FOXP3 and CEACAM6 expression and T cell infiltration in the occurrence and development of colon cancer

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Abstract. The transcription factor forkhead box P3 (FOXP3) is involved in immune cell regulation, and carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) is an adhesion molecule of the immunoglobulin superfamily. These two genes are associated with cancer progression. In the current study, colon tissue specimens from 78 cases of colon cancer (including 40 of stage I-II and 38 of stage III-IV), 30 cases of colonic adenoma and 12 healthy controls were collected from the First Affiliated Hospital of Soochow University between January 2010 and December 2011. The expression of cluster of differentiation (CD) 3, CD4, CD8, CD45RO, CEACAM6 and FOXP3 in colon tissues was examined by immunohistochemical analysis. In addition, a reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay, based on SYBR Green I, was used to detect CD3, CD4, CD8, CD45RO, CEACAM6 and FOXP3 mRNA levels in the paraffin block specimens. $CD3^+$, $CD8^+$ and $CD45RO^+$ T cell infiltrations in colonic adenoma were significantly higher than in normal colonic mucosa ($P<0.001$, $P=0.001$ and $P<0.001$, respectively). However, $CD3^+$, $CD8^+$ and $CD45RO^+$ lymphocytes in stage III-IV colon cancer tissues were lower than in normal control tissues ($P=0.015$, $P=0.002$ and $P=0.041$, respectively); consistently, $CD3^+$, $CD4^+$, $CD8^+$ and $CD45RO^+$ lymphocytes in stage III-IV tissues were even more markedly lower compared with adenoma ($P=0.001$, $P<0.001$, $P<0.001$ and $P<0.001$, respectively). Similarly, $CD3^+$, $CD8^+$ and $CD45RO^+$ T cell infiltration was lower in stage I-II cancer tissues compared with adenoma ($P=0.001$, $P<0.001$ and $P<0.001$). $CD3^+$, $CD4^+$, $CD8^+$ and $CD45RO^+$ T cell infiltrations were also significantly higher in stage I-II compared with stage III-IV cancer tissues ($P<0.001$, $P=0.045$, $P<0.001$ and $P<0.001$, respectively). CEACAM6 was found to gradually increase from normal colon tissue to adenoma and cancer tissue. FOXP3 was expressed more highly in stage I-II compared with normal tissues ($P=0.014$), and was even higher in stage III-IV ($P<0.001$). These results were verified using RT-qPCR, which yielded almost identical results. In summary, the current study demonstrates that FOXP3, CEACAM6 and T cell infiltration are significantly associated with the occurrence and progression of colon cancer, and that immune reactions vary between different stages of colon cancer development.

Introduction

Colon cancer is one of the most prevalent malignant tumors and a leading cause of cancer-associated mortality worldwide, with significant morbidity (1,2). However, the molecular pathogenesis of colon cancer remains poorly understood. Previous studies have suggested that various mechanisms contribute to the progression of colon cancer, including mutations in components of cell cycle or apoptotic pathways, and the processes of signal transduction, angiogenesis, invasion and metastasis (3-6). Recently, growing evidence indicates that immune mechanisms, including infiltrating immune cell activation, immunosurveillance and immunosuppressive pathways, are crucial in colon cancer progression (7).

Recently, research has focused on the study of immune infiltrates, tumor-infiltrating lymphocytes (TILs), which include cluster of differentiation (CD) 3^+, $CD4^+$ and $CD8^+$ T cells, natural killer cells and myeloid cells; these are widely accepted to be the most promising T cell subsets with regard to their effective antitumor response (8,9). In a study on 959 specimens of resected colorectal cancer, Pagès *et al* (10) suggested that T cell activation in colorectal cancer is associated with the absence of pathological evidence of early metastatic invasion

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and with prolonged survival. Another study indicated that $CD8^+$ T cells expressed in colorectal cancer have an important role in antitumor immune responses (11). Furthermore, $CD45RO^+$ T cell densities have been demonstrated to correlate with tumor invasion, disease stage, lymph node metastasis and long-term disease-free survival (12).

The transcription factor forkhead box P3 (FOXP3) is a specific nuclear marker for regulatory T cells (Tregs), which are able to suppress immune responses to tumor antigens, thus maintaining immunological tolerance and contributing to tumor metastasis (13). Accumulating evidence suggests that high levels of tumor-infiltrating Tregs were associated with a poor prognosis in certain types of solid tumor, including oesophageal, pancreatic, breast, hepatocellular and ovarian cancers (14-18). Given the central contribution of FOXP3 to tumor cells, it may represent a novel mechanism by which cancer are able to suppress the immune system to escape destruction.

Carcinoembryonic antigen-related cell adhesion molecule (CEACAM) 6, also known as CD66c or NCA-50/90, is a member of the carcinoembryonic antigen family and an adhesion molecule of the immunoglobulin superfamily (19). It is able to inhibit anoikis (20), increase the ability of tumor invasion, and contribute to promoting tumor occurrence, development, invasion and metastasis (21,22). A growing number of studies have demonstrated that it is widely expressed in numerous types of malignant human tumors, including breast carcinoma, ovarian cancer, gastric carcinoma, colorectal cancer, lung adenocarcinoma and pancreatic cancer (23-28).

