Scandinavian Journal of Clinical and Laboratory Investigation

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/iclb20

Investigation of possible associations between tryptophan/kynurenine status and FOXP3 expression in colorectal cancer

Ebru Nur Ay, Şeyda Demirkol, Mehmet Tolgahan Hakan, Cem Horozoğlu, Soykan Arıkan, Mehmet Baki Doğan, Filiz Akyüz, Ceylan Özsoy Hepokur & İlhan Yaylım

To cite this article: Ebru Nur Ay, Şeyda Demirkol, Mehmet Tolgahan Hakan, Cem Horozoğlu, Soykan Arıkan, Mehmet Baki Doğan, Filiz Akyüz, Ceylan Özsoy Hepokur & İlhan Yaylım (2022) Investigation of possible associations between tryptophan/kynurenine status and FOXP3 expression in colorectal cancer, Scandinavian Journal of Clinical and Laboratory Investigation, 82:3, 185-191, DOI: 10.1080/00365513.2022.2040050

To link to this article: https://doi.org/10.1080/00365513.2022.2040050

Published online: 22 Apr 2022.

SCANDINAVIAN JOURNAL OF CLINICAL AND LABORATORY INVESTIGATION
2022, VOL. 82, NO. 3, 185–191
https://doi.org/10.1080/00365513.2022.2040050

Taylor & Francis
Taylor & Francis Group

ORIGINAL ARTICLE

Investigation of possible associations between tryptophan/kynurenine status and FOXP3 expression in colorectal cancer

Ebru Nur Ay[a,b], Şeyda Demirkol[a,c], Mehmet Tolgahan Hakan[a], Cem Horozoğlu[d], Soykan Arıkan[e], Mehmet Baki Doğan[f], Filiz Akyüz[g], Ceylan Özsoy Hepokur[h] and İlhan Yaylım[a]

[a]Department of Molecular Medicine, Istanbul University, Aziz Sancar Institute of Experimental Medicine, Istanbul, Turkey; [b]Department of Medical Services and Techniques, Vocational School of Health Services, Istinye University, Istanbul, Turkey; [c]Department of Molecular Biology and Genetics, Biruni University, Istanbul, Turkey; [d]Department of Biochemistry, Biruni University Medical Faculty, İstanbul, Turkey; [e]General Surgery Clinic, Cam and Sakura City Hospital, Istanbul, Turkey; [f]General Surgery Clinic, Istanbul Training and Research Hospital, Istanbul, Turkey; [g]Department of Gastroenterology, Istanbul Faculty of Medicine, Istanbul, Turkey; [h]Department of Biochemistry, Faculty of Pharmacy, Sivas Cumhuriyet University, Sivas, Turkey

ABSTRACT

Tryptophan metabolism in the tumor microenvironment exerts immunosuppressive effects by affecting the anti-tumor functions of immune cells. The immunosuppressive roles of tryptophan and tryptophan metabolites and their effects on the FOXP3 gene, highly expressed in regulatory T cells (Tregs), are remarkable. Our study aimed to investigate the relation between tryptophan metabolism and the transcription factor FOXP3 gene in colorectal cancer (CRC). Patients with CRC ($n = 159$) and controls ($n = 112$) were included in the study. The FOXP3 rs3761548 variant genotyping from the isolated genomic DNA was performed by PCR-RFLP. FOXP3 gene expression was determined by Q-PCR in RNAs isolated from resected tissues at the same time. Serum tryptophan, kynurenine, kynurenic acid levels of the cases were determined by HPLC. In serum samples with CRC, tryptophan level was 14.32 ± 1.09 μmol/L, kynurenine level was 1.33 ± 0.02 μmol/L, and the kynurenic acid level was 0.01 ± 0.001 μmol/L. The level of tryptophan was found to be low in CRC compared to control ($p < .001$). In cases with CRC, CC genotype ($p = .048$) and C allele ($p = .012$) frequency for FOXP3 rs3761548 were higher than the control group. It was found that the expression level of the FOXP3 gene was approximately 44 times higher in the advanced tumor stage (T3 + T4) than in the early tumor stage (T1 + T2) ($p = .021$).

We suggest that there may be a possible relationship among serum TRP, TRP metabolites (KYN, KYNA) levels, FOXP3 gene expression, and FOXP3 gene variants in CRC pathogenesis.

