

Dynamics of the IL-33/ST2 network in the progression of human colorectal adenoma to sporadic colorectal cancer

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Abstract Most sporadic colorectal cancers (CRCs) develop from preformed adenomas. Cytokines are involved in the transition from adenoma to CRC. Interleukin-33 (IL-33) is a newly discovered proinflammatory cytokine belonging to the IL-1 cytokine family and involved in the development of chronic inflammation and cancer. The aim of this study was to evaluate the dynamics of the IL-33/ST2 axis during the sequence of progression from normal colorectum to adenoma to carcinoma and to investigate the association of IL-33 and ST2 expression with clinicopathological parameters and prognosis. The results demonstrated that the levels of IL-33 and ST2 in adenomas ($n = 50$), determined by real-time PCR, were significantly higher than those of normal controls ($n = 30$); the levels of both IL-33/ST mRNA in CRCs ($n = 50$) were higher than in normal controls but lower than in adenomas. Further

analysis revealed that the expression level of ST2 in CRCs was associated with tumor/node/metastasis (TNM) stage. The log-rank test showed that neither the IL-33 nor the ST2 expression level was correlated with overall survival in patients with CRC. The increased expression of IL-33/ST2 in adenomas and CRC tissues was confirmed by immunohistochemistry and was observed in both the tumor stromal cells and adenomatous/cancerous cells. Notably, increased densities of IL-33-positive and ST2-positive microvessels were found in the stroma of adenomas and CRCs. In conclusion, increased expression of the IL-33/ST2 axis along the colorectal adenoma–carcinoma sequence might be involved in the neoplastic transformation via the participation of this axis in the regulation of angiogenesis.

Keywords Adenoma–carcinoma transition · Colorectal cancer · Interleukin-33 · ST2

Abbreviations

CRC	Colorectal cancer
CT	Cycle threshold
IBD	Inflammatory bowel diseases
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
LGD	Lower-grade dysplasia
HGD	High-grade dysplasia
HPF	High-power fields
MGD	Moderate-grade dysplasia
MVD	Microvessel densities
PCR	Polymerase chain reaction
SEM	Mean of standard error
Th	T helper
TNF	Tumor necrosis factor
TNM	Tumor/node/metastasis

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Introduction

Human colorectal cancer (CRC) is the third most frequent cancer worldwide. According to the classic genetic model for colorectal tumorigenesis described by Fearon and Vogelstein [1], most sporadic CRCs develop primarily from a preexisting principal precursor—an adenomatous polyp—through a progressive accumulation of a number of genetic mutations and molecular alterations [2, 3]. The transition from an adenoma to a sporadic CRC may take many years and invariably evokes a strong inflammatory response that includes inflammatory cell infiltration and pro-inflammatory cytokine secretion in the tumor microenvironment [4–6]. Because these inflammatory components construct an inflammatory microenvironment and then contribute to the initiation and progression of CRC [7–12], it is important to investigate the dynamic temporal changes in this tumor inflammatory microenvironment. Indeed, we and others have previously demonstrated that the colorectal neoplastic transformation leads to remarkable changes in cytokine profiles in the tumor microenvironment [7, 9, 13, 14], which might have a significance in predicting the progression from adenoma to CRC and the prognosis of the CRC [8, 10, 11, 13–16]. These findings provide new insights into transitional mechanisms of tumor progression and help to design novel interventional strategies.

Interleukin (IL)-33 is a newly discovered pro-inflammatory cytokine belonging to the IL-1 family. The IL-33/ST2 (IL-33 receptor) axis plays an important role in regulating both T helper (Th)1 and Th2 cell responses [17] and is involved in the pathogenesis of human inflammatory diseases [18, 19]. For colorectal diseases, extensive evidence now suggests that IL-33 is activated in the inflamed mucosa of ulcerative colitis and is known to be correlated with the development of chronic inflammation [18, 20]. Because the involvement in chronic inflammation, as seen in inflammatory bowel disease (IBD), has been suggested to be a critical mechanism underlying CRC, the role of IL-33/ST2 in tumors has recently attracted much attention and investigation. Studies have shown that the IL-33/ST2 pathway plays an active role in inhibiting antitumor immunity and subsequently promoting metastasis in breast cancer [19, 21, 22]. In inflammation-associated pancreatic cancer, it might function as a crucial mediator that stimulates the process of carcinogenesis [23]. Moreover, two studies have shown that IL-33 is associated with tumor cell invasion or metastasis in head and neck squamous cell carcinoma [24] and hepatocellular carcinoma [25]. The prognostic significance of IL-33 has also been examined. Several reports have revealed that cancer patients with a high expression of IL-33 have a poor prognosis [24, 26, 27]. However, the involvement of IL-33 in different human tumors is controversial; a protective role of IL-33 in some types of human

cancers has also been reported. For example, a reduced IL-33 plasma level in patients with multiple myeloma is associated with a more advanced stage of disease [28].

