Expression of human glutathione *S*-transferase P1 mediates the chemosensitivity of osteosarcoma cells

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Abstract

Chemoresistance is a major reason that patients with osteosarcoma fail to achieve a lasting chemotherapy response, and it contributes to disease relapse, progression, and death. Human glutathione *S*-transferase P1 (GSTP1), a phase II detoxification enzyme, contributes to chemoresistance in many cancers. However, the role of GSTP1 in osteosarcoma chemoresistance is ill defined. We hypothesized that GSTP1 has cytoprotective effects in human osteosarcoma. To assess this possibility, we used GSTP1 cDNA transfection or RNA interference to overexpress or suppress GSTP1 in osteosarcoma cells, and assessed the cytotoxic effect of chemotherapeutic agents on these cells. Our results showed that GSTP1 expression was up-regulated in osteosarcoma cells when they were treated with doxorubicin or cisplatin. GSTP1 overexpression in SAOS-2 osteosarcoma cells caused the cells to be more resistant to doxorubicin and cisplatin. In contrast, GSTP1 suppression in HOS cells caused more apoptosis and extensive DNA damage in response to doxorubicin and cisplatin. The cytotoxicity assay also showed that GSTP1 suppression caused a 2.5-fold increase in cell growth inhibition resulting from doxorubicin and cisplatin treatments [the IC_{50}s are ~0.16 μmol/L (doxorubicin) and 1.8 μmol/L (cisplatin) for parental HOS versus 0.06 μmol/L (doxorubicin) and 0.75 μmol/L (cisplatin) for GSTP1-silenced HOS]. Moreover, GSTP1 suppression decreased the activation of extracellular signal–regulated kinase 1/2, which is induced by cisplatin and doxorubicin. Taken together, these findings show that GSTP1 contributes to doxorubicin and cisplatin resistance in osteosarcoma, which may be mediated in part by the activation of extracellular signal–regulated kinase 1/2. Targeting of GSTP1 combined with chemotherapy may have synergistic therapeutic effects on osteosarcoma. [Mol Cancer Ther 2007;6(5):1610–9]

Introduction

Osteosarcoma, the most common primary malignant bone tumor, occurs mainly during childhood and adolescence. Although the combination of surgery and aggressive adjuvant chemotherapy has improved disease-free and overall survival for patients with osteosarcoma, >90% of the patients who present with metastatic osteosarcoma and 30% to 40% with nonmetastatic disease will experience relapse (1, 2). Salvage chemotherapeutic regimens have had limited success, and the disease-free survival duration has not improved significantly over the past 20 years (1–3). Intrinsic or acquired drug resistance is one underlying mechanism contributing to the failure of chemotherapy to elicit a lasting response in these patients (2).

Human glutathione *S*-transferase P1 (GSTP1), one of the cytosolic GSTs that belong to a major group of the phase II detoxification enzyme superfamilies, detoxifies a wide variety of electrophilic compounds including exogenous xenobiotics such as mutagens, anticancer agents, and their metabolites (4). Therefore, GSTP1 is believed to play an important protective role in tumor cell pathogenesis and survival, and the overexpression of GSTP1 has been linked to chemoresistance of a number of cancers (4, 5).

In addition to its detoxification function, by regulating mitogen-activated protein kinases (MAPK) including c-*Jun*-NH_2-kinase (JNK), p38 MAPK, and extracellular signal–regulated kinase (ERK), GSTP1 is also involved in stress-induced cell survival and death signaling pathways (6–8). GSTP1-mediated JNK inhibition and p38 MAPK and ERK activation might contribute to the protection of cancer cells against oxidative stress (7, 8). In several types of cancers, JNK and ERK1/2 are up-regulated in response to DNA-damaging chemotherapeutic agents, such as cisplatin or doxorubicin (9–13). Activation of JNK induced by cisplatin in ovarian carcinoma cells (9), or by doxorubicin in leukemia cells (10), correlates with increased apoptosis. Activation of the ERK pathway may lead to an antiapoptotic effect. It has been shown that inhibition of ERK activity increases the sensitivity of ovarian carcinoma cells to cisplatin treatment (11, 12).

Studies of GSTP1 expression in osteosarcoma and its association with chemoresistance are rare. One study of GSTP1 expression in samples from pediatric patients with osteosarcoma showed that GSTP1 expression might have a bearing on the outcome of treatment with chemotherapy (14). Another study by Bruheim et al. (15) showed that the GSTP1 mRNA level was inversely correlated with doxorubicin growth inhibition in human osteosarcoma xenografts.

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However, the role that GSTP1 plays in the resistance of osteosarcoma to chemotherapy remains ill defined. In this study, we sought to elucidate this role. We show that the overexpression of GSTP1 in osteosarcoma cells contributes to chemoresistance to doxorubicin and cisplatin, whereas suppression of GSTP1 by gene silencing results in enhanced chemosensitivity. The protective role of GSTP1 in osteosarcoma cell survival may be mediated in part by enhancing the activation of ERK1/2.

Materials and Methods

Reagents

All chemical reagents and chemotherapeutic agents were purchased from Sigma-Aldrich Corp., except when indicated. Cisplatin, doxorubicin, or methotrexate was dissolved in DMSO to make stock solutions at concentrations of 33 mmol/L, 100 μmol/L, or 100 mmol/L, respectively. An equivalent amount of DMSO was present in the control medium. Primer oligonucleotides were synthesized by Sigma-Genosys. The restriction endonucleases were all purchased from New England Biolabs.

Cell Culture and Transfection

Osteosarcoma cell lines (SAOS-2, HOS, MNNG-HOS, TE-85, MG-63, KRIB, and U2-OS) and neuroblastoma cells (HTB-10) were obtained from the American Type Culture Collection. LM-7, a lung metastatic osteosarcoma cell line, was derived from the SAOS-2 cell line by repeating i.v. recycling through the lungs of nude mice seven times (16). The osteosarcoma cell lines OS-187 and WOL were kindly provided by Dr. Dennis Hughes, M.D. Anderson Cancer Center. Normal human osteoblasts (NHOst) were obtained from Clonetics. The SAOS-2, LM-7, OS-187, WOL, and U2-OS cell lines were cultured in DMEM (supplemented with 10% fetal bovine serum, 1 mmol/L sodium pyruvate, 1 × nonessential amino acids, and 2 mmol/L glutamine). HTB-10 and the other osteosarcoma cell lines were cultured in Eagle's minimal essential medium (supplemented with 10% fetal bovine serum, 1 mmol/L sodium pyruvate, 2× minimal essential medium vitamins, 1× nonessential amino acids, and 2 mmol/L glutamine). NHOst were cultured in osteoblast growth medium (Clonetics) containing 10% FCS and 100 μg/mL ascorbic acid, according to the manufacturer's instructions. All cell lines were incubated at 37°C in humidified 5% CO_2.

