REV3L confers chemoresistance to cisplatin in human gliomas: The potential of its RNAi for synergistic therapy

Huibo Wang, Shu-Yu Zhang, Shuai Wang, Juan Lu, Wenting Wu, Lin Weng, Dan Chen, Yu Zhang, Zhipeng Lu, Jingmin Yang, Yuanyuan Chen, Xu Zhang, Xiaofeng Chen, Caihua Xi, Daru Lu, and Shiguang Zhao

Department of Neurological Surgery, Brain Tumor Research Center, First Affiliated Hospital, Harbin Medical University, Harbin (H.W., X.Z., X.C., S.Z.); State Key Laboratory of Genetic Engineering, School of Life Sciences, Fudan University, Shanghai (H.W., S.-Y.Z., J.L., W.W., D.C., Y.Z., Z.L., J.Y., Y.C., D.L.); State Key Laboratory of Medical Genomics and Shanghai Institute of Hematology, Rui Jin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai (S.W., L.W.); Department of Neurological Surgery, Shanghai 6th People Hospital, Shanghai Jiao Tong University, Shanghai (C.X.); China

The *REV3L* gene, encoding the catalytic subunit of human polymerase ζ, plays a significant role in the cytotoxicity, mutagenicity, and chemoresistance of certain tumors. However, the role of *REV3L* in regulating the sensitivity of glioma cells to chemotherapy remains unknown. In this study, we investigated the expression of the *REV3L* gene in 10 normal brain specimens and 30 human glioma specimens and examined the value of *REV3L* as a potential modulator of cellular response to various DNA-damaging agents. Reverse transcriptase PCR/real-time PCR analysis revealed that *REV3L* was overexpressed in human gliomas compared with normal brain tissues. A glioma cell model with stable overexpression of *REV3L* was used to probe the role of *REV3L* in cisplatin treatment; upregulation of *REV3L* markedly attenuated cisplatin-induced apoptosis of the mitochondrial apoptotic pathway. We therefore assessed the *REV3L*-targeted treatment modality that combines suppression of *REV3L* expression using RNA interference (RNAi) with the cytotoxic effects of DNA-damaging agents. Downregulation of *REV3L* expression significantly enhanced the sensitivity of glioma cells to cisplatin, as evidenced by the increased apoptosis rate and marked alterations in the anti-apoptotic proteins B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma-extra large (Bcl-xl) and proapoptotic Bcl-2-associated x protein (Bax) expression levels, and reduced mutation frequencies in surviving glioma cells. These results suggest that *REV3L* may potentially contribute to gliomagenesis and play a crucial role in regulating cellular response to the DNA cross-linking agent cisplatin. Our findings indicate that RNAi targeting *REV3L* combined with chemotherapy has synergistic therapeutic effects on glioma cells, which warrants further investigation as an effective novel therapeutic regimen for patients with this malignancy.* *Neuro-Oncology 11, 790–802, 2009 (Posted to Neuro-Oncology [serial online], Doc. D08-00174, March 16, 2009. URL http://neuro-oncology.dukejournals.org; DOI: 10.1215/15228517-2009-015)*

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Address correspondence to Daru Lu, State Key Laboratory of Genetic Engineering, School of Life Sciences, Fudan University, Shanghai 200433, China (drlu@fudan.edu.cn) or Shiguang Zhao, Department of Neurological Surgery, Brain Tumor Research Center, First Affiliated Hospital, Harbin Medical University, 23 Youzhen St., Harbin 150001, China (guangsz@hotmail.com).

H.W., S.-Y.Z., and S.W. contributed equally to this work.

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Malignant gliomas, the most common and devastating primary brain tumors striking adults, represent a formidable clinical challenge.[1,2] Despite advances in intensive multimodality therapeutic approaches, such as aggressive surgery, radiotherapy, chemotherapy, and biological therapy, clinical outcome in patients with malignant gliomas remains dismal.[3–5] Thus, the need to investigate the molecular mechanisms governing this malignancy to develop more effective therapeutic regimens based on rational molecular targeting is imperative.

Translesion DNA synthesis (TLS) is one type of DNA damage tolerance mechanism that allows damaged cells to complete genome replication through recruitment of specialized DNA polymerases to stalled replication forks. Most of these polymerases are less stringent and have the capacity to accommodate noncomplementary bases in their active sites. The *REV3L* gene, a human homolog of the *Saccharomyces cerevisiae Rev3* gene, is located on chromosome 6q21.[6] It encodes the catalytic subunit of DNA polymerase ζ, which is thought to be one of the major components of error-prone TLS.[7] The *REV3L* gene appears to be ubiquitously expressed in normal and malignant human tissues, while its expression level varies in different normal and tumor cell lines.[8,9] In vitro studies have shown that *Rev3* or *Rev7* knockout chicken DT40 cells caused TLS deficiency and eventually led to genomic instability in vertebrate cells.[10,11] Similarly, disruption of *Rev3* in mouse embryonic cells may also increase double-strand breaks and chromosomal aberrations, suggesting that *Rev3* is an important contributor to maintain genomic stability in mammalian cells.[12] Also, low-fidelity DNA polymerases are involved in spontaneous and DNA-damage–induced mutagenesis during the course of translesional replication,[10,11,13] which is likely an important contributory cause of malignant transformation.[14,15]

Adjuvant chemotherapy can partially prolong the survival time of patients with malignant gliomas,[16] but the development of resistance to chemotherapeutic agents poses a major impediment that contributes to inevitable tumor recurrence, progression, and certain death.[17] The intrinsic and acquired drug-resistance mechanisms, including reduced intracellular drug concentrations, rapid inactivation of the drug, enhanced DNA repair, and disruption of the apoptotic response to DNA damage,[18–20] are thought to be responsible for the poor response to chemotherapy in malignant gliomas and other recalcitrant tumors. There is accumulating evidence that activation of TLS may be another means of acquiring drug resistance in normal and tumor cells treated with DNA-damaging agents or irradiation, and specific inhibition of DNA polymerases involved in TLS is becoming a promising approach against cancer.[21–23] For example, repression of the expression of *REV3L* in fibroblast cells using antisense RNA can efficiently increase sensitivity to cisplatin and decrease the emergence of drug resistance.[23] In addition, suppression of the expression of either *REV3L* or *REV1L*, a member of the Y-type polymerase family that supports the activity of DNA polymerase ζ, markedly reduced the rate of development of drug resistance in human ovarian or colon carcinoma cells.[22,24] All these reports prompted us to investigate the function of *REV3L* in glioma biology and evaluate its role as a potential therapeutic target for the treatment of gliomas.

In the present study, we examined the expression of *REV3L* in 10 normal brain tissues and 30 human gliomas and investigated whether it would be a key modulator of cellular response to DNA-damaging agents. We found that the *REV3L* gene was highly expressed in gliomas, and its expression level was correlated with tumor grade. Overexpression of *REV3L* in glioma cells was refractory to the cytotoxic effect of cisplatin. The B-cell lymphoma 2 (Bcl-2) antagonist HA14-1, combined with cisplatin, could enhance apoptosis of *REV3L*-overexpressing cells. In contrast, suppression of *REV3L* expression by RNA interference (RNAi) could significantly increase the sensitivity of glioma cells to cisplatin. The sensitization induced by short hairpin RNAi for *REV3L* (sh*REV3L*) was associated with marked induction of cisplatin-induced apoptosis and concomitant marked alterations in apoptosis-related proteins. These results suggest the dysregulation of *REV3L* as a potential component of glioma pathogenesis and reveal that the combination of *REV3L* gene therapy and cisplatin has synergistic antitumor activity against gliomas in vitro.

Materials and Methods

Tissue Samples and Reagents

Ten normal brain tissues and 30 human glioma tissues were obtained postoperatively from the Department of Neurological Surgery, First Affiliated Hospital, Harbin Medical University, China. All patients gave signed, informed consent for their tissues to be used for scientific research. Ethical approval for the study was obtained from the Clinical Ethics Committee, First Affiliated Hospital, Harbin Medical University, China. The histological features of the specimens were confirmed by pathologists based on the WHO criteria.[25] These tissues were resected before chemotherapy and radiation therapy and were immediately frozen and stored at −80°C for reverse transcriptase (RT)-PCR and real-time PCR analysis.

Cisplatin was purchased from Qilu Pharmaceutical Co., Ltd. (Shandong, China). Temozolomide (TMZ) was purchased from Tasly Co., Ltd. (Tianjin, China). Nimustine hydrochloride (ACNU; 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea) was purchased from Sankyo Co., Ltd. (Tokyo, Japan) and diluted in media immediately before cell treatment. Geneticin (G418), hypoxanthine, aminopterin, and thymidine were purchased from Life Technologies (Gaithersburg, MD, USA). 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI), 6-thioguanine (6-TG), and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2) was purchased from Merck (Darmstadt, Germany). Ethyl-2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-

4H-chromene-3-carboxylate (HA14-1) was purchased from Calbiochem (La Jolla, CA, USA). Dimethylsulfoxide (DMSO) and diphenyltetrazolium bromide (MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Amresco (Solon, OH, USA). Antibodies against cleaved caspase-3 (Cell Signaling Technology, Beverly, MA, USA); Bcl-2, B-cell lymphoma-extra large (Bcl-xl), Myeloid cell leukemia-1 (Mcl-1), Bcl-2-associated x protein (Bax), and Bcl-2-homologous antagonist/killer (Bak) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); cytochrome c (BD Pharmingen, San Diego, CA, USA); and β-actin (Sigma-Aldrich) were used for Western blotting analysis.

