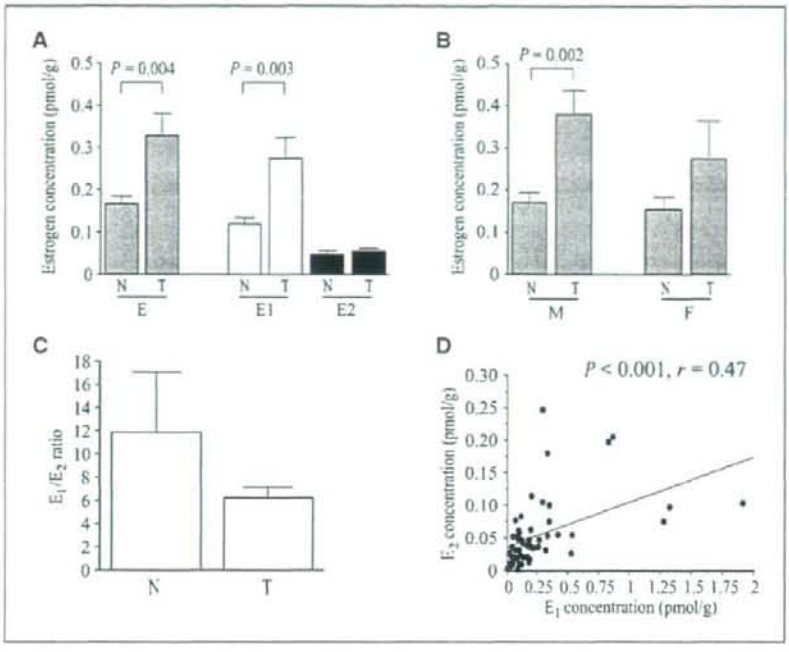

Figure 1. Intratumoral concentrations of estrogens in 53 colon carcinomas as measured by LC-MSI-MS. *A*, tissue concentrations of total estrogen (*E*; $E_1 + E_2$), E_1, and E_2 in colon carcinoma (*T*; $n = 53$) and nonneoplastic colonic mucosa (*N*; $n = 31$). *B*, tissue concentration of total estrogen in males (*M*; $n = 28$) and females (*F*; $n = 25$). *C*, E_1/E_2 ratio in colon carcinoma and nonneoplastic colonic mucosa. *Columns*, mean; *bars*, SE. Statistical analyses were done by Welch's *t* test. *D*, correlation of intratumoral E_1 and E_2 concentrations in the 53 colon carcinomas. Statistical analysis was done by a correlation coefficient (*r*) and regression equation.

Among these 53 cases, specimens of corresponding nonneoplastic colonic mucosa were also available in 31 cases (16 men and 15 women). The mean follow-up time was 22 mo (range, 0–81 mo) and clinical outcome of these patients was available. Specimens for measurement of estrogen concentrations were snap-frozen and stored at −80°C. Tissue concentration of dehydroepiandrosterone-sulfate (DHEA-S) was also measured in both colon carcinoma and corresponding nonneoplastic mucosa in 22 cases (12 men and 10 postmenopausal women). For immunohistochemistry, 10% formalin-fixed and paraffin-embedded tissues were used. Serum samples of the patients were not available in this study.

Another set of colon carcinoma specimens was obtained from 328 consecutive patients (184 men and 144 women including 138 postmenopausal women) from 1994 to 2000. Two hundred six patients were operated on at Miyagi Cancer Center (Natori, Japan) and 122 patients at Tohoku University Hospital. The mean age of the patients was 66.0 y (range, 22–91 y). The mean follow-up time was 78.8 mo (range, 0–161 mo), and survival data of all the patients were available. The specimens were fixed in 10% formalin and embedded in paraffin wax. Snap-frozen tissues and serum were not available in these cases.

Patients clinically suspected to have hereditary nonpolyposis colorectal cancer, carcinoma associated with inflammatory bowel disease, or rectal carcinoma were excluded from this study. Review of the patients' charts revealed that no patients used oral contraceptives or received hormone replacement therapy, irradiation, or chemotherapy before the surgery. Informed consent was obtained from all the patients examined in this study, and the research protocol for this study was approved by the Ethics Committees at both Tohoku University School of Medicine and Miyagi Cancer Center.

LC-ESI-MS analysis. Concentrations of E_1, E_2, and DHEA-S were measured using LC-ESI-MS at Teikoku Hormone Medical (Kawasaki, Japan; refs. 22–24).

Briefly, colon carcinoma specimens (~150 mg for each sample) were homogenized in 1 mL of distilled water. For measurement of E_1 and E_2, ^{13}C-E_1 (100 pg) and ^{13}C-E_2 (100 pg) were added to the homogenate as internal standards. The steroid fraction was extracted with diethyl ether, and the separated organic layer was evaporated. The extracts were subsequently derived with picolinoyl anhydride in tetrahydrofuran. After application to a Bond Elut C_{18} column (Varian, Inc.), the steroid derivatives were eluted with 80% acetonitrile solution.

An API-5000 triple stage quadrupole mass spectrometer equipped with an ESI ion source (Applied Biosystems) and a Shimadzu high-performance liquid chromatography system (Shimadzu Co. Ltd.) were used in our study. The chromatographic separation was done on a Cadenza CD-C_{18} column (150 mm × 3 mm i.d., 3 μm; Imtakt) at 40°C. The mobile phase consisting of CH_3CN-CH_3OH (50:50, v/v; solvent A) and 0.1% HCOOH (solvent B) was used with a gradient elution of A/B = 60:40 to 90:10 (0–5.5 min), 90:10 to 100:0 (5.5–7.5 min), 100:0 (7.5–8.5 min), and 40:60 (8.5–10 min) at a flow rate of 0.4 mL/min. The ESI/MS conditions were as follows: spray voltage, 3,300 V; collision gas, nitrogen, 1.5 psi; curtain gas, nitrogen, 11 psi; ion source temperature, 600°C; and positive ion polarity. The derived E_1 and E_2 were determined using product ions (*m/z* 157 and 264, respectively) produced from their individual protonated molecular ions. The limit of quantification was 4 fmol/g for E_1 and 2 fmol/g for E_2. Absolute recovery was high (97.5–103.1%), and intra-day accuracy and precision were 96% and 9.9% for E_1 and 84.4% and 12.8% for E_2, respectively, in our present study.

For measurement of DHEA-S, the homogenate was treated with acetonitrile for deproteinization, and $^2H_4$-DHEA-S was added as an internal standard. The steroid fraction was extracted using an Oasis HLB cartridge (Waters) and washed with hexane. After the solvent was evaporated under nitrogen gas stream, the residue was dissolved in the LC mobile phase, methanol/5 mmol/L $AcONH_4$ (1:1, v/v; 50 μL). A LC-10AT chromatograph (Shimadzu) coupled with an API 2000 triple-stage quadrupole mass spectrometer (Applied Biosystems) was operated with ESI in negative ion mode. A semi-micro column, Develosil ODS-HG-5 (5 μm, 150 × 2.0 mm i.d.; Nomura Chemical), was used at a flow rate of 0.2 mL/min at 40°C. The ionization conditions were as follows: ion spray voltage, −4 kV; turbo gas temperature, 500°C; ion source gas 1 (nebulizer gas), 40 psi; ion source gas 2 (turbo gas), 80 psi; declustering potential, −71 V; focusing potential, −310 V; entrance potential, −10 V; curtain gas, 55 psi; detection, selected

ion monitoring mode, m/z 367. The analytic recovery was satisfactory (89.4–109.2%) and the limit of quantification was 50 pmol/g.

Immunohistochemistry. The characteristics of STS, EST, and aromatase antibodies used for immunohistochemistry were described previously (16, 25). Briefly, the affinity-purified monoclonal STS (KM1049) antibody was raised against the STS enzyme purified from human placenta and recognizes the peptides corresponding to amino acids 414 to 434. Rabbit polyclonal antibody for EST (PV-P2237) was purchased from the Medical Biological Laboratory. This antibody was raised against the synthetic NH_2-terminal peptides of human EST, corresponding to amino acids 1 to 13. Aromatase monoclonal antibody (#677) was raised against recombinant baculovirus-expressed human aromatase protein. Mouse monoclonal antibodies for ERβ (MS-ERB13-PX1) and Ki-67 (MIB1) were purchased from GeneTex and DAKO, respectively.

A Histofine Kit (Nichirei), which uses the streptavidin-biotin amplification method, was used for immunohistochemistry in this study. Antigen retrieval for ERβ and Ki-67 immunostaining was done by heating the slides in an autoclave at 120°C for 5 min in citric acid buffer (2 mmol/L citric acid and 9 mmol/L trisodium citrate dehydrate, pH 6.0), and antigen retrieval for EST was done by heating the slides in a microwave oven for 15 min in citric acid buffer. The dilutions of the primary antibodies used in this study were as follows: STS, 1:9,000; EST, 1:1,500; aromatase, 1:6,000; ERβ, 1:1,000; and Ki-67, 1:50. 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] and counterstained with hematoxylin. As a negative control, normal rabbit or mouse IgG was used instead of the primary antibodies.

Immunoreactivities for STS, EST, and aromatase were detected in the cytoplasm, and the cases that had >10% of positive carcinoma cells were considered positive (23). Immunoreactivities for ERβ and Ki-67 were detected in the nucleus. These immunoreactivities were evaluated in more than 1,000 carcinoma cells for each case, and subsequently the percentage of positive cells [i.e., labeling index (LI)] was determined (23). Cases with ERβ LI of >10% were considered ERβ-positive colon carcinoma in this study (9).

Statistical analysis. Values for patients' age, estrogen and DHEA-S concentrations, and Ki-67 LI were presented as mean ± SE. Associations between these parameters and STS, EST, and aromatase immunoreactivities were evaluated using Welch's t test. Paired t test was used for analyses of the paired samples. Statistical analysis between E_1 and E_2 concentrations was done using a correlation coefficient (r) and regression equation. Associations between STS and EST immunoreactivities and other clinicopathologic parameters were evaluated in a cross-table using the χ^2 test. Overall survival curves were generated according to the Kaplan-Meier method, and Cox's proportional hazards model was used for univariate and multivariate analyses. StatView 5.0 software (SAS Institute, Inc.) was used for statistical analyses, and differences with $P < 0.05$ were considered significant.

