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Original article

Dynamic stromal cellular reaction throughout human colorectal adenoma-carcinoma sequence: A role of TH17/IL-17A

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ABSTRACT

Background: Accumulating data suggest that the tumour stroma rapidly undergoes dynamic mechanical and cellular changes by which creates a supportive milieu to promote disease progression and metastasis. Cytokines are reported to play a key role in the modulation of tumour stromal response.
Methods: The activation of TH17/interleukin (IL)−17A network in association with tumour stromal proliferative and cellular response in samples from 50 patients with colorectal adenoma, 45 with colorectal cancer (CRCs) were elucidated with quantitative real-time PCR (q-PCR), immunohistochemistry and double immunofluorescence.
Results: q-PCR results showed that retinoic acid-receptor-related orphan receptor-C, a critical transcriptional factor for TH17 cell differentiation, was significantly increased at the adenoma stage and slightly decreased at the CRC stage, but was still higher than that at normal controls. The level of TH17 signature cytokine IL-17A was shown in an increasing gradient throughout the adenoma-carcinoma sequence. Immunohistochemistry revealed an activated proliferative rate evaluated by Ki67 and population expansion of myofibroblasts in the adenoma/CRC stroma. Notably, densities of IL-17A-expressing cells were associated with populations of Ki67-positive cells and myofibroblasts in the adenoma/CRC stroma. Finally, CD146-positive stromal cells are an important participator for stroma remodelling, double immunofluorescence image demonstrated that IL-17 receptor C, one of the key elements for IL-17 receptor complex, was highly expressed in CD146-positive adenoma/CRC stromal cells.
Conclusions: An activated TH17/IL-17A network in the tumour microenvironment is significantly associated with dynamic stromal cellular response throughout the adenoma–carcinoma sequence, which might provide a supportive environment for the initiation and progression of CRC.

1. Introduction

Colorectal cancer (CRC) is the fourth most frequent malignancy and has a high mortality worldwide [1]. Advances of adjuvant chemo-, radio- and bioimmunological therapies have improved clinical outcomes but, unfortunately, metastasis is observed in approximately 20–25% of CRC patients at the time of diagnosis [2] and curative surgery becomes impossible in those patients. Thus, to improve the clinical outcomes, more research is needed to understand the exact mechanisms of progression and metastasis. Recent studies have suggested that during the progression and metastasis, the tumour stroma, together with extracellular matrix and growth factors synthesize, and angiogenesis, undergoes a cellular proliferative and phenotypical change [3–5]. Many types of cells i.e., mesenchymal stromal cells (MSCs) and fibroblasts differentiate to myofibroblasts and construct a supportive cellular milieu to tumour invasion and metastasis by enhancing angiogenesis and immunosuppression, regulating tumour-stromal interaction and epithelial to mesenchymal transition [6–13].

Abbreviations: CRC, colorectal cancer; CT, cycle threshold cross point; DIF, double immunofluorescence; EMT, epithelial-mesenchymal transition; IHC, immunohistochemistry; IL, interleukin; IL-17RC, interleukin 17 receptor C; MSC, mesenchymal stromal cell; qPCR, quantitative real-time PCR; ROR, retinoic acid-receptor-related orphan receptor; TGF, transforming growth factor; TH, T helper; VEGFR2, vascular endothelial growth factor receptor 2.

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The cellular proliferative response and phenotypical change in the tumour stroma are modulated by microenvironmental elements, in which cytokines produced by tumour cells and immune cells play a critical role. T helper (TH) 17, a IL-17-expressing CD4 positive TH cell subset, has been reported to be deeply involved in the pathogenesis of CRC [14]. Its signature cytokine interleukin (IL)− 17A has profound biological functions via a IL-17 receptor A (IL-17RA) and IL-17RC complex in colorectal inflammatory diseases and CRC [15–17] by multiple proposed mechanisms [14,18–22]. One of the potential mechanisms is that IL-17 participates in the modulation of phenotypic differentiation and remodelling of stromal cellular architecture [22–26]. Indeed, several reports have shown that IL-17A stimulates the proliferation, survival and mobilization of stromal fibroblasts [27,28], through a upregulation of the transforming growth factor (TGF)-β receptor to enhance the expression of profibrotic genes [29]. Interestingly, TGF-β is also a upstream stimulator for TH17 cell differentiation [30,31] and increased expression levels of both TGF-β and IL-17A have been previously found in patents with CRC [32–34], indicating a synergistic effect of TGF-β with TH17/IL-17A on the promotion of CRC. Furthermore, MSCs within the tumour microenvironment, as the critical player in the architecture and regulation of tumour-stromal interaction and epithelial to mesenchymal transition (EMT), have been shown to be functionally regulated by IL-17A [26,35], in a dose-dependent manner [25,36,37]. Sivanathan and colleagues [24] reported that exposure of bone marrow-derived MSC with IL-17A could enhance the immunosuppressive properties of MSCs, via the mobilization and recruitment of immune-suppressive cells into the tumour microenvironment. These findings lead us to hypothesize that a TH17/IL-17A microenvironment might contribute to the stromal cellular response in patients with tumours. We have therefore designed this study to investigate the potential involvement of TH17/IL-17 microenvironment on dynamic stromal response along the adenoma – carcinoma sequence.

