Down-regulation of trefoil factor-3 expression in the rectum is associated with the development of ulcerative colitis-associated cancer

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Running title: KONDO et al: TREFOIL FACTOR-3 ASSOCIATED WITH DEVELOPMENT OF ULCERATIVE COLITIS-ASSOCIATED CANCER

Keywords: trefoil factor-3, ulcerative colitis, colitis-associated cancer, field effect, surveillance

Abstract

Diagnostic markers can facilitate more selective screening and treatment strategies for ulcerative colitis (UC)-associated cancer (UCAC). We analyzed trefoil factor-3 (TFF3), which has an important role in mucosal protection and repair in the gastrointestinal tract, and evaluated its significance for UCAC. We enrolled 145 patients with UC who underwent proctocolectomies including 15 patients (10.8%) with UCAC. We assessed TFF3 expression immunohistochemically in their rectal mucosa and in cancer cells, and compared their expression in UCAC and sporadic colorectal cancer. In testing mucinous granules of goblet cells located in crypts, we found that non-cancerous rectal mucosa of UCAC patients had significantly lower ($P < 0.01$) mean TFF3 staining scores (4.53 ± 2.36) than did specimens of patients with UC without UCAC (7.21 ± 3.38) or sporadic cancer (7.58 ± 2.72). TFF3 staining score was an independent predictor of UCAC development (odds ratio: 4.32, confidence interval: 1.01–29.9, $P = 0.05$). These results indicate that low TFF3 expression in rectal mucosa is associated with the development of UCAC. Thus, TFF3 in the rectal mucosa may be a useful biomarker for monitoring patients with UC.

Introduction

Ulcerative colitis (UC) is the most common form of inflammatory bowel disease, and is characterized by chronic inflammation of the gastrointestinal tract. The development of UC-associated cancer (UCAC) is thought to arise from widespread alterations caused by a combination of genetic and epigenetic factors, as well as host and microbial influences, and is sometimes called an "inflammation dysplasia carcinoma sequence," which differs from sporadic colon cancer (1, 2). Patients with UC have an increased risk of developing colorectal cancer; and UC that is persistent or covers a large area is a risk factor for UCAC (3). Reportedly, the cumulative risk probability of UCAC was 2% after 10 years, 8% after 20 years, and 18% after 30 years of disease duration (4). Moreover, Munkholm et al. (5) showed 15% of all deaths among UC patients were from UCAC. In contrast, a more recent meta-analysis by Jess et al. (6) only found 1.6% of UC patients to develop UCAC within 14 years of diagnosis. Whereas some studies of endoscopic surveillance programs for precancerous dysplasia in patients with inflammatory bowel disease have reported that surveillance reduces the mortality rate from colorectal carcinoma (7, 8), other studies have highlighted their failure to do so (6, 9, 10). The influence of these surveillance systems on the mortality rates of UC patients requires clarification. Furthermore, patients with long-standing UC greatly need effective diagnostic markers. Although several biomarkers appear to correlate with colorectal cancer—for example, p53, K-RAS, and APC—they are not effective in detecting UCAC.

Trefoil factors are a family of peptides with a distinct three-loop structure formed by a highly conserved motif of cysteine disulfide bonds that give them remarkable luminal stability (11, 12). The three genes that produce the trefoil family proteins are located within a 55-kb region on human chromosome 21q22.3 (13). Trefoil factor 1 (TFF1, also called pS2) and trefoil factor 2 (or spasmolytic polypeptide) are normally expressed by epithelial cells of the stomach and duodenum. Trefoil factor 3 (TFF3 or intestinal trefoil factor) is

predominantly expressed by goblet cells of the small intestine and colon (14). Trefoil peptides are implicated in the processes of gastrointestinal restitution and repair (13, 15–17); their ability to promote epithelial cell migration (18, 19), while preventing apoptosis and anoikis (17) facilitates these normal physiologic processes.

Apparently, trefoil factors also participate in neoplasia. For example, in the stomach, TFF3 expression is normally weak or absent (14, 20), but is expressed in areas of intestinal metaplasia (21–23) and in approximately half of gastric carcinomas (22, 23). Furthermore, when expressed by gastric carcinomas, it is associated with poor prognosis, independent of tumor stage (22, 23). Reportedly, strong TFF3 expression in cancer cells is also associated with tumor progression in colon cancer (24, 25).

However, to our knowledge, no study has explored the association between TFF3 expression and UCAC. Here, we therefore investigated whether TFF3 expression in the rectal mucosa could predict UCAC development.

