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Dynamics of IL33/ST2 network from the human colorectal adenoma to sporadic colorectal cancer

Short title: IL-33 and sporadic colorectal cancer

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Abstract

Most sporadic colorectal cancers (CRCs) develop from preexisting adenomas. The transition from an adenoma to a CRC leads to disruption in cytokine secretion and immune imbalance. Interleukin-33 (IL-33) is a newly discovered proinflammatory cytokine belonging to the IL-1 cytokine family, and involved in the regulation of immune function and development of chronic colorectal inflammation and cancers. However, the activation of the IL33/ST2 axis alongside the colorectal adenoma-carcinoma sequence is poorly understood. Our aim is to evaluate the dynamics between the IL-33/ST2 axis and the human colorectal normal - adenoma - carcinoma sequence. The expression of IL-33 in adenomas (n=50) determined by real-time PCR was significantly higher than that in the colorectal mucosa from normal individuals. The expression of IL-33 was also increased in CRCs (n=50) when compared to the normal control group. Significantly lower levels of IL-33 where found in the CRCs compared with the adenomas; moreover, the analysis revealed that a high IL-33 level was associated with a low dysplasia degree in the adenomas, as well as with a statistically non-significant increase of survival time in the CRC patients. The increased expression of IL-33 in adenomas/CRCs was confirmed by immunohistochemistry (IHC) which showed that IL-33 immunoreactivity was expressed in the adenomatous/cancerous epithelium, whereas it was undetectable in the normal controls. IHC also confirmed that the IL-33 receptor (ST2) is increased in tumor stromal cells. Double IHCs identified that IL-33 immunoreactivity was in the tumor stromal myofibroblasts and microvessels. In conclusion, the expression of IL-33 in the tumor microenvironment was dynamically altered along the colorectal adenoma-carcinoma sequence; and may be a potential predictor for the progression of the adenoma to the CRC.

Introduction

Human colorectal cancer (CRC) is one of the most common cancers throughout the world. According to the adenoma-carcinoma sequence hypothesis, most sporadic CRCs develop primarily from a pre-existing adenoma in a setting where a multistep molecular and histological carcinogenesis event progresses to a carcinoma (1, 2). The transition from an adenoma to a CRC may take many years and can inevitably result in a series of changes that include altered cytokine secretion and immune cell function in the tumor microenvironment (3-5). Since the cytokines play an essential role in the communication between tumor cell initiation/progression and host anti-tumor immunity (6-11), it is important to investigate the cytokine changes in the tumor microenvironment, in order to better understand the transitional mechanisms and design novel interventional strategies. Indeed, in previous studies, we and others have revealed that the colorectal neoplastic transformation leads to a remarkable alteration of cytokine secretion in the tumor immune microenvironment (6, 8, 12, 13), this may have a significant role in predicting the progression from the adenomas to CRCs (12, 14, 15) and prognosis of the CRCs (7, 9, 10, 12, 13, 16).

IL-33 is a newly discovered proinflammatory cytokine that belongs to the IL-1 family. The IL-33/ST2 (IL-33 receptor) axis plays an important role in regulating both Th1 and Th2 cell responses (17) and is associated with inflammatory diseases in humans (18). The role of IL-33/ST2 in tumors has been investigated; however, the involvement of IL-33 in different human tumors is controversial. It has been reported that the IL-33/ST2 pathway may play an active role in inhibiting antitumor immunity, promoting metastasis in breast cancer (19-21) and acting as a crucial mediator in inflammation-associated pancreatic carcinogenesis (22). Moreover, IL-33 was reported to be associated with the invasion or metastasis of tumors in head and neck squamous cell carcinoma (23) and hepatocellular carcinoma (HCC) (24). The prognostic significance of IL-33 was also examined. Several studies have shown that a high

expression of IL-33 is associated with a poor prognosis in diverse cancers and may be a potential prognostic factor (23, 25, 26). However, opposing findings in different types of cancers have also been reported. In patients with multiple myeloma, reduced IL-33 plasma expression level was associated with a more advanced stage of disease (27). In human HCC, no significant difference in IL-33 serum levels was found in HCC compared to liver cirrhosis and healthy controls (28). The role of IL-33 in inflammatory bowel diseases has been studied, extensive evidence now suggests that IL-33 is activated in the inflamed mucosa of ulcerative colitis and plays a critical role in the development of chronic inflammation (29, 30), which is a risk factor for CRC. However, information is still very limited regarding the IL-33/ST2 axis in sporadic colorectal tumor-bearing hosts, particularly regarding the changes in the colorectal adenoma-carcinoma sequence.

