Published OnlineFirst January 13, 2009; DOI: 10.1158/0008-5472.CAN-08-0906

Research Article

Steroid Sulfatase and Estrogen Sulfotransferase in Colon Carcinoma: Regulators of Intratumoral Estrogen Concentrations and Potent Prognostic Factors

Ryuichiro Sato,[1,2] Takashi Suzuki,[2,3] Yu Katayose,[1] Koh Miura,[1] Kenichi Shiiba,[4] Hiroo Tateno,[5] Yasuhiro Miki,[2] Junichi Akahira,[2] Yukiko Kamogawa,[2] Shuji Nagasaki,[2] Kuniharu Yamamoto,[1] Takayuki Ii,[1] Shinichi Egawa,[1] Dean B. Evans,[6] Michiaki Unno,[1] and Hironobu Sasano[2]

Departments of [1]Surgery and [2]Pathology, Tohoku University School of Medicine; [3]Department of Pathology, Tohoku University School of Health Sciences, Sendai, Japan; Departments of [4]Surgery and [5]Pathology, Miyagi Cancer Center, Natori, Japan; and [6]Novartis Institutes for BioMedical Research Basel, Oncology Research, Basel, Switzerland

Abstract

Previous epidemiologic and *in vitro* studies have indicated a potential involvement of estrogens in the pathogenesis of human colon carcinoma, but the precise roles of estrogens have remained largely unknown. Therefore, in this study, we first measured intratumoral concentrations of estrogens in 53 colon carcinomas using liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS). Tissue concentrations of total estrogen [estrone (E_1) + estradiol] and E_1 were significantly (2.0- and 2.4-fold, respectively) higher in colon carcinoma tissues than in nonneoplastic colonic mucosa (n = 31), and higher intratumoral concentrations of total estrogen and E_1 were significantly associated with adverse clinical outcome. Intratumoral concentration of total estrogen was significantly associated with the combined status of steroid sulfatase (STS) and estrogen sulfotransferase (EST), but not with that of aromatase. Thus, we subsequently examined the STS/EST status in 328 colon carcinomas using immunohistochemistry. Immunoreactivities for STS and EST were detected in 61% and 44% of the cases, respectively. 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 labeling index of the carcinomas. In addition, this −/+ group had significantly longer survival, and a multivariate analysis revealed the STS/EST status as an independent prognostic factor. Results from our present study showed that the STS/EST status of carcinoma tissue determined intratumoral estrogen levels and could be a significant prognostic factor in colon carcinoma, suggesting that estrogens are locally produced mainly through the sulfatase pathway and play important roles in the progression of the disease. [Cancer Res 2009;69(3):914–22]

Introduction

Colon cancer is the third leading cause of cancer-related deaths in both men and women in the United States (1). Although the recent advances in chemotherapy have prolonged the survival of patients with advanced disease (2), the results are still unsatisfactory and further researches are required to understand the disease and to improve the outcome. A number of observational studies (3–5) and a randomized trial (6, 7) have shown that hormone replacement therapy affects the incidence of colon carcinoma and the recurrence of colorectal adenoma (8) in postmenopausal women. In addition, the great majority of colon carcinomas express estrogen receptor β (ERβ; ref. 9), and some colon carcinoma cells are responsive to estrogens (10–12). These findings all suggest a possible involvement of estrogens in the pathogenesis of human colon carcinoma.

It is well known that estrogens are locally produced from circulating inactive steroids in estrogen-dependent tumors, such as breast carcinoma, through sulfatase and aromatase pathways (ref. 13; Supplementary Fig. S1). The sulfatase route synthesizes estrone (E_1) from circulating estrone sulfate (E_1-S) by steroid sulfatase (STS) or inversely inactivates E_1 into E_1-S by estrogen sulfotransferase (EST). E_1 is subsequently converted to a potent estrogen, estradiol (E_2), by reductive 17β-hydroxysteroid dehydrogenases (17βHSD). In the aromatase pathway, aromatase produces E_1 and E_2 from circulating androstenedione and testosterone, respectively (13, 14). In breast carcinoma, STS activity is 50 to 200 times greater than aromatase activity (15), and expressions of STS and EST are both reported as significant prognostic factors (16) whereas aromatase is not (17).

