Prognostic Value of the Cu-Transporting ATPase in Ovarian Carcinoma Patients Receiving Cisplatin-Based Chemotherapy

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ABSTRACT

Purpose: A major obstacle in the treatment of ovarian carcinoma is the intrinsic/acquired resistance to cisplatin-based chemotherapy. Cu-transporting ATPase (ATP7B) has been reported to be associated with cisplatin resistance in vitro. However, the clinical significance of this transporter has not previously been addressed. Our goal was to investigate ATP7B expression in ovarian carcinoma and whether its expression correlates with prognosis and reduced responsiveness to cisplatin treatment.

Experimental Design: We retrospectively examined the expression of ATP7B and p53 in primary ovarian carcinoma and its association with chemotherapeutic effect. Tissues were surgically removed from 104 ovarian carcinomas patients who received cisplatin-based chemotherapy. We performed immunohistochemical analysis of ATP7B and p53 using a monoclonal antibody against ATP7B and DO7 antibody against p53 protein in 104 ovarian carcinomas and adjacent nonneoplastic tissues. The significance of ATP7B and p53 in the prognosis of patients with ovarian carcinomas was also examined in the survival analysis of mortality follow-up data covering the period between 1988 and 2001. Furthermore, mutation analysis at the six Cu-binding domain and ATP-binding domain, which may be important for cisplatin transport, were performed using single-strand conformational polymorphism after reverse transcriptase-PCR.

Results: A variable degree of cytoplasmic staining of ATP7B in tumor cells was observed in 34.6% (36 of 104 cases) of the analyzed carcinomas. ATP7B expression was not observed in adjacent nonneoplastic tissues. ATP7B positivity in poorly/moderately differentiated carcinoma was significantly higher than that in low malignant potential tumor/well-differentiated carcinoma (P = 0.0276). Patients with ATP7B-positive tumors had a significantly inferior response to chemotherapy compared with the patients with ATP7B-negative tumors (P = 0.025). The multivariate Cox regression analysis revealed that ATP7B expression (hazard ratio, 1.8; 95% confidence interval, 1.0–3.2, P = 0.048), as well as International Federation of Gynecologists and Obstetricians stage (hazard ratio, 2.0; 95% confidence interval, 1.1–3.6, P = 0.018), was prognostic for poor disease outcome after adjustment for p53 expression, grade, and residual tumor. p53 expression was detected in 31.5% (26/104 cases). No mutation was observed on the six Cu-binding domain or ATP-binding domain in human ovarian carcinomas expressing ATP7B gene.

Conclusions: This study demonstrates that overexpression of ATP7B in ovarian carcinoma is correlated with unfavorable clinical outcome in patients treated with cisplatin-based chemotherapy. Therefore, ATP7B expression may be considered as a predictive marker of chemoresistance for cisplatin-based chemotherapy in patients with ovarian carcinoma. We further predict that drugs targeting ATP7B might be useful in combination with cisplatin-based regimen for the improvement of patients with ovarian carcinoma.

INTRODUCTION

Ovarian carcinoma is the most lethal gynecological malignancy (1). The incidence and mortality of ovarian carcinoma has not declined in the past decade. This is due to diagnosis during the later stages of this disease and the lack of effective therapy (2). The treatment with cisplatin-based chemotherapy after reductive surgery has improved prognosis of patients with ovarian carcinoma; however, the complete pathological response and 5-year survival rates have not improved. One of the most important clinical problems in the treatment of ovarian carcinoma is the intrinsic/acquired resistance to cisplatin-based chemotherapy. Knowledge of the active mechanism of drug resistance may lead to new treatment strategies and allows the selection of those patients for specific treatment modalities.

In vitro resistance to cisplatin includes decreased drug accumulation, enhanced detoxification, and increased DNA...
repair efficiency. Multidrug resistance (MDR) has been noted as an important mechanism of drug resistance. Several genes, including *MDR1, MRP1* and *LRP*, have been identified (3, 4). *MDR1* and *MRP1* function as a drug efflux pump and are classified as ABC transporter gene family (5, 6) and are expressed in both human solid tumors and hematological malignancies (7, 8). The 110-kDa LRP, the major vault protein, is frequently overexpressed in MDR cells and has an important role(s) in transport of drugs from nuclei to cytoplasm and confers to MDR to *MDR in vitro* (4). Recently, *BCRP (MXR/ABCP)* gene, another member of the ABC transporter family, has been described in breast, colon, gastric, and fibrosarcoma cell lines (3, 4, 9). However, there is no evidence that these molecules are involved in cisplatin resistance in vitro and in clinic.

