Variants in Optineurin Gene and Their Association with Tumor Necrosis Factor-α Polymorphisms in Japanese Patients with Glaucoma

Tomoyo Funayama,¹ Karin Isibika,¹ Yuichiro Ohtake,¹ Tomibiko Tanino,¹ Daijiro Kurosaka,¹ Itaru Kimura,¹ Kotaro Suzuki,¹,² Hidena Ideta,² Kenji Nakamoto,³ Noriko Yasuda,³ Takuro Fujimaki,⁴ Akira Murakami,⁵ Ryo Asaoka,⁵ Yoshibiro Hotta,⁵ Hidenobu Tanibara,⁶ Takashi Kanamoto,⁷ Hiromu Misshima,⁷ Takeo Fukuchi,⁸ Haruki Abe,⁸ Takeshi Iwata,⁹ Naoki Shimada,¹⁰ Jun Kudoh¹¹ Nobuyoshi Shimizu¹¹ and Yukibiko Masbima¹

PURPOSE. To investigate sequence variations in the optineurin (OPTN) gene and their association with TNF-α polymorphisms in Japanese patients with glaucoma.

METHODS. The OPTN gene was analyzed in blood samples from 629 Japanese subjects. There were 194 patients with primary open-angle glaucoma (POAG), 217 with normal-tension glaucoma (NTG), and 218 with no eye disease (control subjects). The gene was screened for mutations by denaturing high-performance liquid chromatography. Genotyping of three polymorphisms of –3086G→A, –857C→T, and –863C→A in the TNF-α promoter region was performed. The associations between the genotypes and age, intraocular pressure (IOP), and visual field defects at the time of diagnosis were examined.

RESULTS. A possible glaucoma-causing mutation, His26Asp, was identified in 1 of the 411 Japanese patients with glaucoma. A c.412G→A (Thr143Thr) polymorphism in the OPTN gene was significantly associated with POAG (allele frequency, P = 0.011; allele frequency, P = 0.003). The frequency of TNF-α/–857T and optineurin/412A carriers was significantly higher (P = 0.006) in patients with POAG than in control subjects. Among the patients with POAG who were carriers of TNF-α/–857T, the optineurin/412A carriers had significantly worse (P = 0.020) visual field scores than the non–optineurin/412A ones. The frequency of TNF-α/–863A and optineurin/603A (or Lys98) carriers was significantly higher in patients with POAG (P = 0.008) or NTG (P = 0.027) than in control subjects. Among the patients with POAG who were carriers of TNF-α/–863A, the ones with optineurin/603A (or Lys98) had significantly worse (P = 0.026) visual field scores than did those with non–optineurin/603A (or Lys98).

CONCLUSIONS. These findings demonstrated that the OPTN gene is associated with POAG rather than NTG in the Japanese. Statistical analysis showed a possible interaction between polymorphisms in the OPTN and the TNF-α genes that would increase the risk for glaucoma. (Invest Ophthalmol Vis Sci. 2004;45:4359 - 4367) DOI:10.1167/ iovs.03-1403

Primary open-angle glaucoma (POAG), the most common form of glaucoma, affects more than 100 million people, which is almost 2% of the world population >40 years of age.¹ This disease is second in importance as a cause of bilateral blindness.² Glaucoma includes a group of conditions that is characterized by progressive optic neuropathy and visual field changes corresponding to the excavation of the optic disc. These changes are usually associated with an elevation of intraocular pressure (IOP). Although the pathogenesis of the glaucomatous optic neuropathy is not well understood, elevated IOP is generally accepted to be a major risk factor for glaucomatous changes.³

In addition to high IOP, the risk factors for development of glaucoma include older age, race (more prevalent in blacks), positive family history, high myopia, and the presence of diabetes or hypertension.⁴ Genetic factors also play a major role in the etiology of POAG,⁵ and, to date, six chromosomal loci have been identified that are associated with POAG. The first gene to be characterized was the trabecular meshwork inducible glucocorticoid response (TIGR) gene on the long arm of chromosome 1. The TIGR gene was mapped to the glaucoma locus GLC1A.⁶ The gene is now known as myocilin,⁷ and mutations in the myocilin (MYOC) gene have been associated with juvenile-onset POAG as well as with adult-onset POAG in 3% to 5% of patients with glaucoma.⁸-¹²

Rezaie et al.¹³ more recently identified a gene, GLC1C, that is associated with adult-onset POAG and normal-tension glaucoma (NTG) at a second locus. This gene was designated as optineurin (OPTN; GenBank accession number AF420371; http://www.ncbi.nlm.nih.gov/genbank; provided in the public
domain by the National Center for Biotechnology Information, Bethesda, MD), and optineurin is located on chromosome 10 at p14 and has been identified by molecular genetic methods in a large family affected by NTG and adult-onset POAG. Sequence alterations in the OPTN gene were found in 16.7% of families with hereditary POAG, including individuals with IOP <22 mm Hg. However, other reports have indicated that alterations of the OPTN gene are only a rare cause of POAG or NTG.

The expression of optineurin transcripts in two human cell lines is induced by tumor necrosis factor (TNF)-α in a time-dependent way. Optineurin is also known to interact with adenovirus E3-14.7K protein, Huntingtin, NF-xB essential modulator (Nemo), transcription factor IIIA, and Rab8. Because optineurin interferes with the protective effect of E3-14.7K protein against TNF-α-mediated cell death, optineurin may be involved in the TNF-α-signaling pathway leading to apoptosis.

