[35] J.A. Nilsson, J.L. Cleveland, Myc pathway provoking cell suicide and cancer, Oncogene 22 (2003) 9007–9021.
[36] K. Malik, K.W. Brown, Epigenetic gene deregulation in cancer, Br. J. Cancer 83 (2000) 1583–1588.
[37] E.R. Greenberg, A.B. Barnes, L. Resseguie, J.A. Barrett, S. Burnside, L.L. Lanza, et al., Breast cancer in mother given diethylstilbestrol in pregnancy, N. Engl. J. Med. 311 (1984) 1393–1398.
[38] S. Li, K.A. Washburn, R. Moore, T. Uno, C. Teng, R.R. Newbold, et al., Developmental exposure to diethylstilbestrol elicits demethylation of estrogen-responsive lactferrin gene in mouse uterus, Cancer Res. 57 (1997) 4356–4359.
[39] C. Cillo, A. Faiella, M. Cantil, E. Boncinelli, Homeobox genes and cancer, Exp. Cell Res. 248 (1999) 1–9.
[40] Y.C. Hung, M. Ueda, Y. Terai, K. Kumagai, K. Ueki, K. Kanda, et al., *Homeobox* gene expression and mutation in cervical carcinoma cells, Cancer Sci. 94 (2003) 437–441.
[41] K. Block, A. Kardana, P. Igarashi, H.S. Taylor, *In utero* diethylstilbestrol (DES) exposure alters Hox gene expression in the developing mullerian system, Fed. Am. Soc. Exp. Biol. J. 14 (2000) 1101–1108.
[42] V. Raman, S.A. Martensen, D. Reisman, E. Evron, W.F. Odenwald, E. Jaffee, et al., Compromised *HOXA5* function can limit p53 expression in human breast tumors, Nature 405 (2000) 974–978.

Research Article

Progesterone Receptor in Non–Small Cell Lung Cancer—A Potent Prognostic Factor and Possible Target for Endocrine Therapy

Hironori Ishibashi,[1,3] Takashi Suzuki,[1] Satoshi Suzuki,[3] Hiromichi Niikawa,[1,3] Liangying Lu,[1] Yasuhiro Miki,[1] Takuya Moriya,[1] Shin-ichi Hayashi,[2] Masashi Handa,[4] Takashi Kondo,[3] and Hironobu Sasano[1]

Departments of [1]Pathology and [2]Molecular Medical Technology, Tohoku University School of Medicine, Sendai, Japan; [3]Department of Thoracic Surgery, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan; and [4]Department of Surgery, Sendai Kousei Hospital, Sendai, Japan

Abstract

A possible involvement of gender-dependent factors has been postulated in development of human non–small-cell lung cancers (NSCLC), but its details remain unclear. In this study, we examined biological significance of progesterone receptor in NSCLCs. Progesterone receptor immunoreactivity was detected in 106 of 228 NSCLCs (46.5%). Progesterone receptor–positive NSCLC was frequently detected in female and adenocarcinoma, and was inversely associated with tumor-node-metastasis stage and histologic differentiation. Progesterone receptor status was also associated with better clinical outcome of the patients, and a multivariate analysis revealed progesterone receptor status as an independent prognostic factor. Progesterone-synthesizing enzymes were detected in NSCLCs, and tissue concentration of progesterone was higher in these cases (n = 42). Immunoblotting analyses showed the presence of progesterone receptor in three NSCLC cell lines (A549, LCSC#2, and 1-87), but not in RERF-LC-OK or PC3. Transcriptional activities of progesterone receptor were increased by progesterone in these three progesterone receptor–positive NSCLC cells by luciferase assays. Cell proliferation was inhibited by progesterone in these progesterone receptor–positive NSCLC cells in a dose-dependent manner, which was inhibited by progesterone receptor blocker. Proliferation of these tumor cells injected into nude mice was also dose-dependently inhibited by progesterone, with a concomitant increase of p21 and p27 and a decrease of cyclin A, cyclin E, and Ki67. Results of our present study suggested that progesterone receptor was a potent prognostic factor in NSCLCs and progesterone inhibited growth of progesterone receptor–positive NSCLC cells. Therefore, progesterone therapy may be clinically effective in suppressing development of progesterone receptor–positive NSCLC patients. (Cancer Res 2005; 65(14): 6450-8)

Introduction

Lung cancer is one of the leading causes of cancer death throughout the world. Despite recent advances in its treatment, its prognosis remains dismal (1–3). Therefore, it becomes very important to examine the details of biological features of lung carcinoma cell proliferation and to develop the targeted therapies aimed at the specific proteins involved in these biological behaviors. Lung cancer is histologically classified into small-cell lung carcinoma and non–small-cell lung carcinoma (NSCLC). NSCLC accounts for ~80% of all the lung cancer cases, and represents heterogeneous groups which include the squamous cell carcinoma, adenocarcinoma, and large cell carcinoma. Squamous cell carcinoma is markedly associated with smoking and more frequency detected in males, but adenocarcinoma tends to occur more frequently in female, suggesting a possible involvement of gender-dependent factors in the pathogenesis and/or development of NSCLCs.

It is well known that sex steroids play important roles in various human tissues as gender-dependent factors. Among sex steroids, estrogens greatly contribute to development of hormone-dependent breast and endometrial carcinomas through an initial interaction with estrogen receptor α and/or β (4), whereas androgens play important roles in the development of prostate cancers. In contrast, progesterone generally promotes differentiation and inhibits cellular proliferation through the progesterone receptor (5). Previous studies showed that progesterone-mediated growth inhibition was mainly preceded by decreased expression of cyclins A, D1, and E and/or induction of cyclin-dependent kinase inhibitors such as p21 and p27 (6–8), and administration of progestins, including medroxyprogesterone acetate, has been currently done as an endocrine therapy in breast and endometrial carcinoma patients (9, 10).

Immunohistochemical examination of sex steroid receptors has been reported in NSCLC tissues by several investigators (11–14). However, the lung tissue is not generally considered a target for sex steroids, and biological significance of sex steroid receptors remains unclear in NSCLCs. In this study, we examined immunolocalization of progesterone receptor and estrogen receptors in 228 cases of NSCLCs, and showed that progesterone receptor was a potent prognostic factor. We also revealed that progesterone inhibited the growth of progesterone receptor–positive NSCLC cell lines, and proposed that progesterone receptor is a possible target for progesterone therapy in NSCLC patients.

Materials and Methods

Patients and tissue specimens. Two-hundred twenty eight specimens of NSCLC were obtained from patients who underwent surgical resection from 1993 to 1995 in the Department of Surgery, Sendai Kousei Hospital, Sendai, Japan. A mean age of the patients was 65.5 years (range 23-82). Patients examined in this study did not receive irradiation or chemotherapy before surgery. The mean follow-up time was 1,600 days (range 17-3,695 days). All specimens were fixed in 10% formalin and embedded in paraffin wax. Frozen tissues were also available in 42 cases. Research protocols for this study were approved by the Ethics Committee at both Tohoku University School of Medicine and Sendai Kousei Hospital, Sendai, Japan.

Requests for reprints: Takashi Suzuki, Department of Pathology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan. Phone: 81-22-717-8050; Fax: 81-22-717-8051; E-mail: t-suzuki@patholo2.med.tohoku.ac.jp (Takashi Suzuki).

Immunohistochemistry. The specimens for immunohistochemistry were fixed in 10% formalin and embedded in paraffin wax, and streptavidin-biotin amplification method was employed for immunostaining using a Histofine Kit (Nichirei Co. Ltd., Tokyo, Japan). Monoclonal antibodies for progesterone receptor (MAB429), estrogen receptor α (6F11), estrogen receptor β (14C8), Ki67 (MIB1), and p21 (15091A) were purchased from Chemicon (Temecula, CA), Novocastra (Newcastle, United Kingdom), GeneTex (San Antonio, TX), DAKO (Carpinteria, CA), and BD Biosciences Pharmingen (San Diego, CA), respectively. p53 (DO-7), p27 (1B4), cyclin A (6E6), cyclin D1 (P2D11F11), and cyclin E (13A3) were purchased from Novocastra. The characteristics of polyclonal antibody for steroidogenic acute regulatory protein (StAR), P450 side chain cleavage (P450scc), and 3β-hydroxysteroid dehydrogenase (3βHSD) were described previously (15, 16). The antigen-antibody complex was subsequently visualized with 3,3′-diaminobenzidine solution and counterstained with hematoxylin.

Immunoreactivity for progesterone receptor, Ki67, p21, p27, p53, and cyclins A, D1, and E was scored in more than 1,000 carcinoma cells for each case, and the percentage of immunoreactivity (i.e., labeling index (LI)] was determined. Cases that have progesterone receptor or estrogen receptor LI less than 10% were determined as progesterone receptor or estrogen receptor negative, according to a report by Allred et al. (17). Statistical analyses were done using a one-way ANOVA and Bonferroni test or a cross-table using the χ^2 test. Overall and disease-free survival curves were generated according to the Kaplan-Meier method. Univariate and multivariate analyses were done by a proportional hazard model (Cox) using PROC PHREG in our SAS software. $P < 0.05$ was considered significant in this study.

Tissue concentrations of progesterone. Tissue concentrations of progesterone were examined in 42 cases of NSCLC in which sufficient amounts of frozen specimens were available for examination. The cytosol and nuclear fractions were prepared by centrifugation of homogenates (1.0 g specimens in 10 mL cold physiologic saline) at 4°C for 60 minutes at 15,000 rpm in an ultracentrifuge and progesterone concentrations were determined by a Progesterone EIA kit (Cayman Chemical Company, Miami, FL). The minimum concentration of progesterone which can be detected by this kit is 10 pg/mL. The specificity is as follows: progesterone, 100%; pregnenolone, 61%; 5α-Pregnan-3α,20α-diol, <0.01%; 5β-Pregnan-3α,20α-diol, <0.01%; and 5β-Pregnan-3α,20α-diol glucuronide, <0.01%, according to the information from the manufacturer.

Cell culture. Five human NSCLC cell lines [A549, LCSC#2, 1-87, RERF-LC-OK, and PC3, which differs from PC-3 prostate cancer cell line (ATCC CRL-1435); refs. 18, 19] were available in this study. Histologic type of the original tumor was adenocarcinoma in these five cell lines, and patient gender of each cell line was as follows: A549, male; LCSC#2, female; 1-87, male; RERF-LC-OK, female; and PC3, female. These cell lines were cultured in RPMI 1640 (Sigma, St. Louis, MO) with 10% fetal bovine serum (FBS). Immunohistochemistry for progesterone-producing enzymes in NSCLC cells was done in the cell blocks which were fixed in formalin and embedded with paraffin wax.

Immunoblotting. The cell protein was extracted in triple detergent lysis buffer (20) at 4°C. Twenty micrograms of the protein (whole cell extracts) were subjected to SDS-PAGE (10% acrylamide gel). Following SDS-PAGE, proteins were transferred onto Hybond P polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ). The blots were blocked in 5% nonfat dry skim milk for 1 hour at room temperature, and were then incubated with primary antibodies for progesterone receptor, estrogen receptor α, or estrogen receptor β for 18 hours at 4°C. After incubation with anti-mouse immunoglobulin G horseradish peroxidase (Amersham Biosciences) for 1 hour at room temperature, antibody/protein complexes on

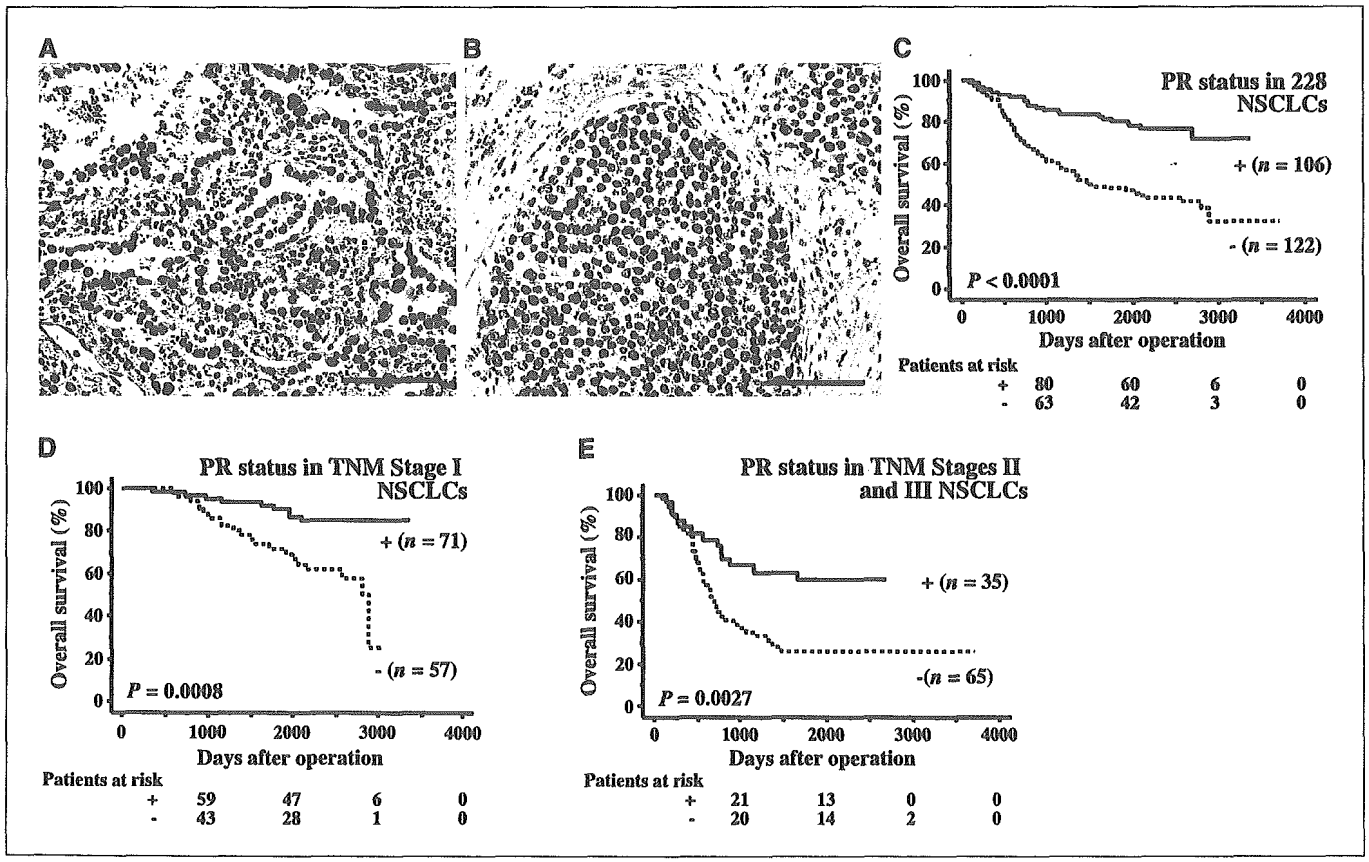

Figure 1. Immunohistochemistry of progesterone receptor in NSCLCs. *A* and *B*, immunoreactivity for progesterone receptor was detected in the nuclei of carcinoma cells in adenocarcinoma (*A*) or squamous cell carcinoma (*B*). *Bar*, 100 μm. *C* to *E*, overall survival curves (Kaplan-Meier method) of NSCLC patients showed that progesterone receptor status was significantly associated with a better clinical outcome of the patients ($P < 0.0001$; *C*), and a similar tendency was also detected in the group of TNM stage I ($n = 128$; $P = 0.0008$; *D*) or TNM stages II and III ($n = 100$; $P = 0.0027$; *E*).

