Clear Cell Tumors Have Higher mRNA Levels of *ERCC1* and *XPB* Than Other Histological Types of Epithelial Ovarian Cancer

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ABSTRACT

Purpose: **The purpose of the present work was to investigate the relationship between mRNA expression of *ERCC1* and *XPB*, two key genes in the nucleotide excision repair pathway, and clinical resistance of platinum-chemotherapy in histological subtypes of epithelial ovarian cancer.**

Experimental Design: **mRNA levels of *ERCC1* and *XPB* in epithelial ovarian cancer specimens from 126 different individuals were assessed using reverse transcription-PCR and followed by Southern hybridization methodology. Data were analyzed by linear regression analyses and by exhaustive regression analyses.**

Results: **Five different histological types of tumors were examined; serous ($n = 76$), mucinous ($n = 11$), clear cell ($n = 9$), poorly differentiated ($n = 9$), and endometroid ($n = 21$). Numerical values for mRNA expression levels were based on internal controls for a stable comparative cell line and for β-*actin*. Median values for *ERCC1* and *XPB* mRNAs within clear cell tumors were, on average, >2-fold higher than the other histological tumor types. Linear regression analyses suggest a continuum of nucleotide excision repair gene expression among these cell types, and exhaustive regression analyses demonstrate that the higher mRNA levels seen in clear cell tumors are highly statistically significant.**

Conclusions: **We conclude that mRNA levels of *ERCC1* and *XPB* tend to be higher in clear cell tumors as opposed to other types of epithelial ovarian cancer. This is consistent with the long-standing observation that clear cell tumors are more likely to show *de novo* drug resistance against DNA damaging agents in the clinic.**

INTRODUCTION

NER[2] is associated with cellular and clinical resistance to platinum compounds and to platinum-based chemotherapy (1, 2). NER is responsible for the repair of covalent bulky damage to DNA caused by polycyclic aromatic hydrocarbons, UV light, and a range of chemical agents, including the platinum therapeutic analogues cisplatin, carboplatin, and oxaliplatin (1–3). Data from several laboratories suggest that *ERCC1* may be a useful molecular marker for NER activity in that higher *ERCC1* mRNA levels are associated with a more active DNA repair process (4–7).

Clear cell carcinoma of the ovary is one of several variants of epithelial histology of this disease (8). Clear cell carcinoma is clinically characterized by *de novo* resistance to platinum-based chemotherapy (8–10). The molecular basis for this longstanding observation is not known. Because NER gene expression is strongly associated with platinum resistance in epithelial ovarian cancer generally, we sought to examine whether NER gene expression might help explain the observed differences in clinical behavior between histological subsets of this disease. We therefore examined a set of 126 tumor specimens obtained from the Cooperative Human Tissue Network (Columbus, OH). This set contained tissues from five different histological subtypes of epithelial disease. These tissues were stripped of clinical identifiers and clinical history information. We used PCR to assess mRNA expression of *ERCC1* and of *XPB* in these tissues.

Although more than three dozen genes are involved in the NER process, the rate-limiting step within NER is the excision of damage from the DNA strand (11). The repairosome responsible for excision includes ~16 proteins, 2 of which are ERCC1 and XPB (12, 13). ERCC1 forms a heterodimer with XPF to execute the incision into the strand, 5′ of the site of damage. ERCC1 also has several other DNA repair roles (14–16) and is essential to life (17). It has been suggested that ERCC1 may play a number of roles that are not yet defined because it is very difficult to maintain cells that do not have a functional *ERCC1* (1, 2, 4, 17).

XPB is a helicase that functions within the repairosome of NER and is necessary for linkage of DNA repair with DNA transcription (18, 19). Two studies have shown an association of XPB with clinical resistance to platinum compounds (20, 21). Also, these and other studies have shown coordinate mRNA expression of *XPB* with *ERCC1*, as well as with other genes in the NER repairosome complex (20–24).

