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ORIGINAL ARTICLE

Estrogen receptor beta expression in colitis-associated carcinoma in comparison with sporadic colonic tumor: An immunohistochemical study

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Abstract

Background and Aim: The rate of ulcerative colitis (UC)-related colorectal cancer (colitis-associated carcinoma) is increasing. Estrogen receptor (ER) beta expression has been studied separately in patients with sporadic colorectal cancer and those with colitis-associated carcinoma. However, no study has compared the expression in both of these cancer types. The present study aimed to evaluate the relationship between colitis-associated carcinoma and ERs and assess whether the expression of ER beta influences cell proliferation.

Methods: This study included 45 surgically operated colitis-associated carcinomas, 43 high-grade dysplasias, 34 low-grade dysplasias, 36 sporadic colorectal cancers, 44 high-grade adenomas, and 34 low-grade adenomas. ER beta expression was evaluated with immunohistochemistry.

Results: Colitis-associated carcinoma showed significantly lower ER beta immunoexpression than sporadic colorectal lesions and high- and low-grade dysplasia. In seven colitis-associated carcinoma harboring both intensity score 3 (strong immunoexpression) and score 1 (weak immunoexpression) areas, the correlation among ER beta intensity, Ki-67, and p21 labeling index was assessed; an area with an ER beta intensity score of 3 showed a higher Ki-67 labeling index than that with score 1. In four out of the seven lesions, p21 labeling index was higher in the area of ER beta score 1 than in that of ER beta score 3.

Conclusions: The data suggest that ER beta expression is an accelerating factor in colorectal tumors. This association may be lower in colitis-associated carcinoma than in sporadic colorectal cancer.

Introduction

Ulcerative colitis (UC) is an unidentified inflammatory bowel disease that affects the mucosa and stratum submucosa of the colorectum.[1] It involves erosions and ulcers in continuity from the rectum and shows repeated remission and chronic relapse.

Previously, steroid therapy and surgery were the primary approaches for treating UC. However, the number of patients receiving medical treatment without surgery for an extended period increased with the use of cytapheresis, immunomodulators, and biologics. The risk of UC-related colorectal cancer (colitis-associated carcinoma) is related to the duration of the disease.[2] Castańo-Milla *et al*. reported that the overall incidence rate of colorectal cancer in patients with UC was 1.67/1000 patients per year of disease (PYD). Incidence rates per decade were estimated to be 1.01/1000 patients PYD, 3.75/1000 patients PYD, and 5.85/1000 patients PYD for the first, second, and third decades, respectively.[3] The reported risk factors for colorectal cancer in patients with UC are extensive disease, a young age at diagnosis,[4,5] a family history of colorectal cancer,[6] a co-existing primary sclerosing cholangitis,[7] and persistent colon inflammation.[8,9] Patients with a high risk of UC should be closely followed up with a colonoscopy.

Several differences between colitis-associated carcinoma and sporadic colorectal cancer have been reported. Studies have mentioned that colitis-associated carcinoma occurs via dysplasia as an early epithelial neoplastic lesion, easily develops poor differentiation with an abnormality of cell adhesion, and frequently shows early *p53* mutations and late Adenomatous polyposis coli (*APC*) mutations.[10–12]

Estrogen is a female hormone that regulates the growth, differentiation, and function of various target tissues within and outside the reproductive system.[13,14] The estrogen receptor (ER) has alpha and beta subtypes. ER alpha and ER beta are expressed at different levels in each human body organ.[15] ER beta is involved in maintaining cell homeostasis, whereas ER beta is considered an antagonist of ER alpha. ER alpha is a positive regulator of cellular growth. In contrast, ER beta has an antagonistic inhibitory function mediated by the downregulation of proto-oncogenes (c-myc and cyclins) and upregulation of oncosuppressants (p21 and p27), resulting in cell cycle arrest.[16] In an inflammatory bowel condition, ER beta was considered to play a role in maintaining epithelial barriers.[17]

