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MASTER THESIS

EVALUATION OF CD70 EXPRESSION RELATED IMMUNE RESPONSE IN TUMOR MICROENVIRONMENT OF COLORECTAL CANCER

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THESIS APPROVAL

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DECLARATION

I declare that this study is an individual work in which there was no unethical behavior during all the stages from planning the thesis until its writing and that all the information in this thesis was obtained according to academic and ethical rules. I declare that I have referenced all the information and interpretations not obtained in this study and that these sources are listed in the list of sourced and that there is no violation of patent or copyrights during the study and the writing of the thesis.

Aylin Seher Uzunoğlu

DEDICATION

I dedicate this thesis

To my Mother, Ayşe Uzunoğlu
To my Father, İbrahim Kemal Uzunoğlu

and

To my Brother, Melih M. Uzunoğlu.

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LIST OF ABBREVIATIONS

CRC: Colorectal cancer

TME: Tumor microenvironment

TNFSF: Tumor necrosis factor super family

sCD27: Soluble CD27

WHO: World Health Organization

MIC: Minimally invasive carcinoma

ECM: Extracellular matrix

CAFs: Cancer-associated fibroblasts

TAMs: Tumor-associated macrophages

VEGF: Vascular endothelial growth factor

FGFs: Fibroblast growth factor

PDGF: Platelet-derived growth factor

TGF-β: Transforming growth factor-beta

SDF1: Stromal cell-derived factor 1

HIFs: Hypoxia-inducible factors

DCs: Dendritic cells

NK cells: Natural killer cells

TILs: T infiltrating lymphocytes

CTLs: Cytotoxic T cells

Tregs: Regulatory T lymphocytes

MDSC: Myeloid-derived suppressor cell population

CTLA-4: Cytotoxic T-lymphocyte antigen-4

PD-1: Programmed cell death protein 1

PD-L1: Programmed cell death protein ligand 1

RCC: Right-sided colon cancer

LCC: Left-sided colon cancer

FOBT: Fecal occult blood test

NSCLC: Non-Small Cell Lung Cancer

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

WB: Western Blot

MRI: Magnetic resonance imaging

ERUS: Endorectal ultrasound

ÖZET

Uzunoğlu AS. CD70/CD27 Molekülleri ile İlişkli Mekanizmaların Kolorektal Kanserli Hastalarda İncelenmesi. İstanbul Üniversitesi Sağlık Bilimleri Enstitüsü, Moleküler Tıp ABD. Yüksek Lisans. İstanbul. 2019

Kolorektal kanser (KRK), dünya genelinde en fazla ölüme sebep olan kanser türlerinden biridir. Immün kontrol noktası moleküllerinden biri olan CD70'in tümör mikroçevrede araştırılması kolorektal kanserin mikeoçevresinin daha iyi anlaşılmasını sağlayacaktır. CD70, B ve T hücrelerinde bulunan kostimülatör bir moleküldür. CD70'in aşırı ekspresyonu ve reseptörü CD27 ile etkileşimi, tümör hücrelerinin bağışıklık sisteminden kaçışını kolaylaştıran sinyaller oluşturabilmektedir. Bu çalışmada, kolorektal kanser hastalarının tümör mikroçevresinde (TMÇ) CD70, CD27, CD3 ve FOXP3 moleküllerinin ekspresyon profilinin araştırılması hedeflenmiştir. Ayrıca, çözünür CD27'nin kolorektal kanser hastaları için teşhis değeri araştırılmıştır. Ekspresyon profili Western Blot ıslak transfer sistemi ile analiz edildi. Toplam protein, 33 hastanın doku örneklerinden TRIzol ile izole edildi. Hastaların serumunda çözünür CD27 protein seviyesi ELISA ile tespit edildi. CD70'in ekspresyonu düşük sayıda kolorektal kanser doku örneklerinde (% 15,15) saptandı, ancak CD27'nin ekpresyonu yüksek sayıda örnekte (% 84,85) bulundu. Sonuçlar, TMÇ'de yüksek oranda CD3+ lenfosit (% 78,79) ve FOXP3+ Treg (% 48,49) olduğunu ortaya koydu. Ayrıca, sCD27 serum seviyesinin hastaların serumlarında anlamlı derecede yüksek ($p<0,0001$) olduğu ve yüksek serum sCD27 düzeyi ile CD70 pozitifliği arasında güçlü bir ilişki olduğu bulunmuştur. Her ne kadar, hastaların klinik sonuçları ile sCD27 serum düzeyi arasında bir ilişki bulunmasa da, kolorektal kanser hastalarında uzak organ metatsisi mevcutken sCD27 düzeyinin anlamlı derecede yüksek olduğu görüldü ($p=0,045$). Sonuç olarak, KRK, TMÇ'de CD70'i güçlü bir şekilde eksprese eden tümörlerden biri değildir, ancak sCD27, iyi bir serum biyobelirteci olarak düşünülebilir.

Anahtar Kelimeler: Kolorektal kanser, CD70, CD27, tümör mikroçevre, Western Blot

Bu çalışma, İstanbul Üniversitesi Bilimsel Araştırma Projeleri Birimi tarafından desteklenmiştir. Proje No: TYL-2018-30123

ABSTRACT

Uzunoğlu AS. Evaluation Of CD70 Expression Related Immune Response In Tumor Microenvironment Of Colorectal Cancer. Istanbul University, Institute of Health Sciences, Department of Molecular Medicine. Master. İstanbul. 2019

Colorectal Cancer is among the highest death-causing cancer types around the world. Immune checkpoint molecules, such as CD70, promise a better understanding of tumor microenvironment in colorectal cancer. CD70 is a costimulatory factor present on B and T-cells. Binding of CD27 to its ligand results in evasion of tumor cells from the immune system. We aim to explore the expression profile of CD70, CD27, CD3, and FOXP3 molecules in the tumor microenvironment (TME) of CRC patients and to detect the recruitment of tumor-infiltrating lymphocytes (TILs) present in TME. Also, we aim to investigate the predictive diagnosing value of soluble-CD27 for monitoring cancer. The expression profile was analyzed by western blot wet transfer system. Soluble CD27 protein level in patients' serum was detected by ELISA. We detected the low expression of CD70 (15.15%) in TME of CRC samples however, high abundance of CD27 (84.85%) was detected. Our results revealed that there is high recruitment of CD3+ lymphocytes (78.79%) and FOXP3+ Tregs (48.49%) in TME. Additionally, sCD27 serum level was found to be significantly high ($p<0.0001.$) in patients' sera and there is a strong correlation between high serum level of sCD27 and CD70 positivity and CD27 negativity in TME. Although no correlation was found between patients' clinical outcome and sCD27 serum level when distant organ metastasis present sCD27 level was significantly high ($p=0.045$). Consequently, CRC is not one of the tumors that are strongly express CD70 in TME however, sCD27 could be considered as a good serum biomarker.

Keywords: Colorectal cancer, CD70, CD27, tumor microenvironment, Western Blot

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1. INTRODUCTION AND AIM

Colorectal cancer (CRC) is one of the cancer types that has a very high burden. CRC incidence and mortality increase every year for both women and men. Genetic and environmental factors strongly influence CRC occurrence and prognosis. It is a slowly developed-type of cancer and it may initiate at a different location in colorectal tissue. Since there are many diagnostic biomarkers, it is important to find and characterize different markers for the diagnosis and prognosis of the disease. [1]–[4]

CD70 and CD27 are both tumor necrosis factor superfamily (TNFSF) molecules and have a costimulatory signaling pathway through their receptor and ligand interaction in the tumor microenvironment (TME) such as differentiation and proliferation of T cells. CD70 expression is restricted with certain immune cells and these are T cells, B cells, and dendritic cells (DCs). However, aberrant expression of CD70 on tumor cells has been found to be related to immune evasion in TME. On the other hand, CD27 expression is only found on T cells, B cells, and natural killer (NK) cells. Also, there is an important mechanism that affects T cell function related to CD27-CD70 interaction. CD27 is cleaved out after binding to its ligand and release into the serum. Several studies show that the level of sCD27 in sera is correlated with patient clinicopathological characteristics. Furthermore, all of those mechanisms cause specific T cell accumulation in TME. One of them is FOXP3+ T regulatory (Treg) cells. Those cells acquire immune suppression in TME and promote tumors. [5]–[10]

The aim was to investigate the potential diagnostic value of CD70/CD27 related mechanisms in the TME of CRC. Also, CD70 expression status was aimed to be revealed in CRC tissues together with the immune cell markers CD27, CD3, and FOXP3 for potential usage of them as a diagnostic biomarker and patient outcome. In addition, the soluble CD27 (sCD27) level in CRC patient sera has not been investigated yet. Thus, sCD27 was aimed to evaluate as a potential diagnostic biomarker in CRC.

2. GENERAL INFORMATION

2.1. Colorectal Cancer And Its Epidemiology

Cancer is a disease involving unregulated cell proliferation and proceeding by time throughout the body. Most of the deaths in the world are caused by cancer and there are more than 100 types of cancer in which almost all cancer types have unique features. [1]–[3]

Colorectal cancer (CRC) constitutes a vital proportion of cancer where genetic and environmental factors have a significant role in the etiology. CRS's development proceeds slowly as a growth called polyps on the inner lining of the colon or rectum. Depend on the location where cancer starts, the name can change as colon cancer or rectal cancer. Adenomatous polyps and hyperplastic polyps are two types of polyps seen in CRC. Hyperplastic polyps are not cancerous while adenomatous polyps may turn into cancer. In the malignant form, it can metastasis into other parts of the body. Environmental risk factors such as obesity, fat-rich, fiber-deficient diet, smoking, and heavy alcohol consumption promote carcinogenesis. [1]–[4], [11]

CRC represents a great part of all cancers world. According to 2018 global cancer statistics, 861.663 (551,269 colon and 310,394 rectum) people have died due to CRC last year. Estimate number for the incidence of CRC was over 1.8 million in 2018 in the world Occurrence of colorectal cancer changes depending on the region of the world. Therefore; it is considered as a marker of socioeconomic development. Statistics show that the incidence rates tend to rise uniformly in countries undergoing major development transition. Moreover, it is known that CRC affects both genders equally. Mortality depends on the stage of diagnosis. [1], [4], [11]–[13]

2.2. Diagnosis Of CRC

Early diagnosis of CRC increases the chance of survival. The screening and diagnostic procedures for CRC are very important. [14]

2.2.1. Fecal occult blood test (FOBT)

This method checks stool samples for the hidden existence of blood. If there is no blood detected in the stool sample then FOBT is negative. Also, there are some kits that are commercially available for people to perform this test at home. It must be

known that neither hospital-based FOBT nor home-based kits detect the location of the bleeding. Thus, additional tests need to be using in order to determine the exact location such as colonoscopy after fecal blood is detected with the FOBT. Colonoscopy is necessary after the test. The test has some limitations because cancer may not cause a loss of blood. No bleeding by polyps may result in wrong assessment. Some certain foods, dietary supplements, and medications such as aspirin and ibuprofen can affect the results of the test. As a result, false-positive and false-negative results can be seen. [14], [15]

2.2.2. Stool DNA test

This test is used for people with no symptoms. Cancer leaves genetic material into the environment as a form of DNA. Besides detecting the mutations in the DNA that shed in the stool of the patient, microscopic amounts of blood in the stool are detected with this test. If any abnormal DNA is detected by this test, further testing such as colonoscopy is necessary. Unusual DNA changes or blood absence means negative test result and otherwise, the test is positive. This test has the highest rates for detection of early-stage colon cancer. [11], [14]–[16]

2.2.3. Flexible sigmoidoscopy

It is a technique in which a device called sigmoidoscope is used to evaluate the rectum and lower colon (sigmoid colon). A small camera at the end of the sigmoidoscope allows visualizing the inside of the colon. The results of a flexible sigmoidoscopy examination are considered negative if there is nothing unusual in the colon and positive if there are polyps or unusual tissue in the colon. It should be noted that this technique has its limitations since it is not possible to screen the whole colon. Hence, cancer or polyps may be overlooked. Patients should not eat the day before the examination. People at age of 50 or older should start to take this test to screen CRC. Sigmoidoscopy exam is faster than colonoscopy which takes about 15 minutes. Also, fewer complications are seen compared to colonoscopy. [15]–[17]

2.2.4. Barium enema X-ray

This method, which is also called lower gastrointestinal (GI) tract radiography, allows examining the rectum, colon, and small part of the patient's small intestine in motion. In this test, barium sulfate ($BaSO_4$) suspension is inserted into the rectum and

visualized by x-ray. The entire examination takes 30 to 60 minutes. If there are no abnormalities found, it means a negative result, otherwise, it is considered a positive result. This test is not appropriate for people who had a recent colonic biopsy. [15], [17], [18]

2.2.5. Colonoscopy

This procedure is similar to sigmoidoscopy where a tube with a camera (colonoscope) is inserted into the rectum. Inside the colon is imaged by the camera. For an accurate result, the colon must be clean. The polyps that are found during the procedure are generally removed to further examine to see if they are benign or malignant. [14]–[17]

2.2.6. Computed tomography (CT) colonography

Special x-ray device is used to monitor the large intestine for polyps in their early stages. This method allows an assessment of the colonic lumen and for oncological staging. It is performed in two different positions; as respectively supine and prone. It usually takes 15 minutes and it has a lower risk of complication comparing the conventional colonoscopy. It provides better and more detailed images than all other methods. [14]–[18]

2.2.7. Imaging scans

Magnetic resonance imaging (MRI) and endorectal ultrasound (ERUS) are the imaging methods that can be used in CRC diagnosis. MRI is an expensive, limited, and less patient-friendly technique. ERUS measures the distance between anorectal junction and the distal part of the tumor, free of error, and it has an advantage for determining the size of the tumor. However, it is not accurate in determining metastasis so it is not a reliable test of CRC in clinical manner. [14]–[17]

2.3. Stages Of CRC

Determining the stage of the CRC is important for a patient for further steps of treatment strategies. Stages are all about the current situation of the tumor. Stages classify according to the TNM system, in which T means tumor size, N is for lymph node metastasis, and M is distant organ metastasis. [2], [19], [20]

Table 2-1. Stages of CRC [2], [19]–[21]

Stages	Features
Stage 0:	Carcinoma in situ is the other way of naming this stage. Tumor locates only the colorectal mucosa of the colon.
Stage I:	Tumor starts to grow in the inner layer of the organ. However, spreading has not occurred yet beyond the colon wall.
Stage II:	Spreading has started to occur inside the colon or rectum wall. Lymph node metastasis has not started yet at this stage.
Stage III:	Tumor has reached the lymph nodes. However, metastasis to distant organs has not seen yet.
Stage IV:	This is the stage where metastasis to other organs has seen.
Recurrent:	It is the place where cancer reoccurs after the last cancer treatment.

