RESEARCH ARTICLE

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Evaluating *CD4* and *Foxp3 mRNA* Expression in Tissue Specimens of Celiac Disease and Colorectal Cancer Patients

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Abstract

Objective: Celiac disease (CD) and colorectal cancer (CRC) are distinct gastrointestinal conditions with a debated association. This study aimed to evaluate the mRNA expression of CD4 and Foxp3 in tissue specimens of CD and CRC patients. The findings can provide valuable insights into the complex connection between these different gastrointestinal conditions. **Methods:** Tissue samples from 100 CRC patients, 50 CD patients, and 50 healthy controls (HCs) were collected. RNA extraction, cDNA synthesis, and quantitative real-time PCR were performed. Statistical analysis was conducted using ANOVA and Pearson's correlation test. **Result:** CD4 mRNA expression was significantly higher in CRC patients compared to CD patients and HCs ($P<0.0001$ for both). *Foxp3 mRNA* expression was significantly higher in CD patients compared to CRC patients and HCs ($P<0.0001$ for both). Clinicopathological characteristics did not correlate significantly with gene expression levels. **Conclusion:** This study reveals differential expression patterns of CD4 and *Foxp3 mRNA* in CRC and CD patients. Upregulated CD4 mRNA suggests its potential role in promoting tumor growth, while increased *Foxp3 mRNA* expression may reflect an immunosuppressive mechanism in CD pathogenesis. These findings provide insights into the molecular and immunological aspects of CRC and CD, warranting further studies for potential therapeutic strategies.

Keywords: Celiac disease- Colorectal cancer- Gene expression

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Introduction

Celiac disease (CD) and colorectal cancer (CRC) are two distinct conditions that can affect the gastrointestinal system [1]. CD is an autoimmune disorder of the small intestine triggered by the ingestion of gluten, a protein found in wheat, barley, and rye, while CRC is a type of cancer that affects the colon or rectum [2, 3]. The association between CD and CRC has been a topic of debate, with some studies suggesting elevated standardized incidence ratios (SIRs) for colon cancer among celiac patients compared to the general population, while others report a low risk [4, 1]. Several factors contribute to the variability in findings on the association between CD and CRC. Varying research methodologies, patient populations, and confounding variables like dietary habits and coexisting conditions influence results. These complexities lead to inconsistent conclusions and ongoing scientific debates. Inflammation and immune dysregulation of the small intestine in CD patients can lead to DNA damage and promote the growth of cancer cells. Genetic factors like HLA-DQ2 and HLA-DQ8

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genotypes, gluten free diet (GFD), as an only therapeutic strategy for CD, are known as risk factors for CRC [5, 1, 6]. The potential link between GFD and CRC risk is multifactorial. GFD products often lack fiber, vitamins, and minerals, which are important for a healthy digestive system and reducing CRC risk. Failure to replace them with high-fiber alternatives may contribute to increased risk. Furthermore, GFD could disrupt gut microbiota, which plays a role in immune function and inflammation regulation, potentially increasing CRC risk. However, evidence is limited and inconclusive regarding GFD as a definitive risk factor for CRC [7-10]. Additionally, immunological changes in the small intestine may play a role in developing CRC in patients with CD [11, 1]. Gluten-specific *CD4*+ T cells, generated in response to gluten exposure, play a crucial role in the pathogenesis of CD [12]. These T cells are responsible for the immune response and the resulting damage to the small intestine in CD patients [13, 14].

Moreover, Regulatory *CD4*+ T cells (Tregs), are the main population involved in the suppressive mechanisms of CD [15]. These subsets express the master transcription factor *Foxp3* [15]. While *Foxp3* expression is associated with anti-inflammatory activity, some studies have shown that circulating gluten-specific *Foxp3*+ Tregs in CD patients have impaired suppressive function [15, 16]. This suggests that the balance between pro-inflammatory and anti-inflammatory mechanisms may be disrupted in CD, contributing to the pathogenesis of the disease [17]. The impaired suppressive function of gluten-specific *Foxp3*+ Tregs in CD may lead to the loss of immune surveillance, allowing for the growth and progression of CRC cells [18, 19]. The balance between effector *CD4*+ T cells and Tregs is also crucial for tumor progression and immune surveillance in CRC patients [20]. In CRC, the role of Tregs and *Foxp3* is more complex and may depend on the tumor microenvironment and the balance between effector T cells and Tregs [21].

