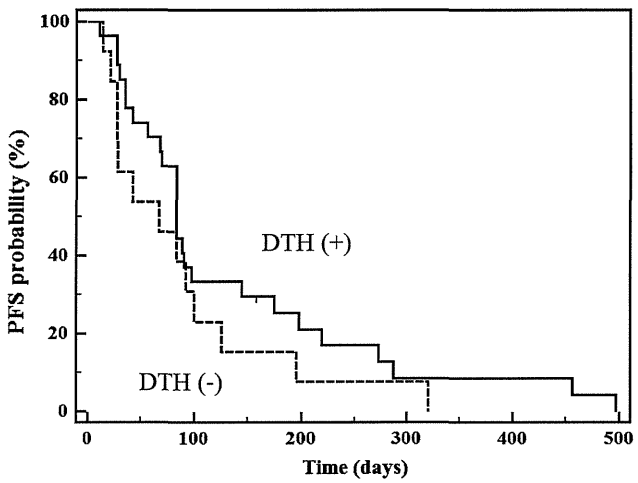

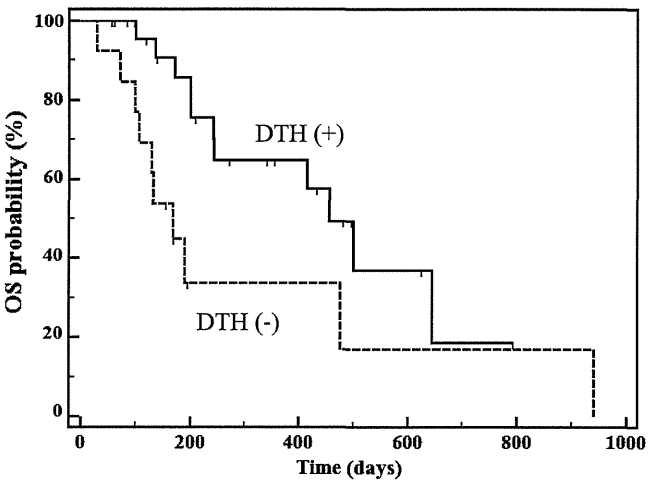

Fig. 1 PFS and OS association with DTH after WT1 vaccination. **a** The PFS tended to be longer in positive DTH cases than in DTH-negative cases ($p = 0.23$ by the log-rank test). **b** The OS was significantly longer in positive DTH cases than in DTH-negative cases ($p = 0.023$ by the log-rank test). *Solid line*: DTH (*plus*), *broken line*: DTH (*minus*)

Izumoto et al. 2008; Ohno et al. 2009). In this current phase II trial, we have tested the efficacy and safety of WT1 immunotherapy for gynecologic malignancies that were progressing, that is, resistant against conventional therapies.

In general, gynecologic tumors, including ovarian, endometrial and cervical carcinomas and uterine sarcomas, are very difficult to further treat, once the disease become resistant to conventional therapies such as chemotherapy or radiotherapy. For example, when ovarian carcinoma is first treated with cytoreductive surgery, the surgery is immediately followed by combination chemotherapy with paclitaxel and carboplatin (TC). If there is a failure of this first-line treatment, a single drug or combination chemotherapy for the recurrent disease, chosen based on the patient's treatment-free interval, can still be performed effectively in some cases (Koensgen et al. 2008; Markman et al. 2003; Harries and Gore 2002; Dizon et al. 2003). However, even though some third-line regimens have been reported to be occasionally effective for second relapses of some of these advanced stage diseases (Vergote et al. 2009; Chiyoda et al. 2010); the efficacy of each attempt becomes progressively lower as the number of previous treatment failures increases.

In the present study, the median number of the previous treatment regimens was 3 (range 1–11 treatments). Since all of the patients in the present trial had exhibited resistance to previous therapies, normally supportive care would have been considered as the only remaining option for them; however, the experimental WT1 vaccination immunotherapy was offered to them as an alternative.

A previous small study showed that stable disease was achieved by WT vaccination in 3 (25 %) of 12 gynecologic malignancies (Ohno et al. 2009). However, that study was so small that a survival effect was not analyzed. The response rate (CR + PR/all) in our study was 0 % (0 of 40 cases). However, the disease control rate (CR + PR + SD/all), which corresponds to disease stabilization lasting at least 3 months from the start of the vaccination, was 40 % (16 of 40 cases). The median PFS was 84 days (11–497), and the median OS of all the patients was 193 days (29–941). Considering that these cases were resistant to various kinds of therapies, and the diseases were progressing prior to the vaccination, these results of disease control rate and PFS time may be favorable, and were consistent with results of the previous smaller study that suggested the therapeutic potential of WT1 vaccine for gynecologic malignancies. Furthermore, surprisingly, in these SD cases, whose tumors had continuously progressed against previous therapies during the median of 185 days of treatments (range 40–1,198 days), the disease was durably controlled, without significant progression of the disease, for the median of longer than 160 days (range 67–427 days) after starting the WT1 immunotherapy (Table 2), implying an improved survival effect of the WT1 peptide vaccine. The adverse effect by the WT1 peptide-based immunotherapy with the dosage and schedule adopted here was limited and largely tolerable.

We next investigated the association of DTH and the efficacy of the WT1 immunotherapy. The OS of the patients with a positive DTH reaction was significantly better than that of those with a negative DTH reaction ($p = 0.023$ by the log-rank test) (Fig. 1). Moreover, the DTH reaction was demonstrated to be an independent factor for overall survival of the patients by multivariate Cox proportional hazards analysis (Table 3). These findings suggested that the induction of WT1-specific immune response, that is, the peptide-specific DTH, is a potential

Table 3 Multivariate Cox proportional hazards analysis on overall survival

Variable	Number of cases	Adjusted HR	95 % CI	*p* value
Age (years)				0.44
<60	24	1		
≥60	16	0.64	0.21–1.96	
Origin of the disease				0.75
Uterus	16	1		
Ovary	24	1.17	0.44–3.14	
Histology				0.98
Carcinoma	35	1		
Sarcoma, carcinosarcoma	5	0.99	0.28–3.42	
Evaluation of the previous therapy				0.39
SD	4	1		
PD	36	1.88	0.46–7.71	
Number of previous therapy regimens				0.034
<3	12	1		
≥3	28	4.28	1.12–16.37	
DTH				0.043
+	27	1		
−	13	2.73	1.04–7.19	

Multivariate Cox proportional hazards analysis (stepwise method) for the factors including age, origin of the disease, histology, evaluation of the previous therapy, number of previous therapy regimens and DTH was performed to evaluate whether DTH was an independently significant factor on OS

SD stable disease, *PD* progressive disease

predictor for the induction of clinical response, leading to a better prognosis.

The number of previous treatment regimens was also demonstrated to be an independent factor for survival prognosis after WT1 immunotherapy. The response rate of the first-line chemotherapy was quite high for ovarian carcinoma, however, that of second-line and the third-line chemotherapy was 34.5 and 27.5 %, respectively (Nishio et al. 2006). Effectiveness of WT1 was demonstrated to be associated with the number of previous treatment regimens, which was similar to that of the cell toxic chemotherapy. As the number of chemotherapy regimen increases, the tumor cells are considered to become resistant to the next line therapy. Furthermore, immunological potentials of the patients treated by chemotherapy with many courses might be dampened, leading to the poor response to the administered cancer vaccine. WT1 peptide vaccination soon after the first-line therapy, including the vaccination to prevent relapse after the operation, chemotherapy or radiation therapy, may be a favorable setting for the next clinical trial.

In the present phase II prospective study with a single arm, we have, for the first time, analyzed the survival effect of the WT1 vaccine for gynecologic malignancies, in addition to its anti-tumor effect conventionally evaluated by RECIST and toxicity, which had previously been reported in a smaller pilot study (Ohno et al. 2009). It was strongly suggested that WT1 peptide vaccination could induce the peptide-specific immune response in patients whose gynecological tumors have become resistant to conventional therapies, leading to a better survival. Larger two-arm randomized studies will be required to confirm the efficacy and clinical usefulness of the WT1 peptide vaccine for gynecologic malignancies.

Acknowledgments We would like to thank Dr. G.S. Buzard, US CDCP, for his editing of our manuscript. We are also grateful to Ms. T. Umeda for her excellent technical assistance.

Conflict of interest The authors have no conflict of interest.

References

Cheever MA, Allison JP, Ferris AS, Finn OJ, Hastings BM, Hecht TT, Mellman I, Prindiville SA, Viner JL, Weiner LM, Matrisian LM (2009) The prioritization of cancer antigens: a National Cancer Institute pilot project for the acceleration of translational research. Clin Cancer Res 15:5323–5337

Chiyoda T, Tsuda H, Nomura H, Kataoka F, Tominaga E, Suzuki A, Susumu N, Aoki D (2010) Effects of third-line chemotherapy for women with recurrent ovarian cancer who received platinum/taxane regimens as first-line chemotherapy. Eur J Gynaecol Oncol 31:364–368

DiSaia PJ, Creasman WT (2002) Clinical gynecologic oncology, 6th edn. St Louis, Mosby

Dizon DS, Dupont J, Anderson S, Sabbatini P, Hummer A, Aghajanian C, Spriggs D (2003) Treatment of recurrent ovarian cancer: a retrospective analysis of women treated with single-agent carboplatin originally treated with carboplatin and paclitaxel. The memorial sloan-kettering cancer center experience. Gynecol Oncol 91:584–590

Dohi S, Ohno S, Ohno Y, Takakura M, Kyo S, Soma G, Sugiyama H, Inoue M (2011) WT1 peptide vaccine stabilized intractable ovarian cancer patient for one year: a case report. Anticancer Res 31:2441–2445

Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, Rubinstein L, Shankar L, Dodd L, Kaplan R, Lacombe D, Verweij J (2009) New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer 45:228–247

Harries M, Gore M (2002) Part II chemotherapy for epithelial ovarian cancer-treatment of recurrent disease. Lancet Oncol 3:537–545

Hashii Y, Sato E, Ohta H, Oka Y, Sugiyama H, Ozono K (2010) WT1 peptide immunotherapy for cancer in children and young adults. Pediatr Blood Cancer 55:352–355

Izumoto S, Tsuboi A, Oka Y, Suzuki T, Hashiba T, Kagawa N, Hashimoto N, Maruno M, Elisseeva OA, Shirakata T, Kawakami M, Oji Y, Nishida S, Ohno S, Kawase I, Hatazawa J, Nakatsuka S, Aozasa K, Morita S, Sakamoto J, Sugiyama H, Yoshimine T (2008) Phase II clinical trial of Wilms tumor 1 peptide vaccination for patients with recurrent glioblastoma multiforme. J Neurosurg 108:963–971

Koensgen D, Stengel D, Belau A, Klare P, Oskay-Oezcelik G, Steck T, Camara O, Mustea A, Sommer H, Coumbos A, Bogenrieder T, Lichtenegger W (2008) North-Eastern german society of gynecological oncology study group-ovarian cancer. Topotecan and carboplatin in patients with platinum-sensitive recurrent ovarian cancer. Results of a multicenter NOGGO: phase I/II study. Cancer Chemother Pharmacol 62:393–400

Markman M, Webster K, Zanotti K, Kulp B, Peterson G, Belinson J (2003) Phase 2 trial of single-agent gemcitabine in platinum-paclitaxel refractory ovarian cancer. Gynecol Oncol 90:593–596

Morita S, Oka Y, Tsuboi A, Kawakami M, Maruno M, Izumoto S, Osaki T, Taguchi T, Ueda T, Myoui A, Nishida S, Shirakata T, Ohno S, Oji Y, Aozasa K, Hatazawa J, Udaka K, Yoshikawa H, Yoshimine T, Noguchi S, Kawase I, Nakatsuka S, Sugiyama H, Sakamoto J (2006) A phase I/II trial of a WT1 (Wilms' tumor gene) peptide vaccine in patients with solid malignancy: safety assessment based on the phase I data. Jpn J Clin Oncol 36:231–236

Nishio S, Katsumata N, Matsumoto K, Tanabe H, Kato Y, Yonemori K, Kouno T, Shimizu C, Ando M, Fujiwara Y. (2006) Analysis of third-line and forth-line chemotherapy for recurrent ovarian cancer treated with first-line platinum/taxane regimens 2006 ASCO Annual Meeting Proceedings Part I. J Clin Oncol 24 (18S June 20 Supplement):15045

Ohno S, Kyo S, Myojo S, Dohi S, Ishizaki J, Miyamoto K, Morita S, Sakamoto J, Enomoto T, Kimura T, Oka Y, Tsuboi A, Sugiyama H, Inoue M (2009) Wilms' tumor 1 (WT1) peptide immunotherapy for gynecological malignancy. Anticancer Res 29:4779–4784

Oji Y, Oka Y, Nishida S, Tsuboi A, Kawakami M, Shirakata T, Takahashi K, Murao A, Nakajima H, Narita M, Takahashi M, Morita S, Sakamoto J, Tanaka T, Kawase I, Hosen N, Sugiyama H (2010) WT1 peptide vaccine induces reduction in minimal residual disease in an imatinib-treated CML patient. Eur J Haematol 85:358–360

