Interleukin-21 and Interleukin-32 gene expression levels and their relationship with clinicopathological parameters in colorectal cancer

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BACKGROUND AND AIMS: *The immune cells in tumor microenvironment release chemokines and cytokines which determine the immune phenotype of the tumor and play an important role in the prognosis. Present study evaluates the gene expression levels of IL-21 and IL-32 and their relations to clinicopathologic parameters in colorectal cancer.*
PATIENTS AND METHODS: *31(17F) patients with colorectal cancer were included. Samples were obtained from normal and tumor tissues. After RNA isolation, IL-21 and IL-32 gene expression levels were measured. Immunohistochemistry was also carried out for CD4+, CD8+ and NKcells to measure cell density. The relations between expression levels, immune cell density and differentiation, stage, presence of vascular, perineural invasion and lymph node metastasis(MLN) were investigated.*
RESULTS: *IL-32 gene expression levels were increased in tumor tissues. IL-21 levels were found to be decreased in 50% of the patients. IL-32 levels were also increased with the stage however, it was decreased significantly with the increased number of the MLN. On the other hand, expression levels of IL-21 increased significantly with the presence of vascular invasion. CD4+ density was decreased with increased T-stage, vascular invasion whereas CD8+ density decreased only with the vascular invasion.*
CONCLUSIONS: *IL-32 expressed by tumor microenvironment reveals that expression increased to control tumor growth, but levels are decreased with the increased number of MLNs which might be due to decreased CD4+ cell density. Changes on IL-21 and IL-32 together with the changes on immune cell density, indicate their role in tumor growth and invasion in colon cancer.*
KEY WORDS: Colorectal Cancer, Cytokines, Immune Cell Density, Interleukin-21, Interleukin-32, Tumor Microenvironment

What does this paper add to the literature? Statement

This paper is investigating the gene expression levels of IL-21 and IL-32 in colorectal cancer. The density of immune cells was also evaluated using immunohistochemistry. Changes on IL-21 and IL-32 together with the changes on immune cell density, indicate the role for cytokines in tumor growth and invasion in colon cancer which might shed light on new researches to understand the mechanism of invasion and also to discover new molecular and immunologic therapies for colorectal cancer.

Introduction

Malignant tumors are caused by genetic and epigenetic changes, the most of which are effected by microenvironment. Tumor specific microenvironment involves many cells including tumor cells, immune cells and stro-

mal cells [1,2]. The location and relations of the cells in stroma and the tumor together with the type of activations and their secretions such as chemokines and cytokines determine the future of the tumor. Interleukins, growth factors and TNF-α are released by immune cells or by tissues act to shape the immune response by inviting the immune cells to the field and also act to increase the communication between immune cells.

Colon cancer is the third most common cancer and also second deadly cancer after lung cancer in USA. Almost 20% of the patients are in stage IV and more than 50% of the patients in stage I and III also have metastasis during the time of diagnosis [3]. It has been shown in invaziv colon cancer that the type and density of immune cells in stroma infiltrating the tumor together with inflammatory microenvironment were very important for the prognosis [4,5].

Interleukin-21 (IL-21) and Interleukin-32 (IL-32) are pro-inflammatory cytokines released from immune cells located in tumor microenvironment. IL-32 is a multifunction pro-inflammatory cytokines released from epithelial cells (colon, stomach, lung), monocytes activated by IL-2, IL-18, IFN-γ, activated natural-killer (NK) cells and T-cells [6-8]. The expression levels of IL-32 was very low in normal colonic mucosal cells whereas, it was found to be highly expressed in inflammatory bowel disease [9]. IL-32 is very important for colon cancer as it inhibits the growth of cancer cells and changes the levels of other cytokines [10,11].

IL-21 is a member of IL-2 cytokine family and expressed by helper T cells (Th), follicular helper T cells (Tfh), Th17 and natural killer T (NKT) cells. The main sources of IL-21 are CD4+ T cells and NKT cells. IL-21 also stimulates the NK cells and increases the cytotoxic effects of CD8+ T cells, decreases the differentiation of Treg cells and induces the release of inflammatory mediators from epithelial cells and fibroblasts [12]. *Jouch et al* has shown in an experimental study that IL-21 stimulated the intestinal carcinogenesis and tumor supported microenvironment also developed by the effects of IL-21 [13-15]. However, the changes on mRNA levels of IL-21 and IL-32 in tumor tissue as compared to normal tissue is not known yet. These cytokines might be an indirect indicator for tumor infiltrating cells including CD4+ Tcell, NK cell and NKT cell.

Therefore, present study is planned to evaluate the gene expression levels of IL-21 and IL-32, immune cell density and their relations to clinicopathologic parameters including tumor stage, tumor differentiation, lymph node involvement, vascular and perineural invasion in patients with colorectal cancer.

Patients and Methods

Tissue samples used in this study obtained from patients who had radical colorectal surgery in 7th Surgical Clinic of Ankara Numune Teaching and Research Hospital and histopathological examination were carried out in the pathology department of the same hospital. Molecular studies carried out in Hacettepe University Cancer institute, Department of Basic Oncology.

The study protocol was approved by the local ethics committee of Hacettepe University, in accordance with the Declaration of Helsinki, and all paticipants approved written informed consent. STROPE-ME statement and guideline is used for collection and handling of samples, analysis, interpretation of data and reporting the results [16].

PATIENTS

Power calculation is used for finding the necessary sample size. However, more saples were collected in order to cover inadequate or low quality material for molecular analysis. Thirty-one patients with the mean (range) age of 61(26-79) years with colorectal cancer who underwent surgery were included in the study. During surgical removal of each tumor an adjacent section of normal tissue was also removed. Samples were put into the RNA-later *(Ambion, ABD)* and kept at -20 °C until RNA isolation. Clinicopathologic parameters of the patients such as stage, size and pathologic grades of tumor, number of metastatic lymph nodes, neuoral and vascular invasion based on pathology reports have all been registered on SPSS 18.00 for Windows.

