

Suppression of hREV1 Expression Reduces the Rate at Which Human Ovarian Carcinoma Cells Acquire Resistance to Cisplatin

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ABSTRACT

Replicative bypass of many DNA adducts is dependent on the interaction of hREV1 with DNA polymerase ζ and potentially with members of the Y family of DNA polymerases. To examine the role of hREV1 in the development of cisplatin (DDP) resistance, a subline (2008-shREV1-3.3) of the ovarian carcinoma cell line 2008 was isolated in which stable expression of a short hairpin RNA suppressed *hREV1* expression to 20% and reduced hREV1 protein level to 43% of that found in the parental cells. The 2008-shREV1-3.3 cells were 1.5-fold more sensitive to the cytotoxic effect of DDP but less sensitive to the mutagenic effect of DDP as evidenced by a 2.6- or 2.7-fold reduction in the ability to induce clones highly resistant to 6-thioguanine or DDP itself, respectively, in the surviving population. Reduction of hREV1 did not alter the initial rate of DDP adduct

removal from DNA but did impair both spontaneous and DDP-induced extra-chromosomal homologous recombination, as measured by the recombination-sensitive reporter vector pBHRF. DDP induced an increase in hREV1 protein level. DDP resistance at the population level evolved 2.8-fold more slowly in the 2008-shREV1-3.3 cells than in the parental cells during repeated cycles of drug exposure. The results indicate that hREV1 functions to enhance both cell survival and the generation of drug-resistant variants in the surviving population. DDP up-regulates hREV1, suggesting that it may enhance its own mutagenicity. Most importantly, hREV1 controls the rate of emergence of resistance to DDP at the population level. Thus, hREV1 is an important contributor to DDP-induced genomic instability and the subsequent emergence of resistance.

Cisplatin (DDP) is used to treat many types of solid tumors; however, its clinical efficacy is limited by the rapid development of resistance. Most tumors that are initially sensitive to this drug become resistant over the course of four to six cycles of treatment. The evolution of resistance seems to involve both selection for pre-existing resistant variants and mutational generation of new highly drug-resistant clones in the surviving population (Schabel et al., 1980; Fink et al., 1998; Lin and Howell, 1999). DDP produces both intra- and interstrand crosslinks in DNA, and these are believed to be important to both the cytotoxicity and the mutagenicity of the drug. DDP is a mutagen in both bacterial (Yarema et al., 1994, 1995) and mammalian cell systems (Turnbull et al.,

1979; Wiencke et al., 1979; Johnson et al., 1980; Cariello et al., 1992; Lin and Howell, 1999; Lin et al., 2001). Whereas DDP triggers recombinational events, most of the mutations generated by DDP seem to result from bypass replication across DDP adducts by the eukaryotic DNA polymerases β , μ , and ζ (Vaisman et al., 1999, 2000; Masutani et al., 2000; Vaisman and Chaney, 2000; Yuan et al., 2000; Havener et al., 2003). When non-error-prone mechanisms for repairing DNA damage (such as base excision repair, nucleotide excision repair, and homologous recombination) are disabled or overwhelmed, bacteria and yeast cells make increased use of specialized low-fidelity, error-prone DNA polymerases to bypass DNA lesions that block normal replicative polymerases (Johnson et al., 1999). Such increased reliance on error-prone translesional bypass seems to contribute to the mutagenicity of DDP adducts (Hoffmann et al., 1997).

In *Saccharomyces cerevisiae*, the majority of DNA damage-induced mutagenesis arises as a result of a translesional replication pathway mediated by REV1 and DNA polymerase ζ (reviewed in Lawrence, 2002). This pathway is required for

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ABBREVIATIONS: DDP, cisplatin [*cis*-diamminedichloroplatinum(II)]; Pol, polymerase; shRNA, short hairpin interfering RNA; PCR, polymerase chain reaction; Ct, cycle number at which fluorescence first exceeds the threshold; 6TG, 6-thioguanine; GFP, green fluorescent protein; EBFP, enhanced blue fluorescent protein.

mutagenesis induced by UV radiation, acetylaminofluorene, and apurinic sites (Morrison et al., 1989; Baynton et al., 1998; Nelson et al., 2000) and for the bypass of adducts at the N² position of guanine situated in the minor groove (Washington et al., 2004). Yeast Rev1 has a unusual dCMP transferase activity that inserts a C opposite not only normal template guanine but also a variety of adducted forms of guanine (Nelson et al., 1996; Lin et al., 1999a; Haracska et al., 2002; Zhang et al., 2002; Guo et al., 2004), and pol ζ has the unique ability to extend nucleotide incorporation from this poorly matched base (Haracska et al., 2002; Guo et al., 2004; Washington et al., 2004). Although yeast Rev1 is required for translesional synthesis by pol ζ , its dCMP transferase activity does not seem to be required for the bypass of all types of lesions (Baynton et al., 1999; Nelson et al., 2000; Lawrence, 2002). The damage-induced mutagenesis pathway in which yeast REV1 operates is evolutionarily conserved in human cells, and the genes of this pathway identified in yeast as controlling platinum-containing drug sensitivity have mammalian homologues that include HHR6A, HHR6B, hRAD18, hREV1, hREV3, and hREV7. In addition to interacting with the REV7 subunit of pol ζ , mouse REV1 binds to pol κ , η , and ι , and all the interactions occur at the same site on REV1 (Guo et al., 2003). This suggests that in mammalian cells, REV1 may have a role in supporting translesional synthesis carried out by multiple DNA polymerases.

Several investigators have previously reported that loss of hREV1 function markedly reduces UV-induced hypoxanthine-guanine phosphoribosyltransferase mutations in human cells engineered to contain reduced levels of hREV1 mRNA through expression of an hREV1 antisense RNA (Gibbs et al., 2000) or a ribozyme that cleaves endogenous hREV1 mRNA (Clark et al., 2003). It has also been demonstrated that inactivation of the *REV1* gene in chicken DT40 cells renders them hypersensitive to a wide variety of DNA-damaging agents, including DDP (Simpson and Sale, 2003).

