Suppression of Glucosylceramide Synthase Restores p53-Dependent Apoptosis in Mutant p53 Cancer Cells

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Abstract

Tumor suppressor p53 plays an essential role in protecting cells from malignant transformation by inducing cell-cycle arrest and apoptosis. Mutant p53 that is detected in more than 50% of cases of cancers loses its role in suppression of tumors but gains in oncogenic function. Strategies to convert mutant p53 into wild-type p53 have been suggested for cancer prevention and treatment, but they face a variety of challenges. Here, we report an alternative approach that involves suppression of glucosylceramide synthase (GCS), an enzyme that glycosylates ceramide and blunts its proapoptotic activity in cancer cells. Human ovarian cancer cells expressing mutant p53 displayed resistance to apoptosis induced by DNA damage. We found that GCS silencing sensitized these mutant p53 cells to doxorubicin but did not affect the sensitivity of cells with wild-type p53. GCS silencing increased the levels of phosphorylated p53 and p53-responsive genes, including p21Waf1/Cip1, Bax, and Puma, consistent with a redirection of the mutant p53 cells to apoptosis. Reactivated p53-dependent apoptosis was similarly verified in p53-mutant tumors where GCS was silenced. Inhibition of ceramide synthase with fumonisin B1 prevented p53 reactivation induced by GCS silencing, whereas addition of exogenous C6-ceramide reactivated p53 function in p53-mutant cells. Our findings indicate that restoring active ceramide to cells can resuscitate wild-type p53 function in p53-mutant cells, offering preclinical support for a novel type of mechanism-based therapy in the many human cancers harboring p53 mutations.

Introduction

p53 protein is a crucial tumor suppressor in preventing tumorigenesis and tumor progression (1, 2). As an essential transcription factor, p53 activates the expressions of p21Waf1/Cip1, Bax, Puma, FAS, and others and consequently regulates cell-cycle arrest and apoptosis to remove abnormal cells (1, 2). The transcription activity of p53 on p53-responsive genes is sequence specific and mainly relies on its DNA-binding domain (DBD, residues 102–292) encoded by exon 5 to exon 8 (residue 126–306) of the core domain (6). p53 mutants that have been found in approximately 50% cases of solid cancers turn this tumor suppressor into an oncogenic factor (5, 6). These mutants are mainly located in exon 5 to exon 8 (residue 126–306) of the core domain (6). Mutant p53 thus loses sequence-specific binding to its responsive genes involved in cell-cycle arrest, senescence, and apoptosis. Mutant p53 is unable to activate MDM2 and degrades protein through ubiquitination, resulting in mutant protein accumulated in cell nucleus (7). Mutation of other tumor suppressors often produces truncated proteins or is unable to synthesize any protein; however, more than 80% of p53 mutants are missense and synthesized as stable and full-length proteins (8). These p53 mutants confer a dominant-negative or oncogenic gain-of-function in cells (9), p53 mutants that promote tumor progression and resistance to therapy become the most common diagnostic indicator for both cancer recurrence and death (10). Restoration of p53 function has been succeeded in regression of lymphomas, sarcomas, and hepatocellular carcinoma and represents a more effective means to cancer treatment (11–13). It has been reported that gene therapy and small molecules (e.g., Prima-1, Ellipticin, peptides) restore p53 function by addition of wild-type or alteration of protein conformation (13–15); however, little is known whether p53 expression can be restored in p53-mutant tumors. Gene therapy could not bring an adequate benefit to ovarian cancers with standard chemotherapy (16),

Note:

Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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which calls for exploration of new approach, restoring the expression of wild-type p53.

