Featured Article

Increase in Expression of the Copper Transporter ATP7A during Platinum Drug-Based Treatment Is Associated with Poor Survival in Ovarian Cancer Patients

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Abstract

Purpose: **The Cu efflux transporter ATP7A is overexpressed in some cisplatin-resistant ovarian carcinoma cell lines. We examined the expression of ATP7A in the major normal human organs and in several types of human malignancies and sought to determine whether ATP7A expression changed during treatment of ovarian carcinomas with Pt-containing regimens.**

Experimental Design: **ATP7A expression was quantified by immunohistochemical staining using microarrays containing normal and malignant tissues, and standard sections of 54 paired tumor samples obtained from ovarian carcinoma patients before and after at least two cycles of platinum-based therapy.**

Results: **ATP7A was expressed in normal endometrium, prostate, testis, and kidney but was not detected in the other major organs. ATP7A was expressed in some of the most common human malignancies, including prostate (7 of 7), breast (10 of 10), lung (8 of 8), colon (5 of 8), and ovary (6 of 7), as well as in a wide variety of other types of malignancy. ATP7A staining was detected in 28 of 54 ovarian carcinomas before treatment. Patients with increased ATP7A expression after treatment (18 of 54) exhibited poorer actuarial survival ($P < 0.0057$ by log-rank test). Expression of ATP7A either before or after treatment was not associated with other clinical factors.**

Conclusions: **Although ATP7A is not detectable in most normal tissues it is expressed in a considerable fraction of many common tumor types. Enrichment of the tumor for ATP7A-expressing cells during platinum drug-based treatment of ovarian cancers is associated with poor survival. These findings are in agreement with results of *in vitro* studies from this laboratory demonstrating that increased expression of ATP7A renders cells resistant to cisplatin and carboplatin.**

Introduction

DDP[4] and CBDCA are essential components of the most effective chemotherapeutic regimens for many types of cancer. However, rapid development of resistance during therapy is common and is a major cause of treatment failure. The mechanisms underlying clinical resistance remain poorly characterized. The cytotoxicity of DDP is related to the amount of drug that enters the cell, the extent to which that drug reaches the nucleus and reacts with DNA, and the ability of the cell to repair or tolerate DDP adducts (1). The major mechanisms of resistance that have been identified thus far involve reduced drug uptake, increased cytoplasmic detoxicification, and increased DNA repair (2–4). Among these, reduced cellular drug accumulation is the most universally identified correlate of acquired DDP resistance (1, 5–12).

The mechanisms by which DDP is transported into cells, distributed to various subcellular compartments, and effluxed from cells are not well defined. DDP and its analogues are highly polar molecules and do not cross lipid bilayer membranes easily. DDP enters cells relatively slowly compared with most anticancer agents, and DDP efflux is characterized by an initial rapid phase followed by a very long terminal half-life that seems to reflect extensive intracellular sequestration or binding (13, 14). Previous studies have suggested that at least one component of DDP influx is mediated by a transporter, and an increased efflux rate has been observed in some DDP-resistant cell lines (15, 16).

Cu is exported from cells by one of two efflux pumps, ATP7A and ATP7B. ATP7A is expressed in most tissues other than liver, whereas ATP7B is expressed predominantly in the liver. A link between Cu transporters and DDP sensitivity was first reported by Komatsu *et al.* (17), who noted that cells engineered to express high levels of ATP7B were resistant to both Cu and DDP. Nakayama *et al.* (18) have recently reported higher levels of ATP7B mRNA in histological subtypes of ovarian carcinoma known to be less responsive to the Pt-containing drugs. Additional evidence implicating Cu homeostasis mechanisms in the cellular pharmacology of DDP was provided by the observation that ovarian carcinoma cells selected *in vitro*

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[4] The abbreviations used are: DDP, cisplatin; CBDCA, carboplatin.

for resistance to DDP are cross-resistant to Cu (19) and that cells selected for resistance to Cu exhibit cross-resistance to DDP (20). Consistent with a possible role for ATP7A in the export of DDP, ATP7A was found to be overexpressed in some ovarian carcinoma cell lines with acquired DDP resistance (19).