Despite extensive data describing the importance of IL-33/ST2 activation in the pathogenesis of chronic colorectal inflammation as seen in IBD [18, 20], the role of the IL-33/ST2 axis in the development of sporadic CRC has yet to be examined. It is known that the inflammatory response occurs throughout the adenoma–carcinoma sequence and contributes to the adenoma–cancer transition through the release of pro-tumor cytokines and other growth factors. In addition, we have previously found that the tumor microenvironment has a Th2 cytokine profile in the adenoma–carcinoma sequence [29]. Moreover, it has been shown that IL-33 enhances Th2 polarization [17]. Given this, we hypothesized that an altered IL-33/ST2 axis might be involved in the pathogenesis of colorectal neoplastic transformation. In the present study, therefore, we characterized the dynamic changes in the IL-33/ST2 axis in the tumor microenvironment and evaluated its significance along the colorectal adenoma–carcinoma sequence.

Patients and methods

Patients

Patients were enrolled at the Departments of Gastrointestinal Surgery and Gastroenterology at the University Hospital of North Norway: 50 colorectal adenomas excised completely by endoscopic polypectomy in patients ages 43–92 years, 50 CRC lesions excised by surgery in patients ages 42–89 years, and 30 morphologically normal colorectal mucosa samples confirmed by colonoscopic and histological examination in patients ages 24–79 years. Patients were recruited between August 2003 and December 2008 (for detailed information, refer to Table 1). None of the included patients or control subjects had a history of regular use of immunomodulation treatment or chemotherapy. All biopsies were prepared and routinely embedded in paraffin. Sections (4 μ m) were cut and stained with hematoxylin and eosin (H&E). Histological diagnosis of all biopsies was determined by experienced pathologists from the Department of Clinical Pathology at the University Hospital of North Norway and was reviewed by senior pathologist SWS. The study was approved by the Regional Ethical Committee of Northern Norway, and written informed consent was obtained from all patients.

Real-time polymerase chain reaction (PCR) quantification of IL-33 and ST2 mRNA

Biopsies were collected in *RNAlater* solution (Ambion Europe, Cambridgeshire, UK), total RNA was extracted

Table 1 Histological data of specimens from normal controls and patients with adenomas and colorectal cancer

	Gender		Pathology			Dysplasia		
	Male	Female	Tubular	Tubulovillous	Villous	LGD	MGD	HGD
Normal	20	10						
Adenoma	31	19	33	15	2	22	24	4
			Adenocarcinoma			TNM stage		
				Mucinous	Signet ring	I	II	III + IV
Cancer	42	8	45	4	1	9	20	21

LGD lower-grade dysplasia; MGD moderate-grade dysplasia; HGD high-grade dysplasia

by the *TRIzol* method (Invitrogen Life Tech., Carlsbad, CA, USA), and reverse transcription was performed with *SuperScript II* (Invitrogen Life Tech., Carlsbad, CA, USA). Real-time PCR was performed on an *ABI-prism 7900* sequence detector with a *TaqMan Gold™* PCR core reagents kit (Applied Biosystems/Roche, Branchburg, NJ, USA) in 25 μ L volume according to our previously published method [30]. The TaqMan primer sequences for the housekeeping gene (beta-actin) were as follows: forward primer 5' TGCCGACAGGATGCAGAAG 3'; reverse primer 5' GCCGATCCACACGGAGTACT 3'; and probe FAM 5' AGATCAAGATCATTGCTCCTCCTGAGCGC 3' TAMRA. Primer sequences for IL-33 were as follows: forward primer 5' TGAGTCTCAACACCCCTCAAATG 3'; reverse primer 5' GGCATGCAACCAGAAGTCTTTT 3'; and probe FAM 5' CAGGTGACGGTGTGATGGTAAGATGTTAATG 3' BHQ. IL-33 receptor ST2 primers were purchased from Life Technologies (Cat. No. 4331182; Grand Island, NY, USA). The expression of IL-33 and ST2 mRNA in tissues obtained from the colorectal adenomas and CRCs was measured by cycle threshold (CT) value relative to that of the normal control mucosa as fold difference (N) = $2^{-\Delta\Delta CT}$, $\Delta CT = CT_{\text{measured gene (IL-33 or ST2)}} - CT_{\text{beta-actin}}$, $\Delta\Delta CT = \Delta CT_{\text{adenoma or CRC}} - \text{average } \Delta CT_{\text{normal}}$ as described in our recent publication [14]. The difference among the normal control, colorectal adenoma and CRC was compared by ΔCT values.

Immunohistochemical examinations of IL-33-positive cells and ST2-positive cells in the tumor microenvironment

To evaluate the distribution and expression patterns of the IL-33/ST2 axis in the tumor microenvironment, immunohistochemistry (IHC) was performed using 4- μ m paraffin sections from the controls, adenomas and CRCs with a *Vectastain Elite ABC Kit* (Vector Lab., Burlingame, CA, USA) according to the manufacturer's instructions and our published methods [31]. The following primary antibodies were used: goat antihuman IL-33 polyclonal antibody (working dilution 1:100; R&D systems, Minneapolis, MN,

USA) and mouse antihuman ST2 monoclonal antibody (working dilution 1:100; Medical & Biological Laboratories Co. Ltd., Nagoya, Japan). Antibodies were incubated at 4 °C overnight. 3-Amino-9-ethylcarbazole (AEC; Vector Laboratories, Burlingame, CA, USA) was used as chromogen, and slides were slightly counterstained with Mayer's hematoxylin. Negative control slides for IHC were used routinely as follows: (1) Primary antibodies were substituted with the isotype-matched control antibodies; (2) secondary antibody was substituted with phosphate buffered saline (PBS).