The purpose of this study was to determine the prevalence of mutations in the OPTN gene in Japanese patients with POAG or NTG. Denaturing high-performance liquid chromatography (DHPLC), an automated heteroduplex detection method with a proven sensitivity and specificity exceeding 95%, was used. In addition, we investigated the distribution of TNF-α promoter polymorphisms in patients with glaucoma and normal control subjects to determine whether a significant association between optineurin polymorphism and TNF-α polymorphism is present in patients with POAG or NTG.

Subjects and Methods

Patients and Control Subjects

Six hundred twenty-nine blood samples were collected at seven institutions in Japan. There were 194 patients with POAG, 217 with NTG, and 218 normal control subjects; none of the subjects was related to others in this study. The patients whose age at diagnosis was <35 years and patients with more than −5.5 D of myopia were excluded. Patients with POAG with MYOC mutations were also excluded.

The procedures used in this research conformed to the tenets of the Declaration of Helsinki. Written, informed consent was obtained after the nature and possible consequences of the study were explained. When applicable, the research was approved by the appropriate institutional Human Experimentation Committee.

All patients received serial ophthalmic examinations, including IOP measurements by Goldmann applanation tonometry, Humphrey (30-2) or Goldmann perimetry, gonioscopy, and optic disc examination including fundus photography. In all patients, glaucoma was diagnosed according to the following criteria: presence of typical optic disc damage with glaucomatous cupping (cup-to-disc ratio, >0.7) and loss of neuroretinal rim; reproducible visual field defects compatible with the glaucomatous cupping; and open angles on gonioscopy.

Among the patients with open-angle glaucoma, POAG was diagnosed in those who had an IOP ≥ 21 mm Hg at any time during the follow-up period. Patients with exfoliative glaucoma, pigmentary glaucoma, and corticosteroid-induced glaucoma were excluded.

Among the patients with open-angle glaucoma, NTG was diagnosed when the untreated peak IOP was ≤21 mm Hg at all times, including the three baseline measurements and during the diurnal testing (every 3 hours from 6 AM to 12 PM); when the peak IOP with or without medication after diagnosis was consistently <22 mm Hg throughout the follow-up period; and when the there was an absence of a secondary cause for glaucomatous optic neuropathy, such as a previously elevated IOP after trauma, a period of steroid administration, or uveitis.

The clinical characteristics that were recorded for the patients with glaucoma were age at diagnosis, untreated maximum IOP (defined as IOP at diagnosis), and visual field defects at the initial examination (defined as visual field defects at diagnosis). The severity of the visual field defects was scored from 1 to 5 according to previously reported criteria. The data obtained by two types of perimetry were combined using a five-point scale: 1, no alterations; 2, early defects; 3, moderate defects; 4, severe defects; and 5, light perception only or no light perception. The first four groups on this severity scale followed Kozaki’s classification based on Goldmann perimetry, or the classification was based on results of visual field perimetry (Humphrey Field Analyzer; Carl Zeiss Meditec, Dublin, CA). Kozaki’s classification is widely used in Japan.

The mean age at diagnosis was 58.4 ± 12.0 years in the patients with POAG and 58.0 ± 11.6 years in the patients with NTG. The mean IOP at diagnosis was 26.7 ± 6.0 mm Hg in the patients with POAG and 16.5 ± 2.5 mm Hg in the patients with NTG. The mean visual field score at diagnosis was 5.1 ± 0.9 in POAG and 2.8 ± 0.7 in NTG. Positive family history was recorded in 61 (31.4%) of the 194 patients with POAG and 70 (32.3%) of the 217 patients with NTG. There were 110 (56.7%) men in the POAG group, 97 (44.7%) in the NTG group, and 92 (42.2%) in the control group.

The two hundred eighteen volunteers in the control group received the same examinations. If there was any doubt whether the subject had glaucoma, the subject was excluded. These volunteers were older than 40 years, had IOPs < 20 mm Hg, had normal optic discs, and had no family history of glaucoma. The mean age at the time of the blood sampling was 65.1 ± 12.0 years in POAG, 60.3 ± 12.4 years in NTG, and 70.6 ± 10.9 years in the control subjects. The mean age of the control subjects was significantly older than that of patients with POAG (P < 0.001) and the patients with NTG (P < 0.001). We purposely selected older control subjects to reduce the probability that a subset of them would eventually have glaucoma.

DNA Extraction and PCR Conditions

All the blood samples were analyzed at Keio University. Genomic DNA was isolated from peripheral blood lymphocytes by phenol-chloroform extraction. The 13 exonic coding regions of the OPTN gene were amplified by polymerase chain reaction (PCR), using the primer sets listed in Table 1. A 20-base GC-clamp was attached to some of the forward primers to detect mutations in the higher-melting-temperature domain by DHPLC analysis.

