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RESEARCH

Elevated Proinflammatory Cytokine IL-17A in the Adjacent Tissues Along the Adenoma-Carcinoma Sequence

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Abstract Considerable evidence has suggested that chronic inflammation is a causative factor in the development of human colorectal cancer (CRC). Interleukin (IL)-17A produced mainly by Th17 cells is a novel proinflammatory cytokine and increased IL-17A is associated with colorectal neoplastic transformation. In this study, we have evaluated the expression of IL-17A in the adjacent tissues along the colorectal adenoma-carcinoma sequence. The expression of IL-17A in the adjacent tissues of colorectal adenoma (adenoma-adjacent, n=32) and sporadic CRC (CRC-adjacent, n=45) was examined. In addition, the expression pattern of Th17 cell differentiation stimulators (IL-1β, IL-6 and IL-23A) in the adjacent tissues were also examined. The results showed that the expression level of IL-17A mRNA was non-statistically increased (4-fold higher) in the adenoma-adjacent tissues and it became significantly increased (9-fold higher) in the CRC-adjacent tissues as compared with the control. The expression level of IL-17A in the CRC-adjacent tissues was not associated with CRC clinicopathological parameters and overall survival. Immunohistochemistry confirmed an increased density of intraepithelial IL-17A expressing cells in the CRC-adjacent tissues. The Th17 cell differentiation simulators IL-1β and IL-6 were also shown in an increase trend from the adenoma-adjacent to CRC-adjacent tissues. These results provide evidence that IL-17A/Th17 response is enhanced in the adjacent tissues during the colorectal neoplastic transformation.

Keywords IL-17A · Adjacent mucosa · Colorectum · Cancer

Abbreviations

CRC	Colorectal cancer
IL	Interleukin
Real-time PCR	Real-time polymerase chain reaction
RT-PCR	Reverse-transcription polymerase chain reaction
IHC	Immunohistochemistry.

Introduction

Colorectal cancer (CRC) remains one of the leading causes of death world-wide. It has long being recognized that CRC is an inflammation related human malignance and a tight pathogenic link has been identified between chronic inflammation and the development of colitis associated carcinogenesis (CAC) as seen in patients with ulcerative colitis (UC) [1–11]. Recent findings suggest that elements of inflammatory pathway can also contribute to the pathogenesis of sporadic CRC [7, 12, 13]. Many clinical and experimental studies have shown that the inflammation is observed in almost all the sporadic colorectal adenoma/CRC specimens, and the presentation of inflammatory components is greatly changed in the tumor microenvironment [13–15]. The tumorigenic promoting effect of inflammation is mediated by the inflammatory mediators. Many mediators such as interleukin (IL)-6, IL-8 and nuclear factor (NF)- B have been found to be increased in the tumor tissues [16–18], these factors have been shown to strongly

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promote the development of CRC in animal studies [12]. Therefore, a proposed mechanism for the development of sporadic CRC is that the pro-inflammatory mediators may significantly contribute to the colorectal neoplastic transformation [6, 12].

IL-17A released mainly from Th17 cells is a novel cytokine with a strong pro-inflammatory capacity and has been linked to the process of both chronic inflammation [19–21] and CRC [17, 22–26]. Several published studies have demonstrated that IL-17A is increased in the CRC tumor tissues [17, 24] and serum [27, 28]. Wang and coworkers found that Th17 cell and Th17 cell differentiation stimulating cytokines in the serum were increased in patients with CRC [28]. We have recently reported that IL-17A was greatly increased along the adenoma-carcinoma sequence, the level of IL-17A was associated with the progression of dysplasia from low grade to high grade in the adenoma [17]. Interestingly, the increase of IL-17A expression has been found to be associated with the clinical outcomes in sporadic CRCs, CRC patients with a high IL-17A level may have a poor prognosis [24, 29]. In addition, several studies have revealed that IL-17A can promote the colorectal tumor formation [22, 30] via an enhanced angiogenesis [29] and the effect on tumor initiating cell [31], whereas disruption of IL-17A signal may suppress the development of CRC in mice [22, 30, 32, 33]. Taken together, the current knowledge links IL-17A to the development of sporadic CRC [26].