Transfection was done with LipofectAMINE 2000 Reagent (Invitrogen), according to the manufacturer's instructions. Stable transfected single cell colonies were selected by incubation of the cells in either 200 μg/mL of hygromycin B for HOS cells transfected with pSilencer plasmids or 500 μg/mL of G418 for SAOS-2 cells transfected with pDsRed2 plasmids. The positive stable transfectants were confirmed by Western blot analysis.

Reverse Transcription-PCR

A reverse transcription-PCR (RT-PCR) was performed to detect the mRNA level of GSTP1 expression, as described previously (17), with modification. Briefly, total RNA was isolated and purified from cultured cells using Trizol Reagent (Invitrogen), and reverse transcription was done using a reverse transcription system kit with oligo-dT primer (Promega Corporation) according to the manufacturer's instructions. PCR was then done using an iTaq DNA polymerase kit (Bio-Rad Laboratories) with the primers 5′-acgtggcaggagggctcactc-3′ (forward) and 5′-tactcagggaggccaggaa-3′ (reverse). The glyceraldehyde-3-phosphate dehydrogenase mRNA level was detected as an internal control for normalization. Densitometric analysis was used to calculate the relative expression of GSTP1.

Construction of GSTP1 Expression Plasmids

RT-PCR was performed to amplify GSTP1 Ile^{105} full-length cDNAs from HOS cells. PCR primers were 5′-ccaagcttaccatgccgccctacacc-3′ (forward) and 5′-ccggatcctgtttcccgttgccat-3′ (reverse), with *Bam*HI and *Hin*dIII restriction endonuclease recognition sites (underlined) on the ends. PCR was performed in 50 μL at 95°C for 2 min, 35 cycles at 95°C for 30 s, 58°C for 1 min, and 72°C for 1 min, and followed by 72°C for 10 min. PCR products were subcloned into the pCR2.1 plasmids (Invitrogen) and expanded in DH5 alpha cells (Invitrogen). Full-length GSTP1 cDNA fragments digested from the pCR2.1 by restriction endonuclease were then ligated to the mammalian expression plasmid pDsRed2-N1 (Clontech) using T4 DNA ligase (Promega). GSTP1 cDNAs subcloned into this vector were expressed as fusions to the NH_2 terminus of DsRed2 (red fluorescent protein). GSTP1 sequences were confirmed by DNA sequencing.

Construction of Small Interfering RNA Expression Plasmids

Five small interfering RNA (siRNA) sequences targeting human GSTP1 mRNA were selected on the basis of the following sequences published in Genbank (gi:6552334): 339 to 357 tacatctccctcatctaca (GSTP1−339si); 377 to 395 ggatgactatgtgaaggca (GSTP1−377si); 451 to 469 agaccttcattgtgggaga (GSTP1−451si); 475 to 493 tctccttcgctgactacaa (GSTP1−475si); and 509 to 527 gctgatccatgaggtccta (GSTP1−509si). The numbers before the sequences indicate the encoding region of the GSTP1 mRNA nucleotide. pSilencer 2.1-U6 hygro plasmid (Ambion) was used for the construction of vector-expressing hairpin siRNA to suppress GSTP1 expression. Five pairs of DNA oligonucleotides were chemically synthesized in accordance with the manufacturer's instructions. The construction procedures and the siRNA control vector used have been described previously (18). All constructs were confirmed by DNA sequencing.

Western Blotting

Cell lysates were prepared using either cell lysis buffer [20 mmol/L HEPES (pH 7.4), 1% Triton X-100, and 10% glycerol] or radioimmunoprecipitation assay lysis buffer (fresh 10 mmol/L sodium fluoride and 1 mmol/L sodium vanadate, added for JNK and ERK1/2 assays) containing 1× protease inhibitor (Calbiochem) and were collected by centrifugation at 12,000 × *g*, at 4°C. Total protein concentration was measured using the bicinchoninic acid assay kit (Bio-Rad Laboratories) with bovine serum albumin as a standard. The protein was denatured by

boiling for 10 min in the presence of the sample buffer [0.5 m Tris (pH 6.8), 10% glycerol, 10% SDS, 5% 2-mercaptoethanol, and 1% bromophenol]. Protein (30 μg) was electrophoresed by SDS-PAGE on a 10% or 12% polyacrylamide separating gel for 90 min at 100 V. After the protein was transferred onto a polyvinylidene difluoride membrane (Millipore), the membrane was first blocked with 5% powdered skim milk in TBST [138 mmol/L NaCl, 2.7 mmol/L KCl (pH 7.4), and 0.1% Tween 20] for 1 h, and then the protein was detected with antihuman polyclonal GSTP1 (Lab Vision Corporation), GSTM1, GSTA1 (Alpha Diagnostic), antibodies, antihuman phospho-JNK, phospho-ERK1/2 antibodies (Cell Signaling Technology), or polyclonal tubulin and monoclonal actin antibodies (Sigma-Aldrich Corp.) overnight at 4°C. The levels of total JNK and total ERK1/2 were measured by reprobing the blots or probing the blots obtained from the same amount of cell lysates with monoclonal antibodies against total JNK and ERK1/2 (Cell Signaling). The second antibody was visualized using an enhanced chemiluminescence detection Western blotting analysis system (Amersham Pharmacia Biotech). Densitometric analyses were performed to quantify Western blotting signals and normalized against that of actin or tubulin.

Cytotoxicity Assay

The [^{3}H]thymidine incorporation assay has been described previously (19). Briefly, osteosarcoma cells were seeded in flat-bottomed 96-well plates at a density of 3,000 (HOS) or 5,000 (SAOS-2) cells/well and allowed to adhere overnight. The cells were treated with chemotherapeutic agents for 48 h and labeled with 0.2 Ci/well of [^{3}H]thymidine (ICN Radiochemicals) during the last 24 h. Triplicate wells were used for each treatment group. Incorporation of the radioactive tracer was quantified, and the percentage of cytostasis was calculated as follows: $[(A - B) / A] \times 100$, where A is the number of counts per minute of cells treated with medium alone and B is the number of counts per minute of cells treated with drugs. IC_{50} was then calculated on the basis of these cytotoxicity results.

