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Increased mRNA Levels of Xeroderma Pigmentosum Complementation Group B (*XPB*) and Cockayne's Syndrome Complementation Group B (*CSB*) without Increased mRNA Levels of Multidrug-Resistance Gene (*MDR1*) or Metallothionein-II (*MT-II*) in Platinum-Resistant Human Ovarian Cancer Tissues

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ABSTRACT. Tumor tissue specimens from human ovarian cancer patients were assessed for relative mRNA abundance levels of several genes thought to be involved in the development of *in vitro* drug resistance in this disease. Higher mRNA levels of Xeroderma pigmentosum group B (*XPB*), which links DNA repair with DNA transcription, and of Cockayne's syndrome group B (*CSB*), which is essential for gene-specific repair, were observed in tumor tissues that were clinically resistant to platinum-based chemotherapy, as compared with tissues from patients responding to therapy. In a cohort of 27 patients, mRNA levels of *XPB* averaged 5-fold higher in platinum-resistant tumors ($P = 0.001$); and for *CSB*, mRNA levels averaged 6-fold higher but with greater variability ($P = 0.033$). Concurrently, these platinum-resistant tumor tissues did not exhibit significantly higher mRNA levels for the *MDR1* (multidrug-resistance) gene ($P = 0.134$) or of the metallothionein-II (*MT-II*) gene ($P = 0.598$). Since these platinum-resistant tumors also show higher mRNA levels of *ERCC1* and *XPA*, platinum resistance appears to be associated with concurrent up-regulation of four genes (*XPA*, *ERCC1*, *XPB*, and *CSB*). These four genes participate in DNA damage excision activity, gene-specific repair, and linkage of DNA repair with DNA transcription. These data suggest that concurrent up-regulation of genes involved in nucleotide excision repair may be important in clinical resistance to platinum-based chemotherapy in this disease. BIOCHEM PHARMACOL **60**;11:1611–1619, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. XPB; *CSB*; ovarian cancer; platinum compounds; *MDR1*; *MT-II*

Platinum compounds (cisplatin and carboplatin) comprise the mainstay of clinical therapy for advanced-stage cancer of the ovary [1, 2]. Cellular resistance to platinum compounds appears to be effected by a number of molecular mechanisms, which appear to vary depending on the level of resistance studied and the cell line selected [3, 4]. At low levels of resistance (10- to 15-fold over baseline), several lines of evidence suggest that DNA repair predominates as the primary mechanism of cellular resistance to these agents. These lines of evidence were obtained in studies of murine L1210 leukemia cells [3, 4], drug-selected human ovarian cancer cells [4–6], and non-drug-selected human T lymphocytes [7]. These studies consistently show that DNA repair is of primary importance at low levels of resistance, regardless of the cell lines studied.

There are several different pathways of DNA repair that exist in mammalian cells. Such pathways include mismatch repair [8, 9], O^6-methyl transferase activity, base excision repair [10], and NER† [11]. Recent data from cell culture studies and from clinical studies strongly suggest that NER may be responsible for the repair of platinum–DNA damage *in vitro* and *in vivo* [7, 12–14].

ERCC1 is the first human gene of the NER pathway to have been cloned and sequenced [15]. Transfection of the *ERCC1* gene into repair-deficient CHO cells of complementation group 1 results in a 5-fold increase in cisplatin

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† *Abbreviations*: NER, nucleotide excision repair; *ERCC1*, human DNA excision repair gene, cross-complementing CHO mutant cell lines of complementation group 1; *XPA*, *XPB*, and *XPF*, human xeroderma pigmentosum correcting gene group A, B, and F, respectively; CHO, Chinese hamster ovary; *CSB*, Cockayne's syndrome complementation group B; RT/PCR, reverse transcription/polymerase chain reaction; *MDR1*, human multidrug resistance gene; and *MT-II*, metallothionein-II gene.

resistance and restores the ability to repair cisplatin–DNA adducts in those cells [13].

ERCC1 gene expression in fresh human ovarian cancer tissues is directly related to clinical resistance to platinum-based therapy [12] and appears to be induced in human ovarian cancer tissues from patients whose disease converts from cisplatin-sensitive to cisplatin-resistant [14]. However, *ERCC1* is only one of several genes that are critical components to the first step in the NER process (DNA damage recognition and excision), which is the rate-limiting step [16].

The precise roles of the specific genes involved in DNA repair form a topic of intense investigation. Current data suggest specific roles for several of these genes. *ERCC1* appears to complex with *XPF*, and this heterodimer has the primary function of incising the DNA strand, at a site 5′ to the covalent DNA base damage [17]. *XPA* appears to have a "DNA damage localizing" function, but not primary DNA incision activity [18]. *XPB* appears to link DNA repair with DNA transcription [17, 19]; and *CSB* appears to effect preferential repair within the NER process [20].