As a first step toward defining a possible role for ATP7A in acquired clinical resistance to DDP and CBDCA, we conducted an immunohistochemical analysis of the expression of ATP7A in normal human organs and in the major classes of human malignancies using tissue microarrays. In addition, we focused more specifically on an analysis of the expression of ATP7A in 54 pairs of ovarian tumor samples obtained from individual patients before and after at least two cycles of platinum-based therapy (21). Associations were sought between ATP7A expression and available clinical data. We report here that ATP7A is expressed in a considerable fraction of many common tumor types, and that enrichment of human ovarian cancers for cells that express ATP7A during the course of treatment with platinum-based therapy is associated with poor survival. These findings are consistent with results of *in vitro* studies showing that increased expression of Cu transporters renders cells resistant to DDP (17).

Materials and Methods

Tissue Microarrays. Tissue arrays containing normal thyroid, spleen, endometrium, prostate, testis, ovary, pancreas, lung, liver, kidney, small intestine, colon, brain, and adrenal tissue and arrays containing tumor tissue from cancers of the prostate, breast, stomach, colon, liver, lung, ovary, thyroid, pancreas, kidney, testis, and endometrium plus samples of lymphomas and melanoma were kindly provided by Dr. Sharon Wilczynski (City of Hope Medical Center, Duarte, CA). Sections of normal breast were provided by Nissi M. Varki (University of California, California, San Diego Cancer Center). In addition, arrays containing tumor tissue from cancers of the lung, liver, kidney, colon, ovary, breast, thyroid, pancreas, prostate, mesothelioma, and samples of Hodgkin's and non-Hodgkin's lymphoma, melanoma, rhabdomyosarcoma, malignant fibrous histiocytoma, Ewing's sarcoma, and epithelioid sarcoma were obtained from DAKO, Inc. (Carpinteria, CA).

Patients. The study population consisted of patients with ovarian cancer who had been entered on one of several different Memorial Sloan-Kettering studies (patients 1–41) or Southwest Oncology Group study 8835 (patients 42–54). All of the cases from these two trials in which paired fixed and paraffin-embedded tumor tissues were available from both before treatment and after at least two cycles of chemotherapy were included. Each patient had received DDP- or CBDCA-based chemotherapy as part of her first line treatment, followed by various second- and third-line treatments. Each patient's age, stage, histological grade, histological subtype, type of chemotherapy, clinical response eventually attained, and survival status was available for analysis. Data on the CA 125 level (pre- and post-first-line treatment) and the number of cycles of treatment received were available for patients 1–41 only. Patient characteristics are summarized in Table 3.

Antibody. A mouse monoclonal antibody to ATP7A was purchased from BD Transduction Laboratories (clone 34; BD Biosciences, La Jolla, CA). This antibody was generated against amino acids 2–122 of the murine protein.

Immunohistochemical Staining. Sections of 3–4 μm were cut from paraffin blocks, deparaffinized with two 10-min soaks in xylene, and hydrated through two 5-min soaks in graded alcohol (100% ethanol followed by 95% ethanol), followed by a 10-min soak in distilled water. Antigen retrieval was performed by boiling the slides at 100°C in citrate buffer for two 5-min periods. The slides were then allowed to cool for 20 min and were rinsed in PBS. Immunohistochemical staining was performed according to the guidelines of the Catalyzed Signal Amplification System (DAKO, Carpinteria, CA; cat. no. K1500). Endogenous peroxidase activity was blocked with a 30-min incubation in 3% hydrogen peroxide followed by a rinse in Tris-buffered saline. Nonspecific protein binding was blocked with a 20-min incubation with serum-free protein in PBS and 0.015 M sodium azide. The slides were incubated with a 1:1500 dilution of anti-ATP7A antibody (clone 34; BD Biosciences). As a negative control, parallel sections were incubated with non-immune mouse IgG1 sera (DAKO); as a technical positive control, slides were incubated with antivimentin antibody (DAKO, cat. no. V1613).

