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Immunohistochemical analysis of P-glycoprotein expression in diverse histological types of epithelial ovarian tumors.

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Abstract

P-glycoprotein is a transmembrane protein which acts as an energy-dependent drug efflux pump for a variety of anti-cancer drugs. The *mdr-1* gene which encodes P-glycoprotein was successfully cloned in 1986. To investigate P-glycoprotein expression in diverse ovarian tumors, including benign, low malignant potential and malignant, immunohistochemical study was done using a monoclonal antibody (C 219). Overall, 8 out of the 59 epithelial ovarian tumors (13.6%) expressed P-glycoprotein. It was noted that 5 of the 12 mucinous tumors were found to express P-glycoprotein, while none of the 31 serous tumors were immunohistochemically positive. In 10 malignant ovarian tumors, P-glycoprotein immunostaining was examined both prior to and after chemotherapy. Nine of them did not express any P-glycoprotein before or after chemotherapy. However, one tumor expressed P-glycoprotein after six courses of multidrug resistance-related drug administration. These findings indicate that P-glycoprotein expression is not so common in ovarian tumors, regardless of their malignant potential. Nevertheless, the results suggest a strong association between P-glycoprotein expression and certain histological cell types in epithelial ovarian tumors. It is also possible that P-glycoprotein appears as a result of chemotherapy, but such a phenomenon can not occur unless chemotherapy is administered at high doses for a long period of time.

KEYWORDS: P-glycoprotein, epithelial ovarian tumor, multidrug resistance, immunohistochemistry

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Immunohistochemical Analysis of P-Glycoprotein Expression in Diverse Histological Types of Epithelial Ovarian Tumors

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P-glycoprotein is a transmembrane protein which acts as an energy-dependent drug efflux pump for a variety of anti-cancer drugs. The *mdr-1* gene which encodes P-glycoprotein was successfully cloned in 1986. To investigate P-glycoprotein expression in diverse ovarian tumors, including benign, low malignant potential and malignant, immunohistochemical study was done using a monoclonal antibody (C 219). Overall, 8 out of the 59 epithelial ovarian tumors (13.6%) expressed P-glycoprotein. It was noted that 5 of the 12 mucinous tumors were found to express P-glycoprotein, while none of the 31 serous tumors were immunohistochemically positive. In 10 malignant ovarian tumors, P-glycoprotein immunostaining was examined both prior to and after chemotherapy. Nine of them did not express any P-glycoprotein before or after chemotherapy. However, one tumor expressed P-glycoprotein after six courses of multidrug resistance-related drug administration. These findings indicate that P-glycoprotein expression is not so common in ovarian tumors, regardless of their malignant potential. Nevertheless, the results suggest a strong association between P-glycoprotein expression and certain histological cell types in epithelial ovarian tumors. It is also possible that P-glycoprotein appears as a result of chemotherapy, but such a phenomenon can not occur unless chemotherapy is administered at high doses for a long period of time.

Key words: P-glycoprotein, epithelial ovarian tumor, multidrug resistance, immunohistochemistry

Chemotherapy after primary cytoreductive surgery has been widely used to treat patients with ovarian cancer. Resistance to chemotherapeutic drugs is one of the most serious problems in the management of patients with ovarian cancer. Some patients who do not show good response to any kind of chemotherapy are considered to be naturally resistant. It is also found that some patients become resistant eventually after several courses of chemotherapy, although they responded well initially.

It is well known that several mechanisms can be involved in the development of drug resistance. One of them, called the multidrug resistance (MDR) phenotype, has been extensively studied since its discovery in 1970 (1). The *mdr* gene encodes for a transmembrane protein of 170 KDa, termed P-glycoprotein (P-gp) (2). This protein acts as an energy-dependent drug efflux pump for a variety of structurally or functionally unrelated cytotoxic drugs, such as doxorubicin, vincristine, etoposide and actinomycin D (3). P-gp expression has been shown in many intrinsically drug-resistant cancers arising from organs where P-gp expression has been confirmed, including the adrenal gland, kidney, liver, colon and pancreas (4, 5). On the other hand, previous studies showed little evidence of P-gp expression in untreated ovarian cancers (6, 7) arising from the surface epithelial cells of the ovary where no P-gp is originally produced (8, 9). It has been found, however, that P-gp expression is seen in several ovarian cancers as a result of the treatment with MDR-related drugs (6, 8, 10, 11). This phenomenon suggests that P-gp plays a crucial role in developing acquired rather than natural resistance in epithelial ovarian cancers.