ARTICLE HISTORY
Received 24 September 2021
Revised 27 January 2022
Accepted 6 February 2022

KEYWORDS
Colorectal cancer (CRC); tryptophan metabolism; PCR-RFLP; gene expression; HPLC

Introduction

Colorectal cancer (CRC) ranks third among cancers in terms of prevalence. Inflammatory bowel diseases, smoking, nutritional type, polyps, and genetic factors are different parts of this process in CRC, which mostly have multifactorial pathogenesis [1]. Immune system and cancer biology studies contribute to the clarification of CRC pathogenesis. Significantly, the number of immunotherapeutic studies, which have recently increased, is vital in revealing new signal mechanisms [2–5]. All metabolic process which is involved in CRC etiopathogenesis has been focused on in recent pieces of literature. Tryptophan metabolism is a crucial mechanism to avoid immune surveillance of tumor cells [6,7]. Tryptophan is the essential amino acid, and 95% of dietary tryptophan is metabolized along the pathway of the kynurenine [KP] [8]. Indoleamine 2,3-dioxygenase 1 (IDO1) enzyme, which is involved in the kynurenine pathway of tryptophan metabolism, is overexpressed in cancer progression. This metabolic pathway is thought to have an important role in gastric, pancreatic, esophageal, and gastrointestinal (GI) malignancies. Therefore, tryptophan metabolites have attracted researchers' attention for immunotherapy and started to be studied in malignancies with gastrointestinal origin [9]. In tumor pathophysiology, rearrangement of the tumor microenvironment is a critical primary mechanism. It is observed that the immune response in the tumor microenvironment by T cells can interfere with specific immunotherapeutic agents [10]. Therefore, it has significant potential to identify cases that may respond to specific immunotherapeutic agents targeting immune cell-derived pathways.

FOXP3 (Forkhead box P3, Transcription factor fork box P3) is a protein with 431 amino acids belonging to the fork-helix transcription factor family, located on the X chromosome p11.23 and encoded from the FOXP3 gene. FOXP3 has been identified as a marker of CD4 + CD25 + regulatory T cells and is an essential protein of immunosuppressive functions. FOXP3 has been shown to limit antitumor immune responses during tumor progression [11].

CONTACT İlhan Yaylım ilhanyaylim@gmail.com Department of Molecular Medicine, İstanbul University Aziz Sancar Institute of Experimental Medicine, 34280 Capa, Istanbul, Turkey

Considering the gene expression levels of the FOXP3 gene that may be related to gene polymorphisms, the reason for the FOXP3 gene expression changes has not been fully elucidated. However, various studies have shown that FOXP3 immune staining in colorectal cancer tissues may decrease due to the different localization of FOXP3 molecule according to the cancer cell type . This issue can be explained due to the its passing of FOXP3 molecule from nucleus to cytoplasm in the tumoral tissues[12]. More studies are needed to understand whether these changes in FOXP3 gene expression levels in tumor cells have the potential of clinical significance as a prognostic factor and its relationship with the differences in FOXP gene expression in tumor cells [3,4].

Increased Tregs activity accelerates tumor growth, while Treg cells decline triggers an effective antitumor immune response. Tryptophan metabolism has effects on the immunosuppressive roles of regulatory T cells through the FOXP3 pathway. Inhibition of CD8 + T cell proliferation and CD4 + T cell apoptosis through the kynurenine pathway of tryptophan by the enzyme IDO (indoleamine 2,3 dioxygenase) secreted from Treg cells increases. Increased IDO expression, which characterizes tryptophan consumption and an overproduction to kynurenine, reduces T effector cell function and activates FOXP3+ Tregs lymphocytes [13]. Thus, the increasing regulatory T cell population leads to active T cell suppression, silencing the immune system. IDO is also produced in cancer cells, which are thought to contribute to the immune breakdown strategy used by many tumors. Tumor development is increased by elevating IDO levels by inactivating both tumor suppressor genes and effective immune tumor surveillance mechanisms [14]. It was reported that the interaction between CTLA 4-B7-1 and/or B7-2 triggers IDO expression and enzymatic activity in dendritic cells since this binding elicits a signal that enhances IDO [15]. In the context of these data, IDO is estimated to be a possible mechanism in CTLA4 + FOXP3+ Tregs mediated immune suppression. Tumor cells expressing IDO secrete tryptophan metabolites, such as kynurenines, suppress cytotoxic effector functions through downregulation of the TCR CD3 chain, and stimulate FOXP3+ Tregs differentiation [16].

Studies have observed that different genes and metabolites may be effective in T cell-mediated immune system interactions in tumor microenvironment regulation [17,18]. Similarly, it has been found that the molecular characterization of the tumor can generally alter the anti-tumor immune response [19,20]. We examined the FOXP3 polymorphism, and its gene expression studies and metabolite levels of TRP metabolism. TRP metabolism immunologically suppresses many cells and tumor microenvironment. This metabolic pathway creates a suppression in the tumor microenvironment by stimulating the differentiation of Treg cells and increasing their proliferation, primarily by increasing the expression of the FOXP3 gene and prepares the ground for the progression of cancer. For this purpose, we wanted to conduct this study to understand whether the FOXP3 gene expression may be changed by Treg cells located in that region and the gene expression status resulting from individual differences because of their FOXP3 gene variants.

Table 1. Distribution of FOXP3 expression, comparing clinicopathological parameters.