The hypothesis of a possible link between colorectal cancer and ERs was advanced after certain epidemiological studies reported that women have a lower rate of colorectal adenomas and cancers than men before menopause, with the differences progressively decreasing after menopause.[18,19] Similarly, both observational and interventional data have shown that hormone replacement therapy decreases the risks of colorectal adenoma and cancer.[20–23] In the last 40 years, a reduction in deaths from large bowel carcinoma was observed in the United States, and this reduction was significantly greater in women (30%) than in men (7%).[24]

Although ER beta expression has been studied in patients with sporadic colorectal cancer[25] and those with UC-associated cancer,[26] no study has compared the expression in these two cancer types. Therefore, the present study aimed to evaluate the relationship between colitis-associated carcinoma and ERs and assess whether the expression of ER beta influences cell proliferation to determine ER beta's involvement in the mechanism of UC cancer progression.

Methods

Patient selection. Sixty-six cases of UC with associated neoplasms (cancer and dysplasia) at Toho University Omori Medical Center, Toho University Sakura Medical Center, and Yokohama Municipal Citizen's Hospital between 2006 and 2016 included 46 cancer lesions, 44 high-grade dysplasia lesions, and 34 low-grade dysplasia lesions. Subsequently, adenomas were selected from 414 cases at Toho University Omori Medical Center from 2010 to 2013, the lesions matching UC-associated dysplasias in histological grade, site, and age. Similarly, sporadic cancer lesions were selected from 649 cases at Toho University Omori Medical Center from 2010 to 2013. The lesions matched UC-associated cancer lesions in histology, site, and depth of invasion. Since the colitis-associated carcinoma cases were more frequently poorly differentiated and younger than sporadic cancer cases, and sporadic, poorly differentiated cancer in younger patients was rare, there were few applicable cases for poorly differentiated sporadic cancer. Finally, only 36 sporadic cancer cases could be collected (Fig. 1). The history of medical treatment prior to surgery for 66 UC cases were as follows: 5-aminosalicylic acid (5-ASA), nine cases; sulfasalazine (SASP), eight cases; 5-ASA + SASP, three cases; 5-ASA + prednisolone (PSL), 10 cases; SASP + PSL, seven cases; 5-ASA + SASP + PSL, three cases; additional cytapheresis (CAP) therapy, 13 cases; additional immunomodulatory therapy, 12 cases; unknown, 1 case. Moreover, the prognosis of the patients, as well as metastasis and recurrence, were examined using medical records.

For neoplastic lesions, this study included 236 lesions, including 45 surgically operated colitis-associated carcinomas, 43 high-grade dysplasia, 34 low-grade dysplasia, 36 sporadic colorectal cancers, and 44 high-grade and 34 low-grade adenomas, which were resected with surgery or polypectomy. Patients who received preoperative chemotherapy and radiation therapy were excluded from this study. The specimens were routinely fixed with formalin and embedded in paraffin. Hematoxylin- and eosin-stained slides were reviewed, and one tissue block for each case was selected. For cancer lesions, tissue blocks that included the deepest parts of the lesions were used for staining. For low- and high-grade dysplasia and adenomas, tissue blocks that primarily represented the lesions were used. After immunohistochemistry, two lesions (one colitis-associated carcinoma and one high-grade dysplasia) that could not be resolved by immunostaining were excluded from this study (Fig. 1). Finally, lesions are summarized in Table 1. The pathological stage of the cancer lesions is expressed using TNM classification.[27]

Immunohistochemistry. For immunohistochemical analysis, 4-μm thick sections were cut from the tissue blocks and stained using a staining kit (Envision, Dako, Glostrup, Denmark) along with the following primary antibodies: Anti-ER beta (dilution 1/50, monoclonal, PPG5/10, Bio-Rad, Hercules, CA), anti-ER alpha (dilution 1/500, monoclonal, 1D5, Thermo Fisher Scientific, Rockford, IL), anti-Ki-67 (dilution 1/100, polyclonal, Ab-4, Thermo Fisher Scientific, Rockford, IL), and anti-$p21^{WAF1}$ (dilution 1/200, monoclonal, EA10, Thermo Fisher Scientific,

