Comparative analysis of the association of HLA-B*51 suballeles with Behçet’s disease in patients of German and Turkish origin

Abstract: The distribution of the different HLA-B*51 suballeles among patients with Behçet’s disease (BD) of German (n = 33) and Turkish (n = 92) origin in comparison to their presence in the respective ethnically matched healthy control groups (German: n = 325, Turkish: n = 93) was studied. HLA-B*51x was significantly increased in both patient groups in comparison to the controls (Germans: 58% vs. 12%, OR 9.76, P < 0.001; Turkish: 75% vs. 25%, OR 9.13, P < 0.001). Molecular subtyping of B*51x revealed HLA-B*51011 and B*5108 as the predominant suballeles in both patient groups and controls although with a slightly increased frequency of HLA-B*5108 in the diseased individuals. HLA-B*5105 was the only further HLA-B*51x subtype detected in one Turkish patient heterozygous also for HLA-B*5101. HLA-B*5107 although present in a Turkish as well as German control was absent in the patient groups. There was also a tendency towards a higher degree of homozygosity for HLA-B*51x in both patient groups versus the matched controls (Germans: 10% in patients vs. 2.5% in controls; Turkish: 27% in patients vs. 13% in controls). Our study further supports previous hypothesis of an association of BD with B51 suballeles which share amino-acid residues at positions 63 and 67 as well as at positions 77–83 for specific peptide binding and natural killer (NK)-cell interactions. This applies to HLA-B*5101 and B*5108, but not to HLA-B*5107 different at position 67, which could be negatively associated with BD.

Behçet’s disease (BD) is a multisystemic inflammatory disorder with histopathologic features of a leukocytoclastic vasculitis. It is mainly characterized by oral and genital aphthous ulcers, skin lesions (papulopustules, erythema nodosum), uveitis/retinal vasculitis, oligoarthritis, thrombophlebitis or major embolism, vascular aneurysms, gastrointestinal ulcerations, and CNS vasculitis (1). The etiology and pathogenesis of BD are still unclear, but the disease is thought to be triggered by environmental factors in patients with a particular genetic background (2). The prevalence of BD is highest in countries along the former Silk Road (10–100/100,000 people) (3). The genetic marker most strongly associated with BD in several ethnic groups is HLA-B51, with a frequency of 50–80% in the different pa-

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It is still an open debate whether the susceptibility to BD is influenced by HLA-B51 itself or by some other non-HLA genes located around HLA-B in linkage disequilibrium with HLA-B51 (4). HLA-B51 has been identified to comprise 26 suballeles at the nucleotide level and 24 subtypes at the protein level (5). It has been reported that HLA-B*5101 is the most frequent suballele (up to 98%) in Japanese, Greek, Spanish, Italian and Arabian patients with BD, followed by HLA-B*5108 (6–11). In this study, we performed polymerase chain reaction (PCR) low- and high-resolution subtyping of the HLA-B locus in order to determine the frequency of HLA-B*51x and its suballeles in German and Turkish patients and ethnically matched healthy controls.

Material and Methods

Patients

Thirty-three German and 92 Turkish patients with BD fulfilling the International Study Group Criteria (12) diagnosed at the University Hospitals of Tübingen and at the University Hospital Benjamin Franklin, Department of Dermatology (Berlin, Germany), as well as at the Department of Medicine, Cerrahapa Medical School (Istanbul, Turkey) were included in the study. EDTA-blood was drawn after informed consent and stored at \(-20°C\) until analysis. The control samples were derived from healthy German blood donors at the Department of Transfusion Medicine and at the Section of Transplantation-Immunology and Immunohematology, University Hospital, Tübingen (German controls, \(n=325\)) as well as from random healthy Turkish donors of the Cerrahapa Medical School in Istanbul (Turkish controls, \(n=93\)).

Preparation of DNA from peripheral blood cells

EDTA blood was used to prepare DNA from peripheral blood cells applying the QIAamp® Blood Kit (Quiagen, Chatsworth, CA, USA) as to the manufacturers instructions.

HLA-B low-resolution genotyping

HLA-B low-resolution typing was performed using Lipa–HLA-B typing kit (Innogenetics, Brussels, Belgium) according to the manufacturer’s instructions.

HLA-B51 subtyping

High-resolution subtyping was applied to all HLA-B*51x-positive patients and controls (Turkish patients: \(n=64\), Turkish controls \(n=23\); German patients \(n=16\), German controls \(n=61\)) by group-specific separate, but overlapping amplification of exon 2 and exon 3 of Bw4-related HLA-B locus alleles using the primers listed in Table 1. Thereafter, subsequent direct non-radioactive cycle-sequencing of the two amplicons was performed.