Cell Culture and Stable Transfection

The human malignant glioma cell lines U251MG and U87MG were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin and incubated at 37°C in a humidified atmosphere with 5% CO_2. To generate stable *REV3L*-overexpressing clones, cells were transfected in a stable manner with the pcDNA3.1/neo-*REV3L* plasmid (provided by Dr. Yoshiki Murakumo, Nagoya University Graduate School of Medicine, Nagoya, Japan) or the pcDNA3.1/neo vector control plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) per the manufacturer's instructions. Forty-eight hours post-transfection, the medium was replaced with DMEM containing 600 μg/ml G418. After 3–4 weeks, G418-resistant colonies were selected and screened for *REV3L* expression by RT-PCR and real-time PCR. The *REV3L*-overexpressing and vector control clones were routinely cultured in DMEM containing 10% fetal bovine serum in the presence of 300 μg/ml G418 at 37°C in humidified air with 5% CO_2.

RT-PCR and Real-Time PCR

Total RNA was extracted according to the manufacturer's instructions for TRIzol Reagent (Invitrogen). First-strand cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) and random primers. The primer pairs of *REV3L* were 5'-TGATGTCTTCAGCTGGTATCATGA-3' and 5'-CCGCCCTTCAGGTTCACTT-3' and for *REV7L*, the processivity subunit of polymerase ζ, were 5'-TGCATCTCATCCTCTACGTG-3' and 5'-TCCTGGATATACTGATTCAGC-3'. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA was also amplified in the same PCR reactions as an internal control using the primers 5'-GAAGGTGAAGGTCGGAGTC-3' and 5'-GAAGATGGTGATGGGATTTC-3'. The PCR reaction conditions were denaturation at 94°C for 5 min and 35 cycles of PCR (94°C, 30 s; 59°C, 30 s; and 72°, 30 s), followed by a final extension of 72°C for 10 min. Ten microliters of PCR products was analyzed by electrophoresis on 1% agarose gel containing ethidium bromide and visualized under UV illumination.

Real-time PCR was performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) in the presence of SYBR Green PCR Master Mix (Toyobo, Osaka, Japan). The primers of *REV3L* described above were used for amplification under the following conditions: an initial denaturation (3 min, 95°C) followed by 40 cycles (95°C, 10 s and 55°C, 45 s). Each sample was tested in triplicate, and melting curve analysis of each sample was used to check the specificity of amplification. Parallel reactions were performed using primers to *GAPDH* as an internal control. The relative gene expression was calculated by using the comparative C_t method.

Cell Viability Assay

Cell viability was evaluated by the MTT assay. We plated 1×10^3 glioma cells per well in 96-well plates with 100 μl maintenance medium. The next day, the cells were treated with various concentrations of anticancer drugs according to the experimental design. The cells were then incubated with 20 μl MTT (5 mg/ml) for 4 h in the dark at 37°C. After removal of the medium, 150 μl DMSO was added into each well and incubated for 20 min. The optical density (OD) at 490 nm was measured using a Microplate Reader (model FL 311; Bio-Tek Instruments, Winooski, VT, USA), and the proliferation index was calculated as experimental OD value/control OD value. Three independent experiments were done in quadruplicate wells.

Colony Formation Assay

The effects of *REV3L* overexpression or *REV3L* knockdown and anticancer drugs on long-term growth of glioma cells were assessed by colony formation assays. Briefly, cells were plated at a density of 1×10^5 in a six-well plate and allowed to attach overnight. The cells were then treated with escalating doses of chemotherapeutic agents as indicated. Twenty-four hours after drug addition, cells were trypsinized, counted, and reseeded at a low density (10,000 in a 10-cm dish) in triplicate. The medium was replaced every 3 days, and the cells were allowed to grow for 2 weeks before being fixed with ice-cold methanol and stained with Giemsa's stain. The survival fraction was determined by dividing the number of colonies formed in the presence of the drugs by the number of colonies formed in the untreated control cells. Each dose was done in triplicate, and the experiments were done at least three times.

RNA Interference Studies

Chemically synthesized pGPU6/GFP/Neo vectors containing short hairpin RNAi (shRNA) against *REV3L* mRNA (5'-TAGTAGTCTGCAGTCACTATCCTTACTGGAAGCTTGCGGTGAGGATAGTGACTGCGGACTATTACATTTTTTT-3') sequence[24] and pGPU6/GFP/Neo vectors containing negative control (NC) shRNA (shNC) were purchased from Shanghai Genepharma Co., Ltd. (Shanghai, China). For shRNA transfection, 1 ×

10^5 cells per well were plated in six-well plates and transfected with 4 μg of shRNA duplex using Lipofectamine 2000 according to the manufacturer's instructions. After 48 h of incubation, the medium was replaced with DMEM containing 600 μg/ml G418. After maintenance for 3–4 weeks in selection media, G418-resistant colonies were selected and screened for *REV3L* expression by RT-PCR and real-time PCR. sh*REV3L*-transfected and shNC-transfected clones were subsequently cultured in DMEM containing 10% fetal bovine serum in the presence of 300 μg/ml G418 at 37°C in humidified air with 5% CO_2.

Detection of Cytochrome C Release into the Cytosol

To determine the release of cytochrome c into the cytosol, glioma cells were collected, washed twice in phosphate-buffered saline (PBS), and resuspended in buffer A (20 mM HEPES [pH 7.5], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, 250 mM sucrose, and protease inhibitors) and homogenized. The cell lysates were then centrifuged at 1,000*g* for 10 min at 4°C to remove large debris, followed by a further centrifugation at 12,000*g* for 15 min at 4°C to pellet the mitochondria. The supernatants containing cytosolic fractions were stored at −80°C. The cytosolic fractions were subjected to Western blotting analysis with an anti-cytochrome c antibody.

Western Blotting

Cell lysates were fractionated by 10%–15% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocking with 5% nonfat milk in PBS-Tween 20 for 1 h at room temperature, the membranes were blotted with primary antibody, followed by incubation with a horseradish peroxidase–conjugated secondary antibody (Santa Cruz Biotechnology). The bound antibodies were detected by using the enhanced chemiluminescence method (Amersham Biosciences AB, Uppsala, Sweden).

Apoptosis Analysis

Cells were subjected to proapoptotic conditions as specified in the text and figure captions. Both adherent and detached cells were collected and subjected to the following apoptosis assays. (1) The percentage of cells with sub-G_1 DNA content was determined by flow cytometric analysis following staining with PI using FACScan flow cytometry (BD Pharmingen) with CellQuest software (BD Pharmingen). (2) The proportion of Annexin V^+ cells was determined using an Annexin V-FITC (fluorescein isothiocyanate) apoptosis detection kit (BD Pharmingen) according to the manufacturer's instructions. Briefly, cells were labeled with FITC-conjugated Annexin V and PI without permeabilization and subsequently analyzed by FACScan flow cytometry with CellQuest software. (3) DAPI staining was used to observe morphological changes such as nuclear condensation and fragmentation. Briefly, cells were washed twice with PBS, fixed in ice-cold acetone and methanol (ratio, 1:1), and then stained with DAPI (0.4 μmol/l) for 5 min in the dark at room temperature. Apoptotic bodies were visualized using a fluorescence microscope (Olympus IX71, Tokyo, Japan). (4) Detection of cytochrome c release, cleaved caspase-3, Bcl-2, Bcl-xl, Mcl-1, Bax, and Bak were examined by Western blotting as described above.

Hypoxanthine Phosphoribosyltransferase Mutation Assay

The frequency of cisplatin-induced mutation at the hypoxanthine guanine phosphoribosyl transferase locus (*HPRT*) was measured as described previously.[13,23,26] To eliminate background *HPRT* mutations, cells were cultured in culture medium supplemented with 100 μM hypoxanthine, 0.4 μM aminopterin, and 0.4 μM thymidine for a minimum of 14 days. *HPRT* mutant-free cells (2.0×10^6) were seeded and treated the following day with one of three doses of cisplatin (10, 20, and 30 μmol/l) for 1 h. After subculturing the treated cells for another 14 days, 1.0×10^5 cells were reseeded on 35-mm dishes in medium containing 6-TG (20 μmol/l) and incubated until colonies were formed. Colonies that consisted of more than 50 cells were counted, and the *HPRT* mutation frequency was defined after correcting for plating efficiency. Each experiment was repeated at least three times.

Statistical Analysis

All values were expressed as means ± SD. Statistical analysis was conducted by Student's *t*-test. Group differences resulting in *p*-values < 0.05 were considered to be statistically significant. All statistical analyses were performed using SPSS version 11.0 (SPSS Inc., Chicago, IL, USA).