Materials and Methods

Patients and samples

We obtained 118 formalin-fixed, paraffin-embedded (FFPE) tissue samples of tumors from patients with sporadic colorectal cancer who underwent colectomies with no pre-surgical treatments from 2010 to 2011 at the Mie University Hospital, and 15 FFPE tissue samples of tumors from patients with UCAC. We excluded patients who had incomplete clinical data, inadequate follow-up information and/or inadequate immunohistochemical analysis. We also examined 50 FFPE tissue samples of adjacent normal rectum from patients with sporadic rectal cancer, and 145 FFPE tissue samples of rectum from patients with UC who underwent proctocolectomies from 2003 to 2011 at the Mie University Hospital,

including 15 UCAC patients. We excluded patients who had incomplete clinical data, inadequate follow-up information and/or inadequate immunohistochemical analysis.

All patients gave written informed consent according to the local ethics guidelines, and the study design was approved by the Ethics Review Board of Mie University Hospital.

Immunohistochemistry

The FFPE tissue samples of tumor and rectum specimens were sliced in 3-μm sections. After deparaffinization by xylene and rehydration by an ethanol gradient, the sections were pretreated in an autoclave at 121°C for 10 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval. Endogenous peroxidase activity was blocked by incubation for 10 min in 3% hydrogen peroxide. Nonspecific-binding sites were blocked in 1 mol/L PBS with 10% normal goat serum and an Avidin/Biotin Blocking Kit (Vector Laboratories, Burlingame, CA, USA). The sections were then incubated with a primary rabbit monoclonal antibody against TFF3 (1:1000; Abcam, Cambridge, UK) in phosphate-buffered saline (PBS) containing 1% bovine serum albumin for 1 h at 20°C. After washing with PBS, sections were loaded with a secondary antibody coupled with peroxidase-conjugated polymers (Envision+Dual Link System-HRP; DakoCytomation, Glostrup, Denmark) for 30 min. Subsequently, the primary antibodies were detected by using 3,3′-diaminobenzidine (DakoCytomation). All sections were counterstained with Mayer's hematoxylin and were dehydrated in an ethanol gradient and mounted. Negative control sections were prepared by omitting the primary antibody. The FFPE specimens of rectum were also sliced in 5-μm sections continually, and stained using the histochemical technique for Mayer's mucicarmine to confirm the presence of mucin.

Evaluation of immunoreactive TFF3 protein

Each slide was observed under a light microscope. The immunoreactivity scoring system was based on the intensity and extent of staining. The criteria were as follows: (i) The

intensity of TFF3 staining was scored as 0 (negative), 1 (weak), 2 (medium), or 3 (strong); and (ii) The percentage of positive-staining cancer cells on tumor specimens or positive-staining epithelial cells in crypt on rectum specimens as 0 (0–5%), 1 (6–25%), 2 (26–50%), 3 (51–75%), or 4 (76–100%). We multiplied (i) and (ii) together to obtain the TFF3 staining scores (range: 0–12), which thus reflected the intensity and extent of stained cells. Each sample was scored in a blinded manner by two independent researchers with no prior information of the originating patient's clinical or pathological parameters. The few discrepancies were resolved using a multi-head microscope; consensus was then reached for each slide.

Statistical Analysis

All statistical analyses were performed using JMP version 12 (SAS Institute Inc., Cary, NC, USA). Contingency tables were analyzed using the chi-square test with Yates' correction. Correlations between continuous and categorical variables were evaluated using the Mann–Whitney U test for two groups. We used logistic regression to analyze whether TFF3 expression in rectal mucosa predicted UCAC development. $P < 0.05$ was considered significant.

Results

Patient demographics and disease characteristics

Characteristics of the patients with UC are shown in Table I. Their median age at UC diagnosis was 28 years (range: 0–82 years), and their median disease duration was 6 years. We found that 69, 61, and 15 patients had mild, moderate, and severe inflammation, respectively.

The UCAC and non-cancer groups did not significantly differ in sex, age at UC diagnosis, or degree of inflammation (Table I). The median age of the UCAC group (48

years) was older than that of the non-cancer group (33 years; $P < 0.01$). The UCAC group also had a significantly longer median disease duration (12 years) than those without cancer (6 years; $P < 0.01$).

Characteristics of patients with sporadic colorectal cancer are shown in Table II. A total of 118 patients (63 men, 55 women) were enrolled in this group. Their mean age was 67 years (range: 35–89 years). Patients were classified according to the TNM classification by Union for International Cancer Control; 25 patients had stage I disease, 33 had stage II, 34 had stage III and 26 had stage IV.

Immunohistochemical findings and evaluation of TFF3 expression

For both UCAC and sporadic cancer, TFF3 expression was localized in cytoplasm, with no expression in nucleus or cell membrane (Figure 1 a, b). Mean TFF3 staining scores were significantly lower for cytoplasm of UCAC cancer cells (2.00 ± 2.24) than for sporadic cancer cells (3.81 ± 2.84; $P < 0.01$; Figure 2).

