INTRODUCTION

By the year 2040, more than 110 million people will be affected with glaucoma worldwide. In the United States, glaucoma is the second leading cause of irreversible blindness and the leading cause among Americans with African heritage. Glaucoma is a heterogeneous group of diseases that share two defining clinical features: structural defects (eg, optic disc cupping) and functional defects (eg, visual field loss). Primary open-angle glaucoma (POAG) is the most common form of glaucoma worldwide. Many clinical risk factors for POAG have been described, including older age, African or Latino ancestry, family history, higher intraocular pressure (IOP), and more recently, central corneal thickness (CCT). Higher IOP confers greater risk for developing glaucoma. However, glaucoma can occur at any IOP. Primary open-angle glaucoma that occurs with IOPs that never rise above an arbitrary threshold of 21 mm Hg is frequently termed normal-tension glaucoma (NTG).

The concept that POAG is heritable is supported by epidemiologic studies, twin studies, and reports of families in which the condition is transmitted as a heritable trait. Formal studies to quantify the role of genes in the development of POAG are extremely limited. One twin study, in which a diagnosis of glaucoma was detected by review of a government registry, estimated the heritability of POAG to be 0.13 (13%). However, this is likely a significant underestimate owing to small sample size and the methods used to identify cases of glaucoma.

Many of the component features of glaucoma, or “endophenotypes,” such as IOP, cup-to-disc ratio, and CCT, are also known to be heritable traits. Several studies of IOP have shown that this risk factor for glaucoma also has a genetic basis. For example, early studies of normal subjects by Armaly indicated that the magnitude of IOP has a heritability of 0.23 to 0.47. The Beaver Dam Eye Study and the Salisbury Eye Evaluation Study also showed a correlation between IOP in family members (heritability of 0.29 to 0.36). Similar studies of cup-to-disc ratio have reported heritability of 0.48 to 0.56. More recently, CCT has been identified as another highly heritable risk factor for glaucoma. A strong correlation of CCT has been detected between first-degree relatives (heritability of 0.6 to 0.7) and between twins (heritability of 0.95). These data show that both POAG and its component features are highly heritable and have a strong genetic basis.

Some cases of POAG are likely caused by the combined actions of several genetic and environmental factors that each contribute a small risk for glaucoma. Such genetic risk factors are commonly observed in both glaucoma patients and normal individuals, but are statistically more frequently observed in POAG patients. Each factor is incapable of causing disease on its own, but the cumulative effects of many of these relatively weak genetic and environmental risk factors may lead to development of POAG. This form of POAG does not have an obvious inheritance pattern as a result of the many independently inherited factors that combine to cause disease. However, because families (and ethnic groups) share many genetic factors, complex genetic forms of glaucoma may be more common within families or specific ethnic groups. Recently, large genome-wide association studies have begun to detect several of these POAG genetic risk factors, including caveolin 1/2 (CAV1/CAV2), cyclin-dependent kinase inhibitor 2B antisense RNA1

**IDENTIFICATION OF TANK-BINDING KINASE 1 AS A NEW NORMAL-TENSION GLAUCOMA GENE**

We recently identified a second NTG gene, TANK-binding kinase 1 (TBK1), with studies of a large four-generation African American family (pedigree GGO-441) in which 10 family members were diagnosed with NTG. Affected family members had clinical features consistent with severe NTG. The mean maximum IOP in the affected family members was 18.2 ± 4.1 mm Hg, and mean cup-to-disc ratio at the time of diagnosis was greater than 0.9. Moreover, there was an early onset of glaucoma in this pedigree, with a mean age at diagnosis of 36 ± 8.2 years. Linkage-based studies of this family and mutation screening identified a large duplication spanning the TBK1 gene as the cause of NTG in this pedigree.

Subsequent studies of many large populations of NTG patients worldwide established that copy-number variations (CNVs), including duplications or triplications of the TBK1 gene, cause approximately 1% of NTG cases (Figure 1). In these studies, another large NTG pedigree was discovered to have a triplication spanning the TBK1 gene. Overall, the frequency of TBK1 CNVs in NTG patients (8 of 1105, 0.72%) in these studies is statistically higher than in control subjects (0 of 1320, 0%). The CNVs ranged in size from 295,000 base pairs (GGJ-414) to 690,000 base pairs (GGO-441), and all of the identified CNVs overlap the entire TBK1 gene. Moreover, TBK1 is the only gene that is spanned by the overlap of the CNVs (Figure 1). Duplications and triplications of TBK1 are associated with both 0.4% and 1.3% of NTG cases in various populations and have a prevalence of 0.72% in NTG patients overall.

