Concise Report

Low prevalence of NOD2 SNPs in Behçet’s disease suggests protective association in Caucasians

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Objective. It has been shown previously that three nucleotide-binding oligomerization domain containing 2 (NOD2) variants (Arg702Trp, Gly908Arg and Leu1007fs) are associated with Crohn’s disease (CD), a disorder clinically resembling Behçet’s disease (BD). We studied the frequency of these variants in BD patients.

Methods. DNA samples of 200 BD patients [59 Caucasians, 139 Middle Eastern (MEs) of Arab descent and 2 Asians] and 520 healthy controls (444 Caucasians and 76 MEs) were genotyped using a Taqman assay.

Results. Both the Arg702Trp and Leu1007fs (frameshift) variants were significantly less frequently present among BD patients compared with healthy controls (0.5 vs 5.8%; P < 1.10-5 and 0.0 vs 1.8%; P < 0.007, respectively). In the Caucasian subpopulation, Arg702Trp was significantly less frequent in the BD group as compared with the controls (P = 0.04); whereas in the ME subpopulation, a trend was observed (P < 0.06).

Conclusions. Of the three CD-associated single nucleotide polymorphisms, one of the variant NOD2 alleles, was found to be present significantly less in Caucasian BD patients.

Key words: Nucleotide-binding oligomerization domain containing 2, Single nucleotide polymorphisms, Genetic association, Peptidoglycan, Neutrophil, NALP3.

Introduction

Behçet’s disease (BD) is an idiopathic systemic vasculitis characterized by recurrent oral and genital aphtous ulcers, skin lesions, ocular inflammation and more seldomly, arthritis, CNS- and gastrointestinal tract inflammation [1, 2].

Prevalence varies from 110–420 per 100 000 in Turkey to about 2 per 100 000 in Western countries [2]. A strong association with HLA-B51, a positive family history in 12% of non-Caucasoid patients and a sibling risk ratio of 11–52 suggest the causal involvement of both genetic and environmental factors [3].

The aetiology of BD is still to be unravelled, but it is widely considered as an excessive inflammatory response, possibly triggered by an (infectious) antigen in a genetically susceptible host. The abundance of neutrophils in early lesions and the association of HLA-B51 with neutrophil activation indicate involvement of innate immunity in BD [1, 2, 4].

Similar symptoms such as uveitis, arthritis, erythema nodosum and colitis with ileocaecal mucosal inflammation and punched-out fissuring ulcers suggest a genetic overlap between BD and Crohn’s disease (CD) [5, 6]. Recently, the latter has been linked with genetic alterations in innate immunity. Increased susceptibility to CD is associated with two single nucleotide polymorphisms (SNPs) (Arg702Trp and Gly908Arg) and a frameshift mutation (Leu1007fs) in the CARD15 gene [7, 8]. This gene encodes the nucleotide-binding oligomerization domain containing 2 (NOD2) protein. In short, NOD2 acts by recognition of muramyl dipeptide (MDP) derived from peptidoglycan, present in the cell wall of all bacteria. NOD2 engagement activates nuclear factor-κB (NF-κB), a transcription factor with a central role in both innate and adaptive immunity. This transcription factor is also considered to play a key role in many inflammatory diseases including BD and CD [7, 9, 10].

Since BD and CD share many clinical features and for both diseases innate immune mechanisms appear crucial, we addressed the hypothesis that NOD2 variants are mechanistically operational in both diseases. We therefore determined the occurrence of the aforementioned NOD2 SNPs in two large and independent BD cohorts.

Materials and methods

Study population

Two cohorts of total 200 unrelated BD patients were included in the study (52 from the Erasmus MC at Rotterdam, 109 from The Jordan Hospital, Amman, Jordan and St John’s Ophthalmic Hospital, Jerusalem, Israel and 39 from St Thomas’ Hospital, London, UK). Of those, 59 were Caucasians, 2 were Asians and 139 from Middle Eastern (ME) origin of Arab descent. All patients fulfilled the International Study Group criteria for the diagnosis of BD [5]. In total, 520 healthy controls were recruited (444 Caucasians and 77 MEs). Dutch controls were healthy Caucasian blood donors, the UK controls were Caucasian transplant donors from which Afro-Caribbean and Far Eastern individuals were excluded. Given the high prevalence of BD in the
Middle East, local controls were either hospital staff or otherwise healthy cataract patients matched with the same population as BD patients from each country. ME controls were >50 years of age as new presentation with BD after this age is unusual in this population. The ethics committees of all centres approved the study, and written informed consent was obtained from the patients.