Results

Intratumoral concentrations of estrogens in 53 colon carcinomas. We first examined tissue concentrations of estrogens in 53 colon carcinomas and 31 nonneoplastic colonic mucosal tissues using LC-ESI-MS. As shown in Fig. 1*A*, mean values ± SE of tissue concentrations of total estrogen ($E_1 + E_2$), E_1, and E_2 were 0.33 ± 0.05, 0.27 ± 0.05, and 0.06 ± 0.01 pmol/g in colon carcinoma and 0.16 ± 0.02, 0.12 ± 0.01, and 0.05 ± 0.01 pmol/g in nonneoplastic colonic mucosa, respectively. Intratumoral concentrations of total estrogen and E_1 were significantly higher ($P = 0.004$ and 2.0-fold, and $P = 0.003$ and 2.4-fold, respectively) than those in nonneoplastic colonic mucosa, whereas E_2 concentration was not significantly different between these two tissues ($P = 0.53$ and 1.2-fold). Similar tendencies were detected regardless of the gender of the patients (Fig. 1*B*), although the P values did not reach statistical significance in women [men: $P = 0.002$ (total estrogen), $P = 0.004$ (E_1), and $P = 0.005$ (E_2), and women: $P = 0.23$ (total estrogen), $P = 0.12$ (E_1), and $P = 0.24$ (E_2); Supplementary Table S1]. An analysis of the 31 paired samples showed that tissue concentrations of total estrogen, E_1, and E_2 were higher in carcinoma tissue than in the corresponding nonneoplastic colonic mucosa in 19 (61%), 19 (61%), and 18 (58%) cases, respectively, and the intratumoral concentrations of total estrogen and E_1 were significantly higher ($P = 0.048$ and 1.3-fold, and $P = 0.006$ and 1.5-fold, respectively) than those in their corresponding nonneoplastic colonic mucosa. On the other hand, DHEA-S concentration in tumor tissue was not significantly different from that in corresponding nonneoplastic colonic mucosa in 22 cases examined ($P = 0.22$). The E_1/E_2 ratio was comparable ($P = 0.30$) between colon carcinoma and nonneoplastic mucosa (6.2 ± 0.9 and 11.8 ± 5.3,

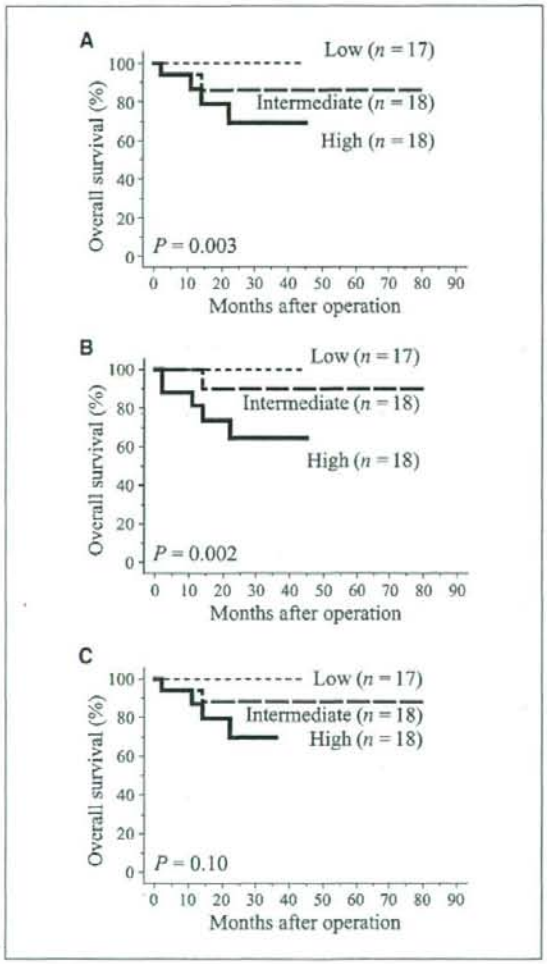

Figure 2. Overall survival curves of the 53 colon carcinoma cases. The cases were divided into three groups of equal size according to the rankings of the intratumoral concentration of total estrogen (*A*), E_1 (*B*), and E_2 (*C*). Cox's proportional hazards model was used for statistical analyses and the data were evaluated as continuous values.

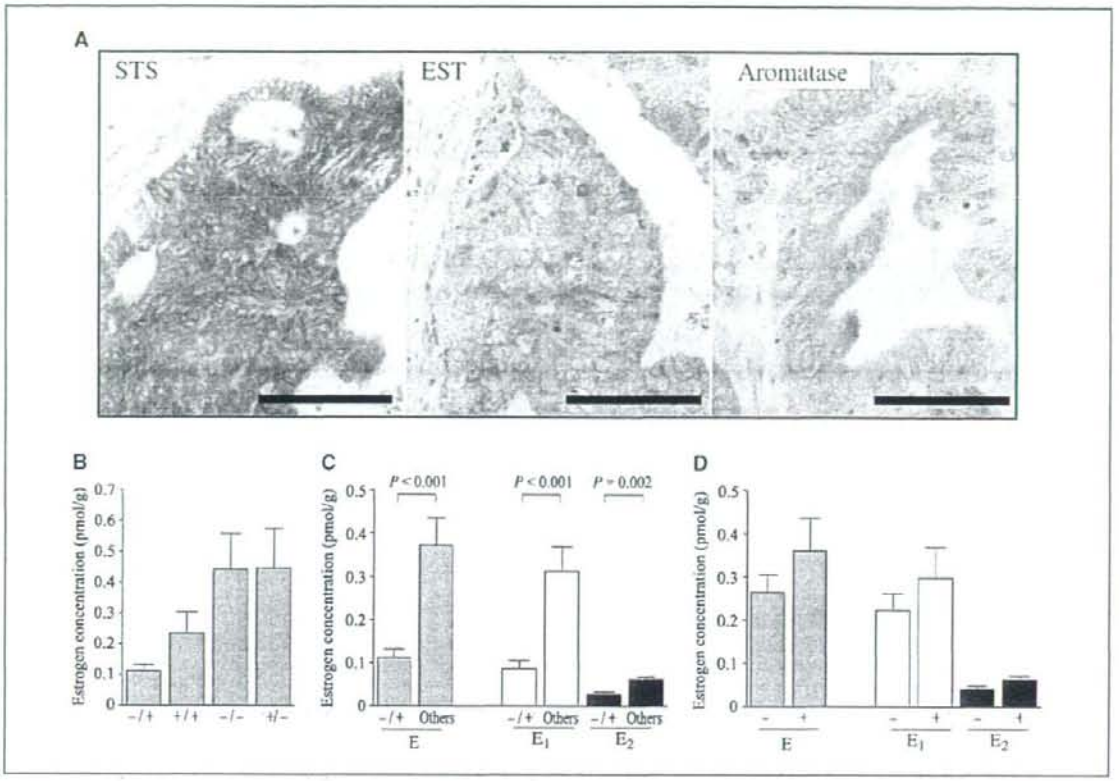

Figure 3. Immunoreactivities for STS, EST, and aromatase in colon carcinoma and their associations with intratumoral estrogen concentrations. *A*, STS, EST, or aromatase immunoreactivity was detected in the cytoplasm of carcinoma cells. *Bar*, 100 mm. *B*, intratumoral concentration of total estrogen in 53 colon carcinomas according to the STS/EST status evaluated by immunohistochemistry. −/+, cases negative for STS and positive for EST (n = 9); +/+, cases positive for both STS and EST (n = 15); −/−, cases negative for both STS and EST (n = 12); and +/−, cases positive for STS but negative for EST (n = 17). *C*, association between intratumoral concentrations of total estrogen (E; $E_1 + E_2$), E_1, and E_2 and the STS/EST status in 53 colon carcinomas. The cases were divided into −/+ (n = 9) and others (n = 44). *D*, association between intratumoral concentrations of estrogens and aromatase immunostaining. *Columns*, mean; *bars*, SE. Statistical analyses were done by Welch's t test.

respectively; Fig. 1*C*). The intratumoral concentrations of E_1 and E_2 were significantly correlated ($P < 0.001$ and $r = 0.47$) in the 53 colon carcinomas (Fig. 1*D*).

Correlations between the intratumoral estrogen levels and clinical outcome of the 53 colon carcinoma patients were shown in Fig. 2. Higher intratumoral concentrations of total estrogen (Fig. 2*A*) and E_1 (Fig. 2*B*) were significantly (P = 0.003 and P = 0.002, respectively) associated with adverse clinical outcome of the patients. A similar tendency was also detected in intratumoral concentration of E_2, but the association did not reach statistical significance (P = 0.10; Fig. 2*C*). This trend was also observed regardless of the gender of the patients [men: P = 0.75 (E), P = 0.82 (E_1), and P = 0.56 (E_2); women: the P values could not be calculated; Supplementary Fig. S2]. Intratumoral concentration of DHEA-S was not associated with clinical outcome of the colon carcinoma patients examined (P = 0.61). No significant correlations were observed between the intratumoral concentrations of estrogens and other clinicopathologic factors examined in this study, such as patients' age, gender, tumor site, and Dukes' stage (data not shown).

Immunohistochemistry for STS, EST, and aromatase in the 53 colon carcinomas and their association with intratumoral estrogen concentrations. We next evaluated associations between the intratumoral estrogen levels and enzymes related to the local estrogen production pathways in the 53 colon carcinomas. Immunoreactivities for STS, EST, and aromatase were detected in the cytoplasm of carcinoma cells in 32 (60%), 24 (45%), and 36 (68%) of the 53 colon carcinomas, respectively (Fig. 3*A*). Nonneoplastic colonic epithelium was positive for EST but negative for STS, as reported previously (26). Aromatase immunoreactivity was weakly detected in nonneoplastic colonic epithelium in approximately one third of the cases examined.

The sulfatase pathway is mediated by both STS and EST, so we subsequently classified the carcinomas into the following four groups according to the STS and EST status of carcinoma tissues: −/+, cases negative for STS and positive for EST (n = 9); +/+, cases positive for both STS and EST (n = 15); −/−, cases negative for both STS and EST (n = 12); and +/−, cases positive for STS but negative for EST (n = 17). As shown in Fig. 3*B*, intratumoral concentration of total estrogen was lowest in the −/+ group

Table 1. Association between STS/EST status and clinicopathologic parameters in 328 colon carcinomas

Value	STS/EST status		P
	−/+ (n = 49)	Others (n = 279)	
Age (y)*	66.4 ± 1.6	65.9 ± 0.7	0.79
[min-max]	[35-85]	[22-91]	
Gender			
Men	26	158	0.90
Premenopausal women	1	5	
Postmenopausal women	22	116	
Tumor site†			
Proximal	32	146	0.09
Distal	17	133	
Dukes' stage			
A + B	34	138	**0.01**
C + D	15	141	
Depth of invasion (T stage)			
Submucosa-muscularis propria (T1 + T2)	18	48	**0.002**
Through muscularis propria (T3 + T4)	31	231	
Lymph node metastasis			
−	35	154	**0.03**
+	14	125	
Distant metastasis			
−	47	226	**0.01**
+	2	53	
Histologic type			
Tubular adenocarcinoma	39	262	**<0.001**
Mucinous adenocarcinoma	10	17	
Histologic differentiation‡			
Well	12	80	0.98
Moderate + poor	27	182	
ERβ			
−	13	86	0.55
+	36	193	
Ki67-LI (%)*	59.7 ± 3.6	48.8 ± 1.2	**0.006**
[min-max]	[6.3-96.5]	[2.3-95.3]	

NOTE: The STS/EST status was evaluated by immunohistochemistry, and "−/+" represents colon carcinomas negative for STS and positive for EST.
*Data are presented as mean ± SE and were evaluated by Welch's t test. All other values represent the number of cases and were evaluated using a cross-table using the χ^2 test. $P < 0.05$ was considered significant, and shown in boldface.
†Proximal colon includes ascending and transverse colon.
‡Cases of mucinous adenocarcinoma were excluded.