2.4. Tumor Microenvironment In CRC

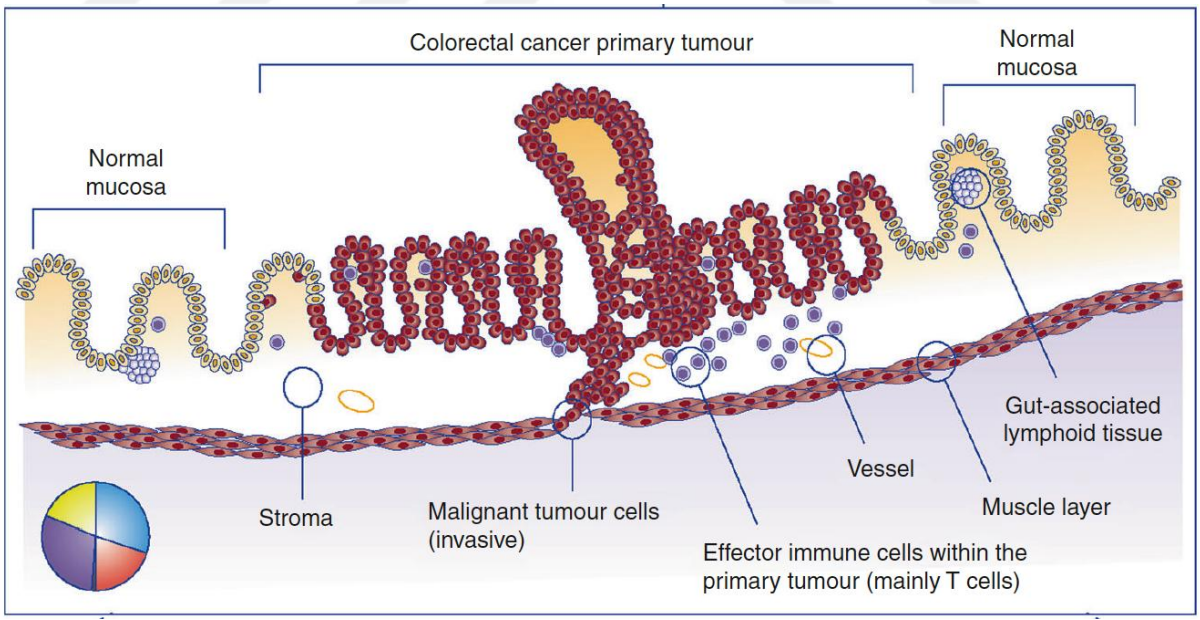
Many research has been shown that genetic changes are not the only reason for progression of tumors or recurrence. Tumor microenvironment (TME) has a vital role in tumor. It can either promote tumors or suppress tumors. TME consists of immune system cells such as dendritic cells (DCs), natural killer (NK) cells, tumor-associated macrophages (TAMs), monocytes, neutrophils, CD8 and CD4 T-cells, and many other cell types such as cancer-associated fibroblasts (CAFs), endothelial progenitor cells (EPCs), platelets, etc.. TME has a promotion effect on tumor in which tumor growth, immune cell suppression, promotion of metastasis and therapy resistance can be enhanced. The primary location of tumor decides the characterization of CRC. CRC where two-third of the transverse colon in which tumor ascends from colon and cecum is called right-sided colon cancer (RCC), whereas another part of the transverse colon which includes splenic flexure, descending colon, sigmoid colon, and rectum is called left-sided colon cancer (LCC). RCC and LCC both have very distinct molecular and clinic features. It is the fact that RCC and RCC have different microbiome and prognosis as well as different TME structure. [21], [22], [31], [23]–[30]

2.4.1. Immune cells

Antitumor immunity is employed by T cells constitutively. Thus, high effector T cell and regulatory T (Treg) cell amount have been found to be related to good prognosis in CRC. Even though Tregs are related to immunosuppression in TME for other types of cancer, recent studies have shown that it is the other way around for CRC. Also, for RCC, infiltration of CD8+ T cells and cytotoxic activity have found higher than LCC. Moreover, roles and effects of B cell in TME are still debated. [22], [26], [29]–[32]

2.4.2. Cancer-Associated Fibroblasts

CAFs are very important part of TME. CAF structure and related signaling pathways are found with the prognosis and aggressiveness of CRC. Several growth factors secreted or regulated by CAFs directly affect the growth of cancer and metastasis. These growth factors promote angiogenesis and as well as carcinogenesis. Distinct receptors and related pathways make CAFs potential target for tumor therapy. [25], [26], [29], [30], [33]



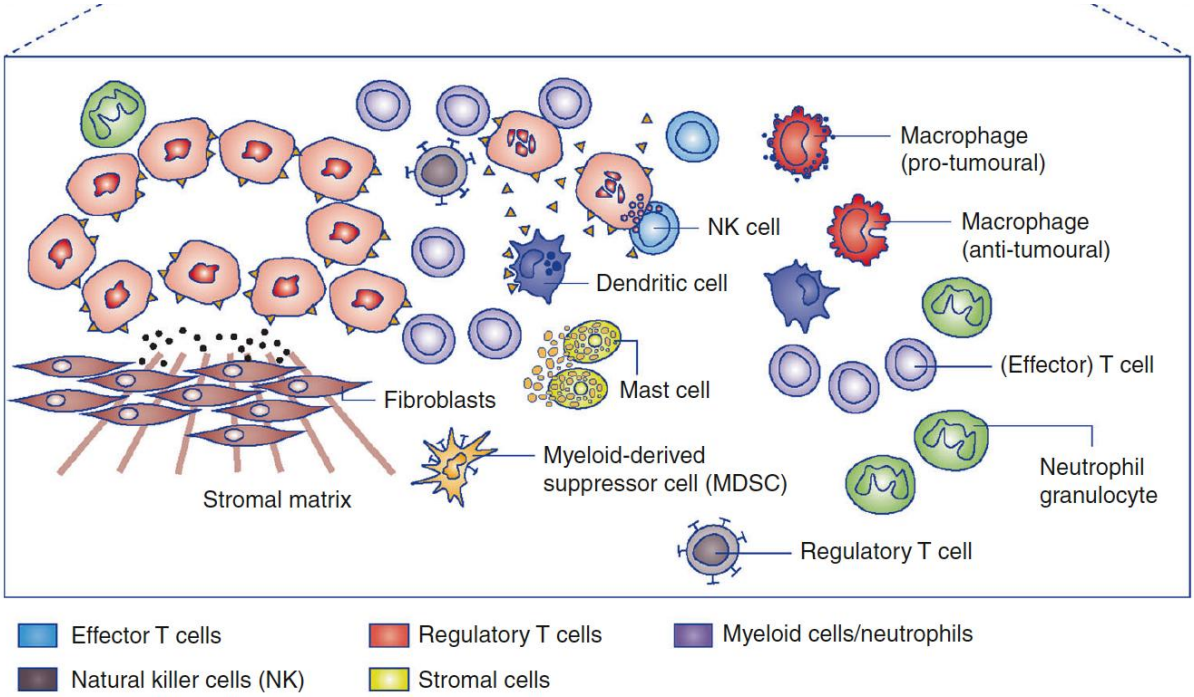


Figure 2-1. A representation of tumor microenvironment of primary CRC with immune cells and structural cells. [24]

2.4.3. CD70 related mechanisms in CRC

CD70, tumor necrosis factor superfamily (TNFSF) molecule, has costimulatory signaling together with its receptor CD27. CD70 expression is controlling very tightly and its transient expression is restricted with T cells and B cells, and DCs. However, CD70 has been found on several solid tumors and hematologic malignancies. Yet, renal cell carcinoma shows the highest expression among solid tumors. [6], [9], [10], [34]–[37]

CD70 is one of the tumor necrosis factor superfamily (TNFSF) molecule and ligand of another TNFSF costimulatory molecule of CD27. They are both employed costimulation of immune cells and their expression is tightly controlled to generate correct signaling at the right time and in the right place. Activated T cells and B cells, NK cells and DCs are the immune cells in which CD70 is expressed transiently, whereas CD70 expression has not been found in normal tissue cells. Different cancer types have been screening for CD70 expression and some solid tumors and hematologic malignancies have been found positive for CD70. Renal cell carcinoma has the highest rate for expression of CD70 among solid tumors followed by glioblastoma. [6], [8]–

[10], [34]–[38]

Receptor molecule CD27 is expressed on T cells, B cells and NK cell, which is responsible for the proliferation of T cells, differentiation of memory T cells, and proper activation of T cells, and also differentiation of plasma cells, maturation of B cells and production of immunoglobulins with CD70/CD27 signaling pathway. [6], [9], [10], [34]–[37]

3. MATERIALS AND METHODS

3.1. Data and Sample Collection

Primary tissue samples from 33 CRC patients, serum samples from 27 CRC patients and 32 healthy controls were collected from Istanbul Training and Research Hospital with the written consent of each patient or healthy subjects. Information about clinicopathological characteristics and demographic data of patients were also obtained to combine with the experimental data. All required procedures were followed and ethical committee approval from Istanbul Training and Research University Hospital was obtained.

3.2. Sample Preparation and Total Protein Extraction from Tissue Samples

3.2.1. Sample preparation with TRIzol® Reagent

The total protein of colorectal tissue samples was extracted by TRIzol® Reagent (Cat. no. 15596-026) at room temperature (RT). The reagent protocol was followed.

Frozen tissues which are normally stored at -80°C were melted on ice. After adding 1 mL of TRIzol® Reagent to 50-100 mg of the tissue sample, samples were then homogenized. Because colorectal tissues contain a high amount of fat, samples were centrifuged at 12,000 x g for 10 minutes at 4°C and the supernatant was collected to a new tube.

200 µl of chloroform was added to each tube and then tubes were shaken thoroughly for 15 seconds. After incubating for 3 minutes at RT, samples were centrifuged at 12,000 x g for 15 minutes at 4°C. This enables the mixture to separate into an aqueous phase on top and interphase & organic phase at the bottom. The upper aqueous phase was discarded and the lower phases were saved for protein extraction.

3.2.2. Protein Isolation

The collected interphase & organic phase was used to extract the total protein. 300 µl of 100% ethanol was added to each tube and samples were mixed by inverting the tubes. After 3 minutes of incubation at RT, the tubes were centrifuged at 2000 x g for 5 minutes at 4°C. The pellet contains the DNA so that the phenol-ethanol supernatant was collected carefully for protein extraction.

In order to precipitate the proteins, 1.5 mL of isopropanol was added to the supernatant. Samples were incubated at RT for 10 minutes and then centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was discarded and the pellet which contains the protein was saved.

The pellet containing the protein was washed with 2 mL of 0.3 M guanidine hydrochloride in 95% ethanol. Samples were incubated for 20 minutes at RT and centrifuged at 7,500 x *g* for 5 minutes at 4°C. After discarding the wash solution, the washing steps were repeated twice.

After three times of washing the pellet, 2 mL of 100% ethanol was added to the pellet and vortexed, and then incubated for 20 minutes at RT. The mixture was centrifuged at 7,500 x g for 5 minutes at 4°C and ethanol wash was discarded. the protein pellet was allowed to air dry for 5-10 minutes.

The isolated protein was resuspended in 200 µl of 1% SDS and mixed by pipetting. Then, samples were centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatant containing the protein was collected into a new tube and saved for further use at -20°C.

3.3. Determination of Concentrations

The concentration was determined by the SMART BCA Protein Assay Kit (Cat. No. 21071). Standards and samples were diluted as designated concentrations and pipetted 25 µl of every standard and samples into a flat-bottom 96-well plate. Two solutions that came with the kit were mixed in 50/1 ratio and after adding standards and samples, 200 µl of this solution was pipetted to all wells. The plate was then covered with a foil and incubated at 37°C for 30 minutes. Absorbance was measured at 562 nm. The concentration of all samples was calculated according to the plotted standard curve.