Apoptosis Detection

Osteosarcoma cells (10^5) were seeded in each well of a six-well plate and allowed to adhere overnight. After treating with chemotherapeutic agents for 72 h, these cells were harvested by trypsinization, labeled with Annexin V and PI using an apoptosis detection kit (BD PharMingen) according to the manufacturer's instructions, and subsequently analyzed using FACScan (Becton Dickinson and Company).

GST Activity Assay

GST enzyme activity was determined by measuring the enzyme's ability to catalyze the conjugation of reduced glutathione with 1-chloro-2,4-dinitrobenzene (20). A GST activity assay kit was purchased from Cayman Chemical. In accordance with the manufacturer's instructions, cell lysates (50 μL) containing 10^7 cells were applied to each reaction. The conjugation resulted in an increase in absorbance at 340 nm. The rate of increase was proportional to the GST activity in the cell lysate samples.

Glutathione Concentration Measurement

A colorimetric assay kit from Cayman Chemical was used to measure the concentrations of intracellular reduced glutathione (GSH) and oxidized glutathione (GSSG). Cells were harvested by gentle scraping and lysate samples were prepared by sonication in MES buffer [0.2 mol/L 2-(*N*-morpholino)ethanesulfonic acid, 50 mmol/L phosphate, and 1 mmol/L EDTA (pH 6.0)]. The assay was done according to the manufacturer's instructions and the absorbance was measured at 5-min intervals for 30 min at 414 nm using a plate reader (Molecular Devices Corp.). The GSH concentration values were calculated and normalized to the protein concentration within each sample. GSH was obtained by subtracting GSSG from total GSH, and the GSSG/GSH ration was calculated.

Alkaline Comet Assay

The extent of DNA damage caused by chemotherapeutic agents in the osteosarcoma cells was measured by an alkaline comet assay (single cell gel electrophoresis), as described previously (21, 22). Briefly, cells embedded in 0.5% agarose gel were spread on a microscope slide precoated with 1% agarose gel. After the cells were lysed with lysing solution [2.5 mol/L NaCl, 10 mmol/L Tris-HCl, 100 mmol/L edetate disodium, 1% Triton, and 10% DMSO (pH 10)] for 1 h at 4°C, the nuclei left on the slides were exposed to the electrophoresis buffer [0.3 mol/L NaOH and 1 mmol/L EDTA (pH 13.5)] for 20 min at 4°C followed by alkaline gel electrophoresis at 300 mA, at 4°C for 20 min. After electrophoresis, DNA was visualized by SYBR Green l (BD PharMingen) staining. The tail moment, which reflected the DNA fragments resulting from DNA damage, was observed under a fluorescence microscope of Nikon Eclipse TE2000-U (Nikon, Japan). The images were captured with a charge-coupled device camera connected to a computer and were analyzed using Scion image software (Scion Corporation). Tail moment was calculated as the tail length multiplied by the fraction of DNA in the comet tail.

Statistical Analysis

Data are expressed as means ± SD. Analysis was done using ANOVA and the two-tailed Student's t test, with $P < 0.05$ being considered statistically significant.

Results

GSTP1 Overexpression in Human Osteosarcoma

As determined by Western blotting, intrinsic GSTP1 expression was found in all 10 available osteosarcoma cell lines. Eight showed strong expression compared with the NHOst in which GSTP1 expression was weakly detectable (Fig. 1).

GSTP1 Expression Was Inducible by Cisplatin and Doxorubicin

DNA-damaging chemotherapeutic agents, such as cisplatin and doxorubicin, are commonly used in chemotherapeutic regimens for osteosarcoma. To investigate how GSTP1 responds to treatment with cisplatin and doxorubicin in osteosarcoma, Western blotting and semiquantitative RT-PCR were done to assess GSTP1 expression in SAOS-2 and

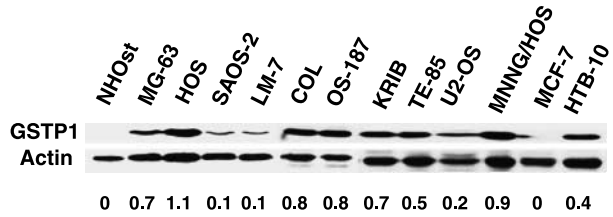

Figure 1. GSTP1 expression in human osteosarcoma cell lines. Whole-cell protein was extracted from the cells of NHOst, 10 human osteosarcoma cell lines, HTB-10 (for positive controls), and MCF (for negative controls). Immunoblotting was performed to detect GSTP1 expressions in the cells. Actin was also detected as a sample loading control.

HOS cells after exposure to these agents. As shown in Fig. 2, GSTP1 expression in SAOS-2 cells treated with doxorubicin or cisplatin was up-regulated at both the mRNA levels (Fig. 2A, *top*) and protein levels (Fig. 2B, *top*). In HOS cells, the GSTP1 protein levels (Fig. 2B, *bottom*) were also increased after 12-h exposure to doxorubicin and cisplatin; however, the mRNA levels (Fig. 2A, *bottom*) did not show an obvious change. In contrast, there was no inducible GSTP1 expression in SAOS-2 or HOS cells treated with methotrexate, a non–DNA-damaging chemoagent, for 48 or 24 h, respectively. The GSTP1 decrease at 72 h is likely a result of cellular cytotoxicity caused by methotrexate treatment.

