Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Gynecologic Oncology 99 (2005) 664 – 670
Gynecologic Oncology
www.elsevier.com/locate/ygyno

Expression of estrogen-responsive finger protein (Efp) is associated with advanced disease in human epithelial ovarian cancer

Michiko Sakuma[a,*], Jun-ichi Akahira[a,b], Takashi Suzuki[b], Satoshi Inoue[c], Kiyoshi Ito[a], Takuya Moriya[b], Hironobu Sasano[b], Kunihiro Okamura[a], Nobuo Yaegashi[a]

[a]Department of Obstetrics and Gynecology, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan
[b]Department of Pathology, Tohoku University Graduate, School of Medicine, Sendai, Japan
[c]Division of Gene Regulation and Signal Transduction, Research Center for Genomic Medicine, Saitama Medical School, Saitama, Japan

Received 28 March 2005
Available online 2 September 2005

Abstract

Objective. The estrogen-responsive ring finger protein (Efp) gene, one of estrogen receptor (ER) target genes, is considered to be essential for estrogen-dependent cell proliferation. To understand the estrogenic action on ovarian cancer, we studied the relationships between Efp and ERs expressions and the correlations of Efp expression with clinicopathological parameters in epithelial ovarian cancer.

Methods. The protein expressions for Efp, ERα and ERβ were examined by immunoblotting in 12 ovarian cancer cell lines. Efp mRNA expressions were evaluated by quantitative RT-PCR in 12 ovarian cancer cell lines. A total of 100 surgical specimens diagnosed as epithelial ovarian cancer were examined immunohistochemically using antibodies for Efp, ERα and ERβ.

Results. Efp protein was detected in 8 out of 12 cell lines. In Efp protein-positive cell lines, Efp mRNA was expressed higher than that in negative ($P = 0.021$). All of the Efp protein-positive cell lines simultaneously expressed either ERα or ERβ protein. By immunohistochemical staining, Efp immunoreactivity was detected in 63 out of 100 ovarian cancer specimens and positive signals were in the cytoplasm of carcinoma cells. There were significant correlations between Efp and ERα, ERβ immunoreactivity (Efp and ERα, $P = 0.022$; Efp and ERβ, $P = 0.032$). Efp expression was significantly higher in a subgroup with serous adenocarcinoma ($P = 0.010$) and with advanced disease ($P = 0.026$). No significant relationship was detected between Efp immunoreactivity and overall survival.

Conclusion. The expression of Efp was detected in human epithelial ovarian cancer and high expression of Efp was correlated with advanced disease and serous adenocarcinoma, and ERs status.
© 2005 Elsevier Inc. All rights reserved.

Keywords: Efp; Ovarian cancer; Immunohistochemistry; Estrogen receptor

Introduction

Estrogen and progesterone are sex steroid hormones that are secreted from the ovary and cause the development of the female sex organs. They are also recognized as a significant modifier of the growth, development, invasion and metastasis of gynecological cancers. The actions of estrogen are mediated through specific ligand receptors. There are two receptor subtypes for estrogen, estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) [1,2]. It is assumed that these receptors, members of the steroid/thyroid hormone receptor superfamily mediate these actions by binding ligand dependently to the estrogen-responsive element (ERE) that is located in the promoter region of target genes, thus directly regulating their transcription [3,4]. A variety of estrogenic functions are characterized by the expression of the estrogen-responsive genes following the binding of receptor protein to EREs [5,6].

Estrogen-responsive finger protein (Efp) is a member of the Ring-finger B-box Coiled-Coil family that is thought to be involved in the regulation of various celluer functions, including cell-cycle regulation and gene transcription [5,7].

* Corresponding author. Fax: +81 22 717 7258.
E-mail address: msakuma@mail.tains.tohoku.ac.jp (M. Sakuma).

0090-8258/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygyno.2005.07.103

Efp has been isolated from human genomic DNA binding-site cloning using a recombinant ER protein [5]. Efp gene has an estrogen-responsive element (ERE) in an exon corresponding with the 3′-untranslated region of mRNA [5,8]. Efp is widely expressed in various organs and structures such as the genital tracts, thyroid gland, aorta, spleen, kidney and brain [9]. Estrogen-induced Efp expression is found in the uterus, brain and mammary gland cells [5,8], and its expression is co-localized with ER [9]. A study of knock-out mice has revealed that Efp is essential for cell growth mediated by estrogen in the uterus [6], suggesting that Efp is essential for estrogen mediated cell growth.

Efp expression in the context of cancer has been studied predominantly in breast cancer. Efp mRNA was detected in the MCF-7 human breast carcinoma cell line, where it was induced by estrogen treatment within 0.5 h [10], suggesting that Efp can mediate estrogen actions such as cell growth as a primary responsive gene in breast cancer [10,11]. Recently, it has been suggested that negative cell-cycle regulators, such as 14-3-3 sigma are reduced in Efp-positive breast cancer cells because Efp targets 14-3-3 sigma for proteolysis as an ubiquitin ligase [11]. Thus, Efp may play roles not only as an estrogen target gene but also as a cell-cycle regulator.

Epithelial ovarian cancer is the leading cause of death due to a gynecological malignancy in the great majority of developed countries [12,13]. Sex steroid hormones have been implicated in the etiology and/or progression of some epithelial ovarian cancers, but the possible biological significance of steroid hormone actions in these cancers remains controversial [14–17]. The expression of Efp has not been examined in human epithelial ovarian cancer tissues, and thus the biological significance of Efp expression and correlation between the expression of Efp and ERs expression in this cancer have not yet been studied. We need to understand the new molecular targets or biological makers related to estrogenic actions for ovarian cancer as well as for those associated with breast cancer. In the current study, we examined the expression of Efp in human ovarian cancer tissues and cell lines.

Materials and methods

Cell lines

We used 12 ovarian carcinoma cell lines, two normal ovarian surface epithelial cell lines and one breast cancer cell line as follows. The seven cell lines OVCAR3, Caov3, SKOV3, TOV112D, TOV21G, OV90 and ES2 (adenocarcinoma OVCAR3, SKOV3; serous adenocarcinoma Caov3, OV90; clear cell adenocarcinoma TOV21G, ES2; endometrioid adenocarcinoma TOV112D) were purchased from American Type Culture Collection. The five cell lines JHOS2, JHOS3, HTOA, OMC3 and JHOC5 (serous adenocarcinoma JHOS2, JHOS3, HTOA; mucinous adenocarcinoma OMC3; clear cell adenocarcinoma JHOC5) were purchased from Riken cell bank (Tsukuba, Japan). Two cell lines OSE2 and OSE4 established from normal ovarian epithelial cells were kindly provided by the Department of Obstetrics and Gynecology, Kumamoto University School of Medicine, Kumamoto, Japan [18]. MCF-7 (the human breast cancer cell line) was provided by the Institute of Department, Aging and Cancer, Tohoku University, Sendai, Japan. Cell lines were maintained in DMEM/F12 medium (Invitrogen, CA, USA), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen) and incubated in a 5% CO_2 atmosphere at 37°C.

Surgical specimens and clinical data

We examined surgical specimens from a total of 100 cases of common epithelial ovarian carcinoma obtained from patients treated between 1988 and 2000 at Tohoku University Hospital, Sendai, Japan. Information regarding age, performance status on admission, histology, stage, grade, residual tumor after primary surgery and overall survival was retrieved from the review of patient charts. Median follow-up time for patients was 59 months (range, 4–120 months). Of the 100 patients, 77 (77%) received optimal cytoreduction at time of surgery, 84 (84%) patients received platinum-based chemotherapy postoperatively. Patients with stage Ia disease, low grade-disease (G1, G2) or poor performance status did not receive platinum-based chemotherapy. Performance status was defined according to WHO criteria (World Health Organization, 1979). Histology, stage and grade were according to FIGO criteria (International Federation of Gynecology and Obstetrics; [19]). Residual tumor was determined by the amount of unresectable tumor left following primary cytoreductive surgery. Optimal cytoreduction was defined as no gross residual tumor or less than 2 cm in diameter, whereas suboptimal cytoreduction was defined as any gross residual tumor remaining 2 cm or residual tumor greater than 2 cm in diameter. Overall survival was calculated from the time of initial surgery to death or the date of the last contact. Survival times of patients still alive or lost to follow-up were censored as of December 2002. All of these archival specimens were retrieved from the surgical pathology files at Tohoku University Hospital, Sendai, Japan. The informed consent was obtained from each patient. These specimens were all fixed in 10% formalin and embedded in paraffin. The research protocol was approved by the Ethics Committee of Tohoku University Graduate School of Medicine, Sendai, Japan.

Quantitative reverse transcription-PCR

Total RNA was isolated from cells by phenol-chloroform extraction using Isogen (Nippon gene, Tokyo, Japan). RNA was treated with RNase-free DNase (Roche Diagnostics; 1 μg/μl) for 2 h at 37°C, followed by heat inactivation at 65°C for 10 min. A reverse transcription (RT)-PCR kit (SUPER-

SCRIPT II First-strand synthesis system, Invitrogen) was used and cDNA synthesis was carried out according to the manufacturer's instructions. cDNAs were synthesized from 5 μg of total RNA using random hexamer and RT was carried out for 50 min at 42°C with SUPERSCRIPT II reverse transcriptase. Quantitative PCR was performed using an iCycler system (Bio-Rad, Tokyo, Japan). For the determination of Efp cDNA content, a 25 μl-reaction mixture consisting of 23 μl iQ™SYBR Green MasterMix, 1 μl each primer and 1 μl of template cDNA was prepared. PCR conditions were as follows: 2-min denaturation at 90°C, 30-s annealing at 60°C (for Efp), 62°C (for β-actin) and 1.5-min extension at 72°C. Primers for PCR reactions were as follows: Efp-F, 5′-CGTGGAGTGGTTCAACAC-3′ and Efp-R, 5′-GAGCAGATGGAGATGGTG-3′ (1689–1923, 234 base pairs, bp); β-actin-F, 5′-CCAACCGCGAG-AAGATGAC-3′ and β-actin-R, 5′-GGAAGGAAGGCTG-GAAGAGT-3′ (382–841, 459 bp). In initial experiments, following amplification, PCR products were purified and subjected to direct sequencing to verify amplification of correct sequences (ABI prism 310 Genetic Analyzer, Applied Biosystems, CA, USA). β-Actin primers were utilized as a positive control and Efp expression level was calculated as value of Efp RT-PCR divided by value of β-actin RT-PCR. Negative controls without RNA and without reverse transcriptase were also performed.

Immunoblotting

Cells were grown to 70% confluence in 10-cm plates and after removal of culture medium with Phosphate-buffered saline (PBS). Whole-cell protein concentration was measured by Model 680 microplate reader (Biorad, USA) using BradFord reagent (Biorad). A rabbit polyclonal antibody against Efp protein was made by one of the authors (SI). Mouse monoclonal antibody for ERα was purchased from NOVOCASTRA (Newcastle, UK). Mouse monoclonal antibody for ERβ was purchased from GeneTex, Inc. (TX, USA). In all, 20 μg of protein of each sample was mixed with an equal volume of 2× concentrated sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, boiled and then electrophoresed on 7% ready-made gels containing SDS (Mini Protein II Western blotting system, Biorad). Proteins were then transferred to nitrocellulose membrane (Hybond PDVF, Biorad). The membranes were incubated in blocking solution (PBS containing 5% nonfat milk and 0.05% Tween-20), then incubated in 1:4000 dilution of Efp antibody (1:100 for ERα, 1:1500 for ERβ and 1:1000 for β-actin) in blocking solution overnight at 4°C. After incubation with horseradish peroxidase (HRP)-labeled anti-rabbit IgG (anti-mouse IgM for ERα and ERβ) (Vector Laboratories, USA), the antigen–antibody complex was visualized with ECL system (Amersham, Germany). The MCF-7 breast cancer cell line was used as positive control. Actin (Ab-1, Oncogene) was used as an internal positive control.