3.4. SDS-PAGE and Western Blot

Isolated total protein lysates were prepared for SDS-PAGE by diluting each sample with pure water. A total of 200 µg of protein was loaded to each well. After dilution, 4X Optiblot LDS Sample Buffer (4X, Abcam, ab119196) was added and then samples were incubated at 95°C for 10 minutes.

SDS-PAGE separation was performed in 10% PAGE gel. Separating and stacking gels we prepared manually as Table 3-1.

Table 3-1: Separating and Stacking gels.

10% Separating Gel	**10 ml**	**5 ml**
Distilled water	4 ml	2 ml
%30 Acrylamide	3.3 ml	1.65 ml
1,5 M Tris-HCl (pH 8.8)	2.5 ml	1.25 ml
%10 SDS	0.1 ml	50 µl
%10 APS	0.1 ml	50 µl
TEMED	4 µl	2 µl
5% Stacking Gel	**5 ml**	**2 ml**
Distilled water	3.4 ml	1360 µl
%30 Acrylamide	0.83 ml	332 µl
1 M Tris-HCl (pH 6.8)	0.63 ml	252 µl
%10 SDS	0.05 ml	20 µl
%10 APS	0.05 ml	20 µl
TEMED	5 µl	2 µl

Mini-Protean® Tetra System (Bio-Rad) and Mini Trans-Blot® Cell System (Bio-Rad) were used for running and transfer. 3.5 µl of protein standard (Bio-Rad, Precision Plus Protein™ Dual Color Standards, #1610374, 10–250 kD) was used. Gels were run at 150V for 70 minutes at room temperature and then blotted onto the Immun-Blot® PVDF Membrane Sandwiches (Bio-Rad, Cat. no. 162-0219) at 100V, 350 mA for 60 minutes at 4°C. Wet transfer system was chosen for blotting. Running and Transfer Buffers used in the experiment are explained in Table 3-2 and Table 3-3.

Table 3-2: Transfer buffer formulation.

Transfer Buffer	**1X**
Tris	3.03 g
Glycine	14.4 g
Methanol 100%	200 ml
ddH_2O	800 ml

Table 3-3: Running buffer formulation.

SDS Running Buffer	10X	1X (pH 8.3)
Tris Base	7.575 g	3 g
Glycine	36 g	14.4 g
SDS	2.5 g	1 g
dH_2O	Up to 250 ml	Up to 1000 ml

After the transfer, membranes were incubated in the blocking solution for 1 hour at room temperature. Then, membranes were incubated overnight in the primary antibody solution on a shaker. Solutions used for blocking and antibody incubations were prepared as Table 3-4. Primary and secondary antibodies and their dilutions are also listed in Table 3-5.

Table 3-4: Blocking solution formulation.

Blocking Solution (Skimmed milk)	%5
1X TBS-T Buffer	50 ml
Skimmed Milk Powder (Pınar #300249)	2.5 g

Table 3-5: Antibodies used for Western Blot.

Antibodies	Concentration
Rabbit polyclonal Anti-CD70 Antibody (Abcam, ab175389)	1:2000
Rabbit polyclonal Anti-beta-actin Antibody (Abcam, ab119716	1:5000
Rabbit polyclonal Anti-CD27 Antibody (Abcam, ab70103)	1µg/1ml
Rabbit polyclonal Anti-FOXP3 Antibody (Abcam, ab70103)	1:500
Rabbit polyclonal Anti-CD3 Antibody (Abcam, ab70103,	0.5 µg/1ml
Goat polyclonal Secondary Antibody to Rabbit IgG-F(HRP) (Abcam, ab97069)	1:5000

After overnight incubation with the primary antibody, membranes were washed for 4 times, 5 minutes for each washing on a shaker. Then, membranes were incubated in the secondary antibody solution for 1 hour at room temperature. Membranes were washed 5 times, 5 minutes for each washing and 10 minutes for last washing with 1X TBS-T solution.

Clarity™ Western ECL Substrate Kit (Bio-Rad, Cat. no. 1705060) was used for imaging the membranes. Chemiluminescent signals were captured using CCD camera-based imager.

3.5. ELISA with Patient Serum

Human sCD27 INSTANT ELISATM Kit (ThermoFisher Scientific) was used to measure serum levels of sCD27. Serum samples were diluted in a ratio of 1:25 before the experiment. Distilled water was added to each standard and samples in designated amounts. After that 50 µl of prediluted samples were added to sample wells. Then the plate was covered with a foil and incubated at room temperature for 3 hours on shaker. Wells were then washed for 6 times with wash buffer. TMB substrate solution was added to each well and the plate was incubated at room temperature for 10-15 minutes. Stop solution was then added to each well to stop the reaction. The absorbance was measured at 450 nm and 620 nm with a microplate reader.

An absorbance graph was plotted for standard OD values and concentration of each sample was calculated according to the graph.

3.6. Statistical Analysis

Chi-square, Student-t, Fisher's Exact, Man Whitney U, Kruskal Wallis tests were performed with SPSS Version 17.0. ROC Curve analysis and two-way ANOVA Test were performed by GraphPad Prism Version 8.2.1 For Windows.

4. RESULTS

4.1. Protein Expression by Western Blot

To detect protein expression profile of CD70, CD27, CD3, and FOXP3 molecules in the colorectal cancer tumor microenvironment, surgically removed the primary tumor and adjacent normal tissue samples from 33 patients were individually analyzed by Western blot. As indicated in Table 4-1, CD70 expression was observed in only 5/33 (15.15%) samples, on the contrary, CD27 expression was highly abundant (84.85%) among tumor samples of all patients. It is found that CD3 protein expression was detected in 78.79% of patients and absent in tumor tissues of only 7 patients while FOXP3 is expressed almost the half of the patients on their tumor tissues (48.48%).

Table 4-1: Number of positive or negative patients for CD70, CD27, CD3, FOXP3 protein expression by Western Blot for only tumor tissue samples (n=33).

Molecules	Positive n (%)	Negative n (%)
CD70	5 (15.15%)	28 (84.85%)
CD27	28 (84.85%)	5 (15.15%)
CD3	26 (78.79%)	7 (21.21%)
FOXP3	16 (48.48%)	17 (51.52%)

Primary tumor and normal tissue samples of 33 CRC patients were individually analyzed for 4 different molecules and Actin (42 kDa) was used as the loading control. Quantified band results were normalized according to the Actin and represented as relative densities in arbitrary units (a.u.). CD70 expression was detected at approximately 65 kDa which was also confirmed by the positive control cell line (P3HR1). The CD27 expression on blots was detected at the molecular weight of 60 kDa and protein bands were seen around 23 kDa for the T-cell marker, CD3. The predicted molecular weight of FOXP3 on WB is 47 kDa, and similarly, approximately 50 kDa bands were detected with the anti-FOXP3 antibody. Band intensities on blots were quantified using ImageJ 1.52a software.

The expression profile and quantitative data with significance were represented for all individual patients as tumor tissue and adjacent normal tissue in the figures below (Figure 4-1 to Figure 4-9). In order to see the differences in protein expression between tumor tissue and normal tissue taken from the same patient, the two-way ANOVA test was used (GraphPad Prism 8.2.1). The difference between tumor and normal tissue expression was found to be significant among all patients ($p<0.0001$).

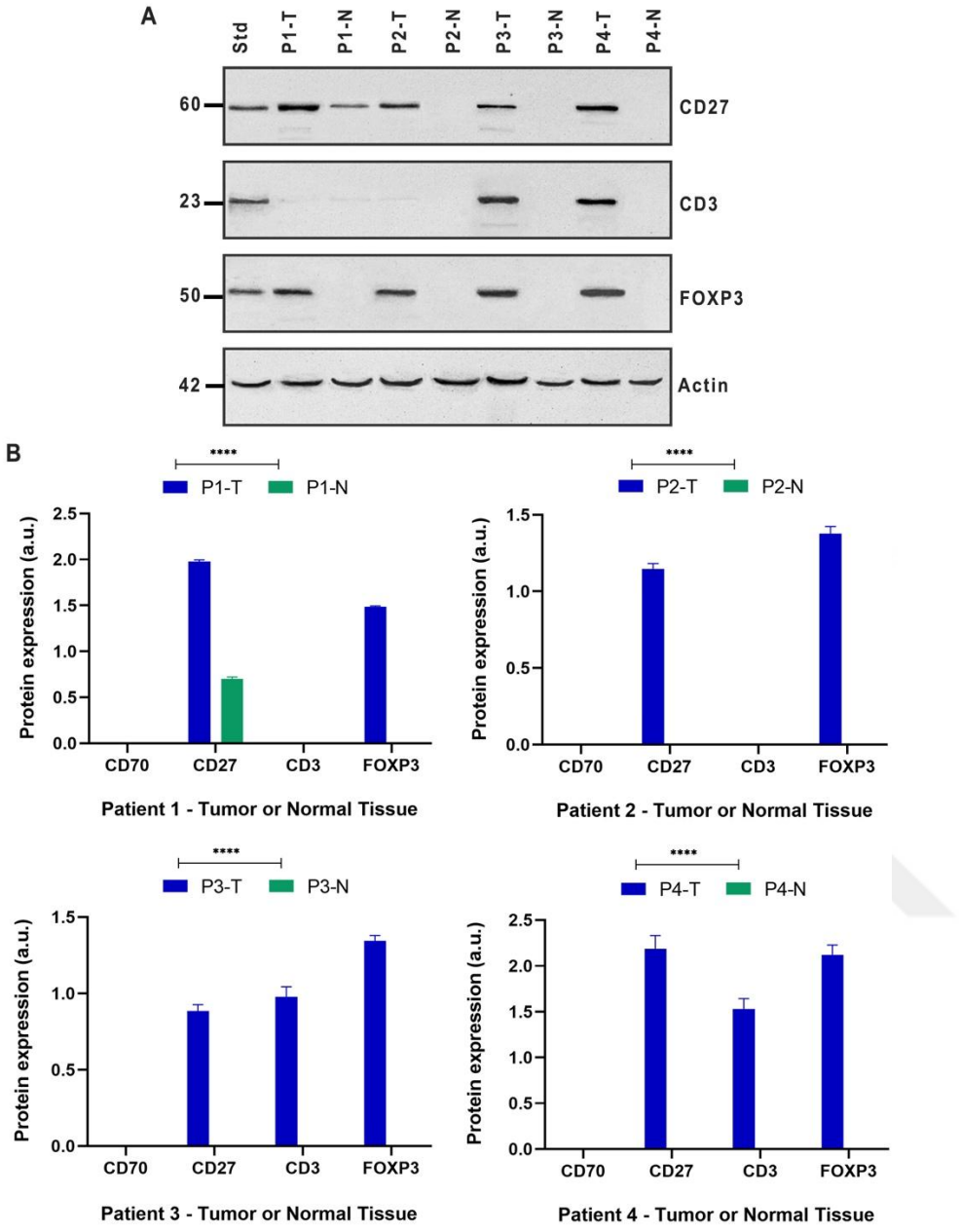


Figure 4-1: Expression profile of CD70, CD27, CD3, and FOXP3 molecules in primary tissue samples of 4 CRC patients (Patients 1-4). (A) Representative Western blot analysis showing CD27, CD3, and FOXP3 expression status among CRC primary tumors and normal adjacent tissues of Patient 1 (P1-T, P1-N), Patient 2 (P2-T, P2-N), Patient 3 (P3-T, P3-N), and Patient 4 (P4-T, P4-N). Std represents the standard sample required for further WB analysis. Actin was used as the loading control. **(B)** Graphs show quantified protein expression of Western blot bands which were quantified by ImageJ and data were analyzed using GraphPad Prism 8.2.0. Protein expression of tumor tissues was significantly higher than the normal tissues for all patients ($p<0.0001$). Error bars, SD (n=3). Protein expression level is represented in arbitrary units (a.u.).