Table 1. Association between progesterone receptor status and clinicopathologic variables in 228 NSCLCs

	Progesterone receptor status		P
	Positive (n = 106)	Negative (n = 122)	
Gender			
Male	67 (29.7%)	98 (42.8%)	
Female	39 (17.0%)	24 (10.5%)	0.0045
Premenopausal	3 (1.3%)	2 (1.6%)	
Postmenopausal	36 (15.8%)	22 (9.6%)	
Age*	65.7 ± 0.9	65.4 ± 0.9	0.7892
TNM stage			
I	71 (31.4%)	57 (25.0%)	
II	11 (4.8%)	14 (6.1%)	
III	24 (10.5%)	51 (22.3%)	0.0085
Histologic type			
Adeno	76 (33.6%)	59 (25.8%)	
Large	11 (4.8%)	11 (4.8%)	
SCC	19 (8.3%)	52 (22.7%)	0.0002
Histologic differentiation†			
Well	31 (15.0%)	21 (10.1%)	
Moderately	22 (10.6%)	40 (19.3%)	
Poorly	43 (20.8%)	49 (23.8%)	0.0355
Estrogen receptor α status			
Positive	47 (20.6%)	40 (17.6%)	
Negative	59 (25.9%)	82 (36.0%)	0.1780
Estrogen receptor β status			
Positive	37 (16.2%)	40 (17.6%)	
Negative	69 (30.3%)	82 (36.0%)	0.7358
Ki67 LI (%)*	18.9 ± 1.7	26.9 ± 1.7	0.0010
p53 status			
Positive	76 (33.3%)	80 (35.1%)	
Negative	30 (13.2%)	42 (18.4%)	0.3202

Abbreviations: Adeno, adenocarcinoma; Large, large cell carcinoma; SCC, squamous cell carcinoma.
*Data were presented as mean ± 95% confidence interval (95% CI), and were statistically evaluated using a one-way ANOVA and Bonferroni test. All other values represented the number of cases and percentage compared with the total number, and were evaluated using a cross-table using the χ^2.
†Cases of Large were excluded (n = 206, in total). Status of steroid receptors was evaluated by immunohistochemistry.

the blots were detected using ECLplus Western blotting detection reagents (Amersham Biosciences). T47D cells were used as positive controls for progesterone receptor and estrogen receptors (21, 22). Equal loading of protein in each lane was confirmed by probing the membrane with anti-human β-actin monoclonal antibody (Sigma).

Luciferase assay. Progesterone-responsive reporter plasmids pHRE (hormone responsive element)-Luc (23) were kindly provided by Dr. Hirotoshi Tanaka (Division of Clinical Immunology, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan). Estrogen-responsive reporter plasmids pERE (estrogen-responsive element)-Luc (24) were also used in this study. Luciferase assay was done according to a previous report (25) with some modifications. Briefly, 1 μg of pHRE-Luc or pERE-Luc plasmids and 200 ng of pRL-TK control plasmids (Promega Co., Madison, WI) were used to measure the transcriptional activity of endogenous progesterone receptor or estrogen receptor. Transient transfections were carried out using *Trans*IT-LT transfection reagents (TaKaRa, Tokyo, Japan), and the luciferase activity of lysates was measured using a Dual-Luciferase Reporter Assay system (Promega) and Luminescencer-PSN (AB-2200; ATTO Co., Tokyo, Japan) after incubation with medium containing 1 μmol/L progesterone or 10 nmol/L estradiol for 24 hours. The cells were also treated with the same volume of ethanol for 24 hours as controls. The transfection efficiency was normalized against Renilla luciferase activity using pRL-TK control plasmids, and the luciferase activity was evaluated as a ratio (%) compared with that of controls. T47D cells were also used as positive controls for the transcriptional activity.

Analysis for progesterone production. Progesterone production in the NSCLC cells was examined according to previous reports (26). Briefly, after 2, 4, 8, and 24 hours of treatment with 40 mg cholesterol/dL of low-density lipoprotein, the medium was removed and purified by Oasis HLB Plus cartridges (Waters Corp. Massachusetts), and progesterone concentration was evaluated by a Progesterone EIA kit (Cayman Chemical).

Cell proliferation assay and cell cycle analysis. NSCLC cells were cultured in phenol red–free RPMI 1640 without FBS for 24 hours following 48 hours of culture with 10% FBS, and the medium was replaced again with either vehicle (0.1%), an indicated concentration of progesterone (Sigma-Aldrich Co., St. Louis, MO), or progesterone (1,000 nmol/L) with RU 38,486 (1,000 nmol/L). Following 12, 24, 48, and 72 hours of incubation, the cells were counted using a Cell Counting Kit-8 system (Dojindo Technologies, Kumamoto, Japan).

Cell cycle perturbations were determined at 72 hours after treatment with progesterone using FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Data were obtained and processed using the Lysis II software (Becton Dickinson). The percentage of cells in each cell cycle phases was evaluated on a DNA linear plot using the CellFit software (Becton Dickinson).

Table 2. Univariate and multivariate analyses for clinical outcome in NSCLC patients

Variable	Overall survival		Relative risk (95% CI)
	Univariate	Multivariate	
	P	P	
TNM stage (II and III/I)	<0.0001*	<0.0001*	3.127 (1.959-4.992)
Progesterone receptor status (negative/positive)	<0.0001*	0.0001*	2.717 (1.657-4.459)
Histologic differentiation (poorly/well and moderately)	0.0028*	0.5809	
Estrogen receptor β status (negative/positive)	0.1463		
Ki67 LI (≥10%/<10%)	0.1729		
Gender (male/female)	0.2827		
p53 status (positive/negative)	0.4513		
Histologic type (large/adeno and SCC)	0.6358		
Estrogen receptor α status (positive/negative)	0.7321		

*Data were considered significant in the univariate analyses, and were examined in the multivariate analyses.

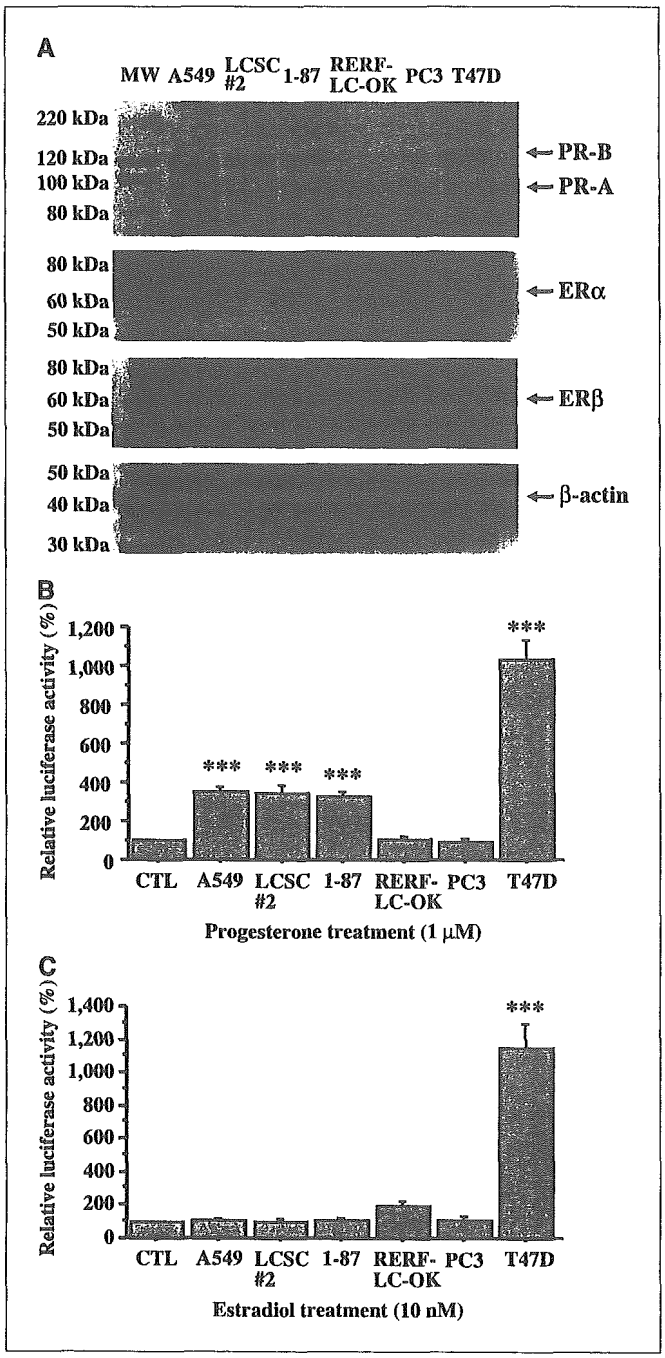

Figure 2. Progesterone receptor and estrogen receptor expression in NSCLC cells. *A*, immunoblotting for progesterone receptor, estrogen receptor α, and estrogen receptor β in A549, LCSC#2, 1-87, RERF-LC-OK, and PC3 cells. Both progesterone receptor-A and progesterone receptor-B immunoreactivities were detected in A549, LCSC#2, and 1-87 cells, whereas estrogen receptor β was present only in RERF-LC-OK cells. Estrogen receptor α was not detected in any of the NSCLC cell lines examined in this study. T47D breast carcinoma cells were used as positive controls. Immunoblotting for β-actin (~42 kDa) was also done as an internal standard of the experiment. Twenty micrograms of protein were loaded on each lane. *B* and *C*, luciferase analyses for transcriptional activity of progesterone receptor (*B*) or estrogen receptor (*C*) in A549, LCSC#2, 1-87, RERF-LC-OK, and PC3 cells. Cells were transiently transfected with pHRE-Luc plasmids (*B*) or pERE-Luc plasmids (*C*), and treated with 1 μmol/L progesterone (*B*) or 10 nmol/L estradiol (*C*) for 24 hours. The luciferase activity was evaluated as a ratio (%) compared with that of controls (*CTL*, treatment without progesterone or estradiol for 24 hours). T47D cells were used as a positive control of this experiment. *Columns*, mean (n = 3); *bars*, SD. ***, $P < 0.001$ versus CTL.

Athymic mouse model. A549, LCSC#2, 1-87, or RERF-LC-OK cells were resuspended in phenol red–free Matrigel [Becton Dickinson, Bedford, MA; 1×10^7 (0.1 mL)/site] and placed on superior side of ovariectomized BALB/c nu/nu athymic female mice (5 weeks of age; Charles River Laboratories, Tokyo, Japan). A progesterone pellet (5, 50, or 200 mg; Innovative Research of America, Toledo, OH) was implanted when tumors measured 5 mm in diameter. Progesterone pellets of 5, 50, and 200 mg used in this study continuously elevate serum progesterone levels up to ~40 nmol/L (equivalent to serum progesterone concentration at the luteal phase of normal cycling premenopausal women), 350 nmol/L, and 1,200 nmol/L, respectively, corresponding to ~8 times higher than the third trimester of pregnancy.[5] The tumor volumes were monitored weekly using the formula for a semiellipsoid ($4/3\ \pi r^3/2$). Tumor tissues were resected at 5 weeks after treatment, and were subsequently fixed in 10% formalin and embedded in paraffin wax to perform immunohistochemistry.

Results

Immunohistochemistry for progesterone receptor and estrogen receptors in non–small-cell lung cancers. Progesterone receptor immunoreactivity was detected in the nuclei of carcinoma cells (Fig. 1*A* and *B*), and progesterone receptor was detected in 106 of 228 (46.5%) NSCLC cases examined in this study. Progesterone receptor immunoreactivity was significantly associated with patient gender ($P = 0.0045$), and was frequently detected in female. Progesterone receptor immunoreactivity was also significantly detected in adenocarcinoma ($P = 0.0002$), and was inversely associated with tumor-node-metastasis (TNM) stage ($P = 0.0085$), histologic differentiation ($P = 0.0355$), and Ki67 LI that indicates activity of carcinoma cell proliferation ($P = 0.0010$; Table 1). Hormone replacement therapy was administrated only in three female patients in this study, and two of that three cases were immunohistochemically positive for progesterone receptor. The mean value of progesterone receptor LI in 228 NSCLCs examined was 15.7% (range 0-93%), and similar statistical association was also detected between progesterone receptor LI and clinicopathologic variables examined in this study (data not shown).

Estrogen receptor α and estrogen receptor β immunoreactivities were detected in the nuclei of carcinoma cells, and the number of estrogen receptor α–positive and estrogen receptor β–positive NSCLCs was 87 (38.2%) and 77 (33.8%) of 228 cases, respectively. Estrogen receptor β immunoreactivity was significantly associated with female ($P = 0.0304$) or adenocarcinoma ($P = 0.0133$) and was inversely correlated with histologic differentiation of carcinoma ($P = 0.0484$). Estrogen receptor β immunoreactivity tended to be positively associated with estrogen receptor α immunoreactivity, but this association did not reach a statistical significance ($P = 0.1139$). No significant association was detected between estrogen receptor β immunoreactivity and other clinicopathologic variables examined. Estrogen receptor α immunoreactivity also tended to be associated with female ($P = 0.1338$), but it was not significantly associated with any clinicopathologic variables of the patients examined in this study.

Correlation between progesterone receptor and estrogen receptor status and clinical outcome of non–small-cell lung cancer patients. Progesterone receptor status was significantly associated with better clinical outcome of the 228 NSCLC patients ($P < 0.0001$; Fig. 1*C*). This association was detected regardless of TNM stage in this study (Fig. 1*D* and *E*). Following a univariate analysis, TNM stage ($P < 0.0001$), progesterone receptor status

[5] Unpublished data from Innovative Research of America, Toledo, Ohio.

($P < 0.0001$), and histologic differentiation ($P = 0.0028$) were shown as significant prognostic factors for overall survival in this study (Table 2). Estrogen receptor β status tended to be associated with better clinical outcome of the patients ($P = 0.1463$), but this association did not reach a statistical significance. No significant association was detected between estrogen receptor α status ($P = 0.7321$) or gender ($P = 0.2827$) and clinical outcome of the patients. A multivariate analysis revealed that TNM stage ($P < 0.0001$) and progesterone receptor status ($P = 0.0001$) were independent prognostic factors with relative risks over 1.0.

Expression of progesterone receptor and estrogen receptors in non–small-cell lung cancer cell lines. We did immunoblotting analyses in A549, LCSC#2, 1-87, RERF-LC-OK, and PC3 cells to examine the expression of progesterone receptor and estrogen receptors in NSCLC cell lines. Two subtypes of progesterone receptor (progesterone receptor-A and progesterone receptor-B), estrogen receptor α, and estrogen receptor β were detected as a specific band of approximately 85 kDa (27), 116 kDa (27), 66 kDa (28), and 65 kDa (14), respectively (Fig. 2*A*). Both progesterone receptor-A and progesterone receptor-B were detected in A549, LCSC#2, and 1-87 cells, but progesterone receptor was not detected in RERF-LC-OK or PC3 cells. Estrogen receptor β was detected only in RERF-LC-OK cells, and estrogen receptor α was not detected in any of the NSCLC cell lines examined in our study.

To further study transcriptional activities of progesterone receptor or estrogen receptor in NSCLC cells, we did luciferase analyses in five NSCLC cell lines. When NSCLC cells were transiently transfected with pHRE-Luc plasmids and were treated with 1 μmol/L progesterone for 24 hours, relative luciferase activity was significantly increased in A549, LCSC#2, and 1-87 cells, compared with the basal level (3.5-fold in A549, 3.4-fold in LCSC#2, and 3.3-fold in 1-87, $P < 0.001$, respectively), but not in RERF-LC-OK or PC3 cells (Fig. 2*B*). A 1.5-fold increase of relative luciferase activity of estrogen-responsive element was detected in RERF-LC-OK cells, when cells were treated with 10 nmol/L estradiol for 24 hours, but this increment did not reach a statistical significance ($P = 0.1128$; Fig. 2*C*).

