Prevalence of myocilin and optineurin sequence variants in German normal tension glaucoma patients

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Purpose: To determine the prevalence of optineurin (OPTN) and myocilin (MYOC) sequence variants in a cohort of German patients with normal tension glaucoma.

Methods: All coding exons of the OPTN and MYOC genes were amplified by PCR from genomic DNA and subjected to direct DNA sequencing. Analysis of sequence variants in controls was done by denaturing high performance liquid chromatography (DHPLC) and restriction fragment length polymorphism (RFLP) analysis.

Results: Sequence variants were identified by DNA sequencing in 10 of 112 cases. The OPTN sequence variant M98K was found in seven patients. In addition, two novel sequence variants (A336G and A377T) in the OPTN gene were identified that were not present in a control group. Mutation screening also identified two potentially pathogenic MYOC mutations (T293K and A445V).

Conclusions: The findings in the current study provide further evidence that MYOC and OPTN gene variants are rare causes of NTG.

Glucoma is a progressive optic neuropathy leading to permanent visual loss. The disease has a high socio-economic impact as it affects over 67 million people worldwide and is the second largest cause of bilateral blindness in the western world [1]. The Rotterdam Study [2] revealed that primary open angle glaucoma (POAG), which represents the most common form of the disease, is one of the main causes of blindness (8%) in the population sample. Other European studies confirmed these results [3].

Reports of aggregation of POAG in families and sometimes segregating as a Mendelian trait have long suggested heritability in POAG [4]. Recent linkage studies have led to the detection of seven different loci in the human genome associated with juvenile open angle glaucoma, adult onset POAG or normal tension glaucoma (GLC1A-G).

Normal tension glaucoma (NTG) is an important subtype of POAG, accounting for approximately 20-50% of all POAG cases. Patients with NTG show intraocular pressures (IOPs) that are within the statistical normal range of the population (10-20 mm Hg). Until recently, only one disease causing gene for POAG has been identified: The MYOC gene, first described by Stone and coworkers [5] in families linked to the GLCIA locus, has been shown to be mutated in about 4% of primary open angle glaucoma cases [6]. Mutations in the optineurin gene (OPTN) were initially reported in 16.7% of families with hereditary POAG, with most of them having NTG [7]. However, other reports have indicated that OPTN sequence variants are only a rare cause of POAG or NTG [8-13]. The role of OPTN in normal ocular physiology and glaucoma is currently unclear.

The purpose of this study was to determine the prevalence of optineurin (OPTN) and myocilin (MYOC) sequence variants in a cohort of unrelated German patients with normal tension glaucoma.

METHODS

Ascertainment of patients: Written informed consent was obtained from all subjects and the study was approved by the ethics committees of the University Hospital Tuebingen and the University Hospital Wuerzburg. It was performed in accordance with the Helsinki Declaration. The patients comprised a cohort of 112 unrelated white patients with NTG that included 67 women and 45 men, with ages ranging from 14 to 84 years. All patients in this study are part of a cohort of 289 patients that has been clinically investigated at the University Eye Hospital in Wuerzburg [14]. Of those, 112 patients with a long term follow-up were selected for molecular genetic analysis in order to ensure diagnosis of NTG with a maximum of certainty. Glaucoma was defined by the following strict criteria: the presence of typical glaucomatous optic neuropathy with compatible visual field loss (according to Aulhorn classification), open drainage angles on gonioscopy, and absence of a secondary cause for glaucomatous optic neuropathy such as a previously raised IOP after trauma, a period of steroid administration, or uveitis. Patients also did not have evidence of high myopia or congenital ocular abnormality, and had no other cause than glaucoma for disc changes and visual field loss. A neurological examination was performed in order to exclude an intracerebral expansion. Stenosis of the Aorta carotis interna was excluded by means of sonography. Patients with NTG had IOPs without treatment that were consistently 21 mm Hg...
or less on diurnal testing and during follow-up. Disc size and disc parameters were evaluated by means of confocal examination (Heidelberg Retina Tomograph). Control DNA samples were obtained from 100 unrelated subjects of German descent without visual complaints. Control subjects had no ophthalmic examination.

