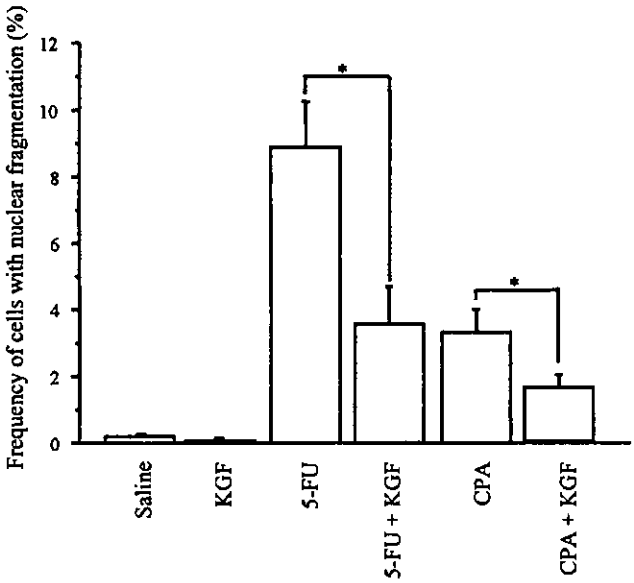

Figure 7 Frequency of cells with nuclear fragmentation in MCF7 treated with anticancer drugs and/or 10 ng/ml KGF. Data are given as the mean ± s.d. from three independent experiments. $^*P<0.05$. 5-FU = 5-fluorouracil (25 μg/ml); CPA = cyclophosphamide (500 μg/ml).

prognosis, though the direct relationship between KGF expression and prognosis remains unclear at present.

In the present study, no correlation was found between coexpression of KGF and KGFR and Ki-67 LI in human breast cancer. In fact, it has been known that KGF induces cell differentiation and suppresses cell proliferation in some cases. For example, KGF is known to promote the differentiation of keratinocytes derived from human neonatal foreskin[24,25] as well as human lung squamous cell carcinoma.[26] In human salivary gland tumors, loss of KGFR expression was observed in the process of malignant transformation.[50,51] Similarly, in prostate cancer, loss of KGFR expression was accompanied by the switch from androgen-sensitive, slow-growing tumors to androgen-insensitive, more aggressive tumors.[52] Therefore, KGF may play a pivotal role in the regulation of cell kinetics.

In conclusion, we have confirmed the expression of KGF and KGFR in human breast cancer cells at the level of both mRNA and protein, and have found a close association of KGF expression with the presence of ER α. And we have further indicated that KGF may play an inhibitory role in the induction of breast cancer cell apoptosis, raising a possibility that KGF may give resistancy against anticancer drugs to the cancer cells.

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Systemic distribution of estrogen-responsive finger protein (Efp) in human tissues

Norihiro Shimada[a,b,*], Takashi Suzuki[a], Satoshi Inoue[c], Katsuaki Kato[b], Akira Imatani[b], Hitoshi Sekine[b], Syuichi Ohara[b], Tooru Shimosegawa[b], Hironobu Sasano[a]

[a] *Department of Pathology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan*
[b] *Department of Gastroenterology, Tohoku University School of Medicine, Sendai, Japan*
[c] *Division of Gene Regulation and Signal Transduction, Research Center for Genomic Medicine, Saitama Medical School, Saitama, Japan*

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Abstract

Estrogen-responsive finger protein (Efp), a target gene product of estrogen receptor (ER), is considered essential for estrogen-dependent cell proliferation. The biological significance of Efp remains unclear in human tissues, and therefore, we examined systemic distribution of Efp in human adult and fetal tissues using RT-PCR and immunohistochemistry. Efp mRNA expression was marked in the placenta and uterus, high in the thyroid gland, aorta, and spleen in adult, and relatively low in other human adult and fetal tissues examined in this study. Efp immunoreactivity was detected in epithelium of various adult tissues, and was also detected in cytotrophoblasts of the placenta and splenic macrophages. Efp immunolocalization in human fetus was generally similar as that in adult. These Efp-positive cells were previously reported to be associated with ERα and/or ERβ expression. Therefore, these results indicate that Efp is widely expressed and may play important roles in various human tissues possibly through ERs.

Keywords: Distribution; Estrogen-responsive finger protein (Efp); Human tissues; Immunohistochemistry; Reverse transcriptase-polymerase chain reaction (RT-PCR)

1. Introduction

It is well known that estrogen plays important roles, not only in female reproductive organs but also in various tissues such as bone, liver, central nervous system (CNS) and cardiovascular system (Dickson and Stancel, 2000; Albertazzi and Purdie, 2001). Biological effects of estrogen are mediated through an interaction with the estrogen receptor (ER). Recently, a second ER, ERβ, has been identified in human (Kuiper et al., 1996; Mosselman et al., 1996), and the previously known human ER has been renamed ERα. ERβ is detected in various human tissues, including in both adult and fetus (Taylor and Al-Azzawi, 2000; Takeyama et al., 2001), while ERα is mainly expressed in female reproductive organs. ERs activate transcription by binding to estrogen-responsive elements (EREs) located in the promoter regions of target genes (Tsai and O'Malley, 1994). A variety of estrogenic functions are characterized by the expression of the estrogen-responsive genes following the binding of receptor protein to EREs (Inoue et al., 1993; Orimo et al., 1999).

Estrogen-responsive finger protein (Efp) belongs to a member of RING- finger B-box Coiled-Coil family (Inoue et al., 1993), which is considered to be involved in the regulation of various cellular functions, including cell-cycle regulation and transcription (Saurin et al., 1996). Efp has been isolated by genomic binding site cloning using a recombinant ER protein, and Efp gene has an ERE at the 3′-untranslated region (Inoue et al., 1993). Efp mRNA was also detected in MCF-7 human breast carcinoma cell line, and was rapidly induced by estrogen treatment within 0.5 h (Ikeda et al., 2000). Efp is mainly expressed in female reproductive organs and brain in mice (Orimo et al., 1995), and the study of Efp knockout mice revealed that Efp is essential for cell growth mediated by estrogen in the uterus (Orimo et al., 1999). In human, Efp expression has been reported

* Corresponding author. Tel.: +81-22-717-8050; fax: +81-22-717-8053.
E-mail address: nshimada@int3.med.tohoku.ac.jp (N. Shimada).

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in breast tissues (Ikeda et al., 2000; Thomson et al., 2001; Urano et al., 2002), and Efp is postulated to be involved in the mammary gland differentiation (Thomson et al., 2001). However, Efp has not been examined in other human tissues, although estrogenic actions have been demonstrated in various human tissues, and the biological significance of Efp in relation to systemic estrogenic actions remains unclear. Therefore, in this study, we examined systemic distribution of Efp in human adult and fetal tissues using reverse transcription/real-time polymerase chain reaction (RT/real-time PCR) and immunohistochemistry.

2. Materials and methods

2.1. Tissue collection and preparation

Nonpathological human adult tissues ($n = 6$, respectively) were retrieved from autopsy files at Tohoku University Hospital (Sendai, Japan). Specimens of nonneoplastic area of human uterus ($n = 5$) were obtained from women who underwent hysterectomy for cervical carcinoma at the Department of Obstetrics and Gynecology, Tohoku University Hospital, and informed consent was obtained from these patients before the surgery. Human fetal tissues ($n = 5$, respectively) were obtained from fetuses aged 12–21 weeks gestation after elective termination in normal pregnant women at Nagaike Maternal Clinic (Sendai, Japan), and informed consent was obtained before the elective termination. The specimens were fixed in 10% formalin and embedded in paraffin wax. Histological examinations revealed no significant pathologic abnormalities in these tissues.

RT/real-time PCR analyses were also performed using snap-frozen samples stored at −80 °C. The type of tissues and the number of specimens examined in this study were listed in Table 2.

This study protocol was approved by the ethics committee of Tohoku University School of Medicine (Sendai, Japan).

2.2. RT/real-time PCR

Total RNAs were isolated using TRIzol regent (Life Technologies Inc., Grand Island, NY, USA), and used for the first-strand cDNA synthesis with Superscript II reverse transcriptase and oligo $(dT)_{12-18}$ primer (Life Technologies Inc., Gaitherburg, MD, USA). The forward and reverse primers for Efp mRNA were designed in the different exon to avoid the amplification of genomic DNA (Table 1). Oligonucleotide primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Tokunaga et al., 1987) were also used as internal standard in this study.

The Light Cycler System (Roche Diagnositics GmbH, Mannheim, Germany) with FastStart DNA Master SYBER Green I (Roche Diagnostics GmbH) was used to obtain relative levels of Efp mRNA expression by real-time PCR (Dumoulin et al., 2000). An initial denaturing step of 95 °C for 10 min was followed by 40 cycles, respectively, of 95 °C for 15 s; 10 s annealing at 64 °C (Efp) or 60 °C (GAPDH); and extension for 10 s at 72 °C. The fluorescence intensity of the double-strand specific SYBER Green I, which reflects the amount of formed specific PCR products, was read by the Light Cycler at 86 °C after the end of each extension step. After PCR, these products were resolved on a 2% agarose ethidium bromide gel. PCR products were purified and subjected to direct sequencing (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 310 Genetic Analyzer, Perkin-Elmer Corp., PE Applied Biosystems, Foster City, CA, USA) to verify amplification of the correct sequences.

As positive controls, breast cancer cell line MCF-7 was used (Ikeda et al., 2000). Negative control experiments lacked cDNA substrate to check for the possibility of exogenous contaminant DNA, and no amplified products were detected. The mRNA levels of Efp were adjusted to the levels of GAPDH and expressed as the ratio per those in MCF-7 (100%).

