Glutathione S-transferase isozyme alpha 1 is predominantly involved in the cisplatin resistance of common types of solid cancer

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Abstract. The roles of glutathione S-transferase pi 1 (GSTP1), glutathione S-transferase mu 2 (GSTM2) and glutathione S-transferase alpha 1 (GSTA1) in cisplatin (DDP)-resistance of solid cancer cells (A549/DDP, SKOV3/DDP and SGC7901/DDP) were compared following expression down-regulation with small interfering RNAs (siRNAs). DDP cytotoxicity was reflected by its half maximal inhibition concentration (IC_{50}) calculated from data using a Cell Counting Kit-8 assay; cell apoptosis was examined using flow cytometry and Hoechst 33342 staining. Higher activities of GST were detected in the cytosol of DDP-resistant cells, compared with those in the parental DDP-susceptible cells. The silencing efficacy of each positive siRNA was supported by western blot analysis. GSTP1 silencing resulted in a 4-fold sensitization of SGC7901/DDP cells to DDP cytotoxicity, but negligible sensitization of SKOV3/DDP and A549/DDP cells. GSTM2 silencing sensitized SKOV3/DDP and A549/DDP cells to DDP cytotoxicity by ~2-fold, but did not sensitize SGC7901/DDP cells. Notably, GSTA1 silencing enhanced DDP cytotoxicity in SGC7901/DDP cells by 6-fold, in A549/DDP cells by 5-fold and in SKOV3/DDP cells by 2-fold. The combined actions of positive siRNAs and DDP increased the percentages of apoptotic cells in the DDP-resistant solid cancer cells compared with the combined actions of DDP and the negative siRNAs. The present findings indicated that GSTA1 is a predominant GST isozyme associated with DDP resistance of SGC7901/DDP, A549/DDP and SKOV3/DDP cells; GSTA1-specific inhibitors may be general sensitizers of SGC7901/DDP, A549/DDP and SKOV3/DDP cells to DDP cytotoxicity through the promotion of cell apoptosis.

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Introduction

Therapeutic resistance of common types of solid cancer to diverse cytotoxic agents is a challenge in medicine, and is primarily acquired through mechanisms including the upregulation of xenobiotic efflux pumps and enhancement of DNA repair (1,2). Glutathione S-transferase (GST) in the cytosol has various isozymes that may be further assigned into sub-isoforms of splice variants (3). The upregulation of these GST isozymes has been proposed to cause drug resistance through the enhanced catalytic detoxification of antineoplastic agents and the modulations of apoptotic signaling pathways (4-6). The suppression of GST activity is thus expected to sensitize drug-resistant solid cancer cells to cytotoxic agents (2,6-12). However, GSTs play complicated physiological roles in cells, and the expression profiles of GST isozymes in cancer cells have been associated with sex, tissues and organs (4,13,14). The incidence of solid cancers in different origins is associated with various GST isozymes (4). Therefore, the cost and time of screening for potent inhibitors against such GST isozymes to sensitize common types of drug-resistant solid cancer is a challenge; no notable progress has been made yet regarding the use of selective GST isozyme inhibitors in the treatment of common types of drug-resistant solid cancer.

The recognition of GST isozymes predominantly involved in drug resistance of common types of solid cancer facilitates the development of potent selective inhibitors to be used as general sensitizers of such types of drug-resistant solid cancer. To recognize a GST isozyme responsible for drug resistance, the effects of selective inhibition of its actions on drug toxicity should be assessed. To this end, the most straightforward method is the detection of drug action after the selective

inhibition of each GST isozyme; however, the selective inhibitors for each GST isozyme are yet to be developed. Current inhibitors for GST isozymes are not satisfactory with regard to their isozyme-selectivity, inhibition potency and membrane permeability (7-12). Therefore, alternative methods have to be sought to determine GST isozymes associated with drug resistance of common types of solid cancer.

Short interfering RNAs (siRNAs) can selectively block the action of a specified protein by inhibiting the expression and/or translation of the target gene. As a result, siRNAs are promising tools for downregulating the expression of GST isozymes in order to detect their roles in drug resistance (15,16) and to identify GST isozymes that may be suitable targets to sensitize common types of drug-resistant solid cancer to cytotoxic agents. Studies on different cancer cells and tissues have identified the contributions of various GST isozymes to the incidence of drug resistance in common types of solid cancer (4,13,14). However, for drug-resistant cancer cells or tissues, the available studies have been investigating the roles of GST isozyme(s) in one type of drug-resistant solid cancer, and few have been examining the roles of different GST isozymes in different types of drug-resistant solid cancer. Lung, ovarian and stomach cancer types exhibit high rates of drug resistance, and therefore high mortality rates (17). Cisplatin (DDP) alone or in combination with other cytotoxic agents is the first-line chemotherapy for such types of solid cancer; however, the therapeutic efficiency is usually hindered due to the development of acquired drug resistance (2,18) and the upregulated expression of glutathione S-transferase pi 1 (GSTP1), glutathione S-transferase mu 2 (GSTM2) and glutathione S-transferase alpha 1 (GSTA1) (4,19-23). To ascertain the hypothesis that there may be a specific GST isozyme that is predominantly involved in the drug resistance of common types of solid cancer to DDP, siRNAs of GSTP1, GSTM2 and GSTA1 were transfected into DDP-resistant solid cancer cells, A549/DDP, SKOV3/DDP and SGC7901/DDP, which were originated from the lungs, ovaries and stomach, respectively; cells after further treatment with DDP were then examined for proliferation and apoptosis.

Materials and methods

Materials. Lipofectamine 2000 was purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Human non-small cell lung cancer cell line A549 (TCHu150) and human gastric cancer cell lines SGC7901 (TCHu 46) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China); Cisplatin-resistant A549/DDP (BNCC341254) and cisplatin-resistant SGC7901/DDP (BNCC342230) were purchased from the BeNa Culture Collection (Beijing Bei Na Chuanglian Biotechnology Research Institute, Beijing, China). Cisplatin-sensitive human ovarian cell line SKOV3 and their cisplatin-resistant clones SKOV3/DDP were obtained from the Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China). Rabbit polyclonal primary antibody against GSTM2 (cat. no. YN2960) and rabbit polyclonal primary antibody against β-actin (cat. no. A283), were purchased from ImmunoWay Biotechnology Co., Ltd. (Plano, TX, USA). Rabbit polyclonal primary antibody against GSTP1 (cat. no. D222453) was purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China), rabbit monoclonal primary antibody against GSTA1 (cat. no. ab207413) was purchased from Abcam (Cambridge, MA, USA). HRP-labeled goat anti-rabbit IgG (cat. no. A25222) was purchased from Abbkine Scientific Co., Ltd. (Wuhan, China). 1-Chloro-2,4-dinitrobenzene (CDNB) was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Reduced glutathione (GSH) was obtained from Beijing Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China). Other chemicals were analytical reagents, unless otherwise stated.