Overall, *CD4*+ T cells and *Foxp3* are involved in the immune response in both CD and CRC and further research is needed to understand the underlying mechanisms and the potential impact of factors such as changes in genes expression on the development of CRC in CD patients. Therefore, this study aimed to evaluate the mRNA expression of *CD4* and *Foxp3* in tissue specimens of celiac disease and colorectal cancer patients to gain a deeper understanding of the molecular aspects of these two gastrointestinal disorders.

Materials and Methods

Study subjects and sample collection

The study group consisted of 200 tissue samples, including 100 from CRC patients, 50 from CD patients, and 50 from healthy control subjects. Controls had no clinical and serological evidences of CD and CRC up to their first-degree relatives. CRC diagnosis was confirmed through clinical examination, colonoscopy, and histopathology tests, while CD diagnosis was based on clinical presentation evaluation, serology assessment, and histological analysis of villous atrophy [22, 23]. The biopsies were preserved in RNA later at -70°C for future evaluation. The ethical committee at the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, approved the study (IR.SBMU.RIGLD.REC.1399.036), and written informed consent was obtained from all participants before their inclusion.

RNA extraction and complementary DNA synthesis

For extracting total RNA from small intestinal biopsy specimens, the YTA Total RNA Purification Mini kit for Blood/Cultured Cell/Tissue (Yekta Tajhiz Azma, Iran) was used in this study. The purity and concentration of the extracted RNA were assessed using the NanoDrop-1000 spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA). To perform reverse transcription of RNA into complementary DNA (cDNA), the 2 Step 2X RT-PCR Premix (Taq) kit (BioFact™, South Korea) was employed.

Primer designing and quantitative real time PCR (RT-qPCR)

Specialized software such as Gene runner and Primer3 were used to design specific primers for *CD4* and *Foxp3* and Beta2 micro-globulin (B2M), as the house-keeping gene. Well-crafted primers were synthesized by Pishgam corporation. Table 1 provides details on the characteristics of the designed primers.

The mRNA expression levels of the target genes were measured using real-time polymerase chain reaction (PCR). The real-time qPCR reactions were conducted in a final volume of 20 µl using SYBR Premix Ex Taq (RealQ Plus 2x Master Mix Green-Amplicon, Japan). The Rotor-Gene Q series was utilized, and the reactions followed these conditions: an initial denaturation for 15 minutes at 95 °C, then 40 cycles of 20 seconds at 95 °C and 60 seconds at 60 °C. The 2-ΔΔCt Method was employed to determine changes in gene expression levels (ΔCt = Cttarget – Cthouse keeping).

Statistical analysis

The data were analyzed using IBM SPSS Version 21 (SPSS Inc., Chicago, IL, USA) and PRISM software version 5.00 (San Diego, CA, USA). The mean ± standard deviation (SD) format was used to express the data. Analysis of Variance (ANOVA) was used to analyze the differences between the means of the studied groups. The correlation coefficient between variables was evaluated using Pearson's correlation test.

Results

Demographic and clinical characteristics of study groups

The demographic and pathological characteristics of the included participants are presented in Table 2. There were no significant differences between the groups in terms of age and sex (P>0.05).

mRNA expression of CD4 and Foxp3

We investigated the tissue mRNA expression of *CD4* and *Foxp3* using B2M as the housekeeping gene. According to our results, *CD4* mRNA expressions was