2. Materials and methods

2.1. Biopsies from patients with adenoma and CRC

Fifty biopsies of colonoscopy resected colorectal adenomas (male/female: 31/19; ages: 43–92 years; Histology, tubular/villous/tubulovillous: 33/2/15; low grade dysplasia (LGD)/moderate grade dysplasia (MGD)/high grade dysplasia (HGD): 22/24/4; and 45 biopsies of surgical resected CRC (male/female: 39/6; ages: 42–89 years; histology: all adenocarcinoma; TNM: I/II/III/IV:7/20/17/1; Node positive/negative:13/32) collected from at the Departments of Gastroenterology and Surgery, University Hospital of North Norway, were included in the study according to standardized diagnostic criteria. Biopsies from 15 subjects (male/female: 10/5; ages: 30–77 years) with normal colonoscopy and histology served as a normal control group. Biopsies were divided into 2 parts: one for gene quantification by quantitative real-time PCR (qPCR), another for histology, immunohistochemistry (IHC) and double immunofluorescence (DIF) staining were embedded in paraffin. Routine haematoxylin and eosin (*H&E*) for histological diagnosis was done at Department of Pathology. The study protocol was approved by the local Regional Ethical Committee of Northern Norway (REK NORD 06/2004) and executed in compliance with the Declaration of Helsinki, written informed consent was obtained from all patients. The permission for the storage of human tissues and data were given by the Norwegian Department of Health and the Norwegian Bureau of Data Surveillance (Norwegian Biobank Register 952/2006).

2.2. Measurement of TH17 key transcriptional factor retinoic acid-receptor-related orphan receptor (ROR)-C and signature cytokine IL-17A by quantitative real-time PCR (qPCR)

ROR-C, a master transcription regulator, has been shown to play a central role in the regulation of TH17 cell differentiation [38–40]. In this study, we quantified the expression level of ROR-C mRNA as a differentiation index of TH17 cells in the adenoma/CRC microenvironment. qPCR was carried out on an *ABI-prism 7900* sequence detector with *TaqMan Gold*™ PCR core reagents kit (Applied Biosystems/Roche, Branchburg, NJ, USA) in 25 μL volume according to our previously published method [41–44]. The primer sequences for ROR-C, IL-17A and housekeeping gene (beta-actin) were listed in Table 1. The expression of ROR-C and IL-17A mRNAs in normal, adenoma and CRC tissues were measured by cycle threshold cross point (CT) value relative to that of normal mucosa as fold difference (N)= $2^{-\Delta\Delta CT}$ as described in our recent publication [43].

2.3. Immunohistochemistry (IHC)

IHCs for stromal proliferation response, stromal myofibroblasts and IL-17A expressing TH17 cells in the adenoma/CRC stroma was performed according to the protocol described in our previous publication [4,45,46]. In brief, after deparaffinized, rehydrated and antigen retrieval routinely, slides were incubated overnight 4 ^{0}C with following antibodies: mouse anti-human Ki67 monoclonal antibody (to label proliferative rate, 1:70; BD Biosciences Pharmingen, San Diego, CA, USA), mouse anti-human smooth muscle actin (SMA)-alpha (Clone 1A4) monoclonal antibody (to label myofibroblasts, 1:100; DAKO, Carpinteria, CA, USA) and goat anti-IL-17A polyclonal antibody (to label TH17 cells, 1:100; R&D System; Minneapolis, MN, USA) respectively. Sections were developed with a commercial *Vectastatin Elite ABC* Kit (Vector Lab., Burlingame, CA, USA) according to the manufacturer's instructions and our published method [46–48]. 3-Amino-9-ethylcarbazole (AEC; Vector Laboratories, Burlingame, CA, USA) was used as chromogen and slides were counterstained with Mayer's haematoxylin.

The negative control slides for IHCs were performed routinely: (1) primary antibodies were substituted with the isotype-matched control antibodies; (2) secondary antibody was substituted with phosphate buffered saline. All the IHC stained slides were examined under a light microscopy (CX31, Olympus Optical Co., LTD, New York, USA).

2.4. Double IHCs for the examination of proliferative capacity of IL-17A expressing cells in the adenoma/CRC stroma

To examine the proliferative capacity of IL-17A expressing cells in the adenoma/CRC stroma, double IHC with Ki67/IL-17A (rabbit polyclonal, Santa Cruz Biotechnology, USA) antibodies was performed in the adenoma and CRC sections using the *EnVision Doublestain System* kit (DAKO, Carpinteria, CA, USA) according to the manufacturer's instructions and our published method [49]. In brief, the slides were incubated overnight at 4°C with mouse anti-Ki67 antibody after antigen retrieval, and then incubated with labelled polymer-horseradish peroxidase-anti-mouse and anti-rabbit antibodies for 30 min at room

Table 1
Primer/probe sequences of house-keeping gene, ROR-C and IL-17A for quantitative real-time PCR.