If REV1 is centrally involved in the translesional bypass of DDP adducts, then there is the possibility that disabling hREV1 function in human cancer cells might both enhance sensitivity to the cytotoxic effect of DDP and also reduce its mutagenesis. In this study, we used constitutive expression of an interfering RNA (Paddison et al., 2002) to suppress the level of hREV1 mRNA in the human ovarian cancer cell line 2008. The sensitivity of these cells to the cytotoxic and mutagenic effects of DDP was compared with that of the parental cells transfected with an empty vector. We report here that reduction in hREV1 mRNA rendered cells somewhat more sensitive to the cytotoxic effect of DDP but substantially decreased its mutagenicity. Most importantly, it significantly reduced the rate at which the whole population of cells acquired resistance to DDP during repeated cycles of drug exposure analogous to the clinical use of this drug. These results support the hypothesis that when a cell is faced with the challenge of replicating its DNA while the genome is burdened with a large number of DDP adducts, it is highly dependent on hREV1 error-prone bypass replication for survival, that the resulting mutations generate highly drug-resistant clones in the surviving population, and that these clones play a central role in the emergence of DDP resistance at the population level.

Materials and Methods

Drugs. DDP was a gift from Bristol-Myers Squibb (Princeton, NJ). A stock solution of 1 mM cisplatin in 0.9% NaCl was stored in the dark at room temperature. 6-Thioguanine was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in 0.2 N sodium hydroxide to form a 20 mM stock solution and stored at -20°C .

Design and Cloning of shRNAs. shRNAs were designed by using software available from the web site of the laboratory of Dr. Gregory Hannon (<http://katahdin.cshl.org:9331/RNAi:web/scripts/mainz.pl>). Two complementary ≈ 72 -nt DNA oligonucleotide shRNA sequences targeted to REV1 mRNA were annealed and cloned directly into the plasmid vector pSHAG-1 (Paddison et al., 2002). When transfected into 2008 cells, this vector constitutively expresses a short interfering RNA targeted to hREV1 mRNA from a RNA polymerase III-specific U6 promoter. The resultant vector has been named pSHAG-REV1. The mRNA targeting sequence is 5'-AATGTCCAACCTCCTGGTAGATTGGTCACGAAGCTTGGTGATCATGTCTACT-AGGAGTT GGACGTTCAATTTTTT-3'.

Cell Culture, Transfection, and Selection. The human ovarian carcinoma cell line 2008 was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cells were cotransfected using Eugene 6 (Roche, Indianapolis, IN) with pcDNA3.1(-) (Invitrogen, Carlsbad, CA) and either pSHAG-REV1 or pSHAG-1 as an empty vector control according to the manufacturer's recommendations. The transfection mixtures contained pcDNA3.1(-) in a 10-fold molar excess to the pSHAG vectors. Cells expressing the G418-resistance marker from pcDNA3.1(-) were then selected by exposure to 400 $\mu\text{g}/\text{ml}$ G418, and individual clones were screened for hREV1 mRNA expression level by real-time PCR and for hREV1 protein level by Western blotting. One clone, designated 2008-shREV1-3.3, was found to have a substantial stable reduction in hREV1 mRNA and protein level and was chosen for all subsequent experiments. 2008-shREV1-3.3 cells were maintained in medium supplemented with 400 $\mu\text{g}/\text{ml}$ G418. The cells transfected with the pSHAG-1 empty vector control were designated 2008-EV.

Quantitation of hREV1 mRNA by Real-Time PCR. Total RNA was extracted with TRIzol reagent (Invitrogen). First-strand cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) and random primers. Real-time PCR was performed using the iCycler iQ detection system in the presence of SYBR-Green I dye (Bio-Rad Laboratory, Hercules, CA). For *hREV1* gene expression, the forward (5'-AAGGCTGATGCAATCG-3') and reverse (5'-CCACCTGGACATTGTCAAGAATAA-3') primers were used for amplification with a iCycler protocol consisting of a denaturation program (95°C for 3 min), an amplification and quantification program repeated 40 times (95°C for 10 s and 55°C for 45 s), and melting curve analysis. A melting curve analysis immediately followed amplification and was executed using 95°C for 1 min then 55°C for 1 min, followed by 80 repeats of heating for 10 s, starting at 55°C with 0.5°C increments. The data were analyzed by using the comparative Ct method, where Ct is the cycle number at which fluorescence first exceeds the threshold. The ΔCt values from each cell line were obtained by subtracting the values for 18 S Ct from the sample Ct. A 1-unit difference in Ct value represents a 2-fold difference in the level of mRNA.

Western Blot Analysis. The nuclear proteins were extracted as described previously (Schreiber et al., 1989) and heated, and a sample containing 10 μg was then subjected to electrophoresis on a 4 to 15% SDS-PAGE gel. The proteins were then transferred to a nitrocellulose membrane that was blocked with 5% skim milk in buffer (0.35 M NaCl, 10 mM Tris-HCl, pH 8.0) containing 0.05% Tween 20 for 1 h at room temperature and then incubated overnight at 4°C with a 1:100 dilution of polyclonal antibody against hREV1 and a 1:500 dilution of a goat polyclonal antibody against histone H1 (Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was then washed and exposed for 1 h to a 1:500 dilution of a horseradish peroxidase-conjugated donkey anti-goat IgG (Santa Cruz Biotechnol-

ogy, Santa Cruz, CA), and bands were detected using the ECL Western blotting detection system (Amersham Biosciences, Buckinghamshire, UK) and analyzed densitometrically by a ChemiImager (Alpha Innotech Corporation, San Leandro, CA).

Immunofluorescent Imaging. Cells were fixed by treatment for 15 min with 3.7% formaldehyde in phosphate-buffered saline and stained as reported previously (Safaei et al., 2005). In brief, cells were stained with 1 μ g/ml Hoechst 33324 and 0.4 μ g/ml Alexa Fluor 647 phalloidin (Molecular Probes, Eugene, OR) along with a primary goat polyclonal antibody to REV1 (Santa Cruz Biotechnology) and Texas red-conjugated donkey anti-goat secondary antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Microscopy was performed at University of California San Diego Cancer Center Digital Imaging Shared Resource using a DeltaVision deconvoluting microscope system (Applied Precision, Inc., Issaquah, WA.). Images were captured from 0.2- μ m sections by 100 \times , 60 \times , and 40 \times lenses, and SoftWorx software (Applied Precision, Inc) was used for deconvolution of data. Image quantification was performed with Data Inspector program in SoftWorx or by NearCount software.