Previously, it was shown that ATP7B is associated with cisplatin resistance in vitro (10). The ATP7B gene was induced by exposure to cisplatin in human prostate cells and the ATP7B-transfected cells showed marked increased in cisplatin efflux (10). Although an active efflux pump for cisplatin has yet to be identified, it is likely that ATP7B may function to efflux cisplatin from some carcinoma cells.

ATP7B is a member of a class of heavy metal-transporting P-type ATPases that pump copper, cadmium, zinc, silver, or lead (11–13). Copper is an essential trace element and is integrated into many enzymatic reactions. Excess level of copper is transported to the extracellular environment by an energy-dependent system (8), and alteration in copper homeostasis can cause severe problems. For example, Wilson disease, an autosomal recessive disease of copper transport, is characterized by chronic liver and/or neurological disorder, sometimes accompanied by kidney damage (14). Detailed understanding of ATP7B is therefore crucial in several diseases, including cancer. The fact that such transporters can also transport small molecule drug is intriguing and could potentially have a significant value in the clinic.

The ATP7B gene is highly conserved across species and contains several motifs such as TGEA/S motif (phosphatase domain), DKTGT/S motif (phosphorylation domain), TGDN motif (ATP-binding domain), and the sequence MXGDXNDXP that connects the ATP binding domain to the transmembrane segment. The six repeated motifs, GMTCXXC located at the NH₂ terminus binds six molecules of copper. Human ATP7B protein is able to rescue the yeast strains lacking the CCC2 gene, the yeast homologue of ATP7B. The experiments with the wild-type or mutated ATP7B cDNA demonstrated that the mutant containing only the sixth Cu-binding domain are sufficient to rescue Δccc2 (15). This suggests the importance of the sixth Cu-binding for ATP7B function. Therefore, the sixth Cu-binding domain may be important for the transport of cisplatin. However, no mutations at the sixth Cu-binding in human ovarian carcinoma have previously been documented.

Because of the importance of p53 function in apoptosis and cell proliferation (see below) and its role in predicting drug efficacy in various cancers, we extended our studies to include the analysis of p53 gene expression in the same samples.

It is now well established that p53 plays important roles in the regulation of the cell cycle and has been implicated in cell differentiation, apoptosis, DNA synthesis, and repair (16). Mutations in p53 gene has been observed in ≥50% of ovarian carcinoma cases (17, 18).

Overexpression of p53 corresponds to a cellular accumulation of a biologically inactive protein stabilized either by a decreased rate of degradation of mutated gene product or by complex formation with certain proteins such as viral oncoproteins or heat-shock protein 70 (19, 20). Recently, particularly in ovarian carcinomas, special attention was attributed to p53 because tumors bearing p53 aberrations were reported to be less sensitive to cisplatin and doxorubicin exposure (21, 22). p53 is one of the most important molecule in the cytotoxicity of DNA-damaging agents (23). p53 and mismatch repair can cooperate to control sensitivity to the cytotoxic effect of cisplatin and to limit its mutagenic potential in the colon cancer cells (24). Although some investigators failed to demonstrate a relation between the course of the disease and p53 expression (17, 25), the results obtained in a number of studies on the prognostic impact of overexpression of p53 protein in ovarian carcinoma tissue (26–28).

The purpose of this study was to provide clinical evidence that ATP7B expression is important for cisplatin resistance. This is the first study suggesting that ATP7B expression should be considered as a predictive marker of chemo resistance to cisplatin in patients with ovarian carcinoma. Thus, in conjunction with the previously identified markers for cisplatin resistance, inclusion of ATP7B expression is of beneficial in selecting the right patient for the right treatment.