Assay		Primer	Sequence
β-actin	TaqMan	Forward	5′ TGCCGACAGGATGCAGAAG 3′
		Reverse	5′ GCCGATCCACACGGAGTACT 3′
		Probe	FAM 5′ AGATCAAGATCATTGCTCCTCCTGAGCGC 3′ TAMRA
ROR-C	TaqMan	Forward	5′GCACACCGTTCCCACATCTC 3′
		Reverse	5′ CAGCGCTCCAACATCTTCT 3′
		Probe	FAM 5′AGGAAGTGACTGGCTACCAGAGGAAGTCCAT 3′ BHQ
IL-17A	TaqMan	Forward	5′ TGATTGGAAGAAACAACGATGACT 3′
		Reverse	5′ ATTGTGATTCCTGCCTTCACTATG 3′
		Probe	FAM 5′ TGGTGTCACTGCTACTGCTGCTGAGC3′ BHG

temperature. Ki67-IR with peroxidase activity was detected with the enzyme substrate 3,3′-diaminobenzidine tetrachloride (DAB). After quenching the enzyme reaction, the slides were incubated in Double-stain Block at room temperature for 5 min to block endogenous phosphatase. The slides were then incubated with rabbit anti-IL-17A antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 2 h at room temperature. After washing, the slides were incubated with labelled polymer-alkaline phosphatase anti-mouse and anti-rabbit antibody for 30 min at room temperature. Fast Red chromogen substrate solution was used to visualize IL-7A immunoreactivity (IR). All the sections were slightly counterstained with Mayer's haematoxylin.

2.5. Double immunofluorescence (DIF) staining

CD146 expressed in stromal cells promotes cancer cell growth and invasion, and induces stromal MSC properties via the activation of EMT [50] and angiogenesis [51]. To define the expression of the critical subunit of IL-17 receptor (IL-17R) complex, IL-17RC [52], in the stromal CD146-positive cells, DIF staining with the antibodies IL-17RC (goat polyclonal antibody from Abcam, UK)/CD146 (rabbit polyclonal antibody from Thermos Fisher Scientific) were performed according to our previously published method [53]. IL-17RA-IR was developed with Texas red-conjugated secondary antibody (Jackson ImmunoRearch Lab., West Grove, PA, USA), CD146-IR was with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Jackson ImmunoRearch Lab., West Grove, PA, USA). Nuclear counterstaining was not applied and the stained slides were observed and photographed with a confocal microscopy (LSM-700, Carl Zeiss, Jena, Germany) under × 200 mediate-power fields. Negative controls were performed with (1) primary antibodies were substituted with the isotype-matched control antibodies; (2) The cross-reactivity was examined by crossing different secondary antibodies.

2.6. Morphometric analysis

The numbers of Ki67-positive cells and IL-17A-positive cells in the stroma were counted in at least 5 five well-orientated fields with abundant positive cell distribution from each slide under 400 × high-power magnifications. SMA-alpha expressing myofibroblasts were found at a very high density in the stroma and were scored with light microscopy using the following criteria: Score 1, 1–25% positive cells; Score 2, 25–50% positive cells; and Score 3: > 50% positive cells [4,54]. The average values were used for the statistical analysis.

2.7. Statistical analysis

The expression of ROR-C and IL-17A mRNAs in normal, adenoma and CRC tissues were measured by cycle threshold cross point value relative to that of normal mucosa as fold difference (N)= $2^{-\Delta\Delta CT}$ as described in our recent publication [43]. All the results were expressed as mean ± SEM unless otherwise stated. Statistical significance was evaluated by the Mann–Whitney and the Kruskal–Wallis tests. Values of $P < 0.05$ were considered significant. The populations of Ki67-positive cells and SMA-positive myofibroblasts in the adenoma/CRC stroma was divided into low and high two groups according to the median of IL-17A-positive cell density in the stroma, the difference between two groups was evaluated by the Mann–Whitney test.

3. Results

3.1. TH17 key transcriptional factor ROR-C and signature cytokine IL-17A were significantly increased throughout the human adenoma-carcinoma sequence

As shown in Fig. 1A, the levels of TH17 transcriptional factor ROR-C mRNA were greatly increased in adenoma and CRC tissues as compared

