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Expression of TFF3 during multistep colon carcinogenesis

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Summary. The pathogenesis of colon cancer is not well understood. This common type of cancer is generally believed to occur in a multistep process which involves alterations of various tumor suppressor genes and oncogenes during the progression through benign lesions towards carcinoma. TFF3 is a product of the colonic epithelium and has been implicated in colonic mucosal protection and also in the aggressiveness of colon cancer cells. The aim of this study was to analyze the expression of TFF3 during propagation towards cancer development in the human colon. Colonic tissues representing colitis, adenomatous polyposis, tubulovillous adenoma, and mucoid/adeno-carcinomas were processed for immunohistochemistry using an antibody specific for human TFF3. The results were correlated with those of PCNA-labeling, quantified, and compared with those of control tissues obtained from the safe margin of macroscopically normal colonic mucosa of patients with colon cancer. The data showed marked down-regulation of TFF3 expression in adenomatous polyposis, then TFF3 expression returns to about control level during adenoma and remains high during mucoid- and adeno-carcinomas. Colonic tissues with highly invasive cancer cells were characterized by statistically significant down-regulation of TFF3 expression. The changes observed in expression of TFF3 showed an inverse correlation with cell proliferation and suggest that it might play a protective role against colon carcinogenesis.

Key words: Colon cancer, Trefoil factor, Cell proliferation, Colonic epithelium, Human colon

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Introduction

The colon is lined by a single layer of epithelial cells which invaginates to form numerous simple tubular glands or crypts. The crypt bottoms contain pluripotent stem cells which perpetually undergo proliferation and migration-associated differentiation to produce three main cell lineages: goblet, absorptive and enteroendocrine. Members of these cell lineages are scattered to populate the entire crypts and can be identified by various morphological criteria (Lipkin, 1973; Karam, 1999; Wright, 2000).

Goblet cells are abundant constituents of the colonic crypts. These cells have been long known to secrete a complex mixture of mucin glycoproteins onto the epithelial surface (Neutra and Leblond, 1966; Specian and Oliver, 1991). Another important product of goblet cells is the intestinal trefoil factor or trefoil factor 3, TFF3 (Suemori et al., 1991). It belongs to a family of peptides which are characterized by six cysteine residues forming three disulfide bonds and hence, the three-loop trefoil domain (Thim, 1997). In the gastrointestinal tract, TFF3 is also expressed in the lining epithelium of the human and mouse stomachs (Karam et al., 2004: Kouznetsova et al., 2004).

It has been shown that TFF3 increases the viscosity of mucin gel and also plays an important role in epithelial restitution and migration in the colonic mucosa (Dignass et al., 1994; Babyatsky et al., 1996). TFF3 is rapidly up regulated at the margins of mucosal injury and in various ulcerative conditions suggesting an important role in mucosal defense and repair (Sands and Podolsky, 1996). Indeed, TFF3 deficient mice had poor epithelial colonic regeneration after oral administration of dextran sulfate sodium (Mashimo et al., 1996).

Colon cancer is one of the most frequent types of cancer worldwide (Jemal et al., 2006). It is believed that colon carcinogenesis involves a multistep process starting with normal mucosa, inflammation, through

early and late adenomas ending with invasive carcinoma (Renehan et al., 2002). This adenoma-carcinoma sequence is associated with accumulation of somatic mutations causing activation of several proto-oncogenes and inactivation of tumor-suppressor genes. Mutations in several genes may be necessary for the development of a malignant tumor, whereas fewer changes may suffice for benign lesions formation (Fearon, 1992; Grady, 2005).

Little is known about the role of TFF3 during colon cancer development. In vitro studies by Uchino et al. (1999), Emami et al. (2001) and Yio et al. (2005) suggested that TFF3 might be implicated in the pathogenesis, progression, and aggressiveness of colon cancer.

To elucidate more on the role of TFF3 in colon cancer development, this study was aimed at examining the expression of TFF3 in colonic tissues known to be precancerous or at high risk of developing colon cancer and, therefore, representative of the multistage process of colon carcinogenesis. TFF3 expression was also examined in various colon cancer tissues. The results demonstrate significant changes in TFF3 expression suggesting a crucial role for TFF3 in colon carcinogenesis and progression.