Studies in ovarian cancer (23, 24), gastric cancer (25), colon cancer (26), and in a range of cell lines (4–7, 27) show that *ERCC1* mRNA levels correlate directly with resistance to platinum-based exposures, *i.e.*, higher *ERCC1* levels are asso-

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[2] The abbreviations used are: NER, nucleotide excision repair; MSPE, mean squared prediction error; MSE, mean squared error.

ciated with tumor growth in the face of therapy. Clinically, lower *ERCC1* levels are associated with improved disease response and improved relapse-free survival (23–26). These data have been interpreted to suggest that lower mRNA levels of *ERCC1* may reflect lower DNA repair activity in these cells and tissues and that higher *ERCC1* levels may reflect higher DNA repair activity. Studies of coordinate expression of NER genes suggest that these genes may share common transcription factors within cells (28) and that high levels of *ERCC1* may reflect high levels of other genes in the repairosome. Likewise, low levels of *ERCC1* may reflect low levels of other genes within the repairosome. Therefore, mRNA levels of *ERCC1* may be a useful molecular marker for NER (1, 2, 28).

Statistical analyses of the data presented below on clear cell carcinoma of the ovary were performed in several ways. We performed analyses of the data based on previous approaches that we have reported (23, 24). We also performed exhaustive regression analyses to thoroughly examine relationships between histological subsets and between the two genes being studied. All analyses support the observation that for *ERCC1* and *XPB*, clear cell tumors show higher mRNA levels of these two genes than the other histological types we examined. We believe that this suggests a more active NER process in ovarian tumors of the clear cell type.

MATERIALS AND METHODS

Specimens Studied and RNA Extraction. One hundred twenty-six tumor specimens from patients with advanced ovarian cancer in stage 3 or stage 4 were obtained from Cooperative Human Tissue Network, Pediatric Division, Children's Hospital. Tumor tissues were collected before drug treatment and flash frozen at −80°C until RNA/DNA extraction. All specimens were diagnosed and classified by pathologists and stratified as 76 serous carcinoma, 11 mucinous, 9 clear cell, 21 endometrioid, and 9 poorly differentiated.

From each of the 126 tumor specimens, total cellular RNA was isolated and purified by the method of hot phenol/chloroform extraction as we have reported previously (23, 24). Purified RNAs were precipitated and dissolved in diethyl pyrocarbonate water.

Reverse Transcription-PCR. Through reverse transcription, using the SuperScript Preamplification System, cDNAs were generated with oligodeoxythymidylic acid primers from 5 μg total RNA/sample (Life Technologies, Inc.). With randomly selected exon primers for the *ERCC1*, *XPB*, and β-*actin*, PCR was carried out among the 126 ovarian samples under optimal conditions, respectively. Oligonucleotide primers for *ERCC1*, for *XPB*, and for β-*actin* were synthesized by Lofstrand Labs Limited (Gaithersburg, MD).

PCR was performed with cDNAs and respective primers at optimal conditions for 35 cycles, using AmpliTaq DNA polymerase (Perkin-Elmer) to amplify desired fragments for each of the targeted genes. *ERCC1* primers were selected to amplify a 239-bp segment from 394 to 633 bp of the *ERCC1* cDNA sequence, primers for *XPB* amplified a 323-bp segment from 1916 to 2238 bp of the *XPB* cDNA sequence, and primers for β-*actin* spanned a 731-bp segment of the coding region of the β-*actin* gene, extending from base 269 of exon 2 to base 1535 in exon 4.