FOXP3 expression (fold change)	Mean ± SD	*p*-Value (*$p<.05$)
Tumor stage		
Advanced tumor stage (T3 + T4)	2.22 ± 0.72	.021*
Early tumor stage (T1+ T2)	0.05 ± 0.03	
Lymph node status		
N1 + N2	2.51 ± 1.07	.968
N0	1.5 ± 0.8	
Distant metastasis		
Present	2.16 ± 1.27	.305
Absent	1.97 ± 0.76	
Perineural invasion		
Present	1.88 ± 1.49	.579
Absent	2.05 ± 0.74	

Materials & methods

Patients

Patient selection, the study group consisted of 159 patients diagnosed with CRC at the General Surgery clinic of the Istanbul Training and Research Hospital and 112 controls. Medical records, and pathological reports were received to confirm the diagnosis and cancer status. The patients received a standard questionnaire regarding the diagnosis time, family history, treatments, and other medical issues. The tissue samples were collected before any chemotherapeutic or radiation therapy treatment had been started. Lymph node metastasis status was categorized as no regional lymph nodes affected (N0) or at least one nodal metastasis (N1 + N2) (Table 1). Advanced tumor stage was categorized as T3 + T4 and early tumor stage was categorized as T1 + T2 (Table 1).

The American Joint Committee on Cancer (AJCC) TNM classification was used for defining the stage of CRC. Istanbul University, Istanbul Faculty of Medicine Clinical Research Ethics Committee approved our study. The protocol of the study was compatible with the World Medical Association Declaration of Helsinki (Ethical Principles for Medical Research Involving Human Subjects). Informed written consent was obtained from all volunteers enrolled in this study.

DNA isolation

Genomic DNA was extracted from peripheral whole blood containing EDTA according to the salting-out technique. DNA was isolated from the blood leukocytes in 10 ml EDTA by the method of Miller et al. based on sodium dodecyl sulfate lysis, ammonium acetate extraction, and ethanol precipitation.

Genotyping

Genotyping was performed by the polymerase chain reaction (PCR) and RFLP. The PCR reactions were carried out

using a Bio-Rad Thermal Cycler (Bio-Rad, USA). For PCR amplification, the primers for FOXP3 (C/A) polymorphism were (forward 5′- GGCAGAGTTGAAATCCAAGC-3′ and reverse 5′-CAACGTGTGAGAAGGCAGAA-3′). Primers were used for another target molecule; For detection of the gene, 20 ng genomic DNA was amplified with 2.5 µl 10 X PCR buffer, 2 µl 2,5 mM dNTP, 1.0 µl 10 pmol/µl of each primer and 0.3 µli-StarTaq™ Taq polymerase (iNtRON Biotechnology, Korea) in a 24 µl reaction volume. The PCR conditions for FOXP3 C/A was initially a melting step of 4 min at 94 °C, then 35 cycles of 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C, and finally, an elongation step of 10 min at 72 °C PCR products (155 bp) were digested with PstI (Fermentas, USA) restriction enzyme (37 °C for 15 min) and electrophoresed on 2% agarose gels and stained with ethidium bromide. Genotypes were determined as CC (80,75 bp), CA (155,80,75 bp), AA (155 bp) for FOXP3 C/A polymorphism.

Detection of gene expression

Tumor tissue and tumor surrounding tissue samples taken from patients with colorectal cancer during the surgical procedure were rapidly placed in cryogenic tubes containing RNA later and freezing at −80 °C was provided. Before analysis, frozen tissue samples were divided into small pieces and 8–10 pieces of stainless-steel beads were added on them and they were homogenized with 700–1000 µl Trizol (Roche) solution in MagnaLyser at 4500 rpm for 20–30 s.