Drug resistance is an outcome of multigene interactions, and p53 mutants impede induced apoptosis when cancer cells are under chemotherapy. Ceramide is an active sphingolipid and plays critical roles in processing of apoptosis and other cellular functions (17–20). Both ceramide and p53 are implicated in DNA damage–induced apoptosis (17, 18). Loss of ceramide production confers resistance to radiation-induced apoptosis in B-lymphoma and myeloid leukemia cells that express wild-type p53 (21, 22). Glucosylceramide synthase (GCS) catalyzes ceramide glycosylation, converting ceramide to glucosylceramide. It is a limiting enzyme controlling intracellular ceramide and glycosphingolipids (23). Overexpression of GCS that confers cell resistance to apoptosis (24, 25) becomes a potential marker that predicts tumor response to chemotherapy and clinical progression (26, 27). Inhibition of GCS by gene silencing or tamoxifen leads p53-mutant cancer cells to apoptosis (25, 28, 29). In this study, we investigate whether disruption of ceramide glycosylation restores p53-dependent apoptosis in p53-mutant cancer cells.

Materials and Methods

Cell culture and treatments

Drug-resistant human NCI/ADR-RES ovary cancer cells (30) and MCF-7 breast adenocarcinoma cells were kindly provided by Dr. Kenneth Cowan (UNMC Eppley Cancer Center, Omaha, NE) and Dr. Merrill Goldsmith (National Cancer Institute, Bethesda, MD; ref. 31). The OVCA-8 and A2780 human ovarian cancer cell lines were kindly provided by Dr. M. Hollingshead (Division of Cancer Treatment and Diagnosis Tumor Repository at National Cancer Institute). Drug-resistant human ovarian cancer A2780ADR (also named A2780-DX3) was purchased from Sigma-Aldrich (32). MCF-12A, a normal human mammary epithelial cell line, was purchased from American Type Culture Collection (ATCC). MCF-7, NCI/ADR-RES, and OVCA-8 cells were cultured in RPMI 1640 medium containing 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 584 mg/L 1-glutamine. A2780ADR cells were cultured in a medium containing 100 mmol/L doxorubicin (Dox) in addition to the aforementioned components. MCF-12A cells were cultured in DMEM/F12 medium containing 5% horse serum, insulin (5μg/mL), hydrocortisone (500 ng/mL), human epidermal growth factor (20 ng/mL), and cholera toxin (100 ng/mL). Cells were maintained in an incubator humidified with 95% air and 5% CO₂ at 37°C. Cell lines were authenticated in November 2010 at the John Hopkins University Fragment Analysis Facility (Baltimore, MD), using Applied Biosystem Identifier System, to test for 16 STR markers and amelogenin for gender determination. Authenticity was confirmed against the ATCC database (http://bioinformatics.istge.it/clima/) and NCI-60 database published (33).

For MBO/casGCS (mixed-backbone oligonucleotide against GCS) pretreatments, experiments were conducted as described previously (29, 34). Briefly, after overnight culture, cells (2 × 10⁵/100-mm dish) were transfected with MBO-casGCS (50–200 n mole/L Integrated DNA Technologies, Inc.), using Lipofectamine 2000 (Invitrogen), and cultured in 10% FBS medium. MBO-asGCS were introduced into cells twice in 7 days to observe p53 expression. Cells were exposed to Dox (1 or 2.5 μmole/L, 48 hours) to induce p53.

GCS inhibitor, N-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol HCl (n-PDMP), C₆-ceramide (N-hexanoyl-N-erythro-sphingosine), and C₆-dihydroceramide (N-hexanoyl-N-erythro-sphinganine) were purchased from Matreya. Dox hydrochloride and acidic sphingomyelinsase (100 units/mg) of human placenta was purchased from Sigma-Aldrich. Ceramide synthase inhibitor, fumonisin B1 (FB1), was purchased from Biomol. Puma siRNA and its scrambled control were purchased from Santa Cruz Biotechnology.

Western blot analysis

After treatments, cells or tissue homogenates were lysed in NP40 cell lysis buffer (Biosource). Equal amount of proteins (50 μg/lane) were resolved using 4% to 20% gradient PAGE (Invitrogen). After transferring, blots were blocked in 5% fat-free milk in PBST (0.05% Tween-20, 20 mmol/L PBS, pH 7.4) and incubated with antibodies against p53 (Invitrogen), phosphorilated p53 (pp53) at Ser15, cleaved PARP (c-PARP), Puma siRNA and its scrambled control were purchased from Santa Cruz Biotechnology, active caspase-7 (a-Casp7; EMD Chemicals), as described previously (34, 35). Endogenous glycerol-dehyes-3-phosphate dehydrogenase (GAPDH) was used as a loading control. The levels of pp53 protein were represented by the ratios of optical densities in pp53 bands normalized against unphosphorylated p53.