Materials and methods

Human tissues

Colonic mucosal tissues were obtained from informed human subjects (n=34) ranging from 25-50 years of age. Tissues were kindly provided by the Pathology Department, National Cancer Institute, Cairo University. The Institute's Ethical regulations of research on human tissues were followed. Patients were diagnosed with colitis (n=9), benign adenoma (n=11), and adenocarcinoma (n=11). According to the grading system of WHO (Hamilton et al., 2000), most of the adenocarcinomas were of grade II or moderately differentiated (n=9). Only 3 colonic samples were of the more invasive grade III, undifferentiated adenocarcinoma. Colonic tissues obtained from a macroscopically normal area (safe margin) distant from adenocarcinoma were taken as control (n=3). Tissues were fixed in 10% formalin solution and processed for paraffin embedding. For general histology some tissue sections of all samples were stained with hematoxylin and periodic acid Schiff (PAS) to visualize nuclei and carbohydrates, respectively. The three control tissues lacked any histopathological changes.

Immunohistochemical studies

To examine the localization of TFF3 in the human colonic mucosa, tissue sections were deparaffinized in xylene, rehydrated in descending grades of alcohol, and treated with 3% H_2O_2 in methanol to block endogenous peroxidase activity. Sections were incubated with blocking buffer (1% bovine serum albumin, 0.5% Tween-20 in phosphate-buffered saline) and then with the rabbit polyclonal antibody specific for human TFF3. This antibody is directed against the last 18 residues of human TFF3 protein sequence. We have previously characterized this antibody (Karam et al., 2004). For negative controls, the primary antibody was omitted or normal rabbit IgG was used instead of the primary antibody.

To label the dividing epithelial cells and relate their location to the TFF3 expressing cells in the colonic mucosal tissues, adjacent sections of the tissue blocks were processed for incubation with mouse monoclonal anti-proliferating cell nuclear antigen, PCNA (Medical & Biological Lab. Co., MA).

Antigen-antibody binding sites were visualized by incubating sections with peroxidase-labeled goat anti-rabbit or anti-mouse IgG and 3,3'-diaminobenzidine (Sigma, MO) as a color reagent.

Estimation of the expression of TFF3 and PCNA

To provide a numerical assessment of the expression of TFF3 in the probed tissues, the colonic epithelial cells were categorized according to the intensity of immunostaining. The most intense brown stained cells were given a score of 3, intermediate brown staining was scored 2, and light brown staining was scored 1. Unstained cells were given 0 score. In each category, the number of cells was multiplied by the corresponding score. The total score value was divided by the total number of cells examined to obtain an estimate of the average TFF3 expression level in each sample. This was termed the "expression score" which was averaged from 3 samples (n=3) in each group of tissues (control, colitis, polyposis, adenoma, mucoid carcinoma, adenocarcinoma, invasive carcinoma) and expressed as mean ± SD. Means of TFF3 expression scores were compared using student t test. $P<0.05$ was considered statistically significant.

The numbers of PCNA-labeled cells were also estimated in the tissues examined. For each group of tissues, 3 samples were analyzed (n=3). In each sample, the PCNA-labeling index (LI) was estimated by dividing the number of labeled cells times 100 by the total number of cells examined. The LI was expressed as mean ± SD. The student *t* test was utilized to determine the significance of the differences; $P<0.05$ was considered statistically significant.

Results

PCNA and TFF3 expression in normal colonic tissues

Immunohistochemical analysis of control tissues using an antibody specific for PCNA demarcated its expression in the nuclei of epithelial cells lining the lower half of the colonic crypts (Fig. 1A,C). The PCNA-LI averaged 22.5±3.6 (Table 1). These PCNA-labeled cells belonged to the dividing epithelial progenitors and