In the present study, the variations in T cell infiltration and FOXP3 and CEACAM6 expression levels between normal colonic mucosa, colonic adenoma, and stage I-II and stage III-IV colon cancer were analyzed. The aims were to investigate the immune reaction in different stages of colon cancer development and to explore the roles that FOXP3, CEACAM6 and various T-cell subsets serve in the occurrence and progression of colon cancer.

Materials and methods

Patient specimens. Tissue specimens from 78 cases of colon cancer (including 40 cases of stage I-II and 38 cases of stage III-IV) and 30 cases of colonic adenoma were collected from the patients who had been treated in the First Affiliated Hospital of Soochow University (Suzhou, China) between January 2010 and December 2011. In addition, 12 healthy colon tissue specimens from patients who underwent colonoscopy as part of colon cancer screening were collected as a normal control group. None of the patients had undergone radiotherapy, chemotherapy or immunotherapy previously. The specimens of colon cancer and adenoma were obtained during surgery and fixed in 10% neutral formalin. Postoperative pathology confirmed colonic carcinoma or colonic adenoma, and tumor-node-metastasis classification and differentiation grading for colon cancer were determined according to the criteria described by the Union for International Cancer Control (29). Tubular, mixed and villous adenoma were all classified into the adenoma group. The study was approved by the research ethics committee of the First Affiliated Hospital of Soochow University, and agreed by each patient with written consent.

Immunohistochemical staining for CD3, CD4, CD8, CD45RO, CEACAM6 and FOXP3. According to routine procedures, specimens were formalin-fixed and paraffin-embedded (FFPE); 4-μm sections were subsequently cut, dried, de-waxed and rehydrated. The antigens were retrieved by incubation in sodium citrate buffer solution (Dako, Glostrup, Denmark) at pH 6.0 and heating in a high-pressure cooker, before being naturally cooled to room temperature. The tissue slides were blocked for 10 min at room temperature (25˚C), washed with phosphate-buffered saline (PBS), then incubated with the following anti-human antibodies at room temperature (25˚C) for 2 h in moist dark chambers: Monoclonal mouse IgG1 against CD3 (#sc-20047), polyclonal rabbit IgG against CD4 (#sc-7219) and CD8 (#sc-7188; all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA; dilution, 1:100); monoclonal mouse IgG2a against CD45RO (#ab86080; dilution, 1:10), monoclonal rabbit IgG against CEACAM6 (#ab134074; dilution, 1:400) and monoclonal mouse IgG3 against FOXP3 (#ab450; 1:50 dilution; all from Abcam, Cambridge, MA, USA). The sections were subsequently washed with PBS and incubated for 1 h in moist dark chambers at room temperature (25˚C) with polyclonal goat anti-mouse/rabbit IgG biotinylated secondary antibodies (#K5007; dilution, 1:2,000; Dako). Finally, the sections were developed with 3,3'-diaminobenzidine tetrahydrochloride hydrate (Dako) and counterstained with hematoxylin (AppliChem GmbH, Darmstadt, Germany). Human tonsillar tissue was used as a positive control, and the negative control was created by omitting primary antibodies.

Scoring system for immunohistochemistry. The expression levels of CD3, CD4, CD8, CD45RO, CEACAM6 and FOXP3 were scored using a semi-quantitative system (30). For each section, the staining intensity was scored as 0 (achromatic), 1 (light yellow), 2 (brownish yellow), or 3 (brown). In addition, the percentage of positive cells was scored as 0 (<5%), 1 (5-25%), 2 (26-50%), 3 (51-75%), or 4 (>75%). The two scores were added together and the specimens were assigned to one of four levels as follows: (-), score 0-1; (+), score 2; (++), score 3-4; or (+++), score ≥5. (-) and (+) were defined as negative expression. In each specimen, 5 randomly selected high-power fields (magnification, x400) were assessed using the Olympus BX53 microscope (Olympus, Tokyo, Japan), avoiding necrotic areas. The specimens were scored by two pathologists independently and the scoring results were strongly consistent.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Purification of total RNA from FFPE tissue sections was performed using an RNeasy FFPE Kit (#73504; Qiagen, Inc., Valencia, CA, USA). Initially, all paraffin was removed from freshly cut FFPE tissue sections by treating with deparaffinization solution (Qiagen, Inc.). Next, samples were incubated in an optimized lysis buffer, containing proteinase K, to release RNA from the sections. A short incubation at 80˚C partially reversed formalin cross-linking of the released nucleic acids. This was followed by deoxyribonuclease treatment that was optimized to eliminate all genomic DNA. Next, the lysate was mixed with Buffer RBC (provided with

Table I. Sequence of the primer pairs used for reverse transcription-quantitative polymerase chain reaction.