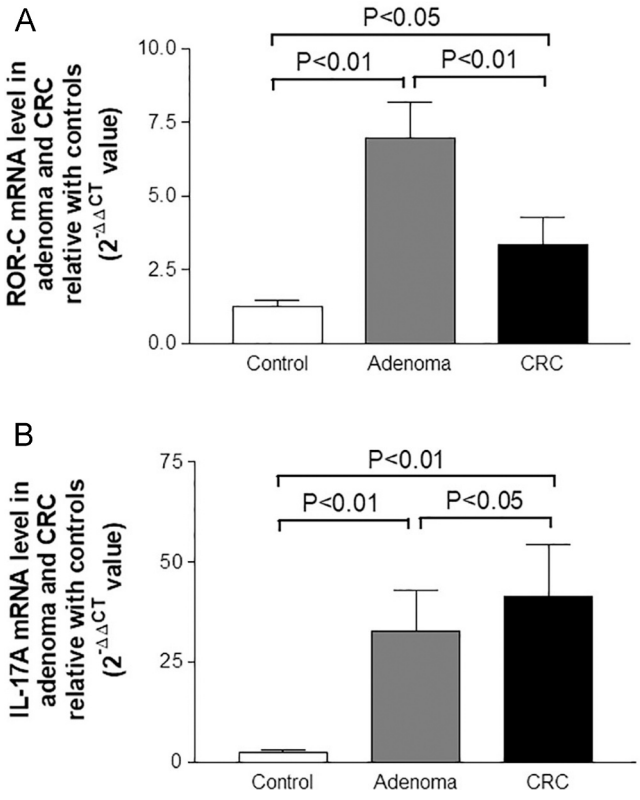

Fig. 1. Dynamic changes of TH17 key transcriptional factor ROR-C and signature cytokine IL-17A transcripts throughout the colorectal adenoma-carcinoma sequence. qPCR data showed that the expression level of ROR-C mRNA was significantly increased in the adenoma tissues (grey bar in Figure A); it remains in a higher in the CRC tissues (black bar in figure A) as compared to the controls (white bar in Figure A), but lower than that in the adenoma tissues. The expression level of TH17 signature cytokine IL-17A mRNA in the adenoma tissues (grey bar in Figure B) as compare to the control (white bar in Figure B); was significantly increased and even higher in the CRC tissues (black bar in Figure B) compared to the adenomas. (Y axes in Figs. 3A and B are fold changes relative to normal controls; P values are derived from the Mann-Whitney test).

with control tissues. Interestingly, the tissue level of ROR-C mRNA at the adenoma stage was higher than the CRC stage. Analysis of ROR-C mRNA against pathological parameters in patients with adenoma and CRCs has been summarized in Table 2 and 3. Data revealed that the levels of ROR-C mRNA did not correlate neither with grading degree of dysplasia in patients with adenoma, with TNM stages, tumour invasion depth, nor node metastasis in patients with CRC.

qPCR data showed that the tissue expression level of IL-17A mRNA was shown in an increasingly gradient throughout the adenoma -

Table 2
Analysis of ROR-C and IL-17A mRNA levels against clinicopathological variables in patients with adenoma.

mRNA	Grading degree of dysplasia	*P1*	Histology	*P2*
	LGD vs. MGD vs. HGD		Tubular vs. Tubulovillous+Villous	
ROR-C	9.27 ± 2.52 vs. 4.99 ± 0.50 vs. 4.80 ± 1.67	>0.05	6.61 ± 0.96 vs. 5.92 ± 1.78	>0.05
IL-17A	31.41 ± 17.09 vs. 26.12 ± 12.02 vs.179.3 ± 70.75	<0.05	31.07 ± 12.71 vs. 35.36 ± 13.96	>0.05

P1 values were from the Kruskal-Wallis test; *P2* values were from the Mann–Whitney test.

Table 3
Analysis of ROR-C and IL-17A mRNA levels against clinicopathological variables in patients with CRC.

mRNA	TNM stage	*P1*	Node involvement	*P2*
	I vs. II vs. III		Positive vs. Negative	
ROR-C	3.76 ± 1.29 vs. 3.98 ± 2.50 vs. 2.77 ± 0.93	>0.05	4.06 ± 1.47 vs. 2.29 ± 0.83	>0.05
IL-17A	26.98 ± 14.88 vs. 42.91 ± 21.27 vs. 41.78 ± 21.46	>0.05	41.78 ± 21.46 vs. 37.28 ± 13.99	>0.05

P1 values were from the Kruskal-Wallis test; *P2* values were from the Mann–Whitney test.

carcinoma sequence (Fig. 1B). Like the analysis data of ROR-C mRNA, the levels of IL-17A mRNA were neither associated with pathological parameters of adenomas nor CRCs (Table 2 and 3).

3.2. IL-17A-positive cells, proliferative rate and myofibroblasts in the adenoma/CRC stroma

Increased IL-17A-positive cells (Fig. 2A–C), proliferative rate (labelled by Ki67) (Fig. 2 D–F) and myofibroblasts (labelled by SMA-α, Fig. G–I) were observed in the adenoma and CRC stroma as compared with the control.

Counting data confirmed IHC observation. As shown in Table 4, populations of Ki67-positive cells, myofibroblasts and IL-17A-positive cells in the adenoma/CRC stroma were increased as compared with the controls.