Genotyping

Genomic DNA was extracted from 5 ml samples of peripheral venous blood using the magnetic bead separation technique from AGOWA (Berlin, Germany) on a Microlab Star pipetting robot (Hamilton Robotics, Reno, NV, USA). Genomic DNA of 1–2 ng was dispensed into 384-well plates using a Caliper Sciclone ALH3000 pipetting robot (Caliper LS, Mountain View, CA, USA). Genotypes were determined using a Taqman allelic discrimination assay. The assay-on-demand service (www.appliedbiosystems.com) was used for the polymorphisms Arg702Trp and Gly908Arg. For Leu1007fs, the primers and probes were designed as described by Inohara et al. [8] (Isogen, IJsselstein, The Netherlands).

The PCR reaction mixture for the assay-on-demand assays included 1–2 ng of genomic DNA in a 2 μl volume and the following reagents: FAM and VIC probes (200 nM), primers (0.9 μM) and 2× Taqman PCR Master Mix (ABgene, Epsom, UK). For Leu1007fs, the PCR reaction mixture included 2 ng of DNA in a 2 μl volume and the following reagents: FAM probes (250 nM), TET probe (500 nM), primers (300 nM) and 2× Taqman PCR Master Mix (Applied Biosystems, Foster City, CA, USA). PCR cycling reaction was performed in 384-well PCR plates in an ABI 9700 PCR system (Applied Biosystems) and consisted of initial denaturation for 15 min at 95°C, 40 cycles with denaturation of 15 s at 95°C and annealing and extension for 60 s at 60°C. Results were analysed by the ABI Taqman 7900HT using the sequence detection system 2.22 software (Applied Biosystems).

Statistics

All data were submitted for Hardy–Weinberg equilibrium calculations before inclusion in the analyses. Subjects were grouped according to genotype for the polymorphisms of interest. Per subject the allele information of both alleles was added. To detect allele frequency differences between cases and controls, Pearson’s chi-square test was used. The analyses were performed in Haploview 4.0. A P-value of < 0.05 was considered as statistically significant.

Results

Of all alleles, <2.5% were excluded for further data processing because genotyping failed. In 200 patients (400 alleles) and 520 controls (1040 alleles), no homozygous variants were present. Both the Arg702Trp and Leu1007fs variants were significantly less frequently present among BD patients compared with healthy controls. The largest difference was found in the Arg702Trp variant: 0.5 vs 5.8%. For the Leu1007fs variant, a difference of 0.0 vs 1.8% was observed. The frequency of the NOD2 Gly908Arg variant was not significantly different between the BD patients and the controls (Table 1).

Differences in frequencies of NOD2 polymorphisms may vary with geographical location [11]. We thus separately re-assessed the SNPs of the Caucasian and ME subpopulations. Though not powered for this analysis, the Arg702Trp variant remained significantly reduced in the Caucasian subpopulation (P = 0.042). In the ME group, a trend (P = 0.058) was seen for a reduced occurrence of Arg702Trp. The Leu1007fs variant was not significantly different in the sub-cohorts (Table 1).

Discussion

In this genetic study consisting of 200 BD patients, three NOD2 variants were genotyped to test the assumption that these variants have a similar distribution as in CD patients. Contrary to this initial hypothesis, we observed a significantly lower frequency of two of the three variant alleles in the BD group as compared with healthy controls.

In the subgroup analysis, a statistically significant difference was obtained for the Arg702Trp variant allele of the Caucasian subpopulation. Our study was appropriately powered to identify frequencies of NOD2 variants in the same magnitude as described for CD. In contrast to this, we observed a lower proportion of variant alleles in our BD population. Apparently, the other subgroups in our study were too low in number to reach significance.

In our study, we focused on individual SNP frequencies in an ethnically mixed cohort and extracted subpopulations. Ahmad et al. [11] described haplotypes of three NOD2 variants in combination with other SNPs in 374 patients and 500 controls and could not find an association with BD. Details on the individual variants, however, were not presented. Uyar et al. [12] used an entirely Turkish cohort (existing of 85 BD patients and 100 healthy controls) and could not demonstrate a significant difference. It has been shown in other studies as well as in our present study that the NOD2 variants are observed at a lower frequency in ME cohorts [11–13]. Therefore, the size of the Turkish cohort might have been too small to detect the observations made in our larger study. A relation between NOD2 variants and HLA-B51 has not been shown in the past, and is therefore not expected to be of added value in the present study, since these genes are located on different chromosomes (16 and 6, respectively) [11, 12].