Gene name	GenBank accession number	Sequence	Product size (bp)
CD3	NM_000732.4	F, 5'-GGGAGTCTTCTGCTTTGCTG-3' R, 5'-TTGTTCCGAGCCCAGTTTC-3'	153
CD4	NM_000616.4	F, 5'-GTGAACCTGGTGGTGATG-3' R, 5'-GAGACCTTTGCCTCCTTG-3'	122
CD8	NM_001768.6	F, 5'-CTGGTGTGAATTGAAGGCTGT-3' R, 5'-GCTGCTGACCTCATTCTTCC-3'	101
CD45RO	NM_002838	F, 5'-TCTGCTGGAACTGACACG-3' R, 5'-CTCATTAACATTTAGCTTTG-3'	168
CEACAM6	BC005008.1	F, 5'-TCCAGCAATCCACACAAGAG-3' R, 5'-R 5-GGACAGGAGCACTTCCAGAG-3'	144
FOXP3	NM_014009.3	F, 5'-TCCCAGAGTTCCTCCACAAC-3' R, 5'-ATTGAGTGTCCGCTGCTTCT-3'	122
β-actin	NM_001101.3	F, 5'-CACTGTGCCCATCTACGAGG-3' R, 5'-AATGTCACGCACGATTTCC-3'	154

CD, cluster of differentiation; CEACAM6, carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen); FOXP3, forkhead box P3; F, forward; R, reverse.

Table II. Patients characteristics (n=120).

Group	n	Age, years		Gender, n		Localization, n	
		Mean ± SD	Range	Male	Female	Left colon	Right colon
Normal controls	12	62.4±14.9	34-83	7	5	6	6
Colonic adenoma	30	58.5±12.9	38-84	16	14	13	17
Stage I-II	40	62.5±12.6	33-83	20	20	18	22
Stage III-IV	38	60.9±13.7	24-85	18	20	13	25

Analysis of variance was used for the comparison of multiple sets of mean ages. There was no difference between the age, gender or tumor localization between the groups (P>0.05). SD, standard deviation.

the RNeasy kit). Ethanol was added to provide appropriate binding conditions for RNA, and then the samples were applied to the provided RNeasy MinElute spin columns, in which the total RNA bound to the membrane and contaminants were washed away. RNA was then eluted in a minimum of 14 μl of RNase-free water. cDNA was subsequently synthesized from the total RNA using RevertAid™ First Strand cDNA Synthesis kit (#K1622; Thermo Fisher Scientific, Pittsburgh, PA, USA), according to the manufacturer's instructions. mRNA levels were quantified by RT-qPCR using a FastStart Universal SYBR Green Master (Rox) kit (#4913914001; Roche Diagnostics, Basel, Switzerland). The PCR cycling conditions were as follows: 1 cycle of starter template degeneration at 95˚C for 1 min; and 45 cycles of template degeneration at 95˚C for 20 sec, annealing at 58˚C for 30 sec and extension at 68˚C for 45 sec. β-actin was used as an internal reference. Three independent experiments were to analyze relative target gene expressions. The expression of RNA was quantified by quantification cycle (Cq) values and normalized by the $2^{-\Delta\Delta Cq}$ method relative to β-actin (31). All primers were supplied by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China) and are shown in Table I.

Statistical analysis. Continuous data is presented as the mean ± standard deviation, and multiple sets of mean values were compared using analysis of variance. The differences in the scores between the groups were analyzed by the non-parametric Wilcoxon Rank Sum test. All statistical analyses were performed using SPSS software, version 18.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate statistically significant differences, and all P-values were two-tailed.

Results

Patient characteristics. A total of 120 patients were included in the study, comprising 40 patients with stage I-II cancer, 38 patients with stage III-IV cancer, 30 patients with colonic adenoma and 12 normal controls. The stage I-II group contained 20 males and 20 females, with a mean age