Given the above background, we hypothesized that altered IL-33 expression in the tumor microenvironment could be an important change accompanying the colorectal neoplastic transformation. In the present study, therefore, we investigate the expression of IL-33/ST2 in the tumor microenvironment and its significance along the colorectal adenoma-carcinoma sequence.

Patients and Methods

Patients

Colorectal biopsies were obtained from 50 colorectal adenomas excised completely by endoscopic polypectomy (age 43-92 years); 50 CRC excised by surgery (age 42-89 years) and 15 morphological normal colorectal mucosa determined by colonoscopic and histological examination (age 30-77 years) in the Departments of Gastrointestinal Surgery and Gastroenterology, University Hospital of North Norway. Patients were recruited between August 2003 and December 2008 (detail information see Table 1). None of the included patients or control subjects had a history of regular use of immunomodulation treatment or chemotherapy. All the biopsies were prepared and routinely embedded in paraffin. Sections (4 μm) were cut and stained with haematoxylin and eosin (*H&E*). Histological diagnosis for all the biopsies was determined by experienced pathologists at the Department of Clinical Pathology, University Hospital of North Norway and reviewed by SWS. The study was approved by the Regional Ethical Committee of Northern Norway and written informed consent was obtained from the patients.

Quantification of IL-33 mRNA in the tissues from the controls, adenomas and CRCs by real-time PCR

Biopsies were collected in *RNAlater* solution (Ambion Europe, Cambridgeshire, UK) and total RNA was extracted by the *Trizol* method (Invitrogen Life Tech., Carlsbad, MA, USA) and reverse transcription was performed with *SuperScript II* (Invitrogen Life Tech., Carlsbad, MA, USA). Real-time PCR was performed 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 (31). The TaqMan primer sequences for housekeeping gene (beta-actin): forward primer 5’

TGCCGACAGGATGCAGAAG 3'; reverse primer 5' GCCGATCCACACGGAGTACT 3'; probe FAM 5' AGATCAAGATCATTGCTCCTCCTGAGCGC 3' TAMRA. For IL-33: forward primer 5' TGAGTCTCAACACCCCTCAAATG 3'; reverse primer 5' GGCATGCAACCAGAAGTCTTTT 3' and probe FAM 5' CAGGTGACGGTGTTGATGGTAAGATGTTAATG 3' BHQ. The expression of IL-33 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)=$2^{-\Delta\Delta CT}$, $\Delta CT = CT_{IL\text{-}8\ gene} - CT_{beta\text{-}actin}$, $\Delta\Delta CT = \Delta CT_{CRA\ or\ CRC} - \text{average}\ \Delta CT_{normal}$ as described in our recent publication (13). The difference among the normal controls, colorectal adenoma and CRC were compared by ΔCT values.

Immunohistochemical examinations of IL-33 (+) cells and IL-33 receptor ST2 in the tumor microenvironment

Immunohistochemistry (IHC) was performed using 4 μm paraffin sections from the controls, adenomas and CRCs with *Vectastatin Elite* ABC Kit (Vector Lab., Burlingame, CA, USA) according to the manufacturer's instructions and our published methods (32). The following primary antibodies were used: goat anti-human IL-33 polyclonal antibody (working dilution 1:100; R&D system, Minneapolis, MN, USA) and mouse anti-ST2 monoclonal antibody (Medical & Biological Laboratories Co. Ldt, Nagoya, Japan). Antibodies were incubated at 4ºC over night. 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 IHCs were performed routinely: (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 type in tumor stroma by double IHCs

To define the phenotypes of IL-33 expressing cells in the tumor microenvironment, double IHC staining with antibodies IL-33 (rabbit polyclonal antibody, from Abcam, UK)/CD34 (monoclonal antibody from Dako Cor., Carpinteria, CA, USA, to label microvessels), and IL-33/SMA-alpha (monoclonal antibody from Dako Cor., Carpinteria, CA, USA, to label myofibroblasts) were performed with *EnVision* Doublestain System kit (Dako Cor., Carpinteria, CA, USA) as described in our publication previously (13). 3-amino-9-ethylcarbazole substrate kit for peroxidase (AEC, Vector Laboratories, Burlingame, CA, USA) was used for the visualization of IL-33 immunoreactivity and Vector blue alkaline phosphatase substrate kit III (Vector blue, Vector Laboratories, Burlingame, CA, USA) for CD34 and SMA-alpha immunoreactivity respectively. Nuclear counterstaining was not applied.