RNA ISOLATION

Total RNA was isolated from 250 mg tumoral and normal tissues using RNeasy Midi Kit (Qiagen, USA) according to manufacturer's instructions. Isolated RNAs were treated with DNAse I enzyme using DNA-Free RNA kit *(Zymo Research, USA)* for degradation of genomic DNA. Purity and concentration of RNAs were calculated on the NanoDrop 1000 spectrophotometer (*Thermo, ABD*). Subsequently cDNA was synthesized using RevertAid First Strand cDNA Synthesis kit (Thermo, ABD).

POLYMERASE CHAIN REACTION (PCR)

Conventinal PCR was done to optimize annealing degrees of designed primers belong to genes and $MgCl_2$ concentration. Primers sequences of IL-21, IL-32 and GAPDH genes are given in Table I. Size of PCR products were evaluated using 2% agarose gel electrophoresis.

REAL-TIME PCR (QPCR)

Expression levels of IL-21, IL-32 and GAPDH genes were evaluated by real-time PCR (qPCR). We used Light

TABLE I - *Primers Sequences for GAPDH, IL-21, IL-32 genes.*

Genes	Primers Sequences (5'-3')	Chromoseme	PCR Products	Gene ID No.
GAPDH	F: CTGAGAACGGGAAGCTTGTCATR: GCCTTCTCCATGGTGGTGAAGA	12p13	143 bp	NM_002046.5
IL-21	F: CAAACTGTAAGTGGTCAGCTR: CTGCATTTGTGGAAGGTGGT	4q26 -q27	132 bp	NM_021803.3
IL-32	F: AGCTGGAGGACTTCAAAGAGR: CCGGGACAGGGGATCTGTTG	16p13.3	141 bp	NM_001012631.1

Cycler-DNA Master SYBR–GreenI (*Roche Diagnostics, Germany*) kit for relative quantification of gene expression in the tumor tissue compared to normal tissue. The qPCR reactions were set up in volume of 20 µl, containing 4 µL of sample cDNA, 1 × SYBR Green I dye, 2 mM MgCl2, and 0,125 µM of IL-21, 0,188 µM IL-32 and GAPDH's specific forward and revers primers (Sequences are given in Table I). The PCR cycling conditions were as follows: initial denaturation at 95°C for 5 min and 95°C for 30 s, 61 °C for 30s, 72°C for 30s for 45 cycles, final extension at 72°C for 10min. Relative expression levels were calculated using the PCR threshold cycle number (CT) for each tissue target genes and internal control gene (GAPDH) using the formula $2^{-(\Delta CT sample - \Delta CT control)}$ [17]. ΔCT represents the difference in CT values between the target gene (IL-21 or IL-32) and internal control gene *GAPDH*. qPCR was performed in duplicate for each sample and average *CT* values were calculated.

IMMUNOHISTOCHEMISTRY

Immunohistochemical staining were carried out in twenty-four patients who had IL-32 and IL-21 gene expression levels analysis. Consecutive 3µm–thick sections were obtained from formalin-fixed paraffin-embedded tissues of each patient. Sections were immunolabelled for CD4, CD8 and CD56.
Procedure was performed using the autostainer (*Bond; Leica Biosystems, UK*) at room temperature. CD4, CD8 and CD56 primary human antibodies (Bond™ Ready to use Primary antibody, Leica Biosystem, UK) were diluted in 1:200 for immunostaining. Density of immune cells was determined from the number of CD4⁺, CD8⁺ and CD56⁺ cells. Cell density assessed in tumor center (TC) and invasive margin (IM) area and five area identified for each tumor site by scanning with light microscopy. In the sequel immune cells were counted in tc and ım area at x400. The mean value of immune cells of five fields was calculated for each tumor SITE.

STATISTICAL ANALYSIS

The data are presented as mean(range). All analysis were conducted by SPSS 18 (*SPSS, Chicago, USA*).
Continuous variables were compared using *Kruskal-Wallis* and *One-way Anova Test*. Differences were considered statistically significant when p-values were <0.05. *Pearson* and *Spearman correlation* analysis were used to evaluate the relationship between clinicopathological parameters, gene expression levels and cell density.

Results

IL-21 AND IL-32 GENE EXPRESSIONS IN TISSUE SAMPLES

Thirty-one patients with the mean (range) age of 61(26-79) years were included in the study. IL-32 levels were studied in 31(17F) and IL-21 levels were studied in 28(15K) patients due to low quantity RNA. Gene expression levels of patients in tumor tissues were compared to their own normal tissue expressions. $2^{-\Delta\Delta CT}$ method is used for calculations. In this method, $2^{-\Delta\Delta CT}$ bigger
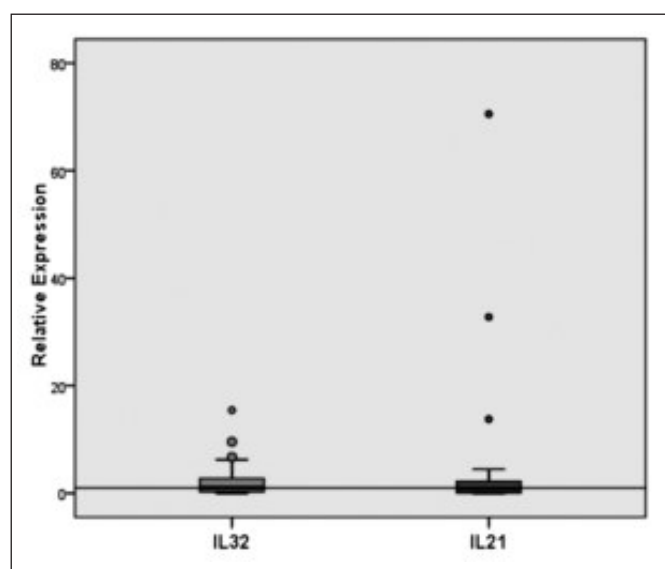

Fig. 1: IL-32 and IL-21 gene expression levels.

