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

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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
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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.

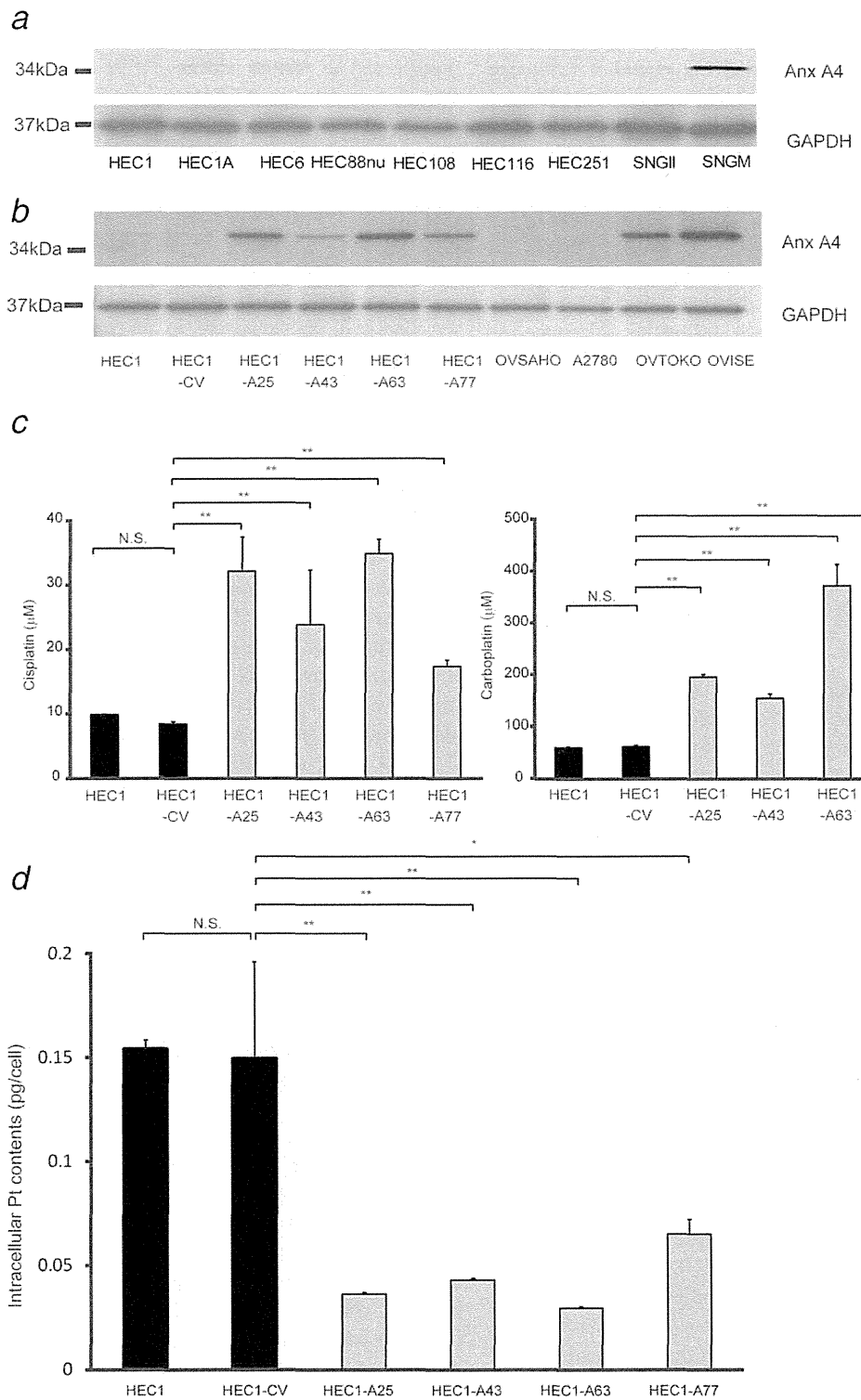

Figure 1. Enforced expression of Anx A4 in HEC1 cells confers platinum resistance *in vitro*. (*a*) Western blot analysis of nine endometrial carcinoma cell lines. Anx A4 was expressed in one cell line. (*b*) Establishment of an Anx A4-stably-expressing HEC1 cell line by transfection with the pcDNA3.1-Anx 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 ± 950 mm^3 in PBS-treated HEC1-CV control mice and 3,554 ± 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 ± 160 mm^3 in the PBS-treated group and only slightly less (7,078 ± 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