Oka Y, Sugiyama H (2010) WT1 peptide vaccine, one of the most promising cancer vaccines: its present status and the future prospects. Immunotherapy 2:591–594

Vergote I, Finkler N, del Campo J, Lohr A, Hunter J, Matei D, Kavanagh J, Vermorken JB, Meng L, Jones M, Brown G, ASSIST-1 Study Group (2009) Phase 3 randomised study of canfosfamide (Telcyta, TLK286) versus pegylated liposomal doxorubicin or topotecan as third-line therapy in patients with platinum-refractory or resistant ovarian cancer. Eur J Cancer 45:2324–2332

IJC
International Journal of Cancer

Annexin A4-conferred platinum resistance is mediated by the copper transporter ATP7A

Shinya Matsuzaki[1,2], Takayuki Enomoto[3], Satoshi Serada[2], Kiyoshi Yoshino[1], Shushi Nagamori[4], Akiko Morimoto[1], Takuhei Yokoyama[1,2], Ayako Kim[2], Toshihiro Kimura[1], Yutaka Ueda[1], Masami Fujita[1], Minoru Fujimoto[2], Yoshikatsu Kanai[4], Tadashi Kimura[1] and Tetsuji Naka[2]

[1] Department of Obstetrics and Gynecology, Osaka University Graduate School of Medicine, Osaka, Japan
[2] Laboratory for Immune Signal, National Institute of Biomedical Innovation, Osaka, Japan
[3] Department of Obstetrics and Gynecology, Niigata University Graduate School of Medicine, Niigata, Japan
[4] Department of Pharmacology, Osaka University Graduate School of Medicine, Osaka, Japan

Although platinum drugs are often used for the chemotherapy of human cancers, platinum resistance is a major issue and may preclude their use in some cases. We recently reported that enhanced expression of Annexin A4 (Anx A4) increases chemoresistance to carboplatin through increased extracellular efflux of the drug. However, the precise mechanisms underlying that chemoresistance and the relationship of Anx A4 to platinum resistance *in vivo* remain unclear. In this report, the *in vitro* mechanism of platinum resistance induced by Anx A4 was investigated in endometrial carcinoma cells (HEC1 cells) with low expression of Anx A4. Forced expression of Anx A4 in HEC1 cells resulted in chemoresistance to platinum drugs. In addition, HEC1 control cells were compared with Anx A4-overexpressing HEC1 cells in xenografted mice. Significantly greater chemoresistance to cisplatin was observed *in vivo* in Anx A4-overexpressing xenografted mice. Immunofluorescence analysis revealed that exposure to platinum drugs induced relocation of Anx A4 from the cytoplasm to the cellular membrane, where it became colocalized with ATP7A, a copper transporter also well known as a mechanism of platinum efflux. ATP7A expression suppressed by small interfering RNA had no effect on HEC1 control cells in terms of chemosensitivity to platinum drugs. However, suppression of ATP7A in Anx A4-overexpressing platinum-resistant cells improved chemosensitivity to platinum drugs (but not to 5-fluorouracil) to a level comparable to that of control cells. These results indicate that enhanced expression of Anx A4 confers platinum resistance by promoting efflux of platinum drugs *via* ATP7A.

Platinum drugs, widely used for treating gynecological cancers, can improve survival rates dramatically, particularly in patients with ovarian and endometrial carcinomas.[1–6] Compared with platinum-sensitive tumors, prognosis is poorer for tumors that are (or become) platinum-resistant; for these tumors, other chemotherapeutic drugs also tend to be less effective. For example, an efficacy of 81% has been demonstrated for chemotherapy regimens that include platinum drugs for treatment of ovarian serous adenocarcinoma (SAC), the most common subtype of ovarian carcinoma; however, the efficacy of these regimens is only 18% for ovarian clear cell carcinomas (CCC), which are frequently resistant to multiple drugs.[7] Compared with advanced SAC, the clinical prognosis of patients with similarly advanced CCC is markedly worse largely because of the considerably higher rate of recurrence after CCC treatment.[7–11] Therefore, determining the mechanism underlying platinum resistance may aid in identification of therapeutic targets for platinum-resistant tumors such as CCC. Studies using proteomic screening approaches have previously demonstrated overexpression of Annexin A4 (Anx A4) protein in ovarian CCC, which is frequently a highly platinum-resistant tumor compared with SAC.[12] Similar findings have been reported in a study comparing SAC and CCC using a genomic screening approach.[13] Anx A4, a previously understudied member of the Annexin protein family, binds to phospholipids in a Ca^{2+}-dependent manner, self-associates on phospholipid

Key words: Annexin A4, ATP7A, platinum resistance, platinum transporter, copper transporter
Abbreviations: 5-FU: 5-fluorouracil; Anx A4: Annexin A4; CCC: clear cell carcinoma; D-MEM: Dulbecco's modified Eagle's medium; FBS: fetal bovine serum; PBS: phosphate-buffered saline; SAC: serous adenocarcinoma; siRNA: small interfering RNA
Additional Supporting Information may be found in the online version of this article.
Grant sponsor: Japanese Ministry of Education, Science, Culture and Sports; **Grant number:** 22791560; **Grant sponsors:** Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation, Ministry of Health, Labour and Welfare of Japan
DOI: 10.1002/ijc.28526
History: Received 4 Dec 2012; Accepted 26 Sep 2013; Online 8 Oct 2013
Correspondence to: Dr. Tetsuji Naka, Laboratory for Immune Signal, National Institute of Biomedical Innovation, 7-6-8 Saito-asagi, Ibaraki, Osaka 567-0085, Japan, Tel.: +81-72-641-9843, Fax: +81-72-641-9837, E-mail: tnaka@nibio.go.jp

What's new?

Although platinum-based drugs are often used in chemotherapy, resistance to these drugs is frequently a problem. The protein Annexin A4 (Anx A4) is known to be involved in platinum efflux in ovarian tumours; however, its precise mechanism of action has been unclear. In this study, the authors demonstrated that the strong platinum-resistance in Anx A4-overexpressing cells involves the transporter protein ATP7A, both in vitro and in vivo. This suggests that Anx A4 may be a highly useful therapeutic target in Anx A4-expressing carcinomas.

membrane surfaces and causes membrane aggregation.[12,14–17] Enhanced expression of Anx A4 has recently shown to increase tumor chemoresistance to carboplatin (a key drug for treating gynecological cancers) *via* increased extracellular efflux of the drug.[12] Another study showed that Anx A4 suppresses NF-κB transcriptional activity, which is significantly upregulated early after etoposide treatment. Anx A4 translocates to the nucleus together with p50 and imparts greater resistance to apoptotic stimulation by etoposide treatment.[18] Anx A4 may also be associated with drug resistance in other types of tumors; enhanced expression of Anx A4 has been reported in colon, renal, lung and pancreatic cancers.[19–23] However, the details of Anx A4-mediated extracellular efflux of platinum drugs remain unclear.

HEC1 is an endometrial carcinoma cell line with low Anx A4 expression levels. In our study, Anx A4-overexpressing derivative HEC1 cell lines were established and their chemosensitivity toward platinum drugs was analyzed both *in vitro* and *in vivo*. Anx A4-conferred platinum chemoresistance was shown to be mediated by the copper transporter ATP7A.[24–28]

Material and Methods

Cell lines

The human endometrial carcinoma cell lines HEC1, HEC1A, HEC6, HEC88nu, HEC108, HEC116 and HEC251; SNGII and SNGM cells, the human ovarian SAC cell line OVSAHO and the ovarian CCC cell lines OVISE and OVTOKO were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan); A2780 cells from the human ovarian SAC cell line were obtained from the European Collection of Animal Cell Culture (Salisbury, Scotland). The identity of each cell line was confirmed by DNA fingerprinting *via* short tandem repeat profiling, as described previously.[29] HEC1, HEC1A, HEC6, HEC88nu, HEC108, HEC116 and HEC251 cells were maintained in Dulbecco's modified Eagle's medium (D-MEM) (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT) and 1% penicillin–streptomycin (Nacalai Tesque, Kyoto, Japan) at 37°C under a humidified atmosphere of 5% CO_2. SNGII and SNGM cells were maintained in Ham's F12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS and 1% penicillin–streptomycin. OVSAHO, A2780, OVISE and OVTOKO cells were maintained in Roswell Park Memorial Institute 1640 medium (Wako Pure Chemical Industries) supplemented with 10% FBS and 1% penicillin–streptomycin.

Generation of Anx A4 stably transfected cell lines

To generate cell lines that stably expressed Anx A4, HEC1 cells were transfected with the pcDNA3.1–Anx A4 expression plasmid, as described previously.[12] Transfected cells were selected with 600 μg/ml of Geneticin (Invitrogen). Clones were maintained in 250 μg/ml of Geneticin for stability of expression. Four stable Anx A4-expressing cell lines were established and designated HEC1-A25, HEC1-A43, HEC1-A63 and HEC1-A77. A control cell line of HEC1 was also established and stably transfected with an empty vector. This cell line was designated as HEC1-CV.

Western blotting

Cells were lysed in radioimmunoprecipitation assay buffer [10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% protease-inhibitor cocktail (Nacalai Tesque) and 1% phosphatase-inhibitor cocktail (Nacalai Tesque)]. After centrifugation (13,200 rpm, 4°C, 15 min), soluble proteins in the supernatant were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as described previously.[12] Additional information can be found in Supporting Information Material and Methods.

Measurement of IC_{50} values after treatment with cisplatin or carboplatin

Cells were suspended in D-MEM medium supplemented with 10% FBS and were seeded in 96-well plates (2,000 cells per well) (Costar; Corning, Corning, NY) for 24 hr. They were then exposed to various concentrations of carboplatin (0–500 μM), cisplatin (0–100 μM) or 5-fluorouracil (5-FU) (0–50 μM) for 72 hr. Cell proliferation was evaluated using the WST-8 assay (Cell Counting Kit-SF; Nacalai Tesque) after treatment at the time points indicated by the manufacturer. The absorption of WST-8 was measured at a wavelength of 450 nm (reference wavelength: 630 nm) using a Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA). Absorbance values for treated cells indicative of proliferation rates were expressed as percentages relative to results for untreated controls, and the drug concentrations resulting in a 50% inhibition of cell growth (IC_{50} values) were calculated.

Small interfering RNA transfection

Two commercial small interfering RNAs (siRNAs) against ATP7A and a nonspecific control siRNA were obtained from

Qiagen (Venlo, The Netherlands) and designated ATP7A siRNA4 and ATP7A siRNA6, respectively. For gene silencing, a specific sense strand 5′-GCAGCUUGUAGUAUUGAA ATT-3′ was used for ATP7A siRNA4, and an antisense strand 5′-UUUCAAUACUACAAGCUGCTA-3′ was also used. For ATP7A siRNA6, a specific sense strand 5′-GCGUA GCUCCAGAGGUUUATT-3′ was used, and an antisense strand 5′-UAAACCUCUGGAGCUACGCAG-3′ was also used. Cells were transfected with siRNA using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Selective silencing of ATP7A was confirmed by Western blot analysis.

In vivo model of cisplatin resistance

All animal experiments were conducted in accordance with the Institutional Ethical Guidelines for Animal Experimentation of our National Institute of Biomedical Innovation (Osaka, Japan). Four-week-old, female Institute of Cancer Research (ICR) nu/nu mice were obtained from Charles River Japan (Yokohama, Japan). For subcutaneous xenograft experiments, 2.5×10^6 HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells were suspended in 100 μl of 1/1 (v/v) phosphate-buffered saline (PBS)/Matrigel (Becton Dickinson, Bedford, MA) and injected subcutaneously into the flanks of the ICR nu/nu mice ($n = 5$ per group). One week after xenograft establishment, tumors measured ~100 mm^3. Mice were then randomly divided into two groups and administered cisplatin (3 mg/kg) or PBS i.p. twice weekly for 4 weeks. Tumor volumes were determined twice weekly by measuring length (L), width (W) and depth (D). Tumor volume was calculated using the formula: tumor volume (mm^3) = $W \times L \times D$. At 56 days after tumor implantation, tumors were removed and weighed.

Quantification of intracellular platinum accumulation

Cisplatin accumulation in cells was analyzed according to a previously established method, with certain minor modifications. In brief, 6×10^6 cells (HEC1, HEC1-CV, HEC1-A25, HEC1-A43, HEC1-A63 and HEC1-A77 cells) were seeded into two 150-mm tissue culture dishes and incubated for 24 hr. The cells were then exposed to 1 mM cisplatin for 60 min at 37°C and then washed twice with PBS. After 3 hr of incubation in cisplatin-free D-MEM medium (supplemented with 10% FBS), whole extracts were prepared and the concentration of intracellular platinum was determined using an Agilent 7500ce inductively coupled plasma mass spectrometer (ICP-MS; Agilent, Santa Clara, CA). The absolute concentration of platinum in each sample was determined from a calibration curve prepared with a platinum standard solution.

