Mechanistic Basis for Overcoming Platinum Resistance Using Copper Chelating Agents


Abstract

Platinum-based antitumor agents are widely used in cancer chemotherapy. Drug resistance is a major obstacle to the successful use of these agents because once drug resistance develops, other effective treatment options are limited. Recently, we conducted a clinical trial using a copper-lowering agent to overcome platinum drug resistance in ovarian cancer patients and the preliminary results are encouraging. In supporting this clinical study, using three pairs of cisplatin (cDDP)-resistant cell lines and two ovarian cancer cell lines derived from patients who had failed in platinum-based chemotherapy, we showed that cDDP resistance associated with reduced expression of the high-affinity copper transporter (hCtr1), which is also a cDDP transporter, can be preferentially resensitized by copper-lowering agents because of enhanced hCtr1 expression, as compared with their drug-sensitive counterparts. Such a preferential induction of hCtr1 expression in cDDP-resistant variants by copper chelation can be explained by the mammalian copper homeostasis regulatory mechanism. Enhanced cell-killing efficacy by a copper-lowering agent was also observed in animal xenografts bearing cDDP-resistant cells. Finally, by analyzing a public gene expression dataset, we found that ovarian cancer patients with elevated levels of hCtr1 in their tumors, but not ATP7A and ATP7B, had more favorable outcomes after platinum drug treatment than those expressing low hCtr1 levels. This study reveals the mechanistic basis for using copper chelation to overcome cDDP resistance in clinical investigations.

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Introduction

Platinum-based drugs have been widely used in chemotherapy for many human solid cancers. Drug resistance is a major barrier to the successful use of these agents. Multiple mechanisms are involved in platinum drugs resistance and reduction of transport capacity has long been recognized as one of the important mechanisms (1, 2). Expression of the high-affinity copper transporter 1 (Ctr1; SCL31A1 or hCtr1 for humans), which also transports platinum drugs including cisplatin (cDDP; ref. 3) and carboplatin (4), are frequently reduced in the cDDP-resistant (cDDPR) variants (5, 6).

The first demonstration that copper chelation can sensitize the cell-killing capacity by cDDP through the upregulation of hCtr1 transporter was observed in cell lines that overproduce glutathione (GSH) established by transfecting recombinant DNA encoding the heavy subunit of γ-glutamylcysteine synthetase, a rate-limiting enzyme for biosynthesis of GSH. GSH is an abundant physiologic copper chelator and elevated GSH levels enhanced hCtr1 expression and cDDP transport (7). Induction of hCtr1 expression by copper chelation was also reported using other copper-lowering agents (8–10). We recently conducted a clinical study using a copper-lowering agent (trientine) in combination of carboplatin for 5 platinum-resistant high-grade epithelial ovarian cancer patients and encouraging results were found (11). However, the mechanistic basis by which copper chelation can overcome platinum drug resistance remains to be elucidated.

In the present study, we showed in multiple cell models that expression of hCtr1 in cDDP-resistant variants can be preferentially upregulated by copper-lowering agents as compared with those in their drug-sensitive counterparts, providing greater sensitivity to the killing by platinum drugs. Enhanced cDDP efficacy by a copper-lowering agent was also observed in animal tumor xenografts bearing cDDP-resistant cells. Furthermore, we analyzed a public dataset and found that ovarian cancer patients with elevated expression levels of hCtr1 in their tumors had more favorable treatment outcomes after platinum drug treatment.
treatment than did those with low hCtr1 levels. Together, our findings provide the mechanistic basis for overcoming cDDP resistance using copper chelation strategy.