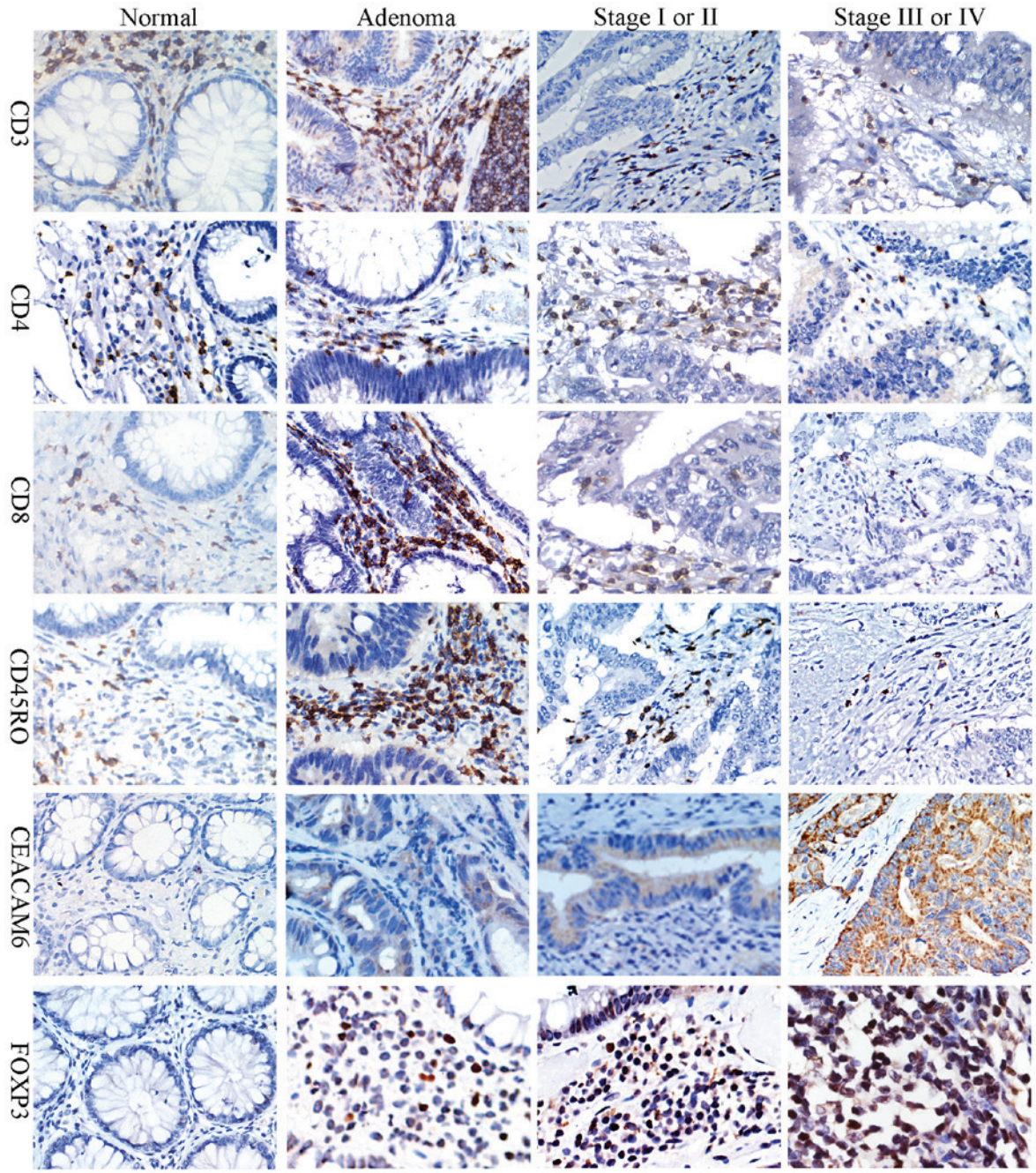

Figure 1. Representative images of CD3, CD4, CD8, CD45RO, CEACAM6 and FOXP3 staining by immunohistochemistry in normal colonic tissue, colonic adenoma and colon cancer. Images show 3,3'-diaminobenzidine tetrahydrochloride hydrate staining of positive cells. CD3, CD4, CD8 and CD45RO were immunolocalized to the membrane, CEACAM6 in the cytoplasmic and FOXP3 in the nucleus. All images were taken at x400 scanning magnification. CD, cluster of differentiation; CEACAM6, carcinoembryonic antigen-related cell adhesion molecule 6; FOXP3, forkhead box P3.

of 62.5 years; the stage III-IV group comprised 18 males and 20 females with a mean age of 60.9 years. The mean ages of the colonic adenoma patients and normal controls were 58.5 years (7 males, 5 females) and 62.4 years (16 males, 14 females), respectively (Table II). There were no differences in age, gender or tumor localization between the four groups (Table II).

Infiltrations of T lymphocyte subsets in normal colonic mucosa, adenoma and stage I-II and III-IV cancer tissues. A semi-quantitative immunohistochemical scoring system was used to assess T cells infiltration in the tissue samples. CD3, CD4, CD8, CD45RO were all localized to the membrane, as shown in Fig. 1. When compared to the normal group, infiltrations of $CD3^+$, $CD8^+$ and $CD45RO^+$ lymphocytes in colonic adenoma were all significantly greater ($P<0.001$, $P=0.001$ and $P<0.001$); however, $CD4^+$ T cell infiltration did not differ significantly between these two groups ($P=0.052$). $CD3^+$ T cell infiltrations in colonic adenoma were significantly higher than in stage I-II cancer tissues ($P=0.001$), and similar results were also found for $CD8^+$ ($P<0.001$) and $CD45RO^+$ ($P<0.001$) T cell infiltration. $CD3^+$, $CD4^+$, $CD8^+$ and $CD45RO^+$ T cells in cancer tissues of stage III-IV were all considerably lower than those of stage I-II ($P<0.001$, $P=0.045$, $P<0.001$ and $P<0.001$,