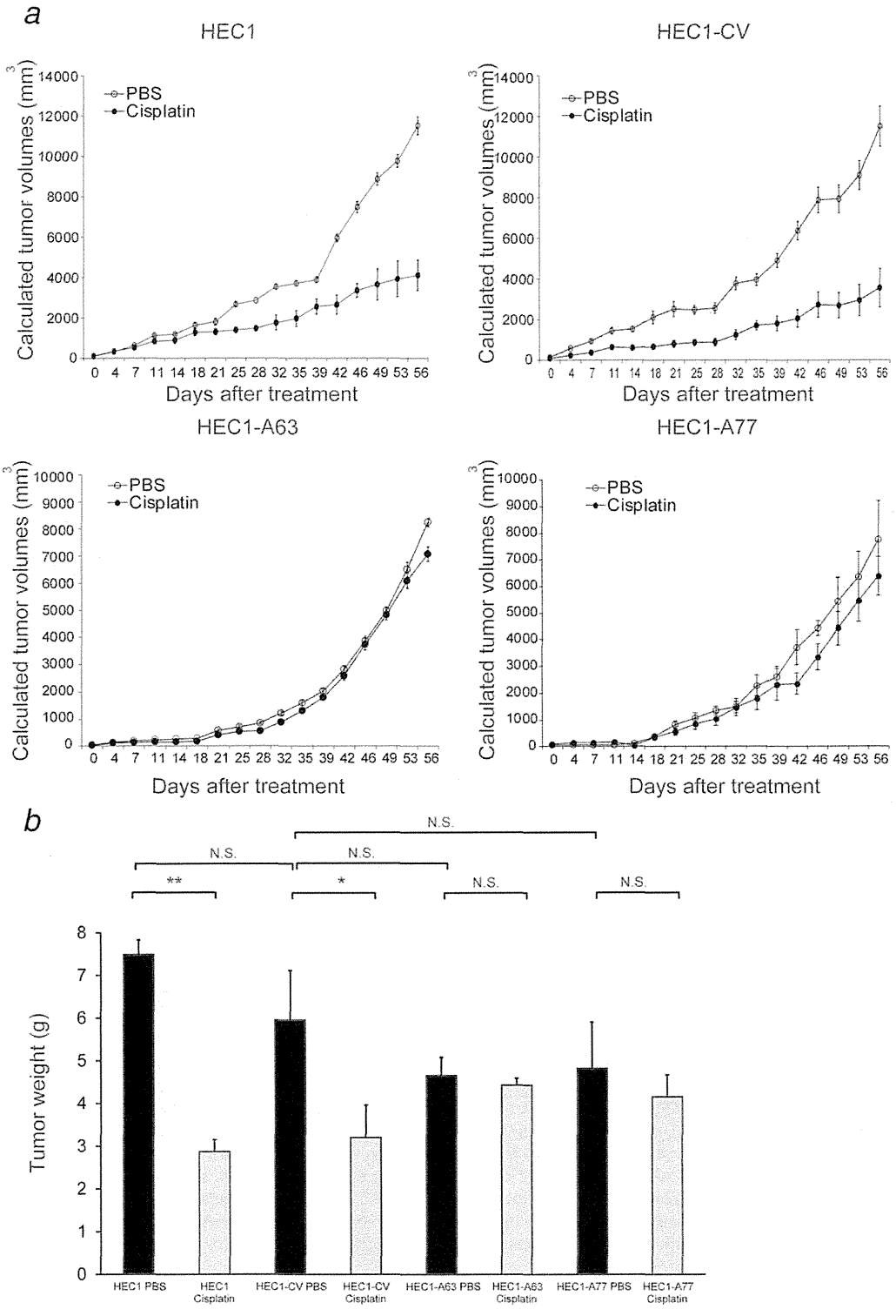

Figure 2. Enforced expression of Anx A4 in HEC1 cells confers platinum resistance *in vivo*. Analysis of Anx A4 as a platinum-resistant protein *in vivo*. (*a*) To determine the resistance of Anx A4-stably-expressing HEC1 cells to platinum *in vivo*, parent HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells 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).

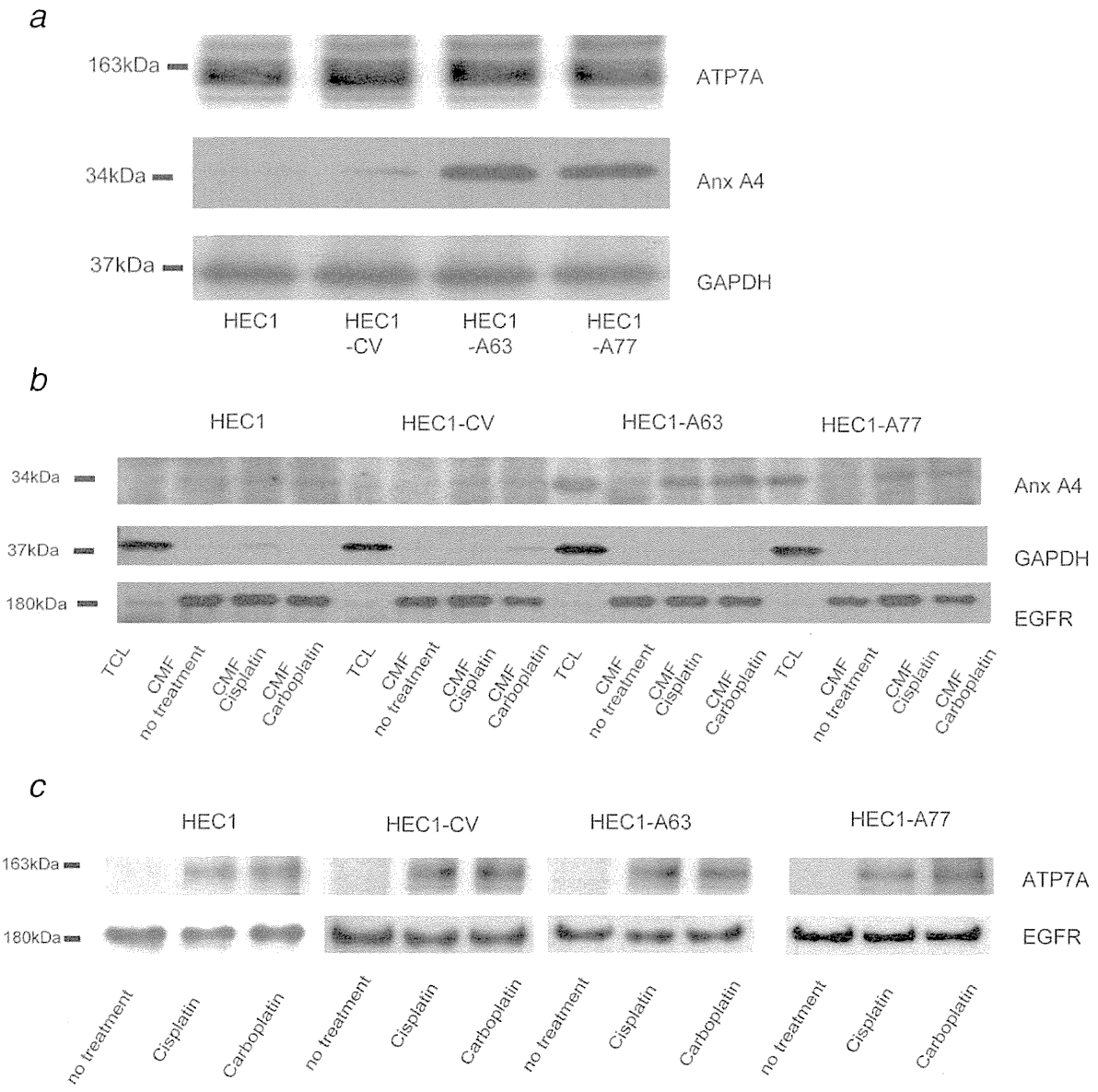

Figure 3. Localization of Anx A4 and ATP7A was investigated using Western blot analysis. The localization of Anx A4 and ATP7A was investigated using two techniques: orthogonal crude membrane fractions and biotinylation of cell surface proteins. (*a*) No significant change in expression levels of ATP7A 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