Evaluation of IL-33-expressing cell phenotypes in the tumor stroma by double IHC staining

To define the phenotypes of IL-33-expressing cells in the tumor microenvironment, double IHC staining with the antibodies IL-33 (rabbit polyclonal antibody from Abcam, UK)/CD34 (monoclonal antibody from Dako Corp., Carpinteria, CA, USA, to label microvessels) and IL-33/SMA-alpha (monoclonal antibody from Dako Cor., Carpinteria, CA, USA, to label myofibroblasts) was performed using the *EnVision Doublestain System kit* (Dako Corp., Carpinteria, CA, USA) as previously described in our publication [14]. The 3-amino-9-ethylcarbazole substrate kit for peroxidase (AEC, Vector Laboratories, Burlingame, CA, USA) was used for the visualization of IL-33 immunoreactivity, and the Vector Blue Alkaline Phosphatase Substrate Kit III (Vector Blue, Vector Laboratories, Burlingame, CA, USA) was used for CD34 and SMA-alpha immunoreactivity, respectively. Nuclear counterstaining was not applied.

Morphometric analysis

All stained slides were examined under light microscopy. IL-33-positive and ST2-positive cells were found to be present in both the lamina propria and the epithelium, and the densities of positive cells were evaluated in the lamina propria and epithelium according to the methods described in

our previous publication [13]. In the stroma, semi-quantified scoring was performed in at least three optional fields with abundant distribution from each slide under $\times 400$ high-power fields (HPF) and was scored as: nil (0), 1–19 cells/field (1+), 20–49 cells/field (2+) and over 50 cells/field (3+). IL-33-positive and ST2-positive microvessel densities (MVDs) in the stroma were quantified according to the previously published method [32]. In the epithelium, IL-33-positive cells and ST2-positive cells were graded on a scale of 0–3, with 0 representing no detectable staining and 3+ representing the strongest staining. The average values were used for statistical analysis.

Statistical analysis

The results were expressed as the mean \pm mean of standard error (SEM) unless otherwise stated. Statistical significance was evaluated by the Mann–Whitney test or the Kruskal–Wallis test. Kaplan–Meier analysis was used to calculate survival rates, and differences in survival curves were determined by the log-rank test. The Cox proportional hazards regression model with a stepwise procedure was used to analyze the simultaneous influence of prognostic factors. Values of $P < 0.05$ and < 0.01 were considered significant.

Results

On the transcript level, the expression of the IL-33/ST2 axis was dynamically altered along the adenomas–carcinoma sequence

The expression level of IL-33 mRNA in the adenoma tissues determined by real-time PCR was significantly increased compared with the normal controls (Fig. 1a). The expression of IL-33 mRNA in the CRC tissues was also significantly higher than in the normal controls but lower than in the adenomas (Fig. 1a).

The expression level of ST2 mRNA along the adenoma–carcinoma sequence was also changed. As shown in Fig. 1B, the change in ST2 expression showed a pattern similar to that in IL-33 expression but to a more moderate degree. ST2 expression was greatly increased in the adenoma tissues but not changed in the CRC tissues compared with the normal controls (Fig. 1b).

Expression levels of IL-33/ST2 mRNA were analyzed against clinical pathological parameters in adenomas and sporadic CRCs

Because the IL-33/ST2 axis has been reported to be involved in the pathogenesis of human cancers [21, 26, 33], the expression levels of IL-33/ST2 mRNA were analyzed