In high-throughput analysis, samples from three patients were pooled. PCR was performed with a thermal cycler (iCycler; Bio-Rad, Hercules, CA) in a total volume of 20 µL containing 45 ng of genomic DNA, 2 µL 10× PCR buffer II (GeneAmp; Applied Biosystems, Inc. [ABI], Foster City, CA), 2 µL of dNTP mix (GeneAmp; ABI) with 2.0 mM concentration of each dNTP, 2.4 µL of a 25-mM MgCl2 solution; 4 pmol of each primer, and 0.1 U of DNA polymerase (AmpliTag Gold; ABI). The PCR conditions were denaturation at 95°C for 9 minutes; 35 cycles at 95°C for 1 minute, 55°C to 60°C for 30 seconds (Table 1), and 72°C for 1 minute, 30 seconds; and a final extension step at 72°C for 7 minutes.

DHPLC Analysis

DHPLC analysis was then performed (Wave System; Transgenomic, Omaha, NE). For heteroduplex formation, products of each PCR (20 µL) were denatured at 95°C for 5 minutes and gradually cooled to 25°C. The annealed PCR products from the three mixed samples were automatically injected into the stationary phase of the DNAsep cartridge (Transgenomic).

Buffer A was made up of 0.1 M triethylammonium acetate (TEAA; Transgenomic), and buffer B of 0.1 M TEAA and 25% acetonitrile. Analysis was performed at a flow rate of 0.9 mL/min and the Buffer B gradient increased by 2%/min for 4.5 minutes. Elution of DNA fragments from the cartridge was detected by absorbance at 260 nm. The temperatures used for the analysis were selected according to the sequences of the DNA fragments. The software (Wavemaker, ver. 4.1.44; Transgenomic) predicted the melting behavior of the DNA fragments at various temperatures. The predicted melting domains within the DNA fragment determined the temperatures for the DHPLC
was detected by using the restriction enzyme assay and by the chromatographic pattern of DHPLC. (317 bp). The polymorphism was confirmed by restriction enzyme
The T-to-A substitution at position c.603 in exon 5 of the
Genotyping the
The G-to-A substitution at position c.412 in exon 4 of the
Genotyping the
were then performed using dye termination chemistry (Prism BigDye Terminator, ver. 3.1 Cycle Sequencing Kit; ABI), according to the manufacturer’s protocol. The data were collected by a gene analyzer (Prism 310; ABI) and analyzed by computer (PRISM Sequencing-Analysis Program, ver. 3.7; ABI).

Genotyping the OPTN c.142G→A
(Thr44Thr) Polymorphism

To detect mutations by direct sequencing, the PCR products were first purified (QiAquick PCR purification kit; Qiagen, Valencia, CA) to remove unreacted primers and precursors. The sequencing reactions were then performed using dye termination chemistry (Prism BigDye Terminator, ver. 3.1 Cycle Sequencing Kit; ABI), according to the manufacturer’s protocol. The data were collected by a gene analyzer (Prism 310; ABI) and analyzed by computer (PRISM Sequencing-Analysis Program, ver. 3.7; ABI).

Genotyping the OPTN c.412G→A
(Thr44Thr) Polymorphism
The G-to-A substitution at position c.412 in exon 4 of the OPTN gene was detected by using the restriction enzyme HpyCH4IV (New England BioLabs, Beverly, MA), with the primers listed in Table 1 for the DHPLC analysis. The G allele sequence was cut into two fragments (192 bp, 20 bp) by HpyCH4IV, whereas the A allele sequence remained intact (212 bp). The polymorphism was confirmed by a restriction enzyme assay and the chromatographic pattern of DHPLC.

Genotyping the OPTN c.1944G→A
(Arg545Gln) Polymorphism
Genotyping the −308G→A Polymorphism
Genotyping the −857C→T Polymorphism
Genotyping the −863C→A Polymorphism

GC-clamp: CGCGCCCGCCGCGCCGCGC.

analysis (Table 1). When abnormal chromatographic patterns were detected in a pool of three samples, each of the three samples was reanalyzed individually in the DHPLC system (Wave System; Transgenic). Then, the PCR product that showed an abnormal chromatographic pattern was sequenced. Once a correlation between abnormal chromatographic patterns and base changes was confirmed by direct-sequencing analysis, additional sequencing analyses were not performed when any of the known abnormal chromatographic patterns were observed in the DHPLC analysis.

Direct DNA Sequencing

To detect mutations by direct sequencing, the PCR products were first purified (QiAquick PCR purification kit; Qiagen, Valencia, CA) to remove unreacted primers and precursors. The sequencing reactions were then performed using dye termination chemistry (Prism BigDye Terminator, ver. 3.1 Cycle Sequencing Kit; ABI), according to the manufacturer’s protocol. The data were collected by a gene analyzer (Prism 310; ABI) and analyzed by computer (PRISM Sequencing-Analysis Program, ver. 3.7; ABI).

Genotyping the OPTN c.142G→A
(Thr44Thr) Polymorphism

The G-to-A substitution at position c.412 in exon 4 of the OPTN gene was detected by using the restriction enzyme HpyCH4IV (New England BioLabs, Beverly, MA), with the primers listed in Table 1 for the DHPLC analysis. The G allele sequence was cut into two fragments (188 bp, 129 bp) by HpyCH4IV, whereas the A allele sequence remained intact (317 bp). The polymorphism was confirmed by restriction enzyme assay and by the chromatographic pattern of DHPLC.