***In situ* production of progesterone in non–small-cell lung cancers.** Progesterone is synthesized from cholesterol through cascades of several steroidogenic enzymes, including StAR, P450scc, and 3βHSD. To examine possible *in situ* production of progesterone in NSCLCs, we examined tissue concentration of progesterone and immunolocalization of these enzymes in 42 cases of NSCLC (Fig. 3). Immunoreactivity for StAR, P450scc, and

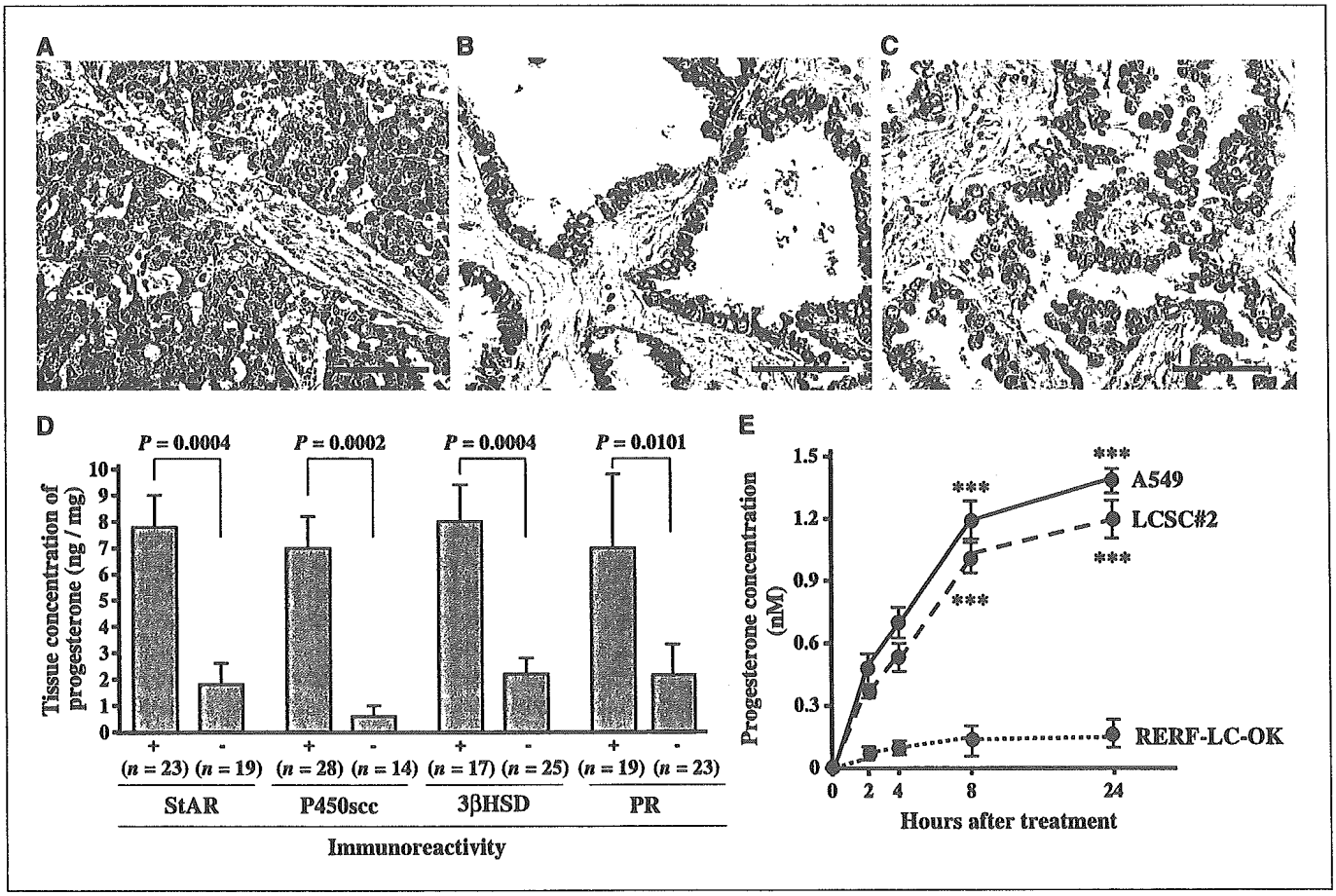

Figure 3. *In situ* production of progesterone in NSCLCs. *A* to *C*, immunoreactivity for StAR (*A*), P450scc (*B*), and 3βHSD (*C*) was detected in the cytoplasms of carcinoma cells in the NSCLC. *Bar*, 100 μm. The specimens used for immunohistochemistry were fixed in 10% formalin and embedded in paraffin wax. *D*, tissue concentration of progesterone in the NSCLC was significantly associated with immunoreactivity for StAR ($P = 0.0004$), P450scc ($P = 0.0002$), 3βHSD ($P = 0.0004$), and progesterone receptor ($P = 0.0101$; $n = 42$). *Columns*, mean of tissue concentrations of progesterone in the group of cases positive (+) or negative (–) for indicated immunoreactivity; *bars*, 95% CI. *E*, A549, LCSC#2, and RERF-LC-OK cells were treated with 40 mg cholesterol/dL of low-density lipoprotein, and subsequently, progesterone concentrations in the medium were evaluated. *Points*, mean ($n = 6$); *bars*, SE. ***, $P < 0.001$, compared with that of RERF-LC-OK at the same treatment period.

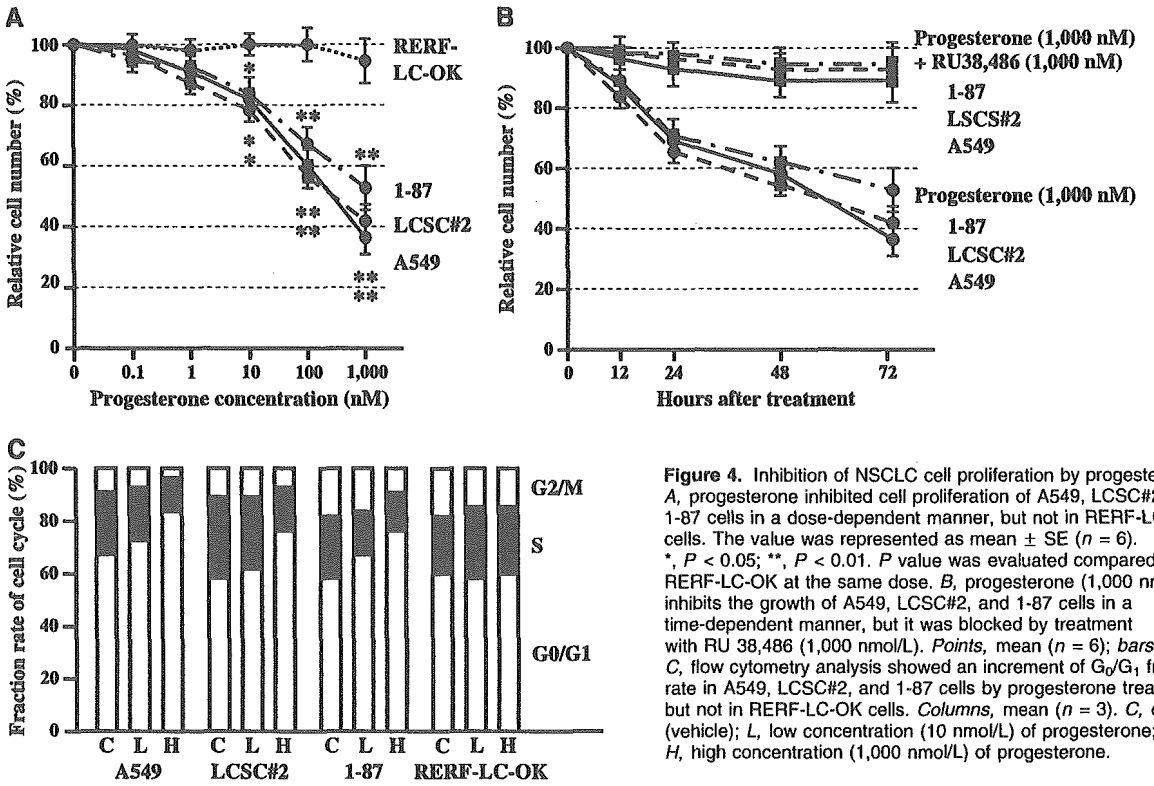

Figure 4. Inhibition of NSCLC cell proliferation by progesterone. *A*, progesterone inhibited cell proliferation of A549, LCSC#2, and 1-87 cells in a dose-dependent manner, but not in RERF-LC-OK cells. The value was represented as mean ± SE (n = 6). *, $P < 0.05$; **, $P < 0.01$. P value was evaluated compared with RERF-LC-OK at the same dose. *B*, progesterone (1,000 nmol/L) inhibits the growth of A549, LCSC#2, and 1-87 cells in a time-dependent manner, but it was blocked by treatment with RU 38,486 (1,000 nmol/L). *Points*, mean (n = 6); *bars*, SE. *C*, flow cytometry analysis showed an increment of G_0/G_1 fraction rate in A549, LCSC#2, and 1-87 cells by progesterone treatment, but not in RERF-LC-OK cells. *Columns*, mean (n = 3). *C*, control (vehicle); *L*, low concentration (10 nmol/L) of progesterone; *H*, high concentration (1,000 nmol/L) of progesterone.

3βHSD was detected in the cytoplasm of carcinoma cells in 23 (54.8%), 28 (66.7%), and 17 (40.5%) of 42 cases, respectively (Fig. 3*A-C*). Tissue concentration of progesterone was positively correlated with immunoreactivity of these enzymes (P = 0.0004 for StAR, P = 0.0002 for P450scc, and P = 0.0004 for 3βHSD) and progesterone receptor status (P = 0.0101; Fig. 3*D*). Immunoreactivity of these enzymes was also significantly associated with progesterone receptor status (P = 0.0035 for StAR, P = 0.0005 for P450scc, and P = 0.0104 for 3βHSD). We then evaluated progesterone synthesis using three NSCLC cell lines. Progesterone was synthesized from cholesterol contained in low-density lipoprotein in A549 and LCSC#2 cells, which were immunohistochemically positive for StAR, P450scc, and 3βHSD, in contrast to RERF-LC-OK cells, which were immunohistochemically negative for these enzymes, after 8 and 24 hours of treatment (Fig. 3*E*; $P <$ 0.001, respectively).

Inhibition of non-small-cell lung cancer cell proliferation by progesterone through progesterone receptor. We then examined the effects of progesterone on NSCLC cell proliferation. Because results of immunoblotting and luciferase assays showed that PC3 cells did not respond to sex steroids in this study, we used three progesterone receptor–positive NSCLC cell lines (A549, LCSC#2, and 1-87) as well as progesterone receptor–negative RERF-LC-OK cells in the experiments. Progesterone significantly suppressed cell proliferation in progesterone receptor–positive A549, LCSC#2, and 1-87 NSCLC cells at 10 nmol/L ($P < 0.05$ versus RERF-LC-OK, respectively) in a dose-dependent manner (Fig. 4*A*). However, no such effects were detected in the growth of progesterone receptor–negative RERF-LC-OK cells. Progesterone did not inhibit the cell proliferation of A549, LCSC#2, and 1-87 cells when these cells were treated with progesterone together with a potent progesterone receptor blocker, RU 38,486 (ref. 29; Fig. 4*B*). Flow cytometry analysis showed that G_0/G_1 fraction was increased in A549, LCSC#2, and 1-87 cells according to the concentration of progesterone, but not in RERF-LC-OK cells (Fig. 4*C*).

Inhibition of non-small-cell lung cancer cell proliferation by progesterone in athymic mice. To further characterize inhibitory effects of progesterone in NSCLC cell proliferation, female nude mice were injected with NSCLC cells, ovariectomized, and treated with a progesterone pellet. Development of progesterone receptor–positive A549 cells was marked (Fig. 5*A*) and the tumor volume reached ~ 10-fold of the original volume in 5 weeks after treatment (Fig. 5*B*). A progesterone pellet, however, significantly reduced the tumor volume of A549 in a dose-dependent manner (Fig. 5*A* and *B*; $P < 0.001$, control versus 200 mg progesterone pellet, and $P < 0.05$, control versus 50 mg progesterone pellet). Similar results were also obtained in other progesterone receptor–positive LCSC#2 (Fig. 5*C*) and 1-87 (Fig. 5*D*) cells, but progesterone pellets did not exert any significant effects on the cell proliferation of progesterone receptor–negative RERF-LC-OK cells (data not shown).

We also did immunohistochemistry for p21, p27, cyclin A, cyclin D1, and cyclin E, as well as Ki67, in NSCLC cells in tumor tissues obtained from nude mice at 5 weeks after treatment with a progesterone pellet (Fig. 6). In A549, 1-87, and LCSC2 cells, LI was dose-dependently increased in p21 (Fig. 6*A*) and p27 (Fig. 6*B*), and was decreased in cyclin A (Fig. 6*C*), cyclin E (Fig. 6*E*), and Ki67 (Fig. 6*F*), with no significant change in cyclin D1 LI (Fig. 6*D*). These changes were not detected in progesterone receptor–negative RERF-LC-OK cells.

Discussion

Several groups of investigators previously reported immunolocalization of estrogen receptors and progesterone receptor in NSCLCs (11, 12, 14) but their biological and/or clinical significance still remains unclear. In our present study, estrogen receptor α, estrogen receptor β, and progesterone receptor immunoreactivities were all positive in 38.2%, 33.8%, and 46.5% of the NSCLCs, respectively, but no significant association was detected among these sex steroid receptors. Transcription of progesterone receptor gene is well known to be regulated by estrogenic actions through estrogen receptor in human breast cancers. Positive progesterone receptor status is generally regarded as one of the markers of functional estrogenic pathways in breast cancers (30), although it does not reflect the patient's circulating progesterone levels (31). However, Su et al. (11) reported that a progesterone receptor (+) estrogen receptor (−) status was detected in 16 of 49 (33%) NSCLC tissues, whereas only one case (2%) was double positive for estrogen receptor and progesterone receptor. Results of our present study are consistent with the study of Su et al. (11), which suggests non-estrogen-induced progesterone receptor expression in NSCLCs. However, it is also true that previous immunohistochemical studies for progesterone receptor have shown great variability of results in NSCLC tissues, and Di Nunno et al. (13) found no immunoreactivity of progesterone receptor in 248 NSCLC tissues. These discrepancies may be partly due to a difference in primary antibody for progesterone receptor used (13).

Progesterone is mainly secreted into circulation from the ovary or placenta in premenopausal women. However, a great majority of NSCLC patients (97.8% in this study) occurs postmenopausal women or men, and the serum progesterone level is in general negligible (less than 6 nmol/L) in these patients. On the other hand, *in situ* biosynthesis of progesterone has been reported in the central nervous system (32). In our present study, we indicated immunolocalization of progesterone-producing enzymes, such as StAR, P450scc, and 3βHSD, in NSCLCs, which were correlated with the tissue concentration of progesterone or progesterone receptor status. Therefore, positive progesterone receptor status may be partly associated with *in situ* production and actions of progesterone, rather than estrogenic actions, in NSCLCs, in contrast to estrogen-dependent breast cancers. Both univariate and multivariate analyses showed that progesterone receptor status is a potent prognostic predictor in NSCLC patients in our study (Table 2). In addition, its effect was similar to that of TNM stage, a well-established diagnostic modality in NSCLCs (33). If progesterone is mainly involved in the growth inhibition through progesterone receptor, residual cancer cells following surgical treatment in progesterone receptor–positive NSCLC possibly grow slowly in the presence of locally produced progesterone, which subsequently results in a better clinical outcome of these patients.