Preparation of crude membrane fractions

To investigate the localization of Anx A4, crude membrane fractions (CMFs) of cells treated in various ways were prepared. Cells were divided into three groups: those that received no treatment, those pretreated with 10 μM cisplatin for 4 hr and those pretreated with 50 μM carboplatin for 4 hr. CMF were prepared as described elsewhere,[30] with modifications. Prepared proteins were investigated using Western blot analysis. Additional information can be found in Supporting Information Material and Methods.

Biotinylation of HEC1 cell membrane surface proteins after cisplatin or carboplatin exposure

To investigate the localization of ATP7A after exposure to platinum drugs, treated or mock-treated HEC1 cells were surface-biotinylated and the presence of ATP7A was investigated by Western blot analysis. Additional information can be found in Supporting Information Material and Methods.

Immunofluorescence for ATP7A and Anx A4

Immunofluorescence staining was performed 2 days after cells had been seeded on cover slips. Before staining, cells in the treatment groups were pretreated with 10 μM cisplatin or 50 μM carboplatin for 4 hr. Cells were then analyzed for localization of Anx A4 and ATP7A. Additional information can be found in Supporting Information Material and Methods.

Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Dunnett's analysis to evaluate the significance of differences. In all analyses, $p < 0.05$ was considered to be statistically significant.

Results

Expression of Anx A4 in endometrial carcinoma cell lines

To investigate Anx A4 expression in nine common endometrial carcinoma cell lines, Western blot analyses were performed. Expression of Anx A4 was strongest in SNGM cells compared with the other eight cell lines (Fig. 1*a*). Thus, enhanced expression of Anx A4 was confirmed in this endometrial carcinoma cell line.

Anx A4 and platinum resistance in HEC1 cell lines

From control HEC1 cells (low Anx A4 expression levels), four stable lines of Anx A4-overexpressing cells (HEC1-A25, HEC1-A43, HEC1-A63 and HEC1-A77 cells) and one line of empty vector transfected cells (HEC1-CV cells) were established. Overexpression of Anx A4 was confirmed using Western blot analysis and was compared with CCC cell lines (OVTOKO and OVISE) used as positive controls (Fig. 1*b*). Significantly higher IC_{50} values for cisplatin were observed in HEC1-A25 (32.1 μM, $p < 0.01$), HEC1-A43 (23.8 μM, $p < 0.01$), HEC1-A63 (34.9 μM, $p < 0.01$) and HEC1-A77 cells (17.3 μM, $p < 0.01$) compared with HEC1 (9.8 μM) and HEC1-CV cells (8.4 μM) (Fig. 1*c*). Similarly, IC_{50} values for carboplatin were significantly increased in HEC1-A25 (194.6 μM, $p < 0.01$), HEC1-A43 (153.3 μM, $p < 0.01$), HEC1-A63 (371.5 μM, $p < 0.01$) and HEC1-A77 cells (158.1 μM, $p < 0.01$) compared with HEC1 (59.1 μM) and HEC1-CV cells (60.9 μM) (Fig. 1*c*). Thus, Anx A4 overexpression conferred platinum resistance in HEC1 cell lines.

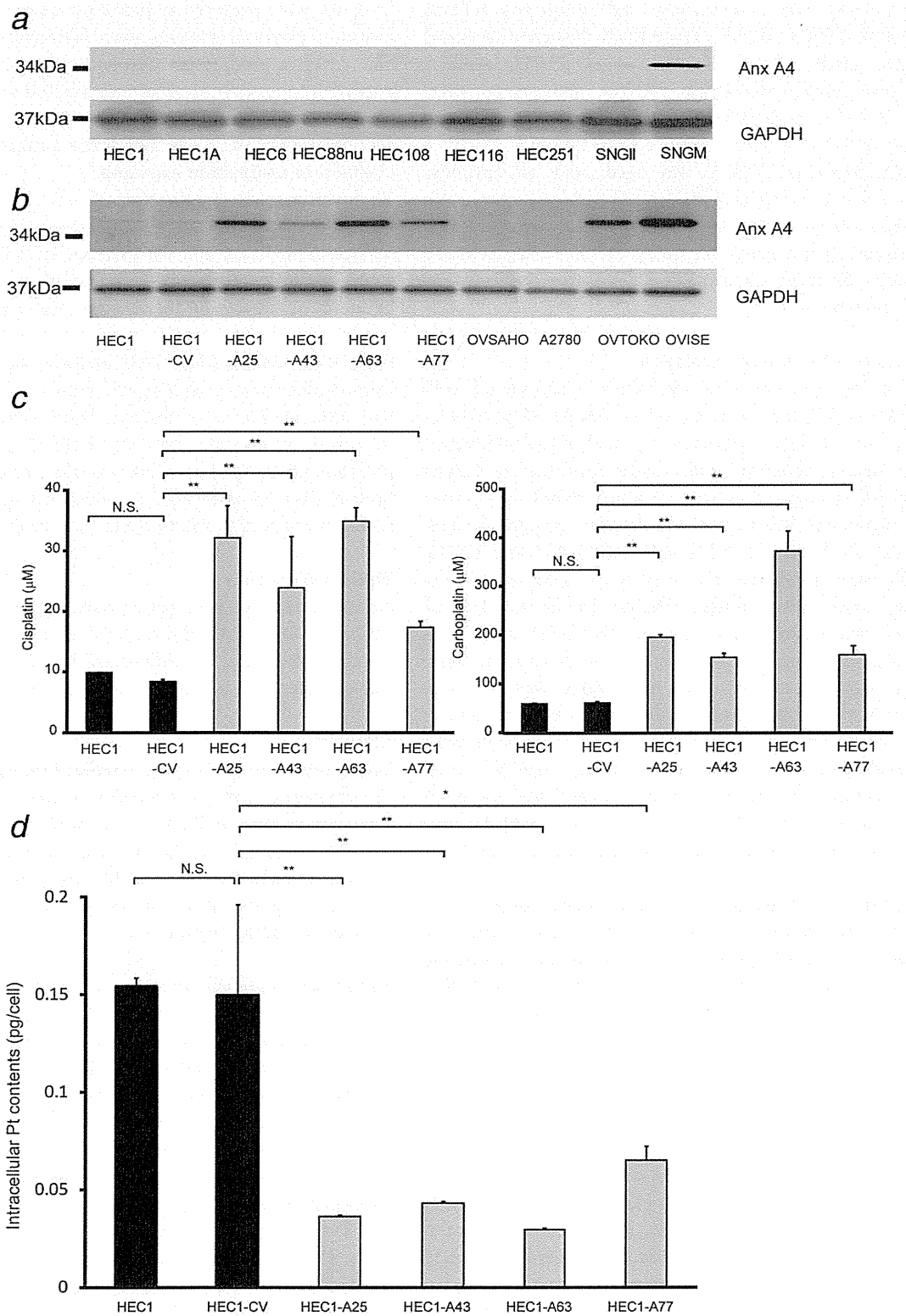

Figure 1. Enforced expression of Anx A4 in HEC1 cells confers platinum resistance *in vitro*. (*a*) Western blot analysis of nine endometrial carcinoma cell lines. Anx A4 was expressed in one cell line. (*b*) Establishment of an Anx A4-stably-expressing HEC1 cell line by transfection with the pcDNA3.1-Anx A4 expression plasmid into a HEC1 cell line with low Anx A4 expression levels. Enforced expression of Anx A4 was confirmed by Western blot analysis. (*c*) The IC_{50} sensitivity to cisplatin or carboplatin was investigated in HEC1, HEC1-CV, HEC1-A25, HEC1-A43, HEC1-A63 and HEC1-A77 cells. (*d*) Intracellular platinum accumulation was investigated after treatment with 1 mM cisplatin for 60 min and further incubation with cisplatin-free medium for 180 min and was determined by ICP-MS analysis.

Intracellular platinum accumulation in Anx A4-overexpressing cells

To elucidate the mechanism underlying platinum resistance induced by Anx A4, intracellular platinum accumulation of HEC1, HEC1-CV, HEC1-A25, HEC1-A43, HEC1-A63 and HEC1-A77 cells after cisplatin exposure was analyzed. Significantly less platinum had accumulated in HEC1-A25, HEC1-A43, HEC1-A63 and HEC1-A77 cells compared with HEC1 and HEC1-CV cells (0.036 pg/cell, $p < 0.01$; 0.04 pg/cell, $p < 0.01$; 0.03 pg/cell, $p < 0.01$; 0.065 pg/cell, $p < 0.05$ and 0.154 and 0.150 pg/cell, respectively) (Fig. 1*d*). Thus, intracellular platinum accumulation was decreased in Anx A4-overexpressing cells.

Anx A4-overexpressing cells and cisplatin in xenograft models

To determine the involvement of Anx A4 in platinum resistance *in vivo*, HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells were subcutaneously injected into nude mice. After the tumor xenograft had been established, cisplatin or PBS was given twice a week for 1 month. On Day 56, average tumor volumes were $11{,}496 \pm 950$ mm^3 in PBS-treated HEC1-CV control mice and $3{,}554 \pm 872$ mm^3 in cisplatin-treated HEC1-CV controls. A significant antitumor effect of cisplatin was therefore observed in HEC1-CV-xenografted mice compared with the PBS-treated group. The parent HEC1 and HEC1-CV xenografts responded similarly to cisplatin (Fig. 2*a*; $p < 0.01$).

In HEC1-A63-xenografted mice, the average tumor volume on Day 56 was $8{,}245 \pm 160$ mm^3 in the PBS-treated group and only slightly less ($7{,}078 \pm 257$ mm^3) in the cisplatin-treated group (Fig. 2*a*; $p = 0.42$). A similar response to cisplatin was observed in the HEC1-A63 and HEC1-A77 xenografts. On Day 56, no significant differences in tumor weight were found in HEC1-A63-xenografted mice between the PBS treatment (4.66 ± 0.42 g) and the cisplatin treatment groups (4.43 ± 0.16 g) (Fig. 2*b*). Similar results were observed in HEC1-A77 xenograft models. In contrast, a significant decrease in tumor weight was observed in HEC1-CV-xenografted mice between the PBS mock treatment (5.95 ± 1.16 g) and the cisplatin treatment groups (3.20 ± 0.76 g; $p < 0.05$) (Fig. 2*b*). Similar results were observed for the HEC1 and HEC1-CV xenografts. No significant differences in tumor weight in the PBS treatment group were observed among HEC1-CV-xenografted (5.95 ± 1.16 g), HEC1-xenografted (7.48 ± 0.34 g), HEC1-A63-xenografted (4.66 ± 0.42 g) and HEC1-A77-xenografted mice (4.82 ± 1.08 g) (Fig. 2*b*). These results indicated that overexpression of Anx A4 in HEC1 endometrial carcinoma cell lines conferred significant platinum resistance to the cells as tumors growing *in vivo*.

Translocation of Anx A4 and ATP7A after platinum exposure

In our study, platinum transporters were the focus of an investigation of the molecular mechanisms of chemoresistance induced by Anx A4. In previous research, intracellular platinum levels were decreased after enhanced expression of Anx A4, and ATP7A and ATP7B are well known as efflux transporters of platinum drugs.[27,28,31] However, the relationship of Anx A4 with ATP7A and ATP7B has not been previously examined. The results of our study demonstrated no change in expression of ATP7A at the protein levels owing to enforced overexpression of Anx A4 (Fig. 3*a*) and no ATP7B expression in HEC1 cells (data not shown). Therefore, the effects of Anx A4 expression on ATP7B in these cells were not investigated.

Because Anx A4 is normally localized to the cytoplasm, we theorized that exposure to platinum drugs may induce translocation of Anx A4 to the cellular membrane, resulting in an increase in chemoresistance owing to the influence of ATP7A. To investigate the possibility of induced translocation of Anx A4 and ATP7A by platinum drugs, CMFs were prepared. By Western blot analysis, Anx A4 expression in CMF of HEC1 and HEC1-CV cells before and after treatment with cisplatin or carboplatin was barely detectable because of its low endogenous expression in these cells (Fig. 3*b*). In contrast, Anx A4 expression was increased in CMF of HEC1-A63 cells and HEC1-A77 cells treated with cisplatin and carboplatin compared with untreated cells (Fig. 3*b*). Biotinylation-based cell surface membrane protein enrichment revealed a marked increase in biotinylation of ATP7A after exposure to cisplatin or carboplatin in HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells (Fig. 3*c*). In the biotinylated samples, no Anx A4 expression was detected on the cell surface, although it had been previously detected in the cell CMF (data not shown). These results suggested that exposure to cisplatin or carboplatin induced massive translocation of Anx A4 to CMF, including the inner surface of the cell membrane (inaccessible to biotinylation). Before exposure of the cell to cisplatin or carboplatin, ATP7A was not expressed in biotinylated samples but after exposure, strong ATP7A expression was detected. These results suggested that exposure to cisplatin or carboplatin induced massive translocation of ATP7A to the outer surface of the cell (accessible to biotinylation).

