



Micronucleus Analysis in Behçet's Disease With and Without HLA-B51

HLA-B51 Pozitif ve Negatif Behçet Hastalığında Mikronükleus Analizi

Doğan Nasır BİNİCİ, Ali KARAMAN*, Melek KADI**

Erzurum Training and Research Hospital, Department of Internal Medicine, Erzurum, Turkey

*Erzurum Nenehatun Obstetrics and Gynecology Hospital, Department of Medical Genetics, Erzurum, Turkey

**Erzurum Training and Research Hospital, Department of Dermatology, Erzurum, Turkey

Summary

Objective: Genetic factors are considered to play important roles in the development of Behçet's disease (BD). Micronucleus (MN) assay is a sensitive indicator of caused genomic damage. Our aim was to investigate whether human leucocyte antigen (HLA)-B51-positive patients had higher MN frequency than those without HLA-B51.

Materials and Methods: This study was conducted between May 2009 and January 2010 in Erzurum Training and Research Hospital. We analyzed MN frequency in lymphocytes of 40 patients with BD and 30 healthy controls. For HLA-B51 typing, DNA was extracted from ethylenediaminetetraacetic acid blood samples and HLA-B5 allele genotyping was performed by the polymerase chain reaction (PCR)-sequence specific primer method.

Results: Twenty-two of 40 patients with BD (55%) and 9 of 30 controls (30%) were HLA-B51-positive. The MN frequency was significantly increased in BD patients compared with controls (3.12 ± 0.81 vs 1.64 ± 0.51 , $p < 0.0001$). The MN frequencies in HLA-B51-positive patients were higher than in HLA-B51-negative ones ($p < 0.001$) with BD, whereas no difference was detected in the control group ($p > 0.05$).

Conclusion: This study revealed that there was a significant association between elevated MN frequency and existence of HLA-B51 in patients with BD. *Turk J Phys Med Rehab 2013;59:36-41.*

Key words: Behçet's disease, HLA-B51, micronucleus

Özet

Amaç: Genetik faktörlerin Behçet hastalığı (BH) gelişiminde önemli rol oynadığı düşünülmektedir. Mikronükleus testi (MN) genomik hasarın sensitif bir belirteçidir. Bu çalışmada HLA-B51 pozitif ve negatif hastalarda yüksek mikronükleus sıklığının farklı olup olmadığını araştırmak amaçlandı.

Gereç ve Yöntem: Bu çalışma Mayıs 2009 ve Ocak 2010 tarihleri arasında Erzurum Eğitim ve Araştırma Hastanesinde yapıldı. 40 BH'li ve 30 sağlıklı kontrolün lenfositlerinde MN sıklığını analiz ettik. HLA-B51 tiplendirmesi için kan örneklerinden etilendiamintetraasetik asit ile DNA elde edildi ve HLA-B51 allel genotiplemesi dizi spesifik primer polimeraz zincir reaksiyonu metodu ile yapıldı.

Bulgular: Kırk BH'li hastanın 22'sinde (%55) ve 30 kontrolün 9'unda (%30) HLA-B51 pozitif bulundu. MN sıklığı BH'li hastalarda kontrollere göre belirgin olarak yüksekti ($3,12 \pm 0,81$ ve $1,64 \pm 0,51$, $p < 0,0001$). MN sıklığı HLA-B51 pozitif hastalarda HLA-B51 negatif olanlardan yüksekti ($p < 0,001$), Kontrol grubunda farklılık tesbit edilmedi ($p > 0,05$).

Sonuç: Bu çalışma HLA-B51 pozitif hastalar ve yüksek MN sıklığı arasında belirgin bir birliktelik olduğunu açıklamaktadır. *Türk Fiz Tıp Rehab Derg 2013;59:36-41.*

Anahtar Kelimeler: Behçet hastalığı, HLA-B51, mikronükleus

Introduction

Behçet's disease (BD) is a recurrent chronic inflammatory, multisystem disease. The main clinical manifestations contains recurrent oral and genital ulceration, uveitis, and erythema

nodosum (1). BD occurs worldwide, however, it is most frequently seen in Turkey and Japan. The exact etiology of BD is unclear. However, many studies suggest that autoimmunity and genetic factors play a role in the pathogenesis (2,3).

Human leukocyte antigen-B51 (HLA-B51), intercellular adhesion molecule-1 (ICAM-1) and tumor necrosis factor- α (TNF- α) and many other gene polymorphisms has been associated with BD (3,4). Among these genes, HLA-B51 is the most strongly associated gene with BD (5-9). HLA-B51 is the primary gene involved in the pathogenesis of BD that can aid the diagnosis (10).