Genotyping the OPTN c.603T→A
(Met98Lys) Polymorphism

The T-to-A substitution at position c.603 in exon 5 of the OPTN gene was detected by the restriction enzyme Stul (TaKaRa, Shiga, Japan), using the same primers as for the DHPLC analysis (Table 1). The A allele sequence was cut into two fragments (175 bp, 102 bp) by Stul, whereas the T allele sequence remained intact (277 bp). The polymorphism was confirmed by a restriction enzyme assay and the chromatographic pattern of DHPLC.

Genotyping the OPTN c.1944G→A
(Arg545Gln) Polymorphism

The G-to-A substitution at position c.1944 in exon 16 of the OPTN gene was analyzed (Invader assay, provided by the Research Department of R&D Center; BML, Saitama, Japan). The polymorphism was confirmed by this assay and by the chromatographic pattern of DHPLC.

Genotyping the TNF-α −308G→A Polymorphism
Genotyping the −857C→T Polymorphism
Genotyping the −863C→A Polymorphism

TABLE 1. Primer Sequences, PCR Product Sizes, and PCR Annealing and DHPLC Analysis Temperatures

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer Sequences (5’ to 3’)</th>
<th>PCR Product Size (bp)</th>
<th>PCR Tm (°C)</th>
<th>DHPLC Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>F CAGTGGTTTGTGGGACTCC</td>
<td>317</td>
<td>60</td>
<td>61.7</td>
</tr>
<tr>
<td></td>
<td>R AAAAGGATTGGCGATTTCCTGCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>F GTCACCTTTCGCGTGCTTCA</td>
<td>277</td>
<td>55</td>
<td>58.7</td>
</tr>
<tr>
<td></td>
<td>R CAACTACAATGTGATCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>F AGCTTTAGTTTTCCTGATTCTC</td>
<td>293</td>
<td>60</td>
<td>57.0, 62.5</td>
</tr>
<tr>
<td></td>
<td>R GTTCTCCTTTCGCAAGGGAGGCT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>F GC-clamp AATCCCTGATTTCTGTTT</td>
<td>188</td>
<td>55</td>
<td>60.4, 61.4, 62.4</td>
</tr>
<tr>
<td></td>
<td>R GTGCAAGCAGCCAGTGACGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>F GC-clamp GTGACTCTCTTGTAGCTTGG</td>
<td>320</td>
<td>57</td>
<td>54.6, 58.5</td>
</tr>
<tr>
<td></td>
<td>R GGGTAAACGTGTAATCTTAAATTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>F GC-clamp GCTATTTCTCTTAAAGCAAGAGA</td>
<td>242</td>
<td>55</td>
<td>57.4, 59.4</td>
</tr>
<tr>
<td></td>
<td>R CATGGGCTGAGACTACTCTGCT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>F GC-clamp GTGAGATGATAATTGTAGATAT</td>
<td>227</td>
<td>55</td>
<td>57.8, 59.8</td>
</tr>
<tr>
<td></td>
<td>R AATGTAATATTTCAAGAGGGAGAAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>F CACATTGCGAGTAAAGGAGCA</td>
<td>286</td>
<td>60</td>
<td>57.5, 59.5</td>
</tr>
<tr>
<td></td>
<td>R CAAATCGAAATTTCAATGTGATAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>F GC-clamp GGTTTGGAGGGAGGAAGTAGT</td>
<td>233</td>
<td>60</td>
<td>60.5, 61.5</td>
</tr>
<tr>
<td></td>
<td>R TCTGTTCATATTGCTACTGAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>F CAGGCGAAATTTATATCAAAACAT</td>
<td>264</td>
<td>60</td>
<td>60.5, 61.5</td>
</tr>
<tr>
<td></td>
<td>R CGGAAATACGTCAGGGCGTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>F GCACACTCCCTCCTACGATAAACCA</td>
<td>260</td>
<td>60</td>
<td>56.7, 59.7</td>
</tr>
<tr>
<td></td>
<td>R GCCATGCTGAGTGGAGCCCTCT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>F GC-clamp GGACTGTGCTGCGACTGTGTC</td>
<td>282</td>
<td>60</td>
<td>56.0, 59.0, 62.0</td>
</tr>
<tr>
<td></td>
<td>R GTTGCCCTTGATTTGGATAATCCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>F GC-clamp CACAACTGCGCTGAGAGACATG</td>
<td>294</td>
<td>60</td>
<td>60.7, 61.7</td>
</tr>
<tr>
<td></td>
<td>R AGGGCAATAATTTGAGTGAAAAAC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Primer Sequences, PCR Product Sizes, and PCR Tm and DHPLC Tm Temperatures
Intron 6 c.863T was present in two control subjects. Thr49Thr was identified in one patient with POAG, and the Pro37Pro was found in 1 patient with NTG. The Met98Lys polymorphism had a higher tendency to be associated with NTG than with POAG. The observed genotype frequencies were in agreement with those predicted by the Hardy-Weinberg equilibrium. Frequency in subjects (%)

