Association of Toll-like Receptor 4 Gene Polymorphisms with Normal Tension Glaucoma

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PURPOSE. Toll-like receptor 4 (TLR4) is a transmembrane receptor that mediates immune responses to exogenous and endogenous ligands and interacts with heat shock proteins, which are reportedly involved in normal tension glaucoma (NTG). This study was undertaken to investigate whether TLR4 polymorphisms are associated with NTG.

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Glaucoma is a degenerative optic neuropathy characterized by the progressive loss of retinal ganglion cells and optic nerve axons, together with visual field damage; it often occurs in relation to elevated intraocular pressure (IOP). It is the second leading cause of blindness worldwide, affecting approximately 70 million people.1,2 Glaucoma is generally a multifactorial disorder initiated as the result of several interacting factors. Factors other than IOP are likely to play a role in the pathogenesis of glaucomatous optic neuropathy, particularly in patients with normal tension glaucoma (NTG). NTG is an important subset of primary open-angle glaucoma (POAG). Although many POAG patients have high IOP,3 patients with NTG have statistically normal IOP.4–6 NTG accounts for approximately 20% to 50% of all POAG cases.7–9 In a recent study of the Japanese population, the reported prevalence of POAG was 3.9%, and 92% of patients with POAG had NTG, in which the IOP was 21 mm Hg or less.10 Because IOP is normal, NTG is underdiagnosed and usually presents late in life, after loss of the visual field. The factors contributing to the development of NTG have not yet been determined.

Recently, it has been suggested that the immune system and heat shock proteins (HSPs) play an important role in glaucoma.11 HSPs are highly immunogenic molecules that are widely distributed in nature; they perform important functions relating to the folding and assembly of protein complexes. Human HSPs are expressed on cell membranes in response to stress, such as physiological shock and microbial challenge. Wax et al.12 observed that patients with NTG have increased...
serum immunoreactivity to bacterial and human HSP60. They also showed that direct application of antibodies to HSPs results in neuronal apoptosis, and patients with NTG have higher titers of antibodies to HSPs, including HSP27 and -60, compared with patients with high IOP and with healthy control subjects.\(^1\)\(^-\)\(^6\)\(^-\)\(^1\)\(^4\) Furthermore, they observed increased expression of HSP27 and -60 in the glaucomatous retina and/or optic nerve head and proposed that immunoregulation of these HSPs is an important component of glaucomatous optic neuropathy.\(^1\)\(^5\) Therefore, it may be important to investigate the HSPs and immune mechanisms involved in glaucoma, particularly NTG.

Toll-like receptor (TLR) proteins are a family of phyllogenetically conserved receptors that recognize both self and nonself molecules and play an important role in both innate and adaptive immunity.\(^1\)\(^6\)\(^-\)\(^1\)\(^8\) Among the TLR family members, TLR4 has been most exhaustively investigated and is known to be a principal lipopolysaccharide (LPS)-recognition receptor; it also interacts with bacterial HSP60. TLR4 interacts with exogenous ligands as well as endogenous HSPs such as HSP60, -70, and -96.\(^1\)\(^7\)\(^-\)\(^1\)\(^8\) Recent studies report associations between single-nucleotide polymorphisms (SNPs) in the TLR4 gene and endotoxin hyporesponsiveness\(^1\)\(^9\)\(^-\)\(^2\)\(^0\) and Gram-negative infections.\(^2\)\(^1\) TLR4 SNPs have also been demonstrated to affect the risk of various multifactorial disorders.\(^2\)\(^2\)\(^-\)\(^2\)\(^6\) These findings suggest that TLR4 gene sequence variants with abnormal function, such as abnormal recognition of HSPs or other ligands, may contribute to the development of various diseases, including multifactorial disorders.

Based on the ability of TLR4 to recognize self- and nonself HSPs, we hypothesized that TLR4 polymorphisms may be associated with the risk of NTG. To test this hypothesis, we performed SNP analysis of the TLR4 gene in patients with NTG and healthy control subjects.