In colon carcinoma, expression of 17βHSDs has been previously examined, and colon carcinoma mainly expresses 17βHSD type 2 and type 4, which metabolize E_2 to E_1 (18, 19). It has been also shown that colon carcinoma tissue has sulfatase (20, 21) and aromatase activities (18). However, to the best of our knowledge, intratumoral concentrations of estrogens and their clinical significance have not been reported. Therefore in this study, we first measured tissue concentrations of estrogens in 53 colon carcinoma tissues and correlated these findings with immunoreactivities for STS, EST, and aromatase. The result showed a strong association between the intratumoral estrogen concentrations and STS/EST status, so we subsequently examined the STS/EST status in 328 colon carcinomas to obtain better understanding of the role and significance of estrogenic actions in colon carcinoma.

Materials and Methods

Patients and tissues. Fifty-three specimens (28 men and 25 postmenopausal women) of colon carcinoma tissues were obtained from patients who underwent surgery from 2000 to 2008 in the Department of Surgery at Tohoku University Hospital, Sendai, Japan (mean age, 70.3 y; range, 45–93 y).

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

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

doi:10.1158/0008-5472.CAN-08-0906

Published OnlineFirst January 13, 2009; DOI: 10.1158/0008-5472.CAN-08-0906

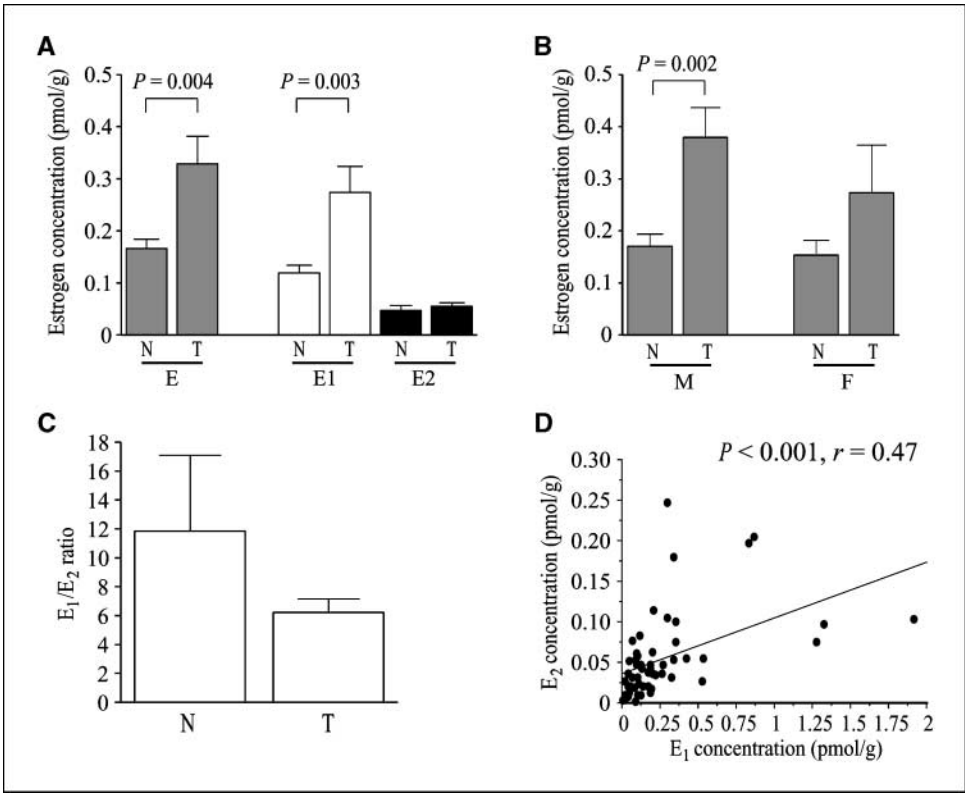

Figure 1. Intratumoral concentrations of estrogens in 53 colon carcinomas as measured by LC-MSI-MS. *A,* tissue concentrations of total estrogen (*E*; E_1 + E_2), E_1, and E_2 in colon carcinoma (*T*; *n* = 53) and nonneoplastic colonic mucosa (*N*; *n* = 31). *B,* tissue concentration of total estrogen 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