Micronucleus (MN) occurs acentric chromosome fragments or whole chromosomes during mitotic cell division and as small additional nuclei, appears in the cytoplasm of interphasic cells (11). The MN frequency test is a sensitive marker of genomic damage and genotoxicity (12). MN frequency may be induced by oxidative stress, exposure to clastogens or aneugens, some genetic defects in cell cycle checkpoint and/or DNA repair genes, and chromosome segregation errors (13-15). Certain reports have measured MN frequency in some skin diseases such as Bloom's syndrome and ataxia telangiectasia. Furthermore, some studies have shown increased MN frequency in some autoimmune diseases such as rheumatoid arthritis, systemic sclerosis and type-1 diabetes mellitus (13,16-19).

The aim of the study was to show if there is a correlation between HLA-B51 and MN frequency in patients with BD.

Materials and Methods

Patients

This study was conducted between May 2009 and January 2010 in Erzurum Training and Research Hospital. The exclusion criteria were prior chemotherapy or radiotherapy, drug use and smoking within the previous 4 months, which may increase MN frequency. The patient and control groups were chosen for their similar habits. The hospital Ethics Committee approved the human study. We obtained written informed consent from each participant. All patients were analyzed prior to treatment.

Cell Culture

For MN analysis, 3 ml of heparinized blood was drawn from each individual. Lymphocyte cultures were established by adding 0.5 ml of whole blood to 5 ml karyotyping medium (Biological Industries, Beit Haemek, Israel) with 2% phytohaemagglutinin M (PHA; Biological Industries) according to standard techniques (20). The cultures were incubated at 37°C for 72 h. All slides were coded and read blind.

Micronucleus Analysis

Cytochalasin B (6 μ g/ml, Sigma, USA) was added after 44 h of culture to block cytokinesis, allowing identifying lymphocytes dividing in culture. The culture was kept at 37°C for 72 h. Cells that have undergone the first mitosis are thus recognized as binucleated cells and are selectively screened for the presence of MN. The cells were then treated hypotonically with 0.075 molar KCl for 5 min at room temperature, and fixed in methanol/acetic acid (3:1). Cells were dropped onto slides and stained with 5% Giemsa in phosphate buffer (pH 6.8) for 5 min. About 1,000 binucleated cells (mean \pm SD=1007.41 \pm 6.95, range: 995-1022) from each case were examined for MN by an experienced observer in the same laboratory (21).

HLA class I Genotyping

HLA-B low-resolution typing was performed using OLERUP-HLA-B (OLERUP SSP AB, Saltsjöbaden, Sweden) typing kit (lot #91) according to the manufacturer's instructions.

Statistical Analysis

Mann Whitney U-test was used to compare the frequency of MN in control and patient groups. To evaluate the correlation of MN frequency with, age and sex Pearson's correlation coefficients were calculated. A p value of less than 0.05 was considered statistically significant.

Results

Twenty-two of 40 patients with BD (55%) and 9 of 30 controls (30%) were HLA-B51-positive. A total of 40 patients with BD (19 women, 21 men) were included in the study. The age of BD patients in HLA-B51-positive and negative groups were similar (ranged from 19 to 65 years) ($p>0.05$). The control group consisted of 30 healthy renal transplant and bone marrow transplant donors (13 women, 17 men). The controls were norelatives. The age of controls ranged from 19 to 52 years. The association of BD with MN frequency in HLA-B51-negative and positive groups is shown in Table 1 and 2. Similarly, the association of controls with MN frequency in HLA-B51-negative and positive groups is shown in Table 3. According to these results, the MN frequency in BD patients

Table 1. Sex, age and micronucleus (MN) frequencies for HLA-B51 positive patients with Behçet disease (BD).

Case	Sex	Age (years)	HLAB51	MN/1,000 BN Mean
1	M	34	+	3.62
2	F	30	+	3.49
3	M	24	+	3.63
4	F	21	+	3.00
5	M	23	+	4.58
6	M	36	+	3.34
7	M	41	+	3.55
8	F	35	+	3.89
9	M	30	+	3.13
10	M	35	+	3.80
11	M	34	+	5.10
12	M	31	+	3.70
13	F	28	+	3.41
14	M	59	+	2.55
15	F	35	+	3.35
16	F	40	+	3.86
17	M	33	+	4.10
18	F	37	+	3.77
19	F	29	+	3.12
20	F	22	+	1.59
21	M	26	+	3.43
22	F	33	+	3.88
Mean \pm SD		35.7 \pm 8.1		3.54 \pm 0.68

BN: Binucleated cells

with positive HLA-B51 was significantly increased, compared to the ones with negative HLA-B51 (Table 4, $p < 0.001$). Regardless of HLA-B51, patients with BD showed an increased frequency of MN as compared with the control group (Table 4, $p < 0.001$). In the control group, no significant difference could be detected between HLA-B51-positive and HLAB51-negative individuals in terms of MN frequency (Table 4, $p > 0.05$). MN frequency did not correlate with age or sex in the BD patients ($r = 0.087$, $p > 0.05$ and $r = 0.114$, $p > 0.05$, respectively).