In non-cancerous rectal mucosa, TFF3 expression was mainly observed in mucinous granules of goblet cells from intestinal crypts (Figure 3 a–c); and was confirmed by Mayer's mucicarmine-stained mucin in adjacent sections (Figure 3 c, d). In mucinous granules of goblet cells in crypts, the mean TFF3 staining score for non-cancer rectal mucosa from patients with UCAC (4.53 ± 2.36) was significantly lower ($P < 0.01$) than that from UC patients without UCAC (7.21 ± 3.38) or with sporadic cancer (7.58 ± 2.72; Figure 4). Weak TFF3 expression was observed in goblet-cell cytoplasm, but not in nuclei or cell membranes.

Correlation of TFF3 expression in the rectal mucosa with clinical outcomes

TFF3 staining scores were significantly lower in patients who were older ($P < 0.01$), had early-onset disease ($P < 0.01$), had longer disease duration ($P < 0.01$), had mild disease severity ($P = 0.04$), or were in the UCAC group ($P < 0.01$; Table III).

We used the median values of TFF3 staining score and the other factors as the cut-off value. In univariate analysis, age (≥ 34 years; odds ratio [OR]: 4.13, 95% confidence interval [CI]: 1.24–18.7, $P = 0.02$), disease duration (OR: 3.88, 95% CI: 1.17–17.6, $P = 0.03$), and TFF3 staining score (OR: 6.70, 95 % CI: 1.76–44.0, $P < 0.01$) were significantly associated with UCAC development (Table IV). In multivariate analysis, TFF3 staining score was retained as an independent predictor of UCAC development (OR: 4.32, CI: 1.01–29.9, $P = 0.05$).

Discussion

In this present study, TFF3 expression was lower in the rectal mucosa and cancer cells of UCAC patients than in the other tested groups. Notably, this relationship differs from those of other cancers, in which up-regulated TFF3 expression correlates with progressive staging and more aggressive malignancies (24–30). For example, strong expression of TFF3 in serum and cancer cells was associated with poor prognosis in gastric cancer (28), breast cancer [29](29), and advanced prostate cancer (30). Additionally, in colon cancer, strong TFF3 expression in cancer cells is reportedly associated with metastasis and early recurrence (24, 25), and strong serum TFF3 expression is associated with TNM stage 3/4 and distant metastasis (26). In contrast, weak TFF3 expression in cancer cells is reportedly associated with colon carcinogenesis (27). The significance of TFF3 expression in colorectal cancer is thus controversial.

TFF3 itself is normally produced by the colorectal mucosa, which is made up of a single layer of simple tubular glands and crypts (14). Pluripotent stem cells in the crypt bottoms differentiate into goblet cells, entero-endocrine cells, and absorptive cells. Goblet cells contain abundant mucinous granules (which can be stained with Mayer’s mucicarmine), and secrete a complex mixture of mucin glycoproteins in the luminal surface (31). Colon goblet cells also secrete TFF3 (32). Thus, TFF3 of colorectal mucosa is predominantly

expressed in mucinous granules of goblet cells. On the other hand, TFF3 synthesis by non-goblet colonocytes, however, is highly conserved in neoplastic differentiation (33). These dissimilar behaviors of TFF3 between those in colorectal mucosa and those in cancer cells, and differing sequences of inflammation–dysplasia–carcinoma and adenoma–carcinoma may explain the discrepancy of down-regulated TFF3 correlating with UCAC development.

TFF3 increases mucin gel viscosity, and also mediates epithelial restitution and migration in colonic mucosa (16, 19). TFF3 rapidly up-regulates at margins of mucosal injury and in various ulcerative conditions, which implies an important function in mucosal defense and repair (34). Indeed, TFF3-deficient mice had poor epithelial colonic regeneration after oral administration of dextran sulfate sodium (35). It might be a possibility that inflammation in the UCAC colon is often inactive, which may lead to a low expression of TTF3, however, not the degree of inflammation but the TFF3 staining score was recorded as an independent predictor of UCAC development.

For patients with long-standing UC, current surveillance guidelines recommend colonoscopies with random biopsies to be collected at 10 cm increments along the colonic mucosa or target biopsy testing every 1–2 years (36–39). Although surveillance colonoscopies help detect early-stage UCAC and reduce the risk of death (40–42), this type of surveillance has several limitations, such as sampling errors and difficulties in macroscopic diagnosis of neoplastic lesions that are flat or diffusely infiltrative (43, 44). Based on the “field effect” concept that neoplastic and non-neoplastic tissues can harbor widespread genetic alterations (45, 46), many investigators have attempted to determine effective biomarkers to identify patients with UC who are at a higher risk of developing UCAC (47-49). However, such biomarkers have not been well established.

