Genomic Organization of *TBK1* Copy Number Variations in Glaucoma Patients

Adam P. DeLuca, PhD,† Wallace L.M. Alward, MD,*†*
Jeffrey Liebmann, MD,‡ Robert Ritch, MD,§ Kazuhide Kawase, MD,‖
Young H. Kwon, MD,† Alan L. Robin, MD,¶#** Edwin M. Stone, MD, PhD,*†*
Todd E. Scheetz, PhD,† and John H. Fingert, MD, PhD*†*

Background: Approximately 1% of normal tension glaucoma (NTG) cases are caused by TANK-binding kinase 1 (*TBK1*) gene duplications and triplications. However, the precise borders and orientation of these *TBK1* gene copy number variations (CNVs) on chromosome 12 are unknown.

Methods: We determined the exact borders of *TBK1* CNVs and the orientation of duplicated or triplicated DNA segments in 5 NTG patients with different *TBK1* mutations using whole-genome sequencing.

Results: Tandemly duplicated chromosome segments spanning the *TBK1* gene were detected in 4 NTG patients, each with unique borders. Four of 5 CNVs had borders located within interspersed repetitive DNA sequences (*Alu* and long interspersed nuclear element-L1 elements), suggesting that mismatched homologous recombinations likely generated these CNVs. A fifth NTG patient had a complex rearrangement including triplication of a chromosome segment spanning the *TBK1* gene.

Conclusions: No specific mutation hotspots for *TBK1* CNVs were detected, however, interspersed repetitive sequences (ie, *Alu* elements) were identified at the borders of *TBK1* CNVs, which suggest that mismatch of these elements during meiosis may be the mechanism that generated *TBK1* gene dosage mutations.

Key Words: *TBK1*, TANK-binding kinase 1, glaucoma, NTG, copy number variation

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Glaucoma is a disease of the optic nerve that is the most common cause of irreversible blindness in the world.[1] The 2 cardinal features of glaucoma are a characteristic appearance of the optic nerve head (optic disc cupping) and corresponding functional loss determined with visual field testing. High intraocular pressure (IOP) is the most important risk factor for glaucoma, but approximately half of primary open-angle glaucoma occurs at low IOP (eg, ≤21 mm Hg); normal tension glaucoma (NTG).[2]

A genetic basis for glaucoma has been established by epidemiology (ie, twin studies), pedigree analysis, and animal studies.[3,4] Association studies have identified several risk factors for NTG including *SRBD1*,[5] *ELOVL5*,[5] *TLR4*,[6] *CDKN2B-AS1*.[7,8] Genes that cause NTG have also been detected with positional cloning studies of large pedigrees, the first having been missense mutations in the optineurin (*OPTN*) gene in 2002.[9] More recently, we discovered copy number variations (CNVs) spanning the TANK-binding kinase 1 (*TBK1*) gene (duplications, triplications, and 1 instance of deletion) in pedigrees with NTG.[10–15] Mutations in *OPTN* or *TBK1* are responsible for ~1% to 2% of NTG cases.[3,16–18]

CNVs spanning *TBK1* have been reported in 10 unrelated NTG pedigrees. The approximate borders of the duplicated and triplicated DNA segments in 9 of these individuals have been coarsely estimated using quantitative analysis of single nucleotide polymorphisms and hybridization probes spanning the chromosomal 12q14 region.[11–14,19] In this report, we use whole-genome sequencing techniques to fine map and identify the precise borders of 5 different CNVs that span the *TBK1* gene. We also determine the genomic organization and orientation of these duplicated and triplicated DNA segments (ie, the presence of tandem repeats, or inversions).

METHODS

Participants

Participants provided informed consent and research was conducted with approval of the institutional review board (IRB) of the University of Iowa or Gifu University. Five subjects (GGO-441-4, GGJ-414-1, GGR-590-1, GGA-1159-1, and GGA-458-1) were diagnosed with NTG using standard criteria including typical glaucomatous optic nerve damage and visual field loss with maximum recorded IOP ≤21 mm Hg as described in prior reports.[10–12] In prior case-control studies, these NTG patients were found to have heterozygous CNVs spanning the *TBK1* gene.[10–12] The precise borders of these CNVs were not previously determined.