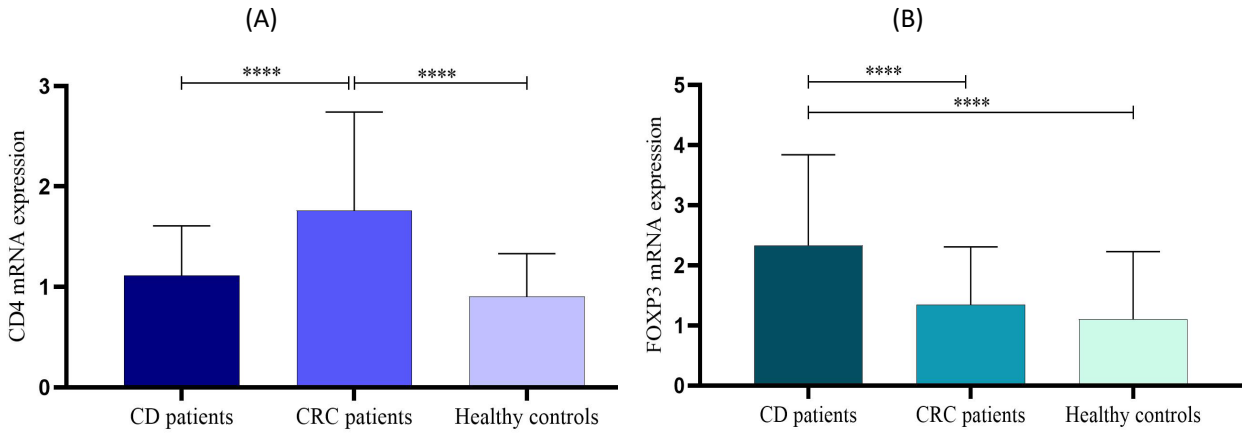

Figure 1. The Relative Expression Level of *CD4* (A), and *Foxp3* (B) Genes in Celiac Disease (CD) and Colorectal Cancer (CRC) Samples Compared with Healthy Controls

Table 1. Primers Used in qPCR

Gene	Primer sequence	Primer length
CD4	F: 5′CCTCCTGCTTTTCATTGGGCTAG3′	23
	R: 5′TGAGGACACTGGCAGGTCTTCT3′	22
Foxp3	F: 5′GGCACAATGTCTCCTCCAGAGA3′	22
	R: 5′CAGATGAAGCCTTGGTCAGTGC3′	22
B2M	F: 5′CCACTGAAAAAGATGAGTATGCCT3′	24
	R: 5′CCAATCCAAATGCGGCATCTTCA3′	23

significantly higher in CRC patients than in CD patients and HCs (P<0.0001 for both). A significant increase in *Foxp3* expression was observed in CD patients' samples compared to CRC patients and HCs (P<0.0001 for both) (Figure 1). The association between *CD4* and *Foxp3* expression levels and the patients' clinicopathological characteristics was also assessed. None of the mentioned features were significantly correlated with the expression levels of the studied genes.

Discussion

The present study aimed to investigate the mRNA expression levels of *CD4* and *Foxp3* in patients with CRC and CD relative to HCs. The findings shed light on the potential role of these genes in the pathogenesis of both conditions. Evaluating *CD4* and *Foxp3 mRNA* levels in CD and CRC tissues is important for understanding the immune response and the role of specific immune cell populations in these diseases. *CD4* and *Foxp3 mRNA* can provide insights into the activation and abundance of *CD4* T-helper cells and Tregs, respectively [24, 25]. These molecular markers can help assess the inflammatory response and immune dysregulation in CD, while also shedding light on the dynamic interplay between effector T cells and Tregs in CRC tumor microenvironment [25, 24].

Our results demonstrated significantly higher *CD4* mRNA expressions in CRC patients compared to both CD patients and HCs. This finding suggests that *CD4* mRNA may play a crucial role in the development and progression of CRC. The upregulation of *CD4* mRNA in CRC patients implies its involvement in the activation and proliferation of immune cells, which could contribute to the tumor microenvironment in CRC [26]. According to the reports, *CD4*+ T lymphocytes are the main type of T cells found in CRC tissues [27, 28].

Table 2. Demographic Characteristics of Study Participants

Groups	Numbers	Age	Sex		Pathology report		
CD patients	50	49±12.24	Male	20 (40%)	Marsh	1	5 (10%)
			Female	30 (60%)		2	15 (30%)
						3	30 (60%)
CRC patients	100	52.17±13.8	Male	60 (60%)	Grade	1	57 (57%)
			Female	40 (40%)		2	25 (25%)
						3	18 (18%)
Healthy controls	50	47±13.2	Male	22 (44%)			
			Female	28 (56%)			