**Materials and Methods**

**Participants**

We recruited 250 unrelated Japanese patients with NTG and 318 unrelated healthy Japanese control subjects at Yokohama City University, Yamanashi University, Gifu University, Kobe University, Yamaguchi University, Kumamoto University, Hokkaido University, Tokyo University, Niigata University, Kanazawa University, Hiroshima University, and Tajimi Municipal Hospital in Japan. NTG was diagnosed in the patients by the following strict inclusion criteria: the presence of glaucomatous optic neuropathy with corresponding visual field loss; normal open angle with angle width of Shaffer grade 2 or higher; absence of IOP greater than 21 mm Hg on repeat measurement with Goldmann applanation tonometry without medication; and lack of a pathologic basis for optic nerve change on neurologic, rhinologic, and general medical examination, including magnetic resonance imaging. Glaucomatous optic nerve abnormality was diagnosed when the vertical cup/disc ratio of the optic nerve head was 0.7 or higher, the rim width at the superior (11 to 1 o’clock) or inferior (5 to 7 o’clock) portion was less than or equal to 10% of the disc diameter, the difference in the vertical cup/disc ratio between eyes was 0.2 or greater, or a nerve fiber layer defect was found. Glaucamatos visual field loss was defined on a hemifield basis using reliable field data examined by a static visual field analyzer (HFA; Humphrey C-30-2 program; Carl Zeiss Meditec, Oberkochen, Germany) according to Anderson and Patella’s criteria.\(^2\)\(^9\) The hemifield was judged abnormal when the pattern deviation probability plot showed a cluster of three or more non-edge contiguous points having sensitivity with a probability of less than 5% in the upper or lower hemifield and in one of these points with a probability of less than 1%.

In addition, the following inclusion and exclusion criteria were used to categorize the patient groups stringently. We excluded individuals in whom NTG was diagnosed at ≤20 years or ≥60 years, as well as patients for whom the refractive error was the spherical equivalent of less than −8.0 D. To correct for the damaging effects of aging on the retinal ganglion (i.e., the mean deviation [MD] measured by HFA C-30-2) the selection criteria were modified based on subject age, as follows: (1) no modification if the patient was diagnosed below the age of 50 years, (2) −10.00 dB or worse in at least one eye if the disease was diagnosed when the patient was between the ages of 50 and 55 years, (3) −15.00 dB or worse in at least one eye if the disease was diagnosed when the patient was older than 55 years. Cases with a comparatively early onset were selected because early onset suggests stronger involvement of genetic factors. During diagnosis, patients whose refractions had changed, because of cataract surgery of refractive surgery, for example, were excluded. In cases in which glaucomatous visual field loss was present in only one eye, the refraction value and glaucomatous visual field loss of the affected eye were used. In cases in which glaucomatous visual field loss was present in both eyes, the refraction and glaucomatous visual field loss of the more severely affected eye were used.

Patient age ranged between 20 and 59 years (mean, 46.1 ± 7.7); 47.6% were men and 52.4% were women. The mean refraction was −4.02 ± 2.85 D, and the MD observed in the static visual field determination (Humphrey; Carl Zeiss Meditec) was −9.15 ± 7.67 dB.

Control subjects were healthy volunteers from a geographic region similar to that for the patients with NTG. They had no glaucoma or local or systemic illness that might cause glaucoma or optic disc changes, and they had no myopia or mild myopia with refractive errors of −5.00 D or less. The control subjects were sex matched (49.4% male and 50.6% female) to the patients, and their age range was 50 to 85 years (mean, 61.2 ± 8.5). The study was approved by the ethics committee of the Yokohama City University School of Medicine and complied with the guidelines of the Declaration of Helsinki. We explained study details to all patients and control subjects before obtaining consent for genetic screening.