(0.11 ± 0.02 pmol/g) and highest in the +/− group (0.45 ± 0.12 pmol/g). STS or EST immunoreactivity alone was not associated with the intratumoral concentration of total estrogen when evaluated as continuous values (i.e., percent of positive carcinoma cells; STS: $P = 0.74$, $r = -0.05$, and EST: $P = 0.11$, $r = -0.22$). When the cases were divided into −/+ and others, the STS/EST status was significantly associated with the intratumoral concentrations of total estrogen ($P < 0.001$), E_1 ($P < 0.001$), and E_2 ($P = 0.002$) in the 53 colon carcinoma tissues examined (Fig. 3C). The intratumoral concentrations of estrogens were higher in aromatase-positive cases, but the differences were not statistically significant ($P = 0.07$ in total estrogen, $P = 0.36$ in E_1, and $P = 0.08$ in E_2; Fig. 3D).

Correlation between the STS/EST status and clinicopathologic factors in 328 colon carcinomas. Because the sulfatase pathway represented by the STS/EST status determined the intratumoral estrogen concentrations rather than the aromatase pathway, we then performed immunohistochemistry for STS and EST in 328 colon carcinoma cases to further examine the clinical significance of the STS/EST status in colon carcinoma. STS and EST immunoreactivities were detected in 200 (61%) and 144 (44%) of 328 cases, respectively, and 49 (15%) cases were in the −/+ group.

As shown in Table 1, the −/+ group was inversely associated with Dukes' stage ($P = 0.01$), depth of invasion ($P = 0.002$), lymph node metastasis ($P = 0.03$), and distant metastasis ($P = 0.01$) and positively correlated with Ki-67 LI ($P = 0.006$). The −/+ group was also frequently ($P < 0.001$) detected in mucinous adenocarcinoma. Other clinicopathologic parameters were not associated with the STS/EST status in this group of patients.

Correlation between the STS/EST status and clinical outcome in the 328 patients. The −/+ group of the STS/EST status was significantly ($P = 0.003$) associated with better clinical

outcome of the 328 colon carcinoma patients examined (Fig. 4*A*). Similar tendencies were detected regardless of the Dukes' stage (Fig. 4*B* and *C*), although the *P* value could not be calculated in the group of Dukes' stages A and B because no patients died in the −/+ group (Fig. 4*B*), and the *P* value did not reach a significant level ($P = 0.08$) in the group of Dukes' stages C and D (Fig. 4*C*). The −/+ cases had significantly longer survival in ERβ-positive cases ($P = 0.009$; Fig. 4*D*) but not in the ERβ-negative group ($P = 0.18$).

Using a univariate analysis (Table 2), distant metastasis ($P < 0.0001$), lymph node metastasis ($P < 0.0001$), depth of invasion ($P = 0.001$), and the STS/EST status ($P = 0.004$) were identified as significant prognostic factors for overall survival in these 328 patients. However, STS alone ($P = 0.10$) or EST alone ($P = 0.09$) did not posses statistically significant prognostic value. Multivariate analysis showed that the STS/EST status ($P = 0.03$) was an independent prognostic factor for overall survival, as well as distant metastasis ($P < 0.0001$), lymph node metastasis ($P = 0.02$), and depth of invasion ($P = 0.03$; Table 2).

Discussion

To the best of our knowledge, this is the first reported study to show intratumoral concentrations of estrogens in colon carcinoma. In the present study, tissue concentration of total estrogen was 0.33 ± 0.05 pmol/g in colon carcinoma, and it was significantly and 2.0-fold higher than that in nonneoplastic colonic mucosa. Previous study in breast carcinoma showed that tissue concentration of total estrogen was 1.9-fold higher in breast carcinoma than in corresponding nonneoplastic breast tissue in postmenopausal women (27), whereas the plasma levels of E_1 and E_2 were similar in normal women and breast carcinoma patients both before and after menopause (28). Therefore, estrogens are considered to be locally produced in breast carcinoma tissue from circulating inactive steroids and to act on the breast carcinoma cells without release into the circulation (13). The intratumoral concentration of total estrogen in colon carcinoma in this study was approximately four times lower than that in breast carcinoma reported previously (15, 27), but the relative ratio of total estrogen between carcinoma and nonneoplastic tissue was similar between these two malignancies. Although we could not examine serum concentrations of estrogens in colon carcinoma patients, our results indicated that estrogens are locally synthesized in colon carcinoma as in breast carcinoma.

Results of our present study also showed that the STS/EST status was significantly associated with the intratumoral concentrations of total estrogen and E_1, whereas aromatase was not. Previously, Suzuki and colleagues (16) reported that STS and EST immunoreactivities were significantly correlated with their mRNA expression levels and enzymatic activities in breast carcinomas using the same antibodies used in the current study. Interconversion of E_1-S and E_1 is regulated by STS and EST, and biologically inactive estrogen E_1-S is a major circulating form of plasma estrogen in both men and women (15, 29). Thus, the sulfatase pathway, rather than the aromatase pathway, may be a potent regulative pathway of intratumoral estrogens in colon carcinoma.

In our present study, E_1 was higher than E_2 in tumor tissue whereas E_2 was reported to be higher in breast carcinoma (15, 27). Interconversion of E_1 and E_2 is mediated by 17βHSDs. To date, 14 isozymes of 17βHSD have been identified (14, 30), and reduction (E_1 to E_2) or oxidation (E_2 to E_1) of estrogen is catalyzed by different 17βHSD isozymes (reduction: 17βHSD types 1, 7, and 12, and oxidation: 17βHSD types 2, 4, and 14). Previous studies have shown that reductive 17βHSD pathway is dominant in breast carcinoma (13) and oxidative 17βHSD activity is a preferential direction in colon carcinoma, which is in good agreement with the result of our present study. Our data also revealed that the E_1/E_2

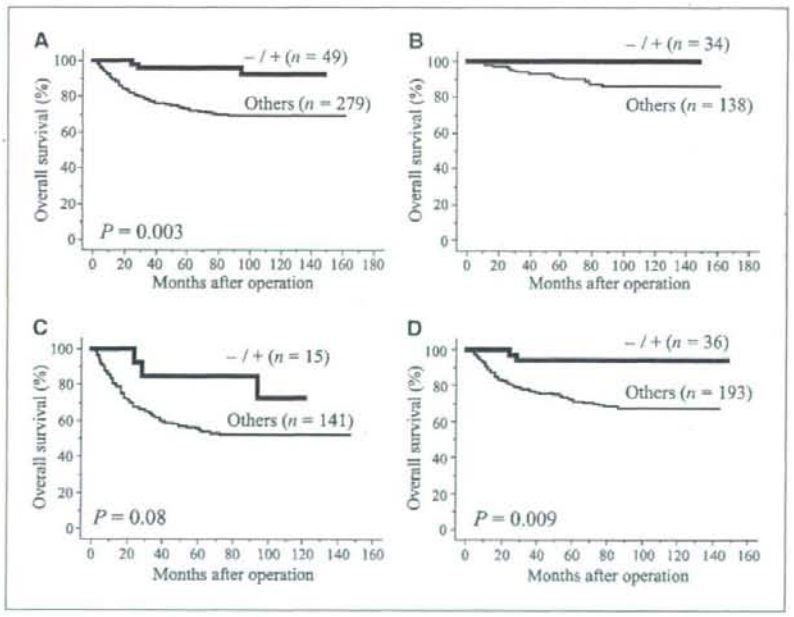

Figure 4. Overall survival curves of 328 colon carcinoma patients according to the STS/EST status. *A*, overall survival curves of all the patients ($n = 328$). *B* and *C*, overall survival curves of patients in the group of Dukes' stages A and B (*B*; $n = 172$) and in the group of Dukes' stages C and D (*C*; $n = 156$). *D*, overall survival curves of ERβ-positive cases ($n = 229$). Cox's proportional hazards model was used for statistical analyses. The *P* value was not available in *B* because no patients died in the −/+ group.

Table 2. Univariate and multivariate analyses of overall survival of 328 colon carcinoma patients

Parameters	Univariate	Multivariate	
	P	*P*	Hazard ratio (95% CI)
Distant metastasis (+ vs −)	**<0.0001***	**<0.0001**	12.3 (7.5–20.2)
Lymph node metastasis (+ vs −)	**<0.0001***	**0.02**	1.1 (1.1–2.7)
Depth of invasion (T3 + T4 vs T1 + T2)	**0.001***	**0.03**	5.0 (1.2–20.8)
STS/EST status (Others vs −/+)	**0.004***	**0.03**	3.5 (1.1–11.2)
Ki67 LI (others vs highest quartile)	0.06		
EST (− vs +)	0.09		
STS (+ vs −)	0.10		
Histologic grade (moderate + poor vs well)†	0.16		
Age‡	0.17		
Ki67 LI‡	0.28		
ERβ (− vs +)	0.59		
Site (distal vs proximal)	0.61		
Gender (women vs men)	0.73		
Histologic type (mucinous vs tubular)	0.98		

NOTE: Statistically significant values ($P < 0.05$) are in boldface.
Abbreviation: 95% CI, 95% confidence interval.
*Data were considered significant in univariate analysis and examined in multivariate analysis.
†Cases of mucinous adenocarcinoma were excluded.
‡Data were evaluated as continuous values.

ratio was similar between colon carcinoma and nonneoplastic colonic mucosa, and the intratumoral concentrations of E_1 and E_2 were closely correlated. Therefore, it is suggested that 17βHSD activity is relatively constant in nonneoplastic and neoplastic colon tissues.