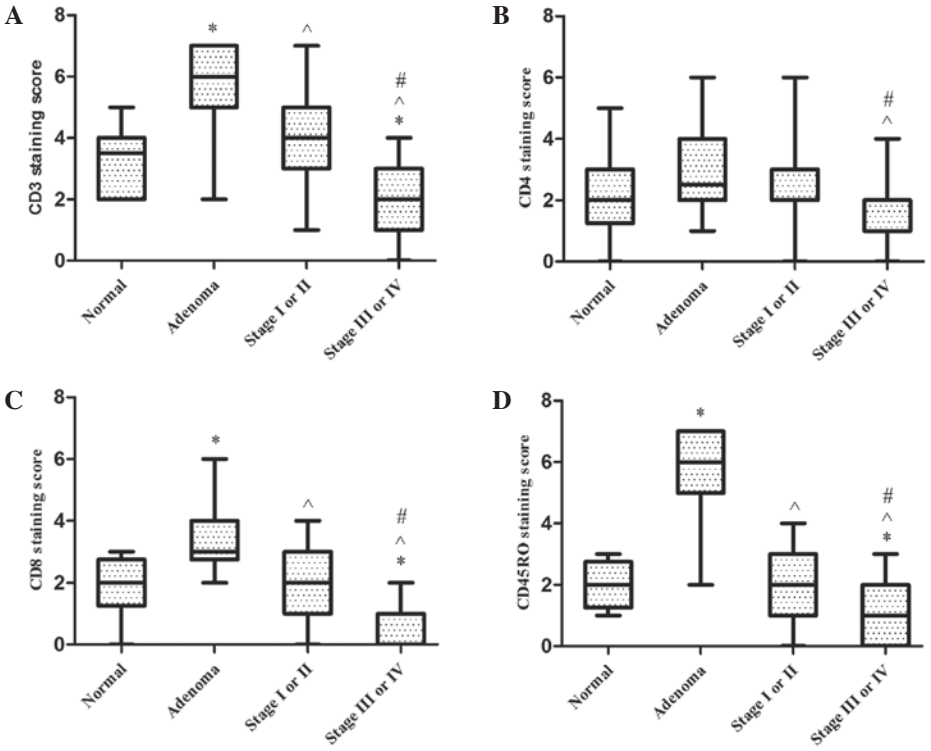

Figure 2. Immunohistochemical staining scores of T cell subsets in normal colonic tissue, colonic adenoma, stage I-II and stage III-IV cancer tissue. (A) CD3, (B) CD4, (C) CD8, and (D) CD45RO staining scores (n=12 for the normal group, n=38 for the adenoma group, n=40 for the stage I-II group, n=38 for the stage III-IV group). Whiskers represent the range and boxes represent the quartile values. *P<0.05 vs. normal group; ^P<0.05 vs. adenoma group; #P<0.05 vs. stage I-II cancer group. CD, cluster of differentiation.

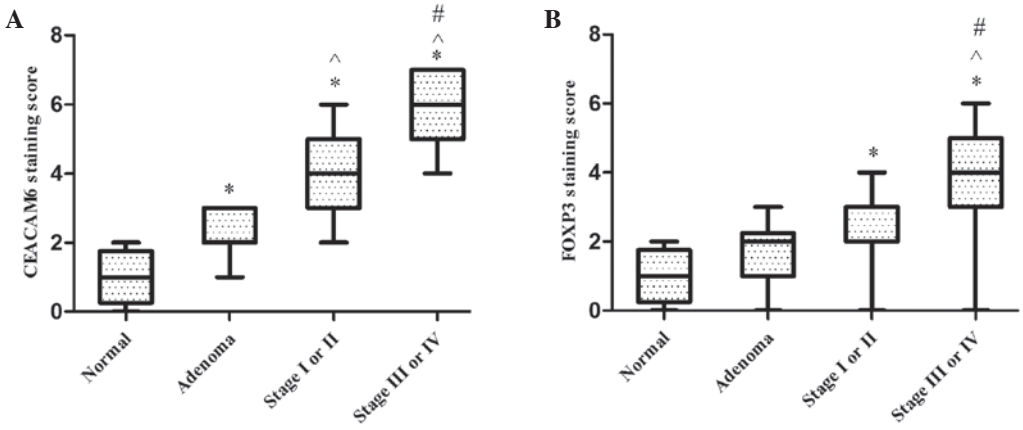

Figure 3. Immunohistochemical staining scores of CEACAM6 and FOXP3 in normal colonic tissue, colonic adenoma, stage I-II and stage III-IV cancer tissues. (A) CEACAM6 and (B) FOXP3 staining scores (n=12 for the normal group, n=38 for the adenoma group, n=40 for the stage I-II group, n=38 for the stage III-IV group). Whiskers represent the range and boxes represent the quartile values. *P<0.05 vs. normal group; ^P<0.05 vs. adenoma group; #P<0.05 vs. stage I-II cancer group. CEACAM6, carcinoembryonic antigen-related cell adhesion molecule 6; FOXP3, forkhead box P3.

respectively). $CD3^+$, $CD8^+$ and $CD45RO^+$ T cell infiltrations in the group of patients with stage III-IV cancer was lower than in normal controls (P=0.015, P=0.002 and P=0.041, respectively). Furthermore, $CD3^+$, $CD4^+$, $CD8^+$ and $CD45RO^+$ T cell infiltrations in stage III-IV cancer tissues were particularly markedly lower than in colonic adenoma tissues (P=0.001, P<0.001, P<0.001 and P<0.001, respectively). However, no significant differences were observed between the normal control and stage I-II groups with regard to $CD3^+$ (P=0.166), $CD4^+$ (P=0.500), $CD8^+$ (P=0.685) or $CD45RO^+$ T cell infiltration (P=0.562) (Fig. 2).