In this study, progesterone significantly induced protein levels of p21 and p27, and decreased those of cyclin A, cyclin E, and Ki67 in progesterone receptor–positive NSCLC cells injected into nude mice (Fig. 6). Previous studies showed that progesterone-mediated growth inhibition was mainly preceded by decreased expression of cyclins A and E and/or induction of p21 and p27 (6–8). Reduction of cyclin D1 protein level by progesterone has been also reported in breast cancer cells (8) but not in smooth muscle cells (34). Results of our study were generally consistent with those of previous reports mentioned earlier, and molecular mechanism of progesterone-induced antiproliferation in NSCLCs

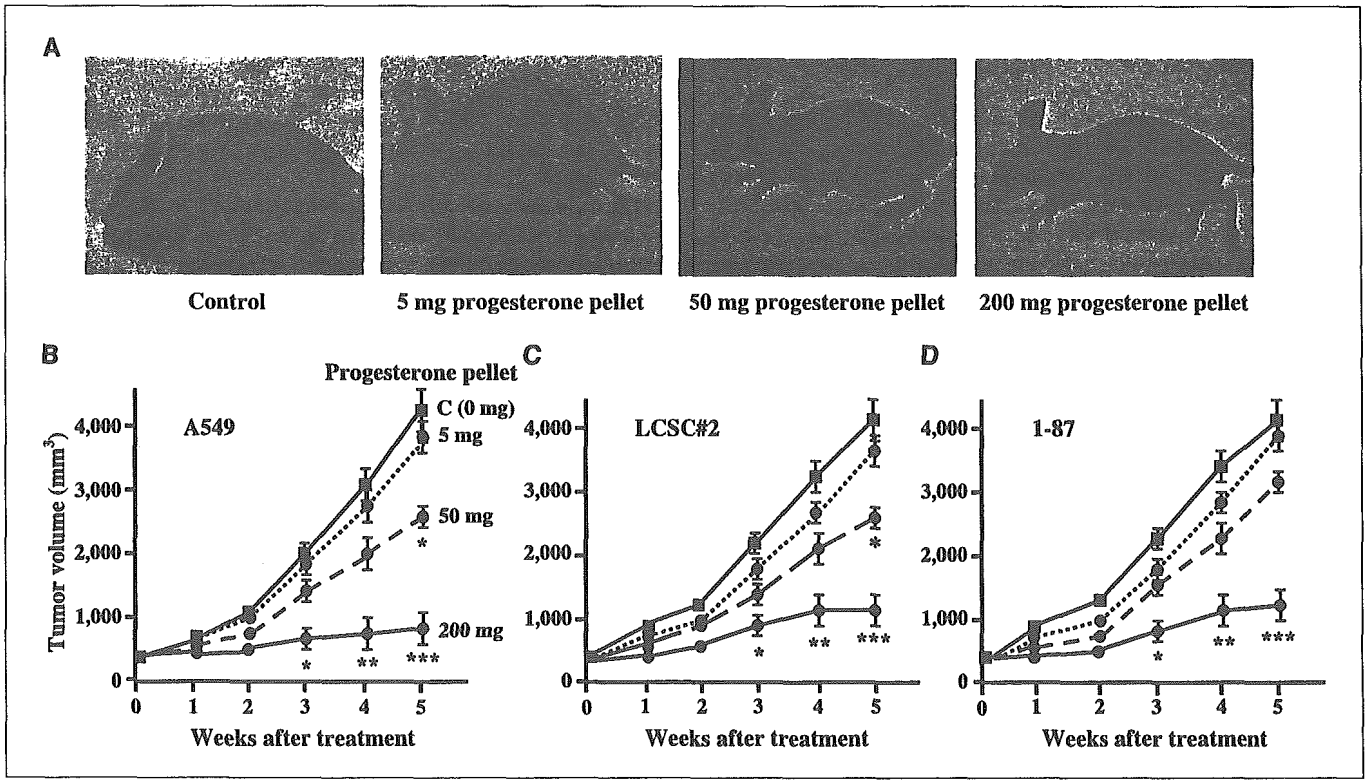

Figure 5. Reduction of NSCLC growth by progesterone in nude mice. *A*, tumor size of A549 in nude mice 5 weeks after treatment with a progesterone pellet. *B* to *D*, tumor volumes of A549 (*B*), LCSC#2 (*C*), and 1-87 (*D*) were dose-dependently reduced by the progesterone pellet. *Points*, mean obtained from six mice ($n = 6$); *bars*, SE. *C*, control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. *P* value was evaluated compared with control at the same time.

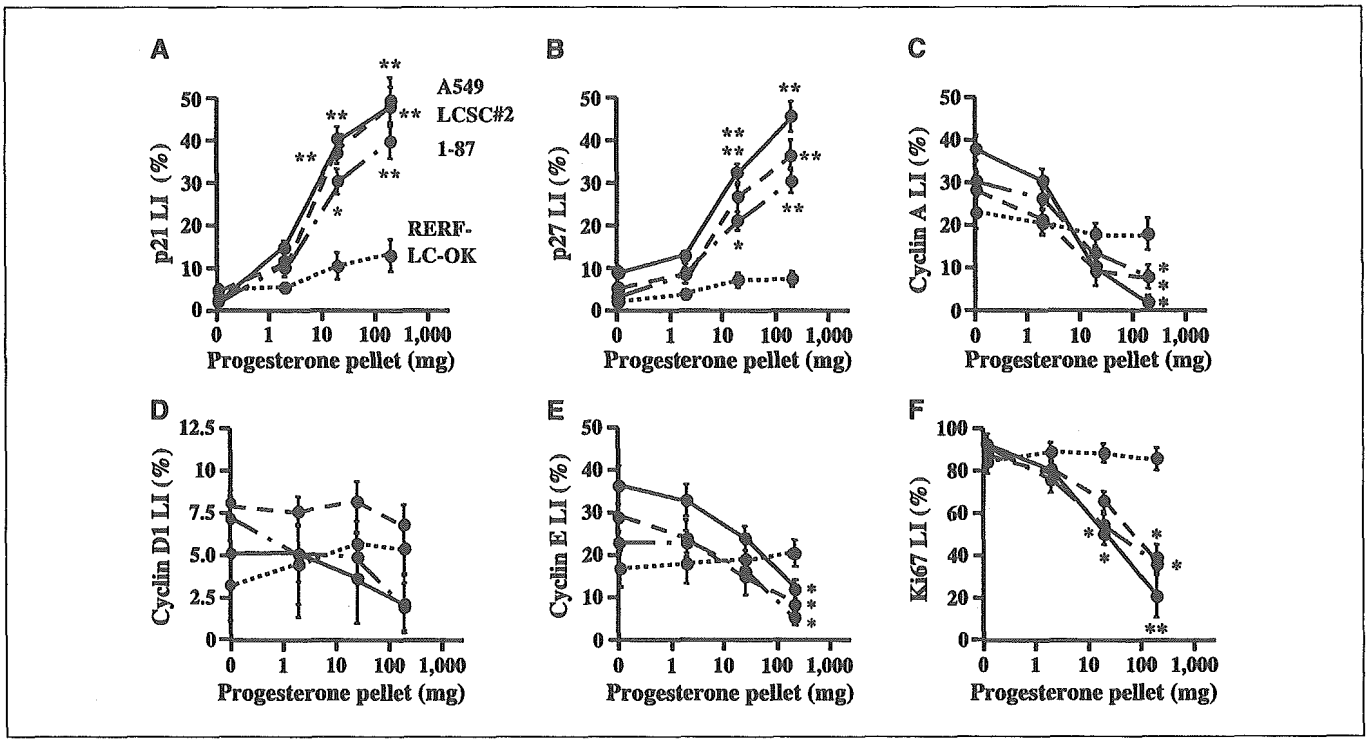

Figure 6. Immunohistochemistry of cell cycle regulators in NSCLC in nude mice. *A*, p21; *B*, p27; *C*, cyclin A; *D*, cyclin D1; *E*, cyclin E; *F*, Ki67. The immunoreactivity was evaluated as LI (%) in NSCLCs in nude mice 5 weeks after treatment with a progesterone pellet. *Points*, mean; *bars*, SE ($n = 6$). *, $P < 0.05$; **, $P < 0.01$. *P* value was evaluated compared with control.

is considered to be generally similar to that previously reported in other progesterone receptor–positive cells. Favorable prognostic values of p21 (35) and p27 (36) or unfavorable values of cyclin A (37) and cyclin E (37) have been reported in NSCLCs. Therefore, the expression of these cell cycle regulators may partly be modulated by *in situ* progesterone actions in NSCLCs.

Inhibition of progesterone receptor–positive NSCLC cell proliferation by progesterone was significantly detected at 10 nmol/L in our *in vitro* study (Fig. 4*A*), similar to that in T47D breast cancer cells (38) or endometrial adenocarcinoma cells (39). Results from our *in vivo* study using nude mice (Fig. 5*A-C*) also showed that progesterone significantly reduced the growth of progesterone receptor–positive NSCLC cells starting from 50 mg of progesterone pellet (~350 nmol/L serum progesterone concentration). Progesterone treatment is an established endocrine therapy in human hormone-dependent breast (9) and endometrial cancers (40), and at present, oral progestins such as medroxyprogesterone acetate are widely employed. Thigpen et al. (10) reported that serum level of medroxyprogesterone acetate became 600 and 1,900 nmol/L in endometrial cancer patients who have received oral medroxyprogesterone acetate in a dose of 200 mg/d (low dose; $n = 145$) and 1,000 mg/d (high dose; $n = 154$), respectively, and the overall clinical response rate was not significantly different in these groups (25% in the low-dose group and 15% in the high-dose group). Effects of medroxyprogesterone acetate were also reported in breast cancer patients when medroxyprogesterone acetate plasma levels were higher than ~500 nmol/L (41), and the overall clinical responsive rate of patients who received high-dose medroxyprogesterone acetate was 54% (42). In addition, Nishimura et al. (43) reported that the clinical responsive rate for oral medroxyprogesterone acetate was not significantly different between 400 mg/d (40%) and 800 mg/d (58%) in breast cancer patients treated with medroxyprogesterone acetate, but four of the six patients who did not respond to the 600 or 800 mg/d dose achieved a clinical response when given 1,200 mg/d of medroxyprogesterone acetate. Therefore, results from our present study as well as from previous reports mentioned above suggest that progestin administration may be effective in suppressing development of the progesterone receptor–positive NSCLC in patients in a therapeutic dose similar to that used in the endocrine treatment of breast and endometrial cancer patients.

Estrogen plays an essential and/or pivotal role in the progression of hormone-dependent breast carcinomas and, therefore, antiestrogens, which block estrogen receptor, have been used in endocrine therapy of patients with breast carcinomas. Tamoxifen is administered to patients with breast cancer, which generally resulted in a 30% to 35% reduction in clinical symptoms, and a 20% to 25% reduction in mortality (44). In NSCLCs, estrogen has been reported to induce estrogen-responsive element activity of RERF-LC-OK cells (14), and to increase cell proliferation of BEAS-2B and DB354 cells (45). In addition, antiestrogen, ICI182,780 or tamoxifen, significantly reduced the colony formation (14) or cell proliferation (45) of these cell lines above. These *in vitro* data suggest a biological function for estrogen in some NSCLC cells, and that estrogen receptors may also have therapeutic potential as an endocrine therapy in NSCLC patients. It awaits further examinations for clarification.

Acknowledgments

Received 8/27/2004; revised 4/20/2005; accepted 4/28/2005.

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.

We appreciate the skillful technical assistance of Chika Kaneko and Dr. Hiroshi Kubo (Departments of Pathology and of Geriatric and Respiratory Medicine, Tohoku University School of Medicine, respectively).