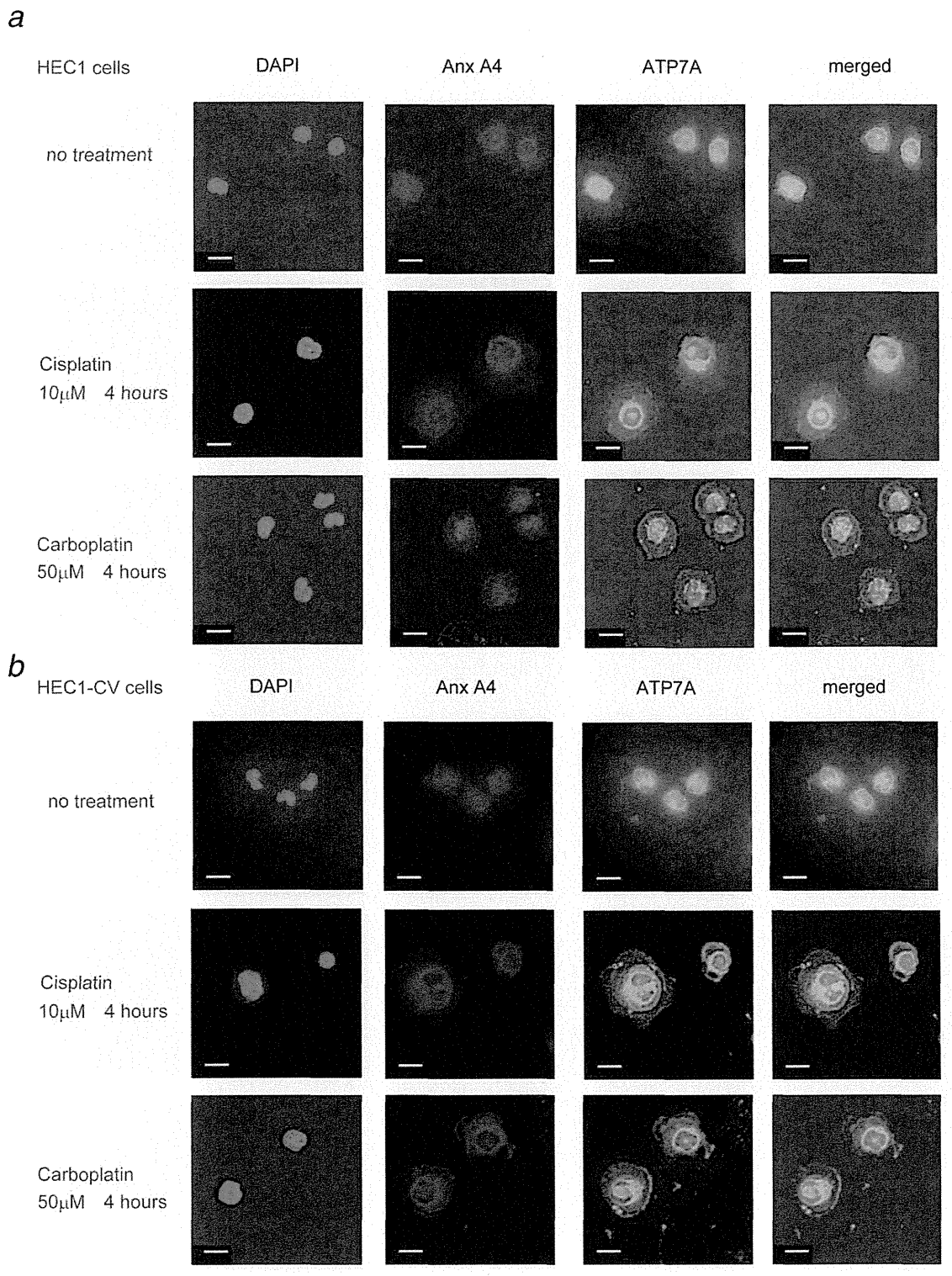

Figure 4. Immunofluorescence staining for ATP7A and Anx A4. HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells were divided into three groups: the no treatment, cisplatin exposure and carboplatin exposure groups. (*a*) HEC1 cells, (*b*) HEC1-CV cells, (*c*) HEC1-A63 cells and (*d*) HEC1-A77 cells. Cells were 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.

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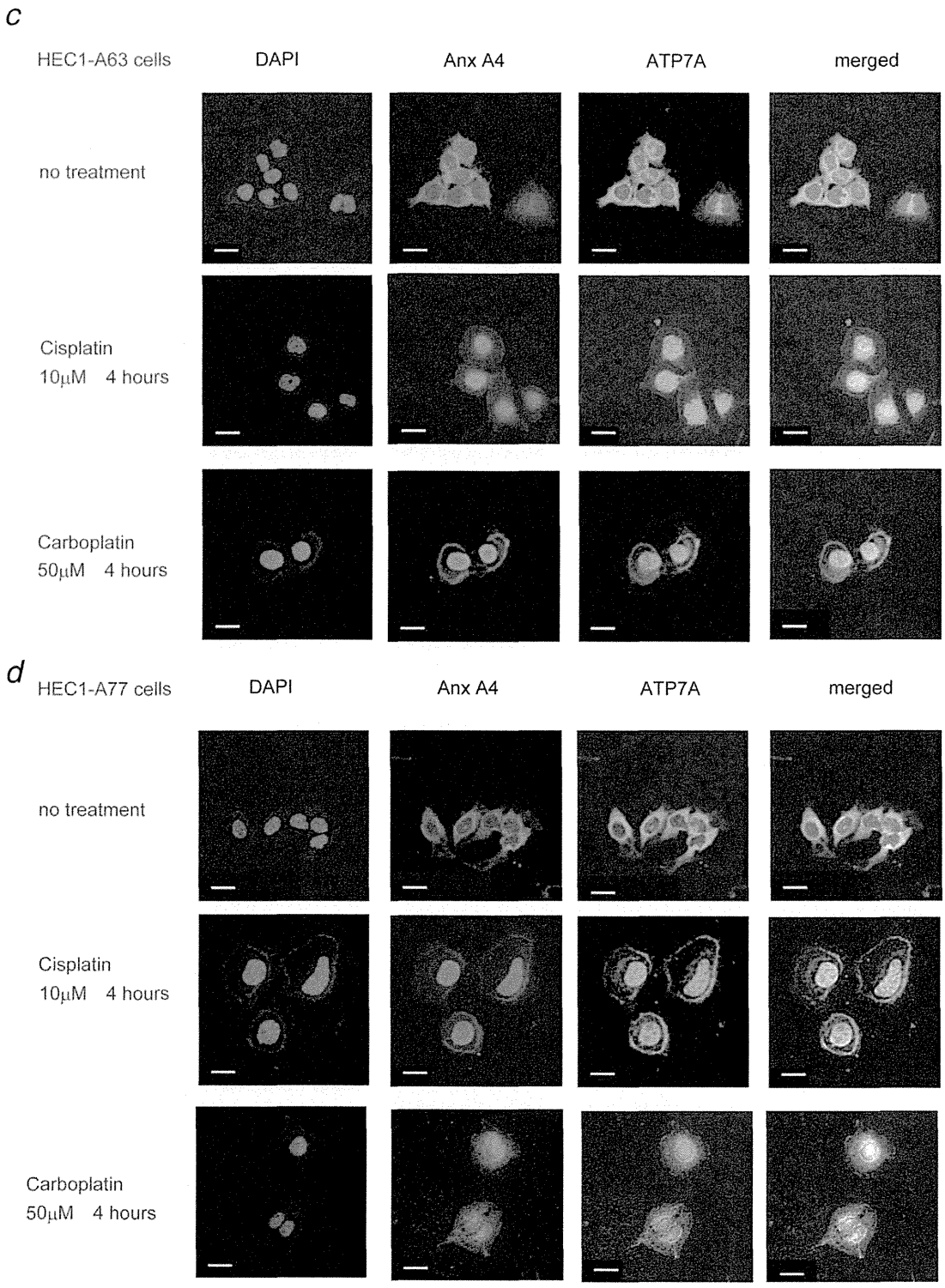