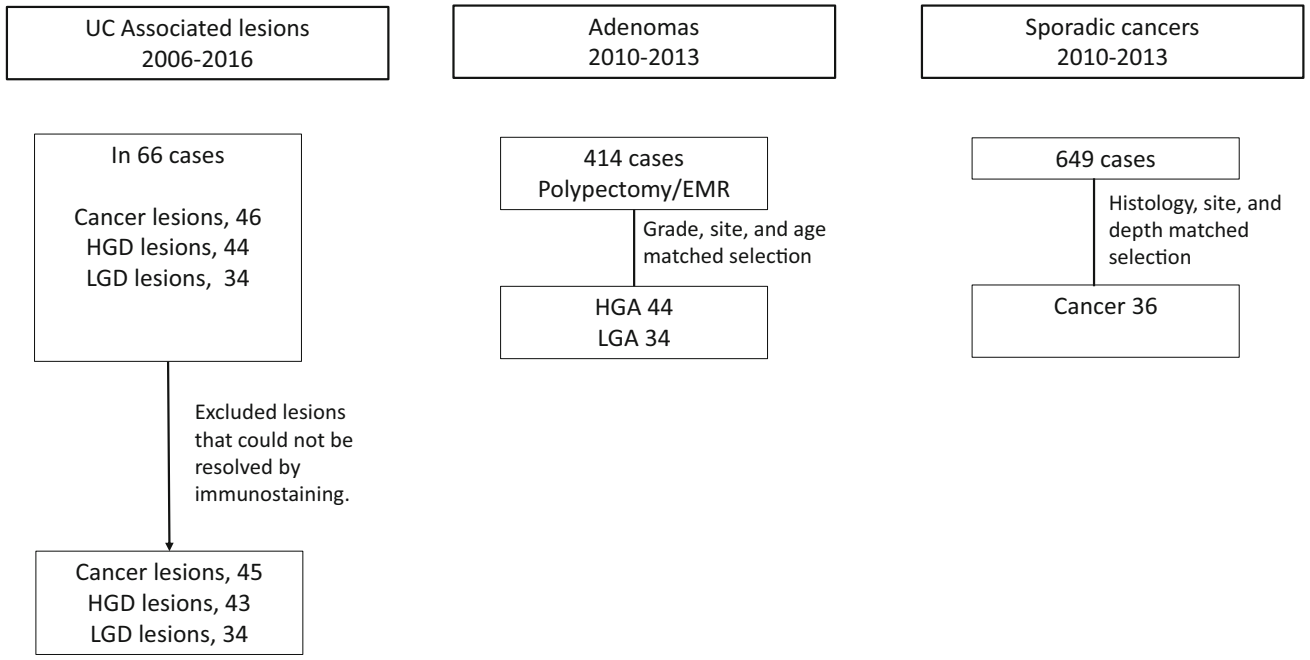

Figure 1 Method of case selection: Ulcerative colitis (UC)- associated lesions were collected from 66 cases between 2006 and 2016: 46 cancer lesions, 44 high-grade dysplasia (HGD) lesions, 34 low-grade dysplasia (LGD) lesions. Adenomas were selected from 414 cases between 2010 and 2013 according to the grade, site, and age of UC-associated HGD or LGD. Cancer lesions were selected from 649 cases from 2010 to 2013, matched to site, histology, and depth of colitis-associated carcinoma. Finally, two lesions (one colitis-associated carcinoma and one high-grade dysplasia) that could not be resolved by immunostaining were excluded. EMR, endoscopic mucosal resection; HGA, high grade adenoma; LGA, low grade adenoma.