Materials and Methods

Cell lines, reagents, and recombinant DNA

The 59M, OVCAR3, IGROV1, and SKOV3 and HEK293 cells were obtained from American Type Culture Collection, 2008 and 2008-Cp cells were from Dr. Z. Siddik (MD Anderson Cancer Center, Houston, TX), A172 and A172-Cp cells were from Dr. A. Gomi (Jichi University, Tokyo, Japan), small cell lung cancer (SCLC) cell line and its cDDP² variant SR2 were from N. Savaraj (University of Miami, Coral Gables, FL). hCtr1-wt and hCtr1-DN cell lines were described previously (10, 12). No authentica-
tion of these cell lines was done by the authors. cDDP, carboplatin, oxaplatin, tratheriomyolobdate (TM), trientine, and D-pencillamide (D-pen) were purchased from Sigma-Aldrich. Cells were grown in Dulbecco’s Modified Eagle’s Media supplemented with 10% FBS at 37°C in a 5% CO₂ atmosphere.

Preparation of anti-hCtr1 antibody, confocal immunofluorescence microscopy, and blockage of copper and cDDP transport assay

A 50-amino acid peptide from the N-terminus of hCtr1 was synthesized and conjugated to the keyhole limpet hemocyanin carrier protein and was used to immunize rabbits. The antipeptide antibody was affinity purified. IGROV-1 cells grown on sterile cover glass slides were immunostained by anti-hCtr1 antibody followed the standard procedure. The cells were counterstained with propidium iodine (PI) and visualized with a Nikon confocal microscope.

For assaying the blockage of copper and cDDP transport, 5 × 10⁵ IGROV-1 cells per well were cultured in the Corning 6-well plates for 16 hours. The cultures were then replaced with a fresh medium containing various concentrations of anti-hCtr1 antibody or control rabbit IgG and cells were grown for another 2 hours. The culture medium was removed, and new medium containing either 10 μmol/L cDDP or 40 μmol/L CuSO₄ was added. After incubation of 1 hour, the cells were washed 3 times with PBS and the amounts of platinum and copper in the cells were measured by atomic absorption spectroscopy.

Animal experiments

Female athymic NCR nu/nu-nude mice (aged 5 weeks, NCI-Frederick Cancer Research and Development Center, Frederick, MD) were housed in a pathogen-free environment. The animals were inoculated subcutaneously with 5 × 10⁶ SR2 cells on the dorsal lumbosacral region. When the tumor volumes reached 150 to 250 mm³, the animals were randomly divided into 4 groups, 4 animals per group. The first group was treated with 100 μL of 0.85% NaCl by gavage. The second group was treated with 100 μL of 400 mmol/L D-pen (in 0.85% NaCl) by daily gavage for 28 days. The third group was intravenously injected with cDDP (5 mg/kg) 4 times at intervals of 7 days. The fourth group was treated with combined D-pen and cDDP following the schedules for the second and third groups, respectively.

Tumor growth rate was evaluated weekly using the described formulat (13). One week after the last treatment, 2 mice from each group were euthanized and necropsied. Blood samples were collected for hematology and biochemistry analysis before termination. At necropsy, the liver, kidney, heart, lung, submandibular and mesenteric lymph nodes, spleen, and the femur and sternum with the bone marrow were examined grossly and collected in 10% neutral buffered formalin for microscopic examination. Total body weight of the mice and the weights of the kidney, spleen, and liver, were recorded for each animal at necropsy. Formalin-fixed tissues were processed in 4-μm-thick sections on glass slides that were stained with hematoxylin and eosin (H&E). The pathologist examined microscopically the H&E-stained slides and evaluated all pathology data including hematology, biochemistry, and organs weight results.

Determination of hCtr1 expression and treatment outcomes in the ovarian cancer database

Microarray gene expression data published by Tothill and colleagues (14) was used for determination of whether ovarian cancer patients with higher levels of hCtr1 expression in their tumors would have better treatment outcomes with cDDP-based regimens. We used a Cox proportional hazard regression model for univariate survival analysis. All statistical analysis and Kaplan–Meier survival plots were conducted using R Software (15).

Other procedures

Procedures for determinations of ⁶⁴Cu (MIR Radiological Sciences; ref. 16) and platinum contents, drug sensitivity (IC₅₀), RNase protection assay (RPA), Western blotting using anti-hCtr1 and anti-Sp1 antibodies (Santa Cruz Biotech; refs. 10, 12) were described previously. All statistical analyses were conducted from at least 4 measure-
ments using the 2-tailed t test and the results were expressed as mean ± standard deviation. A P < 0.05 was considered as statistically significant.