Expression of CEACAM6 and FOXP3 in normal colonic mucosa, adenoma and stage I-II and stage III-IV cancer tissues. CEACAM6 proteins were localized to the cytoplasm (Fig. 1). CEACAM6 expression was markedly higher in stage I-II cancer tissues than in the colonic adenoma or normal control groups (both P<0.001). Additionally, the expression of CEACAM6 was higher in adenoma compared with normal tissues (P<0.001), and higher in stage III-IV in comparison with stage I-II cancer tissues (Fig. 3).

$FOXP3^+$ lymphocytes were observed to have infiltrated the interstitial tissues in the colon tissue specimens, and

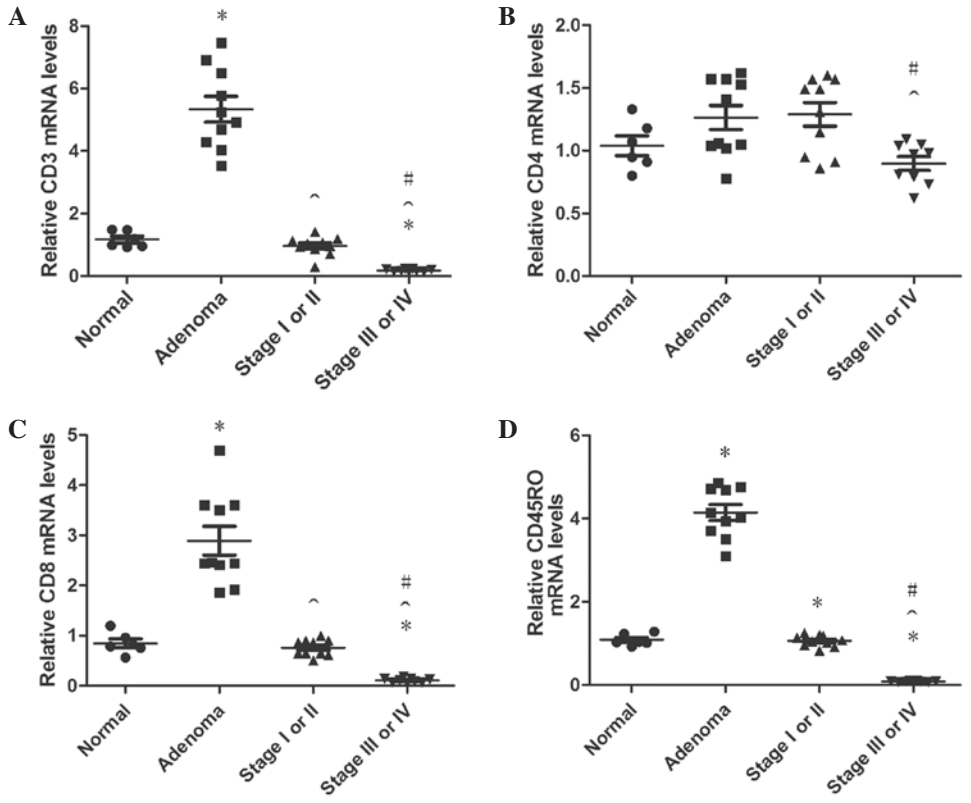

Figure 4. Expression of CD3, CD4, CD8 and CD45RO mRNA in colon tissues of each group. (A) CD3, (B) CD4, (C) CD8 and (D) CD45RO mRNA levels (n=6 for the normal group, n=10 for the adenoma group, n=10 for the stage I-II group, n=9 for the stage III-IV group). *P<0.05 vs. normal group; ^P<0.05 vs. adenoma group; #P<0.05 vs. stage I-II cancer group. CD, cluster of differentiation.