Table 1 Median and mean scores for various cancers

	Clear cell	Serous	Mucinous	Poorly differentiated	Endometrioid
ERCC1					
Median	1.21	0.75	0.05	0.46	0.49
Mean	1.47	0.92	0.31	0.70	0.57
SD	1.07	1.20	0.43	0.84	0.61
n	9	76	11	9	21
XPB					
Median	1.18	0.84	0.0004	0.74	0.68
Mean	1.25	0.86	0.38	0.90	0.77
SD	0.72	0.71	0.73	0.97	1.01
n	9	76	11	9	21

Gel Electrophoreses and Southern Hybridization. Aliquots of amplified DNAs were electrophoresed through a 1.5% agarose gel. Amplified DNAs were visualized by ethidium bromide staining, photographed over an UV transilluminater, and capillary transferred to a Hybond *N* + membrane (Amersham, Amersham, United Kingdom) then fixed by UV crosslink.

Oligonucleotide probes selected to detect the amplified fragments for each of the targeted genes were synthesized by Lofstrand Labs Limited and labeled with the enhanced chemiluminescence 3′-oligolabeling system (Amersham). Southern hybridization was performed with fluorescent-11-dUTP labeled probes for each gene via the terminal transferase reaction. Fluorescent-labeled probes were hybridized respectively to their amplified DNAs on nylon membranes at 42°C overnight. The membranes were then washed and detected with enhanced chemiluminescence detection system.

Densitometry and Statistical Analyses. Numerical values for the expression of the *ERCC1* and *XPB* genes in the studied samples were conducted using the IPLab-Gel software (Scanalytics, Inc., Fairfax, VA). For each specimen, the densitometric readout of *ERCC1* value or *XPB* value was corrected for the corresponding β-*actin* value and presented as relative expression as compared with a human T-lymphocyte cell line control as reported previously (23, 24). The data were analyzed by linear regression analyses and Cricket Graph III software (Computer Associates International, Inc., Islandia, NY). Medians and means with SD are summarized in Table 1.

The relationship between the expression of *ERCC1/XPB* repair genes and pathological type carcinomas was assessed for statistical significance using exhaustive regression analysis (Parabon Computation, Inc.), also called all subsets regression in the literature. The 126 observations were tested with explanatory factors, including the relative expression of *ERCC1* or *XPB*, patient age, and the five types of carcinomas (mucinous, serous, clear cell, endometrioid, and poorly differentiated). A k-fold cross-validation procedure was applied to reduce the probability of overfitting.

Exhaustive Regression Analysis. In addition to ordinary linear regression, exhaustive regression was used to examine relationships between histological subsets and between the two genes being studied. Exhaustive (*i.e.*, all subsets) regression is a

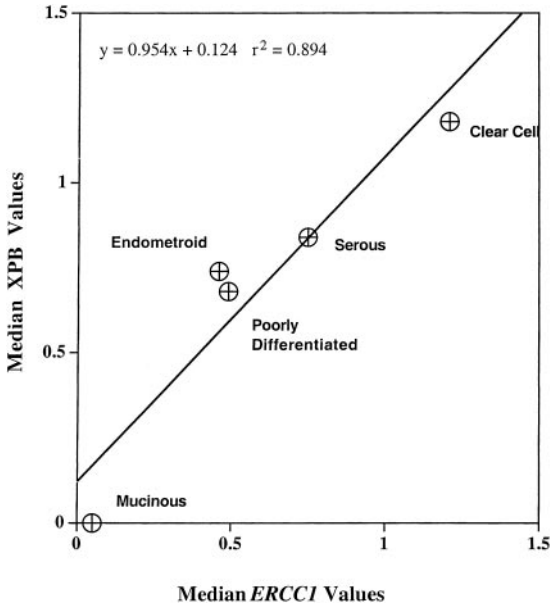

Fig. 1 The median mRNA values obtained by PCR for *ERCC1* and for *XPB* were plotted against one another for each of the five histological subtypes examined. The data were subjected to simple linear regression analysis, and the equation for the line is shown in the figure. On the basis of this analysis, the overall relationship approximates a slope of 1.