Immunohistochemistry and scoring of immunostaining

Immunohistochemical analysis was performed using a streptavidin–biotin amplification method using the Histofine Kit (Nichirei, Tokyo, Japan). For antigen retrieval, slides were heated in an autoclave at 120°C for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate gyhydrate, pH 6.0). The dilutions of primary antibodies for Efp, ERα and ERβ were 1:2000, 1:50 and 1:1500, respectively. The antigen–antibody complex was visualized with 3,3′-diaminobenzidine (DAB) solution (1mM DAB, 50mM Tris–HCl buffer (pH 7.6) and 0.006% H_2O_2), and counterstained with hematoxylin. The ER positive normal breast tissue was used as a positive control. For statistical analysis of Efp immunoreactivity, we classified carcinomas into two groups: +, positive carcinoma cells; and −, no immunoreactivity. For evaluation of ERα and ERβ immunoreactivity, we used the H score system to count carcinoma cells as described previously [20,21]. Scores were generated as follows: (3 × [percentage of strongly staining cells]) + (2 × [percentage of moderately staining cells]) + (1 × [percentage of weakly staining cells]). This scoring system yielded results ranging from 0 to 300. Evaluation was carried out independently by two of the authors (MS and JA) for at least 500 cells.

Statistical analysis

Statistical analysis was performed using Stat View 5.0 (SAS Institute Inc., NC, USA) software. The correlation between expression of Efp mRNA and protein was also assessed using the Mann–Whitney *U* test. The statistical significance between Efp immunoreactivity and clinicopathological parameters was evaluated using Friedman's χ_{2_r}-test. The correlation between Efp and ERα, ERβ immunoreactivity was assessed using the Mann–Whitney *U* test. The univariate analysis of prognostic significance was performed using the log-rank test after each survival curve was obtained by the Kaplan–Meier method. All patients who could be assessed were included in the intention-to-treat analysis. A result was considered significant when the *P* value was less than 0.05.

Results

First, we examined Efp expression in ovarian cancer cells. By immunoblotting with anti-Efp antibody, immunoreactive bands corresponding to Efp, sized at approximately 70 kDa, were detected in 8 out of 12 ovarian cancer cell lines (Fig. 1). Efp expression in cell lines was supported with data obtained by quantitative RT-PCR study. Of the 12 ovarian cancer cell lines, 8 were positive for Efp protein expression by immunoblotting showing relatively higher levels of Efp-mRNA than seen in the 4 cell lines negative for Efp protein expression (Fig. 2) ($P = 0.021$). From these

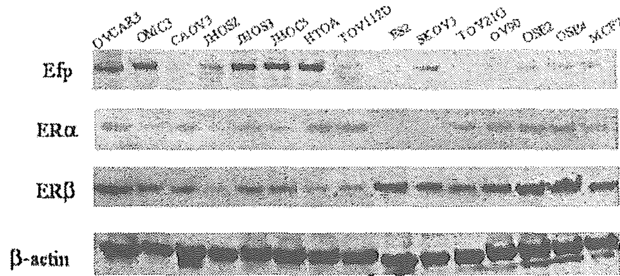

Fig. 1. Immunoblotting with variable cell lines, the top panel with anti-Efp antibody, the second panel with anti anti-ERα antibody, the third panel with anti-ERβ antibody and the bottom panel with anti β-actin antibody. OVCAR3, OMC3, CAOV3, JHOS2, JHOS3, JHOC5, HTOA, TOV112D, ES2, SKOV3, TOV21G and OV90 were derived from ovarian cancer. OSE2 and OSE4 were derived from normal ovarian surface epithelium. MCF-7 was a cell line derived from breast cancer and used as a positive control of Efp and ERs expressions. β-Actin was used as an internal positive control of antibody.

results, we were convinced that cell lines established from not only normal ovarian epithelium but also ovarian cancers expressed Efp genes at various levels similar to results seen with breast cancer cell lines.

Because the Efp gene was expressed by estrones through estrogen receptors, we next examined ERs protein expression in these cell lines (Fig. 2). By immunoblotting with anti-ERα antibody, 10 out of 12 ovarian cancer lines and the 2 cell lines from normal ovarian epithelium showed positive bands. All cell lines positive for Efp protein expression, except SKOV3, were simultaneously positive to ERα. Similarly, all cell lines showed positive bands for ERβ by immunoblotting with anti-ERβ antibody. From these results, we knew that all of the Efp-immunoreactive cell lines simultaneously expressed either ERα or ERβ protein.

Then, we performed immunohistochemical staining with anti-human Efp antibody for the 100 surgical specimens diagnosed as ovarian cancer to confirm Efp expression in ovarian cancer tissues. Efp protein expression was detected in 63 out of 100 specimens (63%). Positive staining was observed in the cytoplasm of ovarian cancer cells (Figs. 3A, D).

We then compared Efp expression and various clinicopathological parameters; results are summarized in Table 1. Differences by histological types were detected in Efp expression, i.e., the subgroup of serous adenocarcinomas showed significantly higher incidence of Efp positivity than other subgroups ($P = 0.010$). Similarly, the subgroup of advanced-stage disease showed a significantly higher incidence of Efp positivity than the subgroup consisting of early-stage disease ($P = 0.026$). There were no significant relationships between Efp immunoreactivity and patient age, performance status, histological grade or residual tumor (Table 1).

We decided to examine simultaneous ER and Efp expression in cancer tissues because Efp is mainly transactivated by ERs. Immunohistochemical studies showed that all ovarian cancer tissues were positive for both ERα and ERβ to a greater or lesser extent, and immunopositive signals were confined exclusively to the nuclei of tumor cells (Figs. 3B, C, E, F). The median H scores for ERα in Efp-immunopositive and Efp-immunonegative tumors were 80.1 ± 70.3 and 39.5 ± 59.4 (mean ± SD), respectively, indicating that Efp-positive cancers expressed significantly higher levels of ERα than Efp-negative cancers ($P = 0.022$). In the same way, the median H score for ERβ in Efp-immunopositive and Efp-immunonegative tumors was 67.7 ± 56.2 and 43.0 ± 43.7 (mean ± SD), respectively, indicating that Efp-positive cancers expressed significantly higher levels of ERβ than Efp-negative cancers ($P = 0.032$). Interestingly, the subgroup of serous adenocarcinomas showed significantly higher H scores (112.1 ± 60.7) than those of the other subgroups (29.7 ± 51.8) ($P < 0.0001$). In contrast, this tendency was not observed in immunoreactivity of ERβ among each histologic subgroup (data not shown).

Finally, we examined the possibility of Efp as a clinical prognostic factor by univariate analysis. As shown in Table 2, clinical variables including histologic type, grade, stage and residual tumor size were all significantly related with overall survival. These results seem to be consistent with data described previously [13,22,23]. With regard to analysis of Efp expression, we did not find any significant correlation between Efp immunoreactivity and overall survival ($P = 0.78$).

Discussion

In this study, we found strong correlations between Efp and ERα and between Efp and ERβ in ovarian cancer tissues; we also found that both ERα and ERβ proteins were expressed in most cancer cell lines with positive for Efp protein. Efp mRNA and protein are up-regulated by

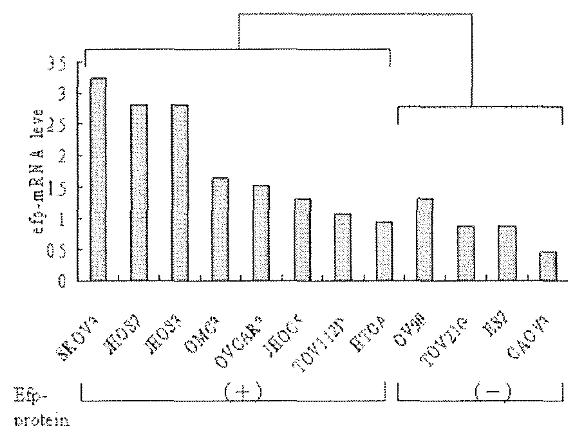

Fig. 2. Quantitative RT-PCR for expression of Efp mRNA in ovarian cancer cell lines. RT-PCR reactions were performed for each samples, and the ratio of Efp; β-actin was calculated and normalized. The left 8 cell lines were positive for Efp protein by immunoblotting, as determined from results of Fig. 1. Efp mRNA expression among cell lines positive for Efp protein was significantly higher than those among cell lines that are negative.

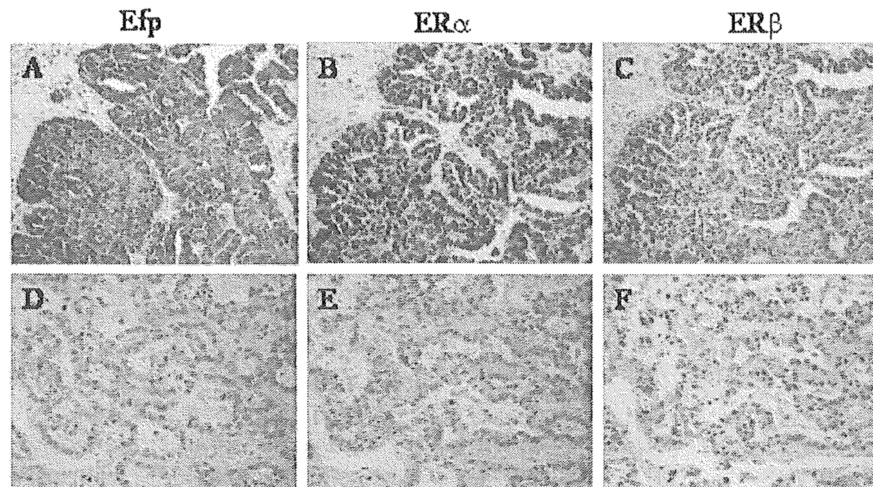

Fig. 3. Immunohistochemistry for Efp, EPα and ERβ in ovarian cancer tissues. Serial sections of each surgical specimens were stained with anti-Efp (A and D), anti-ERα (B and E) and anti-ERβ (C and F) antibodies, respectively. Representative data positive for each antibody (A–C) and negative (D–F) were shown. Positive signals for Efp were detected on the cytoplasm of cancer cells and positive signals for both ERα and ERβ were confined to the nuclei of cancer cells.

estrogen treatment in vivo [5,8]. Estrogen-responsive proliferation of uterine cells which express abundant ERα was impaired in Efp knock-out mice, suggesting that Efp is a mediator of cell proliferation as one of the direct targets of ERα [6]. In breast cancer, which is considered one of the sex steroid hormone-related malignant neoplasms, the Efp promoter could be enhanced by both ERα and ERβ in the setting of estrogen treatment. It has been suggested that Efp responds to estrogen as a common downstream gene of ERα and ERβ and that Efp mediates estrogen action in both ERα- and ERβ-positive breast cancer [10]. Results from our present study are consistent with these previous reports focusing on breast cancer [6,10], and suggest that Efp may mediate estrogen action through ERα and ERβ in some human epithelial ovarian cancer tissues and cell lines.

