

Basic Study

Interleukin-6 compared to the other Th17/Treg related cytokines in inflammatory bowel disease and colorectal cancer

Tsvetelina Veselinova Velikova, Lyuba Miteva, Noyko Stanilov, Zoya Spassova, Spaska Angelova Stanilova

ORCID number: Tsvetelina Veselinova Velikova (0000-0002-0593-1272); Lyuba Miteva (0000-0002-0028-7692); Noyko Stanilov (0000-0002-3536-2154); Zoya Spassova (0000-0002-5142-5450); Spaska Angelova Stanilova (0000-0003-1368-9081).

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Tsvetelina Veselinova Velikova, Clinical Immunology, University Hospital "Lozenetz", Sofia 1407, Bulgaria

Lyuba Miteva, Spaska Angelova Stanilova, Department of Molecular Biology, Immunology and Medical Genetics, Medical Faculty, Trakia University, Stara Zagora 6000, Bulgaria

Noyko Stanilov, Oncoplastic Unit, University College London Hospital, London 235, United Kingdom

Zoya Spassova, Clinic of Gastroenterology, University Hospital "St. Ivan Rilski", Sofia 1431, Bulgaria

Corresponding author: Tsvetelina Veselinova Velikova, MD, PhD, Assistant professor, Clinical Immunology, University Hospital Lozenetz, Kozyak 1 Street, Sofia 1407, Bulgaria. tsvelikova@medfac.mu-sofia.bg

Abstract

BACKGROUND

The connection between inflammatory bowel disease (IBD) and colorectal cancer (CRC) is well-established, as persistent intestinal inflammation plays a substantial role in both disorders. Cytokines may further influence the inflammation and the carcinogenesis process.

AIM

To compare cytokine patterns of active IBD patients with early and advanced CRC.

METHODS

Choosing a panel of cytokines crucial for Th17/Treg differentiation and behavior, in colon specimens, as mRNA biomarkers, and their serum protein levels.

RESULTS

We found a significant difference between higher gene expression of *FoxP3*, *TGFb1*, *IL-10*, and *IL-23*, and approximately equal level of *IL-6* in CRC patients in comparison with IBD patients. After stratification of CRC patients, we found a significant difference in *FoxP3*, *IL-10*, *IL-23*, and *IL-17A* mRNA in early cases compared to IBD, and *IL-23* alone in advanced CRC. The protein levels of the cytokines were significantly higher in CRC patients compared to IBD patients.

CONCLUSION

Our findings showed that *IL-6* upregulation is essential for both IBD and CRC

conflict of interest regarding the publication of this article.

Data sharing statement: Consent from the patients for data sharing was obtained, and additionally, the presented data are anonymized, and the risk of identification is low. No additional data are available.

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development until the upregulation of other Th17/Treg related genes (*TGFb1*, *IL-10*, *IL-23*, and transcription factor *FoxP3*) is a crucial primarily for CRC development. The significantly upregulated *IL-6* could be a potential drug target for IBD and prevention of CRC development as well.

Key words: Inflammatory bowel disease; Colorectal cancer; Cytokines; mRNA; Interleukin-6; Th17/Treg cells

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Core tip: In our paper, we showed that *IL-6* upregulation is essential for both inflammatory bowel disease and colorectal cancer (CRC) development, whereas the upregulation of other Th17/Treg related genes (*TGFb1*, *IL-10*, *IL-23*, and transcription factor *FoxP3*) is a crucial primarily for CRC development. The significantly upregulated *IL-6* could be a potential drug target for inflammatory bowel disease and prevention of CRC development as well.

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INTRODUCTION

Inflammatory bowel disease (IBD), as a group of chronic relapsing inflammatory conditions of the gastrointestinal tract, is characterized by prolonged activation of the intestinal mucosal immune system, along with the system involvement, which promotes the release of biological markers, such as cytokines^[1]. The initiation and aggravation of the inflammatory process seem to be due to dysregulated immune responses with a parallel increase in the expression of pro-inflammatory cytokines, and deficiency of anti-inflammatory cytokines. The dysregulated homeostasis of pro- and anti-inflammatory signals contributes to persistent intestinal inflammation^[2].

Inflammation plays a substantial role in sustaining and promoting colorectal cancer (CRC) development as well. As Virchow described in 1863, cancer can arise from inflammatory sites, where the risk of CRC development may increase in the conditions of chronic intestinal inflammation, *via* the malignant cell transformation in the surrounding tissue. Furthermore, the inflammatory response shares various molecular mechanisms and signaling pathways with the carcinogenic process, such as apoptosis, increased proliferation rate, and angiogenesis^[3]. Nonetheless, the activation of two major oncogenic transcription factors/pathways, NF-κB and STAT3, drives the process of chronic inflammation and carcinogenesis^[4]. Activation of NF-κB is required for the induction of *IL-6* by many cell types, such as lymphocytes, monocytes, macrophages, myeloid cells, and cancerous cells. Through STAT3 signaling, cell proliferation and survival are assured by inhibiting apoptosis, cell adhesion, angiogenesis, *etc.* However, STAT3 can exert opposite roles in colon carcinogenesis depending on the tumor stage^[4].

Dysregulation of the immune response in patients with IBD and CRC triggers infiltration and accumulation of immune cells that provoke the release of several cytokines, chemokines, and growth factors, which may further influence the inflammation and the carcinogenesis process^[2]. Immune cells, such as T regulatory (Treg) cells, Type 2 macrophages, CD4+ T-helper (Th)-17 cells, CD8+ T cells, NK, *etc.*, can have effects either sustaining inflammation in IBD, as well as promoting or inhibiting CRC cell growth. The same is also valid for the cytokines which provide the cross-talk between immune cells and CRC cells^[5]. Epithelial cells of the colon are both producers and responders to cytokines and chemokines, and those signals can modulate epithelial cell activity by affecting their proliferation, migration, and survival programs^[6]. For example, cytokines, such as IL-6, IL-17, IL-21, TNFα, are believed to contribute to the formation of tumor-supportive microenvironment through mitogenic effects on the epithelial cancer cells^[5]. There are many subtle mechanisms of inflammatory responses and malignancy involving cytokines: An

induction of reactive oxygen species (*i.e.*, TNF α , IL-6, TGF β), inflammation-associated tumor growth through NF- κ B and STAT3 (*i.e.*, TNF α , IL-17, IL-6, IL-23, IL-10), inflammation-associated epithelial-mesenchymal transition (*i.e.*, TGF β , TNF α , IL-6), inflammation-associated angiogenesis (*i.e.*, VEGF, TNF α , IL-6, TGF β), and inflammation-associated metastasis (*i.e.*, TNF α , IL-6, TGF β , IL-10)^{6,7}.

