



UPREGULATION OF Treg AND DOWNREGULATION OF TH17 RELATED CYTOKINE EXPRESSION IN PERIPHERAL BLOOD OF COLORECTAL CANCER PATIENTS

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ABSTRACT

The presence of distinct cell types of the innate and adaptive immune system in neoplastic lesion is a marker for local antitumoral responses and tumor elicited inflammation. The aim of the present study was to investigate the gene expression of interleukin (IL)-12A, IL-12B, IL-23A, IL-17, IL-10, IL-6 and TGF- β in peripheral blood cells from colorectal cancer (CRC) patients. The venous blood was collected preoperative and 10 days after surgery from 17 CRC patients and 22 healthy donors. After isolation of total RNA and synthesis of cDNA, the real-time polymerase chain reaction (qRT-PCR) for quantity determination of specific mRNA was performed on a 7500 Real - Time PCR System.

Our results demonstrated that among investigated cytokine genes IL-10 and TGF-b were significantly upregulated in patients with CRC compared to the control group, while the expression of IL-17 and IL23 mRNA was significantly decreased in CRC patients. Based on the results we could assume that peripheral blood gene expression programming in CRC patient's triggers local differentiation of Th cells towards Th17 and Treg instead Th1 anti-tumor subpopulation.

In conclusion we suppose that the established tumor elicited inflammation can contribute to multiple hallmark capabilities by supplying bioactive molecules not only benefit tumor grown, but also affecting epigenetic alternation in immune blood cells resulting in gene expression reprogramming.

Key words: CRC, Th17, mRNA, TGF-betta, IL-17, IL-10, RT-PCR

INTRODUCTION

Colorectal cancer follows definitive genetic changes including at least the adenomatous polyposis coli (APC) tumour suppressor gene and mutations in K-Ras oncogene, providing the reproduction of tumor cells. Genome instability and epigenetic alteration ensure on certain mutant genotypes selectively advantage for tumor growth and present a first enabling hallmark of cancer (1, 2). The second enabling characteristic involves the inflammatory state of premalignant and frankly malignant lesions that is driven by cells of the immune system, some of which serve to promote tumor progression through various means (3-5).

Inflammation is likely to be involved with both forms of sporadic as well as heritable colon cancer. The "Evading immune destruction" as characteristic of tumor illustrated the remarkable progress made in the last decade to describe the role of inflammation in promoting cancer development. The inflammation promote cancer progression and accomplishes the full malignant phenotype, such as tumor tissue remodeling, angiogenesis, metastasis and the suppression of the innate anticancer immune response (6, 7).

The presence of distinct cell types of the innate and adaptive immune system in neoplastic lesion is a marker for local antitumoral responses and tumor elicited inflammation. Additionally,

distinct immune cells can release different cytokines, responsible for pro and anti-tumoral effect (8). Tumor-infiltrating leucocytes and cytokine related signaling pathways are critical components in colorectal cancer initiation and progression. The mixture of cytokines that is locally and systemically produced can either block or facilitate tumor growth (7-9). Most intratumoral immune cells are recruited from peripheral blood. Whether they are already programmed or can change their functions after falling into the tumor microenvironment remains elusive. The ability of immune cell in peripheral blood to produce certain cytokines and possibility for their reprogramming after entering the tumor mass is a main feature for tumor growth or immune destruction. Previously an association with tumor promotion and altered gene expression profiles of cytokines at mRNA level in colorectal cancer monocytes after in vitro stimulation was demonstrated (10).

In view of the above facts, the aim of the present study was to investigate the gene expression of interleukin (IL)-12A, IL-12B, IL-23A, IL-17, IL-10, IL-6 and TGF- β in peripheral blood cells from colorectal cancer patients. We have selected these cytokines considering that they are relevant to the differentiation of Treg and Th17 subpopulations which are involved in tumor promoting inflammation.