Immunocytochemistry

Cells (10,000 cells/chamber) were grown in 4-chamber slides via treatments for 48 hours. After fixation with methanol, cells were blocked with 5% goat serum PBS (block solution) and incubated with antibodies against GCS (1:1,000) or ceramide (1:500; clone MID 15B4 from Sigma) and pp53 (1:1,000) in block solution at 4°C overnight. Cellular GCS and pp53 tagged with antibodies were recognized by Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 555 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG (1:1,000), respectively, at 4°C, overnight. After washing, these blots were incubated with horse-radish peroxide–conjugated secondary antibodies and developed using SuperSignal West Pico ECL (Thermo Scientific), as described previously (34, 35). Endogenous glycerol-dehyes-3-phosphate dehydrogenase (GAPDH) was used as a loading control. The levels of pp53 protein were represented by the ratios of optical densities in pp53 bands normalized against unphosphorylated p53.
respectively. Cell nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole) in mounting solution. Slides were observed under Olympus BX71 fluorescence microscope coupled with digital camera and images were captured using Olympus DP controller software. Confocal images were captured using LSM Pascal confocal microscope (Carl Zeiss Microimaging Inc.).

Tumors were fixed and maintained in paraffin blocks. Microsections of each tumor (5 μm) were stained with hematoxylin and eosin (H&E) and identified by a pathologist. For immunostaining, antigens were retrieved in steaming sodium citrate buffer (10 mmol/L, 0.05% Tween-20, pH 6.0). After blocking, slides were incubated with anti-GCS rabbit serum (1:100) and anti-pp53 mouse primary antibodies at 4°C overnight. Cell nuclei were counterstained with DAPI.

**Flow cytometric assay**

The analyses were done as described previously (29). Cells (5 × 10^5 cells/100-mm dish) were exposed to Dox (2.5 μmol/L) for 48 hours and harvested with trypsinization and centrifugation. Cells resuspended were incubated with 0.01% propidium iodide (PI) in staining solution (0.1% sodium citrate, 0.3% Triton X-100, 2 mg/mL ribonuclease A) at 4°C for 30 minutes. The cells were analyzed using FACSCalibur with CellQuest Pro program (BD Biosciences). For each sample, 10,000 events were counted 3 times and subphase G0/G1 was defined as indicative of apoptotic cells in cell-cycle histograms.

**Tumor xenografts and treatments**

All animal experiments were approved by the Institutional Animal Care and Use Committee, University of Louisiana at Monroe, and were handled in strict accordance with good animal practice as defined by NIH guidelines. Tumor model was established, as described previously (29, 34). Athymic nude mice (Foxn1nu/Foxn1–, 4–5 weeks old, female) were purchased from Harlan. Cell suspension of NCI/ADR-RES (3–5 passages, 1 × 10^6 cells/20 μL/mouse) was subcutaneously injected in the left flank of the mice. Mice were monitored by measuring tumor growth and body weight, under clinical observation. Once tumors were visible (~2 mm in diameter), mice were randomly allotted to different treatment groups (10 mice/group). For treatment, MBO-asGCS (1 mg/kg/3 d) or MBO-SC (scrambled control for MBO-asGCS) was administered intratumorally alone or with Dox (2 mg/kg/wk, i.p.) for 32 days. The control group received saline and Dox combination. Tumor volume was calculated by the formula L/2 × W^2 (where L is the length and W is the width).