Clonogenic and Enrichment Assays. Clonogenic assays were performed as reported previously (Lin et al., 2001) by seeding 250 cells into 35-mm dishes, adding the requisite concentration of DDP for 1 h, washing the drug away, and allowing colonies to form for 10 to 14 days. Each experiment was performed a minimum of three times using triplicate cultures for each drug concentration. IC₅₀ values were determined by log-linear interpolation. For enrichment assays, a cell population containing 10% 2008-EV cells constitutively expressing GFP and 90% 2008-shREV1-3.3 cells was prepared by mixing appropriate numbers of the two cell types, and the actual fraction of 2008-EV cells was documented by flow cytometry. Enrichment assays were performed by plating 10,000 cells from the mixed cell population in 100-mm tissue culture dishes for 24 h and then exposing the cells to increasing concentrations of DDP for 1 h. The untreated cell population served as a control. Flow cytometric analysis was repeated 5 days later to determine the fraction of GFP-expressing 2008-EV cells in the treated and control groups. Each experiment was performed three separate times for each drug concentration.

Measurement of Rate of Generation of Resistant Variants and DDP Mutagenicity. The rate at which highly drug-resistant variants spontaneously appeared in the population was measured using the "maximum likelihood estimation" technique (Glaab and Tindall, 1997) as reported previously (Lin and Howell, 1999). The sensitivity of cells to the mutagenic effects of DDP was measured by determining the frequency of variants highly resistant to either 10 μ M 6TG or to 12 μ M DDP itself in the surviving population 20 days after a 1-h exposure to increasing concentrations of DDP as reported previously (Lin and Howell, 1999; Lin et al., 2001). Each experiment was performed a minimum of three times, and the data are presented as mean \pm S.E.M. When testing for 6TG-resistant variants, the cells were grown in medium containing 0.4 μ M aminopterin, 16 μ M thymidine, and 100 μ M hypoxanthine for a minimum of 14 days before testing to eliminate pre-existing hypoxanthine-guanine phosphoribosyltransferase mutants.

Measurement of Pt in DNA. DNA was isolated using Wizard Genomic DNA Purification Kit (Promega, Madison, WI) according to the manufacturer's instructions. Aliquots of the DNA were digested in 70% nitric acid at 65°C for 2 h and diluted to 5% nitric acid by adding appropriate volumes of double-distilled deionized water. The amount of platinum in the DNA in the hydrolysate (picograms of platinum per microgram of DNA) was quantified by inductively coupled plasma mass spectroscopy as described previously (Katano et al., 2002). When assessing the time course of the loss of Pt from DNA, the cells were treated with 200 μ M DDP for 1 h to obtain quantifiable levels of Pt over the entire period of the experiment. DNA was isolated at 0, 6, 12, 18, and 24 h after drug exposure.

Measurement of the Frequency of Extrachromosomal Homologous Recombination. Homologous recombination was assayed by determining the extent of recombination between two GFP sequences in plasmid DNA as described previously (Slebos and Taylor, 2001). The pBHRF vector contains an intact "blue" variant of GFP (EBFP) that includes a \approx 300-nucleotide sequence with perfect homology to a second truncated nonfunctional copy of GFP. In the absence of homologous recombination within the vector, only EBFP is expressed. However, homologous recombination between the EBFP and truncated GFP sequences creates a functional GFP; if this occurs, the cell expresses GFP as well as EBFP, which is expressed from other plasmids in the cell that have not undergone recombination. Cells were seeded into six-well plates overnight and then exposed to 0 or 10 μ M DDP for 1 h. The untreated or surviving cells were then transfected with pBHRF 24 h later with siPORT XP-1 transfection agent (Ambion Inc., Austin, TX) in the presence of serum according to the manufacturer's instructions. Four hours after transfection, BoosterExpress reagent (Gene Therapy Systems, Inc., San Diego, CA) was added, and the cells were analyzed by two-color flow cytometry 48 h after transfection. The recombination frequency was calculated as $RF = [(EBFP^+ \text{ and } GFP^+) + (GFP^+)] / [(EBFP^+ \text{ and } GFP^+) + (GFP^+) + (EBFP^+)]$, where (EBFP) and (GFP) represent the number of blue and green fluorescent cells, respectively, in the sample.

Relative Rate of Development Resistance to DDP. The rate at which the cell population became resistant to DDP during repeated cycles of 1 h exposure to the drug was determined by measuring the IC₅₀ for DDP by constructing a concentration-survival curve that included a minimum of five DDP concentrations using a clonogenic assay after each round of selection. The degree of resistance was determined by dividing the IC₅₀ for the multiple DDP-treated population by the IC₅₀ for the untreated cells for each cell line individually. The DDP concentration used for selection was the IC₉₀ for the population under study. For each round of selection, 10⁶ cells were exposed to DDP for 1 h. When the cells had recovered to 90% confluence, an aliquot was used to determine cell number and the slope of the DDP concentration-survival curve in a clonogenic assay, and another aliquot was again exposed to DDP. Total cell number and plating efficiency was determined at each step; this information, along with the exact number of cells subcultured, was used to calculate population doubling according to the equation described above. The rate of acquisition of resistance to DDP was then calculated by plotting the slope of the DDP concentration-survival curve as a function of population doubling: population doubling = $(\ln[\text{total number of cells}] - \ln[\text{number of cells plated} \times \text{plating efficiency}]) / \ln 2$. The slope of this plot yields the rate of relative resistance development.

Statistics. All data were analyzed by use of a two-sided paired Student's *t* test with the assumption of unequal variance.

Results

Characterization of 2008-shREV1-3.3 Cells with Stable shRNA-Mediated Knockdown of hREV1. The ovarian carcinoma cell line 2008 was cotransfected with either a pSHAG-1 empty vector (2008-EV cells) or a vector expressing a hREV1-specific shRNA (2008-shREV1-3.3 cells) in combination with pcDNA3.1(-), which provided a selectable marker. Individual clones were expanded, and the resulting populations were screened for stable suppression of hREV1 mRNA level by real-time PCR. The endogenous hREV1 mRNA level in the 2008-shREV1-3.3 clone was found to be reduced to $20 \pm 2\%$ ($p < 0.001$) of that in 2008 cells transfected with the empty pSHAG-1 vector, and this population was used for subsequent studies. Fig. 1A shows that the 80% reduction in level of the hREV1 mRNA was accompanied by

a reduction in the 138-kDa form of REV1 protein in the 2008-shREV1-3.3 cells to 43% of that in the empty vector control cells as determined by Western blot analysis after normalization for histone H1 levels. The effect of stable expression of hREV1 shRNA was also assessed by confocal digital deconvoluting microscopic examination of cells immunofluorescently stained with a polyclonal anti-REV1 antibody. As shown in Fig. 1B, staining for REV1 was abundant in the 2008-EV cells but markedly reduced in the 2008-shREV1-3.3 cells.