3.3. Proliferative capacity of IL-17A-positive cells in the adenoma and CRC stroma

Double IHCs showed that these IL-17A expressing cells had a high

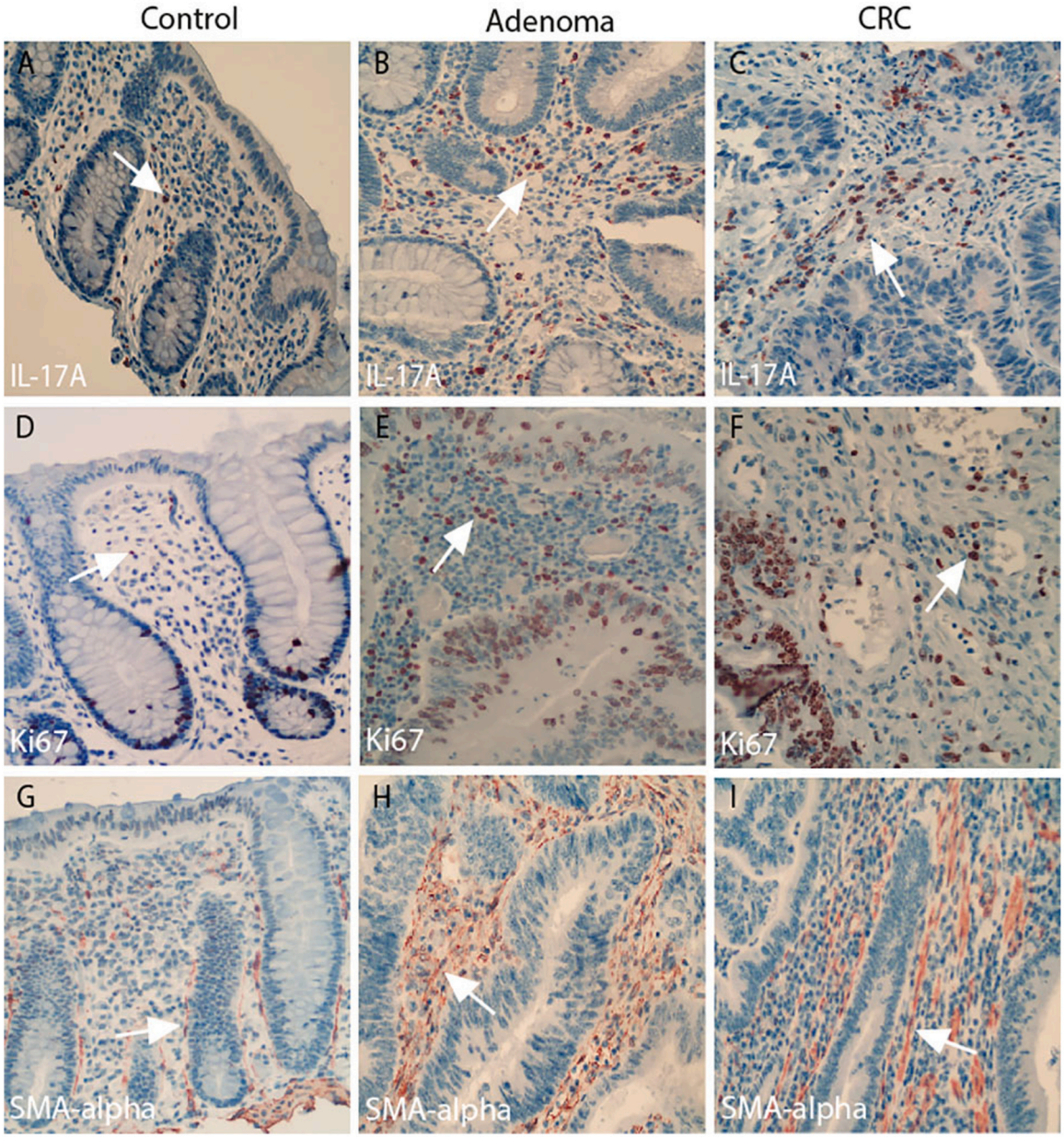

Fig. 2. Photographic representations of IL-17A-, Ki67- and SMA-alpha-positive cells in the adenoma/CRC stroma As compared with the controls (A), increased population of IL-17A-positive cells were observed in the adenoma (B) and CRC (C) stroma. The proliferation labelling index by the Ki67 immunoreactivity was increased throughout the normal-adenoma-carcinoma sequence (D-F). Increased population of SMA-alpha expressing myofibroblasts were detected in the adenoma (Figure H) and CRC (Figure I) stroma as compared with the controls (Figure G). (A-I: IHC, counterstained with haematoxylin, original magnification 200 ×).

Table 4
Populations of stromal IL-17A-positive cells, Ki67-positive cells and SMA-positive myofibroblasts in patients with adenoma and CRC.

Cells	Control	Adenoma	CRC	*P*
IL-17A	10.23 ± 0.88	19.97 ± 1.86	28.27 ± 3.28	<0.01
Ki67	7.0 ± 0.70	26.13 ± 2.98	35.74 ± 4.27	<0.01
Myofibroblasts	1.29 ± 0.11	2.55 ± 0.12	2.71 ± 0.10	<0.01

Values of *P* were from the Kruskal–Wallis test.

proliferative capacity in the active adenoma (Fig. 3A) and CRC stroma (Fig. 3B).

Stromal cellular proliferative response and myofibroblast population correlated with IL-17A-positive cell densities in both patients with adenoma and CRC.

As illustrated in Fig. 4A, analysis revealed that adenoma/CRC patients with a higher IL-17A-positive cell density tended to have a higher Ki67 cell density than those with a lower IL-17A-positive cell density. Similar relationship was observed between IL-17A-positive cell densities and SMA-positive myofibroblasts in the adenoma/CRC stroma (see Fig. 4B).