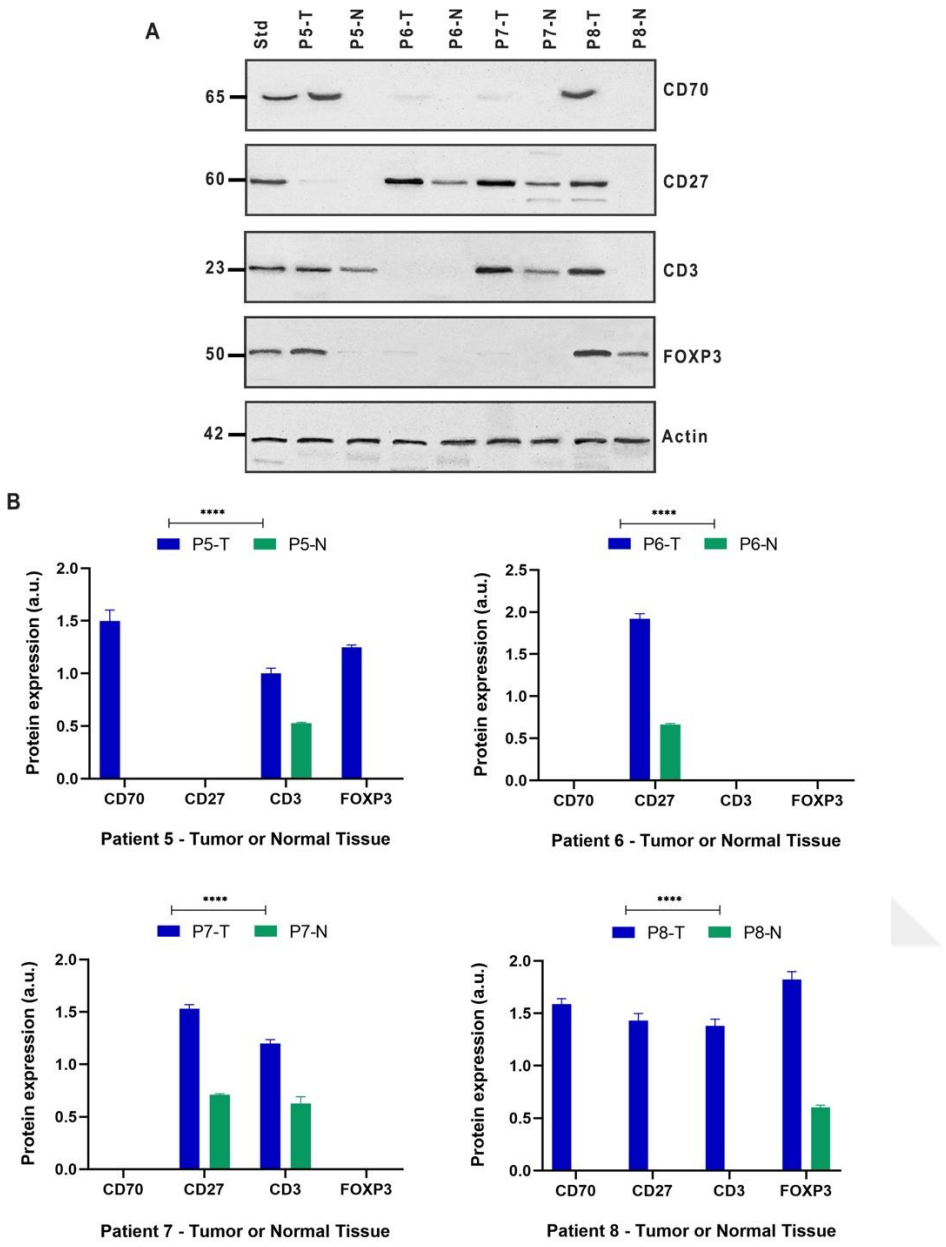


Figure 4-2: Expression profile of CD70, CD27, CD3, and FOXP3 molecules in primary tissue samples of the next 4 CRC patients (Patients 5-8). (A) Representative Western blot analysis showing CD70, CD27, CD3, and FOXP3 expression status among CRC primary tumors and normal adjacent tissues of Patient 5 (P5-T, P5-N), Patient 6 (P6-T, P6-N), Patient 7 (P7-T, P7-N), and Patient 8 (P8-T, P8-N). Std represents the standard sample required for further WB analysis. Actin was used as the loading control. **(B)** Graphs show quantified protein expression of Western blot bands which were quantified by ImageJ and data were analyzed using GraphPad Prism 8.2.0. Protein expression of tumor tissues was significantly higher than the normal tissues for all patients ($p < 0.0001$). Error bars, SD (n=3). Protein expression level is represented in arbitrary units (a.u.).

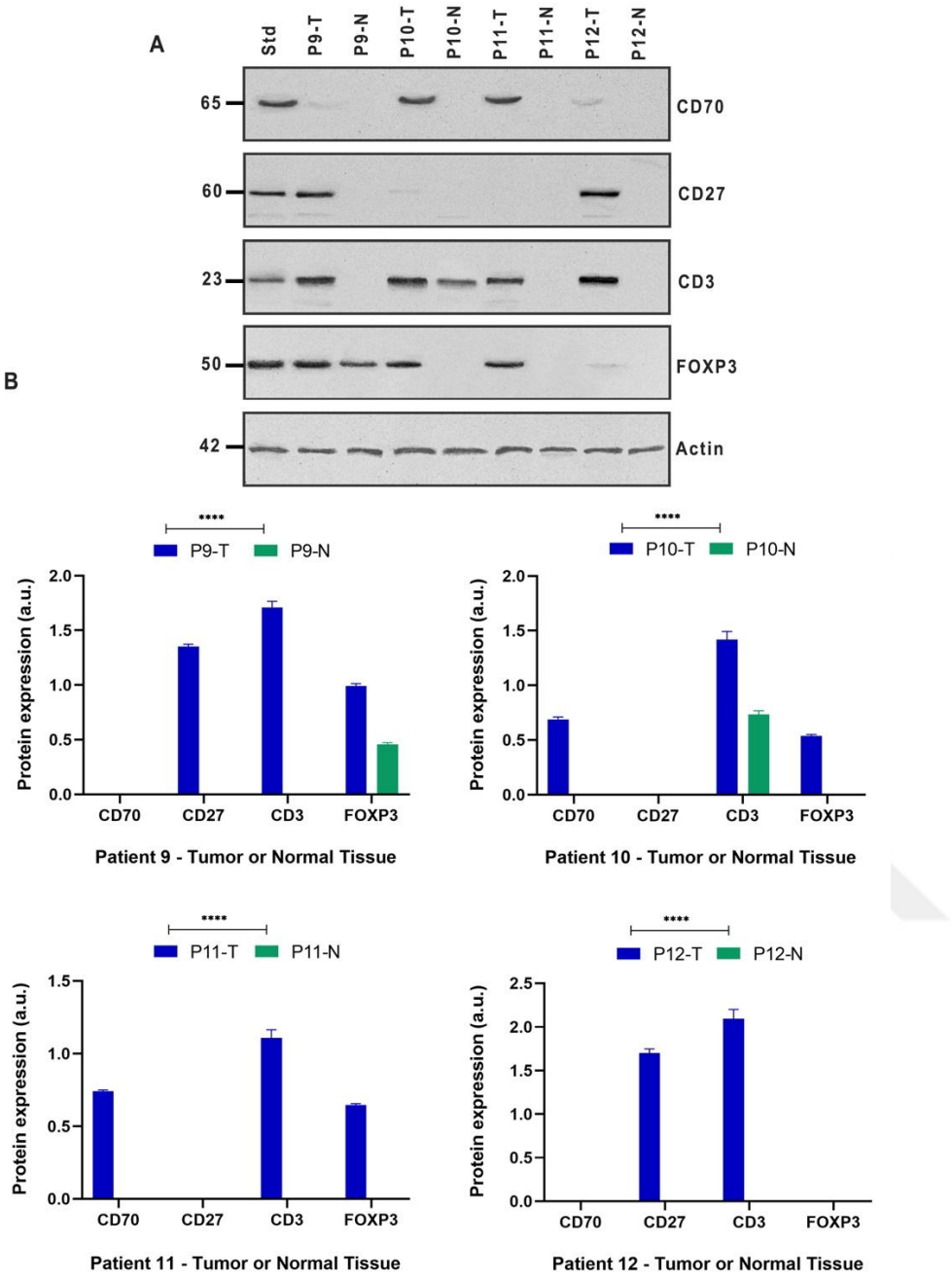


Figure 4-3: Expression profile of CD70, CD27, CD3, and FOXP3 molecules in primary tissue samples of the next 4 CRC patients (Patients 9-12). (A) Representative Western blot analysis showing CD70, CD27, CD3, and FOXP3 expression status among CRC primary tumors and normal adjacent tissues of Patient 9 (P9-T, P9-N), Patient 10 (P10-T, P10-N), Patient 11 (P11-T, P11-N), and Patient 12 (P12-T, P12-N). Std represents the standard sample required for further WB analysis. Actin was used as the loading control. **(B)** Graphs show quantified protein expression of Western blot bands which were quantified by ImageJ and data were analyzed using GraphPad Prism 8.2.0. Protein expression of tumor tissues was significantly higher than the normal tissues for all patients ($p < 0.0001$). Error bars, SD (n=3). Protein expression level is represented in arbitrary units (a.u.).

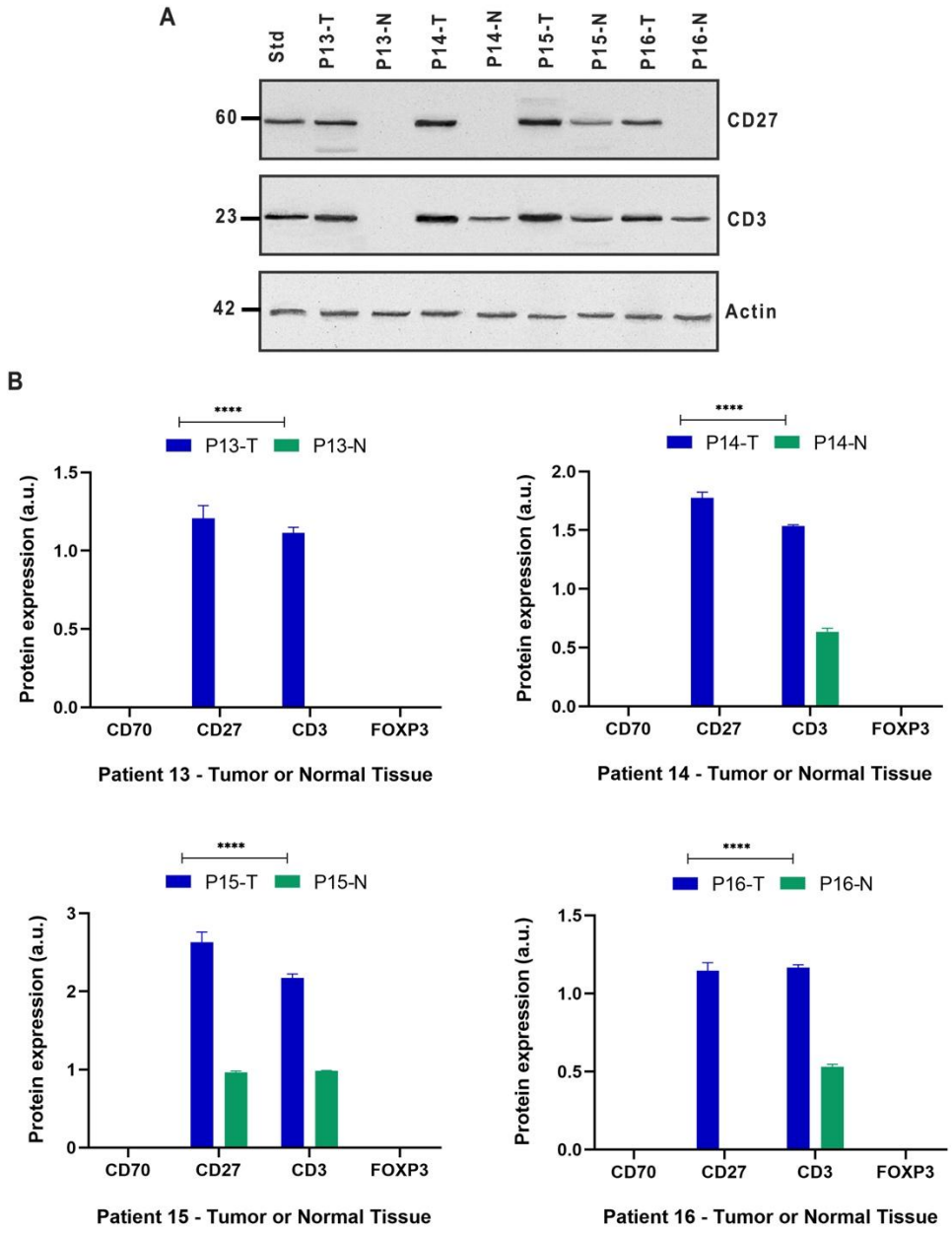


Figure 4-4: Expression profile of CD70, CD27, CD3, and FOXP3 molecules in primary tissue samples of the next 4 CRC patients (Patients 13-16). (A) Representative Western blot analysis showing CD27 and CD3 expression status among CRC primary tumors and normal adjacent tissues of Patient 13 (P13-T, P13-N), Patient 14 (P14-T, P14-N), Patient 15 (P15-T, P15-N), and Patient 16 (P16-T, P16-N). Std represents the standard sample required for further WB analysis. Actin was used as the loading control. **(B)** Graphs show quantified protein expression of Western blot bands which were quantified by ImageJ and data were analyzed using GraphPad Prism 8.2.0. Protein expression of tumor tissues was significantly higher than the normal tissues for all patients ($p < 0.0001$). Error bars, SD (n=3). Protein expression level is represented in arbitrary units (a.u.).