Further controls included staining of cells from patients with Menkes disease who lack expression of ATP7A, as well as the same cells engineered to express ATP7A. Staining was completed as described by the manufacturer, using Tris-buffered saline containing 0.1% Tween 20 for all rinses. After a rinse in distilled water, the slides were counterstained using Mayer's hematoxylin and were mounted and covered.

Immunohistochemical Analysis. Each human ovarian carcinoma sample from the DDP- or CBDCA-treated patients was scored blindly for the percentage of malignant cells that stained positively for ATP7A by an independent pathologist (N. M. V.) who was unaware of the treatment status of the patient from whom the tissue sample was obtained. Detectable staining was estimated in 10% increments, and individual patients were scored as exhibiting an increase of >10%, a decrease of >10%, or no change. The staining in normal tissues and tumor samples on the arrays was scored as either detectable or nondetectable.

Statistical Analysis. On the basis of available clinical and staining data, the paired patient samples were examined for association between percentage staining of ATP7A before and after treatment *versus* age, stage, grade, histological subtype, number of cycles, response to treatment, and type of treatment (two-tailed Fisher's exact test) and overall survival (by log-rank statistic).

Results

The specificity of the commercially available monoclonal antibody used in this study was assessed by immunohistochemical analysis of human cell lines known to express different levels of ATP7A and human liver samples known to express ATP7B. The Me32a fibroblast cell line, established from a Menkes disease patient, does not express any ATP7A whereas the MeMNK subline has been molecularly engineered to express human ATP7A from a transfected vector (22). These cell lines were fixed, embedded in paraffin, and stained in parallel

with the normal and malignant tissue samples obtained from patients. As shown in Fig. 1*A* (*left panel*), no ATP7A staining was detectable in the ATP7A-deficient Me32a cells, whereas perinuclear staining was detectable in the ATP7A-expressing MeMNK cells (*right panel*). It has previously been documented that the MeMNK cells accumulate much less Cu than do the Me32a cells (22). Thus, it appears that a relatively modest degree of staining is likely to be physiologically significant. Normal liver is known to express high levels of ATP7B (23). As shown in Fig. 1*B*, the anti-ATP7A antibody failed to detect staining in the hepatocytes of human normal liver. This establishes that the anti-ATP7A monoclonal antibody is specific for ATP7A.

Glass slide arrays containing tissues from various normal organs were stained with a monoclonal anti-ATP7A antibody to determine expression of ATP7A protein. The results are summarized in Table 1. Expression of ATP7A was detected in normal endometrium, prostate, testis, and kidney. A fraction of the normal pancreatic samples expressed ATP7A protein. ATP7A expression was not detected in the thyroid, spleen, ovary, lung, breast, liver, small intestine, colon, brain, or adrenal tissues. Examples of both positive (prostate and kidney) and negative (ovary, liver, thyroid, and lung) staining for ATP7A are presented in Fig. 2, *A–F*. The results of this analysis of normal tissues demonstrated that, although ATP7A appears to be involved in Cu transport in all tissues except liver, the level of ATP7A protein expression in most normal tissues was not sufficient to be detected by the immunohistochemical assay used in this study.

Tissue microarrays containing various human malignancies were examined to determine whether expression of ATP7A was altered in cancer tissues compared with the normal tissue from which they arose. The results are summarized in Table 2.