This background is the reason why many investigations are concentrated on the molecular patterns and mechanisms for developing IBD and CRC. Since the recent developments for the role of cytokines in the intestinal inflammation are accumulating, there has been an increased interest in the similarities and differences in inflammation and cancer initiation and advances. We were interested in the comparing of cytokine patterns and subtle changes in cytokine milieu in inflamed tissues of IBD patients, as well as in the cancer environment in CRC patients, especially in the clinical context. However, far too little attention has been paid to opportunities for early detection of subtle changes in cytokine milieu in inflamed tissues in IBD and CRC, especially in the clinical context. As the survival of CRC affected patients depends highly on early detection, we were interested in comparing cytokine patterns of IBD patients in active disease with early and advanced CRC. To address this aim, we choose a panel of pro- and anti-inflammatory cytokines with a substantial role in Th17/Treg differentiation and behavior, like IL-6, TGF β , IL-17A, IL-23, IL-10, in colon specimens of IBD and CRC patients, as mRNA biomarkers, and their protein levels in the peripheral blood.

MATERIALS AND METHODS

Patients

By employing standard clinical, laboratory, endoscopic, histopathological, and radiological criteria, two main study groups were distinguished-IBD patients and CRC patients.

Inflammatory bowel disease patients

A total of 18 IBD patients [13 with ulcerative colitis, ulcerative colitis (UC), and 5-with Crohn's disease (CD)]in an active state of disease without immunosuppressive therapy, attending the University Hospital St. Ivan Rilski, Sofia, during 2011-2013, were recruited for the study. The diagnosis of patients was made according to the standard criteria of ECCO Consensus for CD (2010) and UC (2012) based on a set of anamnestic, clinical, laboratory, and instrumental studies. Criteria for the exclusion of the patients from the study were the following but not limited to: any history of colorectal cancer or hereditary cancer, or the presence of any dysplastic or lesions suggestive of tumor tissues during endoscopic and histological examination; any positive result for autoimmune disease markers (*i.e.*, anti-nuclear antibodies), proved infectious diarrhea, any severe systemic or psychiatric illness. The mean age of the IBD patients was 38 ± 14 years, eleven (61%) were women, and seven (39%) were men.

Colorectal patients

A total of 80 patients with CRC, obtained in the University Hospital and Trakia Hospital, Stara Zagora, during 2011-2017, were included in the study. They underwent surgical resection of the tumor with curative intent. The patients had no history of prior surgery for rectal/colon tumors, and no known hereditary cancer, UC, or CD, they did not receive chemotherapy or radiation therapy prior to surgery. The diagnose of CRC was confirmed by the histopathological examination, and TNM classification was performed for tumor grading and staging. According to the TNM classification, CRC patients were stratified as the following: 40 patients with early CRC (8 with 1st stage + 32 in 2nd stage) and 40 patients with advanced CRC (19 with 3rd + 21 with 4th stages). The mean age of the CRC patients was 64.05 ± 9.6 years. The total group of CRC was composed of 49 males (61%) and 31 females (39%). Paired tumoral tissue samples and adjacent non-tumoral mucosa were obtained from 12 patients with early CRC (7 with 1st stage + 5 with 2nd stage) and 18 patients with advanced CRC (10 with 3rd + 8 with 4th stages). The mean age of the patients with early CRC was similar to that of patients with advanced CRC (67.2 ± 7.2 vs 71.1 ± 10.6 ; $P = 0.29$ *t*-test). Participants included 19 men (63%) and 11 women (37%).

Ethical considerations

All patients gave written consent for the study approved by the Ethical Committee of the Medical University of Sofia and University Hospital St. Ivan Rilski. All patients were informed about the purpose of the study.

Specimen collection and preparation

A total of six intestinal samples were collected from each patient with IBD—from paired inflamed (three) and nearby macroscopically non-inflamed areas (three samples). The tissue samples were taken immediately after a routine endoscopic procedure and were stored at -80 °C until processing. Paired tumoral tissue samples and adjacent non-tumoral mucosa of CRC patients were also collected during the surgical procedure and stored at -80 °C until processing.

Three ml of peripheral venous blood from totally 80 CRC patients (40 cases of early CRC and 40 cases of advanced CRC) and 11 IBD patients in the active state of disease without immunosuppressive therapy were collected in sterile tubes, and serum samples were obtained and frozen at -80 °C before use.

RNA extraction, reverse transcription, and quantitative polymerase chain reaction

Total RNA was isolated from tissue samples using a column-based RNA isolation kit (GeneJET RNA purification kit, Thermo scientific). The total amount of RNA was quantified by spectrophotometric analysis (GeneQuant 1300 spectrophotometer, GE Healthcare Life Sciences, Switzerland).

Reverse transcription to cDNA was accomplished manually according to the manufacturer's instructions with the First-strand cDNA Synthesis kit (Thermo Scientific) and High Capacity cDNA Archive kit (Applied Biosystems, United States).

The qRT-PCR was performed on a 7500 Real-Time PCR system (Applied Biosystems, United States) using a TaqMan Universal PCR Mastermix, with cDNA, specific PCR primers sets for our target genes, and 6FAM-labeled TaqMan MGB probes. The ID of Taqman gene expression assays were the following: FoxP3 (Hs00203958_m1), IL-10 (Hs00174086_m1), IL-23 (Hs00372324_m1) from Thermo Scientific; IL-17A (NM_002190), IL-6 (NM_000600) and TGFb (NM_000660) from Primerdesign, United Kingdom. Eukaryotic 18S ribosomal RNA (Hs99999903_m1), from Thermo Scientific, was used as an endogenous control.

Measurement of cytokines

Enzyme immunoassays were performed to measure the protein levels of IL-17A, IL-6, IL-23, TGFb1, and IL-10 in patients' sera (Human ELISA kit, Diaclone, Gene probe, France or Quantikine ELISA Kits, R and D systems, Minneapolis, MN, United States).

Statistical analysis

Triplicated PCR samples were analyzed by Sequence Detection System software v.1.3.1., and the results for mRNA expression were obtained by performing the comparative threshold cycle $\Delta\Delta C_t$ method, after normalization to the endogenous control (as a ratio target mRNA/18S ribosomal RNA). Relative quantification analysis (RQ) represents n-fold mean difference relative to a calibrator (adjacent normal intestinal tissue). Results for local gene expression analyses were expressed as means \pm SD. Cytokine levels were presented as a median and interquartile range (25th percentile and 75th percentile). Statistical differences between groups were analyzed using *t*-test or Mann-Whitney *U*-test. A value of *P* < 0.05 was considered significant. Statistical analysis was performed by using StatSoft software v.6. Dr. Tsvetelina Velikova from the University Hospital Lozenetz reviewed the statistical methods of this study.

RESULTS

Local gene expression of pro-and anti-inflammatory genes in IBD and total CRC patients

Table 1 shows the results of gene expression presented as $dCt \pm SD$ and RQ values of investigated genes in inflamed tissues of IBD patients and tumoral tissue as well. From all studied genes *IL-6* and *IL-17A* were with an approximately equal level of upregulation in both disease-IBD and CRC, although *IL-6* showed a tendency for the increased concentration in IBD and decreased *IL-17A* in comparison to CRC. The upregulation of *IL-23* gene expression was a hallmark only for CRC, where the level of mRNA synthesis was approximately 25 times enhanced than in IBD. The higher expression was also detected for *TGFb1*, *IL-10*, and *FoxP3* in colorectal tissue compared to IBD local expression.