GSTP1 Overexpression Reduced the Chemosensitivity of Osteosarcoma Cells

We have examined the polymorphic phenotype of human GSTP1 allelic variants expressed in the osteosarcoma cell lines and the results showed that GSTP1 Ile^{105} was the allele predominantly expressed in the osteosarcoma cell lines (data not shown). GSTP1 Ile^{105} was thus selected for overexpression. pDsRed2-GSTP1 Ile^{105} was subcloned (data not shown) and transfected into the SAOS-2 cells that have intrinsically low endogenous GSTP1 expression. The stable single cell colonies were then selected by exposing the cells to 500 μg/mL of G418 for 3 months. Western blot analysis (Fig. 3A) and fluorescence microscopy detection (data not shown) confirmed the overexpression of the GSTP1 protein in the SAOS-2 transfectants. This increased expression of GSTP1 protein resulted in a 3- to 4-fold increase in GST enzyme activity compared with SAOS-2 cells transfected with vector alone (Fig. 3B). Parental SAOS-2 cells and transfectants were then used to assess the effect of GSTP1 on chemoresistance. As measured by a [^{3}H]thymidine incorporation assay, the SAOS-2 transfectant of GSTP1 had an ~2-fold increase in IC_{50} for the 48-h treatment of doxorubicin and cisplatin, compared with the IC_{50} of the parental and vector-transfected SAOS-2 cells (Table 1).

RNA Interference Suppressed GSTP1 Expression in HOS Cells

RNA interference (RNAi) is a powerful technique for silencing specific gene expression and has a potential use for therapy. To further investigate the role of GSTP1 in osteosarcoma chemosensitivity, the HOS cell line, which expressed high endogenous GSTP1, was transfected with an individual pSilencer plasmid construct designed to express GSTP1-siRNAs. Stable single-cell colonies of transfected HOS cells were then selected by exposure to 200 μg/mL of hygromycin for 2 weeks. As shown in Fig. 4A, GSTP1 protein expression in three colonies transfected with GSTP1−339si and three colonies transfected with GSTP1−377si were decreased (GSTP1−339si resulted in >90% decrease). In cells transfected with a control vector, GSTP1 expression was unchanged compared with GSTP1 expression in parental HOS cells. The HOS sublines of HOS-339si-3, -6, and -10 were used in subsequent experiments. The mRNA level (Fig. 4B) and activity of GSTP1 (Fig. 4C) in HOS-339si-3, -6, and -10 were also decreased. The GSTP1-339si targets the sequences that contain an encoding region specifically for GSTP1 Ile^{105}, an allele expressed in HOS. In addition, GSTP1-339si transfection did not affect the expression of other GST isoforms (GSTM1 and GSTA1) in HOS cells (Fig. 4D). Taken together, these results imply that GSTP1-339si was able to suppress GSTP1 expression in HOS cells specifically.

GSTP1 Suppression Increased DNA Damage, Apoptosis, and Cytotoxicity of HOS Cells by Doxorubicin and Cisplatin

The DNA-damaging effects of cisplatin result from its interaction with DNA to form DNA adducts, either interstrand or intrastrand cross-links (23), whereas doxorubicin

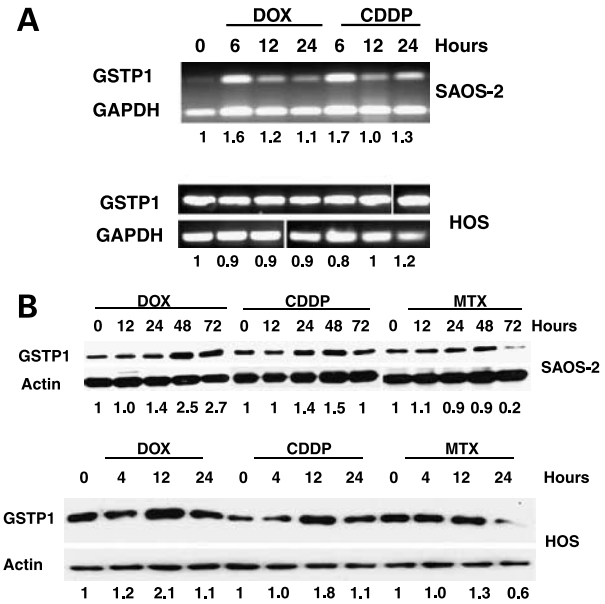

Figure 2. GSTP1 expression was inducible in human osteosarcoma cell lines by doxorubicin (*DOX*) and cisplatin (*CDDP*) treatments. SAOS-2 and HOS osteosarcoma cells were treated with 0.1 μmol/L of doxorubicin, 5 μmol/L of cisplatin, or 100 μmol/L of methotrexate (*MTX*) for the indicated times. **A,** total RNA was extracted from treated and untreated osteosarcoma cells. GSTP1 mRNA expression in the SAOS-2 cells (*top*) or HOS cells (*bottom*) was quantified by RT-PCR, and the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA level was used as an internal control. The relative expression of GSTP1 was normalized against glyceraldehyde-3-phosphate dehydrogenase by densitometric analysis. **B,** whole-cell protein, extracted from SAOS-2 cells (*top*) or HOS cells (*bottom*) was subjected to immunoblotting analyses for GSTP1 expression. The relative expression of GSTP1 was normalized with actin by densitometric analysis.

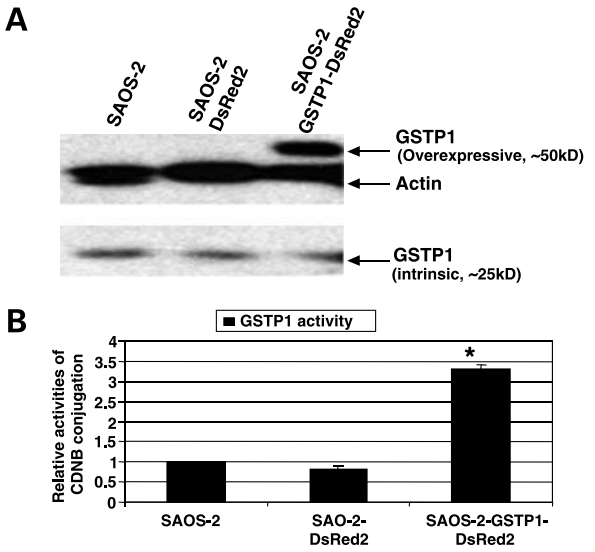

Figure 3. Overexpression of GSTP1 in SAOS-2 cells. **A,** SAOS-2 cells were stably transfected with GSTP1 cDNA (*pDsRed2-GSTP1*) or vector (*pDsRed2*). Whole-cell protein extracts were used for immunoblotting analyses. Overexpression of GSTP1 protein (fusion with the fluorescent protein DsRed2, ~50 kDa) in SAOS-2 cells after transfection was confirmed by Western blotting. The intrinsic GSTP1 (~25 kDa) was expressed at low levels in both parental cells and transfectants. **B,** GST activities of conjugation of reduced GSH with 1-chloro-2,4-dinitrobenzene (*CDNB*) were measured as described in Materials and Methods. As GSTP1 activity of parental SAOS-2 cells was considered as 1, the relative GSTP1 activity of the transfectants was normalized (*, $P < 0.01$).