Recent studies in fresh human tissues show that mRNA levels of expression of *ERCC1* and of *XPA* correlate with clinical resistance to platinum-based therapy in human ovarian cancer [12, 14]. These data show that expression levels of at least two of the genes in the NER process appear to have clinical significance, and suggest greater activity of the NER process in human cancer tissues that are resistant to DNA-damaging agents. In the current study, we have addressed the question of whether such correlations exist for *XPB*, which links DNA repair with DNA transcription, and/or *CSB*, which effects gene-specific repair. Metallothionein overexpression has been observed in some cisplatin-resistant cell lines [21], and *MDR1* gene expression has been linked to clinical resistance in ovarian cancer in some studies [22]. Therefore, we assessed mRNA expression levels for *MDR1*, and *MT-II*, in these same ovarian cancer tissues.

MATERIALS AND METHODS

Tissues Studied

Fresh tumor tissues were obtained from 27 patients with ovarian cancer. These tissues were obtained prior to treatment with cisplatin- or carboplatin-based chemotherapy. Patients from whom tissues were obtained participated in one of three approved experimental treatment protocols for advanced-stage ovarian cancer as detailed in an earlier study [12]. Disease was followed by physical exam and by radiographic means, including abdominopelvic CT scan and/or ultrasound examination.

Complete response was defined as complete eradication of all evaluable disease, confirmed by peritoneoscopy. Partial response was defined as a >50% reduction in the sum of the products of the perpendicular diameters of all measurable lesions lasting at least 1 month. Progressive disease was a >25% increase in the sum of the products of the perpendicular diameters of all measurable lesions or the appearance of new lesions. Stable disease included those clinical circumstances that did not fit the definitions of objective response or progression. Progressive disease and stable disease patients are included in the non-responder category. Complete response and partial response are included in the responder category. By these criteria, there were 15 patients who were responders and 12 non-responders in the cohort.

PCR Analyses

A RT/PCR based assay system was used to determine the level of expression of *XPB*, *CSB*, *MDR1*, *MT-IIa*, and β-actin [12]. Tissues were stored at −80° and extracted for total RNA by hot phenol/chloroform extraction [23]. cDNA was obtained from 10 ng of total RNA, by reverse transcription using oligo-dT primers (Reverse Transcription System, Promega). cDNAs were washed and concentrated by ultrafiltration (Amicon) and resuspended to 100 μL in low TE buffer (10 mM Tris, pH 8.0; 0.1 mM EDTA).

For *XPB*, primers and RT/PCR conditions were selected to effect amplification of a 323-bp segment, from base 1916 to base 2238 of the *XPB* cDNA sequence [24]. For *CSB*, primer and PCR conditions were optimized for amplification of a 510-bp segment, from base 3702 to base 4212 of the *CSB* cDNA nucleotide sequence [20]. Primers chosen for β-actin spanned a 731-base segment of the coding region of the β-actin gene, and extended from base 269 of exon II to base 1535 in exon IV [25].

For *MDR1*, primers and RT/PCR conditions were selected to effect amplification of a 288-bp segment, from base 3544 to base 3831 of the transcribed *MDR1* gene sequence [26]. For *MT-II*, primer and PCR conditions were optimized for amplification of a 177-bp segment, from base 2 to base 178 of the *MT-IIa* cDNA sequence [27].

Aliquots of 3 μL of the cDNA preparation from each sample were amplified by RT/PCR for 40 cycles for *XPB* and *CSB*, and for 30 cycles for *MDR1* and for *MT-II*. Aliquots of 7.5 μL were amplified for 30 cycles for β-actin. The GeneAmp PCR reagent kit with AmpliTaq DNA polymerase (Perkin Elmer Cetus) was used for each gene. Amplification of tissue cDNA to 40 cycles for *XPB* and *CSB* maximized our ability to detect low expressors while remaining in the linear range of PCR. Aliquots of amplified DNA were electrophoresed through a 1.5% agarose gel. Amplified DNA was visualized by ethidium bromide staining, photographed over a UV-transilluminator (Hoefer Scientific Instruments), and transferred to Hybond N+ membrane (Amersham).