It has been previously thought that the immune, histological and molecular alterations were occurred in the malignant lesion and pre-malignant lesion [14, 15, 34, 35], but not in the non-tumor tissue adjacent to the sporadic CRC and adenoma. The non-tumor tissues adjacent to CRC were frequently used as the normal control in the CRC studies. However, recent findings revealed that human cancer can result in a system alteration, many features in the adjacent tissue may also be changed [36–38]. Indeed, Kuniyasu and colleagues [39] have revealed that the expressions of several proangiogenic factors and the vascular density in the hyperplastic mucosa adjacent to CRC were changed as seen in the tumor lesions. They further demonstrated that increased IL-15 in the adjacent hyperplastic mucosa can contribute to tumor angiogenesis and progression [40]. Hao et al. [41] have found that the gene expressions including IL-8 in the normal appearing adjacent mucosa of sporadic CRC were increased as compared with the normal controls. We have recently revealed that density of dendritic cell (DC) and Th1 cytokine IL-12 were significantly reduced in the normal tissues adjacent to sporadic CRCs [42]. Therefore, accumulated data have been gathered in supporting the notion that the immune features in adjacent tissues of sporadic CRC are greatly changed and may be the early risk marker for the development of sporadic CRC [41]. However, there has been remarkably little study of IL-17A signals from the adjacent microenvironment in human adenomas and sporadic CRCs.

Therefore, the present study aimed to evaluate the expression of IL-17A in the adjacent tissues throughout the human colorectal adenoma-carcinoma sequence. Since the Th17 cell is the main cellular source for IL-17A and the differentiation of Th17 cell is regulated by a set of cytokines including IL-1β, IL-6 and IL-23A etc.[43], the expression of these Th17 stimulators were also examined in the adjacent tissues.

Material and Methods

Patients & Biopsies

Adjacent normal appearing biopsies (~10 cm far from the tumor center) were collected from 32 patients (23 males, 9 females, age 49–92 years, and average age 65.9 years) with colorectal adenomas by colonoscopy, 45 patients (male 39, female 6, age 42–88 years, and average age 68.5 year) with CRCs resected by surgery admitted to the Department of Gastroenterology and Surgery, University Hospital of North Norway according to standardized diagnostic criteria. Total 18 (10 males, 8 females, age 33–80 year, and average age 54.6 year) colorectal biopsies without pathological evidence by the colonoscopic and microscopic examinations were served as the controls. Detailed information for each group is presented in Table 1. The Norwegian Regional Ethical Committee of North Norway approved the study and the Norwegian Health Department approved the storage of human biological materials. Informed consent was obtained from the patients.

Tissue Total RNA Extraction and cDNA Synthesis

To avoid RNA degradation, biopsies were collected in *RNA later* solution (Invitrogen Life Tech., Carlsbad, MA, USA) and total RNA was extracted by the *Trizol* method (Invitrogen Life Tech., Carlsbad, MA, USA) [44]. Total RNA quality control was done by the measurement of RNA integrity with RNA 6,000 Nano chips (Agilent Technology, Inc, Böblingen, Germany) according to the manufacturer's instructions. Reverse transcription for cDNA synthesis was performed with *SuperScript II* (Invitrogen Life Tech., Carlsbad, MA, USA) according to our previous report [44].

The mRNA level of IL-17A and Th17 stimulators (IL-1β, IL-6 and IL-23A) in the adjacent tissues of the adenoma (adenoma-adjacent) and sporadic CRC (CRC-adjacent) versus healthy controls quantified by quantitative real-time polymerase chain reaction (PCR).

Primers and probes for cytokines and the housekeeping gene beta-actin were present in Table 2 and the mRNAs of IL-17A, IL-1β, IL-6 and IL-23A in the adjacent tissues from the adenoma (adenoma-adjacent), sporadic CRC (CRC-adjacent) versus healthy controls quantified by quantitative

Table 1 Basic histological information of the patients and the controls

	N	Position		Pathology		TNM stage		
		colon	rectum	tubular	Tubulovillous	I	II	III+IV
Normal	18	8	10					
Adenoma	32	18	14	19	13			
adenocarcinoma								
CRC	45	10	35	45		8	22	15

real-time PCR (*ABI-prism 7900* sequence detector, Applied Biosystems/Roche, Branchburg, NJ, USA) in 25 μL format and the expression levels of these target genes were calculated as relative fold changes ($2^{-\Delta\Delta CT}$ method) according to our previous published method [45, 46].