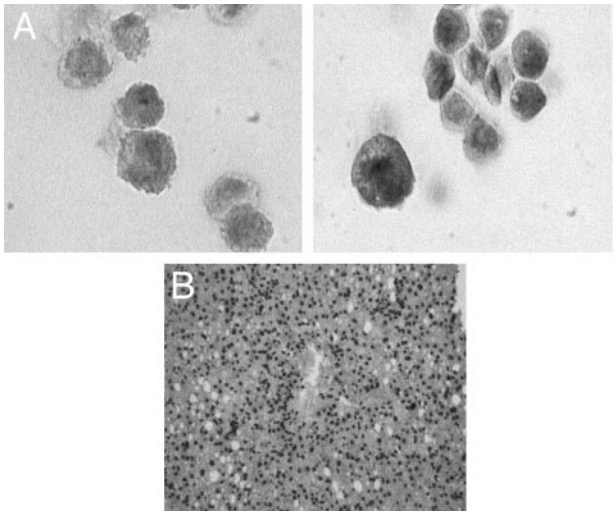

Fig. 1 Immunohistochemical validation of antibody specificity for ATP7A. *A*, anti-ATP7A did not detect staining in fixed and paraffin-embedded ATP7A-deficient Me32a cells (*left panel*), whereas perinuclear staining was observed in MeMNK cells engineered to express ATP7A from a transfected vector (*right panel*). *B*, anti-ATP7A did not stain normal liver, a tissue known to express high levels of ATP7B.

Table 1 ATP7A expression in normal human tissues

Tissue type	No. of positive[a]/no. of examined
Thyroid	0/4
Spleen	0/5
Endometrium	2/2
Prostate	3/3
Testis	5/5
Ovary	0/5
Pancreas	2/5
Lung	0/5
Breast	0/5
Liver	0/5
Kidney	5/5
Small intestine	0/2
Colon	0/2
Brain	0/5
Adrenal	0/4

[a] Tissues were considered positive for ATP7A expression if any staining was detectable.

Expression of ATP7A was detected in a considerable fraction of cancers of the prostate, breast, stomach, colon, lung, ovary, thyroid, and pancreas. In addition, expression was detected in melanomas, mesotheliomas, and non-Hodgkin's and Hodgkin's lymphomas, in both of the rhabdomyosarcomas examined, and in individual samples of malignant fibrous histiocytoma, Ewing's sarcoma, and epithelioid sarcoma. No staining was observed among the five hepatocellular carcinomas in the set of available samples, and no staining was detectable in the single endometrial carcinoma sample or the two benign leiomyomas. Thus, ATP7A expression was detected in a considerable fraction of the most common and in some rare human malignancies. Fig. 2, *G–L* presents examples of tumors that stained positively (ovarian, prostatic, thyroid, and lung carcinoma) and negatively (hepatocellular carcinoma) for ATP7A. Of note is the fact that there are several examples in which the normal tissue did not express ATP7A, whereas the major malignancy arising from that tissue did. This was true for ovary (Fig. 2, *A versus G*), thyroid (Fig. 2, *E versus K*), lung (Fig. 2, *F versus L*), breast, and colon. In addition, normal spleen did not express ATP7A, whereas various non-Hodgkin's and Hodgkin's lymphomas did. Interestingly, neither normal liver nor hepatocellular carcinomas expressed ATP7A (Fig. 2, *C versus I*), supporting the results of previous studies that demonstrated that ATP7A is not expressed in the liver when Cu export is handled by the highly homologous protein ATP7B (reviewed in Ref. 24). The fact that ATP7A is expressed at higher levels in several tumor tissues compared with their cognate tissue of origin indicates that the regulation of this protein is altered at some point during tumorigenesis, and that a high ATP7A level perhaps offers a growth advantage under *in vivo* conditions.