Discussion

The immunopathogenic mechanism of BD remains unclear. However, infectious agents, immune mechanisms and genetic factors have been suggested to be effective for the onset of this disease. Chromosomal instability and HLA-B51 type appear to be strongly associated with the disease. Despite serious efforts to identify the contribution of HLA-B51, its role is still controversial (22). HLA-B51 is the most common HLA allele in BD patients independent of their origin. Moreover, it is known that the HLA-B51 antigens are encoded by 24 alleles. So that by subtyping HLA-B5, its association with the disease became more deviant (23) (01-24).

The main discussion problems whether HLA-B51 is a marker of susceptibility or severity in BD. The role of HLA-B51 is the presentation of endogenous antigens synthesized within the cell to CD8+ cytotoxic suppressor T cell. Whether HLA-B51-

restricted CD8+ T cells exhibit a role in the cause of BD is still unknown (24).

Recent advances in understanding of HLA class I-binding peptide motifs have enabled us to detect and characterize autoreactive CD8+ T cells involved in the pathogenesis of autoimmune diseases (25). For example, in patients with

Table 3. Sex, age, HLA-B51 and micronucleus (MN) frequencies for the controls.

Case	Sex	Age (year)	HLA-B51	MN/1,000 BN Mean
1	M	28		1.60
2	F	32		2.40
3	M	20		1.34
4	M	40	+	1.53
5	M	27		1.10
6	F	36	+	1.90
7	F	29	+	1.40
8	M	34		1.20
9	F	45		1.96
10	F	52		1.60
11	F	21	+	2.10
12	M	34		1.22
13	M	19		1.53
14	M	30	+	1.80
15	F	52	+	1.50
16	M	52		2.44
17	F	40		1.25
18	F	25		0.86
19	M	29		1.63
20	F	28	+	0.97
21	F	58		1.55
22	F	25		1.92
23	F	48	+	1.50
24	M	22		1.45
25	M	26		2.10
26	F	42		1.43
27	F	31		2.12
28	M	30	+	1.46
29	F	52		3.10
30	F	21		1.25
Mean±SD		34.26±11.25		1.67±0.33

BN: Binucleated cells

Table 2. Sex, age and micronucleus (MN) frequencies for HLA-B51 negative patients with Behçet disease (BD).

Case	Sex	Age (year)	HLA-B51	MN/1,000 BN Mean
1	M	48	-	2.30
2	F	50	-	1.53
3	M	32	-	2.14
4	F	37	-	1.85
5	M	44	-	3.10
6	M	24	-	3.15
7	F	19	-	2.25
8	F	32	-	3.20
9	M	37	-	1.98
10	M	28	-	2.80
11	M	33	-	2.62
12	M	48	-	3.14
13	F	33	-	3.50
14	M	31	-	3.00
15	F	65	-	2.38
16	M	24	-	3.96
17	F	35	-	2.28
18	F	31	-	1.87
Mean±SD		36.16±11.2		2.61±0.65

BN: Binucleated cells

Table 4. Mean age, HLA B51 and micronucleus (MN) frequencies across the patients with Behçet Disease (BD) and controls.

		Sex		Age, years		MN/1,000 BN		Total MN/1,000 BN
		Female/Male	Mean±SD	range	Mean±SD	range	Mean±SD	
Patient	HLA-B51 positive	12/10	34.12±9.67	21-59	3.54±0.68*	1.59-5.10	3.12±0.81*	
	HLA-B51 negative	8/10	36.16±11.2	19-65	2.61±0.65*	1.53-3.96		
Control	HLA-B51	17/13	34.26±11.25	19-58	1.67±0.33*	0.86-3.10		

*Significant at P<0.001, BN: Binucleated cells

primary biliary cirrhosis, disease-relevant CD8+ T cells to the E2 component of pyruvate dehydrogenase complexes were detected using synthetic peptides selected according to their binding affinity to HLA-A2 (26). Moreover, recognition of the endogenously generated major histocompatibility complex class I chain-related gene A (MICA) peptide by autoreactive CD8+T cells in the context of HLA-B51 may explain why HLA-B51 is a marker of susceptibility and severity in BD (27,28).

The HLA-B51 molecule is probably primarily involved in BD development because of the presence of epitopes that have a high affinity for the B51 molecule in some BD-provoking extrinsic factors. It has been documented that mononuclear cells exhibited a hypersensitivity response to certain streptococcal antigens in patients with BD but not in healthy controls (29).