MATERIALS AND METHODS

Subject. A group of 17 Bulgarian patients with CRC who underwent surgical resection of the tumor, were included in the study. Cases with new diagnosis of CRC attending the University hospital and St. Ivan Rilsky Hospital in Stara Zagora, Bulgaria between October 2009 and November 2011 were selected. The histopathological examination confirmed the diagnosis of cancer. The mean age of total group of CRC patients was 63.75 ± 7.68 years. Tumor grading and staging was performed according to the tumor-node-metastasis (TNM) classification.

With the consent of the local ethics board, the peripheral venous blood (3 ml) was collected 1 day before surgery (preoperative) and 10 days after surgery (postoperative) from CRC patients and 22 healthy donors in sterile tubes with EDTA and 1 ml was used for RNA isolation immediately.

Criteria's for eligible patients included no history of prior surgery for colon or rectal tumors; no known hereditary cancer, ulcerative colitis or Crohn's disease. All patients and healthy volunteers gave an informed consent for this research.

RNA extraction. Total RNA from peripheral blood (1ml) was isolated using innuPREP blood RNA isolation kit AJ Roboscreen (Leipzig; Germany) with additional step of treatment with DNase I (Fermentas) to remove traces of genomic DNA. The total RNA was quantified by spectrophotometrical analysis. To remove traces of genomic DNA, total RNAs (1 μ g) were treated with RNase-free DNase I (Fermentas) following manufacture's instruction.

Reverse transcription. Synthesis of cDNA was performed manually according to manufacturer's instructions with High-Capacity cDNA Archive kit (Applied Biosystems, USA) that uses random primers and MutliScribe TM MuLV reverse transcriptase enzyme. Incubation conditions for reverse transcription was 10 min at 25°C followed by 2 hours at 37°C and was performed on a GeneAmp PCR System 9700 (Applied Biosystems, USA)

Quantitative real-time polymerase chain reaction (qRT-PCR). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on a 7500 Real - Time PCR System (Applied Biosystems, Foster City, CA, USA). The following validated PCR primers and TaqMan MGB probes (6FAM-labeled) were used: IL-12B (assay ID: Hs00233688_m1); IL-12A (Hs00168405_m1); IL-23A (Hs00372324_m1); IL-10 (Hs00174086_m1); and IL-17A (NM_002190), IL-6 (NM_000600), TGF β 1 (NM_000660) - Primerdesign, UK. As endogenous control was used eukaryotic 18S ribosomal RNA (Hs99999903_m1). An aliquot of 5 μ l of the RT reaction was amplified in duplicate in final volume of 20 μ l using a TaqMan Universal PCR Master Mix and Gene Expression Assay mix, containing specific forward and reverse primers and labeled probes for target genes and endogenous control (Applied Biosystems, USA). The thermocycling conditions were: initial 10 min incubation at 95°C followed by 40 cycles of denaturation for 15 s at 95°C and annealing/extension for 1 min at 60°C. PCR data were collected with Sequence Detection System (SDS) software, version 1.3.1.

Relative quantitative evaluation of cytokine mRNAs was performed by the comparative $\Delta\Delta C_t$ method. The mean ΔC_t obtained in peripheral blood of healthy donors for each cytokine mRNA was used as calibrator, after normalization to endogenous control-18S rRNA. The results are presented as a n-fold difference relative to calibrator ($RQ=2^{-\Delta\Delta C_t}$).

Statistical analysis. The data was expressed as means and standard deviation (SD) of the mean. Student's t-test was used to determine the statistical differences between mean values.

Differences were considered significant when the P value was less than 0.05.

RESULTS

We investigated the expression of cytokine genes IL-12A, IL-12B, IL-23A, IL-17, IL-10, IL-6 and TGF- β in peripheral blood from patients' before surgery (preoperative) and 10 day after surgery (postoperative) of the same patients and results were compared to the data taken from normal healthy volunteers. Results are presented as dCt in **Table 1** and as RQ in **Figure 1**.