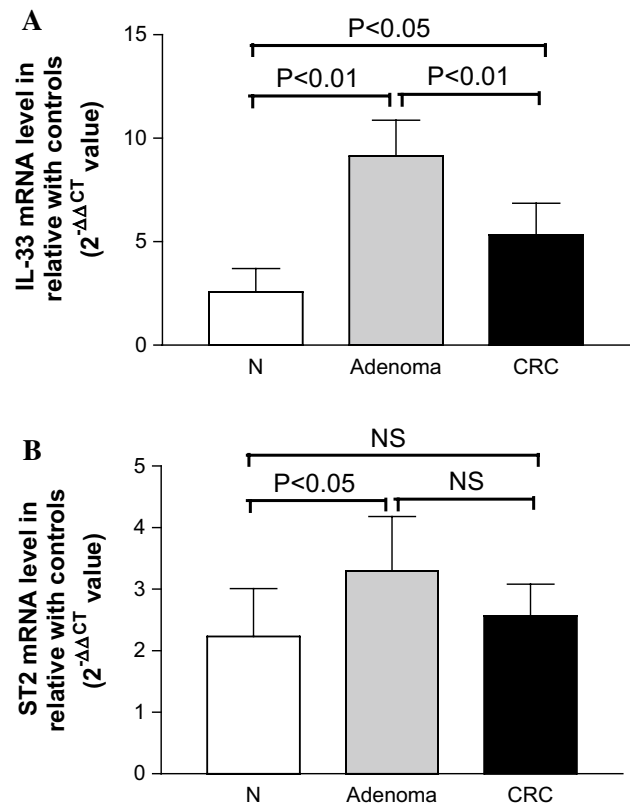


Fig. 1 Dynamic changes in IL-33 and ST2 transcripts along the colorectal adenoma–carcinoma sequence. The quantitative real-time PCR results showed that the expression of IL-33 mRNA was significantly increased to a ~ninefold higher level in adenoma tissues (*gray bar in a*); it was also higher in sporadic CRC tissues (*black bar in a*) than in normal controls (*white bar in a*), but significantly lower compared with the adenomas. The expression of ST2 mRNA in adenoma tissues was increased to a ~threefold higher level and slightly increased in CRC tissues compared with normal controls (*b*). (*Y axes in a, b* are fold changes relative to normal controls; *P* values are derived from the Mann–Whitney test.)

against clinical pathological parameters of adenomas and CRCs.

At the adenoma stage, the expression levels of both IL-33 and ST2 mRNAs were not correlated with degree of dysplasia (low-grade dysplasia vs. moderate-grade dysplasia vs. high-grade dysplasia; IL-33: 6.74 ± 1.69 vs. 10.46 ± 2.65 vs. 11.71 ± 8.65 ; ST2: 2.56 ± 0.94 vs. 4.09 ± 1.81 vs. 1.05 ± 0.35 ; both $P > 0.05$ by the Kruskal–Wallis test). IL-33 and ST2 mRNA levels also did not differ between different histological subtypes/groups (tubular vs. tubulovillous + villous; IL-33: 10.04 ± 2.33 vs. 6.99 ± 2.05 ; ST2: 3.40 ± 1.50 vs. 2.01 ± 0.34 ; both $P > 0.05$ by the Mann–Whitney test).

At the CRC stage, the expression level of IL-33 mRNA was not associated with TNM (tumor/node/metastasis) stage (TNM stage I + II vs. III + VI: 5.61 ± 2.00 vs. 4.89 ± 2.65 ; $P > 0.05$ by the Mann–Whitney test)

or node involvement (node negative vs. node positive: 8.62 ± 2.85 vs. 8.01 ± 4.72 , $P > 0.05$ by the Mann–Whitney test). Similarly, the expression level of ST2 mRNA was not associated with node involvement (node negative vs. node positive: 2.03 ± 0.59 vs. 3.75 ± 1.09 , $P > 0.05$ by the Mann–Whitney test). However, the level of ST2 in CRC patients with advanced TNM stages was significantly higher than in those with early stages (TNM stage I + II vs. III + VI: 1.96 ± 0.66 vs. 3.59 ± 0.95 ; $P < 0.05$ by the Mann–Whitney test).

Significance of IL-33 and ST2 mRNA levels in predicating overall survival of patients with CRC

Previous studies have shown that the expression level of IL-33/ST2 in certain types of human cancers might predicate the survival time [24, 26, 27]. Therefore, we analyzed the relationship between the expression levels of IL-33/ST2 and clinical prognosis in patients with sporadic CRC. The overall survival data were available for 28 patients with CRC. Kaplan–Meier analysis revealed that both IL-33 level (Fig. 2a) and ST2 level (Fig. 2b) in cancer tissues were not associated with the overall survival in patients with CRC.

Immunohistochemistry of IL-33 and ST2 distribution patterns in the tumor microenvironment of adenomas and CRCs

Subsequently, we used IHC to examine the distribution patterns of IL-33 and its receptor, ST2, in the adenomatous and cancerous tumor microenvironments.

In the normal controls, IL-33 immunoreactivity was observed in the lamina propria but rarely in the epithelium (Fig. 3a). In adenomas and CRCs, IL-33 immunoreactivity was observed in both the tumor stroma and the epithelium. Many IL-33-positive cells were located in tumor-associated microvessels (arrow in Fig. 3b, c) and in adenomatous and cancerous epithelium (arrow in Fig. 3b and inserted image in Fig. 3c). The semi-quantitative grading scores of IL-33-positive cells in the adenomatous epithelium (Fig. 4a) and tumor stroma (Fig. 4b) were significantly higher than in the normal control; the grading scores of IL-33 in the CRC epithelium and tumor stroma were also higher than in the normal control, but lower than in the adenomatous epithelium and stroma (Fig. 4a, b).

ST2 immunoreactivity was observed in both the epithelium and lamina propria in normal controls (Fig. 3d), adenomas (Fig. 3e) and CRCs (Fig. 3f). Notably, some of tumor-associated microvessels in the adenoma/CRC tumor stroma (arrow in Fig. 3e for adenoma; arrow in Fig. 3e for CRC) were also positive for ST2 immunoreactivity. The semi-quantitative grading score of ST2 immunoreactivity in the epithelium in all three groups was not significantly

