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Title: THE RELATIONSHIP BETWEEN CD 74 LEVELS, MACROPHAGE MIGRATION INHIBITORY FACTOR GENE POLYMORPHISM AND CLINICAL FEATURES IN PATIENTS WITH ANKYLOSING SPONDYLITIS

Running Title: Macrophage Migration Inhıbitory Factor and CD 74 In Ankylosing Spondylitis

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Abstract

Objective: In this study, the primary objective was to compare CD 74 antigen levels between patients with ankylosing spondylitis (AS) and healthy controls. The secondary objective was to investigate the distribution of Macrophage Migration Inhibitory Factor (MIF) 173 G/C polymorphisms in AS patients and a control group. Finally, it was also aimed to reveal the presence of a relationship between CD 74 antigen levels and MIF 173 G/C polymorphism.

Materials and Methods: 82 healthy blood donors and 79 AS patients were enrolled in this study. MIF 173 G/C polymorphism and CD 74 levels were investigated in the patient and control groups using the ELISA method. Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), Bath Ankylosing Spondylitis Metrology Index (BASMI), Bath Ankylosing Spondylitis Functional Index (BASFI), Bath Ankylosing Spondylitis Radiology Index (BASRI), Visual Analogue Scale (VAS) and Ankylosing Spondylitis Quality of Life (ASQoL) scores were calculated and recorded.

Results: There was no significant difference between the patient and control groups in terms of age, gender, and body mass index. The median CD74 level of the patient group was 1.17 (0.93-2.1), which was significantly lower than that of the control group, i.e. 2.16 (1.6-4.41). There was no correlation between CD 74 antigen levels and BASDAI, BASMI, BASFI, BASRI, VAS and ASQoL scores. The number of patients who had the C allele was higher among the patients with AS in comparison to the control group; however, the difference was not statistically significant ($p>0.05$). There was no correlation between genotypes and BASDAI, BASMI, BASFI, BASRI, VAS and ASQoL scores ($p>0.05$). Comparison of the median CD74 levels of the individuals in the patient group according to their HLA-B27 status and genotypes did not reveal any statistically significant difference.

Conclusion: The CD74 antigen levels of the patients with AS were significantly lower compared to the control group. This implies that CD 74 is a parameter that can be used in the diagnosis of AS. Although the C allele among the MIF 173 G/C polymorphisms was more frequently observed in the patient group, the difference was not statistically significant. Moreover, there was no significant relationship between CD 74 antigen levels and polymorphisms.

Keywords: Ankylosing Spondylitis, CD 74, MIF gene, Polymorphism

Introduction

Ankylosing spondylitis (AS) is a chronic and progressive inflammatory disease characterized by axial skeletal and sacroiliac joint involvement (1). AS is the most prevalent type of spondyloarthropathies

(SpA). While it can cause enthesitis and peripheral joint involvement, it can also exhibit extraarticular involvement (2). AS onset is generally seen during late adolescence and early adulthood. One of the most important characteristics of the disease is axial involvement wherein nearly 90% of the patients exhibit radiographic sacroiliitis as the disease progresses. The diagnosis can be delayed since it can take years to observe the radiographical signs although the clinical signs of the disease are observed earlier (3).

The etiology of AS is not entirely known. However, it is thought to stem from environmental factors connected with genetic factors (4). Among the genetic factors, HLA-B27 (Human Leukocyte Antigen) is used to help diagnose AS. On the other hand, new biomarkers are needed for the diagnosis, follow-up and determination of the prognosis, since a minority of the HLA B-27-positive individuals develop AS and healthy individuals can also be HLA-B27-positive (5). From the genetic viewpoint, besides HLA-B27 AS was also found to be associated with Endoplasmic Reticulum Aminopeptidase I (ERAP1) and Tumor Necrosis Factor Alpha (TNF-α) genes (6,7).

The CD 74 antigen, which is a transmembrane glycoprotein, prevents the early binding of proteins to the MHC class II (Major Histocompatibility Complex). A CD 74 molecule has intracellular regulatory functions such as signal transduction, cell migration and endosomal trafficking (8, 9). It also plays a role in innate and acquired immunity and B cell proliferation. Pro-inflammatory cytokines are released as a result of antibody binding to the CD 74 antigen (8). Therefore, it is thought that CD 74 can be a potential factor in the etiopathogenesis of AS.

