ASODNs were 10.20±0.27% and 11.86±0.38% respectively. The cells transfected with ASODNs or psGCS have a different proliferation rate compared with the MCF-7/AdrR control cells, the apoptosis rate of which was 1.86±0.15% ($p<0.05$). In contrast, the apoptosis rates of cells treated with GCS sense-1

and GCS sense-2 were 2.51±0.31% and 2.60±0.42%, respectively. There was no significant difference between the MCF-7/AdrR control cells and the cells transfected with GCS sense-1 or GCS sense-2 ($p>0.05$).

Discussion

Multidrug resistance is believed to arise from multiple mechanisms, involving alteration of drug influx or efflux by transporters, DNA repair systems, detoxification processes and apoptosis [28–31]. In recent studies, glucosylceramide synthase, which catalizes ceramide glycosylation in the first step in the biosynthesis of glycosphingolipids has been a subject of attention [32, 33]. Overexpression of GCS in MCF-7/AdrR cells conferred resistance to adriamycin and other antitumor agents [13, 34].

RNAi is a novel mean for specific inhibition of a targeted gene. siRNA can be transfected into mammalian cells by various means. First, chemically synthesized siRNA can be introduced into cells [24]. Although synthetic siRNA can achieve very rapid and effective "knockdown", their use is limited to cells that can be transfected at high rates. Moreover, their effects are transient. To circumvent the latter problem, short hairpin RNAs (shRNAs) can be expressed from stably transfected plasmids [26, 35].

In the present study, we used shRNAs targeting GCS to reverse multidrug resistance in multidrug-resistant breast cancer cells. In addition, we used also GCS ASODN to transfect MCF-7/AdrR cells and observed its reversal effect. We selected two shRNAs and cloned them into the expression plasmid pSUPER. As pSUPER vector contains H1-RNA promoter upstream of inserted DNA sequence, the shRNAs can be effectively expressed after being transfected into cancer cells [27]. Our results demonstrated that both shRNAs against GCS expressed by recombinant plasmids were more effective in suppressing GCS mRNA level and GCS protein level in MCF-7/AdrR cells. RT-PCR detection revealed that psGCS1 and psGCS2 reduced GCS mRNA by 86% and 88%, respectively. GCS protein expression decreased 80% and 82%, respectively by using western blot analysis, in contrast, GCS ASODN exhibited less GCS mRNA and GCS protein expression-inhibiting activity.

The effect of psGCS on MDR was assessed by comparison of IC_{50} values using a proliferation assay. Both psGCS1 and psGCS2 showed a pronounced chemosensitizing activity, they restored drug sensitivity in MCF-7/AdrR cells to levels near to the drug-sensitive cells. The drug resistance to adriamycin could be reversed 67% by psGCS1 and 79% by psGCS2, similarly, the drug resistance factor to vincristine and daunorubicin could also be reversed effectively. Moreover, flow cytometry showed that the rates of cell apoptosis increased, which confirmed that psGCS1 and psGCS2 reversed MDR by inducing the apoptosis of multidrug-resistant breast cancer cells. Compared with psGCS1 and psGCS2, GCS ASODN demonstrated less chemosensitizing activity.

Recently, LIU [20] reported that GCS antisense oligonucleotide (ASODN) blocked cellular GCS expression and selectively increased chemosensitivity in multidrug-resistant breast cancer cells and ovarian cancer cells. Due to differences in the experimental procedures, the effectiveness of GCS ASODN and psGCS cannot be compared directly. However, in our work, the results demonstrated that shRNAs showed a more pronounced gene silencing activity in MCF-7/AdrR cells at the mRNA level as well as the protein level, and additionally, they increased the cytotoxicity of different anticancer agents. Although both shRNAs were designed according to the same principle, psGCS2 revealed a more effective GCS reducing activity and MDR reversing activity in multidrug-resistant breast cancer cells. This phenomenon might contribute to the secondary structure of the shRNA target mRNA, and in the future studies, the biologically more effective shRNA should be designed and tested.

In this work, we first constructed plasmids expressing shRNAs targeting glucosylceramide synthase and introduced them to silence MDR-associated gene-GCS. The results revealed that shRNAs expressed from pSUPER can be applied to activate RNAi-mediated cleavage and degradation of the MDR-associated GCS mRNA in target cancer cells. The data demonstrated that shRNA mediated RNAi may be a specific and effective means to prevent and reverse MDR in the clinical treatment. A problem for the utility of plasmid vectors expressing shRNAs arise that a transfection reagent or electroporation introduced to the target cells cannot be applied to human. Lentiviral vectors may provide a more effective and practicable approach in the future studies [36]. These types of vectors can introduce the expression cassette into the chromosome of even non-dividing cells, such as neurons and mature hepatocytes [37–39]. Recently, suppression of MDR1 gene by RNA interference completely reversed the MDR phenotype on drug-resistant human gastric carcinoma cell line EPG85-257RDB [40]. Therefore, the development of lentiviral vectors targeting GCS or other MDR-associated genes such as genes encoding apoptosis, alternative ABC transporter-encoding genes and cell cycle-regulating factors, used singly or in combination therapies could in the future probably offer a novel strategy for the treatment of breast cancer.

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