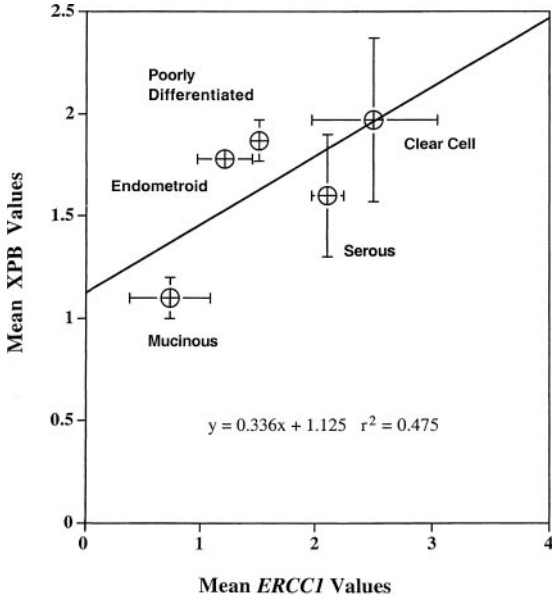

Fig. 2 The mean mRNA values obtained by PCR for *ERCC1* and for *XPB* were plotted against one another for each of the five histological subtypes examined. Also, *error bars* shown in both directions represent the second SD from the mean for that data point. The mean data points were subjected to simple linear regression analysis, and the equation for the line is shown in the figure. On the basis of this analysis, the overall relationship approximates a slope of 0.34.

statistical procedure that examines all possible models (*i.e.,* linear combinations) of a given set of potentially explanatory factors for statistical significance. Because the number of possible models grows exponentially in the number of factors under consideration, exhaustive regression is compute intensive, however, it is superior to traditional heuristic regression procedures (*e.g.,* stepwise or backward regression) that often (particularly in the presence of a large number of potential regressors) fail to report models with the greatest explanatory power (29, 30). Furthermore, unlike traditional heuristic regression procedures, exhaustive regression returns all models in the search space. Numerically unstable and spurious models were culled via several tests. Unstable models with excessive loss of precision during matrix inversions, based upon the condition number with respect to inversion, were culled. Models for which at least one parameter estimate was not significant at the $P = 0.05$ level were culled. For each model in which all parameter estimates were significant at the $P = 0.05$ level (*i.e.,* for each significant model), two statistics were measured: the MSPE, constructed using a 10-fold cross-validation procedure, and the MSE of the regression model (31). For each significant model, we calculated *P*s for the hypotheses that the MSPE and MSE are equal, respectively, to the minimum MSPE and the minimum MSE overall significant models. Lastly, we considered only models for which the *P*s for equivalence to the minimum MSPE and the minimum MSE were both at most 0.20. The single model that passed this series of filters is reported. The exhaustive regression procedure, along with the standard stability and spuriousness tests, achieves a far more robust exploration of the potential model space than do traditional heuristic regression procedures such as backward, forward, and stepwise regression (32, 33). The analysis was performed on ~1000 computers within Parabon Computation's computational grid.[3]

Using exhaustive regression analysis, we examined all possible linear combinations of explanatory factors using *ERCC1* as the dependent variable and selecting explanatory variables from the set *XPB*, age, five histology-specific constant variables, and five histology-specific slope variables. Each of the histology-specific constant variables was set equal to 1 when the specific histology was present and 0 otherwise. Each of the histology-specific slope variables was set equal to *XPB* when the specific histology was present and 0 otherwise. The five histologies used were: mucinous; clear cell; endometrioid; serous; and poorly differentiated.

RESULTS

Table 1 shows summary values that were obtained through the use of semiquantitative reverse transcription-PCR as described above. The highest mRNA values seen in these studies were in clear cell histology. Median values for *ERCC1* and for *XPB* in clear cell tissues were (approximately) 2-fold higher than those seen in poorly differentiated tumors or in endometroid tumors. Median clear cell tumor values for these two genes were ~50% higher than those seen in serous tumors, and many-fold higher than those seen in mucinous tumors. Mean values were higher in clear cell tumors as well. Numerical

[3] Internet address: http://www.parabon.com.