Table 1
Association between Efp immunoreactivity and clinicopathological parameters in human epithelial ovarian cancer

	Total	Efp immunoreactivity		*P* value
		+	–	
		63	37	
Age				
≦50	50	33	17	
>50	50	30	20	NS
Performance status				
0, 1	70	44	26	
2, 3, 4	30	19	11	NS
Histological type				
Serous	43	34	9	
Mucinous	14	10	4	
Endometrioid	15	7	8	
Clear cell	27	11	16	
Squamous cell	1	1	0	0.01
Histological grade				
Grade 1	41	23	18	
Grade 2	35	22	13	
Grade 3	24	18	6	NS
Stage				
I, II	55	23	22	
III, IV	45	40	15	0.026
Residual tumor				
≦2cm	59	34	25	
>2cm	41	29	12	NS
ERα H score		80.1 ± 70.3	39.6 ± 59.4	0.022
ERβ H score		67.7 ± 56.2	43.0 ± 43.7	0.032

Histological type: serous, serous adenocarcinoma; mucinous, mucinous adenocarcinoma; endometrioid, endometrioid adenocarcinoma; clear cell, clear cell adenocarcinoma; squamous, squamous cell carcinoma.

However, responsiveness and prognosis impact of steroid hormone in ovarian cancer may be different from those in breast cancer. Responsiveness to hormonal manipulation clearly results in favorable feature of breast cancer. It is known that most of the ER-positive breast cancers are primarily responsive to endocrine therapy, but breast cancer lacking any ER expression often reveals more aggressive phenotypes and is resistant to endocrine therapy [24]. Human epithelial ovarian cancer is believed to be a sex steroid hormone-related neoplasm, although the biological significance and the prognostic impact of hormone receptors are still controversial [14–17]. Previous studies have suggested a relation between progesterone receptor expression and favorable prognosis of epithelial ovarian cancer; especially, tumors of ER-negative/PR-positive showed a significantly superior prognosis when compared with the other combinations [14,25,26]. These evidences suggest that

Table 2
Univariate analysis of overall survival

Variable	*P* value
Efp immunoreactivity (+ vs. –)	0.78
Histological type	0.018
Histological grade	0.0085
Stage	<0.0001
Residual tumor	<0.0001

estrogen function in ovarian cancer has some differences from that in breast cancer, and this may be a reason why Efp expression was unrelated to survival in this study.

Efp immunoreactivity was associated in the current study with the histologic type, serous adenocarcinoma. Ovarian cancer is a morphologically, histologically and biologically heterogeneous disease, which has likely contributed to difficulties in defining the molecular alterations associated with development and progression. On the basis of morphological and histologic criteria, there are four major types of primary ovarian carcinomas: serous adenocarcinoma, mucinous adenocarcinoma, endometrioid adenocarcinoma and clear cell adenocarcinoma. Some molecular studies have offered support for the hypothesis that different histologic types of ovarian carcinoma likely represent distinct disease entities. For example, serous adenocarcinomas demonstrate frequent *p53* mutations and more than 85% of mucinous ovarian adenocarcinomas show *K-ras* mutations [27,28]. Endometrioid adenocarcinomas preferentially exhibit microsatellite instability and mutations of *CTNNB1* (β-catenin) [29,30]. Considering estrogenic action on ovarian cancer, which still remains controversial, it has been thought that ERs expression and its transcriptional gene expression differ in each histologic type. A higher expression of ERα relative to expression of ERβ has been observed in surface epithelial ovarian cancer compared with normal ovarian surface epithelium [2,31]. As for histologic type, there is a relative paucity of ERα expression in clear cell adenocarcinoma compared with the level of expression in serous, endometrioid and mucinous adenocarcinoma [32]. In epithelial ovarian cancer, especially in serous adenocarcinoma, the germ line mutations of BRCA1 have been observed as frequently as seen in breast cancer. The BRCA1 gene has been found to inhibit signaling by ligand-activated ERα through the estrogen-responsive enhancer element and to block the transcriptional activation domain of ERα, AF-2 [33,34]. Our results, together with those of previous reports, suggest that the expression of Efp might be one of the different characteristics of each histologic type and that Efp overexpression especially correlates with serous histology, as the result of estrogen actions through ERs.

Early stages of ovarian cancer are generally asymptomatic and difficult to detect, thus, by the time clinical diagnosis made, most of patients have widespread tumor dissemination beyond the ovaries. Despite a high response rate to first-line chemotherapy, the prognosis of these women is poor, with the 5-year overall survival only 10–20% [13,22]. We need some markers of ovarian cancer to identify subpopulations of patients whose disease is progressive and behaves differently from those in the majority of patients, and which might therefore benefit from adapted therapeutic options. To our knowledge, this current report is the first to evaluated the relationships between Efp and clinicopathological parameters in epithelial ovarian cancer. In our study, the expression of Efp had no prognostic value but was related to advanced-stage disease. Our results suggest that overexpression of Efp might cause invasive and progressive characteristics of epithelial ovarian cancer, if so Efp might have the potential to be useful as a biological marker.

The benefit of optimal surgery for patients with advanced ovarian cancer is well established [35]. Recently, Levine et al. reported that genetic differences failed to predict for outcome of surgical cytoreduction [36]. Our result that the expression of Efp did not correlate with residual tumor size after primary cytoreductive surgery is consistent with this previous report. With regard to tumor dissemination in the abdominal cavity, intrapelvic location of ovaries and their mobility in relation to adjacent structures might be in more relation to ability of optimal surgery than the genetic changes. These evidences suggest that some genetic differences including Efp expression could clarify a character of tumors but would not predict clinical outcome in epithelial ovarian cancer.

From our experimental data, the expression of Efp was detected in human epithelial ovarian cancer and correlated with ERs status, advanced-stage disease and serous adenocarcinoma histologic type. It has been suggested that the expression of Efp in human epithelial ovarian cancer relates to regulation of cell proliferation and carcinogenesis through ERs and that Efp could be a biological marker of this cancer. Further investigations are required to reveal the roles of Efp related to estrogenic action and cell-cycle regulation in epithelial ovarian cancer.

Acknowledgments

This work was supported in part by a grant-in-aid for Scientific Research from the Ministry of Health and Welfare, a grant-in-aid from the Ministry of Education, Science and Culture, a grant-in-aid from Kurokawa Cancer Research Foundation, a grant-in-aid from Japan Gynecologic Oncology Group (JGOG) and the 21st Century Center of Excellence (COE) Program Special Research Grant from the Ministry of Education Science, Sports and Culture.

References

[1] Enmark E, Gustafsson JA. Oestrogen receptors—An overview. J Int Med 1999;246:133–8.

[2] Li AJ, Baldwin RL, Karlan BY. Estrogen and progesterone receptor subtype expression in normal and malignant ovarian epithelial cell cultures. Am J Obstet Gynecol 2003;189:22–7.

[3] Evans RM. The steroid and thyroid hormone receptor family. Science 1988;240:889–95.

[4] Green S, Chambon P. Nuclear receptors enhance our understanding of transcription regulation. Trends Genet 1988;11:309–14.

[5] Inoue S, Orimo A, Hosoi T, et al. Genomic binding-site cloning reveals an estrogen-responsive gene that encodes a RING finger protein. Proc Natl Acad Sci U S A 1993;99:11117–21.

[6] Orimo A, Inoue S, Minowa O, et al. Underdeveloped uterus and reduced estrogen responsiveness in mice with disruption of the

estrogen responsive finger protein gene, which is a direct target of estrogen receptor α. Proc Natl Acad Sci U S A 1999;96:12027–32.
[7] Ikeda K, Inoue S, Orimo A, et al. Multiple regulatory elements and binding protein of the 5′-flanking region of the human estrogen-responsive finger protein (efp) gene. Biochem Biophys Res Commun 1997;236(3):765–71.
[8] Orimo A, Inoue S, Ikeda K, Noji S, Muramatsu M. Molecular cloning, structure, and expression of mouse estrogen-responsive finger protein Efp. J Biol Chem 1995;270(41):24406–13.
[9] Shimada N, Suzuki T, Inoue S, et al. Systemic distribution of estrogen-responsive finger protein (Efp) in human tissues. Mol Cell Endocr 2004;218:147–53.
[10] Ikeda K, Orimo A, Higashi Y, Muramatsu M, Inoue S. Efp as a primary estrogen-responsive gene in human breast cancer. FEBS 2000;472:9–13.
[11] Horie K, Urano T, Ikeda K, Inoue S. Estrogen-responsive RING finger protein controls breast cancer growth. J Steroid Biochem Mol Biol 2003;85:101–4.
[12] Akahira JI, Aoki M, Suzuki T, et al. Expression of EBAG9/RCAS1 is associated with advanced disease in human epithelial ovarian cancer. Br J Cancer 2004;90:2197–202.
[13] Akahira JI, Yoshikawa H, Shimizu Y, et al. Prognostic factors of stage IV epithelial ovarian cancer: a multicenter retrospective study. Gynecol Oncol 2001;81:398–403.
[14] Akahira J, Inoue T, Suzuki T, et al. Progesterone receptor isoforms A and B in human epithelial ovarian carcinoma: immunohistochemical and RT-PCR studies. Br J Cancer 2000;83(11):1488–94.
[15] Bizzi A, Codegoni AM, Landoni F, et al. Steroid receptors in epithelial ovarian carcinoma: relation to clinical parameters and survival. Cancer Res 1988;48:6222–6.
[16] Masood S, Heitmann J, Nuss RC, Benrubi GI. Clinical correlation of hormone receptor status in epithelial ovarian cancer. Gynecol Oncol 1989;34:57–60.
[17] Rao BR, Slotman BJ. Endocrine factors in common epithelial ovarian cancer. Endocr Rev 1991;12:14–26.
[18] Nitta M, Katabuchi H, Ohtake H, et al. Characterization and tumorigenicity of human ovarian surface epithelial cells immortalized by SV40 large T antigen. Gynecol Oncol 2001;81:10–7.
[19] Shimizu Y, Kamoi S, Amada S, et al. Toward the development of a universal grading system for ovarian epithelial carcinoma: I. Prognostic significance of histopathologic features-problems involved in the architectural grading system. Gynecol Oncol 1998; 70:2–12.
[20] Akahira J, Suzuki T, Ito K, et al. Differential expression of progesterone receptor isoforms A and B in the normal ovary, and in benign, borderline, and malignant ovarian tumors. Jpn J Cancer Res 2002;93:807–15.
[21] McCarty KS Jr, Miller LS, Cox EB, Konrath J, McCarty KS Sr.. Estrogen receptor analysis. Arch Pathol Lab Med 1985;109:716–21.
[22] Bonnefoi H, A'Hern RP, Fisher C, et al. Natural history of stage IV epithelial ovarian cancer. J Clin Oncol 1999;17(3):767–75.
[23] Munkarah AK, Hallum III AV, Morris M, et al. Prognostic significance of residual disease in patients with stage IV epithelial ovarian cancer. Gynecol Oncol 1997;64(1):13–7.
[24] Clarke R, Skaar T, Baumann K, et al. Hormonal carcinogenesis in breast cancer: cellular and molecular studies of malignant progression. Breast Cancer Res Treat 1994;31:237–48.
[25] Lindgren P, Backstrom T, Mahlck CG, et al. Steroid receptors and hormones in relation to cell proliferation and apoptosis in poorly differentiated epithelial ovarian tumors. Int J Oncol 2001;19(1):31–8.
[26] Munstedt K, Steen J, Knauf AG, et al. Steroid hormone receptors and long term survival in invasive ovarian cancer. Cancer 2000;89(8): 1783–91.
[27] Anttila M, Kosma VM, Ji H, et al. Clinical significance of alpha-catenin, collagen IV, and Ki-67 expression in epithelial ovarian cancer. J Clin Oncol 1998;16(8):2591–600.
[28] van der Zee AG, Hollema H, Suurmeijer AJ, et al. Value of P-glycoprotein, glutathione *S*-transferase pi, c-erbB-2, and p53 as prognostic factors in ovarian carcinomas. J Clin Oncol 1995;13(1): 70–8.
[29] Aunoble B, Sanches R, Didier E, Bignon YJ. Major oncogenes and tumor suppressor genes involved in epithelial ovarian cancer [review]. Int J Oncol 2000;16:567–76.
[30] Feeley KM, Wells M. Precursor lesions of ovarian epithelial malignancy. Histopathology 2001;38:87–95.
[31] Pujol P, Rey JM, Nirde P, et al. Differential expression of estrogen receptor-α and β messenger RNAs as a potential maker of ovarian carcinogenesis. Cancer Res 1998;58:5367–73.
[32] Fujimura M, Hidaka T, Kataoka K, et al. Absence of estrogen receptor-α expression in human ovarian clear cell adenocarcinoma compared with ovarian serous, endometrioid, and mucinous adenocarcinoma. Am J Surg Pathol 2001;25(5):667–72.
[33] Aida H, Takakuwa K, Nagata H, et al. Clinical futures of ovarian cancer in Japanese woman with germ-line mutations of BRCA1. Clin Cancer Res 1998;4(1):235–40.
[34] Fan S, Wang JA, Yuan R, et al. BRCA1 inhibition of estrogen receptor signaling in transfected cells. Science 1999;284:1354–6.
[35] Chi DS, Franklin CC, Levine DA, et al. Improved optimal cytoreduction rares for stage IIIc and IV epithelial ovarian, fallopian tube, and primary peritoneal cancer: a change in surgical approach. Gynecol Oncol 2004;94:650–4.
[36] Levine D.A., Bonome T., Olshen A.B., et al. Gene expression profiling of advanced ovarian cancers to predict the outcome of primary surgical cytoreduction. ASCO 2004:448 [abstr 5000].