Effect of REV1 Reduction on the Spontaneous Rate of Generation of Resistant Variants. The spontaneous rate of generation of variants resistant to 6TG was determined by repeatedly measuring the frequency of resistant variants in expanding populations of the 2008-EV and 2008-shREV1-3.3 cells. The results, presented in Fig. 2, show that the hREV1 shRNA-expressing 2008-shREV1-3.3 cells exhibited a 2.9-fold decrease in spontaneous rate of generation of variants resistant to 6TG compared with the empty vector-transfected 2008-EV cells ($p < 0.01$). The population-doubling times were nearly identical for the two cell types: 22.8 ± 1.0 h (mean \pm S.D.) for the 2008-EV cells and 22.9 ± 0.5 h for 2008-shREV1-3.3 cells. There was also no significant difference in cell cycle phase distribution as determined by flow cytometric analysis of propidium iodide-stained cells (2008-EV cells, G₁, 49%; S, 14%; G₂/M, 37%; 2008-shREV1-3.3 cells, G₁, 46%; S, 9%; G₂/M, 45%). Thus, the difference in spontaneous rate of development of 6TG-resistant variant can not be attributed to differences in growth rate. The reduced rate observed in the 2008-shREV1-3.3 cells is consistent with the loss of Rev1 function on spontaneous mutagenesis in a yeast system (Kalinowski et al., 1995) and validates this experimental system for the study of the effect of hREV1 on DDP pharmacodynamics.

Effect of REV1 Reduction on Sensitivity to the Cytotoxic Effect of DDP. Clonogenic assays were used to determine the effect of reduced hREV1 on sensitivity to the cytotoxic effect of DDP. Figure 3A shows the survival of 2008-EV and 2008-shREV1-3.3 cells as a function of drug concentration after a 1-h exposure to DDP. The sensitivity of the

empty-vector-transfected cells did not differ from that of the parental nontransfected 2008 cells (data not shown); however, the 2008-shREV1-3.3 cells were 1.5-fold more sensitive to the cytotoxic effect of DDP than the empty vector-transfected 2008-EV cells [IC₅₀ 5.4 ± 0.69 (mean \pm S.E.M.) μ M versus 8.0 ± 0.62 μ M; $n = 3$; $p < 0.05$]. Thus, impairment of hREV1 function by down-regulation of its mRNA enhanced sensitivity to the cytotoxic effect of DDP. The ability of DDP to select for hREV1-proficient cells in a tumor cell population under conditions in which the hREV1-proficient cells were

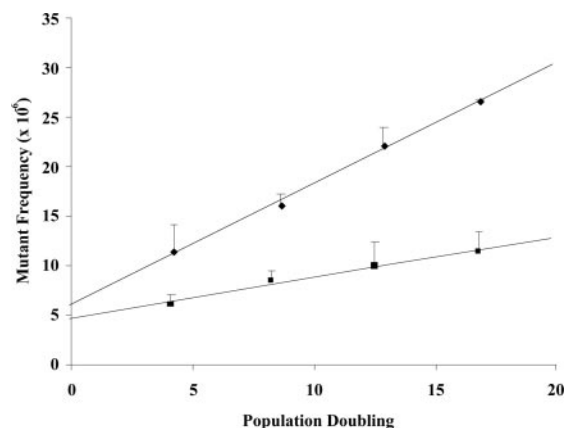


Fig. 2. Effect of reducing hREV1 level on the spontaneous rate of generation of 6TG-resistant variants. \diamond , 2008-EV cells; \blacksquare , hREV1 shRNA-expressing 2008-shREV1-3.3 cells. Each curve shows the change in the frequency of resistant variants with increasing numbers of population doublings. The rate of generation of resistant variants is given by the slope of the linear regression line. Each data point is the mean of two experiments. Vertical bars, S.E.M.

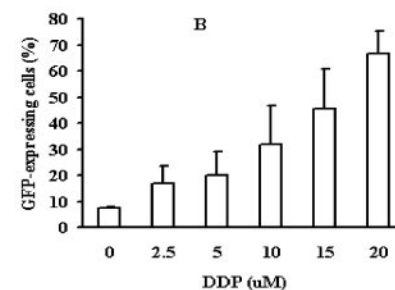
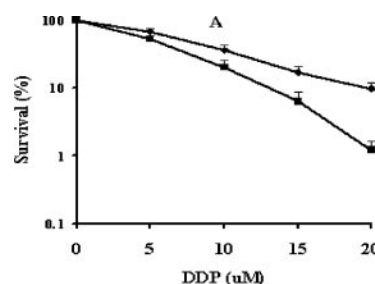


Fig. 3. Effect of hREV1 reduction on sensitivity to the cytotoxic effect of DDP as determined by clonogenic and enrichment assays. A, DDP concentration-survival curves for the hREV1 shRNA-expressing 2008-shREV1-3.3 cells (\blacksquare) and empty vector transfected 2008-EV cells (\diamond). B, DDP concentration-enrichment histogram. Each bar depicts the fraction of GFP-expressing 2008-EV cells remaining in the population 5 days after a 1-h exposure to the indicated concentration of DDP. Each point or bar represents the mean of three experiments performed with triplicate cultures. Vertical bars, S.E.M.

