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The link between serum ACKR2 level and Crohn's Disease and its activity

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Ethics Committee Approval
The study was approved by the Noninvasive Clinical Research Ethics Committee of Kanuni Sultan Suleyman Training and Research Hospital (Decision number: KAEK/2020.06.96, Dated: 06.2020).
All procedures in this study involving human participants were performed in accordance with the 1964 Helsinki Declaration and its later amendments.

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No conflict of interest was declared by the authors.

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Abstract

Background/Aim: Atypical chemokine receptor (ACKR) family suppresses chemokine response and keeps the inflammatory state under control. This study investigated ACKR2 serum levels, which are thought to have an effect on the extreme inflammatory state in Crohn's disease (CD).
Methods: Active and newly diagnosed Crohn's patients under treatment and a healthy control group were included in this prospective case-control study. Patients under the age of 18 years and those with Crohn's disease in remission were excluded. Clinical, demographic, laboratory parameters and serum ACKR2 levels of the patients were examined. Disease activity was evaluated using the simplified endoscopic score for Crohn's disease (SES-CD) and Crohn's disease activity index (CDAI) index. The relationship between disease activity and serum ACKR2 was evaluated using the Spearman correlation analysis.
Results: A total of 119 subjects (66 CD patients and 53 healthy controls) were included in the study. Serum ACKR2 level was significantly lower in the CD group (4.80 ng/mL) compared to the control group (11.15 ng/mL) (P<0.001). In the correlation analysis between ACKR2 level and disease activity indicators, there was a weak positive correlation with SES-CD and CDAI (r = 0.350 P=0.004, r = 0.252, P=0.041, respectively).
Conclusion: Our data show that the ACKR2 level in active CD is quite low compared to the control group. Despite the increase in disease activity, it is not upregulated at a sufficient level and may have adverse effects on the progression of the disease.

Keywords: ACKR2, Chemokine, Crohn's disease, Inflammation

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Introduction

Chemokines are cytokines which interact with target cells by binding to the receptors of the G protein family, which extend transmembranously [1]. Cytokines are the major regulators of the inflammatory response, playing major roles in the recruitment and activation of the immune cells to the inflammatory site, and in the correct development of the adaptive immune response, which determines immunological memory [2]. Homeostatic chemokines are produced under stable conditions and regulate leukocyte migration [3]. However, inflammatory chemokines are mostly produced under pathological conditions and together with pro-inflammatory factors such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α), and they actively participate in the inflammatory response that draws immune cells to the injury site [4].

Among the chemokine receptors are the decoy receptors called atypical chemokine receptors (ACKR) which negatively regulate chemokine function. ACKRs contain four types of receptors including ACKR1, ACKR2, ACKR3 and ACKR4 [5]. They are structurally similar to conventional chemokine receptors (cCKR) and can clear, separate and regulate chemokines to control cCKR-induced responses. Thus, they can limit chemokine responses and inflammation [6]. The removal of chemokines and other inflammatory cytokines is critical during resolution of an inflammation and when evaluated together with lymphatic drainage, ACKRs have an important function in this respect [7]. One of these molecules, ACKR2, is a high-affinity receptor for multiple inflammatory CC-chemokines [8].

Lymphatic endothelial cells (LEC) are the most important source of ACKR2 located in small lymphatics, the villi of the small and large intestines, the large common lymphatics located in the lamina propria of the colon and in the muscle layer [9]. ACKR2 clears CC chemokine ligand-2 (CCL2), which is expressed by LECs and is a chemotactic chemokine for monocytes, reducing the accumulation of macrophages, which has a key role in the development of new lymphatics. In this study conducted on mice, it was emphasized that hyper-branched lymphatics developed in ACKR2 deficiency [7].

There are a limited number of studies examining the relationship between gastrointestinal system diseases and ACKR2 in humans, and one found that ACKR2 expression decreased in patients with colon cancer, which was associated with more invasive tumors [10]. In a study conducted on ACKR2-deficient mice infected with Mycobacterium tuberculosis, which is frequently used in the differential diagnosis of CD and causes a granulomatous disease, survival was lower than that in wild type (WT) mice. Also, a higher mononuclear cell count, as well as higher proinflammatory cytokine and CC chemokine levels were detected [11].

Impaired cytokine balance and impaired interactions between antigen presenting cells are considered the key factors in the chronicity of Crohn's disease [12]. Based on this, we planned the first study in the literature researching the relationship between ACKR2, a member of the chemokine family, and Crohn's disease.

Materials and methods

Ethical approval

The study was approved by the Noninvasive Clinical Research Ethics Committee of Kanuni Sultan Suleyman Training and Research Hospital (Decision number: KAEK/2020.06.96, Dated: 06.2020). Informed consents of all patients were obtained.