Results

The magnitudes of hCtr1 regulation by copper stresses are constrained within upper and lower limits and depend upon the basal hCtr1 levels

Using specifically designed probe in the RPA that can differentiate transcripts between the transfected (exog-
enous, exo-hCtr1) and the endogenous (endo) hCtr1 alleles (Fig. 1A), we previously showed that overexpress-
ing the wild-type exo-hCtr1 mRNA in SCLC cells downregulates endo-hCtr1 mRNA expression (ref. 10; ~85% reduction, Fig. 1B, comparing lanes 1 and 6). In contrast, expression of dominant-negative (dn-) hCtr1
upregulates endo-hCtr1 expression (2.7-fold, Fig. 1D; ref. 12). We used these genetically engineered cell lines to investigate whether different endo-hCtr1 background would affect the magnitudes of hCtr1 regulation by copper stresses. Figure 1B shows that treating hCtr1-wt- and vector-transfected cells with a copper chelator (TM) resulted in a concentration-dependent upregulation of endo-hCtr1 mRNA with maximal induction of approximately 20-fold for the former and approximately 80% for the latter at 20 µmol/L TM. These results showed that magnitudes of hCtr1 induction by copper chelation depend upon the basal endo-hCtr1 levels.

Figure 1. hCtr1 regulation by copper stresses is constrained by upper and lower limits, and the magnitudes of regulation depend upon the basal hCtr1 levels. A, a schematic diagram showing the design of the hybridization probe for the simultaneous detection of endo-hCtr1 and exo-hCtr1 mRNA levels by the RPA (see ref. 9). B, hCtr1-wt or vector-transfected SCLC cells were treated with different concentrations of TM for 16 hours. Endo-hCtr1 mRNA and exo-hCtr1 mRNA levels were determined by the RPA using probe as indicated in A. 18S RNA was used as a loading control. Bottom, densitometric measurements of the RPA results. C, hCtr1-wt-transfected or vector-transfected SCLC cells were treated with TM for 16 hours and then were grown in medium without TM for different time intervals as indicated. Endo- and exo-hCtr1 and endo-Sp1 mRNA, and 18S RNA were measured. Densitometric measurements of RPA results are shown in the bottom. D, hCtr1-DN-transfected or vector-transfected cells were treated with CuSO4 for 16 hours. Endo- and exo-hCtr1 mRNA levels were determined by RPA. Bottom shows densitometric measurements. E, hCtr1-DN-transfected or vector-transfected cells were treated with CuSO4 for 16 hours and were then grown in CuSO4-free medium for different time intervals. Endo- and exo-hCtr1 and Sp1 mRNA levels were determined. Bottom, densitometric measurements of the results. Cu, copper.

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These results also showed that maximal levels of endogenous hCtr1 induction between these 2 cell lines were very similar. We then tested the reversibility of TM-induced hCtr1 upregulation. We first treated the cells with 40 μmol/L TM for 16 hours and then allowed them to grow in a medium without TM for different lengths of time. The endogenous hCtr1 mRNA levels in these 2 cell lines were gradually reduced with similar kinetics and reached levels comparable to those in the respective untreated controls 96 hours after the removal of TM (Fig. 1C). These results further support that the influence of basal hCtr1 mRNA levels in hCtr1 regulation by copper-depleting agents.

To determine whether basal hCtr1 mRNA levels also regulate hCtr1 downregulation by copper overload, we used hCtr1-dn-transfected cells. Figure 1D shows that CuSO4 treatment induces greater reduction of endogenous hCtr1 mRNA in hCtr1-dn-transfected cells (~2.6-fold) than in the vector-transfected cells (~60%), but reaching to levels close to each other when high concentration of CuSO4 was used. The reversibility test revealed that copper-induced hCtr1 mRNA downregulation was restored within 24 hours after copper removal for both cell lines, reduced to levels similar to their respective untreated controls (Fig. 1E). Figures 1C and 1E also show that the expression of Sp1 and hCtr1 was coordinated, consistent with the interregulatory relationship between Sp1 and hCtr1 in response to copper concentration changes (9). These results, taken together, show that there are homeostatic hCtr1 limits that dictate the amplitudes of hCtr1 regulation by copper stresses.