A previous study from this laboratory (19) demonstrated that, whereas expression of ATP7A was detectable in the 2008 ovarian carcinoma cell line, its level was markedly higher in the 2008/C13*5.25 subline selected for DDP resistance. This suggested either that repeated exposure to DDP altered the *ATP7A* gene regulatory controls or that DDP treatment enriched the population for cells that already had high levels of this protein. To determine whether *in vivo* exposure to the Pt-containing

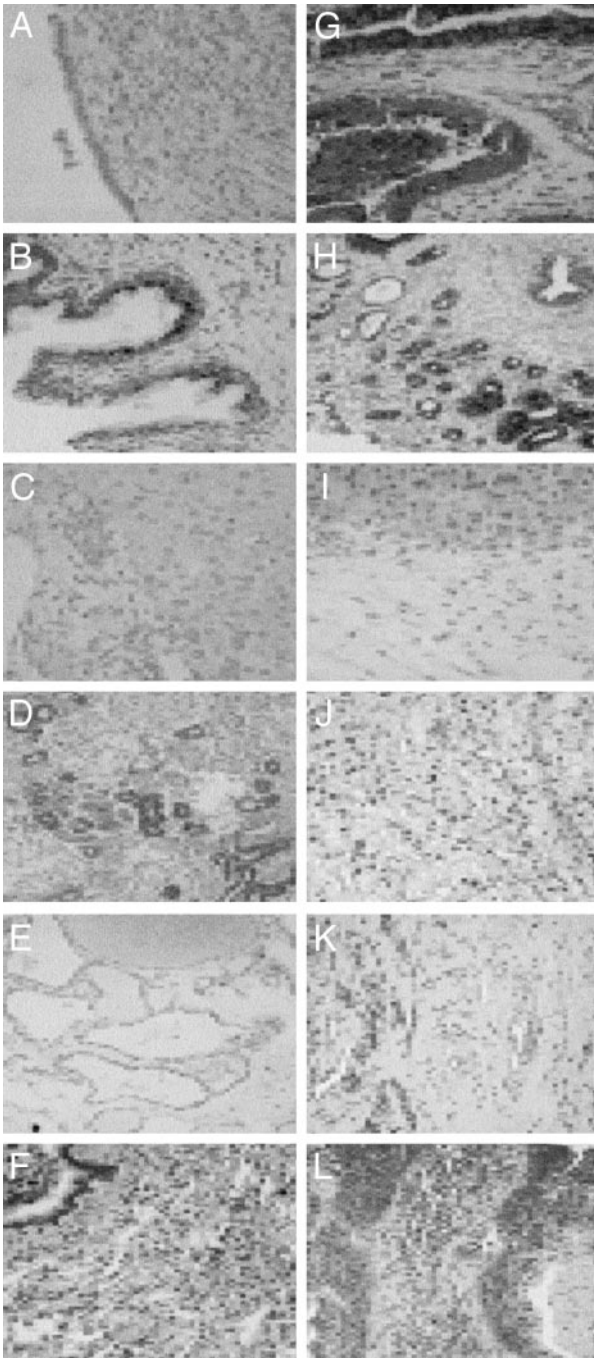

Fig. 2 Immunohistochemical staining of normal and malignant human tissues for ATP7A. Normal tissues (*A–F*); corresponding malignant tissues (*G–L*). *A,* normal ovary; *B,* prostate; *C,* liver; *D,* kidney; *E,* thyroid; *F,* lung. *G,* epithelial ovarian carcinoma; *H,* prostatic carcinoma; *I,* hepatocellular carcinoma; *J,* renal cell carcinoma; *K,* thyroid carcinoma; *L,* non-small cell lung cancer.

drugs also resulted in greater expression of ATP7A in the surviving tumor cell population, sections were stained for ATP7A from pairs of tumor samples that had been obtained from individual ovarian carcinoma patients before the start of initial treatment and again after at least two cycles of DDP or CBDCA-based therapy. All of the clinical data on these patients were collected prospectively because they had all been treated as part of either Memorial Sloan-Kettering Cancer Center or Southwest Oncology Group trials (21). Associations were sought between ATP7A expression and patient and tumor characteristics including age, stage, grade, histological subtype, number of cycles of treatment, response to treatment, type of treatment, and overall survival. The clinical parameters of the 54 patients included in this study are summarized in Table 3, and information on the chemotherapy regimens used and the number of cycles received between biopsies is presented in Table 4. The median time between the time the pretreatment and posttreatment tumor samples were obtained was 6 months (range, 2–63 months), and the median number of cycles of interval treatment was 4 (range, 2–10 cycles).