**DNA and TLR4 Genotyping**

A kit (QiAamp DNA Blood Maxi Kit; Qiagen, Chatsworth, CA) was used to collect peripheral blood lymphocytes and extract genomic DNA from peripheral blood cells. Procedures were performed under standardized conditions to prevent variation in DNA quality. TLR4 comprises four exons and has four transcript isoforms (A, B, C, and D). We evaluated eight SNPs: rs10759930, rs1927914, rs1927911, rs12377632, rs2149356, rs11536889, rs7037117, and rs7045953. These SNPs are located within the TLR4 gene, including 5 kb of the predicted 5’ untranslated region (UTR) and 6 kb of the predicted 3’ UTR, with minor allele frequencies >5% according to the National Center for Biotechnology Information (Bethesda, MD) dbSNP (Table 1). Genotyping of all SNPs was performed by a 5’ exconuclease assay (TaqMan; Applied Biosystems, Inc., [ABI] Foster City, CA) using primers supplied by the manufacturer. The probe fluorescence signal was detected (TaqMan Assay for Real-Time PCR, 7500 Real Time PCR System; ABI), according to the manufacturer’s instructions.

**Statistical Analysis**

We tested the Hardy-Weinberg equilibrium (HWE) of each SNP among the control subjects. Differences in allele and genotype frequencies between cases and control subjects were assessed by using the χ² test. The program Haemoview 3.32 was used to compute pairwise linkage disequilibrium (LD) statistics.\(^3\)\(^0\) D’ and r² were plotted. LD blocks were defined according to the criteria of Gabriel et al.\(^3\)\(^1\) Haploype frequencies were estimated using an accelerated expectation-maximization algorithm similar to the partition-ligation-expectation-maximization method.\(^3\)\(^2\) Statistical significance was defined as P < 0.05. To obtain a measure of significance corrected for multiple testing bias we ran 10,000 permutations to compute P with the Haemoview program.

**Tag SNP Selection**

To avoid overfitting and unbounded haplotype tests in the association analysis, the Tagger algorithm in Haemoview 3.32 was used to select the...
 Allele and Genotype Frequencies

Tag SNPs that optimally capture common haplotypes within the TLR4 gene. The algorithm is based on $\chi^2$, and application of a stringent $\chi^2$ threshold ($\chi^2 > 0.8$) between SNPs would allow the selected tag SNPs to resolve more than 80% of all existing haplotypes.35

RESULTS

HWE Tests and Haplotype Block

Eight SNPs in the TLR4 gene were genotyped. All SNPs were in HWE among both cases and control subjects (data not shown). All eight SNPs were located in 1 haplotype block, and the magnitude of LD between each SNP was extremely high, with pairwise $D' \geq 0.85$ (Fig. 1).

Allele and Genotype Frequencies

Table 1 shows details of 8 SNPs, their genomic locations, and minor allele frequencies in cases and control subjects. The frequencies of the minor alleles of all SNPs but rs11536889 were increased in cases compared with healthy control subjects, and three SNPs (rs10759930, rs1927914, and rs7037117) revealed significant allelic association between cases and control subjects ($P_c < 0.05$). Among these, the minor allele of rs7037117, located in the 3’-UTR of TLR4, had the most significantly increased risk of NTG ($P = 0.0044, P_c = 0.018, OR = 1.51, 95\% CI = 1.14–2.01$). Genotype frequencies of eight SNPs are shown in Table 2. Individuals carrying one or two copies of the minor allele of six SNPs (rs10759930, rs1927914, rs1927911, rs12377632, rs2149356, and rs7037117) had a 1.47- to 1.65-fold increased risk of NTG, with individuals bearing the minor allele of rs7037117 having the most significantly increased risk over that of control subjects ($P = 0.0041$).