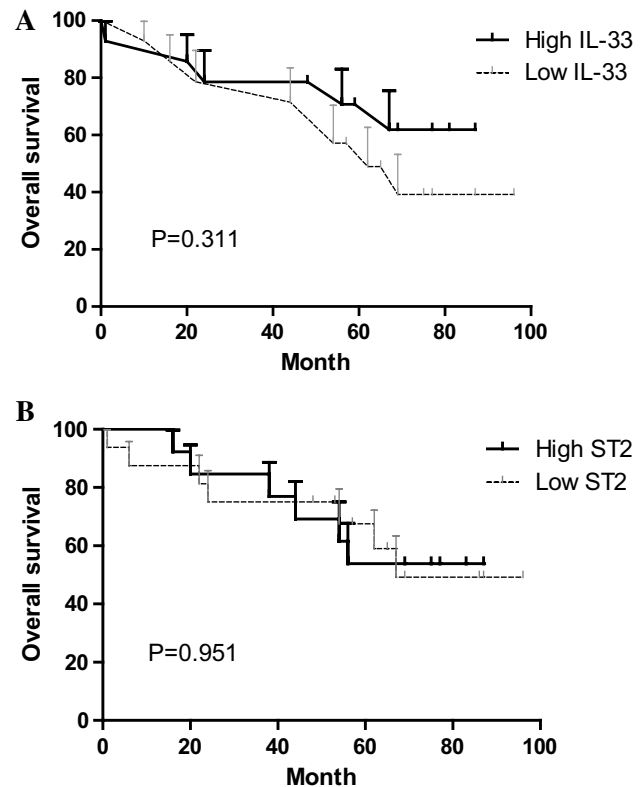


Fig. 2 Kaplan–Meier curves of overall survival differences among patients with sporadic CRC. The Kaplan–Meier analysis shows that the expression levels of IL-33 (a) and ST2 (b) do not predicate the overall survival time in patients with sporadic CRC (both P values determined by the log-rank test)

different (see Fig. 4c). However, the grading score of ST2-positive stromal cells showed an increasing trend from the control to the adenoma stage and to the CRC stage (Fig. 4d).

Because recent studies have demonstrated that vascular endothelial cells are one of the dominant IL-33-expressing cell populations [34], and because we observed an increased IL-33- or ST2-positive microvessels in the tumor microenvironment, we quantified the IL-33-positive microvessel density (MVD) and ST2-positive MVD in the adenoma and the CRC tumor stroma. The results revealed that both IL-33-positive MVD (Fig. 4e) and ST2-positive MVD (Fig. 4f) were significantly increased in the adenoma stroma and nonsignificantly increased in the CRC stroma compared with normal controls.

Phenotypic characterization of IL-33-positive cells in the tumor stroma

To further identify the phenotypes of IL-33-positive cells in the tumor stroma, double IHC using IL-33/CD34 (to mark tumor-associated microvessels) and IL-33/SMA-alpha (to

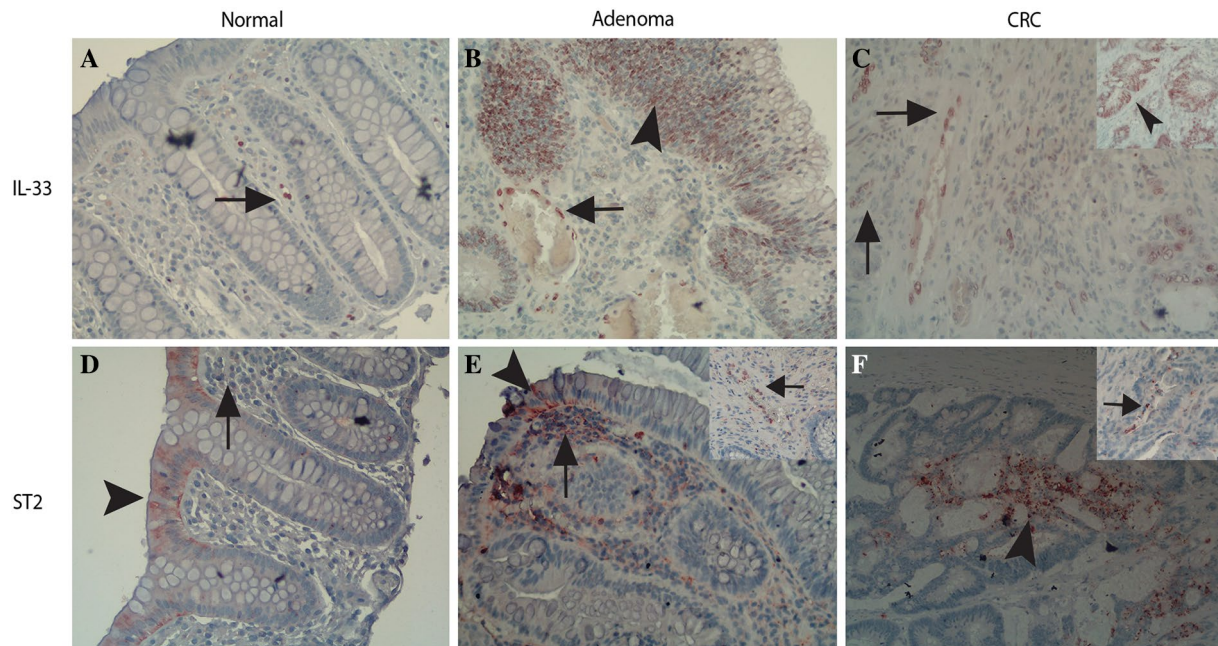


Fig. 3 Photograph presentations of IL-33 and its receptor, ST2, in the tumor stroma and the adenomatous/cancerous epithelium. Immunohistochemical (IHC) results show that IL-33 immunoreactivity was not observed in the normal epithelium, and a low density of IL-33-positive cells was found in the lamina propria in the control (a). In both the adenoma and CRC, IL-33 immunoreactivity was frequently

observed in tumor-associated microvessels (arrow in b, c) and adenomatous/cancerous epithelium (arrow in b for adenoma and inserted image in 3C for CRC). ST2 immunoreactivity was observed in both the epithelium (arrow head) and microvessels (arrow) in all three groups (d for control, e for adenoma and f for CRC). (a–e IHC, counterstained with hematoxylin, original magnification 200 \times .)