Macrophage Migration Inhibitory Factor (MIF) is a multifunctioning mediator protein that consists of 115 amino acids with enzyme, hormone and cytokine properties that can inhibit macrophage migration in vivo (10). After it is secreted extracellularly, it promotes pro-inflammatory activity and increases TNF release by affecting the immune response via autocrine, paracrine and endocrine routes. MIF also acts as a regulator against the immunosuppressive effects of glucocorticoids and hence, it was thought that MIF could be a gene associated with autoimmune-inflammatory diseases (11). It has been demonstrated that MIF is able to reverse the effects of glucocorticoids on inflammation in an antigen-related arthritis model (12). CATT tetra-nucleotide polymorphisms and 173 G/C polymorphism of the MIF gene were found to be associated with inflammation (13,14).

The CD 74 antigen located on the cell surface acts as a receptor for MIF. CD 74 and MIF binding plays an important role in maintaining cell proliferation and cell viability (15).

In designing this study, the primary objective was to determine whether CD 74 antigen levels measured with the ELISA method could be used as a parameter in the diagnosis, treatment and

follow-up of AS. The secondary objective was to investigate the role of 173 G/C polymorphism of the MIF gene in the development of AS. Furthermore, we also aimed to reveal the presence of a relationship between CD 74 antigen levels and MIF gene polymorphisms.

Materials and Methods

82 healthy subjects and 79 patients diagnosed with ankylosing spondylitis (AS) who presented at University Medical Faculty Research Hospital, Physical Medicine and Rehabilitation Department/Rheumatology outpatient clinic between December 2016-April 2017 were enrolled in this study. Patients with ankylosing spondylitis were selected according to the Modified New York criteria. Blood donors who did not have any chronic diseases and/or history of inflammatory disease were recruited as the control group. Patients with an inflammatory disease besides AS, pregnant or breast-feeding women, and those with a history of malignancy were not included in the patient group. Consent was obtained from all individuals included in the study before participating in the study. The study was approved by University Ethics Committee (28.11.2016-308) and the study was performed according to the Declaration of Helsinki.

The patients were asked about their age, gender, height, weight, date of onset of complaints associated with the disease, duration of the disease, involvements, drugs prescribed, family history and extraarticular involvement. The same physician performed the general physical examination and the detailed musculoskeletal examination of all patients. In the patient group, disease activity was evaluated using Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), metrologic evaluation was performed using Bath Ankylosing Spondylitis Metrology Index (BASMI), functional evaluation using Bath Ankylosing Spondylitis Functional Index (BASFI), radiologic changes using Bath Ankylosing Spondylitis Radiology Index (BASRI), nighttime and daytime pain status using Visual Analogue Scale (VAS) and quality of life using Ankylosing Spondylitis Quality of Life (AS-QoL) scale.

Erythrocyte Sedimentation Rate (ESR) and C-Reactive Protein (CRP) values were assayed from blood samples of the patients on the same day and recorded.

Genomic DNA was extracted using the salting out method from mononuclear cells obtained from peripheral venous blood treated with ethylenediaminetetraacetic acid. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to evaluate MIF gene polymorphisms. MIF-173C *rs755622 variant was identified using the following primers: 5'ACTAAGAAAGACCCGAGG-3 ', 5'-TGGAGAAAGGACCAGGAGAC-3'. MIF-173 G/C sequences were amplified to a total reaction volume of 25 µl using 100-500 ng DNA, 1.0 mM of each primer, 250 mM of each nucleotide, 1.5 U Taq polymerase, (Fermentas International, Burlington, Ontario, Canada),

and 2 ml 10x PCR buffer. PCR was performed using GeneAmp® PCR System 9700 (Applied Biosystems, Singapore).