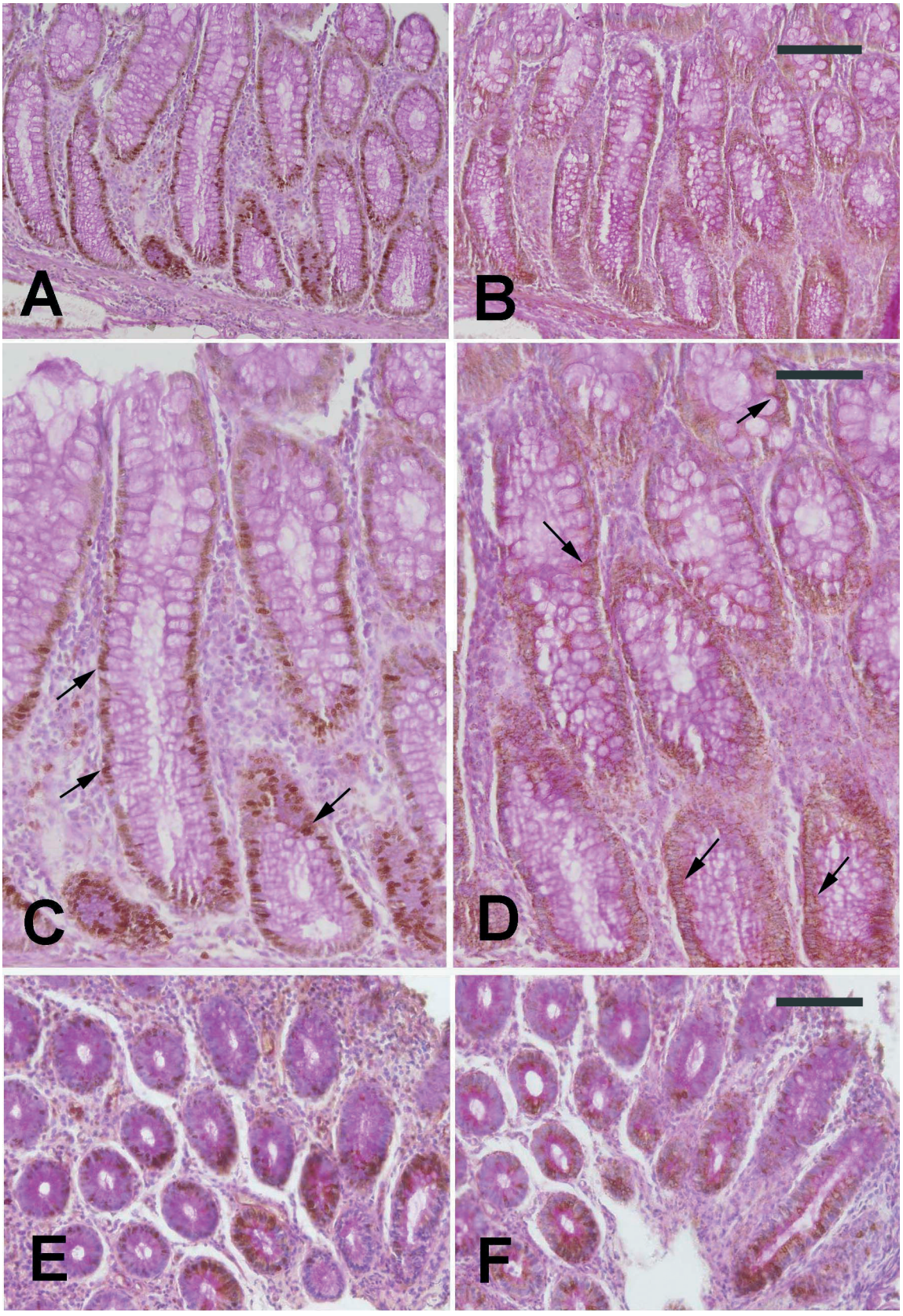

Fig. 1. Immunohistochemical analysis of PCNA (**A, C, E)** and TFF3 (**B, D, F**) in adjacent sections of the colonic mucosae of control (**A-D**) and colitis (**E, F**) tissues. Antigen-antibody binding sites are visualized with DAB which appear brownish. Tissue sections are counterstained with PAS and hematoxylin. **C and D** are montages of a close up view of some crypts seen in **A and B**. In control tissues, PCNA labeling (arrows in **C**) is evident in the nuclei of epithelial cells lining the lower portion of the colonic crypts of both control and colitis tissues. TFF3 labeling is seen in the supra- and peri-nuclear cytoplasm (arrows in **D**) of epithelial cells in both the basal and luminal portions of the colonic crypts. In tissues with colitis (**E and F**), note the absence of surface (inter-crypt) epithelial cells and massive infiltration of the lamina propria with lymphoid cells. PCNA (**E**) and TFF3 (**F**) labeling are evident mainly at the basal portions of the crypts. Bar: A, B, E, F, 80 μm; C, D, 40 μm.