Figure 4. (Continued)

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

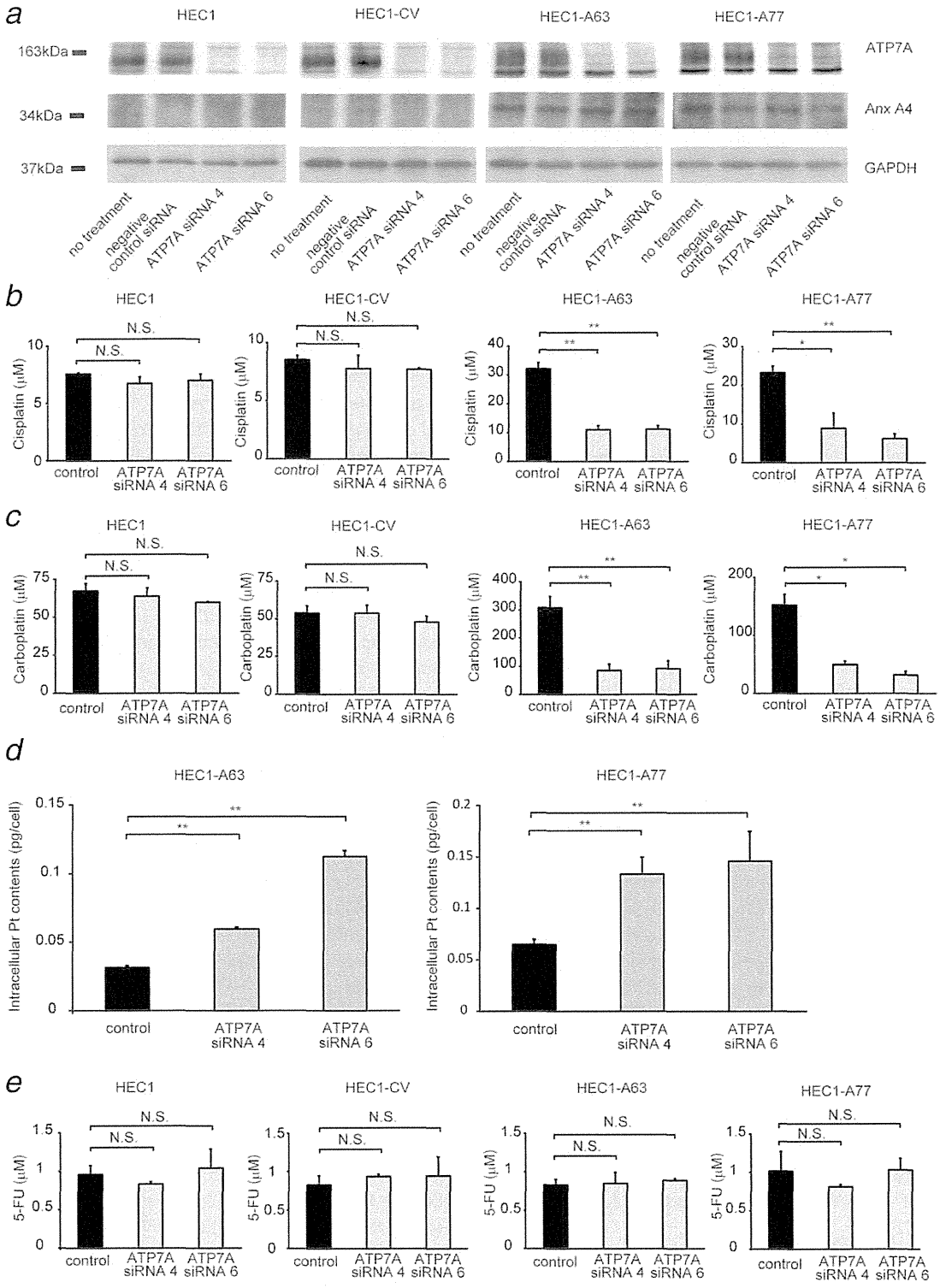

Figure 5. Knockdown of ATP7A expression improves platinum chemosensitivity in Anx A4-overexpressing cells. (*a*) Knockdown expression of ATP7A by siRNA in HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells by Western blot analysis. (*b*) IC_{50} values are shown for cisplatin in HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 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.

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Original Article

Evaluation of a Free-Coupon Program for Cervical Cancer Screening Among the Young: A Nationally Funded Program Conducted by a Local Government in Japan

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ABSTRACT

Background: Finding ways to improve the cervical cancer screening rates among young women has been seen as a critical national health problem in many countries, including Japan. The aim of the present study was to evaluate the effects of a free-coupon program for cervical cancer screening conducted by a local government under financial support from the Japanese national government.