Figure 1 presents a relative quantity of mRNA levels of the investigated genes. It is visible that the highest RQ difference between IBD and CRC patients was for the *IL-23* gene, as well as for the *FoxP3* and *IL-10* genes and the lowest – for the *TGFb1*, while for the gene expression of *IL-6* and *IL-17A* the differences were not significant.

Local gene expression of pro-and anti-inflammatory genes in IBD and early and

Table 1 Local gene expression of pro and anti-inflammatory cytokines in paired inflamed vs non-inflamed tissues of inflammatory bowel disease patients and paired tumoral vs adjacent non-tumoral mucosa of colorectal cancer patients

Genes	IBD			CRC			P value IBD vs CRC
	dCt ± SD		RQ (min-max)	dCt ± SD		RQ (min-max)	
	Inflamed tissue	Non-inflamed tissue		Tumoral tissue	Non-tumoral tissue		
<i>Foxp3</i>	14.94 ± 2.65	15.81 ± 2.59	1.82 (0.11-74.85)	18.62 ± 5.5	21.30 ± 4.3	6.42 (0.47-97.2)	0.025
<i>TGFB1</i>	9.52 ± 4.13	8.97 ± 4.4	0.68 (0.03-2.27)	17.27 ± 2.9	18.63 ± 1.9	2.37 (0.211-93.7)	0.007
<i>IL-10</i>	16.87 ± 1.76	17.7 ± 1.5	1.78 (0.22-28.5)	16.43 ± 5.5	19.17 ± 4.4	6.66 (0.43-202.3)	0.023
<i>IL-6</i>	17.13 ± 3.05	19.53 ± 2.7	5.28 (0.27-61.6)	19.11 ± 3.8	21.07 ± 2.5	3.88 (0.03-512.4)	0.784
<i>IL-23A</i>	18.0 ± 2.09	18.1 ± 2.8	1.07 (0.07-6.61)	12.56 ± 2.1	17.55 ± 2.5	28.5 (2.04-219.5)	0.0003
<i>IL-17A</i>	18.9 ± 2.65	20.3 ± 2.9	2.70 (0.07-20.5)	24.7 ± 4.3	26.9 ± 3.0	4.69 (0.034-494.9)	0.574

The results are presented as dCt ± SD and RQ (min-max). IBD: Inflammatory bowel disease; CRC: Colorectal cancer; dCt: Normalized to the endogenous control; RQ: Relative quantity of mRNA levels.

advanced CRC patients

Turning now to the comparison of the local gene expression of target genes in early (1st and 2nd stages) and advanced (3rd and 4th stages) cases of CRC to that in active IBD patients, the results are shown in **Figure 2A-F**. The figure illustrates that the gene expression of all genes was higher in CRC cases (nevertheless early or advanced) except *IL-6* and *IL-17A* for advanced CRC cases.

Comparing the two stages (early and advanced) of CRC patients, the t-test revealed significant elevation of *Foxp3*, *IL-10*, *IL-23A*, and *IL-17A* mRNAs in early cases of CRC compared to the IBD cases (**Table 2**). The *TGFB1* mRNA was increased in early CRC compared to the IBD cases with borderline significance ($P = 0.057$). *IL-6* was upregulated in both groups of patients in approximately equal amounts (**Table 2**). When we compared the gene expression of IBD patients and advanced cases of CRC, we saw that the gene expression of *FoxP3*, *TGFB1*, *IL-10*, and *IL-23* was upregulated, although the statistical significance was reached only for *IL-23*, whereas the mRNA expression of *IL-6* and *IL-17* were downregulated (**Table 3**). Moreover, the mRNA expression of *FoxP3* (RQ = 10.74), *IL-10* (RQ = 8.44), *IL-23A* (RQ = 61.93), and *IL-17A* (RQ = 26.83) in early cases of CRC and *IL-23A* alone in advanced CRC (RQ = 13.96) were overexpressed.

Serum level of pro- and anti-inflammatory cytokines in IBD and CRC patients

The results from the analysis of serum cytokine levels in IBD and CRC patients are compared in **Table 4**. All cytokines were at a significantly higher level in CRC patients compared to IBD patients. The levels of *TGFB1* were doubled in CRC compared to IBD patients, and about 20 times higher for *IL-10* and almost 40 times higher for *IL-23*.

Further, we have analyzed the cytokine serum levels in IBD patients in the active state of disease without immunosuppressive therapy ($n = 11$) compared to early CRC ($n = 40$) and advanced CRC ($n = 40$) (**Figure 3**). Non-parametric statistical analysis revealed that all cytokine levels were elevated significantly in early and advanced CRC patients compared to IBD patients, except *IL-17A*, which was reduced in advanced CRC to the similar to IBD levels. Moreover, the levels of *IL-6*, *IL-10*, and *IL-23* were gradually elevated from IBD - to early - advanced CRC (**Table 5**).

Our previous data on serum levels of the investigated cytokines in healthy individuals were the following: For *TGFB1*: 10028.82 ± 2250.15 pg/mL, for *IL-6*: 0.03 ± 0.02 , for *IL-17*: 0.16 ± 0.14 pg/mL, for *IL-10*: 0.68 ± 0.05 pg/mL, and for *IL-23*: 0.33 ± 0.03 pg/mL^[8].

DISCUSSION

Since the common feature between IBD and CRC-chronic inflammation was established, we aimed to compare some aspects of the molecular signature of immune cells-inflammatory in IBD and tumor-infiltrated in CRC, like the gene expression pattern for cytokines related to the Th17/Treg differentiation. Our findings showed that several cytokines were significantly upregulated in CRC patients when compared

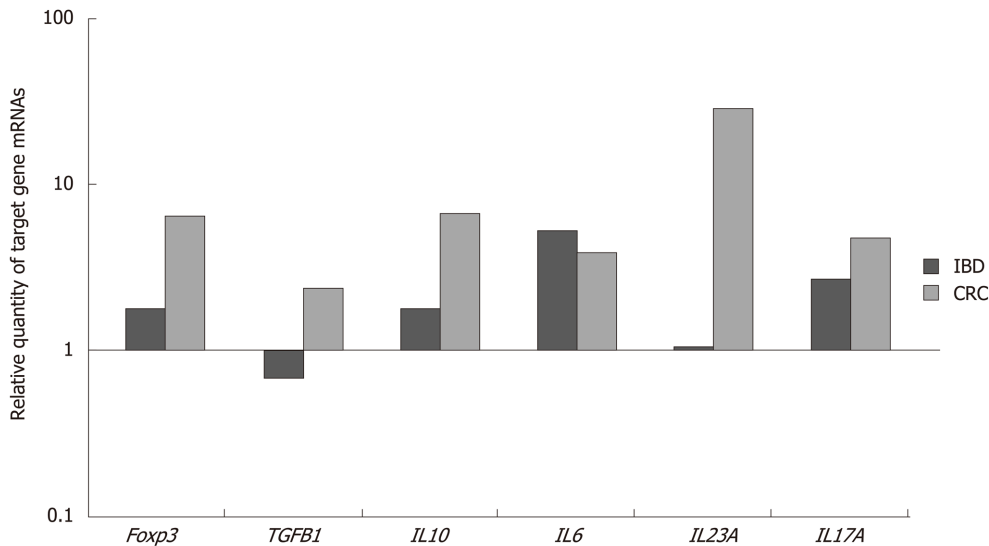


Figure 1 The relative quantity of mRNA levels in inflamed and tumoral tissue calibrated to their adjacent regular counterparts after normalization to endogenous control-18S rRNA. IBD: Inflammatory bowel disease; CRC: Colorectal cancer.