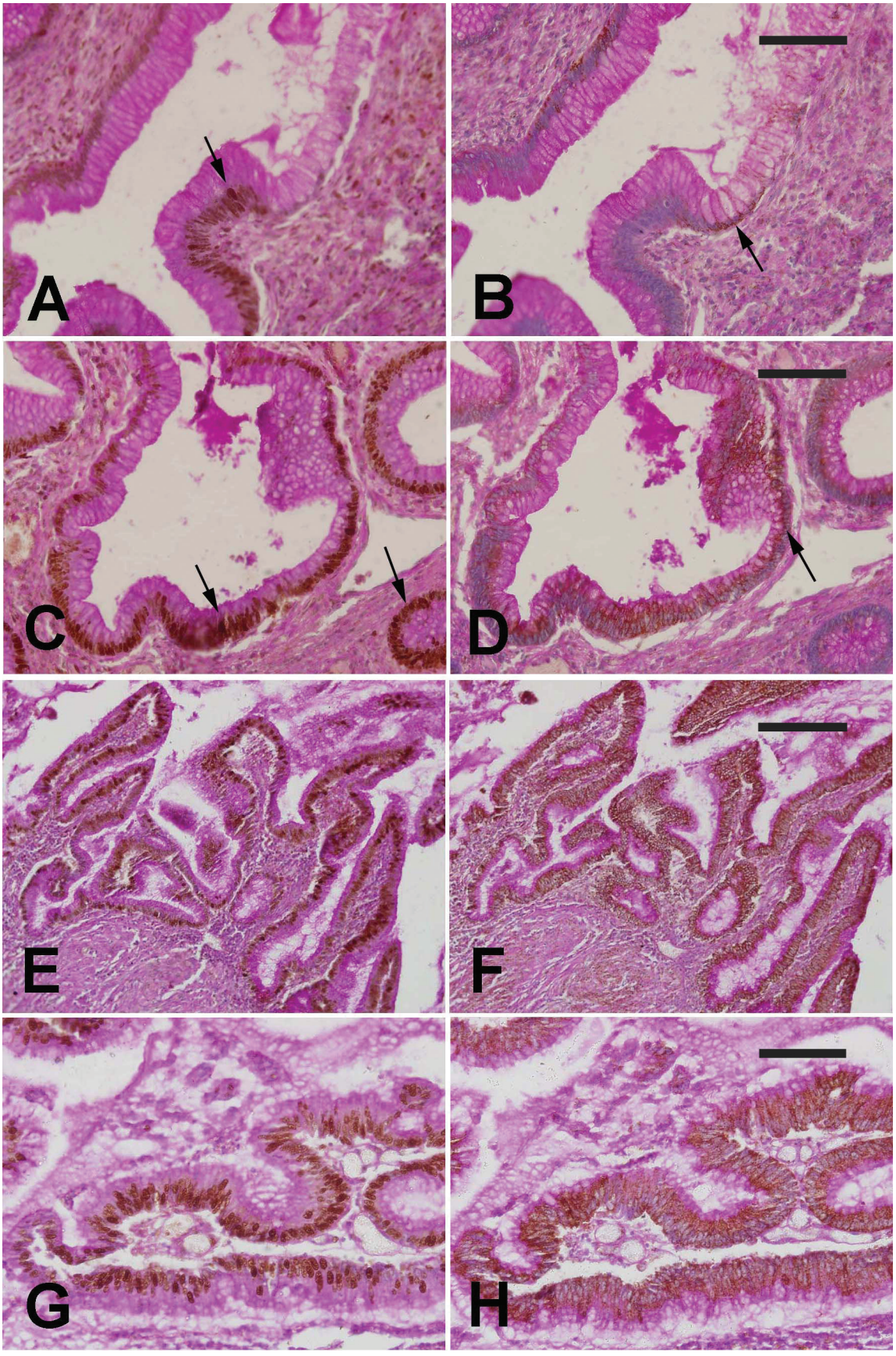

Fig. 2. Immunohistochemical analysis of PCNA and TFF3 in benign adenomas. In juvenile adenomatous polyposis **(A-D),** many PCNA labeled cells are detected (arrows in **A and C**). Some TFF3 labeled cells are seen in **B** and **D** (arrows). In tubulovillous adenoma (**E-H**), almost all lining epithelial cells are PCNA-labeled (**E, G**), and many show supranuclear TFF3 labeling (**F, H**). Bar: A-D, G, H, 40 μm; E, F, 80 μm

the differentiating absorptive and goblet (oligo-mucous) cells which maintained some capacity for mitosis.