**MATERIAL AND METHODS**

**Patients and Samples.** After obtaining informed consent in accordance with each institutional guideline, 104 tumor specimens were collected and embedded in OCT compound (Sakura Fine technical Co., Ltd., Tokyo, Japan). Diagnosis was based on conventional morphological examination of the sections stained with H&E staining, and tumors were classified according to the WHO classification. Staging of tumor was performed according to the International Federation of Gynecology and Obstetrics (FIGO) classification. The clinicopathological characteristics of the patients included in this study are summarized in Table 1. The patients had no other form of malignancies and performance status with grade 0 or 1. All patients were primarily treated with reductive surgery and postoperative chemotherapy, which basically consisted of a cisplatin-based regimen [cisplatin (75 mg/m²), doxorubicin (30 mg/m²), and cyclophosphamide (500 mg/m²)]. All of the cases performed 4–6 course cisplatin regimens. Response to induction chemotherapy was assessed by second-look surgery or clinical and/or radiographic evaluation according to WHO criteria after chemotherapy. Patients with incomplete response to induction chemotherapy and patients with recurrent tumors were treated with a variety of second-line chemotherapy regimens. Follow-up for all of the patients included in the survival analysis was updated in June 1, 2002 (median follow-up was 51 months; range, 26–165 months). At that time, 27 patients had died of ovarian carcinoma and 77 were alive.

**Tissue Staining and Evaluation of Stained Sections.** A 2.5-μm section of each submitted frozen block was first
stained with H&E to verify the histopathological diagnosis and the quality of fixation for immunohistochemical analysis. Immunostaining was performed on cryostat sections using the standard immunoperoxidase procedure ( vectastain Elite ABC kit; Vector, Burlingame, CA). After recovering from OCT compound (Sakura Fine Technical Co., Ltd.), the sections were fixed in 10% neutral buffered formalin, incubated in 0.03% H2O2 in absolute methanol for 30 min at room temperature, and blocked in 3% skim milk in PBS for 30 min at room temperature. The sections were then incubated with 100-fold diluted monoclonal antibody against the NH2-terminal region of ATP7B, which included the six Cu-binding domains (amino acid residues 21–623; Ref. 29) for 15 h at 4°C. After rinsing with PBS, the sections were incubated in biotinylated horse antimouse IgG at 1:200 in 1.5% normal horse serum for 30 min at room temperature. Sections were then rinsed in PBS and incubated for 30 min at room temperature in the avidin-biotin horseradish peroxidase macromolecular complex. After rinsing with PBS, the sections were incubated for 6 min in 0.05% diaminobenzidine in PBS with 0.03% H2O2. The slides were counterstained with hematoxylin, dehydrated, and mounted. The serial sections were routinely incubated with irrelevant mouse IgG as a negative control.

The slides were examined and scored independently by two observers (K. Nakayama and Y. Takebayashi) without knowledge of clinical information of the patients in the evaluation of ATP7B expression. If >10% of the tumor cells were stained, the samples were considered to be ATP7B-positive carcinomas. The 10% cutoff level was chosen for the following reasons: (a) 10% positive cells were considered the lowest level of expression that could be most consistently detected in cryostat sections; and (b) Chan et al. (30) demonstrated that a small percentage of cells positive for multidrug-resistance-related proteins (i.e., P-glycoprotein) could have clinical significance. The x test of reliability between the two observers was 0.750, indicating fair to good agreement for the evaluation of ATP7B and p53 expression in ovarian carcinoma (data not shown). When the two observers’ report differed from each other, they would evaluate the images of stained sections on a TV-captured image station. Finally, the evaluation of p53 expression was performed essentially as described previously (31).

Reverse Transcriptase-PCR. We examined mutation after analyzed ATP7B mRNA expression level using reverse transcriptase-PCR. Of the total of 104 patients, we could only obtain RNA from 82 patients because of tissue availability.