### Resensitization of cDDP-resistant cell lines to platinum drugs by copper-lowering agents

Given the observations that cDDPR variants often display reduced hCtr1 expression (5, 6), we then tested whether hCtr1 expression in cDDPR variants would be reduced hCtr1 expression (5, 6), we then tested whether hCtr1 expression in cDDP-resistant variants would be greatly induced by copper-lowering agents as compared with their cDDPS counterparts. Three cDDP-resistant/cDDP-susceptible pairs, that is, 2008-Cp (ovarian cancer cells), A172-Cp (glioma cells), and SR2 (SCLC), and 3 different copper chelators (TM, trientine, and D-penicillamine) were used.

All these cDDPR cells exhibited reduced hCtr1 expression in reference to their corresponding parental cell lines (Fig. 2A–C, comparing the nontreated lanes in each pair). These cDDPR cells also show reduced platinum accumulation (Fig. 2E), therefore, these are platinum transport variants. Treating pairwise cDDP-resistant/cDDP-susceptible cell lines with different copper depletors enhanced hCtr1 mRNA expression. Strikingly, hCtr1 mRNA expression levels were increased more in the cDDPR cells (ranging from 15- to 20-fold increase from basal levels) than in their respective drug-sensitive cells (<50% increase) by densitometry analysis (data not shown). Higher hCtr1 expression in the treated cells generally correspond with larger increases in 64Cu (Fig. 2D) and cDDP (Fig. 2E) uptake, although there may not be strictly correlated in all cases, suggesting the complexity of cDDP resistance mechanisms. Preferentially enhanced hCtr1 expression is generally correlated with an increased sensitivity to cDDP (Fig. 2F) and carboplatin albeit to a lesser extent (Fig. 2G), but much reduced sensitivity to oxaliplatin (Fig. 2H).

We prepared a polyclonal antibody using the N-terminal 50 amino acid residues of hCtr1 as antigen. This peptide sequence is extracellularly located and is important for the hCtr1-mediated copper and cDDP transport (12, 17). Three protein species (~55, 28, and 23 kDa) were seen in the Western blot analyses of whole cell extracts prepared from SCLC and TM-treated SCLC cells by this antibody (Fig. 3A). The intensity of 23-kDa signal, which corresponds to the molecular mass of unmodified monomeric hCtr1 protein, was increased in the TM-treated sample. This 23-kDa protein is highly expressed in the hCtr1-dn-transfected cells. Further characterizations of this anti-hCtr1 antibody included immunofluorescent confocal microscopy on cultured cells, which revealed that this anti-hCtr1 antibody heavily stains the cell membrane, with minor staining inside the cytoplasm, consistent with primary cytologic location of hCtr1 (Fig. 3B). Furthermore, this antibody can block copper and cDDP transport in concentration-dependent manner using nonimmunized rabbit IgG as a control (Fig. 3C), showing the functionality of this antibody. Whether the 55-kDa and 28-kDa signals represent posttranslationally modified hCtr1 or nonspecific proteins have yet to be determined. Given the consideration that Ctr1 is an evolutionarily conserved protein and good anti-hCtr1 antibody is difficult to obtain (18, 19), our hCtr1 antibody can be reliably used for Western blot analyses.

Western blotting of hCtr1 expression in 3 pairs of cDDP-resistant/cDDP-susceptible cells treated with various concentrations of TM for 16 hours were conducted. hCtr1 levels in all 3 cDDP-resistant variants were markedly less than those in the corresponding cDDP-susceptible cell lines (Fig. 3D), between lanes 1 and 6) and all the cDDP-resistant cell lines exhibited greater hCtr1 induction (~20-fold above basal levels) than their respective parental cell lines (~2-fold; Fig. 3D). Overall, the hCtr1 protein results were consistent with the mRNA results (Figs. 2A–C). No induction of copper exporters ATP7A and ATP7B at mRNA and protein levels between 2008 and 2008-Cp were seen by the RPA and Western blotting, respectively (Supplementary Fig. 1S).