Table 2 Difference of means test: clear cell *versus* others

H_0: $\mu_{clear\ cell}$ μ_{other}
H_a: $\mu_{clear\ cell} > \mu_{other}$

	Serous	Mucinous	Poorly differentiated	Endometrioid
ERCC1				
Difference in means	0.55	1.16	0.77	0.90
SD of difference in means	0.37	0.37	0.44	0.37
Degrees of freedom	11	10	15	10
P	0.086	0.005	0.052	0.018
XPB				
Difference in means	0.40	0.87	0.35	0.48
SD of difference in means	0.25	0.33	0.40	0.33
Degrees of freedom	10	17	15	21
P	0.075	0.008	0.197	0.076

Table 3 Wilcoxon rank-sum test: clear cell *versus* others

H_0: Population $median_{clear\ cell}$ population $median_{other}$
H_a: Population $median_{clear\ cell}$ > population $median_{other}$

	Serous	Mucinous	Poorly differentiated	Endometrioid
ERCC1				
Summed rank for clear cell	578.5	144	114	206
Expected summed rank	387	94.5	85.5	139.5
SD of summed rank	70.01	13.16	11.32	22.10
Test statistic	2.74	3.76	2.52	3.01
P	0.003	0.0001	0.006	0.001
XPB				
Summed rank for clear cell	512	129	95	190.5
Expected summed rank	387	94.5	85.5	139.5
SD of summed rank	70.01	13.16	11.32	22.10
Test statistic	1.79	2.62	0.84	2.31
P	0.037	0.004	0.201	0.010

differences are given in the table, and the visual representations of these differences are shown in Figs. 1 and 2.

Table 2 shows the *P*s for comparisons of the mean for clear cell tumors to the means of other histological types studied. For *ERCC1*, clear cell tumors had higher levels of mRNA with *P*s ranging from 0.086 to 0.005. For *XPB*, clear cell tumors had higher levels of mRNA at statistically significant levels for mucinous tumors but showed only a trend for serous, poorly differentiated tumors, and endometroid tumors. Given the small number of observations for clear cell and poorly differentiated histologies combined with data that is (possibly) nonnormal, a nonparametric test for differences in medians may be more appropriate. Table 3 shows the *P*s for comparisons of the median for clear cell tumors to the medians of other histological types using the Wilcoxon rank-sum test. The nonparametric tests yield stronger results than the difference of means test. For *ERCC1*, clear cell tumors had higher levels of mRNA with *P*s ranging from 0.006 to 0.0001. For *XPB*, clear cell tumors had higher levels of mRNA than serous, mucinous, and endometrioid histologies with *P*s ranging from 0.037 to 0.01 and showed only a trend for poorly differentiated. Figs. 1 and 2 show that the median for serous tumors was higher than the medians for poorly differentiated and endometroid tumors, whereas the mean for serous tumors was lower than for the other two cell types.

Fig. 1 shows the linear regression evaluation of the median values for *ERCC1* and *XPB*, as assessed by histological type. As shown, this relationship closely approximates a straight line, with a r^2 of 0.894. This suggests that these cell types may lie on a biological continuum with respect to mRNA expression levels of these genes. Implications of this observation will be discussed below. Fig. 2 shows a similar plot of the mRNA values for these genes when assessed as means with SDs. Again, a biological continuum is suggested, although the fit to a straight line is less impressive; r^2 is 0.475. For Figs. 1 and 2, the best regression fit was to a line representing a polynomial relationship. For Fig. 1, the best relationship was $y = -0.701x^2 + 1.858x - 0.064$, $r^2 = 0.973$. For Fig. 2, the best relationship was $y = -0.412x^2 + 1.677x + 0.195$, $r^2 = 0.662$.