Mutation detection by direct sequencing: Patient DNA was extracted from peripheral blood lymphocytes using a standard salting-out procedure. Individual exons of the MYOC and the OPTN gene were amplified by polymerase chain reaction (PCR) using appropriate amplification protocols. Primer pairs for amplification and sequencing are available upon request. PCR fragments were purified by ExoSAP-IT treatment (USB, Cleveland, OH), sequenced using Big Dye Termination chemistry (Applied Biosystems, Weiterstadt, Germany), and products separated on a DNA capillary sequencer (ABI 3100 Genetic Analyzer).

Detection of nucleotide variants by DHPLC: In control subjects, OPTN Exon 11 was screened for the A336G and the A377T sequence variants with the WAVE denaturing high performance liquid chromatography system (Transgenomic, Inc., Omaha, NE) at 57.7 °C. The resulting DHPLC trace profiles were examined with Navigator™ software. The chromatograms were compared with the profile of wildtype DNA fragments. Samples with aberrant profiles were sequenced.

Detection of nucleotide variants by RFLP: A 341 bp PCR product encompassing exon 5 of the OPTN gene was digested with 1 U Stul restriction enzyme (NEB, Beverly, MA). The M98K missense change results in the gain of a Stul restriction site, that upon cleavage of the amplicon produces two DNA fragments of 190 bp and 151 bp.

RESULTS
We sequenced the complete coding sequence plus flanking intron/UTR sequences of MYOC and OPTN in 112 NTG patients. Two sequence alterations were detected in exon 3 of MYOC and 3 sequence alterations were detected in exons 5 and 11 of OPTN (Table 1).

The two point mutations in exon 3 of the MYOC gene are heterozygous mutations at the second nucleotide of codon 293 (c.878C>A), which substitutes threonine with lysine (T293K), and at the second nucleotide of codon 445 (c.1334C>T), which substitutes alanine with valine (A445V), respectively. Both mutations have been described before and were judged as probable disease causing mutations [15]. The point mutation in exon 5 of the OPTN gene affects the second nucleotide of codon 377 (c.1317C>G), resulting in an alanine to glycine change (A336G), and at the first nucleotide of codon 377 (c.1439G>A), resulting in an alanine to threonine change (A377T), respectively. These mutations have not been described yet and were also excluded in 100 ethnically matched controls. We did not find any of the other OPTN variants (E50K, c.691_692_insAG, and R545Q) described in the original publication by Rezaie and coworkers [7]. Screening of both the MYOC and the OPTN gene also led to the identification of a number of polymorphisms (c.412G>A, c.433G>A, and c.863-5C>T in OPTN, c.227G>A, c.366C>T, and c.1041T>C in MYOC) that have already been described in other studies [7,9,15,17,18] and a new polymorphism (c.524G>A) in the MYOC gene (Table 1). Neither a deletion nor a sequence alteration close to splice donor/acceptor sites were found.

DISCUSSION
The study presented here is the first large scale analysis of OPTN and MYOC gene variants in German patients with the NTG subtype of POAG. (1) Our cohort comprises a clinically homogenous group of patients based on the application of a strictly defined clinical protocol and examination by a single physician. (2) The patients were enrolled solely based on clinical criteria. Without any selection bias for familial cases of glaucoma our sample is representative for NTG as a whole. (3) All patients were screened for OPTN and MYOC gene mutations by DNA sequencing of the complete coding sequence plus flanking intron/UTR sequences. DNA sequencing represents the gold standard in mutation screening as it offers higher detection rates compared with other techniques. Taking into account these points, our study provides reliable and representative data for the prevalence of OPTN and MYOC gene variants in German NTG patients.