Study design

Patients with active CD and a Crohn's Disease Activity Index (CDAI) above 150 points, and those referred to our center with a pre-diagnosis of CD between June 2020 and January 2021 were evaluated in this prospective case-control study. All patients with luminal CD confirmed according to the standard criteria were included [13].

Endoscopies were performed by a gastroenterologist using Fujinon Video Colonoscope EC-580RD-L (Fujifilm ™ Europe, Düsseldorf, Germany) colonoscopy devices.

Montreal classification was used to define disease involvement site (L1: Ileal, L2: Colonic, L3: Ileocolonic, L4: Isolated upper disease) and disease behavior (B1: Non-stricturing, non-penetrating, B2: Stricturing, B3: Penetrating, p: Modified perianal disease) [14].

Simplified endoscopic score for Crohn's disease (SES-CD) [15] and CDAI [16] were used to assess disease activation. CDAI score was calculated and recorded electronically on the day of the endoscopy. Blood samples were obtained from the patients for analysis on the same day. The current treatments and additional diseases of the patients were recorded by querying the hospital online data and patient files.

Inclusion and exclusion criteria

Inclusion criteria: Presence of active Crohn's disease meeting the criteria [13].

Exclusion criteria: 1) Patients under the age of 18 years, 2) Crohn's disease in remission, 3) Patients diagnosed with malignancy.

Laboratory tests

Blood samples of the patients were taken into tubes with Ethylenediaminetetraacetic acid (EDTA), and hematological analysis of the samples was performed on the XN-900 (Sysmex™, Japan) device. The biochemistry parameters were analyzed in the SF-8200 (Roche™ Cobas 8000, USA) device with the serum obtained by centrifuging the blood samples taken into the gel tube at 3500 rpm for 10 minutes at room temperature. A part of the sample was immediately separated and stored at -80ºC.

The ACKR2 level was quantitatively determined from the serum samples of the patients stored at -80°C in the central laboratory of the second hospital with the ELISA method (Addcare ELISA 200, P.R. China) using the ELISA kit on the device (SinoGeneClon Biotech, Hangzhou, China-SG-16750). ACKR2 analysis was performed by the method described:

Purified ACKR2 antibody was used to coat the plate, solid phase antibody was made, then ACKR2 was added to the wells, and the ACKR2 antibody was combined with the labeled Horseradish peroxidase (HRP) to form an antibody-antigen-enzyme-antibody complex. After complete washing, Tetramethylbenzidine (TMB) substrate solution was added. TMB substrate turns blue when catalyzed by the enzyme HRP,

the reaction was terminated by adding stop solution and the color change was measured at 450 nm wavelength. The ACKR2 concentration in the samples was then determined by comparing the optical density of the samples with the standard curve.

Statistical analysis

The data were analyzed using the Statistical Package for the Social Sciences v22 (SPSS, Inc, Chicago IL, USA). Conformity of continuous variables to normal distribution was evaluated using visual (histogram and probability graphs) and analytical methods (Kolmogorov-Smirnov / Shapiro-Wilk tests). In the descriptive statistics, categorical variables were given as numbers and percentages, data showing normal distribution were presented as arithmetic mean and standard deviations, data not suitable for normal distribution were given as median (minimum-maximum) values. Continuous variables among independent groups were analyzed with the Kruskal Wallis test and the Mann-Whitney U test. Pearson chi-square ($\chi 2$) test was used in the comparison analysis for categorical variables among independent groups. In cases where the variables were not normally distributed, a nonparametric test of Spearman correlation was used instead.

Results

A total of 119 subjects were included in the study, including 66 CDs and 53 healthy controls. The mean age of the control group was 31.56 (7.07) years, and the mean age of CD was 35.89 (11.42) years. Gender distribution in the control (47.2% male, n=25 and 52.8% female, n=28) and the CD groups (77.3% male, n=51 and 22.7% female, n=15) significantly differed (P=0.001).

Clinical findings in CD

In the CD patient group, the number of newly diagnosed and untreated patients was 38 (57.6%) and the number of patients receiving treatment was 28 (42.4%). The median disease duration in the treated group was 3 (1-20) months.

According to the Montreal classification, the disease type and the rates of involvement were as follows: B1 (non-stricturing, non-penetrating: 50%, n= 33), B2 (stricturing: 24%, n = 16), B3 (penetrating: 25%, n = 17), L1 (ileal: 19.7%, n = 13), L2 (colonic: 33%, n = 22) and L3 (ileocolonic: 47%, n = 31). Perianal disease was present in 4.5% (n = 3) of the cases. While 89.4% (n = 59) of the patients did not have any rheumatological disease, 10.6% (n = 7) had an additional rheumatological disease. Of the patients who received treatment, 15 (53.6%) were treated with Azathioprine (AZT), 10 (35.7%) with AZT + anti-TNF, 1 (3.5%) with AZT + steroid and 2 (7.1%) were taking only anti-TNF (Table 1).