The Density of IL-17A Expressing Cells in the Adenoma-Adjacent and CRC-Adjacent Tissues Evaluated by Immunohistochemistry

The biopsies were prepared and embedded in paraffin routinely. Sections were cut at 4 μm. IL-17A expressing cells were examined in the adjacent mucosa from adenomas and sporadic CRCs. Immunohistochemistry was performed with Vectastatin *Elite* ABC Kit (Vector Lab., Burlingame, CA, USA) according to the manufacturer's instructions and our published methods [45]. Antigen retrieval was achieved using EDTA buffer (PH 8.0) through microwave processing. Goat anti-human IL-17A polyclonal antibody (Catalog No: AF-317-NA, clone Ile20Ala155, working dilution 1:100, R&D System; Minneapolis, MN, USA) was added to the prepared tissue slides and incubated overnight at 4ºC, 3-Amino-9-ethylcarbazole (AEC; Vector Laboratories, Burlingame, CA, USA) was used as chromogen and then slides were counterstained with Mayer's hematoxylin.

Morphometric Analysis

Well-oriented sections were examined with a light microscope (CX31, Olympus Optical Co., LTD, New York, USA). Semi-quantification of IL-17A expressing cell density in both the lamina propria and epithelium was done in the stained slides. The density of IL-17A expressing cells was counted in at least 5 optical fields from each slide under × 400 high-power magnification by the method described our previous publication [13] and average values were used for statistical analysis.

Statistics

Results were expressed as mean ± SEM (standard error mean) unless otherwise stated. Statistical significance was evaluated

Table 2 Real-time PCR primer sequences for IL-17A and Th17 cell differentiation stimulators quantification

Assay		Primer	Sequence
β-actin	TaqMan	Forward	5' TGCCGACAGGATGCAGAAG 3'
		Reverse	5' GCCGATCCACACGGAGTACT 3'
		Probe	FAM 5' AGATCAAGATCATTGCTCCT CCTGAGCGC 3' TAMRA
IL1β	TaqMan	Forward	5' CCTGAGCTCGCCAGTGAAA 3'
		Reverse	5' TTTAGGGCCATCAGCTTCAAA 3'
		Probe	FAM 5' ATGGCTTATTACAGTGGCAATG AGGATGACTTG 3' TAMRA
IL6	TaqMan	Forward	5' CCAGGAGCCCAGCTATGAAC 3'
		Reverse	5' CCCAGGGAGAAGGCAACTG 3'
		Probe	FAM 5' CCTTCTCCACAAGCGCCTTCGGT 3' TAMRA
IL17A	TaqMan	Forward	5' TGATTGGAAGAAACAACGATGACT 3'
		Reverse	5' ATTGTGATTCCTGCCTTCACTATG 3'
		Probe	FAM 5' TGGTGTCACTGCTACTGCTGCTGAGC3' BHQ
IL-23A	TaqMan	Forward	5' CCCAAGGACTCAGGGACAAC 3'
		Reverse	5' TCCTAGCAGCTTCTCATAAAAAATCA 3'
		Probe	FAM 5' TCAGTTCTGCTTGCAAAGGATCCACCAG 3' BHQ

by the Mann–Whitney test and the Kruskal–Wallis test. The Kaplan–Meier analysis with the log-rank test was used to calculate survival rates and differences in survival curves and p values were determined by the log-rank test. The Cox proportional hazards regression model with a stepwise procedure was used to analyse the simultaneous influence of prognostic factors. Values of $P<0.05$ and <0.01 were considered significant.