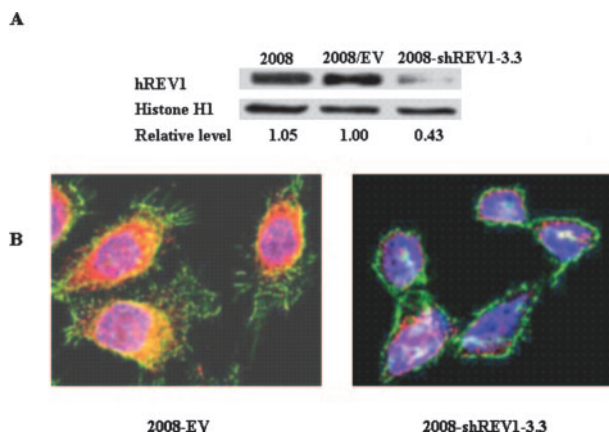


Fig. 1. shRNA-mediated suppression of hREV1 in the 2008-shREV1-3.3 cells. A, Western blot analysis of hREV1 expression. Fold reduction was calculated as the ratio of hREV1 level in the parental 2008 and 2008-shREV1-3.3 cells relative to that in the 2008-EV cells after normalization to the level of histone H1. B, shRNA-induced suppression of hREV1 at the single-cell level by immunofluorescence. hREV1 was stained with hREV1-specific antibody (red), β -actin with Alexa Fluor 647 phalloidin (green), and nuclei with Hoechst 33342 (blue).

only 1.5-fold less sensitive was tested by preparing a population containing 10% GFP-expressing 2008-EV cells and 90% hREV1 shRNA-expressing 2008-shREV1-3.3 cells. This mixed population was exposed to increasing concentrations of DDP for 1 h, and the fraction of hREV1-proficient GFP-expressing cells was determined by flow cytometric analysis after 5 days of subsequent growth in drug-free medium. Figure 3B shows that the fraction of GFP-expressing hREV1-replete 2008-EV cells increased in a DDP concentration-dependent manner. Five days after a single 1-h exposure to the highest concentration of DDP tested (20 μ M), the treated populations contained up to 66.6% GFP-expressing 2008-EV cells ($p < 0.001$). The greater sensitivity of 2008-shREV1-3.3 cells as measured by clonogenic assay and the marked selection against these cells in the enrichment assay indicate that hREV1 is a determinant of the ability of the cell to tolerate damage induced by DDP.

Effect of REV1 Reduction on the Ability of DDP to Generate Resistant Variants. DDP is a mutagen in human cells and generates mutations that result in high-level resistance to many classes of drugs (Lin and Howell, 1999; Lin et al., 1999b, 2001). To determine the role of hREV1 in DDP-induced mutagenesis, the 2008-EV and 2008-shREV1-3.3 cells were exposed to 10 μ M DDP for 1 h and then, 20 days later, the fraction of clonogenic cells demonstrating high-level resistance to 6TG or to DDP itself was determined. As shown in Fig. 4A, the frequency of 6TG-resistant variants induced by exposure to 10 μ M DDP was significantly lower in the 2008-shREV1-3.3 cells (2.6-fold) than in the 2008-EV cells ($p < 0.01$). Figure 4B shows that after exposure to DDP, the hREV1-proficient cells yielded 2.7-fold more colonies that were highly resistant to DDP itself than the hREV1-shRNA expressing cells ($p < 0.01$). Thus, DDP was able to generate

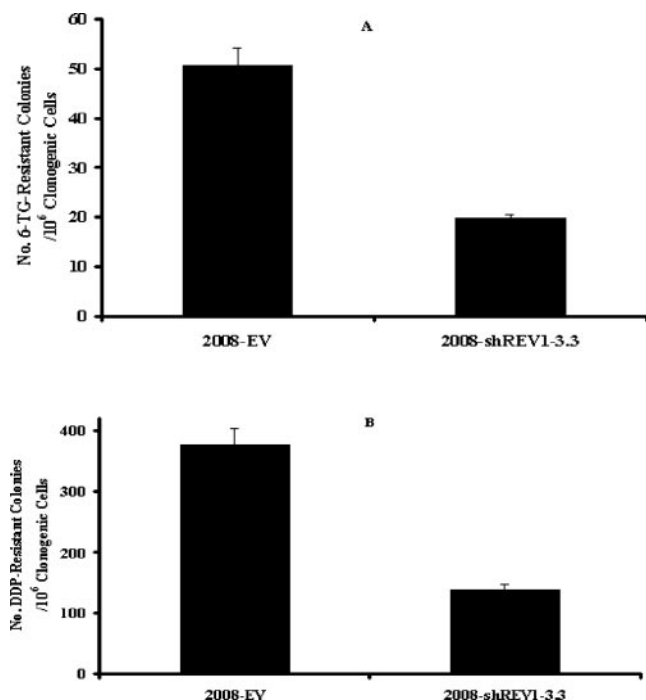


Fig. 4. Effect of hREV1 reduction on the ability of DDP to generate drug resistant variants in the surviving population. A, number of 6TG-resistant colonies per 10^6 clonogenic cells scored on day 20 after a 1-h exposure to 10 μ M DDP. B, number of DDP-resistant colonies. Each data point represents the mean of three experiments. Vertical bars, S.E.M.

variants in the surviving population that were highly resistant to 6TG or to DDP itself, and reduction in hREV1 level decreased this mutagenic effect. This indicates that hREV1 plays an important role in generating mutations that lead to drug resistance when DDP adducts are present in DNA.

Effect of hREV1 Reduction on the Removal of Platinum from DNA. The changes in sensitivity to the cytotoxic and mutagenic effects of DDP could be explained by differences in initial adduct levels or their persistence if loss of hREV1 interferes with DNA adduct removal. The rate of disappearance of platinum from the DNA accurately mirrors the rate of removal of the most common DDP adducts (Djit et al., 1988; Johnson et al., 1994). The initial Pt/DNA ratio and the rate of disappearance of platinum from total cellular DNA were measured in both cell lines after a 1-h exposure to 200 μ M DDP. The initial adduct levels were nearly identical: 6.64 ± 1.21 (mean \pm S.E.M.) and 6.79 ± 1.03 pg of Pt/ μ g of DNA, respectively, for the 2008-EV and 2008-shREV1-3.3 cells. Figure 5 shows that there was no difference in the kinetics of platinum disappearance from DNA in the two cell lines for the initial period of 12 h. More platinum remained in the 2008-shREV1-3.3 cells at 18 and 24 h (52.3 and 48.5%, respectively) than in the 2008-EV cells (41.2 and 32.4%, respectively), but this difference did not reach the level of statistical significance ($p > 0.05$). Because nucleotide excision repair is responsible for removal of the majority of DDP adducts during the initial phase of repair, this suggests that the degree of impairment of hREV1 function in the 2008-shREV1-3.3 cells did not interfere significantly with this pathway of DNA repair.