Published OnlineFirst January 13, 2009; DOI: 10.1158/0008-5472.CAN-08-0906

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,

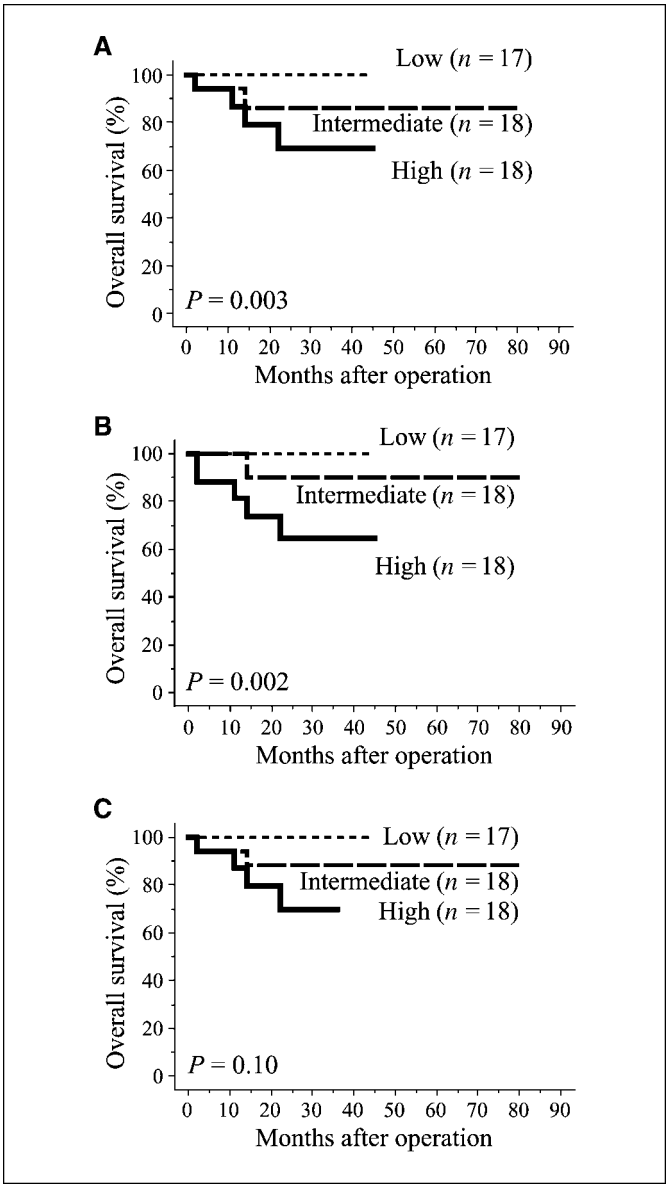

Figure 2. Overall survival curves of the 53 colon carcinoma cases. The cases were divided into three groups of equal size according to the rankings of the intratumoral concentration of total estrogen (*A*), E_1 (*B*), and E_2 (*C*). Cox's proportional hazards model was used for statistical analyses and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