Next, exhaustive regression analyses were performed to assess the statistical variation of *ERCC1* as a function of *XPB* and as a function of histological type. The model that best fit the data for these 126 tissue samples was: ERCC1 = 0.172 + (XPB) (0.505 + 0.585 CC + 0.369 SC); CC = clear cell histology, SC = serous histology.

The data show that under baseline conditions, each 1.0 unit increase in *XPB* mRNA expression is associated with a 0.505 unit increase in *ERCC1* mRNA expression. This suggests tight coordinate expression of these two genes, as have been previously reported in smaller cohorts (20–24).

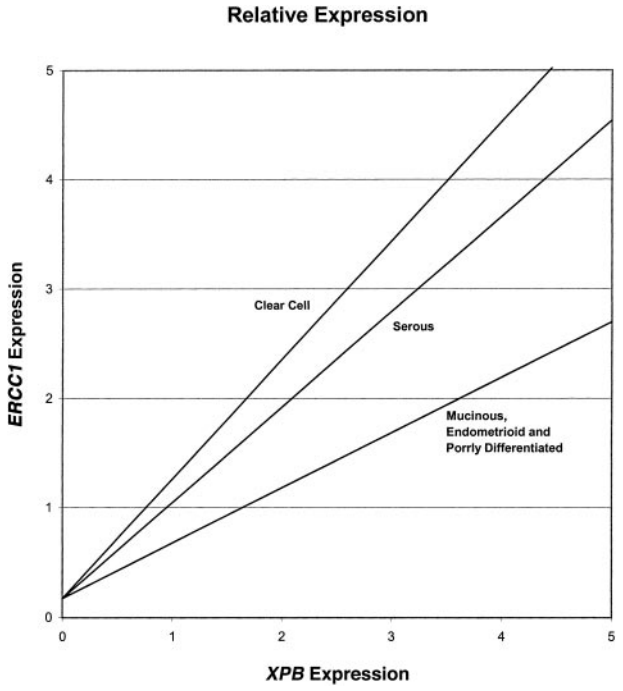

Fig. 3 The best linear fit for the data, are shown in the figure for: all baseline data ($n = 126$); all serous tumors ($n = 76$); and all clear cell tumors ($n = 11$). The respective slopes of the lines are: all baseline data, slope = 0.505; all serous tumors, slope = 0.874; all clear cell tumors, slope = 1.09.

Futhermore, under exhaustive regression analyses, in clear cell tumors there was an increase of 1.09 units for *ERCC1* mRNA for every 1.0 unit increase in *XPB*. This is an additional 0.585 unit increase over the baseline situation (p2 = 0.05, comparing clear cell tumors to baseline). This suggests that within the context of coordinate mRNA expression of NER genes, the relative increase in expression among genes in the repairosome may differ in magnitude within some cell types. Additional evidence of this comes from the data from serous tumors. In the case of serous tumors, there was a 0.874 increase in *ERCC1* with every 1.0 increase in *XPB* or an additional 0.369 increase over the baseline situation (p2 = 0.05, comparing serous tumors to baseline). Fig. 3 shows the linear relationship for *XPB* expression *versus ERCC1* expression for the baseline situation for clear cell tumors and for serous tumors.

Although mucinous tumors were of substantially lower mRNA values than the baseline situation, there was no statistically significant difference between mucinous tumors and baseline. Furthermore, age of the patient had no statistically significant impact in any of the models examined by exhaustive regression analyses (data not shown).

DISCUSSION

Itamocha *et al.* (34) studied 41 cases of clear cell carcinoma of the ovary and compared those data to those of 90 cases with serous carcinoma of the ovary. This group also conducted *in vitro* studies of cell lines derived from patients with clear cell carcinoma (10). Clinically, clear cell carcinoma patients showed significantly poorer response rates than patients with serous carcinoma. The response rate was 14.6% for clear cell carcinoma patients *versus* 72.2% for patients with serous tumors (34). Five-year survival rates were significantly poorer for patients with clear cell tumors as well. Ki-67-labeling data on tissues from these patients suggested that clear cell tumors may have a prolonged tumor doubling time, as compared with serous tumors. Tissue culture data were obtained from 11 clear cell carcinoma cell lines and compared with five cell lines derived from serous tumors (10). Tissue culture data confirmed that clear cells had prolonged doubling times, as compared with serous tumors; $P < 0.05$.