<table>
<thead>
<tr>
<th>Location</th>
<th>Sequence Changes</th>
<th>Codon Changes</th>
<th>POAG</th>
<th>NTG</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 4</td>
<td>c.863C→G</td>
<td>His26Asp</td>
<td>1/194 (0.5)</td>
<td>0/217 (0)</td>
<td>0/218 (0)</td>
</tr>
<tr>
<td>Exon 4</td>
<td>c.449_451delCTC</td>
<td>Leu475del</td>
<td>0/194 (0)</td>
<td>0/217 (0)</td>
<td>1/218 (0.5)</td>
</tr>
<tr>
<td>Exon 5</td>
<td>c.603T→A</td>
<td>Met98Lys</td>
<td>33/194 (17.0)</td>
<td>48/217 (22.1)</td>
<td>36/218 (16.5)</td>
</tr>
<tr>
<td>Exon 16</td>
<td>c.1944G→A</td>
<td>Arg545Gln</td>
<td>11/194 (5.7)</td>
<td>15/217 (6.9)</td>
<td>11/218 (5.0)</td>
</tr>
<tr>
<td>Exon 4</td>
<td>c.412G→A</td>
<td>Thr34Thr</td>
<td>69/194 (35.6)</td>
<td>69/217 (31.8)</td>
<td>52/218 (23.9)</td>
</tr>
<tr>
<td>Exon 4</td>
<td>c.412G→A</td>
<td>Pro37Pro</td>
<td>0/194 (0)</td>
<td>1/217 (0.5)</td>
<td>0/218 (0)</td>
</tr>
<tr>
<td>Exon 4</td>
<td>c.457C→T</td>
<td>Thr49Thr</td>
<td>1/194 (0.5)</td>
<td>0/217 (0)</td>
<td>0/218 (0)</td>
</tr>
<tr>
<td>Exon 16</td>
<td>c.2023C→T</td>
<td>His571His</td>
<td>0/194 (0)</td>
<td>0/217 (0)</td>
<td>2/218 (1.0)</td>
</tr>
<tr>
<td>Intron 4</td>
<td>c.476 + 15C→A</td>
<td></td>
<td>0/194 (0)</td>
<td>1/217 (0.5)</td>
<td>0/218 (0)</td>
</tr>
<tr>
<td>Intron 6</td>
<td>c.863 → 10G→A</td>
<td></td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>Intron 6</td>
<td>c.863 → 5C→T</td>
<td></td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>Intron 8</td>
<td>c.1089 + 20G→A</td>
<td></td>
<td>2/147 (1.4)</td>
<td>10/163 (6.1)</td>
<td>4/126 (3.2)</td>
</tr>
<tr>
<td>Intron 9</td>
<td>c.1192 + 19C→T</td>
<td></td>
<td>0/147 (0)</td>
<td>4/163 (2.5)</td>
<td>3/130 (2.5)</td>
</tr>
<tr>
<td>Intron 11</td>
<td>c.1458 + 28C→C</td>
<td></td>
<td>2/147 (1.4)</td>
<td>3/163 (1.8)</td>
<td>0/157 (0)</td>
</tr>
<tr>
<td>Intron 15</td>
<td>c.1922 + 10G→A</td>
<td></td>
<td>1/147 (0.7)</td>
<td>4/163 (2.5)</td>
<td>1/157 (0.6)</td>
</tr>
<tr>
<td>Intron 15</td>
<td>c.1922 + 12G→C</td>
<td></td>
<td>0/147 (0)</td>
<td>1/163 (0.6)</td>
<td>0/157 (0)</td>
</tr>
<tr>
<td>Intron 15</td>
<td>c.1923 - 48C→A*</td>
<td></td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
</tbody>
</table>

NC, not checked.

* Sequence variation was found by direct sequencing analysis.

Statistical Analyses

The frequencies of the genotypes and alleles in patients and control subjects were compared with the χ² test or the Fisher exact test. The odds ratio and 95% confidence intervals (CIs) were also calculated. The Hardy-Weinberg equilibrium for the observed frequencies was also calculated. Comparisons of the clinical characteristics between the two groups were performed using the Mann-Whitney test or Student’s unpaired t-test when appropriate. Logarithmic transformation was performed on skewed-distribution clinical data, which were the IOP at diagnosis of POAG, visual field score at diagnosis of NTG, and POAG, to obtain a normal distribution for performing analysis of variance (ANOVA). One-way ANOVA was used to compare three clinical characteristics among patients with four different combinations of the TNF-α/–857C→T and optineurin/412G→A genotypes, or the TNF-α/–863C→A and optineurin/603T→A genotypes (see Table 6).

Statistical analyses were performed on computer (SPSS software; SPSS Inc., Chicago, IL). P <0.05 was considered to be significant.

RESULTS

OPTN Variants in Japanese Subjects

Six hundred twenty-nine Japanese subjects were studied, and the results are presented in Table 2. Seventeen sequence changes were identified in the patients with glaucoma and control subjects. Among these, three were missense changes, one was a deletion of one amino acid residue, four were synonymous codon changes, and nine were changes in non-coding sequences. One possible disease-causing mutation, His26Asp, was identified in one POAG proband and was not present in the 218 normal Japanese control subjects. Her brother, aged 55, harbored the mutation and received a diagnosis of NTG. Her niece, aged 23, also had the mutation and showed cupping of the optic nerve head with a cup-to-disc ratio of 0.7, with no sign of visual field defect by perimetry (Humphrey 30-2 program; Carl Zeiss Meditec).