Results

REV3L *mRNA Was Significantly Higher in Malignant Gliomas, and Its Expression Level Correlated with Tumor Grade*

To investigate *REV3L* gene expression in a cohort of human gliomas and normal brain samples (Table 1), the mRNA level of *REV3L* was initially analyzed by RT-PCR using 10 normal brain tissues and 30 human glioma tissues (grade I, $n = 3$; grade II, $n = 5$; grade III, $n = 9$; grade IV, $n = 13$). A slight increase of *REV3L* mRNA was observed in grade I and grade II gliomas compared with normal brain tissues (Fig. 1A, lanes 1–4). In grade III and grade IV gliomas, all cases showed a significant increase of *REV3L* mRNA (Fig. 1A, lanes 5–16). To further confirm our results, real-time PCR was used to measure *REV3L* gene expression in these samples. As shown in Fig. 1B, the relative expression of *REV3L* was significantly elevated in malignant gliomas compared with normal brain tissues, and its expression

Table 1. Patient demographics for the PCR human glioma samples

	Normal	Grade I	Grade II	Grade III	Grade IV	All Tumors
Number	10	3	7	8	12	30
Mean age (years)	28.2	37	42.7	42.6	42.7	42.1
Age range (years)	15–43	10–61	33–58	35–53	8–72	8–72
Sex						
Male	8	3	4	5	3	15
Female	2	0	3	3	9	15
Surgery	SLTC	MC	MC	MC	MC	
Pathological classification	N/A	Polycystic astrocytoma (2) Choroid plexus papilloma (1)	Astrocytoma (4) Oligodendroglioma (2) Ependymoma (1)	Anaplastic astrocytoma (5) Anaplastic oligodendroglioma (3)	Glioblastoma (10) Medulloblastoma (2)	

Abbreviations: SLTC, standard large trauma craniotomy; MC, microneurosurgical craniotomy; N/A, not applicable.

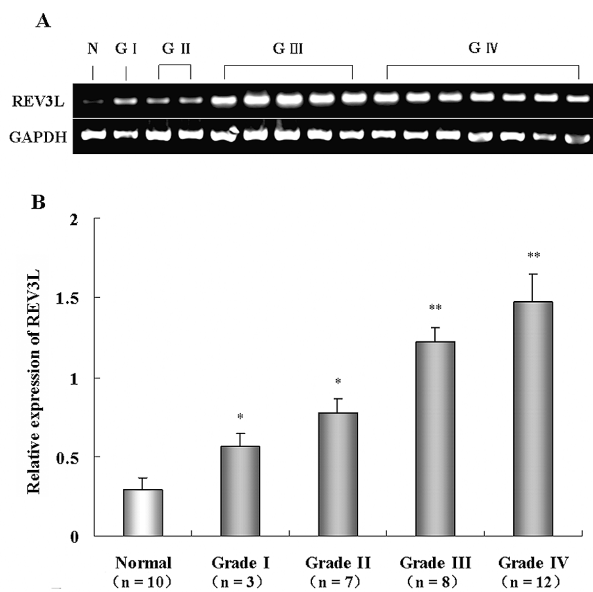

Fig. 1. mRNA expression of *REV3L* in normal brain specimens and human glioma specimens. (A) Representative images of reverse transcriptase PCR analysis for *REV3L* gene expression in 15 specimens of human gliomas (G). *REV3L* gene expression was significantly elevated in malignant gliomas compared with normal brain tissue (N). (B) mRNA expression of *REV3L* measured by real-time PCR analysis. *REV3L* expression was significantly upregulated in malignant gliomas compared with normal brain tissues. Data are means of three independent experiments + SD. *$p < 0.01$; **$p < 0.001$.

level was noted to be highest in grade IV gliomas ($p < 0.001$). There were no significant differences between sex and age for *REV3L* expression. At the present time, no sufficiently specific antibody is available to permit accurate immunoblot analysis of REV3L protein levels.

Establishment of Glioma Cell Lines with REV3L *Overexpression or Knockdown*

To gain insight into the function of *REV3L* in human glioma cells, we set up cell line models derived from the parental U251 and U87 glioma cells genetically manipulated for *REV3L* expression. As shown in Fig. 2B, *REV3L* expression was significantly increased in the *REV3L*-overexpressing cells compared with the parental cells and the vector control cells. In contrast, *REV3L* expression was significantly suppressed in glioma cell lines stably transfected with sh*REV3L* (Fig. 2A) compared with the parental cells and shNC cells (Fig. 2B). We found that enhancement or suppression of *REV3L* gene expression in established cell lines did not influence *REV7L* mRNA expression levels (Fig. 2C) and did not have a significant effect on cell proliferation by MTT assay (Fig. 2D) or cell cycle distribution as examined by flow cytometry (Fig. 2E).

REV3L *Overexpression Conferred Resistance to DNA-Damaging Agents*

To evaluate the effect of *REV3L* on the chemosensitivity of human glioma cells to chemotherapeutic drugs, we first examined the sensitivity of U251 cells to four DNA-damaging agents, including a DNA cross-linking agent (cisplatin), DNA-alkylating agents (TMZ and ACNU), and hydrogen peroxide (H_2O_2). The colony formation assays showed that overexpression of *REV3L* rendered the cells resistant to various doses of cisplatin (Fig. 3A) but not to TMZ, ACNU, or H_2O_2, which have different mechanisms of action. Consistent with clonogenic data, the *REV3L*-overexpressing U251 cells showed a marked decrease in cisplatin-induced apoptosis, as judged by decreased Annexin V-FITC–positive staining (Fig. 3B,C) and morphological alteration of nuclei (Fig. 3D). Similar apoptotic results were also obtained in the *REV3L*-overexpressing U87 cells (Fig. 3B,C). To further test whether *REV3L* overexpression conferred resistance to cisplatin that was attributable to inhibition of apoptosis, Western blotting was done to analyze the expression of cleaved caspase-3. After exposure to two relatively low doses of cisplatin (1 and 2 μmol/l) for 48 h, the expression levels of cleaved caspase-3 were lower in *REV3L*-overexpressing cells compared with the parental U251 cells and the vector control U251 cells (Fig. 4A). Taken together, these data support the notion that *REV3L* overexpression decreased cisplatin-induced apoptosis in glioma cells.