Published OnlineFirst January 13, 2009; DOI: 10.1158/0008-5472.CAN-08-0906

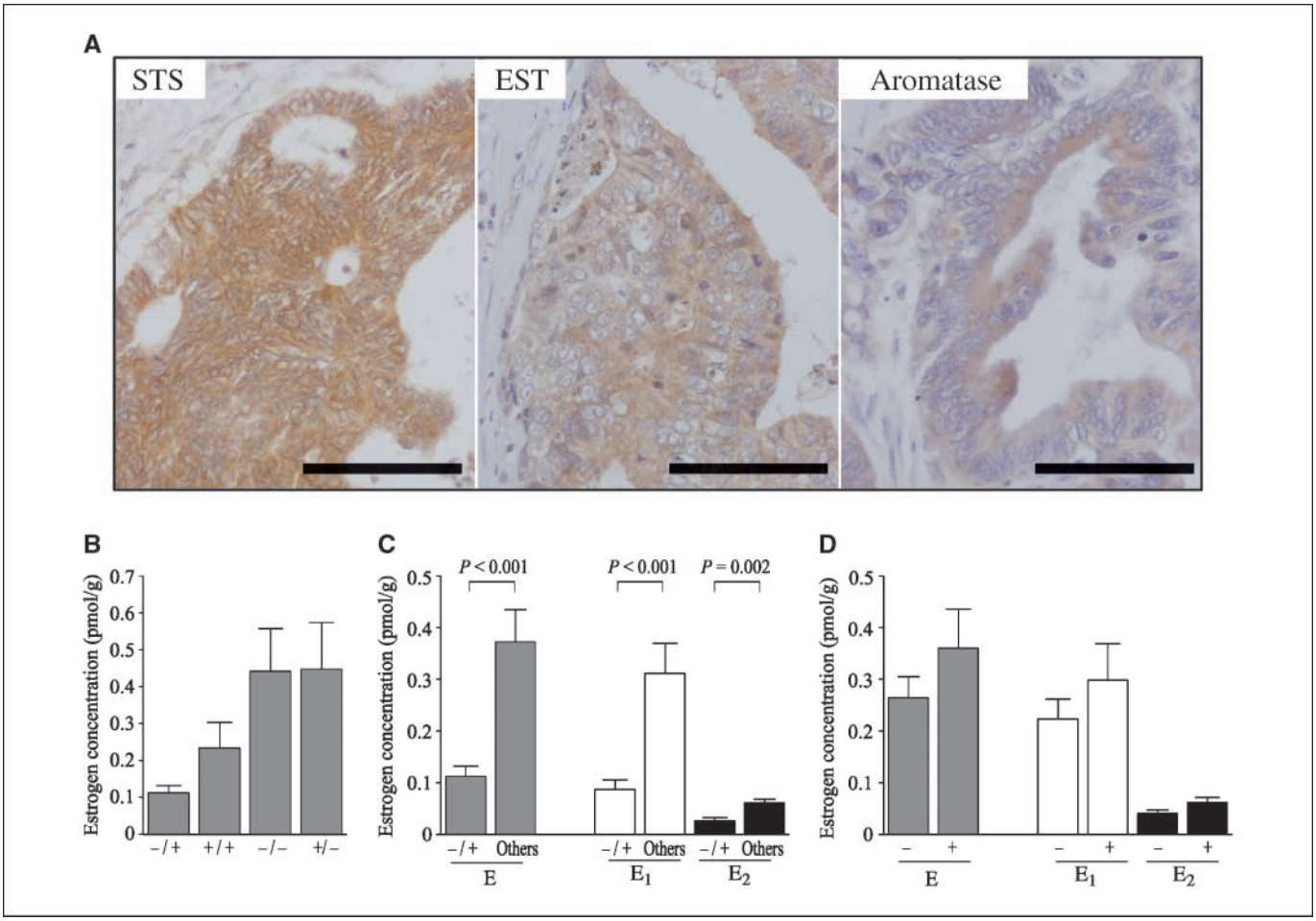

Figure 3. Immunoreactivities for STS, EST, and aromatase in colon carcinoma and their associations with intratumoral estrogen concentrations. *A,* STS, EST, or aromatase immunoreactivity was detected in the cytoplasm of carcinoma cells. *Bar,* 100 mm. *B,* intratumoral concentration of total estrogen 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

Published OnlineFirst January 13, 2009; DOI: 10.1158/0008-5472.CAN-08-0906

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. 3*C*). 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. 3*D*).