Oligonucleotides (26-mer for *XPB*, *CSB*, and *MDR1*, a 24-mer for *MT-II*, and a 46-mer for β-actin) from the central region of each amplified sequence were end-labeled with [^{32}P]rATP (Amersham) using T4 polynucleotide kinase (Stratagene), and were used as the respective probes. Oligonucleotides used as primers and probes for RT/PCR-

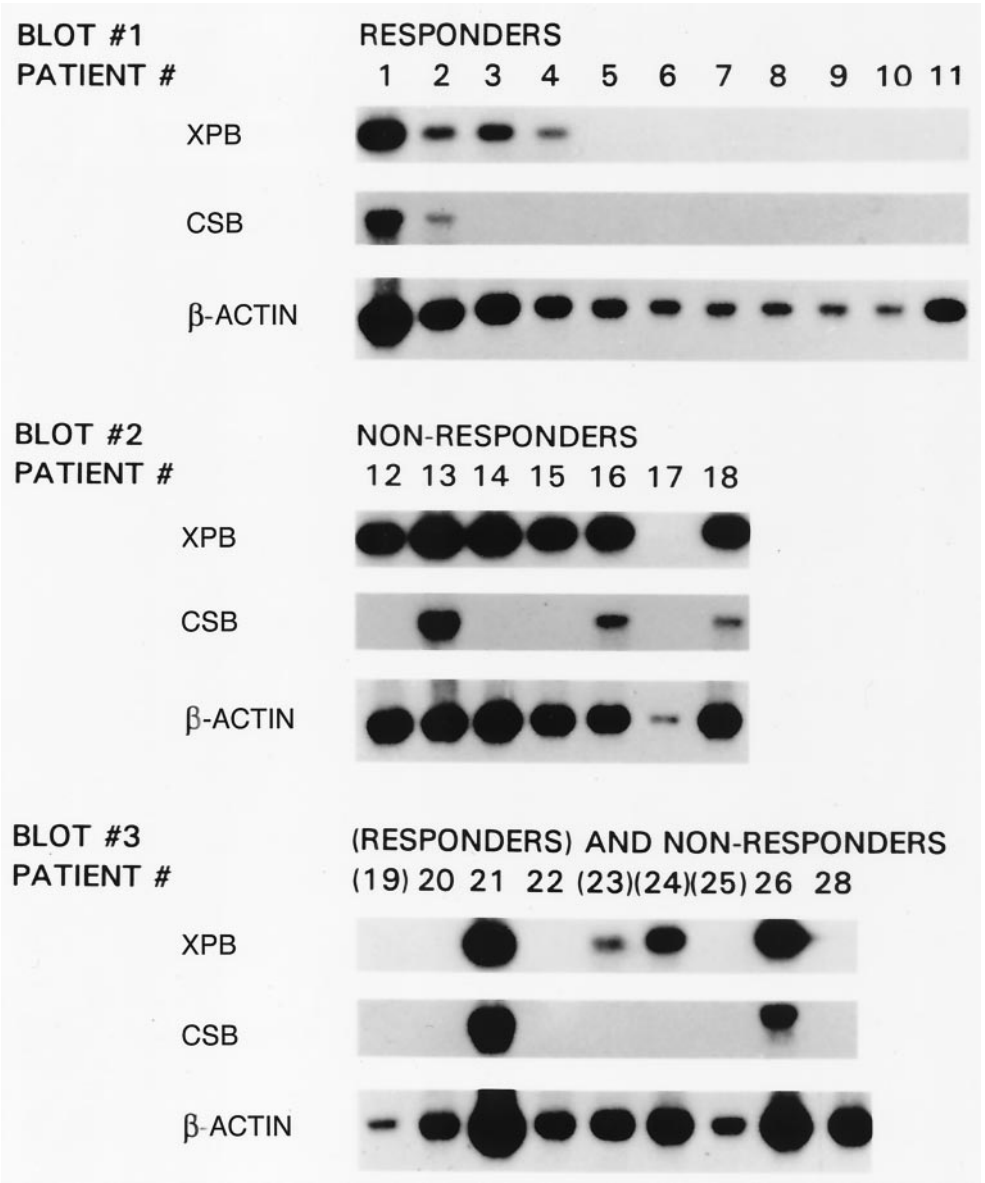

FIG. 1. Autoradiographs of RT/PCR-amplified mRNA for *XPB*, *CSB*, and β-actin from 27 ovarian cancer tissues, following hybridization of amplified segments with the respective radiolabeled probes. Samples 1–11, 19, and 23–25 (blots 1 and 3) represent 15 tumor tissues from patients responding to platinum-based therapy; samples 12–18, 20–22, 26, and 28 (blots 2 and 3) represent 12 tumor tissues from patients resistant to platinum-based therapy.

based analysis of *XPB*, *CSB*, *MDR1*, *MT-II*, and β-actin were synthesized by Lofstrand Laboratories.