References

1. Jemal A, Tiwari RC, Murray T, et al. American Cancer Society. Cancer statistics, 2004. CA Cancer J Clin 2004; 54:8–29.
2. Jemal A, Chu KC, Tarone RE. Recent trends in lung cancer mortality in the United States. J Natl Cancer Inst 2001;93:277–83.
3. Rom WN, Hay JG, Lee TC, Jiang Y, Tchou-Wong KM. Molecular and genetic aspects of lung cancer. Am J Respir Crit Care Med 2000;161:1355–67.
4. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A 1996; 93:5925–30.
5. Clarke CL, Sutherland RL. Progestin regulation of cellular proliferation. Endocr Rev 1990;11:266–301.
6. Lee WS, Harder JA, Yoshizumi M, Lee ME, Haber E. Progesterone inhibits arterial smooth muscle cell proliferation. Nat Med 1997;3:1005–8.
7. Groshong SD, Owen GI, Grimison B, et al. Biphasic regulation of breast cancer cell growth by progesterone: role of the cyclin-dependent kinase inhibitors, p21 and p27(Kip1). Mol Endocrinol 1997;11:1593–607.
8. Musgrove EA, Hunter LJ, Lee CS, Swarbrick A, Hui R, Sutherland RL. Cyclin D1 overexpression induces progestin resistance in T-47D breast cancer cells despite p27(Kip1) association with cyclin E-Cdk2. J Biol Chem 2001;276:47675–83.
9. Santen RJ, Manni A, Harvey H, Redmond C. Endocrine treatment of breast cancer in women. Endocr Rev 1990; 11:221–65.
10. Thigpen JT, Brady MF, Alvarez RD, et al. Oral medroxyprogesterone acetate in the treatment of advanced or recurrent endometrial carcinoma: a dose-response study by the Gynecologic Oncology Group. J Clin Oncol 1999;17:1736–44.
11. Su JM, Hsu HK, Chang H, et al. Expression of estrogen and progesterone receptors in non-small-cell lung cancer: immunohistochemical study. Anticancer Res 1996;16:3803–6.
12. Kaiser U, Hofmann J, Schilli M, et al. Steroid-hormone receptors in cell lines and tumor biopsies of human lung cancer. Int J Cancer 1996;67:357–64.
13. Di Nunno L, Larsson LG, Rinehart JJ, Beissner RS. Estrogen and progesterone receptors in non-small cell lung cancer in 248 consecutive patients who underwent surgical resection. Arch Pathol Lab Med 2000;124: 1467–70.
14. Omoto Y, Kobayashi Y, Nishida K, et al. Expression, function, and clinical implications of the estrogen receptor β in human lung cancers. Biochem Biophys Res Commun 2001;285:340–7.
15. Clark BJ, Combs R, Hales KH, Hales DB, Stocco DM. Inhibition of transcription affects synthesis of steroidogenic acute regulatory protein and steroidogenesis in MA-10 mouse Leydig tumor cells. Endocrinology 1997; 138:4893–901.
16. Sasano H, Nagura H, Harada N, Goukon Y, Kimura M. Immunolocalization of aromatase and other steroidogenic enzymes in human breast disorders. Hum Pathol 1994;25:530–5.
17. Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Mod Pathol 1998;11: 155–68.
18. Yamashita J, Tashiro K, Yoneda S, Kawahara K, Shirakusa T. Local increase in polymorphonuclear leukocyte elastase is associated with tumor invasiveness in non-small cell lung cancer. Chest 1996;109:1328–34.
19. Imai M, Nakamura T, Akiyama T, Horii A. Infrequent somatic mutations of the ICAT gene in various human cancers with frequent 1p-LOH and/or abnormal nuclear accumulation of β-catenin. Oncol Rep 2004; 12:1099–103.
20. Shibahara S, Yoshizawa M, Suzuki H, Takeda K, Meguro K, Endo K. Functional analysis of cDNAs for two types of human heme oxygenase and evidence for their separate regulation. J Biochem (Tokyo) 1993;113:214–8.
21. Savouret JF, Fridlanski F, Atger M, Misrahi M, Berger R, Milgrom E. Origin of the high constitutive level of progesterone receptor in T47-D breast cancer cells. Mol Cell Endocrinol 1991;75:157–62.
22. Vladusic EA, Hornby AE, Guerra-Vladusic FK, Lakins J, Lupu R. Expression and regulation of estrogen receptor β in human breast tumors and cell lines. Oncol Rep 2000;7:157–67.
23. Kodama T, Shimizu N, Yoshikawa N, et al. Role of the glucocorticoid receptor for regulation of hypoxia-dependent gene expression. J Biol Chem 2004;278: 33384–91.
24. Saj S, Okumura N, Eguchi H, et al. MDM2 enhances the function of estrogen receptor α in human breast cancer cells. Biochem Biophys Res Commun 2001;281: 259–65.
25. Sakamoto T, Eguchi H, Omoto Y, Ayabe T, Mori H, Hayashi S. Estrogen receptor-mediated effects of tamoxifen on human endometrial cancer cells. Mol Cell Endocrinol 2002;192:93–104.
26. Lopezde Alda MJ, Barcelo D. Use of solid-phase extraction in various of its modalities for sample preparation in the determination of estrogens and progestogens in sediment and water. J Chromatogr A 2001;938:145–53.
27. Clarke CL, Zaino RJ, Feil PD, et al. Monoclonal antibodies to human progesterone receptor: characterization by biochemical and immunohistochemical techniques. Endocrinology 1987;121:1123–32.
28. Nakamura Y, Igarashi K, Suzuki T, et al. E4F1, a novel estrogen-responsive gene in possible atheroprotection, revealed by microarray analysis. Am J Pathol 2004;165: 2019–31.
29. Wiechert R, Neef G. Synthesis of antiprogestational steroids. J Steroid Biochem 1987;27:851–8.
30. Horwitz KB, McGuire WL. Estrogen control of progesterone receptor in human breast cancer. Correlation with nuclear processing of estrogen receptor. J Biol Chem 1978;253:2223–8.
31. Jacobsen BM, Richer JK, Sartorius CA, Horwitz KB. Expression profiling of human breast cancers and gene regulation by progesterone receptors. J Mammary Gland Biol Neoplasia 2003;8:257–68.
32. Baulieu EE, Robel P. Neurosteroids: a new brain function? J Steroid Biochem Mol Biol 1990;37:395–403.
33. Gail MH, Eagan RT, Feld R, et al. Prognostic factors in patients with resected stage I non-small cell lung cancer. A report from the Lung Cancer Study Group. Cancer 1984;54:1802–13.
34. Lee WS, Liu CW, Juan SH, Liang YC, Ho PY, Lee YH. Molecular mechanism of progesterone-induced anti-proliferation in rat aortic smooth muscle cells. Endocrinology 2003;144:2785–90.
35. Caputi M, Esposito V, Baldi A. p21waf1/cip1mda-6 expression in non-small-cell lung cancer: relationship to survival. Am J Respir Cell Mol Biol 1998;18:213–7.
36. Esposito V, Baldi A, De Luca A, et al. Prognostic role of the cyclin-dependent kinase inhibitor p27 in non-small cell lung cancer. Cancer Res 1997;57:3381–5.
37. Dobashi Y, Shoji M, Jiang SX, Kobayashi M, Kawakubo Y, Kameya T. Active cyclin A-CDK2 complex, a possible critical factor for cell proliferation in human primary lung carcinomas. Am J Pathol 1998; 153:963–72.
38. Murphy LC, Alkhalaf M, Dotzlaw H, Coutts A, Haddad-Alkhalaf B. Regulation of gene expression in T-47D human breast cancer cells by progestins and antiprogestins. Hum Reprod 1994;1:174–80.
39. Terakawa N, Ikegami H, Shimizu I, Inoue M, Tanizawa O, Matsumoto K. Inhibitory effects of danazol and medroxyprogesterone acetate on [^{3}H]thymidine incorporation in human endometrial cancer cells. J Steroid Biochem 1988;31:131–5.
40. Kelley RM, Baker WH. Progestational agents in the treatment of carcinoma of the endometrium. N Engl J Med 1961;264:216–22.
41. Pannuti F, Camaggi CM, Strocchi E, Martoni A. MPA at high doses in advanced breast cancer: a statistical evaluation. Chemioterapia 1986;5:159–63.
42. Martoni A, Longhi A, Canova N, Pannuti F. High-dose medroxyprogesterone acetate versus oophorectomy as first-line therapy of advanced breast cancer in premenopausal patients. Oncology 1991;48:1–6.
43. Nishimura R, Nagao K, Matsuda M, et al. Predictive value of serum medroxyprogesterone acetate concentration for response in advanced or recurrent breast cancer. Eur J Cancer 1991;33:1407–12.
44. Pasqualini JR, Chetrite G, Blacker C, et al. Concentrations of estrone, estradiol, and estrone sulfate and evaluation of sulfatase and aromatase activities in pre- and postmenopausal breast cancer patients. J Clin Endocrinol Metab 1996;81:1460–4.
45. Mollerup S, Jorgensen K, Berge G, Haugen A. Expression of estrogen receptors α and β in human lung tissue and cell lines. Lung Cancer 2000;37:153–9.

Estrogen-Responsive Finger Protein as a New Potential Biomarker for Breast Cancer

Takashi Suzuki,[1] Tomohiko Urano,[3] Tohru Tsukui,[4] Kuniko Horie-Inoue,[4] Takuya Moriya,[1] Takanori Ishida,[2] Masami Muramatsu,[4] Yasuyoshi Ouchi,[3] Hironobu Sasano,[1] and Satoshi Inoue[3,4]

Abstract

Purpose: Estrogen-responsive finger protein (Efp) is a member of RING finger-B box-Coiled Coil family and is also a downstream target of estrogen receptor α. Previously, Efp was shown to mediate estrogen-induced cell growth, which suggests possible involvement in the development of human breast carcinomas. In this study, we examined expression of Efp in breast carcinoma tissues and correlated these findings with various clinicopathologic variables.

Experimental Design: Thirty frozen specimens of breast carcinomas were used for immunohistochemistry and laser capture microdissection/real-time PCR of Efp. Immunohistochemistry for Efp was also done in 151 breast carcinoma specimens fixed with formalin and embedded in paraffin wax.

Results: Efp immunoreactivity was detected in breast carcinoma cells and was significantly associated with the mRNA level ($n = 30$). Efp immunoreactivity was positively associated with lymph node status or estrogen receptor α status and negatively correlated with histologic grade or 14-3-3σ immunoreactivity ($n = 151$). Moreover, Efp immunoreactivity was significantly correlated with poor prognosis of breast cancer patients, and multivariate analyses of disease-free survival and overall survival for 151 breast cancer patients showed that Efp immunoreactivity was the independent marker.

Conclusions: Our data suggest that Efp immunoreactivity is a significant prognostic factor in breast cancer patients. These findings may account for an oncogenic role of Efp in the tumor progression of breast carcinoma.

Breast cancer is the most common type of cancer and continues to be the most frequent cause of cancer-related deaths in women in the Western world. Whether or not human primary breast cancers are estrogen dependent is a critical factor that determines patient prognosis and availability of antiestrogenic endocrine therapy (1). Two thirds of breast carcinomas are positive for estrogen receptor α (ERα) and a great majority of these tumors initially respond to antiestrogens such as tamoxifen and aromatase inhibitors. However, it is also true that these ERα-positive breast carcinomas frequently acquire resistance to endocrine therapy, although ERα remains to be expressed (1, 2). The molecular mechanisms through which breast carcinomas become hormone-refractory are still largely unclear.

Identification and functional studies of ERα target molecules may provide a clue for understanding the mechanism that alters tumor phenotypes. We have previously isolated estrogen-responsive finger protein (Efp), which is a member of RING finger-B box-Coiled Coil family (3). Efp also is one of the downstream targets of ERα (3–6). Efp-deficient mice displayed underdeveloped uteri and reduced estrogen responsiveness (7), and therefore, Efp is considered to be essential for estrogen-dependent proliferation. It has also been shown that Efp promotes the growth of breast tumor by functioning as a ubiquitin ligase (E3) that targets the negative cell cycle checkpoint 14-3-3σ (8).

Expression of Efp was previously reported in breast carcinoma tissues at mRNA (5) and protein levels (9). However, information on the expression of Efp in human breast carcinoma tissues is still very limited, and the biological significance of Efp remains unclear at this juncture. Therefore, in this study, we examined expression of Efp in 30 cases of breast carcinoma tissues using immunohistochemistry and laser capture microdissection/real-time PCR. We subsequently examined immunolocalization of Efp in 151 cases of human breast carcinoma tissues and correlated these findings with various clinicopathologic factors including clinical outcome of the patients.

Authors' Affiliations: Departments of [1]Pathology and [2]Surgery, Tohoku University School of Medicine, Aoba-ku, Sendai, Miyagi-ken, Japan; [3]Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo, Japan; and [4]Research Center for Genomic Medicine and Department of Molecular Biology, Saitama Medical School, Yamane, Hidaka-shi, Saitama, Japan

Received 1/6/05; revised 6/9/05; accepted 6/28/05.

Grant support: Ministry of Health, Labor, and Welfare Japan, the Ministry of Education, Culture, Sports, Science and Technology Japan, the Core Research for Evolutional Science and Technology, and the Princess Takamatsu Cancer Research Fund (02-23402).

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.

Requests for reprints: Takashi Suzuki, Department of Pathology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, 980-8575, Japan. Phone: 81-22-717-8050; Fax: 81-22-717-8051; E-mail: t-suzuki@patholo2.med.tohoku.ac.jp.

doi:10.1158/1078-0432.CCR-05-0040

Materials and Methods

Patients and tissues. Thirty specimens of invasive ductal carcinoma were obtained from patients who underwent mastectomy in 2001 in the Department of Surgery at Tohoku University Hospital, Sendai, Japan. Specimens for RNA isolation were snap-frozen and stored at −80°C, and those for immunohistochemistry were fixed with 10% formalin and embedded in paraffin wax. Informed consent was obtained from all patients before their surgery and examination of specimens used in this study.

One hundred fifty-one specimens of invasive ductal carcinoma of the breast were obtained from female patients who underwent mastectomy from 1982 to 1989 in the Department of Surgery, Tohoku University Hospital, Sendai, Japan. All specimens were fixed with 10% formalin and embedded in paraffin wax, and snap-frozen tissues were not available for examination in these cases. These patients did not receive any preoperative radiotherapy and chemotherapy as well as any postoperative hormone therapy. Information on patient age, menopausal status, stage, tumor size at operation, lymph node status, histologic grade, and relapse and survival times was retrieved from the review of patient charts. The mean follow-up period was 105 months (3-157 months).

Research protocols for this study were approved by the Ethics Committee at Tohoku University School of Medicine.

Immunohistochemistry. Anti-human Efp antibody was generated as previously described (3). Polyclonal antibody for 14-3-3σ (N-14) and monoclonal antibodies for ERα (1D5), progesterone receptor (MAB429), Ki-67 (MIB-1), and p53 (DO7) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Immunotech (Marseille, France), Chemicon (Temecula, CA), DAKO (Tokyo, Japan), and Novocastra Laboratories (Newcastle, United Kingdom), respectively. A Histofine Kit (Nichirei, Tokyo, Japan), which employs the streptavidin-biotin amplification method, was used for immunohistochemistry, and the antigen-antibody complex was visualized with 3.3′-diaminobenzidine solution [1 mmol/L 3,3′-diaminobenzidine, 50 mmol/L Tris-HCl buffer (pH 7.6), and 0.006% H_2O_2]. For a negative control for Efp immunohistochemistry, an immunohistochemical preabsorption test was done.

Efp immunoreactivity was classified into three groups: ++, >50% positive carcinoma cells; +, 1% to 50% positive cells; and −, no immunoreactivity, according to a previous report (10). Immunoreactivity of ERα, progesterone receptor, and Ki-67 was scored in more than 1,000 carcinoma cells for each case, and the percentage of immunoreactivity [i.e., labeling index (LI)] was determined. Cases that were found to have ERα LI of more than 10% were considered ERα-positive breast carcinomas (11).

Laser capture microdissection/real-time PCR. Laser capture microdissection was conducted using the Laser Scissors CRI-337 (Cell Robotics, Inc., Albuquerque, NM). A detailed procedure has been described previously (12, 13). Briefly, ~1,000 carcinoma or intratumoral stromal cells were separately collected under the microscope from breast carcinoma frozen tissue sections embedded in Tissue-Tek O.T.C. The Light Cycler System (Roche Diagnostics GmbH, Mannheim, Germany) was used to semiquantify the level of Efp mRNA expression in this study. The primers used for real-time PCR were the following: Efp sense, 5′-CGTGGAGTGGTTCAACAC-3′, and Efp antisense, 5′-GAGCAGATGGAGAGTGTGG-3′; glyceraldehyde-3-phosphate dehydrogenase sense, 5′-TGAACGGGAAGCTCACTGG-3′, and glyceraldehyde-3-phosphate dehydrogenase antisense, 5′-TCCACCACCCTGTTGCTGTA-3′. To verify amplification of the correct sequences, PCR products were purified and subjected to direct sequencing. Efp mRNA levels were normalized to those of glyceraldehyde-3-phosphate dehydrogenase, and subsequently, the fold change of Efp mRNA level in each sample was evaluated using the mRNA level in MCF7 cells as a positive control. Negative control experiments lacked cDNA substrate to check for the possibility of exogenous contaminant DNA, and no amplified products were detected under these conditions.

Statistical analyses. Statistical analyses were done using one-way ANOVA and Bonferroni test or a cross-table using χ^2 test. Overall and disease-free survival curves were generated according to the Kaplan-Meier method, and statistical significance was calculated using log-rank test. Univariate and multivariate analyses were evaluated by a proportional hazard model (Cox) using PROC PHREG in our SAS software. Differences with $P < 0.05$ were considered significant.

Results

Expression of Efp in 30 breast cancer tissues: Immunohistochemistry. Efp immunoreactivity was detected in the cytoplasm of breast carcinoma cells, but not in intratumoral

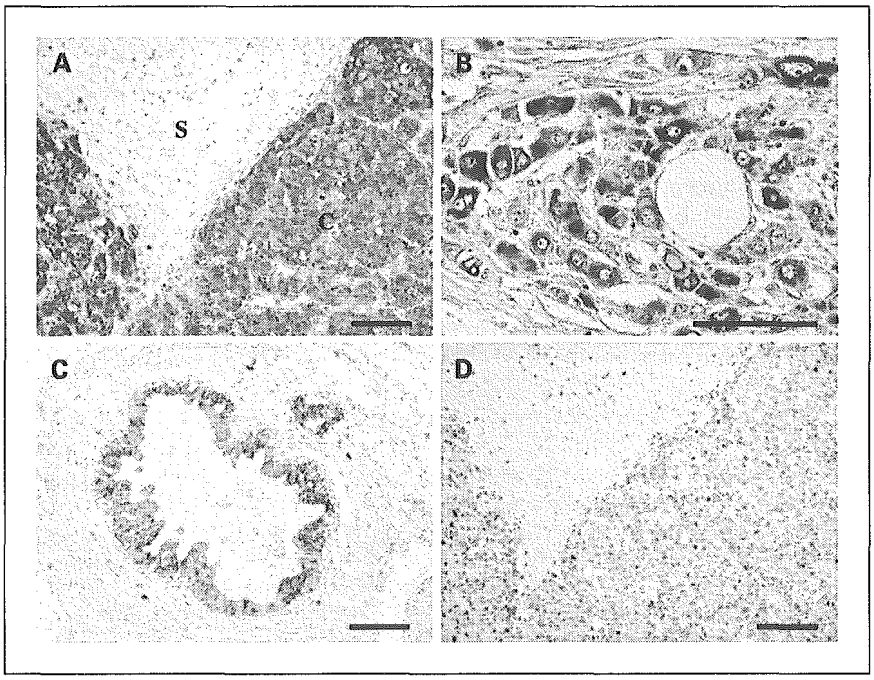

Fig. 1. Immunohistochemistry for Efp in breast carcinoma (invasive ductal carcinoma). *A* and *B*, Efp immunoreactivity was detected in the cytoplasm of the carcinoma cells (*C*), but not in intratumoral stromal cells (*S*). *C*, in morphologically normal mammary glands, Efp immunoreactivity was also detected in the epithelial cells. *D*, no significant immunoreactivity of Efp was detected in a section analyzed by immunohistochemical preabsorption test as a negative control. Same area as *A*. *Bar*, 100 μm.

stromal cells (Fig. 1A and B). The number of cases expressing immunoreactive Efp in each group of 30 breast carcinoma tissues is summarized as follows: ++, n = 9 (30.0%); +, n = 13 (43.3%); and −, 17 (56.7%). Efp immunoreactivity was also positive in epithelial cells of morphologically normal mammary glands (Fig. 1C). Immunohistochemical preabsorption test for Efp showed no specific immunoreactivity in a negative control (Fig. 1D).