TABLE II - *IL-32 and IL-21 expression levels according to clinicopathologic parameters (*)*

Clinicopathologic Parameters T Stage (pT)	n	IL-32	n	IL-21
T2	6	3,37 (0,051-9,58)	4	9,71 (0,027-32,79)
T3	11	4,33 (0,142-15,45)	10	2,84 (0,007-13,784)
T4	14	1,02 (0,019-4,169)	14	5,64 (0,003-70,520)
	31	p> 0.05	28	p> 0.05
Differentiation				
Grade 1 (well)	18	1,96 (0,019-9,579)	17	1,77 (0,008-13,784)
Grade 2 (medium)	10	3,28 (0,127-15,454)	8	5,22 (0,007-32,785)
Grade 3 (undifferentiated)	1	6,75 (6,75)	1	2,62 (2,62)
	29	p> 0.05	26	p> 0.05
Astler-Coller Classification				
A	1	0,58 (0,58)	1	4,44 (4,438)
B1	3	3,14 (0,929-6,475)	1	32,79 (32,785)
B2	13	3,76 (0,031-15,454)	13	2,42 (0,007-13,784)
C2	13	1,44 (0,19-6,75)	12	6,35 (0,003-70,520)
D	1	0,64 (0,639)	1	0,049
	31	p> 0.05	28	p> 0.05
Lymph Node Metastasis				
none	16	3,233 (0,031-15,454)	14	4,61 (0,007-32,785)
PLN 3	7	2,978 (0,858-6,750)	6	1,21 (0,017-3,784)
PLN >3	7	0,668 (0,019)	7	1,448 (0,005-1,851)
	30	p> 0.05	27	p> 0.05
Vascular invasion				
present	7	4,180 (0,019-15,454)	6	13,494 (0,625-70,520)
absent	20	2,25 (0,024-9,579)	18	2,58 (0,003-32,785)
unknown	4	0,85 (0,168-1,328)	4	4,42 (0,049-13,784)
	31	p> 0.05	28	p=0.02
Perineural invasion				
present	6	1,524 (0,019-6,475)	5	3,50 (0,075-13,784)
absent	22	3,021 (0,024-15,454)	20	6,18 (0.003-70,520)
unknown	3	0,711 (0,168-1,328)	3	1,30 (0,49-3,784)
	28	p> 0.05	28	p> 0.05

(*) All values are mean(range), ( )p=0.01 for patients with PLN>3, PLN: positive lymph nodes.

than 1 means increased gene expression in tumor tissue, $2^{-\Delta\Delta CT}$ smaller than 1 means decreased gene expression as compared to normal tissue and $2^{-\Delta\Delta CT}$ equal 1 reflects equal gene expressions in both normal and tumor tissues.

IL-32 expression levels were found to be increased, whereas IL-21 gene expression levels were decreased in 50% of the patients and increased in remaining 50% of patients in tumor tissues as compared to normal tissue (Fig. 1).

IL-32 and IL-21 gene expression levels were also evaluated according to tumor volume, differentiation, patologic T grade (pT), Astler-Coller stage, presence of lymph node metastasis, vascular invasion, perineural invasion and metastasis (Table II).

IL-32 AND IL-21 EXPRESSION LEVELS ACCORDING TO DIFFERENTIATION

There was no correlation between the degree of differentiation and IL-32 gene expression levels. IL-21 gene expressions were not increased in well-differentiated group but, increased in medium-differentiated and undifferentiated group without any statistical significance (p>0.05).