Table 1 Characteristics of the lesions in this study

	Colitis associated carcinoma	High-grade dysplasia	Low-grade dysplasia	Sporadic cancer	High-grade adenoma	Low-grade adenoma
Number of cases	45	43	34	36	44	34
Patient sex, male: female	32:13	26:17	23:11	26:10	27:17	23:11
Patient age, years (mean ± standard deviation)	53.7 ± 13.9	54.8 ± 13.8	52 ± 14.3	59.1 ± 10.6	63.9 ± 13.8	59.5 ± 11.2
Lesion location						
C to A	7	5	5	5	6	5
T to D	16	18	5	11	18	8
S to R	22	20	24	20	20	21
Lesion invasion depth[†]						
pT1(SM)-2(MP)	26		20			
pT3(SS or A)-4(SE)	19		16			

Abbreviations: A, ascending colon; C, cecum; D, descending colon; R, rectum; S, sigmoid colon; T, transverse colon.
[†]TNM classification (Reference 27).

Rockford, IL). Before incubation with the primary antibody, the slides were treated with a pressure cooker (anti-ER beta, anti-Ki-67), hot water (anti-ER alpha), and microwave oven (anti-$p21^{WAF1}$). Chromogenic fixation was carried out by immersing the sections in a 3,3′-diaminobenzidine solution. Counterstaining was done with hematoxylin.

For ER beta and ER alpha, both the stained area and intensity of the lesions were evaluated, and the immunoreactivity score was calculated. The proportion of positivity of the stained area was scored as follows: 0, no staining; 1, 1–25%; 2, 25–50%; 3, 50–75%; and 4, >75%. In addition, the staining intensity was scored as follows: 1, weak; 2, moderate; 3, strong. The positivity proportion and intensity scores were multiplied to obtain the immunoreactivity score (range, 0–12) (Fig. 2). For Ki-67 and p21, more than 500 cancer cells were assessed for immunoexpression at the target area. The stained