**TUNEL staining**

Apoptotic cells of tumor tissues were detected by measurement of nuclear DNA fragmentation using terminal deoxynucleotide transferase–mediated dUTP nick end labeling (TUNEL) system (Promega), following the manufacturer’s instruction, as described previously (29). Briefly, after antigen retrieval, slides were put in 0.2 mg/mL proteinase K in 10 mmol/L Tris-HCl, pH 8.0, for 20 minutes for digestion and labeled for 90 minutes with fluorescein-12-dUTP terminal deoxynucleotide transferase reaction mixture at 37°C in a humidified chamber. After mounting with DAPI, the sections were observed.

**Statistical analysis**

Cell experiments in triplicate were repeated twice. All data represent the mean ± SD. Student’s t test was employed to compare mean values, using a Prism 4 program (GraphPad software).

**Results**

**Silencing of GCS by MBO-asGCS sensitized mutant p53 cells to Dox**

Mutant p53, particularly the deletion mutant, is highly associated with poor response to chemotherapy (10, 11). NCI/ADR-RES and OVCAR-8 cells are mutant p53 cell lines that dominantly express the p53 with deleted 21-bp and 18-bp within the DBD (36, 37). NCI/ADR-RES has an additional point mutation, arginine instead of proline, at codon 72 of p53 (36). A2780ADR cells do not respond to cisplatin-induced p53 activation, even though the mutation has not been determined (32; Table 1). NCI/ADR-RES, OVCAR-8, and A2780ADR display considerable resistance to several anticancer drugs including

![Table 1. p53 status and cell response to anticancer drugs](image-url)

Note: A2780ADR does not respond to CDDP-induced p53 activation, but mutation type has not been determined (32). GCS mRNA was measured by real-time reverse transcriptase PCR (35).
Silencing of GCS sensitized mutant p53 cancer cells to Dox. A, cell response to Dox. NCI/ADR-RES cells were pretreated with MBO-asGCS for 7 days and exposed to Dox for additional 72 hours. *, \( P < 0.01 \) compared with vehicle control; **, \( P < 0.001 \) compared with vehicle control. B, \( E_{50} \) values for Dox. Cells were pretreated with MBO-asGCS (50 nmol/L) or vehicle (Lipofectamine 2000) for 7 days and exposed to Dox in 5% FBS medium for additional 72 hours. \( E_{50} \) was calculated using Prism software after measurements. *, \( P < 0.01 \) compared with vehicle control.

Dox and cisplatin (31, 37; Table 1). To examine whether disruption of ceramide glycosylation restores p53-dependent apoptosis, we treated NCI/ADR-RES cells with MBO-asGCS to silence GCS and then tested cell response to Dox. As shown in Figure 1A, MBO-asGCS treatments significantly increased cell response to Dox, as suppressed GCS expression in a dose-dependent fashion (Supplementary Fig. S1A). At 200 nmol/L, MBO-asGCS decreased the \( E_{50} \) for Dox by 17-fold (12.9 \( \mu \)mol/L vs. 0.8 \( \mu \)mol/L) as compared with vehicle control. To test whether this sensitization is associated with p53 status, we silenced GCS with MBO-asGCS (50 nmol/L, 7 days) in cell lines with variant p53 status (Table 1). OVCAR-8 and NCI/ADR-RES cells sharing mutant p53 displayed Dox resistance, and their \( E_{50} \) values for Dox were 22-fold (5.2 \( \mu \)mol/L vs. 0.23 \( \mu \)mol/L) and 53-fold (12.4 \( \mu \)mol/L vs. 0.23 \( \mu \)mol/L) greater than p53 wild-type cells, either MCF-12A or MCF-7 (Fig. 1B). Interestingly, silencing of GCS with MBO-asGCS sensitized p53-mutant cells but not p53 wild-type cells. With decreases of GCS protein levels (Supplementary Fig. S1B), MBO-asGCS treatments decreased \( E_{50} \) values for Dox in OVCAR-8, NCI/ADR-RES, and A2780ADR by 4-fold, 8-fold, and 4-fold, respectively. However, MBO-asGCS minimally reduced GCS protein (Supplementary Fig. S1B) and the \( E_{50} \) values in MCF-12A, MCF-7, and A2780 cells (Fig. 1B).