IL-32 AND IL-21 GENE EXPRESSIONS ACCORDING TO TUMOR GRADE

IL-32 and IL-21 expression levels were evaluated according to *Astler-Coller* classification of the tumors. IL-32 gene expressions were decreased on stage A but increased when tumor passes mucosa on stage B1 and B2. Expression levels were decreased to its half with the involvement of lymph nodes in stage C. Although it was also decreased in stage D, it was not as low as it was in stage A (Fig. 2A). When all the stages were compared, the changes did not reach the statistical significance (p. 0.05) which might be the result of low patient volume and unequal distribution in the groups.
The expressions of IL-21 also decreased with the increased stage of the disease (Fig. 2B).
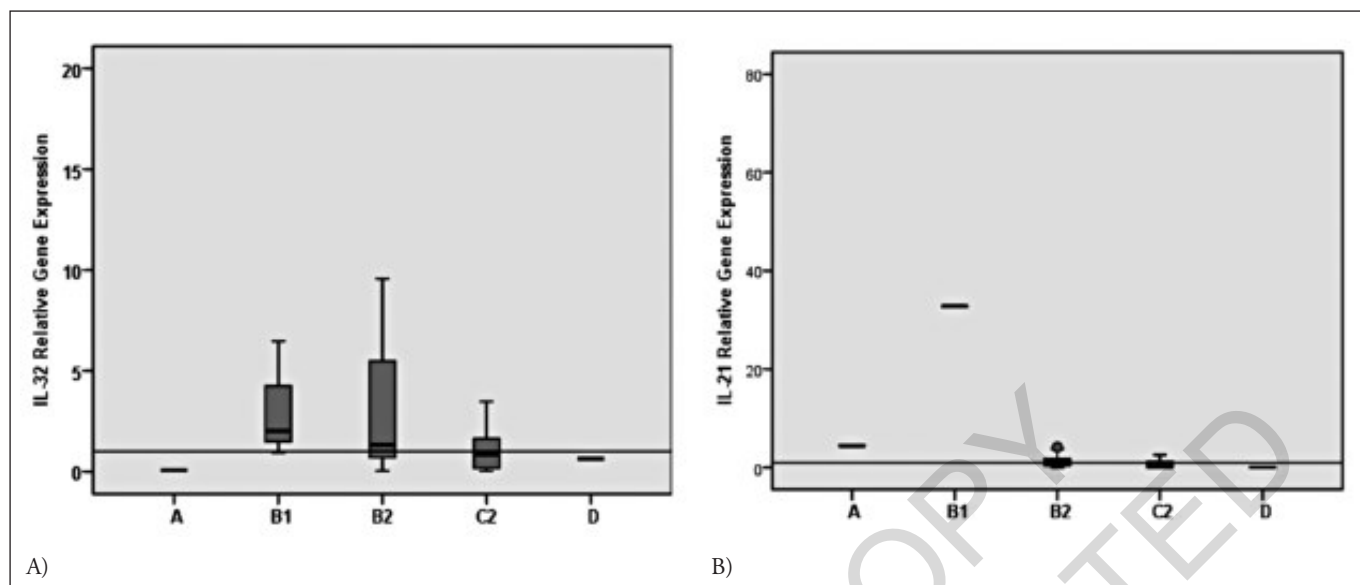
Fig. 2: Gene expression levels according to Astler-Coller classification A) IL-32 gene expression B) IL-21 gene expression.
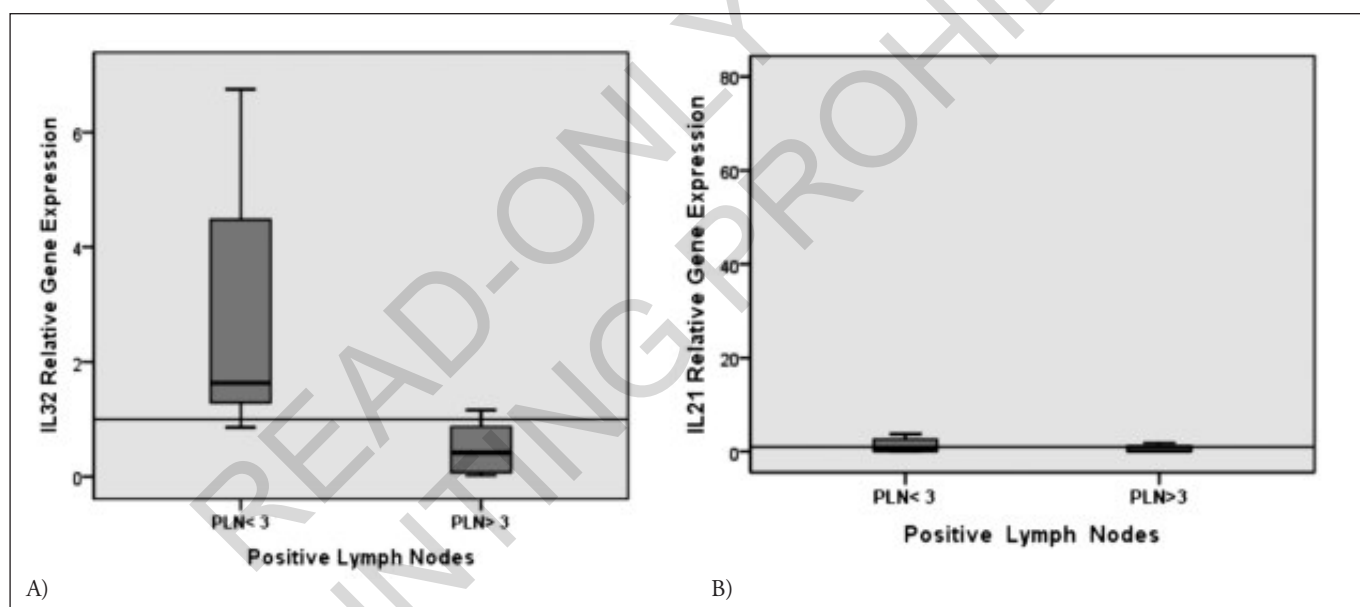

Fig. 3: Gene expressions according to number of PLNs. A) IL32 gene B) IL-21 gene, PLN: Positive Lymph Nodes.

IL-21 AND IL-32 GENE EXPRESSIONS ACCORDING TO T-STAGE

Gene expressions of IL-21 and IL-32 according to T-stage were similar to the expressions according to Astler-Coller classification. IL-32 expressions were increased with T-stage as compared to normal tissue and decreased when tumor passed the serosal wall and started to invade periphery. Although IL-21 expressions were found to be higher in T2 stage, it was found to be significantly decreased in T3 and T4.

IL-21 AND IL-32 GENE EXPRESSIONS ACCORDING TO LYMPH NODE INVOLVEMENT

Expression levels were not changed with the lymph node involvement. Hovewer, IL-32 levels were significantly higher when the number of tumor positive lymph nodes(PLN) is less or equal to 3, and decreased significantly when PLN is over 3. (p<0.05) (Fig. 3A). IL-21 gene expressions did not change sigificantly with the number of PLNs. (Fig. 3B).
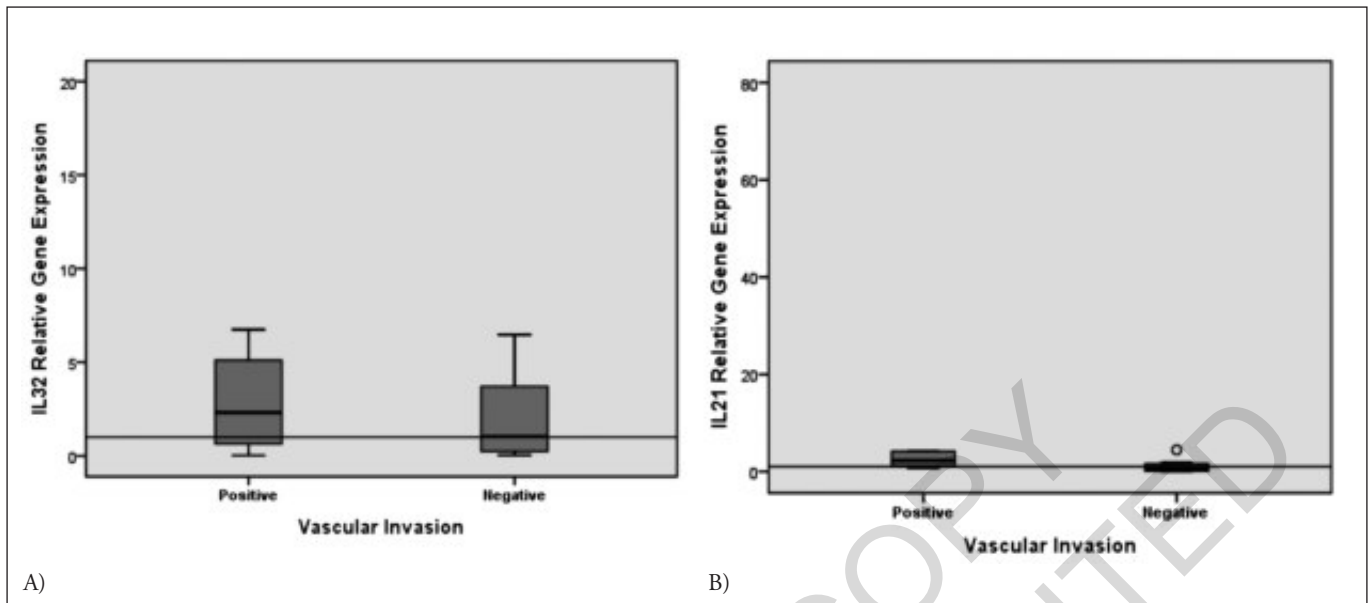
Fig. 4: Gene expressions according to vascular invasion A) IL-32 gene B) IL-21 gene.