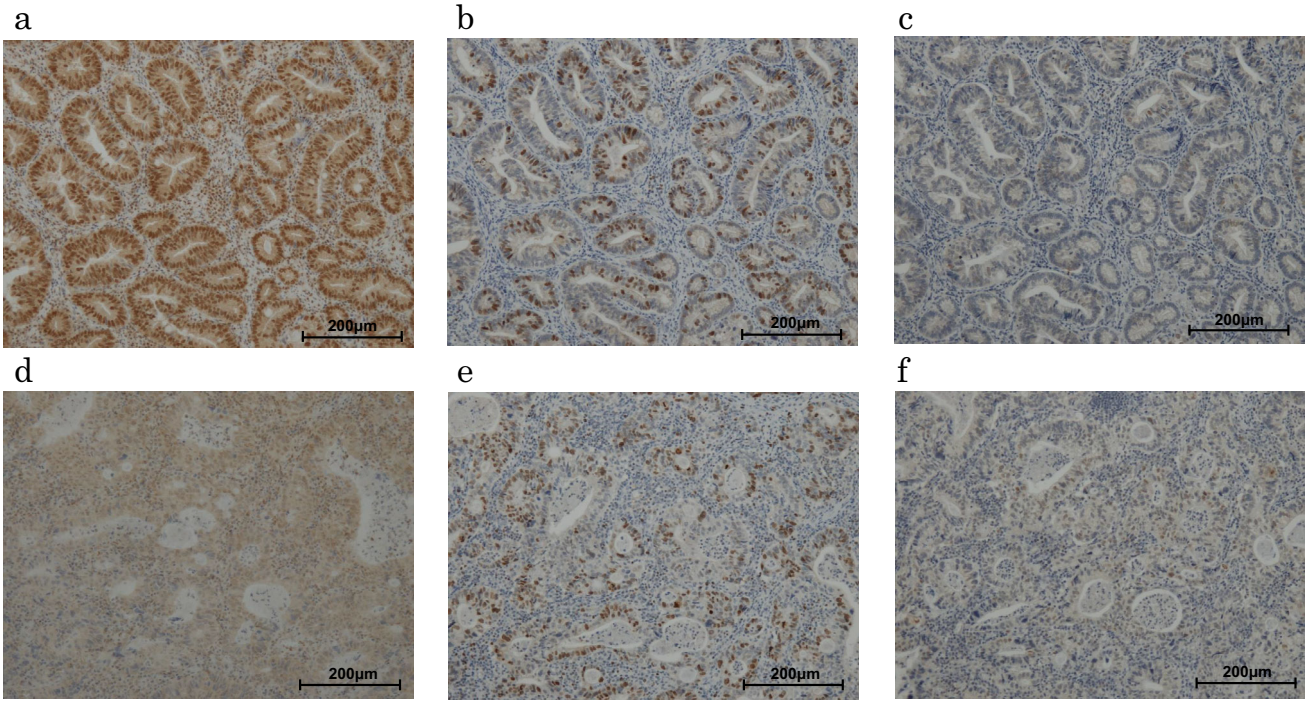

Figure 2 Representative photomicrographs showing expression of estrogen receptor (ER) beta (a and d). A case showing ER beta intensity score 3 (a); the same area as (a) with Ki-67 expression (b) and p21 expression (c). The other case showing low expression of ER beta, intensity score 1 (d); the same region as (d) with Ki-67 expression (e) and p21 expression (f). (×20 objective lens; scale bar: 200 μm).

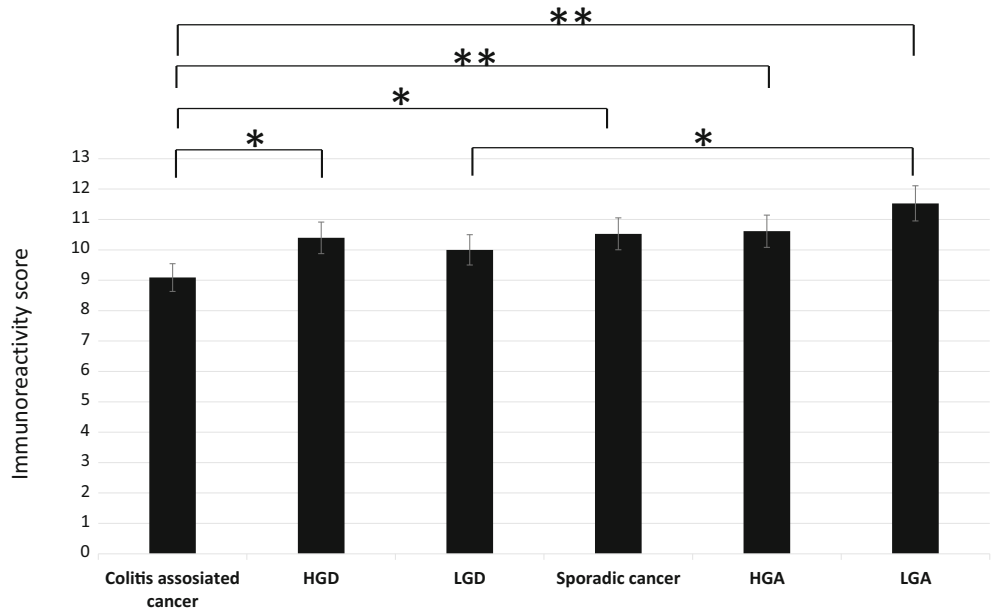

Figure 3 Estrogen receptor (ER) beta expressions in colitis-associated lesions and sporadic colorectal lesions. Colitis-associated carcinoma (9.02 ± 2.29) and low-grade adenoma (LGA) (11.52 ± 1.16) ($P < 0.0001$), colitis-associated carcinoma and high-grade adenoma (HGA) (10.6 ± 1.71) ($P = 0.0021$), and colitis-associated carcinoma and sporadic colorectal cancer (10.5 ± 1.84) ($P = 0.0088$). There is a significant difference in ER beta expression between LGA and low-grade dysplasia (10.0 ± 2.23) ($P = 0.0187$) and between colitis-associated carcinoma and high-grade dysplasia (10.4 ± 2.18) ($P = 0.0142$). *$P < 0.05$, **$P < 0.01$.

nuclear ratio was calculated as a percentage, considered the labeling index (LI).

The LIs of Ki-67 and p21 were compared to a lesion area with an ER beta intensity score of 3 and a score of 1 to examine whether ER beta immunoexpression influences cell proliferation. In this comparison, 7 colitis-associated carcinomas with a score of 3 and 1 were examined for areas of ER beta.