In our study, lower concentrations of intratumoral total estrogen and E_1 and the −/+ group of the STS/EST status were significantly associated with better clinical outcome of the colon carcinoma patients, with the STS/EST status being an independent prognostic factor. Previous reports showed that hormone replacement therapy reduced the risk of colon cancer (4, 5) and improved the survival of the patients with colon cancer (31), which seems to be inconsistent with our present findings. However, it is also true that hormone replacement therapy often contains progestin with estrogens. The randomized controlled trial in the Women's Health Initiative revealed that increasing age was significantly ($P = 0.048$) associated with increasing risk of colon cancer in the postmenopausal women who used conjugated equine estrogen alone (6). Newcomb and colleagues (3) recently showed that the risk of colon cancer was increased in women who previously received estrogen monotherapy (odds ratio, 1.5; 95% confidence interval, 1.0–2.2). Therefore, the results of our present study are not necessarily discrepant with those of previous studies. An alternative interpretation of the results is that higher serum estrogen levels repress STS expression, and the STS^-/EST^+ status is a marker of the patients with higher circulating estrogen levels. Further studies including an analysis of serum concentrations of estrogens are required to clarify the clinical and/or biological significance of the patients with STS^-/EST^+ carcinoma of the colon.

ER consists of ERα and ERβ in humans. ERα is considered to mainly mediate estrogenic actions, and the great majority of breast carcinomas are positive for ERα (32). On the other hand, ERβ is predominantly expressed in some malignancies such as colon (9), lung (33), and prostate (34) carcinomas. Among these, E_2 stimulated the proliferation of ERβ-positive lung carcinoma cells (35), whereas ERβ adopted a regulatory role in estrogen signaling, mediating antiproliferative effects in prostate carcinoma cells (36). Regarding ERβ-mediated estrogenic effects on colon carcinoma cell lines, Fiorelli and colleagues (10) showed that physiologic concentration (1–100 pmol/L) of E_2 stimulated the proliferation of HCT8 cells but inhibited the growth of LoVo cells. On the other hand, Qiu and colleagues (11) reported estrogen-induced apoptosis in COLO205 cells, but Arai and colleagues (12) observed that estrogen had no effects on four colon carcinoma cell lines examined. Results of these previous *in vitro* studies were thus inconsistent, and the significance of estrogens remains largely unclear in colon carcinoma cells. In our present study, the STS/EST status was significantly associated with clinical outcome of ERβ-positive colon carcinoma patients, which implies that estrogens locally produced through the sulfatase pathway contribute to the progression of colon carcinoma mainly through ERβ. Antiestrogen therapies including STS inhibitors, which are currently being developed by several groups (37, 38), may be clinically effective in a selected group of colon carcinoma patients.

The biological activity of E_1 is weaker than E_2, and E_1 is generally considered as a precursor of E_2. However, it was reported that E_1 could also bind to ERs, and its binding affinity relative to E_2 was 60% for ERα and 37% for ERβ (39). In addition, E_1 induced the transcriptional activity of ERs, and its relative potency compared with E_2 was 65% to 110% in ERα and 55% to 70% in ERβ (40). Because the intratumoral concentration of E_1 was ~6-fold higher than that of E_2 in colon carcinoma in this study, E_1 might also act on colon carcinoma cells in addition to serving as a precursor of E_2. Oduwole and colleagues (41) reported that higher mRNA expression of 17βHSD type 2 was significantly associated with shorter survival in female patients with distal colorectal carcinoma,

and suggested a possible protective effect of E_2 against colon carcinoma. However, considering our present results, it is also possible to interpret this finding as evidence that increment of intratumoral E_1 by 17βHSD type 2 is, at least in part, involved in the adverse clinical outcome of colon carcinoma patients. Further examinations are required to clarify the biological significance of E_1 in colon carcinoma.

Results of our present study showed that STS^- and EST^+ colon carcinoma was frequently detected in mucinous adenocarcinoma, which was reported to have worse prognosis (41, 42). However, the American Committee on Cancer Prognostic Factors Consensus Conference concluded that histologic type is of no prognostic significance in colon carcinoma (42), and such an association between histologic type and clinical outcome of the patients was not detected in our present study, as summarized in Table 2.

The −/+ group of the STS/EST status was inversely associated with Dukes' stage, depth of invasion, lymph node metastasis, and distant metastasis and positively correlated with Ki-67 LI. Ki-67 LI has been frequently used as a marker for the proliferative activity in various human malignancies, and higher Ki-67 LI in tumor tissues is associated with shorter survival of the patients with breast and lung carcinomas, astrocytoma, and meningioma (43). However, overexpression of Ki-67 induced growth arrest (44), and Hilska and colleagues (45) suggested that strongly stained tumor cells had a slow cell cycle and a low proliferation rate. Furthermore, several investigators reported that higher Ki-67 LI was associated with longer survival in colon carcinoma patients (45–47). Therefore, taken together with these previous reports and our present results, it is indicated that the decrement of intratumoral total estrogen concentration in the STS^- and EST^+ colon carcinoma may contribute to lower proliferative activity of carcinoma cells, diminished invasive and metastatic potentials, and better clinical outcome in colon carcinoma despite elevated Ki67-LI, but it awaits further investigations for clarification.

In summary, we showed that the intratumoral concentrations of total estrogen and E_1 were significantly higher than those in nonneoplastic colonic mucosa, and higher intratumoral concentrations of total estrogen and E_1 were significantly associated with adverse clinical outcome of the patients. Immunoreactivities for STS, EST, and aromatase were detected in 61%, 44%, and 68% of colon carcinomas, respectively, and the STS/EST status determined the intratumoral concentration of estrogens in colon carcinoma. The −/+ group of the STS/EST status was inversely associated with Dukes' stage, depth of invasion, lymph node metastasis, and distant metastasis. Moreover, this −/+ group was significantly associated with better clinical outcome of the patients, and a multivariate analysis revealed the STS/EST status as an independent prognostic factor. Results from our present study suggest that estrogens are locally produced in colon carcinoma mainly through the sulfatase pathway and play important roles in the progression of the disease.

Disclosure of Potential Conflicts of Interest

D.B. Evans: ownership interest, Novartis Pharma, AG. The other authors disclosed no potential conflicts of interest.

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Estrogen signaling ability in human endometrial cancer through the cancer–stromal interaction

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Abstract

The estrogen pathway plays an important role in the etiology of human endometrial carcinoma (EC). We examined whether estrogen biosynthesis in the tumor microenvironment promotes endometrial cancer. To examine the contribution of stromal cells to estrogen signaling in EC, we used reporter cells stably transfected with the estrogen response element (ERE) fused to the destabilized green fluorescent protein (GFP) gene. In this system, the endometrial cancer stromal cells from several patients activated the ERE of cancer cells to a variable extent. The GFP expression level increased when testosterone, a substrate for aromatase, was added. The effect was variably inhibited by aromatase inhibitors (AIs), although the response to AIs varied among patients. These results suggest that GFP expression is driven by estrogen synthesized by aromatase in the endometrial cancer stromal cells. In a second experiment, we constructed an adenovirus reporter vector containing the same construct as the reporter cells described above, and visualized endogenous ERE activity in primary culture cancer cells from 15 EC specimens. The GFP expression levels varied among the cases, and in most primary tissues, ERE activities were strongly inhibited by a pure anti-estrogen, fulvestrant. Interestingly, a minority of primary tissues in endometrial cancer showed ERE activity independent of the estrogen-ER pathway. These results suggest that AI may have some therapeutic value in EC; however, the hormonal microenvironment must be assessed prior to initiating therapy.

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Introduction

Endometrial carcinoma (EC) is one of the most common gynecological cancers found in women worldwide (Landis *et al.* 1999). Estrogen contributes to endometrial carcinogenesis (Hecht & Mutter 2006, Ito 2007) and malignant transformation. Estrogen influences the activation and/or expression of growth factors such as insulin-like growth factor (IGF-I; Rutanen *et al.* 1993, O'Toole *et al.* 2005) and vascular endothelial growth factor (O'Toole *et al.* 2005). These growth factors play important roles in the development and progression of EC (Mochizuki *et al.* 2006).

Recent studies reveal that estrogen receptors (ERs) are activated not only by estrogen but also by protein phosphorylation by kinases such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt (Campbell *et al.* 2001, Stoica *et al.* 2003). Activated ER contributes to the proliferation, anti-apoptosis, and metastasis of tumor cells. This effect is the result of the induction of its downstream genes whose promoter regions contain the estrogen response element (ERE). The molecular mechanisms of the regulation of transcriptional activity by ER have been well investigated in breast cancer cells. Although both EC and breast cancer are

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considered as estrogen-dependent carcinomas, they differ in their responses to anti-estrogens, particularly tamoxifen (TAM; Ito 2007). Thus, the actions of estrogen in EC might be different from those in breast carcinoma. The molecular mechanisms of the actions of estrogen, the target genes of the ER, and the pathway of estrogen signaling in EC have not been elucidated.

Cancer–stromal interactions play important roles in the genesis and progression of malignancies. We reported previously that stromal cells obtained from individual primary breast cancer patients activate the estrogen signaling pathway in breast cancer cells through tumor–stromal interaction (Yamaguchi *et al.* 2005). ER-activating activity is correlated with menopausal status and histological grade.

The estrogen level in EC tissue is higher than that in the endometrial tissue of healthy women (Naitoh *et al.* 1989, Berstein *et al.* 2003). Furthermore, estrogen-metabolizing enzymes such as aromatase, sulfatase, sulfotransferase, and 17β-hydroxysteroid dehydrogenases (17β-HSDs) exist in stromal cells adjacent to the tumor. These convert androgens and inactive estrogens (estrone (E1), estrone sulfate) into active estrogen (17β-estradiol, E_2; Pasqualini & Chetrite 2006, Takase *et al.* 2006).

Aromatase is a key enzyme that catalyses the conversion of androgens to estrogen (Pasqualini & Chetrite 2006, Takase *et al.* 2006). Aromatase mRNA is expressed in various tissues, such as adipose tissue, bone, brain, skin, and breast cancer (Bulun *et al.* 2005). The promoter driving the expression of aromatase mRNA is specifically activated in tissues where the gene is normally expressed (Bulun *et al.* 2005). The mRNA and protein of aromatase are detectable in EC but are absent in disease-free endometrium (Bulun *et al.* 1994, Watanabe *et al.* 1995). Furthermore, the endometrial cancer patients with aromatase-positive stromal cells have poor survival prognosis compared with patients with aromatase-negative stromal cells (Segawa *et al.* 2005). This evidence also suggests that aromatase is a key enzyme in the etiology of EC; however, the participation of local estrogen in the activation of ER in individual EC tumor cells has not been evaluated. Hormonal therapies such as anti-estrogens and aromatase inhibitors (AIs) are widely used in breast cancer patients, particularly ER-positive cases. However, with the exception of medroxyprogesterone acetate (MPA) in advanced disease, the use of hormonal therapies in endometrial cancer is not prevalent.