Tohoku J. Exp. Med., 2005, **206**, 31-40

Ber-EP4 and Anti-Calretinin Antibodies: A Useful Combination for Differential Diagnosis of Various Histological Types of Ovarian Cancer Cells and Mesothelial Cells

Satoshi Okamoto, Kiyoshi Ito, Hironobu Sasano,[1] Takuya Moriya,[1] Hitoshi Niikura, Yukihiro Terada, Shinji Sato, Kunihiro Okamura and Nobuo Yaegashi

Departments of Obstetrics and Gynecology, and [1]Pathology, Tohoku University School of Medicine, Sendai, Japan

Okamoto, S., Ito, K., Sasano, H., Moriya, T., Niikura, H., Terada, Y., Sato, S., Okamura, K. and Yaegashi, N. *Ber-EP4 and Anti-Calretinin Antibodies: A Useful Combination for Differential Diagnosis of Various Histological Types of Ovarian Cancer Cells and Mesothelial Cells.* Tohoku J. Exp. Med., 2005, **206** (1), 31-40 —— The differential diagnosis between reactive mesothelial cells and ovarian carcinoma cells is often difficult in cytologic specimens. Immunocytochemical procedures have been utilized in assisting this differential diagnosis, with limitations. Furthermore, previous studies examined only serous type but not other histological types of ovarian carcinoma cases. Therefore, we evaluated the practical value of various epithelial and mesothelial markers in differential diagnosis of these two types of cells. Various types of ovarian carcinoma (serous, $n = 22$; mucinous, $n = 10$; endometrioid, $n = 7$; clear cell, $n = 10$) and benign mesothelial tissues ($n = 15$) were studied by immunohistochemistry. We then studied effective panels of antibodies by immunohistochemistry in 43 cytologic specimens of ascites or peritoneal lavage fluid consisting of 20 reactive mesothelium and 23 adenocarcinomas of the ovary. In the tissue specimens, Ber-EP4, a monoclonal antibody of epithelial antigen, and a polyclonal antibody against calretinin, which is expressed in mesothelium, are used in differentiating reactive mesothelial cells from ovarian carcinoma. In cytologic specimens, the sensitivity and specificity of Ber-EP4 were 100% and 90%, respectively. The sensitivity and specificity of the anti-calretinin antibody were 90% and 91%, respectively. Using multiple regression analysis, the correlation coefficient between epithelial antigen and calretinin reactivity was $r = 0.938$, with a significance level of $p < 0.0001$. In conclusion, the combined immunostaining of cytologic specimens for Ber-EP4 and the anti-calretinin antibody is helpful for the differential diagnosis between mesothelial cells and not only serous type, but also mucinous, endometrioid and clear cell types of ovarian cancer cells. ——— peritoneal cytology; reactive mesothelial cell; ovarian carcinoma; Ber-EP4; calretinin
© 2005 Tohoku University Medical Press

Received July 6, 2004; revision accepted for publication February 10, 2005.
Correspondence: Kiyoshi Ito, M.D., Department of Obstetrics and Gynecology, Tohoku University School of Medicine, 1-1 Seiryomachi, Aoba-ku, Sendai 980-8574, Japan.
e-mail: kito@mail.tains.tohoku.ac.jp

Cytologic examination of peritoneal fluids is an important tool in detecting intraperitoneal dissemination of genital tract cancers. Ovarian cancer patients are well-known to show the highest percentage of malignant peritoneal cytology, which reflects clinical stage, intraperitoneal metastasis and patient survival (Keettel et al. 1974; Zuna and Behrens 1996). In many cases, however, the differentiation of reactive mesothelial cells (RM) from neoplasms in peritoneal fluids cannot always be made definitively on morphology alone (Fox 1993). Zuna and Behrens (1996) demonstrated that the sensitivity and specificity of cytologic examination of peritoneal fluids were 88% and 83%, respectively. Some studies have examined the criteria for distinguishing RM from adenocarcinoma cells, but the effectiveness of these criteria are limited, especially with respect to cytologic specimens (Covell et al. 1985; Mulvany 1996; Pisharodi et al. 1996; Weir and Bell 2001).

Immunohistochemical procedures have been widely utilized in aiding the differential diagnosis of numerous gynecological pathologies. However, no specific markers have been found to be diagnostic in differentiating RM from adenocarcinomas. Some of the markers that proved to be useful in separating RM from lung cancer cells have a different value in differentiating between RM and ovarian cancer cells (Ordoñez 1998). Therefore, the use of a panel of antibodies provides the highest degree of accuracy. Many studies have attempted to apply immunocytochemical staining in the differentiation of these two types of cells. However, large differences exist in the results of these published studies, regarding both the value of some of the markers and which markers should be included in the routine diagnostic panel for differentiating them (Delahaye et al. 1997; Fetsch et al. 1998; Ordoñez 1998; Lozano et al. 2001; Attanoos et al. 2002). In addition, previous studies examined only serous type but not other histological types of ovarian carcinoma cases. The aim of this investigation was to assess the practical value of markers for identifying mesothelial cells including calretinin, vascular cell adhesion molecule-1 (VCAM-1) and mesothelial cell antigen and of markers for identifying epithelial cells including carcinoembryonic antigen (CEA), CA-125, cytokeratin 18, epithelial membrane antigen (EMA), epithelial related antigen and epithelial antigen in distinguishing RM from various types of ovarian cancer cells. Our findings suggest that epithelial antigen and calretinin immunoreactivity are highly effective in differentiating RM from ovarian carcinoma than other carcinoma and mesothelial markers, when employed in immunohistochemical study. We then confirmed the effectiveness of these antibodies in the immunocytochemical diagnosis of RM and ovarian cancer cells in cytologic specimens.

Materials and Methods

Forty-nine cases of ovarian carcinoma (serous, n = 22; mucinous, n = 10; endometrioid, n = 7; clear cell, n = 10) and fifteen normal mesothelial tissues were retrieved from surgical pathology files at Tohoku University Hospital, Sendai, Japan. Clinicopathological findings for these patients were retrieved by review of patient charts. None of the patients described in this study had received preoperative chemotherapy and/or hormonal therapy or pelvic radiation. None of the patients used oral contraceptives. The lesions were classified according to the Histological Typing of Female Genital Tract Tumors by WHO (Tavassoli and Devilee 2003). All specimens were routinely processed (i.e., 10% formalin fixed for 24 to 48 hours), paraffin embedded, and thin sectioned (3 μm).

Immunohistochemical analysis was performed employing the streptavidin-biotin amplification method using a Histofine Kit (Nichirei, Tokyo), as previously described (Suzuki et al. 1994). Table 1 shows a summary of the immunomarkers used, the suppliers and specifications. All antibodies used in this study are mouse monoclonal antibodies, except for an anti-calretinin polyclonal antibody. The sections were pretreated with trypsin for Ber-EP4, proteinase K for anti-CEA, or pronase for anti-calretinin, or treated in citrate buffer at pH 6.0 in a microwave oven for 30 min for anti-CA125. The sections for MOC-31, anti-cytokeratin 18 and anti-VCAM-1 stainings were pretreated by autoclaving in citrate buffer (pH 6.0) at 121°C for 10 min. The antigen-antibody complex was visualized with 3,3′- diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris-HCl buffer [pH 7.6], and 0.006% H_2O_2), and counterstained with hematoxylin. Suitable positive control tissues were used to confirm antibody specificity. As a negative control, nor-

TABLE 1. *Characteristics of primary antibodies employed in immunohistochemistry*

Antibody to:	Clone	Source	Dilution	Antigen retrieval
Epithelial cell markers				
Epithelial antigen	Ber-EP4	DakoCytomation*	1:25	Trypsin
EMA	E29	DakoCytomation	1:50	
Epithelial related antigen	MOC-31	DakoCytomation	1:50	Autoclave
Cytokeratin18	DC10	DakoCytomation	1:50	Autoclave
Tumor markers				
CA125	M11	DakoCytomation	1:20	Microwave
CEA	II-7	DakoCytomation	1:50	Proteinase K
Mesothelial cell and mesothelioma markers				
Mesothelial cell	HBME-1	DakoCytomation	1:50	
VCAM-1	1.4C3	DakoCytomation	1:50	Autoclave
Calretinin	-	Swant**	1:8000	Pronase

*DakoCytomation, Glostrup, Denmark ; ** Swant corp., Bellinzona, Switzerland.

EMA, epithelial membrane antigen; CEA, carcinoembryonic antigen; VCAM-1, vascular cell adhesion molecule-1.

mal rabbit or mouse IgG was used instead of the primary antibodies. No specific immunoreactivity was detected in these sections.

In addition, we examined a total of 43 cytologic specimens of ascites or peritoneal lavage fluid retrieved from the cytology files at Tohoku University Hospital, Sendai, Japan. These specimens consisted of 20 RMs and 23 adenocarcinomas of the ovaries (serous, n = 18; mucinous, n = 1; endometrioid, n = 2; clear cell, n = 2). All primary tumors were confirmed by histology. Cytologic specimens consisting of RMs were retrieved from the cases of ovarian carcinoma. The diagnoses of cytologic specimens were confirmed by board-certified cytopathologists (Sasano H. and Ito K). These cases are different from previous ones used in immunohistochemical analysis, in order to avoid bias. The collected cells obtained from ascites or peritoneal lavage fluid were fixed onto glass slides using an auto-smear method (Sakura, Inc., Tokyo). All slides were Papanicolaou-stained and reviewed to ensure that they were adequate for diagnosis. For immunocytochemistry, one representative Papanicolaou-stained smear was selected in each case and destained with 1% hydrochloric acid in 95% ethanol. We then performed all immunocytochemical procedures with primary antibodies, anti-calretinin and Ber-EP4, employing the streptavidin-biotin amplification method using a Histofine Kit. Staining was performed following either our procedure or the procedure proposed by Doglioni et al. (1996; Okamoto 1996).