Epithelial ovarian tumors are usually classified according to the predominant pattern of histologic differentiation.

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To our knowledge, there has been little study investigating P-gp expression in diverse types of epithelial ovarian tumors. In this study, immunohistochemical staining was carried out using a monoclonal antibody (C 219) to investigate P-gp expression in 59 patients with benign, low malignant potential, or malignant epithelial ovarian tumors, including five common histological cell types: serous, mucinous, endometrioid, clear cell, and Brenner. Here we found P-gp expression in several specimens from epithelial ovarian cancers before any treatment with anti-cancer drugs was administered. In 10 epithelial malignant ovarian tumors, paired specimens from pre and post-chemotherapy surgical tissue samples were available to detect the appearance of P-gp expression.

Materials and Methods

Tissues and patients. Fifty-nine patients with a diagnosis of epithelial ovarian tumor were included in this study. All patients underwent laparotomy at the Department of Obstetrics and Gynecology of Okayama University Medical School, and tumor specimens were obtained at the time of surgery. The histological cell types of tumors were assigned according to the WHO classification: 6 were classified as benign, 8 as low malignant potential and 45 as malignant epithelial ovarian tumors. All malignant tumors were surgically staged using Federation of International Gynecology and Obstetrics (FIGO) staging: 12 were stage I, 3 were stage II, 24 were stage III and 2 were stage IV. Specimens were immediately fixed in 10% neutral buffered formalin and embedded in paraffin.

Immunohistochemical method. Four-micrometer sections from several representative areas of the tumor specimens were put onto glass slides and immunostained according to the avidin-biotin-peroxidase complex (ABC) procedure using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) as follows. After slides were dewaxed in xylene and rehydrated in alcohol, they were incubated with 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity. They were then incubated with 10% normal horse serum for 20 min at room temperature to prevent non-specific binding of the second antibody, followed by incubation with C 219 (Centocor, Malvern, VA, USA) at a concentration of 10 µg/ml for 18 h at 4°C. C 219 is a murine monoclonal antibody, subclass IgG 2a, which recognizes a highly conserved epitope in the cytoplasmic domain of

the P-gp molecule near the nucleotide binding site (12). Since Rubin *et al.* reported the contamination of some C 219 lots with anti-A blood groups antibodies (13), we eliminated the possibility of such contamination by performing the hemagglutination test for all lots we used prior to the immunohistochemical procedures (data not shown). Then the slides were incubated for 30 min with biotinylated horse anti-mouse IgG at room temperature, followed by incubation for 45 min with ABC at room temperature. After the slides were incubated for 5 to 10 min in 0.05% 3, 3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Osaka, Japan) containing 0.05% hydrogen peroxide, they were rinsed in deionized water and counterstained with Mayer's hematoxylin, then dehydrated and mounted. The slides were washed carefully in phosphate-buffered saline (pH 7.4) between each step. As negative controls, the sections were incubated with normal mouse serum (DAKO PATTS, Copenhagen, Denmark) at a concentration of 10 µg/ml instead of the primary antibody. As positive controls, we used SBC-3/ADM cells, a generous gift from the Second Department of Internal Medicine of Okayama University Medical School, which were an adriamycin-resistant phenotype of parental cell line SBC-3 derived from small-cell lung cancer (14).

Staining evaluation. Sections were examined with a light microscope and C 219 reactivity was estimated. It was defined as positive when membranous and/or paranuclear staining was observed.

Results

Expression of P-gp in untreated epithelial ovarian tumors. Fifty-nine tumor specimens were examined and the results are summarized in Table 1. Overall, 8 out of the 59 untreated epithelial ovarian tumors (13.6%) were C 219-positive. Five of them were mucinous tumors. In contrast, none of the 31 serous tumors showed any C 219 immunoreactivity. The incidence of P-gp expression in mucinous tumors was significantly higher than that in serous tumors (Fisher's exact test, $P=0.0008$). As to malignant epithelial ovarian tumors, 5 out of the 45 epithelial ovarian cancers (11.1%) expressed P-gp. These positive cases were limited to three particular histological types (mucinous cystadenocarcinoma, clear cell carcinoma and endometrioid adenocarcinoma), but we failed to observe P-gp staining in any of the 24 cases of serous cystadenocarcinomas and 3 undifferentiated adenocarcinomas. The incidences of P-gp