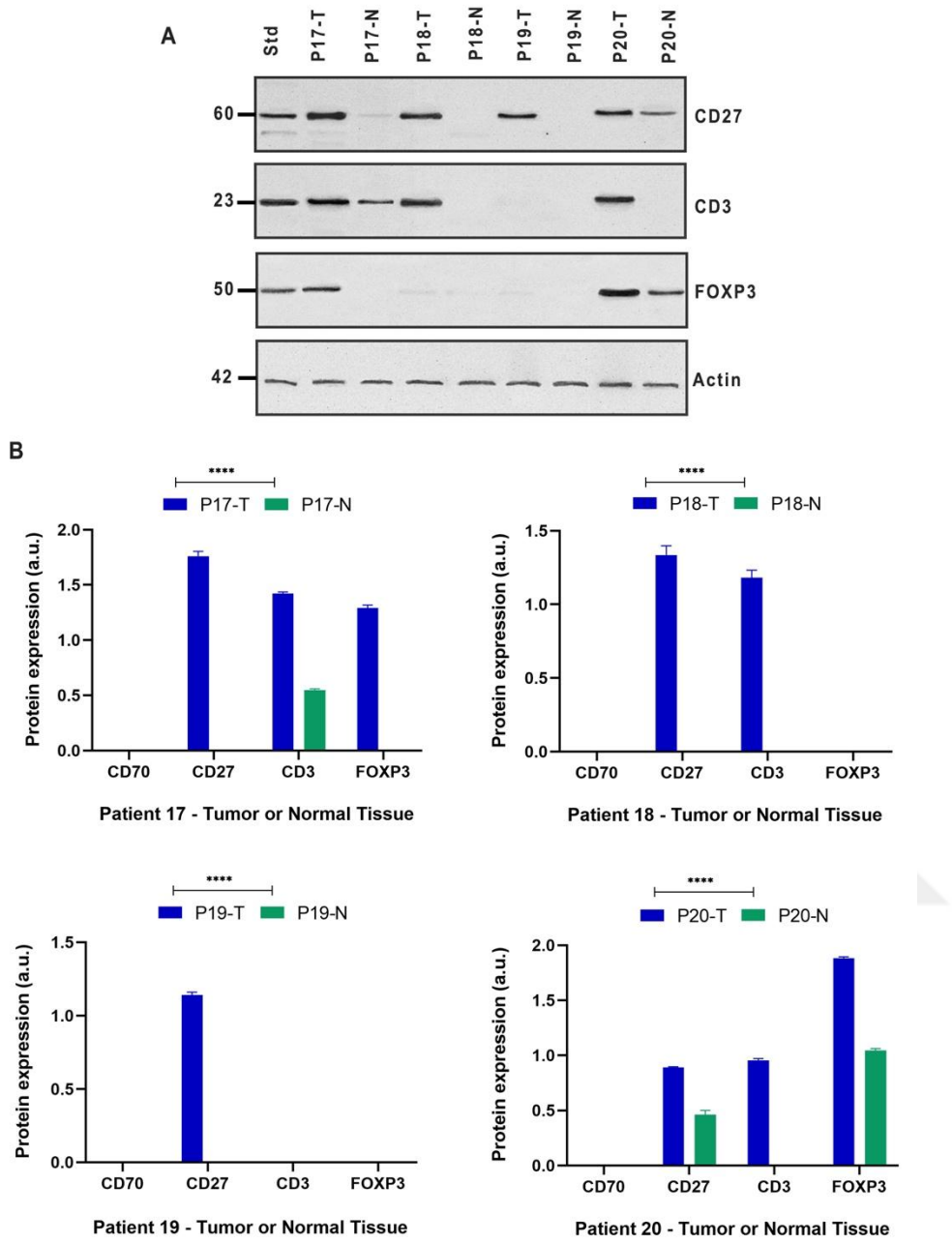


Figure 4-5: Expression profile of CD70, CD27, CD3, and FOXP3 molecules in primary tissue samples of the next 4 CRC patients (Patients 17-20). (A) Representative Western blot analysis showing CD27, CD3, and FOXP3 expression status among CRC primary tumors and normal adjacent tissues of Patient 17 (P17-T, P17-N), Patient 18 (P18-T, P18-N), Patient 19 (P19-T, P19-N), and Patient 20 (P20-T, P20-N). Std represents the standard sample required for further WB analysis. Actin was used as the loading control. **(B)** Graphs show quantified protein expression of Western blot bands which were quantified by ImageJ and data were analyzed using GraphPad Prism 8.2.0. Protein expression of tumor tissues was significantly higher than the normal tissues for all patients ($p < 0.0001$). Error bars, SD (n=3). Protein expression level is represented in arbitrary units (a.u.).

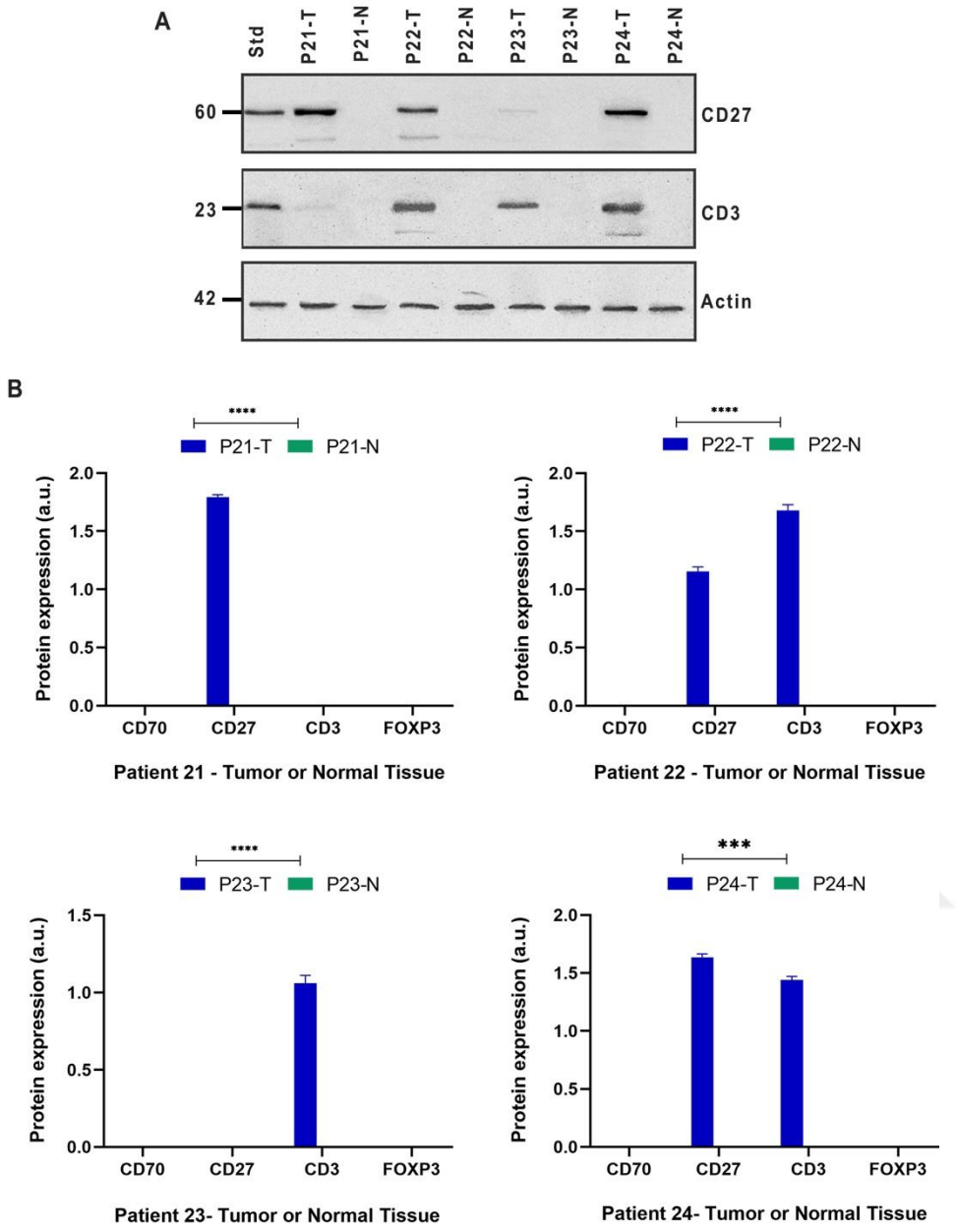


Figure 4-6: Expression profile of CD70, CD27, CD3, and FOXP3 molecules in primary tissue samples of the next 4 CRC patients (Patients 21-24). (A) Representative Western blot analysis showing CD27 and CD3 expression status among CRC primary tumors and normal adjacent tissues of Patient 21 (P21-T, P21-N), Patient 22 (P22-T, P22-N), Patient 23 (P23-T, P23-N), and Patient 24 (P24-T, P24-N). Std represents the standard sample required for further WB analysis. Actin was used as the loading control. **(B)** Graphs show quantified protein expression of Western blot bands which were quantified by ImageJ and data were analyzed using GraphPad Prism 8.2.0. Protein expression of tumor tissues was significantly higher than the normal tissues for all patients ($p < 0.0001$). Error bars, SD (n=3). Protein expression level is represented in arbitrary units (a.u.).

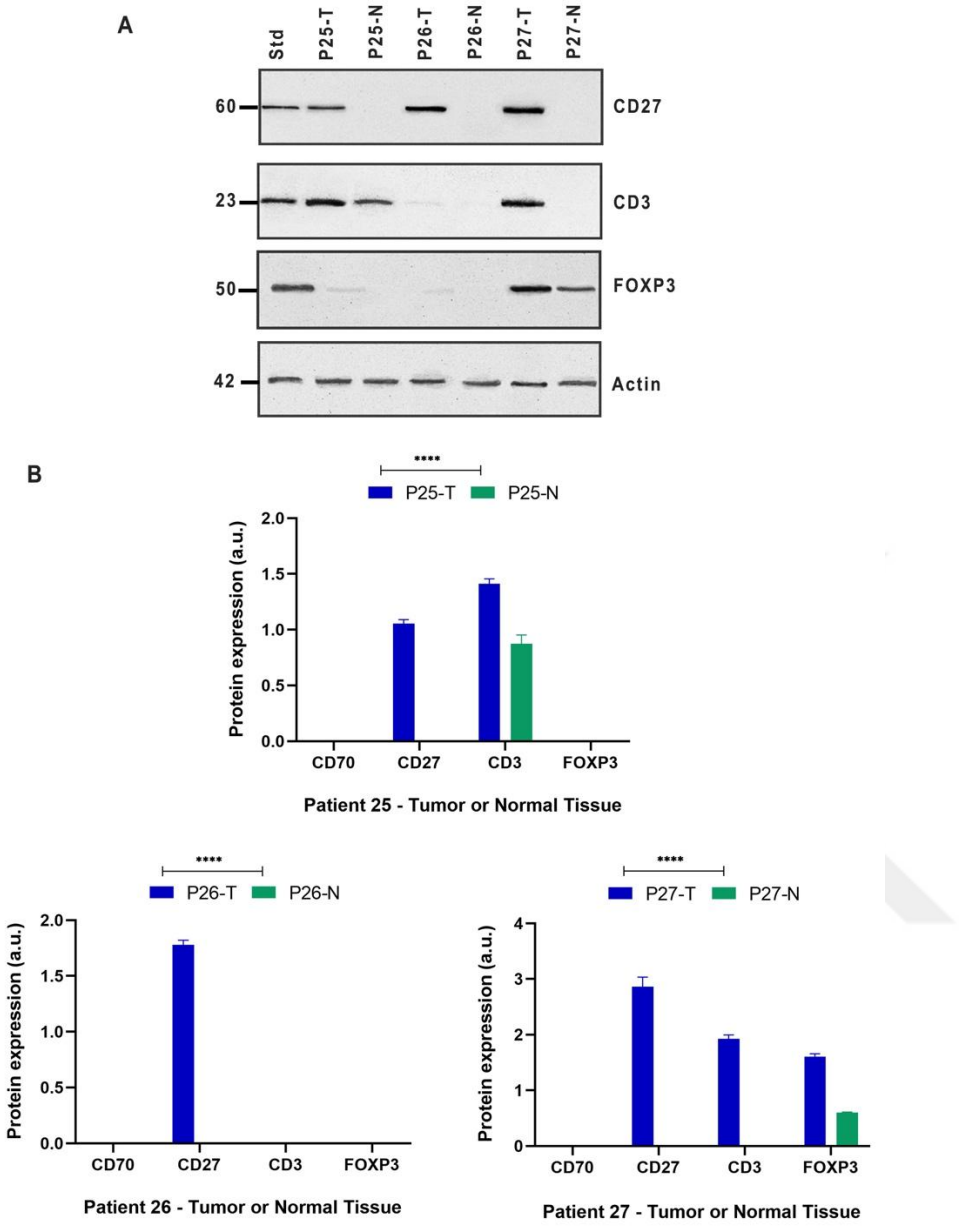


Figure 4-7: Expression profile of CD70, CD27, CD3, and FOXP3 molecules in primary tissue samples of the next 3 CRC patients (Patients 25-27). (A) Representative Western blot analysis showing CD27, CD3, and FOXP3 expression status among CRC primary tumors and normal adjacent tissues of Patient 25 (P25-T, P25-N), Patient 26 (P26-T, P26-N), and Patient 27 (P27-T, P27-N). Std represents the standard sample required for further WB analysis. Actin was used as the loading control. **(B)** Graphs show quantified protein expression of Western blot bands which were quantified by ImageJ and data were analyzed using GraphPad Prism 8.2.0. Protein expression of tumor tissues was significantly higher than the normal tissues for all patients ($p < 0.0001$). Error bars, SD (n=3). Protein expression level is represented in arbitrary units (a.u.).