In this study, we analyzed tumor–stromal interactions in EC and examined whether estrogen biosynthesis functions importantly in the local environment of tumor tissue. We also succeeded in measuring estrogen-mediated ER activation of primary tumor cells obtained from individual EC patients. Our results identify AIs as another potential hormonal treatment for endometrial cancer. Our method of measuring estrogen activity may be used as a diagnostic tool for identifying estrogen-dependent endometrial cancer. Finally, our results underscore the importance of tailoring therapy to individual patients, and our assay provides a way to accomplish this.

Materials and methods

Cells and cultures

ERE-tk-green fluorescent protein (GFP)-MCF-7 cells (E10 cells) were established from a human breast cancer cell line, MCF-7, by the introduction of a plasmid carrying the ERE fused with the ERE-GFP gene, as described previously (Yamaguchi *et al.* 2005). MCF-7 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The human EC cell lines, Ishikawa, was provided by Dr Masato Nishida, Kasumigaura National Hospital, Japan; Sawano (RCB1152), HHUA (RCB0658), A431 (RCB0202), JHUEM2 (RCB1551), and JHUAS1 (RCB1544) were purchased from RIKEN (Ibraki, Japan); and Hec1A (HTB-112) and RL95-2 (CRL-1671) were purchased from ATCC. MCF-7 cells and the human EC cell lines were cultured in RPMI 1640 (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS; Tissue Culture Biologicals, Turale, CA, USA). For co-culturing with stromal cells or treatment with AIs, the cells were cultured in phenol red-free (PRF) RPMI 1640 supplemented with 10% dextran-coated charcoal-treated FCS (DCC-FCS). The culture media contained 0.1% penicillin/streptomycin (GIBCO BRL). Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. 293A cells, a line of human kidney cells, were purchased from Invitrogen (Carlsbad, CA, USA), cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), and used to propagate the adenovirus.

Drugs

Anastrozole (aromatase inhibitor) and ICI 182 780 (fulvestrant, pure anti-estrogen) were kindly provided by AstraZeneca Pharmaceuticals; letrozole (aromatase inhibitor) was from Novartis Pharmaceuticals (Basel, Switzerland); and exemestane (aromatase inhibitor) from Pharmacia Co. (Bridgewater, NJ, USA; currently Pfizer Inc., New York, NY, USA). Testosterone and E_2 were purchased from Sigma.

Tumor samples

Specimens of human EC tissues were obtained from female patients who underwent hysterectomy from 2004 to 2006 in the Department of Gynecology at Tohoku University Hospital (Miyagi, Japan). Informed consent was obtained from all 18 patients prior to their surgery and examination of the specimens (Table 1). Human breast cancer tissues were obtained from surgical specimens at the Saitama Cancer Center Hospital (Saitama, Japan) after obtaining informed consent from the patients. The Tohoku University Ethics Committee and the Saitama Cancer Center Ethics Committee approved this study.

Isolation of primary stromal cells from cancer tissue

The isolation procedure of stromal cells was as described previously by Ackerman *et al.* (1981) with slight modifications. Briefly, tissue specimens were minced to $\sim 1\ mm^3$ in size after being rinsed with PBS and digested with collagenase solution (2.5 mg/ml collagenase, 40 mg/ml BSA, 2 mg/ml glucose, 1× antibiotic–antimycotic, and 50 μg/ml gentamicin in HBSS) for 20–30 min at 37 °C. The cells, including stromal cells, were washed several times with PBS after centrifugation and cultured at 37 °C in 5% CO_2–95% air following suspension in PRF-RPMI 1640 containing 10% FCS. Outgrowth of cells was observed after 5–10 days, and the medium was renewed twice weekly.

Table 1 Clinicopathologic variables of cancer patients

	Total no.
Age	
<50	6
≥50	12
Menopausal	
Pre	6
Post	10
Unknown	2
Grade	
1	11
2	2
3	4
Unknown	1
Stage	
1	14
2	1
3	3
Metastasis	
Positive	3
Negative	15
Histology	
Endometrioid	15
Serous	2
Carcinosarcoma	1
Muscular invasion	
Negative	8
≤1/2	6
≥1/2	4
Vascular invasion	
Negative	15
Positive	3

Co-culture of MCF-7 cells with primary stromal cells

Co-culturing of E10 cells plus stromal cells was done as described previously (Yamaguchi *et al.* 2005). Briefly, 5×10^4 stromal cells were seeded onto 24-well plates following pre-culturing in PRF-RPMI 1640 containing 10% DCC-FCS for 96 h. After 2 h, 5×10^4 E10 cells were seeded on top of the stromal cells in media containing testosterone at 1×10^{-7} mol/l as a substrate for aromatase. After further culturing for 4 days, the co-cultured cells were collected by centrifugation after 0.05% trypsinization, and the GFP-expressed E10 cells were counted on glass slides using fluorescence microscopy. Regardless of the fluorescence intensity, all GFP-expressed E10 cells were identified as GFP-positive cell to avoid wrong evaluation. E10 and stromal cells were easily discriminated by their morphology. To avoid the effects of aging, stromal cells were used within ten passages.

Quantitative reverse transcription-PCR for aromatase, 17β-HSD-type 2 and RPL13A

Total RNA of stromal cells was prepared using ISOGEN (Nippon Gene Co., LTD, Toyama, Japan) by the method of Chomczynski & Sacchi (1987). Reverse transcription and quantitative PCR were performed using SuperScript III RT (Invitrogen) and LightCycler FastStart DNA Master SYBR Green I with LightCycler DX400 (Roche Diagnostics AG, Rotkreuz, Switzerland) respectively. The oligonucleotides used in quantitative PCR were as follows: 5′-CTT CTG CGT CGT GTC ATG CT-3′ and 5′-GGA GAG CTT GCC ATG CAT CAA-3′ for aromatase; 5′-CAA AGG GAG GCT GGT GAA T-3′ and 5′-TCA CTG GTG CCT GCG ATA-3′ for 17β-HSD type 2; and 5′-CCT GGA GGA GAA GAG GAA AGA GA-3′ and 5′-TTG AGG ACC TCT GTG TAT TTG TCA A-3′ for ribosomal protein L13a (RPL13A), internal control (Vandesompele *et al.* 2002).

Luciferase assays

ERE activity in tumor cell lines was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The estrogen reporter plasmid ERE-tk-Luci was described previously (Omoto *et al.* 2001). The control vector pRL-TK (Promega) was used as an internal control of transfection efficiency in reporter assays. Transient transfection was performed using the method described previously by Omoto *et al.* Briefly, after 4 days of culture in PRF-RPMI 1640 with 10% DCC-FCS, 5×10^5 cells were seeded per well onto a 6 well plate in the same medium and incubated for 24 h. One microgram of pERE-tk-Luci plasmid and 0.1 μg pRL-TK were mixed with 5 μl TransIT LT-1 reagent (Mirus Co., Madison, WI, USA) in 300 μl serum-free medium and subjected to transfection according to the manufacturer's instructions. Plasmid-transfected cells were cultured with/without 1×10^{-8} mol/l E_2 for 24 h, and luciferase activity (i.e., ERE activity) was measured according to the manufacturer's instructions using the Dual-Luciferase Reporter Assay System.

Reverse transcription-PCR for ERα and β-actin

Total RNA of the endometrial cancer cell lines, Ishikawa, Sawano, HHUA, A431, Hec1A, RL95-2, JHUEM2, JHUAS1, and a breast cancer cell line, MCF-7, was prepared using ISOGEN. Reverse transcription and PCR were performed using SuperScript III RT and ExTaq (Takara, Tokyo, Japan) respectively. Oligonucleotides used for PCR were as follows: 5′-CAT GAT CAA CTG GGC GAA GA-3′ and 5′-ACC GAG ATG ATG TAG CCA GC-3′ for ERα; 5′-CCA ACC GCG AGA AGA TGA C-3′and 5′-GGA AGG AAG GCT GGA AGA GT-3′ for β-actin as a control.

Construction of Ad-ERE-tk-GFP and Ad-cytomegalovirus (CMV)-DsRed

The consensus estrogen-responsive element and TK promoter gene cassette (ERE-tk) was spliced out from pRC-ERE-tk-Luci (Omoto *et al.* 2001) and was inserted into the multi-cloning site (MCS) in front of the GFP cDNA of pEGFP-1 (pEGFP-1-ERE-tk; Clontech Laboratories Inc). After the ERE-tk-GFP cassette was spliced out from pEGFP-1-ERE-tk, it was inserted into the MCS of the pENTER 1A vector (pENTER-ERE-tk-GFP; Invitrogen). The ERE-tk-GFP cassette was inserted into the adenovirus vector (pAd/PL-DEST; Invitrogen) by homologous recombination using the pENTER 1A vector, and the resultant vector was named pAd-ERE-tk-GFP. The ampicillin and pUC ori region was removed from pAd-ERE-tk-GFP, and the resultant vector was transfected into human kidney 293A cells using TransIT (Takara). After a few days, the virus Ad-ERE-tk-GFP was recovered in the medium from the 293A cells. The Ad-ERE-tk-GFP used in the experiments was propagated by culturing in PRF-RPMI 1640 supplemented with 10% DCC-FCS at 37 °C in 5% CO_2–95% air.

Ad-CMV-DsRed was constructed to assess the infectivity of the adenovirus in primary tumor cells as a control for transfection of Ad-ERE-tk-GFP. The immediate early promoter of CMV and the DsRed gene of red fluorescent protein were spliced out from pCMV-DsRed-Express (BD Biosciences, Palo Alto, CA, USA). Thereafter, they were inserted into the pENTER 1A vector (pENTER-CMV-DsRed), and pENTER-CMV-DsRed was transfected together with the adenovirus vector and recovered as Ad-CMV-DsRed virions using the same strategy described above for Ad-ERE-tk-GFP.

Assay of ERE activity in primary tumor cells

To assess ERE activation in primary tumor cells, we used Ad-ERE-tk-GFP. Cancer tissue specimens were minced to ~1 mm^3 after rinsing with PBS and digested with collagenase solution for 20–30 min at 37 °C. The cells, including tumor cells, were washed several times with PBS after being recovered by centrifugation, and incubated in 24-well plates using 400 μl PRF-RPMI 1640 supplemented with 10% DCC-FCS. The cells were then promptly or 1 day later infected with 2×10^9 PFU (in 293A cells) Ad-ERE-tk-GFP, and incubated for 3 days at 37 °C in 5% CO_2–95% air. To examine the infectivity of the adenovirus in primary tumor cells, the primary cells were infected with 2×10^9 PFU Ad-CMV-DsRed or Ad-ERE-tk-GFP. GFP- or DsRed-expressing cells were counted by fluorescence microscopy after incubation for 3 days at 37 °C in 5% CO_2–95% air.