Expression of ATP7A was detected in 28 of the 54 patients before treatment, and 25 of the 54 patients after treatment. Among the 25 patients who expressed ATP7A after treatment, there was greater staining after treatment than before treatment in 18 cases. In 10 of these 18 cases, no staining at all was detectable before treatment, but there was clear staining after treatment. In the other 8 of these 18 cases, some staining was detectable before treatment, but ATP7A expression was higher after treatment. Fig. 3 depicts increased ATP7A staining in a sample of ovarian carcinoma obtained after platinum-based therapy compared with staining in a sample of the same tumor obtained before the start of chemotherapy. As shown in Fig. 4, Kaplan-Meier analysis indicated that the 18 patients with new or increased ATP7A staining after treatment had reduced actuarial survival compared with patients who did not display increased ATP7A expression after treatment ($P < 0.0057$, log-rank test). Thus, in those patients in whom treatment with a DDP or CBDCA-based program resulted in a greater number of

Table 2 ATP7A expression in representative human tumor samples

Cancer type	No. of positive[a]/total examined
Prostate	7/7
Breast	10/10
Stomach	5/5
Colon	5/8
Hepatocellular carcinoma	0/5
Lung	8/8
Ovary	6/7
Thyroid	3/3
Pancreas	2/2
Renal cell carcinoma	1/3
Testis	1/3
Endometrial carcinoma	0/1
Melanoma	4/4
Mesothelioma	2/2
Non-Hodgkin's lymphoma	3/8
Hodgkin's lymphoma	4/9
Rhabdomyosarcoma	2/2
Malignant Fibrous Histiocytoma	1/1
Ewing's sarcoma	1/1
Epithelioid sarcoma	1/1
Leiomyoma	0/2

[a] Tissues were considered positive for ATP7A expression if and staining was detectable in malignant cells.

Table 3 Patient characteristics

Characteristic	No. of patients	%
Histological subtype		
Clear cell	5	9
Endometrioid	8	15
Mucinous	2	4
Serous	21	39
Not otherwise specified	18	33
FIGO[a] grade		
2	12	22
3	34	63
NA[b]	8	15
FIGO stage		
1	1	2
3	41	76
4	9	16
NA	4	7
Response		
Progression of disease	11	20
Stable/Persistent	11	20
Partial response	23	43
Complete response	8	15
NA	1	2
ATP7A staining pretreatment		
Positive	28	52
Negative	26	48
ATP7A staining posttreatment		
Positive	25	46
Negative	29	54

[a] FIGO, International Federation of Gynecologists and Obstetricians.
[b] NA, relevant clinical information not available.

ATP7A-expressing cells in the surviving tumor, there was reduced survival. There was no significant association between ATP7A staining either before or after treatment with either response or survival or any of the other clinical parameters examined including age, stage, grade, or histological subtype.

Discussion

Evidence is rapidly accumulating to suggest that DDP enters cells, is distributed to subcellular compartments, and is effluxed from mammalian cells via transporters and chaperones that mediate Cu homeostasis. Selection for resistance to DDP frequently results in cross-resistance to a variety of metalloids including Cu (19, 25), and in at least one case, selection of cells for resistance to Cu resulted in cross-resistance to DDP (20). Ishida *et al.* (26) reported that DDP enters cells predominantly via the Cu influx transporter CTR1, and this had been confirmed in this laboratory (27). Loss of CTR1 expression in *Saccharomyces cerevisiae* impairs the accumulation not only of DDP but also of CBDCA and oxaliplatin (27). Studies of cells selected for either DDP or Cu resistance *in vitro* indicate that parallel changes occur in the cellular pharmacology of both DDP and Cu such that net cellular accumulation of both is reduced (19, 20). Interestingly, although CTR1 seems to be responsible for most DDP and CBDCA influx in yeast, no mutations were detected in the CTR1 exons in three DDP-resistant ovarian carcinoma cells examined (27). On the other hand, among four DDP-resistant cell lines studied, all had increased expression of either ATP7A or ATP7B (19). Thus, enhanced export may be the most important contributor to the reduced DDP accumulation so commonly observed in DDP-resistant cells.

