Oxaliplatin Resistance Induced by ERCC1
Up-regulation Is Abrogated by siRNA-mediated Gene Silencing in Human Colorectal Cancer Cells

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Abstract. Background: Oxaliplatin is used to treat patients with colorectal cancer (CRC); however, half the patients fail to benefit. The excision repair cross-complementing group-1 (ERCC1) gene was studied and it was hypothesized that its inducible expression contributes to cellular resistance. Materials and Methods: Thirty CRC cell lines were treated with oxaliplatin and sensitivity was determined by apoptosis. Four sensitive and resistant cell lines were analyzed for oxaliplatin effect on ERCC1 expression and two resistant cell lines were subjected to siRNA-mediated gene silencing. Results: There was no correlation of basal ERCC1 mRNA expression with response to oxaliplatin. ERCC1 mRNA was induced at 24, 48, and 72 hours (71-264%, p<0.05) and ERCC1 protein at 48 hours (123-521%, p<0.05) post-oxaliplatin treatment in resistant cells only. siRNA-mediated silencing of ERCC1 sensitized the CRC cells to oxaliplatin-induced apoptosis, and increased cleaved PARP. Conclusion: ERCC1 gene expression is inducible, contributes to oxaliplatin resistance, and is reversible by targeted suppression of ERCC1, identifying ERCC1 as a potential target for drug development.

The platinum group of cancer chemotherapeutic agents includes cisplatin and its analog carboplatin, but neither of these drugs has been shown to be effective in the treatment of colorectal cancer (CRC). In contrast, oxaliplatin (trans-L-1,2-diamino cyclohexane oxalatoplatinum), a third- generation platinum compound is highly effective when used in combination with 5-fluorouracil (5-FU), and has become a standard treatment option in lymph node-positive colon cancer as adjuvant therapy, and as frontline therapy in the advanced setting (1-4). CRC is the second leading cause of cancer-related mortality in men and women in the United States, with 146,970 new diagnoses and 49,920 deaths in 2009 (5). In addition to CRC, the platinum drugs are an important class of therapeutic agents for a wide variety of other types of cancer including lung, breast, esophageal, gastric, ovarian, testicular, cervical, endometrial, and bladder cancer; however their efficacy is limited by the development of resistance (6).

Tissue resistance to oxaliplatin appears to be multifactorial, with the nucleotide excision repair (NER) pathway playing a major role (7). NER is carried out by a multienzyme complex and is a stepwise process of recognition, incision, excision, repair synthesis and ligation (8, 9). Excision repair cross complementing group 1 (ERCC1), along with xeroderma pigmentosa (XPF), forms a critical heterodimer active in the NER pathway, cleaving DNA 5’ to the damage site (8, 10-17). There are indications that the relative level of ERCC1 mRNA is a good marker for NER activity in human cancer cells, but it is unclear whether expression of this gene is important in other pathways of DNA repair (18). It has been shown previously that a higher basal expression of ERCC1 significantly lowers survival of patients with advanced CRC treated with oxaliplatin, although surprisingly there was no difference in tumor response (19). Furthermore, the expression of ERCC1 and XPA demonstrated that ERCC1 expression was predictive of oxaliplatin sensitivity (20). It has been shown that the suppression of ERCC1 gene expression in Hela S3 cells led to decreased cell viability against platinum drugs (21).

Moreover, studies in colorectal and ovarian cancer cells have suggested that ERCC1 is an inducible protein upon insult by a platinum agent (22). The human CRC cell line HT29 showed an induction in ERCC1 on exposure to cisplatin and oxaliplatin. Thus, it has been suggested that relative ERCC1 expression may be responsible for rendering a cell sensitive or resistant to oxaliplatin. In this study it was
hypothesized that a cancer cell in which ERCC1 is induced by oxaliplatin will be resistant to the cytotoxic effects of the drug, and in contrast, inhibition of this gene by siRNA will eliminate cellular resistance to oxaliplatin.