In conclusion, our results associate TFF3 down-regulation in rectal mucosa with UCAC development. Thus, the combination of current surveillance and evaluation of TFF3

expression in rectal mucosa may usefully and safely identify high-risk patients with UCAC. However, these data should be interpreted with some caution. First, we had a selection bias, as the patients in this study were not representative of a typical patient population under surveillance. This study also included relatively few UCAC patients. We therefore plan further studies—possibly prospective multicenter studies of biopsy samples from UC patients—to assess the utility of these markers for routine clinical use.

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Figure legends

Figure 1. Immunohistochemical analysis of TFF3 expression (×100) in tumor cells. TFF3 expression was observed in the cytoplasm of cancer cells. Patient (a) had ulcerative colitis-associated cancer, and patient (b) had sporadic colorectal cancer.

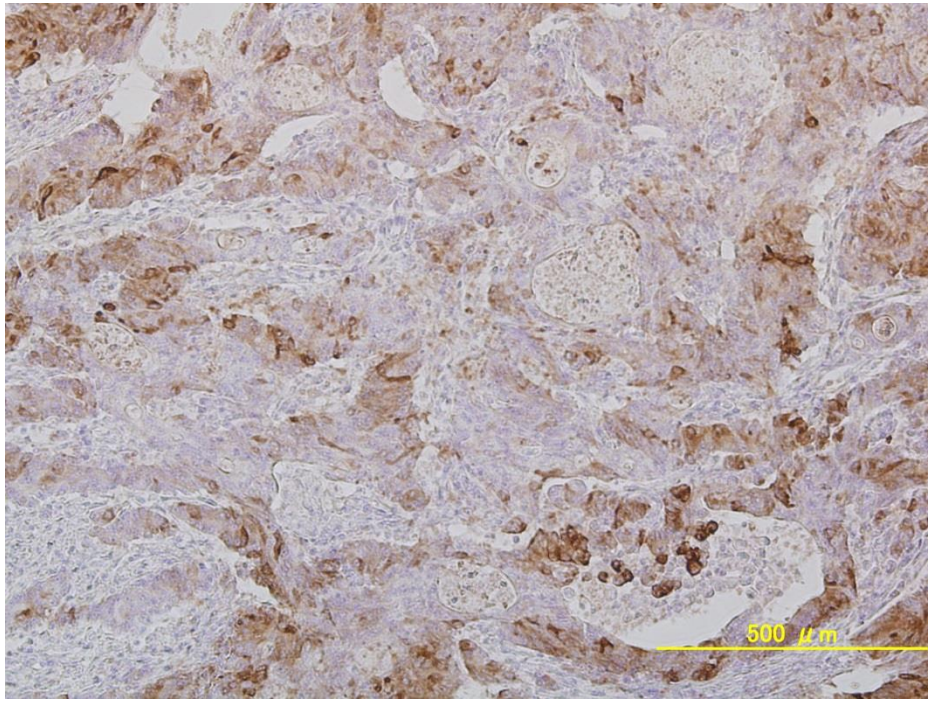
Figure 2. Mean TFF3 staining score of cytoplasm in UCAC tumor cells was significantly lower than that in sporadic rectal cancer cells.

Figure 3. Representative slides of rectal mucosa from patients with ulcerative colitis, comparing expression patterns for mucinous granules and TFF3 in goblet cells. (a–c): TFF3 staining (anti-TFF3 monoclonal antibody in brown, ×100). All patients showed very extensive TFF3 staining in the rectal mucosa. Patient (a) had TFF3 staining of weak intensity; patient (b) had TFF3 staining of medium intensity; and patient (c) had TFF3 staining of strong intensity. (d): Mucinous granules in goblet cells, from serial section of (c) (Mayer's mucicarmine stain, ×100).

Figure 4. In goblet cells in crypts, the mean TFF3 staining score of UCAC was significantly lower than that of UC without UCAC or sporadic cancer.