2.3. Immunohistochemistry

Immunohistochemical analysis was performed by employing the streptavidin–biotin amplification method using a Histofine Kit (Nichirei, Tokyo, Japan). The dilution of human Efp monoclonal antibody (Inoue et al., 1993) was 1/5000 in this study. 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 methyl green or hematoxylin. Human breast tissues were used as positive control for Efp immunostaining (Ikeda et al., 2000; Thomson et al., 2001). For negative controls, normal mouse IgG instead of the primary antibody was used, and no specific immunoreactivity was detected in these sections. To identify Efp positive cells in the spleen, we also performed immunohistochemistry for CD68 (DAKO, Carpinteria, CA, USA) using the serial sections.

Table 1
Primer sequences utilized in RT/real-time PCR analysis

cDNA		Primer sequences (5′–3′)	cDNA position	Size (bp)
Efp (D21205)	Forward	AACATCTCTCAAGGCCAAGGT	1338–1358	287
	Reverse	AGATGCCTACCCCACAGAAGT	1604–1624	
GAPDH (M33197)	Forward	TGAACGGGAAGCTCACTGG	731–750	307
	Reverse	TCCACCACCCTGTTGCTGTA	1018–1038	

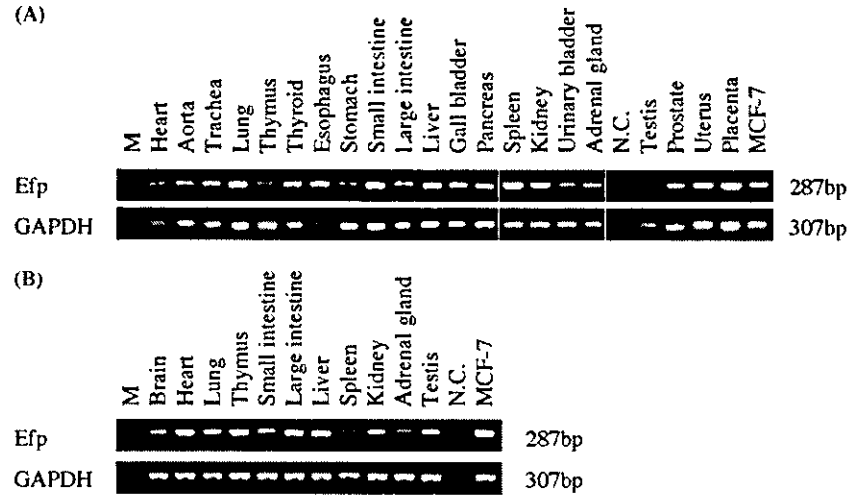

Fig. 1. Reverse transcription/real-time polymerase chain reaction (RT/real-time PCR) analysis for Efp in various human tissues in adult (A) and fetus (B). mRNA expression for Efp was detected as a specific single band (287 bp) in various human tissues both in adult (A) and fetus (B) (upper panels, respectively). Efp mRNA was not detected in adipose tissue in adult. In this study, mRNA expression for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also detected as a specific single band (307 bp) in each specimen examined (lower panels, respectively). NC: negative control (no cDNA substrate); MCF-7: positive control. Representative samples were shown in this agarose gel photo.

3. Results

3.1. RT/real-time PCR

As shown in Fig. 1A and B, mRNA expression for Efp and GAPDH was detected as a specific single band (287 bp for EFP and 307 bp for GAPDH) by RT/real-time PCR analysis. Results of Efp mRNA expression levels in various human tissues were summarized in Table 2. Efp mRNA expression was abundantly detected in the placenta (159.2%) and uterus (95.7%). Efp mRNA expression was also high in the thyroid gland, aorta, and spleen in adult (12.5–12.8%). Efp mRNA expression was relatively low in other human adult and fetal tissues examined (≤10.5%).

3.2. Immunohistochemistry

Results of immunohistochemistry for Efp were summarized in Table 3. In adult, Efp immunoreactivity was detected in the cytoplasm of epithelium in various tissues (Fig. 2A–F). In some glandular epithelia, Efp immunoreactivity was tended to be marked at the luminal side (Fig. 2F). In addition, Efp immunoreactivity was detected in the cytotrophoblasts, but not syncytiotrophoblasts (Fig. 2G) of the placenta and vascular endothelium. Efp immunoreactivity was also positive in CD68-positive splenic macrophages in the spleen (Fig. 2H and I).

Patterns of Efp immunolocalization in fetus were generally similar as these in adult (Fig. 2J–L), however, no significant immunoreactivity was detected in the fetal lung, and spleen in this study. Efp immunoreactivity was detected in chondrocytes of cartilage in fetus, whereas it was not significant in adult cartilage.

Table 2
Efp mRNA expression in human tissues

Tissues	Adult		Fetus	
	n	mRNA level (%)	*n*	mRNA level (%)
Placenta	4	159.2 ± 98.6	NA	
Uterus	5	95.7 ± 34.4	NA	
Testis	1	7.9	1	2.0
Prostate	3	4.9 ± 3.6	NA	
Thyroid gland	6	12.5 ± 7.5	NA	
Adrenal gland	5	3.5 ± 2.0	1	0.2
Brain	NA		1	1.4
Heart	4	7.1 ± 3.0	2	3.7 ± 0.9
Aorta	2	12.8 ± 7.9	NA	
Trachea	3	8.4 ± 4.2	NA	
Lung	5	5.3 ± 3.4	2	8.2 ± 3.8
Thymus	1	3.6	2	8.2 ± 3.1
Esophagus	3	1.1 ± 1.1	NA	
Stomach	4	0.5 ± 0.4	NA	
Small intestine	5	1.3 ± 0.8	1	0.8
Large intestine	5	3.1 ± 1.8	1	9.5
Liver	5	4.6 ± 3.1	3	5.9 ± 2.3
Gall bladder	3	2.0 ± 1.6	NA	
Pancreas	3	6.1 ± 3.4	NA	
Spleen	6	12.8 ± 7.6	1	0.1
Kidney	6	10.5 ± 5.9	2	1.2 ± 0.7
Urinary bladder	1	0.6	NA	

The mRNA level of Efp was summarized as a ratio of GAPDH and evaluated as a ratio (%) compared with that of positive control (MCF-7 cells = 100%). Data represent mean ± S.E.M. (more than two samples) or mean ± percent difference (two samples). NA: samples were not available.

4. Discussion

In this study, Efp mRNA was abundantly detected in the placenta (cytotrophoblasts) and uterus (endometrial glandular or epithelial cells). In the placenta, estrogen is gen-

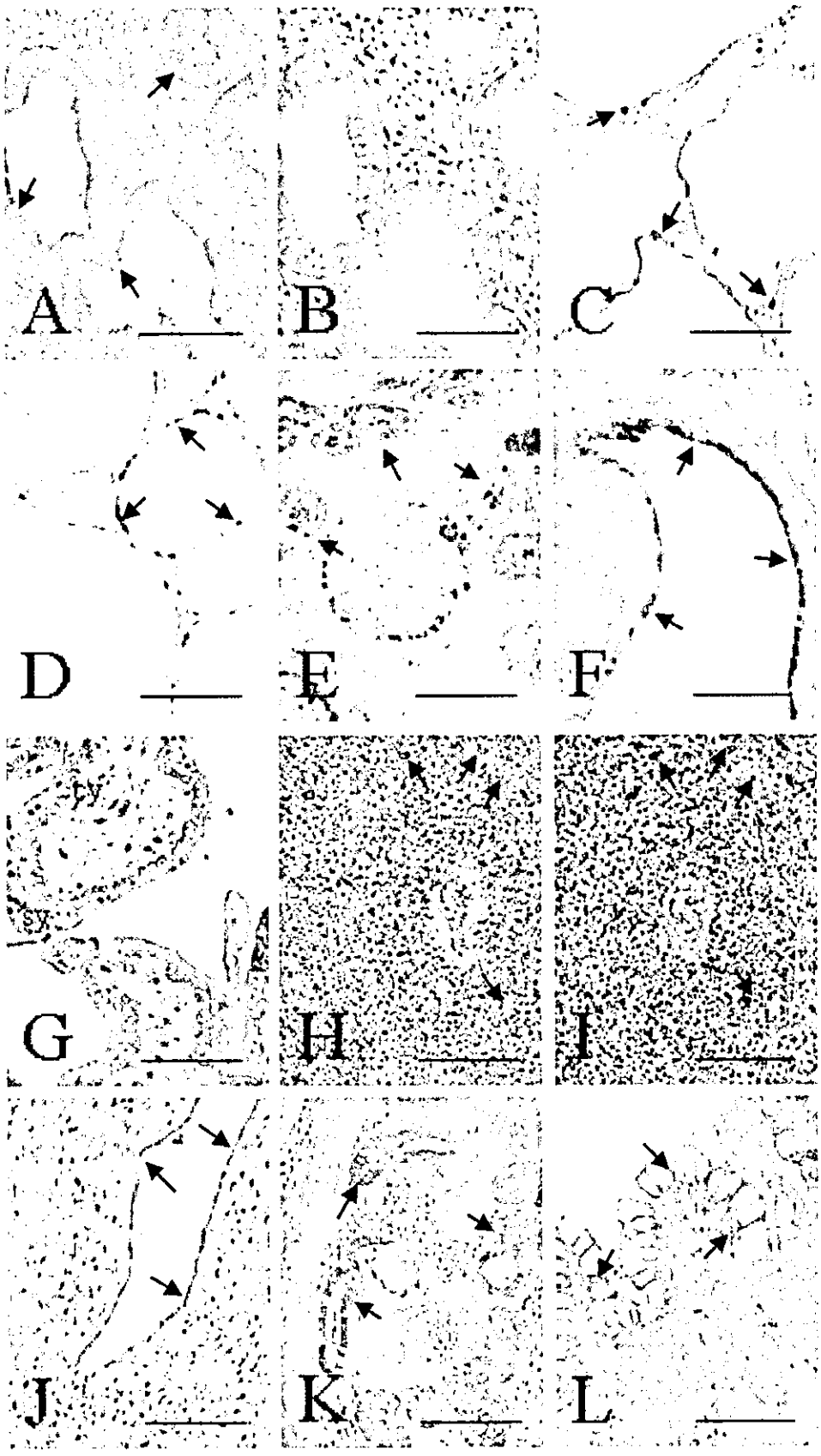
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Table 3
Immunolocalization of Efp in human tissues