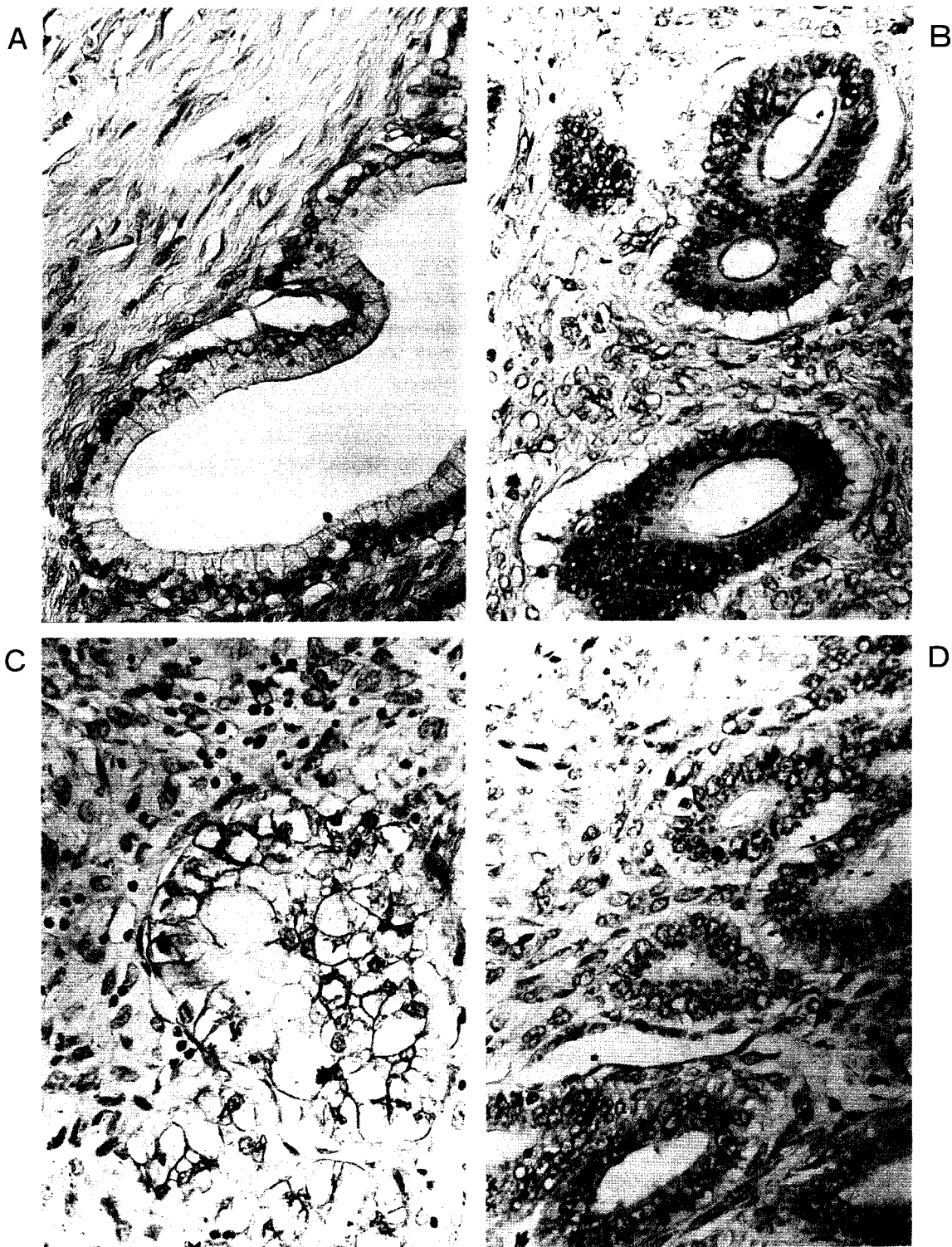


Fig. 1 C 219 immunoreactivity in epithelial ovarian tumors. **A:** Benign mucinous cystadenoma showing discrete luminal membranous and supranuclear staining for P-glycoprotein (P-gp). **B:** Mucinous cystadenocarcinoma showing luminal membranous and paranuclear staining for P-gp. **C:** Clear cell adenocarcinoma showing membranous staining for P-gp. **D:** Endometrioid adenocarcinoma showing paranuclear staining for P-gp.