As a negative control, a slide incubated with only buffer solution instead of the primary antibody was used in each immunohistochemical procedure.

As positive controls, for ER beta, a case of colorectal cancer confirmed to express ER beta previously was used. For ER alpha, breast cancer tissue confirmed to express ER alpha was used. For Ki-67 and p21, colorectal cancer tissue confirmed to express p21 was used. In addition, normal mucosa adjacent to the examined neoplastic lesion was used as an internal positive control for ER beta, Ki67, and p21. Two researchers (Matsuno T and Mikami T) independently scored the immunoreactivity scores. If the scores of the two did not match, the final score was the score agreed upon by the two looking at the discussion microscope.

Statistical analysis. Differences in the immunoreactivity scores were assessed using analysis of variance and Tukey–Kramer's honestly significant difference test as a post-hoc test using JMP software (version 13, SAS Institute Inc., Cary, NC). The LIs of Ki-67 and p21 were determined using the paired *t*-test; *P*-values <0.05 were considered statistically significant.

Ethical consideration. The study protocol was approved by the Ethics Committee of the Faculty of Medicine, Toho University (No. A16048 and A22048_A21082) and the Toho University Omori Medical Center (No. M21067). The retrospective observational study was performed with the opt-out method to guarantee the chance for refusal and data anonymization without individual informed consent.

Results

ER beta expression. ER beta was expressed in the nucleus of colorectal epithelial cells of background normal mucosa in sporadic cancer cases, particularly epithelial cells of the lower half of the colorectal crypts. In neoplastic lesions, nuclear immunoexpression was observed (Fig. 3). On comparing the immunoreactivity scores, we noted significant differences between colitis-associated carcinoma (9.02 ± 2.29) and low-grade adenoma (11.52 ± 1.16) ($P < 0.0001$), between colitis-associated carcinoma and high-grade adenoma (10.6 ± 1.71) ($P = 0.0021$), between colitis-associated carcinoma and sporadic colorectal cancer (10.5 ± 1.84) ($P = 0.0088$), between low-grade adenoma and UC-associated low-grade dysplasia (10.0 ± 2.23) ($P = 0.0187$), and between colitis-associated carcinoma and UC-associated high-grade dysplasia (10.4 ± 2.18) ($P = 0.0142$) (Fig. 3). However, there were no significant differences concerning the site, sex, and age (<60 years and ≥60 years) (Table 2). The number of cases scored as 12 in sporadic cancer was significantly higher than that in colitis-associated carcinoma. Similarly, the number in low-grade adenomas was significantly higher than that of low-grade dysplasia (Table 3).

Table 2 Comparison of ER beta expression between men and women

	Colitis associated carcinoma	High-grade dysplasia	Low-grade dysplasia	Sporadic cancer	High-grade adenoma	Low-grade adenoma
Total lesions	$n = 45$	$n = 43$	$n = 34$	$n = 36$	$n = 44$	$n = 34$
Men	$n = 32$, 9.09 ± 2.24	$n = 28$, 10.3 ± 2.27	$n = 28$, 10.2 ± 1.73	$n = 26$, 10.3 ± 1.92	$n = 26$, 10.1 ± 1.81	$n = 32$, 11.6 ± 1.10
Women	$n = 13$, 8.85 ± 2.51	$n = 16$, 10.8 ± 2.02	$n = 16$, 9.43 ± 2.61	$n = 10$, 11.0 ± 1.63	$n = 18$, 11.3 ± 1.28	$n = 12$, 11.3 ± 1.36
<60 years	$n = 31$, 8.63 ± 2.14	$n = 25$, 10.4 ± 2.29	$n = 23$, 10.0 ± 2.13	$n = 11$, 10.7 ± 1.79	$n = 15$, 9.80 ± 1.66	$n = 15$, 11.5 ± 1.25
>60 years	$n = 14$, 9.93 ± 2.46	$n = 18$, 10.4 ± 2.09	$n = 11$, 9.82 ± 2.52	$n = 25$, 10.4 ± 1.89	$n = 29$, 11.0 ± 1.61	$n = 19$, 11.5 ± 1.12

Abbreviations: ER, estrogen receptor; *n*, number of cases.