Numerical values for the expression of the *XPB*, *CSB*, *MDR1*, and *MT-II* genes in tumor tissue specimens were obtained as follows. For each sample, the densitometric readout of the autoradiographic signal generated by the RT/PCR-amplified DNA when hybridized to the respective ^{32}P-labeled probe was divided by the densitometric reading for β-actin. For purposes of comparison, the sample with the highest XPB:actin value was assigned the value of "1," and XPB:actin values for all other samples were expressed relative to that value. For *CSB* expression, the sample with the highest CSB:actin value was assigned the value of 1, and the CSB:actin values for all other samples were expressed relative to this value. The numerical values for *MDR1* and *MT-II* expression were similarly obtained. When assessing XPB, CSB, and MT-II mRNA, the human T lymphocyte cell line H9 [28] was used as an internal control. For MDR1, the human ovarian cancer cell line A2780/CP70 [6] was used as an internal control.

Statistical Analyses

The relationship between response to therapy and expression of *XPB*, *CSB*, *MDR1*, and *MT-II* was examined for statistical significance using Student's *t*-test with the Statworks program (Cricket Software, Inc.). Two-sided *P* values are shown in the tables and text. The relationships between expression levels of *XPB*, *CSB*, *XPA*, and *ERCC1* were assessed by mathematical regression analyses using the

TABLE 1. Relative levels of *XPB* and *CSB* gene expression in ovarian tumor tissue in relation to response

	Range	Median	Mean ± SD
XPB			
Responders ($N = 15$)	< 0.01–0.43	< 0.01	0.08 ± 0.13
Non-responders ($N = 12$)	< 0.01–1.00	0.53	0.44 ± 0.35
			$P = 0.001$
CSB			
Responders ($N = 15$)	< 0.01–0.41	< 0.01	0.04 ± 0..11
Non-responders ($N = 12$)	< 0.01–1.00	0.02	0.27 ± 0.38
			$P = 0.033$

CricketGraph program (Computer Associates International, Inc.) and the DeltaGraph program (DeltaPoint Inc.).

RESULTS

Figure 1 shows autoradiographs of RT/PCR-amplified mRNA for *XPB*, *CSB*, and β-actin, from the 27 patients studied in the cohort. For *XPB*, detectable levels of mRNA were seen in 8 of 12 tumors that were resistant to therapy, and in 6 of 15 tumors that were clinically sensitive to therapy. For *CSB*, detectable mRNA levels were seen in 5 of 12 non-responders, and 2 of 15 responders. Numerical values for mRNA levels for *XPB* and *CSB* in responders and non-responders, corrected for β-actin (see Materials and Methods), are summarized in Table 1. In ovarian cancer tissues that were resistant to platinum-based therapy, there was a 5-fold higher mean level of expression of the *XPB* gene ($P = 0.001$), and a 6-fold higher mean level of expression of the *CSB* gene ($P = 0.033$). *CSB* expression appeared to be more variable than *XPB* expression (Table 1).

The relative mRNA levels of the three genes *XPA*, *XPB*, and *CSB* were examined in three-dimensional plots (Fig. 2). In this way, one can concurrently assess mRNA levels associated with DNA damage recognition, gene-specific repair, and linkage of DNA repair with DNA transcription. As shown, patients with tumors that were sensitive to platinum-based therapy (responders, panel A) tended to have comparatively low levels of mRNA expression for all three genes. In contrast, tumors that were resistant to platinum-based therapy (non-responders, panel B) tended to have high levels of expression of all three genes. When a similar analysis was performed using *ERCC1* instead of *XPA*, the same pattern was observed (data not shown). This suggests that within platinum-resistant tissues, there is greater activity of the NER genes that are responsible for each of these essential properties within the process.

Figure 3 shows autoradiographs of RT/PCR-amplified mRNA for *MDR1* and *MT-II* from 25 patients studied in the cohort. Detectable levels of mRNA for *MDR1* were seen in 7 of 10 tumors that were resistant to therapy, and in 6 of 15 tumors that were clinically sensitive to therapy. For *MT-II*, detectable mRNA levels were seen in 9 of 10 non-responders, and in 14 of 15 responders. Numerical values for mRNA levels of *MDR1* and *MT-II* in responders and non-responders, corrected for β-actin (see Materials and Methods), are shown in panels A and B of Fig. 4. These data are summarized in Table 2. In ovarian cancer tissues