Laser capture microdissection/real-time PCR. To examine the localization of Efp mRNA in breast carcinoma tissues, we did laser capture microdissection/real-time PCR analyses. Expression of Efp mRNA was detected in carcinoma cells, but not in intratumoral stromal cells (Fig. 2A). As shown in Fig. 2B, a significant association was detected between Efp immunoreactivity and Efp mRNA level (P = 0.0012, ++ versus − and ++ versus +, respectively) in 30 cases of breast cancer tissues examined.

Correlation between Efp immunoreactivity and clinicopathologic variables in 151 breast carcinomas. Results of associations between Efp immunoreactivity and clinicopathologic variables in 151 breast carcinomas were summarized in Table 1. The number of cases expressing immunoreactive Efp in each group is summarized as follows: ++, n = 46 (30.5%); +, n = 64 (42.4%); and −, n = 41 (27.2%). Efp immunoreactivity was significantly associated with lymph node status (P = 0.0027), ERα status (P = 0.0013), or ERα LI (P = 0.0023, ++ versus −; P = 0.0045, + versus −). On the other hand, negative correlation was detected between Efp immunoreactivity and histologic grade (P = 0.0064) or 14-3-3σ immunoreactivity (P < 0.0001). There was, however, no significant relationship between Efp immunoreactivity and other clinicopathologic variables, including patient age, menopausal status, stage, tumor size, progesterone receptor LI, Ki-67 LI, and 53 status, in this study. Similar tendencies described above were confirmed in increased rankings of positivity for Efp immunoreactivity into five groups (0%, 1-25%, 26-50%, 51-75%, and 76-100% positive cells; data not shown).

Correlation between Efp immunoreactivity and clinical outcome of the 151 breast cancer patients. Efp immunoreactivity was significantly associated with an increased risk of recurrence (P < 0.0001; Fig. 3A). A similar tendency was also detected when Efp immunoreactivity was further categorized into five groups (0%, 1-25%, 26-50%, 51-5%, and 76-100% positive cells; Fig. 3B). Following univariate analysis by Cox (Table 2), lymph node status (P < 0.0001), Efp immunoreactivity (P < 0.0001), tumor size (P = 0.0019), and 14-3-3σ immunoreactivity (P = 0.0314) were shown as significant prognostic variables for disease-free survival in 151 breast carcinoma patients examined. A multivariate analysis revealed that lymph node status (P < 0.0001), Efp immunoreactivity (P = 0.0011), and tumor size (P = 0.0349) were independent prognostic factors with relative risks over 1.0, whereas 14-3-3σ immunoreactivity was not significant (P = 0.0681; Table 2).

Overall survival curve was shown in Fig. 3C. A significant correlation was detected between Efp immunoreactivity and adverse clinical outcome of the patients (P < 0.0001), and a similar tendency was also detected when Efp immunoreactivity was categorized into five groups (Fig. 3D). Using a univariate analysis (Table 3), Efp immunoreactivity (P < 0.0001), lymph node status (P < 0.0001), tumor size (P = 0.0084), and p53 status (P = 0.0230) turned out to be

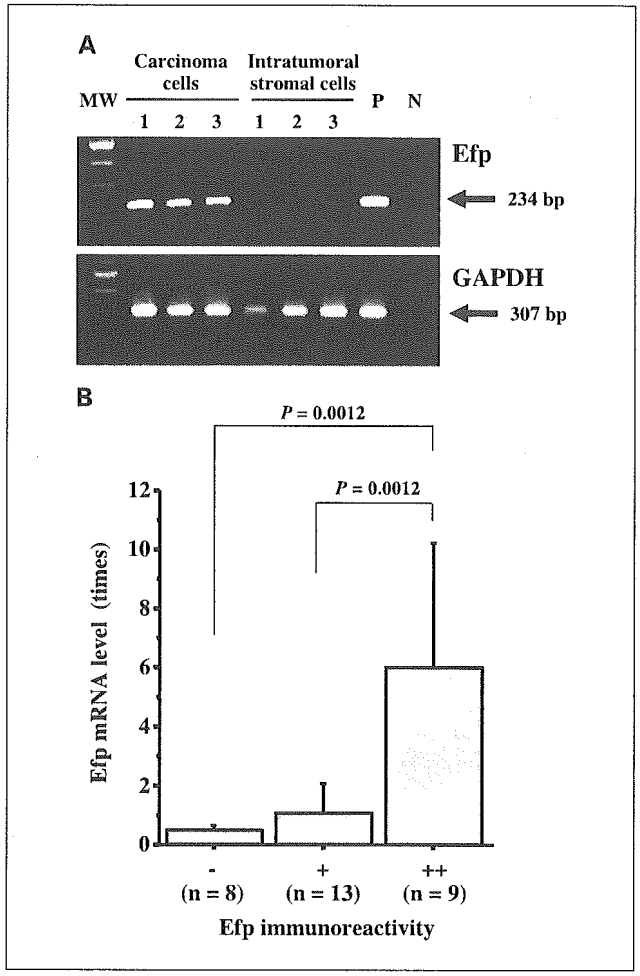

Fig. 2. Laser capture microdissection/real-time PCR analysis for Efp in breast carcinoma tissues. *A*, expression for Efp mRNA (234 bp) was detected only in the component of breast carcinoma cells, whereas that of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA (307 bp) was detected in both components of carcinoma cells and intratumoral stromal cells. Three cases are represented in this agarose gel photo. PCR was done for 40 cycles. *P*, positive control (MCF7 cells); *N*, negative control (no cDNA substrate). *B*, association between Efp immunoreactivity and Efp mRNA level in the carcinoma cell component in 30 breast carcinoma tissues. A significant positive correlation was detected (P = 0.0012, ++ versus + and ++ versus −, respectively). Expression level of Efp mRNA in the carcinoma cell component of each case was represented as a ratio of that of glyceraldehyde-3-phosphate dehydrogenase, and was subsequently evaluated as the ratio (%) compared with that of MCF7 cells.

significant prognostic factors for overall survival in this study. However, multivariate analysis revealed that only Efp immunoreactivity (P = 0.0030) and lymph node status (P = 0.0065) were independent prognostic factors with a relative risk over 1.0; other factors were not significant in this study (Table 3).

The significant association between Efp immunoreactivity and clinical outcome of the patients was detected regardless of ERα status in this study (Fig. 4A-D).

Discussion

In this study, Efp immunoreactivity was significantly associated with Efp mRNA level, and was detected in carcinoma cells in 110 of 151 human breast carcinomas (72.8%).

Table 1. Association between Efp immunoreactivity and clinicopathologic variables in 151 human breast carcinomas

	Efp immunoreactivity			*P*
	++ (*n* = 46)	+ (*n* = 64)	− (*n* = 41)	
Age* (y)	52.8 ± 1.9	53.5 ± 1.6	52.4 ± 1.3	>0.05
Menopausal status				
Premenopausal	23 (15.2%)	33 (21.9%)	15 (9.9%)	
Postmenopausal	23 (15.2%)	31 (20.5%)	26 (17.2%)	>0.05
Stage				
I	11 (7.3%)	16 (10.6%)	11 (7.3%)	
II	26 (17.2%)	42 (27.8%)	26 (17.2%)	
III	9 (6.0%)	6 (31.6%)	4 (21.0%)	>0.05
Tumor size* (mm)	29.5 ± 2.5	24.1 ± 1.3	26.9 ± 3.0	>0.05
Lymph node status				
Positive	29 (19.2%)	20 (13.2%)	15 (9.9%)	
Negative	17 (11.3%)	44 (29.1%)	26 (17.2%)	0.0027
Histologic grade				
1	17 (11.3%)	17 (11.3%)	3 (2.0%)	
2	16 (10.6%)	22 (14.6%)	14 (9.3%)	
3	13 (8.6%)	25 (16.6%)	24 (15.9%)	0.0064
ERα status				
Positive	39 (25.8%)	49 (32.5%)	21 (13.9%)	
Negative	7 (4.6%)	15 (9.9%)	20 (13.2%)	0.0013
ERα LI*	48.9 ± 4.8	46.0 ± 4.2	27.0 ± 4.9	− vs ++; 0.0023 − vs +; 0.0045
Progesterone receptor LI*	46.8 ± 11.5	47.6 ± 4.3	34.6 ± 5.6	>0.05
14-3-3α immunoreactivity				
Positive	9 (6.0%)	20 (13.2%)	29 (19.2%)	
Negative	37 (24.5%)	44 (29.1%)	12 (7.9%)	<0.0001
Ki-67 LI*	23.9 ± 2.2	25.8 ± 2.2	27.2 ± 2.9	>0.05
P53 status				
Positive	13 (8.6%)	13 (8.6%)	17 (11.3%)	
Negative	33 (21.9%)	51 (33.8%)	24 (15.9%)	>0.05

*Data are presented as mean ± 95% confidence interval (95% CI). All other values represent the number of cases and percentage.

Previously, Ikeda et al. (5) reported Efp mRNA expression in 9 of 15 (60.0%) breast carcinoma tissues using RNase protection assay, and Thomson et al. (9) reported Efp immunoreactivity in breast carcinoma cells in 64 of 91 (70.3%) cases. The frequency and cellular localization of Efp in our present study were in good agreement with these reports (5, 9), and widespread distribution of Efp may suggest an important role of Efp in breast carcinomas. Efp immunoreactivity was also detected in normal glandular epithelia in our study. It is also consistent with previous studies (5, 9) and Thomson et al. (9) suggested the involvement of Efp in mammary gland differentiation.

Efp is known as a downstream product of ERα (3–7). *Efp* gene has an estrogen-responsive element at the 3′-untranslated region (3). The estrogen-responsive element of *Efp* responded to ERα in transfected estrogen receptors in 293T cells (5), and Efp mRNA was rapidly induced by estrogen treatment within 0.5 hour in MCF7 cells (5). In this study, Efp immunoreactivity was significantly associated with ERα status and ERα LI in 151 breast carcinoma tissues. Therefore, it is suggestive that Efp is mainly produced in carcinoma cells through ERα as a result of estrogenic action in breast carcinoma. On the other hand, we also found Efp immunoreactivity in 22 of 42 ERα-negative breast carcinomas. It may be partly explained that Efp expression was induced by a low or undetectable level of ERα in these cases. However, Ikeda et al. (14) analyzed human 5′-flanking region of human Efp gene, and reported the possible regulation of Efp promoter by multiple elements and/or interacting factors. Therefore, other factors rather than ERα may be also involved in the expression of Efp in some breast carcinomas.

In our study, Efp immunoreactivity was significantly associated with an increased risk of recurrence or worse prognosis ($P < 0.0001$, respectively). Both univariate and multivariate analyses have shown that Efp immunoreactivity was a potent prognostic factor for both recurrence and overall survival in breast carcinomas, and that the effect is similar to that of lymph node status, a well-established diagnostic modality (15). Efp knockout mice showed a smaller increase in uterine weight and a lower cell cycle progression from G_0/G_1 to S phase compared with the wild-type (7), suggesting a pivotal role of Efp in ERα-induced cell growth in the uterus. In addition, Urano et al. (8) showed that overexpression of Efp caused tumor cell growth in MCF7 breast cancer cells. Therefore, taken together with these previous reports and our present results, it is suggested that Efp

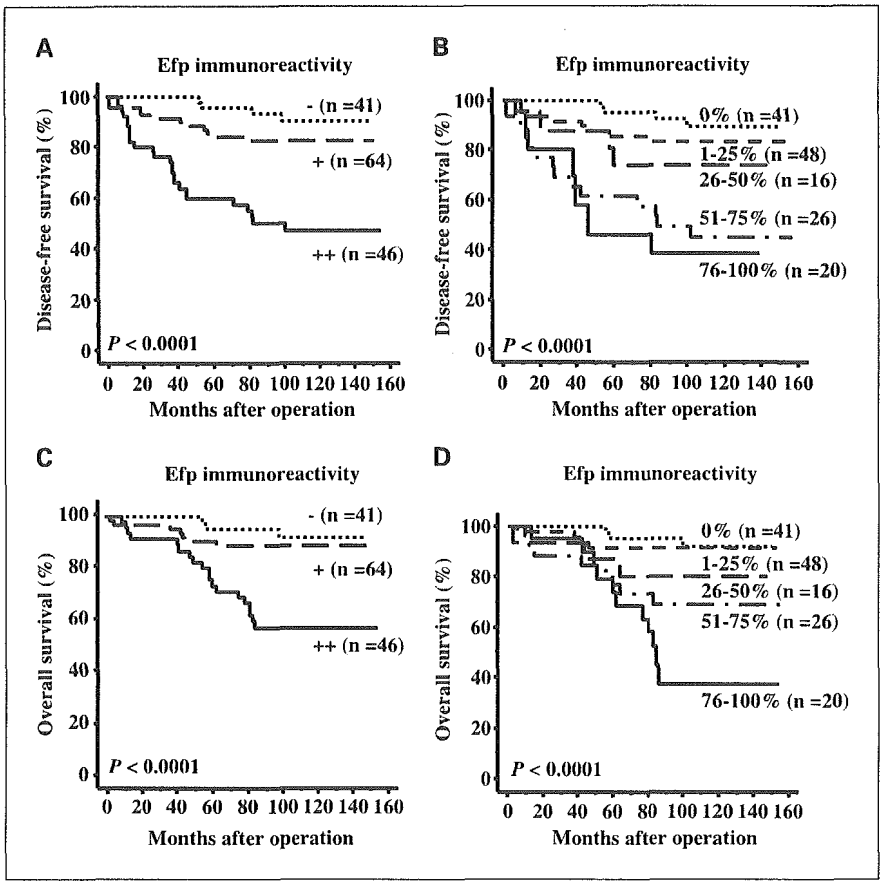

Fig. 3. Disease-free (*A* and *B*) and overall (*C* and *D*) survival of 151 patients with breast carcinoma according to Efp immunoreactivity (Kaplan-Meier method). *A*, Efp immunoreactivity was significantly ($P < 0.0001$, log-rank test) associated with an increased risk of recurrence. *B*, association between Efp immunoreactivity and increased risk of recurrence was also detected in increased rankings of positivity for Efp immunoreactivity into five groups (0%, 1-25%, 26-50%, 51-75%, and 76-100% positive cells). *C* and *D*, Efp immunoreactivity was significantly ($P < 0.0001$, log-rank test) associated with worse overall survival when Efp immunoreactivity was classified into three (*C*) or five (*D*) groups.

plays an important role in the proliferation of breast carcinoma cells. It is well known that biologically active estrogen, estradiol, is locally produced in breast carcinoma tissues from circulating inactive steroids, and acts on these cells via ERα (16). Therefore, residual cancer cells following surgical treatment in Efp-positive breast carcinomas may grow rapidly in the presence of local estrogens, thereby resulting in an increased recurrence and poor prognosis in these patients.