Homogenized samples are stored at room temperature for 5 min. After waiting, 150 µl chloroform (200 µl chloroform into 1 ml trizol) was added to the tube, and vigorously mixed for 15 s. After 2–3 min at room temperature, tissue samples were centrifuged at 15 min at +4 °C at a speed of 12.000 g. The supernatant part of the tube was transferred to another tube with a pipette at an angle of 45°. The protein extract is in the middle phase and is preserved for possible future experiments. 375 µl of 100% isopropyl alcohol (500 µl of 100%isopropyl alcohol for 1 ml of trizol) was added onto the supernatant taken into a clean Eppendorf tube. At room temperature again 10 min, tissue samples were centrifuged at 10 min at +4 °C at a speed of 12.000 g after waiting. It was centrifuged again. The supernatant was discarded and 750 µl of 75% ethyl alcohol (1 ml of 75% ethyl alcohol for 1 ml of trizol) was added to the pellet. The RNA obtained in this step can be used by storing at −20 °C for at least 1 year or at +4 °C for 1 week. With the addition of ethyl alcohol, the RNA is vortexed for a very short time and at a speed of 7.500 g at +4 °C for 5 min. It was centrifuged throughout. In the next step, the supernatant part is discarded, and the pellet is 5–10 min. allowed to dry, then dissolved in 20 µl RNase free water. In the water bat hat 55–60 °C or 10–15 min on the heat block. At the end of the retention period, the RNAs were stored at −80 °C. RT-PCR (Roche, Light Cycler® 480 System) instrument was used for the quantitative Real-Time Polymerase Chain Reaction (Q-PCR) measurement of FOXP3 gene expression. Amplifications were performed using cDNA, site-specific primers, gene expression RT2 SYBR Green Master mix in a total reaction volume of 20 µL. The cDNA samples obtained to normalize FOXP3 gene expression were studied using GADPH gene-specific primer and FOXP3 gene expression primer. For Q-PCR expression, the primers for FOXP3 gene were: (forward 5′- TCCCAGAGTTCCTCCACAAC-3′ and reverse 5′- ATTGAGTGTCCGCTGCTTCT-3′ Primers were used for another target molecule the primers for GAPDH gene were forward 5′-TGCACCACCAACTGCTTAGC-3′ and reverse 5′- GGCATGGACTGTGGTCATGAG-3′. Primers were used for another housekeeping molecule for expression of the gene, 12.5 µl RT^2 SYBR Green Master mix, 1 µl cDNA reaction, 1 µl RT^2 qPCR, 10.5 µl Primer Assay RNase-free dH_2O in 25 µl reaction volume. The qPCR conditions for FOXP3 expression were initially stepped of 10 min at 95 °C, then 35 cycles of 15 s at 95 °C, 1 min at 60 °C. Analysis of relative gene expression data was performed according to the threshold cycle (CT) method.

The level of FOXP3 mRNA expression in tumor and tumor surrounding tissues of 20 cases in our study group was analyzed by qPZR. The delta Ct values were calculated by subtracting the duplicated studied average Ct values of the obtained target gene, FOXP3, from the average Ct values of GAPDH determined as housekeeping. Then, delta delta Ct values were obtained to be subtracted from the Ct value of the control tissue for the delta Ct value of the tumor tissue. The expression of the fold change was found by calculating the delta delta Ct values above 2, the negative or positive delta delta Ct value was expressed as a relatively lowor high expression of the gene to this fold change. Standard errors of duplicated samples were also calculated.

HPLC analysis

The determination of plasma tryptophan (TRP), kynurenine (KYN) and kynurenic acid (KYNA) levels were performed by high pressure liquid chromatography-fluorescent detector (HPLC-FD) (Shimadzu LC-20A, Japan) according to the method developed by Xiang et al. Chromatographic conditions were determined for the metabolites to be analyzed by HPLC. Preparation of the HPLC samples was done according to the procedure. 100 µL of 0.624 mol/L perchloric acid solution was added and vortexed to precipitate proteins on 100 µL of plasma. 10 min at 10,000 g at +4 °C. After centrifugation, the separated supernatant was injected into the HPLC system in a volume of 20 µL.

The derivatized amino acids by the solid-phase specific extraction (presented under 2.4) were separated using an ODS column (5 µm, 150 × 4.6 id) (Shimadzu, Japan). The mobile phase consisted of Zinc Acetate Buffer (15 mmol/l; pH: 4.0): Acetonitrile (95:5). The flow rate was set at 1.5 ml/min and the column temperature was kept at 20 °C. Run-to-run time was set at 24 min. and the injected volume was 20 µL. HPLC detector UV λ values of plasma tryptophan (TRP), kynurenine (KYN), and kynurenic acid (KYNA) were defined as 365–480 nm for kynurenine, 254–404 nm for tryptophan and 344–404 nm for kynurenic acid. Moreover, retention times of metabolites were determined

Table 2. Concentrations of calibration solutions.

	TRP (μmol/L)	KYN (μmol/L)	KYNA (μmol/L)
Mix 1	195.8	96	2.116
Mix 2	97.9	48	1.058
Mix 3	48.95	24	0.529
Mix 4	24.48	1.2	0.0153
Mix 5	0.245	0.048	0.00105

as 5.8 min. for kynurenine, 13.0 min. for tryptophan and 16.7 min. for kynurenic acid.

Standard solutions were prepared from tryptophan, kynurenine and kynurenic acid (Sigma-Aldrich) stock solutions with HPLC grade distilled water and stored at −20 C until the working time. Standard solutions were prepared as 979 μmol/L for tryptophan, 192 μmol/L for kynurenine, and 211.6 μmol/L for kynurenic acid, resulting in a standard calibration curve. To draw a 5-point calibration graph of 3 molecules to be analyzed simultaneously, mixing standards of these stock standard solutions were prepared with 0.312 mol/L perchloric acid at the following concentrations. The concentrations of the calibration solutions of the standard samples prepared for HPLC analysis are given in Table 2.