Probing of adjacent sections of the control tissues with the antibody specific for human TFF3 revealed an epithelial pattern of its cellular distribution similar to that of PCNA. However, TFF3 was expressed mainly in the supranuclear cytoplasm (Fig. 1B,D). Also, the epithelial cells labeled with anti-TFF3 antibodies were present throughout the upper and lower portions of the crypts. A decreasing gradient of TFF3 labeling was demonstrated with the highest level of expression at the bottom of the crypts (Fig. 1D). Analysis of the labeling intensity in control samples obtained from 3 individuals revealed that the TFF3 expression score was 159.3±13.6 (Table 2).

PCNA and TFF3 expression in benign colonic tissues

During colitis, with abundant inflammatory cells in the lamina propria and loss of integrity of the luminal inter-crypt epithelial cells, a slight increase was detected in the PCNA-LI and the average TFF3 expression score (Fig. 1E,F). They were estimated at 26.5±4.1 and 162±3.0, respectively (Tables 1, 2). The differences between PCNA labeling and TFF3 expression in control and colitis tissues were not statistically significant (p=0.27 and 0.76, respectively).

In benign colonic tumors, such as juvenile polyposis coli, many proliferating cells were intensely labeled with PCNA, whereas TFF3-positive cells were highly variable (Fig. 2A-D). Measurements showed that PCNA-LI and TFF3 expression score averaged 65.6±5.5 and 84.3±6.1, respectively (Tables 1, 2). Therefore, while PCNA labeling was increased very significantly as compared with that of control tissues (p<0.001), TFF3 expression score was highly reduced (p<0.001).

In the colonic tissues with tubulovillous adenoma, the nuclei of most of the surface epithelial cells were stained positive for PCNA (Fig. 2E,G) and the LI was 62.8±6.1, which was highly significant as compared to that of control tissues, p<0.001 (Table 1). Similarly, immunoprobing of adjacent tissue sections revealed that most of the epithelial cells expressed TFF3 in their supranuclear cytoplasm (Fig. 2F,H). The expression score averaged 144.3±10.0 which was not significantly different from that of control tissues, p=0.20 (Table 2).

PCNA and TFF3 expression in malignant colonic tissues

In malignant lesions, such as mucoid carcinoma, PCNA-positive epithelial cells were abundant in the glandular profiles which were surrounded with PAS-positive mucoid secretions (Fig. 3A,C). The LI was much higher than that of control (p<0.001) and averaged

Table 1. PCNA labeling indices (LI) in control colonic mucosal tissues distant from cancer and in different pathological colonic tissues varying from colitis to invasive carcinoma. Each line represents data from one patient.

	No. of cells examined	PCNA-labeled cells	LI	p value
Control	1806	409	22.6	
	1749	454	26.0	
	1946	368	18.9	
			22.5±3.6	
Colitis	2016	535	26.5	
	1967	441	22.4	
	1540	470	30.5	
			26.5±4.1	p=0.27
Juvenile polyposis coli	2898	1953	67.4	
	2456	1460	59.4	
	1955	1366	69.9	
			65.6±5.5	p<0.001
Tubulovillous adenoma	3994	2572	64.4	
	2456	1670	68.0	
	3873	2172	56.1	
			62.8±6.1	p<0.001
Mucoid carcinoma	2541	1575	62.0	
	2322	1320	56.8	
	2766	1464	52.9	
			57.2±4.6	p<0.001
Adenocarcinoma	3017	2079	68.9	
	2414	1755	72.7	
	2049	1160	56.6	
			66.0±8.6	p<0.001
Invasive carcinoma	1537	1149	74.8	
	1230	805	65.4	
	989	870	88.0	
			76.1±11.4	p<0.01