A deletion of Leu475 (3-bp deletion, CTC) was found in 1 control. A Met98Lys was identified in 33 patients with POAG, 48 patients with NTG, and 36 control subjects, and an Arg545Gln was identified in 11 patients with POAG, 15 patients with NTG, and 11 control subjects.

Four synonymous nucleotide substitutions, c.412G→A (Thr34Thr), c.421G→A (Pro37Pro), c.457C→T (Thr49Thr), and c.2023C→T (His571His), were found. The Thr34Thr substitution was present in 69 (35.6%) patients with POAG, 69 (31.8%) patients with NTG, and 52 (25.9%) control subjects, and the Pro37Pro was found in 1 patient with NTG. The Thr49Thr was identified in one patient with POAG, and the His571His was present in two control subjects.

Distribution of OPTN Variants in Japanese Subjects

The Thr34Thr (c.412G→A) polymorphism was significantly associated with POAG and NTG (Table 3). A significant association was found in patients with POAG (P = 0.009 in genotype frequency: G/G versus G/A+A/A, and P = 0.003 in allele frequency). No significant difference was detected between patients with glaucoma and control subjects in either genotype or allele frequency for the Met98Lys (c.603T→A) or the Arg545Gln (c.1944G→A) polymorphisms. However, the Met98Lys polymorphism had a higher tendency to be associated with NTG than with POAG. The observed genotype frequencies were in agreement with those predicted by the Hardy-Weinberg equilibrium.

Three clinical characteristics of the patients with glaucoma—age, IOP, and visual field score at diagnosis—were examined for association with the c.412G→A (Thr34Thr) or c.603T→A (Met98Lys) polymorphisms (Table 4). The patients with glaucoma did not show an association with the clinical characteristics with the c.412G→A polymorphism. Patients with POAG with the G/A+A/A genotype (or 412A carriers) tended to have more advanced visual field scores than those with the G/G genotype (or non-412A carriers; P = 0.093). Patients with POAG with the 603T→A polymorphism showed a weak association with age at diagnosis (P = 0.046).
Association between the OPTN and TNF-α Polymorphisms in Patients with Glaucoma

No significant difference in genotype or allele frequency was noted between patients and control subjects for the three polymorphisms of the 5’ flanking region of the TNF-α gene (Table 5). In addition, the patients with glaucoma did not show an association with the clinical characteristics for the three polymorphisms (data not shown). The observed genotype frequencies were in agreement with those predicted by the Hardy-Weinberg equilibrium.

However, among individuals with the C/T→T/T genotype (or −857T carriers) in the TNF-α gene, 44.1% of patients with POAG were G/A/A genotypes (or 412A carriers) of the OPTN gene compared with 21.6% of control subjects (Table 6). This difference in frequency was significant (P = 0.006). Among individuals with the C/A/A+G/A genotypes (or −863A carriers) in the TNF-α gene, 603A carriers (or Lys98 carriers) in the OPTN gene were significantly associated with POAG as well as NTG (P = 0.008 and 0.027, respectively).

The clinical characteristics of these combined genotypes, such as age, IOP, and visual field score at diagnosis are shown in Table 7. The patients with POAG who were TNF-α/−857T and optineurin/412A carriers had significantly worse (P = 0.020) visual field scores than those who were TNF-α/−857T and non–optineurin/412A carriers. However, there was no significant difference in the three clinical features of patients with POAG among the four genotypes of combined −857T→A and c.412G→A polymorphisms (Table 6) by one-way ANOVA: P = 0.823 for age at diagnosis; P = 0.692 for IOP at diagnosis; and P = 0.152 for visual field score at diagnosis.

Patients with POAG who were TNF-α/−863A and optineurin/603A carriers had significantly worse (P = 0.026) visual field scores than those who were TNF-α/−863A and non–optineurin/603A carriers. However, there was no significant difference in the visual field score of patients with POAG among the four genotypes of combined −863C→A and c.603T→A polymorphisms (Table 6, one-way ANOVA: P = 0.200).

**DISCUSSION**

Rezaie et al. detected two missense mutations, Glu50Lys and Arg545Gln, and one truncating mutation due to a 2-bp insertion (c.691_692 ins AG), in 9 (16.7%) of 54 families with hereditary POAG. Most of the family members presented with IOPs ≤ 21 mm Hg, and only 3 of the 23 affected members had a higher IOP (23, 26, and 40 mm Hg). These researchers also identified a risk-associated sequence change (Met98Lys) in 23 (13.6%) of 169 index cases and in 9 (2.1%) of 422 control subjects. This difference in the frequencies between patients and control subjects was significant.

In England, a Glu50Lys mutation was identified in 2 (1.5%) of 132 patients with NTG, and in the Chinese population, two probable disease-causing mutations, Glu103Asp and His486Arg, were found in 2 (1.6%) of 119 patients with sporadic-occurring POAG. However, the results of other studies suggested that alterations of the OPTN gene were rare causes of POAG or NTG.