acts via the induction of either single- or double-stranded DNA breaks by stabilizing the topo II-DNA complex (24). The alkaline comet assay indicated that the GSTP1-silenced HOS cells were more sensitive to doxorubicin-induced DNA strand breaks, as shown by more DNA tail moments (Fig. 5A). Because cisplatin induced DNA cross-linking adduction, which would decrease DNA tail moments, the cisplatin-treated HOS cells were exposed to cesium irradiation to break the DNA, and then the alkaline comet assay was performed. The results showed that GSTP1-silenced HOS cells had decreased DNA tail moments, which could reflect the more extensive cross-linking of DNA induced by cisplatin. Moreover, as shown in Fig. 5B, GSTP1-silenced HOS cells were more sensitive to apoptosis induced by doxorubicin or cisplatin than were HOS cells expressing GSTP1. As measured by the [^{3}H]thymidine incorporation assay (Table 1), the HOS-339si cells were more sensitive to the treatment of doxorubicin (IC_{50} ~0.06 μmol/L) or cisplatin (IC_{50} ~0.7 μmol/L) than the parental HOS (IC_{50} ~0.16 μmol/L) or HOS-control cells (IC_{50} ~1.8 μmol/L). These results suggested that GSTP1 suppression amplifies the DNA damage induced by doxorubicin and cisplatin, and results in increased apoptosis and cytotoxicity in osteosarcoma cells.

GSTP1 Suppression Decreases the Level of Phospho-ERK1/2 Induced by Doxorubicin and Cisplatin

JNK and ERK1/2 MAPK pathways are involved in the cellular response of osteosarcoma to the treatments of cisplatin and doxorubicin. The activation of ERK1/2 or JNK was measured by phosphorylation via Western blot analysis. As shown in Fig. 6, doxorubicin and cisplatin were able to up-regulate activated forms of phospho-JNK and phospo-ERK1/2 in parental, HOS-control, and HOS-339si-3 cells as expected. Interestingly, GSTP1 suppression in HOS-339si-3 resulted in decreases of the levels of phospho-ERK1/2 induced by cisplatin and doxorubicin (Fig. 6A). However, GSTP1 did not alter the levels of phospho-JNK induction (Fig. 6B). These results suggest that the protective roles of GSTP1 against apoptosis triggered by doxorubicin and cisplatin may be mediated by the activation of ERK1/2 rather than JNK in HOS cells.

Doxorubicin or Cisplatin Causes Oxidative Stresses in HOS Cells

Either doxorubicin or cisplatin could lead to intracellular oxidative stress (25), which may serve to induce MAPK activations (26). We thus assayed the intracellular oxidative stress caused by doxorubicin or cisplatin treatment via determining intracellular GSH and GSSG levels in HOS cells. In untreated HOS-control and HOS-339si-3 cells, GSH concentrations were similar (30.77 ± 5.03 versus 30.7 ± 0.11 nmol/mg protein), whereas GSSG concentrations were very low or were not detectable in some experiments. As shown in Fig. 6C–F, either 0.5 μmol/L of doxorubicin or 5 μmol/L of cisplatin treatment for 2 to 12 h could cause a decrease in the GSH level and an increase in the GSSG/GSH ratio in both HOS-control and HOS-339si-3 cells, indicating the generation of oxidative stress by doxorubicin or cisplatin. HOS-339si-3 cells treated with cisplatin have a higher GSSG/GSH ratio than that in HOS-control cells; however, no significant difference in the GSSG/GSH ratio was observed between HOS-control and HOS-339si-3 cells treated with doxorubicin. These results suggest that suppression of GSTP1 in HOS-339si-3 cells was responsible for higher-level oxidative stress caused by cisplatin rather than by doxorubicin. In addition, although doxorubicin causes the same levels of intracellular

Table 1. Cytotoxic effect of doxorubicin and cisplatin on SAOS-2 and HOS cells

	IC_{50} (μmol/L)	
	Doxorubicin	Cisplatin
SAOS-2	0.06	1.1
SAOS-2-DsRed2	0.05	1.3
SAOS-2-GSTP1-DsRed2	0.13*	2.5*
HOS	0.16	1.8
HOS-control	0.15	1.5
HOS-339si-3	0.06†	0.7†
HOS-339si-6	0.05†	0.75†

*Differences between SAOS-2-GSTP1-DsRed2 and SAOS-2 or SAOS-2-DsRed2 were considered statistically significant at $P < 0.05$.
†Differences between HOS-339si and HOS or HOS-control were considered statistically significant at $P < 0.05$.