expression both in mucinous cystadenocarcinomas and clear cell carcinomas were significantly higher than that in

Table 1 Expression of P-glycoprotein in epithelial ovarian tumors

Cell type	Tumor grade			Total
	Benign	LMP	Malignant	
Serous	0/4	0/3	0/24	0/31
Mucinous	1/2	2/4	2/6	5/12
Brenner	—	0/1	—	0/1
Endometrioid	—	—	1/8	1/8
Clear cell	—	—	2/4	2/4
Undifferentiated	—	—	0/3	0/3
Total	1/6	2/8	5/45	8/59

Fraction represents positive cases/examined cases.
LMP: low malignant potential

serous cystadenocarcinomas (Fisher's exact test, $P=0.034$ and $P=0.016$, respectively). Statistical analysis revealed no correlation between P-gp expression and FIGO stage (data not shown).

In eight specimens which showed positive C 219 immunoreactivity, membranous staining was observed in six cases and the remaining two showed paranuclear staining. Positive cells were distributed in a focal scattered fashion within given tumor specimens and less than 20 % of the total tumor cell populations in most cases. Fig 1A illustrates a P-gp-positive specimen of benign mucinous cystadenoma and Figs. 1B, 1C and 1D illustrate the P-gp-positive specimens in epithelial ovarian cancers.

Expression of P-gp in epithelial ovarian cancers after chemotherapy. Specimens from the 10 patients with malignant epithelial ovarian tumors