Anx A4 and ATP7A localization

By immunofluorescence analysis, Anx A4 was localized in the perinuclear and cytoplasmic regions of untreated cells, whereas ATP7A was localized mainly in the perinuclear and cytoplasmic regions and slightly less in the cellular membrane in HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells (Figs. 4*a*–4*d*). After 4-hr exposure to cisplatin or carboplatin, Anx A4 and ATP7A were found to be colocalized to the cellular membrane in HEC1-A63 cells (Fig. 4*c*). Similar findings were observed in HEC1-A77 cells (Fig. 4*d*). Because of the low expression of Anx A4 in HEC1 and HEC1-CV cells, no Anx A4 was detected in the cellular membranes in these cells (Figs. 4*a* and 4*b*). Thus, the results of the immunofluorescence analysis were in accordance with those of both Western blot analysis of CMF preparations and biotinylation

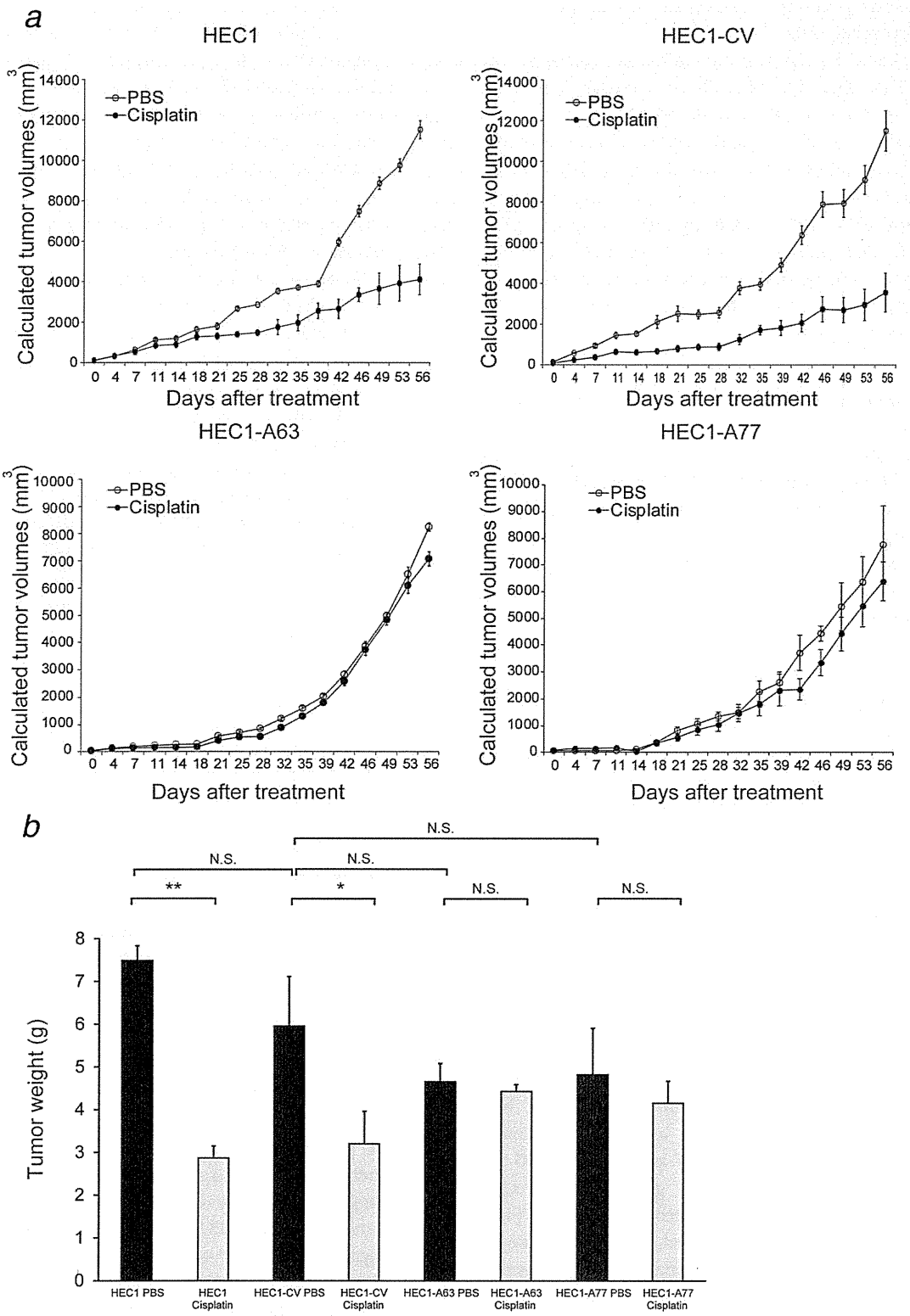

Figure 2. Enforced expression of Anx A4 in HEC1 cells confers platinum resistance *in vivo*. Analysis of Anx A4 as a platinum-resistant protein *in vivo*. (*a*) To determine the resistance of Anx A4-stably-expressing HEC1 cells to platinum *in vivo*, parent HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells were subcutaneously injected into nude mice ($n = 5$ per group). After tumor xenografts were established, cisplatin (3 mg/kg) or PBS was administered i.p. twice weekly for 1 month. Figure shows the average (points) for five animals ± SD (bars). (*b*) Fifty-six days after implantation, tumors were removed and weighed. Values shown are the means (±SD) of five mice. NS: not significant (*$p <$ 0.05; **$p < 0.01$; one-way ANOVA, followed by Dunnett's analysis).

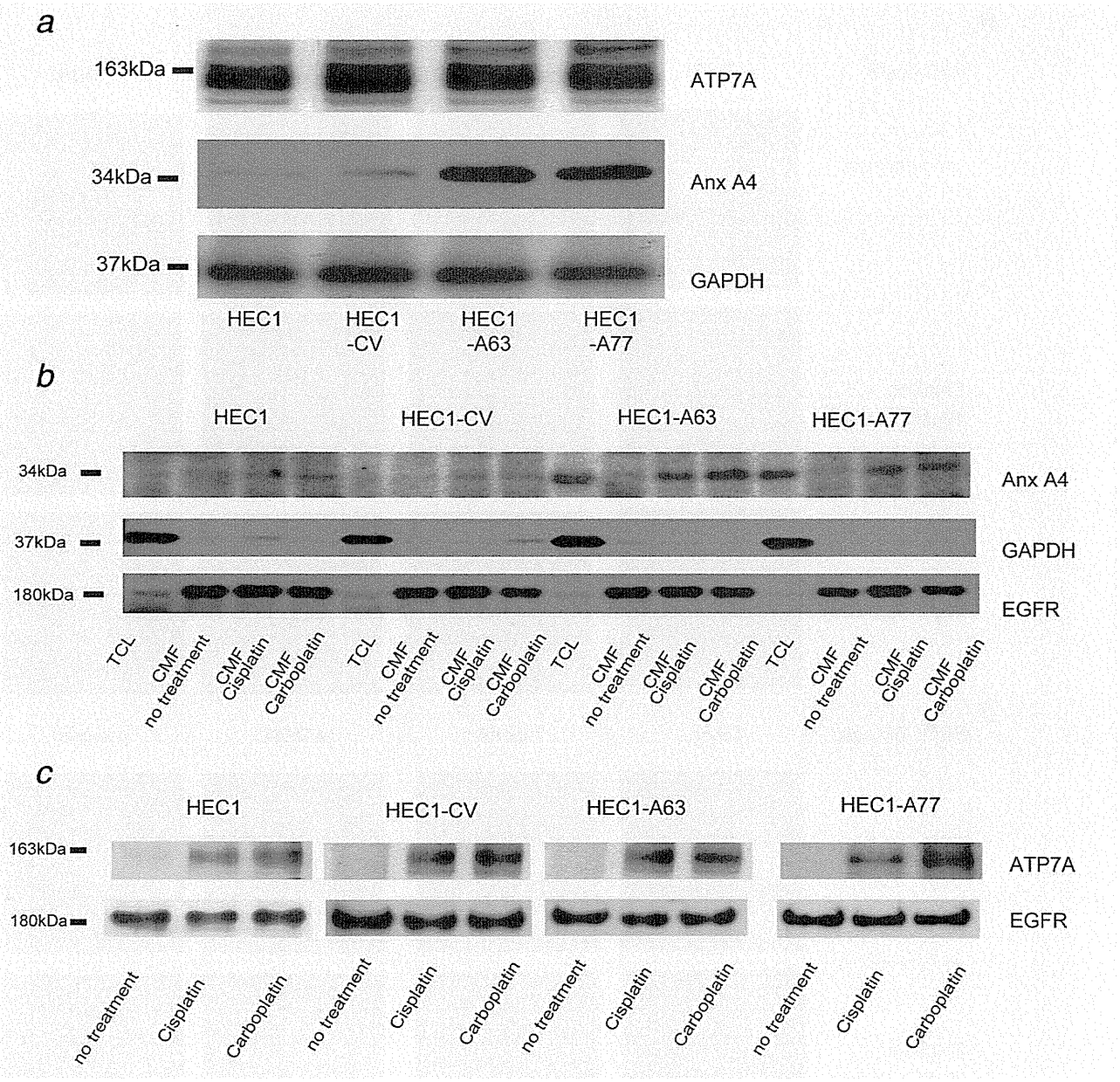

Figure 3. Localization of Anx A4 and ATP7A was investigated using Western blot analysis. The localization of Anx A4 and ATP7A was investigated using two techniques: orthogonal crude membrane fractions and biotinylation of cell surface proteins. (*a*) No significant change in expression levels of ATP7A was observed in HEC1, HEC1-CV, HEC1-A63 or HEC1-A77 cells. (*b*) In both HEC1-A63 and HEC1-A77 cells (but not in HEC1 and HEC1-CV cells), the drug-induced translocation of Anx A4 into the crude membrane fraction was shown by Western blot analysis after exposure to 10 μM cisplatin or 50 μM carboplatin for 4 hr. TCL: total cell lysate. Epidermal growth factor receptor was used as the control for cell surface protein labeling. (*c*) In HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells, translocation of ATP7A to the cell surface was shown by Western blot analysis. Cells were treated with 25 μM cisplatin or 150 μM carboplatin for 4 hr, and cell surface proteins were biotinylated with 500 μM sulfo-NHS-SS-biotin. Biotinylated surface proteins were enriched with UltraLink Immobilized Neutroavidin (Thermo Fisher Scientific, Waltham, MA) and analyzed by Western blot analysis using anti-ATP7A. Levels of epidermal growth factor receptor, a surface protein, are shown as loading controls.

assays (Figs. 3*b* and 3*c*). Anx A4 and ATP7A were localized in the cytoplasm before cisplatin or carboplatin exposure; Anx A4 and ATP7A were then translocated to the cellular membrane after cisplatin or carboplatin exposure. Thus, Anx A4 and ATP7A are colocalized to the cellular membrane in platinum-treated HEC1-A63 and HEC1-A77 cells but not in HEC1 and HEC1-CV cells.

Effect of ATP7A expression on resistance to platinum drugs

The mechanism of platinum resistance conferred by Anx A4 overexpression was explored further by suppression of ATP7A expression using siRNA. The suppression of ATP7A was confirmed using Western blot analysis (Fig. 5*a*). Anx A4 expression was unchanged by silencing ATP7A (Fig. 5*a*). The