Assuming that the findings in the current study can be confirmed by future studies, it is of interest to speculate how the low frequency of NOD2 variants in BD might contribute to the understanding of the underlying mechanism of the disease. It is clear that variation in many genes will contribute to BD susceptibility and disease progression. Candidates are PTPN22 620W, IL-10 and TNF-α genes, which support an increased inflammatory response and a failure of regulation [14]. Similar mechanisms in CD are postulated to explain the involvement of NOD2 variants leading to decreased NF-κB activation (loss of function), and thus reduced defensin production resulting in an increased bacterial load in the gut of CD patients [9, 15, 16]. Moreover, NOD2

<table>
<thead>
<tr>
<th>SNP</th>
<th>Allele</th>
<th>BD (frequency)</th>
<th>Control (frequency)</th>
<th>P-value</th>
<th>BD (frequency)</th>
<th>Control (frequency)</th>
<th>P-value</th>
<th>BD (frequency)</th>
<th>Control (frequency)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg702Trp</td>
<td>Arg</td>
<td>388 (0.995)</td>
<td>961 (0.942)</td>
<td>1.35E–05</td>
<td>112 (0.980)</td>
<td>811 (0.934)</td>
<td>0.0421</td>
<td>272 (1.000)</td>
<td>150 (0.987)</td>
<td>0.0579</td>
</tr>
<tr>
<td></td>
<td>Trp</td>
<td>2 (0.005)</td>
<td>59 (0.058)</td>
<td></td>
<td>2 (0.018)</td>
<td>57 (0.066)</td>
<td></td>
<td>0 (0.000)</td>
<td>2 (0.013)</td>
<td></td>
</tr>
<tr>
<td>Gly908Arg</td>
<td>Gly</td>
<td>357 (0.982)</td>
<td>1011 (0.980)</td>
<td>0.7533</td>
<td>116 (1.000)</td>
<td>864 (0.982)</td>
<td>0.1432</td>
<td>267 (0.974)</td>
<td>147 (0.967)</td>
<td>0.6606</td>
</tr>
<tr>
<td></td>
<td>Arg</td>
<td>7 (0.018)</td>
<td>21 (0.020)</td>
<td></td>
<td>0 (0.000)</td>
<td>16 (0.018)</td>
<td></td>
<td>7 (0.026)</td>
<td>5 (0.033)</td>
<td></td>
</tr>
<tr>
<td>Leu1007fs</td>
<td>fs</td>
<td>392 (1.000)</td>
<td>1017 (0.982)</td>
<td>0.0069</td>
<td>116 (1.000)</td>
<td>866 (0.980)</td>
<td>0.1209</td>
<td>272 (1.000)</td>
<td>151 (0.993)</td>
<td>0.1805</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 (0.000)</td>
<td>19 (0.018)</td>
<td></td>
<td>0 (0.000)</td>
<td>18 (0.020)</td>
<td></td>
<td>0 (0.000)</td>
<td>1 (0.007)</td>
<td></td>
</tr>
</tbody>
</table>

Significantly less Arg702Trp and Leu1007fs variants are seen in the BD cohort compared with the healthy controls.
activation inhibits the stimulation of Toll-like receptors by pathogen products, and as NOD2 function is impaired, TLR signalling is enhanced and generates an exaggerated inflammatory response inducing tissue damage [9, 15].

In a second model, transgenic expression of the Leu1007fs variant shows a gain of function, with a direct increase in NF-kB, pro-inflammatory cytokines and tissue damage [9]. A potential connection between these paradoxical results is the mechanism of the NOD2 response. Acute stimulation of human blood-derived macrophages with the NOD2 agonist, MDP, induces a pro-inflammatory cytokine response. By comparison, persistent treatment of cells with MDP before activation through TLR2 or TLR4 ligands decreases cytokine responses, possibly through induction of IRAK-M, in support of control of TLR signalling. However, this tolerance is lost in cells from Leu1007fs homozygous patients [17]. As gut epithelial cells, dendritic cells and macrophages are in constant contact with bacterial products of the normal gut flora, it is proposed that such tolerance is a protective mechanism [18]. Failure to control responses would lead to tissue damage as seen in CD.

Our findings suggest that NOD2 variants react in some cases with a decreased production of inflammatory cytokines to an exogenous stimulus. This is supported by the observation that NOD2 gene-deficient mice show reduced joint inflammation, and are protected against early cartilage damage after IA injection of Streptococcus pyogenes cell wall fragments [19]. In addition to this, human peripheral blood mononuclear cells of Leu1007fs variant donors exposed to cell wall fragments produce less TNF-α and IL-1β than healthy controls [19]. However, this is contradictory to the macrophage data discussed above, and may be due to the in vivo nature of these experiments, route of challenge or the type of antigen used. The complex role of NOD2 in response to bacterial challenge in different cell types should be addressed in BD and other conditions.

In conclusion, our observations indicate that at least Caucasian carriers of the NOD2 variant allele have a reduced risk of developing BD. Our study contributes to the general assumption that carriers of the NOD2 variant allele have a reduced risk of developing BD. Our study contributes to the general assumption that carriers of the NOD2 variant allele have a reduced risk of developing BD. It will therefore be of considerable interest to investigate other variants in BD patients in order to gain more insight into the pathophysiology and potentially identify the new leads for treatment options.

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### References