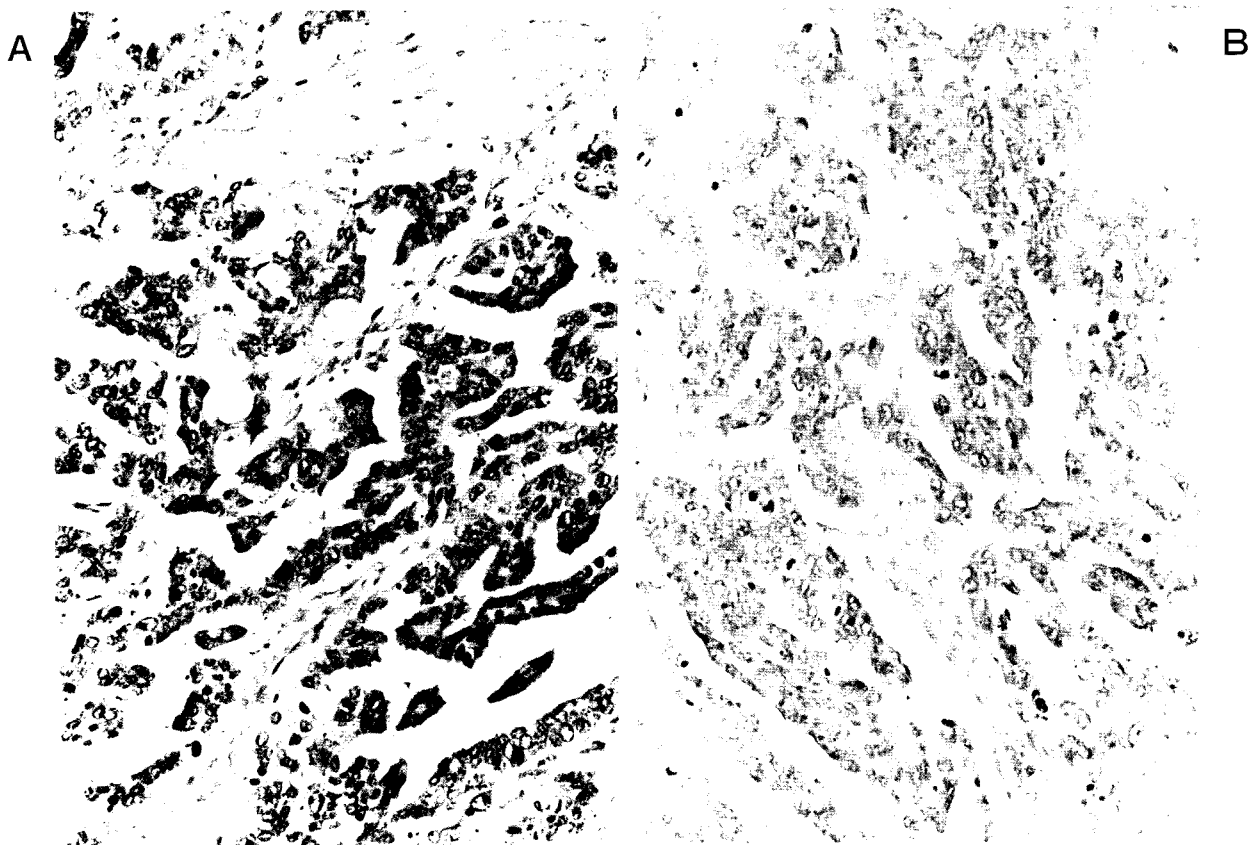


Fig. 2 P-gp immunostaining in paired specimens from pre and postchemotherapy surgical tissue samples of serous cystadenocarcinoma
A: Absence of P-gp immunostaining before chemotherapy
B: Paranuclear and diffuse cytoplasmic staining was observed in recurrent tumor after six courses of chemotherapy with multidrug resistance (MDR) related drugs. Membranous staining is focal. P-gp: See Fig.1.

Table 2 Expression of P-glycoprotein in epithelial ovarian cancers after administration of multidrug drug resistance (MDR)-related drugs

Case no	FIGO stage	Histologic cell type	Chemotherapy ^a	P-gp expression
1	IIIc	Serous	CAP (6)	+
2	IIIc	Serous	CAP (4)	-
3	IIIc	Serous	CAP (3)	-
4	IIIc	Serous	CAP (3)	-
5	Ic	Serous	CAP (3)	-
6	IIIb	Serous	AP (2)	-
7	IIIb	Serous	EP (2)	-
8	IIIc	Endometrioid	CAP (4)	-
9	IIIb	Endometrioid	CAP (3)	-
10	IIIc	Undifferentiated	CAP (1) EP (1)	-

All cases were confirmed to be P-glycoprotein-negative prior to chemotherapy. FIGO: Federation of International Gynecology and Obstetrics; CPM: cyclophosphamide; ADR: adriamycin; CDDP: cisplatin; and VP-16: etoposide.

CAP = CPM + ADR + CDDP EP = VP-16 + CDDP

a: Numbers in parentheses indicate numbers of courses of drug administration.

were available at the second laparotomy after chemotherapy. All these patients were treated with MDR-related drugs and P-gp expression was investigated in paired tumor specimens from the same patients before and after chemotherapy. We found only one patient whose P-gp expression became positive as a result of MDR-related drug administration (case 1 shown in Table 2).

Case 1 is a 51-year-old Japanese patient with ovarian cancer (stage IIIc, serous cystadenocarcinoma). Total abdominal hysterectomy, bilateral salpingo-oophorectomy and partial omentectomy were performed and an intraperitoneal infusion of cisplatin (CDDP) 100mg was administered. This was followed by six courses of intravenous administration of a multidrug regimen consisting of CDDP 60mg/m², adriamycin 35mg/m² and cyclophosphamide 350mg/m² every 4 weeks. The patient was followed-up closely, and 13 months after initial therapy, she presented with a tumor in the pelvic region. She underwent unsatisfactory removal of the recurrent tumor and additional chemotherapeutic treatment was tried with eventual increase of the tumor size and an appearance of another metastatic lesion. She died 6 months after resection of the recurrent tumor. Fig. 2A illustrates the P-gp-negative tumor specimen at the time of the first

operation and Fig. 2B illustrates the specimen from the recurrent tumor showing membranous and paranuclear staining of P-gp.

Discussion

We surveyed a relatively wide variety of epithelial ovarian tumors for the presence of P-gp by immunohistochemical techniques. Although several monoclonal antibodies have been developed for the investigation of P-gp, we used monoclonal antibody C 219 which is applicable to paraffin-embedded sections. The procedures are technically simple and the pathological structure of the tissue can be preserved, making it possible to detect the localization of the positive cells. It implies that there can not be any overestimation of the expression of P-gp, which could happen in the blotting systems. In this regard, *in situ* hybridization with *mdr-1*-specific probes on tissue sections might be a better tool, if the stringency and sensitivity can be controlled well despite its technical complexity. C 219 immunoreactivity within a cell was observed as membranous and/or paranuclear staining, which is compatible with previous studies (15-19). Willingham *et al.* confirmed the existence of P-gp on plasma membrane and on Golgi stack membrane by electron microscopic immunocytochemistry (20). Weinstein *et al.* demonstrated that the amount of P-gp at the colonic luminal membrane is significantly lower than at the Golgi apparatus (17). They also reported difficulty in detecting membranous staining when immunohistochemistry is applied to paraffin-embedded sections (19), nevertheless we were able to demonstrate membranous staining in most cases. C 219 staining within a single section was always variable and heterogeneous. It might be due to a relatively brief half-life of P-gp or differences in levels of expression or could be a reflection of complexity of P-gp *per se*.