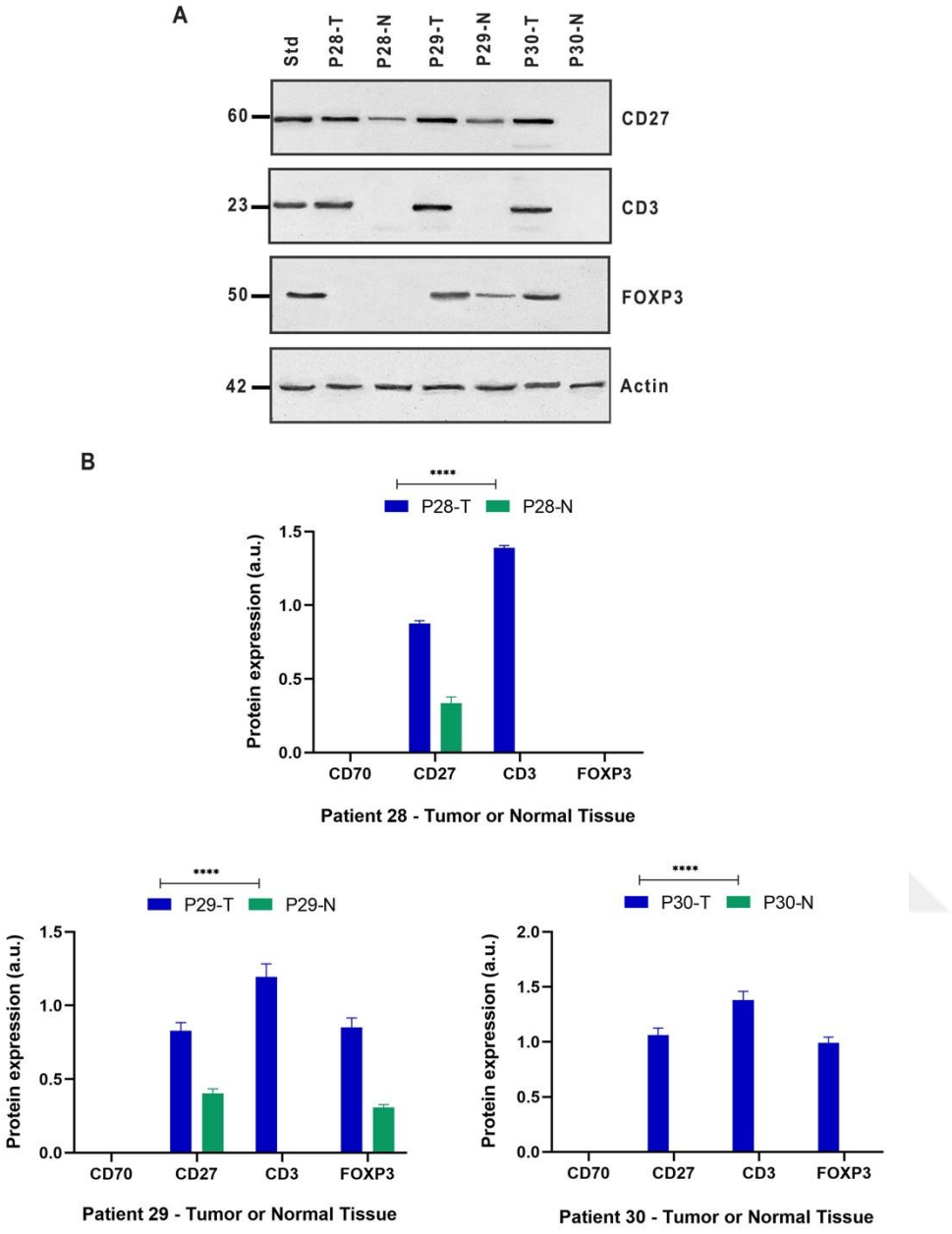


Figure 4-8: Expression profile of CD70, CD27, CD3, and FOXP3 molecules in primary tissue samples of the next 3 CRC patients (Patients 28-30). (A) Representative Western blot analysis showing CD27, CD3, and FOXP3 expression status among CRC primary tumors and normal adjacent tissues of Patient 28 (P28-T, P28-N), Patient 29 (P29-T, P29-N), and Patient 30 (P30-T, P30-N). Std represents the standard sample required for further WB analysis. Actin was used as the loading control. **(B)** Graphs show quantified protein expression of Western blot bands which were quantified by ImageJ and data were analyzed using GraphPad Prism 8.2.0. Protein expression of tumor tissues was significantly higher than the normal tissues for all patients ($p < 0.0001$). Error bars, SD (n=3). Protein expression level is represented in arbitrary units (a.u.).

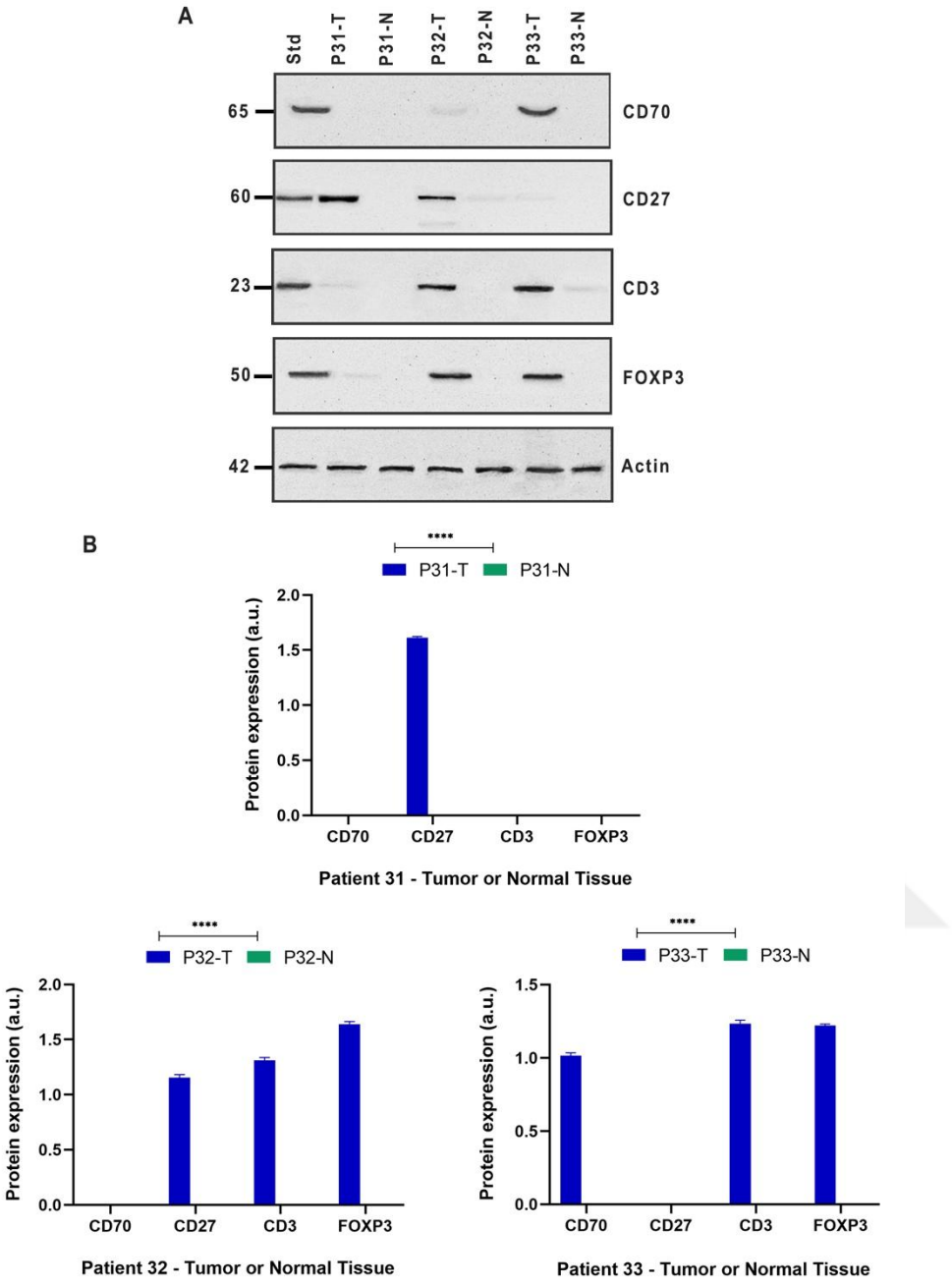


Figure 4-9: Expression profile of CD70, CD27, CD3, and FOXP3 molecules in primary tissue samples of the next 3 CRC patients (Patients 31-33). (A) Representative Western blot analysis showing CD70, CD27, CD3, and FOXP3 expression status among CRC primary tumors and normal adjacent tissues of Patient 31 (P31-T, P31-N), Patient 32 (P32-T, P32-N), and Patient 33 (P33-T, P33-N). Std represents the standard sample required for further WB analysis. Actin was used as the loading control. **(B)** Graphs show quantified protein expression of Western blot bands which were quantified by ImageJ and data were analyzed using GraphPad Prism 8.2.0. Protein expression of tumor tissues was significantly higher than the normal tissues for all patients ($p < 0.0001$). Error bars, SD (n=3). Protein expression level is represented in arbitrary units (a.u.).

4.2. Demographic Distribution And Clinicopathological Characteristics Of The Study Group

A total of 39 patients and 32 healthy subjects were included in the study group. Although tumor and normal tissues of 33 patients were analyzed for CD70, CD27, CD3, and FOXP3 expression by Western Blot, only 21 patients were in common and investigated for both serum sCD27 levels and expression profile. A total of 27 patients were screened for sCD27 levels in sera by ELISA. In Table 4-2, age and gender distribution are shown for the study group.

Table 4-2: Age and gender parameters for the study group.

Parameters	Colorectal Cancer Patients n=39 (%)	Healthy Controls n=32 (%)	p
Age (years) Mean ± SD	62.83 ± 12.49	60.65 ± 16.84	0.675
Gender			
Female	10 (25.6%)	7 (21.9%)	0.711
Male	29 (74.4%)	25 (78.1%)	

Colorectal cancer patients in the study group were classified according to their clinicopathological characteristics. As seen in Table 4-3, tumor location and staging, lymph node and distant organ metastasis, perineural invasion, differentiation, and mucinous component presence were indicated.

Table 4-3: Clinicopathological characteristics of colorectal cancer patients in the study group.

Parameters	Colorectal Cancer Patients n (%)
Tumor Location	
Right-sided colon	7 (20.0%)
Left-sided colon	2 (5.7%)
Rectum	9 (25.7%)
Sigmoid	13 (37.1%)
Rectosigmoid	4 (11.4%)

Tumor Stage	
T1	2 (6.1%)
T2	3 (9.1%)
T3	15 (45.5%)
T4	13 (39.4%)
Lymph node metastasis	
N0	19 (57.6%)
N1	7 (21.2%)
N2	6 (18.2%)
N3	1 (3.0%)
Distant organ metastasis	
Yes	6 (18.2%)
No	27 (81.8%)
Perineural invasion	
Yes	11 (32.4%)
No	23 (67.6%)
Differentiation	
Weak	15 (48.4%)
Moderate	9 (29.0%)
Strong	7 (22.6%)
Mucinous component	
Yes	13 (40.6%)
No	19 (59.4%)

Association between patients' clinicopathological characteristics and expressions of CD70, CD27, CD3, and FOXP3 molecules in the tumor microenvironment of CRC patients was evaluated in Table 4-4.

Table 4-4: Relations between clinicopathological characteristics of patients and protein expression status of CD70, CD27, CD3, and FOXP3 in the TME of CRC patients.

Parameters	CD70 n (%)		CD27 n (%)		CD3 n (%)		FOXP3 n (%)	
	positive	negative	positive	negative	positive	negative	positive	negative
Tumor Stage								
T1 +T2	—	4 (100%)	4 (100%)	—	4 (100%)	—	2 (50.0%)	2 (50.0%)
T3 +T4	2 (12.5%)	14 (87.5%)	13 (81.2%)	3 (18.8%)	11 (68.8%)	5 (31.2%)	—	—
Lymph node metastasis								
N0	1 (6.32%)	15 (93.8%)	14 (87.5%)	2 (12.5%)	12 (75%)	4 (25%)	7 (43.8%)	9 (56.2%)
N1, N2, N3	2 (16.7%)	10 (83.3%)	10 (83.3%)	2 (16.7%)	10 (83.3%)	2 (16.7%))	7 (58.3%)	5 (41.7%)
Distant organ metastatis								
Yes	1 (25%)	3 (75%)	3 (75%)	1 (25%)	4 (100%)	—	2 (50%)	2 (50%)
No	2 (8.7%)	21 (91.2%)	20 (87%)	3 (13%)	17 (73.9%)	6 (26.1%)	12 (52.2%)	11 (47.8%)
Perineural invasion								
Yes	—	9 (100%)	9 (100%)	—	7 (77.8%)	2 (22.2%)	4 (44.4%)	5 (55.6%)
No	3 (15.8%)	16 (94.2%)	15 (78.9%)	4 (21.1%)	15 (78.9%)	4 (21.1%)	10 (52.6%)	9 (47.4%)
Differentiation								
Weak + Moderate	2 (10.5%)	17 (89.5%)	16 (84.2%)	3 (15.8%)	14 (73.7%)	5 (26.3%)	10 (52.6%)	9 (47.4%)
Strong	—	6 (100%)	6 (100%)	—	5 (83.3%)	1 (16.7%)	1 (16.7%)	5 (83.3%)
Mucinous component								
Yes	1 (8.3%)	11 (91.7%)	11 (91.7%)	13 (81.2%)	9 (75%)	3 (25%)	6 (50%)	6 (50%)
No	2 (12.5%)	14 (87.5%)	1 (8.3%)	3 (18.8%)	13 (81.2%)	3 (18.8%)	8 (50%)	8 (50%)

4.3. Detection Of sCD27 Level On Patients Sera Of CRC Patients

Serum levels of sCD27 were detected for 27 CRC patients and 32 healthy controls. As seen in Table 4-5, the mean level (mean ± SD) of sCD27 is 101.8 ±28.02 U/ml with a range of 19.90-139.43 U/ml.