Cell culture and siRNA transfection. Three candidate sequences of siRNA for silencing GSTP1, GSTM2 and GSTA1 and negative siRNA were designed and synthesized by Suzhou GenePharma Co., Ltd. (Suzhou, China) and the most potent siRNA was screened. Cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (FBS; Capricorn Scientific GmbH Ebsdorfergrund, Germany), 100 kU/l penicillin and 100 mg/l streptomycin in an atmosphere containing 5% CO_2 at 37˚C. Cells were grown in the complete medium for 24 h and subsequently transfected with GST isozyme siRNAs. Following pre-incubation at room temperature for 20 min, cells were seeded in 6-well plates to 60-70% confluence and treated with a solution of 0.2 μg siRNA and 5 μl Lipofectamine 2000 in Opti-MEM (Gibco; Thermo Fisher Scientific, Inc.). The final concentration of siRNA used was 50 pmol/l. Cells were incubated with medium containing siRNA and Lipofectamine complex for 6 h. Following this, the transfection medium was replaced with complete medium free of antibiotics. The transfected cells were incubated at 37˚C in an atmosphere containing 5% CO_2 for 48 h.

Candidate sequences for siRNA of GSTP1, GSTM2 and GSTA1. GSTP1 sequence 1: Sense, 5'-CCUACACCGUGGUCUAUU UTT-3' and antisense, 5'-AAAUAGACCACGGUGUAGGTT-3'; GSTP1 sequence 2: Sense, 5'-CCUCAUCUACACCAACUA UTT-3' and antisense, 5'-AUAGUUGGUGUAGAUGAGGTT-3'; GSTP1 sequence 3: Sense, 5'-GCUGAUCCAUGAGGUCCU ATT-3'; and antisense, 5'-UAGGACCUCAUGGAUCAGCTT-3'; GSTM2 sequence 1: Sense, 5'-GGAUUUCAUCGCUUAUGA UGUTT-3' and antisense, 5'-ACAUCAUAAGCGAUGAAA UCCTT-3'; GSTM2 sequence 2: Sense, 5'-GCACUCCCUGAA AUGCUGAAGTT-3' and antisense, 5'-CUUCAGCAUUUC AGGGAGUGCTT-3'; GSTM2 sequence 3: Sense, 5'-GAU UUGAGGCUUGGAGAAGATT-3' and antisense, 5'-UCU UCUCCAAGCCCUCAAAUCTT-3'; GSTA1 sequence 1: Sense, 5'-CCACAGUGAAGAAGUUUCUTT-3' and antisense: 5'-AGAAACUUCUUCACUGUGGTT-3'; GSTA1 sequence 2: Sense, 5'-CCAAGCUUGCCUUGAUCAATT-3' and antisense, 5'-UUGAUCAAGGCAAGCUUGGTT-3'; GSTA1 sequence 3: Sense, 5'-GGAGCUUGACUCCAGUCUUTT-3' and antisense, 5'-AAGACUGGAGUCAAGCUCCTT-3'.

Western blot analysis. DDP-susceptible or DDP-resistant cells were detached with trypsin, centrifuged at 4˚C and washed three times with pre-chilled phosphate-buffered saline (PBS). RIPA lysis buffer (cat. no. P0013K; Beyotime Institute of Biotechnology, Haimen, Jiangsu, China) plus

PMSF (cat. no. ST506; Beyotime Institute of Biotechnology), was subsequently added and the cells were incubated on ice for 30 min. The supernatants containing proteins were collected by centrifugation at 13,000 x g for 20 min. Protein concentration in each cell lysate was determined using the Bradford assay with BSA as the reference (24). A total of 50 μg protein was separated from each group using SDS-PAGE (10% gels) and transferred to a polyvinylidene fluoride (PVDF) membrane (0.45 μm; GE Healthcare, Chicago, IL, USA). The membrane was soaked in 5% bovine serum albumin (BSA) (in TBS at pH 7.4 containing 0.1% Tween-20) for 2 h and incubated with rabbit polyclonal/monoclonal antibodies against GST isozymes and β-actin (all 1:1,000 dilution) at 4˚C overnight. Following this, the PVDF membrane was washed and subsequently incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (dilution 1:5,000; cat. no. A25222; from Abbkine Scientific Co., Ltd., Wuhan, China) at 37˚C for 1 h. The ChemiDoc MP Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and BeyoCEL Plus (Beyotime Institute of Biotechnology, Haimen, China) were used to detect chemiluminescence under the catalytic action of horseradish peroxidase conjugated to the adsorbed goat anti-rabbit IgG. Densitometry analysis was performed using ImageJ software (version 1.46; National Institutes of Health, Bethesda, MD, USA).

Assay of GST activity in lysates. As described by Habig *et al* (25), in 1.0 ml of 20 mmol/l phosphate buffer (pH 6.5) containing GSH and CDNB (final concentration, 1.0 mmol/l, 30 μl of cell lysate was added to initiate the reaction, and the absorbance at 340 nm was recorded for 3 min at 10-sec intervals, following a lagging time of 15 sec. One unit of GST activity was defined as the amount of GST enzyme that resulted in 1 μmol product/min at 25˚C (pH 6.5). Apparent specific activities in cell lysates were calculated using the concentrations of total proteins (24).