In the context of CRC, these cells may contribute to the development and progression of the disease through various mechanisms, including the production of pro-inflammatory cytokines and the activation of other immune cells [29, 30]. As *CD4*+ T cells are also crucial in the development of CD, their uncontrolled activation within the CD context may lead to the emergence of CRC [31]. Wahbi et al. [32] analyzed the presence of *CD4*+ and CD8+ Tumor Infiltrating Lymphocytes (TILs) in oral squamous cell carcinoma (OSCC) and their correlation with malignancy grade. *CD4*+ TILs were more frequent in poorly differentiated specimens, while CD8+ TILs were more frequent in well-differentiated specimens. The *CD4*+/CD8+ ratio was higher in low-grade specimens, suggesting that *CD4*+ and *CD4*+/CD8+ ratio are independent prognostic factors in OSCC [32]. Salama et al. [33] investigated the correlation between PDL1 expression and clinicopathological parameters in endometrial cancer (EC) cases, focusing on *CD4* and CD8 immune cells. They found that PDL1 was expressed in tumor cells in 67% of cases and in immune cells in 61% of cases. Significant correlations were observed between PDL1 expression and patient age, LVSI, TILS score, and *CD4*+/CD8+ expression. The findings suggest that these variables may serve as predictive biomarkers for successful immune therapy in EC [33].

On the other hand, the expression of *Foxp3*, exhibited a significantly increased level in CD patients when compared to both CRC patients and HCs. This suggests the activation of Tregs in CD patients, which are known to have immunosuppressive properties [27]. The higher expression of *Foxp3* in CD may reflect an attempt to counterbalance the inflammatory response and limit tissue damage in the context of CD pathogenesis [34, 35]. This is consistent with the role of Tregs in preventing inappropriate inflammatory responses and maintaining intestinal immune tolerance [36]. However, certain studies have revealed that gluten-specific *Foxp3*+ Tregs found in CD patients may exhibit compromised suppressive capabilities [18]. This impairment could potentially have negative consequences for patients, as it may heighten the risk of cancer development. Rachmadi et al. [37] investigated the expression of FOXP3, *CD4*, CD8, and p53 in the transformation of Sinonasal Inverted Papilloma (SIP) into sinonasal carcinoma. According to their results, FOXP3 and p53 were significantly overexpressed in SIP with dysplasia compared to SIP without dysplasia. *CD4* and CD8 expression did not show significant differences between the two groups. They concluded that, FOXP3 and p53 may serve as potential biomarkers for the malignant transformation of sinonasal inverted papilloma, particularly in the presence of dysplasia [37].

The results of this study contribute to our understanding of the underlying molecular mechanisms involved in CRC and CD. By identifying *CD4* mRNA and *Foxp3* as potential markers for disease progression and immune modulation, these findings open new avenues for further investigations and therapeutic interventions. It is worth noting that our study has certain limitations. The sample size was relatively small, and the study population was limited to a specific geographical region. Therefore, the generalizability of these findings to other populations should be interpreted with caution. Future large-scale studies encompassing diverse populations are warranted to validate and expand upon these initial observations. It would be valuable to investigate the localization of *CD4* and *Foxp3* expression within specific cell types or compartments of the affected tissues, as well as to explore the potential interplay between *CD4*-positive effector T cells and *Foxp3*-positive regulatory T cells in both CD and CRC microenvironments [38, 39].

In conclusion, this research demonstrates the differential expression patterns of *CD4* mRNA and *Foxp3* in CRC patients, CD patients, and HCs. The upregulation of *CD4* mRNA in CRC suggests its possible involvement in promoting tumor growth, highlighting a novel target for therapeutic intervention in CRC treatment. Additionally, the increased expression of *Foxp3* in CD implies an immunosuppressive mechanism in CD pathogenesis, indicating a potential avenue for immune-modulating therapies. These findings have considerable value as they deepen our understanding of the complex interplay between the immune system and these diseases. By identifying specific molecular players and their roles, this research opens up new opportunities for the development of targeted treatments that specifically address the underlying mechanisms of CRC and CD.

Author Contribution Statement

[MRN], [MRT], [ENM] and [HH] contributed to the study conception and design. Material preparation, data collection and analysis were performed by [NA], [MYT], [MSN], [SJS], [MK] and [AS]. The first draft of the manuscript was written by [NA], [AN], [MJEA] and all authors commented on previous versions of the manuscript. All authors read and approved the final attest that all listed authors meet the authorship criteria and that no other authors meeting the criteria have been omitted

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Approval

It was approved by the ethical committee at the Shahid Beheshti University of Medical Sciences (IR.SBMU.RETECH.REC.1402.418).

Conflict of Interest

The authors declare there is no conflict of interest.

Ethical Declaration

The ethical committee at the Shahid Beheshti University of Medical Sciences, approved the study (IR.SBMU.RETECH.REC.1402.418).

Data Availability

Data is available on demand by reaching out to the author Dr. Rostami-Nejad In by email at: m.rostamii@gmail.com

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