### Copper-lowering agents preferentially sensitize cDDP-resistant ovarian cancer cell lines derived from patients failed cDDP-based chemotherapy

We further asked whether cells lines derived from ovarian cancer patients who failed cDDP-based treatments would contain reduced hCtr1 levels, and if so, whether these cells could be preferentially resensitized to cDDP treatment by copper-lowering agents in comparison with those derived from patients who had not been treated with platinum drugs. We randomly chose 4 cell lines, including the 59M cell line that was established from the ascites of an ovarian cancer patient who had never been treated with cDDP but failed ifosfamide/melphane treatment (20), the
IGROV1 line that was established from a patient before chemotherapy (21), and the OVCAR3 (22) and the SKOV3 (23) cell lines that were established from patients with relapsed cDDP-based treatments. These cells lines were treated with various concentrations of TM, D-pen, or trientine for 16 hours and following results were obtained: (i) without treatment, levels of hCtr1 mRNA (Fig. 4A, lanes 1) and protein (Fig. 4B) were lower in cell lines derived from patients who had been treated with platinum drug (OVCAR3 and SKOV3) than those derived from patients without platinum drug exposure (M59 and IGROV1). Although it is tempting to infer

Figure 2. hCtr1 mRNA levels in cDDP-resistant cells and their parental cells treated with different copper-lowering agents. A–C, RPA of endo-hCtr1 mRNA expression levels in 3 pairs of cells, that is, 2008 (cDDP)/2008-cp (cDDP-resistant), A172 (cDDP)/A172-CP (cDDP-resistant), and SCLC (cDDP)/SR2 (cDDP-resistant), treated with different concentrations of TM (top row), trientine (middle), or D-pen (bottom) as indicated for 16 hours. 18S RNA was used as an internal control. D, measurements of 64Cu uptake in 3 pairs of cDDP-resistant cell lines with or without copper-lowering agent added. E, cDDP uptake measurements. Cells were treated with TM (5 μmol/L), trientine, or D-Pen (100 μmol/L each) for 16 hours. Greater sensitization of cDDP-resistant cells to cDDP (F), carboplatin (G), but not oxaliplatin (H) than their parental cells, after treatment with copper-lowering agents was shown. *F < 0.05; Student t test. Pt, platinum.
that the in situ low hCtr1 expression was associated with the cDDP-refractory outcome in patients, we realize that these cell lines have been in cultures for so long and results must be treated with great care. (ii) The sensitivities of these tumor cell lines to cDDP treatment were inversely correlated with their hCtr1 expression levels (Fig. 4B bottom, IC50 values), implicating that cell lines derived from non–cDDP-treated patients were more sensitive to cDDP than those derived from patients that had been treated with cDDP. (iii) RPA assays showed that hCtr1 mRNA levels were induced in all 4 ovarian cancer cell lines by all 3 copper-lowering agents; and all the copper-lowering agents induces more hCtr1 mRNA expression in the low hCtr1-expressing cells (OVCAR3 and SKOV3, 9- to 15-fold) than in the high hCtr1-expressing cells (59M and IGROV1, 1.5- to 2-fold; Fig. 4A). (iv) D-Pen induced hCtr1 protein expression in all 4 cell lines (Fig. 4C), and greater induction of protein expression was found in low-hCtr1 expressing cells (OVCAR3 and SKOV3, 2- to 3-fold) than in the high hCtr1-expressing cells (59M and GROV1, ~50% increases; Fig. 4D). Although the fold changes in protein expression analyzed by Western blotting were not strictly agreeable with the mRNA results, the trend that the copper-lowering agent induces hCtr1 more in the low hCtr1-expressing cells than in the high hCtr1-expressing cells remained consistent. The discrepancy in the fold of induction determined between Western blotting and RNA assays may reflect differences in sensitivities of the 2 assay systems and/or possible
involvement of translational/posttranslational regulation. And (v), we also found that greater induction of hCtr1 expression was correlated with greater sensitivities to cDDP (Fig. 4E). These results, collectively, suggest that hCtr1 expression levels are correlated to cDDP sensitivity and that copper-lowering agents can preferentially resensitize ovarian cancer cells with reduced hCtr1 expression from patients-derived cell lines.