Tissues	Types of Efp positive cell			
	n	Adult	*n*	Fetal
Placenta	3	Cytotrophoblasts, decidual cell	0	NA
Uterus	3	Endometrial epithelial cell	2	Endometrial epithelial cell
Testis	3	Ductal cell, leydig cell	3	Ductal cell, mesenchymal cell
Prostate	2	Grandular epithelium	0	NA
Thyroid gland	3	Follicular epithelium	1	Follicular epithelium
Adrenal gland	5	None	2	None
Brain	0	NA	5	Nerve cell
Heart	2	None	2	None
Aorta and blood vessel	2	Endothelial cell	2	Endothelial cell
Trachea and bronchus	2	Epithelial cell	5	Epithelial cell
Lung	4	Alveolar epithelium	4	None
Thymus	2	Thymic epithelial cell	3	Thymic epithelial cell
Esophagus	2	Glandular epithelium	1	None
Stomach	2	None	3	None
Small and large intestine	3	Epithelial cell	4	Epithelial cell
Liver	2	Hepatocyte	3	Hepatocyte
Gall bladder	5	None	0	NA
Pancreas	2	Ductal epithelium	2	Ductal epithelium
Spleen	2	Splenic macrophage	2	None
Kidney	3	Epithelium of proximal tubule	2	Epithelium of proximal tubule
Urinary bladder	2	Transitional epithelium	3	Transitional epithelium
Cartilage	2	None	5	Chondrocyte
Salivary gland	2	Ductal epithelium	0	NA

NA: samples were not available; none: there was not significant immunoreactivity.

erally considered to play an important role in the regulation of placental function. ERα was expressed in cytotrophoblast cells, not in syncytiotrophoblast cells, and diminution of ERα and appearance of ERβ were demonstrated to be associated with the development of syncytiotrophoblast cells (Bukovsky et al., 2003a,b). Orimo et al. (1995) demonstrated that Efp was mainly expressed in female reproductive organs in mice, including uterus, ovary, and mammary gland, and was colocalized with ER. The study of Efp knockout mice subsequently revealed that Efp is essential for estrogen-induced cell proliferation as one of the direct targets of ERα (Orimo et al., 1999). Therefore, it is suggested that Efp is closely involved in the estrogenic actions, including the growth and/or differentiation, in these female reproductive organs through ERα.

High level of Efp mRNA expression was also detected in the aorta (endothelial cells) and spleen (splenic macrophages). Estrogen modulates vascular functions, and vascular endothelium is associated with expression of both ERα and ERβ (Suzuki et al., 2003). Estrogen reduces the vascular injury in wild-type, ERα knockout (ERαKO) (Sullivan et al., 1995; Iafrati et al., 1997), and ERβKO (Karas et al., 1999), but could not reduce the injury in ERαβ double KO mice (Karas et al., 2001). ERs were also detected in splenic macrophages (Kramer and Wray, 2002). Erlandsson et al. (2001) further demonstrated that deletion of ERα led to hypoplasia of spleen in their study using ERαKO, ERβKO, and ERαβ double KO mice. However the detailed estrogenic functions remain unclear in human aorta or spleen. Therefore the estrogenic actions in these tissues above may be partly through the Efp expression.

In our study, relatively low levels of Efp mRNA expression were detected in various tissues including those in which the expression of ERs has been reported. Among human tissues examined, Efp immunoreactivity was mainly detected in the epithelial cells. Previous studies demonstrated the widespread distribution of ERβ in various human tissues both in adult and fetus (Taylor and Al-Azzawi, 2000; Takeyama et al., 2001). ERβ immunoreactivity was strongly detected in the follicular epithelium of human thyroid gland

Fig. 2. Immunohistochemistry for Efp in human tissues. Efp immunoreactivity was detected in the endometrial epithelial cells in the uterus (A). No immunoreactivity was detected in the negative control using the serial section (B). In the lung, Efp immunoreactivity was detected in the alveolar epithelium (arrows) (C). Efp immunoreactivity was detected in follicular epithelium of the thyroid gland (arrows) (D). In the kidney, Efp immunoreactivity was detected in epithelium of the proximal tubule (arrows) (E). Glandular epithelial cells (arrows) were positive for Efp in the esophagus (F). In the placenta, Efp was positive in the cytotrophoblast (cy) but not syncytiotrophoblasts (sy) (G). In the spleen, Efp immunoreactivity was sporadically found in the marginal zone (H). These cells were also positive for CD68 (arrows) in the serial section (I), suggesting the splenic macrophages. Hematoxylin was used for counterstaining in (I). In fetal aorta, Efp immunoreactivity was detected in endothelial cells (arrows) (J). Efp immunoreactivity was detected in epithelium of the proximal tubule (arrows) in the fetal kidney (K). Efp was immunolocalized in the epithelium of the fetal colon (L): bar = 100 μm, respectively.

from 11 gestational weeks, suggesting the important estrogen actions in the development and/or maintenance of thyroid follicular functions through ERβ (Kawabata et al., 2003). ERβ was also reported in glandular epithelium of the prostate (Tsurusaki et al., 2003), epithelium of respiratory system (Taylor and Al-Azzawi, 2000), and epithelium of the stomach (Matsuyama et al., 2002) and colon (Konstantinopoulos et al., 2003), where Efp immunoreactivity was detected in our present immunohistochemical study. On the other hand, ERβ was also detected in interstitial cells such as adipocytes, smooth muscle cells and fibroblasts (Taylor and Al-Azzawi, 2000; Joyner et al., 2001; Haczynski et al., 2002), while Efp immunoreactivity was not detected in these cells in our present study. Therefore, Efp is considered to be involved in the regulation of estrogen related functions in epithelial cells in these tissues, possibly through ERβ, but not in that of stromal cells.

In summary, our present results demonstrated that Efp is widely expressed in human adult and fetal tissues. The Efp-positive cells were generally associated with the expression of ERα and/or ERβ previously, and therefore, it is suggested that Efp plays important roles in various human tissues possibly through ERs. Further examinations, including functional studies, are required to clarify these hypotheses or to understand the detailed biological significance of Efp in human tissues.

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Decreased Expression of 14-3-3σ Is Associated with Advanced Disease in Human Epithelial Ovarian Cancer: Its Correlation with Aberrant DNA Methylation

Jun-ichi Akahira,[1,2] Youko Sugihashi,[1] Takashi Suzuki,[2] Kiyoshi Ito,[1] Hitoshi Niikura,[1] Takuya Moriya,[2] Makoto Nitta,[3] Hitoshi Okamura,[3] Satoshi Inoue,[4] Hironobu Sasano,[2] Kunihiro Okamura,[1] and Nobuo Yaegashi[1]

Departments of [1]Obstetrics and Gynecology, and [2]Pathology, Tohoku University Graduate School of Medicine, Sendai, Japan; [3]Department of Obstetrics and Gynecology, Kumamoto University School of Medicine, Kumamoto, Japan; and [4]Division of Gene Regulation and Signal Transduction, Research Center for Genomic Medicine, Saitama Medical School, Saitama, Japan

ABSTRACT

Purpose: **In this study, we examined the promoter methylation status and expression of 14-3-3σ and evaluated its clinical significance in epithelial ovarian cancer.**

Experimental Design: **Twelve ovarian cancer cell lines; 2 ovarian surface epithelial cell lines; and 8 normal, 8 benign, 12 borderline, and 102 ovarian cancer tissues were examined. Methylation-specific PCR, quantitative reverse transcription-PCR, and immunohistochemistry were used to evaluate methylation status and expression of *14-3-3σ* gene and protein.**

Results: **Among the 12 ovarian cancer cell lines, the presence of a methylated band was detected in seven cell lines. Median values of relative *14-3-3σ* gene expression in cancers with methylation (3.27) were significantly lower than those without methylation (16.4; $P < 0.001$). Treatment of 5-aza-2′-deoxycitidine resulted in the demethylation of the promoter CpG islands and reexpression. All of the normal, benign, and borderline tissues were positive for 14-3-3σ protein, and in ovarian cancer tissues, 73.5% (75 of 102) were positive for 14-3-3σ protein and was almost consistent with methylation status. Negative immunoreactivity of 14-3-3σ was significantly correlated with high age and serous histology, high-grade, advanced-stage residual tumor of >2 cm, high serum CA125, high Ki-67 labeling index, and positive p53 immunoreactivity. 14-3-3σ immunoreactivity was significantly associated with overall survival ($P = 0.0058$).**

Conclusions: **Our findings suggest that 14-3-3σ is inactivated mainly by aberrant DNA methylation and that it may play an important role in the pathogenesis of epithelial ovarian cancer.**

INTRODUCTION

Epithelial ovarian cancer is the leading cause of death from gynecological malignancies in the great majority of developed countries (1). This high mortality is considered to be, in large part, due to the advanced stage of the disease commonly present at the time of diagnosis, but many clinical studies have reported that there are some prognostic factors in ovarian cancer other than clinical stages, such as histology, the degree of primary surgical cytoreduction, and response to chemotherapy (1–3). Other prognostic parameters have been also proposed in addition to those relatively established parameters. These include Ki67 index, progesterone receptor, and the preoperative serum maker CA125, and others (4–6). The identification of new prognostic factors may further contribute to improve treatment and clinical outcome of ovarian cancer patients.

The cause of epithelial ovarian cancer is still unknown. Although *BRCA1* and *BRCA2* mutation have been identified as associated with susceptibility to ovarian cancer (7, 8), mutations in these genes account for only 2–3% of all ovarian cancers. The remaining cases are considered to be sporadic in nature and arise as a result of acquired alterations in oncogenes and tumor suppressor genes such as *TP53* and *PTEN* (9–11).