Table 4-5: sCD27 serum levels of colorectal cancer patients and healthy controls. SPSS Version 17.0 software was used to analyze the data (p=0.001).

Parameters	Colorectal Cancer Patients n=27 (mean ± SD)	Healthy Controls n=32 (mean ± SD)	p
sCD27 U/ml	101.8 ± 28.02	82.06 ± 21.47	0.001

The cut-off value was determined as 89.15 U/ml by ROC analysis for sCD27. In addition to this, sensitivity and specificity were detected as 0.74 and 0.72 respectively (Figure 4-10).

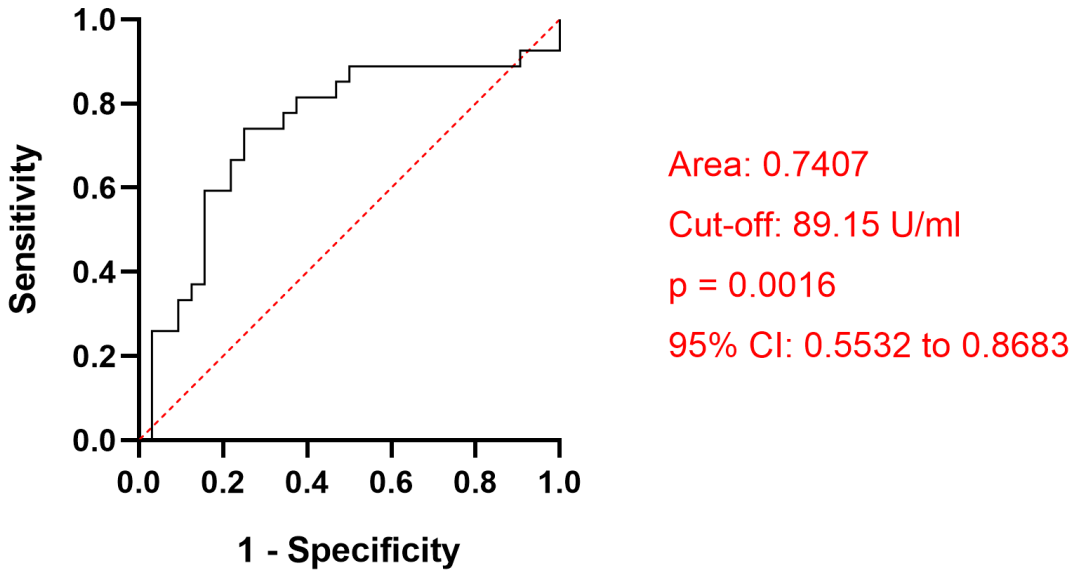


Figure 4-10: ROC Curve analysis of the sCD27 level between CRC patients and healthy controls. Above the value of 89.15 U/ml for sCD27, the patient group has a sensitivity of 74%

and specificity of 72% (p=0.0016). ROC Curve analysis was performed by GraphPad Prism 8.2.1.

No relation was found between high serum level of sCD27 and tumor stage, lymph node metastasis, differentiation status, perineural invasion, or mucinous component. Distant organ metastasis presence is significantly higher with the higher sCD27 level (Table 4-6).

Table 4-6: Relations between clinicopathological characteristics of patients and serum sCD27 levels. The correlation was calculated in the SPSS Version 17.0 software.

Parameters	sCD27 level (U/ml) (mean ± SD)	p
Tumor Stage		
T1 +T2	103.12	0.855
T3 +T4	97.63 ± 29.08	
Lymph node metastasis		
N0	99.92 ± 38.09	
N1, N2, N3	96.28 ± 19.69	0.768
Distant organ metastasis		
Yes	121.88 ± 15.59	**0.045**
No	93.83 ± 27.78	
Differentiation		
Weak + Moderate	99.69 ± 27.06	0.545
Strong	89.94 ± 36.18	
Perineural invasion		
Yes	85.70 ± 40.13	0.139
No	105.07 ± 17. 15	
Mucinous component		
Yes	94.50 ± 37.23	0.897
No	96.19 ± 22.92	

A strong correlation was found between high serum level of sCD27 and CD70 expression positivity in the tumor microenvironment of CRC patients ($p<0.0001$) as seen in Table 4-7 below. Exact the opposite, low sCD27 serum level was found to be significantly related to high CD27 expression in the tumor microenvironment ($p=0.006$). No correlation was found between high sCD27 serum level and CD3 expression or FOXP3 expression positivity on the tumor microenvironment of CRC patients.

Table 4-7: Relation between sCD27 serum levels and expression status of CD70, CD27, CD3, and FOXP3 in the tumor microenvironment of CRC patients. Correlation analysis was performed in the SPSS Version 17.0 software.

Parameters	Expression status		p
sCD27 U/ml	**CD70 (mean ± SD)**		p<0.0001
	Positive (n=5)	**Negative (n=16)**	
	128.62 ± 4.74	88. 95 ± 26.82	
	CD27 (mean ± SD)		0.006
	Positive (n=16)	**Negative (n=5)**	
	90.60 ± 28.78	123.35 ± 9.47	
	CD3 (mean ± SD)		0.075
	Positive (n=16)	**Negative (n=5)**	
	105.35 ± 24.69	76.14 ± 33.45	
	FOXP3 (mean ± SD)		0.702
	Positive (n=12)	**Negative (n=9)**	
	98.66 ± 34.85	98.04 ± 20.93	

The ROC curve analysis indicated that the cutoff value was 89.15 U/ml for the sCD27 level in the sera of CRC patients. According to this data, the correlation between

the number of patients who are positive or negative for sCD27 serum level and clinicopathological characteristics or expression status was investigated (Table 4-8).

Table 4-8: Relation between sCD27 serum levels and clinicopathological characteristics or expression status of CD70, CD27, CD3, and FOXP3 in the tumor microenvironment of CRC patients. The patients who have higher sCD27 serum levels than the cutoff value, 89.15 U/ml, considered positive, on the other hand, lower serum levels than 89.15 U/ml taken as negative.

Parameters	**sCD27 level (U/ml) n (%)**		
	Positive	**Negative**	**p**
Gender			
Male	16 (80.15%)	4 (20%)	0.328
Female	4 (57.1%)	3 (42.9%)	
Tumor location			
Right-sided colon	2 (40%)	3 (60%)	
Left-sided colon	2 (100%)	—	
Rectum	6 (100%)	—	1.00
Sigmoid	7 (70%)	3 (30%)	
Rectosigmoid	1 (50%)	1 (50%)	
Tumor stage			
T1	1 (100%)	—	
T2	—	—	1.00
T3	8 (72.7%)	3 (27.3%)	
T4	7 (63.6%)	4 (36.4%)	
Lymph node metastasis			
N0	8 (61.5%)	5 (38.5%)	
N1	4 (%)	—	1.00
N2	3 (60%)	2 (40%)	
N3	1 (100%)	—	
Distant organ metastasis			
Yes	4 (100%)	—	0.273
No	12 (63.2%)	7 (36.6%)	

Differentiation			
Weak + Moderate	15 (83.3%)	3 (16.7%)	**0.046**
Strong	1 (25%)	3 (75%)	
Perineural invasion			
Yes	4 (50%)	4 (50%)	0.146
No	11 (84.6%)	2 (15.4%)	
Mucinous component			
Yes	5 (7.14%)	2 (28.6%)	1.00
No	10 (66.7%)	5 (33.3%)	
CD70 expression			
Yes	5 (100%)	—	0.262
No	10 (62.5%)	6 (37.5%)	
CD27 expression			
Yes	10 (62.5%)	6 (37.5%)	0.262
No	5 (100%)	—	
CD3 expression			
Yes	13 (81.2%)	3 (18.8%)	0.115
No	2 (40%)	3 (60%)	
FOXP3 expression			
Yes	9 (75%)	3 (25%)	1.00
No	6 (66.7%)	3 (33.3%)	

Only the differentiation status was found to be significant in which sCD27 negativity in patients seems to be related to strong differentiation (p=0.046). Aggressive tumors have a lower sCD27 level in the serum with an odd ratio of 4.5 (95% CI: 1.386-14.612).

CD70, CD27, CD3, and FOXP3 expressions in the tumor microenvironment of CRC patients showed no correlation with the sCD27 level positivity or negativity. All patients whose CD70 expression was detected in their tumor microenvironment also have a higher sCD27 level in their serum, although CD27 expression status was quite the contrary.

5. DISCUSSION

In this study, CD70, CD27, CD3, and FOXP3 molecules in colorectal cancer were examined and data were correlated with clinicopathological characteristics of the patient group. Also, the sCD27 level was evaluated with those expression profiles and patient characteristics.

Expression of CD70, TNFSF member protein, is very limited to activated T cells and B cells, and matured DCs. In spite of the fact that CD70 expression is absent on normal tissues, several hematological malignancies and solid tumors have been found to be positive for CD70. Renal cell carcinoma has the highest rate for expression of CD70 among solid tumors followed by glioblastoma. Also, several other tumor types have been analyzing for CD70 expression as a potential prognostic and clinicopathological biomarker. Unusual CD70 expression has been correlated with an increase in tumor cell proliferation when it interacts with its receptor in TME. [39]–[42]

While ovarian cancer patients showed resistance to chemotherapy who are found to be positive for CD70 expression, in B cell lymphoma, expression of CD70 was found that there is an association between poor outcome and CD70 expression. However, most of the cancer types have not been analyzed for CD70 expression regarding patient outcome and disease progression. Colorectal cancer is among those types of cancer. Thus, it is important to investigate the expression profile and potential usage of CD70 and related mechanisms as a diagnostic biomarker in CRC. [34], [39]–[41], [43]–[45]

On the other hand, CD70 is the ligand of another TNFSF member protein, CD27 which is one of the immune checkpoint molecules plays an important role in TME through its specific interaction with its ligand. CD27 receptor expression is also restricted with only immune cells such as T cells, B cells, and NK cells as well. It is responsible for the proliferation of T cells, differentiation of memory T cells, and proper activation of T cells, and also differentiation of plasma cells, maturation of B cells and production of immunoglobulins through CD70/CD27 signaling pathway. However, normal or tumor tissue expression of CD27 has not seen. Thus, costimulatory effect of this receptor on immune cells and tumor cells in TME makes it a very promising target together with especially the interaction through its ligand, CD70. Furthermore, CD70 is involved in suppression of the immune system governed by

tumor to escape the immune system in TME with its related mechanism through other immune cells such as CD27+ TILs. Since CD27 expression in TME is restricted by only immune cells, the presence of its ligand on tumor cells and the interaction of CD27+ TILs or other immune cells with that CD70 ligand can generate an antitumor effect in TME. On the other hand, once CD70 interacts with its receptor, CD27 is cleaved out from the surface of the T cells and released into serum and soluble CD27 level also found as correlated with clinicopathological features of patients. [34], [39]–[42], [44], [46]

FOXP3 molecule is known as the Treg marker. Treg cells are suppressive immune cells and their presence in TME is correlated with immune suppression. Not only antitumor immune response is governed by Tregs, but also Tregs accumulation promotes tumor cells to evade the immune system. Moreover, expression of CD70 and its signaling pathway have been found that have an effect on tumor through the immune cell by the expansion of Tregs and reducing cytotoxic T cell response on tumor to help the tumor to escape immune response and promote the growth. Therefore, CD70 related mechanism promotes FOXP3+ Tregs accumulation in TME. [39]–[41], [47]

CD3 is T cell marker that represents the presence of T cell in TME when CD3 positivity is found in TME. Determining the accumulation of T cells in TME is important because T cells can directly kill tumor cells which are correlated with the regulation of immune response. Additionally, CD70 overexpression in TME of several cancer types correlates to abnormal behavior of TILs such as the accumulation of CD3+ T cells in TME. The presence of CD70 expression and accumulation of CD27+/CD3+ TILs and FOXP3+ Tregs in TME is shown to be associated with poor prognosis and tumor aggressiveness. Thus, it is important to investigate CD70 expression in TME together with accumulation of CD3+, CD27+, and FOXP3+ TILs. [5], [39], [41]

In this study, only 5 patients showed CD70 positivity among 33 total patients which firstly indicates that CRC is not a high CD70+ cancer type. Also, CD70 expression was also analyzed not only for tumor cells but also for adjacent normal tissues. There were no positive results for CD70 expression on normal tissues. A significant difference between tumor tissue and normal tissue expression was detected ($p<0.0001$). Consequently higher number of patients should have screened for better analysis. On the other hand, Western Blot analysis limits the evaluation of the molecule

of which there is no way to understand the origin of the CD70 in TME. In further analysis, colorectal cancer needs to be screened with another technique such as immunohistochemistry. [39]–[41], [48]

Although CD70 expression was detected at approximately 65 kDa by Western Blot, the expected molecular weight of the antibody used for the experiment is 21 kDa. However, the positive control cell line confirmed that the detected band belongs to CD70 presence in tissues. When a highly specific anti-CD70 antibody was used, 3 different bands reflecting the monomer (20.9 kDa), dimer (42 kDa), and trimer (63 kDa) bands were detected by Western Blot in Renal Cell Carcinoma Cell lines (Jilaveanu et al. 2012). Thus, we are suggesting that detected 65 kDa bands are the trimeric form of CD70. Patients with CD70+ tumors showed no positivity on adjacent normal tissues ($p<0.0001$). [44]

Any correlation or significance was found between the expression of CD70, CD27, CD3, and FOXP3 and clinicopathological features of patients. On the other hand, all CD70 positive patients showed CD3 and FOXP3 positivity supporting the assumption of accumulation of TILs in TME when CD70 expression was present. A total of 21 patients out of 33 have both CD27+ and CD3+ tumor tissues in common. Ruf et al, 2015 showed that CD27 accumulation in TME of Renal Cell Carcinoma comes from CD3+ TILs by immunohistochemistry. Although our data support this finding, Western Blot analysis alone does not approve where CD27 TILs were originated.