Table 3 The number of cases scored as 12 for ER beta immunostaining

	UC lesions	Sporadic lesions	*P*
Adenocarcinoma	14/45 (31.1%)	21/36 (58.3%)	<0.05
High grade dysplasia/ adenoma	26/43 (60.5%)	25/44 (56.3%)	NS
Low grade dysplasia/ adenoma	16/34 (47.1%)	29/34 (85.3%)	<0.05

Abbreviations: NS, not significant, UC, ulcerative colitis.

ER alpha expression. There was no ER alpha immunoexpression in all examined colonic lesions, although a breast cancer sample used as a positive control showed its immunoexpression in the nucleus.

Ki-67. In the seven colitis-associated carcinoma cases having intensity scores of both 1 and 3 for ER beta, the LI of Ki-67 was 25.8 ± 12.11 (%) at the area of ER beta intensity score 1 and 36.87 ± 14.63 (%) at the area of ER beta intensity score 3, with a significant difference ($P = 0.039$).

p21. In four among the seven lesions, the LI of p21 was higher at the area of ER beta intensity score 1 than at that of score 3. p21 immunoexpression was not noted in two lesions; the LI of p21 was higher at the area of ER beta intensity score 3 than at that of score 1 in the remaining one lesion.

Relation between ER expression and patient's outcome. Data for 5-year overall survival and 5-year disease-free survival by ER beta expression level were examined. The mean ER beta score for all cases of sporadic and colitis-associated carcinoma was 9.73. Therefore, a high ER beta expression group was defined as a group with an expression score of 10 or higher, and a low ER beta expression group was defined as a group with an expression score of 9 or lower. Sporadic carcinoma was detected in 22 lesions in the high-expression group and 14 lesions in the low-expression group. Colitis-associated carcinoma was detected in 23 lesions in the high-expression group and 22 lesions in the low-expression group. No significant differences in 5-year overall survival and 5-year disease-free survival by ER beta expression level were observed in either sporadic or colitis-associated cancer (Figure S1).

In addition, a publicly available data set (GSE 17536) was obtained from the Gene Expression Omnibus (GEO https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE17536) Reference. This data set contains gene expression data and disease-free survival information from 177 patients with primary CRC. The gene expression levels of *ER alpha* and *ER beta* in this data set were analyzed. No difference in expression level was observed between cases with and without recurrence for both *ER alpha* and *ER beta*.

Discussion

The present study found that ER beta immunoexpression was lower in UC-associated neoplasia than in sporadic colorectal cancer. Cell proliferation activity was higher in lesion areas with high ER beta immunoexpression than in those with low ER beta immunoexpression.

Principi *et al.* investigated ER beta immunoexpression in 48 patients with UC from four groups (UC with normal mucosa, UC with low-grade dysplasia, UC with high-grade dysplasia, and UC-associated cancer) by immunostaining. They reported a significant decrease in ER beta immunoexpression in high-grade dysplasia and UC-associated cancer.[26] In addition, Campbell-Thompson *et al.* investigated *ER beta* mRNA expression in the normal mucosa and colorectal cancer and reported that *ER beta* mRNA expression level was lower in colorectal cancer than in the normal mucosa among both men and women.[28] In addition, we showed that there were no significant differences in ER beta immunoexpression with regard to sex and age.