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

Published OnlineFirst January 13, 2009; DOI: 10.1158/0008-5472.CAN-08-0906

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

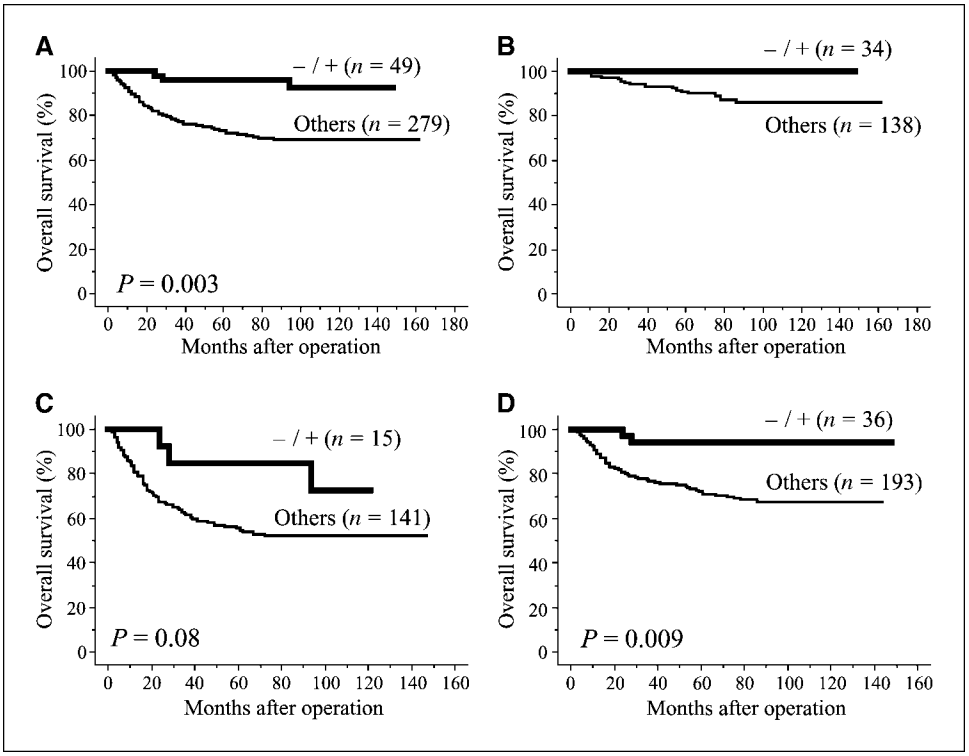

Figure 4. Overall survival curves of 328 colon carcinoma patients according to the STS/EST status. *A,* overall survival curves of all the patients ($n = 328$). *B* and *C,* overall survival curves of patients in the group of Dukes' stages A and B (*B*; $n = 172$) and in the group of Dukes' stages C 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.

Published OnlineFirst January 13, 2009; DOI: 10.1158/0008-5472.CAN-08-0906

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,

Published OnlineFirst January 13, 2009; DOI: 10.1158/0008-5472.CAN-08-0906

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.

Acknowledgments

Received 3/14/2008; revised 9/18/2008; accepted 10/24/2008.

Grant support: Japanese Ministry of Health, Labor, and Welfare for Research on Intractable Diseases, Risk Analysis Research on Food and Pharmaceuticals, and Development of Multidisciplinary Treatment Algorithm with Biomarkers and Modeling of the Decision-making Process with Artificial Intelligence for Primary Breast Cancer. This work was also partly supported by Grant-in-Aid for Scientific Research (18390109) from the Japanese Ministry of Education, Culture, Sports, Science, and Technology, and the Yasuda Medical Foundation.

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 thank Katsuhiko Ono and Miki Mori (Department of Pathology, Tohoku University School of Medicine) and Keiko Inabe, Emiko Shibuya, and Midori Chiba (Department of Surgery, Tohoku University School of Medicine) for their skillful technical assistance.