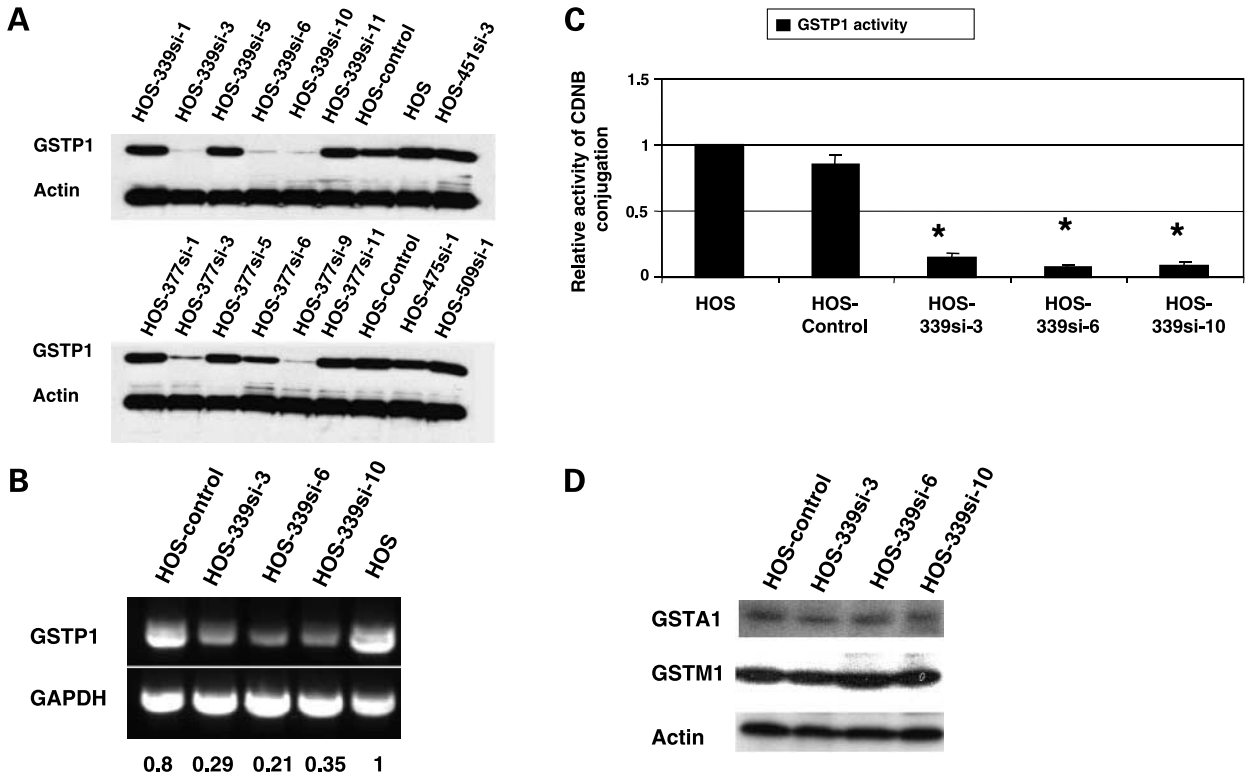

Figure 4. GSTP1 expression was suppressed by vector-based siRNA transfection in HOS cells. **A,** HOS cells were stably transfected with pSilencer constructs (*GSTP1–339si, 377si, 451si, 475si, 509si*, or *control*). GSTP1 suppression in stable colonies resulting from transfection of 339si (*HOS-339si-3, -6, -10*) or 377si (*HOS-377si-3, -6, -9*) was confirmed by immunoblotting analyses. **B** and **C,** GSTP1 mRNA levels and GST activities for parental HOS and transfectants were determined by RT-PCR and an activity assay as described in Materials and Methods. The relative levels of GSTP1 mRNA and GST activity were shown and compared with those of parental HOS and HOS-control. *Columns,* mean; *bars,* SD; *, $P < 0.01$. **D,** the expression of GSTA1 or GSTM1 (other GST isoforms) in HOS cells was not affected by GST-339si transfection.

oxidative stresses between HOS-control and HOS-339si-3 cells, the ERK1/2 activation induced by doxorubicin in HOS-control cells was higher than that in HOS-339si-3 cells.

Discussion

Our findings show that the overexpression of GSTP1 in osteosarcoma cells contributes to chemoresistance to doxorubicin and cisplatin, which may be mediated by the induction of phospho-ERK1/2. Indeed, GSTP1 was overexpressed and could be up-regulated by doxorubicin and cisplatin in osteosarcoma cell lines. The forced expression of GSTP1 enhanced the chemoresistance of SAOS-2 cells to doxorubicin and cisplatin. RNAi-mediated GSTP1 suppression enhanced HOS cell sensitivity to doxorubicin- or cisplatin-induced DNA damage, apoptosis, and cytotoxicity. Furthermore, GSTP1 suppression decreased the level of phospho-ERK 1/2 induction by cisplatin and doxorubicin.

GSTP1 has been associated with the chemoresistance and poor overall survival of patients with several types of cancer. Specifically, it has been reported that GSTP1 contributes to cisplatin resistance in ovarian cancer (27), colon cancer (28), head and neck cancer (29), breast cancer (30), and lung cancer (31) as well as to doxorubicin resistance in ovarian cancer (27) and laryngeal carcinoma cell lines (32). Because doxorubicin and cisplatin are commonly used for treating osteosarcoma, the overexpression of GSTP1 detected in all of our osteosarcoma cell lines strongly suggests that GSTP1 also contributes to chemoresistance in osteosarcoma.

The mechanism by which GSTP1 contributes to chemoresistance to doxorubicin and cisplatin remains unclear. Recently, several studies have shown that GSTP1 participates in the MAPK pathways by either inhibiting JNK phosphorylation or augmenting ERK1/2 and p38 MAPK phosphorylations (6–8), protecting the cells from cell death or apoptosis (7, 8). Because chemotherapeutic DNA-damaging agents such as doxorubicin and cisplatin induce phosphorylations of ERK, JNK, and p38 MAPK, it was expected that GSTP1 would modulate these MAPK activities in osteosarcoma cells treated with doxorubicin and cisplatin, which would in turn affect chemoresistance.

Indeed, both ERK1/2 and JNK phosphorylations were induced by the treatment of either doxorubicin or cisplatin in a time-dependent manner in HOS osteosarcoma cells. Compared with parental and HOS-control cells, HOS-339si-3 cells with GSTP1 suppression by RNAi had a lower level of ERK1/2 phosphorylation after 2- to 12-h treatments with

cisplatin or doxorubicin. However, the levels of JNK phosphorylation induced by doxorubicin and cisplatin treatment were increased equally in parental, HOS-control, and HOS-339si-3 cells. It is known that either cisplatin or doxorubicin treatment leads to cellular oxidative stress (25). The accumulation of reactive oxygen species caused by UV light, H_2O_2, or certain chemotherapeutic compounds could induce the activation of multiple stress kinase cascades including ERK and JNK signaling pathways (7, 26). Our data also showed that cisplatin or doxorubicin could cause oxidative stress in HOS cells. Therefore, the activations of ERK1/2 and JNK in HOS cells may be induced by oxidative stress resulting from doxorubicin or cisplatin treatment. However, the extent of ERK1/2 activation is not correlated to intracellular oxidative stress in HOS cells treated with doxorubicin. We assume that the oxidative stress generated in HOS cells may serve to initiate, but not sustain, ERK1/2 and JNK activation. The effect of GSTP1 on increasing ERK1/2 activation may be independent of oxidative stress, and its mechanism needs further investigation. On the other hand, it has been shown that GSTP1 inhibits JNK activation by direct protein-protein interactions of GSTP1 and JNK in nonstressed cells. Under conditions of oxidative stress; however, the dissociation of GSTP1-JNK complex occurs and the effect of GSTP1 on the inhibition of JNK activation is reversed (6). Thus, our results show that GSTP1 did not inhibit JNK activation in HOS cells treated with cisplatin or doxorubicin, which may be due to the dissociation of GSTP1-JNK complex resulting from oxidative stress caused by chemotherapeutic agents.