Results

The Expression Level of IL-17A mRNA is Increased from the Colorectal Adenoma-Adjacent Tissues to the CRC-Adjacent Tissues

Relative to the controls, the expression of IL-17A mRNA in the adenoma-adjacent tissues was non-statistically (~4–fold) increased ($P>0.05$ as compared with the controls, see *gray bar* in Fig. 1a). But it was significantly (~9–fold) increased in the CRC-adjacent tissues (compared with the control and the adenoma-adjacent tissue, both $P<0.05$, see *black bar* in Fig. 1a).

The Expression Levels of Th17 Stimulators in Adjacent Tissues were Changed

Since the production of IL-17A from Th17 cells is stimulated by a set of cytokines including IL-1β, IL-6 and IL-23A [43], the mRNA expression level of IL-1β, IL-6 and IL-23A in the adjacent tissues were also examined. The results showed that the expression of IL-1β and IL-6 mRNA were non-statistically increased in the adenoma-adjacent tissues (*gray bar* in Fig. 1b and c) and both became significantly increased in the CRC-adjacent tissues verses the controls (*black bar* in Fig. 1b and c). Whereas the expression level of IL-23A was non-statistically decreased in the adenoma-adjacent tissues and further decreased in the CRC-adjacent tissues verses the controls (Fig. 1d).

The Density of Intraepithelial IL-17A Expressing Cells was Increased from the Adenoma-Adjacent Tissues to the CRC-Adjacent Tissues

Increased number of IL-17A expressing cells was observed in both the lamina propria (arrow pointed in Fig. 2b and c) and the epithelium (arrow head pointed in Fig. 2b and c) in the adenoma-adjacent sections and CRC-adjacent sections;