**FUNCTIONAL STUDIES OF TBK1 DUPLICATION ON GENE EXPRESSION**

We investigated the functional effect of an extra copy of the TBK1 gene by comparing gene expression in a fibroblast cell culture system. Fibroblasts were obtained from six NTG patients from pedigree GGO-441 who were known to have a TBK1 gene duplication and from six control subjects. The expression level of TBK1 mRNA in these cells was measured using three independent methods: Northern blot analysis, quantitative reverse transcription polymerase chain reaction (PCR) assays, and microarray analysis.

In all cases, TBK1 gene duplications were found to result in increased TBK1 mRNA expression. In the microarray-based studies, TBK1 mRNA was found to be 1.60-fold higher in fibroblast cells from patients with a TBK1 gene duplication than in controls (P = .00074), demonstrating a functional consequence of the gene duplication. Increased transcription of TBK1 mRNA suggests that duplication of the TBK1 gene dysregulates its expression and may lead to increased production and/or kinase activity of TBK1 as a step in the pathogenesis of glaucoma.

Although increased TBK1 gene expression was detected in cells carrying a TBK1 gene duplication, the expression levels of other genes of interest in autophagy, including PTEN-induced putative kinase 1 (PINK1), parkin RBR E3 ubiquitin protein ligase (PARK2), optineurin (OPTN), and microtubule-associated protein 1 light chain 3 alpha (LC3), beclin (BECN1), and sequestosome (SQSTM1) were not significantly altered.

**TBK1 GENE DISCOVERY PROVIDES INSIGHTS INTO NORMAL-TENSION GLAUCOMA PATHOGENESIS**

The human TBK1 gene is composed of 21 exons and encodes a 729 amino acid, 84 kDa protein that has several known functional domains, including a kinase domain (AA 1-308), a ubiquitin-like domain (AA 309-383), a scaffold/dimerization domain (AA 407-657), and a TANK-binding domain (AA 688-729). Several key structures in the TBK1 protein have been identified that regulate its function. Kinase function is activated by phosphorylation of Ser172 and ubiquination of Lys30 and Lys401. TBK1 participates in several signaling pathways (NF-κB, autophagy, and others) by phosphorylating downstream target proteins. Several substrates for
TBK1’s kinase activity have been identified, including interferon regulatory factor 3 (IRF3), interferon regulatory factor 7 (IRF7), p62, and most notably, OPTN.

FIGURE 1
Extent of TBK1 copy-number variations (CNVs) on chromosome 12q14 detected in normal-tension glaucoma (NTG) patients and pedigrees. The overlap of these CNVs spans a single gene, TBK1.

Two genes, TBK1 and OPTN, are known to cause high-penetrance, autosomal dominant forms of NTG. Copy-number variations in TBK1 are associated with approximately 1% of NTG cases, whereas missense mutations in OPTN are associated with 1% to 2% of NTG cases. Remarkably, these genes encode proteins that have been shown to directly interact with each other. Early studies by Morton and coworkers and by others used the yeast two-hybrid system, glutathione-S-transferase pull-down assays, and co-immunoprecipitation to show that TBK1 interacts with OPTN. The TBK1-binding domain of OPTN was subsequently localized to its N-terminus (amino acid residues 78-121), and the OPTN-binding domain of TBK1 was mapped to the C-terminus (amino acid residues 688-729). In addition to interacting with OPTN, TBK1 phosphorylates and activates OPTN. Not only have both of the two known Mendelian NTG genes been shown to interact with each other, but glaucoma-causing mutations have also been shown to alter this interaction. The E50K mutation in OPTN enhances binding with TBK1, and recent cell culture studies have suggested that the pathogenicity of OPTN mutations is dependent on interactions with TBK1. These data suggest a common biological pathway and mechanism of disease for NTG caused by mutation of TBK1 or OPTN.

TBK1 FUNCTION AND INTERACTION WITH OPTINEURIN SUGGESTS A ROLE FOR AUTOPHAGY IN NORMAL-TENSION GLAUCOMA PATHOGENESIS

Genetic studies of NTG have identified mutations or variations in three genes known to participate in autophagy, an important catabolic biological process. In addition to the TBK1 and OPTN genes, which have been linked with autosomal dominant, Mendelian forms of NTG, the toll-like receptor 4 (TLR4) gene has also been associated with NTG. In some, but not all, association studies of NTG, TLR4 has been associated with complex genetic forms of NTG. The known roles of TBK1, OPTN, and TLR4 in autophagy (described below) and their association with NTG suggest that autophagy has an important role in this form of glaucoma.