Laboratory findings

The comparison between ACKR2 and some laboratory parameters in the control and CD groups is summarized in Table 2. According to the analysis, ACKR2, albumin, hemoglobin and lymphocyte values of the CD group were significantly lower, while CRP, neutrophil and platelet (PLT) counts were significantly higher compared to the controls (P<0.05 for all).

The correlation analysis between ACKR2 and some laboratory parameters, SES-CD and CDAI is summarized in Table 3. There was a weak positive correlation between ACKR2, SES-CD and CDAI (r = 0.350, P=0.004; r = 0.252, P=0.041, respectively), and a weak negative correlation between ACKR2 and albumin (r = -0.253, P=0.040, respectively). No correlation was found between other laboratory parameters and ACKR2 (P>0.05).

Table 1: Clinical findings of the study population

Variables	n (%)
Age (years)	35.89 (11.42)
Females	15 (22.7)
Males	51 (77.3)
Disease location	
L1 (ileal)	13 (19)
L2 (colonic)	22 (33)
L3 (ileocolonic)	31 (47)
L4 (isolated upper GI)	0 (0)
Luminal disease behavior	
B1 (non-stricturing, non-penetrating)	33 (50)
B2 (stricturing)	16 (24)
B3 (penetrating)	17 (25)
Perianal involvement	3 (4.5)
Disease duration, median, (months)*	3 (1-20)
Current smoker	5 (7.57)
Treatment	28 (42.4)
Azathioprine	15 (53.6)
Anti-TNF alfa + Azathioprine	10 (35.7)
Corticosteroids + Azathioprine	1 (3.5)
Anti-TNF alfa	2 (7.1)

Table 2: Comparison of laboratory parameters in CD and control group

	Control (n=53)	CD (n=66)	*P*-value
ACKR2 (ng/mL)	11.15 (3.43-67.08)	4.80 (0.42-23.10)	<0.001
Albumin (g/L)	40.00 (33.00-45.00)	35.50 (24.00-40.00)	<0.001
CRP (mg/L)	0.85 (0.09-5.16)	27.50 (0.60-248.0)	<0.001
Hemoglobin(gr/dL)	13.9 (11.00-16.40)	12.50 (4.90-16.20)	0.001
Platelet ($x10^9$/L)	264 (164-369)	360 (155-791)	<0.001
Neutrophil ($x10^9$/L)	3.53 (1.95-8.71)	6.15 (2.00-16.00)	<0.001
Lymphocyte ($x10^9$/L)	2.30 (1.10-4.70)	1.80 (0.40-3.80)	0.001

Mann-Whitney U test. CD: Crohn's disease, ACKR2: atypical chemokine receptor 2, CRP: C-reactive protein

Table 3: Examining the relationship between ACKR2 and clinical-laboratory parameters

	r	*P*-value
ACKR2		
CDAI score	0.252	0.041
SES-CD score	0.350	0.004
Disease duration (month)	0.092	0.463
Albumin (g/L)	-0.253	0.040
CRP (mg/L)	0.210	0.091
Hemoglobin (gr/dL)	-0.033	0.794
Platelet ($x10^9$/L)	-0.067	0.595
Neutrophil ($x10^9$/L)	0.127	0.308
Lymphocyte ($x10^9$/L)	-0.239	0.053

Spearman correlation analysis. ACKR2: atypical chemokine receptor 2, CDAI: Crohn's disease activity index, SES-CD: simplified endoscopic score for Crohn's disease, CRP: C-reactive protein.

Comparison of serum ACKR2, C-reactive protein (CRP), albumin levels and SES-CD and CDAI scores between the groups according to the Montreal classification is presented in Table 4. In the comparison made according to disease behavior, no statistically significant difference was found between these parameters (P>0.05). Serum ACKR2, CRP and SES-CD scores were significantly lower in the L1 (ileal) group when compared in terms of disease localization (P<0.05).

The median (min-max) ACKR2 levels in the newly diagnosed and treated groups were 4.48 ng/mL (0.42-23.10), and 5.05 ng / mL (1.20-22.08), respectively. According to this analysis, although serum ACKR2 levels were higher in Crohn's patients receiving treatment, the difference was not significant (P=0.673).

Table 4: Comparison of clinical-laboratory parameters according to Montreal classification on CD.