3.4. The expression of IL-17RC in CD146-positive stromal cells

DIF images showed that IL-17RC (Fig. 5A for adenoma, 5D for CRC) was expressed in CD146-positive stromal cells (Fig. 5B for adenoma, 5E for CRC) in the adenoma (Fig. 5C for merged image) and CRC (Fig. 5F for merged image), indicating a possible modulation pathway of IL-17A on CD146-positive stromal cells in the adenoma/CRC.

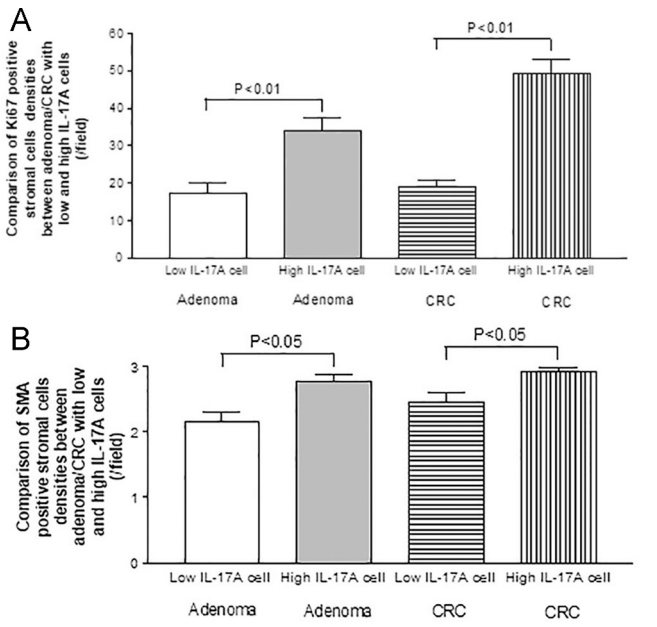

Fig. 4. The correlation between IL-17A-positive cell density and Ki67 cell and SMA-positive myofibroblasts densities in the adenoma/CRC stroma Adenoma/CRC patients with a higher IL-17A-positive cell density tended to have a higher Ki67 cell density than those with a lower IL-17A-positive cell density in the adenoma/CRC stroma (A). Similarly, a high IL-17A-positive cell density also correlated to a high SMA-positive myofibroblasts in the adenoma/CRC stroma (B).

4. Discussion

Considerable evidence suggests that TH17/IL-17A plays an essential role in the development, progression, and metastasis of human CRC [14,18,20,21,55]. Current study has evaluated the correlation between the TH17/IL-17A and dynamic response of cellular stroma throughout the adenoma-carcinoma sequence. We were able to show that an activated TH17/IL-17A milieu was associated with the dynamic stromal cellular response in reaction to tumour signals and created a supportive microenvironment for the disease progression and metastasis.

To evaluate the dynamic of TH17/IL-17A along the adenoma-carcinoma sequence, we have firstly determined dynamic of key transcriptional factor ROR-C for TH17 cell differentiation and TH17 main cytokine IL-17A. q-PCR results showed that significantly increased expression levels of ROR-C were started from the adenoma stage and slightly decreased at the CRC stage, but still higher than the controls. Since the promoting effect of IL-17A on the development of CRC has been reported [21,52], the current finding might imply that TH17/IL-17A is an important element involving in the progression of adenoma to CRC in human. Whereas the expression levels of IL-17A were continuedly increased from the adenoma stage to the CRC stage. Such inconsistent changing trend between transcriptional factor for TH17 differentiation and IL-17A levels might suggest that TH17 cell differentiation from normal to the adenoma stage is highly activated and becomes stable when the CRC is developed. We postulated that the