Effect of hREV1 Reduction on Spontaneous and DDP-induced Homologous Recombination. DDP induces sister chromatid exchange, and homologous recombination may play a role in the ability of DDP to generate highly drug-resistant variants. The pBHRF recombination-sensitive reporter vector described by Slebos and Taylor

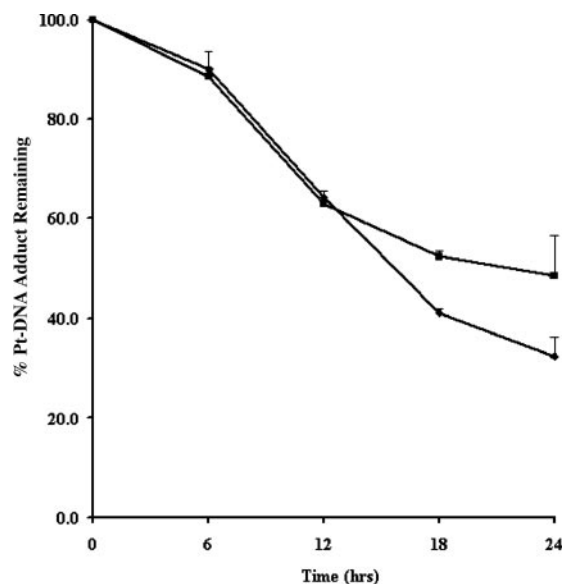


Fig. 5. Effect of hREV1 reduction on the disappearance of platinum from DNA. The 2008-EV and 2008-shREV1-3.3 cells were treated with 200 μ M DDP for 1 h. DNA was isolated at the indicated times after treatment and the platinum content quantified. \blacklozenge , 2008-EV cells; \blacksquare , hREV1 shRNA-expressing 2008-shREV1-3.3 cells. Each data point represents the mean of three experiments. Vertical bars, S.E.M.

(2001) was used to assess the effect of reduced hREV1 function on basal and DDP-induced rates of homologous recombination. This plasmid constitutively expresses an intact, emission-shifted, "blue" variant of GFP (EBFP), and also contains a carboxyl-terminally truncated form of GFP in which there exists a 300-bp homologous sequence for recombination with identical nucleotide stretch within the intact EBFP sequence. In the absence of homologous recombination within or between the GFP sequences in the vector, only EBFP is expressed in transfected cells; however, a homologous recombination event can create a functional GFP, in which case the cell expresses both EBFP and GFP, because most cells acquire multiple copies of the vector during transfection, only some of which recombine. The hREV1-proficient 2008-EV and hREV1-shRNA expressing 2008-shREV1-3.3 cells were exposed to 10 μ M DDP for 1 h or left untreated, and the pBHRF was transfected into the cells 24 h later. After a further 48 h, the fraction of BFP-positive cells that also expressed GFP was determined by flow cytometry. As shown in Fig. 6, the spontaneous homologous recombination frequency was 1.4-fold lower in the 2008-shREV1-3.3 cells than the empty vector-transfected 2008-EV cells ($p < 0.05$). Exposure to 10 μ M DDP for 1 h increased the recombination frequency in both cell types; however, the frequency was still significantly lower (1.6-fold, $p < 0.05$) in the 2008-shREV1-3.3 than in the 2008-EV cells. Thus, hREV1 function seems to be important to both spontaneous and DDP-induced extra-chromosomal homologous recombination.

Effect of DDP on hREV1 Protein Levels. Given its ability to promote both survival and mutagenicity after a DDP exposure, it was of interest to determine whether DDP induces an increase in hREV1 level during the injury response after drug exposure. Figure 7A shows that DDP induced a concentration-dependent increase in hREV1 protein level in the 2008-EV cells when measured 24 h after a 1-h drug exposure. It is interesting that, although smaller in magnitude, DDP also induced an increase in hREV1 protein

level in the hREV1 shRNA-expressing cells, suggesting that the ability of the shRNA to mediate hREV1 degradation may be overwhelmed by an increase in endogenous hREV1 mRNA production or that DDP impairs the production or action of the interfering RNA. Figure 7B shows the change in hREV1 protein level as a function of time after exposure to 10 μ M DDP for 1 h. The hREV1 protein level continued to increase for up to 24 h in both cell lines by 3.3- and 1.9-fold in the 2008-EV and 2008-shREV1-3.3 cells, respectively. However, the increase was generally lower in the shRNA-expressing 2008-shREV1-3.3 cells than in the 2008-EV cells. By 48 h, the hREV1 protein levels had declined in the both cell lines. These results indicate that DDP induced an increase in hREV1 protein level that peaked at a time similar to the time of maximum cell cycle arrest produced by DDP (Lin et al., 2001).

Effect of REV1 Reduction on the Rate of Development of DDP Resistance. The development of drug resistance during repeated cycles of DDP exposure may be caused by enrichment for resistant clones already present in the population, it may be the result of DDP-induced generation of new resistant clones, or both. As shown above, loss of hREV1 function reduced the ability of DDP to generate drug-resistant variants in the surviving population. If this ability of DDP is central to the emergence of acquired DDP resistance in the whole population, then reduction of hREV1 activity would be expected to reduce the rate at which resistance emerges. We measured the rate of development of resistance in the whole population of 2008-EV and 2008-shREV1-3.3 cells starting with a total 500,000 cells. The cells were exposed to an IC_{90} concentration of DDP for 1 h, and the exposure was repeated again as soon as log phase growth resumed. After each round of drug treatment, the sensitivity of the whole population to DDP was measured by determining survival over 2 logs of cell kill as a function of DDP concentration in a clonogenic assay. The degree of resistance after repeated cycles of DDP exposure was expressed as the ratio of the IC_{50} values for the treated cells relative to the untreated cells for each line separately. Figure 8 shows that after the first drug treatment, the degree of resistance rela-