To clarify the influence of decreased ER beta expression, cell proliferation activity was measured using Ki-67 immunostaining. The Ki-67 positivity rate was significantly higher in lesion areas with an ER beta intensity score 3 than in those with score 1, indicating that higher ER beta immunoexpression was associated with higher cell proliferation activity. Furthermore, p21 immunoexpression was investigated and found to be higher in lesion areas with an ER beta intensity score 1 than in those with score 3 in over half of the cases. p21 is known to inhibit cellular turnover.[16] Popp *et al.* investigated p21 immunoexpression in the epithelial cells from biopsy specimens in 45 patients with UC and reported that p21 and p53 immunoexpression were positive in the inflammatory mucosa.[29] Mitsuhashi *et al.* reported that the p53 and p21 LI in non-neoplastic colorectal epithelial cells increased with UC duration and were significantly elevated in individuals with neoplasia.[30] ER beta exhibited an antagonistic inhibitory function mediated by the downregulation of proto-oncogenes (c-myc and cyclins) and upregulation of oncosuppressants (p21 and p27), resulting in cell cycle arrest.[16] These studies dealt with non-neoplastic epithelial cells.[29,30] However, in this study, neoplastic lesions, namely cancer lesions, were investigated, and at least in about half of the cancer lesions, higher immunoexpression of ER beta and down-regulation of p21 may regulate cell proliferation.

Unlike ER beta, ER alpha was not expressed in sporadic colorectal cancer or colitis-associated carcinoma in the present study. Campbell-Thompson *et al.* reported that *ER alpha* mRNA expression was lower than *ER beta* expression in general patients with colorectal cancer and that there were no significant differences in the *ER alpha* expression between normal and neoplastic cells among both men and women.[28] Rath-Wolfson *et al.* reported that the expression of ER alpha was negative in all cases of colorectal cancer similar to the present study.[31] Studies from Waliszewski *et al.* uncovered that *ER alpha* was not expressed in the isolated colorectal cancer cells and cell lines.[32] In contrast, Principi *et al.* reported higher ER alpha immunoexpression in high-grade dysplasia and colitis-associated carcinoma than in low-grade dysplasia.[26] Kaklamanos *et al.* report that ER alpha expression was found in 21 of 65 colorectal cancer cases.[33] These different results might be related to the optimization of immunostaining or specificity of the antibody. The monoclonal antibody used in this study was widely used for breast cancer, the specificity of which is considered reliable. Data from the study by Campbell-Thompson *et al.* indicate that *ER alpha* mRNA was much lower than *ER beta* in colorectal cancer.[28]

Such a low expression should likely be considered negative since the expression determined by immunostaining has been optimized for breast cancer.

Söderlund *et al*. reported that the risk of developing colorectal cancer was 60% higher in men with inflammatory bowel disease than in women.[34] Further, to the best of our knowledge, no previous study has investigated age differences. In the present study, we found no significant differences in ER beta immunoexpression with respect to sex and age, suggesting a weak relationship between hormone replacement therapy and the cancer progression process in UC.

The relation between ER beta expression and prognosis was not significant in this study. In literature, Wang *et al*. and Peng *et al*. showed similar results.[35,36] However, Rath-Wolfson *et al*. reported that ER beta expression was significantly high in fatal cases.[31] Rudolph *et al*. showed that ER beta expression-negative cases had a poor prognosis.[37] With these controversial points, we cannot offer a clear explanation. However, in these four studies, the immunoexpression was evaluated with immunohistochemistry. The difference in the detection method may influence the results. Further examination may be necessary in the future.

There are some limitations to our study. First, this study was histopathological. Therefore, we could only examine the expression and proliferative capacity of the cells in a limited manner. Second, the number of cancer cases that showed both ER beta immunoexpression score 1 and score 3 was so small (seven cases) that the analysis of the relation between Ki67 LI and p21 expression was limited.

In conclusion, our study found lower ER beta immunoexpression in UC-associated neoplasia than in usual colorectal neoplasia. Higher cell proliferation activity was associated with higher ER beta immunoexpression in both sporadic and UC-associated cancer lesions. These findings suggest that ER beta expression is an accelerating factor in colorectal tumors. In addition, this association may be lower in colitis-associated carcinoma than in sporadic colorectal cancer. Hormone replacement therapy may have fewer effects on colitis-associated carcinoma and the cancer progression process in UC.

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Ethics statement

The study protocol was approved by the Ethics Committee of Faculty of Medicine, Toho University (No. A16048 and A22048_A21082) and Toho University Omori Medical Center (No. M21067).

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's website:

Figure S1. Supporting information.