Cell proliferation assay. Cell proliferation was assessed using the Cell Counting Kit-8 assay (CCK-8; Biotool, Houston, TX, USA). Following transfection with GST isozyme positive siRNAs or negative siRNAs (sicontrol), A549/DDP, SKOV3/DDP and SGC7901/DDP cells were seeded in 96-well plates at a density of $5.0x10^7$ cells/l. Following 24 h of incubation, DDP was added at a final concentration of 0-80 μmol/l for A549/DDP, 0-40 μmol/l for SKOV3/DDP and 0-20 μmol/l for SGC7901/DDP. Cells were subsequently incubated for 72 h (15,16,20-22). CCK-8 was added according to the manufacturer's instructions in order to detect the absorbance at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Apoptosis assay. Cells transfected with a positive siRNA against an indicated GST isozyme or negative siRNA (sicontrol) were further treated for 72 h with DDP (10 μmol/l for A549/DDP but 5 μmol/l for SKOV3/DDP and SCG7901/DDP). To examine apoptosis, DDP concentrations were set to maintain >60% survival of the tested solid cancer cells when treated with DDP alone (15,16,20-22). Cells after the treatment were collected and washed with pre-cooled PBS prior to staining using Annexin Cy5 apoptosis assay kit (cat. no. ab14150) and propidium iodide flow cytometric kit (cat. no. ab139418; Abcam, Cambridge, MA, USA) according to the manufacturer's instructions. Following this, analysis was conducted using flow cytometry (FACSVantage SE system; BD Biosciences, Franklin Lakes, NJ, USA). Data acquisition and analysis were performed using CellQuest Pro software (version 5.1; BD Biosciences).

Table I. Comparison of the GST activity in cell lysates.

Cell lines	GST activity in cell lysates (U/mg)	Ratio[a]
A549	0.19±0.02	
A549/DDP	0.24±0.02	1.3
SGC7901	0.14±0.01	
SGC7901/DDP	0.18±0.02	1.3
SKOV3	0.43±0.04	
SKOV3/DDP	1.25±0.10[b]	2.9

Data were presented as the mean ± standard deviation (n=3). [a]Ratio of GST activity in DDP-resistant cells to that in the DDP-susceptible cells. [b]P<0.05 vs. those in the DDP-susceptible cells. GST, glutathione S-transferase; DDP, cisplatin.

Hoechst 33342 staining. Cells after silencing of an indicated GST isozyme and the control cells were seeded in 24-well plates at a density of 5,000 cells/well for 24 h-incubation and subsequently treated with DDP further as described above for 72 h. Cells were fixed with 50% methanol at 4˚C for 30 min and stained with 10 μl Hoechst 33342 (Invitrogen; Thermo Fisher Scientific, Inc.) for 10 min at 4˚C in the dark, according to the manufacturer's instructions. The supernatant was discarded and the cells were washed three times with pre-cooled PBS. Cells were then observed under an inverted microscope (Nikon Corp., Tokyo, Japan).

Statistical analysis. SPSS software (version 19.0; IBM Corp., Armonk, NY, USA) was used for statistical analysis. Experimental data were performed in triplicate and presented as the mean ± standard deviation. The Student's t-test was used for analyzing the differences between groups and one-way ANOVA followed by a Newman-Keuls post hoc test was used for the analysis of the differences among groups. P<0.05 was considered to indicate a statistically significant result.

Results

Expression and activity of GSTs. As upregulated GST expression levels are commonly associated with the incidence of DDP-resistance, the activities and expression levels of GSTP1, GSTM2 and GSTA1 in the three tested types of DDP-resistant cancer cells were compared with those in their parental DDP-susceptible cells. All DDP-resistant cancer cells exhibited some increased GST activities compared with the parental DDP-susceptible cells (Table I). Notably, a significant increase of GST activity was observed in SKOV3/DDP cells compared with that in SKOV3 cells. Compared with those in the parental DDP-susceptible cells, the protein expression levels of GSTP1, GSTM2 and GSTA1 were upregulated in SKOV3/DDP according to western blot analysis (Fig. 1B); the protein

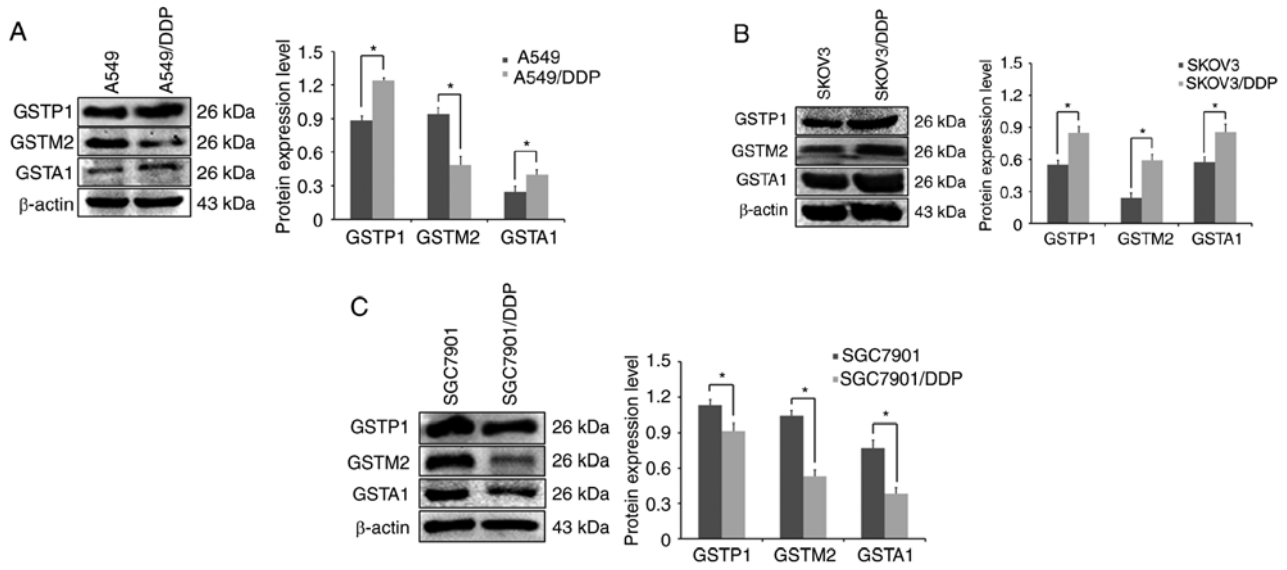

Figure 1. Comparison of the expression levels of GST isozymes in DDP-susceptible and DDP-resistant cancer cells. The expression levels of GSTP1, GSTM2 and GSTA1 were determined using western blotting, in (A) A549 and A549/DDP, (B) SKOV3 and SKOV3/DDP, and (C) SGC7901 and SGC7901/DDP cells. β-actin served as the reference protein. Relative expression values in triplicate were expressed as the mean ± standard deviation. Student's t-test was used for comparison. *P<0.05 vs. those in the DDP-susceptible cells. GST, glutathione S-transferase; DDP, cisplatin.

expression levels of GSTP1 and GSTA1 were increased, while that of GSTM2 was decreased in A549/DDP cells (Fig. 1A). These results were in line with data revealing GSTP expression to be significantly upregulated in a variety of drug-resistant solid cancer types (originating from the breast, colon, pancreas, liver, lung, ovary and stomach), and GSTA and GSTM expression to be induced by lower extents in some types of such drug-resistant solid cancers (4,12-15,18). However, the protein levels of GSTM2 and GSTA1 were decreased in SGC7901/DDP cells compared with those in parental DDP-susceptible cells. Furthermore, the protein expression of GSTP1 was decreased to a lower degree in SGC7901/DDP cells compared with that in parental DDP-susceptible cells (Fig. 1C), potentially due to lower quantification accuracy of western blot analysis.