In our Japanese subjects, 17 sequence changes in the OPTN gene were identified. The missense mutation, His26Asp, in exon 4 was found in only one (0.24%) of 411 patients with open-angle glaucoma and not in 218 normal subjects. In Japan, this mutation has recently been reported in a patient with open-angle glaucoma and not in 218 normal subjects. In Japan, this mutation has recently been reported in a patient with POAG. Thus, His26Asp may be a disease-causing mutation. However, our results and those of two other studies on Japanese patients suggested that OPTN gene mutations are rare...
as a glaucoma-causing gene in Japanese patients with POAG or NTG.

Our observations showed that the frequency of the A allele in c.603T→A (Met98Lys) was slightly higher in patients with NTG (A: 12.2%, P = 0.071) than in patients with POAG (A: 8.8%) or control subjects (A: 8.5%). The Met98Lys change was observed as 22.1% in NTG (P = 0.139), 17.0% in POAG (P = 0.893), and 16.5% in control subjects. In the United Kingdom, a significant association of Met98Lys with NTG but not POAG has been reported.19 These results suggest that there may be genetic differences between the two phenotypes. Tang et al.16 reported no significant difference in allele frequency between Japanese patients with POAG or NTG and control subjects for the c.412A→G (Thr34Thr) polymorphism. Arland et al.20 observed a significantly higher prevalence (P = 0.01) of the Met98Lys change in 51 (20.7%) of 247 Japanese patients with NTG, compared with 8 (9.0%) of 89 Japanese control subjects. However, the number of control subjects in their study was too few to perform a case-control association study.

In patients with POAG in France and Morocco, the Met98Lys frequency was similar to that of control subjects.39 However, a Met98Lys variant was reported to be significantly associated with a lower initial IOP: There was a downward shift of the initial IOP in patients with POAG harboring Met98Lys.39 In our study, a Met98Lys variant was not associated with a lower initial IOP, but was weakly (P = 0.046) associated with an older age at diagnosis in patients with POAG.

No significant difference in the frequency of the Arg545Gln variant was found between Japanese patients with glaucoma and control subjects. In a Chinese population, the Met98Lys and Arg545Gln variants were reported to have similar frequencies in patients with glaucoma and control subjects.18 Arg545Gln is a common polymorphism in the Japanese and Chinese populations, but may be rare in whites.20

The distribution of c.412G→A (Thr34Thr) genotype in the OPTN gene differed significantly between POAG (P = 0.011) and control subjects in our Japanese population, with the A allele being significantly more frequent than the G allele (POAG, P = 0.003). This polymorphism is associated with POAG more than NTG (P = 0.078 in genotype frequency and P = 0.034 in allele frequency). This finding is new, although the c.412G→A polymorphism has been identified in the United States,15 Finland,14 Hong Kong,18 and Japan.16,20 Previous studies of this polymorphism in Japanese patients did not find an association with glaucoma.16,20 Our Japanese subjects resided throughout the nation and consisted of a larger number of subjects, which may account for the differing results.

Although the reason for the significant association of the c.412G→A polymorphism with patients with glaucoma is unknown, it may be linked to another unknown single-nucleotide polymorphism that exists in the promoter region and may alter the activity of the protein or may affect the stability or splicing accuracy of the mRNA.40 Alternatively, the c.412G→A polymorphism may be linked to another unknown gene that lies near the OPTN gene.41
Optineurin is induced by TNF-α and interacts with several proteins to regulate apoptosis, inflammation, and vasoconstriction. For example, optineurin interacts with adenosinergic E3-14.7K protein which protects cells from the cytolytic activity of TNF-α.25 Huntingtin is linked to the Rab8 protein through optineurin and interacts with several genes. Especially, the OPTN c.412G→A (Thr34Thr) polymorphism and TNF-α receptor-1 was upregulated in the retina and optic nerve head.43,44 Yuan and Neufeld45 reported that the expression of TNF-α receptor-1 was upregulated in the retina and optic nerve degeneration. An association of TNF-α c.603T→C polymorphism with POAG has been reported in the Chinese.46 In this study, we examined three single-nucleotide polymorphisms (c.308G→A, c.603T→C, and c.863C→A) in the TNF-α promoter region in a Japanese population. Transcriptional activity of the −857T allele or −863A allele was significantly greater than that of the −857C allele or −863C allele.37 However, no significant difference in genotype or allele frequency was noted between patients and control subjects for the three single-nucleotide polymorphisms of the TNF-α gene. Especially, the −308G→A polymorphism is rare in the Japanese.37

The genotype frequency of the c.412G→A (Thr34Thr) polymorphism in the OPTN gene was significantly associated with POAG, and the frequency of 412A carriers was significantly greater in patients with POAG than in control subjects (P = 0.009). This association was influenced by TNF-α −857C→T genotypes (Table 6). Among individuals with the C/T+T/T genotype (or −857T carriers) in the TNF-α gene, the frequency of optineurin/412A carriers was significantly greater in patients with POAG than in control subjects (odds ratio 2.86, P = 0.006). The visual field scores at diagnosis in patients with POAG were significantly worse in patients with optineurin/412A when they were TNF-α−857T carriers (P = 0.020; Table 7), although we found no significant difference in the scores between the c.412G→A genotypes in the OPTN gene (P = 0.093, Table 4).