Methods: The personal cervical cancer screening information was analyzed for all female residents of Toyonaka City, including any past screening history and clinical results since the year 2009, when a free-coupon program for screening was started. These results were compared to results from 2008, prior to implementation of the free-coupon screening program.

Results: The screening rates of women eligible for the free-coupon peaked dramatically compared to women of similar age who paid for their screening; however, the rates for the ineligible-age population also increased significantly in parallel to those in the free-coupon program, possibly by indirect peer and publicity effects. In women aged 20 to 25 years, the consecutive screening rate after a free-coupon screening was significantly lower than for those women who received a regular residential screening. After a free-coupon screening, the rate for participating in consecutive screenings depended significantly on the institution where the participant received her first screening test.

Conclusions: These results suggest that, for a generation of young women 20–25 years of age, a free-coupon program for cervical cancer screening was effective in increasing the first-time participation rate for screening; however, the increase in first-time participation did not lead to the expected increase in consecutive screenings.

Key words: cervical cancer screening; free-coupon; screening rate; consecutive screening

INTRODUCTION

Cancer of the cervix is the second most common cancer in women worldwide, with about 500 000 new cases and 250 000 deaths each year.[1] Almost 80% of cases occur in low-income countries.[1] Although a vaccine against the human papillomavirus (HPV) effectively prevents human papillomavirus infection and thus reduces the risk of cervical cancer by around 70%,[2] about 30% will still develop cervical cancer.

In some countries, including the United States and the United Kingdom, the cervical cancer screening rate is roughly 80%; however, in Japan it is only 25%.[3] Of particular concern, the screening rate for women aged 20–29 years is less than 10%.[4] Further, the incidence of cervical cancer among this 20- to 29-year age group has recently been increasing dramatically.[5] Finding ways to improve the screening rates among this younger generation has been seen as a critical national health problem.

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In Japan, it is recommended that women start receiving cervical cancer screening at age 20, to be repeated every 2 years. Even if women skip a screening test in the appropriate second year, they can still undergo a screening test the following year. The local government covers part of the screening costs, and the participant pays the rest, which usually amounts to ¥500 to ¥2000 (approximately $5 to $20 in United States' dollars [USD]). In 2009, a free-coupon program for screening for cervical and breast cancers was introduced in Japan as a national policy. In this program, a coupon or voucher for a free cervical cancer screening was sent by mail to women aged 20, 25, 30, 35, and 40. The program costs were covered by local governments, with financial support from the national government. Because this free-coupon program was terminated at the end of 2013, all citizens aged 20–44 years in Toyonaka had received a free-coupon only once between 2009 and 2013. A woman aged 20 in 2009, for example, would have received a free-coupon screening in 2009 and undergone a regular screening in 2011 and 2013.

There is an evidence gap as to whether removal of out-of-pocket costs and receipt of an individual invitation letter would be effective for increasing the cervical cancer screening rate, especially in Asia.[6–9] However, the reason for this inconsistency is unclear.

Toyonaka is an urban city located in Osaka prefecture. In October 2013, Toyonaka had an area of 38.6 km^2 and a population of 394 004. Toyonaka is officially acknowledged by the national government of Japan as a core city. In the present study, we evaluated the effectiveness of the free-coupon program in improving cervical cancer screening rates among the younger population of Toyonaka.

It was recently reported that removal of out-of-pocket costs for cervical cancer screening was an effective means of increasing the screening attendance of eligible women.[6] In the present study, we analyzed for the first time the effects of the free-coupon on the screening rate not only for the eligible women but also for the coupon-ineligible women, as well as the results of the screening tests and the consecutive screening rates following the free-coupon screening.

MATERIALS AND METHODS

The personal screening information of all female residents aged 20–49 in Toyonaka, including screening history and test results since 2009 (when the registration system was renewed and the free-coupon program was started), was available at an individual level. Only the screening rates aggregated by age groups of 20–24, 25–29, 30–34, 35–39, and 40–44 years were recorded for the year 2008. In Toyonaka, participants in the regular cervical cancer screening program typically paid ¥600 (about $6 USD) for a standard cervical cancer screening.

The rate of cervical cancer screening among the young generation of women (defined here as women aged 20–44 years) for each year between 2009 and 2012 was analyzed. During the period from 2009 to 2012, a free-coupon program was conducted for women at 5-year age intervals, beginning at the recommended starting age of 20 years (ie, ages 20, 25, 30, 35, and 40 years). These screening rates were compared to that of each age group during the index year of 2008, which was just prior to the start of the free-coupon program. A comparison of the rates for those requiring further diagnostic workups and for cancer detections between the free-coupon and regular screening programs was also conducted. The screening histories of the free-coupon group and regular screening program group were analyzed for changes in consecutive screening rates and any links between those rates and the screening sites where the previous screening was performed.

This study was approved by the Institutional Review Board and the Ethics Committee of the Osaka University Hospital.

Statistical analysis

MedCalc software (MedCalc Software, Mariakerke, Belgium) was used for the statistical analysis. Increases in the screening rate for each age or age group were evaluated by the logistic regression model. Differences in the rates of further diagnostic workups and cancer detection between the free-coupon group and the regular screening group were evaluated using Fisher's exact test. Differences in consecutive screening rates between a free-coupon group and a regular screening group and between screening sites were also evaluated using Fisher's exact test. Results were considered to be significant when the *P*-value was less than 0.05.

RESULTS

Effect of a free-coupon on young women's participation in cervical cancer screening

Figure and Table 1 show the yearly rate of cervical cancer screening for 20- to 44-year-old women between the years of 2009 and 2012, when the free-coupon program was being conducted. The screening rates for free-coupon-eligible 20-, 25-, 30-, 35-, and 40-year-old women formed peaks. Compared to screening rates in the year 2008 (prior to the free-coupon program), which were calculated for the age groups of 20–24, 25–29, 30–34, 35–39, and 40–44 years, the screening rates for the 20-, 25-, 30-, 35-, and 40-year-old women exhibited statistically significant increases (rate ratio [RR] 7.1, 95% confidence interval [CI] 5.9–8.6; RR 6.4, 95% CI 5.2–7.1; RR 3.1, 95% CI 2.9–3.3; RR 3.3, 95% CI 3.1–3.5; and RR 3.0, 95% CI 2.8–3.2, respectively; Table 2). The RRs of the 20- and 25-year-olds were especially high, relative to those of the 30-, 35-, and 40-year-olds.