Disruption of ceramide glycosylation increased pp53 and induced the expressions of p53-responsive genes in mutant p53 cells

To examine whether disruption of ceramide glycosylation alters p53, we used NCI/ADR-RES cells that dominantly express mutant p53 and high level of GCS (25, 36). It was found that suppression of GCS by MBO-asGCS increased the expression levels of wild-type p53 and p53-responsive genes. After 48 hours of treatment, MBO-asGCS increased the levels of pp53 (at Ser15 in DBD) by more than 4-fold with p21\(^{Waf1/Cip1}\) and Bax, as GCS was significantly suppressed in NCI/ADR-RES cells (Fig. 2A). Silencing of GCS by MBO-asGCS reactivated p53 response to Dox-induced DNA damage, as pp53 levels were increased with a decrease in GCS protein (Fig. 2B). As expected, GCS protein levels were suppressed by MBO-asGCS in a dose-dependent manner; the pp53 levels were significantly increased by greater than 2-fold, even though at 50 nmol/L MBO-asGCS, whereas unphosphorylated p53 protein levels were constant in all treatments (Fig. 2B). Consequently, the levels of p21\(^{Waf1/Cip1}\) and Bax were enhanced with pp53 augmenting. The association of pp53 with GCS suppression was confirmed in NCI/ADR-RES cells treated with \( \nu \)-PDMP, a GCS inhibitor. Disruption of ceramide glycosylation by \( \nu \)-PDMP significantly increased the levels of pp53, p21\(^{Waf1/Cip1}\), and Bax in a dose-dependent fashion (Fig. 2B). Furthermore, immunofluorescent staining revealed that suppression of GCS restored pp53 response to Dox-induced DNA damage. MBO-asGCS treatment dramatically decreased GCS protein in Golgi apparatus where ceramide glycosylation occurred, which, consequently, increased nuclear pp53 in more than 90% of NCI/ADR-RES cells exposed to Dox (Fig. 2C). Different from wild-type p53 A2780 cells (Fig. 2C, left panel), pp53 particles were also detected in cytoplasm, even though most pp53 were concentrated in nucleus of NCI/ADR-RES.

Disruption of ceramide glycosylation induced p53-dependent cell growth arrest and apoptosis in mutant p53 cells

To examine the effects of GCS silencing on p53-responsive genes, we further assessed cell division and apoptosis. Puma is a major p53-responsive gene involved in induced apoptosis in response to DNA damage, and cleaved PARP (c-PARP) and active caspase-7 (a-Casp7) are effectors in p53-dependent apoptosis processing (38, 39). We found that the protein levels...
of Puma were significantly increased in NCI/ADR-RES cells exposed to Dox after MBO-asGCS (Fig. 3A). The levels of c-PARP and a-Casp7 were increased by 2-fold to 3-fold, respectively. Silencing of Puma with siRNA eliminated the effects of reactivated p53 on Puma, c-PARP, and a-Casp7 (Fig. 3A). Flow cytometric assays revealed that MBO-asGCS pretreatments significantly affected cell division and apoptotic cells. The numbers of G2/M phase cells that were under division and proliferation in histogram of flow cytometry significantly decreased with increasing concentrations of MBO-asGCS (50–200 nmol/L); however, G1 phase cell numbers were increased by 2-fold, 3-fold, and 5-fold, respectively (Fig. 3B and C). Furthermore, flow cytometry detected that the numbers of G0/G1 phase cells that are under apoptosis were 3-fold (9.4% vs. 3.4% of total cells), 7-fold (24.8% vs. 3.4% of total cells), and 15-fold (52.6 vs. 3.4% of total cells) at 50 to 200 nmol/L MBO-asGCS pretreatment as compared with vehicle control, respectively. Silencing of Puma eliminated the apoptotic effects of restored p53 (Fig. 3B and D). These data indicate that silencing of GCS resulted in cell growth arrest and apoptosis through p53-responsive genes.