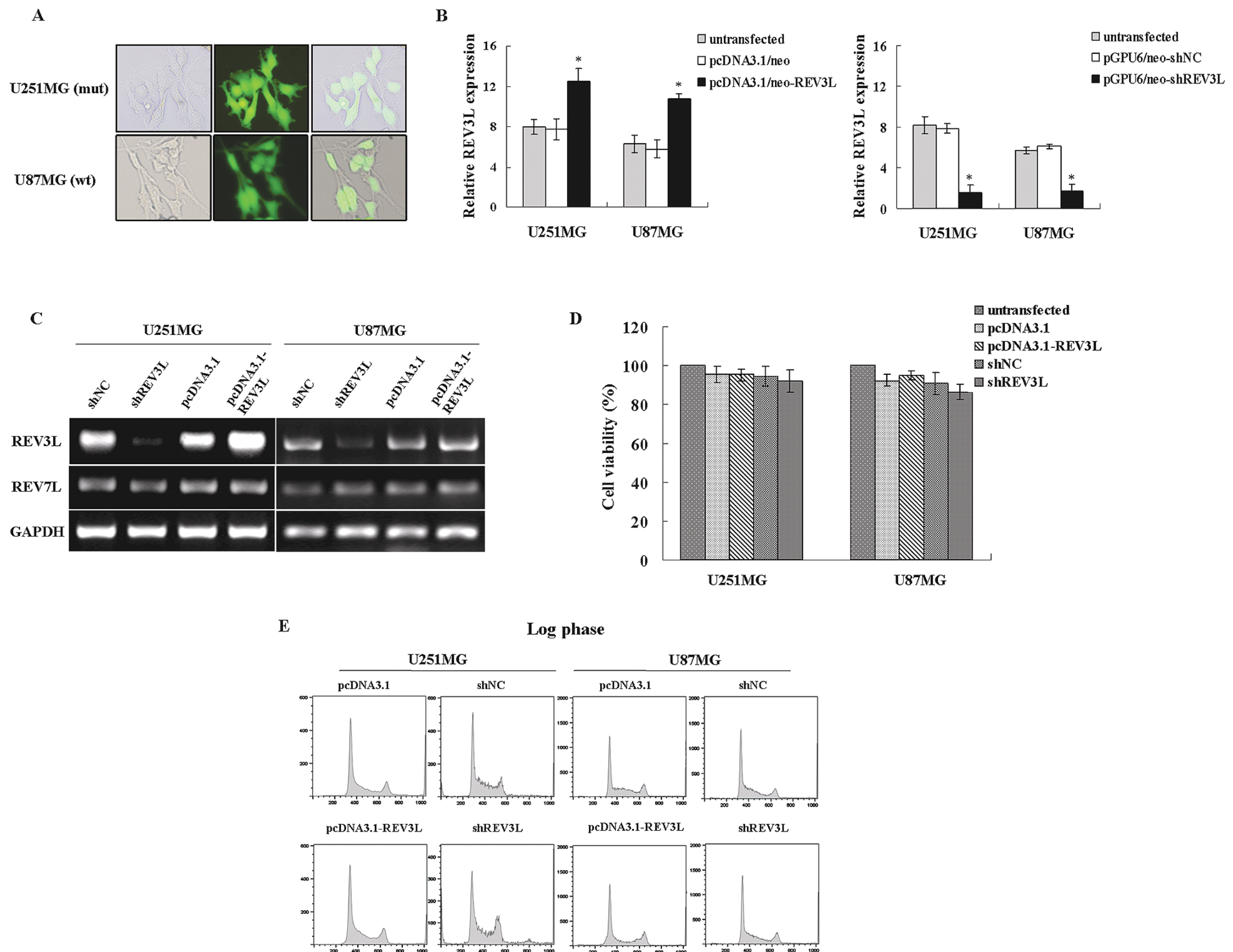

Fig. 2. Influence of upregulation or downregulation of *REV3L* on *REV7L* expression, cell proliferation, and cell cycle distribution in established glioma cells. (A) Representative images of stable transfectants of short hairpin interfering RNA for *REV3L* (sh*REV3L*) U251 cells [U251MG (mt)] and sh*REV3L* U87 cells [U87MG (wt)] in selection media under a fluorescence microscope. (B) Real-time PCR analysis for *REV3L* mRNA expression in U251 and U87 glioma cells. *REV3L* expression was significantly increased in the *REV3L*-overexpressing cells (left) and was significantly decreased in sh*REV3L* cells (right) compared with control cells and untransfected cells. Data are means of three independent experiments ± SD. $*p < 0.01$. (C) Reverse transcriptase PCR analysis for *REV3L* and *REV7L* mRNA expression in short hairpin RNA-transfected negative control cells (shNC), sh*REV3L* cells, vector control cells, and *REV3L*-overexpressing cells. Suppression or enhancement of *REV3L* expression in established cell lines did not influence *REV7L* mRNA expression. (D) The effect of *REV3L* expression on cell viability by diphenyltetrazolium bromide assay. Enhancement or suppression of *REV3L* expression did not have a significant effect on cell survival. Data are means of three independent experiments ± SD. (E) The effect of *REV3L* expression on cell cycle distribution by flow cytometry. Enhancement or suppression of *REV3L* expression did not affect cell cycle distribution.

REV3L *Overexpression Conferred Resistance to Cisplatin via Inhibition of Cisplatin-Induced Apoptosis of the Mitochondria-Mediated Apoptotic Pathway*

The mitochondria-mediated apoptotic pathway is involved in the cellular response of tumors to treatment with anticancer drugs.[27] We hypothesized that overexpression of *REV3L* rendered glioma cells resistant to activation of the mitochondrial-mediated apoptotic pathway induced by cisplatin. To test this possibility, we detected the release of cytochrome c by Western blotting analysis. As shown in Fig. 4A, cytochrome c release was decreased in the *REV3L*-overexpressing U251 cells after exposure to two different doses of cisplatin for 48 h compared with the parental U251 cells and the vector control U251 cells. Since the antiapoptotic and proapoptotic activities of Bcl-2 family members occupy an important position in the apoptotic response leading to mitochondrial membrane permeabilization and caspase activation,[28] we then performed an analysis of antiapoptotic effectors (Bcl-2, Bcl-xl, and Mcl-1) and proapoptotic effectors (Bax and Bak) in the vector control U251 cells and the *REV3L*-overexpressing U251 cells in the absence or presence of cisplatin. Interestingly, no significant differences in the expression levels of these apoptosis-related proteins were observed in untreated cells (Fig. 4B). Conversely, after treatment with cisplatin (10 μmol/l) for 48 h, a decrease in the amounts of Bcl-2 and Bcl-xl and a concomitant

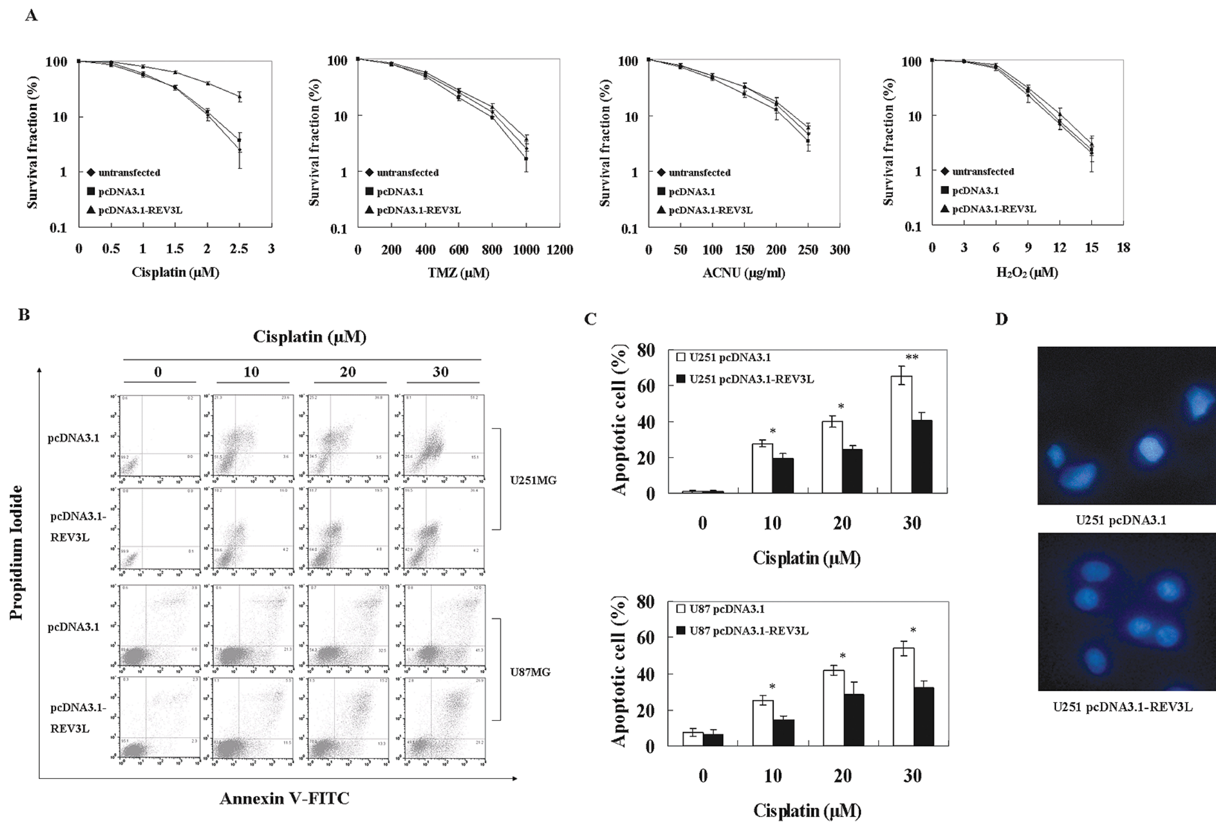

Fig. 3. *REV3L* overexpression rendered glioma cells resistant to DNA cross-linking agents. (A) Colony formation ability of the untransfected U251 cells, the vector control U251 cells, and the *REV3L*-overexpressing U251 cells after continuous treatment with cisplatin, temozolomide (TMZ), nimustine hydrochloride (ACNU), and H_2O_2. Overexpression of *REV3L* rendered glioma cells resistant to cisplatin but not to TMZ, ACNU, or H_2O_2. Data are means of three independent experiments ± SD. (B) Overexpression of *REV3L* inhibited apoptosis induced by cisplatin. Cells were treated with the indicated concentrations of cisplatin for 24 h, followed by staining with Annexin V–fluorescein isothiocyanate (FITC) and propidium iodide (PI) for the detection of dying (Annexin V^+ PI^-) or dead (Annexin V^+ PI^+) cells. Apoptotic rates of the *REV3L*-overexpressing cells were significantly lower than those of the vector control cells exposed to similar conditions. (C) Apoptosis results of three independent experiments. Data are means of three independent experiments ± SD. *$p < 0.01$; **$p < 0.001$. (D) Morphological examination to detect apoptosis by 4',6-diamidino-2-phenylindole dihydrochloride staining. Nuclear condensation and fragmentation of the *REV3L*-overexpressing U251 cells were not more prominent than vector control U251 cells after treatment with cisplatin (2 μmol/l) for 24 h.

increase in the amounts of Bax were observed in the vector control U251 cells, whereas the *REV3L*-overexpressing U251 cells showed minor alterations of these proteins in response to the same dose of cisplatin. However, no detectable changes in Mcl-1 and Bak expression were observed in these two cell types (Fig. 4B). In addition, there were no significant differences in the expression levels of these apoptosis-related proteins between the vector control U251 cells and *REV3L*-overexpressing U251 cells in the presence of TMZ, ACNU, or H_2O_2 (data not shown). Our findings suggest that overexpression of *REV3L* attenuated the mitochondria-mediated apoptosis induced by the DNA cross-linking agent cisplatin, and the protective role of *REV3L* against apoptosis triggered by cisplatin may be mediated, in part, by minor alterations in Bcl-2, Bcl-xl, and Bax protein levels.