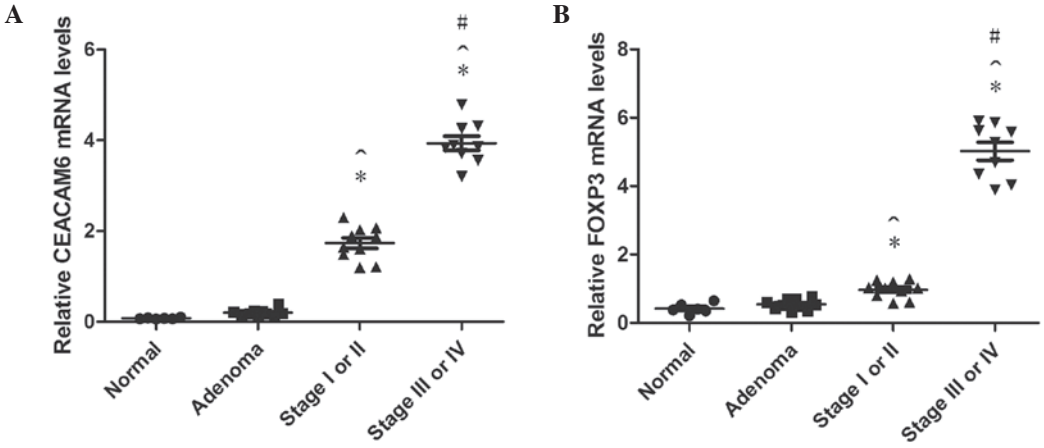

Figure 5. Expression of CEACAM6 and FOXP3 mRNA in colon tissues of each group. (A) CEACAM6 and (B) FOXP3 mRNA levels (n=6 for the normal group, n=10 for the adenoma group, n=10 for the stage I-II group, n=9 for the stage III-IV group). *P<0.05 vs. normal group; ^P<0.05 vs. adenoma group; #P<0.05 vs. stage I-II cancer group. CEACAM6, carcinoembryonic antigen-related cell adhesion molecule 6; FOXP3, forkhead box P3.

FOXP3 expression was localized to the cell nuclei (Fig. 1). FOXP3 expression was significantly stronger in stage I-II (P=0.014) and stage III-IV (P<0.001) cancer tissues than in normal controls. The expression of FOXP3 was marginally stronger in stage I-II than in colonic adenoma; however, this difference was not significant (P=0.169). There was also no significant difference between normal and adenoma tissues (P=0.156). However, the expression of FOXP3 in patients with stage III-IV was significantly greater than in adenoma (P<0.001), and marked differences between stage III-IV and stage I-II cancer tissues were also observed (P<0.001) (Fig. 3).

Expression of CD3, CD4, CD8, CD45RO, CEACAM6 and FOXP3 mRNA. A total of 50 randomly selected paraffin-embedded colon tissues were obtained from 120 specimens, which including 9 normal colon tissues, 13 adenoma tissues, 14 stage I-II colon cancer tissues and 14 stage III-IV colon cancer tissues. The overall mRNA extraction yield from the formalin-fixed, paraffin-embedded colon

tissues was 70% (35/50), and the extraction yields of mRNA from the normal colon, adenoma, stage I-II colon cancer and stage III-IV colon cancer tissue specimens were 66.7% (6/9), 76.9% (10/13), 71.4% (10/14) and 64.3% (9/14), respectively. Compared with the normal colon tissues, $CD3^+$, $CD8^+$ and $CD45RO^+$ T cell infiltrations increased significantly in colonic adenoma (all $P<0.001$); however, there were no significant differences in $CD4^+$ T cell infiltration density between these two groups ($P=0.098$). $CD3^+$, $CD8^+$ and $CD45RO^+$ T cell infiltrations in colonic adenoma were higher than in stage I-II cancer tissue (all $P<0.001$), and $CD3^+$, $CD8^+$ and $CD45RO^+$ T cell infiltrations in stage III-IV were lower than in the normal group ($P=0.015$, $P=0.015$ and $P<0.001$, respectively). In agreement with the previous findings, the infiltration densities of $CD3^+$, $CD4^+$, $CD8^+$ and $CD45RO^+$ T cells were also significantly lower in stage III-IV than in stage I-II cancer tissues ($P<0.001$, $P=0.045$, $P<0.001$ and $P<0.001$, respectively). $CD3^+$, $CD4^+$, $CD8^+$ and $CD45RO^+$ T cell infiltration in the adenoma specimens was higher than in stage III-IV specimens ($P=0.026$, $P=0.026$, $P=0.009$ and $P<0.001$, respectively); however, there were no statistically significant differences between normal and stage I-II specimens ($P=0.583$, $P=0.583$, $P=0.735$, $P=0.895$, respectively; Fig. 4).

CEACAM6 mRNA expression was higher in stage III-IV than stage I-II cancer and adenoma tissues (both $P<0.001$), and higher in stage I-II compared with adenoma and normal (both $P<0.001$), while no obvious differences were identified between normal and adenoma tissues ($P=0.443$; Fig. 5).

The relative FOXP3 mRNA expression in stage I-II and III-IV cancer tissues was significantly higher than in the normal group ($P=0.021$ and $P<0.001$, respectively). In addition, FOXP3 mRNA in stage I-II was higher than the adenoma group ($P=0.036$); however, there was no statistically significant difference between adenoma and normal tissues ($P=0.599$; Fig. 5).

Discussion

Colorectal cancer is a multistep process, and growing evidence suggests that cancer progression is not solely determined by the characteristics of the tumor, but also by the host response (32). Tumors of the colon are generally immunogenic and often infiltrated by immune cells, particularly those of the lymphoid lineage, including effector/cytotoxic ($CD3^+$ and $CD8^+$) and memory ($CD45RO^+$) T cells; these cells have been extensively studied and are associated with the destruction of tumor cells, reduction of tumor burden and improved clinical prognosis (33,34). A study on 215 colorectal cancer patients confirmed that TILs are indeed important clinical and prognostic indicators in colorectal cancer, irrespective of microsatellite instability status (9).