The applied amplification and sequencing primers were synthesised on a LKB Gene Assembler Plus (Pharmacia, Uppsala, Sweden). For amplification of exon 2 (amplicon 1) one HLA-B locus-specific forward primer located in the first intron (BIN1–45) with a Bw4 (BEX2–4.0)-specific reverse primer from the 3’ end of exon 2 was combined. For amplification of exon 3 (amplicon 2) the Bw4 (BEX2–4.2)-specific forward primer located in the second exon was combined with the locus-specific reverse primer from the 5’ end of intron 3 (BIN3–37). For sequencing of exon 2 the same HLA-Bw4-related reverse primer (BEX2–4.0) as well as the HLA-B locus-speci-

### Table 1

<table>
<thead>
<tr>
<th>Primer designation</th>
<th>DNA sequence</th>
<th>Localization</th>
<th>(T_m) (°C)</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplification of exon 2 (Amplicon 1)</td>
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<tr>
<td>BIN1–45 F</td>
<td>5’GAG GGG ACC GCA GGC GG 3’</td>
<td>45–62 (Intron 1)</td>
<td>62°C</td>
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<tr>
<td>BEX2–4.0 R</td>
<td>5’GCT CTG GTT GTA GTC GCG GA 3’</td>
<td>317–336 (Exon 2)</td>
<td>56.9°C</td>
<td>20</td>
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<tr>
<td>Amplification of exon 3 (Amplicon 2)</td>
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<tr>
<td>BEX2–4.2 F</td>
<td>5’GAA CCT GCG GAT CGC GCT CC 3’</td>
<td>300–319 (Exon 2)</td>
<td>71.7°C</td>
<td>20</td>
</tr>
<tr>
<td>BIN3–37 R</td>
<td>5’GGCCATCCCAGCGACATAT 3’</td>
<td>36–55 (Intron 3)</td>
<td>71°C</td>
<td>20</td>
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<tr>
<td>Sequencing primer exon 3</td>
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<tr>
<td>BIN2–165 F</td>
<td>5’CCC GGT TTC ATT TTC AGT TG 3’</td>
<td>151–170 (Intron 2)</td>
<td>67.1°C</td>
<td>20</td>
</tr>
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</table>

The nucleotide positions of the primers located in the introns refer to the numbering by Gomez-Casado et al. (16), those located in the exons to the numbering given by Robinson et al. (13)
fic forward primer used for amplification were applied. For sequencing of exon 3 the locus-specific forward primer (BEX2–4.2) as well as another forward primer located in intron 2 (BIN2–165) and the same reverse primer located in intron 3 (BIN3–37) used for amplification were applied.

The PCR reactions were carried out in a GeneAmp PCR system 9600 (Perkin-Elmer Cetus, Norwalk, CT, USA) in a final volume of 50 μl consisting of the following mixture: 5 μl GeneAmp® 10× PCR Buffer (Perkin Elmer, Roche Molecular Systems, Branchburg, NJ, USA), leading to a final concentration of 10 mM Tris-HCL, pH 8.3, 1.5 mM MgCl₂ and 0.001% (w/v) gelatine, 350 μM of each dNTP, 1 Unit Amplitaq® DNA polymerase (Perkin Elmer, Roche Molecular Systems), 30 pMol each of the group-specific exon primers, 200 ng DNA and ddH₂O. After an initial denaturation step at 94°C for 1 min, 10 cycles consisting of denaturation at 94°C for 20 s, annealing at 66°C for 50 s and extension at 72°C for 22 s were carried out. In 22 subsequent cycles with denaturation at 94°C for 20 s the annealing temperature was decreased to 65°C for 50 s followed by an extension at 72°C for 22 s. The last cycle was terminated by an extension at 72°C for 10 min. Five μl of the amplification products were routinely separated on a 1.5% agarose gel and controlled for the presence or absence of appropriately sized bands of amplicon 1 and 2.

For direct sequencing, the PCR products were purified by the QUAIquick PCR Purification Kit® (Quiagen). Cycle-sequencing of amplicon 1 and 2 was performed on an Applied Biosystems 377A DNA sequencer using the Big DyeTerminator Ready Reaction Mix-AmpliTaq FS® (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions and as previously described (13).

Raw data collection and evaluation of the chromatographic sequencing results was performed using the software package provided with the ABI 377A sequencing system. For the group of specifically amplified Bw4-related HLA-B alleles data files including all the respective known Bw4-positive B-locus sequences were created to facilitate sequence comparison and automated subtyping of the determined HLA-B locus alleles.

**Data analysis**

For statistical analysis of BD patients and controls in a case-control setting, the odds ratio (OR) ((a×d)/ (c×b)) was determined using the Mantel-Haenszel 2-tailed test. For statistical analysis of associations of parameters with BD, Chi-square/Fisher’s exact test was used. All statistical calculations were performed with the use of the SAS program for Windows software, version 6.11.