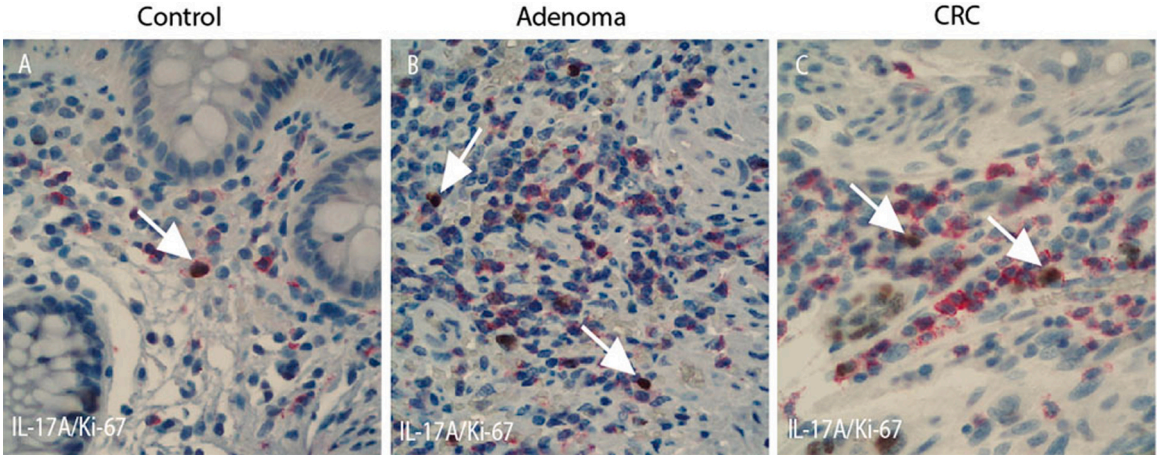

Fig. 3. Double immunohistochemical photograph presentation of proliferative capacity in TH17 cells (labelled by IL-17A immunoreactivity) in the adenoma/CRC stroma Double IHC with Ki67/IL-17A antibodies further showed that increased proliferation LI (Ki67 visualized by DAB, *Brown colour* in Figure B for Adenoma and 3C for CRC) was frequently observed in TH17 cells (IL-17A visualized by Fast Red, *Red colour* in Figure B for Adenoma and 3C for CRC), but less in the controls (A). (Figure A-C, double immunohistochemistry images, original magnification 400 ×; counterstaining was haematoxylin). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

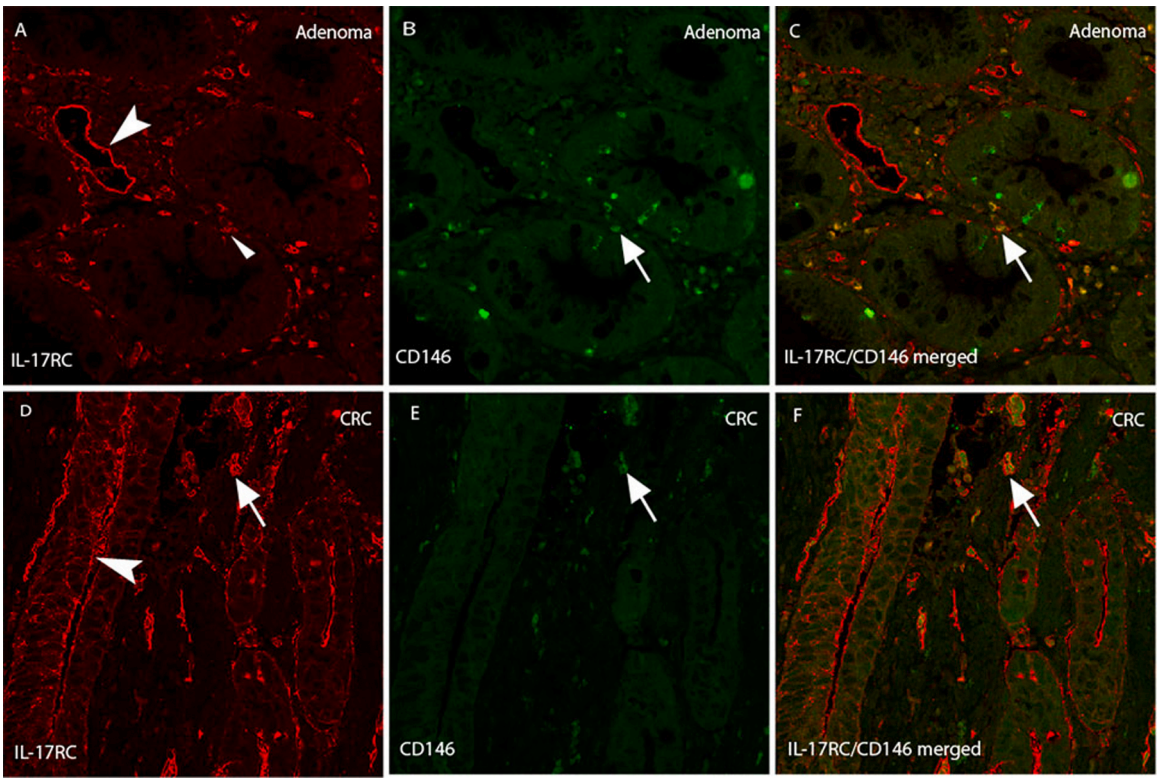

Fig. 5. Double immunofluorescence visualize the expression of IL-17RC on CD146-positive cells in the adenoma/CRC stroma Double immunofluorescence images showed that IL-17RC-immunoreactivity (visualized by Texas red, *red colour*) was frequently expressed on CD146-positive cells (visualized by FITC, *green colour*) in the adenoma (arrows in A-C) and CRC (arrows in D-F) stroma. Interestingly, IL-17RC-immunoreactivity could be also observed in tumour associated microvessels (arrowhead in Figure A) and CRC epithelium (arrowhead in D) respectively. (Figure A-F, confocal images, original magnification 200 ×; counterstaining was not applied). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

cellular source of IL-17A has been altered along the adenoma – carcinoma sequence. In the adenoma stage, TH17 cells could be the main cellular source for IL-17A, however in the CRC stage other cells might join the production of IL-17A. For instance, studies have shown that IL-17 could be produced from stromal cells such as fibroblast [56] and FoxP3-positive regulatory T cells [57], these cells are significantly increased and activated in the CRC microenvironment [4,13,48,58,59].