Effect of a free-coupon program on participation rates in cervical cancer screening by the ineligible population

Interestingly, the screening rates for the coupon-ineligible population also increased during the study period (Figure).

Table 1. Yearly rate of cervical cancer screening for 20- to 44-year-old women between the years of 2008 and 2012

Age (years)	2008	2009	2010	2011	2012
20		174/2016 (8.6%)	183/1868 (9.8%)	220/1731 (12.7%)	175/1778 (9.8%)
21		24/1921 (1.2%)	40/1994 (2.0%)	25/1879 (1.3%)	69/1746 (4.0%)
22	137/9573 (1.4%)	34/1989 (1.7%)	54/1950 (2.8%)	63/2006 (3.1%)	76/1910 (4.0%)
23		51/2077 (2.5%)	76/2015 (3.8%)	60/1960 (3.1%)	101/2004 (5.0%)
24		44/2082 (2.1%)	86/2071 (4.2%)	65/1997 (3.3%)	108/1925 (5.6%)
25		408/2290 (17.8%)	409/2049 (20.0%)	495/2091 (23.7%)	440/2003 (22.0%)
26		78/2240 (3.5%)	89/2237 (4.0%)	79/2068 (3.8%)	133/2104 (6.3%)
27	360/11 031 (3.3%)	85/2293 (3.7%)	154/2255 (6.8%)	136/2241 (6.1%)	168/2110 (8.0%)
28		100/2335 (4.3%)	151/2328 (6.5%)	156/2246 (6.9%)	184/2311 (8.0%)
29		145/2473 (5.9%)	205/2364 (8.7%)	185/2385 (7.8%)	239/2279 (10.5%)
30		578/2628 (22.0%)	639/2494 (25.6%)	616/2393 (25.7%)	593/2518 (23.6%)
31		235/2793 (8.4%)	249/2578 (9.7%)	199/2541 (7.8%)	282/2390 (11.8%)
32	1032/13 232 (7.8%)	170/2836 (6.0%)	247/2765 (8.9%)	220/2627 (8.4%)	226/2602 (8.7%)
33		278/2952 (9.4%)	349/2858 (12.2%)	284/2775 (10.2%)	317/2654 (11.9%)
34		208/3233 (6.4%)	269/3019 (8.9%)	174/1896 (6.0%)	274/2801 (9.8%)
35		874/3574 (24.5%)	873/3219 (27.1%)	863/3054 (28.3%)	736/3016 (24.4%)
36		244/3404 (7.2%)	219/3468 (6.3%)	158/3283 (4.8%)	212/3079 (6.9%)
37	1334/16 753 (8.0%)	381/3558 (10.7%)	389/3460 (11.2%)	362/3415 (10.6%)	375/3308 (11.3%)
38		223/3335 (6.7%)	299/3579 (8.4%)	238/3480 (6.8%)	319/3465 (9.2%)
39		322/3314 (9.7%)	352/3357 (10.5%)	352/3526 (10.0%)	374/3462 (10.8%)
40		807/3422 (23.6%)	832/3309 (25.1%)	865/3361 (25.7%)	773/3599 (21.5%)
41		346/3223 (10.7%)	312/3362 (9.3%)	294/3308 (8.9%)	266/3386 (7.9%)
42	1277/15 900 (8.0%)	196/2607 (7.5%)	240/3234 (7.4%)	239/3379 (7.1%)	242/3293 (7.3%)
43		349/2932 (11.9%)	262/2594 (10.1%)	330/3240 (10.2%)	395/3371 (11.7%)
44		214/3014 (7.1%)	233/2954 (7.9%)	174/2612 (6.7%)	244/3248 (7.5%)

Compared with the screening rate in 2008, the screening rates in the off years from 2009 to 2012 for the coupon-ineligible women in the 21–24, 26–29, 31–34, 36–39, and 41–44 year age groups also significantly increased at the same time that the free-coupon was sent to the eligible 20-, 25-, 30-, 35-, and 40-year-old women (Table 1). The RRs for the 21–24 and 26–29 year age groups were around 2.0 (RR 2.2, 95% CI 1.8–2.6 and RR 1.9, 95% CI 1.7–2.1, respectively); however, those of the 31–34, 36–39, and 41–44 year age groups were around 1.1 (RR 1.2, 95% CI 1.1–1.2; RR 1.1, 95% CI 1.1–1.2; and RR 1.1, 95% CI 1.0–1.2; Table 2).

In order to analyze the reasons for the increased screening rates observed among coupon-ineligible women, the screening history of members of the ineligible population (ie, 21-, 22-, 23-, 24-, 26-, 27-, 28-, and 29-year-old women) post-2009, when the free-coupon program started, who attended screening in 2012 ($n = 799$) was investigated (Table 3). Among 799 women, excluding in-migrants, 531 (66%) had no prior history of screening, while 156 (20%) had a history of an ordinary program screening alone, and 111 (14%) had a history of a free-coupon program screening.

Quality evaluation of cervical cancer screening in a free-coupon program

In order to compare the characteristics of women who received a free-coupon screening and those who were screened in a regular program, the rate of further diagnostic workups and that of cancer detection were analyzed in both groups. The women aged 20, 25, 30, 35, and 40 years were all eligible for a free-coupon, so there were no women among these groups who received a regular program screening and who paid for the costs. The rates of further diagnostic workups and cancer detection during 2009 to 2012 were compared between the women aged 20, 25, 30, 35, and 40 years who received screening with a free-coupon versus those aged 21, 26, 31, 36, and 41 years who received screening in a regular paid program. The rate of requiring further diagnostic workups was 2.0% (240/11 793) in the free-coupon group and 2.3% (80/3553) in the regular program group, indicating no significant difference between the two groups ($P = 0.43$ by Fisher's exact test). The rate of cancer detection was 8.4 per 100 000 (10/11 793) in the free-coupon group and 8.9 per 100 000 (3/3553) in the regular program group, indicating no significant difference between the two groups ($P = 1.0$ by Fisher's exact test).