The immunostaining of tumor and/or mesothelial cells was evaluated for cytoplasmic staining. Calretinin expression was demonstrated by nuclear and cytoplasmic immunostaining. Staining for Ber-EP4 and the anti-calretinin antibody was considered positive if any number of tumor and/or mesothelial cells showed positive staining. The slides were assessed by two of the authors (K.I. and S.O.) Statistical analysis was performed using Stat View 5.0 (SAS Institute Inc., Cary, NC, USA) software. With the association between immuno-positive and -negative epithelial antigen and calretinin as explanatory variables, the judgment of whether ovarian carcinoma or mesothelial cells were detected as a dependent variable was evaluated with correlation coefficient (r) and regression equation.

RESULTS

Results of carcinoma and epithelial cell markers in 49 ovarian carcinomas and fifteen normal mesothelial tissues are summarized in Table 2. More than 90% of ovarian carcinoma cells reacted positively with epithelial antigen, EMA, epithelial related antigen and cytokeratin 18. On the other hand, EMA, epithelial related antigen and cyto-

TABLE 2. *Immunoreactivity for epithelial and tumor markers in tissue specimens*

	Epithelial related					
	Epithelial antigen	EMA	Antigen	CK	CA125	CEA
Serous (22)	86% (19/22)	100% (22/22)	100% (22/22)	100% (22/22)	100% (22/22)	27% (6/22)
Mucinous (10)	100% (10/10)	80% (8/10)	100% (10/10)	90% (9/10)	50% (5/10)	90% (9/10)
Endometrioid (7)	100% (7/7)	100% (7/7)	100% (7/7)	100% (7/7)	86% (6/7)	29% (2/7)
Clear (10)	90% (9/10)	100% (10/10)	100% (10/10)	100% (10/10)	100 (10/10)	10% (1/10)
Positivity (%) (Positive No./Total No.)	92% (45/49)	96% (47/49)	100% (49/49)	98% (48/49)	88% (43/49)	37% (18/49)
Normal mesothelial tissue section (15)	0% (0/15)	20% (3/15)	33% (5/15)	100% (15/15)	100% (15/15)	7% (1/15)

EMA, epithelial membrane antigen; CK, Cytokeratin 18; CEA, carcinoembryonic antigen.

keratin 18 immunoreactivities were demonstrated in 20%, 33% and 100% of mesothelial cells, respectively. Only epithelial antigen immunoreactivity was not detected in these cells.

Results of mesothelial cell markers in ovarian carcinomas and others are summarized in Table 3. Sixty-seven to 100% of mesothelial cells demonstrated immunoreactivity of mesothelial cell antigen, VCAM-1 and calretinin. In comparison, mesothelial cell antigen, VCAM-1 and calretinin immunoreactivity was expressed in 84%, 39% and only 6% of ovarian carcinoma cells, re-

TABLE 3. *Immunoreactivity for mesothelial cell and mesothelioma markers in tissue specimens*

	Mesothelial antigen	VCAM-1	Calretinin
Serous (22)	100% (22/22)	68% (15/22)	5% (1/22)
Mucinous (10)	30% (3/10)	20% (2/10)	10% (1/10)
Endometrioid (7)	86% (6/7)	0% (0/7)	14% (1/7)
Clear (10)	100% (10/10)	20% (2/10)	0% (0/10)
Positivity (%) (Positive No./Total No.)	84% (41/49)	39% (19/49)	6% (3/49)
Normal mesothelial tissue section (15)	100% (15/15)	67% (10/15)	100% (15/15)

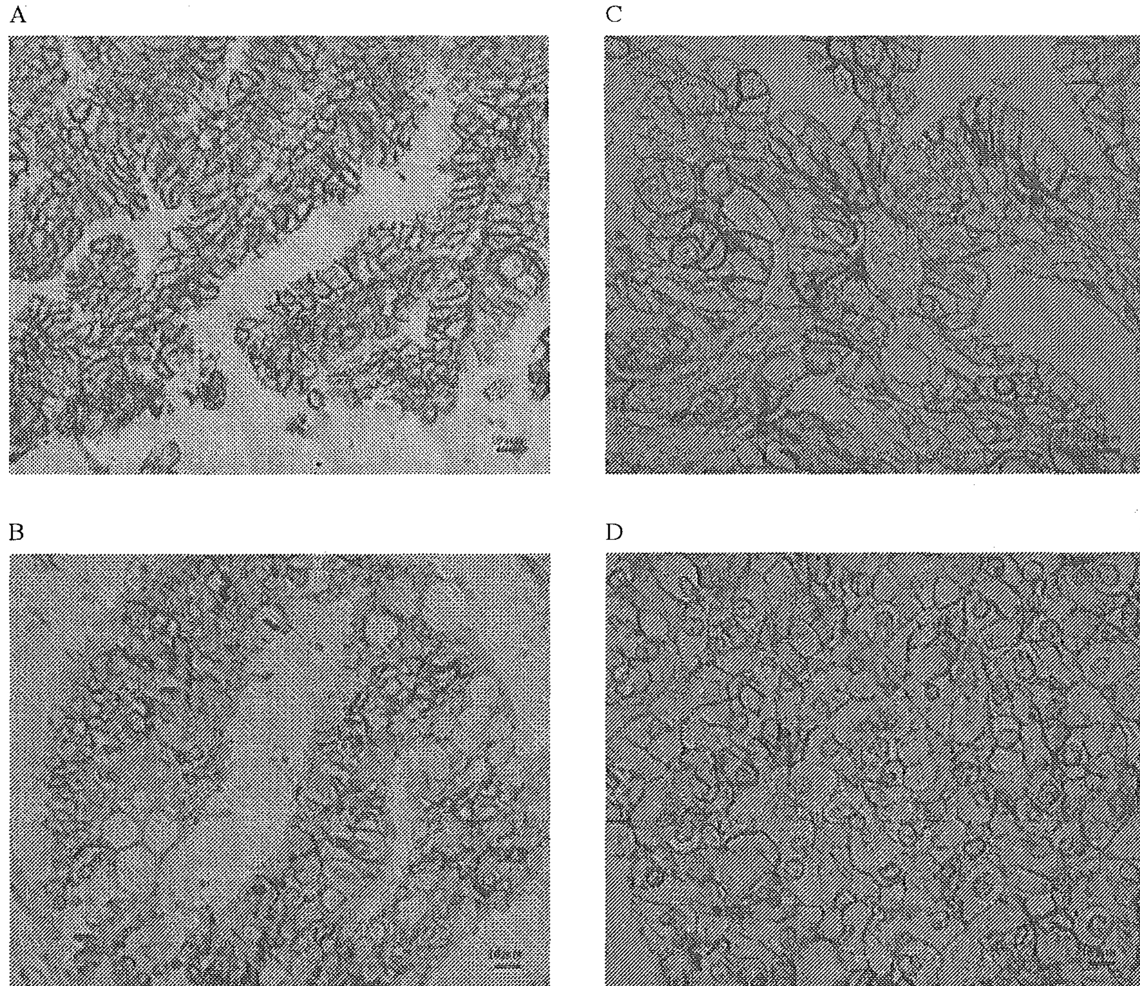

Fig. 1. Immunohistochemistry for epithelial antigen in surgical resections of: A, serous adenocarcinoma; B, mucinous adenocarcinoma; C, endometrioid adenocarcinoma; D, clear cell adenocarcinoma. Brown staining was detected in the cell membranes of all specimens. Original magnification × 400.

spectively. These results suggest that epithelial antigen and calretinin immunoreactivity are highly effective in differentiating RM from ovarian carcinoma than other epithelial and mesothelial markers when employed in immnohistochemical study (Figs. 1, 2 and 3). We therefore examined the effectiveness of these antigens (epithelial antigen and calretinin) in the diagnosis of RM and ovarian carcinoma cells in cytologic specimens (Fig. 4). Immunoreactivity for epithelial antigen in cytologic specimens is summarized in Table 4. The sensitivity and specificity of Ber-EP4 in these specimens were 100% and 90%, respectively. Immunoreactivity for calretinin in cytologic specimens is summarized in Table 5. The sensitivity and specificity of the anti-calretinin antibody in these specimens were 90% and 91%, respectively. Only two (one is serous type and another is clear cell type) of 23 ovarian carcinomas expressed immuno-reactive calretinin. Using multiple regression analysis with immuno-positive and -negative epithelial antigen and calretinin as explanatory variables, and the judgment of whether ovarian carcinoma or mesothelial cells were detected as a dependent variable, we determined the correlation coefficient to be r = 0.938, with a significance

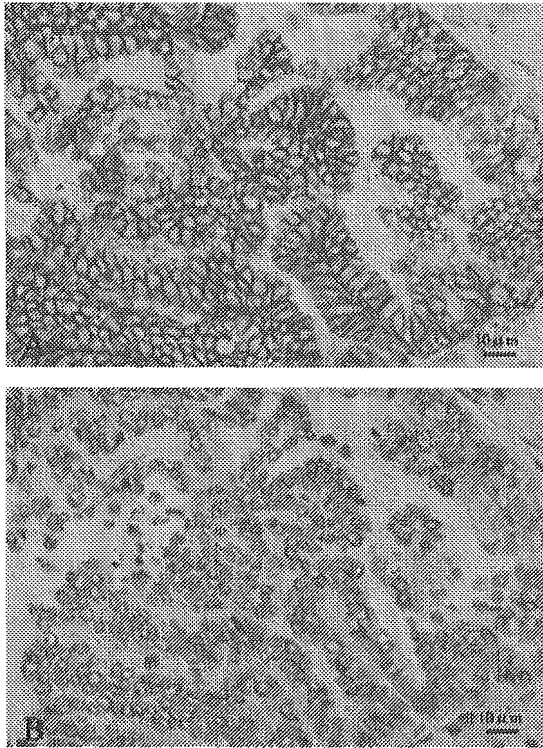

Fig. 2. Immunohistochemistry for epithelial antigen and calretinin in serial sections of serous ovarian carcinoma. A: Epithelial antigen immunoreactivity was predominantly detected in the cell membranes of cells. B: Calretinin immunoreactivity was negative. Original magnification x 400.

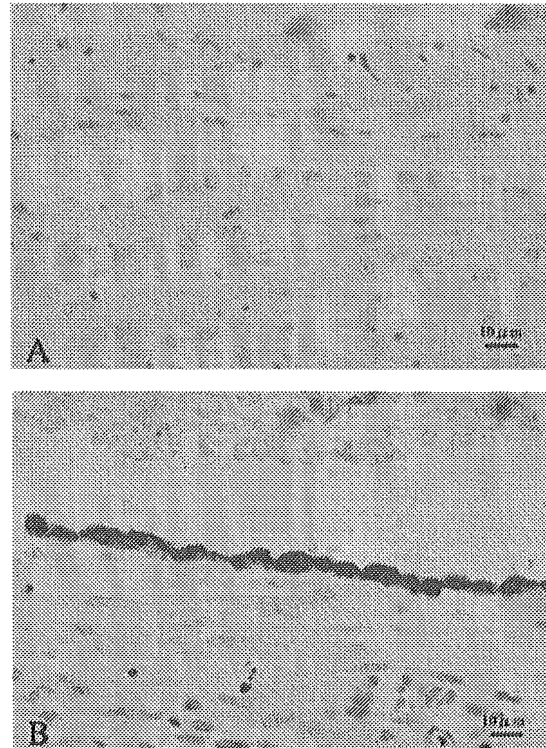

Fig. 3. Immunohistochemistry for epithelial antigen and calretinin in serial sections of a normal mesothelium. A: Epithelial antigen immunoreactivity was negative. B: Calretinin immunoreactivity was detected in both the nuclei and cytoplasm. Original magnification x 400.

level of $p < 0.0001$. These findings match well into an estimated multiple regression model (Table 6).