Morphometric analysis

All the stained slides were examined under light microscopy. IL-33 and ST2 positive cells were found to be present in both the lamina propria and epithelium, their numbers were evaluated in the lamina propria and epithelium respectively according to the method described in our previous publication (12). In the stroma, semi-quantified scoring was done in at least 3 optional fields with abundant distribution from each slide under ×400 high-power magnifications and scored as: nil (0), 1-19 cells/field (1+), 20-49 cells/field (2+) and over 50 cells/field (3+). 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 mean ± SEM unless otherwise stated. Statistical significance was evaluated 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

On the transcript level, the increase of IL-33 was demonstrated in the adenomas and sporadic CRCs

The increase of IL-33 mRNA in adenomas, determined by real-time PCR, was significantly higher than that in the normal colorectal mucosa (see Fig. 1). The expression of IL-33 transcript in sporadic CRCs was also significantly higher than in normal controls, but lower than that in adenomas (see Fig.1).

The predicating significance of IL-33 level in the adenomas and CRCs

The expression levels of IL-33 with pathological parameters in the adenomas and sporadic CRCs were analyzed. It showed that high IL-33 levels were significantly associated with the low grade of dysplasia in the adenomas (see Fig. 1B). Adenoma patients with a high grade of dysplasia had a lower IL-33 expression level than compared with patients with a low grading score of dysplasia; but it did not differ between different histological subtypes/groups (see Fig. 1C).

The relationship between IL-33 and CRC pathological parameters was also analyzed. The results have shown that the expression level of IL-33 did not significantly differ in CRC patients with different TNM stages (TNM stage I vs. II vs. III+VI: 8.37±3.96 vs. 9.39±4.44 vs. 7.40±3.62: $P>0.05$, the Kruskal–Wallis test) and node involvements (node negative vs. node positive: 8.62±2.85 vs. 8.01±4.72, $P>0.05$, the Mann–Whitney test) in sporadic CRCs.

It has been shown that increased IL-33 expression may influence the survival in certain types of cancer patients (23, 25, 26, 28). Hence, we also analyzed the relationship between IL-33

expression and clinical prognosis in CRC patients. In this study, the overall survival data were available in 28 CRC patients. As shown in Fig. 1D, the Kaplan–Meier analysis revealed that the overall survival in CRC patients with a high IL-33 expression was in an increasing trend than those with a low IL-33 expression, although the difference was statistically not significant (Fig. 1E).

IL-33 and its receptor ST2 were expressed in both the tumor stromal cells and adenomatous/cancerous cells

To examine the expression patterns of IL-33 and its receptor ST2 in the adenomas and sporadic CRCs, IHCs with specific IL-33 and ST2 antibodies were performed. The IHC results demonstrated that IL-33 immunoreactivity was only observed in the normal lamina proporia, but not in the controls epithelium (Fig, 2A); whereas it was observed in both the in the microvessels (arrow pointed in Fig. 2B & C respectively) in the tumor stroma and adenomatous/cancerous epithelium (arrow head pointed in Fig. 2B and inserted image in Fig. 2C respectively). When the IL-33 positive cells were semi-quantified, the grading score of IL-33 positive cells were shown to be significantly increased in the adenomatous epithelium than that in the control epithelium (Fig. 2G); it was also increased in the cancerous epithelium compared to the control epithelium but lower than in the adenomatous epithelium (see Fig. 2G).