Immunohistochemistry of the ER

ER expression in individual EC patients was assayed with immunohistochemistry. To activate paraffin sections, the slides were heated at 120 °C for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate (pH 6.0)) by autoclaving. Analysis was performed using the streptavidin–biotin amplification method using a Histofine kit (Nichirei, Tokyo, Japan). The antigen–antibody complex was visualized with 3,3′-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris–HCl buffer (pH 7.6), and 0.006% H_2O_2), and counterstaining was performed using hematoxylin. Monoclonal antibodies for the ER (ER1D5) were purchased from Immunotech

(Marseille, France) and used at a dilution of 1:50. For evaluation of ERα immunoreactivity, more than 1000 tumor cells from three different representative fields per case were counted, and the percentage immunoreactivity (i.e., labeling index (LI)) was determined.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U* test for comparison of two independent groups using the StatView 5.0 software program (SAS Institute Inc., Cary, NC, USA). For comparison among three or more groups, the Kruskal–Wallis test was used to assess the differences. Data were expressed as mean ± S.D. $P<0.05$ was considered statistically significant.

Results

Detection of ER-activating ability of stromal cells in endometrial cancers

E10, an ER activity reporter cell line, was previously established from a clone of the human breast cancer cell line MCF-7 by stable transfection with the ERE-tk-GFP gene. E10 cells showed a high ERα expression level, and specifically expressed GFP upon treatment with E_2 (Fig. 1A). Testosterone alone had no effect on the induction of GFP expression in E10 cells (Fig. 1A). Using E10 cells, we developed a system to visualize the ERE-activating ability of stromal cells in breast cancers based on tumor–stromal interactions (Yamaguchi *et al.* 2005).

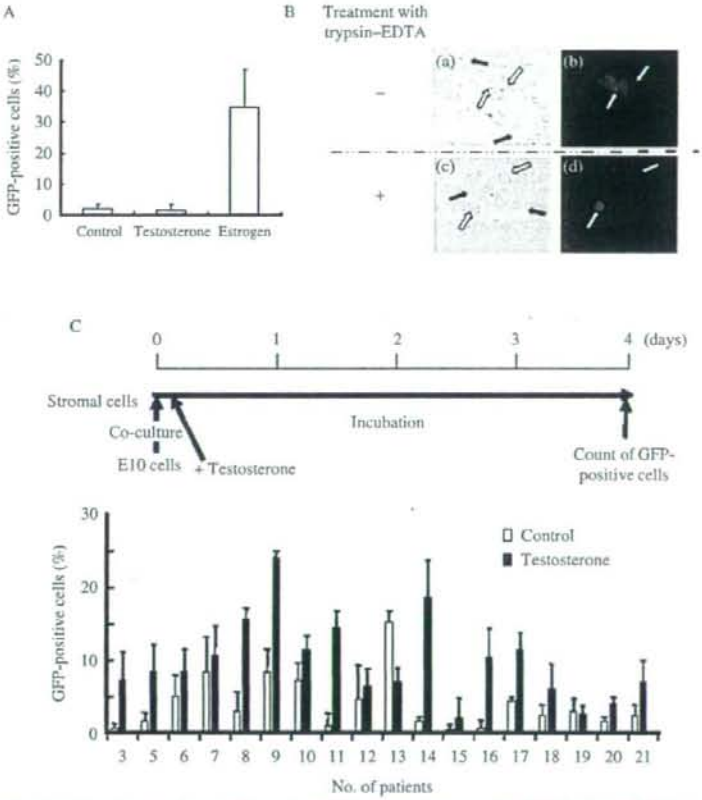


Figure 1 GFP expression in E10 cells co-cultured with endometrial cancer stromal cells. (A) 17β-Estradiol (E_2), but not testosterone, induced GFP expression in E10 cells. After 3 days of culture, E10 cells were cultured in the presence of testosterone, E_2, or ethanol (control) for 4 days. The E10 cells expressing GFP were then counted. (B) Detection of GFP expression in E10 cells co-cultured with stromal cells. After 3 days of culture, E10 cells were cultured with stromal cells in the presence of testosterone or ethanol (control) for 4 days ((a) bright field, (b) dark field). The E10 cells expressing GFP were counted after treatment with trypsin–EDTA ((c) bright field, (d) dark field). Solid and open arrows show stromal cells of the endometrial tissue and E10 cells respectively. (C) GFP expression in E10 cells co-cultured with stromal cells obtained from individual endometrial cancers. After 3 days of culture, E10 and stromal cells were co-cultured in the presence or absence of testosterone for 4 days. E10 cells expressing GFP were counted, and the data are shown as percentages of GFP-expressing cells. All experiments were done in triplicate. Bars, average; error bars, S.D.

In this study, we examined the ability of stromal cells obtained from EC to activate the ERE. Figure 1B shows a case in which E10 cells co-cultured with stromal cells from EC expressed GFP in the presence of testosterone, a substrate of aromatase. GFP expression in E10 cells was usually analyzed after mild trypsinization, which enabled easy discrimination of E10 cells from stromal cells based on their morphology (Fig. 1B). Using this system, we analyzed whether the stromal cells obtained from various ECs activated estrogen signaling (Fig. 1C). The induction of GFP-positive cells was observed in most cases, though the percentage of GFP-positive cells was variable. In some cases, the presence of testosterone significantly increased GFP expression by 10- to 15-fold. This suggests that stromal cells in EC convert testosterone to E_2 and activate estrogen signaling in tumor cells expressing ER. Interestingly, in one case (patient 13), the GFP-positive levels were high in the absence and low in the presence of testosterone. When the percentages of GFP-positive cells were analyzed with respect to clinicopathological variables, no statistically significant differences were found.

Expression of aromatase and 17β-HSD type 2 mRNA in primary endometrial cancer stromal cells

The concentration of intratumoral E_2 is higher in the endometrial cancer tissues than in normal endometrium (Naitoh *et al.* 1989, Berstein *et al.* 2003), and is regulated by estrogen-metabolizing enzymes such as aromatase, 17β-HSD types 2 and 5 (Ito *et al.* 2006; Fig. 2C). Aromatase and 17β-HSD type 5 increase local estrogen production, whereas 17β-HSD type 2 decreases (Fig. 2C). To analyze the relationship between these enzymes and ER-activating ability in stromal cells, we examined the expression levels of aromatase and 17β-HSD type 2 genes by real-time PCR (Fig. 2A and B). We did not analyze 17β-HSD type 5 because our

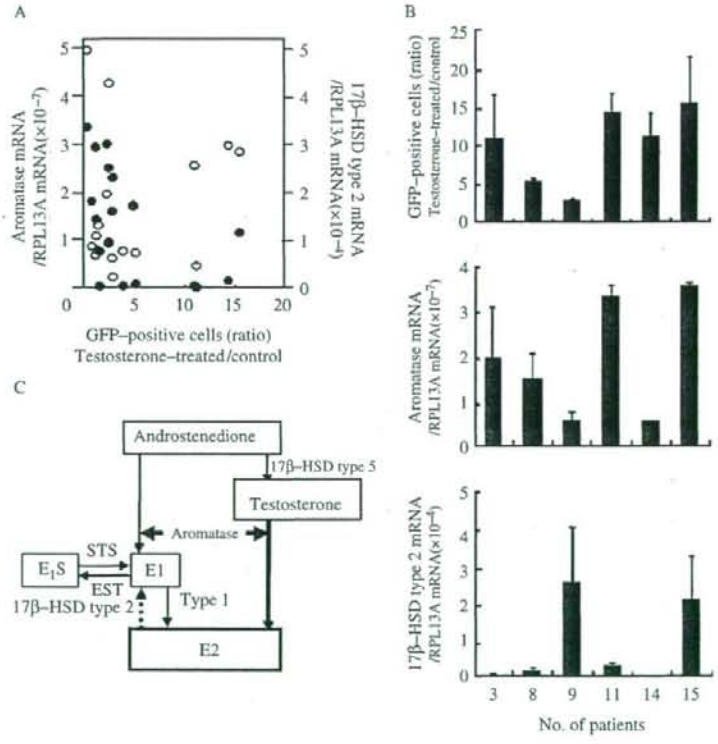


Figure 2 Local estrogen biosynthesis in stromal cells of endometrial cancers. (A) Influence of aromatase and 17β-HSD type 2 gene expressions in stromal cells. The ratios of GFP-expressing cells increased following the addition of testosterone. Aromatase (○) and 17 β-HSD type 2 (●) gene expressions in stromal cells were analyzed by real-time PCR. The expression levels were compared with the increase in the ratios of GFP-expressing cells following the addition of testosterone to E10 cells co-cultured with stromal cells. (B) Six representative samples showing aromatase and 17β-HSD type 2 gene expressions in stromal cells and GFP expression in the co-culture system. (C) Estrogen biosynthesis pathways in the microenvironment of endometrial cancer.

co-culture system contained testosterone, a reaction product of 17β-HSD type 5. The GFP expression level (the fold increase in estrogen signaling by the addition of testosterone) was not correlated ($R=0.19$) with aromatase, but was inversely correlated ($R=-0.51$) with 17β-HSD type 2 mRNA expression levels (Fig. 2A). The data from individual patients suggested that ER activation in EC was greatly affected by estrogen biosynthesis, mediated by aromatase in the stromal cells, as shown in Fig. 2B. However, intratumoral E_2 levels may also be regulated by other estrogen-metabolizing enzymes. The percentage of GFP-positive cells was low in cases such as patient 9. This case had a low expression level of aromatase and a high expression level of 17β-HSD type 2. This may have resulted in low synthesis of E_2 in the local cells (Fig. 2B). In patient 14, stromal cells showed high ERE-activating ability, although they had a low level of aromatase gene expression. This might have been due to the lack of 17β-HSD type 2 expression.

Effects of AIs on the induction of GFP in the co-culture system

Our results suggest that aromatase in stromal cells plays a significant role in the regulation of local estrogen signaling; therefore, we examined the effects of AIs on ER activation in a co-culture system with endometrial cancers. AIs widely applied in breast cancers, including anastrozole, exemestane, and letrozole (Brueggemeier *et al.* 2005), were tested in 18 cases of EC. Figure 3A shows that all of these inhibitors significantly inhibit the induction of GFP expression (Kruskal–Wallis test: $P<0.01$). As in the case of breast cancers (Yamaguchi *et al.* 2005), the sensitivity of stromal cells to the drugs was variable among the cases (Fig. 3B). These results suggest that endocrine therapy using AIs might be effective for EC, especially those who have high sensitivity to AI; however, a method to select the most suitable drug for an individual patient needs to be developed first.