Materials and Methods

Cell culture and determination of sensitivity of CRC cells to oxaliplatin. Each of 30 CRC cell lines was cultured in 6-well plates at densities 50,000 to 750,000 cells/well and exposed to oxaliplatin at concentrations of 10-500 μM for a period of 72 hours as reported previously (23). The data from this panel of cell lines were used to select four sensitive and four resistance CRC cell lines for further investigation, using an oxaliplatin concentration of 10 μM. As sensitive cells, four lines were randomly chosen that had an apoptotic rate of >20% in response to oxaliplatin (Caco-2, RKO, SW480 and RW2982), although that for RKO was somewhat lower but was chosen to avoid using three cell lines derived from the same patient (i.e. the SW series). For the resistant cells, those with induced apoptotic rates of <4% (Colo201, HCT-8, HT29 and KM12, again avoiding cell lines derived from the same patient) were selected. The source and maintenance of the cells have been described previously (24). Cells were harvested for mRNA determination by quantitative real-time reverse-transcriptase polymerase chain reaction (qPCR) and for protein by Western blotting at baseline and after exposure to oxaliplatin for 24-72 hours.

Fluorescence-activated cell sorting (FACS). Oxaliplatin effects on the cell cycle were determined by FACS analysis following staining of cells overnight at 4°C with 50 μg/ml propidium iodide. A total of 10,000 cells were evaluated using a Becton Dickinson FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). The percentage of cells with a subdiploid DNA content was quantified using Modfit LT (Verity Software House, Topsham, ME, USA).

RNA isolation and qPCR. For isolation of RNA for qPCR experiments, cells were harvested in PBS and pellets were frozen at −80°C. RNA was isolated using the RNeasy kit (Qiagen, Germantown, MD, USA) and expression level of ERCC1 mRNA measured by qPCR. RNA aliquots (5 μg total) from each cell line were reverse-transcribed using Roudned Parenthesis III (Invitrogen, Carlsbad, CA, USA). PCR primers for specific target genes were designed using Primer 3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). cDNA (10 ng) from each cell line was amplified with specific primers using the SYBR green Core Reagents Kit and a 7900HT real-time PCR instrument (Applied Biosystems, Foster City, CA, USA). Expression of the ERCC1 gene was standardized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference, and relative levels of expression across the panel of cell lines were quantified by standard curve method and the ratio of ERCC1 to GAPDH was computed. The primers used in the qPCR assay were as follows: ERCC1 forward (F): GGAGGCTGTTTGTAGTCTCG, and ERCC1 reverse (R): TTACACTGGGGGTTTCCTTG, and GAPDH F: TCAAGAGA GGTTGTTGGCGCAG and GAPDH R: AAAGG TGAGGAGGTGTTGCT.

Western blotting and quantification of signal intensity. Cells were harvested and washed in PBS, and then lysed at 4°C for 30 min in 200 μl lysis buffer (50 mM Tris-HCL pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA pH 8, and protease inhibitor cocktail (Sigma catalog number P-8340). Final protein content was determined by spectrometry using a Bio-Rad dye-binding assay (Bio-Rad, Hercules, CA, USA). Samples were boiled for 5 min in a Laemmli sample buffer and 50 μg of protein, in equal loading volumes, were fractionated by electrophoresis on 12% SDS-polyacrylamide gels. The proteins were transferred at 4°C onto the Hybond ECL membranes (GE Healthcare, Amersham HybdoTM-P). Rainbow molecular weight markers (GE Healthcare UK Ltd, Cat no. RPN 800E) were used as standards. After the transfer, the membranes were blocked with Tris-buffered saline with Tween 20 (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) containing 10% non-fat milk for one hour at room temperature, and incubated overnight at 4°C with anti-human ERCC1 (sc-53281, at 1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-human PARP/cleaved PARP (sc-8007, at 1:200 dilution) antibodies. Membranes were then washed, incubated with anti-mouse secondary antibody (GE Healthcare UK Ltd, Cat. no. NA-931V) in a 2.5% blocking buffer for 1 hour, rewashed and proteins visualized using ECL Plus Western Blotting Detection System (GE Healthcare UK Ltd, Cat. no. RPN 2132). To control for equal loading, blots were reprobed with an anti-β-actin antibody (Sigma, 1:10,000 for 1 hour).