Macrophagocytosis, hereafter referred to as autophagy, is a catabolic process used to digest proteins and cellular structures such as organelles for energy in times of nutritional deprivation. In autophagy, a double membrane structure (the autophagosome) forms around materials in the cytoplasm. Autophagosomes then fuse with lysosomes and their contents are degraded into basic building blocks (ie, amino acids), which support cell survival in times of starvation. The catabolic mechanisms of autophagy have been shown to serve a variety of disparate purposes beyond adapting to nutritional stress. Autophagy may support cellular homeostasis by degrading proteins that accumulate from basal metabolism. The innate immune system may also utilize autophagy to capture and eliminate intracellular pathogens. Finally, autophagy has a key role in the pathophysiology of many neurodegenerative diseases, such as amyotrophic lateral sclerosis, Parkinson disease, Huntington disease, and Alzheimer disease. The role of autophagy in the neuronal cell death in these diseases suggests that it may be involved in other neurodegenerative diseases, such as glaucoma.
Autophagy has been implicated as a contributor to retinal ganglion cell death in several experimental animal models of glaucoma. Activation of autophagy in some of these animal models increases the rate of cell death. For example, increased autophagy has been linked with retinal ganglion cell death observed in a rat glaucoma model with chronic elevation of IOP\textsuperscript{57} and in a rat model of retinal ischemia following high IOP.\textsuperscript{77} In contrast, in other animal models of glaucoma, activation of autophagy appears to be neuroprotective. In one mouse model of glaucoma that employs an acute, severe injury to the optic nerve (optic nerve transection), induction of autophagy appears to be protective to retinal ganglion cells.\textsuperscript{78} The range of influences that autophagy may exert on retinal ganglion cell survival suggests complex interactions that depend heavily on biological context. In some circumstances autophagy may promote retinal ganglion cell survival (such as following optic nerve transection), perhaps by clearing damaged or toxic cellular components.\textsuperscript{79,80} However, in other circumstances, activation of autophagy may promote apoptosis and retinal ganglion cell death, possibly by excessive degradation of cellular structures necessary for survival.\textsuperscript{76} These data also raise the possibility that dysregulation of autophagy by either overstimulation (too much autophagy) or by defects that block its function (too little autophagy) may be pathologic. Tight regulation of autophagy may be necessary for cell survival. Moreover, mitophagy is a subset of autophagy in which the mitochondria are targeted for degradation, and recent studies have suggested that \textit{TBK1} has a role in this specific type of autophagy.\textsuperscript{81,82} Although the evidence linking \textit{TBK1}-related NTG to autophagy is compelling, it is possible that mutations in \textit{TBK1} cause disease via another mechanism. \textit{TBK1} also has important roles in NF-κB signaling and other biological processes that could also be involved in the pathogenesis of NTG.\textsuperscript{26}

### CURRENT STUDIES OF \textit{TBK1} AND JUVENILE-ONSET OPEN-ANGLE GLAUCOMA AND SECONDARY FORMS OF GLAUCOMA

Family-based genetic studies of glaucoma have successfully identified glaucoma-causing genes such as myocilin and optineurin and provided important insights into the pathophysiology of glaucoma. Here we present additional research into the mechanisms of NTG using research that began with genetic studies of large glaucoma pedigrees and has been confirmed with population-based experiments. It is our general hypothesis that genetic studies of open-angle glaucoma pedigrees will facilitate identification of new glaucoma-causing genes and will provide novel insights into the biological pathways that are important in the pathogenesis of glaucoma. With this approach, we have identified \textit{TBK1} as a gene that causes NTG. Frequently, a gene discovered to cause one form of a disease is later shown to cause similar forms of disease as well. For example, mutations in myocilin were initially discovered to be a common cause of juvenile-onset open-angle glaucoma (JOAG)\textsuperscript{42} and were later shown to also cause 3% to 4% of POAG.\textsuperscript{42,45} We have already searched for \textit{TBK1} CNVs in some forms of glaucoma, ie, POAG. However, no \textit{TBK1} CNVs were detected in patients with POAG that have IOP > 21 mm Hg.\textsuperscript{54,59,83} Here we extend this research by hypothesizing that mutations in \textit{TBK1} might cause other forms of open-angle glaucoma, such as JOAG, pigmentary glaucoma (PG), exfoliation glaucoma (XFG), or steroid-induced glaucoma (SIG). To test this hypothesis, cohorts of patients with JOAG, PG, XFG, and SIG were investigated for the presence of \textit{TBK1} mutations.