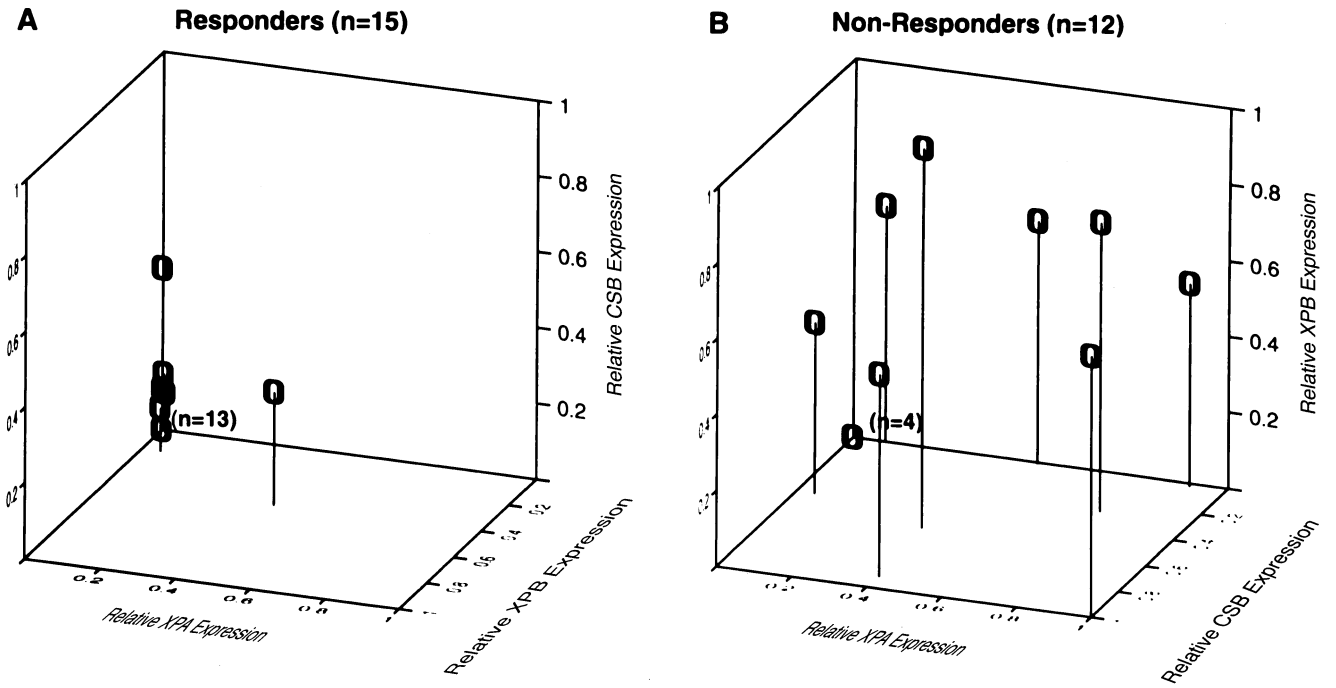

FIG. 2. Three-dimensional plots concurrently analyzing *XPA*, *XPB*, and *CSB* expression levels in ovarian tumor tissues from responders (panel A) and non-responders (panel B).