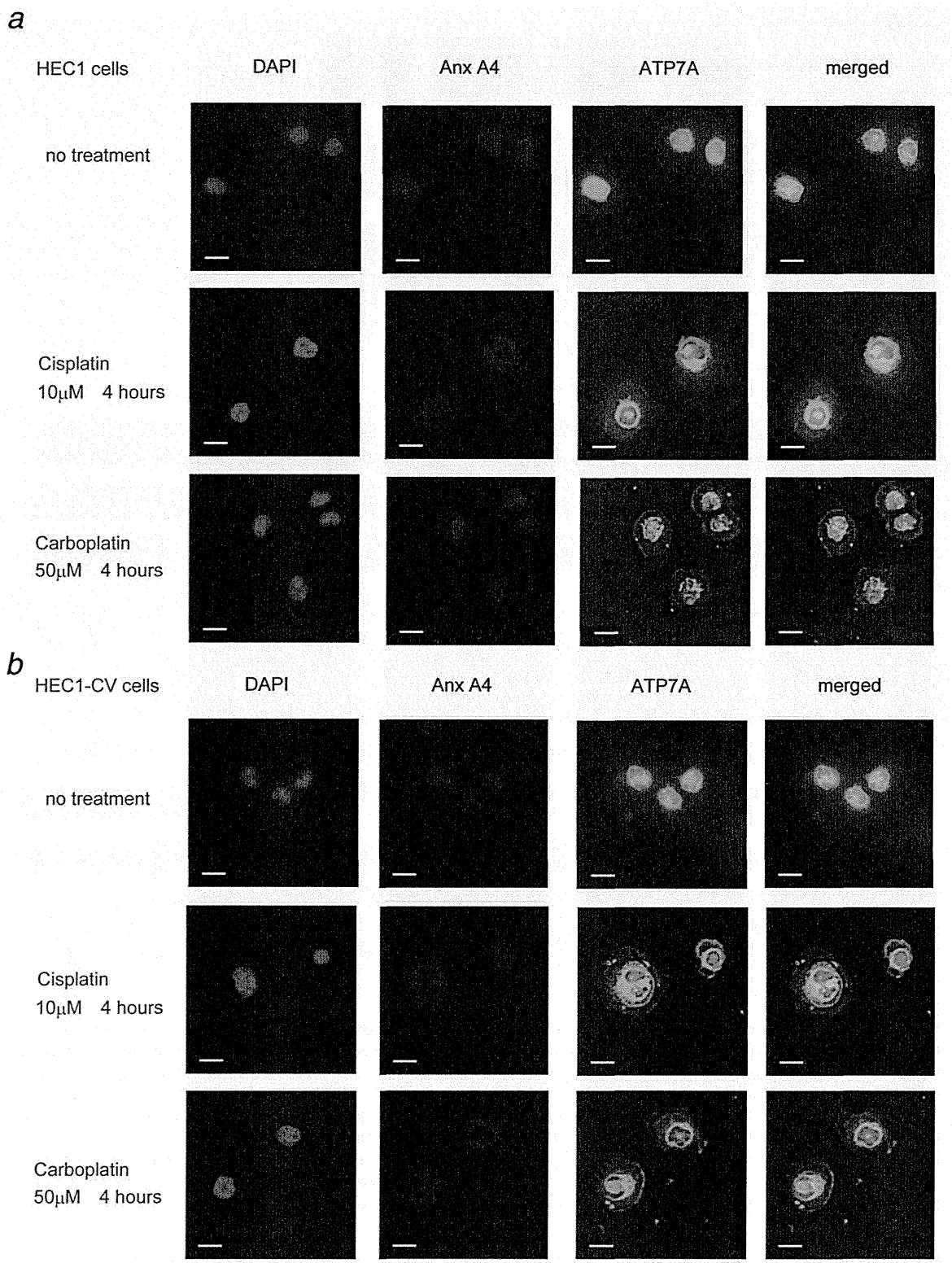

Figure 4. Immunofluorescence staining for ATP7A and Anx A4. HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells were divided into three groups: the no treatment, cisplatin exposure and carboplatin exposure groups. (*a*) HEC1 cells, (*b*) HEC1-CV cells, (*c*) HEC1-A63 cells and (*d*) HEC1-A77 cells. Cells were incubated with anti-Anx A4 antibody (red) or anti-ATP7A antibody (green). Nuclei were stained with DAPI (blue). In the no treatment group for each cell, Anx A4 was localized in perinuclear and cytoplasmic regions and ATP7A was strongly localized in perinuclear regions. In HEC1 and HEC1-CV cells, after exposure to cisplatin or carboplatin, ATP7A was relocalized in the cellular membrane, although some ATP7A remained in the cytoplasm; however, no change in location of Anx A4 was observed. In HEC1-A63 and HEC1-A77 cells, Anx A4 and ATP7A were newly colocalized in the cellular membrane as well as remaining in the cytoplasm. In a comparison of HEC1 and HEC1-CV cells with HEC1-A63 and HEC1-A77 cells, expression of Anx A4 in HEC1-A63 and HEC1-A77 cells was stronger in the cytoplasm and cellular membrane. Scale bar = 30 μm.

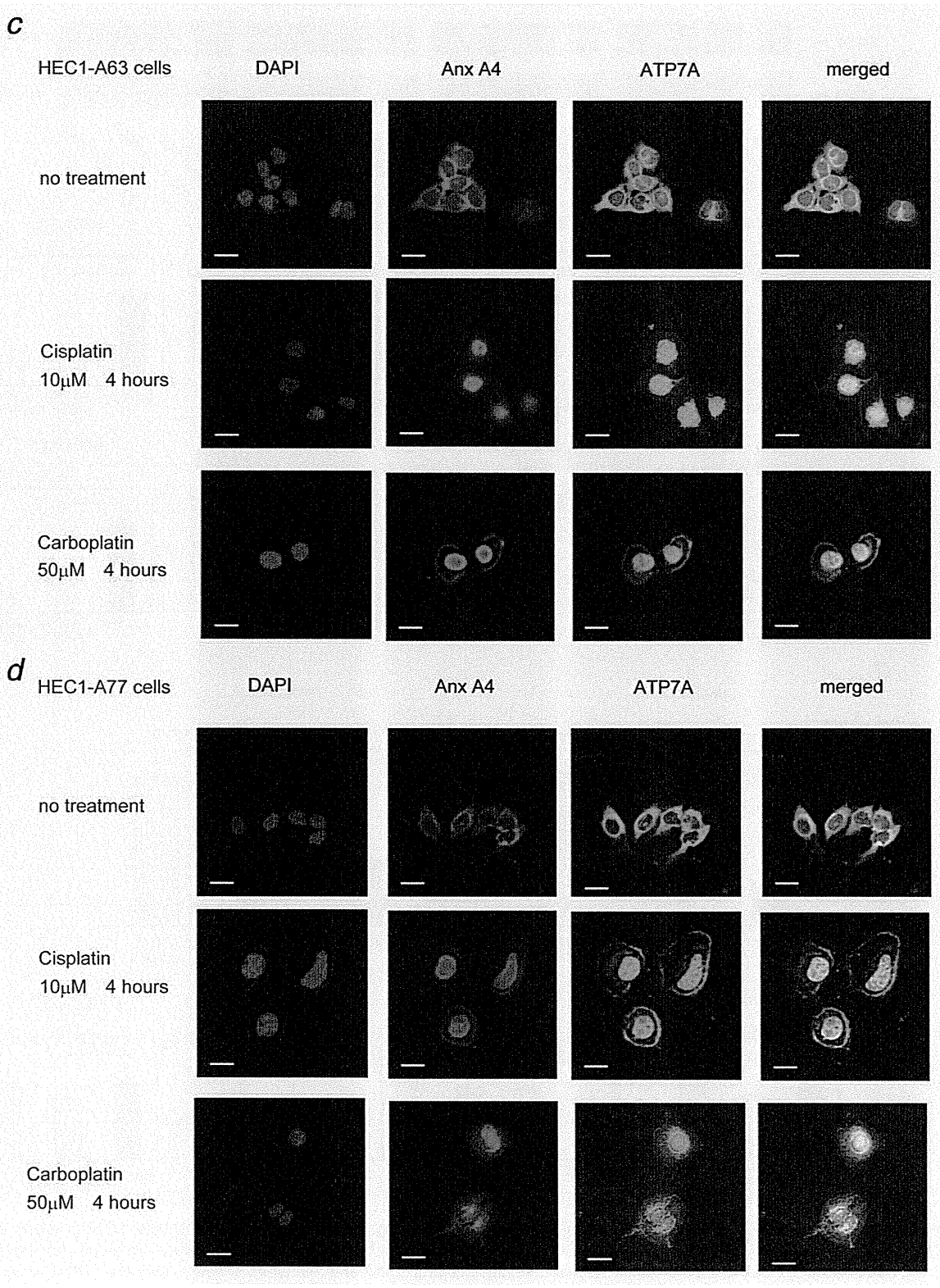

Figure 4. (Continued)

control and commercial siRNAs against ATP7A were transfected and the IC_{50} values of cisplatin and carboplatin were determined for each cell line. The IC_{50} value for cisplatin was significantly lower for the two kinds of ATP7A-silenced HEC1-A63 cells (ATP7A siRNA4, $IC_{50} = 11.0$ μM, $p < 0.01$; ATP7A siRNA6, $IC_{50} = 11.2$ μM, $p < 0.01$) compared with

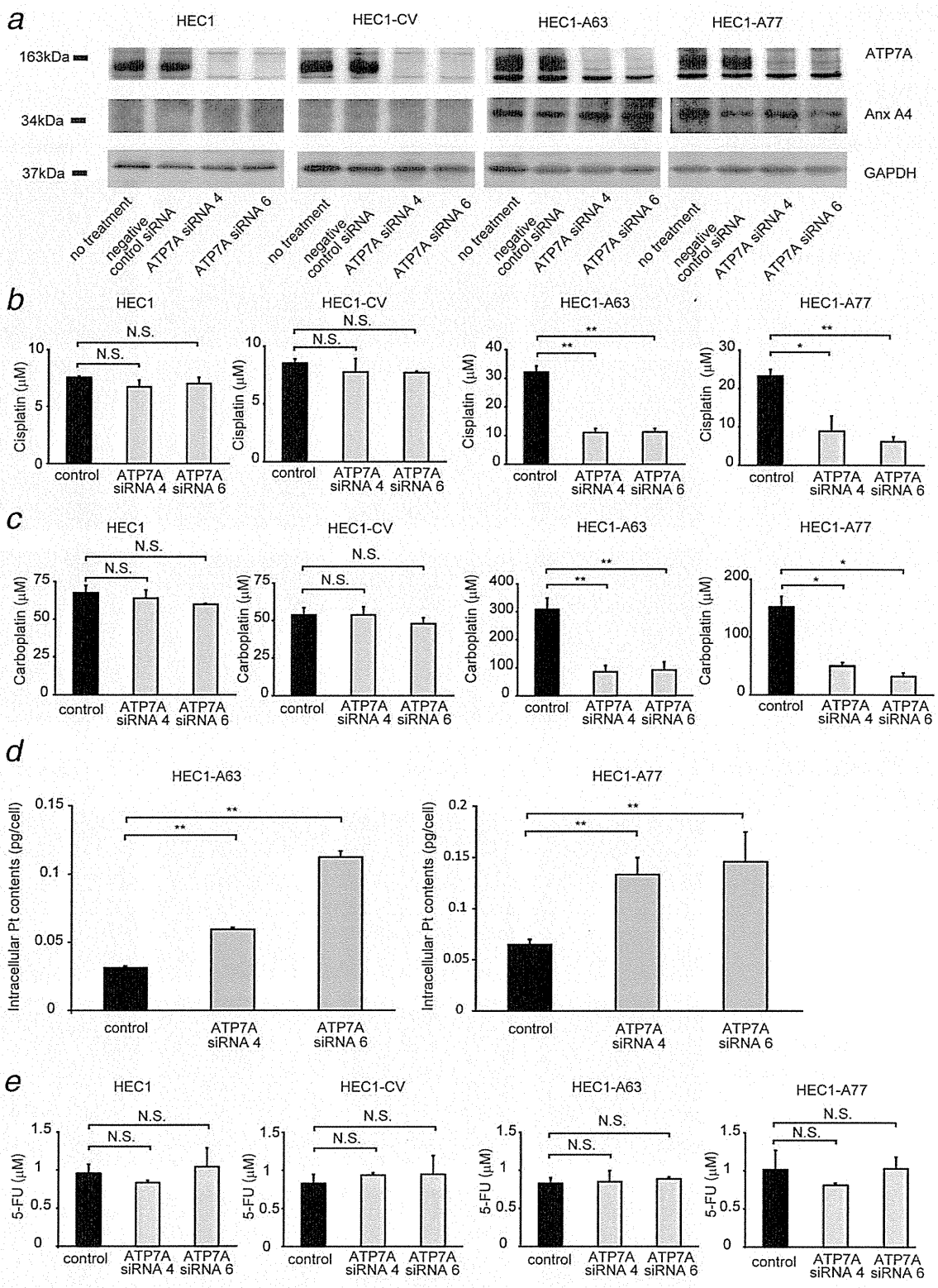

Figure 5. Knockdown of ATP7A expression improves platinum chemosensitivity in Anx A4-overexpressing cells. (*a*) Knockdown expression of ATP7A by siRNA in HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells by Western blot analysis. (*b*) IC_{50} values are shown for cisplatin in HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells transfected with negative control siRNA and two types of siRNA targeting ATP7A. A significant decrease in IC_{50} value for cisplatin was observed for the two types of ATP7A-silenced HEC1-A63 and HEC1-A77 cells but not for the HEC1 and HEC1-CV cells. (*c*) IC_{50} values are shown for carboplatin in HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells transfected with negative control siRNA and two kinds of siRNA targeting ATP7A. A significant decrease in IC_{50} value for carboplatin was observed for the two types of ATP7A-silenced HEC1-A63 and HEC1-A77 cells but not for the HEC1 and HEC1-CV cells. (*d*) Intracellular platinum content after treatment with 1 mM cisplatin for 60 min and further incubation with cisplatin-free medium for 180 min in D-MEM medium in HEC1-A63 cells and HEC1-A77 cells transfected with negative control siRNA and ATP7A-targeting siRNA, as determined by ICP-MS analysis. Significantly higher intracellular platinum accumulation was observed in HEC1-A63 cells and HEC1-A77 ATP7A-silencing cells than in control siRNA-transfected HEC1-A63 cells and HEC1-A77 cells. (*e*) No significant differences in IC_{50} values for 5-FU were noted between HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells. Similar results were observed in ATP7A-silenced cell lines for HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells (*$p < 0.05$; **$p < 0.01$; one-way ANOVA followed by Dunnett's analysis).

the HEC1-A63 control siRNA-transfected cells ($IC_{50} = 32.2$ μM) (Fig. 5*b*).