In preparation for studies on changes in ATP7A expression during treatment with DDP or CBDCA, we sought to identify the normal human tissues that express ATP7A at the protein level and the major categories of human malignancy in which expression can be found by immunohistochemical staining. Although most normal tissues examined did not express ATP7A at detectable levels, several organs did express this protein including endometrium, prostate, testis, and kidney. It is possible that cells of these tissues have a greater physiological need to export Cu because several of these, and particularly the proximal renal tubules, are known to be at high risk for excessive Cu accumulation in patients with Menkes disease (reviewed in Ref. 28). A recent *in situ* hybridization study of ATP7A mRNA levels has confirmed expression in the mouse kidney (29). To our knowledge, there are no prior immunohistochemical studies of the expression of ATP7A in normal human tissues. However, mRNA expression has been examined; expression was detected in heart, brain, placenta, lung, muscle, kidney, and pancreas but not in liver (30, 31).

As anticipated, based on its expression in the cognate normal tissue, all seven cases of prostate cancer expressed ATP7A as did one of the three renal cell and testicular carcinomas. Of perhaps greater interest is the observation that some of the most common human tumors expressed easily detectable levels of ATP7A even though their tissues of origin did not. This was true for carcinomas of the thyroid, ovary, lung, breast, and colon. In a parallel manner, many cases of both non-Hodgkin's lymphomas and Hodgkin's disease expressed ATP7A, whereas normal spleen did not. More detailed mapping of the tissues that are concordant *versus* discordant with respect to ATP7A expression in their normal and malignant state is needed, but these results place *ATP7A* among the genes whose expression is commonly up-regulated at some point during tumorigenesis.

The key clinical question is whether staining for ATP7A could identify tumors that are resistant to the Pt-containing drugs, and whether increased expression of this protein is one of the factors that contributes to the acquired DDP and CBDCA-resistant phenotype that so commonly emerges in ovarian carcinoma patients during therapy. No significant correlation between ATP7A staining prior to the start of therapy and any of the measures of patient outcome was observed. However, the total number of ovarian cancer patient samples available for this study was insufficient to detect such an association unless it was extremely strong. Despite the limited size, however, a significant association was observed between survival and the change that occurred in ATP7A expression between the first and the second biopsies. The poorer survival in patients with greater ATP7A expression after treatment is consistent with the hypothesis that enhanced ATP7A expression does in fact endow the tumor cell with a certain level of DDP resistance. Previous studies have established that even the modest degree of resistance generated by loss of DNA mismatch repair is sufficient to result in enrichment of the deficient cells during exposure to DDP *in vitro* or during the treatment of tumors in *in vivo* experimental systems (32) and in humans (21). It is important to note that because several clinical variables were tested for association with ATP7A expression, there is a chance that the

Table 4 Types of chemotherapeutic regimens and distribution of number of treatment cycles administered between biopsies

Treatment type	Total no. of patients receiving specified chemotherapy regimen	No. of cycles between biopsies	No. of patients receiving specified number of cycles
Cisplatin, cyclophosphamide	29	2	15
		3	1
		4	1
		5	11
		6	1
Cisplatin, paclitaxel	4	2	2
		5	2
Carboplatin, paclitaxel	1	9	1
Cyclophosphamide, carboplatin	5	4	2
		5	2
		6	1
Cyclophosphamide, cisplatin, doxorubicin	2	6	1
		10	1
Platinum-based[a]	13	>2[b]	13

[a] Not otherwise specified.
[b] Information on total number of cycles not available.