The calibration graph of the HLPC device with LC Solution program (Shimadzu, Japan) was prepared by injecting a 20 ml volume of 5 prepared standard solutions into HPLC system. In the calculated calibration graph, R^2 values of TRP, KYN and KYNA were calculated as 0.9994, 0.9973, and 0.9999, respectively. Detection limits (LOD) of the method selected for the analysis were found to be 0.001 μmol/L for TRP, 0.0245 μmol/L for KYN, and 0.05 μmol/L for KYNA. With the results obtained, the calibration graph was drawn, and the relevant calibration graph values were calculated, then the metabolite amounts of the patient and control samples were calculated based on these calibration values.

Statistical analysis

Statistical analysis was performed by using the SPSS software package (version 21.0; SPSS Inc., Armonk, NY, USA). Descriptive statics include the mean, standard deviation, and percentages. Mean values of differences in clinical parameters between patients and controls were compared with unpaired t-test and expressed. The Hardy-Weinberg equilibrium was tested for all polymorphisms. For comparing the distributions and differences of genotypes and alleles between patients and controls, the Chi-square test was used. Values of p less than .05 were statistically considered as significant.

Non-parametric Man Whitney U analysis was performed using GraphPad Prism (version 8.0.0) program for FOXP3 expression level comparisons of patients and controls. Expression analysis results of FOXP3 gene in tumor and tumor surrounding tissues were determined by GraphPad Prism (version 8.0.0) program.

Table 3. Genotype and allele frequencies of FOXP3 polymorphism in CRC patients and controls.

Genotype and allele	CRC patients n (%)	Controls n (%)	p-Value (*p<.05)
Genotype			
CC (%)	61 (38.4)	28 (25)	.048*
AC (%)	70 (44)	55 (49.1)	
AA (%)	28 (17.6)	29 (25.9)	
Allele			
C (%)	192 (60.4)	111 (49.6)	.012*
A (%)	126 (39.6)	113 (50.4)	

Results

The median age was 55.5 ± 17.5 years for the control group and 59.93 ± 13.3 years in CRC patients. 22 patients (27.6%) were diagnosed with stage I ($n = 6$) and II ($n = 16$) disease, whereas 103 patients (82.4%) were diagnosed with stage III ($n = 64$) and IV ($n = 39$) disease. The FOXP3 genotype distribution was 25.9% for AA, 49.1% for AC, and 25% for the control group. The FOXP3 genotype distribution was 17.6% for AA, 44% for AC, and 38.4% for CC in CRC patients. The frequencies of CRC patients who have CC genotype ($p = .048$) and C allele ($p = .012$) were higher than the control group. Allele and genotype distributions for FOXP3 are shown in Table 3. When the cumulative gene expression levels were compared between tumor and non-tumor tissues, it was statistically determined that the expression of the FOXP3 gene was expressed 2.4 times in tumor tissue compared to normal tissue, and this difference was significant ($p = .048$). The cumulative gene expression chart is shown in Figure 1. FOXP3 gene expression level was found to be approximately 44.4 times higher in the advanced tumor stage (T3 + T4) compared to the early tumor stage (T1 + T2) ($p = .021$). Gene expression levels according to other histopathological parameters are shown in Table 1.

Tryptophan level in CRCs was determined as 14.32 ± 1.09 μmol/L, and in controls, it was determined as 26.82 ± 1.04 μmol/L. It was determined that the tryptophan level of the control group was 1.87 times higher than the CRCs ($p < .001$). While the level of kynurenine was determined as 1.33 ± 0.02 μmol/L in CRC, it was determined as 1.42 ± 0.02 in controls. In the control group, the level of kynurenine was found to be higher than CRC ($p = .003$). Kynurenic acid levels were found to be 0.01 ± 0.001 μmol/L, in CRCs and 0.008 ± 0.001 μmol/L in control ($p = .163$). Metabolite levels for our subjects are shown in Table 4. There was no statistically significant difference between the advanced tumor stage (T3 + T4) and early tumor stage (T1 + T2) in terms of serum tryptophan level ($p = .853$), kynurenine level ($p = .263$), and kynurenic acid level ($p = .674$).

Discussion

Cancer cells represent many characteristics because they escape from host immune responses, and one of these is the active suppression of the immune response. However, detailed mechanisms in colorectal cancer remain to be elucidated [21]. Interactions between cancer cells and the tumor microenvironment are thought to be important for tumor

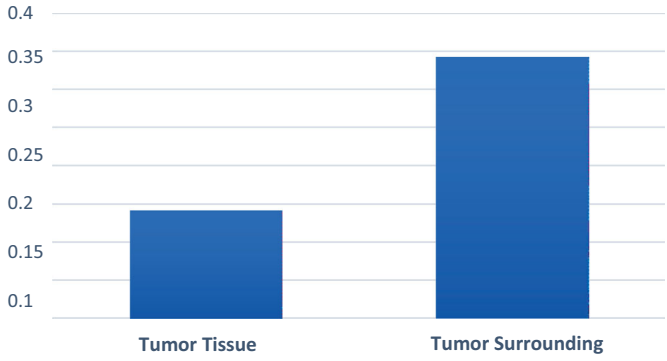

Figure 1. Foxp3 mRNA expression levels of CRC patients compared to control.