### METHODS AND MATERIALS

#### STUDY DESIGN

Patients from Iowa diagnosed with JOAG, PG, XFG, or SIG were tested for CNVs in the \textit{TBK1} gene in a prospective case-control study. Glaucoma patients were tested for \textit{TBK1} CNVs in a prospective manner, and 608 matched control subjects from Iowa that had been previously tested for \textit{TBK1} mutations were also used as controls for this study.\textsuperscript{44,58}

#### ENROLLED STUDY PATIENTS

All participants provided informed consent for this research project, which was conducted under the approval of the University of Iowa’s Internal Review Board for human subjects research. All patients received a comprehensive eye examination by glaucoma fellowship–trained, board-certified ophthalmologists that included visual acuity, gonioscopy, tonometry, perimetry, and ophthalmoscopy. Most patients also underwent optic nerve and retinal nerve fiber layer examination with spectral domain optical coherence tomography. In some cases, family members who were unavailable for examination shared records from community ophthalmologists that were reviewed by the research team. Patients were judged to have open-angle glaucoma based on standard criteria as we have previously described.\textsuperscript{44,84} Briefly, patients with glaucomatous cupping of the optic disc and corresponding visual field defects with a maximum IOP > 21 mm Hg were diagnosed with POAG. Patients with POAG diagnosed at age < 40 years were diagnosed with JOAG. All JOAG patients had been previously tested for myocilin mutations, and those with myocilin mutations were not included in this study. A total of 30 JOAG patients from Iowa were included in the study.

Patients were judged to have pigment dispersion syndrome (PDS) if Krukenberg spindle, iris transillumination defects, pigment on zonules or lens equator, or dark pigmentation of the trabecular meshwork were identified on examination, and they were further diagnosed with PG if they met criteria for glaucoma as described above. A total of 297 patients with PDS were enrolled (221 from Iowa and 76 from New York), and of these PDS patients, 209 also had PG (165 from Iowa and 44 from New York). Patients were judged to have exfoliation syndrome (XFS) if they had signs of exfoliation material on the anterior surface of the lens, iris margin, or corneal endothelium; peripupillary iris transillumination defects; or brown sugar–colored pigmentation of the trabecular meshwork. Patients were further judged to have XFG if they had signs of XFS and also met criteria for glaucoma as described above. A total of 267 patients with XFS were enrolled (167 from Iowa and 100 from New York), and of these XFS patients, 225 had XFG, 167 from Iowa and 58 from New York. Patients were judged to have SIG if they had glaucoma associated with ocular hypertension following...
administration of topical, oral, periocular, or intraocular steroids (79 from Iowa). Normal control subjects had a complete eye examination and were judged not to have glaucoma by an ophthalmologist. Control subjects in this report were part of previous studies.44,58 DNA samples were prepared from blood samples using standard techniques.85

Power calculations were conducted to assess our ability to detect \( \text{TBK1} \) CNVs in each cohort. Given the individual cohort sizes, we had 80% power to detect CNVs if they occurred with a frequency of 7% or greater in the JOAG population (\( n=30 \)), 0.94% or greater in the PG population (\( n=209 \)), 0.86% or greater in the XFG population (\( n=225 \)), and 2.5% or greater in the SIG population (\( n=79 \)).

GENETIC STUDIES (MUTATION DETECTION)

A TaqMan Copy Number Assay, a quantitative polymerase chain reaction (qPCR) assay (TaqMan, Probe Hs06980763_cn specific for \( \text{TBK1} \) exon 3, Applied BioSystems, Carlsbad, California) was used to assess \( \text{TBK1} \) gene dosage in JOAG, PG, XFG, and SIG patients as well as in previously tested control subjects.44,58 The TaqMan assay was conducted using the manufacturer’s protocol on a BioRad C1000 thermocycler with the CFX96 real-time PCR adapter, and a positive control was used in every experiment. All experiments were conducted in triplicate. Confirmation of positive tests for \( \text{TBK1} \) CNVs was conducted with two additional independent Taqman primer sets (Probe Hs02974732_cn specific for \( \text{TBK1} \) exon 13 and Probe Hs07396069_cn specific for \( \text{TBK1} \) exon 1, Applied BioSystems). In all CNV experiments, an RNAseP probe was used as a control.