Restoration of functional p53 expression induced apoptosis in mutant p53 tumors

We further investigated whether silencing of GCS restores p53 expression and eliminates mutant p53 tumor growth in vivo. After tumors of NCI/ADR-RES were apparent, mice were treated with MBO-asGCS (1 mg/kg twice a week, intratumoral injection) combined with Dox (2 mg/kg once a week, i.p.) for 32 days. MBO-asGCS combined with Dox decreased tumor volume to 42% (157 mm3 vs. 376 mm3, \( P < 0.01 \)) as compared with MBO-SC combined with Dox or Dox-alone treatment (Fig 4A). Western blotting detected that silencing of GCS substantially decreased GCS, which, in turn, increased pp53 (red) antibodies with Alexa Fluor 488- and Alexa Fluor 555–conjugated goat antibodies. Nucleus was counterstained with DAPI (blue).
the ratio of pp53/p53 > 4-fold \((P < 0.001)\), as compared with vehicle control or MBO-SC. Consequently, the reactivated p53 substantially induced the expression of\( p21^\text{Waf1/Cip1}, \text{Bax}, \text{Puma} \), and \(\alpha\)-Casp7 (Fig. 4B). Immunostaining confirmed these findings and showed that MBO-asGCS, but not MBO-SC, significantly increased nuclear pp53 in tumors whereas decreased GCS protein in cytoplasm (Fig. 4C). TUNEL analysis indicated that approximately 80% of tumor cells underwent apoptosis in tumors treated with MBO-asGCS, combined with Dox, compared with only 3% and 9% of apoptotic cells in Dox alone and Dox combined with MBO-SC (Fig. 4D). Thus, these data indicate that GCS suppression restores wild-type p53 expression and leads mutant p53 tumors to induced apoptosis.

### Ceramide Mediated the Restoration of p53 Expression

To determine whether ceramide or glucosylceramide after disruption of ceramide glycosylation is the factor restoring p53, we assessed its effects on cellular ceramide and glycosphingolipids. In NCI/ADR-RES cells, MBO-asGCS treatments (50–200 nmol/L) decreased globotriaosylceramide (Gb3) and significantly increased ceramide (Supplementary Fig. S2A) in a dose-dependent fashion. At 200 nmol/L MBO-asGCS, endogenous ceramide increased more than 160% (1.01 vs. 1.65 ng/100 µg protein, \(P < 0.001\); Supplementary Fig. S2C). In addition, MBO-asGCS significantly enhanced ceramide in other p53-mutant OVCAR-8 and A2780ADR cells but not p53 wild-type cells (Supplementary Fig. S2D). Furthermore, we examined the expression of pp53 and p53-responsive genes in NCI/ADR-RES cells treated with ceramide synthase inhibitor and cell-permeable ceramide, respectively. As shown in Figure 5A, FB1 treatment (25 µmol/L, 28 hours) that inhibited ceramide synthesis in \textit{de novo} pathway eliminated the effects of MBO-asGCS on restoration of p53. MBO-asGCS alone increased pp53 protein level by approximately 9-fold, but MBO-asGCS combined with FB1 could not enhance pp53 level or \(p21^\text{Waf1/Cip1}\) and Bax. Exogenous cell-permeable C6-ceramide (C6-Cer, 25 µmol/L, 48 hours) substantially increased pp53 level by 9-fold; however, C6-dihydroceramide (C6-DH-Cer; 25 µmol/L, 48 hours) that had less bioactivity enhanced pp53 by 2-fold and could not significantly increase \(p21^\text{Waf1/Cip1}\) or Bax (Fig. 5A). Immunofluorescent staining confirmed that MBO-asGCS dramatically increased cellular ceramide and pp53 dramatically appeared in nucleus in cells exposed to Dox (Fig. 5B). FB1 that inhibited ceramide synthase prevented cellular ceramide and pp53 reactivated in nucleus, even though MBO-asGCS disrupted ceramide glycosylation. C6-Cer treatment significantly increased pp53 in nucleus, as
C6-Cer was detected mostly in cytoplasm and nuclear envelope (Fig. 5B).