Copper-lowering agent enhances the antitumor activity of cDDP in an animal tumor model

We established SR2 tumor xenografts in nu/nu mice. These animals were treated with vehicle, D-pen, cDDP, or D-pen plus cDDP more than 4 weeks. In comparison with those in the untreated group, tumor growth rates in the D-pen–treated group were reduced, consistent with previous findings that anti-copper agents have antitumor activities (24). Tumor growth rates were also reduced in animals treated with cDDP alone, and strikingly, were further reduced in the group treated with D-pen and cDDP combination (Fig. 5A). Examples of tumor sizes in each group at the end of treatment are shown in Fig. 5B. These results show that D-pen can enhance the antitumor activity of cDDP in this animal model.

hCtr1 mRNA and human Sp1 mRNA levels in the residual tumors from each animal at the end of treatments in reference to those in the SR2 cells were determined. The human hCtr1 and Sp1 probes do not cross-react with the murine counterparts (data not shown). No significant difference of hCtr1 and Sp1 expression levels between tumors from the untreated animals and those in SR2 cells was found (Fig. 5C and D). Tumors from all 4 animals in the D-pen– and cDDP-treated groups showed higher expression of hCtr1 and Sp1 than those in the untreated group, showing that hCtr1/Sp1 expression can be induced by D-pen and cDDP in the xenograft model. The observation of cDDP treatment alone can also induce hCtr1/Sp1 expression can be explained that cDDP functions as an antagonist for hCtr1-mediated copper transport (Liang et al., manuscript in preparation). We found further induction of hCtr1 expression in the D-pen plus cDDP-treated group, although no apparent additional induction of Sp1 mRNA expression as determined by

![Figure 4. hCtr1 expression levels and resensitization to cDDP in 4 ovarian cancer cell lines treated with copper-lowering agents. A, RPA of hCtr1 mRNA levels in cells treated with different concentrations of TM (top), D-pen (middle), and trientine (bottom). B, Western blotting analysis of hCtr1 expression (top) and sensitivity of these cell lines to cDDP (IC50 values shown in bottom). C, Western blotting of ovarian cancer cell lines treated with D-Pen as indicated using β-actin as loading control. D, Densitometric analyses of hCtr1 expression blotting results as shown in C. E, sensitivity of ovarian cancer cells to cDDP in the presence and absence of copper-lowering agents as indicated.](https://www.aacrjournals.org/doi/figlink/10.1158/1535-7163.MCT-12-0580.s4)
densitometry (Fig. 5C and D). These results are consistent with the observed additional antitumoric effects in combination therapy using D-pen and cDDP (Fig. 5A). Therefore, we conclude that hCtr1 expression can be induced in animal tumor model by a copper-lowering agent. hCtr1 protein levels could not be conclusively determined in the tumor tissues because our anti-hCtr1 antibody cross-reacts with the mouse counterpart.

No treatment-related abnormalities were observed clinically during the in-life period of animals of this study. Postmortem pathologic investigations from each group of treated animals revealed no differences in hematological and biochemical results among the 4 groups of animals. Furthermore, gross and microscopic investigations of liver, kidney, heart, lung, submandibular and mesenteric lymph nodes, spleen, and bone marrow of the femur and sternum revealed no significant treatment-related adverse effects in animals of this study. Only a mild to moderate reduction of erythropoiesis of bone marrow was observed in 50% of the mice from groups treated with cDDP and cDDP plus D-pen but not in mice from D-pen treated or control groups. Overall, the pathology results support the no observed adverse effect level of the D-pen, cDDP, and combination of cDDP plus D-pen treatments in athymic nude mice under the doses and conditions described in this study.