In addition to cisplatin, improved chemosensitivity associated with ATP7A silencing was observed with carboplatin. Significantly lower IC_{50} values for carboplatin were observed in both types of ATP7A-silenced HEC1-A63 cells (siRNA4, $IC_{50} = 85.9$ μM, $p < 0.01$; siRNA6, $IC_{50} = 92.8$ μM, $p < 0.01$) compared with the HEC1-A63 control siRNA-transfected cells ($IC_{50} = 300.7$ μM) (Fig. 5*c*). Similar results were found for HEC1-A77 ATP7A-silenced cells, where a significantly lower IC_{50} value for cisplatin was observed (siRNA4, $IC_{50} = 8.9$ μM, $p < 0.05$; siRNA6, $IC_{50} = 6.2$ μM, $p < 0.01$) compared with that for HEC1-A77 control siRNA-transfected cells ($IC_{50} = 23.3$ μM). IC_{50} values for carboplatin were also significantly lower for the two kinds of ATP7A-silenced HEC1-A77 cells (siRNA4, $IC_{50} = 49.8$ μM, $p < 0.05$; siRNA6, $IC_{50} = 31.9$ μM, $p < 0.05$) compared with the HEC1-A77 control siRNA-transfected cells ($IC_{50} = 152.1$ μM, $p < 0.01$) (Fig. 5*c*). In contrast, siRNA treatments targeting ATP7A were ineffective in HEC1 and HEC1-CV cells treated with cisplatin or carboplatin (Figs. 5*b* and 5*c*). Intracellular platinum accumulation after cisplatin exposure was significantly increased in HEC1-A63 cells treated with ATP7A siRNA (0.060 pg/cell, $p < 0.01$ to 0.113 pg/cell, $p < 0.01$) compared with control siRNA-transfected cells (0.030 pg/cell) (Fig. 5*d*). Similarly, a significant increase in intracellular platinum accumulation was observed in HEC1-A77 cells treated with ATP7A siRNA (0.133 pg/cell, $p < 0.01$ to 0.146 pg/cell, $p < 0.01$) compared with control siRNA-transfected cells (0.065 pg/cell) (Fig. 5*d*).

To investigate the relationship between resistance to drugs other than platinum drugs and Anx A4 or ATP7A expression, IC_{50} values for 5-FU were determined for each cell line. No significant change in IC_{50} values for 5-FU was observed in HEC1 ($IC_{50} = 0.96$ μM), HEC1-CV ($IC_{50} = 1.00$ μM), HEC1-A63 ($IC_{50} = 0.83$ μM) or HEC1-A77 cells (IC50 = 1.01 μM) (Fig. 5*e*). Similar results were observed in the ATP7A-silenced cell lines for HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells as well as in ATP7A-silenced cell lines (Fig. 5*e*). These results suggested that platinum resistance induced by enforced expression of Anx A4 was mainly dependent on the platinum transporter ATP7A and that expression of Anx A4 and ATP7A was unrelated to resistance to 5-FU.

Discussion

In our study, overexpression of Anx A4 in HEC1 cells decreased cell sensitivity to platinum drugs *in vitro*. Increased drug efflux was the mechanism underlying this change. In addition, an association between Anx A4 and platinum resistance was demonstrated for the first time *in vivo*. The mechanism of Anx A4-induced drug efflux may prove to be a promising therapeutic target because blockage of that mechanism may improve the prognosis of patients with Anx A4-associated platinum-resistant tumors.

Anx A4 itself is not a drug transporter, but it does bind to phospholipids in a Ca^{2+}-dependent manner and self-associates onto phospholipid membrane surfaces, causing membrane aggregation.[12,14–17] Thus, we assumed an indirect mediating effect of Anx A4 on drug efflux through an association between an unidentified drug transporter and Anx A4. Recently, MRP2 (an ABC ATPase-like multidrug-resistant protein) and ATP7A and ATP7B (two P-type Cu-transporting ATPases) were identified as platinum efflux transporters strongly associated with platinum resistance.[32,33] In an analysis of clinical gynecological samples, expression of MRP2 failed to predict tumor response to chemotherapy and did not correlate with overall survival.[34–36] In contrast, poor survival rates were associated with overexpression of ATP7A in patients with ovarian cancer.[27] Similarly, a correlation was found between ATP7B overexpression in endometrial carcinomas and an unfavorable clinical outcome in patients treated with cisplatin-based chemotherapy.[37] Therefore, we focused on the platinum transporters ATP7A and ATP7B and investigated their relationships with expression of Anx A4. In normal, unchallenged cells, ATP7A and ATP7B are localized in the Golgi apparatus and are involved in copper homeostasis, using ATP hydrolysis to transport copper ions across cellular membranes. They function in both the export of excess copper and its delivery to copper-dependent enzymes. ATP7A and ATP7B are also known to be efflux transporters of platinum drugs.[8,27,28,31,38,39] In one study, only a slight increase in expression of transfected ATP7A was seen in a human ovarian cancer cell line; however, that small increase was sufficient to confer significant resistance to cisplatin or carboplatin.[40] In a similar study in another human cisplatin-resistant ovarian cancer cell line, silencing of ATP7B by siRNA transfection resulted in a 2.5-fold decrease in cisplatin IC_{50} levels and a significant increase in DNA-platinum adduct formation.[41] Preparing CMF of treated cells facilitated the localization of Anx A4 expression in cells before and after exposure to platinum drugs. The abundance of AnxA4 in the membrane fraction along with the translocation to the membrane was increased. Using the orthogonal method of cell surface protein labeling to monitor proteins appearing on the cell surface, biotinylated ATP7A was increased after cisplatin or carboplatin exposure both in HEC1 and HEC1-CV cells (cells expressing low levels of Anx A4) and HEC1-A63 and HEC1-A77 cells (cells overexpressing Anx A4). Taken together, these results suggest that platinum drug exposure causes relocalization of Anx A4 expression to the membrane fraction and relocalization of ATP7A transporters (to a minimum) to the external surface of the cellular membrane. Unfortunately, no similar analysis of ATP7B was possible because it is not expressed in HEC1 cells (data not shown). However, in cells that express both ATP7A and ATP7B proteins, other immunofluorescence studies have shown similar changes in localization of both proteins after cisplatin exposure.[42] After cisplatin or carboplatin exposure in HEC1-A63 and HEC1-A77 Anx

A4-overexpressing cells, immunofluorescence showed that Anx A4 expression was relocated from the perinuclear and cytoplasmic Golgi regions to the cellular membrane. This relocalization was not observed in HEC1 and HEC1-CV cells, in which overexpression of Anx A4 does not occur.

ATP7A also relocates from the perinuclear and cytoplasmic regions to the cellular membrane after cisplatin or carboplatin exposure. However, this occurs both in HEC1 and HEC1-CV cells (cells expressing low levels of Anx A4) and HEC1-A63 and HEC1-A77 cells (cells overexpressing Anx A4). Although no direct interaction between ATP7A and Anx A4 was detected by coimmunoprecipitation analysis (data not shown), immunofluorescence analysis showed colocalization of ATP7A and Anx A4 at least within the cellular membrane in Anx A4-overexpressing cells. These results suggested that Anx A4 is not required for ATP7A translocation and that ATP7A translocation is unrelated to expression of Anx A4.

Translocation of Anx A4 to plasma membranes is reportedly mediated by an increase in intracellular free Ca^{2+}, which is increased by exposure to platinum drugs.[43,44] In addition to the translocation of ATP7A and Anx A4 to the plasma membrane, our results also showed translocation of ATP7A to the nucleus in HEC1 and HEC1-CV cells. Translocation to the nucleus and colocalization of both ATP7A and Anx A4 were also observed in HEC1-A63 and HEC1-A77 cells after exposure to cisplatin or carboplatin in the immunofluorescence staining analysis in our study (Fig. 4). Anx A4 translocates to the nucleus after etoposide treatment and suppresses NF-κB transcriptional activity, which induces expression of Bax, a proapoptotic Bcl-2 family protein.[18] In addition, a correlation has been reported between nuclear staining of Anx A4 and poor survival in patients with ovarian cancer.[45] However, the role of ATP7A in the nucleus and its relationship with NF-κB transcriptional activity has not been investigated. Further investigation is needed to elucidate the role of nuclear colocalization of Anx A4 and ATP7A in platinum resistance.

In our study, translational silencing of ATP7A in HEC1 and HEC1-CV (Anx A4-nonexpressing cells) and HEC1-A63 and HEC1-A77 cells (Anx A4-overexpressing cells) was performed. Western blot analysis demonstrated no detectable changes in protein expression of Anx A4 when ATP7A was silenced in any of these four cell lines.

In HEC1 and control HEC1-CV cells (low Anx A4 expression levels), IC_{50} values for cisplatin or carboplatin cells after the knockdown of ATP7A expression caused no improvement in the sensitivity of these cells to cisplatin or carboplatin. Similar results were observed in a previous study in which no improvement in sensitivity to cisplatin resulted from silencing of ATP7A in platinum-resistant or -sensitive ovarian cancer cell lines.[41] However, Mangala *et al.* reported improved sensitivity to cisplatin in both platinum-resistant ovarian cancer cells and parental cells expressing ATP7B as a result of silencing of ATP7B expression.[41] An important discovery related to ATP7A was communicated in our study: in cells overexpressing both Anx A4 and ATP7A, silencing of ATP7A significantly improved sensitivity to cisplatin and carboplatin, thus restoring them to sensitivity levels comparable to those of HEC1 and HEC1-CV cells. These results were supported by a quantitative analysis of the accumulation of intracellular platinum, demonstrating that siRNA silencing of ATP7A in Anx A4-overexpressing HEC1-A63 and HEC1-A77 cells resulted in greater intracellular platinum accumulation than HEC1-A63 and HEC1-A77 cells transfected with a control siRNA. On the other hand, the analysis of IC_{50} values for 5-FU showed no relationship between overexpression of Anx A4 and resistance to 5-FU. In addition, no improvement in sensitivity to 5-FU was observed as a result of ATP7A silencing. These results suggested a specific relationship of Anx A4 with ATP7A and resistance to platinum drugs but with to nonplatinum drugs such as 5-FU. Differences in efficacy and improvement in drug sensitivity of ATP7A silencing were observed between cell lines (HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells). These variations may be related to the colocalization of Anx A4 and ATP7A in the cellular membrane after cisplatin or carboplatin exposure. Colocalization of Anx A4 and ATP7A after exposure to platinum drugs was specific to changes in Anx A4-overexpressing cells, which are probably related to drug efflux. These results suggest that in conjunction with higher Anx A4 expression levels, ATP7A had a positive effect on efflux of platinum drugs, resulting in significantly increased platinum resistance. Because overexpression of Anx A4 had no effect on ATP7A expression and because no direct interaction between ATP7A and Anx A4 was detected in the coimmunoprecipitation analysis, Anx A4 seems to promote ATP7A activity in a manner which is currently unexplained.

In addition to the effects of Anx A4 on drug resistance in ovarian cancer, similar findings have been reported for other overexpressed members of the Annexin family such as Annexin A3 (Anx A3).[46,47] Intracellular platinum concentrations of cisplatin and levels of platinum DNA binding in that study were significantly lower in Anx A3-overexpressing cells than in control cells, suggesting a more general involvement of the Annexin family in platinum resistance.[46] From the results of these related reports and those of our study, we conclude that the Annexin family may potentially enhance the activity of numerous drug transporters. Identifying these enhancement mechanisms may be extremely useful for developing additional therapeutic targets for drug-resistant tumors.

In summary, our study demonstrated that enhanced expression of Anx A4 induces chemoresistance by promoting platinum drug efflux *via* ATP7A. These findings suggested that Anx A4 is a potential therapeutic target for chemosensitization, particularly in tumors with higher expression of both Anx A4 and ATP7A. Thus, our study provides a clear example of applied genotoxicology. However, platinum resistance induced by overexpression of Anx A4 may occur as a

result of multiple processes, including regulation of apoptosis and efflux of platinum drugs. Thus, other unknown chemoresistant mechanisms may be induced by overexpression of Anx A4. Because overexpression of Anx A4 has been reported in several other types of clinically important cancers, such as rectal, renal, lung and pancreatic cancer,[19–23] targeting Anx A4 may lead to the development of an effective therapy for overcoming chemoresistance in more types of cancer.

Acknowledgements

The authors thank Y. Kanazawa and S. Sugiyama for their secretarial assistance, M. Urase for technical assistance and Dr. G.S. Buzard for helpful editing.