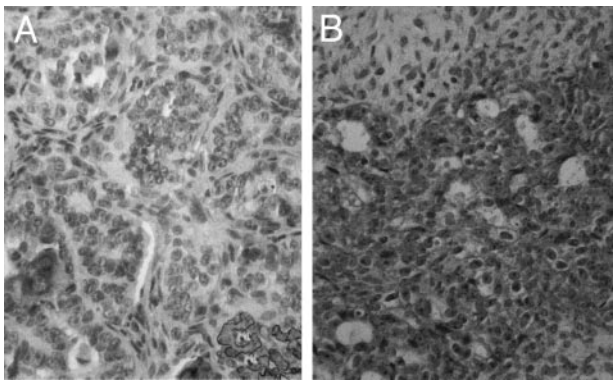

Fig. 3 Immunohistochemical staining for ATP7A in samples of an ovarian carcinoma from the same patient obtained before (*A*) or after (*B*) platinum-based chemotherapy. Staining is observed in psammoma bodies but not the epithelial cells of the pretreatment carcinoma, whereas strong perinuclear staining is present in the posttreatment samples.

correlation with survival may be an artifact. Both the existence of this association and its biological relevance require confirmation. However, a recent study by Nakayama *et al.* (18) demonstrated an association between high levels of mRNA for ATP7B, the alternate Cu exporter, in the tumor and poor survival in ovarian cancer patients, and this provides additional evidence that the Cu export pathway plays an important role in determining the outcome of treatment in ovarian cancer patients. It is also important to note that it remains possible that the enrichment of the tumor for cells expressing ATP7A was caused by cyclophosphamide, doxorubicin, or paclitaxel rather than the Pt-containing drug that the patient received, and that this study examined only the expression of ATP7A, whereas current evidence would suggest that ATP7B and hCTR1 may also be important to the DDP-resistant phenotype.

The increase in the number of ATP7A-expressing cells in some cases of ovarian cancer may be related to the enrichment by DDP or CBDCA for cells somatically mutated to express more of this protein. However, DDP is a good mutagen, and we have demonstrated that even a single exposure results in the generation of clones in the surviving population that are highly resistant to DDP itself, as well as to all of the other drugs commonly used in combination with DDP in the clinic (33, 34). Thus, it is possible that the pathway that regulates the expression of the *ATP7A* gene is perturbed by DDP-induced mutagenesis, thus creating a situation in which the treatment is both generating the resistant cell and then selecting for its enrichment. Finally, it remains possible that increased expression of ATP7A offers some important growth advantage under *in vivo* conditions and that the number of ATP7A-expressing cells increases in some tumors independently of any effect of the therapy.

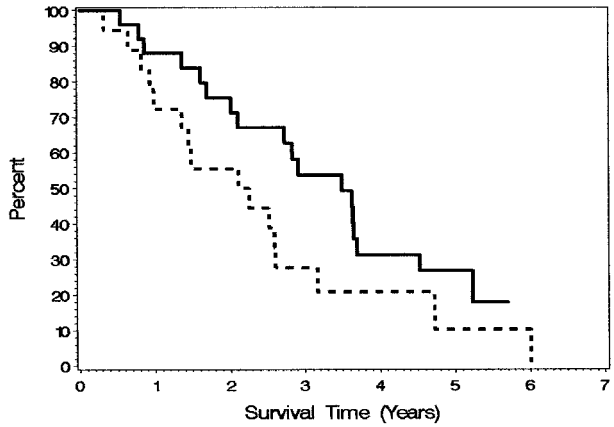

Fig. 4 Kaplan-Meier plot of survival of ovarian carcinoma patients for whom new or increased staining of ATP7A was observed after Pt-based chemotherapy (- - -) *versus* those in whom it was not (—). $P < 0.0057$, log-rank test.

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