**Role of hCtr1 expression in sensitivity to cDDP chemotherapy in ovarian cancer patients**

Ishida and colleagues (8) analyzed an array-based hCtr1 expression dataset consisting of 91 patients with stage III or IV serious epithelial ovarian cancer who had been treated with a cytoreductive surgery followed by adjuvant chemotherapy of a platinum drug and a taxane deposited in The Cancer Genome Atlas. The investigators reported that high expression of hCtr1 was associated with longer disease-free survival in these patients.
The therapeutic values of hCtr1 and other potential biomarkers for cDDP in clinical settings require additional substantiation. To this end, we analyzed an independent dataset derived from 285 serous and endometrioid tumors of ovary, peritoneum, and fallopian tube using Affymetrix U133 Plus 2 arrays (14). The patient characteristics and clinicopathologic features were presented (14). Of the 285 patients, 243 patients received first-line platinum/taxane-based chemotherapy and were analyzed in the present study. While 3 SLC31A1 probes that encode hCtr1 (203971_at, 235013_at, and 236217_at) were included in the microarray, however, using the BLAST tool from NCBI, we found that only 203971_at and 235013_at probes contain hCtr1 mRNA-encoded sequences located at the 3'-untranslated region; whereas probe 236217_at contains a sequence outside the transcriptional unit of SLC31A1 locus. Therefore, 236217_at probe is not an hCtr1 probe, but we used it as a negative control for the comparison.

To determine the relationship between the treatment outcomes and hCtr1 expression levels, we compared patients whose hCtr1 expression levels were in the upper 30th percentile (hereafter referred to a high hCtr1) with those in the lower 30th percentile (low hCtr1). We arbitrarily chose these cut-off lines so that the possibility of overlap between the high-hCtr1 and low-hCtr1 groups would be minimized. Figure 6A shows that patients with high hCtr1 expression levels had significantly longer progression-free survival (PFS, top) and overall survival (OS, bottom) times than those with low hCtr1 expression detected by both 203971_at and 235013_at probes but not by the 236217_at probe (Fig. 6B).

The roles of copper/cDDP efflux exporters ATP7A and ATP7B were similarly analyzed. Two ATP7A probes were present in the dataset, but only 1 (204624_at) probe but not the other (205197_s_at) showed a statistically significant correlation in relation to reduced ATP7A expression and longer PFS and OS in the patients (Fig. 6C). Only 1 ATP7B probe (204624_at) is present in the dataset, but its expression has no significance for the treatment outcomes (Fig. 6D). We also analyzed the following cDDP resistance biomarkers (25) but no significant correlation was observed. These include the redox regulator γ-glutamylcysteine synthetase (2 probes, 1555330_at and 202922_at), ABCB1 (205197_s_at and 243951_at) and ABCC1 (202804_at and 202805_at), which encode MDR transporters, BRCA1 (204531_s_at and 211851_x_at) and DNA polymerase δ (208070_s_at and 208070_s_at), encoding proteins for repair/tolerance of cDDP-induced DNA damages (data not shown). These results, taken together, support the association of hCtr1 expression with favorable treatment outcomes in ovarian cancer patients receiving the cDDP/taxane protocol.

Discussion

In this communication, we used multiple cultured cell models including genetically engineered, established cDDPR, and patient-derived cell lines to show that reduced hCtr1-associated cDDP resistance can be overcome by copper-lowering agents. We also present supportive results from animal work and bioinformatics from independent ovarian cancer patient dataset demonstrating the role of hCtr1 expression and cDDP sensitivity. The mechanism underlying differential upregulation of hCtr1 in cDDP variants over cDDPS cells by copper-lowering agents can be explained by the transcriptional regulation of hCtr1 expression within the context of the overall copper homeostasis regulation network, which consists of the Cu-Sp1-hCtr1 loop (9, 18). Here, we showed that copper homeostatic regulation is confined within upper and lower ranges that constrain the magnitudes of hCtr1 regulation in response to copper-stressed conditions. The cDDPR cells with reduced hCtr1 levels possess high capacity of hCtr1 upregulation (~20-fold) by copper chelation, whereas cDDPS cells that already express high hCtr1 levels, only have limited capacity by which hCtr1 can be further upregulated (general <2-fold). These findings provide the mechanistic basis for the use of copper chelation in overcoming cDDP resistance.