DNA methylation has an essential regulatory function in mammalian development, suppressing gene activity by changing chromatin structure (12, 13). It has become apparent that aberrant DNA methylation of promoter region CpG islands may serve as an alternate mechanism to genetic defects in the inactivation of tumor suppressor genes in human cancers (14, 15). Accordingly, the identification of gene targets of methylation-associated silencing could lead to novel genes involved in the initiation and progression of human neoplasia.

14-3-3σ was originally identified as a p53-inducible gene that is responsive to DNA damaging agents (16). Recent study demonstrated that 14-3-3σ protein plays a crucial role in the G_2 checkpoint by sequestering the mitotic initiation complex, cdc2-cyclin B1, in the cytoplasm after DNA damage (17). This prevents cdc2-cyclin B1 from entering the nucleus in which the protein complex would normally initiate mitosis. In this manner, 14-3-3σ induces G_2 arrest and allows the repair of damaged DNA (16, 17). The expression of 14-3-3σ is reported to be frequently lost in human breast, gastric, and lung cancers, and the inactivation is due to

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Requests for reprints: Jun-ichi Akahira, Department of Obstetrics and Gynecology, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai, 980-8574, Japan. Phone: 81-22-717-7254; Fax: 81-22-717-7258; E-mail: jakahira-tohoku@umin.ac.jp.

aberrant DNA methylation (18–20). However, the expression of 14-3-3σ and its mechanism have not been examined in epithelial ovarian cancer. Therefore, in this study, we examined the promoter methylation status and expression of 14-3-3σ in epithelial ovarian cancer cells. We also evaluated the correlation between 14-3-3σ expression and clinicopathological parameters in patients with epithelial ovarian cancer.

MATERIALS AND METHODS

Tissues and Cells. Eight normal ovaries, 8 ovarian serous cystadenomas, 12 serous borderline tumors, and 102 ovarian cancer cases were obtained from patients after surgical therapy from 1988 to 2000 at Tohoku University Hospital, Sendai, Japan. In ovarian cancer patients, information regarding age, performance status on admission, histology, stage, grade, residual tumor after primary surgery, and overall survival were retrieved from the review of patient charts. Median follow-up time of the patients in this study was 59 months (4–120 months). Eighty-four (82.3%) of 102 patients received platinum-containing chemotherapy after operation. Patients who have early-stage (stage Ia) and low grade-disease (G1, G2) and patients who have poor performance status did not receive platinum-based chemotherapy. Performance status was defined according to WHO criteria (21). Histology and stage were determined according to FIGO (International Federation of Gynecology and Obstetrics) criteria. Grade was evaluated by one of the authors (T. M.) using universal grading system in epithelial ovarian cancer (22). Residual disease was determined by the amount of unresectable tumor that remained after primary cytoreductive surgery. Optimal cytoreduction was defined as no gross residual tumor greater than 2 cm in diameter, and suboptimal cytoreduction was defined as any gross residual disease remaining greater than 2 cm in diameter. Overall survival was calculated from the time of initial surgery to death, or the date of last contact. Survival times of patients still alive or lost to follow-up were censored in December 2002. All of these archival specimens were retrieved from the surgical pathology files at Tohoku University Hospital, Sendai, Japan. 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.

OVCAR3, Caov3, SKOV3, TOV112D, TOV21G, OV90, and ES2 (adenocarcinoma, OVCAR3, SKOV3; serous adenocarcinoma, Caov3, OV90; clear cell adenocarcinoma, TOV21G, ES2; endometrioid adenocarcinoma, TOV112D) cell lines were purchased from American Type Culture Collection. JHOS2, JHOS3, HTOA, OMC3, and JHOC5 (serous adenocarcinoma, JHOS2, JHOS3, HTOA; mucinous adenocarcinoma, OMC3; clear cell adenocarcinoma, JHOC5) cell lines were purchased from Riken cell bank (Tsukuba, Japan). Normal ovarian surface epithelial cell lines (OSE2 and OSE4) were established by one of the authors (M. N.; Ref. 23). Cell lines were maintained in DMEM/F12 (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen), and were incubated in 5% CO_2 at 37°C. For 5-aza-2′ deoxycytidine (5azaC) treatment, 1×10^6 cells were seeded into T75 flasks and were treated with 0.5 μM or 1.0 μM 5azaC (Sigma) for 72 h.

Methylation-Specific PCR. Methylation status of the samples was investigated by methylation-specific PCR as described in the literature (24). Genomic DNA of ovarian cancer tissue was extracted using a laser capture microdissection and treated with proteinase K (0.5 mg/ml) for 48 h at 37°C. Genomic DNA from ovarian cancer cell lines was extracted using AquaPure Genomic DNA kit (Bio-Rad). The quality and integrity of the DNA was determined by the A260:280 ratio. One μg of genomic DNA was treated with sodium bisulfite using CpGenome DNA modification kit (Intergen) according to the instructions. Amplification was achieved in a 20-μl reaction volume containing 2 μl of 10× Ex Taq Buffer, 1.5 μl of 25 mM $MgCl_2$, 1 μM each primer, 1.5 μl of 2.5 mM dNTPs, and 1 unit of Takara Ex Taq polymerase (Takara, Japan). Hot-start PCR was performed in a thermal cycler (Takara) for 35 cycles, each of which consisted of denaturation at 96°C for 30 s, annealing at 64°C for 30 s, and extension at 72°C for 90 s, followed by a final 10 min extension at 72°C. Primers used were 5′-TGGTAGTTTTTATGAAAGGCGTC-3′ and 5′-CCTCTAACCGCCCACCACG-3′ (104-bp) for the methylated reaction (M primers), and 5′-ATGGTAGTTTTTATGAAAGGTGTT-3′ and 5′-CCCTCTAACCACCCACCACA-3′ (106-bp) for the unmethylated reaction (U primers; Ref. 18). Universal methylated human male genomic DNA (Intergen) was used as a positive control for methylated reaction. Genomic DNA purified from MCF-7 breast cancer cell line was used as a positive control for unmethylated reaction (18). A blank control containing all of the PCR components except template DNA was also included in all of the PCRs. Reaction products were separated by electrophoresis on a 3% agarose gel, stained with ethidium bromide, and visualized under UV illumination. Specimens with purely unmethylated promoters have positive PCR products by U primers but not with the M primers. Specimens that contain purely methylated promoters will have PCR products by using M primers but not with the U primers. Specimens that contain heterogeneous status of both methylated and unmethylated promoters have PCR products from both U primers and M primers.

Reverse Transcription and Real-Time Quantitative PCR. Total RNA was isolated from cell lines 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-PCR kit (SUPERSCRIPT II First-strand synthesis system, Invitrogen) was used and cDNA synthesis was carried out according to the instructions. cDNAs were synthesized from 2 μg of total RNA using random hexamer, and reverse transcription was carried out for 50 min at 42°C with SUPERSCRIPT II reverse transcriptase. Real-time quantitative PCR was performed using the iCycler system (Bio-Rad). For the determination of 14-3-3σ cDNA content, a 25-μl reaction mixture consisted of 23 μl iQ SYBR Green MasterMix, 1 μM each primer, and 1 μl of template cDNA were prepared. The PCR conditions were as follows: initial duration at 96°C for 60 s, and followed by 35 cycles with denaturation at 96°C for 30 s, annealing at 64°C (both for 14-3-3σ and β-actin) for 30 s, and extension at 72°C for 30 s. The fluorescence intensity of the double-strand specific SYBR Green I, reflecting the amount of formed PCR-product, was read at 88°C after the end of each elongation step. Primers used were

as follows: 14-3-3σ, 5′-CCTGCTGGACAGCCACCTCA-3′ and 5′-TGTCGGCCGTCCACAGTGTC-3′ (397-bp; Ref. 20); β-actin, 5′-CCAACCGCGAGAAGATGA-3′ and 5′-GGAAGGAAGGCTGGAAGAGT-3′ (459-bp; Ref. 25). β-actin cDNA fragments were amplified as internal positive controls. Normal human ovarian cDNA library (Stratagene, La Jolla, CA) was used as a normal control, cDNA from MCF-7 was used as a positive control, and water blank was used as a negative PCR control (data not shown). Control reactions in which reverse transcriptase was omitted were amplified under the same conditions to exclude DNA contamination (data not shown). Two independent quantitative PCR reactions were performed for each sample.

Immunohistochemistry. Immunohistochemical analysis was performed using the streptavidin-biotin amplification method using a Histofine kit (Nichirei, Tokyo, Japan), and have been previously described in detail (26). Polyclonal antibody for 14-3-3σ (N-14), monoclonal antibody for p53 (B20.1), and monoclonal antibody for Ki-67 (MIB-1) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), Biomeda (Foster City, CA), and DAKO (Tokyo, Japan), respectively.

For antigen retrieval, the 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 dyhydrate (pH 6.0)]. The dilutions of primary antibodies for 14-3-3σ, p53 and Ki-67 were 1:100, 1:40, and 1:300, respectively. 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. Nonneoplastic breast epithelial tissue was used as a positive control (18). As negative controls, 0.01 M PBS and normal mouse IgG were used in place of primary antibodies.

The immunohistochemical expression of 14-3-3σ, p53, and Ki-67 was independently reviewed by two of the authors (J. A. and T. M.) who had no knowledge of the clinicopathological data. As immunoreactivities of 14-3-3σ and p53 were relatively homogeneous and clearly distinguished for positive and negative, they were classified into two groups: +, positive carcinoma cells; and −, no immunoreactivity. Scoring of Ki-67 in carcinoma cells was counted independently by these same two authors, and the percentage of immunoreactivity in at least 500 carcinoma cells, *i.e.*, labeling index, was determined. Whenever a difference of greater than 5% was observed between the two readings, slides were reviewed jointly, and a consensus was reached.