Total RNA was prepared by Trizol (Life Technologies, Inc., Gaithersburg, MD) from the frozen block after observing whether the tissues contain the carcinoma cells. The tissues containing at least 80% of carcinoma cells were used in this study. cDNA was synthesized with 3 μg of total RNA and random hexadeoxynucleotide primer (Life Technologies, Inc.) in 20 μl of a solution containing reverse transcriptase. After synthesis the cDNA was diluted 1:4 with water and stored at −20°C until use. PCR was performed with cDNA derived from 30 ng of RNA. PCR reactions were carried out in a total volume of 25 μl containing cDNA, dGTP, dATP, dTTP, and dCTP at a concentration of 200 μM, 4 μM of each primer, and 0.25 unit of ExTaq polymerase (Takara Shuzo, Otsu, Shiga, Japan). The PCR cycling parameters were as follows: 10 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C, and a final cycle at 72°C for 10 min. The PCR products were electrophoresed on 1.5%-agarose gel. The PCR primer sequences of ATP7B for ATP-/sixth copper-binding domains were shown in Fig. 4B.

Single-Strand Conformational Polymorphism and Mutation Analysis of PCR Products. PCR single-strand conformational polymorphism assays were performed as described. PCR amplification was performed as described above. PCR products were loaded onto a 12% polyacrylamide gel containing 8% glycerol. Electrophoresis was performed at 15°C for 8 h at 100 V. After electrophoresis, gels were stained by silver staining kit (Bio-Rad, Hercules, CA). Sequence analysis of a fraction of each PCR product was performed by 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Statistical Analysis. Data analysis was performed using Statview version 5 statistical software package. Continuous variables were analyzed using Student t test and χ2 test as appropriate. Overall survivals were measured in months from the month of surgery to a reported death. Survival curves were determined using Kaplan-Meier method, and differences in survival between subgroups were compared with log-rank test. Multivariate prognostic analysis was performed using Cox proportional hazards model. P values <0.05 were hypothesized to be significant. All reported P values were two-sided.

RESULTS

Expression of ATP7B Protein in Human Ovarian Carcinoma. A total of 104 primary ovarian carcinoma tissues was used for the detection of ATP7B protein by immunohistochemistry using an antibody that specifically reacts with ATP7B as evidenced by immunoblotting analysis (32). A granular staining of ATP7B was observed in the cytoplasm of ovarian carcinoma cells (Fig. 1A). ATP7B-negative tumor stained with anti-ATP7B monoclonal antibody (Fig. 1B). Although, a weak to moderate cytoplasmic staining could be observed in some stromal cells (Fig. 1C), in the normal ovary, the ATP7B expression was not detectable (Fig. 1D). The immunostaining results are summarized in Fig. 1E. A variable degree of cytoplasmic staining of tumor cells was observed in 35.6% (37 of 104 cases) of the analyzed tumors.

Expression of p53 Protein in Human Ovarian Carcinoma. The central role of p53 in cell growth and differentiation as well as apoptosis and efficacy of drugs in vivo prompted us to investigate its expression in the same ovarian carcinoma tissues as above. As expected, the p53 staining was observed only in the nuclei of carcinoma cells (Fig. 2A). Of the analyzed tumors, we observed 25.0% (26 of 104) of cases as p53 positive (Fig. 2B).

Relationship between Clinicopathologic Findings, Chemotherapeutic Response, and ATP7B and p53 Expression. Table 1 summarizes the relationship between clinicopathologic features and ATP7B and p53 expression in ovarian carcinomas. No significant association was found between ATP7B expression and age, FIGO stage, and histological subtype (Table 1). ATP7B positivity was correlated with tumor grade. ATP7B
positivity in poorly/moderately differentiated carcinoma was significantly higher than that in low malignant potential tumor/well-differentiated carcinoma (Table 1, P = 0.0276). Interestingly, the p53 expression was independent of all of the examined clinicopathological variables (Table 1).

Patients with ATP7B-positive tumors had a significantly inferior response to chemotherapy (59% complete and partial response and 41% no response) as compared with the patients with ATP7B-negative tumors (80% complete and partial response and 20% no response; Table 2; P = 0.025). No significant association was found between p53 expression and response to cisplatin-based chemotherapy (Table 2; P = 0.933).