To further address the significance of the apoptosis pathway in the *REV3L*-induced chemoresistance, we treated these cells with HA14-1, a small organic compound that interacts with the surface pocket of Bcl-2 and can be used as a cell-permeable agent to affect the Bcl-2–regulated apoptotic pathway.[29] Current evidence indicates that low concentrations of HA14-1 can efficiently sensitize human glioblastoma multiforme cells in vitro and in vivo to induction of cell death by chemotherapy and radiotherapy.[30] To determine whether HA14-1 could enhance apoptosis in the *REV3L*-overexpressing cells, we treated the *REV3L*-overexpressing U251 cells with HA14-1 and cisplatin at different concentrations and for different time periods and measured the expression of cleaved caspase-3 and cell viability. HA14-1, in combination with cisplatin (2 μmol/l) at different time points (Fig. 4C) or three different doses of cisplatin (1, 2, and 3 μmol/l; Fig. 4D) significantly enhanced cleavage of caspase-3 in the *REV3L*-overexpressing cells compared with the cells treated with cisplatin only. The MTT assay also showed that the HA14-1 treatment led to decreased cell viability in the *REV3L*-overexpressing cells in a dose-dependent manner compared with the cells without HA14-1 treatment ($p < 0.01$; Fig. 4E). Thus, inhibition of Bcl-2 by HA14-1 in the *REV3L*-overexpressing cells led to increased sensitivity to cisplatin-induced apoptosis.

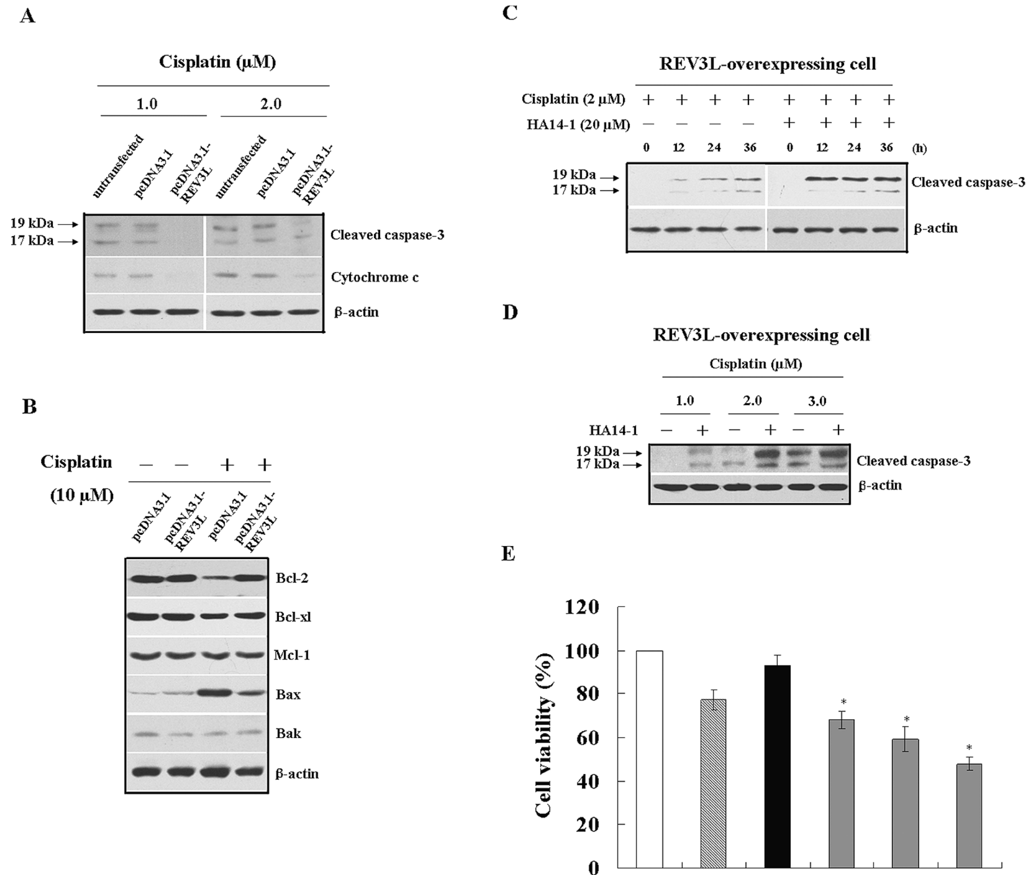

Fig. 4. *REV3L* overexpression conferred resistance to DNA cross-linking agents via inhibition of cisplatin-induced cell death of mitochondria-mediated apoptotic pathway. (A) The expression of cleaved caspase-3 and cytochrome c after exposure to two doses of cisplatin (1 and 2 μmol/l) for 48 h in untransfected U251 cells, vector control U251 cells, and *REV3L*-overexpressing U251 cells. Expression levels of cleaved caspase-3 and cytochrome c were lower in the *REV3L*-overexpressing U251 cells compared with the untransfected U251 cells and the vector control U251 cells. (B) Western blotting analysis of Bcl-2 family members in vector control U251 cells and *REV3L*-overexpressing U251 cells. Cells were cultured in the absence or presence of cisplatin (10 μmol/l) for 48 h. Expression levels of apoptosis-related effectors in treated cells did not significantly differ from those in untreated cells. The vector control cells that were treated with cisplatin (10 μmol/l) showed significant changes in the levels of Bcl-2 and Bax and a slight change in Bcl-xl. In contrast, the *REV3L*-overexpressing U251 cells treated with the same dose of cisplatin were indistinguishable from untreated cells, which suggests that no significant activation of these proteins occurred. Only a slight increase in Bax was observed in the *REV3L*-overexpressing U251 cells treated with cisplatin. (C) Effect of HA14-1 combined with cisplatin on the *REV3L*-overexpressing U251 cells. Cells were treated with cisplatin (2 μmol/l) in the absence or presence of HA14-1 (20 μmol/l) for the indicated periods. Expression levels of cleaved caspase-3 were significantly increased in *REV3L*-overexpressing U251 cells compared with cells treated with cisplatin only. (D) Effect of HA14-1 in combination with cisplatin on *REV3L*-overexpressing U251 cells. Cells were treated with HA14-1 (20 μmol/l) and three doses of cisplatin (1, 2, and 3 μmol/l) for 48 h. Expression levels of cleaved caspase-3 were significantly increased in *REV3L*-overexpressing U251 cells compared with levels in cells treated with cisplatin only. (E) Effect of HA14-1 treatment on cell viability after exposure to cisplatin by the diphenyltetrazolium bromide assay. Cells treated with HA14-1 alone were used as a toxicity control in the cell viability studies. HA14-1 treatment led to decreased cell viability in response to cisplatin. Data are means of three independent experiments ± SD. $^*p < 0.01$.

Suppression of REV3L *Expression by RNAi Could Efficiently Synergize with Cisplatin to Induce Glioma Cell Apoptosis via the Mitochondria-Mediated Apoptotic Pathway*

It has been suggested that cisplatin can induce an increase in *REV3L* mRNA levels in normal cells.[23] Thus, we next investigated whether cisplatin also increases *REV3L* mRNA levels in glioma cells. As shown in Fig. 5A, concentration-dependent increases in *REV3L* mRNA levels in the parental U251 cells were observed when measured at 24 h after a 1-h exposure to various concentrations of cisplatin (1.31-, 1.71-, 2.92-, and 3.78-fold increases with 10, 20, 30, and 40 μmol/l, respectively). Interestingly, a slight increase of *REV3L* mRNA levels in sh*REV3L* cells was also induced by cisplatin, suggesting that the ability to downregulate *REV3L* by RNAi may be antagonized by an increase in endogenous *REV3L* mRNA production. We also found that *REV3L* mRNA levels continued to increase and peaked at 24 h after a 1-h exposure to 20 μmol/l cisplatin in both cell lines but was significantly lower in sh*REV3L* cells compared with the parental U251 cells (Fig. 5A). Thus, these