Screening for siRNA against GST isozymes. Three candidate sequences of siRNA against GSTM2, GSTP1 and GSTA1 were designed by Suzhou GenePharma Co., Ltd. A549/DDP, SKOV3/DDP and SGC7901/DDP cells were separately transfected with each of those three candidates of siRNA for GSTP1, GSTM2, or GSTA1 in liposome, and the expression profiles of GSTs were compared by western blot analysis for screening the sequence causing the largest reduction in the protein expression of each GST isozyme. Compared with the untreated cells or those treated solely with Lipofectamine 2000, different candidate sequences of siRNAs resulted in varying reductions of each targeted GST isozyme (Fig. 2). Accordingly, those producing the most significant reductions of protein expression in each of the three types of cancer cells, i.e., sequence 1 for si-GSTP1, sequence 2 for si-GSTM2, and sequence 2 for si-GSTA1 in A549/DDP (Fig. 2A), sequence 3 for si-GSTP1, sequence 2 for si-GSTM2, and sequence 1 for si-GSTA1 in SKOV3/DDP (Fig. 2B), and sequence 3 for si-GSTP1, sequence 1 for si-GSTM2 and sequence 2 for si-GSTA1 in SGC7901/DDP (Fig. 2C), were selected for subsequent experiments.

Effects of silencing GST isozymes on DDP toxicity to DDP-resistant cancer cells. To investigate whether siRNAs of GSTP1, GSTM2 and GSTA1 modulate differently the susceptibility of A549/DPP, SKOV3/DDP and SGC7901/DDP cells to DDP, those cells after the transfection with positive or negative siRNAs were further treated with DDP. Of the three tested types of DDP-resistant cancer cells transfected with positive and negative siRNAs, the half maximal inhibition concentration (IC_{50}) values of DDP were compared. For each of the three types of DDP-resistant cancer cells, the treatment with Lipofectamine 2000 alone or the transfection with a negative siRNA resulted in a survival rate consistent with that of the untreated cells (data not shown). In comparison with cells after the combination treatment with DDP and a negative siRNA against a certain GST isozyme, IC_{50} of DDP was decreased differently in each DDP-resistant cell type after the combination treatment with DDP and the positive siRNA against the indicated GST isozyme (Fig. 3). In detail, in A549/DDP cells, the silencing of GSTA1 resulted in a 5-fold decrease whereas the silencing of GSTM2 resulted in a 2-fold decrease in the IC_{50} of DDP, but the silencing of GSTP1 had no significant effect on the IC_{50} of DDP (Fig. 3A). In SKOV3/DDP cells, the silencing of GSTM2 and GSTA1 resulted in 2-fold decreases in the IC_{50} of DDP, whereas the silencing of GSTP1 had negligible effect on the IC_{50} of DDP (Fig. 3B). In SGC7901/DDP cells, the silencing of GSTA1 and GSTP1 resulted in 6- and 4-fold decreases, respectively, whereas the silencing of GSTM2 provided a marginal reduction in the IC_{50} of DDP (Fig. 3C). Therefore, these GST isozymes may have different roles in DDP resistance of the tested three types of DDP-resistant solid cancer. Notably, the silencing of GSTA1 produced the highest sensitizing effects on DDP cytotoxicity in the examined three types of DDP-resistant solid cancer cells, indicating that GSTA1 may be a suitable target for the development of potent selective inhibitors to sensitize various types of drug-resistant solid cancer.