Genome Sequencing and Analysis

DNA from each subject was prepared for whole-genome sequencing and was processed on an Illumina HiSeqX following the manufacturer's protocol and as we

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From the *Department of Ophthalmology and Visual Sciences, Carver College of Medicine; †Stephen A. Wynn Institute for Vision Research, University of Iowa, Iowa City, IA; ‡Bernard and Shirlee Brown Glaucoma Research Laboratory, Columbia University Medical Center; §Einhorn Clinical Research Center, New York Eye and Ear Infirmary of Mt. Sinai, New York, NY; ‖Department of Ophthalmology, Gifu University Graduate School of Medicine, Gifu, Japan; ¶Department of Ophthalmology, School of Medicine; #Department of International Health, and the Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD; and **Department of Ophthalmology, University of Michigan, Ann Arbor, MI.
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Reprints: John H. Fingert, MD, PhD, University of Iowa, 3111B Medical Education Research Facility, 375 Newton Road, Iowa City, IA 52242 (e-mail: john-fingert@uiowa.edu).
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previously described.[20] DNA sequences were aligned using the burrows-wheeler aligner (BWA) and were examined with the genome analysis toolkit (GATK) and a custom genome analysis pipeline.[21–23] The previously reported, approximate locations of CNVs for each study participant[10–12] were used to guide manual inspection of genomic regions of chromosome 12q14 for CNV breakpoints and orientation of DNA segments. Exhaustive inspection of genomic sequences in targeted chromosome 12q14 regions was used to manually map the precise beginnings and endings of each duplicated (or triplicated) DNA segment and to accurately measure the extent of the duplicated DNA segments. The number of sequencing reads was used to identify duplicated and/or triplicated DNA segments. Standard Sanger DNA sequencing was using to confirm internal borders of the identified CNVs. In some cases (GGR-590-1 and GGA-1159-1), we were unable to use polymerase chain reaction (PCR) amplification and Sanger sequencing for confirmation due to challenges with flanking repetitive sequences.

RESULTS

CNVs spanning the *TBK1* gene have been detected in ~1% of NTG cases. We previously described *TBK1* CNVs in several unrelated NTG patients.[10–14] Microarray-based assessment using panels of genetic markers (single nucleotide polymorphisms) and/or comparative genome hybridization of chromosome 12q14 was previously used in several of these patients to identify the approximate locations of the CNV borders. However, this mapping approach was too coarse to reveal the exact chromosomal location of the borders of the duplicated or triplicated DNA segments. Consequently, next-generation DNA sequencing was used to obtain genomic sequences spanning the chromosome 12q14 locus in DNA samples available from 5 unrelated NTG patients with unique *TBK1* CNVs.

Whole-genome sequencing was obtained from 5 previously reported patients (GGO-441-4, GGJ-414-1, GGR-590-1, GGA-1159-1, and GGA-458-1) with NTG caused by a *TBK1* CNV.[10–12] DNA sequences were obtained spanning chromosome 12q14 of each individual with a mean coverage exceding 30×. We manually inspected DNA sequence alignments with reference genome sequence (GRCh37/hg19) in the 12q14 region using the integrative genomics viewer (IGV) software (http://software.broadinstitute.org/software/igv/). Manual inspection focused on the regions identified by coarse mapping techniques was used to locate the specific coordinates of CNV boarders.