A 330 bp fragment was amplified for MIF-173 G/C, and then digested with Alu I restriction endonuclease (Invitrogen, Carlsbad, CA, USA) at 37°C overnight. Digestion products were dissolved in 3% agarose gel and viewed under UV light. 330 bp PCR products have a consistent restriction region that leads to 62 and 268 bp fragments. GG genotype did not have a second cutting point for Alu I. CC genotype had a second cutting point and led to three fragments, i.e. 205, 62 and 63 bp fragments. The experiment was repeated for 20% of the samples in order to prevent sampling or reading errors.

The CD 74 concentration was measured using Elabscience brand ELISA kits. The measurement was carried out in accordance with the user manual in the kit. The microplates in the kit were spectrophotometrically read at 450 nm with an EL 312 Microplate brand ELISA reader. The obtained OD (Optical Density) values were used to create standard curves and concentration values were obtained in ng/ml.

Statistical Analysis

Normal distribution of numerical variables was tested using the *Shapiro Wilk test* when n<50, and the *Kolmogorov Smirnov* test when n>50. Independent *Samples t-test*, which is a parametric test, was used in comparing two independent groups when numerical variables had a normal distribution, and the *Mann Whitney U test* was used in the case of non-normal distribution. The *Kruskal Wallis test* was used to compare more than two independent groups when numerical variables did not have a normal distribution. In non-parametric tests, the differences between the two groups were compared using the *Mann Whitney U test* and evaluated using the *Bonferroni correction*. The Pearson Chi-Square test was used to compare the differences between categorical variables in 2x2 tables, and the *Fisher Freeman Halton Test* in RxC tables. Relationships between the numerical variables that did not have a normal distribution were evaluated using the *Spearman Rho Correlation Coefficient*. Statistical analyses were performed using R 3.3.2 v (open source) software and 0.05 (p-value) was used as the level of significance in statistical analyses.

Results

79 patients and 82 healthy subjects were enrolled in this study. Age, gender and body mass index distributions were similar in the patient and control groups (p>0.05 for each) (Table 1).

The mean time until diagnosis was 6.87±5.55 years in the patient group. Of the 79 patients included in the study, 29 were on Disease Modifying Drugs (DMARD) and 50 were receiving anti-TNF therapy.

The median CD74 level of the patient group was 1.17 (0.93-2.1), which was significantly lower than that of the control group, i.e. 2.16 (1.6-4.41) ($p<0.001$). The prevalence of C allele presence was higher in the patient group (29.12%) as compared to the control group (24.39%); however, the difference was not statistically significant ($p>0.05$) (Table 2).

CRP scores increased in parallel to the CD 74 levels in the patient group ($p=0.015$). Comparison of the CD 74 scores with BMI, time until diagnosis, ESR, nighttime VAS, daytime VAS, basdai, basmi, basri vertebra, basri hip, basri total and AS-QoL in the patient group did not reveal a significant linear relationship ($p>0.05$ for each).

49 individuals (62%) in the patient group were HLA-B27-positive, whereas 30 individuals (38%) were HLA-B27-negative. Comparison of the median CD74 levels of the individuals in the patient group according to their HLA-B27 status and genotypes did not reveal any statistically significant difference ($p>0.05$ for each) (Table 3).

Comparison of the CD74 levels of the individuals in the patient group according the drugs prescribed, presence or absence of arthritis, uveitis and enthesitis did not reveal any statistically significant difference in terms of the median values ($p>0.05$ for each). There was no statistically significant relationship between the genotypes and the clinical parameters of the subjects in the patient group ($p>0.05$ for each).

Discussion

AS is a chronic and progressive inflammatory disease that initially manifests with axial skeletal and sacroiliac joint involvement, and its etiology is not entirely known. Delayed diagnosis is one of the most important problems in AS. The diagnosis can be delayed for 7 to 10 years, since there is no test that can directly establish a diagnosis of AS. Therefore, new biomarkers are required for the diagnosis, follow-up and estimation of the prognosis of AS.

It is thought that many different factors may play a role in the etiology of AS together with its genetic causes. A minority of HLA-B27-positive patients develop AS. Therefore, it is asserted that HLA-B27 can only partly account for a genetic susceptibility, although it is the most commonly recognized genetic factor for the disease (16).