Discussion

The differential diagnosis between RM and adenocarcinoma in pleural and/or peritoneal effusion continues to be a diagnostic problem in routine cytology practice. Immunocytochemistry is considered useful as a diagnostic aid in this type of differential diagnosis. To date, a significant number of antibodies have been applied to differential diagnosis of cytology specimens from pleural and/or peritoneal effusion, with various degrees of efficacy. However, the usefulness of immunocytochemistry in peritoneal cytology has been less well explored than in pleural effusions. Studies of peritoneal immunocytochemistry with respect to the origin of ovarian carcinoma are markedly limited. Although ovarian cancer patients have the highest percentage of malignant peritoneal cytology, which represents the clinical stage, intraperitoneal metastasis and patient survival, the number of the patient specimens was small and insufficient (Khoury 1990; Ordoñez 1998a; Lozano et al. 2001; Attanoos et al. 2002). Furthermore, these studies examined only serous type but not other histological types of ovarian carcinoma cases. The differentiation of RM from ovarian carcinoma is very important for management of ovarian cancer patients. To our knowledge, the present study is the first report that summarizes data from the largest number and on various types of ovarian cancer cases demonstrat-

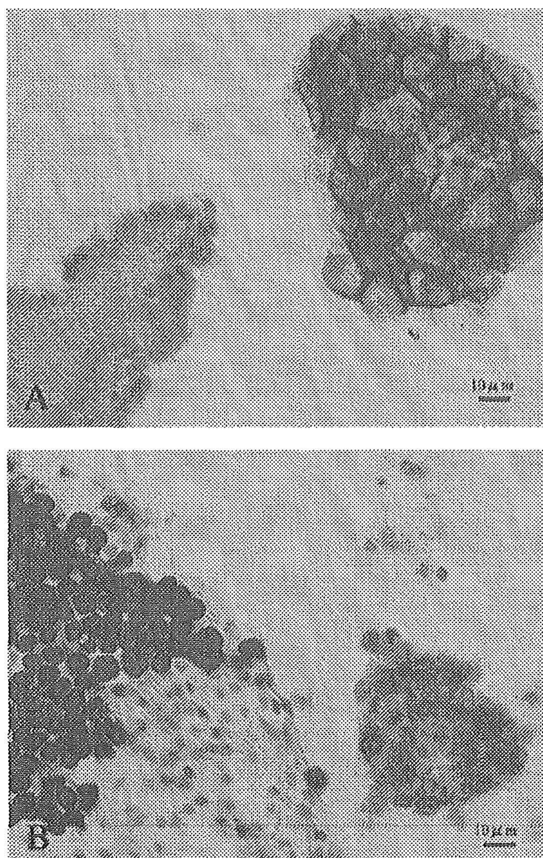

Fig. 4. A: Immunostaining for epithelial antigen in an ascites sample diagnosed as positive following cytodiagnosis. The cell membranes of this serous ovarian carcinoma cell are intensely stained, whereas normal mesothelial cells are negative for epithelial antigen immunostaining. B: Immunostaining for calretinin in an ascites sample diagnosed as positive following cytodiagnosis. Mesothelial cells are stained positive for calretinin, whereas ovarian carinoma cells are negative. Original magnification × 400.

ing the usefulness of immunocytochemistry in the differentiation of RM from ovarian carcinoma.

In our immunohistochemical study, epithelial antigen and calretinin were found to be effective discriminant markers in distinguishing RM from ovarian carcinomas, compared to other epithelial and mesothelial markers in surgical pathology specimens described previously (Sato et al. 2000). Ber-EP4 is a monoclonal antibody that recognizes an epitope present on 2 glycopeptides of 30 kDa and 34 kDa molecular weight, respectively (Latza et al. 1990). This recognized antigen is considered to be present in human epithelial cells and carcinomas but almost absent in the mesothelium (Latza et al. 1990; Sheibani et al. 1991; Ordoñez 1999). In 1990, Latza et al. reported epithelial antigen reactivity in 142 (98.6%) of 144 carcinomas of various organs. However, no reactivity was detected in 88 nonepithelial tumors, including 14 mesotheliomas. In another report, Sheibani and colleagues detected epithelial antigen immunoreactivity in 72 (87%) of 83 adenocarcinomas of various organs but in only one (1%) of 115 mesotheliomas (Sheibani et al. 1991). These studies appear to suggest that epithelial antigen could be of use in the differentiation of mesothelium from adenocarcinomas. According to the literature, epithelial antigen immunoreactivity depends on the primary site of the tumor (Ordoñez 1998b, 1999). Several studies have reported epithelial antigen immunoreactivity in ovarian carcinoma. Ordoñez (1998a) found that epithelial antigen reactivity was detected in all 30 serous ovarian carcinomas but in only four (11%) of 35 mesotheliomas. In addition, Attanoos et al. (2002) reported that epithelial antigen showed 95% sensitivity and 91% specificity in 32 diffuse peritoneal mesotheliomas,

TABLE 4. *Immunoreactivity for epithelial antigen in cytologic specimens*

	Ov. ca.	Mesothelial cell
Epithelial antigen positive (No.)	23	2
Epithelial antigen negative (No.)	0	18
Sensitivity: 100%	Specificity: 90%	

Ov. ca., ovarian carcinoma cells.

TABLE 5. *Immunoreactivity for calretinin in cytologic specimens*

	Ov. ca.	Mesothelial cell
Calretinin positive (No.)	2	18
Calretinin negative (No.)	21	2
Sensitivity: 90%		Specificity: 91%

Ov. ca., ovarian carcinoma cells.

TABLE 6. *Multiple regression analysis*

	Ov.ca.	Mesothelial cell
Epithelial antigen positive calretinin positive (No.)	2	2
Epithelial antigen positive calretinin negative (No.)	21	0
Epithelial antigen negative calretinin positive (No.)	0	17
Epithelial antigen negative calretinin negative (No.)	0	1
	23	20

Ov.ca., ovarican carcinoma cells.
The correlation coefficient between epithelial antigen and calretinin reactivity was r = 0.938, with a significance level of $p < 0.0001$.

20 serous papillary ovarian carcinomas and three primary peritoneal serous papillary carcinomas. Results from our present study are also consistent with these previous reports above. Epithelial antigen immunoreactivity was detected not only in serous ovarian carcinomas, but also in all mucinous, endometrioid and clear cell ovarian carcinomas.

Calretinin, which is a 29-kDa calcium-binding protein normally present in neurons of the central and peripheral nervous system, has recently been recognized as an immunohistochemical marker of reactive mesothelium and malignant mesothelioma in tissue sections (Andressen et al. 1993; Doglioni et al. 1996). In 1996, Doglioni et al. reported calretinin immunoreactivity in all of 44 mesotheliomas, but only focally in 28 (10%) of 294 adenocarciomas of various organs including one (6%) of 16 serous papillary ovarian carcinomas. Ordoñez (1998a), who used a different commercial antibody, reported that all 35 mesotheliomas demonstrated calretinin expression, whereas only three (10%) of 30 serous papillary ovarian carcinomas expressed calretinin immunoreactivity. The distribution of calretinin immunoreactivity has recently been suggested to be useful as the only nuclear reactivity marker that is highly specific for the mesothelium (Cury et al. 2000; Attanoos et al. 2002). These studies demonstrated rates of immunoreactivity for normal and neoplastic mesothelium ranging from 80% to 100%, with only rare adenocarcinomas exhibiting nuclear immunoreactivity. Interpreting only nuclear immunoreactivity as being unequivocally positive, a study by Attanoos et al. (2002) recently reported that calretinin was identified as the most sensitive (88%) and specific (100%) mesothelial marker in the distinction of peritoneal mesothelioma from

serous papillary ovarian carcinoma with.

CEA has been widely applied in immunohistochemistry due to its ability to distinguish adenocarcinomas from reactive and neoplastic mesothelium. Although CEA is considered to be highly specific, it has a relatively low sensitivity in serous ovarian carcinomas (Brown et al. 1997; Ordoñez 1998a). Our findings were consistent with those previous reports. Results from our study described the immuno-reactive expression of immunoreactivity of epithelial related antigens, cytokeratin 18, EMA and CA125 in the great majority of ovarian carcinomas and mesothelial tissues, a finding consistent with previous reports (Delahaye et al. 1991, 1997; Bateman et al. 1997; Ordoñez 1998a; Attanoos et al. 2002). These results suggest that these markers are not necessarily helpful in distinguishing adenocarcinomas of the ovary from a reactive and/or neoplastic mesothelium. VCAM-1 is a cytokine-induced adhesion molecule which recognizes ligands that are abundantly present on leukocytes. Ruco et al. (1996) recently reported that VCAM-1 was detected in 14 of 16 malignant mesotheliomas and in only one of 58 epithelial malignant tumors. However, in our study, VCAM-1 was expressed in ovarian carcinomas, especially in the serous papillary type. HBME-1 was generated using a suspension of human mesothelioma cells from patients diagnosed with malignant epithelial mesothelioma. No mesothelial antigen reactivity has been detected in normal cells other than mesothelial cells, but this antibody has been reported to react with adenocarcinomas in some organs (Fetsch and Abati 2001). Our results have confirmed that mesothelial antigen immunostaining has no practical value in distinguishing between these two tumors.

From our findings we conclude that epithelial antigen and calretinin are useful discriminant markers in the mesothelia for not only serous but also mucinous, endometrioid and clear cell types of ovarian carcinomas. We also examined the effectiveness of these antibodies (Ber-EP4, anti-calretinin) in the diagnosis of mesothelial cells and ovarian cancer cells in cytologic specimens. Immunocytochemical results from the present study suggest that the combined immunostaining for Ber-EP4 and the anti-calretinin antibody is helpful for the differential diagnosis between mesothelial cells and not only serous type, but also mucinous, endometrioid and clear cell types of ovarian cancer cells in cytologic specimens.

References

Andressen, C., Blumcke, I. & Celio, M.R. (1993) Calcium-binding proteins: selective markers of nerve cells. *Cell Tissue Res.*, **271**, 181-208.

Attanoos, R.L., Webb, R., Dojcinov, S.D. & Gibbs, A.R. (2002) Value of mesothelial and epithelial antibodies in distinguishing diffuse peritoneal mesothelioma in females from serous papillary carcinoma of the ovary and peritoneum. *Histopathology*, **40**, 237-244.

Bateman, A.C., al-Talib, R.K., Newman, T., Williams, J.H. & Herbert, A. (1997) Immunohistochemical phenotype of malignant mesothelioma: predictive value of CA125 and HBME-1 expression. *Histopathology*, **30**, 49-56.

Brown, R.W., Campagna, L.B., Dunn, J.K. & Cagle, P.T. (1997) Immunohistochemical identification of tumor markers in metastatic adenocarcinoma. A diagnostic adjunct in the determination of primary site. *Am. J. Clin. Pathol.*, **107**, 12-19.

Covell, J.L., Carry, J.B. & Feldman, P.S. (1985) Peritoneal washings in ovarian tumors. Potential sources of error in cytologic diagnosis. *Acta Cytolol.*, **29**, 310-316.

Cury, P.M., Butcher, D.N., Fisher, C., Corrin, B. & Nicholson, A.G. (2000) Value of the mesothelium-associated antibodies thrombomodulin, cytokeratin 5/6, calretinin, and CD44H in distinguishing epithelioid pleural mesothelioma from adenocarcinoma metastatic to the pleura. *Mod. Pathol.*, **13**, 107-112.

Delahaye, M., Van der Ham, F. & Van der Kwast, T.H. (1997) Complementary value of five carcinoma markers for the diagnosis of malignant mesothelioma, adenocarcinoma metastasis, and reactive mesothelium in serous effusions. *Diagn. Cytopathol.*, **17**, 115-120.