Image quantification for the protein estimates obtained by Western Blots was performed using the ‘Image J’ software program downloaded from the website of the National Institutes of Health (25).

siRNA transfection and gene silencing. Two oxaliplatin-resistant cell lines, HT29 and KM12, were selected for siRNA-mediated gene silencing. ERCC1 siRNA was obtained from Santa Cruz Biotechnology, Inc. (sc-35331) as a pool of three target-specific 20-25 nt siRNAs designed to inhibit expression of the human ERCC1 gene. Non-targeting siRNA (sc-37007) was used as a negative control. The cells were transfected with the siRNA duplexes using LipofectamineTM 2000 reagent (Invitrogen Cat no. 11668-027) at a final siRNA concentration of 50 μM. Six hours post-transfection, cells were treated with 10 μM oxaliplatin or left untreated, with cells harvested after 72 hours to analyze ERCC1 mRNA by qPCR, and for cell viability using FACS.

Identification of p53 and KRAS mutations in the cell lines. The mutation status of the cell lines of the SW series. For the resistant cells, those with induced apoptotic rates of <4% (Colo201, HCT-8, HT29 and KM12, again avoiding cell lines derived from the same patient) were selected for siRNA-mediated gene silencing. ERCC1 siRNA was obtained from Santa Cruz Biotechnology, Inc. (sc-35331) as a pool of three target-specific 20-25 nt siRNAs designed to inhibit expression of the human ERCC1 gene. Non-targeting siRNA (sc-37007) was used as a negative control. The cells were transfected with the siRNA duplexes using LipofectamineTM 2000 reagent (Invitrogen Cat no. 11668-027) at a final siRNA concentration of 50 μM. Six hours post-transfection, cells were treated with 10 μM oxaliplatin or left untreated, with cells harvested after 72 hours to analyze ERCC1 mRNA by qPCR, and for cell viability using FACS.

Statistical analysis. To analyze differences between two groups, an unpaired Student’s t-test was utilized. When the same data was utilized for multiple comparisons, a Bonferroni correction was used to account for potential errors from multiple analyses. Since the baseline ERCC1 expression and the apoptosis data was used for 3 different analyses, a p-value of ≤0.0167 (calculated as 0.05/3) was considered statistically significant. Correlation was calculated using Spearman’s correlation coefficient. All statistical calculations were carried out using Microsoft Excel (MS Office 2003).

Results

Baseline ERCC1 gene expression does not stratify CRC cells as being sensitive or resistant to oxaliplatin. A panel of 30 CRC cell lines was previously screened with low- (10 nM), intermediate-(100 nM) or high-dose (500 nM) oxaliplatin for
72 hours and measured apoptosis induction by PI staining and FACS analysis (23). To determine whether basal ERCC1 expression was linked to oxaliplatin response, basal ERCC1 mRNA expression was determined in the same cell lines by qPCR. While the ERCC1/GAPDH ratio expression levels ranged from 0.059 to 2.401 across the panel, the mean ERCC1/GAPDH mRNA ratio in the 15 most sensitive cell lines was not significantly different from levels in the 15 most resistant cell lines (0.65 versus 0.68, p=0.88). Likewise, a regression analysis failed to demonstrate a significant association between response and baseline ERCC1 mRNA level (r²=0.014, p=0.52, Figure 1). Similar to the qPCR data, baseline ERCC1 protein expression by Western blot experiments did not have any association with sensitivity to oxaliplatin (data not shown).

The lack of an inverse correlation between baseline ERCC1 mRNA expression and sensitivity to oxaliplatin was supported by the observation that the highly sensitive RKO cell line was in the upper quartile for both mRNA (ERCC1/GAPDH ratio of 1.38) and apoptosis (19%). Moreover, the protein expression as quantified by Image J was the highest (ERCC1/β-actin ratio of 2.9) among the panel of 30 cell lines.

**Baseline ERCC1 gene expression is dependent on p53 but not on KRAS mutation.** The mean ERCC1/GAPDH gene expression of cells mutated in p53 was 0.43 and was significantly less than the median value of 1.08 in cells with wild-type p53 (p=0.003). It has previously been shown that the response to oxaliplatin is not dependent on p53 mutation (23). When KRAS status was analyzed, its mutation status did not correlate with sensitivity to oxaliplatin or baseline ERCC1 gene expression. The mean oxaliplatin-induced apoptosis of cells mutated in KRAS was 11.7% versus 11.5% in cells with wild-type KRAS (p=0.95). Similarly, the mean ERCC1/GAPDH gene expression of cells line mutated in KRAS was 0.57 versus 0.75 in cell lines with wild-type KRAS (p=0.43).