14-3-3σ induces G_2 arrest and inhibits the progression of cell cycle (17) by sequestering the mitotic initiation complex Cdc2-cyclin B1 in the cytoplasm, blocking nuclear entry (18). Expression of 14-3-3σ was examined by several groups in breast carcinoma tissues. However, results of these studies seem to be inconsistent. Ferguson et al. (19) reported that 14-3-3σ mRNA was detected only in 3 of 48 (6.3%) breast carcinoma tissues by Northern blot analysis. Ferguson et al. (19) and Umbricht et al. (20) showed hypermethylation of CpG islands in the 14-3-3σ gene in more than 90% of breast carcinomas, and postulated that loss of 14-3-3σ expression was an early event in neoplastic transformation of the breast. Simooka et al.

Table 2. Univariate and multivariate analyses of disease-free survival in 151 breast cancer patients examined

Variable	Univariate	Multivariate	
	P	*P*	Relative risk (95% CI)
Lymph node status (positive/negative)	<0.0001*	<0.0001	6.053 (2.549-14.379)
Efp immunoreactivity (++/+, −)	<0.0001*	0.0011	3.090 (1.572-6.073)
Tumor size (≥20 mm/<20 mm)	0.0019*	0.0349	3.157 (1.085-9.184)
14-3-3α immunoreactivity (negative/positive)	0.0314*	0.0681	
Ki-67 LI (≤10/<10)	0.1149		
ERα status (positive/negative)	0.1392		
Histologic grade (3/1, 2)	0.2362		
p53 status (positive/negative)	0.4094		

*Data were considered significant in the univariate analyses and were examined in the multivariate analyses.

Table 3. Univariate and multivariate analyses of overall survival in 151 breast cancer patients examined

Variable	Univariate	Multivariate	
	P	*P*	Relative risk (95% CI)
Efp immunoreactivity (++/+, −)	<0.0001*	0.0030	5.343 (1.725-16.175)
Lymph node status (positive/negative)	<0.0001*	0.0065	7.783 (1.773-34.166)
Tumor size (≥20 mm/<20 mm)	0.0084*	0.1514	
p53 status (positive/negative)	0.0230*	0.0832	
ERα status (positive/negative)	0.2890		
Ki-67 LI (≥10/<10)	0.3562		
Histologic grade (3/1, 2)	0.3827		
14-3-3α immunoreactivity (negative/positive)	0.6286		

*Data were considered significant in the univariate analysis and were examined in the multivariate analyses.

(21) detected 14-3-3σ immunoreactivity in 23% of invasive ductal carcinomas and reported that loss of 14-3-3σ expression was relatively low compared with the methylation status of 14-3-3σ gene in breast carcinoma previously reported. On the other hand, Urano et al. (8) showed that 14-3-3σ is a primary target for proteolysis by Efp, and 14-3-3σ protein was regulated by Efp-mediated posttranslational modification. However, Moreira et al. (22) recently reported that expression level of 14-3-3σ was similar in nonmalignant breast epithelial tissue and matched malignant tissue with only sporadic loss of expression observed in 3 of the 68 (4.4%) tumors examined. The lack of expression of 14-3-3σ in the three breast carcinomas was not associated with increased expression of Efp, and they suggested that loss of expression of 14-3-3σ protein was a sporadic event in the breast carcinoma (22). In our present study, immunoreactivity of 14-3-3σ was detected in 58 of 151 (38.4%) breast carcinomas, and was inversely associated with Efp immunoreactivity. These results seem to support the down-regulation of

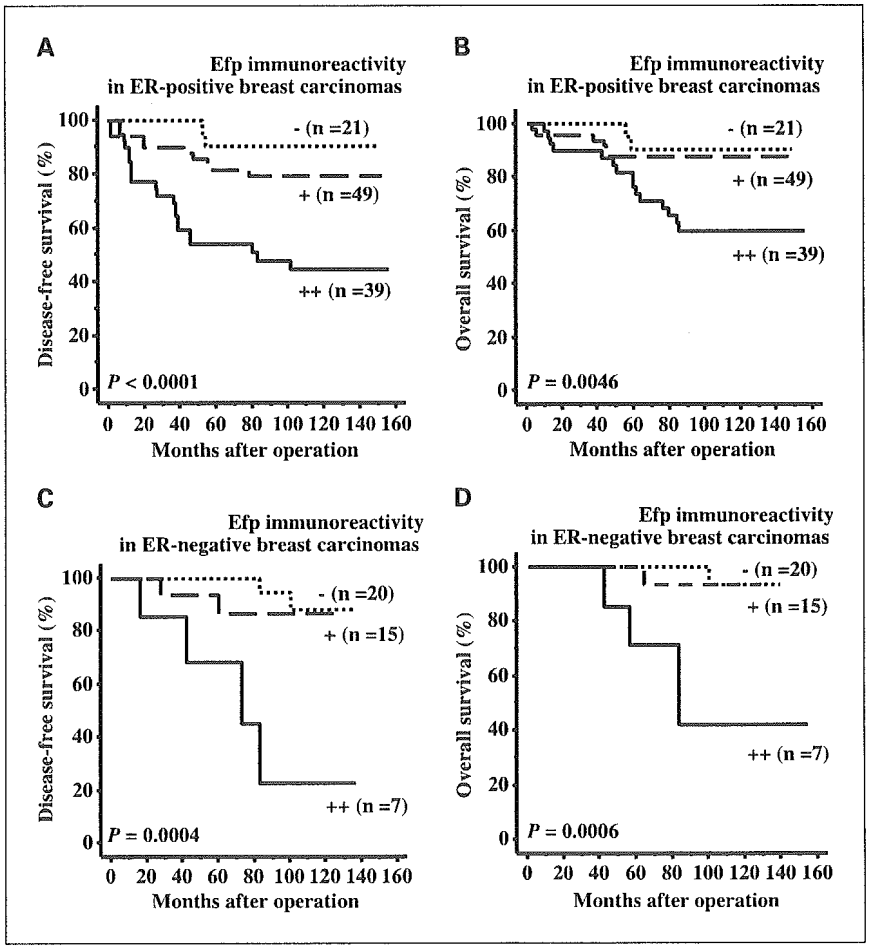

Fig. 4. Association between Efp immunoreactivity and disease-free (*A* and *C*) or overall (*B* and *D*) survival of 151 patients according to ERα status (Kaplan-Meier method). Efp immunoreactivity was significantly associated with poor clinical outcome of 151 breast cancer patients regardless of ERα status. Statistical association was evaluated by log-rank test.

14-3-3σ by methylation of the gene and/or proteolysis by Efp in breast carcinoma tissues; however, these are not necessarily consistent with the findings by Moreira et al. (22). Further examinations, including validation of the immunohistochemical results by another laboratories, are required to clarify the expression of 14-3-3σ in breast carcinoma tissues.

In summary, Efp immunoreactivity was detected in carcinoma cells in 72.8% of breast cancer tissues, and it was associated with the mRNA level. Efp immunoreactivity was significantly associated with lymph node status or ERα status, and was inversely correlated with histologic grade or 14-3-3σ immunoreactivity. Moreover, Efp immunoreactivity was significantly associated with poor clinical outcome of the patients. These present results suggest that Efp is mainly involved in the estrogen-dependent growth of breast carcinomas, and Efp immunoreactivity is a potent prognostic factor in breast carcinoma patients.

Acknowledgments

We appreciate the skillful technical assistance of Dr. Yasuhiro Miki (Department of Pathology, Tohoku University School of Medicine, Sendai, Japan) and Toshiki Hishinuma (Saitama Medical School, Hidaka, Japan). We thank Dr. Bruce Blumberg (Department of Developmental and Cell Biology, University of California, Irvine, CA) for critical reading and comments on the manuscript.

References

1. Ali S, Coombes RC. Endocrine-responsive breast cancer and strategies for combating resistance. Nat Rev Cancer 2002;2:101–12.
2. Jordan VC. Fourteenth Gaddum Memorial Lecture. A current view of tamoxifen for the treatment and prevention of breast cancer. Br J Pharmacol 1993;110:507–17.
3. 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;90:11117–21.
4. Orimo A, Inoue S, Ikeda K, Noji S, Muramatsu M. Molecular cloning, structure, and expression of mouse estrogen-responsive finger protein Efp. Co-localization with estrogen receptor mRNA in target organs. J Biol Chem 1995;270:24406–13.
5. Ikeda K, Orimo A, Higashi Y, Muramatsu M, Inoue S. Efp as a primary estrogen-responsive gene in human breast cancer. FEBS Lett 2000;472:9–13.
6. Muramatsu M, Inoue S. Estrogen receptors: how do they control reproductive and nonreproductive functions? Biochem Biophys Res Commun 2000;270:1–10.
7. 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.
8. Urano T, Saito T, Tsukui T, et al. Efp targets 14-3-3σ for proteolysis and promotes breast tumour growth. Nature 2002;417:871–5.
9. Thomson SD, Ali S, Pickles L, et al. Analysis of estrogen-responsive finger protein expression in benign and malignant human breast. Int J Cancer 2001;91:152–8.
10. Suzuki T, Nakata T, Miki Y, et al. Estrogen sulfotransferase and steroid sulfatase in human breast carcinoma. Cancer Res 2003;63:2762–70.
11. Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Mod Pathol 1998;11:155–68.
12. Emmert-Buck MR, Bonner RF, Smith PD, et al. Laser capture microdissection. Science 1996;274:998–1001.
13. Niino YS, Irie T, Takaishi M, et al. PKCθ II, a new isoform of protein kinase C specifically expressed in the seminiferous tubules of mouse testis. J Biol Chem 2001;276:36711–7.
14. Ikeda K, Inoue S, Orimo A, et al. Multiple regulatory elements and binding proteins of the 5′-flanking region of the human estrogen-responsive finger protein (efp) gene. Biochem Biophys Res Commun 1997;236:765–71.
15. Dowlatshahi K, Fan M, Snider HC, Habib FA. Lymph node micrometastases from breast carcinoma: reviewing the dilemma. Cancer 1997;80:1188–97.
16. Suzuki T, Moriya T, Ishida T, Ohuchi N, Sasano H. Intracrine mechanism of estrogen synthesis in breast cancer. Biomed Pharmacother 2003;57:460–2.
17. Hermeking H, Lengauer C, Polyak K, et al. 14-3-3σ is a p53-regulated inhibitor of G_2/M progression. Mol Cell 1997;1:3–11.
18. Chan TA, Hermeking H, Lengauer C, Kinzler KW, Vogelstein B. 14-3-3σ is required to prevent mitotic catastrophe after DNA damage. Nature 1999;401:616–20.
19. Ferguson AT, Evron E, Umbricht CB, et al. High frequency of hypermethylation at the 14-3-3σ locus leads to gene silencing in breast cancer. Proc Natl Acad Sci U S A 2000;97:6049–54.
20. Umbricht CB, Evron E, Gabrielson E, Ferguson A, Marks J, Sukumar S. Hypermethylation of 14-3-3σ (stratifin) is an early event in breast cancer. Oncogene 2001;20:3348–53.
21. Simooka H, Oyama T, Sano T, Horiguchi J, Nakajima T. Immunohistochemical analysis of 14-3-3σ and related proteins in hyperplastic and neoplastic breast lesions, with particular reference to early carcinogenesis. Pathol Int 2004;54:595–602.
22. Moreira JM, Ohlsson G, Rank FE, Celis JE. Down-regulation of the tumor suppressor protein 14-3-3σ is a sporadic event in cancer of the breast. Mol Cell Proteomics 2005;4:555–69.

Estrogen Inhibits Cell Proliferation through *In situ* Production in Human Thymoma

Hironori Ishibashi,[1,2,4] Takashi Suzuki,[1] Satoshi Suzuki,[2] Takuya Moriya,[1] Chika Kaneko,[1] Taisuke Nakata,[5] Makoto Sunamori,[4] Masashi Handa,[3] Takashi Kondo,[2] and Hironobu Sasano[1]

Abstract

Purpose: We showed previously estrogen receptor (ER) α as an independent prognostic marker in human thymoma. Estrogen sulfotransferase (EST), steroid sulfatase (STS), 17β-hydroxysteroid dehydrogenase (17β-HSD), and aromatase are considered to play important roles in hormone metabolism of estrogen-dependent tumors.

Experimental Design: We examined estrogen production using primary cultures of human thymoma epithelial cells (TEC), intratumoral estradiol (E_2) concentrations, and status of these enzymes above using immunohistochemistry or semiquantitative reverse transcription-PCR. We then correlated these findings with clinicopathologic variables and/or clinical outcome in 132 patients.

Results: E_2 inhibited cell proliferation via ERα in TEC, which synthesized estrone and E_2. Intratumoral E_2 concentrations were inversely correlated with EST, positively correlated with STS or 17β-HSD type 1, and significantly higher in lower-grade or early-stage thymoma. EST status was positively correlated with tumor size, clinical stage, histologic differentiation, and Ki-67 labeling index and significantly associated with adverse clinical outcome and turned out to be a potent independent prognostic factor. STS and/or 17β-HSD type 1 status was inversely correlated with Ki-67 labeling index and associated with lower histologic grade or early clinical stages.

Conclusions: E_2 inhibits proliferation of TEC through ERα, which suggests that E_2 may be effective in treatment of thymoma, especially inoperable tumor, possibly through suppressing its cell proliferation activity. EST status is a potent prognostic factor in thymoma through inactivating estrogens. *In situ* estrogen synthesis through intracrine mechanism therefore may play important roles in tumorigenesis and/or development of thymoma through regulation of cell proliferation in an intracrine manner.

Estrogen regulates cell proliferation and/or other biological functions in various neoplasms derived from hormone-dependent tissues, such as breast and endometrial cancer (1). *In situ* estrogen levels are also considered to play very important roles in the pathogenesis of these neoplasms (2). We reported previously that estrogens play an important role via binding to estrogen receptor (ER) α in the development of human thymomas (3).

A major circulating form of plasma estrogen is estrone sulfate (E_1-S), a biologically inactive form of estrogen. E_1-S has a relatively long half-life in the peripheral blood (4), in which serum levels of E_1-S are 10-fold higher than those of unconjugated E_1 or estradiol (E_2; ref. 5). E_1-S is transformed into a biologically active form, E_1, by steroid sulfatase (STS; refs. 6, 7), whereas aromatase catalyzes circulating androgen androstenedione into E_1 (8). E_1 is subsequently converted to E_2 by 17β-hydroxysteroid dehydrogenase (17β-HSD) type 1 in several estrogen target tissues, including breast cancer cells (9). E_2 acts on breast cancer cells primarily via its binding to ERα and/or ERβ. STS, aromatase, and 17β-HSD type 1 have been all considered to be involved in *in situ* production of estrogen in neoplastic tissues, such as breast cancer. Estrogen sulfotransferase (EST), *SULT1E1* or *STE* gene, is a member of the superfamily of cytosolic steroid sulfotransferases (10) and catalyzes E_1 into biologically inactive E_1-S.