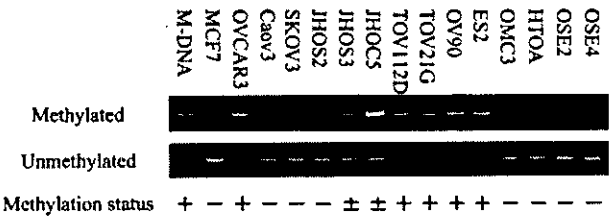

Fig. 1 Methylation-specific PCR (MSP) for 14-3-3σ in ovarian cancer and surface epithelial cell lines. Specimens with methylated promoters have positive PCR products in the *Methylated Lane*, and specimens with unmethylated promoters have positive PCR products in the *Unmethylated Lane*. Specimens that contain heterogeneous status of both methylated and unmethylated promoters have PCR products in both the *Methylated* and the *Unmethylated Lanes*. Methylation statuses of each cell line are defined as; +, purely methylated: −, purely unmethylated: ±, both methylated and unmethylated, and are shown in the *Methylation status Lane*. *M-DNA* (universal methylated human male genomic DNA) was used as a positive control for methylated reaction. MCF-7 (*MCF7*) breast cancer cell line was used as a positive control for unmethylated reaction.

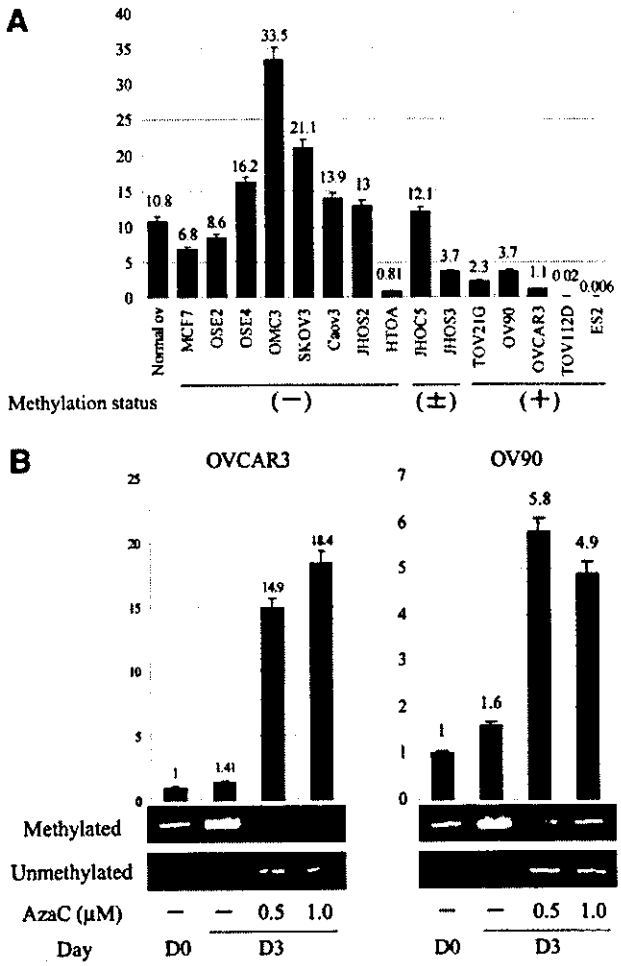

Fig. 2 *A*, expression of the *14-3-3σ* gene in ovarian cancer cells. Mean of the two independent results of real-time quantitative reverse transcription (RT)-PCR of ovarian cancer and surface epithelial cell lines are shown. *The number on top of each bar*, relative *14-3-3σ* gene expression standardized by the amount of internal positive control (β-actin). Normal ov (normal human ovarian cDNA library) was used as a normal control. MCF-7 was used as a positive control. *B*, reexpression of the *14-3-3σ* gene by 5-aza-2′-deoxycitidine (5azaC) treatment. OVCAR3 and OV90 cell lines that have only methylated bands were treated with 5azaC for 3 days. Methylation status and mRNA expression of 14-3-3σ was assessed by methylation-specific PCR (MSP) and quantitative RT-PCR on day 0 (*D0*) and day 3 (*D3*) with or without 5azaC (0.5 μM and 1.0 μM) treatment (*AzaC*). Complete and partial demethylation was observed in OVCAR3 and OV90, respectively. *The number on the top of each bar*, relative *14-3-3σ* gene expression standardized by the amount of internal positive control (β-actin).

Statistical Analysis. Statistical analysis was performed using Stat View 5.0 (SAS Institute Inc.) software. The statistical significance between 14-3-3σ and characteristics of the patients was evaluated using Mann-Whitney *U* test, Kruskal-Wallis test, and Scheffe analysis. Correlation between 14-3-3σ and Ki-67, p53 immunoreactivity was also assessed using Mann-Whitney

U test. Univariate analysis of prognostic significance for prognostic factors was performed using a log-rank test, after each survival curve was obtained by the Kaplan-Meier method. Multivariate analysis was performed using Cox regression model to evaluate the predictive power of each variable independently of the others. All of the patients who could be assessed were included in the intention-to-treat analysis. A result was considered significant when the P was less than 0.05.

RESULTS

Methylation Status and Expression of 14-3-3σ in Ovarian Cancer Cells. Among the 12 ovarian cancer cell lines in which 14-3-3σ promoter methylation was investigated, the presence of a methylated band was detected in 7 cell lines, 2 of which were together with unmethylated band as shown in Fig. 1. The methylated band was detected in all of the cell lines derived from clear cell adenocarcinoma (TOV21G, ES2, JHOC5), two of five of serous adenocarcinoma (Caov3, OV90, JHOS2, JHOS3, HTOA), one of one of endometrioid adenocarcinoma (TOV112D), and none of one of mucinous adenocarcinoma (OMC-3). Both of the OSE cells were negative for methylated band.

The expression of *14-3-3σ* gene is shown in Fig. 2*A*. Quantitative reverse transcription-PCR was performed and the ratio of 14-3-3σ:β-actin was calculated to allow for comparison between the cell lines. Median values of relative *14-3-3σ* gene expression in cancers with methylation (3.27) were significantly lower than those without methylation (16.4; $P < 0.001$, Kruskal-Wallis test). In HTOA, the expression of *14-3-3σ* gene was relatively decreased, although this cell line did not have methylated promoter alleles. The expression of *14-3-3σ* gene was relatively high in OSE2 (8.6), OSE4 (16.2), and in the normal ovarian cDNA library (10.8).

To further confirm that aberrant DNA methylation contributed to loss of expression of *14-3-3σ* gene, we assessed the effect of 5azaC, a demethylating agent, on 14-3-3σ mRNA expression by quantitative reverse transcription-PCR. Treatment of OVCAR3 and OV90 cells with 5azaC for 3 days resulted in the complete and partial demethylation of the promoter CpG islands and reexpression of *14-3-3σ* gene, respectively (Fig. 2*B*). The amount of expression of mRNA after treatment (14.9 and 5.8 for 0.5 μM, and 18.4 and 4.9 for 1.0 μM in OVCAR and OV90, respectively) was significantly higher than that before

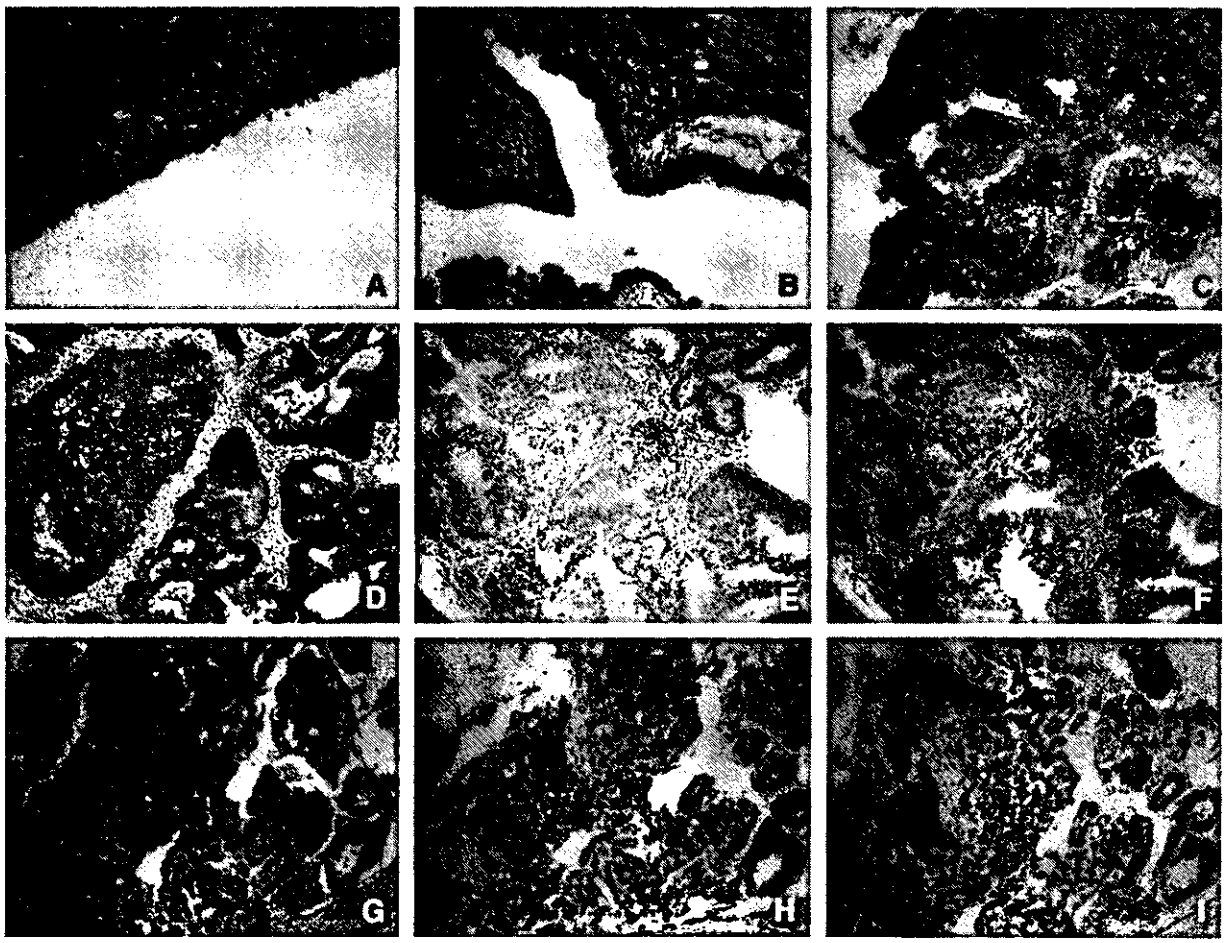

Fig. 3 Immunohistochemistry for 14-3-3σ in normal ovary, and in benign, borderline, and malignant ovarian tumors. Representative cases of immunohistochemistry for 14-3-3σ in the ovarian surface epithelium (*A*), benign adenoma (*B*), borderline tumor (*C*), two cases of epithelial ovarian cancer (*D, G*), and serial sections of each case for Ki-67 (*E, H*) and p53 (*F, I*) are shown. Note that positive 14-3-3σ case (*D*) is negative for Ki-67 (*E*) and p53 (*F*), whereas negative 14-3-3σ case (*G*) is positive for Ki-67 (*H*) and p53 (*I*), ×200 for all figures.