to IBD ones. In our study, the cytokines included *TGFB1*, *IL-10*, and *IL-23* in the local microenvironment (tumor tissue vs. inflamed tissue), and *TGFB1*, *IL-10*, *IL-17A*, *IL-6* and *IL-23* in the systemic circulation of patients. The unequal distribution of the cytokines mentioned above supports the general hypothesis that local changes do not mirror the systemic level of inflammation. Nevertheless, a chronic inflammatory response may create an environment which is not only permissive towards cancer development, but that indeed sustains tumor promotion and progression by means of cytokines and growth factor release^[9].

Along with the crucial cytokines, we investigated the mRNA expression of transcription factor *FoxP3*. We found that the expression of *FoxP3* was significantly elevated in the whole CRC group (RQ = 6.42), as well as in the early CRC cases (RQ = 10.74), but not significantly in the advanced stages of CRC (RQ = 4.55). The role of Tregs in inflammation-associated CRC remains elusive. However, the suppression of the immune system may actively hamper host immune surveillance against tumors^[5]. Nevertheless, *FoxP3* expression may suppose a pivotal role for tumor growth, invasion, and spread, in addition to maintaining tumor escape from immune surveillance. Under chronic inflammatory conditions, *FoxP3* + Treg are increased in circulation and accumulate in large numbers in lymph nodes and surrounding tumors. Tumor-infiltrating *FoxP3* + cells are seen primarily during regression of the tumors, *i.e.*, after biologic therapy^[10].

On the other hand, Treg cells may help prevent and delay inflammation-mediated tumor growth as Treg cells exert anti-tumor activity in colitis-associated CRC. In our study, *FoxP3* mRNA levels were higher in the inflamed tissue of IBD patients and even higher in tumoral tissue, especially in the early stages of CRC. This elevation suggests that in the early stages of CRC, there is a more abundant accumulation of *FoxP3* + cells locally, similarly to IBD inflamed tissue.

Treg phenotype contributes significantly to CRC progression, as previously published by Miteva *et al*^[11] colleagues in 2017. The authors claimed that the upregulation of *FoxP3*, *IL-10*, *TGFB1*, and *IL-6* might be a transcriptional hallmark for CRC metastases, and the gene expression of Treg and Th17 related cytokines in the primary tumor and regional lymph nodes might provide suitable microenvironment sufficient for promoting metastasis^[11]. Previous studies have also shown a significant upregulation of *FoxP3* and *IL-23* in tumor CRC tissue and elevated *IL-10* mRNA on the systemic and local levels of CRC patients^[12].

Recently, in human CRC patients, intratumoral *FoxP3* cells were correlated with a favorable outcome. However, *FoxP3* may be expressed in many cell types, including Th17 cells or tumor cells. Furthermore, Treg cells may not only have diminished efficacy during inflammation but may also differentiate directly into *IL-17*-producing cells^[10]. This way, they may fuel carcinogenic events by contributing to hosting pro-inflammatory responses. Miteva *et al*^[11] also suggested that the observed elevation of mRNA *IL-17A* with *TGFB1*, *IL-10*, and *IL-6* genes in tumor tissue and regional lymph nodes could be associated with the presence of *FoxP3* + Th17 cells.