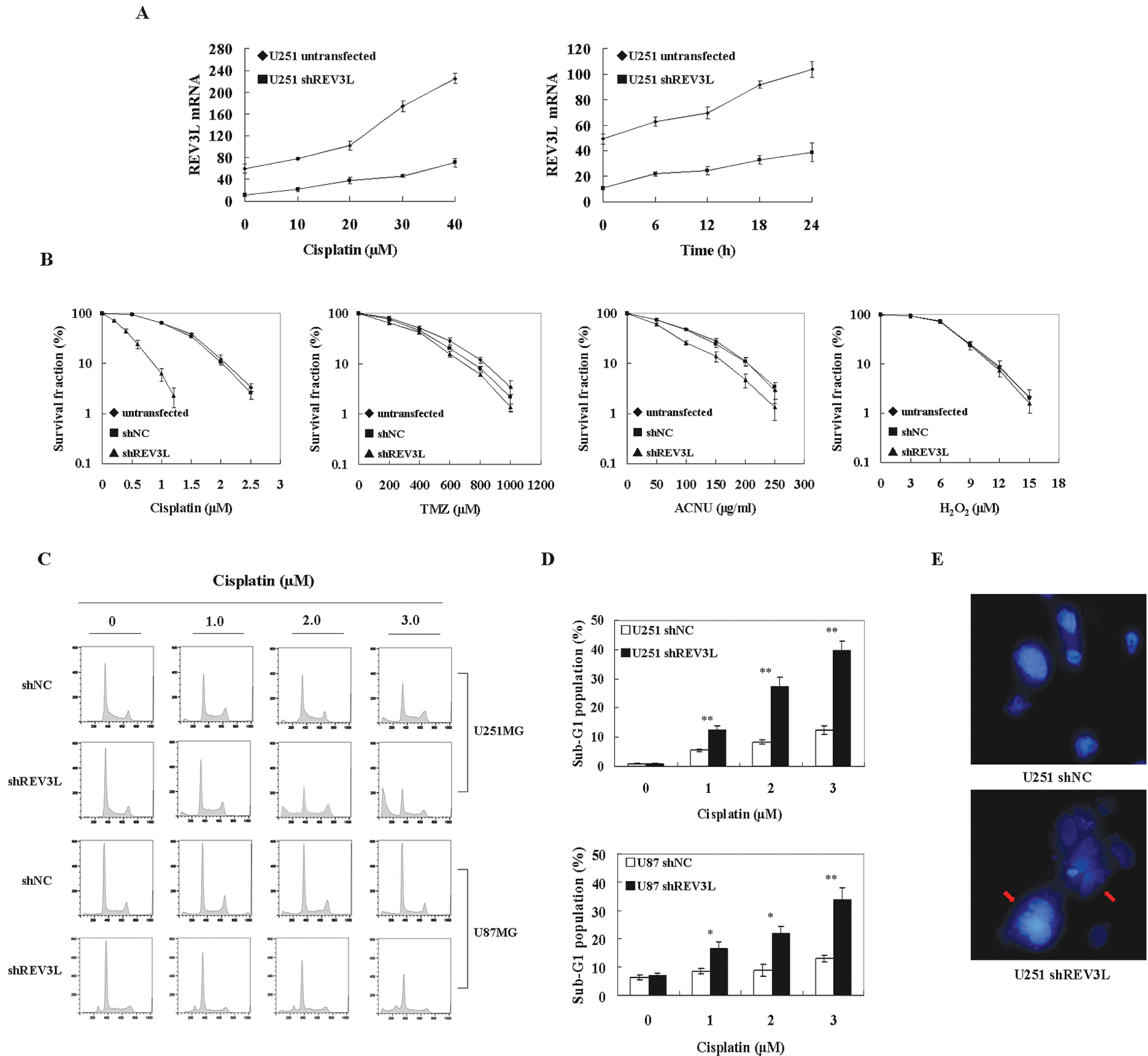

Fig. 5. *REV3L* knockdown can efficiently synergize the apoptosis response to treatment with cisplatin in glioma cells. (A) Real-time PCR analysis for *REV3L* mRNA level in the untransfected U251 cells and short hairpin interfering RNA for *REV3L* (sh*REV3L*) U251 cells induced by various doses of cisplatin (10, 20, 30, and 40 μmol/l) at 24 h after a 1-h exposure (left), and time course change of *REV3L* mRNA level in both cell lines after exposure to 20 μmol/l cisplatin for 1 h (right). Data are means of three independent real-time PCR measurements ± SD. (B) Colony formation ability of the untransfected U251 cells, short hairpin RNA-transfected negative control (shNC) cells, and sh*REV3L* cells after continuous treatment with cisplatin, temozolomide (TMZ), nimustine hydrochloride (ACNU), and H_2O_2. Suppression of *REV3L* expression conferred hypersensitivity to cisplatin but not to TMZ, ACNU, or H_2O_2. Data are means of three independent experiments ± SD. (C) Detection of apoptotic cells by flow cytometry. shNC U251, sh*REV3L* U251, shNC U87, and sh*REV3L* U87 cells were treated with three doses of cisplatin (1, 2, and 3 μmol/l) for 24 h. A sub-G_1 peak was significantly higher in sh*REV3L* cells than in shNC cells. (D) Sub-G_1 peak results. Data are means of three independent experiments ± SD. $*p < 0.01$; $**p < 0.001$. (E) Morphological examination to detect apoptosis by 4',6-diamidino-2-phenylindole dihydrochloride staining. Nuclear condensation and fragmentation of sh*REV3L* cells were more prominent than shNC cells after treatment with cisplatin (2 μmol/l) for 24 h.

results indicate that *REV3L* might be activated by exposure to cisplatin in a dose- and time-dependent manner, and effective repression of *REV3L* expression could be a potential therapeutic target for the treatment of human gliomas.

To determine whether suppression of *REV3L* expression could enhance the chemosensitivity of human glioma cells to chemotherapeutic drugs, we adopted the strategy of *REV3L* RNAi to repress the expression of *REV3L*. Colony formation assays showed that reduced expression of *REV3L* rendered glioma cells more sensitive to the cytotoxic effect of cisplatin but not to TMZ, ACNU,

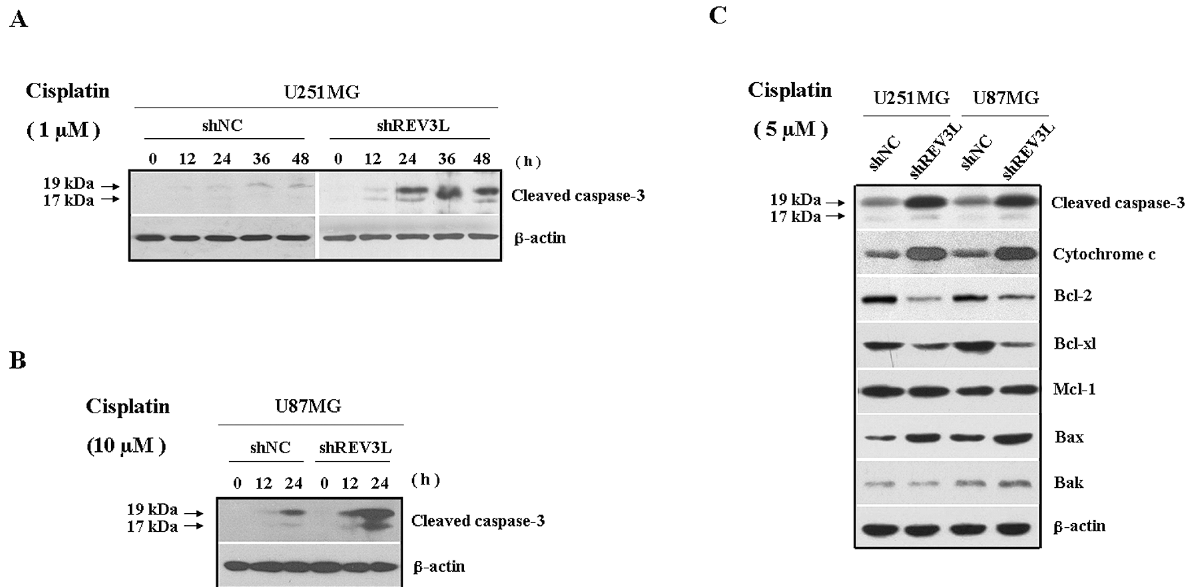

Fig. 6. *REV3L* knockdown could efficiently synergize with cisplatin to induce glioma cell apoptosis via the mitochondria-mediated apoptotic pathway. (A) Time-dependent expression of cleaved caspase-3 after exposure to a single dose of cisplatin (1 μmol/l) in short hairpin RNA-transfected negative control (shNC) and short hairpin interfering RNA for *REV3L* (sh*REV3L*) U251 cells. Expression levels of cleaved caspase-3 were significantly higher in sh*REV3L* U251 cells compared with shNC U251 cells in response to the same dose of cisplatin. (B) Time-dependent expression of cleaved caspase-3 after exposure to a single dose of cisplatin (10 μmol/l) in shNC and sh*REV3L* U87 cells. Expression levels of cleaved caspase-3 were significantly higher in sh*REV3L* U87 cells compared with shNC U87 cells in response to the same dose of cisplatin. (C) The expression levels of cleaved caspase-3, cytochrome c, and Bcl-2 family members after exposure to a single dose of cisplatin (5 μmol/l) for 48 h in shNC and shREV3L cells. Expression levels of Bcl-2 and Bcl-xl were significantly lower and levels of cleaved caspase-3, cytochrome c, and Bax were significantly higher in sh*REV3L* cells compared with shNC cells in response to the same dose of cisplatin. In contrast, the expression levels of Mcl-1 and Bak were indistinguishable from those of shNC cells.

or H_2O_2 (Fig. 5B). Changes in cell cycle distribution as well as cell death, reflected by the sub-G_1 cells, were then analyzed. The data showed that cisplatin evoked reproducible and significant levels of death in sh*REV3L* U251 cells as reflected by emerging sub-G_1 cells of 12%, 27.8%, and 43.1% in response to three doses of cisplatin (1, 2, and 3 μmol/l) for 24 h (Fig. 5C,D). Similar results were also obtained in sh*REV3L* U87 cells treated with three same doses of cisplatin for 24 h (Fig. 5C,D). In contrast, cisplatin did not induce significant levels of apoptosis in shNC U251 and shNC U87 cells. DAPI fluorescence nuclear staining showed that the nuclear condensation and fragmentation of sh*REV3L* U251 cells were more prominent than shNC U251 cells at 24 h exposure to 2 μmol/l cisplatin (Fig. 5E). Furthermore, after exposure to cisplatin (1 μmol/l) for 48 h, a time-dependent increase in the amounts of cleaved caspase-3 was observed in sh*REV3L* U251 cells (Fig. 6A). Similar results were also obtained in sh*REV3L* U87 cells treated with cisplatin (10 μmol/l) for 24 h (Fig. 6B).