Stromal response in reaction to tumorigenesis includes a series cellular and functional changes i.e. stromal cell proliferation, population expansion and phenotypical differentiation, growth factor production, enhanced angiogenesis and extracellular matrix modification [60]. Studies have demonstrated that fibroblasts, as the major cell type composing the stromal cell population, within the adenoma/CRC stroma are greatly activated and differentiated [4]. Studies have also suggested that resident fibroblasts in the tumour stroma contribute to a significant increased population of SMA-alpha expressing (myofibroblasts) in the adenoma/CRC stroma and play a critical biophysical role in cancer invasion and metastasis [58,59,61]. Fibroblast-myofibroblast transition significantly contribute to the generation of an inflammatory microenvironment that supports the progression and metastasis of CRC [62]. In this study, we were able to show that increased proliferative rate of stromal cells and stromal myofibroblast populations (a subpopulation of fibroblasts) were observed in the adenoma/CRC stroma, suggesting a strong stromal response in reaction to the tumorigenesis process.

The proliferation, migration and expansion of stroma cells are regulated by many factors including cytokines [63,64]. McGeachy and colleagues have previously shown that IL-17 could significantly promote the survival and proliferation of fibroblastic reticular cells in inflamed lymph nodes by enhancing their metabolic activity [28]. In this study, we found that densities of IL-17A-positive cells correlated either with Ki67 positive cells and densities of myofibroblasts in the adenoma/CRC stroma. Analysis revealed that the adenoma/CRC patients with higher IL-17A expressing cells tended to have a higher proliferative rate and population of myofibroblasts in the stroma, suggesting a possible impact of IL-17 on myofibroblast expansion in the adenoma/CRC stroma. In addition, MSCs are one of the putative cellular sources for stromal fibroblasts [65]. There is strong evidence to suggest that MSCs are regulated by their surrounding microenvironmental elements [66]. In which, IL-17A has been shown to be a promoting factor for MSCs [26,37]. CD146 is a cell membrane protein whose expression has been implicated in multiple human cancers. High expression of CD146 is reported in stromal MSCs [51,67–69], and involved in the tumour stroma remodelling [70]. CD146 has also been hypothesized to be an EMT inducer in breast cancer [50], and also participates in the modulation of tumour angiogenesis [71]. Recent studies revealed that CD146 could be a cellular surface receptor of miscellaneous ligands, including some growth factors and extracellular matrixes [72]. Jiang et al. found that CD146 is a coreceptor for vascular endothelial growth factor receptor 2 (VEGFR2) and participates in the regulation of tumour angiogenesis [51], Espagnolle et al. reported that CD146 expression on MSCs is associated with their vascular smooth muscle commitment [68]. These lines of evidence strongly suggest that CD146 is a critical molecule involved in the stromal remodelling. We have therefore examined the potential pathway of IL-17A on CD146-positive stromal cells. Our data showed that IL-17RC was highly expressed in the CD146-positive stromal cells in both the adenoma and CRC stroma. This finding implies a possible action pathway for IL-17A on the adenoma/CRC stromal cells. Since recent progressions have supportively indicated that TH17/IL-17 network plays an essential role in promoting CRC progression and metastasis, monoclonal antibodies targeting the IL-17/IL-17RA axis has been considered as a potential biotherapeutic that might improve the efficiency of anti-VEGF therapy in metastatic CRC patients [73]. Indeed,

we and others have previously demonstrated that administration of anti-IL-17A antibody could significantly suppress the development of CRC in mice [74,75].

Some of the limitations for this study must be discussed here. Firstly, we only examined the co-expression of IL-17 in Ki67 positive stromal cells and did not look at the co-staining for IL17RC in Ki67 positive stromal cells. Which only indicate a possible paracrine pathway. However, autocrine IL-17A–IL-17RC neutrophil activation has been shown in the pathological condition [76]. Thus, a possible autocrine pathway of IL-17A-IL-17RC in tumour stromal cells need to be validated in further studies. Furthermore, we mainly focus on the potential modulation of IL-17A on CD146 positive stromal cells in this study and show a co-staining of CD146 with IL-17RC in adenoma/CRC stromal cells. However, previous studies have demonstrated an increased expression of CD146 in tumour microvessels in breast carcinoma indicated that CD146 is a potentially useful prognostic marker for breast cancer [77]. Such inconsistent results might be due to the limited sections used for double immunofluorescence of CD146/IL-17RC and different antibody sources used in the current study. Extension studies with larger samples need to be conducted in the future.

5. Conclusion

Although exact mechanisms for TH17/IL-17A in promoting adenoma/CRC progression and metastasis are so far undetermined, however taken together with previous findings, current observations along the adenoma–carcinoma sequence could suggest a potential modulatory effect of TH17/IL-17A on stromal reaction and cellular response, which might provide a supportive environment for the initiation and progression of CRC. In the future, the blocking efficacy of IL-17 signal in modulating stromal components involved in the adenoma-carcinoma transition and the process of metastasis should be evaluated.

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CRediT authorship contribution statement

GC had the idea for this project and performed the most experiments, ZL and RG performed rest of experiments and data analysis. JF joined the data analysis and discussion. All the listed authors contributed to this manuscript in writing and final approvement.

Conflict of interest statement

The authors declare no conflicts of interests.

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