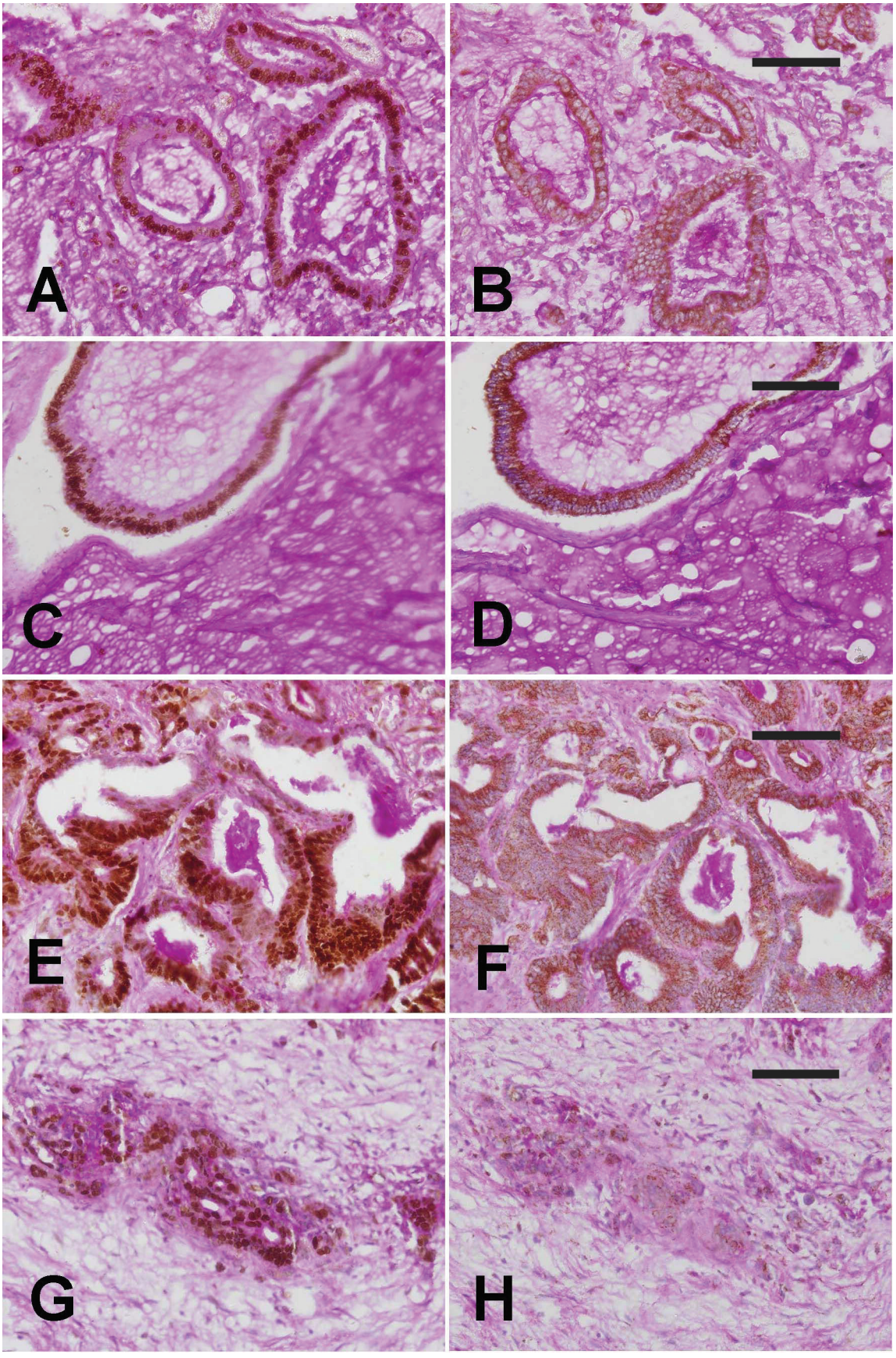

Fig. 3. PCNA and TFF3 labeling in mucoid carcinoma **(A-D)** and adenocarcinoma (**E-H**) of the colon. In mucoid carcinoma, note the abundant PAS-positive mucoid material inside and around the cancerous glandular epithelial profiles. Many PCNA-labeled cells are seen in **A and C**. Similarly, TFF3 labeled cells are numerous in **B and D**. In adenocarcinoma, PCNA-labeled cancer cells are abundant, and TFF3 labeling is mainly seen in the supranuclear cytoplasm (**F**). In areas with invasive cancer cells, PCNA labeling is prominent (**G**), whereas TFF3 is down-regulated **(H)**. Bar: 40 µm.