To further confirm that sh*REV3L*-induced sensitization to cisplatin was a result of increased apoptosis via mitochondria-mediated apoptotic pathway, we performed analysis of Bcl-2 family members mentioned above in shNC cells and shREV3L cells without cisplatin treatment. However, suppression of *REV3L* expression did not affect this apoptosis-related protein expression in untreated cells (data not shown). We then determined levels of cleaved caspase-3, cytochrome c, and Bcl-2 family members in the presence of cisplatin. As expected, after treatment with cisplatin (5 μmol/l) for 48 h, cleaved caspase-3, cytochrome c, and Bax levels increased and Bcl-2 and Bcl-xl levels concomitantly decreased, without leading to any detectable changes in Mcl-1 and Bak expression, in sh*REV3L* U251 and U87 cells compared with shNC U251 and U87 cells (Fig. 6C). In addition, there were no differences in the expression levels of these proteins between shNC cells and sh*REV3L* cells in response to TMZ, ACNU, or H_2O_2 (data not shown). Thus, these results suggest that suppression of *REV3L* expression by RNAi could efficiently synergize the mitochondrial apoptotic response to treatment with cisplatin in glioma cells. This effect may be partially mediated by marked alterations in Bcl-2, Bcl-xl, and Bax protein levels.

Suppression of REV3L *Expression Reduced Mutation Rates at the HPRT Locus*

To further elucidate the molecular mechanism responsible for sh*REV3L*-induced chemosensitization, we determined if suppression of *REV3L* expression reduced the frequency of cisplatin-induced mutations in surviving glioma cells. Cells were treated with three doses of cisplatin (10, 20, and 30 μmol/l) for 1 h and then cultured for 2 weeks. An *HPRT* mutation assay was then carried out to assess the frequency of cisplatin-induced 6-TG–resistant mutants, which reflect the mutation frequencies at the *HPRT* locus.[31] As shown in Fig. 7, the frequen-

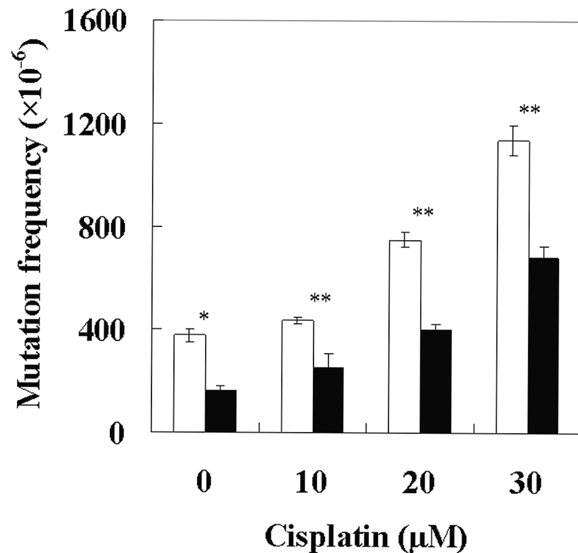

Fig. 7. *REV3L* knockdown reduced mutation frequency at the hypoxanthine guanine phosphoribosyl transferase locus (*HPRT*): frequency of cisplatin-induced generation of 6-thioguanine (6-TG) mutants after a 1-h exposure to one of three doses of cisplatin (10, 20, and 30 μmol/l) and 2 weeks of culture. Cisplatin-induced generation of 6-TG mutants was significantly reduced in short hairpin interfering *REV3L* RNA (sh*REV3L*) cells compared with short hairpin RNA-transfected negative control (shNC) cells in response to cisplatin. Data are means of three independent experiments ± SD. *$p < 0.01$; **$p < 0.001$.

cies of 6-TG–resistant mutants induced by cisplatin were significantly reduced in the surviving sh*REV3L* U251 cells compared with shNC U251 cells, suggesting that suppression of *REV3L* expression significantly reduced the frequencies of cisplatin-induced mutations at the *HPRT* locus. Thus, these data indicate that suppression of *REV3L* expression by RNAi could synergize the effect of cisplatin on inducing apoptosis of glioma cells through repressing the TLS pathway and thus contribute to less frequent mutations in those surviving glioma cells.

Discussion

DNA polymerase ζ, together with DNA polymerases η, ι, and κ, are responsible for carrying out TLS, which includes error-prone or error-free DNA repair processes past fork-blocking lesions.[32] The TLS pathway may play an important role in the initiation and progression of tumors.[33–35] Previous studies have revealed that these specialized low-stringency DNA polymerases may contribute to spontaneous and DNA-lesion–triggered mutations during translesional DNA replication, thereby contributing to the accumulation of genetic damage.[10,11,13] However, the relationships between those family members and tumors are uncertain. It has been reported that the expression level of the *REV3L* gene appeared to be similar in lung, gastric, colon, and renal tumors compared to normal-tissue counterparts.[8] *REV7L* was found to be highly upregulated in breast and colon cancers.[36,37] O-Wang et al.[38] reported that polymerase κ was significantly elevated in human lung cancers, whereas polymerase η was unaltered. In another study, however, transcription levels of polymerases η, ι, and κ and the *REV3L* gene were significantly reduced in various lung, stomach, and colorectal cancers.[39] In the present study, using RT-PCR and real-time PCR, we showed that the expression of *REV3L* was significantly upregulated in malignant gliomas and basically correlated with tumor grade. These findings indicate that the specialized DNA polymerases of TLS in human malignancies are deregulated, and their potential roles in tumorigenesis and progression may differ depending on the type, origin, and tissue specificity of tumors. Therefore, further studies are needed to elucidate the role of these specialized DNA polymerases in tumorigenesis and tumor progression.

Chemoresistance is a major obstacle for the treatment of malignant gliomas, which are unable to be totally resected because of their diffusely infiltrative nature.[40] Many different mechanisms may account for this chemoresistance, including multidrug resistance, upregulation of antiapoptotic pathways, enhanced DNA repair, and increased metabolic inactivation and subsequent elimination of the applied drugs.[41,42] Recently, *REV3L* has been suggested to regulate cytotoxicity, mutagenicity, and chemoresistance of cisplatin in both human fibroblast cells and human colon carcinoma cells.[23,24] To investigate *REV3L* function in human glioma cells, we generated stable transfectants overexpressing *REV3L* and found that overexpression of *REV3L* may inhibit the sensitivity of glioma cells to cisplatin. *REV3L* overexpression conferred resistance to cisplatin and decreased cisplatin-induced apoptosis of the mitochondria-mediated apoptotic pathway due to, in part, minor alterations in Bcl-2, Bcl-xl, and Bax expression levels. However, how *REV3L* regulates apoptosis of glioma cells through DNA repair pathway and whether the regulation involves a DNA-repair-independent pathway remain to be investigated.

Previous studies have demonstrated that cisplatin treatment in human normal cells and tumor cells can induce an increased expression of certain DNA polymerases involved in the TLS of cisplatin-mediated DNA damage.[22,23] In our system, we also showed that cisplatin induced a concentration- and time-dependent increase in the *REV3L* mRNA level in glioma cells, indicating that *REV3L* may play an important role in the DNA damage response in both normal and tumor cells and that there may be a negative loop regulation mechanism for the resistance of tumor cells to cisplatin treatment. By expressing shRNAs targeted to *REV3L* mRNA, we established stable human glioma cell lines with very low levels of *REV3L* mRNA and found that disruption of *REV3L* function by downregulation of its mRNA increased cellular sensitivity to cisplatin. In addition, activation of caspase-3 and cytochrome c, as well as marked alterations in the expression levels of Bcl-2, Bcl-xl, and Bax, confirmed that suppression of *REV3L* expression facilitated cisplatin-induced activation of

the mitochondrial apoptotic pathway in glioma cells. These results are clearly reminiscent of those of *REV7L* knockdown, which was previously reported to confer hypersensitivity to certain types of chemotherapeutic agents, especially cisplatin, through activation of apoptosis.[13] Although the redundant functions of specialized DNA polymerase family members in TLS remain elusive, it seems that *REV3L* may play a predominant role in regulating the sensitivity to the DNA cross-linking agent cisplatin in human gliomas. RNAi is emerging as a powerful approach for gene target therapy.[43] With recent advances in RNAi delivery strategy, the shRNA complex could be efficiently delivered to the brain.[44–46] Therefore, downregulation of *REV3L* expression by RNAi in combination with cisplatin could enhance the clinical efficacy of chemotherapy for glioma patients.

In summary, we have shown for the first time that *REV3L* is overexpressed in human gliomas, especially in high-grade gliomas. Enhancement of *REV3L* expression in glioma cells resulted in reduced sensitivity to cisplatin-induced cell death. Inhibition of Bcl-2 in *REV3L*-overexpressing cells by HA14-1 significantly promoted cisplatin-induced apoptosis. Furthermore, suppression of *REV3L* expression by RNAi enhanced the sensitivity of glioma cells to cisplatin, led to more pronounced apoptosis in association with marked alterations in Bcl-2, Bcl-xl, and Bax expression levels, and reduced frequencies of cisplatin-induced mutations in glioma cells. Future studies are therefore warranted to determine the function of *REV3L* in vivo, especially its role in tumorigenesis and chemoresistance. Whether other DNA polymerases involved in TLS might be responsible for chemoresistance to DNA-damaging agents in human malignancies remains to be examined.

Acknowledgments

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The current affiliation of H.W. is Department of Pathology and Cancer Center, Medical College of Georgia, Augusta, GA, USA.