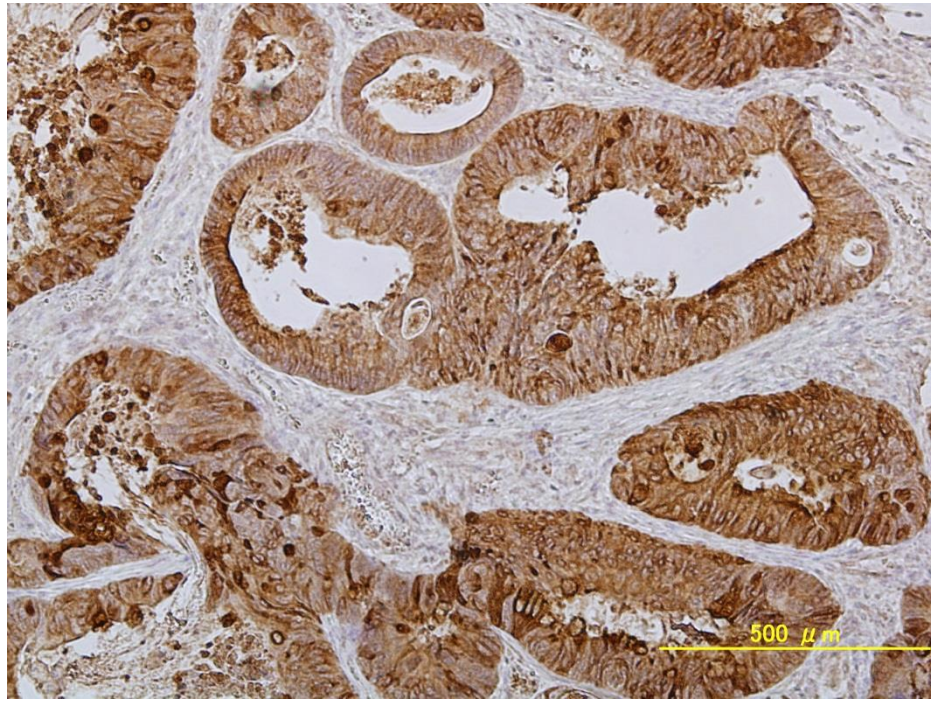
Figure 1

a



UCAC

b



sporadic colorectal cancer

Figure 2