Table. 1 Gene expression levels in peripheral blood before and 10 days after surgery of CRC patients and healthy controls. The gene expression level is presented as average dCt value with standard deviation (SD)

Target gene	Average dCt \pm SD			P value		
	Pre-operative blood	Post operative blood	Healthy controls blood	Pre vs. post	Pre vs. HC	Post vs. HC
IL12A	16.37 \pm 1.4	16.86 \pm 0.52	16.8 \pm 0.93	0.341	0.264	0.847
IL12B	21.48 \pm 1.8	22.34 \pm 3.08	21.43 \pm 1.06	0.409	0.919	0.196
IL23	15.59 \pm 1.6	14.96 \pm 0.94	14.55 \pm 0.86	0.298	0.010	0.233
IL10	15.58 \pm 1.9	16.29 \pm 1.4	16.87 \pm 1.65	0.358	0.034	0.349
TGF- β	15.71 \pm 0.94	16.76 \pm 0.94	16.78 \pm 0.74	0.019	0.0048	0.955
IL6	23.55 \pm 1.85	24.19 \pm 1.18	23.39 \pm 0.97	0.374	0.789	0.105

We observed significantly increased mRNA in CRC patients' blood before surgery for IL-10 and TGF- β ($p=0.034$ and $p=0.0048$ respectively) in comparison with healthy donors. When compared data from pre and post operative blood, significantly decreased mRNA quantity postoperatively was detected for TGF- β alone.

Our results demonstrated that among investigated cytokine genes IL-10 and TGF- β were significantly upregulated in patients with CRC compared to the control group, while the expression of IL-17 and IL23 mRNA was significantly decreased in CRC patients. Although the expression of IL-23A was downregulated, the other gene of IL-23 cytokine for subunit IL-12p40, IL-12B was not suppressed in preoperative blood compared to healthy donors.

IL17A gene expression was undetectable in 69% pre-operative patients' blood while in post operative blood was undetectable among 22% post-operative CRC patients' and 8% of healthy donors' blood. We detected a significant downregulation of IL17A expression in preoperative blood of CRC compared to both post-operative blood and healthy control group ($p=0,03$; $p=0,007$ respectively according to χ^2 test).

None statistically significant differences were obtained for expression of the other investigated genes between three studied groups. Overall analysis showed that the expression at mRNA level of investigated IL-12A, IL-12B and IL-6 genes in patients' blood did not alter depending on development of colorectal cancer.