Table 4. TRP, KYN, and KYNA plasma levels in CRC patients and controls.

Parameters (μmol/L)	CRC patients Mean ± SD	Controls Mean ± SD	*p*-Value (*$p<.001$;**$p<.01$)
Plasma TRP	14.32 ± 1.09	26.82 ± 1.04	.000*
Plasma KYN	1.33 ± 0.02	1.42 ± 0.02	.003**
Plasma KYNA	0.01 ± 0.001	0.008 ± 0.001	.163

growth. It is known that these communications between tumor cells and infiltrating lymphocytes show a crucial network that affects disease progression and prognosis [22]. Tumor immune escape during carcinogenesis is a necessary process that disrupts cellular immune response. FOXP3, one of the regulators of immunosuppression in the tumor microenvironment, contributes to the active inhibition process by expressing in T cells. [23]. There are some important hypotheses that the effect of tryptophan metabolism in carcinogenesis is due to the suppression of the proliferation and activation of T cells due to the disturbances of tryptophan catabolism in the tumor microenvironment. Some of this catabolite suppress *in vitro* T cell proliferation with increased Foxp3 + Tregs cells, causing T cell apoptosis and affecting the function of natural killer cells [24,25]. Changes in the tryptophan kynurenine pathway are associated with inflammation and neoplasia in many cancers [20]. Therefore, the examination of FOXP3 and tryptophan metabolism in colorectal cancers is critical in terms of its role in pathogenesis, its importance as a therapeutic target, and its prognostic effect.

Immunohistochemical studies of FOXP3 high gene expression show that it is associated with poor prognosis in CRC [26]. In terms of different malignancies, FOXP3 gene expression was positively correlated with lymph node metastasis [27,28]. In cases with gastric and bladder tumors with a similar histological origin, FOXP3 gene expression is associated with poor survival and prognosis [29,30]. Our study found that FOXP3 gene expression level was higher in tumor tissue than in the healthy part of the resected tissue.

Numerous research has been conducted on the polymorphism of FOXP3. The frequency of the C allele for FOXP3 rs3761548 is observed between 4 and 57% in different populations. The C allele frequency was observed as 49.6% in our control subjects. At the same time, the C allele and CC genotype carriage of patients with CRC were statistically higher than the control group. However, it has been found that the AC genotype is high in colorectal cancers for the variant rs3761548 in patients with CRC in different populations and that the A allele carrier is an independent risk factor for disease susceptibility [31,32]. Our literature reviews for other malignancies determined the relationship between the AA genotype for prostate cancers and the A allele for thyroid cancers with disease pathogenesis [31,33]. Besides, data were determined that A allele carriage is characterized by aggressive tumor growth. Despite the data in the literature, the high frequency of C allele we observed in our study groups. This status can be explained by the fact that we carry allele frequencies different from other populations. In all cases with CRC, it may be thought that the C allele carrier is an independent risk factor specific to the Turkish population. The data we receive is compatible with the data in the literature.

Different FOXP3 SNPs have been reported, and their role in lung, breast, hepatocellular, and colorectal cancer susceptibility has been investigated. Some FOXP3 SNPs have been associated with an increased risk of lung, colon, and colorectal cancers. In a study of 312 Iranian participants, it was reported that the T allele at rs3761549 (T/C) correlates with susceptibility to lung cancer [34]. In another study, the rs3761548 (A/C) polymorphism was investigated in the Chinese population, and it was found that the frequency of A alleles in endometrial cancer women was significantly lower than in controls. [35]. In another study, on a sample taken from 1049 breast cancer patients, it was not found that there was a significant relationship between the allele C mutation in FOXP3 rs2280883 (C/T) and breast cancer risk. [36]. In a different study, susceptibility to colorectal cancer was determined in individuals with FOXP3 rs3761548 polymorphism in the Chinese population. [32]. Although there have been conflicting publications regarding the role of FOXP3 in cancer, a meta-analysis was published in 2014 to clarify this situation, and FOXP3 rs3761549 (T/C) and rs3761548 (A/C) polymorphisms are not associated with breast cancer risk but lung and hepatocellular cancer risk [37].

In the literature, tryptophan, kynurenine, and kynurenic acid levels were investigated in different pathological cases in terms of tryptophan catabolism, and data were found to be related to immune system pathologies and tryptophan catabolism in colorectal cancers [38–40]. Similarly, a relationship has been found in tryptophan catabolism with advanced tumor stage in other malignancies [41,42]. In our study, the tryptophan level was found lower in cases with CRC than in the control group, and the data are compatible with the literature. However, no statistically significant difference was found between the advanced tumor stage (T3 + T4) and early tumor stage (T1 + T2) in terms of serum tryptophan, kynurenine and kynurenic acid levels. Increased Tregs activity accelerates tumor growth, while Tregs cell decline triggers an effective antitumor immune response [11]. Increased IDO expression, which characterizes tryptophan consumption and an overproduction to kynurenine, reduces T effector cell function and is found in studies that activate FOXP3 + Tregs lymphocytes [14]. In our study, a high FOXP3 level in tumor tissue and low

tryptophan level in patient sera in the same sample support the related hypothesis in terms of CRC. Thus, we think that low levels of tryptophan and tryptophan metabolites contribute to immunomodulation in terms of T cell suppression in colorectal cancer. Similarly, we think that high FOXP3 expression shows an immunosuppressive effect in the advanced tumor stage. It may also affect immune suppression via tryptophan metabolism in the early stages of cancer pathogenesis and FOXP3 in the late stages.