RESULTS

TESTING COHORTS OF JUVENILE-ONSET OPEN-ANGLE GLAUCOMA PATIENTS FOR COPY-NUMBER VARIATIONS THAT SPAN THE \( \text{TBK1} \) GENE

Juvenile-onset open-angle glaucoma patients have not been evaluated for \( \text{TBK1} \) mutations in prior reports. In this study, a cohort of 30 patients diagnosed with JOAG was tested for CNVs that span the \( \text{TBK1} \) gene using a qPCR assay that measures copies of the 5' end of the gene, exon 3. In this novel assessment of JOAG patients, no \( \text{TBK1} \) CNVs were detected, suggesting that \( \text{TBK1} \) gene dosage variants are not commonly involved in the pathogenesis of JOAG.

TESTING COHORTS OF SECONDARY GLAUCOMA PATIENTS FOR COPY-NUMBER VARIATIONS THAT SPAN THE \( \text{TBK1} \) GENE

The role of \( \text{TBK1} \) CNVs in secondary forms of glaucoma has not previously been investigated. Here we tested patients with three different types of secondary open-angle glaucomas, including PG, XFG, and SIG (Table 1), for mutations that alter \( \text{TBK1} \) gene dosage using the same qPCR assay described above.

<table>
<thead>
<tr>
<th>( \text{TBK1} ) CNV</th>
<th>PIGMENTARY ( n=209 )</th>
<th>EXFOLIATION ( n=225 )</th>
<th>STEROID-INDUCED ( n=79 )</th>
<th>CONTROLS ( n=608 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{TBK1} ) duplications</td>
<td>0</td>
<td>1 (0.44%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>( \text{TBK1} ) triplications</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>0</td>
<td>1 (0.44%)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

CNV, copy-number variation.

Pigmentary Glaucoma

A cohort of 297 patients with PDS, 209 of which also had secondary PG, was tested for deletions or duplications of the \( \text{TBK1} \) gene. No \( \text{TBK1} \) deletions or duplications were detected.

Exfoliation Glaucoma

A cohort of 267 patients with XFS, 225 of which also had secondary XFG, was tested for deletions or duplications of the \( \text{TBK1} \) gene. A \( \text{TBK1} \) gene duplication was detected in one (0.44%) of 225 XFG patients, patient GGR-623-1. This CNV was subsequently confirmed by two additional qPCR probes specific for additional segments of the \( \text{TBK1} \) gene (exon 1 and exon 13).

Steroid-Induced Glaucoma

A cohort of 79 patients with steroid-induced ocular hypertension and SIG were tested for deletions or duplications of the \( \text{TBK1} \) gene. No \( \text{TBK1} \) deletions or duplications were detected.

A \( \text{TBK1} \) gene duplication was detected in one of 225 patients with XFG, and no such mutations were identified in PG or steroid-induced ocular hypertension/glaucoma patients. These data indicate that mutations involving \( \text{TBK1} \) gene dosage may be involved in a subset of XFG cases.
CLINICAL FEATURES OF A PATIENT WITH EXFOLIATION GLAUCOMA AND A TBK1 GENE DUPLICATION

Patient GGR-623-1 tested positive for a TBK1 gene duplication. He was diagnosed with XFG at 59 years of age based on the bilateral pattern of exfoliation material observed on his anterior lens capsules and gonioscopy, which showed heavy pigmentation in the trabecular meshwork and Sampaolesi lines in both eyes. He has markedly thin central corneas (450 μm in the right eye and 460 μm in the left eye), severe optic nerve damage with large cup-to-disc ratios (Figure 2), and corresponding visual field defects consistent with end-stage glaucoma. Both a trabeculectomy with mitomycin C and a Baerveldt implant were ultimately required in both eyes for control of IOP. The maximum recorded IOPs prior to filtering surgeries, but on topical medications, were 44 mm Hg in the right and 40 mm Hg in the left eye. Patient GGR-623-1 had a positive history of glaucoma in his mother, but no information about XFS was available.

FIGURE 2
Optic disc photos of right eye (on left) and left eye (on right) of exfoliation glaucoma patient GGR-623-1 with a TBK1 gene duplication, taken when patient was 74 years old. Glaucomatous cupping is seen.