Overall, the results of this study show that GSTP1 modulates ERK1/2 rather than JNK activation in HOS osteosarcoma cells triggered by doxorubicin or cisplatin. Conversely, GSTP1 suppression in HOS-339si cells resulted in increased cell cytotoxicity and apoptosis in response to doxorubicin and cisplatin. These results suggest that the protective role of GSTP1 in osteosarcoma cell survival may be mediated in part by promoting the activation of ERK1/2.

GSTP1 expression can be regulated by an epigenetic mechanism. The hypermethylation of CpG islands in the GSTP1 gene promoter results in down-regulation of GSTP1 expression in prostate cancers (33). On the other hand, GSTP1 expression can be up-regulated by certain chemical agents (34), chemoagents (35), and oxidants (hydrogen peroxide; ref. 36). In our study, GSTP1 protein expression was elevated in SAOS-2 and HOS cells and the GSTP1 mRNA level was also elevated in SAOS-2 cells while the cells were being treated with 0.1 μmol/L of doxorubicin or 5 μmol/L of cisplatin, although there was no change of GSTP1 mRNA level in HOS cells, which may be due to the higher level of intrinsic GSTP1 mRNA in HOS cells so that RT-PCR was unable to detect the change of up-regulated GSTP1. Therefore, it seems that elevated GSTP1 expression in osteosarcoma in response to chemotherapy represents a mechanism of acquired drug resistance for osteosarcoma. Several transcription factors, including activator protein 1, nuclear factor κB, and nuclear factor E2–related factor 2, which are responsible for cellular oxidative stress, have been shown to be involved in GSTP1 gene up-regulation (35, 37–39). Oxidative stress resulting from treatments with the DNA-damaging agents doxorubicin and cisplatin, rather than methotrexate, may provide an explanation for GSTP1 induction in osteosarcoma cells. In addition, Usami et al. (40) reported that down-regulation of the proteosome by either inhibitor or RNAi resulted in the induction of GSTP1 and activation of MAPKs and suggested that JNK was likely responsible for the GSTP1 induction. Our results also showed that cisplatin and doxorubicin induced both

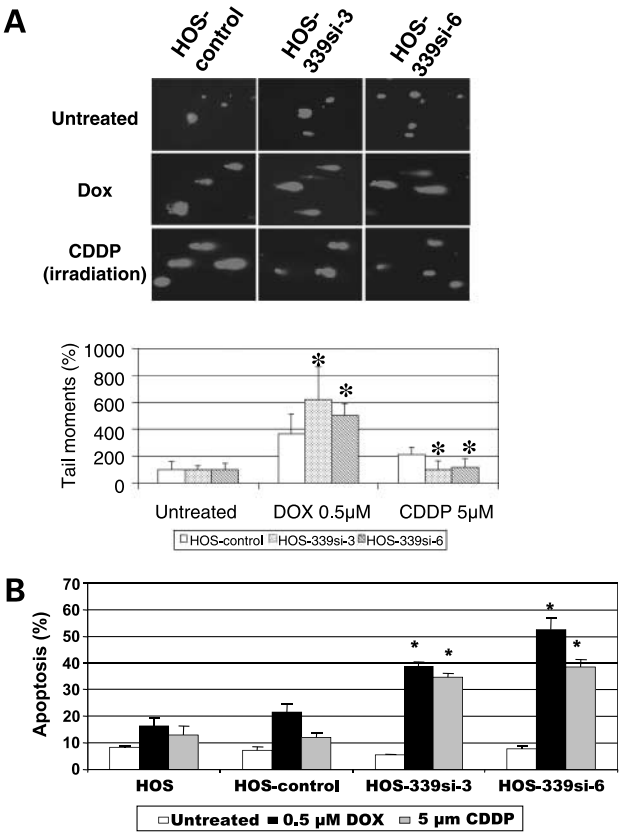

Figure 5. GSTP1 suppression increased DNA damage and apoptosis by doxorubicin (*DOX*) or cisplatin (*CDDP*) in HOS cells. **A** and **B,** parental HOS cells and transfectants were treated with doxorubicin or cisplatin at the indicated concentrations for 48 h. **A,** a comet assay was done to assess DNA damage (*top*), as described in Materials and Methods. Before undergoing electrophoresis, the cells harvested after cisplatin treatment were exposed to cesium irradiation at 15 Gy to break DNA that was cross-linked by cisplatin, whereas the cells harvested after doxorubicin treatment were subjected to electrophoresis directly. SYBR green staining visualized DNA, and DNA strand breakage or cross-linking was determined by assessing the DNA tail moment. The extent of the DNA breakage or cross-linking was quantified by assessing the tail moment (*bottom*). The percentage of tail moments for the cells treated with doxorubicin or cisplatin was normalized to that of untreated cells, which was expressed as 100%. Twenty to 30 cells were scored for each treatment. *Columns,* mean; *bars,* SD; *, $P < 0.05$ when compared with HOS-control cells treated with doxorubicin or cisplatin. **B,** cells were collected by trypsinization and then labeled with Annexin V-FITC and propidium iodide. Apoptotic cells staining with Annexin V and propidium iodide were determined by fluorescence signals of Annexin V-FITC and propidium iodide on a flow cytometer.