IL-32 AND IL-21 GENE EXPRESSIONS ACCORDING TO VASCULAR INVASION

Although the number of patients with the vascular invasions were too small to comment, (n=7), both IL-32 and IL-21 gene expressions were found to be increased in patients with vascular invasion (Figg. 4A-B). This increase was even more than 4-folds for IL-21 and reached statistical significance (p=0.023).

GENE EXPRESSIONS ACCORDING TO PERINEURAL INVASION AND DISTANT METASTASIS

There was no correlation between gene expression levels and perineural invasion. There was only one patient with distant metastasis and both IL-21 and IL-32 gene expressions were found to be decreased in this patient.

IMMUNE CELL DENSITY IN TUMOR TISSUE

The density of CD4+ T, CD8+ T and NK cells were counted at tumor invasive margin (IM) and tumor center (TC) in 24 patients (Fig. 5). The density of CD4+ and CD8+ at tumor invasive margin were found to be significantly increased as compared to their density at center (*p<0.05*). (Table III)
CD8+ T cells were significantly higher than CD4+ T cells at center and invasive margin of the tumor(*p<0.05*). The density of both cells were correlated to each other and increased at center and invasive margin of the tumor (*p<0.05*). NK cells were found at tumor center in only one patient (Fig. 5C). When the correlation between IL-32 and IL-21 gene expression levels and the density of

CD4+ were evaluated no significant correlation was found. There was a strong correlation between patologic T-stage and CD4+ cell density which was significantly decreased both at the tumor center and invasive margin with the increased T-stage. (*p<0.05 with Pearson correlation= -, 630 and -, 602, respectively*). Similar results were found with Astler-Coller classification. When the relation between cell density and vascular invasion is assessed, density of both CD4+ and CD8+ cells were decreased at invasion margin of the tumor in patient with vascular invasion (p< 0.05 *with Pearson correlation = -, 572 and -, 470 respectively*). Whereas, no correlation was found between density of CD8+ T cell and T-stage. No correlation or relationship also found between CD4+, CD8+ cell density and differantiation, number of PLNs and perineural invasions.

Discussion

Determination of the prognosis and response to treatment of multifactorial diseases such as cancer is very difficult. Untill now various biomarkers have been developed to determine the prognosis of cancer patients however; very few of them have found a use in clinical practice. Currently, tumor microenvironment affecting the oncogenesis is subject to active research in order to determine a suitable biomarker for prognosis and response to therapy. Furthermore, tumor microenvironment is currently an attractive target for personalized cancer therapy.
In order for the tumor cells to adopt and proliferate in the microenvironment; an interaction with various microenvironmental components such as endothelial cel-
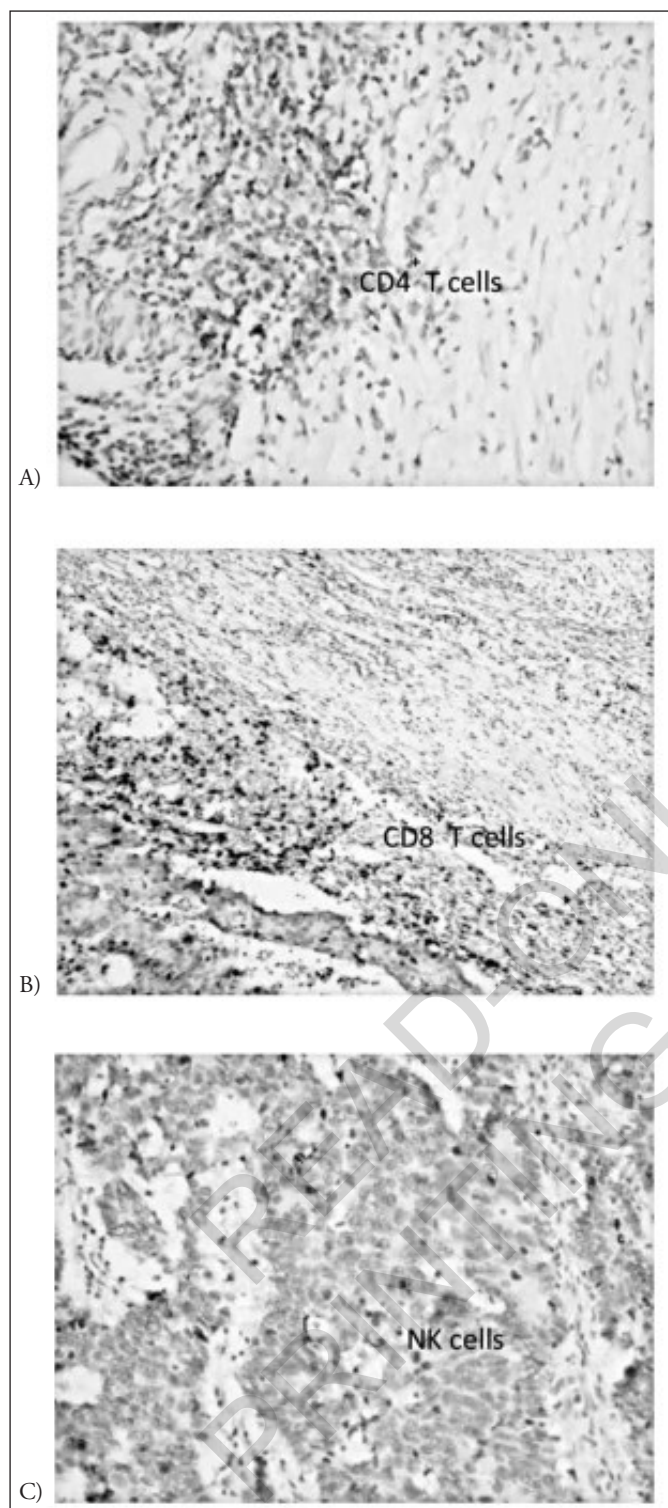
A)

B)

C)

Fig. 5: Immunohistochemistry A) CD4+ T cells at invasive margin (X 200) B) CD8+ T cells at invasive margin (X 100) C) CD56+ NK cells at tumor center (X 200).