Establishment of a new reporter system to analyze ERE activities in endometrial cancer

The results obtained with the co-culture system indicate that stromal cells in EC locally produce estrogen signals. The significance of ER signaling in endometrial tumor cells has not been studied to date. We analyzed ERE activity in various endometrial cell lines (Ishikawa, Sawano, A431, RL95-2, JHUEM2, JHUAS1) and a breast cancer cell line (MCF-7) as a control, using a luciferase reporter assay. Somewhat unexpectedly, the ERE activities of the endometrial cancer cells were much lower than that in MCF-7 cells (Fig. 4).

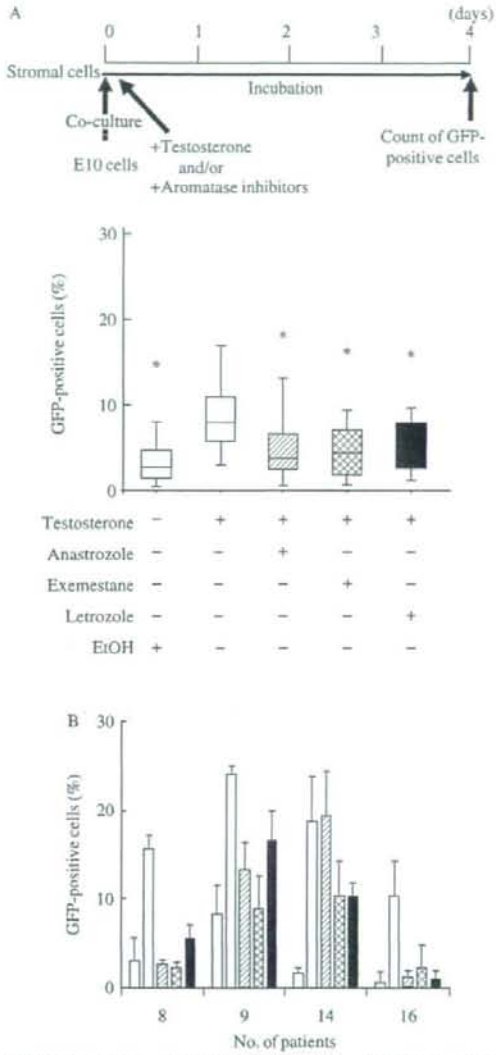


Figure 3 Aromatase in endometrial cancer stromal cells plays a significant role in local estrogen synthesis. (A) Effect of aromatase inhibitors on GFP expression in E10 cells co-cultured with stromal cells from 18 endometrial cancers. After culture in PRF-RPMI with 10% DCC-FCS, E10 and stromal cells were co-cultured in the absence (open bars) or presence (grey bars) of testosterone (1×10^{-7} mol/l) with or without anastrozole (0.1 μM, hatched bars), exemestane (0.1 μM, cross hatched bars) or letrozole (0.1 μM, solid bars). After 4 days of co-culture, E10 cells expressing GFP were counted. The data are shown as percentages of GFP-expressing cells. $^*P<0.05$. (B) Effects of aromatase inhibitors on the induction of GFP expression in the co-culture system for individual patients. Representative data from four patients are shown. Symbols are the same as in (A). All experiments were done in triplicate. Bars, average; error bars, S.D.

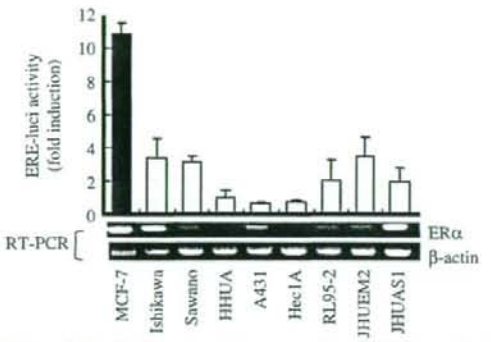


Figure 4 Luciferase reporter assay for ERE-dependent transcriptional activity in various cell lines. The MCF-7 breast cancer cell line, and Ishikawa, Sawano, HHUA, A431, Hec1A, RL95-2, JHUEM2, and JHUAS1 endometrial cancer cell lines were co-transfected with the ERE-luciferase reporter or with the pRL-luciferase plasmid as a control, and cultured in PRF-RPMI with 10% DCC-FCS for 4 days. After further culture for 24 h with or without 17β-estradiol (10 nmol/l), luciferase activities were assayed. Data are shown as the ratio of 17β-estradiol:vehicle (ethanol). Expression of ERα in these cell lines was analyzed by reverse transcription-PCR as indicated in the bottom part of the figure.

Given the relatively lower ERE expression in the EC lines, we realized that the efficiency of the luciferase assay was not sufficient to measure the ERE activity in primary endometrial tissues containing a mixture of cells. Therefore, we developed a new reporter system in which the ERE-tk-GFP gene was transfected into tumor cells using an adenovirus vector. This system enabled us to directly detect the ER activity in individual endometrial cancers by monitoring GFP expression. We first examined the validity of this system using MCF-7 cells (Fig. 5). When MCF-7 cells were infected with Ad-ERE-tk-GFP, GFP expression was induced by E_2 in a dose-dependent manner. GFP expression was detected at 3 pmol/l, and reached a maximal level at 100 pmol/l (Fig. 5A). GFP expression increased for 72 h and then decreased. Fulvestrant, an ER antagonist, inhibited the GFP expression, indicating that the expression was induced in an estrogen-specific manner (Fig. 5A and B). Adenovirus infectivity in MCF-7 cells was examined using Ad-CMV-DsRed, and a minimum of 95% of cells were infected (Fig. 5A and B). We then analyzed estrogen signals in primary endometrial cancer cells with this new reporter assay system.

Detection of estrogen signal activity in primary endometrial cancer cells

Using the above adenovirus system, we characterized the ERE activity in primary tumor cells obtained from EC after treatment with collagenase (Fig. 6A). Three days after infection with Ad-ERE-tk-GFP, the GFP expression was observed (Fig. 6A). Primary tumor cells significantly expressed GFP, but the percentage of GFP-positive cells varied among individual cases.

The adenovirus infectivity of primary tumor cells, as estimated by infection with Ad-CMV-DsRed, was not less than 75% (data not shown). In some cases, primary EC tumor cells showed high percentages of GFP-positive cells comparable with the percentages seen in breast cancer tumor cells. ER expression in cancer cells was detected by immunohistochemical analysis and compared with the percentage of GFP-positive cells. The GFP expression levels of endometrial tumor cells were related to their ER expression levels, suggesting that estrogen signaling was conducted via the ER in endometrial cancers (Fig. 6B).

We then examined whether the ERE activities in the endometrial cancer cells were linked to estrogen/testosterone activation of the ER. Cancer tissues were prepared from four patients for Ad-ERE-tk-GFP infection. The infection was performed immediately after collagenase treatment or performed after overnight culture in estrogen-depleted medium. In three of four specimens, the GFP expression levels decreased in the cells infected immediately after the collagenase treatment compared with the cells infected after overnight culture (Fig. 7, gray bars). This might have been due to the decrease in intracellular estrogen during culture, since the addition of estrogen induced high GFP expression (Fig. 7, hatched bars). GFP expression was also induced by the addition of testosterone in the cell infected after overnight culture, and this induction was inhibited by fulvestrant. These results indicate that stromal cells, present in primary cell cultures, could supply estrogen via their expression of aromatase. In one specimen (patient 35), the GFP expression levels did not differ between the cells infected immediately after the collagenase treatment and the cells infected after overnight culture, even after the addition of estrogen, testosterone, and fulvestrant. In this specimen, the ERE activity was independent of the estrogen-ER signal pathway. Overall, our adenovirus vector system enabled us, for the first time, to detect the ER activity in primary endometrial cancer cells. This confirms that endometrial tumor cells can respond to estrogen signals.

Discussion

Interaction among tumor and stromal cells has recently been shown to influence carcinogenesis or malignant transformation of cancer cells. Estrogen is one of the most important mediators of tumor–stromal interaction

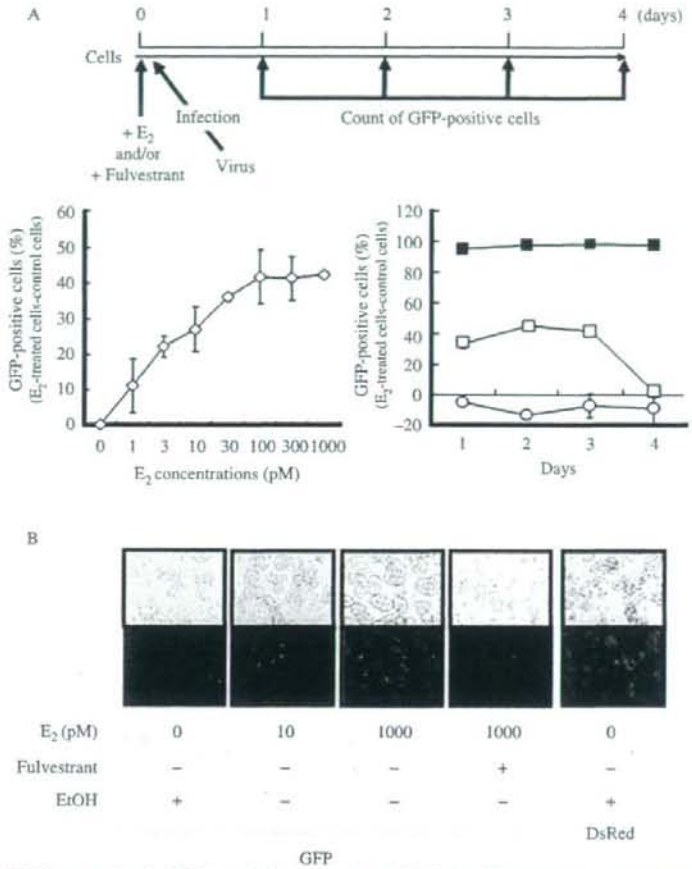
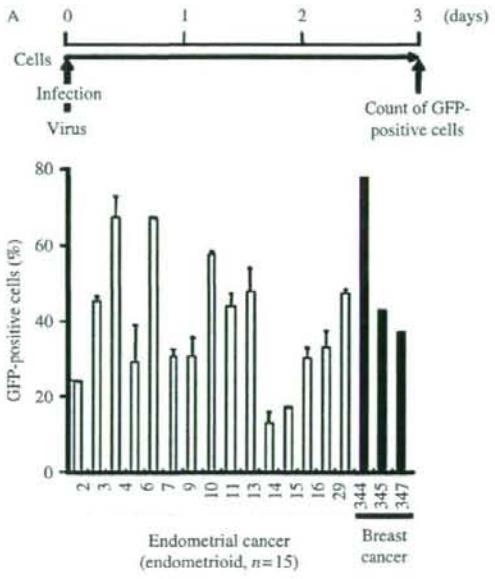