Our current findings may explain some seemingly contradictory published results. It has been reported that no induction of hCtr1 expression in the livers and intestines (26) in the rats fed copper-deficient diets, despite these organs showed more than 69% reduced copper contents as correspondingly compared with those in animals fed copper adequate diet. In another study, levels of mCtr1 expression were elevated in cervical tumors developed in the HPV16/E2 transgenic mice as compared with those in the cervix of wild-type animals. TM treatment did not further induce mCtr1 expression in these tumors (8). These results can be explained because these tissues already produce elevated Ctr1 levels. Alternatively, it remains possible that different Ctr1 regulation mechanisms may exist between in vivo and in vitro systems, and between tumor tissues and normal counterparts. Further studies are needed to address these important issues.

hCtr1 is located on human chromosome 9q32. Another transcription unit with opposite direction encoding an FK506-binding protein-like transcript is located ~201 bp upstream of the transcription start site of hCtr1 locus (10). This intergenic sequence controls the expression of both genes in response of copper bioavailability (our unpublished data). While we found no single nucleotide polymorphism associated with the Sp1 binding sites within this region from the NCBI database; however, whether other genetic polymorphisms in the promoter region of hCtr1 that may contribute to differential regulation of hCtr1 by copper chelation, particularly in the patient-derived ovarian cancer cells used in this study, remains to be critically evaluated.

Posttranslational regulation of hCtr1 expression by copper stresses has also been described (18, 27). Nonetheless, this study showed that hCtr1 mRNA levels are mostly correlated with hCtr1 protein levels that in turn correlated with cDDP sensitivity, although we also observed no strict correlations in some cases. These results...
suggest that transcriptional regulation may remain the major mechanism of hCtr1 regulation by copper chelation.

While copper chelation strategy targets cDDP-resistant tumor cells with reduced hCtr1 expression. It is important to note that mechanisms of cDDP resistance are multifactoral (28, 29). Notably, it has been reported that hCtr2 (for copper storage) and ATP7A/ATP7B (for copper efflux) can also transport cDDP (5) and their overexpression is associated with cDDP resistance (5, 30, 31). The complex cDDP resistance mechanisms suggest the need of using hCtr1 expression level as a guidance for the copper chelation strategy in platinum drug chemotherapy.

Figure 6. Correlation between copper expression and the treatment outcomes in ovarian cancer patient using different gene probes. A, hCtr1. B, hCtr1-unrelated DNA sequence. C, ATP7A. D, ATP7B. In all cases, probes for the given genes are indicated in parentheses, PFS results are shown in the top, and the corresponding OS results are shown in the bottom; high expressers in each panel are shown as red lines and lower expressers are shown in blue. *, P ≤ 0.05 is considered as significant and is underlined by a red line.
Copper-lowering agents have been used for treating Wilson’s disease resulting from copper accumulation because of genetic defect in ATP7B. These chelating agents have also been used in clinical studies by targeting tumor angiogenesis (32, 33) because many angiogenic modulators require copper as cofactor (34, 35). Combination therapy using copper-lowering agents and platinum drugs may have additive antitumor effects but may also produces additional adverse cytotoxicities (18). Carboplatin, which is the second-generation antitumor platinum drug, has reduced cytotoxicities in many organs as compared with cDDP. The major adverse event in the trientine/carboplatin trial is myelosuppression but is medically manageable (11). Several approaches have been proposed for improving the therapeutic index of copper chelation therapy by enhancing the treatment efficacies of platinum drug through upregulating hCtr1 expression and reducing the unwanted adverse events (18). Further research in this area may eventually lead to the development of effective strategies for using copper chelation to combat platinum drug resistance in cancer chemotherapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Molecular Cancer Therapeutics

Mechanistic Basis for Overcoming Platinum Resistance Using Copper Chelating Agents
Zheng D. Liang, Yan Long, Wen-Bin Tsai, et al.

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