57.2±4.6 (Table 1). These glandular epithelial cells also exhibited positive supranuclear immunostaining specific for TFF3 (Fig. 3B,D). Even though the expression score reached the highest level, which was 175.3±4.0 (Table 2), it was not significantly different from that of control, p=0.12. In the more common type of malignant lesions, adenocarcinoma, when we examined the tumor parts where the glandular structure was dysplastic, there were numerous PCNA-positive cells (Fig. 3E). The LI averaged 66.4±8.6 which was significantly higher than that of control, p=0.001 (Table 1). In addition, TFF3 was slightly up-regulated in the malignant cells forming these dysplastic glandular profiles (Fig. 4F). The expression score, 137.7±8.6 (Table 2), was not significantly different from that of control tissue, p=0.06. However, analysis of the more invasive grade III adenocarcinoma where colonic cancer cells have totally lost their glandular pattern (Hamilton et al., 2000) showed an apparent down-regulation in TFF3 expression (Fig. 3H) and were intensely labeled with anti-PCNA antibodies (Fig. 4G). The PCNA-LI averaged 76.1±11.4 and their TFF3 expression score was 69.7±10.7 (Tables 1, 2). These values were significantly different (p<0.01) from those of control tissues.

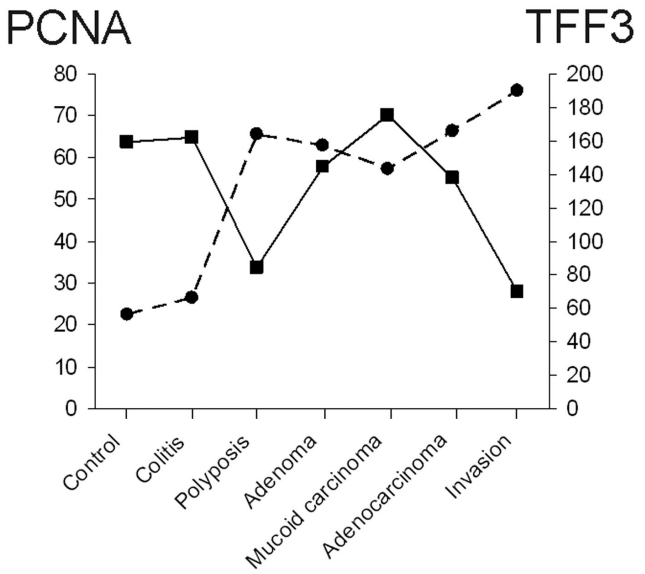

Fig. 4. The pattern of PCNA labeling indices (dashed line) and TFF3 expression scores (solid line) in colonic tissues of control, colitis, and various benign and malignant lesions. Note the significant down-regulation of TFF3 expression in tissues showing polyposis and invasive carcinoma. PCNA labeling is significantly increased in all benign and malignant lesions as compared to control.

Table 2. Intensity of TFF3 expression in control colonic tissues and in various pathological colonic tissues. Each line represents data from one patient.

	No. of cells examined	Total score	Expression score	p value
Control	246	396	161	
	246	424	172	
	193	279	145	
			159.3±13.6	
Colitis	240	396	165	
	234	378	162	
	157	249	159	
			162.0±3.0	p=0.76
Juvenile polyposis coli	276	219	79	
	303	251	83	
	343	311	91	
			84.3±6.1	p<0.001
Tubulovillous adenoma	380	564	148	
	441	587	133	
	452	688	152	
			144.3±10.0	p=0.20
Mucoid carcinoma	242	414	171	
	287	514	179	
	327	574	176	
			175.3±4.0	p=0.12
Adenocarcinoma	286	413	144	
	326	433	133	
	376	513	136	
			137.7±5.7	p<0.06
Invasive carcinoma	290	168	58	
	185	134	72	
	245	194	79	
			69.7±10.7	p<0.01