Figure 5 E_2-induced GFP expression in MCF-7 cells infected with Ad-ERE-tk-GFP. (A) Flow chart of the experiment and GFP expression in MCF-7 cells. MCF-7 cells, pre-cultured in PRF-RPMI with 10% DCC-FCS, were infected with Ad-ERE-tk-GFP in the presence of various concentrations of E_2 for 4 days (◇), or in the presence of E_2 (1000 pmol/l) with (○) or without (□) 1 μmol/l fulvestrant for the indicated days. Cells expressing GFP were then counted. Virus infectivity was estimated using Ad-CMV-DsRed (■). All experiments were done in triplicate. Bars, average; error bars, s.d. (B) GFP expression in Ad-ERE-tk-GFP-infected MCF-7 cells cultured in the presence of various concentrations of E_2, with or without fulvestrant, a pure anti-estrogen, for 3 days. Virus infectivity was inferred from the expression of DsRed in Ad-CMV-DsRed-infected cells.

in breast cancer (Yamaguchi *et al.* 2005) and endometrial cancer (Ito 2007). Immunohistochemical studies of the biosynthesis and metabolism of estrogen in EC confirm that estrogen-metabolizing enzymes, such as 17β-HSD types 2 and 5, are present in the cytoplasm of tumor cells but not stromal cells (Ito *et al.* 2006). By contrast, aromatase is more highly expressed in stromal than tumor cells (Watanabe *et al.* 1995, Sasano *et al.* 1996), and its expression is correlated with poor survival (Segawa *et al.* 2005). To date, there has been no direct confirmation of stromal–tumor interactions mediated by estrogen signaling and the transactivation of ERE by endometrial stromal cells.

In this study, we demonstrated for the first time, the ability of stromal cells obtained from individual EC patients to stimulate the estrogen-signaling pathway in previously established ER-positive reporter cells (Yamaguchi *et al.* 2005). Furthermore, we evaluated the estrogen signaling sensitivity of primary tumor cells from individual patients using a unique system developed to visualize ER activity.

The ability of stromal cells to activate the ER varied among patients. In all cases except one, the expression of GFP was induced by the addition of testosterone, which was utilized as a substrate of aromatase (Fig. 1C). Aromatase is a key enzyme for estrogen



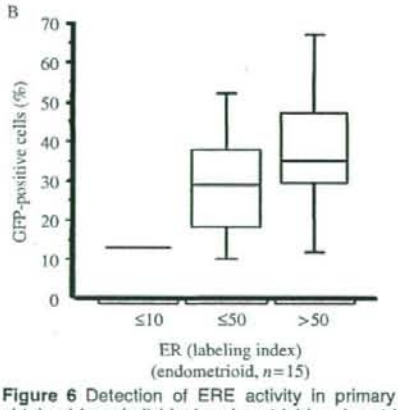


Figure 6 Detection of ERE activity in primary tumor cells obtained from individual endometrioid endometrial cancers. (A) Primary tumor cells obtained from 15 individual endometrioid endometrial cancers (open bars) and three breast cancers (closed bars) after treatment with collagenase were infected with Ad-ERE-tk-GFP. Virus-infected cells were cultured in PRF-RPMI containing 10% DCC-FCS for 3 days, and GFP-expressing cells were counted. The experimental flow chart is shown at the top. All experiments were done in duplicate. Bars, average; error bars, error. (B) ER expression in 15 primary endometrial cancers was scored using the labeling index by immunohistochemical staining. Numbers of cases in each group of ≤10, ≤50, and >50 were 1, 3, and 11 respectively. GFP expression levels were correlated with immunohistochemical scores for the ER in endometrioid endometrial cancer.

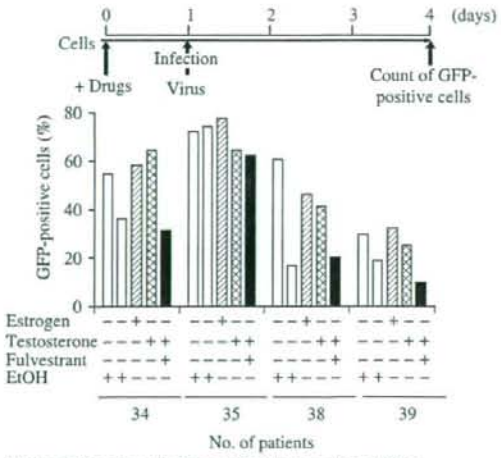


Figure 7 Change of ERE activity following the addition of estrogen, testosterone, and fulvestrant. Primary tumor cells obtained from four endometrioid endometrial cancers were infected with Ad-ERE-tk-GFP immediately (open bars) or after 1 day of culture (gray bars), after treatment of the tissue with collagenase. Virus-infected cells were incubated in PRF-RPMI containing 10% DCC-FCS for 3 days with E_2 (10 nmol/l, hatched bars), testosterone (0.1 μmol/l, cross hatched bars), testosterone and fulvestrant (1 μmol/l, solid bars), or ethanol as a control (white or gray bars). Cells expressing GFP were counted on day 4.

synthesis. The estrogen activity in tissues, however, is regulated not only by aromatase but also by various estrogen-metabolizing enzymes, as shown in Fig. 2C. From our data, the local estrogen level in cancer tissues seemed to be determined by the balance of all of these estrogen-metabolizing enzymes. However, the expression of GFP significantly decreased when AIs were added to the co-culture of stromal and E10 cells treated with testosterone (Fig. 3A). This indicates that the provision of estrogen from endometrial stromal cells is strongly dependent on aromatase activity. Testosterone levels in the ovarian vein are higher in patients with endometrioid EC than those in healthy subjects (Nagamani *et al.* 1986), and the testosterone levels in tumor tissues are 10–40 times higher than those in serum (Ito 2007). Therefore, it is conceivable that conversion in the local environment may increase the local concentration of estrogen in the EC tissues.

Interestingly, the expression level of GFP in patient 13 was high even without the addition of testosterone (Fig. 1C). In addition, the expression level of GFP was not decreased by the addition of AIs in this case (data not shown). We did not identify similar outliers in our previous study of breast cancer patients (Yamaguchi *et al.* 2005). In addition to estrogen, growth factors such as epidermal growth factor and IGF-I, which activate downstream kinases including MAPK and PI3K/Akt,

activate ER via its phosphorylation (Kato *et al.* 1995, Bunone *et al.* 1996, Ignar-Trowbridge *et al.* 1996, Lian *et al.* 2006). MAPK also stimulates the activity of ER via the phosphorylation of the ER-associating coactivators, amplified in breast cancer 1 (AIB1; Font de Mora & Brown 2000) and human steroid receptor coactivator 1 (SRC-1; Rowan *et al.* 2000). In the mouse endometrium, loss of phosphate and tensin homologue deletedon chromosome 10 (PTEN) activates the PI3K/Akt pathway and results in the activation of the ER (Dickson & Lippman 1995). In patient 13 (Fig. 1C), ER activation may also have depended on ligand-independent activation. The stimulating factors supplied from adjacent stromal cells may be important for this estrogen independence. Our system has the advantage of being able to evaluate overall estrogen signaling in primary cells of individual cases.

We developed a unique system that was able to assess the ER-activating ability of individual stromal cells using E10 cells. Here, we showed that all three AIs tested, anastrozole, letrozole, and exemestane, significantly suppressed the ER-activating ability of stromal cells in EC (Fig. 3A). Furthermore, the sensitivity to each aromatase inhibitor differed among individual EC patients (Fig. 3B). The individual variation of AI sensitivity may be attributed to individual differences in ligand-independent ER activation. Moreover, the differing sensitivity to each AI may be a reflection of individual differences in metabolizing enzymes such as cytochrome P450s (Grimm & Dyroff 1997). Thus, a system to predict the effectiveness of therapy is necessary for individual patients with estrogen-dependent cancers.

This study was the first to document ER activity in the EC cells from individual surgical specimens (Fig. 6A). In order to examine the state of ER activity in EC *in vivo*, a novel assay system using Ad-ERE-tk-GFP, which has an ERE-tk-GFP reporter gene in an adenovirus expression vector, was constructed and used to infect primary culture cells. Although most of the established, widely distributed, the EC cell lines showed extremely low ER activity in comparison with the ER-positive breast cancer cell line (Fig. 4), the cells isolated from individual ECs showed expression levels of GFP equivalent to those in breast cancers. Furthermore, as in breast cancer, the levels varied in individual patients (Fig. 6A). The levels seemed to be related to the expression levels of ER in the tumor cells (Fig. 6B). In most cases, except patient 35, tumor cells infected with Ad-ERE-tk-GFP after one night of precultivation showed reduced GFP expression compared with cells infected immediately after collagenase treatment. When estrogen or testosterone was added to the medium, the GFP expression was restored. The induction by testosterone was decreased by fulvestrant. These observations suggest that these EC cells are estrogen responsive, and probably depended on endogenous ER and local estrogen biosynthesis.

Interestingly, one case (patient 35) did not show sensitivity to estrogen, although the GFP expression was high. A similar phenomenon was also observed in patient 13, as shown in Fig. 1C. An explanation might be other factors that activate transcription through ERE in a ligand-independent fashion in EC. Therefore, our system may be useful for predicting the effectiveness of hormone therapy for estrogen-dependent cancers.

In this study, we demonstrated that a tumor–stromal interaction through local estrogen biosynthesis is active and important in endometrial cancer. Presently, there are limited options for endocrine therapy of EC. High-dose progestin therapy using MPA is currently applied in advanced cancer. TAM, an anti-estrogen, which is widely used for breast cancer treatment, however, increases the risk of EC (Fisher *et al.* 1994, Grilli 2006). By contrast, AIs do not increase the risk of EC carcinogenesis in breast cancer patients, and have an excellent safety profile in postmenopausal women with breast cancer (Duffy & Greenwood 2003). In our study, AIs decreased estrogen production by stromal cells and suppressed ER activation in tumor cells (Fig. 3A). Although efficacy is variable based on a few case studies (Rose *et al.* 2000, Berstein *et al.* 2002, Burnett *et al.* 2004, Leunen *et al.* 2004), AIs do show promise as an alternative endocrine therapy for EC especially who has high sensitivity to AI. Our system suggests that AIs may be effective in a subset of carefully screened patients with estrogen-dependent EC.

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