mark tumor-associated myfibroblasts) was performed. Compared with the control, the co-expression of IL-33 with CD34 (Fig. 5a–c) and SMA-alpha (Fig. 5d–f) in the stroma of adenomas/CRCs was frequently observed. This finding indicates that most IL-33-positive cells in the stroma of adenomas/CRCs were tumor-associated microvessels and myfibroblasts.

Discussion

Accumulated evidence has suggested that inflammatory cytokines, including tumor necrosis factor (TNF)- α , IL-6 and IL-17A, are involved in the pathogenesis of the adenoma–carcinoma transition [9, 13, 14, 35]. In this study, we found that the expression profile of the IL-33/ST2 axis within the tumor microenvironment was altered throughout the adenoma–carcinoma sequence and might be involved in the process of CRC. In particular, IHC examination demonstrated that high IL-33/ST2 expression is likely to be partially produced by the tumor-associated microvessels, fueling an autocrine pathway involving IL-33/ST2 expression and angiogenesis and ultimately influencing the development of colorectal adenomas and CRCs.

IL-33 is a novel cytokine with a potential pro-inflammatory capacity. The importance of IL-33 in the development

of the colorectal inflammatory disease ulcerative colitis has been addressed [18, 20]. Recent studies have also revealed that the elevation of IL-33 is involved in the pathogenesis of pancreatic cancer [23], gastric cancer [27] and liver cancer [25, 36], and ST2 is expressed in both the tumor cells and stromal cells [23]. However, there is very limited information available regarding the change in the IL-33/ST2 axis during the development of CRC. To our best knowledge, the current study is the first of its kind to demonstrate a dynamic change in the IL-33/ST2 axis from colorectal adenomas to sporadic CRCs. The increase in IL-33 began at the adenoma stage, and, although it declined slightly at the cancer stage compared with the adenoma stage, it was higher in both stages compared with controls. This finding might indicate that IL-33 is an early-response inflammatory element and might serve as an alarm index for the colorectal neoplastic transformation. Although the exact mechanism for this expression pattern is currently unclear, it might be related to the changed expression patterns of IL-33 regulatory factors at different time periods during the colorectal adenoma–carcinoma sequence. For instance, cytokines such as interferon (IFN)- γ and TNF- α are the well-documented stimulators for IL-33 production [37–39], and we have previously demonstrated that the expression of IFN-gamma and TNF- α is remarkably elevated in adenoma tissues but reduced in CRC tissues [13]. This might