DISCUSSION

MUTATIONS INVOLVING TBK1 GENE DOSAGE ARE MOST FREQUENTLY DETECTED IN PRIMARY OPEN-ANGLE GLAUCOMA THAT OCCURS AT LOW INTRAOCULAR PRESSURE

Our prior genetic study of a large African American pedigree demonstrated that duplication of the TBK1 gene causes a familial, autosomal dominant NTG in rare extended pedigrees. Subsequent population studies of NTG further showed that TBK1 CNVs (TBK1 duplications or triplications) are responsible for between 0.4% and 1.3% of NTG cases worldwide.\(^{44,57-59}\) Previous investigations of NTG with family-based studies had identified another glaucoma-causing gene, optineurin (OPTN).\(^{43}\) Missense mutations in OPTN (ie, E50K mutation) have been shown to cause 1% to 2% of NTG cases. These data indicate that TBK1 CNVs are one of the two most common known mutations that cause Mendelian forms of NTG (ie, after OPTN E50K mutations).

PHENOTYPE OF TBK1-RELATED NORMAL-TENSION GLAUCOMA

Eight unrelated NTG probands with TBK1 CNVs had been previously identified.\(^{44,57-59}\) Several features of NTG related to TBK1 CNVs have been observed. Patients with TBK1-related NTG have four chief clinical features: early onset, strong family history of NTG, low IOP, and large cup-to-disc ratio at diagnosis. The average age at diagnosis of these patients is 37 years. The mean maximum observed IOP for patients with TBK1-related glaucoma was 17 mm Hg, although three NTG patients had IOP slightly above 21 mm Hg, which suggests that low IOP is not an absolute feature of this form of glaucoma. Six (75%) of the eight probands had a positive family history of NTG. In all TBK1-related NTG pedigrees, the inheritance pattern is consistent with autosomal dominant inheritance of glaucoma. Finally, many of the TBK1-related NTG patients had nearly completely cupped optic discs at the time of diagnosis, demonstrating that these patients have severe glaucoma. Overall, patients with TBK1-related NTG have an unremarkable mean CCT of 530 μm; however, some patients have CCT <500 μm, and one patient had a CCT >600 μm. The phenotype of the XFG patient with a TBK1 mutation described in this report, patient GGR-623-1, differs from that of patients with
TBK1-related NTG. Patient GGR-623-1 had a later age at diagnosis and a higher maximum IOP. However, identification of more XFG patients with a TBK1 mutation will be necessary to make any definitive comments about the phenotype of TBK1-related XFG.

Genetic testing for TBK1 CNVs is not currently widely available for diagnosis of NTG. However, given the relatively low prevalence of TBK1 CNVs (ie, 1% of NTG), testing for diagnostic purposes should be considered only in high-risk patients, that is, those patients with features of TBK1-related NTG (early onset, strong family history of NTG, low IOP, and large cup-to-disc ratio at diagnosis) or in relatives of NTG patients known to carry a TBK1 CNV. Moreover, genetic testing should always be discussed with experienced physicians and/or genetic counselors to determine whether such investigations should be done and to help interpret results.86

**COPY-NUMBER VARIATIONS OF THE TBK1 GENE ARE NOT A COMMON CAUSE OF JUVENILE-ONSET OPEN-ANGLE GLAUCOMA, PIGMENTARY GLAUCOMA, OR STEROID-INDUCED GLAUCOMA**

We also evaluated the role of TBK1 CNVs in other open-angle glaucomas. In two prior studies, we examined 1,045 POAG patients and no TBK1 CNVs were detected.44,59 In the current study, we expanded our study of TBK1 and open-angle glaucoma by investigating other types of glaucoma for TBK1 CNVs, including JOAG, PG, XFG, and SIG. A single instance of a TBK1 gene duplication was detected in a patient with XFG, and no such TBK1 CNVs were identified in JOAG, PG, or SIG patients. This study suggests that TBK1 CNVs are not commonly associated with JOAG, PG, or SIG. However, it is possible that with larger cohorts, rare instances of TBK1 CNVs might be detectable among these types of glaucoma patients.