Understanding the molecular basis of drug resistance is an important part of our efforts to improve cancer chemotherapy. Drug resistance is a multifactorial process, and the specific mechanism(s) that may be responsible for resistance to one agent may be totally different from the mechanism(s) responsible for resistance to another. For platinum compounds and for platinum-based therapy, DNA repair is an important contributor to clinically relevant levels of drug resistance in tissue culture and in the clinic (1, 2, 42–45). Schwartz *et al.* (35) studied gene expression in ovarian cancer using a more global genomic method. Although they observed increased expression in clear cell tumors for a total of 73 genes, they did not place their findings in the context of clinical resistance to therapy.

The possible role of mismatch repair in determining resistance to platinum compounds has recently come under some debate (36, 37). However, it is clear that the ability to repair that damage rests with NER (1–3). Increased expression of *ERCC1* and other NER genes has been shown to exist in cell lines that are resistant to platinum-based compounds (4–7). Conversely, low *ERCC1* expression is seen in cell lines and tissues that are platinum sensitive (4–7). In the clinic, higher levels of *ERCC1* are associated with clinical resistance to platinum-based therapy in several diseases, which is believed to represent up-regulation of NER in those tissues (24–26).

In addition to ovarian cancer cells and tissues (20, 23, 24), coordinate expression of genes involved in NER has been reported in nonmalignant bone marrow from patients with a range of malignancies (38). Coordinate expression of these genes have been reported in malignant and nonmalignant brain tissues (21, 22), as well as human leukemia cells (39, 40). Although coordinate mRNA expression is a term that depends on mathematical assessment of data generated, it implies that biologically, the cells and/or tissues have a way of handling the expression of these genes so that they can work together in an efficient manner. We know that among the things these genes have in common are untranslated region binding sites for the same positive and negative transcription factors such as AP1, MZF1, and so on (28, 41). This suggests the mechanism through which coordinate expression may be exacted. These studies show that when *ERCC1* is up-regulated in a tissue so are other genes involved in the NER repairosome such as *XPB* (20–24, 38–40).

In the studies presented in this article, *ERCC1* and *XPB* provide biological cross validation of the relative up- or down-regulation of genes involved within NER. The notion that one histological subtype of disease might have consistently higher or lower mRNA expression levels of these genes is compatible with the fact that clinically some subtypes of this disease appear

to behave differently from others. Clear cell tumors of the ovary tend to be diagnosed at earlier stage than most other epithelial cell types (8). However, when seen in advanced stage, clear cell tumors are notoriously difficult to treat with platinum-based therapy or other DNA damaging chemotherapy (8, 9, 34). The data presented above suggests that clear cell tumors may have markedly enhanced DNA repair activity, as compared with other histological types of ovarian cancer. Enhanced DNA repair is the hallmark of cellular resistance to platinum compounds at clinically relevant levels of drug exposure (1, 2).

We have shown in a collection of tumor tissues from 126 different individuals that clear cell tumors have higher mRNA levels of *ERCC1* and of *XPB* than other common subtypes of epithelial ovarian cancer. We believe that this is evidence that enhanced NER is one major factor that contributes to clinical drug resistance in this disease.

The current studies represent a first-step investigation of the relationship between expression of NER genes and clinical resistance of platinum chemotherapy in histological tumor type of ovarian cancer. They are exploratory investigations that generate hypotheses for future studies. Studies using a larger sample size that include clear cell tumors, mucinous tumors, and poorly differentiated tumors, with data collected from patients responsive to platinum-chemotherapy, are warranted.

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