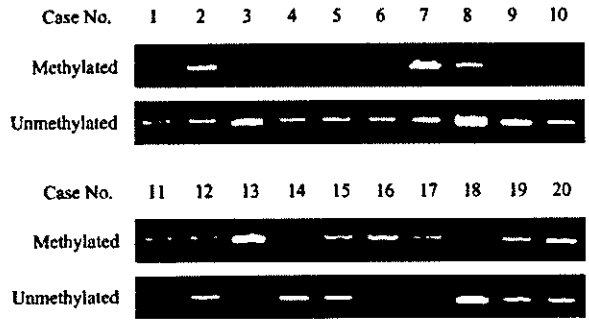

Fig. 4 Methylation-specific PCR (MSP) for *14-3-3σ* gene in ovarian cancer tissues. The methylation status of *14-3-3σ* gene in microdissected ovarian cancer tissues was evaluated by MSP in the 10 cases of positive (*Lanes 1–10*), and 10 cases of negative (*Lanes11–20*) immunoreactivity. Definition of *Methylated* and *Unmethylated Lanes* are the same as in Fig. 1.

treatment ($P < 0.001$, Kruskal-Wallis test). The decreased expression of 14-3-3σ in this cell line is, therefore, not attributable to abnormalities at the gene level or to the inability to express 14-3-3σ, but rather is directly related to the methylation.

Immunohistochemistry and Methylation Status of 14-3-3σ in Ovarian Cancer Tissues. Positive immunoreactivity for 14-3-3σ was detected in the cytoplasm of epithelial cells, although p53 and Ki-67 were confined exclusively to the nuclei of epithelial cells (Fig. 3). In normal, benign, and borderline tissues, all of the cases were positive for 14-3-3σ immunoreactivity. In ovarian cancer tissues, 73.5% (75 of 102) and 36.3% (37 of 102) were positive for 14-3-3σ and p53, respectively. Median Ki-67 labeling index was 17.2%.

To further clarify the relationships between 14-3-3σ immunoreactivity and aberrant DNA methylation in ovarian cancer tissues, we analyzed the methylation status in microdissected ovarian cancer tissues (Fig. 4). In the 10 cases of positive immunoreactivity, unmethylated bands were detected in all of the cases, and methylated bands were detected in 3 cases. In the another 10 cases of negative immunoreactivity, methylated bands were detected in 8 cases, although only unmethylated bands were detected in 2 cases.

Correlation between Clinicopathological Parameters and Immunohistochemistry in Ovarian Cancer Patients. Results of immunohistochemistry for 14-3-3σ, p53, and Ki-67 and correlation with clinicopathological parameters are summarized in Table 1. Negativity of 14-3-3σ was significantly correlated with high age, serous histology, high-grade, advanced-stage, residual tumor of >2 cm, high serum CA125, and high Ki-67 labeling index. Interestingly, p53 expression was inversely correlated with that of 14-3-3σ.

Results of univariate analysis of prognostic significance for

Table 1 Correlation between 14-3-3 σ immunoreactivity and clinicopathological parameters in epithelial ovarian cancer

			14-3-3 σ immunoreactivity		
	Total (n = 102)	%	+ (n = 75)	− (n = 27)	P
Age (median)	51		48.3	60.4	0.039
≦50	50	49.0	41	9	
>50	52	51.0	34	18	
Performance status[a]					
0–1	72	70.6	56	16	0.13
2–4	30	29.4	19	11	
Histology					
Serous	45	44.1	24	21	0.0003
Endometrioid	16	15.7	14	2	
Mucinous	14	13.7	14	0	
Clear cell	27	26.5	23	4	
Grade					
1	43	42.2	39	4	0.0022
2	35	34.3	23	12	
3	24	23.5	13	11	
Stage					
I/II	48	47.1	45	3	0.0001
III/IV	54	52.9	30	24	
Residual tumor[b]					
≤2 cm	62	60.8	55	7	0.0001
>2 cm	40	39.2	20	20	
CA125 (median, IU/liter)	255		43.5	63.9	0.0016
p53 immunoreactivity					
Positive	37	36.3	21	16	0.0038
Negative	65	63.7	54	11	
Ki67 LI (median)	17.2		15.6	24.4	0.047

[a] Performance status score: 0, asymptomatic and fully active; 1, symptomatic, fully ambulatory, restricted in physically strenuous activity; 2, symptomatic, ambulatory, capable of self-care, more than 50% of waking hours are spent out of bed; 3, symptomatic, limited self-care, spends more than 50% of time in bed, but not bedridden; 4, completely disabled, no self-care, bedridden.

[b] Residual tumor is defined in the "Materials and Methods."

[c] LI, labeling index.

Table 2 Univariate analysis of overall survival in epithelial ovarian cancer

Variable	*P*
14-3-3 σ (positive *vs.* negative)	0.0058
Age (≦50, >50)	0.029
PS[a] (0–1 *vs.* 2–4)	<0.0001
Histological type	0.913
Histological grade	0.05
Stage (I/II *vs.* III/IV)	<0.0001
Residual tumor (≤2 cm *vs.* >2 cm)	<0.0001
p53 (positive *vs.* negative)	0.36
Ki67 (<15% *vs.* >15%)	0.011

[a] PS, performance status.

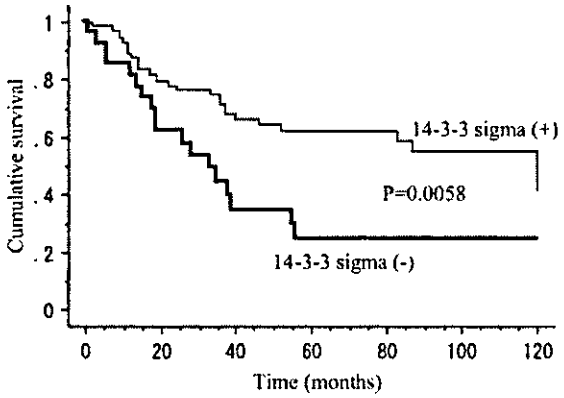

Fig. 5 Correlation between 14-3-3σ expression and overall survival in patients with epithelial ovarian cancer.

each variable with respect to survival are summarized in Table 2. In this analysis, we determined the positive cases of Ki-67 as those with a labeling index of more than 15%. Among the clinicopathological factors examined, those significantly associated with overall survival were 14-3-3σ immunoreactivity, age, performance status, grade, stage, residual tumor, and Ki-67. Negative 14-3-3σ cases had significantly worse overall survival rates than positive cases (Fig. 5; $P = 0.0058$). In multivariate analysis, 14-3-3σ immunoreactivity turned out not to be an independent prognostic indicator (Table 3). Among the variables examined, only stage and residual tumor turned out to be independent prognostic factors.

DISCUSSION

The aberrant methylation of 14-3-3σ was associated with loss of RNA expression, and the expression was restored by treatment with the demethylating agent 5azaC, indicating that aberrant DNA methylation is the main pathway of transcriptional silencing of *14-3-3σ* gene in ovarian cancer cells. Also, decreased expression of 14-3-3σ occurs in substantial proportion, and it turned out to be a prognostic factor in univariate analysis in epithelial ovarian cancer tissues. These results suggest the importance of the *14-3-3σ* gene in the development and progression of this tumor. To our knowledge, this is the first report on epigenetic silencing of 14-3-3σ in human ovarian cancer.

The *14-3-3σ* gene is demonstrated to be induced after DNA damage in a p53-dependent manner (16), and to play a role in the G_2 checkpoint by sequestering the Cdc2/cyclin B1 complex (17). Similar to our results, several authors demonstrated epigenetic inactivation of the *14-3-3σ* gene in human cancers (18, 20, 27). Experimental inactivation of the *14-3-3σ* gene causes a G_2 checkpoint defect, and results in the accumulation of chromosomal aberrations that increase the sensitivity to the DNA-damaging events (28, 29). In this context, it is of interest that serous histology in which significantly decreased expression of 14-3-3σ protein was observed is highly sensitive to chemotherapeutic agents. The histological type-specific expression of the *14-3-3σ* gene suggests that serous adenocarcinomas develop unique differentiation, different from other histological subtypes. Although a high proportion of human cancers are likely to have a chromosomal instability phenotype, mutations of the genes involving the G_2-M-phase checkpoint have rarely been found, and the mechanism of chromosomal instability in most of them is still unknown. Further study on the correlation between genetic instability and 14-3-3σ is needed.