	ACKR2 (ng/mL)		CRP (mg/L)		Albumin (g/L)		CDAI		SES-CD	
	Median (min-max)	*P*-value	Median (min-max)	*P*-value	Median (min-max)	*P*-value	Median (min-max)	*P*-value	Median (min-max)	*P*-value
B1	3.96 (0.42-23.10)	0.076	26.00 (0.60-217)	0.569	36.0 (25.0-40.0)	0.108	210 (160-490)	0.146	7 (4-12)	0.083
B2	6.54 (1.20-22.98)		28.50 (1.00-152)		34.5 (28.0-38.0)		250 (180-460)		9.5 (7-12)	
B3	6.51 (1.70-18.13)		34.00 (3.00-248)		33.0 (24.0-40.0)		310 (180-580)		8 (5-12)	
L1	3.22 (0.42-7.18)	0.002	12.00 (0.60-72.0)	0.010	38.0 (34.0-40.0)	0.066	190 (166-420)	0.104	6 (4-10)	0.029
L2	5.10 (1.81-23.10)		45.00 (1.0-217)		34.0 (25.0-38.0)		233 (160-490)		7.5 (4-12)	
L3	6.51 (1.20-22.98)		32.00 (3.0-248)		34.0 (24.0-40.0)		280 (160-580)		10 (5-12)	

The Kruskal Wallis test was used. CD: Crohn's disease, ACKR2: atypical chemokine receptor 2, CRP: C-reactive protein, CDAI: Crohn's disease activity index, SES-CD: simplified endoscopic score for Crohn's disease, Montreal classification: B1: non-stricturing, non-penetrating, B2: stricturing, B3: penetrating, L1: ileal, L2: colonic, L3: ileocolonic

Discussion

There are important studies investigating autoimmune and inflammatory mechanisms related to ACKR 2. It has become an interesting molecule over time due to its contribution to the regulation of T lymphocyte functions and since it prevents inappropriate accumulation of chemokines and immune cells in inflamed tissues [17].

Mc Kimme et al. stated that ACKR2 in LECs was a necessary molecule for the development of mature dendritic cells and the efficiency of antigen presentation [18].

In a study conducted to investigate susceptibility to autoimmunity, experimental autoimmune encephalomyelitis modeling was performed. It has been reported that in mice with ACKR2 deficiency and disease symptoms, this may occur because of dendritic cell migration and T-cell priming [19].

Hansell et al. stated that ACKR2 deficiency in mice impaired autoreactive T cell priming, could not suppress autoimmune pathology and caused subtle changes in the development of the disease by inducing arthritic and neuropathic autoimmunity [20].

ACKRs also have important roles in the control of inflammatory events. Lymphatic endothelial cells in the skin express ACKR2 to prevent them from being coated with inflammatory chemokines. This is especially important in terms of stopping the leukocytes from accumulating around the vessels and interfering with the flow of tissue fluid and mature dendritic cells [21]. In a study using human dermal LECs, ACKR2 was upregulated by the inflammatory mediators IL-6, type-I interferon (IFN) and IFN- and noted that ACKR2 plays a role in suppressing inflammatory leukocyte binding to lymphatic endothelial surfaces [18]. In an animal model, psoriasiform lesions could be controlled with ACKR2 stimulated by IFN- applied to the lesion edges on the skin. The authors also emphasized that a systemic ACKR2 induction could be used as a therapeutic strategy [22].

In a mouse model of dextran sulfate sodium (DSS)-induced colitis, ACKR2-deficient mice were found to have increased levels of inflammatory chemokines and increased intestinal inflammation, weight loss, and disease activity index compared to WT. In WT mice, on the other hand, ACKR2 was overexpressed by the lymphatic venules and had a protective role against intestinal inflammation [23].

In our study, ACKR2 level was significantly lower in the CD group compared to the control group ($P<0.05$). There was a weak positive correlation between ACKR2 level, SES-CD and CDAI. This shows us that despite the increase in disease activity, there is not enough ACKR2 upregulation. Serum ACKR2 level was significantly lower in the L1 (ileal) group ($P<0.05$). This can be explained by the lower CDAI and SES-CD scores in the L1 group compared to the other groups.

Limitation

Assessment could be more accurate with evaluation of ACKR2 gene polymorphisms among the CD and control groups. Thus, both the effects of ACKR2 deficiency on susceptibility to CD and why it could not be induced at a sufficient level despite the increase in disease activity could be partially answered, and its effects on the course of the disease could be discussed from another perspective. This could be the subject of future research.

Conclusion

ACKR2 level was lower in active CD in our study. This evidence may conclude that ACKR2-mediated chemokine clearance is not sufficient in CD, which has a negative effect on the course of the disease. Given its regulatory role during inflammation and T-cell priming, ACKR2 could be seen as a novel therapeutic target that could be used to suppress chemokine-induced inflammation in chronically inflamed tissues.

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