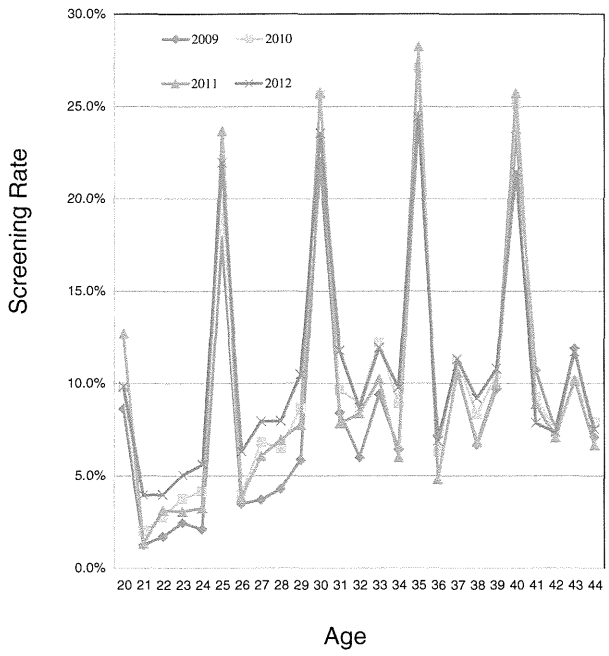

Figure 1. The rate of cervical cancer screening in women 20 to 44 years old in Toyonaka between 2009 and 2012.

Rate of consecutive cervical cancer screening after a free-coupon screening

The screening rates of the women aged 20 and 25 years were dramatically increased by the free-coupon program (Figure and Table 1). To assess whether these increased screening rates resulted in increased rates of consecutive screening, the data were analyzed regarding whether or not those women who underwent a free-coupon screening at the ages of 20 or

Table 2. Comparison of the cervical cancer screening rate between the index year of 2008 and the free-coupon program years of 2009–2012

Age, years	2008	2009–2012		
	Rate of screening	Rate of screening	Rate ratio	95% CI
20	1.4%	10.2%	7.1	5.9–8.6
21–24		3.1%	2.2	1.8–2.6
25	3.3%	20.8%	6.4	5.7–7.1
26–29		6.3%	1.9	1.7–2.2
30	7.8%	24.2%	3.1	2.9–3.3
31–34		9.0%	1.2	1.1–1.2
35	8.0%	26.0%	3.3	3.1–3.5
36–39		8.8%	1.1	1.1–1.2
40	8.0%	23.9%	3.0	2.8–3.2
41–44		8.7%	1.1	1.0–1.2

Table 3. Past screening history of the population ineligible for a free coupon who received a screening in a regular local program in 2012

Age, years	Number screened (in 2012)	Fixed domicile resident	No history of screening	History of screening with free-coupon	History of screening without free coupon
21	69	65	56 (86%)	9 (14%)	0 (0%)
22	76	65	56 (86%)	9 (14%)	0 (0%)
23	101	79	54 (68%)	19 (24%)	6 (8%)
24	108	90	72 (80%)	0 (0%)	18 (20%)
Subtotal	354	299	237 (80%)	37 (12%)	24 (8%)
26	133	112	81 (72%)	5 (4%)	26 (23%)
27	168	90	28 (31%)	40 (44%)	22 (24%)
28	184	134	88 (66%)	29 (22%)	17 (13%)
29	239	164	97 (59%)	0 (0%)	67 (41%)
Subtotal	724	500	294 (59%)	74 (15%)	132 (26%)
Total	1078	799	531 (66%)	111 (14%)	156 (20%)

Table 4. Rates of consecutive cervical cancer screening after a free-coupon screening and a regular screening

	Screening number	Out-migrant within 2 years	Repeated screening within 2 years
Free coupon in 2009			
20 years old	174	19/174 (11%)	10/152[a] (6.5%)[b]
25 years old	408	92/408 (23%)	40/311[a] (13%)[c]
Total	582	111/582 (19%)	50/463[a] (11%)[d]
Regular program in 2009			
21 years old	24	3/24 (13%)	7/21[a] (33%)[b]
26 years old	78	17/78 (22%)	18/61[a] (30%)[c]
Total	102	20/102 (20%)	25/82[a] (30%)[d]

[a]Cases that required further diagnostic workups on initial screening are excluded.
[b,c,d]$P < 0.001$ by Fisher's exact test.

Table 5. Differences in rates of consecutive screening are related to the screening sites where the previous screening was performed

	Clinic A	Other institutions	*P*-value
Free coupon in 2009			
Subsequent screening within 2 years	22/88 (25%)[b]	28/375[a] (7%)[b]	<0.001
Ordinary program in 2009			
Subsequent screening within 2 years	13/32 (41%)[c]	12/50[a] (24%)[c]	0.11

[a]The cases that required further diagnostic workups on initial screening were excluded.
[b]$P < 0.001$ by Fisher's exact test.
[c]$P = 0.07$ by Fisher's exact test.

25 years returned for a subsequent screening. The rate of consecutive cervical cancer screening was compared between the women aged 20 and 25 years who received screening with a free-coupon in the year 2009 and those aged 21 and 26 years who received screening in a regular program in 2009.

In order to investigate the rate of consecutive screening, we excluded from analysis women who out-migrated after a free-coupon screening. In the urban city of Toyonaka, the number of out-migrants was relatively high. Among 582 women aged 20 or 25 years who received a free-coupon screening in the year 2009, 111 persons (19%) moved out of the city within 2 years (Table 4). Among the 102 coupon-ineligible women aged 21 or 26 years who received a screening in a regular program in the year 2009, 20 persons (20%) moved out of the city within 2 years.

After excluding the out-migrants, the continuous screening rate was analyzed. In Japan, women aged 20 years or older are invited for cervical cancer screenings at consecutive two-year intervals, with financial support from their local government. The consecutive screening rate of women aged 20 and 25 within the 2-year interval following the introduction of the free-coupon screening program in 2009 was 6.5% for the 20-year-olds (10/152) and 13% for the 25-year-olds (40/311). On the other hand, the rates of re-visits for women aged 21 or 26 years within a similar 2-year period following a screening in the regular program in the year 2009 were significantly higher: 33% for the 21-year-olds (7/21; $P < 0.001$) and 30% for the 26-year-olds (18/61; $P < 0.001$).

When for some reason a person does not receive a screening after a 2-year interval, she can still undergo a screening in the 3rd year with the same financial support. The consecutive screening rate of women aged 20 and 25 within the 3-year interval following the introduction of the free-coupon screening program in 2009 was 16% for the 20-year-olds (24/142) and 22% for the 25-year-olds (63/277; data not shown). On the other hand, the rates of re-visits for women aged 21 or 26 years within a similar 3-year period following screening in the regular program in the year 2009 were significantly higher: 56% for the 21-year-olds (10/18; $P < 0.001$) and 60% for the 26-year-olds (31/52; $P < 0.001$; data not shown).