**ERCC1 is an inducible gene in oxaliplatin resistant, but not in sensitive cells.** To investigate effects of oxaliplatin on expression of ERCC1, mRNA levels were analyzed after treating four resistant and four sensitive cell lines with 10 μM oxaliplatin for 24, 48 and 72 hours. As shown in Figure 2A, oxaliplatin treatment resulted in induction of ERCC1 gene expression in all of the resistant cell lines, ranging from 71 to 264% (1.7- to 3.6-fold). The increase was seen at all time points studied, and the maximum increase of 3.6 fold was observed in the KM12 cells after 48 hours exposure to oxaliplatin. In contrast, a minimal decrease in ERCC1 gene expression was observed in the 4 sensitive cell lines at all time points examined (Figure 2B). As shown in Table 1, there was a statistically significant difference in ERCC1 expression pre- and post-oxaliplatin treatment in resistant cell lines (at all three time points studied), but not in sensitive cell lines.

Samples isolated at 48 hours of oxaliplatin treatment were used for estimation of protein content by Western blot experiments in order to compare the levels of ERCC1 protein in each of the eight cell lines. Similar to the qPCR results, the level of ERCC1 protein was increased post-oxaliplatin treatment in all four resistant cell lines but not in sensitive cell lines (Figure 3). Image quantification by Image J confirmed this, with a statistically significant increase in the ERCC1/β-actin ratio in the resistant cell lines (p=0.008), while no change was observed in the sensitive cell lines (p=0.239) (Table 1).

**Down-regulation of the ERCC1 mRNA by siRNA-mediated silencing sensitizes formerly resistant cells to oxaliplatin.** To determine directly the role of ERCC1 in oxaliplatin-induced apoptosis, ERCC1 mRNA expression was down-regulated in two oxaliplatin-resistant cell lines, HT29 and KM12, using siRNA. The siRNA treatment was effective in down-regulating ERCC1 gene expression, reducing basal levels by 73% and 62% in HT29 and KM12, respectively (Figure 4A). To confirm gene silencing, the KM12 cell line was probed for ERCC1 protein following siRNA treatment. There was a clear reduction in ERCC1 protein expression after ERCC1 siRNA treatment as compared to nt siRNA (Figure 4B). Furthermore, in the presence of reduced expression by siRNA of ERCC1 in HT29 cells, oxaliplatin did not increase in ERCC1 mRNA levels. Thus, siRNA-mediated gene silencing abrogated oxaliplatin-mediated induction of ERCC1 in this cell line.
Figure 2. Effect of oxaliplatin treatment on ERCC1 mRNA expression in CRC cells. From the panel of 30 CRC cells, four sensitive and four resistant cells were selected. Cells were treated with 10 μM oxaliplatin for 24, 48 and 72 hours, mRNA was then extracted from treated and untreated cells, and ERCC1 expression determined by real-time PCR. The four resistant cell lines KM12, Colo 201, HCT8 and HT29, demonstrate a clear induction of ERCC1 mRNA expression in response to oxaliplatin treatment (A). The four sensitive cell lines RKO, SW480, Caco2, and RW 2982, do not demonstrate a difference in the ERCC1 mRNA expression (B).
Cells transfected with ERCC1 or nt siRNA were then exposed to 10 μM oxaliplatin for 72 hours and the apoptotic response was determined. ERCC1 silencing significantly enhanced oxaliplatin-induced apoptosis in the two resistant cell lines examined (Figure 5A). For HT29 cells, apoptosis increased from 0.1% in nt siRNA-transfected cells to 17.6% in ERCC1 siRNA-transfected cells (p=0.0015). Similarly, in KM12 cells, apoptosis increased from 3.3% in the nt siRNA-transfected cells to 11.3% in ERCC1 siRNA-transfected cells (p=0.03). To confirm that this increase in apoptosis was due to oxaliplatin treatment and not an effect of ERCC1 gene silencing, FACS analysis was also performed for cells transfected with NT and ERCC1 siRNA without oxaliplatin treatment, with no change in apoptosis rates being observed.