References

1. American Cancer Society. Cancer facts & figures 2007. Atlanta: American Cancer Society 2007.
2. Colucci G, Gebbia V, Paoletti G, et al. Phase III randomized trial of FOLFIRI versus FOLFOX4 in the treatment of advanced colorectal cancer: a multicenter study of the Gruppo Oncologico Dell'Italia Meridionale. J Clin Oncol 2005;23:4866–75.
3. Newcomb PA, Zheng Y, Chia VM, et al. Estrogen plus progestin use, microsatellite instability, and the risk of colorectal cancer in women. Cancer Res 2007;67:7534–9.
4. Grodstein F, Newcomb PA, Stampfer MJ. Postmenopausal hormone therapy and the risk of colorectal cancer: a review and meta-analysis. Am J Med 1999;106:574–82.
5. Prihartono N, Palmer JR, Louik C, Shapiro S, Rosenberg L. A case-control study of use of postmenopausal female hormone supplements in relation to the risk of large bowel cancer. Cancer Epidemiol Biomarkers Prev 2000;9:443–7.
6. Anderson GL, Limacher M, Assaf AR, et al. Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. JAMA 2004;291:1701–12.
7. Rossouw JE, Anderson GL, Prentice RL, et al. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. JAMA 2002;288:321–33.
8. Woodson K, Lanza E, Tangrea JA, et al. A. Hormone replacement therapy and colorectal adenoma recurrence among women in the Polyp Prevention Trial. J Natl Cancer Inst 2001;93:1799–805.
9. Wong NA, Malcomson RD, Jodrell DI, Groome NP, Harrison DJ, Saunders PT. ERβ isoform expression in colorectal carcinoma: an *in vivo* and *in vitro* study of clinicopathological and molecular correlates. J Pathol 2005;207:53–60.
10. Fiorelli G, Picariello L, Martineti V, Tonelli F, Brandi ML. Functional estrogen receptor β in colon cancer cells. Biochem Biophys Res Commun 1999;261:521–7.
11. Qiu Y, Waters CE, Lewis AE, Langman MJ, Eggo MC. Oestrogen-induced apoptosis in colonocytes expressing oestrogen receptor β. J Endocrinol 2002;174:369–77.
12. Arai N, Strom A, Rafter JJ, Gustafsson JA. Estrogen receptor β mRNA in colon cancer cells: growth effects of estrogen and genistein. Biochem Biophys Res Commun 2000;270:425–31.
13. Suzuki T, Miki Y, Moriya T, et al. *In situ* production of sex steroids in human breast carcinoma. Med Mol Morphol 2007;40:121–7.
14. Mindnich R, Moller G, Adamski J. The role of 17β-hydroxysteroid dehydrogenases. Mol Cell Endocrinol 2004;218:7–20.
15. 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.
16. Suzuki T, Nakata T, Miki Y, et al. Estrogen sulfotransferase and steroid sulfatase in human breast carcinoma. Cancer Res 2003;63:2762–70.
17. Silva MC, Rowlands MG, Dowsett M, et al. Intratumoral aromatase as a prognostic factor in human breast carcinoma. Cancer Res 1989;49:2588–91.
18. English MA, Kane KF, Cruickshank N, Langman MJ, Stewart PM, Hewison M. Loss of estrogen inactivation in colonic cancer. J Clin Endocrinol Metab 1999;84:2080–5.
19. English MA, Stewart PM, Hewison M. Estrogen metabolism and malignancy: analysis of the expression and function of 17β-hydroxysteroid dehydrogenases in colonic cancer. Mol Cell Endocrinol 2001;171:53–60.
20. Koudstaal J. The histochemical demonstration of arylsulphatase and acid hydrolases in carcinoma of the colon and the breast. Eur J Cancer 1975;11:479–82.
21. Double JA, Cooper EH, Goligher JC. Hydrolytic enzymes in colorectal cancer. Biomedicine 1977;27:11–3.
22. Yamashita K, Okuyama M, Watanabe Y, Honma S, Kobayashi S, Numazawa M. Highly sensitive determination of estrone and estradiol in human serum by liquid chromatography-electrospray ionization tandem mass spectrometry. Steroids 2007;72:819–27.
23. Suzuki T, Miki Y, Moriya T, et al. 5α-Reductase type 1 and aromatase in breast carcinoma as regulators of *in situ* androgen production. Int J Cancer 2007;120:285–91.
24. Mitamura K, Setaka M, Shimada K, et al. Determination of sulfates of androsterone and epiandrosterone in human serum using isotope diluted liquid chromatography-electrospray ionization-mass spectrometry. Biomed Chromatogr 2005;19:796–801.
25. Sasano H, Anderson TJ, Silverberg SG, et al. The validation of new aromatase monoclonal antibodies for