Individuals with weakened *IL-10* and Treg cells mediated inhibitory mechanism,

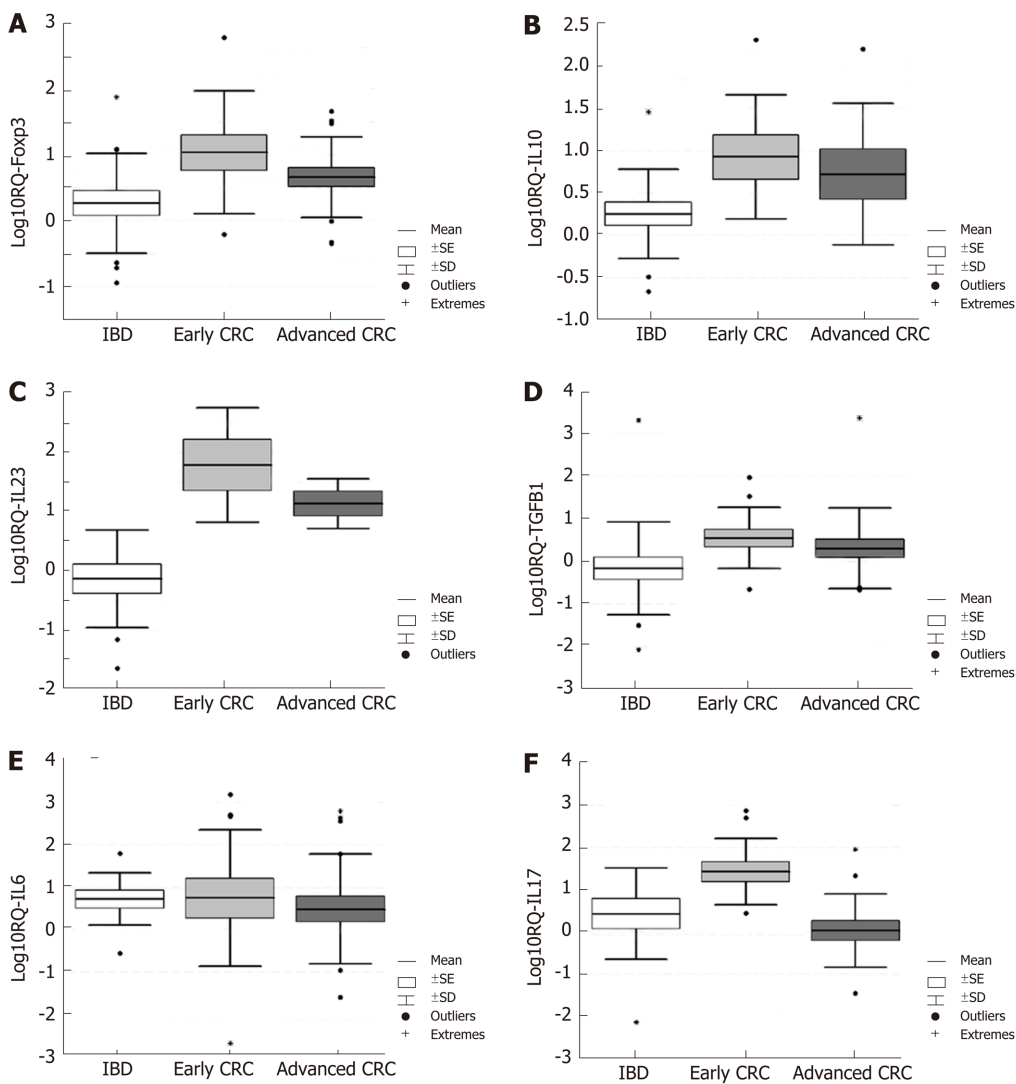


Figure 2 Local gene expression of *Foxp3*, *IL-10*; *IL-23A*; *TGFB1*; *IL-6* and *IL-17A* in inflammatory bowel disease patients and in early and advanced colorectal cancer cases. A: *Foxp3*; B: *IL-10*; C: *IL-23A*; D: *TGFB1*; E: *IL-6*; F: *IL-17A*. The relative quantity of target mRNAs in inflamed and tumoral tissue is calibrated to their normal counterparts after normalization to endogenous control-18S rRNA. The results are presented as mean (line) with standard error (box), and standard deviation (whiskers) of log-transformed fold changes (base 10). IBD: Inflammatory bowel disease; CRC: Colorectal cancer.

and elevated IL-6 and IL-17, would be more likely to develop uncontrollable inflammation in response to proinflammatory challenges, and thus, more frequently susceptible to inflammation-associated cancers later in life^[10]. In line with these are our results regarding *IL-10* mRNA expression, which we found higher in CRC compared to IBD patients. However, its role remains controversial due to its pro- and anti-tumoral effects. It can be secreted not only by Tregs but also by tumor cells themselves, as well as by tumor-infiltrating macrophages^[13]. *IL-10* inhibits NF- κ B signaling; therefore, it can downregulate pro-inflammatory cytokine expression and acts as an anti-tumoral cytokine. *IL-10* can also dampen antigen presentation, cell maturation, and differentiation, allowing tumor cells to evade immune surveillance mechanisms^[14]. STAT3 can also be activated by *IL-10*, depending on the time frame of STAT3 activation. *IL-6* leads to a temporary, rapidly declining STAT3 phosphorylation, whereas *IL-10* induces a sustained one, leading to a pro-tumorigenic effect involving Bcl-2 upregulation and apoptosis resistance activation^[15].

Some authors reported the average levels of *IL-10* in CRC and CD patients. In our CRC patients, serum levels of *IL-10* were significantly higher than in the IBD patients ($P < 0.0001$) in both early and advanced stages. We suppose that elevated *IL-10* production in tumor tissue is associated with its pro-tumoral activities rather than anti-inflammatory ones. CD4⁺ cells insufficient in *IL-10* were preferentially recruited to a Th17 phenotype when faced with a robust pro-inflammatory challenge^[10]. Under conditions of low *IL-10* and elevated *IL-6*, there is weak regulation of inflammation, which may contribute to cancer growth. Individuals with insufficient *IL-10* are more susceptible to the effects of *IL-6* and developing uncontrollable inflammation. In these

Table 2 The local gene expression in inflammatory bowel disease patients compared to that in early colorectal cancer cases

Genes	IBD			Early stages CRC (1 + 2)			P value IBD vs Early CRC
	dCt ± SD		RQ (min-max)	dCt ± SD		RQ (min-max)	
	Inflamed tissue	Non-inflamed tissue		Tumoral tissue	Non-tumoral tissue		
<i>Foxp3</i>	14.94 ± 2.65	15.81 ± 2.59	1.82 (0.11-74.85)	16.98 ± 5.8	20.41 ± 4.5	10.74 (0.615-97.2)	0.023
<i>TGFB1</i>	9.52 ± 4.13	8.97 ± 4.4	0.68 (0.03-2.27)	16.99 ± 2.4	18.83 ± 2.3	3.59 (0.222-93.7)	0.057
<i>IL-10</i>	16.87 ± 1.76	17.7 ± 1.5	1.78 (0.22-28.5)	16.17 ± 5	19.25 ± 4.1	8.44 (1.253-202.3)	0.019
<i>IL-6</i>	17.13 ± 3.05	19.53 ± 2.7	5.28 (0.27-61.6)	18.12 ± 4.7	20.59 ± 1.9	5.55 (0.03-512.4)	0.97
<i>IL-23A</i>	18.0 ± 2.09	18.1 ± 2.8	1.07 (0.07-6.61)	11.69 ± 1.9	17.65 ± 2.9	61.93 (2.04-219.5)	0.001
<i>IL-17A</i>	18.9 ± 2.65	20.3 ± 2.9	2.70 (0.07-20.5)	22.42 ± 3.6	27.17 ± 2.9	26.83 (2.73-494.9)	0.029

The results are presented as dCt ± SD and RQ (min-max). IBD: Inflammatory bowel disease; CRC: Colorectal cancer; dCt: Normalized to the endogenous control; RQ: Relative quantity of mRNA levels.

conditions, Treg cells had increased invasion of neoplastic epithelia^[2,10].

It has been reported of upregulation of IL-10 and TGFB1 cytokines in PBMCs of CRC patients preoperative with consecutive downregulation of their expression postoperative suggesting that tumor induces aberrant changes in gene expression, and indicate that the mRNA levels of cytokines were associated with the presence of CRC^[16,17].

The TGFB1 expression under inflammatory conditions may be due to the down-regulation of the body's immune responses in an attempt to control inflammation. As a critical component for both Th17 and Treg differentiation, for us, it was essential to find the expression of this cytokine locally in inflamed (IBD) and tumor (CRC) tissue samples of patients. We observed that TGFB1 was higher in CRC (early and advanced cases) compared to IBD patients. The role of TGFB1 in cancer, however, is complicated and ambiguous, varying by cell type and stages of tumorigenesis. In the early stages of CRC, TGFB1 acts as a tumor suppressor, inhibiting cell cycle progression, and promoting apoptosis. Later, TGFB1 enhances invasion and metastases by inducing epithelial-mesenchymal transition^[18], as well as helps tumor growth by creating an immunotolerant tumor environment^[19]. In our recent study, we showed that the expression of TGFB1 is involved in CRC development and metastasis but depends on gender and genotype^[20]. Thus, we can speculate that increasing the level of TGFB1 is a marker for cancer development or progression.

In our study, the only cytokine, which was approximately equally upregulated in both CRC and IBD, was IL-6. Previously, we found a higher expression of IL-6 in inflamed mucosa of IBD patients^[8] and tumor tissue and regional lymph nodes of CRC patients^[10]. Here, we observed that the differences between mRNA expression of *IL-6* locally in IBD and CRC were not significant. We could suggest that this crucial cytokine is of equal importance for both inflammation and tumor development.