To further confirm these findings, the extent of PARP cleavage was examined in the KM12 cell line following ERCC1 down-regulation and oxaliplatin treatment. Consistent with the FACS data, the extent of PARP cleavage observed in response to oxaliplatin treatment was higher in ERCC1-transfected cells compared to nt siRNA transfected controls (Figure 5B).

**Discussion**

The outcome for patients with metastatic (stage IV) CRC has improved considerably over the past decade, from a median survival of 6 months with best supportive care to 24 months with the incorporation of newer cytotoxic drugs (including irinotecan and oxaliplatin) and the monoclonal antibodies bevacizumab, panitumumab and cetuximab (28). The combination of oxaliplatin with 5-FU and leucovorin is a standard frontline treatment for patients with metastatic disease and as adjuvant therapy for lymph node-positive stage III CRC (3, 4). However, response rates in patients

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**Table I. Effect of oxaliplatin treatment on ERCC1 mRNA and protein expression in four sensitive and four resistant cell lines.** The control level of ERCC1 mRNA and protein, and ERCC1 mRNA at 24, 48 and 72 hours and protein expression at 48 hours following oxaliplatin treatment in the four sensitive and resistant cell lines are shown. The baseline protein level was normalized to 1. Statistical significance was assessed by a Student’s t-test.

<table>
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<th>Time (h)</th>
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<th>Resistant mRNA by RT-PCR</th>
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<td>Treated Mean (±SEM)</td>
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<td>48 hours</td>
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<td>0.92 (0.03)</td>
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<td>72 hours</td>
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<tr>
<th>Time (h)</th>
<th>Sensitive Protein estimation by J Image</th>
<th>Resistant Protein estimation by J Image</th>
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<tr>
<td></td>
<td>Control Mean (±SEM)</td>
<td>Treated Mean (±SEM)</td>
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<tr>
<td>48 hours</td>
<td>1</td>
<td>0.79 (±0.16)</td>
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**Figure 3. Effect of oxaliplatin treatment on ERCC1 protein expression in CRC cells.** Oxaliplatin-sensitive and -resistant cells were treated with 10 μM oxaliplatin for 72 hours, following which total protein was extracted from treated and untreated cells and ERCC1 expression determined by Western blot. The four resistant cell lines KM12, Colo 201, HCT8 and HT29, demonstrate a clear increase in ERCC1 protein, whereas the four sensitive cell lines RKO, SW480, Caco-2, and RW2982, do not demonstrate any difference in ERCC1 expression in response to oxaliplatin treatment.
remain at 40%, and 5-year survival for node-positive CRC remains at 75%. Thus, inherent resistance to platinum drugs continues to be a major detrimental factor in the therapeutic outcomes of patients with cancer. ERCC1 is an important component of the NER pathway which may have a major impact on the emergence of resistance and normal tissue tolerance to platinum drugs (29).

While multiple clinical trials have suggested that high baseline tumor expression of ERCC1 protein as assessed by immunohistochemistry or mRNA (19) expression may serve as a biomarker for relative resistance to platinum compounds, little work has been performed to formally test this hypothesis using mRNA from cell lines. Based on a panel of 30 CRC cell lines, this study failed to find a link between baseline ERCC1 mRNA expression and response to oxaliplatin at all three concentrations of oxaliplatin tested (10, 100 and 500 μM). To further validate this finding, baseline ERCC1 protein expression was measured in all 30 cell lines by quantitative methods and again it was not possible to find an association with response to oxaliplatin.