Tag SNPs Selection and Haplotype Analysis

To estimate haplotype frequencies and to analyze the haplotype association with NTG, we selected tag SNPs using the Tagger algorithm in the program Haploview to select a comparatively nonredundant set of SNPs. Four tag SNPs, including rs10759930, rs11536889, rs7037117, and rs7045953, were selected that captured most of the allelic diversity of the 8 TLR4 SNPs. As shown in Table 3, Haplotype CGGCA, which included minor alleles of rs10759930 and rs7037117, was a susceptibility haplotype and significantly increased association with an increased risk of NTG ($P = 0.0090, P_c = 0.041, OR = 1.59, 95\% CI = 1.12–2.28$), although the overall comparison of haplotypes consisting of four tag SNPs rendered a slightly significant difference between cases and control subjects ($P = 0.044$). On the other hand, the overall comparison of haplotypes created by two tag SNPs, rs10759930 and rs7037117, associated with NTG was significantly different between cases and control subjects ($P = 0.010$), with one of the haplotypes showing an increased risk for NTG (Haplotype CG: $P = 0.0038, P_c = 0.0095, OR = 1.53, 95\% CI = 1.15–2.03$). These results suggest that only Haplotype CG created by minor alleles of rs10759930 and rs7037117 was a susceptibility haplotype and the significant increase of minor alleles of rs10759930, rs1927914, rs1927911, rs12377632, and rs2149356 in patients with NTG may arise from a strong linkage disequilibrium with rs7037117.

DISCUSSION

The purpose of this study was to investigate whether TLR4 polymorphisms affect the development of NTG, which is considered a multifactorial disorder. To our knowledge, this is the first attempt to investigate a series of common SNPs located in the TLR4 gene. We demonstrated that multiple SNPs in the
TABLE 2. Genotype Frequencies of SNPs of the TLR4 Gene among NTG Patients and Control Subjects

<table>
<thead>
<tr>
<th>dbSNP</th>
<th>Alleles (1/2)</th>
<th>rs10759930 T/C</th>
<th>Cases 81 (32.4)</th>
<th>Controls 137 (43.1)</th>
<th>1/2</th>
<th>127 (50.8)</th>
<th>42 (16.8)</th>
<th>P = 0.028</th>
<th>0.0094 OR (1.58 [1.12–2.23])</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>rs1927914 A/G</td>
<td>Cases 82 (32.8)</td>
<td>Controls 137 (43.1)</td>
<td>1/2</td>
<td>126 (50.4)</td>
<td>42 (16.8)</td>
<td>P = 0.036</td>
<td>0.012 OR (1.55 [1.10–1.29])</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs1927911 G/A</td>
<td>Cases 87 (34.8)</td>
<td>Controls 141 (44.3)</td>
<td>1/2</td>
<td>122 (48.8)</td>
<td>41 (16.4)</td>
<td>P = 0.067</td>
<td>0.021 OR (1.49 [1.06–2.10])</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs12377632 G/T</td>
<td>Cases 86 (34.4)</td>
<td>Controls 140 (44.0)</td>
<td>1/2</td>
<td>122 (48.8)</td>
<td>42 (16.8)</td>
<td>P = 0.053</td>
<td>0.020 OR (1.50 [1.07–2.11])</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs2149356 G/T</td>
<td>Cases 87 (34.8)</td>
<td>Controls 140 (44.0)</td>
<td>1/2</td>
<td>122 (48.8)</td>
<td>41 (16.4)</td>
<td>P = 0.070</td>
<td>0.026 OR (1.47 [1.05–2.07])</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs11536889 G/C</td>
<td>Cases 146 (58.4)</td>
<td>Controls 146 (58.4)</td>
<td>1/2</td>
<td>93 (37.2)</td>
<td>11 (4.4)</td>
<td>P = 0.42</td>
<td>0.51 OR (1.23 [0.92–1.66])</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs7037117 A/G</td>
<td>Cases 138 (55.2)</td>
<td>Controls 138 (55.2)</td>
<td>1/2</td>
<td>98 (39.2)</td>
<td>14 (5.6)</td>
<td>P = 0.015</td>
<td>0.0041 OR (1.65 [1.17–2.32])</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs7045953 A/G</td>
<td>Cases 203 (81.2)</td>
<td>Controls 269 (84.6)</td>
<td>1/2</td>
<td>45 (18.0)</td>
<td>2 (0.8)</td>
<td>P = 0.19</td>
<td>0.28 OR (1.26 [0.86–1.87])</td>
</tr>
</tbody>
</table>

 Patients n = 250 and controls n = 318. P was calculated by χ² test 3 x 2 or 2 x 2 contingency table. 1, major allele; 2, minor allele.