Published OnlineFirst January 13, 2009; DOI: 10.1158/0008-5472.CAN-08-0906

immunohistochemistry-a correlation with biochemical activities in 46 cases of breast cancer. J Steroid Biochem Mol Biol 2005;95:35–9.
26. Miki Y, Nakata T, Suzuki T, et al. Systemic distribution of steroid sulfatase and estrogen sulfotransferase in human adult and fetal tissues. J Clin Endocrinol Metab 2002;87:5760–8.
27. Chetrite GS, Cortes-Prieto J, Philippe JC, Wright F, Pasqualini JR. Comparison of estrogen concentrations, estrone sulfatase and aromatase activities in normal, and in cancerous, human breast tissues. J Steroid Biochem Mol Biol 2000;72:23–7.
28. Pasqualini JR. The selective estrogen enzyme modulators in breast cancer: a review. Biochim Biophys Acta 2004;1654:123–43.
29. Ranadive GN, Mistry JS, Damodaran K, et al. Rapid, convenient radioimmunoassay of estrone sulfate. Clin Chem 1998;44:244–9.
30. Haeseleer F, Palczewski K. Short-chain dehydrogenases/reductases in retina. Methods Enzymol 2000; 316:372–83.
31. Mandelson MT, Miglioretti D, Newcomb PA, Harrison R, Potter JD. Hormone replacement therapy in relation to survival in women diagnosed with colon cancer. Cancer Causes Control 2003;14:979–84.
32. Ali S, Coombes RC. Estrogen receptor α in human breast cancer: occurrence and significance. J Mammary Gland Biol Neoplasia 2000;5:271–81.
33. 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.
34. Leav I, Lau KM, Adams JY, et al. Comparative studies of the estrogen receptors β and α and the androgen receptor in normal human prostate glands, dysplasia, and in primary and metastatic carcinoma. Am J Pathol 2001;159:79–92.
35. Hershberger PA, Vasquez AC, Kanterewicz B, Land S, Siegfried JM, Nichols M. Regulation of endogenous gene expression in human non-small cell lung cancer cells by estrogen receptor ligands. Cancer Res 2005;65: 1598–605.
36. Stettner M, Kaulfuss S, Burfeind P, et al. The relevance of estrogen receptor-β expression to the antiproliferative effects observed with histone deacetylase inhibitors and phytoestrogens in prostate cancer treatment. Mol Cancer Ther 2007;6:2626–33.
37. Nussbaumer P, Billich A. Steroid sulfatase inhibitors. Med Res Rev 2004;24:529–76.
38. Reed MJ, Purohit A, Woo LW, Newman SP, Potter BV. Steroid sulfatase: molecular biology, regulation, and inhibition. Endocr Rev 2005;26:171–202.
39. Kuiper GG, Carlsson B, Grandien K, et al. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 1997;138:863–70.
40. Pennie WD, Aldridge TC, Brooks AN. Differential activation by xenoestrogens of ERα and ERβ when linked to different response elements. J Endocrinol 1998; 158:R11–4.
41. Oduwole OO, Makinen MJ, Isomaa VV, et al. 17β-Hydroxysteroid dehydrogenase type 2: independent prognostic significance and evidence of estrogen protection in female patients with colon cancer. J Steroid Biochem Mol Biol 2003;87:133–40.
42. Compton C, Fenoglio-Preiser CM, Pettigrew N, Fielding LP. American Joint Committee on Cancer Prognostic Factors Consensus Conference: Colorectal Working Group. Cancer 2000;88:1739–57.
43. Brown DC, Gatter KC. Ki67 protein: the immaculate deception? Histopathology 2002;40:2–11.
44. Duchrow M, Ziemann T, Windhovel U, Bruch HP, Broll R. Colorectal carcinomas with high MIB-1 labelling indices but low pKi67 mRNA levels correlate with better prognostic outcome. Histopathology 2003;42:566–74.
45. Hilska M, Collan YU, O Laine VJ, et al. The significance of tumor markers for proliferation and apoptosis in predicting survival in colorectal cancer. Dis Colon Rectum 2005;48:2197–208.
46. Garrity MM, Burgart LJ, Mahoney MR, et al. Prognostic value of proliferation, apoptosis, defective DNA mismatch repair, and p53 overexpression in patients with resected Dukes' B2 or C colon cancer: a North Central Cancer Treatment Group Study. J Clin Oncol 2004;22:1572–82.
47. Allegra CJ, Paik S, Colangelo LH, et al. Prognostic value of thymidylate synthase, Ki-67, and p53 in patients with Dukes' B and C colon cancer: a National Cancer Institute-National Surgical Adjuvant Breast and Bowel Project collaborative study. J Clin Oncol 2003;21:241–50.

Cancer Research
The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

AACR American Association for Cancer Research

Steroid Sulfatase and Estrogen Sulfotransferase in Colon Carcinoma: Regulators of Intratumoral Estrogen Concentrations and Potent Prognostic Factors

Ryuichiro Sato, Takashi Suzuki, Yu Katayose, et al.

Cancer Res 2009;69:914-922. Published OnlineFirst January 13, 2009.