<table>
<thead>
<tr>
<th>Location</th>
<th>Sequence change</th>
<th>Codon change</th>
<th>Genotype</th>
<th>NTG (n=112)</th>
<th>Controls (n=100)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MYOC:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 1</td>
<td>c.227G&gt;A</td>
<td>R76K</td>
<td>3/37/ 72</td>
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<td>NA</td>
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<tr>
<td>Exon 1</td>
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<td>G122G</td>
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<td>NA</td>
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<tr>
<td>Exon 1</td>
<td>c.524G&gt;A</td>
<td>R168R</td>
<td>0/ 1/111</td>
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<td>NA</td>
</tr>
<tr>
<td>Exon 3</td>
<td>c.1041T&gt;C</td>
<td>Y347T</td>
<td>0/ 8/104</td>
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<td>NA</td>
</tr>
<tr>
<td>Exon 3</td>
<td>c.878C&gt;A</td>
<td>T293K</td>
<td>0/ 1/111</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Exon 3</td>
<td>c.1334C&gt;T</td>
<td>A445V</td>
<td>0/ 1/111</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td><strong>OPTN:</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Exon 4</td>
<td>c.412G&gt;A</td>
<td>T34T</td>
<td>14/35/ 63</td>
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<tr>
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<td>M98K</td>
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<td>0/ 7/ 93</td>
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</tr>
<tr>
<td>Intron 6</td>
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<td>NA</td>
</tr>
<tr>
<td>Exon 11</td>
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<td>A336G</td>
<td>0/ 1/111</td>
<td>0/0/100</td>
<td>0/0/100</td>
</tr>
<tr>
<td>Exon 11</td>
<td>c.1439G&gt;A</td>
<td>A377T</td>
<td>0/ 1/111</td>
<td>0/0/100</td>
<td>0/0/100</td>
</tr>
</tbody>
</table>

Potentially pathogenic sequence changes are in red. Under the “Genotype” column, the numbers are the counts of homozygotes, heterozygotes, and wildtype. Some mutations were not analyzed (NA).
Two missense mutations in the MYOC gene were detected in our mutational screening of 112 NTG patients. This is consistent with the recent figures that about 2-4% of POAG patients worldwide have MYOC mutations [19]. We also detected three missense mutations in the OPTN gene. The M98K variant has been reported in multiple studies [7,9-11,16-18], while the other two are novel mutations (A336G and A377T). M98K was found to have intriguingly similar frequencies among glaucoma patients and control subjects in several studies [9-11], although it was reported to be associated specifically with NTG [7,12] and was shown to be associated with a decreased intraocular pressure in patients with POAG [20]. In our study, the M98K variant was present in 6.3% of patients with NTG and in 7% of control subjects. Therefore, our results do not support the notion that the M98K variant represents a risk associated mutation in normal tension glaucoma. However, because the average age in the control group was significantly lower than in the patient cohort, it remains possible that a subset may eventually undergo development of glaucoma and consequently affect the statistical results of the current study.

In addition, we could identify two novel coding changes in the OPTN gene; A336G and A377T. We consider them as disease causing because they are absent in the control group and the mutated amino acid residues are fully conserved in OPTN sequences of five different mammalian species (human, macaque, mouse, rat, and pig). Both affected patients are sporadic cases of NTG suggesting either reduced penetrance or de novo mutations.

So far, no mutations that affect splicing have been identified in the OPTN gene. Although the A336G and A377T sequence changes are located relatively close to exon/intron boundaries, these changes were not predicted to introduce a cryptic splice site. However, the A336G and A377T sequence changes may represent exonic mutations that affect exonic splicing enhancer (ESE) sequences. Bioinformatic modeling of the ESE site shows that both A336G and A377T altered the potential ESE sites in exon 11 of OPTN, resulting in the gain of a SF2/ASF site (data not shown). There is growing evidence supporting the role of the splicing machinery as a potentially potent modifier of the phenotypic outcome of a genotype [21]. Additional studies will be necessary to evaluate the role of optineurin sequence variants in the pathogenesis of normal tension glaucoma.

REFERENCES