References

1. Omura G, Blessing JA, Ehrlich CE, et al. A randomized trial of cyclophosphamide and doxorubicin with or without cisplatin in advanced ovarian carcinoma. *A Gynecologic Oncology Group Study. Cancer* 1986;57:1725–30.
2. Thigpen T, Vance R, Puneky L, et al. Chemotherapy in advanced ovarian carcinoma: current standards of care based on randomized trials. *Gynecol Oncol* 1994;55:S97–S107.
3. Vaughan S, Coward JI, Bast RC, Jr, et al. Rethinking ovarian cancer: recommendations for improving outcomes. *Nat Rev Cancer* 2011;11: 719–25.
4. Fleming GF, Brunetto VL, Cella D, et al. Phase III trial of doxorubicin plus cisplatin with or without paclitaxel plus filgrastim in advanced endometrial carcinoma: a Gynecologic Oncology Group Study. *J Clin Oncol* 2004;22:2159–66.
5. Hoskins PJ, Swenerton KD, Pike JA, et al. Paclitaxel and carboplatin, alone or with irradiation, in advanced or recurrent endometrial cancer: a phase II study. *J Clin Oncol* 2001;19:4048–53.
6. Obel JC, Friberg G, Fleming GF. Chemotherapy in endometrial cancer. *Clin Adv Hematol Oncol* 2006;4:459–68.
7. Enomoto T, Kuragaki C, Yamasaki M, et al. Is clear cell carcinoma and mucinous carcinoma of the ovary sensitive to combination chemotherapy with paclitaxel and carboplatin? *Proc Am Soc Clin Oncol* 2003;22:(abstr 1797).
8. Nakayama K, Kanzaki A, Terada K, et al. Prognostic value of the Cu-transporting ATPase in ovarian carcinoma patients receiving cisplatin-based chemotherapy. *Clin Cancer Res* 2004;10: 2804–11.
9. Pectasides D, Fountzilas G, Aravantinos G, et al. Advanced stage clear-cell epithelial ovarian cancer: the Hellenic Cooperative Oncology Group experience. *Gynecol Oncol* 2006;102: 285–91.
10. Goff BA, Sainz de la Cuesta R, Muntz HG, et al. Clear cell carcinoma of the ovary: a distinct histologic type with poor prognosis and resistance to platinum-based chemotherapy in stage III disease. *Gynecol Oncol* 1996;60:412–17.
11. Sugiyama T, Kamura T, Kigawa J, et al. Clinical characteristics of clear cell carcinoma of the ovary: a distinct histologic type with poor prognosis and resistance to platinum-based chemotherapy. *Cancer* 2000;88:2584–9.
12. Kim A, Enomoto T, Serada S, et al. Enhanced expression of Annexin A4 in clear cell carcinoma of the ovary and its association with chemoresistance to carboplatin. *Int J Cancer* 2009;125: 2316–22.
13. Miao Y, Cai B, Liu L, et al. Annexin IV is differentially expressed in clear cell carcinoma of the ovary. *Int J Gynecol Cancer* 2009;19:1545–9.
14. Gerke V, Moss SE. Annexins: from structure to function. *Physiol Rev* 2002;82:331–71.
15. Kaetzel MA, Hazarika P, Dedman JR. Differential tissue expression of three 35-kDa annexin calcium-dependent phospholipid-binding proteins. *J Biol Chem* 1989;264:14463–70.
16. Kaetzel MA, Mo YD, Mealy TR, et al. Phosphorylation mutants elucidate the mechanism of annexin IV-mediated membrane aggregation. *Biochemistry* 2001;40:4192–9.
17. Kim A, Serada S, Enomoto T, et al. Targeting annexin A4 to counteract chemoresistance in clear cell carcinoma of the ovary. *Expert Opin Ther Targets* 2010;14:963–71.
18. Jeon YJ, Kim DH, Jung H, et al. Annexin A4 interacts with the NF-kappaB p50 subunit and modulates NF-kappaB transcriptional activity in a Ca2+-dependent manner. *Cell Mol Life Sci* 2010; 67:2271–81.
19. Alfonso P, Canamero M, Fernandez-Carbonie F, et al. Proteome analysis of membrane fractions in colorectal carcinomas by using 2D-DIGE saturation labeling. *J Proteome Res* 2008;7:4247–55.
20. Duncan R, Carpenter B, Main LC, et al. Characterisation and protein expression profiling of annexins in colorectal cancer. *Br J Cancer* 2008; 98:426–33.
21. Sitek B, Luttges J, Marcus K, et al. Application of fluorescence difference gel electrophoresis saturation labelling for the analysis of microdissected precursor lesions of pancreatic ductal adenocarcinoma. *Proteomics* 2005;5:2665–79.
22. Zimmermann U, Balabanov S, Giebel J, et al. Increased expression and altered location of annexin IV in renal clear cell carcinoma: a possible role in tumour dissemination. *Cancer Lett* 2004;209:111–18.
23. Wei R, Zhang Y, Shen L, et al. Comparative proteomic and radiobiological analyses in human lung adenocarcinoma cells. *Mol Cell Biochem* 2012;359:151–9.
24. Furukawa T, Komatsu M, Ikeda R, et al. Copper transport systems are involved in multidrug resistance and drug transport. *Curr Med Chem* 2008;15:3268–78.
25. Gourdon P, Liu XY, Skjorringe T, et al. Crystal structure of a copper-transporting PIB-type ATPase. *Nature* 2011;475:59–64.
26. Owatari S, Akune S, Komatsu M, et al. Copper-transporting P-type ATPase, ATP7A, confers multidrug resistance and its expression is related to resistance to SN-38 in clinical colon cancer. *Cancer Res* 2007;67:4860–8.
27. Samimi G, Varki NM, Wilczynski S, et al. Increase in expression of the copper transporter ATP7A during platinum drug-based treatment is associated with poor survival in ovarian cancer patients. *Clin Cancer Res* 2003;9:5853–9.
28. Safaei R, Holzer AK, Katano K, et al. The role of copper transporters in the development of resistance to Pt drugs. *J Inorg Biochem* 2004;98: 1607–13.
29. Iwahori K, Serada S, Fujimoto M, et al. SOCS-1 gene delivery cooperates with cisplatin plus pemetrexed to exhibit preclinical antitumor activity against malignant pleural mesothelioma. *Int J Cancer* 2013;132:459–71.
30. Khunweeraphong N, Nagamori S, Wiriyasermkul P, et al. Establishment of stable cell lines with high expression of heterodimers of human 4F2hc and human amino acid transporter LAT1 or LAT2 and delineation of their differential interaction with (alpha)-alkyl moieties. *J Pharmacol Sci* 2012;119:368–80.
31. Rabik CA, Maryon EB, Kasza K, et al. Role of copper transporters in resistance to platinating agents. *Cancer Chemother Pharmacol* 2009;64: 133–42.
32. Galluzzi L, Senovilla L, Vitale I, et al. Molecular mechanisms of cisplatin resistance. *Oncogene* 2012;31:1869–83.
33. Kelland L. The resurgence of platinum-based cancer chemotherapy. *Nat Rev Cancer* 2007;7:573–84.
34. Arts HJ, Katsaros D, de Vries EG, et al. Drug resistance-associated markers P-glycoprotein, multidrug resistance-associated protein 1, multidrug resistance-associated protein 2, and lung resistance protein as prognostic factors in ovarian carcinoma. *Clin Cancer Res* 1999;5:2798–805.
35. Guminski AD, Balleine RL, Chiew YE, et al. MRP2 (ABCC2) and cisplatin sensitivity in hepatocytes and human ovarian carcinoma. *Gynecol Oncol* 2006;100:239–46.
36. Materna V, Pleger J, Hoffmann U, et al. RNA expression of MDR1/P-glycoprotein, DNA-topoisomerase I, and MRP2 in ovarian carcinoma patients: correlation with chemotherapeutic response. *Gynecol Oncol* 2004;94:152–60.
37. Aida T, Takebayashi Y, Shimizu T, et al. Expression of copper-transporting P-type adenosine triphosphatase (ATP7B) as a prognostic factor in human endometrial carcinoma. *Gynecol Oncol* 2005;97:41–5.
38. Katano K, Kondo A, Safaei R, et al. Acquisition of resistance to cisplatin is accompanied by changes in the cellular pharmacology of copper. *Cancer Res* 2002;62:6559–65.
39. Kuo MT, Chen HH, Song IS, et al. The roles of copper transporters in cisplatin resistance. *Cancer Metastasis Rev* 2007;26:71–83.
40. Samimi G, Safaei R, Katano K, et al. Increased expression of the copper efflux transporter ATP7A mediates resistance to cisplatin, carboplatin, and oxaliplatin in ovarian cancer cells. *Clin Cancer Res* 2004;10:4661–9.
41. Mangala LS, Zuzel V, Schmandt R, et al. Therapeutic targeting of ATP7B in ovarian carcinoma. *Clin Cancer Res* 2009;15:3770–80.
42. Kalayda GV, Wagner CH, Buss I, et al. Altered localisation of the copper efflux transporters ATP7A and ATP7B associated with cisplatin

resistance in human ovarian carcinoma cells. *BMC Cancer* 2008;8:175.
43. Al-Bahlani S, Fraser M, Wong AY, et al. P73 regulates cisplatin-induced apoptosis in ovarian cancer cells via a calcium/calpain-dependent mechanism. *Oncogene* 2011;30:4219–30.
44. Splettstoesser F, Florea AM, Busselberg D. IP(3) receptor antagonist, 2-APB, attenuates cisplatin induced Ca2+-influx in HeLa-S3 cells and prevents activation of calpain and induction of apoptosis. *Br J Pharmacol* 2007;151: 1176–86.
45. Choi CH, Sung CO, Kim HJ, et al. Overexpression of annexin A4 is associated with chemoresistance in papillary serous adenocarcinoma of the ovary. *Hum Pathol* 2013;44:1017–23.
46. Yan X, Yin J, Yao H, et al. Increased expression of annexin A3 is a mechanism of platinum resistance in ovarian cancer. *Cancer Res* 2010;70:1616–24.
47. Yin J, Yan X, Yao X, et al. Secretion of annexin A3 from ovarian cancer cells and its association with platinum resistance in ovarian cancer patients. *J Cell Mol Med* 2012;16:337–48.

VOLUME 31 · NUMBER 28 · OCTOBER 1 2013

JOURNAL OF CLINICAL ONCOLOGY

DIAGNOSIS IN ONCOLOGY

Distinguishing Between Lymphangioleiomyomatosis and Carcinomatous Peritonitis in a Patient With Ovarian Cancer

Case Report

A 48-year-old woman with no notable medical or family history visited our hospital with a complaint of abdominal bloating. An ultrasonography scan showed severe accumulation of ascites in her pelvis, and magnetic resonance imaging revealed a solid mass measuring 15 cm in diameter. The mass was assumed to be ovarian cancer (OC) accompanied by carcinomatous peritonitis.

We performed abdominal total hysterectomy, bilateral salpingo-oophorectomy, pelvic lymphadenectomy, para-aortic lymphadenectomy, and omentectomy as staging laparotomy and debulking surgery for OC. The left ovary measured 15 cm in diameter, and the tumor was present on its surface. The volume of ascites was 8,250 mL, although neither peritoneal metastasis nor carcinomatous peritonitis was noted. A careful examination of the peritoneal cytology, involving the centrifuged pellet from almost all of the ascites, failed to detect any malignant cells. Pathologic examination confirmed that the pelvic mass was OC (endometrioid adenocarcinoma, G1), and the International Federation of Gynecology and Obstetrics stage was Ic(a). No metastasis was detected in the omentum, lymph nodes, and lymph duct or vessels. However, pathologic examination incidentally revealed lymphangioleiomyomatosis (LAM) in the lymph nodes and lymphatic vessels (Fig 1). Hematoxylin and eosin staining revealed oval to short spindle-shaped smooth muscle-like cells (LAM cells) aggregating and proliferating in a nodular pattern within lymph ducts and vessels (Fig 1A). The LAM cells were positive for smooth muscle actin, HMB-45 (Fig 1B), estrogen receptor, and progesterone receptor. High-resolution computed tomography of the chest revealed six small (2 to 5 mm), thin-walled cysts in the upper lobes of both lungs before the operation (Fig 2A, arrows). Pathologic examination of the lymph nodes and lymphatic vessels indicated that the pulmonary cysts were LAM lesions. In addition, a sarcoid-like reaction, which contained noncaseating epithelioid cells, was occasionally detected in regional lymph nodes (Fig 1C). This case did not fulfill the criteria for systemic sarcoidosis, and there was no evidence of metastatic ovarian adenocarcinoma.

Cell count, biochemical analysis, culture, polymerase chain reaction for *Mycobacterium tuberculosis*, and cytologic examination of ascitic fluid were performed for differential diagnosis of the massive ascites. Abdominal ultrasonography and computed tomography were also performed to identify the cause of ascites or findings suggestive of portal hypertension. Bacterial peritonitis, tuberculous peritonitis, and portal hypertension were not considered likely causes. Abdominocentesis was frequently performed postoperatively to prevent abdominal distention and to facilitate peritoneal cytology. No malignant cells were detected after any of these procedures.

This patient was a suitable candidate for adjuvant chemotherapy.[1-5] Paclitaxel (180 mg/m^2) and carboplatin (area under the curve, 6) were administered once every 3 weeks. A severe adverse event (grade 3 neuropathy as per the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0) occurred after three cycles had been administered, and chemotherapy was therefore discontinued.

In this patient, ascites had markedly diminished at 3 months and disappeared at 6 months after the operation. Moreover, lung cysts and pulmonary function did not subsequently worsen. No remarkable changes in either the size or number of cysts were observed 5 years later (Fig 2B, arrows). Figure 3 shows the postoperative changes in pulmonary function expressed as a percentage of predicted forced vital capacity (FVC) and forced expiratory volume in the first second (FEV1). These pulmonary function tests subsequently improved. The patient remained well 5 years after the operation with neither recurrence nor LAM symptoms.