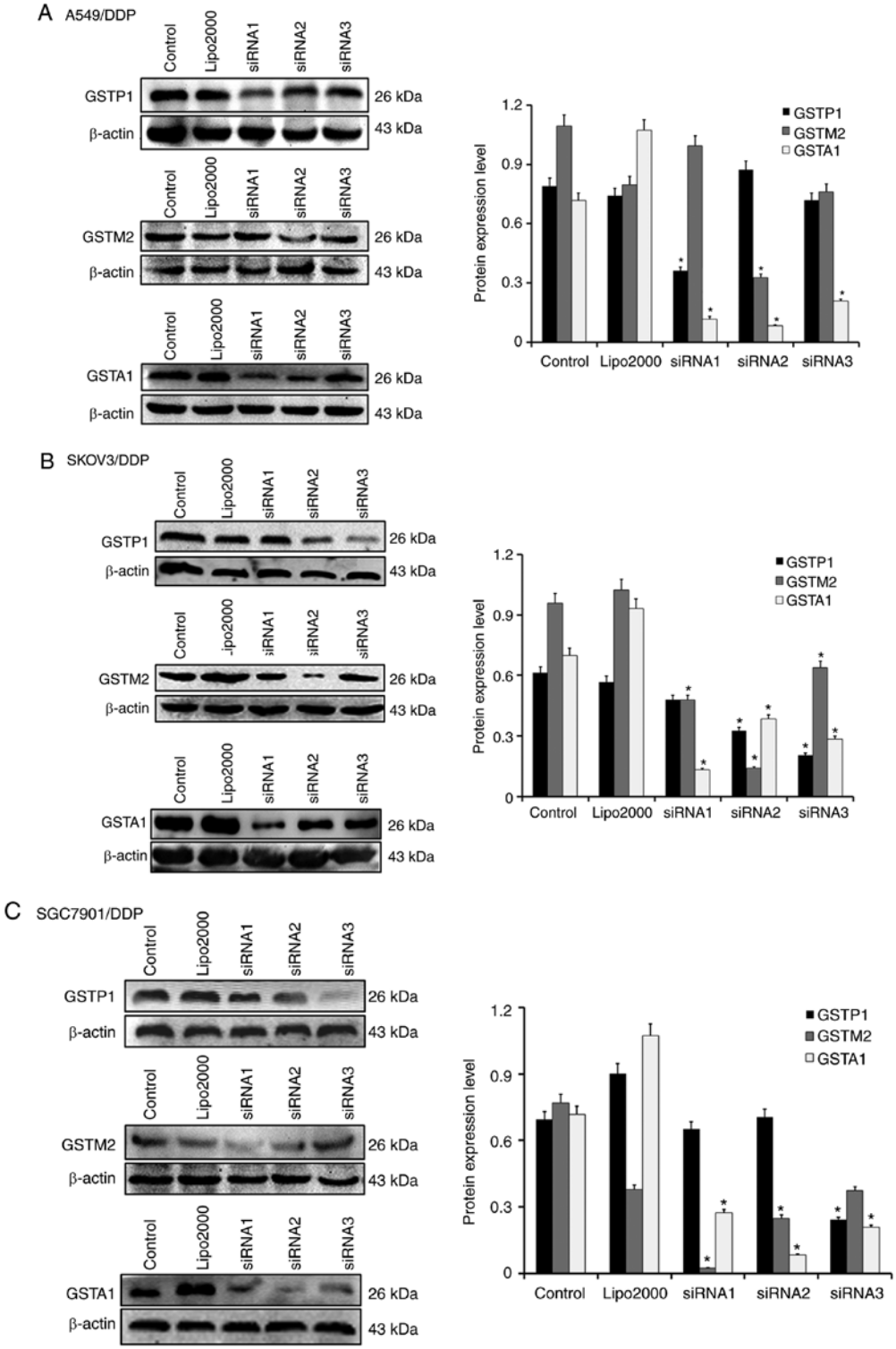

Figure 2. Effects of siRNA on protein expression levels of GSTP1, GSTM2 and GSTA1 in each type of cisplatin-resistant cells. The levels of GST proteins in (A) A549/DDP(A), (B) SKOV3/DDP and (C) SGC7901/DDP after the transfection of siRNAs were determined by western blotting with β-actin as the reference (left panel) and quantitative analyses (right panel). Cells untreated or treated solely with Lipofectamine 2000 were used for comparison. One-way ANOVA followed by a Newman-Keuls post hoc test was used for analyzing, and $^{*}P<0.05$ vs. the untreated cells and those treated solely with Lipofectamine 2000.

Effects of silencing of GSTs on cell apoptosis. To assess the cellular mechanism associated with the sensitization effects of GST silencing on DDP toxicity, the apoptotic rates of A549/DDP, SKOV3/DDP and SGC7901/DDP cells were determined using flow cytometry and the morphological changes were observed using Hoechst 33342 staining. On the apoptosis rate of each tested type of DDP-resistant cells, the effect of the combined treatment with a positive siRNA against a certain GST isozyme and DDP at a final concentration smaller than the IC_{50} was compared with that of the combined treatment with DDP at the same level and a negative siRNA. Of either of those three tested types of DDP-resistant