Effect of screening site on rate of repeating cervical cancer screening

Next, we investigated the effect of where the screening tests were performed on the consecutive screening rate of women aged 20 or 25 years who received a free-coupon screening and that of those aged 21 or 26 years who received a screening through the regular program in 2009. There were 22 clinics and 6 screening centers where cervical screening test were provided in Toyonaka; however, only 18 of the 22 clinics participated in the 2009 program.

Interestingly, the consecutive screening rates of the 20- and 25-year-olds screened for free at clinic A within the 2-year interval was 25% (22/88), which was significantly higher than the 7% (28/375) reported from the other institutions ($P < 0.001$; Table 5). On the other hand, the consecutive screening rates for 21- and 26-year-olds after a paid screening were slightly (but not significantly) higher at clinic A than at the other screening sites ($P = 0.11$).

The consecutive screening rates of the 20- and 25-year-olds screened for free at clinic A within the 3-year interval was 46% (37/80), which was significantly higher than the 15% (50/339) reported from the other institutions ($P < 0.001$; data not shown). On the other hand, the consecutive screening rates for 21- and 26-year-olds after a paid screening were slightly (but not significantly) higher at clinic A than the other screening sites ($P = 0.07$).

DISCUSSION

There is a critical need to improve the rate of cervical cancer screening among younger women in Japan, as well as in many developing countries. The screening rate of women aged 20 to 29 years is still less than 10%,[4] despite the increasing incidence of cervical cancer in this group.[5] In addition, due to a media blitz about adverse events following HPV vaccination and a statement by the Ministry of Health, Labor, and Welfare of Japan in June 2013 regarding the suspension of an aggressive recommendation for HPV vaccination, the rate of HPV vaccination has dramatically decreased. Given these

situations, the need for improvement in the cervical cancer screening rate among younger women is attracting serious attention. National and local governments therefore enacted a program in which a free cervical screening coupon was sent to 20-, 25-, 30-, 35-, and 40-year-old women to address this problem.

Although many interventions have attempted to remove some of the barriers to cervical cancer screening,[10–16] out-of-pocket costs for screening remain a barrier to access in the United States and Japan.[7] Recently, Tabuchi et al. demonstrated that removal of the out-of-pocket costs by providing a free-screening coupon improved cervical cancer screening participation in Japan.[6] However, they did not analyze how the screening rate was affected for women who had out-of-pocket costs (because of ineligible age for the free screening). In the present study, the screening rates during 2009 to 2012 were shown to rise sharply among those receiving free screening compared to the rates among those of the same age during the pre-program index year of 2008, especially in the two youngest age groups studied (ie, the women aged 20 or 25; Figure and Table 1). However, the screening rate among coupon-eligible women did not increase significantly between 2009 and 2012 (data not shown). This might imply a limitation of the effect of removal of out-of-pocket costs.

We demonstrated for the first time that the screening rates of the population who were paying for their screening (because they were an ineligible age) also increased significantly during the period of this program. While the rates among coupon-ineligible women did not increase as dramatically as those among coupon-eligible women, there was still a significant improvement over 2008 rates.

Possible reasons for the increased screening rates of the youngest of the free-coupon ineligible population during the free-coupon program might be an return visit for screening in a regular program 1 to 3 years after an initial free-coupon screening, or due to indirect effects of the free-coupon program, including improved education and understanding of cervical (and breast) cancer and enhanced motivation for cancer screening. Peer pressure from family, friends, and colleagues to participate in screening between members of the two groups is also likely.

The rate of repeat screening after receiving a previous free-coupon screening among the women who received a regular screening in 2012 was only 14%. This low rate of repeat screening suggests that the significant increase of screening rates seen among 21- to 24-year-old and 26- to 29-year-old women (RR 2.2 and 1.9, respectively; Table 2) cannot be explained by return visits for a regular screening 1 to 3 years after initial free-coupon screening. The increased screening rates of the ineligible population after the free-coupon program started might be caused by indirect publicity effects of the free-coupon program, including improved understanding of cervical cancer and enhanced motivation for cancer screening in young women (Table 2). This somewhat unexpected effect of the free-coupon program should be confirmed in the future.

It was also demonstrated that the rate of requiring a diagnostic workup and the rate of cancer detection due to the screenings were not markedly different between the free-coupon and paid screening program groups. Perhaps more importantly, it was demonstrated for the first time that the follow-up screening rates were significantly lower in the free-coupon group than in the regular screening group (Table 4). This result shows that the complete removal of out-of pocket costs for cervical cancer screening dramatically inspires young women to attend an initial screening; however, it does not translate to following through for a repeat screening 2 years later. This may be a limitation of the effect of a free-coupon cervical cancer screening program. On the other hand, the women who paid some amount of money for a regular screening program were shown to have a consecutive screening than those who attended a free-coupon screening. These results suggest that the largest problem now is how to inspire women to maintain a regular schedule of subsequent screenings. Understanding why the free-coupon group failed to improve rates of consecutive screening will help in providing a solution.

Interestingly, the consecutive screening rate after a free-coupon screening varied depended on where the participants received their previous screening test. This link to the screening experience may provide a partial explanation for the lack of improvement in consecutive screening rates. In the clinic where the rate of follow-up screening was significantly higher, the doctors and staff had spent enormous time and effort to educate the patient about the importance of the screening test to detect cervical cancer; however, it is difficult to statistically compare these educational efforts with those of other institutions. Education is but a part of the screening experience. Institutional reputation, location, scheduling convenience, and waiting room and screening room ambiance all play a role in whether the patient perceives the screening experience as worth repeating. These features of the screening experience are all difficult to quantify and compare statistically.

The Community Preventive Services Task Force demonstrated effectiveness of removal of out-of-pocket costs for breast cancer screening in increasing screening rates for breast cancer; however, evidence with respect to improving cervical cancer screening rates was insufficient.[17] The present study provided some evidence that a free-coupon program is also effective in improving cervical cancer screening rates.

In the present study, the effects of a free-coupon program on the screening rate of both eligible and ineligible women, the rates of requiring further diagnostic workups and cancer detection of a free-coupon screening, and the consecutive screening rate following a free-coupon screening in Toyonaka were analyzed. However, data from only one urban city were analyzed, which is a limitation of the present study. A larger, nation-wide study is necessary to confirm our findings.