In CRC patients, serum level of sCD27 was not evaluated to date, although some studies have shown that sCD27 serum level in Renal Cell Carcinoma and NSCLC would be a prognostic biomarker. Thus, in this study sCD27 level was measured by ELISA and correlated to patients' clinicopathological characteristics and expression status of CD70, CD27, CD3, and FOXP3 in the TME for 27 CRC patients. sCD27 level was significantly higher when distant organ metastasis was present ($p=0.045$).

Moreover, the serum level of sCD27 was found significantly higher in CD70 positive patients compared to CD70 negative ones ($p<0.0001$). It may refer to the cleavage and release of sCD27 to sera due to CD70 overexpression of CD70 and interaction CD70-CD27 in CRC. Interestingly, the higher sCD27 level in patients' sera is strongly correlated to CD27 negativity in TME ($p=0.006$).

In this study, expression analysis was carried out by Western Blot. It is a good technique for the detection of protein expression but it is insufficient to determine the location and the source of the expression of a specific protein. Thus, only 5 patients showed CD70 positivity out of 33 patients. It must require further investigation with a higher number of patients. Also, immunohistochemistry may be a better technique to detect the localization of the expressions. Western Blot could say there is an expression of CD70 in TME but it is impossible to say whether CD70 positivity comes from tumor cells, tumor-associated fibroblasts or TILs. None of the normal tissues showed CD70 positivity which suggests CD70 related mechanisms play a role in the tumor microenvironment.

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ETHICS COMMITTEE APPROVAL

T.C.
İSTANBUL ÜNİVERSİTESİ
İSTANBUL TIP FAKÜLTESİ
KLİNİK ARAŞTIRMALAR ETİK KURULU

Sayı : 912 Tarih : 23.08.2017
Konu: Prof. Dr. İlhan YAYLIM hk.

Sayın Prof. Dr. İlhan YAYLIM
Aziz Sancar Deneysel Tıp Araştırma Enstitüsü

İlgi : Aziz Sancar Deneysel Tıp Araştırma Enstitüsünün 07/08/2017 gün ve 296974 sayılı yazısı

Sorumlu araştırıcılığını üstlendiğiniz ve Aylin Seher UZUNOĞLU' nun yürüteceği 2017/883 dosya numaralı "CD27/CD70 molekulleri ile ilişkili mekanizmalarin kolorektal kanserli hastalarda incelenmesi" başlıklı çalışma kurulumuzun 11/08/2017 gün ve 13 sayılı toplantısında görüşülerek etik yönden uygun bulunmuş olup, tutanaklar ekte sunulmuştur.

Bilgilerinizi rica ederim.

Prof. Dr. A.Yağız ÜRESİN
İstanbul Tıp Fakültesi Klinik Araştırmalar
Etik Kurul Başkanı

Eki: İstanbul Tıp Fakültesi Klinik Araştırmaları Etik Kurulu Karar Formu

İSTANBUL TIP FAKÜLTESİ KLİNİK ARAŞTIRMALARI ETİK KURULU KARAR FORMU

ETİK KURUL BİLGİLERİ	ETİK KURULUN ADI	**İSTANBUL TIP FAKÜLTESİ KLİNİK ARAŞTIRMALARI ETİK KURULU**
	AÇIK ADRESİ:	**İ.Ü.İSTANBUL TIP FAKÜLTESİ HULUSİ BEHÇET KÜTÜPHANESİ KAT:3 FATİH/İSTANBUL**
	TELEFON	**0 (212) 414 21 53**
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BAŞVURU BİLGİLERİ	ARAŞTIRMANIN AÇIK ADI	**"CD27/CD70 molekulleri ile ilişkili mekanizmalarin kolorektal kanserli hastalarda incelenmesi"**			
	ARAŞTIRMA PROTOKOL KODU	---			
	KOORDİNATÖR/SORUMLU ARAŞTIRMACI UNVANI/ADI/SOYADI	**Prof. Dr. İlhan YAYLIM**			
	KOORDİNATÖR/SORUMLU ARAŞTIRMACININ UZMANLIK ALANI	**Moleküler Tıp**			
	KOORDİNATÖR/SORUMLU ARAŞTIRMACININ BULUNDUĞU MERKEZ	**İstanbul Üniversitesi Aziz Sancar Deneysel Tıp Araştırma Enstitüsü Moleküler Tıp Anabilim Dalı**			
	DESTEKLEYİCİ	**İstanbul Üniversitesi Bilimsel Araştırma Projeleri**			
	DESTEKLEYİCİNİN YASAL TEMSİLCİSİ	---			
	ARAŞTIRMANIN FAZI	FAZ 1	☐		
		FAZ 2	☐		
		FAZ 3	☐		
		FAZ 4	☐		
	ARAŞTIRMANIN TÜRÜ	Yeni Bir Endikasyon	☐		
		Yüksek Doz Araştırması	☐		
		Diğer ise belirtiniz :			
	ARAŞTIRMAYA KATILAN MERKEZLER	TEK MERKEZ ☐	ÇOK MERKEZLİ ■	ULUSAL ■	ULUSLARARASI ☐

İSTANBUL TIP FAKÜLTESİ KLİNİK ARAŞTIRMALARI ETİK KURULU KARAR FORMU

ARAŞTIRMANIN AÇIK ADI	"CD27/CD70 molekulleri ile ilişkili mekanizmalarin kolorektal kanserli hastalarda incelenmesi"

	Belge Adı	Tarihi	Versiyon Numarası	Dili
DEĞERLENDİRİLEN BELGELER	ARAŞTIRMA PROTOKOLÜ	21/07/2017		Türkçe ■ İngilizce ☐ Diğer☐
	BİLGİLENDİRİLMİŞ GÖNÜLLÜ OLUR FORMU	■		Türkçe ■ İngilizce ☐ Diğer☐
	OLGU RAPOR FORMU	☐		Türkçe ☐ İngilizce ☐ Diğer☐
	ARAŞTIRMA BROŞÜRÜ	☐		Türkçe ☐ İngilizce ☐ Diğer☐

	Belge Adı		Açıklama
DEĞERLENDİRİLEN DİĞER BELGELER	TÜRKÇE ETİKET ÖRNEĞİ	☐	
	SİGORTA	☐	
	ARAŞTIRMA BÜTÇESİ	■	
	BİYOLOJİK MATERYEL TRANSFER FORMU	☐	
	HASTA KARTI/GÜNLÜKLERİ	☐	
	İLAN	☐	
	YILLIK BİLDİRİM	☐	
	SONUÇ RAPORU	☐	
	GÜVENLİLİK BİLDİRİMLERİ	☐	
	DİĞER:	■	Anabilim Dalı Başkanlığından Üst Yazı ve Akademik Kurul Kararı, Literatür Kaynağı, Sorumluluk Paylaşım Belgesi, Olgu Rapor Formu, İlgili Elemanların Bilgilendirildiğine Dair Belge, CV, CD

KARAR BİLGİLERİ	**Karar No:13**	**Tarih: 11/08/2017**
	İstanbul Üniversitesi Aziz Sancar Deneysel Tıp Araştırma Enstitüsü Moleküler Tıp Anabilim Dalında görevli **Prof. Dr. İlhan YAYLIM' ın** sorumluluğunda ve Aylin Seher UZUNOĞLU' nun yürüteceği yukarıda bilgileri verilen araştırma başvuru dosyası ile ilgili belgeler araştırmanın gerekçe, amaç, yaklaşım ve yöntemleri dikkate alınarak incelenmiş, gerçekleştirilmesinde etik ve bilimsel sakınca bulunmadığına toplantıya katılan Etik Kurul üye tam sayısının salt çoğunluğu ile karar verilmiştir.	

İSTANBUL TIP FAKÜLTESİ KLİNİK ARAŞTIRMALARI ETİK KURULU

ÇALIŞMA ESASI	**19.08.2011 tarihli, 28030 sayılı Resmi Gazetede yayınlanan Klinik Araştırmalar Hakkındaki Yönetmelik**
BAŞKANIN UNVANI / ADI / SOYADI:	**Prof. Dr. A. Yağız ÜRESİN**

Unvanı/Adı/Soyadı	Uzmanlık Alanı	Kurumu	Cinsiyet		Araştırma ile ilişki *		Katılım **		İmza
Prof. Dr. A. Yağız ÜRESİN	Farmakoloji ve Klinik Farmakoloji	İstanbul Tıp Fakültesi (Etik Kurul Başkanı	E ■	K ☐	E ☐	H ■	E ■	H ☐	
Prof. Dr. Berrin UMMAN	Kardiyoloji	İstanbul Tıp Fakültesi (Etik Kurul Başkan Yardımcısı)	E ☐	K ■	E ☐	H ■	E ☐	H ■	İZİNLİ
Prof. Dr. Ahmet GÜL	Romatoloji	İstanbul Tıp Fakültesi	E ■	K ☐	E ☐	H ■	E ■	H ☐	
Prof. Dr. Oğuzhan ÇOBAN	Nöroloji	İstanbul Tıp Fakültesi	E ■	K ☐	E ☐	H ■	E ■	H ☐	
Dr. Sevda ÖZEL YILDIZ	Biyoistatistik	İ.Ü. İstanbul Tıp Fakültesi Biyoistatistik	E ☐	K ■	E ☐	H ■	E ☐	H ■	İZİNLİ

* :Araştırma ile İlişki
** :Toplantıda Bulunma

İ.Ü. İstanbul Tıp Fakültesi Klinik araştırmalar Etik kurulu 13.04.2013 tarih, 28617 sayılı Resmi Gazetede yayınlanan Klinik Araştırmalar Hakkında Yönetmelik çerçevesinde kurulmuş ve T.C. Sağlık Bakanlığı Türkiye İlaç ve Tıbbi Cihaz Kurumu tarafından onaylanmıştır. İlgili yönetmelik kapsamında kalan araştırmalar Sağlık Bakanlığından izin almak zorundadır. Yönetmelik kapsamı dışında kalan araştırmalar ise Etik Kurul bünyesinde oluşturulmuş 5 kişilik alt komisyon tarafından değerlendirilmekte olup Sağlık Bakanlığı iznine tabi değildir.

FIRST PAGE OF PLAGARISM REPORT

EVALUATION OF CD70 EXPRESSION RELATED IMMUNE RESPONSE IN TUMOR MICROENVIRONMENT OF COLORECTAL CANCER

ORIJINALLIK RAPORU

%4	%3	%1	%3
BENZERLIK ENDEKSI	İNTERNET KAYNAKLARI	YAYINLAR	ÖĞRENCI ÖDEVLERI

BIRINCIL KAYNAKLAR

1	www.openaccess.hacettepe.edu.tr:8080 İnternet Kaynağı	<%1
2	Submitted to Universiti Sains Malaysia Öğrenci Ödevi	<%1
3	Submitted to UC, San Diego Öğrenci Ödevi	<%1
4	tessera.spandidos-publications.com İnternet Kaynağı	<%1
5	soft-tox.org İnternet Kaynağı	<%1
6	Submitted to University of Nevada Reno Öğrenci Ödevi	<%1
7	Submitted to Imperial College of Science, Technology and Medicine Öğrenci Ödevi	<%1
8	Submitted to Middle East Technical University	

CURRICULUM VITAE

Kişisel Bilgiler

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	Mezun Olduğu Kurumun Adı	**Mez. Yılı**
Doktora		
Yük.Lis.		
Lisans		
Lise		

İş Deneyimi (Sondan geçmişe doğru sıralayın)

	Görevi	**Kurum**	**Süre (Yıl - Yıl)**
1.			-
2.			-
3.			-

Yabancı Dilleri	**Okuduğunu Anlama***	**Konuşma***	**Yazma***	**KPDS/ÜDS Puanı**	**(Diğer) Puanı**

***Çok iyi, iyi, orta, zayıf olarak değerlendirin

	Sayısal	**Eşit Ağırlık**	**Sözel**
LES Puanı			
(Diğer) Puanı			

Bilgisayar Bilgisi

Program	**Kullanma becerisi**
Microsoft Office	Çok İyi
GraphPad Prism	Çok İyi
Image J	Çok İyi

Yayınları/Tebligleri Sertifikaları/Ödülleri

Özel İlgi Alanları (Hobileri):