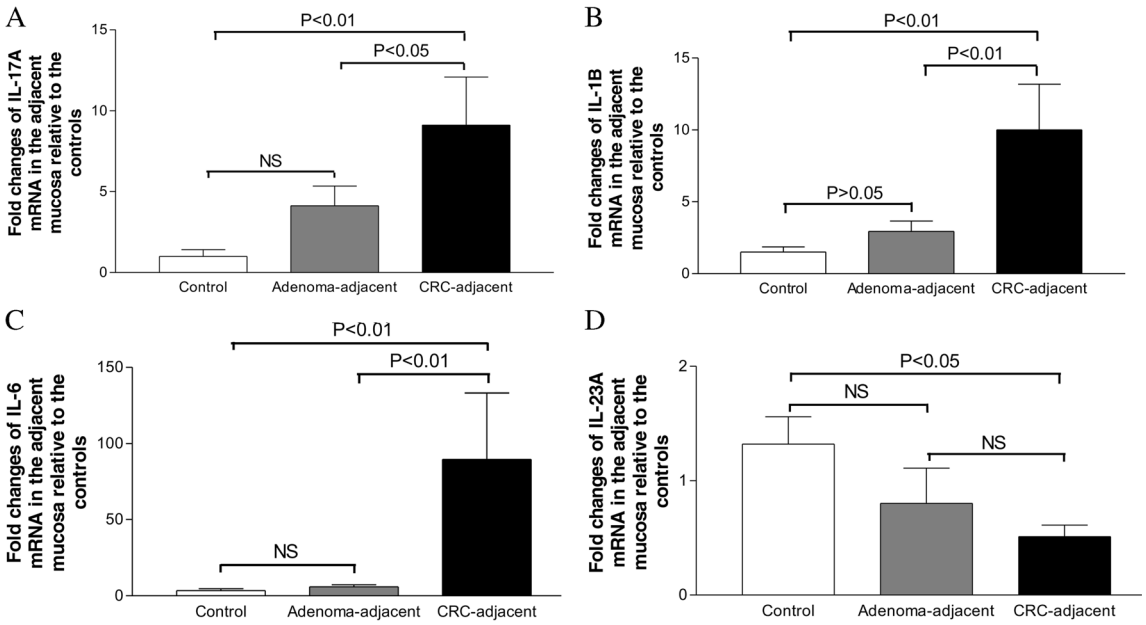

Fig. 1 Graphic analysis of mRNA levels of IL-17A and Th17 cell differentiation stimulators IL-1beta, IL-6 and IL-23A in the adjacent tissues of adenomas and sporadic CRCs. The quantitative real-time PCR results showed that the expression level of IL-17A mRNA (Fig. 1a) in the adjacent tissues of adenomas (adenoma-adjacent) was non-statistically increased (*gray bar* in Fig. 1a), but it in the adjacent tissues of CRC (CRC-adjacent) became significantly (*black* bar in Fig. 1a). The expression level of IL-1β (see Fig. 1b) mRNA in the adenoma-adjacent started to be increased and became significantly in the CRC-adjacent, whereas the expression of IL-6 mRNA in the CRC-adjacent (see Fig. 1c) was significantly increased, but not in the adenoma-adjacent. The expression level of IL-23A mRNA was non-statistically decreased from the adenoma-adjacent to the CRC-adjacent (Fig. 1d). (Y axes are fold change relative to the controls. P values are from the Mann–Whitney test)

however, the cells were mostly located in the lamina propria of normal controls (see Fig. 2a). Semi-quantification results confirmed a significantly increased IL-17A expressing cell density in the epithelium (Fig. 2e), but not in the lamina propria in the CRC-adjacent sections (Fig. 2d).

The Relationship Between the Expression Level of IL-17A in the CRC-Adjacent Tissues and the TNM Stages, Node Involvement and Overall Survival

The analysis showed that IL-17A mRNA expression level in the adjacent tissues neither influenced the CRC TNM stage (TNM stage I vs. II vs. III+IV: 10.64±7.21 vs. 9.49±4.27 vs. 8.96±6.30, $P>0.05$, the Kruskal-Wallis test) and nor lymph node involvement (with node metastasis vs. without: 8.96± 6.30 vs. 9.76±3.54, $P>0.05$, the Mann Whitney test).

It has been shown that the expression level of IL-17A in patients with CRC is associated with the prognosis, CRC patients with higher IL-17A level have a worse prognosis [27, 29]. Hence, we further analyzed the relationship between IL-17A expression level in adjacent tissues and the overall survival of CRC. In this study, the overall survival data were available in 19 CRC patients. As shown in Fig. 3, the Kaplan–Meier analysis revealed that the survival time was not different between CRC patients with higher IL-17A expression level and those with lower IL-17A expression level. This finding indicated that the expression level of IL-17A in the adjacent tissues did not significantly influence the CRC patients' overall survival.

Discussion

It has previously been demonstrated that the expression of IL-17A is increased in both the serum [27] and adenomatous/cancerous tissues [17, 29]; The number of IL-17A positive cells is also increased and related to the progression of sporadic CRCs [28]. In the current study, we were able to show that IL-17A is also increased in the adjacent tissues along the adenoma-carcinoma sequence, which likely reflects an enhanced IL-17A/Th17 immune response in the adjacent tissues of sporadic CRC.

Recent studies with mouse models have also revealed a role for IL-17/TH17 signaling in the pathogenesis of CRC [22, 26, 32, 33], while blocking or defecting of IL-17A signaling can significantly suppress the growth of colon cancer cell implanted in murine models [30] and colitis associated CRC [32]. In the investigation of IL-17A in human CRC, we have previously found an increase of IL-17A in the tumor center of adenomatous/cancerous lesions [17], the expression level of IL-17A was 33-fold elevated in the adenomatous