IL-6 was shown to promote T cell accumulation in the colon lamina propria by upregulation of anti-apoptotic factors such as BCL-2 and BCL-xl^[21]. IL-6 was also demonstrated to downregulate the tumor suppressor p53 simultaneously, but to upregulate the oncogene c-myc in colon epithelial cells, epithelial-mesenchymal transition, and resistance to cytotoxic stress^[6], and upregulates *Oct4* gene expression by activating IL-6R/JAK/STAT3 signaling pathway^[3]. IL-6 and its related cytokines directly support the growth of colon epithelial cells and repair of intestinal wounds but can also promote the development of CRC^[3]. In summary, IL-6 is a crucial tumor promoter during early CRC. Thus, it arises as a potential target in both IBD and CRC.

Some studies showed that CRC patients presented with high levels of IL-6 and VEGF^[22]. Elevated expression of IL-6, which can be detected in patient serum, is linked to increased risk of development of colorectal adenomas^[23] and poor prognosis in CRC^[24,25]. Our CRC patients had elevated serum levels of IL-6 ($P = 0.044$). However, limited studies are existing that might be used to define cut off values for IL-6 as a diagnostic tool.

TGFB1 and IL-6 as crucial cytokines for Th17 differentiation were upregulated in

Table 3 The local gene expression in inflammatory bowel disease patients compared to that in advanced colorectal cancer cases

Genes	IBD			Advanced stages CRC (3 + 4)			P value IBD vs Advanced CRC
	dCt ± SD		RQ (min-max)	dCt ± SD		RQ (min-max)	
	Inflamed tissue	Non-inflamed tissue		Tumoral tissue	Non-tumoral tissue		
<i>Foxp3</i>	14.94 ± 2.65	15.81 ± 2.59	1.82 (0.11-74.85)	19.71 ± 5.2	21.89 ± 4.2	4.55 (0.45-45.1)	0.101
<i>TGFB1</i>	9.52 ± 4.13	8.97 ± 4.4	0.68 (0.03-2.27)	17.46 ± 3.3	18.5 ± 1.6	2.05 (0.211-14.56)	0.176
<i>IL-10</i>	16.87 ± 1.76	17.7 ± 1.5	1.78 (0.22-28.5)	16.69 ± 6.2	19.09 ± 5	5.26 (0.43-158.8)	0.113
<i>IL-6</i>	17.13 ± 3.05	19.53 ± 2.7	5.28 (0.27-61.6)	19.78 ± 3.1	21.39 ± 2.8	3.05 (0.025-434.7)	0.608
<i>IL-23A</i>	18.0 ± 2.09	18.1 ± 2.8	1.07 (0.07-6.61)	13.64 ± 2.1	17.44 ± 2.4	13.96 (3.93-36.28)	0.012
<i>IL-17A</i>	18.9 ± 2.65	20.3 ± 2.9	2.70 (0.07-20.5)	26.62 ± 3.9	26.73 ± 3.2	1.07 (0.034-89.26)	0.350

The results are presented as dCt ± SD and RQ (min-max). IBD: Inflammatory bowel disease; CRC: Colorectal cancer; dCt: Normalized to the endogenous control; RQ: Relative quantity of mRNA levels.

the inflamed tissue of IBD, as well as in tumor tissue of CRC patients. However, the expression of *TGFB1* was higher in CRC patients compared to IBD patients, unlike the *IL-6* expression. Based on these results, we could speculate that the additive upregulation of *TGFB1* expression to the *IL-6* during inflammation could drive to CRC transition. Regarding *IL-17* mRNA expression, as a hallmark of Th17 cells, we found that the cytokine was upregulated in the whole CRC group (RQ = 4.69), especially in early cases in comparison with IBD (RQ = 26.83 *vs* 2.70; *P* = 0.029). Conversely, the RQ level of *IL-17* in advanced CRC cases was lower (RQ = 1.07) than in the IBD group (RQ = 2.70).

It is known that *IL-17A* bridges the adaptive and innate immune system and plays a role in the maintenance of epithelial barrier homeostasis, but in colitis and CRC, its expression is elevated and worsens disease progression^[3]. The role of *IL-17A* as an antitumor or tumor-promoting factor is still incompletely understood; however, increasing evidence support that *IL-17A* is involved in the development of CRC^[26]. *IL-17* family members demonstrated distinct expression patterns in CRC, suggesting a differential role exerted by each member in colon carcinogenesis^[27]. It is tempting to speculate that an inflamed environment dominated by Th17 cells might facilitate cancer development. The involvement of Th17 cells in tumor growth is further sustained by the demonstrated role of *IL-6* in this process^[9].

IL-17A has shown a dual role in controlling neoplastic cell growth – it can inhibit tumor growth in some murine models, while it promotes malignant cells in mouse models of spontaneous intestinal cancer^[28,29]. It can also stimulate tumor growth by its proangiogenic effect via enhancing the production of VEGF, basic fibroblast growth factor, and hematopoietic growth factor, and it has an impact on cancer-infiltrating stem cells^[26]. *IL-17A* contributes to the tumor-initiating stage in the advancement of colitis-associated CRC due to the STAT3 *IL-6* induced Th17 differentiation^[26]. *IL-17* overexpressed in tumors from colitis-associated cancer patients and is associated with angiogenesis and poor prognosis markers. It is secreted in tumors by macrophages/monocytes CD68+; Th17 and Treg FoxP3+*IL-17*+ cells^[30].

The clinical implication of *IL-17A* is investigated by some authors, as they stated that measuring *IL-17A* in serum samples from CRC patients might be a valuable tumor marker^[31], but it is not correlated with the TNM parameters of CRC. We have also found elevated levels of *IL-17A* in the serum of CRC patients compared to IBD patients (*P* = 0.034), which may be useful in the follow up of IBD patients and predicting CRC progression.

The blockade of *IL-17A* leads to a substantial modification in the microenvironment of *IL-17A* related inflammation and tumors^[26,28,29]. And also, targeting *IL-17A* with anti-angiogenic therapeutics may be beneficial for the patients^[26,32].

IL-6 alone can be sufficient to drive Th17 cells to secrete cytokines, whereas *IL-23* is required for providing Th17 cells, a pathogenic phenotype^[6]. In our study, *IL-23* was the only cytokine for which mRNA expression was significantly higher in CRC (both early and advanced cases) than in IBD patients. Despite that different animal models suggest the protective role of *IL-23* in induced CRC in mice, recently published papers have been shown the neoangiogenesis property of *IL-23*, particularly for human CRC development^[16,33,34]. One possible mechanism could be the enhancement of the intestinal inflammation mediated by the Th17 axis maintained from *IL-23*. Moreover, *IL-23* has been found to be overexpressed in a number of different human cancers

Table 4 Serum levels of pro and anti-inflammatory cytokines in inflammatory bowel disease and colorectal cancer patients

Cytokines	IBD patients	CRC patients	P value (U-test)
TGFB1 (ng/mL)	11.19 (9.36-20.99)	22.57 (18.08-29.65)	0.0008
IL-10 (pg/mL)	0.4 (0.0-2.8)	8.29 (6.75-15.12)	0.000001
IL-6 (pg/mL)	0.0 (0.0-2.7)	3.21 (2.03-4.19)	0.012
IL-23A (pg/mL)	0.7 (0.0-3.7)	27.55 (25.35-33.0)	0.000001
IL-17A (pg/mL)	0.0 (0.0-0.26)	2.85 (0.0-9.0)	0.034

The results are presented as median (IQR: 25%-75% percentile). IBD: Inflammatory bowel disease; CRC: Colorectal cancer.

compared to normal adjacent tissues, suggesting that IL-23 is essential for tumor-promoting pro-inflammatory processes and as well as the failure of the adaptive immune surveillance to infiltrate tumors^[12,33]. However, IL-12p40 cytokine was evaluated as a useful prognostic marker for survival, unlike IL-23, which had no outcome prognostic value^[16].