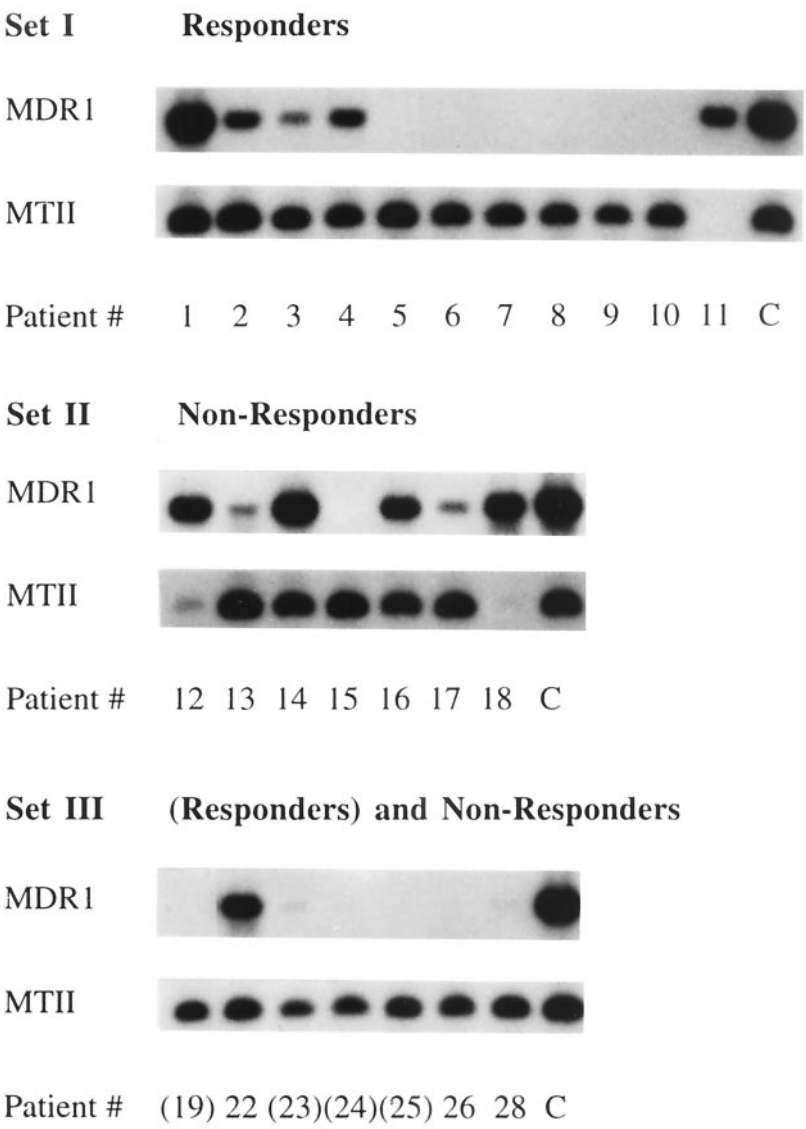

FIG. 3. Autoradiographs of RT/PCR-amplified mRNA for *MDR1* and *MT-II* from 25 ovarian cancer tissues, following hybridization of amplified segments with the respective radiolabeled probes. Samples 1–11, 19, and 23–25 (blots 1 and 3) represent 15 tumor tissues from patients responding to platinum-based therapy; samples 12–18, 22, 26, and 28 (blots 2 and 3) represent 10 tumor tissues from patients resistant to platinum-based therapy.

that were resistant to platinum-based therapy, there was a 3-fold higher mean level of mRNA expression of the *MDR1* gene. The difference in mean *MDR1* expression between tissues from responders and non-responders, however, was not statistically significant ($P = 0.134$). Mean levels of expression of the *MT-II* gene did not differ between tissues from responders and non-responders ($P = 0.598$).

In an earlier study with these ovarian cancer tissues, elevated levels of mRNA for *ERCC1* and *XPA* were observed in tissues that were clinically resistant to platinum-based therapy [12]. Coordinate expression of human DNA repair genes has been reported previously by this group in non-malignant tissues [29], as well as in malignant tissues [30, 31]. Therefore, we performed similar mathematical regression analyses of coordinate expression in these tissues for *ERCC1*, *XPB*, *CSB*, and *XPA*. For platinum-sensitive tumor tissues, the relationship between the mRNA expression of these genes was strongest between *XPA* and *CSB* ($r^2 = 0.940$, $P < 0.001$; Table 3). In tumor tissues from responders, weak correlations were observed between *ERCC1* and *XPB* ($P = 0.041$), and between *ERCC1* and *CSB* ($P = 0.048$).

For platinum-resistant tissues, the relationships between the expression levels of these DNA repair genes was strongest between *ERCC1* and *XPB* ($r^2 = 0.587$, $P = 0.004$; Table 3). A good correlation was also observed in non-responder tissues between *XPA* and *XPB* ($P = 0.019$). However, no correlation was observed between *XPA* and *CSB* levels ($P = 0.113$) or between *ERCC1* and *CSB* ($P = 0.065$, Table 3).

Mathematical regression analyses were also performed for possible coordinate expression in ovarian cancer tissues between *MDR1* and *MT-II*, and the DNA repair genes *ERCC1*, *XPB*, *CSB*, and *XPA*. Correlation coefficients obtained for expression of *MDR1* with the excision repair genes ranged from 0.000 to 0.106, with *P* values ranging from 0.111 to 0.925. Correlation coefficients obtained for the expression of *MT-II* with the excision repair genes