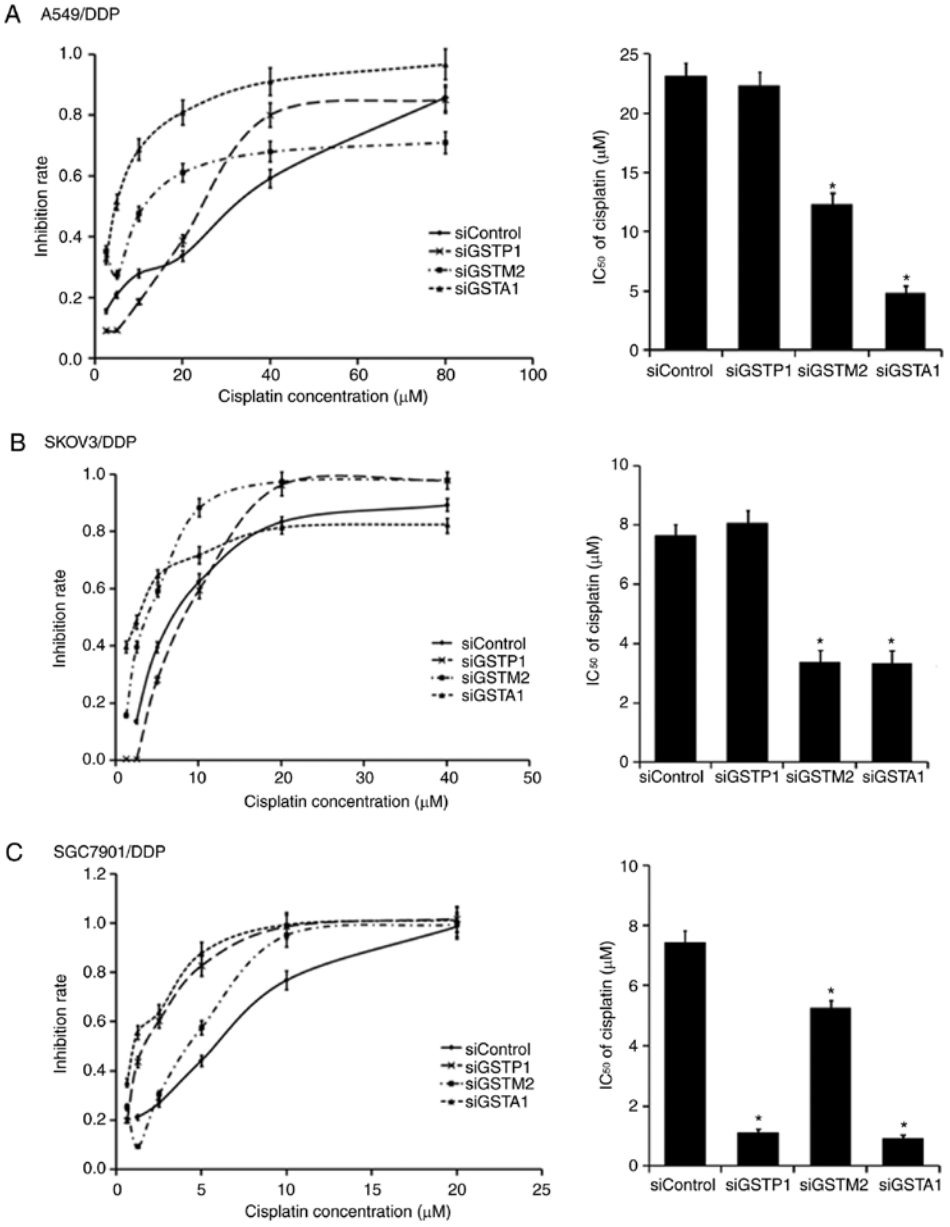

Figure 3. Silencing of GST isozymes has different roles in sensitizing DDP-resistant cells to DDP. Following the silencing of GST isozymes and DDP treatment for 72 h, the proliferation of (A) A549/DDP, (B) SKOV3/DDP or (C) SGC7901/DDP cells was detected using the Cell Counting Kit-8 assay (left panel) and analyzed using SPPS 19.0 software to quantitatively compare the IC_{50} values (right panel) with that combinedly treated with the negative siRNAs (siControl) and DDP. IC_{50} values in triplicate were presented as the mean ± standard deviation. One-way ANOVA followed by a Newman-Keuls post hoc test was used for comparison. *P<0.05 vs. cells after treatment with DDP in combination with negative siRNA. GST, glutathione S-transferase; DDP, cisplatin; IC_{50}, half maximal inhibitory concentration.

cancer cells, the treatment with only a negative siRNA against a certain GST isozyme or Lipofectamine 2000 alone produced an apoptosis rate consistent with that of the untreated cells (data not shown). However, the effective silencing of GSTP1, GSTM2 and GSTA1 with positive siRNAs had different synergistic effects with DDP treatment on the apoptosis rates of the tested types of DDP-resistant cancer cells (Fig. 4). In detail, in A549/DDP cells, the treatment with DDP after the effective silencing of GSTP1, GSTM2 and GSTA1 resulted in apoptosis rates that were 1- (insignificant), 3- and 4-fold of those in the same cells after the combination treatment with DDP and the negative siRNAs, correspondingly (Fig. 4A). In SKOV3/DDP cells, the combination treatment with DPP and the positive siRNAs of GSTP1, GSTM2 and GSTA1 resulted in apoptosis rates that were ~1.2- (insignificant), 6- and 13-fold of those in the same cells after the combination treatment with DDP and the negative siRNAs, respectively (Fig. 4B). In SGC7901/DDP cells, the combination treatment with DDP and the positive siRNAs of GSTP1, GSTM2 and GSTA1 resulted in apoptosis rates that were 2-, 1.4- and 3-fold of those in the same cells treated with DDP and the negative siRNAs, correspondingly (Fig. 4C). Clearly, of the tested DDP-resistant cancer cells after the combination treatment with DDP and the positive siRNAs, the apoptotic rates were inversely associated with their IC_{50} values of DDP (Fig. 3), among which the silencing of GSTA1 consistently exhibited the highest percentage of cell apoptosis. Furthermore, staining with Hoechst 33342 revealed some characteristic features of