Most epithelial ovarian tumors are derived from the ovarian surface epithelium, a specialized type of mesothelium. Other investigators have reported that a normal ovary does not express any detectable P-gp (8, 9) and in fact we did not detect P-gp in normal ovaries other than in the corpus luteum (data not shown). To our knowledge, there have been no reports of P-gp expression in benign and low malignant potential epithelial ovarian tumors. Here, we showed that mucinous cystadenomas in both benign and low malignant potential epithelial ovarian tumors could express P-gp. Overall, 8 of 59

epithelial ovarian tumors (13.6 %) were positive for P-gp. Little research has focused on P-gp expression in diverse histological cell types of untreated epithelial ovarian cancers. Only two other investigators reported positive cases (histological type; one was endometrioid adenocarcinoma and the other was unknown) of P-gp expression (6, 7). In our study, 5 out of the 45 (11.1 %) untreated epithelial ovarian cancers expressed P-gp. This low incidence of P-gp expression in untreated epithelial ovarian cancers was compatible with other smaller studies (6, 7). It should be noted that P-gp-positive ovarian cancers were limited to three histological cell types: mucinous cystadenocarcinoma, clear cell carcinoma and endometrioid adenocarcinoma. In contrast, no P-gp staining was observed in 24 serous cystadenocarcinomas. Apparently, it contributed to the low incidence of P-gp reactivity in this study.

It is known that ovarian serous tumors are the most common histological cell type among ovarian tumors and they morphologically resemble fallopian tube cells. We examined the fallopian tube cells for the presence of P-gp and it was negative (data not shown). Cell types similar to these three C 219-positive cell types in terms of histological appearance and pathway of differentiation are cervical adenocarcinoma for mucinous cystadenocarcinoma, endometrial adenocarcinoma for endometrioid adenocarcinoma and clear cell carcinoma arising from the endometrium or cervical gland for clear cell carcinoma (21, 22). Interestingly, it was revealed that all of them are capable of producing P-gp (18, 23). Although the regulation of P-gp expression is still under investigation, these data strongly suggest that there is an association between P-gp expression and histological cell types. Furthermore, our result partially explains the natural resistance of some tumors because it is widely known that serous cystadenocarcinomas are susceptible to chemotherapy, while mucinous cystadenocarcinomas and clear cell carcinomas are often intrinsically resistant to any kind of chemotherapy. Recently, Arao *et al.* reported that *mdr1* gene expression by reverse transcriptase polymerase chain reaction is higher in serous and mucinous adenocarcinomas than in clear cell and endometrioid carcinomas (24). We do not have any clear explanation for this discrepancy, but it might be that each histological cell type has its own translational regulation of P-gp. Another explanation is differences in methodology and the relative small population of each cell type in both studies.

As to the appearance of P-gp expression after chemo-

therapy, we found one tumor that had converted from negative to positive P-gp staining as a result of anti-cancer drug administration among the 10 patients treated with MDR-related drugs. We believe that since cells producing a considerable amount of P-gp had advantage of cell growth, they could survive to make the recurrent tumor in this patient. We should state here that this patient received six courses of chemotherapy with MDR-related drugs, but others received no more than four courses of such chemotherapy. There was a possibility that some cells might have produced P-gp under the limit of detection by immunohistochemistry in the remaining patients. The tumors in which we failed to detect any P-gp might have become positive if further chemotherapy with MDR-related drugs had been administered.

From our present data, it was indicated that P-gp expression is not common in epithelial ovarian tumors, except for mucinous type ovarian tumors, regardless of its malignant potential. That P-gp expression was limited to three histological cell types suggested that P-gp expression in epithelial ovarian tumors might originate from the surface epithelium of the ovaries with differentiation to the particular histological cell types. The amount of P-gp could be increased as a result of chemotherapy, but high doses and long-term administration of MDR-related drugs is required for this phenomenon. Thus, P-gp plays a role not only in acquired resistance but also in natural resistance in some epithelial ovarian tumors.

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