MIF is a molecule that plays a role in the regulation of innate and acquired immunity as well as carcinogenesis and inflammation (17, 18). Suppression of the biological activity of MIF can

significantly limit TNF-α, interferon-γ and matrix metalloproteinase production in the large intestinal tissues in an experimentally induced murine colitis model (19). The MIF gene and its polymorphisms in inflammatory diseases have been widely investigated due to the close relationship between MIF and inflammation and its role as a regulator against glucocorticoids.

MIF gene polymorphism was most commonly investigated in Rheumatoid Arthritis (RA). It was found that MIF gene polymorphism resulted in a susceptibility to the disease and was correlated with radiological progression in patients with RA. Martinez et al. found that the MIF-173 C allele in the promotor was especially associated with a susceptibility in early-onset RA patients (20). In another study, it was reported that the MIF-173 C allele or MIF CATT alleles were correlated with serum MIF levels and they could be used as prognostic factors (21).

Zheng et al. investigated MIF levels as well as MIF-173 G/C and -794 CATT polymorphism in 600 healthy subjects and 600 patients with Behçet's disease. They found that the prevalence of the C allele was higher in the patient group compared to the control group. They also found that MIF levels were higher in the patient group compared to the control group and that polymorphism could have an effect on MIF expression (22).

Przybyłowska et al. investigated MIF gene polymorphism in inflammatory bowel disease (IBD) in their study conducted on 58 ulcerative colitis patients, 41 patients with Crohn's disease and 436 healthy subjects. They found that the IBD risk was 2.02- and 1.89-fold higher in the presence of the G/C genotype and C allele, respectively. Considering the genotype distribution according to the subgroups, they found a relationship between Ulcerative Colitis and the C allele, but no statistically significant relationship between Crohn's Disease and polymorphism (23).

Gürel et al. investigated AS and MIF-173 G/C polymorphism in the Turkish population. As a result of the study, they found that there was no relationship between AS and MIF genotype and alleles, whereas the onset of the disease was earlier in patients who had the C allele. They asserted that the C allele could lead to earlier onset of the disease (24). According to the present study, there was no significant relationship between genotype and duration of the disease, but it was found that the patients who had the C allele experienced a longer duration of the disease in comparison to the control group.

CD 74 is a critical molecule that plays a role in various processes such as cell migration, premature antigen binding, B cell maturation and continuity of cell viability wherein its association with inflammation has been clearly shown (25, 26). Antibody binding to CD74 molecules in vivo was found to cause the production of pro-inflammatory cytokines and activation in target cells (26).

Baerlecken et al. conducted a study on 216 SpA patients and 325 control patients, and evaluated the presence of antibodies against CD74. The control group consisted of 40 patients with psoriatic arthritis, 40 patients with systemic lupus erythematosus, 40 patients with HIV, 80 patients with RA and 125 blood donors, wherein the anti-CD74 antibody level was 67% in the SpA group and 6% in the control group. They asserted that antibody positivity could be used especially in the early stage of SpA and in HLA-B27-negative patients for the diagnosis of the disease with high sensitivity and specificity (8).

Baraliakos et al. conducted a study on 94 patients with axial SpA and 51 non-SpA patients (13 RA, 17 fibromyalgia, 17 degenerative spine disease, 3 psoriatic arthritis without axial involvement and 1 polymyalgia rheumatica) to investigate the prevalence of the IgG antibody against the CLIP region of CD74. They found that the prevalence of the anti-CLIP antibody was 85.1% in axial SpA patients and 7.8% in the control group. The sensitivity and specificity of anti-CLIP were 85.1% and 92.2%, respectively in axial SpA patients. In addition, anti-CLIP antibody positivity was also detected in HLA-B27-negative patients (27). Baraliakos and Baerlecken did not observe a relationship between antibody positivity, and radiological progression and disease activity in their studies. Similarly, there was no relationship between CD74 antigen levels and disease activity, radiological involvement, drug use and extraarticular involvement according to this study.