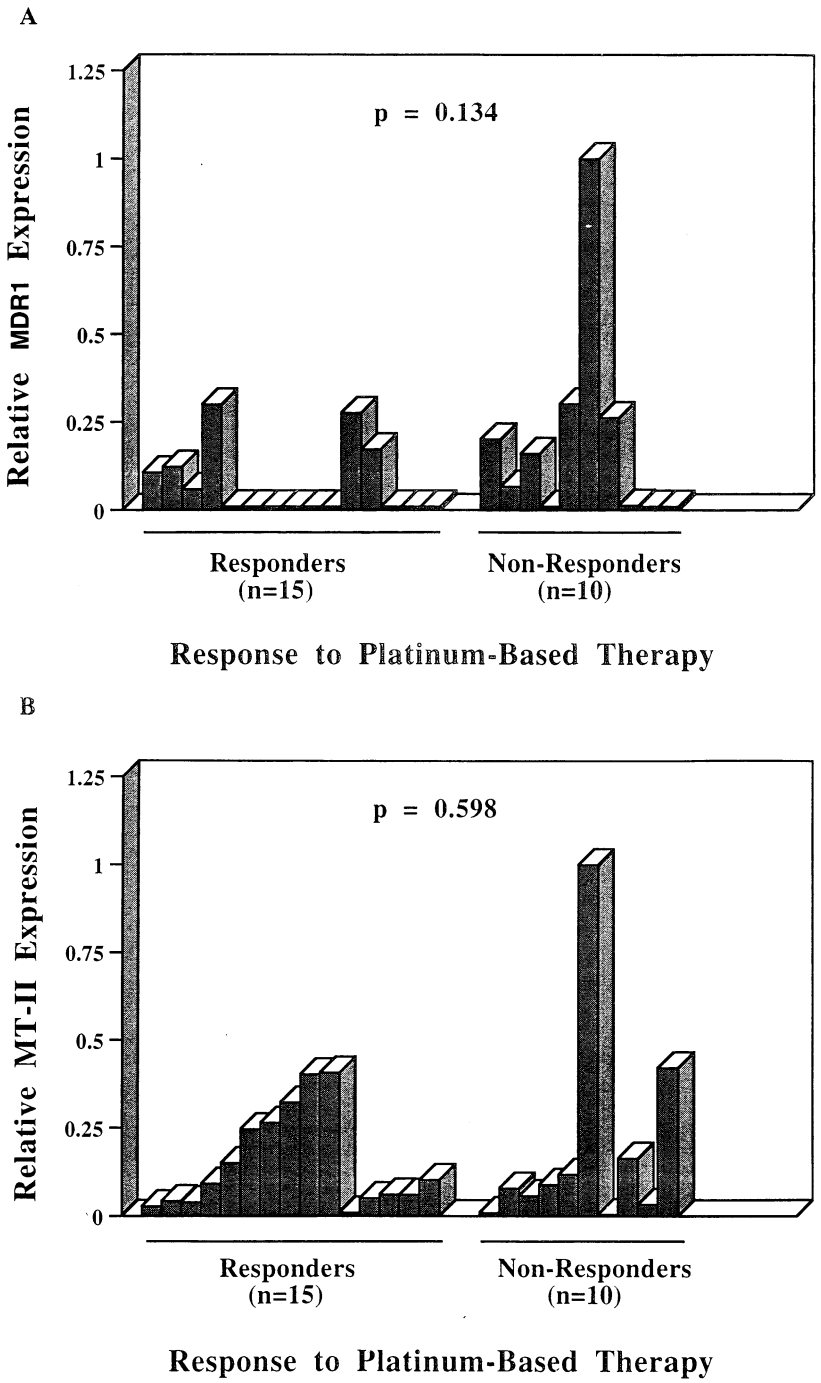

FIG. 4. Relative expression levels are shown for *MDR1* (A) and *MT-II* (B) in tumor tissue from 25 patients with ovarian cancer in relation to their response to platinum-based therapy.

ranged from 0.057 to 0.167, with negative slopes, and *P* values ranging from 0.045 to 0.253. Considering the positive linear regression relationships between the excision repair genes, these data suggest that mRNA levels of *MDR1* or *MT-II* in these ovarian cancer tissues are not coordinately expressed with the mRNA levels of *ERCC1*, *XPB*, *CSB*, and *XPA*.

DISCUSSION

We have shown previously that in human ovarian cancer tissues, platinum-resistant tissues show higher levels of expression of the NER genes *ERCC1* and *XPA* [12]. These two genes are involved directly in DNA damage recognition and incision of the DNA strand at sites flanking the DNA damage. We now show that the genes involved in preferential DNA repair (*CSB*) and involved in the linkage of DNA repair with DNA transcription (*XPB*) are also up-regulated significantly in malignant tissues clinically resistant to DNA-damaging chemotherapy. We believe that this is strong evidence that the nucleotide excision repair process is strongly associated with clinical resistance to platinum compounds in this disease.

This view is consistent with *in vitro* data reported from

TABLE 2. Relative levels of *MDR1* and *MT-II* gene expression in ovarian tumor tissue in relation to response

	Range	Median	Mean ± SD
MDR1			
Responders (N = 15)	< 0.01–0.30	< 0.01	0.07 ± 0.11
Non-responders (N = 10)	< 0.01–1.00	0.11	0.20 ± 0.30
			$P = 0.134$
MT-II			
Responders (N = 15)	< 0.01–0.41	0.09	0.15 ± 0.14
Non-responders (N = 10)	< 0.01–1.00	0.08	0.20 ± 0.31
			$P = 0.598$

this laboratory, from the laboratory of Eastman and colleagues [3, 6], and from the laboratory of Hamilton and colleagues [5]. We have reported previously that in human ovarian cancer cell lines in tissue culture DNA repair appears to be the primary effector of cellular resistance to cisplatin [6]. Platinum-resistant cell lines show increased overall genomic repair [6] and increased gene-specific repair [32].