TABLE III - *Immune Cell Density in Tumor Tissue*

	Number of CD4+ T Cell(n=24)	Number of CD8+ T cell(n=24)	p value
Tumor Center	30,17 (0-96)	63,46 (2-134)	p< 0.05
Tumor Invasive Margine	49,58 (0-98)	99,08 (22-238)	p< 0.05
P value	p< 0.05	p< 0.05	

(*)All Values are mean(range).

to circumstances and localization it can support or suppress tumor growth. Immune system can protect the host from tumor development by the so called "immune surveillance" mechanism. The incidence of spontaneous or mutagen induced carcinogenesis is found to be increased in immune suppressed animals; which emphasizes the role of innate and adoptive immune system in antitumor resistance in the organism. Tumor cells develope resistance to immune surveillance by "immune-shaping" and therefore reducing the antigenic profile [19,20]. Process of changing from immune surveillance to a tumor supporting immune cell requires complex signal transduction mechanisms. This process is affected by the cytokines that are released from immune cells and other nonneoplastic cellular components (*epithelial cells, cancer associated fibroblasts*). Cyokines associated signal transduction cascade during Immuno-editing can either suppress or support tumor proliferation. Elucidation of this complex interaction will contribute to our understanding of tumor growth and propagation. Cytokines are an indirect indicator of the immunocytes infiltrating the tumor [21,22]. The localization, density and behavior of these cells show changes according to stages of the tumor. Clinical studies have shown that in different individuals with cancers in same histopathologic stage can show diverse prognoses. Furthermore; an advanced stage disease can be stable for a long time and a disease with multiple metastasis can show spontaneous regression or an early stage disease can relapse immediately after R0 resection and lead to death of the patient. This is due to the fact that classical tumor stage considers the tumor cells but not the components of the microenvironment such as immune cells that infiltrate the tumor. Recents studies that are peformed support this hypothesis [23]. Immune cells are the most important components of the tumor stroma and they provide the optimal cytokine and inflammatory support to the milleu. Histopathologic analysis demonstrate that tumor is infiltrated by various innate and adoptive immune system cells. Macropahges are frequently found in the stroma and are responsible for the fibrosis. Lymphocyte type and distribution show differences according to the tumor type. Natural Killer cells (NK cells) are usullay present in the stroma of many tumors except in renal cell carcinoma (RCC) where it is in close relationship with the tumor cells 24. Present

ls, fibroblasts, blood and lymphatic vessels, immune cells, cytokines and chemokines and cellular metabolites are required [18]. Immune system is one of the most important factors that determine tumor progression. According

study supports this information as we couldn't find NK cells at center and margin of the tumor in any patient but one (Fig. 5C). T cells are present both in the tumor center and invasive edge. Naïve T cells are usually localized in the neighbouring lymphoid tissue around the tumor. CD8+ T cells are both in contact with the tumor cells and also present in the stroma of the tumor cells. We also found CD4+ and CD8+ T cells at margin and center of the tumor and the density of CD8+ T cells was much higher than CD4+ T cells. This might be a resemblance of the active role for CD4+ and CD8+ T cell subgroups. Regulatory T cells (Treg), Tfh, Th17 and B lymphocytes are localized in the stroma and lymphoid islands around the tumor. The distribution of these cells change according to tumor type and patient characteristics and this determines the individual prognosis. The studies indicate that NK, CD8+ T cells, Th1, M1 type macrophages and dendritic cells are indicators of good prognosis. On the contrary; Treg, Th2, myeloid-derived suppressor cell (MDSC) and M2 type macrophages are related with poor prognosis. An intense lymphocyre infiltration is indicative of with strong antitumor immune response. Studies have shown a clear relationship between intratumoral immune cell count and clinical prognosis of the patients with head and neck cancers, ovarian, transitional cell, breast, colorectal, renal, prostate, lung cancers and malignant melanoma. When the subtype analysis of the immune cells were performed; it was found that increased infiltration of CD3+T cell, CD8+T cell ve memory T cell in the tumor was related with prolonged disease free survival (DFS) and overall survival (OS) of the patients [25,26]. In the present study proinflammatory cytokines IL-21 and IL-32 gene expression was analyzed in the patients with colorectal cancer as an indicator of the innate and adoptive immune process. Furthermore; the IL-21 and IL-32 gene expression levels were correlated with patient and tumor related prognostic factors such as differentiation level, clinical and pathologic stage, volume, lymph node metastasis, vascular, lymphatic and perineural invasion. IL-32 gene expression was found to be increased in the tumor tissue in comparison to the normal counterpart. However, its expression level was reduced in 48% of the patients. IL-21 expression was enhanced in 50% of the patients and reduced in the remaining 50%. Although the number of the patients is not enough to reach a definitive conclusion and also to evaluate the results of the study, the variation of the expression levels of IL-32 and IL-21 genes suggests the variation of the immune function in different individuals which has also been emphasized by Angell et al [18]. Furthermore, in the present study the tumor samples have been taken from the center of the tumor; hence the results indicate only a part of the immune reaction in the organism due to the fact that the immunocytes are scatterded throughout the tumor microenvironment. In previous immunohistochemical studies increased IL-32 expression was found to be cor-

related with poor prognosis in certain cancers [27]. However, we have not come a cross any study regarding the gene expressions of IL-32 and IL-21 in colorectal cancer.