**Prognostic Relevance of ATP7B Expression.** Kaplan-Meier estimate of overall survival excluding low malignant potential tumor tumors is plotted in Fig. 3. The patients with ATP7B-positive carcinomas had a significantly poorer overall survival than those with ATP7B-negative tumors (P = 0.0161; Fig. 3A). The median overall survival of ATP7B-positive or negative carcinomas are 33 or 65 months. p53

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**Fig. 1** Immunohistochemical staining of ovarian carcinoma specimens using antibodies to ATP7B from cryostat sections. A, ATP7B-positive tumor stained with anti-ATP7B monoclonal antibody. Note the distinct cytoplasmic staining in ovarian carcinoma cells (immunoperoxidase stain; original magnification, ×200). B, ATP7B-negative tumor stained with anti-ATP7B monoclonal antibody (magnification, ×200). C, ATP7B-positive normal ovarian stromal tissue stained with anti-ATP7B monoclonal antibody (immunoperoxidase stain; original magnification, ×400). D, normal ovarian tissue stained with anti-ATP7B monoclonal antibody (immunoperoxidase stain; original magnification, ×400). E, percentage of cells expressing ATP7B protein in ovarian carcinoma.
expression was also prognostic indicator by Kaplan-Meier curve analysis (\( P = 0.0073 \); Fig. 3B). To determine whether ATP7B and/or p53 positivity were a prognostic factor(s) independent of FIGO stage, an established prognostic factor, we conducted an overall survival analysis with the use of the Cox proportional hazards model. Although it was unlikely that the other clinicopathological variables shown in Table 1 affected patient survival, we included them in our analysis to confirm the absence of their associations with survival. Univariate analysis revealed that FIGO stage, grade, residual tumor, and ATP7B and p53 positivity significantly affected on the survival of the patients with ovarian carcinomas (Table 3). Therefore, we performed multivariate analysis with FIGO stage, grade, residual tumor, p53 positivity, and ATP7B positivity, and the results are shown in Table 3. FIGO stage and ATP7B positivity were independent prognostic factors (\( P = 0.0184 \) and 0.0477, respectively) in patients with ovarian carcinoma treated with cisplatin-based chemotherapy.

**DISCUSSION**

The observation that ATP7B is associated with cisplatin resistance in vitro as reported by Komatsu et al. (10) has now been confirmed in tissues from ovarian carcinoma patients treated with cisplatin-based chemotherapy. Herein, we demonstrated that ATP7B protein is expressed in human ovarian

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<tr>
<th>Table 1 Relationship of ATP7B or p53 expressions and clinicopathological variables in patients with ovarian carcinoma (n = 104)</th>
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<td>Variables</td>
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<td>Age (yr)</td>
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<td>Stage (International Federation of Gynecology and Obstetrics)</td>
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<td>I, II</td>
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<td>Histology</td>
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<td>Moderate</td>
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<td>Poor</td>
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\( ^a \) \( P \) are obtained from the \( \chi^2 \) test (two-sided).

\( ^b \) NS, not significant; LMP, low malignant potential tumor.

\( ^c \) LMP, well versus moderate, poor.
carcinoma as assessed by immunohistochemistry (Fig. 1). A significant ATP7B protein expression 35.6% (37 of 104 cases) was observed in ovarian carcinoma (Fig. 1E). ATP7B immunoreactivity was detected as characteristic granular cytoplasmic staining. In agreement with this observation, ATP7B has been reported to be abundant in the Golgi apparatus (9). These findings are the first evidence that ATP7B, which is involved in Wilson disease, is expressed in human ovarian carcinoma.

The present study demonstrates that the ATP7B expression in poorly differentiated ovarian carcinoma is more frequent than well to moderately differentiated carcinoma (Table 1). Very low expression of ATP7B protein could be detected in adjacent nonneoplastic tissues. The fact that the expression level of ATP7B is very low in normal ovarian tissue raises the possibility that ATP7B might be involved in transformation of a normal cell to a malignant tumor cell and/or differentiation of carcinoma cell.

Patients with ATP7B-positive tumors had a significantly inferior response to chemotherapy as compared with patients with ATP7B-negative tumors (Table 2). The fact that ATP7B expression assessed by immunohistochemistry was an independent prognostic factor in ovarian carcinoma attests to the diagnostic value as a marker for prediction of response to cisplatin-based chemotherapy. However, this study had one limitation that the patients were treated with combination regimens, including cisplatin, doxorubicin, and cyclophosphamide. Therefore, it is not entirely sure that the effect of ATP7B on cisplatin plays the primary role in this study. To explore this limitation, additional studies with analyzing ATP7B function for other anticancer agents such as doxorubicin and cyclophosphamide should be required.