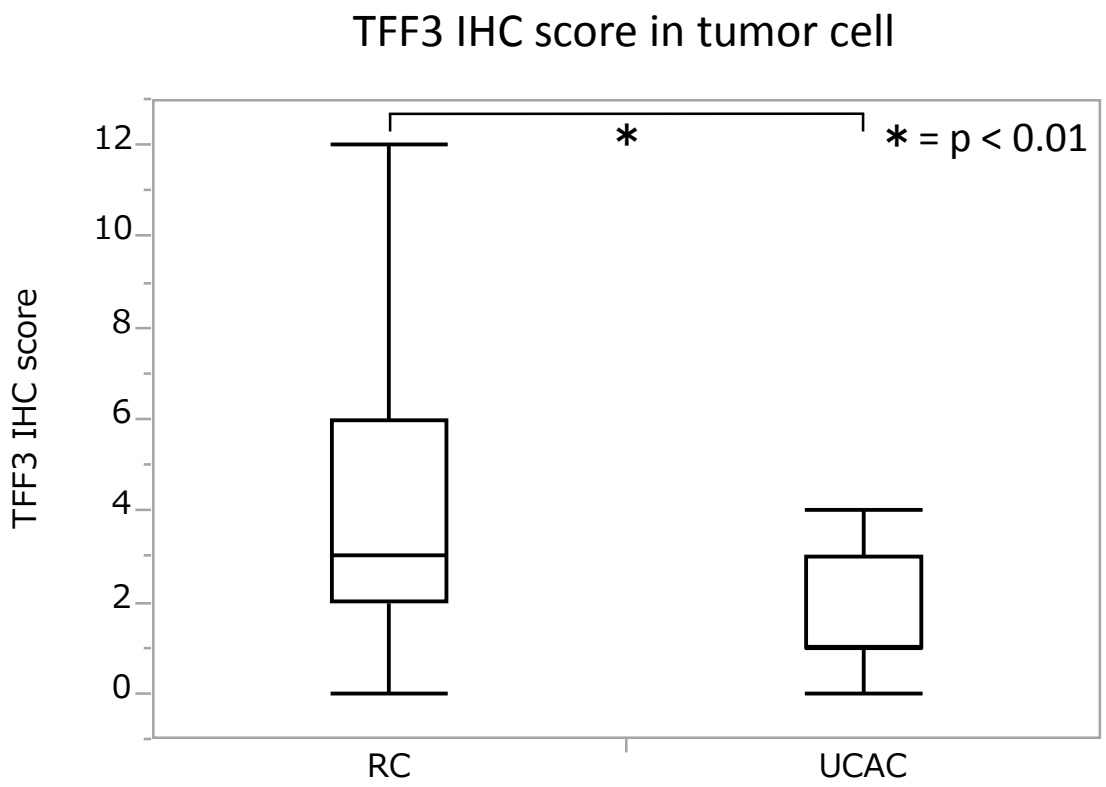
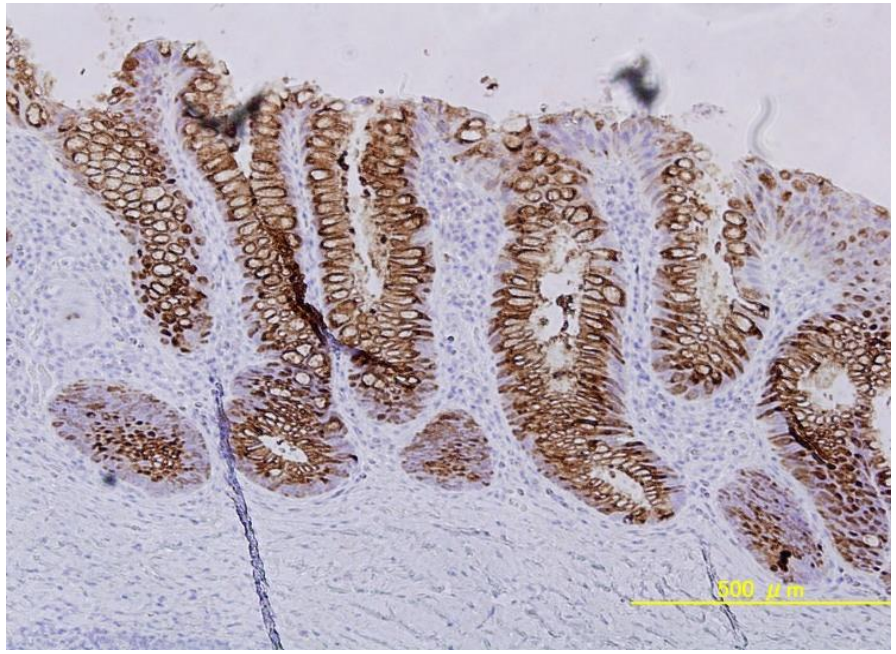
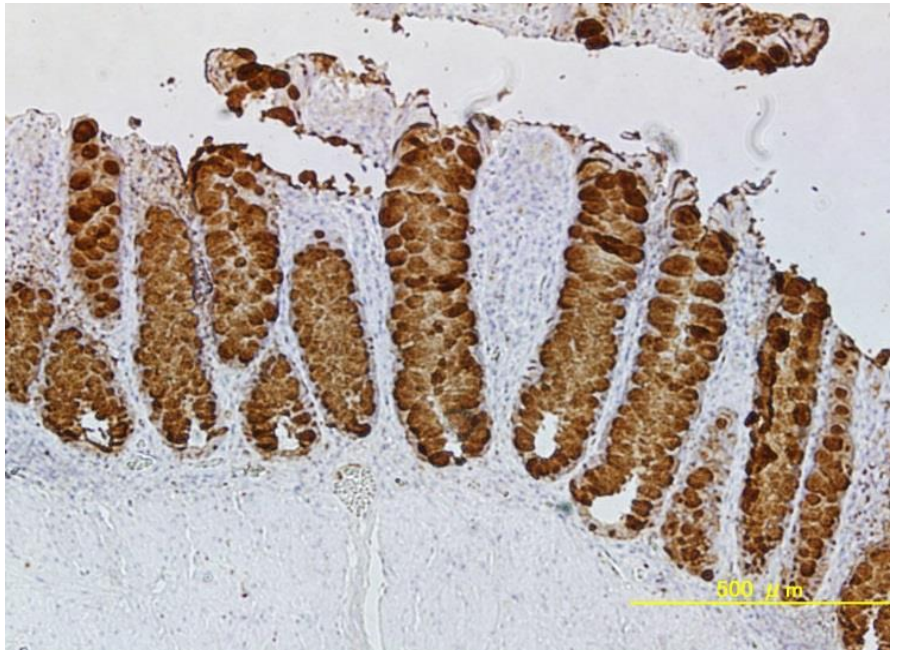