Nevertheless, we found that the level of IL-23 was about 40 times higher in CRC patients than IBD patients ($P < 0.001$). Recent data have shown that CRC progression was closely associated with infiltration with Th17 cells, and the central cytokines related IL-6, TGFB1, and IL-23^[35,36]. CRC generates not only the local inflammatory microenvironment, named as tumor-elicited inflammation, but also promotes systemic changes that are favorable for cancer progression. Here, if we compared the serum levels of IL-23 in CRC patients to the average levels of healthy volunteers, the concentrations were 80 times higher in CRC.

Suppression of these cytokines was found to improve the symptoms of IBD to CRC progression^[21,37,38]. This can be accomplished either with anti-cytokine drugs or immunosuppressive agents. Chemoprevention with anti-inflammatory agents and immunomodulatory drugs has been shown to reduce the risk of developing CRC on the grounds of inflammation by lowering the level of produced cytokines^[39,40]. We have also proven the results regarding treatment with immunosuppressive drugs as more beneficial in reducing inflammation in IBD patients via driving cytokine expression to restore immune regulation^[20].

The current study was limited by a relatively small number of IBD patients included. We used a convenient sample of IBD patients with no immunosuppressive therapy to avoid bias. However, caution must be applied, and the hypothesis and findings should be tested with a larger sample size.

Secondly, our study explores two cohorts of patients-IBD and sporadic CRC. Although sporadic colon carcinogenesis and colitis-associated carcinogenesis share similar immune-related mechanisms^[41], we cannot exclude differences in the critical molecular mechanisms underlying these two types of CRC.

In conclusion, with this cytokine panel, we documented significant changes in genes related to Treg/Th17 development when comparing mRNA expression profiles of IBD and CRC (early and advanced) patients. Our findings showed that IL-6 upregulation is essential for both IBD and CRC, whereas the upregulation of genes related to Th17/Treg differentiation and behavior (TGFB1, IL-10, IL-23, and transcription factor FoxP3) is a crucial primarily for CRC development. Altogether we recorded marked differences in the distribution of investigated gene local expression, and the significance of these results could be used mainly in the effort to establish reliable and efficient methods for personalized therapies. Thus, the significantly upregulated IL-6 could be a potential drug target for IBD and prevention of CRC development as well.

Table 5 Serum levels of anti and pro-inflammatory cytokines in inflammatory bowel disease patients compared to early and advanced colorectal cancer cases

Cytokines	IBD	Early CRC	<i>P</i> value (<i>U</i> -test), IBD vs early CRC	Advanced CRC	<i>P</i> value (<i>U</i> -test), IBD vs advanced CRC
TGFB1 (ng/mL)	11.19 (9.36-20.99)	22.74 (18.17-27.98)	0.001344	21.51 (15.5-31.05)	0.0031
IL-10 (pg/mL)	0.4 (0.0-2.8)	7.73 (6.0-12.71)	0.000006	8.84 (7.2-19.81)	0.000002
IL-6 (pg/mL)	0.0 (0.0-2.7)	3.06 (2.03-4.19)	0.0153	4.19 (2.15-8.4)	0.044
IL-23A (pg/mL)	0.7 (0.0-3.7)	25.7 (24.6-32.5)	0.000024	30.5 (26.3-33.5)	0.000009
IL-17A (pg/mL)	0.0 (0.0-0.26)	3.62 (0.0-8.23)	0.0086	1.2 (0.0-12.08)	0.178

The results are presented as median (IQR, 25%-75% percentile). IBD: Inflammatory bowel disease; CRC: Colorectal cancer.