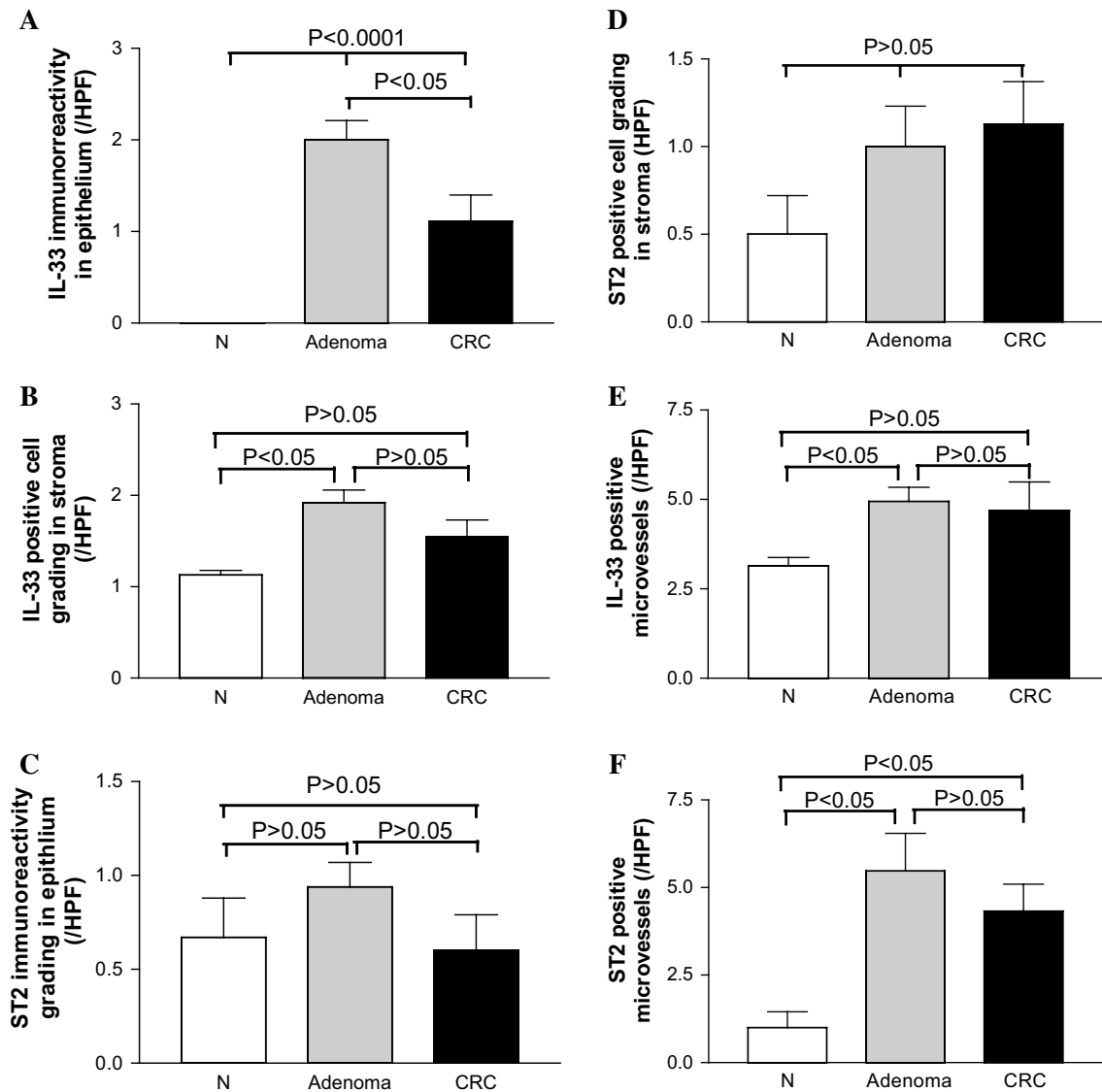


Fig. 4 Density grading scores of IL-33- and ST2-positive cells in adenomas and CRC tissues. The semi-quantitative results showed that the density of IL-33-positive cells in the adenomatous epithelium (gray bar in **a**) was higher than in the controls. The density of IL-33-positive cells was also higher in the CRC epithelium (black bar in **a**) compared with the controls, but lower than the adenomatous epithelium (Fig. **a**). The density of IL-33-positive stromal cells was increased in the adenoma stroma, with a smaller increase in magnitude of density in the CRC stroma (**b**). The density of ST2-positive cells in the adenomatous epithelium was non-statistically increased and was unchanged in the CRC cancerous epithelium (**c**). The density of ST2-positive cells in the stroma showed a gradually increasing

trend from the control to adenoma to CRC, but these differences were not statistically significant (**d**). To examine the expression of IL-33/ST2 in endothelial cells, the number of IL-33-positive tumors associated with microvessel density (MVD) and the number of ST2-positive tumors associated with MVD were counted. The results show that the IL-33-positive MVD was significantly increased in the adenoma stroma and non-statistically increased in the CRC tumor stroma compared with normal lamina propria (**e**). The number of ST2-positive microvessels was greatly increased in both the adenoma stroma and the CRC tumor stroma compared with the normal lamina propria (**f**). (HPF high-power field.)

partially explain the dynamic changes in IL-33 expression along the adenoma–carcinoma sequence. The expression of ST2 along the adenoma–carcinoma sequence was shown a pattern similar to that of IL-33; the expression level of ST2 mRNA was greatly increased at the adenoma stage but was unchanged from normal at the CRC stage. Taken together,

our quantitative findings indicate that the greatest change in the IL-33/ST2 axis along the adenoma–carcinoma sequence occurred at the adenoma stage.

With respect to the relationship between IL-33/ST2 and clinicopathological parameters of patients with adenomas and CRCs, we have found that although the difference