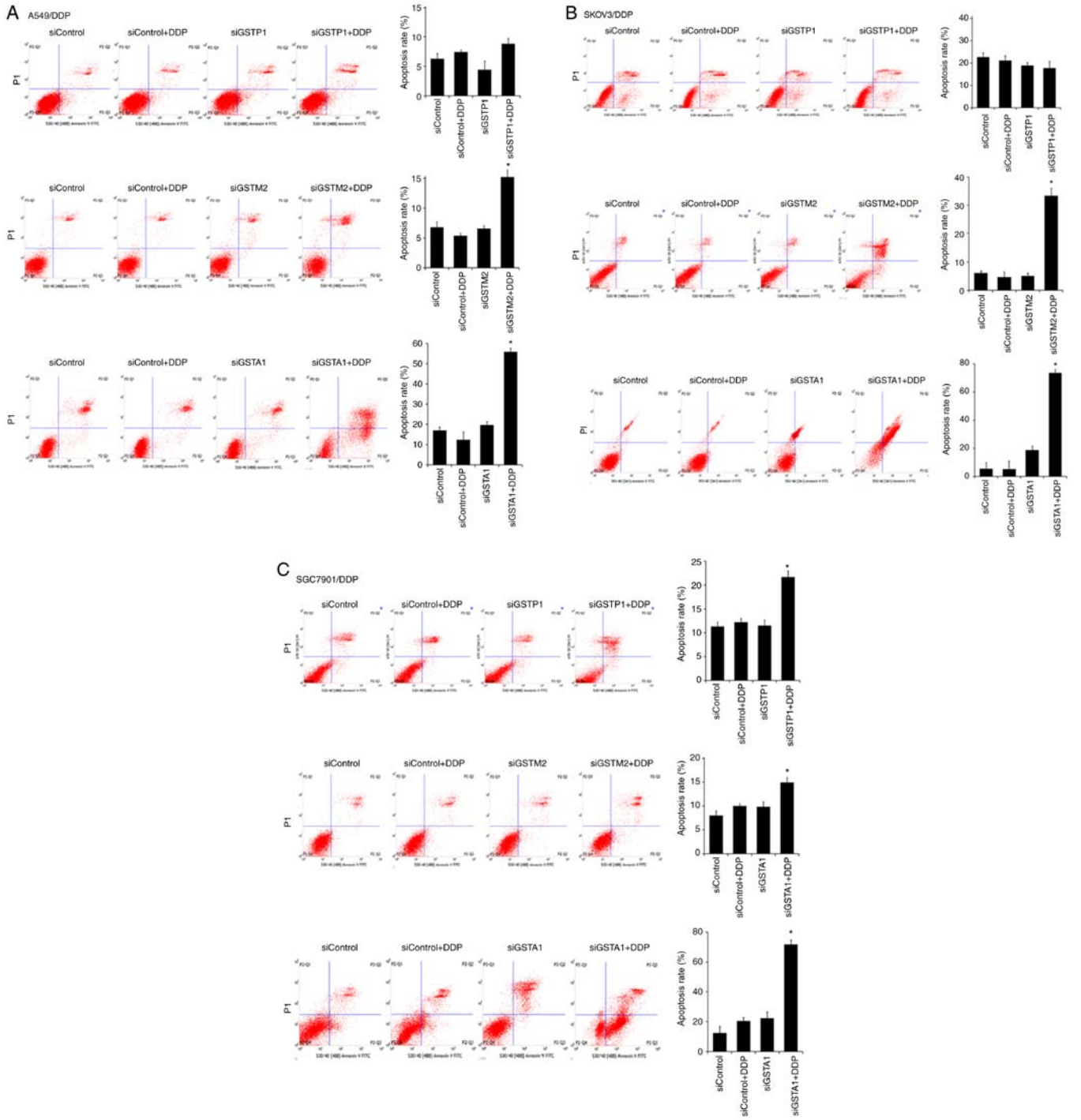

Figure 4. Apoptosis rates of three examined types of GST-silenced cells treated with DDP. Flow cytometry was used to analyze the percentages of apoptotic cells (left panel) for quantitative comparison (right panel) against those treated with DDP and the negative siRNAs (siControl). (A) Apoptosis profiles of A549/DDP cells treated with GST silencing and 10 μmol/l DDP for 72 h. (B) Apoptosis profiles of SKOV3/DDP cells treated with GST silencing and 5 μmol/l DDP for 72 h. (C) Apoptosis profiles of SGC7901/DDP cells treated with GST silencing and 5 μmol/l DDP for 72 h. Apoptosis rates in triplicate were presented as the mean ± standard deviation. One-way ANOVA followed by a Newman-Keuls post hoc test was used for comparison. *P<0.05 vs. cells treated with DDP in combination with negative siRNA and those solely with the negative siRNAs. GST, glutathione S-transferase; DDP, cisplatin.

apoptosis, such as pyknotic and condensed nuclei, in each type of DDP-resistant cells subjected to the combination treatment of the positive siRNAs and DDP (Fig. 5). Therefore, of the three tested types of DDP-resistant solid cancer cells, GSTA1 was the predominant GST isozyme associated with their DDP-resistance and the downregulation of GSTA1 expression levels may enhance DDP cytotoxicity by the promotion of cell apoptosis.

Discussion

DDP is a first-line anticancer agent that is widely used for the treatment of various types of solid cancer, but frequently confronts with the challenge of DDP-resistance. Inhibition of the expression of a specified GST isozyme by chemicals or siRNA has been found to reverse DDP-resistance in A549/DDP, SCG7901/DDP or SKOV3-/DDP cells, but there

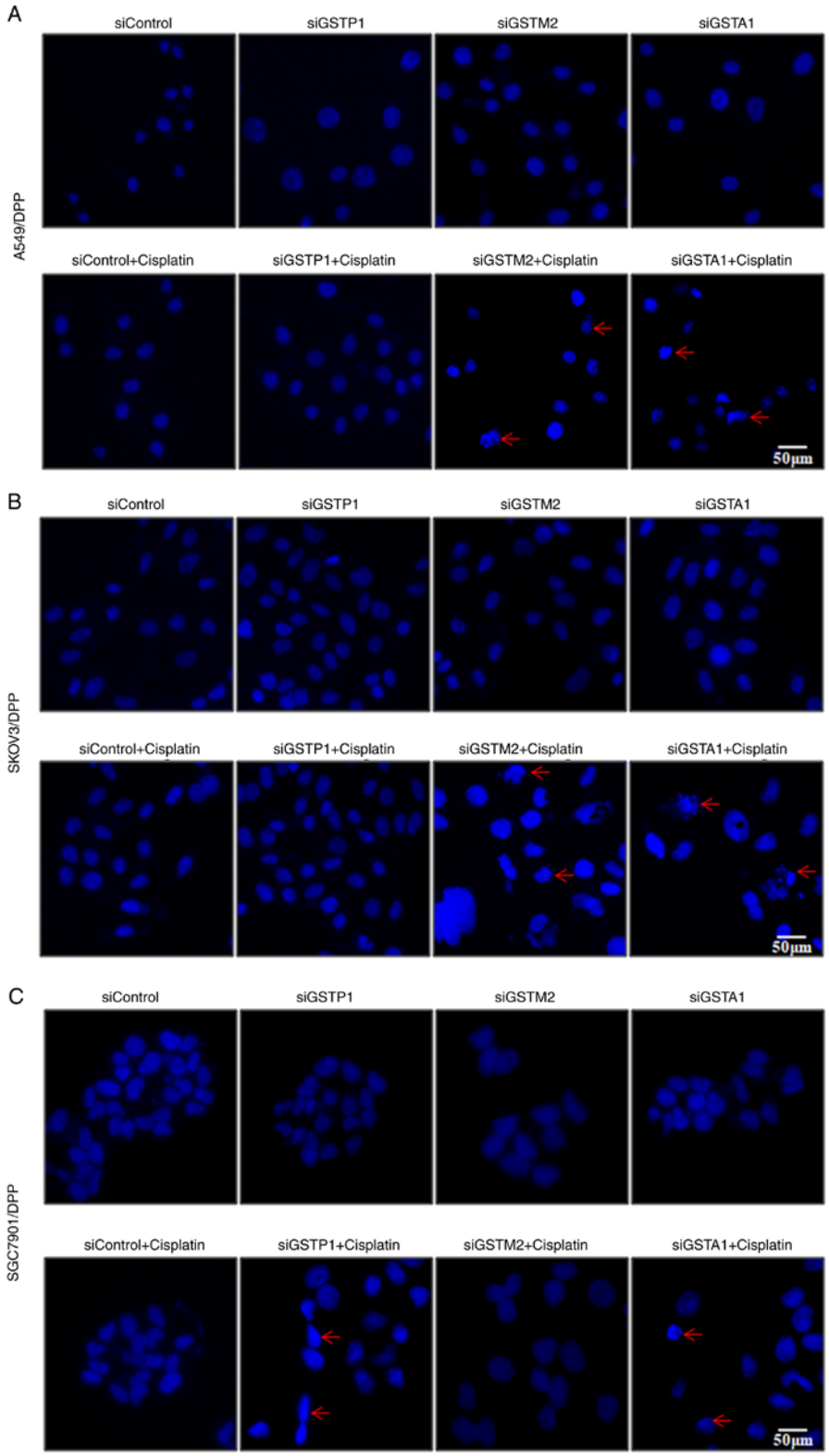

Figure 5. Hoechst 33342 staining of examined GST-silenced cells treated with DDP. After GST-silencing and further treatment with DDP for 72 h, including the treatment with negative siRNAs (siControl) and DDP, cells were fixed, stained with Hoechst 33342 and observed under an inverted microscope. Apoptosis profiles of (A) GST-silenced A549/DDP cells after further treatment with 10 μmol/l DDP, (B) GST-silenced SKOV3/DDP cells after further treatment with 5 μmol/l DDP, and (C) GST-silenced SGC7901/DDP cells after further treatment with 5 μmol/l DDP. The red arrows indicate the representatives of apoptotic cells. GST, glutathione S-transferase; DDP, cisplatin; si, small interfering.

are few studies on any of the same GST isozymes potentially involved in DDP-resistance of different types of solid cancer (15,16,20-22). The present study indicated that there were some increases in the activities of GSTs and the differently induced expression of tested GST isozymes in SGC7901/DDP, A549/DDP and SKOV3/DDP cells. Notably, the transfection with positive siRNA against GSTA1 resulted in the largest enhancements of DDP toxicity and apoptotic rates of all

the tested three types of DDP-resistant solid cancer cells, in comparison to the transfections with siRNAs against GSTP1 and GSTM2. Therefore, GSTA1 may be a rational target for the development of GSTA1-selective inhibitors to sensitize all the three types of, and even other types of, DDP-resistant cancer cells.