TFF3 IHC score in tumor cell
TFF3 IHC score
12
10
8
6
4
2
0
*
* = p < 0.01
RC
UCAC

Figure 3

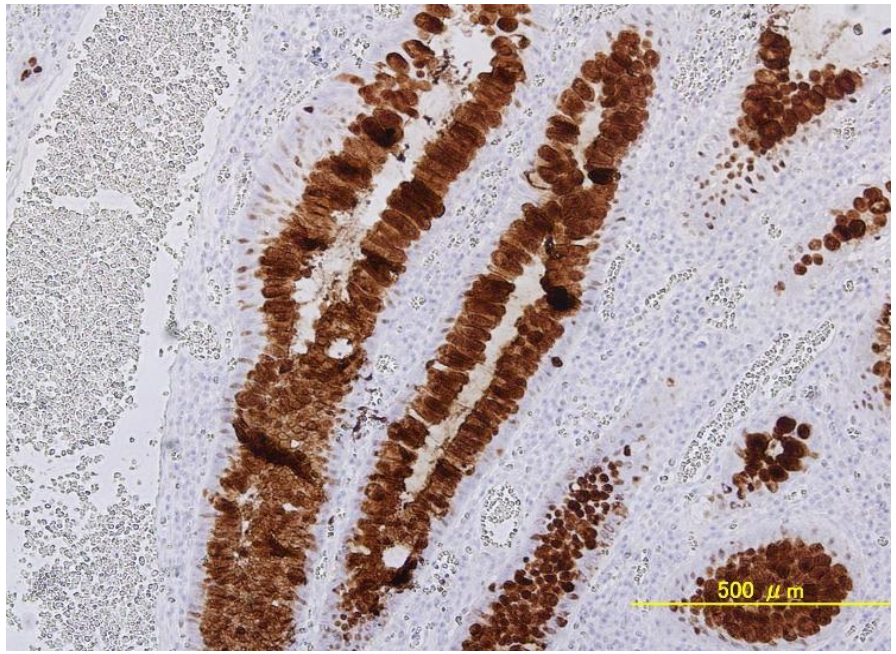
a



b



c



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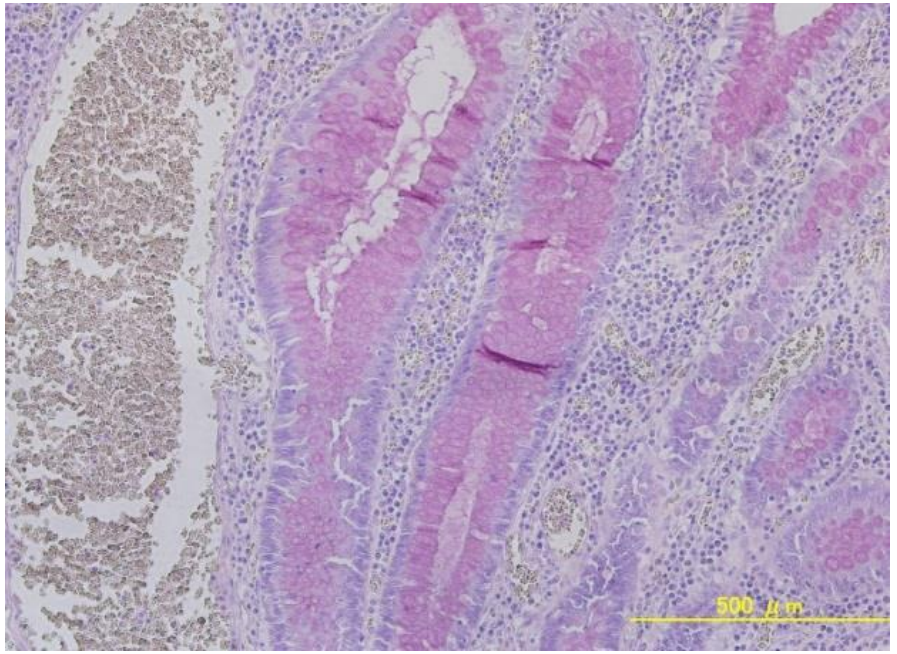

Figure 4

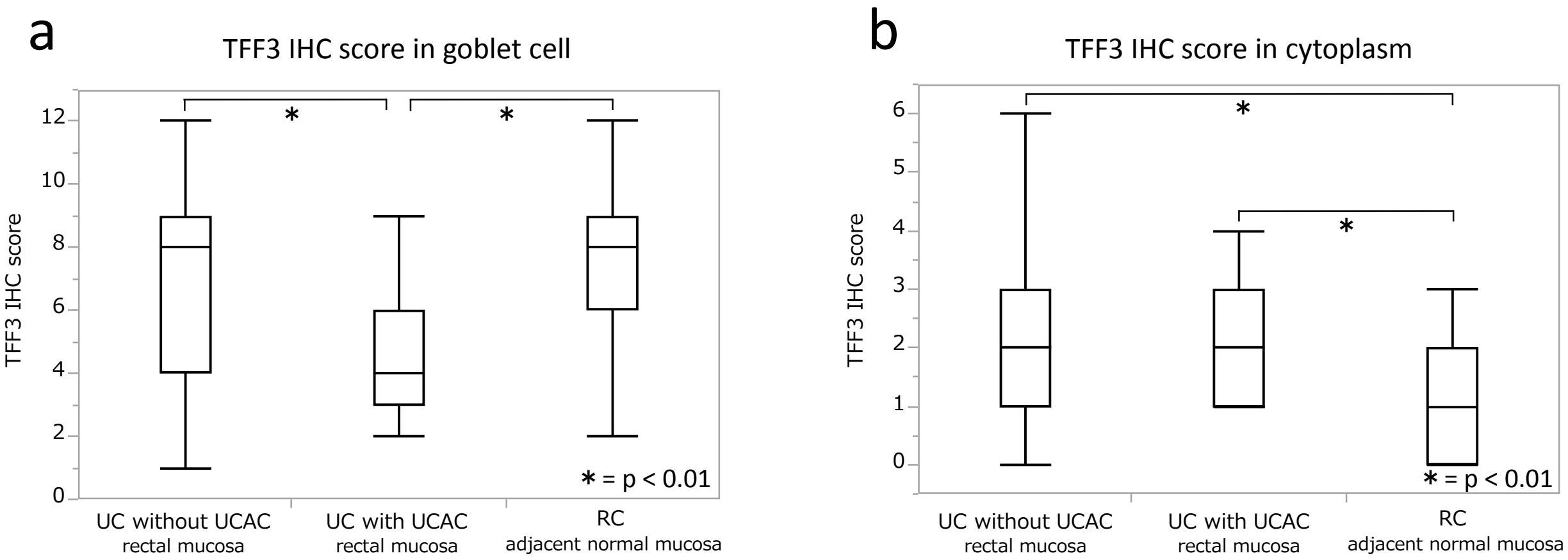
a
TFF3 IHC score in goblet cell
TFF3 IHC score
12
10
8
6
4
2
0
*
*
* = p < 0.01
UC without UCAC
rectal mucosa
UC with UCAC
rectal mucosa
RC
adjacent normal mucosa
b
TFF3 IHC score in cytoplasm
TFF3 IHC score
6
5
4
3
2
1
0
*
*
* = p < 0.01
UC without UCAC
rectal mucosa
UC with UCAC
rectal mucosa
RC
adjacent normal mucosa