When the expression levels were analyzed according to the T stage of the disease it was found that IL-32 mRNA levels were higher in stages T2 and T3 and showed a tendency towards reduction in the T4 stage. On the other hand; IL-21 mRNA level was highest in stage T2 disease and was lower in the stage T3 and T4 disease. Milecnik et al have shown a reduced CD8+ T cell density as the T-stage (invasion level) of the disease increased (Tis/T1 and T4). In adition to all memory T cell (CD45RO+) and cytotoxic effector cells (GRZMB+) density was reduced as the T-stage of the disease was increased [28]. Present study revealed that in patients with advanced stage tumor, CD4+ T-cell density was decreased both at center and the margin of the tumor, which might be an important indicator for the deficiency of immune system with the growth of the tumor. In adition, density of both CD4+ and CD8+ cells were also decreased at invasion margin of the tumor in patient with vascular invasion.

Galon et al have shown a close relation between perineural and lymphovascular invasion by phenotypic and genotypic analysis of the immune function in patients with colorectal cancer [19]. Furthermore, they also demonstrated that in the absence of tumor embolism immunocyte infiltration was enhanced together with mRNA levels of the markers that define Th1 and effector T cells. Among these markers were CD8, T-box transcription factor-21 (Tbet), IFN regulatory factor-1 (IRF-1), IFN-γ, granulysin, granzymes. The subtypes of the lymphocytes significantly differ in the presence of tumor emboli in the patients [19]. The main lymphocytes that increased were the CD8+ memory T cells [29]. In our study it was found that IL-32 mRNA levels were increased in cases without lymph node metastasis such as stage B1 and B2; however in stage C2 where there is an extensive lymph node metastasis, expression levels were decreased. These cytokines are especially expressed in Th and NK cells and might be an indirect marker of immune infiltration. However, we failed to find any significant relation between Th and NK cell density and IL-32 and IL-21 gene expression levels. On the other hand we found that IL-21 gene expression levels were also decreased with advanced T-stage as the density of CD4+ T cells. One of the short commings of the study is the fact that there are not enough patients in the early and advanced stage disease which makes the interpretation of the results harder. Together with increased patient number and longer follow-up periods for patient survival analysis could yeild to more extrapolatable results. Once the IL-21 and IL-32 expression levels were analyzed in groups with and without lymph node metastasis no significant difference was observed. However; once the expression levels were compared among the patients with <3 and ≥3 lymph

node metastasis; a reduction in expression levels of the IL-32 was observed which was statistically significant (p<0.05). In a study by Milenick et al on tissue samples of 599 colorectal cancer cases a reduction in the immunocyte density was determined with lymph node metastasis [28]. In the present study, reduction in the expression levels of IL-32 may indicate a mechanism of immune infiltration which suggests immune reaction in the tumor microenvironment is a factor that controls tumor progression starting from the early phases of the disease.

Determination of IL-32 gene expression in colon cancer may provide information regarding lymp node metastasis and tumor microenvironment. IL-21 is released from the follicular T cells in the tumor microenvironment and regulates the function of the Th1, memory T cell and cytotoxic T-cell response which is important in the patient's overall and disease free survival [30]. Analysis of the immunocyte density, subtype and localization in the tumor microenvironment have been shown to be useful prognostic criteria regarding patient survival and recurrences and it was independent from and superior to the TNM staging system [31,32]. However; the exact mechanisms of change in intratumoral imunocyte density and subtype are not clearly defined. As the knowledge in these areas are increased more personalized immunotherapy for different cancers will be developed.

In conclusion, present study indicates that IL-21 and IL-32 are not directly related with differentiation, perineural invasion and distant metasastasis. However, IL-32 expressed by tumor microenvironment reveals that IL-32 expression increased to control tumor growth, but levels are decreased with the increased number of involved lymph nodes which might be due to decreased CD4+ cell density. Changes on IL-21 and IL-32 together with the changes on immune cell density, indicate the role for cytokines in tumor growth and invasion in colon cancer. As the cytokine networks are very important for cancer we need to know which and how cytokine networks effect the tumor progress in patients, for which further studies are required.

Riassunto

Le cellule immunitarie nel microambiente tumorale rilasciano chemochine e citochine che determinano il fenotipo immunitario del tumore e svolgono un ruolo importante nella prognosi. Il presente studio valuta i livelli di espressione genica di IL-21 e IL-32 e le loro relazioni con i parametri clinicopatologici nel carcinoma del colon-retto.

Sono stati inclusi nello studio 31pazienti (di cui 17 donne) affetti da carcinoma del colon-retto. I campioni sono stati ottenuti da tessuti normali e tumorali. Dopo l'isolamento dell'RNA, sono stati misurati i livelli di espressione genica di IL-21 e IL-32. L'immunoistochimica è stata eseguita anche per le celle CD4 +, CD8 + e NK per misurare la densità cellulare. Sono state studiate le relazioni tra livelli di espressione, densità e differenziazione delle cellule immunitarie, stadio, presenza di invasione vascolare, perineurale e metastasi dei linfonodi (MLN).

Risultati: i livelli di espressione genica dell'IL-32 sono aumentati nei tessuti tumorali. È stato riscontrato che i livelli di IL-21 sono diminuiti nel 50% dei pazienti. Anche i livelli di IL-32 sono stati aumentati con lo stadio, ma è stato significativamente ridotto con l'aumento del numero di MLN. D'altra parte, i livelli di espressione di IL-21 sono aumentati significativamente con la presenza di invasione vascolare. La densità di CD4 + è stata ridotta con un aumento dello stadio T, invasione vascolare mentre la densità di CD8 + è diminuita solo con l'invasione vascolare.

Conclusioni: IL-32 espresso dal microambiente tumorale rivela che l'espressione è aumentata per controllare la crescita del tumore, ma i livelli sono diminuiti con l'aumento del numero di MLN che potrebbe essere dovuto alla diminuzione della densità cellulare CD4 +. I cambiamenti su IL-21 e IL-32 insieme ai cambiamenti sulla densità delle cellule immunitarie, indicano il loro ruolo nella crescita del tumore e nell'invasione nel cancro del colon.