In this study, we examined the expression and biological significance of EST, STS, 17β-HSD type 1, and aromatase in human thymoma to further elucidate the status of *in situ* estrogen production in thymoma. We first studied effects of estrogens on cell proliferation and *in situ* E_1 or E_2 production from E_1-S using primary cultures of thymoma epithelial cells (TEC). We then determined E_2 concentrations and immunolocalized EST, STS, and 17β-HSD type 1 in human thymoma. In addition, mRNA levels of EST, STS, 17β-HSD type 1, and aromatase were examined using semiquantitative reverse

Authors' Affiliations: [1]Department of Pathology, Tohoku University School of Medicine; [2]Department of Thoracic Surgery, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan; [3]Department of Thoracic Surgery, Iwate General Hospital, Morioka, Japan; [4]Thoracic Cardiovascular Surgery, Graduate School, Tokyo Medical and Dental University; and [5]Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan

Received 12/6/04; revised 4/26/05; accepted 5/31/05.

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.

Requests for reprints: Hironori Ishibashi, Department of Thoracic Surgery, Institute of Development, Aging and Cancer, Tohoku University, 4-1 Seiryo-machi Aoba-ku, Sendai, Miyagi Prefecture 980-0875, Japan. Phone: 81-22-717-8521; Fax: 81-22-717-8526; E-mail: hishiba@kf6.so-net.ne.jp.

doi:10.1158/1078-0432.CCR-04-2495

transcription-PCR in human thymoma. We then correlated EST, STS, or 17β-HSD type 1 immunoreactivity with clinicopathologic factors and clinical outcome in 132 patients with thymomas to establish clinical significance of these findings.

Materials and Methods

Cell culture analysis

Establishment of thymoma-derived neoplastic epithelial cellsin primary culture. Fresh thymoma tissues (five thymoma cases: two cases for type A, two cases for type B2, and one case for type B3), which expressed ERα, STS, or 17β-HSD type 1 but not ERβ were repeatedly washed in PBS and carefully minced and digested in a collagenase-containing mixture composed of collagenase type I (1 mg/mL; Wako, Osaka, Japan), DMEM, 10% fetal bovine serum (FBS), and antibiotics (penicillin and streptomycin) for 8 hours at 37°C (11). These minced and digested thymoma tissues were subsequently filtrated through three layers of sterile gauze and cultured by a 10-day culture in DMEM supplemented with 10% FBS and antibiotics but without any growth factors. On confluence, cells were mechanically dislodged by 0.25% trypsin and 0.02% EDTA and subjected to secondary cultures. Cells were subsequently characterized by their morphology and immunoreactivity of cytokeratin using immunocytostaining. Coexisting fibroblasts were eliminated by anti-fibroblast microbeads (MACS, Miltenyi Biotec, Gladbach, Germany) according to the manufacturer's instructions. The purity of the TEC, which expressed ERα, STS, or 17β-HSD type 1 but not ERβ, was examined by flow cytometry and the proportion of cytokeratin-positive cells was nearly always >97% (data not shown). TEC was grown to confluence in DMEM in the presence of 10% FCS and antibiotics under standard concentrations.

Cell proliferation assay. TEC was seeded at a density of 5×10^3 per mL into 24-well plates (Falcon; Becton Dickinson Labware, Lincoln Park, NJ) in a final volume of 1 mL phenol red-free DMEM with 5% charcoal-stripped FBS. Medium was then replaced with phenol red-free DMEM without FBS to arrest the growth in 24 hours after seeding. The medium was replaced again with phenol red-free DMEM with 5% charcoal-stripped FBS together with either a vehicle (0.1%), increasing concentration of E_2 (0.1, 1, 10, 100, and 1,000 nmol/L), and/or 100 nmol/L ICI 182,780 in 24 hours later. Following 72-hour incubation with these agents, the cells were trypsinized and resuspended. E_2 was purchased from Sigma (St. Louis, MO) and ICI 182,780 from Tocris Cookson Ltd. (Ellisville, MO). Estrogens and anti-estrogens were dissolved in absolute ethanol (Sigma) and added to the medium daily. Cell cultures that were not treated with estrogenic compounds received absolute ethanol as a vehicle control. Total additive ethanol concentrations never exceeded 0.2% throughout the culture period. The cells were refed with freshly prepared medium every other day. We then used a Cell Counting Kit-8 system (Dojindo Technologies, Kumamoto, Japan) to determine the cell number. Then, 100 μL from each sample was aliquoted into a 96-well microtiter plate with 10 μL of the working solution containing WST-8 and then incubated for an additional 2 hours in a CO_2 incubator at 37°C. The absorbance of each well was measured at 450 nm with a reference wavelength at 650 nm with a M-UVmax microscope reader (Molecular Devices Corp., Menlo Park, CA). An aliquot was taken from the medium to count the number of cells with a Burker-Turk counter (Nitirin, Tokyo, Japan).

Table 1. Summary of clinical data in 132 patients and in 20 patients with thymoma examined in this study

	n = 132	*n* = 20
Age (y)*	54.0 ± 15.1	54.4 ± 13.2
Sex		
Male	58 (43)	10 (50)
Female	74 (57)	10 (50)
Premenopausal	38 (29)	6 (30)
Postmenopausal	36 (28)	4 (20)
Myasthenia gravis		
+	24 (18)	3 (15)
−	108 (82)	17 (85)
Tumor size (mm)*	60.0 ± 25.5	61.3 ± 17.0
Clinical stage		
I	67 (51)	10 (50)
II	28 (21)	4 (20)
III	21 (16)	3 (15)
IV	16 (12)	3 (15)
WHO classification		
A	28 (21)	4 (20)
AB	23 (17)	4 (20)
B1	29 (22)	4 (20)
B2	39 (30)	5 (25)
B3	13 (10)	3 (15)

*Mean ± SD. All other values represent *n* (%).

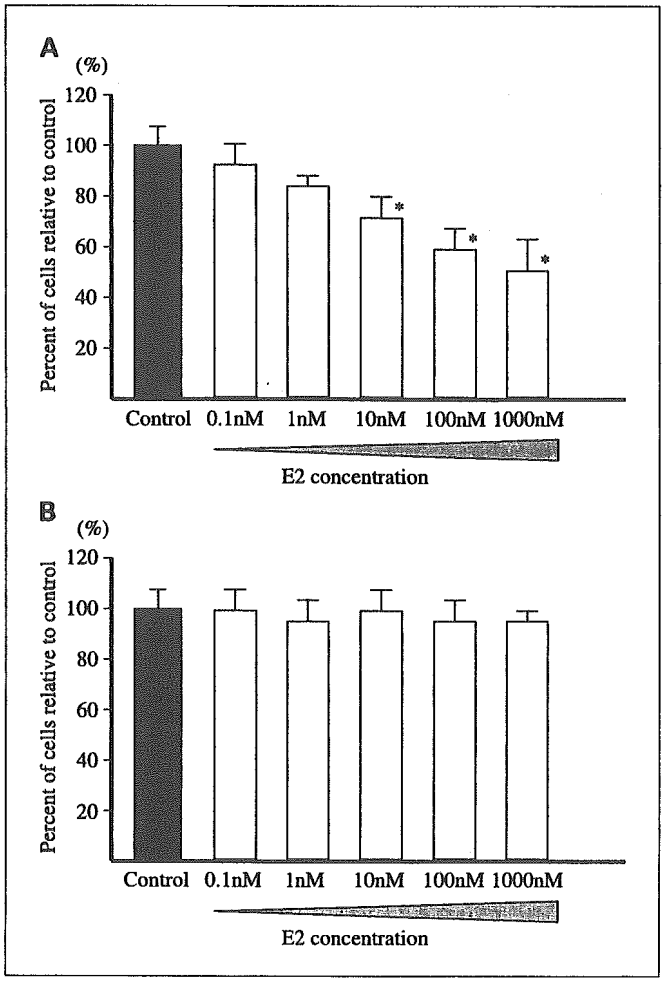

Fig. 1. Effects of E_2 and anti-estrogen on cell growth of TEC. Cells (5×10^3/mL) were plated in triplicate well onto a 24-well plates. After 24 hours for cell attachment, cells were treated for 73 hours with E_2 at various concentrations with or without anti-estrogen, ICI 182,780. Cells treated with vehicle (absolute ethanol) were used as control. Columns, mean (n = 5); bars, SD. *, $P < 0.001$, compared with control.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling analysis. Visualization of apoptotic cells in chamber slides was done using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling method (Apoptosis *In situ* Detection kit, Wako; ref. 12). As a negative control, fixed and permeabilized cells incubated without terminal deoxynucleotidyl transferase, but with secondary antibodies and 3,3'-diaminobenzidine, were used. As a positive control, permeabilized cells were incubated with bovine pancreatic DNase I before terminal deoxynucleotidyl transferase treatment to induce DNA fragmentation. The presence of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling positivity was determined by visual inspection of cultures using light microscopy.

Estrone and estradiol production from thymoma epithelial cells. E_1-S was added after a 48-hour culture in phenol red and FCS-free DMEM (final concentration, 0.5, 2.5, and 12.5 nmol/L). Medium (2 mL) was extracted twice with diethyl ether. Extracts were evaporated to dryness under nitrogen and reconstituted in 100 μL dextran-coated, charcoal-treated FBS. E_1 and E_2 levels of the extracts were then determined by RIA using an assay kit (Diagnostic Products, Tokyo, Japan). The sensitivity of the assay was 3 pmol/L.

Estradiol concentrations in human thymoma and thymus. E_2 concentrations were examined in 20 cases of fresh frozen thymoma. These specimens were carefully dissected to eliminate all adjacent adipose and connective tissues. Cytosol and nuclear fractions were prepared by centrifugation of the homogenates (1.0 g specimens in 10 mL cold physiologic saline) at 4°C for 60 minutes at 15,000 × *g* in an ultracentrifuge. Tissue E_2 concentrations were determined according to the methods described above.

Patients and tissue specimens. Thymoma cases (n = 132) were retrieved from surgical pathology files at Sendai Kousei Hospital (Sendai, Japan) and Tokyo Medical and Dental University (Tokyo, Japan). All specimens were fixed for 24 hours in 10% formalin at room temperature and embedded in paraffin wax. Relevant clinical findings, including patient age, sex, menopausal status, presence or absence of myasthenia gravis, tumor size, clinical stage (13), and WHO histologic classification (14) are summarized in Table 1.

Freshly frozen specimens were also available for reverse transcription-PCR analysis, real-time PCR, and E_2 concentration analysis in 20 cases (Table 1) among these 132 cases of thymoma examined for immunohistochemistry. Specimens for RNA isolation were immediately frozen in liquid nitrogen and stored at −80°C until used for RNA isolation. RNA was extracted within 2 weeks following surgery. Informed consent was obtained from all the patients. The research protocols used in this study were approved by the Ethics Committee at Tohoku University School of Medicine, Sendai Kousei Hospital and Tokyo Medical and Dental University School of Medicine.

Antibodies. Rabbit polyclonal antibody for EST was raised against the synthetic NH_2-terminal peptide of human EST corresponding to amino acids 1 to 13 (PV-P2237, Medical Biological Laboratory, Nagoya, Japan; 1:750 dilution). STS antibody was raised against the enzyme purified from human placenta, which recognizes the STS peptide corresponding to amino acids 420 to 428 (KM1049, kindly provided by Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan; 1:3,000 dilution) and has been reported previously in the evaluation of human breast cancer (15). 17β-HSD type 1 antibody was a rabbit polyclonal antibody against the enzyme purified from human placenta (kindly provided by Dr. Van Luu-The, Laboratory of Molecular Endocrinology, CHUL Research Center, Quebec, Quebec, Canada; ref. 16).

The antibodies used were mouse monoclonal ERα (NCLER-6F11, Novocastra Lab, Newcastle, United Kingdom; 1:50 dilution), progesterone receptor-B (PR-B; hPRa2, NeoMarkers Co. Ltd., Fremont, CA; 1:200 dilution), and Ki-67 (MIB-1, Immunotech, Marseilles, France; 1:50 dilution) described in our previous study (3).

Immunohistochemistry. Immunohistochemical procedures employed in this study have been described previously in detail (2, 17, 18). Briefly, immunohistochemical staining was done by the streptavidin-biotin method with a Histofine kit (Nichirei Co. Ltd., Tokyo, Japan). The antigen-antibody complex was then visualized with 3,3'-diaminobenzidine solution and counterstained with hematoxylin. Antigen retrieval for ERα, PR-B, and Ki-67 immunostaining was done by heating the slides in an autoclave at 120°C for 5 minutes in citric acid buffer. Similarly, antigen retrieval for EST immunostaining was done by heating the slides in a microwave (500 W) for 15 minutes in citric acid buffer. No antigen retrieval was done for STS and 17β-HSD type 1 immunostaining. As a positive control, normal liver was used for EST (18), normal full-term placenta for STS (18) and 17β-HSD type 1 (19), breast cancer for ERα, and endometrium for PR-B (3). Normal rabbit and mouse IgG was used instead of the primary antibody as a negative control. No specific immunoreactivity was detected in these tissue sections.

Scoring of immunoreactivity. Immunoreactivity of EST, STS, and 17β-HSD type 1 was analyzed according to a previously described method (20). After completely reviewing all the slides of immunostained sections for each thymoma, two of the authors (H.I. and T.S.) independently and blindly divided the thymomas into the following three groups: ++, >50% immunopositive cells; +, 1% to 50% immunopositive cells; and −, no immunoreactivity. Cases with discordant results among the observers were simultaneously reevaluated using a multiheaded microscope. Semiquantitative analysis of

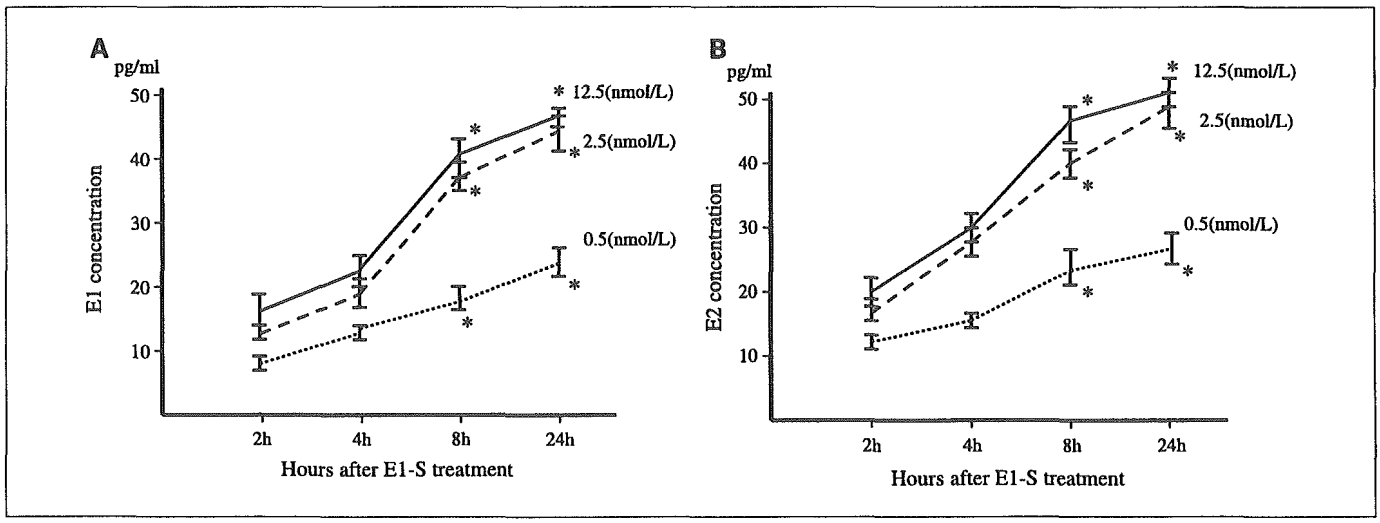

Fig. 2. Time course of E_1 (*A*) and E_2 (*B*) concentration produced from E_1-S in primary culture of TEC. E_1 and E_2 concentrations were significantly increased in proportion to the duration and concentration of E_1-S. Points, mean of three determinations; bars, SE. *, $P < 0.05$.