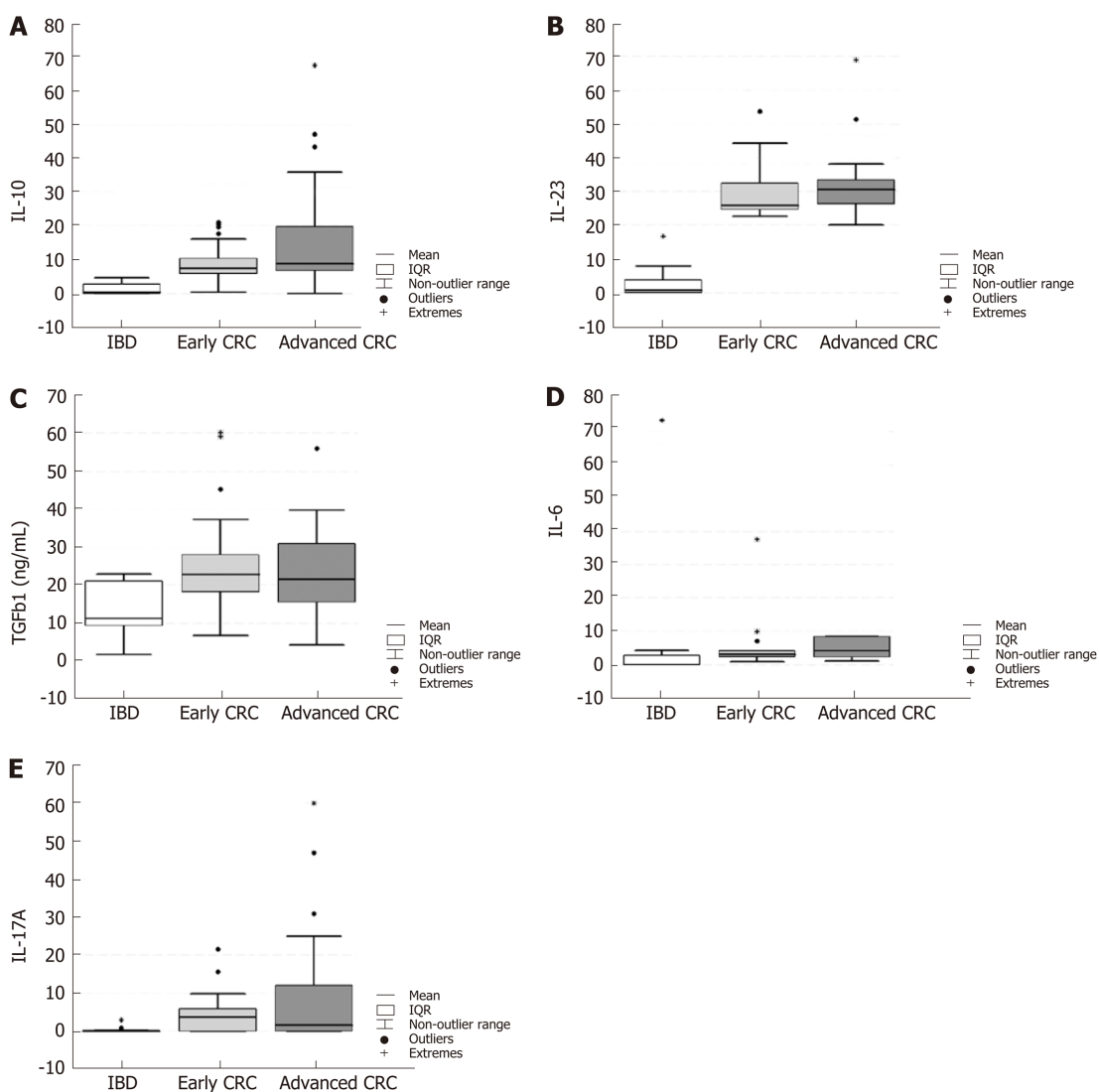


Figure 3 Serum levels of anti- and pro-inflammatory cytokines. A: IL-10; B: IL-23A; C: TGFB1; D: IL-6; E: IL-17A. In inflammatory bowel disease patients compared to early and advanced colorectal cancer cases. The results are presented as median with 25%-75% percentile. IBD: Inflammatory bowel disease; CRC: Colorectal cancer.

ARTICLE HIGHLIGHTS

Research background

Since various molecular mechanisms and signaling pathways are common for the carcinogenic process and inflammatory bowel disease, including accumulation of immune cells and the release of several cytokines, chemokines, and growth factors, we were interested in the comparing of cytokine patterns and subtle changes in cytokine milieu in inflamed tissues of inflammatory bowel disease (IBD) patients, as well as in the cancer environment in colorectal cancer (CRC) patients. However, far too little attention has been paid to opportunities for early detection of subtle changes in cytokine milieu in inflamed tissues in IBD and CRC, especially in the clinical context. In line with this, we were searching for mRNA cytokine patterns of IBD patients in active disease compared to early and advanced CRC, to obtain data on in the similarities and differences in inflammation and cancer initiation and advances.

Research motivation

Dysregulation of the immune response in patients with IBD and CRC triggers infiltration and, which may further influence the inflammation and the carcinogenesis process. Since the recent developments for the role of cytokines in the intestinal inflammation are accumulating, there has been an increased interest in their role. Furthermore, the survival of CRC affected patients depends highly on early detection. Thus, establishing some of the parameters that can be detected and followed-up and used as prognostic factors or determining the treatment options can improve the survival rate and quality of life of CRC patients.

Research objectives

Our objectives were: (1) To assess the mRNA cytokine levels in inflamed tissues of IBD patients, as well as in the cancer environment in CRC patients (at early and advanced stages); and (2) To compare serum protein levels of the respective cytokines in both groups of patients. The objectives were chosen to compare cytokine patterns of active IBD patients with early and advanced CRC by selecting a panel of cytokines crucial for Th17/Treg differentiation and behavior (IL-6, TGF β , IL-17A, IL-23, IL-10), as well as the transcription factor FoxP3, in colon specimens, as mRNA biomarkers, and their serum protein levels. Future directions of this study could be the follow-up of IBD patients for developing dysplastic lesions or CRC, where the proposed biomarkers can be evaluated as a prognostic factor.

Research methods

To address the aims of the study, we choose a panel of pro- and anti-inflammatory cytokines with a substantial role in Th17/Treg differentiation and behavior, in colon specimens of IBD and CRC patients, as mRNA biomarkers, and their protein levels in the peripheral blood. We used RNA extraction, reverse transcription, and quantitative polymerase chain reaction, as well as enzyme immunoassays to measure the protein levels of IL-17A, IL-6, IL-23, TGF β 1, and IL-10 in patients' sera.

Research results

We found a significant difference between higher gene expression of *FoxP3*, *TGF β 1*, *IL-10*, and *IL-23*, and approximately equal level of *IL-6* in CRC patients in comparison with IBD patients. After stratification of CRC patients, we found a significant difference in *FoxP3*, *IL-10*, *IL-23*, and *IL-17A* mRNA in early cases compared to IBD, and *IL-23* alone in advanced CRC. The protein levels of the cytokines were significantly higher in CRC patients compared to IBD patients.

Our findings contribute to the research in the field by finding that *IL-6* upregulation is essential for both IBD and CRC development until the upregulation of other Th17/Treg related genes (*TGF β 1*, *IL-10*, *IL-23*, and transcription factor *FoxP3*) is a crucial primarily for CRC development. The significantly upregulated *IL-6* could be a potential drug target for IBD and prevention of CRC development as well. However, more research are needed regarding the role and targeting of *IL-6* in CRC patients.

Research conclusions

With the chosen cytokine panel, we documented significant changes in genes related to Treg/Th17 development when comparing mRNA expression profiles of IBD and CRC (early and advanced) patients. Our findings showed that *IL-6* upregulation is essential for both IBD and CRC, whereas the upregulation of genes related to Th17/Treg differentiation and behavior (*TGF β 1*, *IL-10*, *IL-23*, and transcription factor *FoxP3*) is a crucial primarily for CRC development.

In our study, the only cytokine, which was approximately equally upregulated in both CRC and IBD, was *IL-6*. Previously, we found a higher expression of *IL-6* in inflamed mucosa of IBD patients and tumor tissue and regional lymph nodes of CRC patients. Here, we observed that the differences between mRNA expression of *IL-6* locally in IBD and CRC were not significant. We could suggest that this crucial cytokine is of equal importance for both inflammation and tumor development, *i.e.*, we hypothesized that *IL-6* is a critical tumor promoter during early CRC.

Thus, the significantly upregulated *IL-6* could be a potential drug target for IBD and prevention of CRC development as well, with a significant potential for the clinical practice.

However, since we used a convenient sample of IBD patients with no immunosuppressive therapy to avoid bias, caution must be applied, and the hypothesis and findings should be tested with larger sample size. Secondly, although sporadic colon carcinogenesis and colitis-associated carcinogenesis share similar immune-related mechanisms, we cannot exclude differences in the critical molecular mechanisms underlying these two types of CRC.

Research perspectives

The most important lesson learned from this study is the crucial role of IL-6 for both IBD and CRC. It is well-known that elevated IL-6 is linked to increased risk of development of colorectal adenomas and poor prognosis in CRC, but limited data is available regarding the role of IL-6 in IBD towards CRC. Besides, inadequate studies are existing that might be used to define cut off values for IL-6 as a diagnostic tool. In line with this, future directions should include studies exploring the diagnostic and therapeutic potential of IL-6.

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