**DETECTION OF A TBK1 GENE DUPLICATION IN A CASE OF EXFOLIATION GLAUCOMA WITH HIGH INTRAOCULAR PRESSURE**

The current study reports the first instance of a TBK1 CNV in a patient with a secondary glaucoma, XFG. This is also the first case of a TBK1 gene duplication detected in a patient with markedly elevated IOPs, ie, 44 mm Hg. Prior to this finding, mutations in TBK1 had been solely associated with autosomal dominant inheritance of NTG.26 The discovery of a mutation in a glaucoma-causing gene in a patient with XFG is a novel finding. Previous studies of XFG have primarily investigated a complex genetic basis of disease in which many genetic and environmental “risk factors” can each increase the likelihood of disease but are unable to cause disease on their own. Common genetic mutations in the lysyl oxidase–like 1 (LOXL1) gene87 and the calcium channel, voltage dependent, P/Q type, alpha-1A subunit (CACNA1A) gene88 are important contributors to the development of XFS. More recently, environmental risk factors have also been implicated in the pathogenesis of XFS. There is an association between latitude and potentially between sun exposure and the risk for developing XFS.89,90 Other potentially important dietary influences for XFS, such as caffeine and folate consumption, have also been reported.91,92 While the preponderance of the available data suggests a complex genetic basis for XFS and XFG, there is some evidence that rare cases may have a simple genetic (single gene) basis. A few rare pedigrees with familial/autosomal dominant inheritance of XFS have been reported.93-95 These large XFS families suggest that a subset of the disease might be caused by a single gene as a Mendelian trait, such as by a TBK1 CNV mutation.

The detection of a TBK1 gene duplication in one (0.44%) of 225 XFG patients (patient GGR-623-1) suggests that mutations that alter TBK1 gene dosage (ie, a gene duplication) might have a role in rare cases of XFS and XFG. Alternatively, it is also possible that patient GGR-623-1 had independent factors predisposing NTG and XFG (ie, NTG caused by a TBK1 gene duplication and XFG due to different genetic and environmental factors). Such concurrent conditions would likely result in a single diagnosis of XFG and is the most plausible explanation for this patient’s disease. In either case, the results of this study suggest that one might consider TBK1 CNVs as a possible mechanism of disease in patients diagnosed with XFG. Studies of additional cohorts of patients with XFG will be necessary to establish a role for the TBK1 gene in this condition.

**MODEL OF TBK1-RELATED GLAUCOMA**

Most cases of glaucoma associated with TBK1 CNVs occur with lower IOP. Consequently, we have developed a model that focuses on the pathophysiology of NTG described below. The possible role of the TBK1 gene in XFG and glaucoma that occurs at higher IOP remains unclear and will require further study and more data.

Two genes known to cause Mendelian cases of NTG (TBK1 and OPTN) and one gene associated with complex genetic forms of NTG (TLR4) also have important roles in autophagy. The interaction of these three genes to activate autophagy has been best described with studies of the innate immune system’s response to intracellular pathogens.64,96 Intracellular bacteria (eg, Salmonella enterica) may be recognized and cleared via a form of autophagy known as xenophagy. Cells respond to bacteria in the cytosol by coating them with the protein ubiquitin. A cascade of events involving TLR4, TBK1, and OPTN leads to formation of an autophagosome around the ubiquitin-coated bacterium and ultimately to fusion of the autophagosome with a lysosome, in which it will be degraded. When exposed to lipopolysaccharide from Salmonella, TLR4 stimulates TBK1 to phosphorylate OPTN. Via its multiple binding domains, phosphorylated OPTN then brings together key components of the autophagosome, including the microtubule-associated protein LC3, and facilitates envelopment of ubiquitin-coated Salmonella within the autophagosome. Finally, the autophagosome fuses with the lysosome and its cargo Salmonella is digested. Importantly, OPTN’s function in autophagy requires phosphorylation of its LC3 binding domain by TBK1.64,96 These data are significant because they define a mechanistic link between known glaucoma genes, autophagy, and NTG pathogenesis.

Although it is remarkable that three NTG genes participate in xenophagy, there is little evidence to suggest an infectious component to the pathophysiology of glaucoma. Instead, we hypothesize that the same key molecules that activate xenophagy (TLR4, TBK1, and OPTN) interact in a similar fashion to stimulate autophagy in retinal ganglion cells of patients with NTG. We have begun...
to investigate the mechanism by which TBK1 gene duplications and triplications cause NTG using cell culture approaches. Early studies using fibroblast cell lines obtained from NTG patients in pedigree GGO-441 with a TBK1 gene duplication have shown that the TBK1 gene duplication results in increased transcription of TBK1 mRNA.\(^4\) This data suggests that the gene duplication may result in increased TBK1 kinase function and ultimately in increased activation of autophagy.

More recently, we examined the activation of autophagy in cell cultures from NTG patients with TBK1 gene duplications. Fibroblast cells from these patients were reprogrammed to become induced pluripotent stem cells (iPSCs) and then differentiated into retinal ganglion cell–like neurons.\(^7\) Both fibroblast cell cultures and retinal ganglion cell–like neurons were assessed for activation of autophagy. During the formation of the autophagosome, LC3 becomes lipidated (termed LC3-II). Increasing levels of LC3-II is a reliable marker of activation of autophagy.\(^8\) Elevated levels of LC3-II were detected in retinal ganglion cell–like neurons from an NTG patient with a TBK1 gene duplication when compared to controls.\(^7\) These data provide additional evidence that duplication of TBK1 may activate autophagy in cell types most relevant to NTG.