$CD3^+$, $CD8^+$ and $CD45RO^+$ T cells that are present in colon cancers have been suggested to correlate with disease stage and contribute to a protective role by preventing tumor metastasis and recurrence (12); concurrently, the current study demonstrated that T cell infiltrations were markedly higher in normal tissues compared with advanced colon cancer tissues. Most importantly, the current findings indicated that $CD3^+$, $CD8^+$ and $CD45RO^+$ T cell infiltrations were all notably higher in the colonic adenoma group. $CD3^+$, $CD8^+$ and $CD45RO^+$ T cell subsets are widely thought to be indicators of host immune response to tumor cells; in other words, as the immune response in adenoma was strongest of the four tissue groups, the microenvironment of adenoma tissue may induce an appropriate antitumor response to prevent the progression of the tumor (9). The magnitude of the immune response was gradually increased from the normal mucosa to adenoma; but, once the colonic adenoma progressed to colon cancer, the infiltrations of T cell subsets fell into decline, and T cell infiltration was lower in stage III-IV cancer than in normal colonic mucosa. We hypothesize that the constant changes in the immune reaction from normal to adenoma to colon cancer tissues may be due to the varying tumor microenvironment, which contributes to the genomic and epigenomic aberrations of malignant cells, enhancing carcinoma cell survival, invasion and metastasis.

Recently, there has been an increase research focusing on FOXP3 and $CD4^+CD25^+FOXP3^+$ Tregs. Xu *et al* (35) suggest that the appearance of $CD4^+CD25^+FOXP3^+$ Treg infiltration in a cancer nest is a potential independent risk factor for overall survival, and that FOXP3-positive cancer cells may be a risk factor for overall survival in colon cancer. Another study demonstrated that a significantly higher demethylation rate of the Treg-specific demethylated region of the FOXP3 gene, and increased expression levels of FOXP3 mRNA and protein, may be detected in tumor sites compared with adjacent normal tissues (36). In the present study, FOXP3 expression was upregulated distinctly in colon cancer compared with normal colonic mucosa; however, there were no significant differences in normal vs. adenoma or adenoma vs. cancer tissues. The association between FOXP3 expression and T-cell infiltration was further analyzed, revealing that high FOXP3 expression was associated with lower $CD3^+$, $CD8^+$ and $CD45RO^+$ T cell infiltrations. This suggests that FOXP3 may be involved in escaping immunological surveillance, and promoting the generation and progression of colon cancer. $FOXP3^+$ Tregs have the ability to inhibit the T cell-mediated immune response against tumors in colorectal cancer (37). A previous study reported that $FOXP3^+$ T cells in colorectal cancer act as suppressors of cytotoxic T lymphocytes ($CD8^+$ T cells; CTLs) (38). Functional studies have also determined the existence of a tight interplay between the degree of antitumor immune responses mediated by CTLs and the genetic instability of tumor cells. CTLs have the ability to kill target cells upon being exposed to a tumor cell antigen/human leukocyte antigen complex for which the T cell receptor is specific (39).

In the current study, CEACAM6 expression was observed to be gradually increased from normal colon to colonic adenoma to colon cancer tissues. Stefan *et al* (40) reported that CEACAM6 exhibited a broad expression zone in proliferating cells in colorectal hyperplastic polyps and adenomas compared with normal mucosa, which was also observed in the current study. In addition, the previous study suggested that upregulation of CEACAM6 and downregulation of CEACAM7 in polyps and adenomas may represent some of the earliest observable molecular events leading to colon cancer (40). Furthermore, Blumenthal *et al* (22) suggested that CEACAM6 is directly involved in the adhesion and invasion of colon cancer cells, and monoclonal antibodies against CEACAM6 may prevent tumor metastasis. It follows from

this that CEACAM6 is a crucial biological marker associated with disease stage and progression. In a previous study, Witzens-Harig *et al* (41) found that CEACAM6 expression in multiple myeloma patients is important in the inhibition of $CD8^+$ T cell responses. Combining all of these results, it is apparent that overexpression of CEACAM6 as a specific antigen cannot induce an antitumor immune response, and instead may decrease immune responses.

In summary, T cell infiltrations were observed to be greater in colonic adenoma compared with normal tissue, and began to decrease in colon cancer, indicating that the immune response varies between different stages of colon cancer development and progression. FOXP3 and CEACAM6 expression gradually increased from normal colonic mucosa to colonic adenoma to colon cancer; both molecules function in promoting tumor growth and metastasis. The results obtained in the present study indicate that FOXP3, CEACAM6 and T cell infiltration are closely associated with the occurrence and development of colon cancer. Thus, they may be potential surrogate biomarkers of colon cancer. Targeted drugs against CEACAM6 and FOXP3, as well as other related biological treatments, may prove to be promising in future research.

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