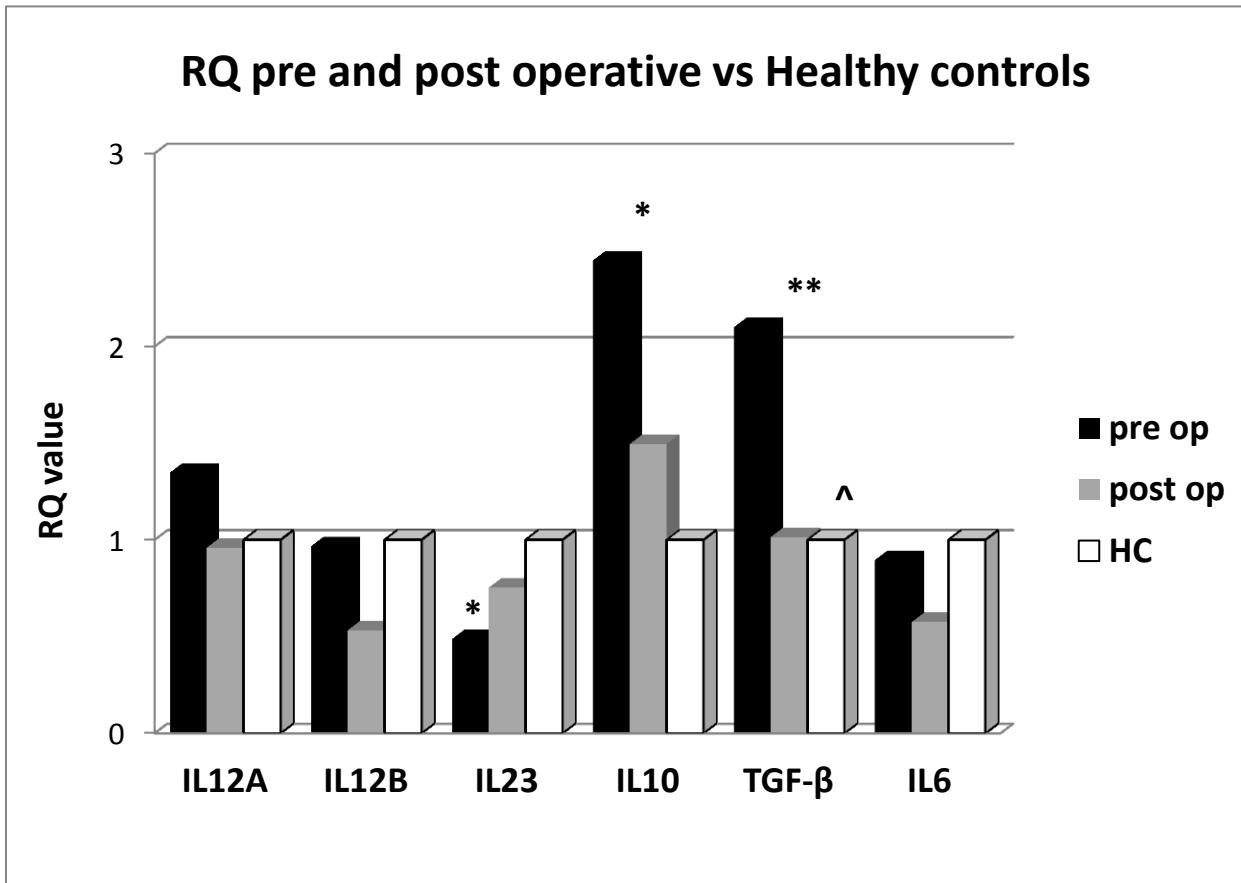


Figure 1. Relative quantity of cytokine gene expression in peripheral blood before and 10 days after surgery of CRC patients and healthy controls.

* $p < 0.05$; ** $p < 0.01$ – pre-operative vs. healthy controls

$\wedge p < 0.05$ – post-operative vs. pre-operative levels

DISCUSSION

In this study, we aimed to show that CRC is causally involved in the induction of aberrant systemic cytokine expression and to clarify which cytokine genes from peripheral blood are involved. mRNA levels of expression of following cytokines: IL-6; IL-12A; IL-12B; IL-23A; IL-10; IL-17 and TGF- β was investigated. Colorectal cancer affected tissue is invaded by immune cells from the host, suggesting that the activity of these cells may play a potential impact upon cancer development. Within the tumor mass a huge infiltration of white blood cells, as pivotal players in the tumor microenvironment is well documented (11, 12).

The critical role of inflammation in colon tumorigenesis, including initiation, promotion, progression, and metastasis has been shown (7, 8). Colorectal cancer mucosa invaded immune cells synthesized plenty of cytokines, which drive inflammation and have a huge impact upon both cancer development and anti-tumor immune

response. The molecular signature of tumor infiltrated immune cells includes gene expression pattern for cytokines that they produced. Thus far, a strong association has been shown between CRC development and changes of cytokine gene expression in tumor mucosa (13-15). Recently data showed that colorectal cancer progression was closely associated with infiltration of Th17 inflammatory cells, and main cytokines related with their differentiation and function are IL-6, IL-23, TGF- β and IL-17 (16, 17).

Apart from local tumor elicited inflammation, cancer patients frequently present with changes in numerous systemic parameters. These include changes in peripheral blood cell number, and alternations in the level of serum inflammatory cytokines (18, 19). Here, we demonstrated a well expressed downregulation of IL-23A and IL-17, and upregulation of IL-10 and TGF- β cytokine in peripheral immune cells of CRC patients (preoperative blood) compared to healthy

individual. In addition, eradication of tumor (post-operative blood) leads to a significantly decreased expression of IL-10 and TGF- β . These findings suggest that tumor induces aberrant changes in gene expression and indicate that levels of cytokine gene expression are associated with presence of CRC. However, it is unclear whether these changes in gene expression reflect the anti-cancer immune response or the cancer itself.