The results for ST2 IHC showed that ST2 immunoreactivity could be observed in both the epithelium and lamina propria in the control (Fig. 2D), the adenoma (Fig. 2E) and the CRC (Fig. 2F). ST2 immunoreactivity can also be seen in the tumor associated microvessels in the tumor stroma in both the adenomas (arrow pointed in inserted image in Fig. 2E) and the CRCs (arrow pointed in inserted image in Fig. 2E). However, when the ST2 positive cells were semi-quantitatively graded, only the grading score of ST2 positive cells in the stroma showed

a non-significant gradually increasing trend from the controls to adenomas and sporadic CRCs (see Fig. 2I). The grading score of ST2 immunoreactivity in the epithelium in all three groups was not changed (see Fig. 2H).

The phenotypic characterization of IL-33 expressing cells in the tumor stroma

To identify the IL-33 positive cell types in the tumor stroma, double IHCs with IL-33/CD34, IL-33/SMA-alpha were performed. As compared with the control group sections, the co-expression of IL-33 with CD34 (Fig. 3A-C) and SMA-alpha (Fig. 3D-F) in the tumor stroma was frequently observed in the adenomatous and CRC sections and revealed that most IL-33 positive cells in the adenomatous and cancerous stroma were tumor associated microvesells and myofibroblasts.

Discussion

Previously we and other groups have demonstrated that certain cytokines may play an important role in the adenoma-carcinoma transition (8, 12, 14, 15). In this study we have examined the dynamics of the novel proinflammatory cytokine IL-33 along the colorectal adenoma-carcinoma sequence. We were able to demonstrate that IL-33 was significantly elevated at the adenomatous stage, and became lower at the CRC stage as compared with the adenomatous stage, but still higher than the controls. The expression level of IL-33 was associated with the degree of dysplasia in the adenomas, and also statistically non-significant with the increase trend of overall survival time in the CRCs. These findings may suggest that IL-33 could be a potential prognostic indicator along the colorectal adenoma-carcinoma sequence.

Recent studies have revealed that the elevation of IL-33 is involved in several types of digestive system cancers that include pancreatic cancer (22), gastric cancer (26) and HCC (24, 28). In the investigation of IL-33 in colonic diseases, it has been found that the increase of IL-33 is related to the development of colorectal chronic inflammation (ulcerative colitis) (29, 30), whereas the epidemiological studies from different countries have revealed that ulcerative colitis may significantly raise the CRC risk (33-39). Our current study, in our best knowledge, is the first study to demonstrate a dynamic change of IL-33 from the colorectal adenomas to the sporadic CRCs. The increase of IL-33 is an early event in the colorectal adenoma-carcinoma sequence, which was significantly increased at the adenomatous stage and became lower at the cancer stage as compared to the adenomas; however it is still higher than that in the controls. The exact reason for this expression pattern is currently unclear. However, it may be related to the dynamic changes observed in IL-33 regulatory factor expressions in the same microenvironment at different stages of the colorectal adenoma-

carcinoma sequence. It has been shown that some cytokines i.e. interferon (IFN)-gamma and tumor necrosis factor (TNF)-alpha can stimulate the expression of IL-33 in human skin cell and skin inflammation (40-42), we have previously demonstrated that levels of IFN-gamma and TNF-alpha are elevated in the adenomatous tissues, but reduced in the CRC tissues (12). This may partially explain the dynamic change of IL-33 expression along the colorectal adenoma-carcinoma sequence. In this study we have found that the expression level of IL-33 in the adenomatous tissues is associated with the grade of dysplasia, which is an important histological transitional indicator from the adenoma to the CRC; adenomas with higher IL-33 level had a lower dysplasia degree. This finding may indicate that IL-33 is an early response cytokine and may serve as an alarm cytokine during the colorectal neoplastic transformation. Interestingly, the survival time was shown in an increasing trend in CRC patients with a higher IL-33 level had a longer survival time than those with lower IL-33 level, although the difference was not statistically significant. Since we have only got the survival data from 28 CRC patients, it is necessary to investigate its prognostic significance further in a large scale study of a CRC patient group.