To understand how silencing of GCS reactivates p53, we examined protein, mRNA and heteronuclear RNA (hnRNA) of mutant p53 and wild-type p53 in cell lines that dominantly express wild-type p53 or mutant p53 after GCS silencing. As shown in Supplementary Figure S3A, MBO-asGCS pretreatment (50 nmol/L, 7 days) did not significantly affect pp53 levels in MCF-12A and A2780 cells, as p53 expression in these wild-type p53 responded to Dox exposure. In contrast, MBO-asGCS considerably increased pp53 by approximately 3-fold in mutant p53 OVCAR-8 and NCI/ADR-RES cells. The mRNA that included p53 exon 5 could not be detected in mutant p53 OVCAR-8 and NCI/ADR-RES cells, even under Dox exposure (Supplementary Fig. S3B). However, we detected p53 exon 5 in mutant p53 cells after silencing of GCS with MBO-asGCS pretreatment (Supplementary Fig. S3B). Analysis of p53 hnRNA found that p53 exon 5 was transcribed in both wild-type and mutant p53 cell lines (Supplementary Fig. S3C). These data indicate that silencing GCS may restore p53 at the level of posttranscriptional processing.

**Discussion**

Reactivation of mutant p53 can trigger massive cell death and efficiently eliminate abnormal cells. Previous works from those of others have shown that both introduction of wild-type p53 by gene transfection and modulation of the protein conformation by small molecules activate p53-responsive genes and restore p53-dependent apoptosis in mutant p53 cancer cells (11, 14, 15). This study, for the first time, has shown that suppression of GCS restores p53 expression and its functions in mutant p53 ovarian cancer cells. Suppression of GCS expression by MBO-asGCS disrupts ceramide...
glycosylation and increases ceramide, which, in turn, leads cell death and eliminates tumor progression through p53-dependent apoptosis.

Mutant p53 has been reported as a marker of poor prognosis and a cause of chemotherapy failure in many types of cancers (3, 6). More than 85% of point mutants occur in the DBD encoded by exon 3 to exon 8 (5, 9). Exon 5 mutations are prognostic indicators of shortened survival in non–small cell lung cancer (40). Small-fragment deletion is less common than point mutant detected in p53 mRNA and protein; however, all deletion mutants in DBD completely lose p53 functions (5). In OVCAR-8 and NCI/ADR-RES cells, the 18-bp and 21-bp deletions in exon 5 confer these cells resistance to anticancer drugs, particularly those inducing DNA damage, including Dox and cisplatin (25, 36). It has been shown that ceramide activates apoptosis processing in response to DNA damage stress, when cells are exposed to anticancer drugs, cytokines, and irradiation (19, 41). Ceramide can independently induce apoptosis in testicular germ-cell tumor cells with null or mutant p53 (42), although ceramide response to DNA damage stress is p53 dependent in others (18). Enhancing endogenous ceramide by disrupting ceramide glycosylation sensitizes mutant p53 cells to drug-induced apoptosis, even though it does not significantly alter p53 protein levels, as reported previously (25) and detected by Western blotting in this study (Figs. 2A and B, pp53 and ceramide in cells. Merged fluorescence microphotographs (>200) were captured by confocal microscopy. Green, cells were incubated with anti-pp53 (green) and anti-ceramide (red) following addition of Alexa Fluor 488– and Alexa Fluor 555-conjugated goat antibodies. Nucleus was counterstained with DAPI.)