References

1. Macarthur M, Hold GL, El-Omar EM: *Inflammation and Cancer II. Role of chronic inflammation and cytokine gene polymorphisms in the pathogenesis of gastrointestinal malignancy.* Am J Physiol Gastrointest Liver Physiol, 2004; 286(4):G515-20.

2. Coussens LM, Werb Z: *Inflammatory cells and cancer: Think different!.* J Exp Med, 2001; 193(6):F23-26.

3. Siegel R, Naishadham D, Jemal A: *Cancer statistics, 2012.* CA Cancer J Clin, 2012; 62(1):10-29.

4. Canna K, McArdle PA, McMillan DC, et al.: *The relationship between tumour T-lymphocyte infiltration, the systemic inflammatory response and survival in patients undergoing curative resection for colorectal cancer.* Br J Cancer, 2005; 92(4):651-54.

5. Galon J, Costes A, Sanchez-Cabo F, et al.: *Z. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome.* Science, 2006; 313(5795):1960-964.

6. Kim SH, Han SY, Azam T, Yoon DY, Dinarello CA.: *Interleukin-32: a cytokine and inducer of TNFalpha.* Immunity, 2005; 22(1): 131-42.

7. Kobayashi H, Lin PC: *Molecular characterization of IL-32 in human endothelial cells.* Cytokine, 2009; 46(3):351-58.

8. Nishida A, Andoh A, Inatomi O, Fujiyama Y: *Interleukin-32 expression in the pancreas.* J Biol Chem, 2009; 284(26):17868-76.

9. Shioya M, Nishida A, Yagi Y, et al.: *Epithelial overexpression of interleukin-32alpha in inflammatory bowel disease.* Clin Exp Immunol, 2007; 149(3): 480-86.

10. Yun HM, Park KR, Kim EC, Han SB, Yoon do Y, Hong JT:

IL-32 γ enhances TNF-α-induced cell death in colon cancer. Oncotarget, 2015; 6(11):9061-9072.

11. Park ES, Yoo JM, Yoo HS, Yoon DY, Yun YP, Hong J: *IL-32 γ enhances TNF-α-induced cell death in colon cancer.* Mol Carcinog, 2014; 53 (Suppl 1):E23-35.

12. Tangye S SG: *Advances in IL-21 biology-enhancing our understanding of human disease.* Curr Opin Immunol, 2015; 34:107-15.

13. Jauch D, Martin M, Schiechl G, et al.: *Interleukin 21 controls tumour growth and tumour immunosurveillance in colitis-associated tumorigenesis in mice.* Gut, 2011; 60(12):678-86.

14. Mantovani A, Allavena P, Sica A, Balkwill F: *Cancer-related inflammation.* Nature, 2008; 454(7203):436-44.

15. Stolfi C, Rizzo A, Franzè E, et al.: *Involvement of interleukin-21 in the regulation of colitis-associated colon cancer.* J Exp Med, 2011; 208(11): 2279-290.

16. Gallo V, Egger M, McCormack V, Jet al.: *Strengthening the reporting of observational studies in epidemiology. Molecular epidemiology STROBE-ME: An extension of the STROBE statement.* J Clin Epidemiol, 2011; 64(12):1350-363.

16. Livak KJ, Schmittgen TD: *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001; 25(4): 402-08.

17. Angell H, Galon J: *From the immune contexture to the Immunoscore: the role of prognostic and predictive immune markers in cancer.* Curr Opin Immunol, 2013; 25(2): 261-67.

18. Galon J, Fridman WH, Pagès F: *The adaptive immunologic microenvironment in colorectal cancer: A novel perspective.* Cancer Res, 2007; 67(5): 1883-886.

19. Dunn GP, Old LJ, Schreiber RD: *The three Es of cancer immunoediting.* Annu Rev Immunol. 2004; 22: 329-60.

20. Burkholder B, Huang RY, Burgess R, et al.: *Tumor-induced perturbations of cytokines and immune cell networks.* Biochim Biophys Acta, 2014; 845(2): 182-201.

21. West NR, McCuaig S, Franchini F, Powrie F: *Emerging Cytokine networks in colorectal cancer.* Nat Rev Immuno, 2015; 15(10): 615-629.

22. Bindea G, Mlecnik B, Fridman WH, Galon J: *The prognostic impact of anti-cancer immune response: A novel classification of cancer patients.* Semin Immunopathol, 2011; 33(4):335-40.

23. Fridman WH, Dieu-Nosjean MC, Pagès F, et al.: *The immune microenvironment of human tumors: General significance and clinical impact.* Cancer Microenviron, 2013; 6(2):117-22.

24. Terrazas JM, Val-Bernal JF, Buelta L: *Staging of carcinoma of the colon and rectum.* Surg Gynecol Obstet, 1987; 165(3): 255-59.

25. Fridman WH, Pagès F, Sautès-Fridman C, Galon J: *The immune contexture in human tumours: Impact on clinical outcome.* Nat Rev Cancer, 2012; 12(4): 298-306.

26. Lee HJ, Liang ZL, Huang SM, et al.: *Overexpression of IL-32 is a novel prognostic factor in patients with localized clear cell renal cell carcinoma.* Oncol Lett. 2012; 3(2): 490-96.

27. Mlecnik B, Tosolini M, Kirilovsky A, et al.: *Histopathologic-based prognostic factors of colorectal cancers are associated with the state of the local immune reaction.* J Clin Oncol, 2011; 29(6): 610-18.

28. Pagès F, Berger A, Camus M, et al.: *Effector memory T cells, early metastasis, and survival in colorectal cancer.* N Engl J Med, 2005; 353(25): 2654-666.

29. Bindea G, Mlecnik B, Angell HK, Galon: *The immune landscape of human tumors: Implications for cancer immunotherapy.* Oncoimmunology, 2014; 3(1): e27456.

30. Galon J, Pagès F, Marincola FM, et al.: *The immune score as a new possible approach for the classification of cancer.* J Transl Med, 2012; 10:1.

31. Bindea G, Mlecnik B, Tosolini M, et al.: *Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer.* Immunity, 2013; 39(4):782-95.