Delahaye, M., Hoogsteden, H.C. & Van der Kwast, T.H. (1991) Immunocytochemistry of malignant mesothelioma: OV632 as a marker of malignant mesothelioma. *J. Pathol.*, **165**, 137-143.

Doglioni, C., Dei Tos, A.P., Laurino, L., Iuzzolino, P., Chiarelli, C., Celio, M.R. & Viale, G. (1996) Calretinin: A novel immunocytochemical marker for mesothelioma. *Am. J. Surg. Pathol.*, **20**, 1037-1046.

Fetsch, P.A., Abati, A. & Hijazi, Y.M. (1998) Utility of the Antibodies CA19-9, HBME-1, and Thrombomodulin In the Diagnosis of Malignant Mesothelioma and Adenocarcinoma in Cytology. *Cancer Cytopathol.*, **84**, 101-108.

Fetsch, P.A. & Abati, A. (2001) Immunocytochemistry in effusion cytology: a contemporary review. *Cancer*, **93**, 293-308.

Fox, H. (1993) Primary neoplasia of the female peritoneum. *Histopathology*, **23**, 103-110.

Keettel, W.C., Pixley, E.E. & Buchsbaum, H.J. (1974) Experience with peritoneal cytology in the manegement of gynecologic malignancies. *Am. J. Obstet. Gynecol.*, **120**, 174-182.

Khoury, N., Raju, U., Crissman, J.D., Zarbo, R.J. &

Greenawald, K.A. (1990) A comparative immunohistochemical study of peritoneal and ovarian serous tumors, and mesotheliomas. *Hum. Pathol.*, **21**, 811-819.

Latza, U., Niedobitek, G., Schwarting, R., Nekarda, H. & Stein, H. (1990) Ber-EP4: New monoclonal antibody which distinguishes epithelia from mesothelia. *J. Clin. Pathol.*, **43**, 213-219.

Lozano, M.D., Panizo, A., Toledo, G.R., Sola, J.J. & Pardo-Mindan, J. (2001) Immunocytochemistry in the differential diagnosis of serous effusions. *Cancer Cytopathol.*, **93**, 68-72.

Mulvany, N. (1996) Cytohistologic correlation in malignant peritoneal washings. Analysis of 75 malignant fluids. *Acta Cytol.*, **40**, 1231-1239.

Okamoto, S., Konno, R., Takano, T., Wagatsuma, S., Nagase, S., Yoshida, Y., Igarashi, T., Sato, S. & Yajima, A. (1998) Immnohistochemistry as a method of differential diagnosis between malignant ovarian tumors cells and mesothelial cells in ascites. *J. Jpn. Soc. Clin. Cytol.*, **37**, 389-393. (in Japanese with English abstract)

Ordoñez, N.G. (1998a) Role of immunohistochemistry in distinguishing epithelial peritoneal mesotheliomas from peritoneal and ovarian serous carcinomas. *Am. J. Surg. Pathol.*, **22**, 1203-1214.

Ordoñez, N.G. (1998b) Value of Ber-EP4 antibody in differentiating epithelial pleural mesothelioma from adenocarcinoma. The M.D. Anderson experience and a critical review of the literature. *Am. J. Clin. Pathol.*, **109**, 85-89.

Ordoñez, N.G. (1999) Role of immunohistochemistry in differentiating epithelial mesothelioma from adenocarcinoma. *Am. J. Clin. Pathol.*, **112**, 75-89.

Pisharodi, L.R. & Bedrossian, C.W.M. (1996) Cytopathology of serous neoplasia of the ovary and the peritoneum: differential diagnosis from mesothelial proliferations. *Diagn. Cytopathol.*, **15**, 292-295.

Ruco, L.P., de Laat, P.A., Matteucci, C., Bernasconi, S., Sciacca, F.M., Van der Kwast, T.H., Hoogsteden, H.C., Uccini, S., Mantovani, A. & Versnel, M.A. (1996) Expression of ICAM-1 and VCAM-1 in human malignant mesothelioma. *J. Pathol.*, **179**, 266-271.

Sato, S., Okamoto, S., Ito, K., Konno, R. & Yajima, A. (2000) Differential diagnosis of mesothelial and ovarian cancer cells in ascites by immunocytochemistry using Ber-EP4 and calretinin. *Acta Cytolol.*, **44**, 485-487.

Sheibani, K., Shin, S.S., Kezirian, J. & Weiss, L.M. (1991) Ber-EP4 antibody as a discriminant in the differential diagnosis of malignant mesothelioma versus adenocarcinoma. *Am. J. Surg. Pathol.*, **15**, 779-784.

Suzuki, T., Sasano, H., Kimura, N., Tamura, M., Fukaya, T., Yajima, A. & Nagura, H. (1994) Immunohistochemical distribution of progesterone, androgen and oestrogen receptors in the human ovary during the menstrual cycle: relationship to expression of steroidogenic enzymes. *Human Reprod.*, **9**, 1589-1595.

Tavassoli, F.A. & Devilee, P. (2003) Pathology and genetics of tumours of the breast and female genital organs. In: *World Health Organization Classification of Tumours*, World Health Organization, Lyon, pp. 113-145.

Weir, M.M. & Bell, D.A. (2001) Cytologic identification of serous neoplasms in peritoneal fluids. *Cancer Cytopathol.*, **93**, 309-318.

Zuna, R.E. & Behrens, A. (1996) Peritoneal washing cytology in gynecologic cancers: long-term follow-up of 355 patients. *J. Natl. Cancer Inst.*, **88**, 980-987.

The Clinical Value of Somatic *TP53* Gene Mutations in 1,794 Patients with Breast Cancer

Magali Olivier,[1] Anita Langerød,[2] Patrizia Carrieri,[3] Jonas Bergh,[4] Sigrid Klaar,[4] Jorunn Eyfjord,[5] Charles Theillet,[6] Carmen Rodriguez,[6] Rosette Lidereau,[7] Ivan Bièche,[7] Jennifer Varley,[8] Yves Bignon,[9] Nancy Uhrhammer,[9] Robert Winqvist,[10] Arja Jukkola-Vuorinen,[11] Dieter Niederacher,[12] Shunsuke Kato,[13] Chikashi Ishioka,[13] Pierre Hainaut,[1] and Anne-Lise Børresen-Dale[2]

Abstract To investigate the clinical value of somatic *TP53* mutations in breast cancer, we assembled clinical and molecular data on 1,794 women with primary breast cancer with long-term follow-up and whose tumor has been screened for mutation in exons 5 to 8 of *TP53* by gene sequencing. *TP53* mutations were more frequent in tumors of ductal and medullar types, aggressive phenotype (high grade, large size, node positive cases, and low hormone receptor content) and in women <60 years old. *TP53* mutations within exons 5 to 8 conferred an elevated risk of breast cancer – specific death of 2.27 (relative risk >10 years; $P < 0.0001$) compared with patients with no such mutation. The prognostic value of *TP53* mutation was independent of tumor size, node status, and hormone receptor content, confirming and reconciling previous findings in smaller series. Moreover, an interaction between *TP53* mutation and progesterone receptor (PR) status was revealed, *TP53* mutation combined with the absence of progesterone receptor being associated with the worst prognosis. Whereas previous studies have emphasized the fact that missense mutations in the DNA-binding motifs have a worse prognosis than missense mutations outside these motifs, we show that non – missense mutations have prognostic value similar to missense mutations in DNA-binding motifs. Nonetheless, specific missense mutants (codon 179 and R248W) seem to be associated with an even worse prognosis. These results, obtained on the largest series analyzed thus far, show that *TP53* mutations identified by gene sequencing have an independent prognostic value in breast cancer and could have potential uses in clinical practice.

The tumor suppressor gene *TP53* plays a key role in many cellular pathways controlling cell proliferation, cell survival, and genomic integrity. It acts as a proliferation brake when cells experience stress conditions, such as DNA-damage, hypoxia, or oncogene activation. Disrupting *TP53* function promotes checkpoint defects, genomic instability, and inappropriate survival, leading to the uncontrolled proliferation of damaged cells. The proliferative advantage given by its inactivation, and the fact that it is ubiquitously expressed, explains why it is frequently found to be mutated in almost every type of cancer (1). It has been shown in various experimental *in vitro* systems, as well as in mouse models, that cell cycle arrest or apoptosis induced by radiotherapy and various chemotherapeutic drugs depends on an intact *TP53* pathway (2, 3). These results have raised the hypothesis that *TP53* could be a key player in defining tumor sensitivity to a broad range of anticancer treatments in patients with cancer. Moreover, the presence of a *TP53* mutation could be one of the underlying causes of drug resistance, the major cause of treatment failure and cancer death.

Several studies have investigated the predictive value of *TP53* mutation status for tumor response to treatment and patient outcome in various cancers. Different clinical and methodologic

Authors' Affiliations: [1]IARC, Lyon, France; [2]Department of Genetics, The Norwegian Radium Hospital, and Faculty Division, University of Oslo, Oslo, Norway; [3]INSERM U379, ORS PACA, Epidémiologie Sociale Appliquée à l'Innovation, Marseille, France; [4]Department of Oncology (Radiumhemmet), Karolinska Hospital and Institute, Stockholm, Sweden; [5]The Icelandic Cancer Society, Molecular and Cell Biology Research Laboratory, and Faculty of Medicine, University of Iceland, Reykjavik, Iceland; [6]INSERM E 229, CRLC Val d'Aurelle/Paul Lamarque, Montpellier Cedex, France; [7]INSERM U735/ Oncogénétique, Centre René Huguenin, St. Cloud, France; [8]Paterson Institute for Cancer Research, Manchester, United Kingdom; [9]Department of Oncogenetics, Centre Jean Perrin, Clermont-Ferrand Cedex, France; Departments of [10]Clinical Genetics and [11]Oncology, Oulu University Hospital, University of Oulu, Oulu, Finland; [12]Molekulargenetisches Labor, Frauenklinik des Universitätsklinikums Düsseldorf, Gebäude, Düsseldorf, Germany; and [13]Department of Clinical Oncology, Institute of Development Aging and Cancer, Aoba-ku, Sendai, Japan

Received 5/10/05; revised 11/4/05; accepted 12/8/05.

Grant support: EC FP6 funding. This publication reflects the author's views and not necessarily those of the EC. The Community is not liable for any use that may be made of the information contained herein.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Requests for reprints: Pierre Hainaut, IARC, 150 Cours Albert Thomas, F-69372 Lyon, France. Phone: 33-472-738-532; Fax: 33-472-738-322; E-mail: hainaut@iarc.fr.

©2006 American Association for Cancer Research.

doi:10.1158/1078-0432.CCR-05-1029

settings have been used and the results are often contradictory. A majority of studies have relied on immunohistochemistry to assess p53 alterations. This approach is, however, a poor surrogate for gene mutation detection because many mutations do not lead to protein accumulation, and because accumulation of wild-type p53 may also occur. Hence, the use of immunohistochemistry leads to an unacceptable number of misclassified cases and to a greater interstudy variability. By contrast, in studies that have screened *TP53* mutations by gene sequencing to precisely identify the mutation, the presence of a mutation has been correlated with a shorter survival or a poor response to treatment in several cancers (http://www-p53.iarc.fr/Somatic.html). Moreover, a number of studies have described specific types of mutation that were associated with a worse prognosis compared with other mutations. This is the case for mutations within the DNA-binding domain that have been repeatedly associated with poor prognosis in several types of cancer (4-7). These clinical results are substantiated by *in vitro* experimental evidence showing that different missense mutations have different functional consequences (see *TP53* Function Database, http://www-p53.iarc.fr/). Wild-type TP53 activities rely mainly on the capacity to transactivate specific target genes by binding to specific response elements. In human cancers, >1,800 different *TP53* missense mutations have been reported and functional assays have shown that mutant proteins show a great variability in their transactivation activities. Whereas hotspot missense mutations in the DNA-binding domain lead to a general loss of specific transactivation capacity, missense mutations outside the DNA-binding domain more often retain transcriptional activity on a variety of promoters (8, 9).