Table I. Characteristics of patients with and without UC-associated cancer.

Characteristics	All patients *n* = 145	UCAC *n* = 15	Non-cancer *n* = 130	*P*
Sex, *n* (male/female)	81/64	10/5	71/59	0.37
Age, years (range)	35 (15–82)	48 (28–74)	33 (15–82)	< 0.01*
Age at diagnosis, years (range)	28 (0–82)	29 (17–63)	26 (0–82)	0.12
Disease duration, years (range)	6 (0–37)	12 (0–26)	6 (0–37)	< 0.01*
Degree of inflammation				
Mild, *n* (%)	69 (48)	9 (60)	59 (45)	0.28
Moderate or severe, *n* (%)	76 (52)	6 (40)	71 (55)	

UC, ulcerative colitis; UCAC, ulcerative colitis-associated cancer.

*Statistically significant.

Table II. Characteristics of patients with sporadic cancer.

Characteristics	
Sex, *n* (male/female)	63/55
Age, years (range)	67 (35–89)
T category, *n* (1/2/3/4)	23/12/58/25
Lymph node metastasis, *n* (negative/positive)	64/54
Liver metastasis, *n* (negative/positive)	107/11
Peritoneal carcinomatosis, *n* (negative/positive)	107/11
Distant metastasis, *n* (negative/positive)	99/19
UICC stage, *n* (1/2/3/4)	25/33/34/26

Table III. Correlation of TFF3 expression in the rectal mucosa with clinical outcomes.

Characteristics	n	TFF3 staining score	P
Sex			
Male	81	6.93 ± 3.24	0.88
Female	64	6.88 ± 3.64	
Age, years			
< 34	69	8.06 ± 2.88	< 0.01*
≥ 34	76	5.86 ± 3.53	
Age at UC diagnosis, years			
< 27	70	7.69 ± 2.92	< 0.01*
≥ 27	75	6.17 ± 3.68	
Duration of disease, years			
< 6	67	8.07 ± 3.13	< 0.01*
≥ 6	78	5.90 ± 3.34	
Degree of inflammation			
Mild	68	6.29 ± 3.33	0.04*
Moderate or severe	77	7.44 ± 3.40	
UCAC			
UCAC	15	4.53 ± 2.36	< 0.01*
Non-cancer	130	7.18 ± 3.41	

TFF3, trefoil factor-3; UC, ulcerative colitis; UCAC, ulcerative colitis-associated cancer.

*Statistically significant.

Table IV. Univariate and multivariate analyses of factors that influence UCAC.

Factors	Univariate			Multivariate		
	OR	95% CI	*P*	OR	95% CI	*P*
Sex						
Male vs. female	1.66	0.55–5.58	0.37			
Age, years						
≥ 34 vs. < 34	4.13	1.24–18.7	0.02*	1.83	0.13–18.7	0.62
Age at UC diagnosis, years						
< 27 vs. ≥ 27	2.84	0.92–10.7	0.07*	1.25	0.18–14.6	0.84
Duration of disease, years						
≥ 6 vs. < 6	3.88	1.17–17.6	0.03*	2.45	0.66–11.8	0.19
Degree of inflammation						
Mild vs. moderate or severe	1.81	0.62–5.66	0.28			
TFF3 staining score						
< 7 vs. ≥ 7	6.7	1.76–44.0	< 0.01*	4.32	1.01–29.9	0.05*

CI, confidence interval; OR, odds ratio; TFF3, trefoil factor-3; UC, ulcerative colitis; UCAC, ulcerative colitis-associated cancer.

*Statistically significant.