References

1. Behin A, Hoang-Xuan K, Carpentier AF, Delattre JY. Primary brain tumours in adults. *Lancet*. 2003;361:323–331.
2. Furnari FB, Fenton T, Bachoo RM, et al. Malignant astrocytic glioma: genetics, biology, and paths to treatment. *Genes Dev*. 2007;21:2683–2710.
3. Barzon L, Zanusso M, Colombo F, Palu G. Clinical trials of gene therapy, virotherapy, and immunotherapy for malignant gliomas. *Cancer Gene Ther*. 2006;13:539–554.
4. DeAngelis LM. Brain tumors. *N Engl J Med*. 2001;344:114–123.
5. Okada H, Pollack IF. Cytokine gene therapy for malignant glioma. *Expert Opin Biol Ther*. 2004;4:1609–1620.
6. Morelli C, Mungall AJ, Negrini M, Barbanti-Brodano G, Croce CM. Alternative splicing, genomic structure, and fine chromosome localization of REV3L. *Cytogenet Cell Genet*. 1998;83:18–20.
7. Gibbs PE, McGregor WG, Maher VM, Nisson P, Lawrence CW. A human homolog of the Saccharomyces cerevisiae REV3 gene, which encodes the catalytic subunit of DNA polymerase zeta. *Proc Natl Acad Sci U S A*. 1998;95:6876–6880.
8. Kawamura K, O-Wang J, Bahar R, et al. The error-prone DNA polymerase zeta catalytic subunit (Rev3) gene is ubiquitously expressed in normal and malignant human tissues. *Int J Oncol*. 2001;18:97–103.
9. Xiao W, Lechler T, Chow BL, et al. Identification, chromosomal mapping and tissue-specific expression of hREV3 encoding a putative human DNA polymerase zeta. *Carcinogenesis*. 1998;19:945–949.
10. Okada T, Sonoda E, Yoshimura M, et al. Multiple roles of vertebrate REV genes in DNA repair and recombination. *Mol Cell Biol*. 2005;25:6103–6111.
11. Sonoda E, Okada T, Zhao GY, et al. Multiple roles of Rev3, the catalytic subunit of polzeta in maintaining genome stability in vertebrates. *EMBO J*. 2003;22:3188–3197.
12. Van Sloun PP, Varlet I, Sonneveld E, et al. Involvement of mouse Rev3 in tolerance of endogenous and exogenous DNA damage. *Mol Cell Biol*. 2002;22:2159–2169.
13. Cheung HW, Chun AC, Wang Q, et al. Inactivation of human MAD2B in nasopharyngeal carcinoma cells leads to chemosensitization to DNA-damaging agents. *Cancer Res*. 2006;66:4357–4367.
14. Nelson JR, Lawrence CW, Hinkle DC. Thymine-thymine dimer bypass by yeast DNA polymerase zeta. *Science*. 1996;272:1646–169.
15. Albertella MR, Lau A, O'Connor MJ. The overexpression of specialized DNA polymerases in cancer. *DNA Repair*. 2005;4:583–593.
16. Grossman SA, Wharam M, Sheidler V, et al. Phase II study of continuous infusion carmustine and cisplatin followed by cranial irradiation in adults with newly diagnosed high-grade astrocytoma. *J Clin Oncol*. 1997;15:2596–2603.
17. DeAngelis LM. Chemotherapy for brain tumors—a new beginning. *N Engl J Med*. 2005;352:1036–1038.
18. Bredel M, Zentner J. Brain-tumour drug resistance: the bare essentials. *Lancet Oncol*. 2002;3:397–406.
19. Fruehauf JP, Brem H, Brem S, et al. In vitro drug response and molecular markers associated with drug resistance in malignant gliomas. *Clin Cancer Res*. 2006;12:4523–4532.
20. Nagane M, Levitzki A, Gazit A, Cavenee WK, Huang HJ. Drug resistance of human glioblastoma cells conferred by a tumor-specific mutant epidermal growth factor receptor through modulation of Bcl-XL and caspase-3-like proteases. *Proc Natl Acad Sci U S A*. 1998;95:5724–5729.
21. Lin X, Okuda T, Trang J, Howell SB. Human REV1 modulates the cytotoxicity and mutagenicity of cisplatin in human ovarian carcinoma cells. *Mol Pharmacol*. 2006;69:1748–1754.

22. Okuda T, Lin X, Trang J, Howell SB. Suppression of hREV1 expression reduces the rate at which human ovarian carcinoma cells acquire resistance to cisplatin. *Mol Pharmacol*. 2005;67:1852–1860.
23. Wu F, Lin X, Okuda T, Howell SB. DNA polymerase zeta regulates cisplatin cytotoxicity, mutagenicity, and the rate of development of cisplatin resistance. *Cancer Res*. 2004;64:8029–8035.
24. Lin X, Trang J, Okuda T, Howell SB. DNA polymerase zeta accounts for the reduced cytotoxicity and enhanced mutagenicity of cisplatin in human colon carcinoma cells that have lost DNA mismatch repair. *Clin Cancer Res*. 2006;12:563–568.
25. Kleihues P, Louis DN, Scheithauer BW, et al. The WHO classification of tumors of the nervous system. *J Neuropathol Exp Neurol*. 2002;61:215–229.
26. Chiu RK, Brun J, Ramaekers C, et al. Lysine 63-polyubiquitination guards against translesion synthesis-induced mutations. *PLoS Genet*. 2006;2:e116.
27. Asakura T, Ohkawa K. Chemotherapeutic agents that induce mitochondrial apoptosis. *Curr Cancer Drug Targets*. 2004;4:577–590.
28. Robertson JD, Orrenius S. Role of mitochondria in toxic cell death. *Toxicology*. 2002;181–182:491–496.
29. Wang JL, Liu D, Zhang ZJ, et al. Structure-based discovery of an organic compound that binds Bcl-2 protein and induces apoptosis of tumor cells. *Proc Natl Acad Sci U S A*. 2000;97:7124–7129.
30. Manero F, Gautier F, Gallenne T, et al. The small organic compound HA14-1 prevents Bcl-2 interaction with Bax to sensitize malignant glioma cells to induction of cell death. *Cancer Res*. 2006;66:2757–2764.
31. Maher VM, McCormick JJ. The HPRT gene as a model study for mutation analysis. In: Pfeifer GP, ed. *Technologies for Detection of DNA Damage and Mutations*. New York: Plenum; 1996:381–390.
32. Lehmann AR. Translesion synthesis in mammalian cells. *Exp Cell Res*. 2006;312:2673–2676.
33. Andersen PL, Xu F, Xiao W. Eukaryotic DNA damage tolerance and translesion synthesis through covalent modifications of PCNA. *Cell Res*. 2008;18:162–173.
34. Lawrence CW, Hinkle DC. DNA polymerase zeta and the control of DNA damage induced mutagenesis in eukaryotes. *Cancer Surv*. 1996;28:21–31.
35. Liu G, Chen X. DNA polymerase eta, the product of the xeroderma pigmentosum variant gene and a target of p53, modulates the DNA damage checkpoint and p53 activation. *Mol Cell Biol*. 2006;26:1398–1413.
36. Yuan B, Xu Y, Woo JH, et al. Increased expression of mitotic checkpoint genes in breast cancer cells with chromosomal instability. *Clin Cancer Res*. 2006;12:405–410.
37. Rimkus C, Friederichs J, Rosenberg R, Holzmann B, Siewert JR, Janssen KP. Expression of the mitotic checkpoint gene MAD2L2 has prognostic significance in colon cancer. *Int J Cancer*. 2007;120:207–211.
38. O-Wang J, Kawamura K, Tada Y, et al. DNA polymerase kappa, implicated in spontaneous and DNA damage-induced mutagenesis, is overexpressed in lung cancer. *Cancer Res*. 2001;61:5366–5369.
39. Pan Q, Fang Y, Xu Y, Zhang K, Hu X. Downregulation of DNA polymerases kappa, eta, iota, and zeta in human lung, stomach, and colorectal cancers. *Cancer Lett*. 2005;217:139–147.
40. Lu C, Shervington A. Chemoresistance in gliomas. *Mol Cell Biochem*. 2008;312:71–80.
41. Bredel M. Anticancer drug resistance in primary human brain tumors. *Brain Res Brain Res Rev*. 2001;35:161–204.
42. Bronger H, Konig J, Kopplow K, et al. ABCC drug efflux pumps and organic anion uptake transporters in human gliomas and the blood-tumor barrier. *Cancer Res*. 2005;65:11419–11428.
43. Kim DH, Rossi JJ. Strategies for silencing human disease using RNA interference. *Nat Rev Genet*. 2007;8:173–184.
44. Grzelinski M, Urban-Klein B, Martens T, et al. RNA interference-mediated gene silencing of pleiotrophin through polyethylenimine-complexed small interfering RNAs in vivo exerts antitumoral effects in glioblastoma xenografts. *Hum Gene Ther*. 2006;17:751–766.
45. Medarova Z, Pham W, Farrar C, Petkova V, Moore A. In vivo imaging of siRNA delivery and silencing in tumors. *Nat Med*. 2007;13:372–377.
46. Pardridge WM. shRNA and siRNA delivery to the brain. *Adv Drug Deliv Rev*. 2007;59:141–152.