In a number of previous reports, it is strongly postulated that the inactivation of 14-3-3σ might play an important role in tumor progression. Ostergaard *et al.* (30) showed that less-differentiated bladder squamous cell carcinoma is characterized by decreased expression of some proteins, including 14-3-3σ. Suzuki *et al.* (19) reported that aberrant methylation of the *14-3-3σ* gene was frequently observed in poorly differentiated gastric adenocarcinoma. Umbricht *et al.* (31) reported that decreased expression of 14-3-3σ was observed in 24 (96%) of 25 carcinomas, 15 (83%) of 18 of ductal carcinoma *in situ*, and 3 (38%) of 8 of atypical hyperplasias, and concluded that inactivation of 14-3-3-sigma occurs at an early stage in the progression of invasive breast cancer. Osada *et al.* (20) recently demonstrated frequent and type-specific inactivation of the *14-3-3σ* gene in small cell lung cancer. In our study, loss of 14-3-3σ expression was significantly correlated with high-grade advanced-stage bulky residual tumor and high serum CA125 and high Ki-67 levels, all of these factors represent fundamental difference in pathogenesis in ovarian cancer. Our results, together with previous reports, suggest that the loss of 14-3-3σ expression in ovarian cancer may have invasive and progressive characteristics.

Recent study suggests that promoter methylation increases with age in several genes in normal tissues, although the mechanism of age-related methylation is unknown (32, 33). Several factors may modulate age-related methylation, such as exogenous carcinogens, endogenously generated reactive oxygen species, and genetic differences in individual susceptibility (33). In our study, 14-3-3σ protein expression was decreased in elderly

Table 3 Multivariate analysis of overall survival using Cox's proportional hazard model

Variable	*P*
14-3-3 σ (positive *vs.* negative)	0.34
Age (≦50, >50)	0.15
PS[a] (0–1 *vs.* 2–4)	0.29
Histological grade	0.73
Stage (I/II *vs.* III/IV)	0.0081
Residual tumor (≤2 cm *vs.* >2 cm)	0.023
Ki67 (<15% *vs.* >15%)	0.11

[a] PS, performance status.

patients by immunohistochemistry. Although age-related methylation of *14-3-3σ* has not been reported, it is possible that expression of this gene is suppressed by methylation with age, and it may contribute to carcinogenesis in ovarian cancer.

In the present study, a clear correlation between aberrant methylation and silencing of 14-3-3σ was observed except for one cell line (HTOA) and 2 of 10 primary ovarian cancer tissues, which showed unmethylated patterns despite loss of 14-3-3σ expression. These results suggest that DNA methylation-independent mechanism may also be involved in the loss of 14-3-3σ expression. Several other ways of gene inactivation such as loss of transcription factor are assumed, and Osada *et al.* (20) considered that *14-3-3σ* gene silencing might occur without methylation in only primary tissues. From our results, positive p53 immunoreactivity, which suggests loss of functional p53 protein (34, 35), seems to contribute to the loss of 14-3-3σ expression in primary ovarian tissues. Possible methylation-independent mechanism remains to be elucidated to better understand the regulation of 14-3-3σ expression.

In conclusion, aberrant CpG island methylation is an epigenetic change that is largely responsible for silencing of the *14-3-3σ* gene. Inactivation of 14-3-3σ occurs in substantial proportion and may play a role as a potential tumor suppressor gene in epithelial ovarian cancer.

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Expression of EBAG9/RCAS1 is associated with advanced disease in human epithelial ovarian cancer

J-i Akahira*[,1,2], M Aoki[1], T Suzuki[2], T Moriya[2], H Niikura[1], K Ito[1], S Inoue[3], K Okamura[1], H Sasano[2] and N Yaegashi[1]

[1]Department of Obstetrics and Gynecology, Tohoku University Graduate School of Medicine, Sendai, Japan; [2]Department of Pathology, Tohoku University Graduate School of Medicine, Sendai, Japan; [3]Department of Biochemistry, Saitama Medical School, Saitama, Japan

Oestrogen receptor-binding fragment associated gene 9, EBAG9, is an oestrogen-responsive gene that was identified in MCF-7 human breast carcinoma cell line. It is identical to RCAS 1, a cancer cell surface antigen possibly involved in immune escape. In the present study, we examined the expression of EBAG9/RCAS1 in human epithelial ovarian cancer using immunohistochemistry, immunoblotting and reverse transcription–polymerase chain reaction (RT–PCR). A total of 90 epithelial ovarian cancer cases were examined immunohistochemically by means of the antibodies for EBAG9 and ERα. The correlation between EBAG9 immunoreactivity and clinicopathological parameters was examined. mRNA expression of EBAG9 and ERα were evaluated by RT–PCR in 22 cases. The expression for EBAG9 and ERα was examined by immunoblotting in 12 ovarian cancer cell lines. EBAG9 immunoreactivity was detected in the surface and cytoplasm of carcinoma cells in 46 out of 90 cases (51.1%). EBAG9 expression was significantly higher in serous histology ($P=0.0402$) and advanced disease ($P=0.0206$). No significant relationship was detected between EBAG9 immunoreactivity and overall survival ($P=0.689$). There was a highly significant correlation between EBAG9 and ER immunoreactivity ($P<0.0001$). The EBAG9 mRNA was detected in 20 out of 22 cases. In all of the cases that were positive for ERα mRNA, they were also positive for EBAG9 mRNA. Immunoreactive band corresponding to EBAG9 was detected in 11 out of 12 of ovarian cancer cell lines, and was consistent with ERα expression. In conclusion, the wide distribution of EBAG9 and its relation to advanced disease suggest that this protein may play important roles in epithelial ovarian cancer.
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Keywords: EBAG9; ovarian cancer; immunohistochemistry; oestrogen receptor

Epithelial ovarian cancer is the leading cause of death from gynaecological malignancies in the great majority of developed countries (Akahira J *et al*, 2001; Akahira JI *et al*, 2001,). Sex steroid hormones have been implicated in the aetiology and/or progression of some epithelial ovarian cancers. Both oestrogen (ER) and progesterone receptors (PR) have been reported in human epithelial ovarian cancer (Rao and Slotman, 1991; Akahira *et al*, 2002). In endometrial and breast carcinomas, steroid hormone receptor status correlates well with response to hormonal manipulation and prognosis (McGuire, 1978; Benraad *et al*, 1980; Ehrlich *et al*, 1981; Kaupilla, 1984). However, in epithelial ovarian carcinoma, the prognostic significance of tumour ER status among patients still remains controversial (Bizzi *et al*, 1988; Masood *et al*, 1989; Sevelda *et al*, 1990; Rao and Slotman, 1991; Hempling *et al*, 1998).

Recently, oestrogen receptor-binding fragment associated gene 9 (EBAG9) has been identified as an oestrogen-responsive gene from a cDNA library of MCF-7 human breast cancer cell line (Watanabe *et al*, 1998). Oestrogen receptor-binding fragment associated gene 9 is identical to the receptor-binding cancer antigen expressed in SiSo cells (RCAS1) (Nakashima *et al*, 1999). The EBAG9/RCAS1 is a membrane molecule that acts as a ligand for a putative receptor present in cells (Nakashima *et al*, 1999). *In vitro* studies have also demonstrated that EBAG9/RCAS1 inhibits growth of activated $CD3^+$ T lymphocytes, suggesting a possible involvement in the immune escape of neoplastic cells (Nakashima *et al*, 1999). Endocrine-immune interactions are considered to play an important role in the development and/or progression of various hormone-dependent neoplasms, but the details of these interactions remain unclear.

Oestrogen receptor-binding fragment associated gene 9 is demonstrated to be widely distributed in human breast carcinoma, and may play an important role in the development of this oestrogen-dependent cancer (Suzuki *et al*, 2001). Others reported that RCAS1 is associated with poor prognosis and/or advanced stage in various human cancers (Sonoda *et al*, 1996; Kaku *et al*, 1999; Izumi *et al* (2001); Nakakubo *et al*, 2002). However, the expression of EBAG9 and its clinical significance have not been examined in epithelial ovarian cancer. Therefore, in this study, we examined the expression of EBAG9 in human epithelial ovarian cancer using immunohistochemistry, reverse transcription–polymerase chain reaction (RT–PCR) and immunoblotting. We also evaluated the correlations of EBAG9 immunoreactivity with various clinicopathological parameters and ER status.

*Correspondence: J-i Akahira, Department of Obstetrics and Gynecology, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan;
E-mail address: jakahira@ob-gy.med.tohoku.ac.jp
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MATERIALS AND METHODS

We studied a total of 90 cases of common epithelial ovarian carcinoma. Information regarding age, performance status on admission, histology, stage, grade, residual tumour after primary surgery and overall survival was retrieved from the review of patient charts. The median follow-up time of the patients in this study was 54 months (18–112 months). Of 90 patients, 76 (84.4%) received platinum-containing chemotherapy after operation. Performance status was defined according to WHO criteria (World Health Organization, 1979). Histology, stage and grade were determined according to FIGO (International Federation of Gynecology and Obstetrics) criteria (Shimizu *et al*, 1998). Residual disease was determined by the amount of unresectable tumour left following primary cytoreductive surgery. Optimal cytoreduction was defined as no gross residual tumour greater than 2 cm in diameter, whereas suboptimal cytoreduction was defined as any gross residual disease remaining greater than 2 cm in diameter. Overall survival was calculated from the time of initial surgery to death, or the date of last contact. Survival times of patients still alive or lost to follow-up were censored in December 2002. All of these archival specimens were retrieved from the surgical pathology files at Tohoku University Hospital, Sendai, Japan. These specimens were all fixed in 10% formalin and embedded in paraffin. Among these 90 cases, 22 cases were available for examination by RT-PCR analysis. These specimens were dissected immediately into small pieces following gross dissection, quickly transferred to liquid nitrogen, and then stored at −80°C until further use. The research protocol was approved by the ethics committee of Tohoku University Graduate School of Medicine, Sendai, Japan.