In breast cancer, more than 20 studies have analyzed the prognostic or predictive value of *TP53* mutation (10). In 18 of these studies, *TP53* mutation was clearly associated with poor prognosis, mutations at residues involved in DNA contacts being of worse prognosis in several of them. However, it is not clear from these studies whether *TP53* is a factor of prognosis that is independent of other clinicopathologic factors. Also, there is no clear consensus on the specific type of mutations carrying a worse prognosis because different classifications of mutations have been used and because the comparison of individual mutations was limited by a lack of statistical power.

Using a more powerful analysis in order to assess whether the identification of *TP53* mutation presents a real benefit over currently available factors of prognosis (such as tumor size, node status, and estrogen and progesterone receptor contents), we collected and pooled clinical and molecular data from 1,794 European patients with breast cancer who were followed-up for at least 10 years and whose tumors were screened for somatic *TP53* gene mutation.

Materials and Methods

Patients and selection criteria. Patients were from 10 hospitals in seven European countries (Norway, Finland, Iceland, France, Sweden, United Kingdom, and Germany). Breast cancer cases were selected from cohorts of patients included in previous studies related to *TP53* gene analysis that have been approved by local ethical committees. The largest cohorts have previously been described in detail in refs. (11-16), whereas clinical and molecular data from 600 patients have not been reported before. Updated follow-up information has been obtained on about half of the previously reported cases. Patients were considered eligible for the present study if they had a primary unilateral breast cancer and if their tumor has been screened for *TP53* mutation by gene sequencing. Patients in whom *TP53* mutation had not been confirmed were not considered eligible (84 patients were excluded). Three patients whose tumors carried more than one mutation were also excluded because they could not be grouped according to the type of mutation (mutations of different type in the same tumor). A total of 1,794 patients were eligible. Clinical data were recovered from hospital pathologic records and included, histopathologic subtype, histopathologic grade, nodal status, tumor size, estrogen receptor (ER) and progesterone receptor (PR) status, age at diagnosis, presence of a *TP53* mutation and characteristics of *TP53* mutation if present, time elapsed between surgery and death or last follow-up, and cause of death (breast cancer or other). ER and PR status were assessed in laboratory hospitals by standard biochemical assays in 90% of the cases, with a cutoff value of 10 fmol/mg of protein, and by immunohistochemistry in 10% of the cases (see details in Supplementary Table S1).

Tumor material and TP53 mutation screening. The samples analyzed were either biopsies or surgery specimens, either fresh-frozen or paraffin-embedded. Histopathology, grading, and hormone receptor contents were determined independently for each cohort at their respective institutions. *TP53* mutation screening was done on genomic DNA, except for 311 patients from the Swedish cohort, in which RNA was analyzed. Constant denaturing gel electrophoresis/denaturing gradient gel electrophoresis/temporal temperature gradient electrophoresis, single-strand conformational polymorphism, or denaturing high-pressure liquid chromatography prescreening methods were used to detect mutations and sequencing was done to precisely identify the mutation in all cohorts except for the Swedish cohort, in which no prescreening was done (direct sequencing of cDNA was applied). The entire coding sequence of *TP53* gene (exons 2-11) was screened in 651 tumors, whereas only exons 5 to 8 were analyzed in 1,124 samples, and exons 5 to 11 in 19 samples. Details of methods and PCR primers have been described previously (13-15, 17).

TP53 mutation classifications. Mutations in exons 5 to 8 (including introns) were classified according to their position, nature, and suspected effect on protein structure and activity (18, 19). The following groups were defined:

- Silent. Mutations in the coding sequence that do not change the amino acid sequence or mutation in introns, excluding splice sites and branch sites;
- Non-missense. Any mutation other than missense, including nonsense (introducing a stop codon), deletions and insertions (in-frame or producing a frame shift), and substitutions at splice sites.
- Missense. Mutation resulting in a single amino acid change. This category was further subdivided as:

  (a) Missense DNA-binding motifs. Missense mutations in DNA-binding motifs (DBM) formed by the L2 and L3 loops (codons 164-194 and 237-250, respectively) involved in DNA contacts in the minor groove, and by the LSH motif (codons 119-135 and codons 272-287) involved in DNA contacts in the major groove.
  (b) Missense non-DBM. Missense mutations outside the above-defined DBMs (L2/L3 and LSH).
  (c) Missense Zn. Missense mutations affecting residues involved in binding a zinc atom (codons 176, 179, 238, and 242).
  (d) Missense DNA. Missense mutations affecting residues involved in DNA contacts (codons 119, 120, 121, 239, 241, 243, 247, 248, 273, 275, 276, 27, 280, and 283).

- Codons. Codons most frequently affected by missense mutations in the present series (codons 163, 175, 179, 220, 245, 248, 249, 273, and 282).
- Mutants. Frequent substitutions at "hotspot" codons in the present series as well as in many other cancer types (R248Q, R248W, R175H, R273H, R273C, and G245S).

- Missense conserved/nonconserved. Missense mutation at an amino acid residue conserved (or not conserved) during evolution (conservation based on CLUSTALW alignment of 35 p53 protein sequences from vertebrate species).
- Functional classes. Results of yeast-based functional assays have been used to classify missense mutations according to their capacity to transactivate the promoters of several p53 target genes (9). The promoters used were the p53-binding elements of *WAF1, MDM2, BAX, GADD45, 14-3-3σ, p53AIP1*, and *Noxa* genes. Functional groups were defined as follows: 1, active or partially active on all promoters; 2, inactive on one to two promoters; 3, inactive on three to five promoters; 4, inactive on six to seven promoters. For individual promoters, three groups were considered: 1, inactive; 2, partially active; 3, activity similar to wild-type.
- Structural classes. Results of a systematic computerized prediction model of the effects of *TP53* mutations on the structure of the core domain of the protein were used to classify *TP53* missense mutations into two classes: 1, mutations predicted to impair correct folding of the core domain (structurally explained); 2, mutations predicted to have no effect on the folding of the core domain (not structurally explained). Criteria taken into account include hydrogen bonding, structural clashes, mutation from glycine or to proline, direct contact with DNA or zinc binding (20).

Statistical analysis. Statistics were done using SPSS and Minitab software. To avoid a possible selection bias, patients with missing data for clinical prognostic variables were included by creating a category labeled as "missing" for each variable. Patient follow-ups were computed as the time interval between surgery date and the date of last follow-up, or as the time interval between the date of surgery and the date of death. To reduce heterogeneity among hospitals for duration of follow-up (three hospitals, accounting for <30% of the patients, had <10 years of maximum follow-up), follow-up was censored at 120 months (10 years – censoring time). Date of follow-up was set at 10 years after surgery for patients whose survival exceeded 10 years. Death from breast cancer within 10 years after surgery was considered as the primary outcome variable. Due to censoring, all breast cancer deaths after 10 years of follow-up were not taken into consideration. Patients who died from causes other than breast cancer were censored at the time of their death when in the 10-year follow-up period or at 10 years if survival exceeded 10 years.

Mortality rates were computed with a censoring at 10 years and using the cumulated number of person years (PY) in each category as a denominator. Kaplan-Meier survival curves and hazard rates estimated by a Cox proportional hazard model were computed to quantify the effect of *TP53* mutation on breast cancer-specific mortality after adjustment for known clinical cofactors (tumor size, node status, ER and PR contents, and age at diagnosis). Histopathologic subtypes and grading were also considered in a descriptive analysis but were not used as predictors of survival because of missing data and possible differences in the classification systems used by participating hospitals.

All Cox models were stratified by hospital using a different baseline hazard function for each hospital to adjust for differences between centers that may be attributable to differences in tumor classification methods, hormone receptor measurements, treatment regimen (surgery and/or chemotherapy), or disease severity (some centers may be more likely than others to admit patients with more severe disease). Patients were grouped according to the mutation present in their tumor as described above. Proportionality assumption for the Cox model was verified by looking at parallelism of survival curves in Kaplan-Meier analysis. To identify possible interactions with *TP53*, Kaplan Meier curves were also stratified according to the presence of a *TP53* mutation.

Because the detection of *TP53* mutation outside exons 5 to 8 was done in a limited number of cases, we focused on mutations occurring in exons 5 to 8 and two distinct analyses were done. In the first analysis, all patients were considered and patients with a mutation outside exons 5 to 8 were included in the "no-mutation" group. Multivariate analysis was restricted to patients who had complete data for the variables that remained associated in the final Cox model. Several sensitivity analyses were previewed to validate the results obtained in the final Cox model under the presence of missing data for some clinical cofactors or when using a different definition of the outcome measure:

(a) The estimated coefficients in the final Cox model were compared with those obtained when using the whole population, which included individuals with missing values for some cofactors who were included in the analyses and labeled as "unknown" or "missing information."

(b) Estimated coefficients in the final Cox model were compared with those obtained when death for breast cancer was replaced by death for all causes.

(c) The set of predictors and the estimated coefficients in the Cox model were compared with those obtained when censoring time was set at 5 years instead of 10 years.

In the second analysis, only patients with mutations in exons 5 to 8 were included to compare the prognostic value of specific *TP53* mutations. Mortality rates and Kaplan-Meier curves and log rank test were computed.

Results

Associations between TP53 mutations and clinicopathologic variables. Clinical and molecular data on primary breast cancer patients from 10 European hospitals were gathered to investigate the relationship between *TP53* mutation status and patient outcome (breast cancer survival) after adjustment for known clinical predictors. A total of 1,794 patients were eligible for the present study. All patients underwent surgery between 1987 and 1997 and were followed-up for a median period of 78 months (interquartile range, 46-121). The number of eligible patients by center varied from 41 to 389 with follow-up varying from 95 to 262 months. *TP53* gene was sequenced from exons 5 to 8 in 1,143 tumors and from exons 2 to 11 in 651 tumors. The clinical and molecular characteristics of patients and tumors are summarized in Table 1. The median age at surgery was 59 years (interquartile range, 48-70). The majority of cases were invasive ductal carcinomas of size <5 cm without node invasion and with positive ER and PR expression. A total of 308 samples had a single mutation within exons 5 to 8 (17%, 308 of 1,794), whereas 26 had a single mutation outside exons 5 to 8 (4%, 26 of 651).

The association of *TP53* mutation with clinical and molecular variables was investigated (Table 1). For this analysis, patients whose tumors carried a mutation outside exons 5 to 8 (26 of 651 patients analyzed for exons 2-11) were included in the no-mutation group because mutations in exons 2 to 4 and exons 9 to 11 should also be expected to occur in the 1,143 patients whose tumors were analyzed only for exons 5 to 8. *TP53* mutation was significantly associated with age at diagnosis ($P = 0.037$), histopathologic subtype ($P < 0.001$), size ($P < 0.001$), histopathologic grading ($P < 0.001$), node status ($P = 0.016$), and hormone receptor status (ER and PR, $P < 0.001$). *TP53* mutation was more prevalent in patients <60 years old, tumors of ductal and medullar histology, high grade, large size, in node-positive cases, and in tumors with low hormone receptor contents.

Prognostic value of TP53 gene status. In univariate analysis, known prognostic factors of survival such as tumor size, histopathologic subtype, grading, node status, and hormone