Discussion

In the normal colonic epithelium, TFF3 is expressed prominently in goblet cells and also in some absorptive columnar cells (Podolsky et al., 1993). It has been shown that TFF3 contributes to the physiological protection of the colonic mucosa. It acts as a potent motogen and has been shown to accelerate migration of colonocytes (Dignass et al., 1994). Moreover, TFF3 knockout mice were more susceptible to intestinal mucosal damage than the normal mice (Mashimo et al., 1996). Recently, *in vitro* studies showed that TFF3 was implicated in the invasiveness of colon cancer cells (Emami et al., 2004; Yio et al., 2005). However, little is known concerning the role of TFF3 in the pathogenesis of colon cancer *in vivo*.

The present study demonstrates the changes that occur in TFF3 expression as compared to normal control tissues, in a series of colonic tissues that are precancerous or at high risk of colon cancer development, such as ulcerative colitis, polyposis coli, and tubulovillous adenoma. In addition, TFF3 expression was examined in different colon cancer tissues: mucoid carcinoma, adenocarcinoma, and grade III invasive adenocarcinoma. Macroscopically normal colonic tissues obtained from the safe marginal areas of colon cancer patients are taken as control.

In the control tissues, in addition to TFF3 expression in mucus-secreting goblet cells and absorptive columnar cells, it is also found in the bottom of the colonic crypts which includes the epithelial stem cells and their immediate descendants. This finding was not described earlier in normal colonic crypts and, therefore, could reflect some of the molecular changes of the colonic epithelium that formed the background for cancer development. This also might explain the up-regulation of TFF3 observed in the bottom of the colonic crypts during colitis, which could then be viewed as an early change in the multistep process of colon carcinogenesis. This is not unexpected since patients with colitis are considered to be at high risk of developing colon cancer (Seidelin, 2004; Ullman, 2005).

An interesting aspect in the expression of TFF3 is its highly significant down-regulation during juvenile polyposis coli. The lowest level of TFF3 expression score occurs during this polyposis stage. The amount of protein is about one-half of that in control tissues.

In colonic tubulovillous adenoma, the expression of TFF3 is not significantly different from that of control, but the PCNA-LI is significantly elevated.

When colitis and adenomas are left without repair, major biological consequences may develop. Development of colitis and lack of an adequate repair system, as exemplified by our observation concerning expression of TFF3, may be an important contributor to the propagation of the carcinogenic cascade in the colon. It has been shown that the expression of TFF3 is up-regulated in tissues with colonic adenocarcinoma (Taupin et al., 1996; Efstathiou et al., 1998). In the present study, the expression of TFF3 in adenocarcinoma is confirmed. However, examination of colonic tissues with more invasive grade III adenocarcinoma shows a significant down regulation in the expression of TFF3.

That the reduction in TFF3 is an early event in colon carcinogenesis, during the polyposis stage, makes it less likely that it is simply an epiphenomenon of an already altered genotype. This notion is supported by evidence that changes in TFF3 can play a role in invasion of colon cancer cells (Rivat et al., 2005; Yio et al., 2005).

Finally, it is interesting to note that the expression of TFF3 and PCNA shows some inverse correlation (Fig. 4). The reduction of TFF3 expression in the polyposis stage is associated with a significant increase in the number of PCNA-labeled cells as expected from mucosal growth and polyp formation. The down-regulation of cellular TFF3 and its association with increased PCNA labeling in polyposis stage may suggest an initial role for TFF3 in the alteration of cell proliferation and progression toward carcinogenesis. This inverse correlation between PCNA and TFF3 expression is also observed in the highly invasive tumors, once again suggesting that *in vivo* TFF3 is not required for increased cell proliferation, but rather required for cell differentiation and maintaining glandular structure.

In conclusion, our findings show that human colonic carcinogenesis is associated, at an early stage and during invasion, with down-regulation of TFF3 expression. The features of these changes in human colon diseases and the biological role of TFF3 in maintaining normal mucosal cell proliferation and differentiation showed previously *in vitro* or in experimental in vivo models suggest that its under-expression can be crucial to the development of colon cancer cells and their aggressive behavior.

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