TLR4 gene significantly affect the development of NTG and that increased frequency of the minor allele of these SNPs, minor TLR4 polymorphism, increases the risk of NTG. rs7037117 had the most pronounced effect on the development of NTG, and haplotype CG, created by minor alleles of rs10759930 and rs7037117, conferred a significantly increased risk of NTG. LD analysis showed that the eight SNPs were in high LD and located in one haplotype block. These results suggest that rs7037117 is principally associated with risk of NTG and that strong LD with rs7037117 is responsible for the increased frequency of the minor allele of other SNPs in patients with NTG.

rs7037117 is located in the 3' UTR of the TLR4 gene. Since the sequence and structural motifs of the 3' UTR are known to participate in the regulation of mRNA stability, translation, and localization, it is possible that the 3' UTR sequence regulates the expression of the TLR4 gene and that rs7037117 may play a critical role in the gene expression process. Recently, we found that Behcet's disease, is probably triggered by HSPs or LPS and is significantly associated with rs7037117.

Among the various ligands of TLR4, HSPs have been reported to be associated with the development of NTG.

The main function of TLRs is the induction of various inflammatory cytokines and effector molecules; TLR4 is known to recognize antigens such as HSPs and LPS and to induce the production of many cytokines, including interleukin (IL)-1, IL-6, IL-10, IL-13, tumor necrosis factor-α, and interferon-β.

The IL-1β levels in the rat retina are increased after retinal ischemia and reperfusion; they proposed that IL-1 is an important mediator of ischemic and excitotoxic retinal damage in glaucoma. Several recent studies investigating the association between the IL-1 gene and NTG or high tension glaucoma (HTG) have shown no significant association between NTG or HTG and IL-1α or IL-1β, although one study demonstrated a significant association between the IL-1α polymorphism and HTG. Thus, the increased IL-1 in the ischemic retina may be due to a sequence variant of the inducer gene, such as TLR4, that is involved in the expression of IL-1 rather than to an IL-1 gene polymorphism itself. In addition, Baudouin et al. showed that IL-6 and IL-10 are overexpressed in patients with glaucoma. Thus, a TLR4 signaling network comprising HSPs and IL may play a significant role in the development of NTG.

TABLE 3. Haplotype Frequencies of Tag SNPs of the TLR4 Gene among NTG Patients and Control Subjects

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Frequency, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tag SNPs rs10759930, rs11536889, rs7037117 and rs7045953</td>
<td>0.044</td>
</tr>
<tr>
<td>TGAA</td>
<td>35.0</td>
</tr>
<tr>
<td>TCAA</td>
<td>22.6</td>
</tr>
<tr>
<td>CGAA</td>
<td>16.6</td>
</tr>
<tr>
<td>CGGA</td>
<td>15.4</td>
</tr>
<tr>
<td>CGGG</td>
<td>9.6</td>
</tr>
<tr>
<td>Tag SNPs rs10759930 and rs7037117</td>
<td>0.010</td>
</tr>
<tr>
<td>TA</td>
<td>57.5</td>
</tr>
<tr>
<td>CG</td>
<td>24.9</td>
</tr>
<tr>
<td>CA</td>
<td>17.5</td>
</tr>
</tbody>
</table>

Haplotypes with frequency less than 1% are not listed. P values were calculated by χ² test 2 x 2 contingency table. These probabilities (Pc) for multiple testing were corrected by Haploview program using 10,000 permutations. Overall P was calculated by χ² test 5 x 2 or 3 x 2 contingency table.
In conclusion, we found that multiple SNPs within the high-ld region of the TLR4 gene are associated with the risk of NTG. We also determined that 1 SNP in the 3’ UTR of the TLR4 gene is principally associated with NTG. Our findings suggest that TLR4 ligands such as HSPs and cytokines induced by TLR4 (e.g., IL-1) play an important role in NTG. Therefore, it is imperative to identify the ligands associated with the TLR4 sequence variant and the TLR4 signaling network that affects the development of NTG.

References