Eastman and colleagues [3] have studied low levels of cisplatin resistance, as well as high levels of resistance (>20-fold), in murine L1210 cells. Eastman, Hamilton, and their colleagues have also studied low levels of cisplatin resistance, as well as high levels of resistance (>20-fold), in human ovarian cancer cells [3, 6, 33–36]. In both cases, DNA repair was the primary effector of cisplatin resistance at low levels, and glutathione pathways became an important effector at high levels of cellular resistance.

In vitro, high levels of cisplatin resistance are developed by continuous stepwise increases in cisplatin exposure over many months. In the clinic, our ability to increase cisplatin exposure is limited by toxicity to the human host. Therefore, it is not possible to expose patients to cisplatin doses that are many-fold over the baseline doses that we currently administer, even in bone marrow transplant settings. Therefore, we believe that at this time, levels of cisplatin resistance that are important to the human situation are of the magnitude discussed here. This would suggest that tissue culture work is totally consistent with the clinical work we currently present, suggesting that DNA repair is of major importance in determining clinical outcome in this disease.

TABLE 3. Analysis of the relationship between relative mRNA abundance of DNA repair genes in malignant ovarian tissue from responders and non-responders

	Responder tissue (N = 15)		Non-responder tissues (N = 12)	
	Linear curve fit			
mRNA transcripts	r^2	*P* value	r^2	*P* value
ERCC1 and *XPB*	0.284	0.041	0.587	0.004
XPA and *XPB*	0.233	0.068	0.438	0.019
ERCC1 and *CSB*	0.269	0.048	0.300	0.065
XPA and *CSB*	0.940	< 0.001	0.232	0.113

Modrich and colleagues [37] and Howell and colleagues [38] have investigated the possible role of mismatch repair in effecting cisplatin resistance in human ovarian cancer cells or in colon cancer cells, respectively. Modrich studied cells where major differences have already been shown for NER [6, 32], whereas Howell studied cells that appear to be NER-intact [38]. In both sets of studies, data showed that the intact mismatch repair system appeared to be essential for the linkage of DNA damage/repair with the initiation of apoptosis. In the Howell studies, the difference between cell lines in cisplatin resistance was only 2-fold. Because of the previously reported NER differences in cell lines used in the Modrich studies (>8-fold differences in gene specific repair, as an example), the true contribution of mismatch repair in his studies is not clear.

Whereas NER appears to be of primary importance for cisplatin and carboplatin, mismatch repair (MMR) may take on a greater role for oxaliplatin in respect to cellular resistance. One group has shown that the carrier ligand for oxaliplatin may cause a difference in DNA damage recognition by DNA repair proteins. Chaney and colleagues studied the roles of hMLH1, hMSH3, and hMSH6 in the replicative bypass of DNA lesions caused by cisplatin and by oxaliplatin [39, 40]. Defects in hMLH1 or in hMSH3 resulted in 1.5- to 4.8-fold increases in cisplatin resistance, and a 2.5- to 6-fold increase in replicative bypass of cisplatin–DNA adducts. In the same cell lines, no differences in replicative bypass were seen for oxaliplatin–DNA adducts under any conditions studied. It has been hypothesized that these differences may be due to the fact that the 1,2-diaminocyclohexane ring of oxaliplatin tends to protrude into the major groove of the damaged DNA, forming a less polar major groove in the area of the DNA adduct [41].

The precise role(s) for these four genes (*ERCC1*, *XPA*, *XPB*, *CSB*) in the NER process is the current topic of intense study. Based on current concepts, ERCC1 appears to have DNA damage recognition and strand incision functions [14, 17, 18, 39, 42], and XPA appears to have a DNA damage localization function [18]. ERCC1 and XPA function within the same complex along with other proteins encoded by the genes *XPF*, *ERCC11*, and others [17, 18, 37, 42, 43]. XPB and CSB appear to have helicase activities [20, 24, 44], but they appear to differ in their primary functions. XPB appears to effect "linkage" between DNA repair and DNA transcription [19, 44, 45], and CSB appears to be essential to the process of preferential DNA repair [20].

The concurrent up-regulation of *ERCC1*, *XPB*, *CSB*, and *XPA* in platinum-resistant human ovarian cancer tissues, along with concurrent down-regulation of these genes in platinum-sensitive tissues, strongly suggest intact coordinated regulation of these genes in most human ovarian cancer tissues. It is unclear at this time whether coordinated regulation within NER is effected by one gene in this

pathway such as *ERCC1*, or by some other gene that affects DNA repair, such as *p53*. Such questions are the subject of current studies in our laboratory.

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