OVCAR3, Caov3, SKOV3, TOV112D, TOV21G, OV90 and ES2 (adenocarcinoma: OVCAR3, SKOV3; serous adenocarcinoma: Caov3, OV90; clear cell adenocarcinoma: TOV21G, ES2; endometrioid adenocarcinoma: TOV112D) cell lines were purchased from American Type Culture Collection. JHOS2, JHOS3, HTOA, OMC3 and JHOC5 (serous adenocarcinoma: JHOS2, JHOS3, HTOA; mucinous adenocarcinoma: OMC3; clear cell adenocarcinoma: JHOC5) cell lines were purchased from Riken cell bank (Tsukuba, Japan). Cell lines were maintained in DMEM/F12 (Invitrogen, CA), supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin (Invitrogen, CA), and incubated in 5% CO_2 at 37°C.

Immunohistochemistry

Immunohistochemical analysis was performed using the streptavidin-biotin amplification method using a Histofine Kit (Nichirei, Tokyo, Japan), and have been previously described in detail (Akahira JI *et al*, 2001, Akahira J *et al*, 2001). Oestrogen receptor-binding fragment associated gene 9 antibody was a rabbit polyclonal antibody against a GST-EBAG9 fusion protein (Tsuchiya *et al*, 2001). The characterisation of this antibody was confirmed by Western blotting (Suzuki *et al*, 2001). Monoclonal antibody for ERα was purchased from Immunotech (Marseille, France). For antigen retrieval, the 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 dyhydrate, pH 6.0). The dilutions of primary antibodies for EBAG9 and ERα were 1:200 and 1:2, respectively. The antigen-antibody complex was visualised 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 haematoxylin. The ER-positive breast carcinoma tissue was used as a positive control for EBAG9 (Suzuki *et al*, 2001). As negative controls, 0.01 M phosphate-buffered saline (PBS) and normal mouse IgG were used in place of primary antibodies. No specific immunoreactivity was detected in these tissue sections.

Scoring of immunostaining

For statistical analyses of EBAG9 immunoreactivity, carcinomas were classified independently by two of the authors (MA and JA) into two groups: +, positive carcinoma cells; and −, no immunoreactivity. Cases with disconcordant results among the observers were re-evaluated. For evaluation of ERα immunoreactivity, labeling index (LI) was obtained in carcinoma cells as described by Sasano *et al* (1996). In brief, two of the authors (JA and TM) independently evaluated at least 500 carcinoma cells microsopically and the percentage of immunoreactivity was determined. In the present study, interobserver differences were less than 5%, and the mean of the two values was obtained.

Reverse transcription-PCR

Total RNA was isolated from tissues by phenol-chloroform extraction using Isogen (Nippon Gene, Japan), and was treated by DNase I (Roche, Germany). The RT-PCR kit (SUPERSCRIPT Preamplification system, Invitrogen) was employed and cDNA synthesis was carried out according to the instructions. cDNAs were synthesised 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. After an initial 1-min denaturation step at 94°C, 35-cycle PCRs were carried out on a DNA thermal cycler (PTC-200 DNA Engine, MJ Research, Inc., USA) under the following conditions: 1-min denaturation at 94°C, 1-min annealing at 58°C for EBAG9, 62°C for ERα and 2-min extension at 72°C. Primers for PCR reactions were as follows: EBAG9: 5′sense - GCTACACAAGATTCTGCCT and 3′ antisense - CTTCTTCATT AGCCGTTGTG (680–892, 213 bp); ERα: 5′ sense - AAGAGCTGCC AGGCCTGCC and 3′ antisense - TTGGCAGCTCTCATGTCTCC (702–869, 168 bp); β-actin: 5′ sense - CCAACCGCGAGAAGAT GAC and 3′ antisense - GGAAGGAAGGCTGGAAGAGT (382–841, 459 bp). In initial expreriments, following amplification, PCR products were purified and subjected to direct sequencing to verify amplification of the correct sequences (ABI prism 310 Genetic Analyzer, Applied Biosystems, CA, USA). β-Actin primers were utilised as positive controls. 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 PBS, whole-cell protein was extracted by conventional method. The protein concentration was measured by Model 680 microplate reader (Biorad, USA) using BradFord reagent (Biorad). In all, 20 μg of protein of each sample was mixed with an equal volume of 2× concentrated SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, boiled, and then electrophoresed on 7% ready-made gels containing SDS (Mini Protian 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), and then incubated in 1:200 dilution of EBAG9 antibody (1:2 for ERα and 1:1000 for Actin) in blocking solution overnight at 4°C. After incubation with HRP-rabelled anti-rabbit IgG (Vector Laboratories, Inc., USA), the antigen-antibody complex was visualised with ECL system (Amersham, Germany). MCF-7 breast cancer cell line was used as a positive control (Watanabe *et al*, 1998). Actin (Ab-1, Oncogene) was used as internal positive controls.

Statistical analysis

Statistical analysis was performed using Stat View 5.0 (SAS Institute Inc., NC, USA) software. The statistical significance

between EBAG9 and characteristics of the patients was evaluated using Mann–Whitney *U*-test, Kruskal–Wallis and Scheffe analysis. The correlation between EBAG9 and ERα immunoreactivity was also assessed using Mann–Whitney *U*-test. Univariate analysis of prognostic significance for prognostic factors was performed using a 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

Results of immunohistochemistry and their correlation with clinicopathological parameters are summarised in Table 1. Immunoreactivity for EBAG9 was detected on the surface and in the cytoplasm of epithelial ovarian cancer tissues. Oestrogen receptor α immunoreactivity was confined exclusively to the nuclei of tumour cells (Figure 1). The number of cases immunopositive for EBAG9 was 46 out of 90 cases (51.1 %). The median LI for ERα was 12.8 % (0–85.2%). As shown in Table 1, EBAG9 expression was significantly higher in serous histology ($P=0.0402$) and advanced disease ($P=0.0206$). There was no significant relationship between EBAG9 immunoreactivity and patient age, histological grade, residual tumour or performance status. There was a highly significant correlation between EBAG9 immunoreactivity and ERα LI ($P<0.0001$).

Results of univariate analysis of prognostic significance for each variable, with respect to survival, are summarised in Table 2. Among the clinicopathological factors examined, those significantly associated with overall survival were histology, grade, stage and residual tumour. No significant relationship was detected between EBAG9 immunoreactivity and overall survival ($P=0.689$).

Relationships between EBAG9 immunoreactivity and EBAG9, ERα mRNA in 22 cases are summarised in Table 3. Oestrogen receptor-binding fragment associated gene 9 mRNA was positive in 20 cases. In four cases, EBAG9 immunoreactivity was not detected although its mRNA was present. In all of the cases that

Table 1 Association between EBAG9 immunoreactivity and clinicopathological parameters in human ovarian cancer

	EBAG9 immunoreactivity		
	+($n=46$)	**−($n=44$)**	***P*-value**
Age (years)	52.8 ± 1.8	49.5 ± 1.5	NS
Histological type			
Serous	27	14	
Mucinous	7	9	
Endometrioid	3	10	
Clear cell	9	11	0.0402
Histological grade			
Grade 1	15	16	
Grade 2	11	15	
Grade 3	12	6	NS
Stage			
I,II	17	27	
III,IV	29	17	0.0206
Residual tumour			
<2 cm	27	34	
>2 cm	19	10	NS
PS			
0,1	30	32	
2,3,4	16	12	NS
ER LI	18.8	11.4	<0.0001

Performance status score: 0 = asymptomatic and fully active; 1 = symptomatic, fully ambulatory, restricted in physically strenous activity; 2 = symptomatic, ambulatory, capable of self-care, more than 50% of waking hours are spent out of bed; 3 = symptomatic, limited self-care, spends more than 50% of time in bed, but not bedridden; 4 = completely disabled, no self-care, bedridden.

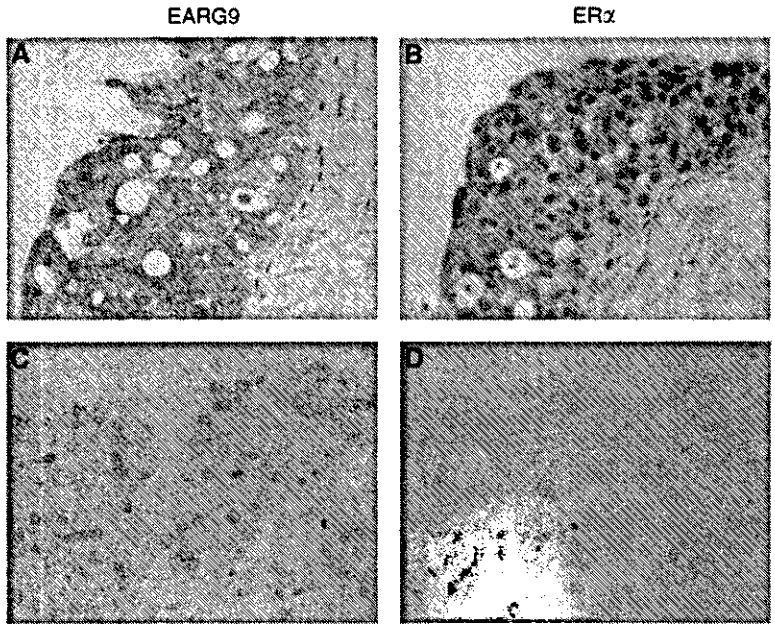

Figure 1 Serial sections of positive and negative cases of immunohistochemistry for EBAG9 and ERα in epithelial ovarian carcinoma (**A**,**B**) a case of serous adenocarcinoma, positive for both EBAG9 and ERα; (**C**,**D**) a case of clear-cell adenocarcinoma, negative for both EBAG9 and ERα). Immunoreactivity for EBAG9 was detected on the surface, and in the cytoplasm of epithelial ovarian cancer tissues. ER-α immunoreactivity was confined exclusively to the nuclei of tumour cells.