Thus, several observations suggest that TBK1 gene dosage mutations (duplications and triplications) might cause NTG by activating autophagy. One of TBK1’s functions is to stimulate autophagy. Moreover, extra copies of the TBK1 gene in NTG patients have been shown to result in increased transcription of TBK1 mRNA in fibroblasts and increased production of the key marker of autophagy, LC3-II, in iPSC-derived retinal ganglion cell–like neurons. Together, these data suggest that mutations that increase TBK1 gene dosage (ie, TBK1 gene duplications or triplications) cause optic nerve damage and glaucoma by increasing autophagy in retinal ganglion cells. This discovery has important implications in the study of NTG pathogenesis and the potential development of new glaucoma therapies that target autophagy in the eye.

**FUTURE DIRECTIONS**

**Transgenic Mouse Studies of TBK1-Related Normal-Tension Glaucoma**

Compelling evidence suggests that mutations that alter TBK1 gene dosage (ie, TBK1 gene duplications or triplications) cause NTG. TBK1 CNVs have been detected in NTG patients from the United States,\(^44\), Japan,\(^57\) and Australia,\(^59\) but have never been detected in control subjects with no glaucoma or in public databases. Moreover, TBK1 gene duplications have been shown to cause increased TBK1 gene activity in cultured fibroblast cells, indicating that these mutations have functional consequences on gene transcription (at least in this cell culture system).\(^44\) Moreover, TBK1 is known to be specifically expressed in retinal ganglion cells of human retina, a key site of NTG pathology.\(^44\) Finally, TBK1 is known to interact with the only other gene (OPTN) known to cause autosomal dominant, Mendelian forms of NTG. Together, these data strongly suggest that TBK1 CNVs cause NTG. However, the most definitive proof that CNVs spanning TBK1 cause NTG would be to generate transgenic mice that carry extra doses of the TBK1 gene and to see if they develop signs of glaucoma. Such mice have been engineered and are currently being evaluated for signs that TBK1 CNVs in mice recapitulate human glaucoma. Moreover, ocular tissue from transgenic TBK1 mice might also be studied for signs of activation of autophagy (ie, increased production of LC3-II and other markers of autophagy) to solidify the role of this process in TBK1-related glaucoma.

**Cell Culture Studies of TBK1-Related Normal-Tension Glaucoma**

Preliminary studies of iPSC-derived retinal ganglion cell–like neurons have suggested that TBK1 gene duplications activate autophagy. However, more rigorous studies to investigate autophagy in these cells are necessary, including confirmatory studies with cell lines from more NTG patients with TBK1 CNVs. Moreover, these iPSC-derived cells provide an ideal system to test our hypothesis that TBK1 CNVs increase signaling via TBK1 and OPTN to activate autophagy and lead to retinal ganglion cell death. The same iPSC-derived cells will also be a powerful tool for testing new therapies directed at targets in the autophagy pathway.

In conclusion, although we found a TBK1 gene duplication in only one patient with XFG, our sample size was not large enough to conclude that TBK1 gene duplication is more common in NTG than in secondary open-angle glaucomas. Because NTG is generally considered to be on a continuum with POAG, we felt that it would be interesting to determine whether TBK1 mutations are present in patients with POAG and other forms of open-angle glaucoma (JOAG, PG, XFG, and SIG). This had been the case with MYOC mutations, which were initially discovered in patients with JOAG and were later found to cause 3% to 4% of POAG cases\(^45\) as well as rare instances of other forms of glaucoma.\(^59\) In this report, we did not detect TBK1 mutations in JOAG, PG, or SIG patients. Prior reports similarly did not detect TBK1 mutations in POAG patients.\(^44\) We did, however, detect a TBK1 gene duplication in a single XFG patient with markedly high IOP, which suggests that defects in TBK1 may also be involved in rare cases of this type of glaucoma. To date, TBK1 mutations have been primarily found in NTG patients. This subset of patients may have a primary destruction of their retinal ganglion cells mediated through autophagy. Further investigations of autophagy, therefore, may suggest new ideas for the treatment of NTG and other glaucomas.

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REFERENCES


Rodríguez-Muela N. Axonal damage and neuronal survival. *Autophagy* 2012;8(2):286-288.