Possible reasons for these differences are that in cell culture, one can evaluate the direct cytotoxic effect of a drug on cell growth and survival; while in patients, there are multiple other confounding factors such as preceding or subsequent therapy, immune modulation, and gene expression unrelated to cancer that can influence ultimate survival. Second, ERCC1 may serve as a prognostic marker rather than a predictive one, and lower expression itself may be reflected by better overall outcomes. Studies have shown that low ERCC1 expression is predictive of better survival when patients are treated with oxaliplatin-based regimens, and more recently, one study demonstrated that radiological response is higher in patients whose tumors have low ERCC1 mRNA (30). However, to date, no clinical study has been successful in establishing a link between ERCC1 expression and progression-free survival, the one parameter that is not affected by subsequent therapies. Another remote possibility is that too few cell lines were used, since apoptosis was not found to be higher with lower ERCC1 mRNA (14.2% versus 8.9%), although this difference did not reach statistical significance.

This is the first demonstration, to the Authors’ knowledge, that the inducible expression of ERCC1 is a determinant of resistance to oxaliplatin. Prior clinical studies suggest that baseline ERCC1 expression can predict benefit from platinum...
agents in non-small cell lung cancer and CRC, but not in gastric cancer (31-34). However, none have analyzed changes in gene expression in response to oxaliplatin treatment. This study has successfully shown in a selected series of 8 CRC cell lines that the ERCC1 gene is induced by oxaliplatin in resistant cells, but in contrast, remains uninduced by the drug in sensitive cells. Thus, cells which have the capacity to activate this regulatory switch are capable of converting to a resistant phenotype, while those in which this gene expression is unaffected cannot undergo this alteration, and are susceptible to drug-induced apoptosis. In regards to previous work on inducibility of ERCC1, it has been shown that cisplatin induces this gene in ovarian cancer cell lines, mediated by the AP1 transcription factor (35), and that oncogenic HRAS can induce ERCC1 through the AP1 binding site (36). However, in this study, no association was found between KRAS mutation status (the principal mutated KRAS allele found in CRC) and sensitivity to oxaliplatin or to basal ERCC1 gene expression.

An unexpected observation, however, was that baseline ERCC1 mRNA expression level is p53 dependent, with cells that are p53 wild-type demonstrating a significantly higher expression of ERCC1 than the p53 mutant cells. The p53 gene is well known as a transcription factor and thus could directly regulate ERCC1 expression in this manner (37). While ERCC2 and ERCC3 are known to functionally interact with p53, there are no data for ERCC1 (37). It has been reported that a different NER gene, namely p48 xeroderma pigmentosa, is p53 dependent; its mRNA expression depends on basal p53 expression and increases after DNA damage in a p53-dependent manner (38). Further work to study the mechanisms of p53 and ERCC1 interaction remains to be undertaken.

To further support the hypothesis that ERCC1 is the crucial protein determining resistance, this study showed that down-regulation of ERCC1 by siRNA transforms a resistant cell to a sensitive one, demonstrated by both increased induction of apoptosis by oxaliplatin, as well as PARP cleavage. This may be explained by the fact that induction of ERCC1 helps repair DNA damage induced by oxaliplatin, which results in cell survival. As a corollary, failure to repair the damage leads to apoptosis. Therefore, ERCC1 function, and not only altered regulation, which could have been a surrogate marker for other changes in expression of other genes, is directly linked to the mechanism of resistance.

This observation that up-regulation of ERCC1 is a determinant of oxaliplatin sensitivity, and a prior finding that gemcitabine is synergistic with cisplatin 

\[ viu \] inhibition of repair of DNA-induced damage, suggest that effects on damage-induced DNA repair can be exploited therapeutically (39). For example, patients who are resistant to oxaliplatin may respond to the drug by approaches that first inhibit ERCC1. Thus, efforts to develop pharmacological or biological inhibitors of this enzyme may be highly fruitful.

**Conclusion**

Oxaliplatin exhibits broad-spectrum antitumor activity, including activity against a subset of cisplatin-resistant cell lines, but the outcome of oxaliplatin treatment is linked to development of drug resistance. Prior reports strongly suggested that ERCC1 gene expression was critical in efficacy of oxaliplatin treatment. The current results clearly demonstrate the importance of the dynamics of ERCC1 expression in developing oxaliplatin resistance, as the resistant cell lines up-regulate the ERCC1 mRNA levels by 2- to 3-fold upon oxaliplatin treatment in contrast to lack of induction in sensitive cell lines. Moreover, cells initially resistant to oxaliplatin were sensitized to the drug by siRNA-mediated inhibition of ERCC1 expression.

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**References**


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