The TGF- β signaling pathway plays an important role in controlling cell proliferation and differentiation involved in colorectal carcinogenesis. In addition, there is evidence that excess production and/or activation of TGF- β by cancer cells can contribute to the tumor progression by paracrine mechanisms involving neoangiogenesis, production of stroma and proteases, and subversion of immune surveillance mechanisms in tumor hosts (20). Moreover, the activity of TGF- β on stromal cells increases the efficiency of organ colonization by CRC (21). Within the tumor microenvironment TGF- β also exerts a predominantly immunosuppressive effect on CD8⁺ cytotoxic T-lymphocytes and has been shown an active player in tumor immune evasion. In light of these findings upregulation of TGF β 1 expression in CRC patients' blood sustain our hypothesis that changes in gene expression reflect the cancer itself. In the same direction are data for participation of TGF β 1 in differentiation of Treg and Th17 subpopulations which are involved in tumor promoting inflammation (22). It has been demonstrated that TGF- β and IL-10 maintain differentiation of Treg cell, while IL-23 and IL-17 cytokines are closely connected to differentiation and function of Th17 cells (23, 24)

In view of the importance of cytokines, which are responsible for the differentiation of Treg and Th17 subpopulations which are involved in tumor promoting inflammation we aimed to investigate the levels of these cytokines in peripheral blood cell from CRC patients. Among the inflammation-related genes analyzed, the expression levels of IL-10 and TGF- β were upregulated in CRC blood cells and decreased after tumor eradication, almost paralleling those of healthy donors' levels. Downregulated expression was observed in pre-operative level of IL-23 and IL17 genes, while IL-10 and TGF- β

genes was up-regulated in pre-operative CRC patients' blood compared to healthy donors. We hypothesize that observed changes in peripheral blood cells could be associated with epigenetic modification, particularly with DNA methylation. Moreover those of genes with low transcription are known to be susceptible to methylation (25). Thus, methylation of these genes will maintain the same pattern of expression in lymphocytes after entry into the tumor microenvironment. In a mouse model of inflammation-promoting intestinal cancer the inflammation is associated with increased global aberrant DNA methylation, (26, 27) As a final argument for role of methylation, it was shown an overexpression of DNA methyl transferases (Dnmts), which are implicated in methylation in various human cancers (28). Nevertheless those enzymes may mediate silencing of numerous target genes in mouse and human colon cancers (29)

These assumptions are confirmed by our previous findings that monocytes isolated from colorectal cancer patients exhibit a hyporesponsiveness to LPS stimulation compared with healthy people and that hyporesponsiveness was strongly expressed in monocytes from advanced then early stages of CRC (10). Further studies are needed to clarify whether gene expression pattern in blood lymphocytes and monocytes are related to epigenetics alteration.

Obviously, CRC generates not only local inflammatory microenvironment named as tumor-elicited inflammation, but also promoting systemic changes that are favorable for cancer progression. A part of these systemic effects, that cancer development induces, include reprogramming of gene expression in blood immune cells. Here we demonstrated an upregulation of TGF- β and IL-10 simultaneously with downregulation of IL-17 and IL-23 cytokine mRNA expression in peripheral immune cell. Moreover IL-23 mRNA suppression was specific as tumoral expression of other IL-12/23 family members (IL-12A and IL-12B) was not substantially affected.

Bearing in mind the above we could assume that peripheral blood gene expression programming in CRC cancer patients triggers local differentiation of Th cells towards Th17 and Treg instead Th1 anti-tumor subpopulation. At the same time TGF- β produced by peripheral

immune cell of CRC patients additionally depresses anti-tumor immune responses at the level of Th cells, cytotoxic T lymphocytes and natural killer cells, while increasing the numbers of Tr cells.

In conclusion we suppose that the established tumor elicited inflammation can contribute to multiple hallmark capabilities by supplying bioactive molecules not only benefit tumor grown, but also affecting epigenetic alternation in immune blood cells resulting in gene expression reprogramming leading to ineffective antitumor immune response.

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