**Results**

The phenotype frequency of the group specificity HLA-B*51x in German and Turkish patients with BD in comparison to respective ethnic control groups was determined by low-resolution PCR-SSOP typing of HLA-B locus alleles (Table 2). HLA-B*51x was observed to be significantly associated with BD in the Turkish (75%, OR 9.13, \(P<0.001\)) but also in the German patients of Caucasian origin although with a comparative reduced frequency (58%, OR 9.76, \(P<0.001\)). High-resolution genotyping of the HLA-B*51x suballeles (HLA-B*5101-B*5126) was subsequently performed in 16 German and 64 Turkish HLA-B*51x-positive patients as well as in 61 German and 23 Turkish healthy controls selected for HLA-B*51x expression (Table 3). The applied direct sequencing technique of overlapping Bw4-related PCR fragments of exon 2 and exon 3 allowed to define all known HLA-B*51x suballeles (B*51011–B51026). HLA-B*51x-positive German patients carried either HLA-B*5101 (87.5%) or HLA-B*5108 (14.2%). The HLA-B*51x-positive Turkish patients also predominantly typed for HLA-B*51011 (81.2%) or...
HLA-B*5108 (17.1%), but additionally showed presence of HLA-B*5105 in one Turkish patient (1.5%). In the German and Turkish controls, a very similar distribution of the HLA-B*51x suballeles was found, with HLA-B*51011 being the most frequent allele in both ethnic control groups (Germans: 93.4%, Turkish: 86.9%) followed by HLA-B*5108 (Germans: 3.5%, Turkish: 10%). HLA-B*5108 showed a slightly higher frequency in both patient groups than in the respective control groups. In addition, HLA-B*5107 present in a healthy German and a Turkish individual was another rare suballele only in the investigated ethnic healthy controls, but not found in the patient groups. All other defined HLA-B*51x suballeles were negative in the German and Turkish healthy and diseased populations. In both patient groups, there was a tendency towards a higher frequency of HLA-B*51x-homozygous individuals in comparison to their respective controls (Germans: 10% of the patients vs. 2.5% of the controls; Turkish: 27% of the patients vs. 13% of the controls) (Table 4).

**Discussion**

In this study, we evaluated the frequency of HLA-B*51x suballeles in Caucasian patients with BD originating from Germany, a European Caucasian country, where the disease is rare (estimated prevalence 0.55/100,000) in comparison to patients from Turkey, a country where BD is a much more prevalent disease (estimated prevalence 80–370/100,000) (13), and to ethnically matched healthy controls. Like in previous studies of other populations of Southern Europe and of the Middle East, a strong association of HLA-B*51x with BD was found in both patient groups. In both German and Turkish patients, HLA-B*51011 was the most common suballele, HLA-B*5108 and HLA-B*5105 were the only two other suballeles observed to be present in either one of the two analysed patient groups. The distribution of the HLA-B*51x suballeles in the patients in comparison to the matched healthy controls revealed a similar frequency of HLA-B*51011. However, in order to also prove significance of the slightly higher number of HLA-B*5108-positive individuals in both patient groups, still larger numbers of patients and controls have to be investigated. The same also holds true for a possible negative association of B*5107 represented in the German and Turkish controls, but not in the respective patient groups. HLA-B*5105 was inherited with HLA-B*5108 in a Turkish patient and thus can not be considered as possible independent susceptibility factor for BD. Among the 26 defined HLA-B*51x suballeles only HLA-B*5102 has been described as another B*51x associated with BD in a Japanese patient (14).

In spite of the still limited number of HLA-B*51x-positive German and Turkish patients, this study further strengthens that BD is associated with at least two subtypes of HLA-B*51x, B*5101 and B*5108, as it has been also reported previously for BD patients in Japanese, Greek, Spanish, Saudi Arabian and Italian populations. In addition there is a first hint that HLA-B*5107 might be negatively associated with BD. HLA-B*5101 and B*5108 share two amino acid substitutions at position 63 and 67 as the only distinguishable sequence difference in comparison to HLA-B*5201 which is clearly not associated with BD. These amino acid residues have been suggested to be primarily responsible for the development of BD (10). The same amino acid substitutions are present in all other HLA-B*51x suballeles except in B*5107 and B*5122. B*5107 shares Ser at position 67 with HLA-B*5201 whereas B*5122 carries Cys at this position. Thus, the association of with BD of specific HLA-B*51x suballeles appears to be identical in different populations irrespective of its occurrence as endemic (Japan, Middle East, Mediterranean) or rare disease (Germany). The higher incidence of homozygosity for HLA-B*51x in both patient groups could point to an increased risk for BD in patients with a “double” dose of associated HLA-B*51x alleles. However, further analysis of larger patient and control groups is needed to clarify these observations and their relevance for differences in clinical manifestations. In summary, these data further support HLA-B*5101 and B*5108 as predisposing genes for BD in populations of different ethnic origins.
References