In a study by Ranganathan et al. evaluating CD74 antigen levels in AS patients, it was found that the CD74 level was statistically significantly lower in AS patients compared to the control group. Results of this study are consistent with the results of the present study. In the mentioned study, the CD 74 level was measured from monocytes obtained from peripheral blood using the flow cytometry method (28). It is suggested that this method is not suitable for routine daily use as it is more expensive and requires advanced laboratory conditions.

The present study also showed that the CD74 levels were significantly higher compared to the control group. The researchers think this can be explained by way of several mechanisms. It is thought that the N-terminal telopeptide part, i.e. the part that activates NF-Kβ, of the CD74 molecule undergoes several proteolysis processes. It is also thought that the pathway referred to as the regulated intramembrane proteolysis is more active in AS patients. Another possible mechanism could be the fact that continuous and increased MIF signal production may lead to the cleavage and consumption of the CD74 antigen (28).

Another objective of this study was to investigate the relationship between CD 74 levels and MIF gene polymorphisms due to the close association between the CD74 molecule and MIF. However, a

significant difference between polymorphism distributions and CD 74 antigen levels could not be identified. Moreover, a similar comparison could not be found in the literature.

Limitations

This study had some limitations. The mean duration of symptoms was high in the patient group and the study was a cross-sectional study. The patient group consisted of individuals who were taking drugs, and a pre- and post-treatment evaluation was not performed. Another limitation of the study was the relatively low number of patients.

Finally it is contended that CD 74 antigen levels and MIF gene polymorphisms can be used in the diagnosis, follow-up and determination of the prognosis of the disease after conducting further prospective studies with a greater number of patients.

Conclusion

Consequently, although MIF 173 G/C polymorphism is more frequently encountered in AS patients, there was no statistically significant difference. The afore-mentioned difference can be significant in studies that may be conducted in the future with larger patient groups and on different patient populations. It is suggested that different samples should be studied since the only study that investigates MIF gene polymorphism in AS patients has been conducted on the Turkish population. It is asserted herein that CD 74 antigen levels can be used in the diagnosis and follow-up of AS, whereby the ELISA method can be used in the measurement as a suitable method in daily practice. It is also proposed that such studies can prevent possible complications and delays in diagnosis as well as contributing to the development of new treatment options.

Conflict of Interest: None

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Table 1. Comparison of the demographic characteristics between the patient group and the control group

		Group		
		Patient (n=79)	**Control (n=82)**	**p**
Gender	Male	64 (81.01)	73 (89.02)	0.154*
	Female	15 (18.99)	9 (10.98)	
Age		36.01±9.14	36.63±10.31	0.687**
BMI		27.95±4.61	26.76±4.09	0.084**

*: Chi-square test was used. Descriptive statistics were provided in numbers (%).

**: Independent samples t test was used. Descriptive statistics were expressed as mean±standard deviation.

Table 2. Comparison of CD 74 antigen levels and polymorphisms between the patient group and the control group

		Group		
		Patient (n=79)	**Control (n=82)**	**p**
CD74		1.17 (0.93-2.1)	2.16 (1.6-4.41)	**p<0.001****
Genotype	GG	56 (70.89)	62 (75.61)	0.581*
	GC	20 (25.32)	19 (23.17)	
	CC	3 (3.8)	1 (1.22)	

*: Chi-square test was used. Descriptive statistics were provided in numbers (%).

**: Mann Whitney U test was used. Descriptive statistics were expressed as median values (Q1-Q3).

Table 3. Comparison of CD 74 antigen levels according to HLA-B27 and polymorphism status in the patient group

		CD 74	
		M(Q1-Q3)	**p**
HLA-B27	HLA-B27+ (n=49)	1.39 (0.89-2.27)	0.377*

	HLA-B27- (n=30)	1.08 (0.97-1.48)	
Genotype	GG (n=56)	1.17 (0.9-2.06)	
	GC (n=20)	1.1 (0.93-1.855)	0.077**
	CC (n=3)	5.24 (1.77-11.82)	

*: Mann Whitney U test was used. Descriptive statistics were expressed as median values (Q1-Q3).

**: Kruskal Wallis test was used. Descriptive statistics were expressed as median values (Q1-Q3).