However, the exact action mechanisms of GST isozymes, especially GSTA1, in DDP resistance of SGC7901/DDP, A549/DDP and SKOV3/DDP cells remain unclear. The mechanism of DDP action which can be intervened by GST isozymes putatively involves two ways; one is the initiation of some toxic consequences that can be alleviated by the catalytic actions of GST isozymes, the other is the induction of apoptosis primarily through the activation of the mitogen-activated protein kinase signaling pathway involving the interactions with c-Jun N-terminal kinase (JNK) whose actions can be modulated by GST isozymes (2,18,26). In general, the effects of siRNAs cannot discriminate the roles of these two ways, since siRNA reduced the protein expression levels of GST isozymes, and thus decreased both their catalytic actions and their interactions with signaling proteins involved in cell apoptosis. In fact, GSTs are a family of detoxification enzymes that can be upregulated in response to xenobiotics stress (4,5), and putatively catalyze the conjugation of cytotoxic drugs with GSH to drive the efflux of their conjugates through transporters (2,6,7). With regard to biotransformations, GSTP1 and GSTA1 preferrentially act on DDP and doxorubicin, while GSTM1 has stronger actions on nitrosourea and nitrogen mustards (4-7,27). In cancer cells, DDP produces a toxic product of lipid peroxidation, 4-hydroxyheptanene (4HNE), which at high levels activates JNK and caspase-3 to induce apoptosis (28). When GSH is heavily consumed, 4HNE acts as a small electrophile to form conjugates with proteins and DNA that also induce apoptosis (29). Notably, various studies on GSTA subtypes have suggested that they regulate the physiological effects of oxidative stress by limiting the actions of 4HNE (28,30). For instance, GSTA1-1 and GSTA2-2 were found to limit the formation of 4HNE by catalyzing the metabolism of lipid hydroperoxides that were the precursors of 4HNE, and GSTA4-4 was identified to reduce 4HNE levels via the direct catalysis on the conjugation of 4HNE and GSH (4,28,30). As a result, the reductions of 4HNE-mediated cell apoptosis may account principally for the catalysis-dependent mechanism of GSTA1 in DDP resistance, indicating that GST isozymes are indirect determinants of DDP resistance of solid cancer cells. On the other hand, there has also been increasing evidence to suggest that GST isozymes are direct determinants of drug resistance of solid cancer cells since such enzymes as proteins are components in protein-protein interaction networks regulating cell apoptosis (3,4,18). The formation of GSTP1-JNK-c-Jun and GSTA-JNK complexes may inhibit c-Jun phosphorylation and thus inhibit the JNK signaling pathway that promotes cell apoptosis; the binding of GSTP1-1 to TRAF2, and GSTM1-1 to ASK1, as both TRAF2 and ASK1 are the upstream activators of JNK, blocked the MAPK/JNK signaling cascade that promotes apoptosis of cancer cells (3,4,27,31). The interactions of GSTP or GSTA with signaling proteins can be disrupted upon ligand binding in the active sites of GSTs through the induced changes of GST conformations. For example, membrane-permeable GST inhibitor 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)-hexanol (NBDHEX) (32) and GSTP1 inhibitors TLK199 and TLK286 have been found to be bound in the active sites of GSTs and induce cell death through both the inhibition of their catalytic activities and the dissociation of the JNK:GSTP1 complex (6,7). Notably, significant allosteric conformation changes in GSTs were observed upon the binding of NBDHEX into their active sites (32,33), indicating that the sensitization actions of isozyme-selective inhibitors of GST on DDP-resistant solid cancer cells may be initiated by allosteric effects upon their bindings to activate signaling pathways promoting cell apoptosis. The action mechanisms of GST isozymes in DDP resistance should thus be associated with both their catalytic detoxification actions on cytotoxic agents and/or small apoptosis signaling mediators, and their direct interactions with signaling proteins in pathways promoting cancer cell apoptosis; the contributions of such two ways may vary with regard to types of solid cancers and cytotoxic agents.

The development of selective GST isozyme inhibitors as general sensitizers on a wide spectrum of drug-resistant solid cancer cells is surely absorbing in the field. However, no such isozymes have been demonstrated as rational targets. Data in the present study indicated that GSTA1 was involved in DDP resistance of cancer cells originated from the lung, ovary and stomach; GSTA-specific inhibitors may be useful in sensitizing the three tested types of and even other types of DDP-resistant solid cancer cells to DDP. Certainly, other pathways associating with cellular responses to the stress of cytotoxic agents, such as the downregulation of MRP/P-gp and the inhibition of other GST isozymes, may also play important roles in the resistance of cancer cells to cisplatin (3,18); other types of solid cancers resistant to cisplatin and/or other chemotherapy agents still warrant studies of the roles of GST isozymes in drug resistance. The development of GSTA-selective inhibitors bearing demonstrated actions to sensitize diverse DDP-resistant solid cancers are highly desired to support the conclusion in this study and the handling of DDP-resistance of some types of solid cancer in practice. Collectively, with the use of siRNAs to compare the roles of GSTP1, GSTM2 and GSTA1 in three representative cisplatin-resistant solid cancer cells, it is preliminarily concluded that GSTA predominates over the other two isozymes in the DDP resistance of the tested types of solid cancer cells; GSTA may be a rational target for the development of potent isozyme-selective inhibitors to overcome drug resistance of common types of solid cancer.

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Availability of data and materials

The datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

MZ, XH, BX, XW, FL and XY made substantial contributions to the conception, design and intellectual content of this study. MZ and TT performed the experiments, and MZ wrote the manuscript. YJ, LX, WZ, JL, XW and FL made key contributions to the acquisition, analysis, and interpretation of the data. TT, YJ, LX, WZ, JL, XY and FL revised critically the manuscript for important intellectual content. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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