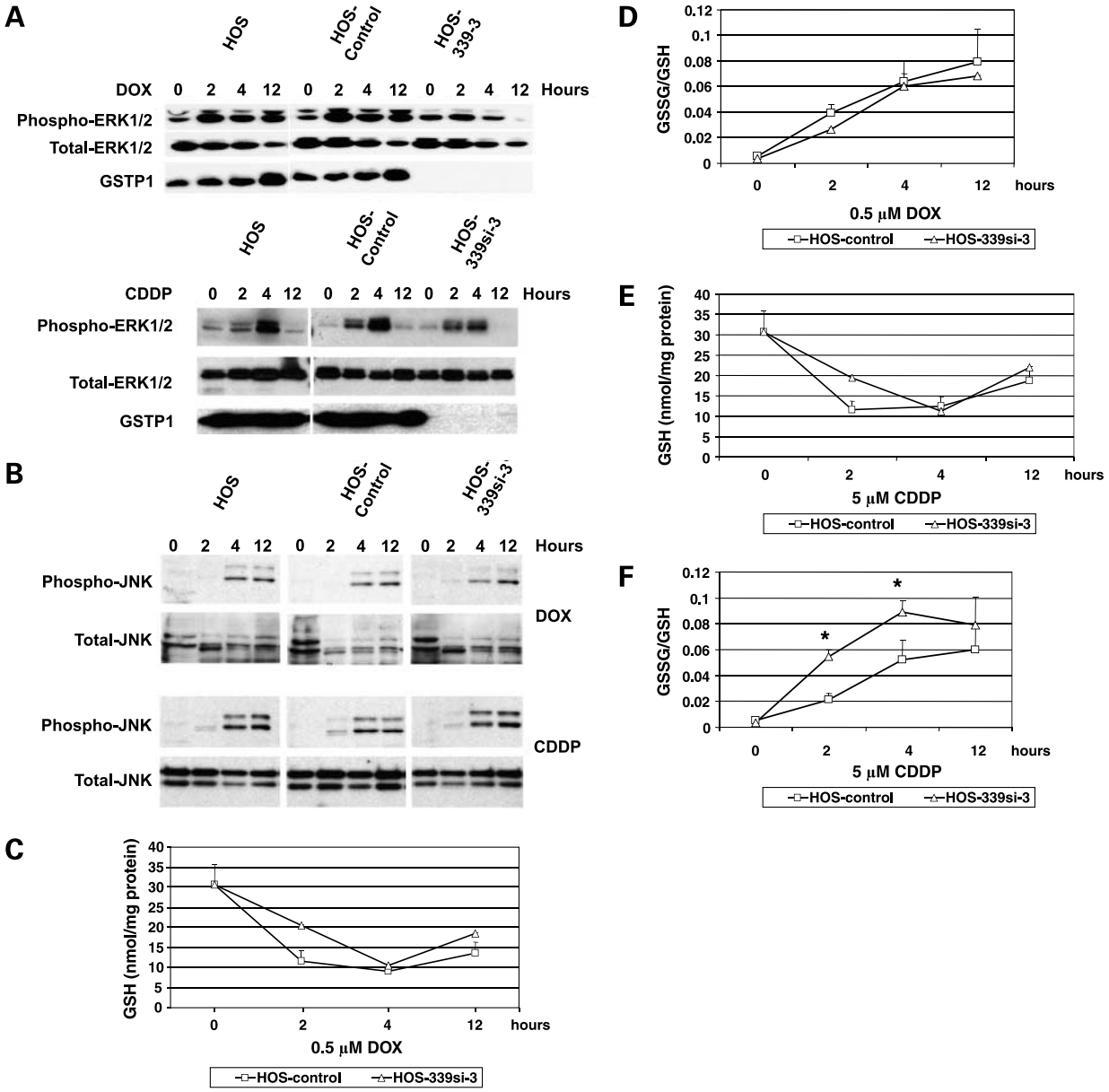

Figure 6. Doxorubicin (*DOX*) or cisplatin (*CDDP*) induced ERK1/2 and JNK phosphorylations and oxidative stress in HOS cells. Cells were treated with 0.5 μmol/L of doxorubicin or 5 μmol/L of cisplatin for the indicated times. Cell lysates were subjected to immunoblotting analyses or assay for GSH and GSSG, as described in Materials and Methods. **A,** phospho-ERK1/2 was induced by treatment with doxorubicin (*top*) or cisplatin (*bottom*) in parental, HOS-control, and HOS-339si-3 cells. The inductions of phospho-ERK1/2 were decreased in the HOS-339si-3 cells in which GSTP1 expression was suppressed compared with that in HOS or HOS-control cells. **B,** treatment of HOS, HOS-control, and HOS-339si-3 cells with doxorubicin or cisplatin increased phospho-JNK by 4 to 12 h. However, the inductions of phospho-JNK were similar in all cell lines and independent on GSTP1 expression. **C** to **F,** GSH and GSSG concentrations were measured, and the GSSG/GSH ratio was calculated. The concentrations are expressed as nmol of GSH equivalents/mg of protein. GSSG concentration and GSSG/GSH ratio were considered as 0 when GSSG was undetectable. The levels of intracellular GSH and GSSG/GSH ratio in HOS-control and HOS-339si-3 cells untreated or treated with doxorubicin or cisplatin for 2 to 12 h are shown. *Columns,* mean; *bars,* SD; *, $P < 0.05$.

GSTP1 expression and activation of JNK and ERK1/2 in HOS osteosarcoma cells. Collectively, we assume that both cellular oxidative stress and activation of the MAPK signaling pathway in response to cisplatin and doxorubicin treatments may play roles in GSTP1 induction in osteosarcoma cells.

Inhibiting GSTP1 has emerged as a therapeutic strategy for overcoming chemoresistance. GSTP1 inhibitor TER 199 (4), a glutathione analogue, has been used to increase the chemosensitivity of tumor cells in patients. Turella et al. (41) reported that a glutathione *S*-transferase inhibitor, 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol, had

cytotoxic effects on tumor cells by triggering apoptosis and thus was a promising anticancer drug. RNAi has also emerged as a potential therapy for various diseases, including cancers, by silencing specific pathologic genes. Our findings showed that a vector-based RNAi was capable of silencing GSTP1 expression almost completely in HOS cells at the protein level, resulting in HOS cells that were more sensitive to doxorubicin and cisplatin. These results suggest that the RNAi of GSTP1 combined with chemotherapeutic agents have synergistic effects against osteosarcoma.

In conclusion, we showed here that GSTP1 was overexpressed and inducible in osteosarcoma cells, and increased GSTP1 expression enhanced ERK1/2 activation and drug resistance of osteosarcoma cells in response to doxorubicin and cisplatin. Moreover, suppression of GSTP1 by RNAi enhanced the osteosarcoma cells' sensitivity to doxorubicin and cisplatin. These findings suggest that GSTP1 contributes to osteosarcoma chemoresistance and that the targeting of GSTP1 by RNAi combined with chemotherapy provides a potential synergistic therapy for patients with osteosarcoma.

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