The same interactions were more clearly observed between the c.603T→A (Met98Lys) polymorphism in the OPTN gene and the −863C→A polymorphism in the TNF-α gene. Although there was no significant association between c.603T→A (Met98Lys) polymorphism and POAG or NTG, the frequency of optineurin/603A carriers was significantly greater in patients with POAG (odds ratio, 4.11; P = 0.008) than in control subjects and in patients with NTG (odds ratio, 3.31; P = 0.027) in control subjects among individuals with the C/A+A/A genotype (or −863A carriers) in the TNF-α gene. The visual field scores at diagnosis in patients with POAG were significantly worse in patients with optineurin/603A (or Lys98) when they were TNF-α−863A carriers (P = 0.026). However, there was no significant difference in visual field score at

### Table 6. Distribution of Optineurin Genotypes (c.412G→A and c.603T→A) According to TNF-α Genotypes (−857C→T and −863C→A) in Glaucoma Patients and Control Subjects

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>c.412G→A</th>
<th>c.603T→A</th>
<th>c.863C→A</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.412G→A (Thr34Thr)</td>
<td>G/G</td>
<td>G/A + A/A</td>
<td>G/A + A/A</td>
</tr>
<tr>
<td>POAG</td>
<td>92 (68.1)</td>
<td>43 (31.9)</td>
<td>0.024</td>
</tr>
<tr>
<td>Control</td>
<td>108 (75.0)</td>
<td>36 (25.0)</td>
<td>1.00</td>
</tr>
<tr>
<td>NTG</td>
<td>97 (65.5)</td>
<td>51 (34.5)</td>
<td>0.077</td>
</tr>
<tr>
<td>POAG</td>
<td>112 (83.0)</td>
<td>23 (17.0)</td>
<td>0.811</td>
</tr>
<tr>
<td>Control</td>
<td>124 (77.0)</td>
<td>37 (23.0)</td>
<td>1.00</td>
</tr>
<tr>
<td>NTG</td>
<td>111 (75.0)</td>
<td>37 (25.0)</td>
<td>0.056</td>
</tr>
<tr>
<td>POAG</td>
<td>125 (78.6)</td>
<td>34 (21.4)</td>
<td>0.636</td>
</tr>
<tr>
<td>Control</td>
<td>130 (80.7)</td>
<td>31 (19.3)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

**Phenotype**
- c.412G→A (Thr34Thr)
- c.603T→A (Met98Lys)
- c.863C→A

**Odds Ratio**
- G/G
- G/A + A/A
- G/A + A/A

**95% CI**
- G/G
- G/A + A/A
- G/A + A/A

**P**
- c.412G→A (Thr34Thr)
- c.603T→A (Met98Lys)
- c.863C→A

* P by χ² test.
† P < 0.05.
‡ P < 0.01.
diagnosis in patients with POAG among the four different genotypes of combined TNF-α/857T→A and optineurin/412G→A polymorphisms, or TNF-α/863C→A and optineurin/603T→A polymorphisms in Table 6, by one-way ANOVA (P = 0.152 or P = 0.200, respectively). These results suggest an association between the visual field scores at diagnosis and combination of the TNF-α/857C→T and optineurin/412G→A genotypes, or TNF-α/863C→A and optineurin/603T→A genotypes.

In conclusion, the His26Asp mutation in the OPTN gene is a possible disease-causing mutation in Japanese patients with open-angle glaucoma. The c.412G→A polymorphism was significantly associated with POAG and NTG, and the c.603T→A (Met98Lys) polymorphism tended to be associated with NTG. Optineurin expression is directly induced by TNF-α. Genetic statistical analysis showed an interaction between single-nucleotide polymorphisms in the TNF-α gene (−857C→T and −863C→A) and those in the optineurin gene (c.412G→A and c.603T→A), which increases the risk for the development and probably progression of glaucoma in patients with POAG.

Acknowledgments

The authors thank Makoto Nagano in the Research Department of R&D Center, BML for excellent technical assistance with the Invader assay.

References


---

**Table 7. Comparison of Clinical Characteristics of Glaucoma Patients According to TNF-α Genotypes (−857T and −863A) and Optineurin Genotypes (c.412G→A and c.603T→A)**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G/G</td>
<td>G/A + A/A</td>
</tr>
<tr>
<td>c.412G→A (Thr34Thr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>POAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis (y)</td>
<td>57.1 ± 10.7 (n = 32)</td>
<td>57.6 ± 13.1 (n = 26)</td>
</tr>
<tr>
<td>IOP at diagnosis (mm Hg)</td>
<td>26.4 ± 6.1 (n = 30)</td>
<td>26.4 ± 5.5 (n = 20)</td>
</tr>
<tr>
<td>Visual field score</td>
<td>2.9 ± 0.9 (n = 33)</td>
<td>3.3 ± 0.8 (n = 26)</td>
</tr>
<tr>
<td>NTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis (y)</td>
<td>58.4 ± 11.1 (n = 51)</td>
<td>59.3 ± 10.5 (n = 18)</td>
</tr>
<tr>
<td>IOP at diagnosis (mm Hg)</td>
<td>16.4 ± 2.6 (n = 46)</td>
<td>16.1 ± 2.3 (n = 17)</td>
</tr>
<tr>
<td>Visual field score</td>
<td>2.8 ± 0.8 (n = 51)</td>
<td>2.6 ± 0.5 (n = 18)</td>
</tr>
</tbody>
</table>

*P < 0.05, Mann-Whitney test.