Functional studies for FOXP3 and tryptophan catabolism will detail the effects of two candidate biomarkers in terms of CRC. Thus, two candidate biomarkers will contribute to clinicopathological processes such as treatment, diagnosis, and prognosis in candidates with CRC.

Ethical approval

This study has an ethical approval from 'Clinical Research Ethics Committee of İstanbul University School of Medicine' İstanbul, Turkey. (Ethical Code: 2018/479)

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This study was funded by Scientific Research Projects Coordination Unit of Istanbul University. Project number: TDK-2018-30121.

ORCID

Ebru Nur Ay http://orcid.org/0000-0001-7121-4362
Şeyda Demirkol http://orcid.org/0000-0003-1211-0736
Mehmet Tolgahan Hakan http://orcid.org/0000-0002-0622-7148
Cem Horozoğlu http://orcid.org/0000-0001-8998-2028
Soykan Arıkan http://orcid.org/0000-0001-7132-6161
Mehmet Baki Doğan http://orcid.org/0000-0003-0767-8327
Filiz Akyüz http://orcid.org/0000-0001-7498-141X
Ceylan Özsoy Hepokur http://orcid.org/0000-0001-6397-1291
İlhan Yaylım http://orcid.org/0000-0003-2615-0202

References

[1] Terry P, Giovannucci E, Michels KB, et al. Fruit, vegetables, dietary fiber, and risk of colorectal cancer. J Natl Cancer Inst. 2001;93(7):525–533.
[2] Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer. 2012;12(4):252–264.
[3] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646–674.
[4] Warburg O, Wind F, Negelein E. The metabolism of tumors in the body. J. Gen. Physiol. 1927;8(6):519–530.
[5] Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. Nat Rev Cancer. 2011;11(2):85–95.
[6] Löb S, Königsrainer A, Rammensee H-G, et al. Inhibitors of indoleamine-2,3- dioxygenase for cancer therapy: can we see the wood for the trees? Nat Rev Cancer. 2009;9(6):445–452.
[7] Platten M, Knebel Doeberitz N, Oezen I, et al. Cancer immunotherapy by targeting IDO1/TDO and their downstream effectors. Front Immunol. 2014;5:673.
[8] Peters JC. Tryptophan nutrition and metabolism: an overview. Adv Exp Med Biol. 1991;294:345–358.
[9] Austin CJD, Rendina LM. Targeting key dioxygenases in tryptophan-kynurenine metabolism for immunomodulation and cancer chemotherapy. Drug Discov Today. 2015;20(5):609–617.
[10] Santhanam S, Alvarado DM, Ciorba MA. Therapeutic targeting of inflammation and tryptophan metabolism in colon and gastrointestinal cancer. Transl. Res. 2016;167(1):67–79.
[11] Grimmig T, Kim M, Germer CT, et al. The role of FOXP3 in disease progression in colorectal cancer patients. Oncoimmunology. 2013;2(6):e24521–e24523.
[12] Uva V, Sfondrini L, Triulzi T, et al. FOXP3 expression in tumor cells and its role in cancer progression. Atlas Genet. Cytogenet. Oncol. Haematol. 2015;19:234–239.
[13] Löb S, Königsrainer A, Zieker D, et al. IDO1 and IDO2 are expressed in human tumors: levo- but not dextro-1-methyl tryptophan inhibits tryptophan catabolism. Cancer Immunol Immunother. 2009;58(1):153–157.
[14] Bellanti JAI. IV: Clinical applications in health and disease; 2019. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3651157.
[15] Nugent JL, McCoy AN, Addamo CJ, et al. Altered tissue metabolites correlate with microbial dysbiosis in colorectal adenomas. J Proteome Res. 2014;13(4):1921–1929.
[16] Chou C, Pinto AK, Curtis JD, et al. c-Myc-induced transcription factor AP4 is required for host protection mediated by CD8+ T cells. Nat Immunol. 2014;15(9):884–893.
[17] Llosa NJ, Cruise M, Tam A, et al. The vigorous immune microenvironment of microsatellite instable colon cancer is balanced by multiple counter-inhibitory checkpoints. Cancer Discov. 2015;5(1):43–51.
[18] Gentles AJ, Newman AM, Liu CL, et al. The prognostic landscape of genes and infiltrating immune cells across human cancers. Nat Med. 2015;21(8):938–945.
[19] Giannakis M, Mu XJ, Shukla SA, et al. Genomic correlates of immune-cell infiltrates in colorectal carcinoma. Cell Rep. 2016;15(4):857–865.
[20] Caro D, Marchesi G, Laghi F, et al. F. Immune cells: plastic players along colorectal cancer progression. J Cell Mol Med. 2013;17(9):1088–1095.
[21] De la Cruz-Merino L, Henao Carrasco F, Vicente Baz D, et al. Immune microenvironment in colorectal cancer: a new hallmark to change old paradigms. Clin Dev Immunol. 2011;2011:174149.
[22] Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Nat Med. 2013;19(11):1423–1437.
[23] Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. Nat Immunol. 2002;3(11):999–1005.
[24] Beaugerie L, Itzkowitz SH. Cancers complicating inflammatory bowel disease. N Engl J Med. 2015;372(15):1441–1452.
[25] Arthur JC, Gharaibeh RZ, Mühlbauer M, et al. Microbial genomic analysis reveals the essential role of inflammation in bacteria-induced colorectal cancer. Nat. Commun. 2014;5:4724.
[26] Kim M, Grimmig T, Grimm M, et al. Expression of Foxp3 in colorectal cancer but not in treg cells correlates with disease progression in patients with colorectal cancer. PLOS One. 2013;8(1):e53630.
[27] Dimitrakopoulos F, Papadaki H, Antonacopoulou AG, et al. Association of FOXP3 expression with non-small cell lung cancer. Anticancer Res. 2011;31(5):1677–1683.
[28] Fu H, Li C, Yang W, et al. FOXP3 and TLR4 protein expression are correlated in non-small cell lung cancer: implications for tumor progression and escape. Acta Histochem. 2013;115(2):151–157.
[29] Winerdal ME, Marits P, Winerdal M, et al. FOXP3 and survival in urinary bladder cancer. BJU Int. 2011;108(10):1672–1678.
[30] Yoshii M, Tanaka H, Ohira M, et al. Expression of forkhead box P3 in tumour cells causes immunoregulatory function of