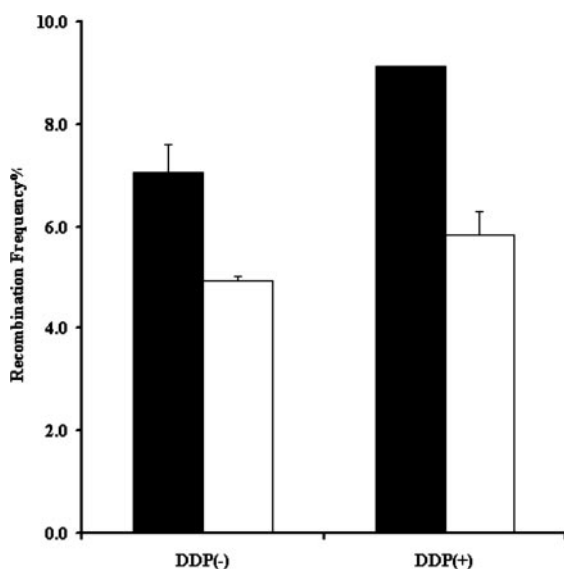


Fig. 6. Effect of hREV1 reduction on the spontaneous and DDP-induced homologous recombination frequency. Closed bars, 2008-EV cells; open bars, 2008-shREV1-3.3 cells. Each data point represents the mean of three experiments each performed with duplicate cultures. Vertical bars, S.E.M; where not shown, bar is too small to be visible.

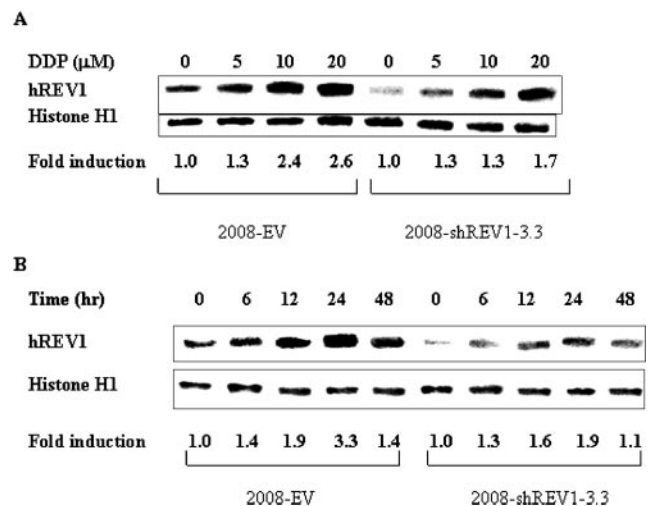


Fig. 7. The effect of DDP on hREV1 protein level. A, induction of hREV1 protein as a function of DDP concentration at 24 h after a 1-h exposure to DDP. B, time course of change in hREV1 protein levels after exposure to 10 μ M DDP for 1 h.

tive to non-DDP-treated cells increased linearly, with population doubling for the 2008-EV cells. In the case of the 2008-shREV1-3.3 cells, there was an abrupt development of 2.1-fold resistance relative to non-DDP-treated 2008-shREV1-3.3 cells after just a few population doublings in the face of DDP treatment; thereafter, however, resistance was acquired 2.8-fold more slowly than for the 2008-EV cells ($p < 0.05$). Thus, hREV1 plays a central role in the acquisition of DDP resistance at the population level. Because loss of hREV1 does not seem to alter the extent of adduct formation or the time course of platinum removal from DNA, these results are consistent with the concept that mutagenic translesional synthesis across DDP adducts is responsible for generating drug-resistant variants that become enriched in the population by subsequent rounds of DDP exposure.

Discussion

The biochemical pathways that mediate resistance to DDP are poorly defined, as are the mechanisms by which these pathways become altered in the resistant cells. The DDP-resistant phenotype is stable over many cell generations, indicating that it is the result of genetic changes. The results of the studies reported here indicate that hREV1 plays a role in producing these genetic changes and that disabling its function slows the emergence of resistance to this important chemotherapeutic agent.

The DNA adducts produced by DDP are important to its ability to kill the cell. Cytotoxicity is proportional to the extent of adduct formation, and cells with defects in nucleotide excision repair, the major DNA repair mechanism that removes these adducts, are hypersensitive to DDP (Damia et al., 1998; Furuta et al., 2002). REV1-deficient chicken DT40 lymphocytes have been shown to be hypersensitive to the cytotoxic effect of a variety of mutagens, including DDP. These cells have normal resting and damage-induced sister chromatid exchange but have an increased frequency of chromosome and chromatid breaks after exposure to UV irradi-

ation (Simpson and Sale, 2003). The results of the current studies indicate that impairment of hREV1 function in human ovarian carcinoma cells caused a moderate increase in sensitivity to the cytotoxic effect of DDP without altering the rate at which total platinum adducts were removed from DNA during the early phase of DNA repair. This suggests that, as for other types of adducts that block the progression of the replicative polymerases (Bradley et al., 1993; Chaney and Vaisman, 1999), hREV1 is involved in a pathway that normally carries out enough translesional synthesis to allow some cells to complete DNA synthesis and survive.

The mechanisms that generate mutations in eukaryotes have been studied most intensively in the budding yeast *S. cerevisiae*. In this organism, translesional bypass replication mediated by REV1 and pol ζ accounts for a large fraction of the mutations induced by DNA-damaging agents (Lawrence and Hinkle, 1996). Pol ζ , consisting of the REV3 and REV7 subunits, is required for translesion replication. REV1 participates in bypass replication but also has an independent dCMP transferase activity (Nelson et al., 1996; Lin et al., 1999a; Masuda and Kamiya, 2002; Zhang et al., 2002). Deletion of the genes coding for yeast REV1, REV3, or REV7 results in a similar phenotype that includes failure to generate as many mutations after exposure to a variety of mutagenic agents. It has been reported that both pol ζ and REV1 are important for error-prone translesion synthesis across bulky guanine adducts in yeast cells (Guo et al., 2004; Washington et al., 2004).

The pol ζ mutagenesis pathway also exists in human cells (Gibbs et al., 1998, 2000; Lin et al., 1999a). We have reported previously that reduction of the expression of hREV3 mRNA in human fibroblasts produces a phenotype with many of the same characteristics as that observed in the 2008-shREV1-3.3 cells, including hypersensitivity to the cytotoxic effect of DDP and a marked reduction in sensitivity to its mutagenic effects (Wu et al., 2004). The similarity of the phenotypes produced by reduction of hREV1 and hREV3 mRNA, at least with respect to cytotoxic sensitivity and mutagenesis, suggests that these two proteins act in the same process, possibly as elements of a single complex. However, because reduction of either of these proteins may destabilize the other, it is not possible to determine whether it is pol ζ activity or a pol ζ -independent activity of REV1 that is essential to error-prone bypass replication across DDP adducts. Likewise, because REV1 has been shown to interact with other DNA polymerases, they, too, may play a role (Guo et al., 2003).