Our further IHC results clearly showed that the adenomatous/cancerous epithelium may contribute to the increased expression of IL-33 in those patients, because the IL-33 immunoreactivity seen in the transformed (adenomatous/cancerous) epithelium, which was not observed in the control epithelium. In the tumor stroma IL-33 immunoreactivity can also be identified in microvessels and myfibroblasts, this observation is consistent with previous findings from studies in patients with ulcerative colitis (30, 43-45). In those studies, IL-33 immunoreactivity was observed in both the inflamed colonic epithelium and lamina propria. It has been revealed that IL-33 can stimulate angiogenesis and cell growth via its receptor ST2 expressed in the effecting cells (20, 46). In this study we have observed that ST2 was expressed in both the adenomatous/cancerous epithelium and microvessels, whether IL-

33/ST2 has such regulating functions in the tumor microenvironment needs to be investigated in the future.

In conclusion our data outlines a dynamic change of IL-33 expression along the colorectal adenoma-carcinoma sequence in which IL-33 is increased at the adenomatous stage with a declining expression towards the CRC stage. The level of IL-33 was associated with the progression of dysplastic degree in patients with adenomas and survival time in patients with CRCs. Additional studies are needed to give insight into the role of IL-33/ST2 in the initiation of adenomas and sporadic CRCs.

Table 1. Histological data of specimens from patients and normal controls

	Gender		Pathology			Dysplasia		
	male	female	tubular	tubulovillous	villous	LGD	MGD	HGD
Normal	10	5						
Adenoma	31	19	33	15	2	22	24	4
						TNM stage		
			adenocarcinoma	mucinous	signet-ring	I	II	III+VI
Cancer	42	8	45	4	1	9	20	21

LGD: Lower grade dysplasia.

MGD: Moderate grade dysplasia.

HGD: High grade dysplasia.

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Legends

Figure 1 legend

The dynamic change of tissue IL-33 mRNA expression along the colorectal adenoma-carcinoma sequence.

The quantitative real-time PCR results showed that the tissue level of IL-33 mRNA in the adenoma tissues was significantly increased to a ~33-fold higher level (*grey bar* in Fig. 1), The expression of IL-33 transcript in sporadic CRCs (*black bar* in Fig. 1A) was also higher, but significantly lower compared to the adenomas, than that in the normal controls (*white bar* in Fig. 1A). In the adenomas, the level of IL-33 mRNA was higher in patients with low grading score of dysplasia than those with high grading score (Fig. 1B), and did not differ between histological types (Fig. 1C). In the CRCs, the Kaplan–Meier analysis showed that high IL-33 expression level was associated with a longer survival time (Fig. 1D), although the difference was statistically not significant (Fig. 1E).

(Y axes in Fig. 1A are fold change relative to normal controls; P values are from the Kruskal–Wallis test or the Mann-Whitney test or log-rank test).

Fig. 2 legend

Photograph presentations of IL-33 and its receptor ST2 in the tumor stroma and the adenomatous/cancerous epithelium

Immunohistochemical (IHC) results showed that low density of IL-33 positive cells was only observed in the lamina propria in the controls (Fig. 2A), but not in the normal epithelium. In both the adenomas and CRCs, it was observed in the tumor stroma (arrow pointed in 2B&C) and adenomatous/cancerous epithelium (arrow head pointed in Fig. 2B and inserted image in2C respectively). The semi-quantitative results showed that IL-33 expression in the adenomatous epithelium was higher than that in the controls (*gray bar* in Fig. 2G); it was also higher in the CRC epithelium (*black bar* in Fig. 2G), but lower than that in the adenomatous epithelium, compared to the controls (see Fig. 2G).

The IHC results for ST2 IHC showed that ST2 immunoreactivity could be observed in the epithelium and lamina propria in the controls (Fig. 2C), adenomas (Fig. 2D) and CRCs (Fig. 2E). The grading score results showed that the expression of ST2 in the epithelium was not changed (Fig. 2H), but it was shown in an increase trend in the tumor stroma (Fig. 2I).

(A-E: IHC, counterstained with hematoxylin, original magnification 200×).

Fig. 3

The phenotypic characterization of IL-33 expressing cells in the tumor stroma

Double IHC results with IL-33/CD34, IL-33/SMA-alpha showed that some IL-33 positive cells were frequently colocalized with CD34 labeled microvessels (see Fig. 3B&C) and with SMA-alpha labeled myofibroblasts (see Fig. 3E&F) in the adenomatous and cancerous stroma as compared with the normal lamina propria in the controls (Fig. 3A & D respectively).

(A-F, double IHC, original magnification 400×; all counterstained with hematoxylin)