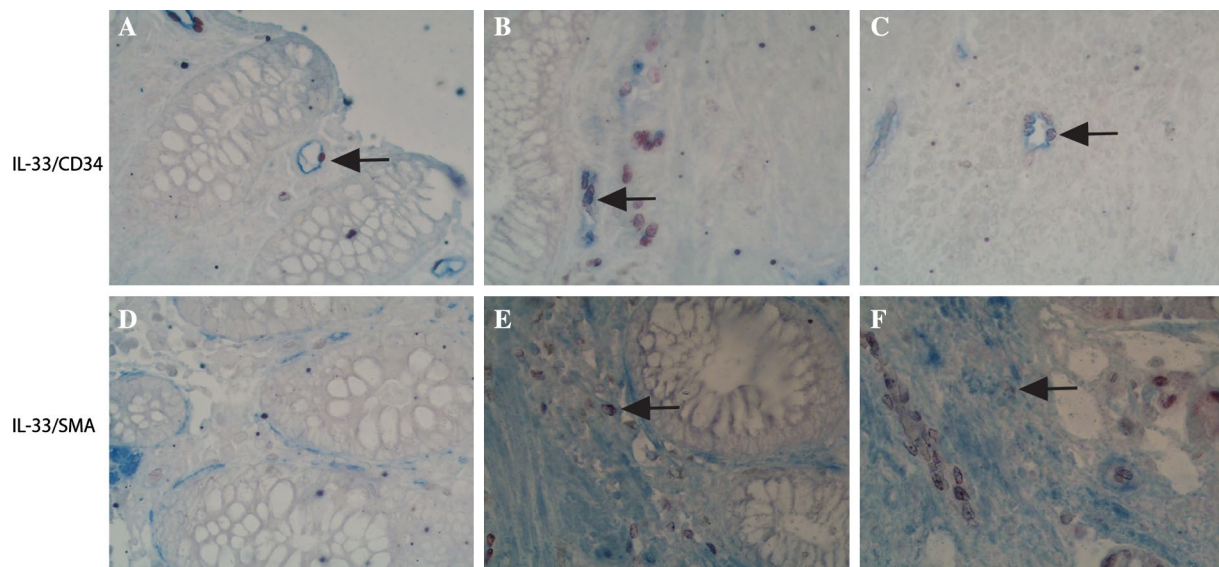


Fig. 5 Phenotypic characterization of IL-33-expressing cells in the adenoma and CRC tumor stromas. Double IHC with IL-33/CD34 and IL-33/SMA-alpha revealed that an increase in IL-33-positive cells (red) in the adenoma and CRC tumor stromas was frequently

co-localized with CD34-labeled microvessels (blue) (b, c) and SMA-alpha-labeled myofibroblasts (blue) (e, f) compared with the normal lamina propria (a, d). (a–f) double IHC, original magnification 400 \times ; counterstaining was not applied.)

did not reach statistical significance, the increased IL-33 expression in the adenoma tissues was associated with degree of dysplasia. Because the progression of dysplastic severity in adenomas is associated with a high malignant potential [40], our observation might imply that IL-33 is a potential stimulating factor for the disease stage progression. The expression level of ST2, but not IL-33, in the CRC tissues was associated with TNM stage; patients with advanced stages had a higher ST2 level than those with early stages. This finding is consistent with the Jovanovic and colleagues' data observed in breast cancer [33] and suggests that ST2 could be a potential modulator in CRC progression by increasing binding avidity for IL-33. Previous publications have also reported that the expression level of IL-33/ST2 is related to survival in patients with certain types of cancer [24, 26, 27]. However, we could not confirm this relationship between the expression levels of IL-33/ST2 and survival in patients with sporadic CRC. The overall survival was not influenced by either IL-33 or ST2. However, given that the number of patients with CRC and survival data in this study was rather small (only 28 patients), it is necessary to investigate the prognostic significance of IL-33/ST2 in a large-scale study of patients with CRC.

The expression of IL-33 and the ST2 receptor has been studied in several types of digestive system cancers [23, 25, 27, 36] and has been found to be localized in various cell types within specific microenvironments [23]. Our IHC results clearly showed increased IL-33 and ST2 immunoreactivity in the adenomatous/cancerous

epithelium, supporting the possibility that the IL-33/ST2 axis is involved in regulating the transformation of colorectal epithelium. Our double IHC observations confirmed IL-33 immunoreactivity in myofibroblasts in the tumor stroma, which is consistent with previous findings from studies of patients with ulcerative colitis demonstrating that myofibroblasts are an important cellular source of IL-33 [18, 29, 41, 42]. In this study, our IHC observations of increased IL-33 immunoreactivity in CD34-positive microvessels in the adenoma/CRC tumor stroma confirmed that vascular endothelial cells are the dominant IL-33-expressing cell population [34]. It has been recently reported that the pro-tumor mechanism of IL-33/ST2 is via enhanced angiogenesis in the mouse breast cancer model [33] and is mediated by its receptor, ST2, expressed in the effecting cells [19, 43]. In this study, we found that IL-33 was expressed in adenomatous/cancerous epithelium, tumor-associated myofibroblasts and microvessels and that ST2 was expressed in tumor-associated microvessels. By further quantifying IL-33-positive and ST2-positive MVDs, we found that both were significantly increased in adenoma/CRC tumor stroma. Our present findings might strongly support the hypothesis that increased expression of the IL-33/ST2 axis along the adenoma–carcinoma sequence enhances angiogenesis via paracrine and autocrine mechanisms. Because the expression of ST2 in adenomatous/cancerous epithelium has also been observed, a direct effect of IL-33 on transformed epithelial cells cannot be excluded, and further investigation is required.

In conclusion, our data reveal a dynamic change in the IL-33/ST2 axis along the colorectal adenoma–carcinoma sequence in which IL-33 and ST2 are more significantly increased at the adenoma stage than at the CTC stage. The expression levels of IL-33 and ST2 were not correlated with overall survival in patients with CRCs. Immunohistochemical identification of IL-33/ST2 phenotypes in adenoma/CRC might suggest a mixed cellular source in which tumor-associated microvessels are an important source of IL-33 and possess ST2 receptors in the tumor stroma; this might imply an autocrine mechanism of angiogenesis stimulation during the process of CRC. Additional studies are needed to further refine the exact functions and mechanisms of IL-33/ST2 contributing to the pathogenesis of adenomas and CRCs.

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Conflict of interest All authors declare no conflicts of interest.

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