Figure 5. Ceramide restored p53 expression in mutant p53 cells. After pretreatments of MBO-asGCS (50 nmol/L, 7 days), FB1 (25 µmol/L, 48 hours), C6-ceramide (5 µmol/L, 48 hours; C6-Cer), and C6-dihydroceramide (5 µmol/L, 48 hours; C6-dih-Cer), NCI/ADR-RES/asGCS cells were exposed to Dox (2.5 µmol/L, 48 hours). A, Western blotting. Equal amount proteins extracted from tumors (100 µg/lane) were resolved by 4% to 20% PAGE and immunoblotted with antibodies. *, $P < 0.001$ compared with vehicle. B, pp53 and ceramide in cells. Merged fluorescence microphotographs (>200) were captured by confocal microscopy. Green, cells were incubated with anti-pp53 (green) and anti-ceramide (red) following addition of Alexa Fluor 488– and Alexa Fluor 555-conjugated goat antibodies. Nucleus was counterstained with DAPI.
mitochondrial alterations, may be dominant. It is more likely that ceramide-processing apoptosis is associated with p53-dependent pathways, as we have found that ceramide increased after MBO-asGCS treatment (Supplementary Fig. S2; ref. 29) or exogenous C6-ceramide reactivates pp53 in nucleus and enhances p53-responsive genes including p21^{Waf1/Cip1}, Bax, and Puma (Figs. 2–5).

Mutant p53 is a defined target for cancer therapies; however, discovering an effective approach to restore functional p53 is a great challenge. Mutant p53 is often expressed at high levels in cancers, and it affects tumor progression and treatment outcome in terms of loss-of-function in tumor suppression and gain-of-function in oncogenic effects (12, 43). Introduction of wild-type p53 by gene therapy can correct the loss-of-function in tumor suppression, but it cannot diminish the oncogenic effects of mutant p53 on tumors. It has been reported that a group of small molecular compounds (Ellipticine, CP-31398, Prima-1) and peptides (C369–382, C361–382) modulate protein conformations of mutant p53, reactivating its transcription function (15, 44). CP-31398, a styrylquinazoline compound, upregulates the expression of p53-responsive genes and represses tumor growth through binding to DBD and stabilizing the active protein conformation in mutant p53 cells (45, 46). Prima-1 reactsivate mutant p53 (His^{175}, His^{273}) by covalent binding to and modification of thiol groups in the core domain in the H1299-His^{175} lung adenocarcinoma or Saos-2-His^{273} osteosarcoma cells (15). On the other hand, peptide C361 to 382 restores the transcriptional activity of mutant p53 by an allosteric mechanism of negative regulation (44, 47). In this study, we have found that ceramide mediates p53 restoration, as silencing of GCS with MBO-asGCS can increase endogenous ceramide (29) and C6-ceramide leads to the same restorative effects (Figs. 2, 4, and 5).

It is not clear how ceramide restores functional p53 in deletion mutants of p53. In addition to direct effects on processing of apoptosis, ceramide mediates gene expressions including MDR1, telomerase, c-fos, p21^{Waf1/Cip1}, GCS, caspase-9, and Bcl-x (34, 35, 48, 49). Ceramide might reactivate functional p53 by modulating either mutant p53 conformation or wild-type p53 expression, but on the basis of this study, it is more likely that ceramide restores wild-type p53 expression at posttranscriptional processing. Suppression of GCS restores p53 reactivation in 12 hours to several days (Fig. 2A) and wild-type p53 is detected in mRNA and protein (Figs. 2, 4, and 5, Supplementary Fig. S1). In addition, suppression of GCS does not have any effect on p53 trans-activation (data not shown). These strongly suggest that ceramide-mediated posttranscriptional processing may play a critical role in regulating expression of wild-type or mutant p53.

High levels of GCS and mutant p53 are coincidently detected in several drug-resistant ovarian cancer cells lines, such as OVCAR-8, NCI/ADR-RES, and A2780ADR, in this study. MBO-asGCS, a specific agent for GCS suppression, effectively restores p53-dependent apoptosis in vitro and in vivo. Further investigating the association of GCS and mutant p53 in drug resistance and the molecular mechanism by which ceramide mediates p53 restoration in mutant p53 barrier cancers may lead to discovering effective approaches to improve cancer treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Ceramide Restores p53-Dependent Apoptosis


Suppression of Glucosylceramide Synthase Restores p53-Dependent Apoptosis in Mutant p53 Cancer Cells

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