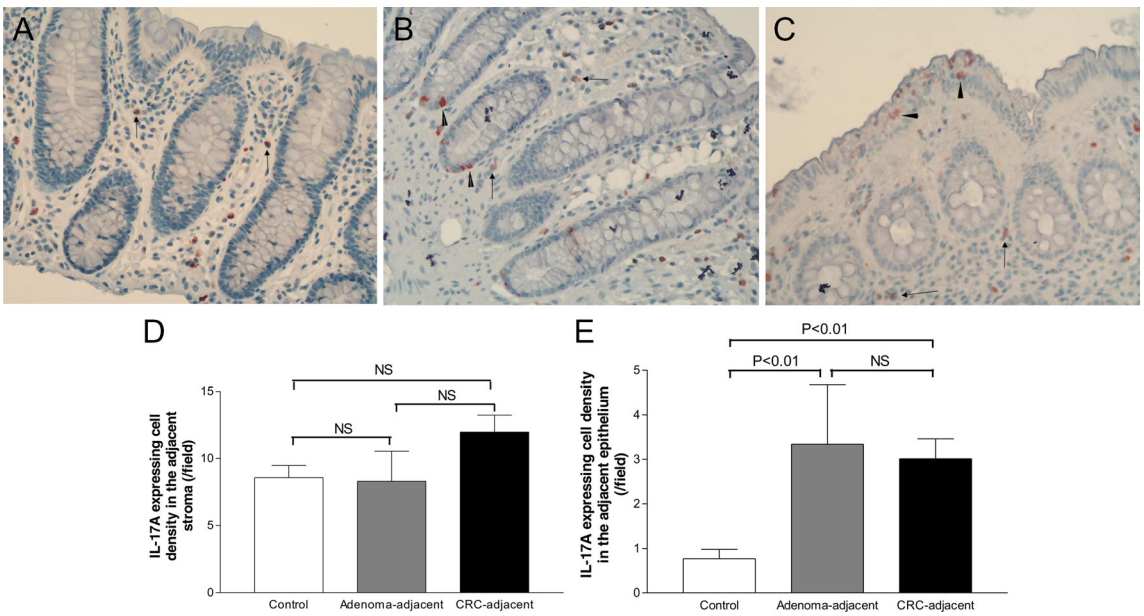

Fig. 2 Increased IL-17A expressing cells in the adenoma-adjacent and CRC-adjacent tissues. In the control section, low density of IL-17A expressing cells was mostly observed in the lamina propria (arrows in Fig. 2a). In the adenoma-adjacent and CRC-adjacent sections, the IL-17A expressing cells were observed in both the lamina propria (arrows in in Fig. 2b for adenoma-adjacent, arrows in Fig. 2c for CRC-adjacent) and epithelium (arrow heads in Fig. 2B for adenoma-adjacent, arrow heads in Fig. 2c for CRC-adjacent). Morphometric analysis revealed that the density of IL-17A expressing cells in the lamina propria in both the adenoma-adjacent and the CRC-adjacent was not changed as compared with the control (see Fig. 2d). But the density of intraepithelial IL-17A expressing cells in both the adenoma-adjacent and the CRC-adjacent was significantly increased as compared with the control (see Fig. 2e). (Immunohistochemistry, counterstained with hematoxylin, original magnification 200×; P values obtained from the Mann–Whitney test)

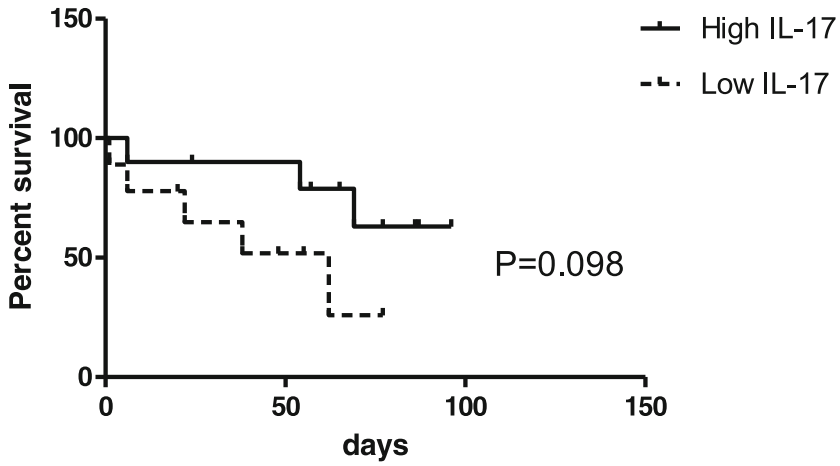

Fig. 3 The association between the expression level of IL-17A in adjacent tissues and CRC patients' overall survival time The Kaplan–Meier analysis revealed that the expression level of IL-17A in adjacent tissues did not influence the CRC patients' overall survival