The observation that reduction in the level of hREV1 decreased the ability of DDP to generate highly drug-resistant clones in the surviving population provides strong evidence that the translesional synthesis pathway in which hREV1 functions is error-prone when it bypasses DDP adducts in mammalian cells. It seems that this pathway normally fosters the development of resistance to DDP both by permitting the survival of cells that contain mutagenic adducts in their DNA and by generating new mutations in genes that mediate the resistant phenotype.

The role hREV1 plays in mammalian DNA repair mechanisms has not been clearly defined. Despite being 1.5-fold hypersensitive to the cytotoxic effect of DDP, the time course of removal of the majority of platinum from DNA was the same in the hREV1-proficient and deficient cells, which suggests that nucleotide excision repair is not highly dependent

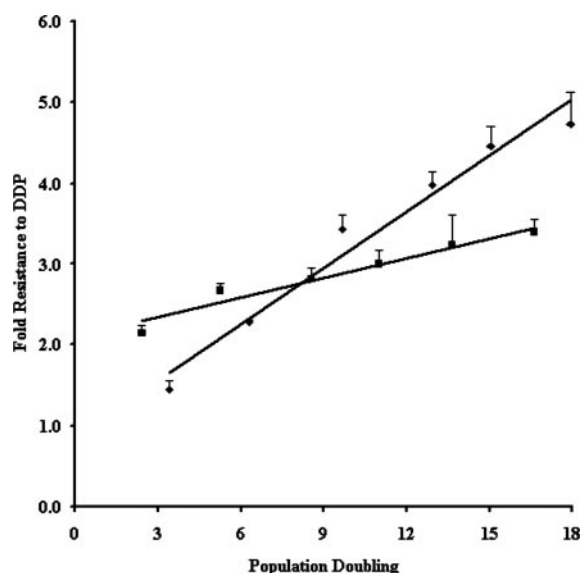


Fig. 8. Effect of hREV1 reduction on the rate of development of DDP resistance. ♦, 2008-EV cells; ■, hREV1 shRNA-expressing 2008-shREV1-3.3 cells. Each data point represents the mean of 3 experiments. Vertical bars, S.E.M.

on hREV1. However, reduction in hREV1 level did have an effect on both the endogenous and DDP-induced frequency of homologous recombination as detected by the pBHRF vector. This suggests a role for hREV1, pol ζ , or one of the other proteins with which hREV1 interacts in homologous recombination, consistent with another recent report (Sonoda et al., 2003). Homologous recombination is important to the survival of cells after DDP exposure (Zhong et al., 1999; Aloyz et al., 2002; Stracker et al., 2002), and it may be essential for repair of interstrand crosslinks (Keller et al., 2001; McHugh et al., 2001; Thompson and Schild, 2001). The finding that DDP exposure enhances pBHRF recombination suggests that DDP up-regulates this putatively nonmutagenic repair mechanism, an effect expected to improve the ability of the cell to survive the DNA damage produced by this agent.

The expression of a large number of genes is known to be altered after DDP exposure (Johnsson et al., 2001), and the current study indicates that *hREV1* belongs to the family of genes whose expression is up-regulated by DDP-induced injury. hREV1 protein levels increased in proportion to the extent of injury, and the level peaked at 24 h after a 1-h exposure to 10 μ M DDP. It is not known whether the increase in hREV1 protein directly reflects increased enzyme activity, but the data are consistent with the concept that DDP increases hREV1 activity and thus further enhances its own mutagenicity. A completely independent line of evidence suggesting the importance of hREV1 in platinum drug resistance has emerged from cDNA microarray-based studies of genes differentially expressed in isogenic oxaliplatin-sensitive and -resistant cells (Manorek et al., 2004). Among the 12,300 genes assayed, hREV1 was one of a very small number that were statistically significantly up-regulated in the resistant member of three or more of the five pairs of cells examined. Of even greater interest was the discovery that the two genes that lie immediately adjacent to hREV1 in the human chromosome are also significantly up-regulated in the oxaliplatin-resistant cells (Samimi et al., 2005).

The studies reported here were performed on a clonal population of 2008 cells in which REV1 expression was suppressed. Caution is needed in generalizing the results of these studies because the RNAi may have produced off-target effects, and integration of the vector may have produced an effect on other genes as well. Nevertheless, the phenotypic characteristics of the 2008-shREV1-3.3 are consistent with those of other cell systems in which the expression of REV1 has been disabled (Simpson and Sale, 2003) and cannot be attributed to differences in population growth rate or cell cycle phase distribution.

Perhaps the single most important observation to emerge from these studies is that reduction of hREV1 impedes the development of resistance to DDP at the population level. Preventing the emergence of resistance during DDP treatment is a key clinical goal. Previous studies have established that exposure to DDP results in the generation of clones in the surviving population that are highly resistant to DDP (Lin and Howell, 1999), and this was confirmed in the current study. However, whether these clones play a central role in the change in sensitivity of the entire population of tumor cells remained unknown. The results reported here indicate that acquisition of resistance by the entire population is not just the result of enrichment for drug-resistant clones that existed in small numbers before drug exposure. They also

indicate that the genes involved in high level DDP resistance are susceptible to attack by DDP with the formation of adducts that can be mutagenically bypassed by hREV1 acting in combination with pol ζ or other polymerases. It is noteworthy that the magnitude of the effect of REV1 depletion on the rate of resistance development was quite large. Thus, hREV1 is of interest as a target for the development of drugs capable of stabilizing the genome after DNA damage and reducing the rate of development of resistance in patients treated with DDP.

The finding that inhibition of hREV1 expression in the human cancer cells markedly reduces the mutagenicity of DDP indicates an important role for hREV1 in DNA damage-induced mutagenesis and identifies hREV1 as being of particular interest with respect to the mechanism underlying emergence of the multidrug resistant phenotype that so frequently accompanies the development of DDP resistance. The hREV1 protein is an attractive target for therapeutic intervention to simultaneously enhance DDP sensitivity and reduce the risk of development of drug resistance. Studies are now needed of the extent to which expression of hREV1 varies among different types of tumors and whether its expression is linked to clinical response.

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