On the other hand, structural similarity between dominant determinants in a foreign and self molecule has also been reported (30). A mycobacterial 65-kDa heat shock protein (hsp), having significant homology with the human 60-kDa hsp, has been shown to cross-react with strains of *Streptococcus sanguinis* and is furthermore able to up-regulate the expression of T cells in patients but not in controls (30,31). Thus, an immune response against self antigens generated by T-cell activation against bacterial antigens (the molecular mimicry model), may account for the clinical observation (32). In a new study (33), it was shown that HLA-B51 restricted CD8 T cell response was correlated with the target tissues expressing MICA*009 by stress in active BD patients with HLA-B51. Bes-1 gene and HSP-65 derived from oral streptococcus *sanguinis*, which is the uncommon serotype (KTH-1, strain BD113-20), are supposed to play important roles in BD pathogenesis. The peptides of the Bes-1 gene are highly homologous with the retinal protein Brn3b. Furthermore, the Bes-1 peptides were homologous with HSP-65 derived from microorganisms in association with the counterpart human HSP-60, which appeared reactively in BD patients (33).

The etiopathogenesis of BD is associated with numerous intrinsic and extrinsic factors. The intrinsic factors are HLA-B51 and antigen presentation, cellular and humoral immunity, neutrophil activation, antigenic stimuli, heat shock proteins, retinal-S antigen, antiendothelial cell antibodies and gender. The extrinsic factors are infections, such as with Herpes simplex virus, *Streptococcus sanguis*, *Streptococcus pyogenes*, *Streptococcus faecalis* and *Streptococcus salivarius*, *Escherichia coli*, *Klebsiella pneumoniae*, *Mycoplasma fermentans* and environmental pollution (28,34-36).

In our study, nine of 30 controls were HLA-B51-positive (30%). This result was in agreement with the study of İkbal et al (37). Similarly, in some studies, HLA-B51 frequency is observed in 18-30% of Turkish normal population (38-41). On the other hand, in our study, 22 of 40 patients with BD were HLA-B51-positive (55%). The association of HLA-B51 with BD has been confirmed in different ethnic groups and the association between HLA-B51 and some clinical features of BD have been described (42-44). The high frequency of HLA-B51 in the Turkish population may be attributed to the high prevalence of BD in Turkey.

MN test provides a measure of both chromosome breakage and chromosome loss or nondisjunction in clastogenic and aneugenic events (13). In this study, we found significantly elevated MN frequency in BD patients compared with controls. This finding was in agreement with the study of Hamurcu et al (45). On the other hand, HLA-B51-positive patients had higher MN frequency than HLA-B51-negative ones, whereas no significant difference was present between HLA-B51-positive and -negative controls. In our study, no significant relationship of MN frequency with age or sex was found in patients with BD. Age and sex, however, have been reported to influence MN frequency in the cultured peripheral blood lymphocytes of humans (46). Furthermore, some authors have examined the sister chromatid exchange (SCE) frequencies in BD patients to understand the genetic mechanism. They reported that increased SCE frequencies were observed in patients with BD (37,47).

Molecular studies have strengthened the basic association of HLA-B51 with BD. However, the exact pathogenic mechanism of the HLA-B51 molecule is still unknown. Spontaneous and/or induced over expression of pro-inflammatory cytokines from various cellular sources seems responsible for the enhanced inflammatory reaction in BD. It may be associated with the genetic susceptibility (48).

Recently, the genotoxicity of reactive oxygen species (ROS) is well established, and oxidative stress can cause genomic damage (49,50). Some results indicate that defective repair of DNA damage may lead to multisystem inflammation in BD patients. There is an increased genomic DNA damage and an increased susceptibility to cytotoxic killing by oxidative stress in lymphocytes of the patients with certain autoimmune diseases including BD (51). ROS produced in excess may cause toxic effects described as oxidative damage on biological molecules, membranes and tissues. The oxidation of membrane lipids has been implicated as one of the primary events in the oxidative

cellular damage (52). Many studies suggest that oxidative stress plays an important role in the pathogenesis of BD. In the previous studies, plasma MDA levels were found to be higher in patients with BD in the active stage of the disease (53,54).

In conclusion, the chronic inflammation production in BD may be caused by result of molecular mimicry. This situation may lead to increased oxidative stress. Increased oxidative stress may impair genetic stability, as reflected by higher MN frequency in BD. Thus, increased frequency of MN in BD patients with HLA-B51 allele may be the result of molecular mimicry. Furthermore, our results indicate that there was an increased DNA damage in BD, and this situation may be associated with the pathogenesis of BD.

Conflict of Interest

Authors reported no conflicts of interest.

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