signet ring cell carcinoma of the ll carcinoma of the stomach. Br J Cancer. 2012;106(10):1668–1674.
[31] Chatrabnous N, Ghaderi A, Ariafar A, et al. Serum concentration of interleukin-35 and its association with tumor stages and FOXP3 gene polymorphism in patients with prostate cancer. Cytokine. 2019;113:221–227.
[32] Chen L, Yu Q, Liu B, et al. Association of FoxP3 rs3761548 polymorphism with susceptibility to colorectal cancer in the Chinese population. Med. Oncol. 2014;31:1–4.
[33] Jiang W, Zheng L, Xu L, et al. Association between FOXP3, FOXE1 gene polymorphisms and risk of differentiated thyroid cancer in Chinese Han population. Mol Biol. 2015;4:3.
[34] Haghighi MF, Ghayumi MA, Behzadnia F, et al. Investigation of FOX. P3 Genetic variations at positions -2383 C/T and IVS9 + 459 T/C in Southern Iranian patients with lung carcinoma. Iran J Basic Med Sci. 2015;18(5):465–471.
[35] You D, Wang Y, Zhang Y, et al. Association of Foxp3 promoter polymorphisms with susceptibility to endometrial cancer in the Chinese Han women. Medicine. 2018;97(18):e0582.
[36] Zheng J, Deng J, Jiang L, et al. Heterozygous genetic variations of FOXP3 in Xp11.23 elevate breast cancer risk in Chinese population via skewed X-chromosome inactivation. Hum Mutat. 2013;34(4):619–628.
[37] Jiang LL, Ruan LW. Association between FOXP3 promoter polymorphisms and cancer risk: a meta-analysis. Oncol Lett. 2014;8(6):2795–2799.
[38] Engin AB, Karahalil B, Karakaya AE, et al. Helicobacter pylori and serum kynurenine- tryptophan ratio in patients with colorectal cancer. WJG. 2015;21(12):3636–3643.
[39] Liu X, Shin N, Koblish HK, et al. Selective inhibition of IDO1 effectively regulates mediators of antitumor immunity. Blood. 2010;115(17):3520–3530.
[40] Ferdinande L, Decaestecker C, Verset L, et al. Clinicopathological significance of indoleamine 2,3-dioxygenase 1 expression in colorectal cancer. Br J Cancer. 2012;106(1):141–147.
[41] De Jong RA, Nijman HW, Boezen HM, et al. Serum tryptophan and kynurenine concentrations as parameters for indoleamine 2,3-dioxygenase activity in patients with endometrial, ovarian, and vulvar cancer. Int. J. Gynecol. Cancer. 2011;21:1320–1327.
[42] Schroecksnadel K, Winkler C, Fuith LC, et al. Tryptophan degradation in patients with gynecological cancer correlates with immune activation. Cancer Lett. 2005;223(2):323–329.