Discussion

LAM is a rare progressive disease that occurs almost exclusively in women.[6] Although most patients with LAM demonstrate pulmonary

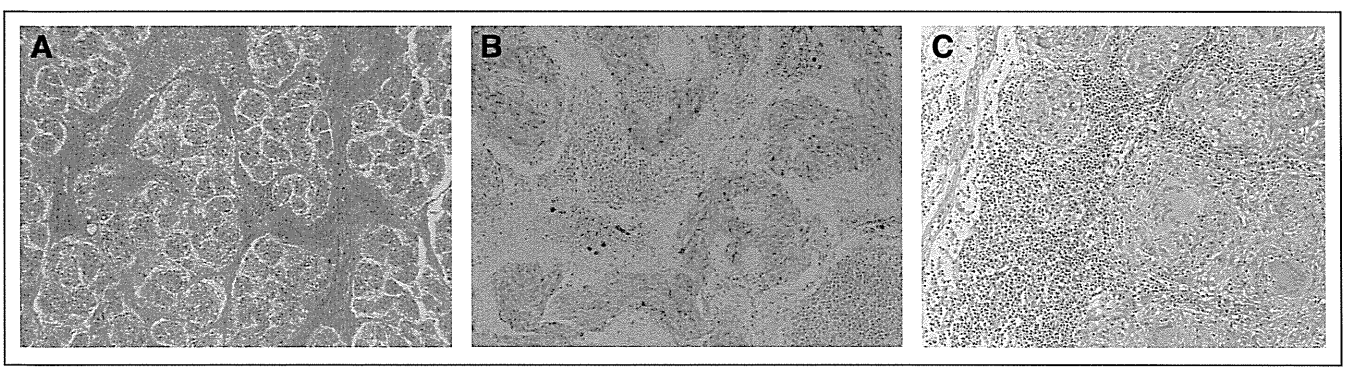

Fig 1.

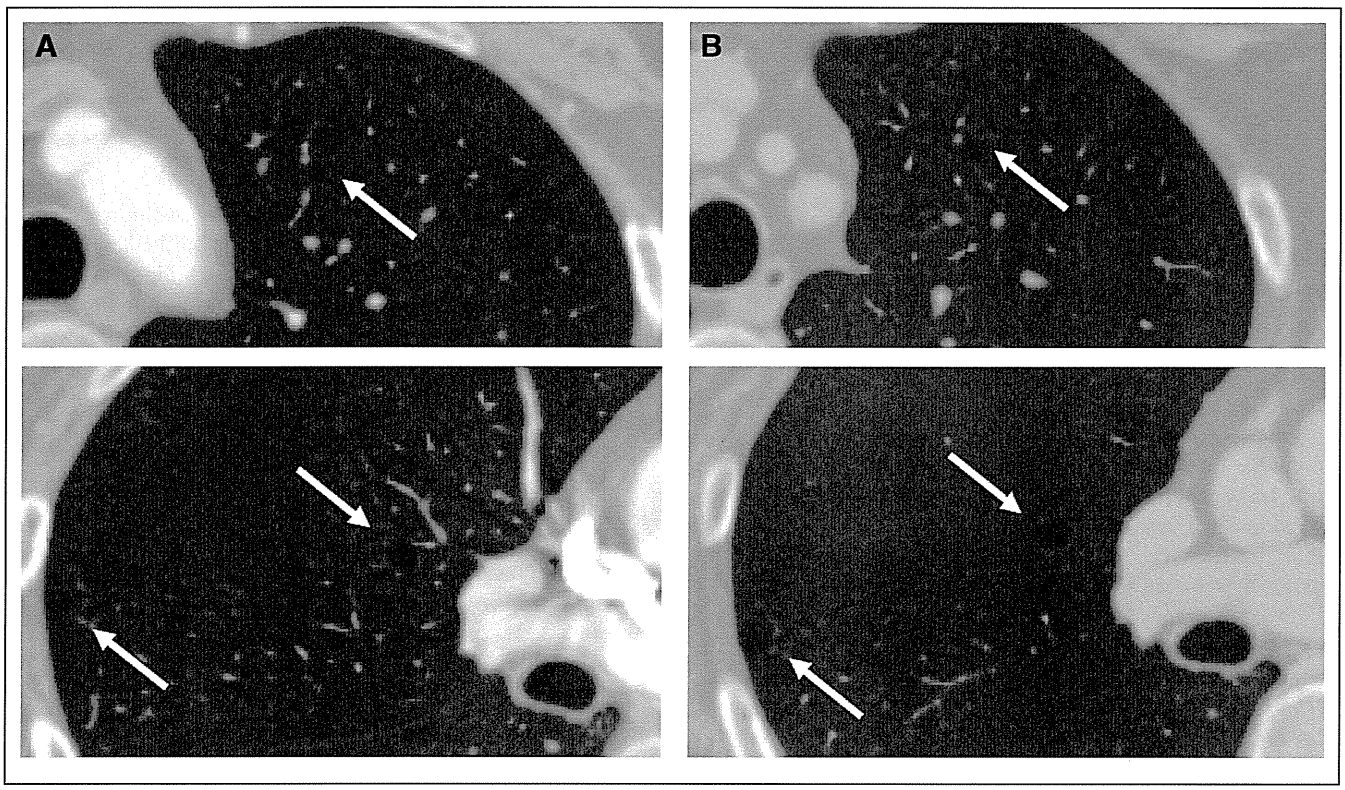

Fig 2.

involvement, patients with retroperitoneal or pelvic involvement at lymph node sites have been reported.[7] Gynecologic diseases can sometimes display symptoms that are similar to those of LAM when ascites is present, and a careful differential diagnosis is warranted.[7,8] Furthermore, LAM is occasionally complicated by tuberous sclerosis,[6] but tuberous sclerosis findings were not noted in our patient.

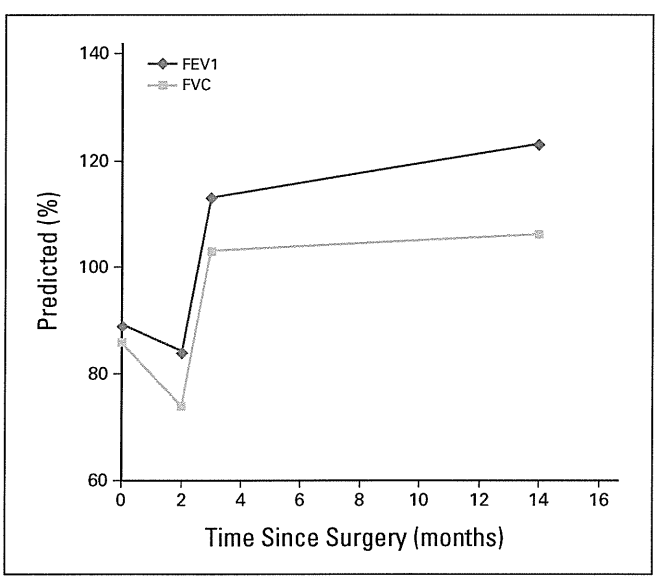

Fig 3.

To date, there is no consensus regarding the most appropriate therapeutic strategies for LAM, although hormone suppression therapy such as oophorectomy, gonadotropin-releasing hormone agonist treatment, or progesterone treatment can relieve the symptoms of this disease. Female sex hormones are probably associated with the occurrence and progression of LAM; thus, hormone suppression therapy has frequently been performed, which reportedly inhibits deterioration of respiratory function and may even stabilize it.[9,10] Although an effective therapy for reliably preventing the progression of LAM has not been identified, hormone suppression therapy is still administered. LAM frequently occurs in women of reproductive age, and the application of oophorectomy for the treatment of LAM has been strictly limited, given that an attempt must be made to preserve fertility. However, in our patient, oophorectomy was performed as the standard therapy for OC, which in turn resulted in female hormone suppression and apparently contributed to the alleviation of LAM symptoms, improved respiratory function, and reduced ascites. Because the patient was a never-smoker and had no symptoms of Sjögren's syndrome or any other cystic lung disease, we concluded that the cystic lesion in the lung was probably related to LAM.

Recently, a number of clinical trials on sirolimus (rapamycin) for the treatment of OC have been undertaken.[11] Sirolimus inhibits the mammalian target of rapamycin pathway and has an immunosuppressive effect, and its efficacy and safety for LAM have also been tested.[12]

OC is often referred to as a silent killer because it is usually only detected in advanced stages, given that the early stages of the disease are frequently asymptomatic. Clinicians should therefore consider the

possibility of LAM in the differential diagnosis of OC and tumors with unknown primary origin, especially when ascites is present.[7,8] Although Pseudo Meigs' syndrome was a possible cause of ascites accumulation in our patient, it was relatively unlikely because large amounts of ascites are not usually associated with OC. Given that repeated cytologic examinations of the ascites failed to detect any malignant cells and that the lung lesions did not progress, we speculate that the massive ascites accumulation was caused not by OC but by LAM, and that the ascites diminished as a result of the oophorectomy.

Sarcoid-like reaction refers to the development of noncaseating epithelioid cell granulomas in patients who do not fulfill the criteria for systemic sarcoidosis. It is occasionally detected in a variety of malignant diseases, occurring in 4% to 6% of carcinomas, and is most commonly observed in the lymph nodes draining the cancer.[13,14] However, it has been reported in association with OC in only a few cases.[15,16]

There are a few reports of LAM in women in whom OC was initially suspected because of massive ascites.[8] The case presented here may be a rare example of OC coexisting with LAM and sarcoid-like reaction, in which massive ascites resulting from LAM led to the diagnosis of OC. In addition, it demonstrates that oophorectomy may inhibit deterioration of respiratory function and may even stabilize it. Although LAM is rare, we suggest that clinicians should include it as part of the differential diagnosis of massive ascites in women patients.

Akira Hirasawa, Takashi Sato, Mari Ueno, Tomoko Akahane, Nobuyuki Susumu, Tomoko Betsuyaku, and Daisuke Aoki

School of Medicine, Keio University, Tokyo, Japan

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

REFERENCES

1. Trimbos JB, Parmar M, Vergote I, et al: International Collaborative Ovarian Neoplasm trial 1 and Adjuvant ChemoTherapy in Ovarian Neoplasm trial: Two parallel randomized phase III trials of adjuvant chemotherapy in patients with early-stage ovarian carcinoma. J Natl Cancer Inst 95:105-112, 2003

2. Colombo N, Guthrie D, Chiari S, et al: International Collaborative Ovarian Neoplasm trial 1: A randomized trial of adjuvant chemotherapy in women with early-stage ovarian cancer. J Natl Cancer Inst 95:125-132, 2003

3. Trimbos JB, Vergote I, Bolis G, et al: Impact of adjuvant chemotherapy and surgical staging in early-stage ovarian carcinoma: European Organisation for Research and Treatment of Cancer Adjuvant ChemoTherapy in Ovarian Neoplasm trial. J Natl Cancer Inst 95:113-125, 2003

4. Vasey PA, Jayson GC, Gordon A, et al: Phase III randomized trial of docetaxel-carboplatin versus paclitaxel-carboplatin as first-line chemotherapy for ovarian carcinoma. J Natl Cancer Inst 96:1682-1691, 2004

5. Japan Society of Gynecologic Oncology: Ovarian cancer treatment guidelines 2004. Tokyo, Japan, Kanehara, 2001, pp 12-29

6. Blei F: Lymphangiomatosis: Clinical overview. Lymphat Res Biol 9:185-190, 2011

7. Matsui K, Tatsuguchi A, Valencia J, et al: Extrapulmonary lymphangioleiomyomatosis (LAM): Clinicopathologic features in 22 cases. Hum Pathol 31:1242-1248, 2000

8. Yamashita S, Nakamura K, Shinozaki H, et al: Lymphangioleiomyomatosis suspected to be a gynecologic disease. J Obstet Gynaecol Res 37:267-269, 2011

9. Eliasson AH, Phillips YY, Tenholder MF: Treatment of lymphangioleiomyomatosis: A meta-analysis. Chest 96:1352-1355, 1989

10. Rossi GA, Balbi B, Oddera S, et al: Response to treatment with an analog of the luteinizing-hormone-releasing hormone in a patient with pulmonary lymphangioleiomyomatosis. Am Rev Respir Dis 143:174-176, 1991

11. US National Institutes of Health: Clinical Trials.gov. http://clinicaltrials.gov/

12. McCormack FX, Inoue Y, Moss J, et al: Efficacy and safety of sirolimus in lymphangioleiomyomatosis. N Engl J Med 364:1595-1606, 2011

13. Cohen PR, Kurzrock R: Sarcoidosis and malignancy. Clin Dermatol 25:326-333, 2007

14. Brincker H: Sarcoid reactions in malignant tumours. Cancer Treat Rev 13:147-156, 1986

15. Volckaert A, Van der Niepen P, De Hou MF, et al: Association of sarcoid like lesions in the bone marrow with an adenocarcinoma of the ovary: Report of a case. Acta Clin Belg 41:426-430, 1986

16. Montag TW, Dyer LL, Spirtos NM, et al: Sarcoid-like lesions associated with epithelial ovarian adenocarcinoma. Obstet Gynecol 78:978-980, 1991

DOI: 10.1200/JCO.2012.45.3019; published online ahead of print at www.jco.org on June 10, 2013