tissues and 39-fold in the sporadic CRC tissues [17]. In this study, the IL-17A expression level in the CRC-adjacent tissues was only ~9-fold increased and lower than that in the CRC tumor tissues. We postulated that such difference might be partially explained by the different expression levels of Th17 differentiation stimulators between CRC tumor center and CRC-adjacent tissue. Indeed, the quantification results of Th17 cell differentiation stimulators confirmed our postulation and showed that IL-6, one of the main IL-17A stimulators, was ~90-fold elevated in the normal tissue adjacent to sporadic CRC, which was 10 times lower than that that in the CRC tissues (it was ~900-fold elevated in the CRC tissues [17]). It has been demonstrated that IL-6 is a potent pro-tumor factor and is essential for the growth, expansion and metastasis of human CRC [16, 47, 48]. Thus, the increased IL-6 in adjacent non-tumor tissues may not only stimulate Th17 cell differentiation and IL-17A production, and can also inhibit the host anti-tumor immunity and promote the expansion and metastasis. Apart from the IL-6, we have found in this study that another Th17 stimulator - IL-1β was ~10-fold higher in the CRC-adjacent tissue than that in the control, but it was also lower than that in the CRC tumor tissues [17]. In previous studies performed in the sporadic CRC, IL-23A was shown to be increased in the adenoma/CRC tumor tissues [17, 49, 50]. However, in this study IL-23A in adjacent non-tumor tissues was shown in a gradually decreasing trend from the adenoma to CRC. The exact mechanisms for such decrease change trend of IL-23A expression in the adjacent mucosa are currently unclear; it may be related to the individual difference between different studies. Since the Th17 cell is the main cellular source for IL-17A [43], our current findings taken together with our previous results may reflect that an enhanced Th17 differentiation was occurred not only in the tumor center, but also in the adjacent non-tumor region.

Since it has been reported that the expression level of IL-17A is associated with the survival in patients with CRC [27, 29], we therefore examined the relationship between IL-17A level in the CRC-adjacent tissues and CRC clinicopathological parameters in this study. However, our data showed that the expression level of IL-17A in adjacent tissues did not affect the TNM stage, lymph node involvement and the CRC patients' overall survival.

As the role of other proinflammatory cytokines in the neoplastic transformation and tumor progression has been appreciated [17, 32, 51], the mechanisms and significance of IL-17A in the development and progression of sporadic CRC have also been investigated [29, 32]. It is well known that the growth of tumor cells is highly dependent on angiogenesis [52]. Studies have shown that the impact of IL-17A on the CRC tumor growth is via an enhanced angiogenesis [53, 54], this was verified by a clinical study in patients with CRC, in which increase of IL-17A was associated with an increased production of proangiogenic factors like vascular endothelial growth factor (VEGF) [55]. The importance of IL-17 in angiogenesis is illustrated by the proangiogenic efficacy of IL-17A to the mouse tumor locally [56], it was shown that the increase of IL-17 in the local tumor microenvironment by the generation of a fusion protein consisting of the F8 antibody (a marker of angiogenesis) and of murine IL-17 (mIL-17) can selectively promote tumor angiogenesis in both immunocompetent and immunodeficient mice [56]. Interestingly, an enhanced angiogenesis has been previously observed in the human CRC-adjacent hyperplastic mucosa [39], while cytokine from adjacent hyperplastic tissues has been demonstrated to stimulate angiogenesis in the CRC tumor center [39, 40]. Although the effect of IL-17A from the CRC-adjacent tissues on angiogenesis in the CRC tumor center remains unclear, one previous study has revealed that microvessel density in adjacent normal tissues of CRC is increased [57]. Thus, the increased IL-17A in the adjacent tissues of CRC may contribute to the growth and invasion of CRC.

In addition, the adjacent non-tumor tissues were frequently used as the normal controls for the measurements of cytokines in patients with CRC. However, it must be aware that adjacent

tissues taken from patients with CRC are not really normal tissues, both of biochemical, angiogenesis and immunological parameters are changed as compared with the normal tissues [41, 49–51].

In conclusion, the present study provides evidence that the expression of IL-17A is elevated in the adjacent tissues from the adenoma to sporadic CRC; this may reflect an enhanced IL-17A/Th17 immune response in the adjacent tissues during the colorectal neoplastic transformation. Future studies are required to shed light on the exact pathogenic role of increased IL-17A signal in the adjacent tissues of sporadic CRC.

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