Because of its central role in a variety of cellular mechanisms, analysis of p53 status in our sample was of paramount importance. In fact, it was shown previously that p53 is a very important molecule in the activity of DNA-damaging agents, including cisplatin (23). However, the results obtained in a number of studies on the prognostic impact of overexpression of p53 protein in ovarian carcinoma tissue were controversial. Whereas some investigators reported impaired clinical outcome in patients with p53 overexpressing tumors (22, 27, 28), others failed to demonstrate a relation between the course of the disease and p53 expression (17). Actually, p53 expression was a prognostic indicator in univariate analysis but was not in multivariate analysis. This suggests that the detection of p53 by immunohistochemistry may be difficult in clinical samples. Because unlike p53 missense mutations, which result in a stable mutant protein detected by immunohistochemistry, other mutations such as deletions, additions, or splice site mutations do not result in a stable protein (33, 34).

The sixth Cu-binding domain is very important for ATP7B function (15), and by extension, it might also be important for cisplatin transport. We were interested to see whether there are
any significant polymorphisms among patients in the regions highly critical for ATP7B function and possibly cisplatin efflux. When we looked for mutations in either ATP-binding motif or the sixth copper-binding domain, we did not observe any (Fig. 4, D and E).

In conclusion, we demonstrated that ATP7B, a transporter associated with chemoresistance, is expressed in a subset of ovarian carcinomas. Of especial interest is the finding that the expression is more frequent in undifferentiated carcinomas that are usually more refractory to therapy. Furthermore, ATP7B expression is an independent prognostic factor in human ovarian carcinoma treated with cisplatin-based chemotherapy. Therefore, ATP7B expression may be considered as a predictive marker of chemoresistance for

Table 3  Univariate and multivariate Cox proportional hazard analysis for overall survival of 98 patients with ovarian carcinoma treated with cisplatin-based chemotherapy

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<tr>
<th>Factors</th>
<th>Univariate</th>
<th>Multivariate</th>
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<tr>
<td>Age (yr)</td>
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<td>≤54 (median; n = 49)</td>
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<td>&gt;54 (n = 49)</td>
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<td>others (n = 47)</td>
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<td>Histologic grade</td>
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<tr>
<td>Poorly differentiated (n = 31)</td>
<td>0.0455</td>
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<td>versus others (n = 67)</td>
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<td>FIGO stage</td>
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<td>I, II (n = 37)</td>
<td>0.003</td>
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<td>versus III, IV (n = 61)</td>
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<td>Status of residual tumors</td>
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<td>Negative (n = 72)</td>
<td>0.0359</td>
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<td>versus positive (n = 26)</td>
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<td>p53 expressions</td>
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<td>Negative (n = 72)</td>
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<td>versus positive (n = 26)</td>
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<td>ATP7B expressions</td>
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<tr>
<td>Negative (n = 61)</td>
<td>0.0161</td>
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<td>versus positive (n = 37)</td>
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a* Hazard ratios, 95% CIs, and two-sided *P were obtained from the Cox proportional hazards models.

*CI, confidence interval; NA, not analyzed; FIGO, International Federation of Gynecology and Obstetrics.

Fig. 4  A, sequence of ATP7B gene at ATP-/sixth Cu-binding domain. B, primers to detect PCR products including sixth Cu-binding and ATP-binding domain. C, expression of ATP7B gene determined by reverse transcriptase-PCR, as described in “Materials and Methods.” Cu; PCR product including sixth Cu-binding domain. ATP: PCR product including ATP-binding domain. Mutation analysis of ATP7B gene. D, single-strand conformational polymorphism analysis (left panel); sixth Cu-binding domain (right panel); ATP binding domain. E, sequence analysis (left panel); sixth Cu-binding domain, (right panel); ATP binding domain.
cisplatin-based regimen in patients with ovarian carcinoma. We further predict that drugs targeting ATP7B might be useful in combination with cisplatin-based chemotherapy for the improvement of patients with ovarian carcinoma.

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