Patient GGO-441-4 (668,758 bp Duplication)

Patient GGO-441-4 is a member of an African American NTG pedigree from Maryland that we studied with linkage analysis and used to identify *TBK1* as a glaucoma-causing gene.[10] A large segment of chromosome 12q14 spanning the *TBK1* gene, ~668,000 bp, was previously detected in 10 members of this pedigree with NTG using microarray analyses. We used genome sequencing to localize the precise breakpoints of this duplication to chromosome 12 at 64,723,496 bp and 65,392,254 bp with a total span of 668,758 bp (Table 1). We confirmed the locations of the breakpoints with Sanger sequencing. Genomic sequence analysis also indicates that this segment of chromosome 12 is tandemly repeated in the same orientation. We also determined that the 5′ breakpoint of the duplicated DNA

TABLE 1. CNVs Spanning *TBK1*

	CNV Size (Estimated by Microarray-based Data)					CNV (Measured by Genome Sequencing)				
NTG Patient	CHR 12 Start	CHR 12 End	Length of CNV Segment	Overall Length	CNV Type	CHR 12 Start	CHR 12 End	Length of CNV Segment	Repetitive Elements	CNV Orientation
GGO-441-4	64,724,000	65,392,000	668,000	1,336,000	Duplication	64,723,496	65,392,254	668,758	LINE L1	Tandem duplication
GGJ-414-1	64,804,000	65,099,000	295,000	590,000	Duplication	64,803,234	65,098,920	295,686	Alu	Tandem duplication
GGR-590-1	64,557,000	64,934,000	377,000	754,000	Duplication	64,562,069	64,934,989	372,920	AluSx/AluSx1	Tandem duplication
GGA-1159-1	64,774,000	65,074,000	300,000	600,000	Duplication	64,759,677	65,072,864	313,187	$(TA)_n$/Alu	Tandem duplication
GGA-458-1	64,713,000	64,946,000	233,000	699,000	Triplication	64,475,956	65,121,870	645,914	AluYb8/AluSq2	Triplication with an internal inversion

Initial coarse estimates of the start, end, and extent of CNVs were previously determined by microarray mapping.[13] Genome sequencing identified the precise location of the CNVs start, end, and extent along chromosome 12q14 down to the nucleotide. Inspection of DNA sequence alignments across the CNVs identified the orientation of duplicated/triplicated DNA segments. CNVs were mapped against the hg19 genome build.
CNV indicates copy number variation; NTG, normal tension glaucoma; TBK1, TANK-binding kinase 1.

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segment lies within a long interspersed nuclear element (*LINE*)-*L1* repetitive sequence (Fig. 1A).

Patient GGJ-414-1 (295,686 bp Duplication)

Patient GGJ-414-1 is a member of a Japanese NTG pedigree that was identified with a case-control study of the *TBK1* gene. A duplication spanning the *TBK1* gene was detected in patient GGJ-414-1 with real-time PCR and was estimated to encompass 295,000 bp by microarray studies.[11] In this study, we mapped the exact location of the breakpoints for this duplication to 64,803,234 and 65,098,920 bp on chromosome 12 with a total span of 295,686 bp with genome sequencing (Table 1). Moreover, genomic sequencing indicated that this duplication is tandemly repeated in the same orientation and that the breakpoints lie within *Alu* short interspersed nuclear element (SINE) repetitive sequence elements (Fig. 1B). The Sanger DNA sequencing confirmed the location of the borders of the duplicated DNA segments identified by genome sequencing experiments.

Patient GGR-590-1 (372,920 bp Duplication)

We detected a large duplicated segment of chromosome 12 that spans the *TBK1* gene in patient GGR-590-1 as part of a case-control study of NTG patients from New York. Initial analyses of this duplication with microarray studies estimated its extent to be 377,000 bp.[12] We used genome sequencing to more precisely map the borders of this duplicated DNA sequence to be at 64,562,069 and 64,934,989 bp, which spans 372,920 bp (Table 1). Our analysis further demonstrated that this DNA segment is tandemly repeated in the same orientation and that the borders of this DNA segment are located within *Alu* repeats (Fig. 1C).

Patient GGA-1159-1 (313,187 bp Duplication)

A duplication was detected in NTG patient from Iowa (GGA-1159-1) and was initially estimated to span a 300,000 bp segment of chromosome 12 that includes the *TBK1* gene using microarray analyses.[10] In this study, genome sequencing mapped the borders of this CNV to 64,759,677 and 65,072,864 bp, which define a 313,187 bp

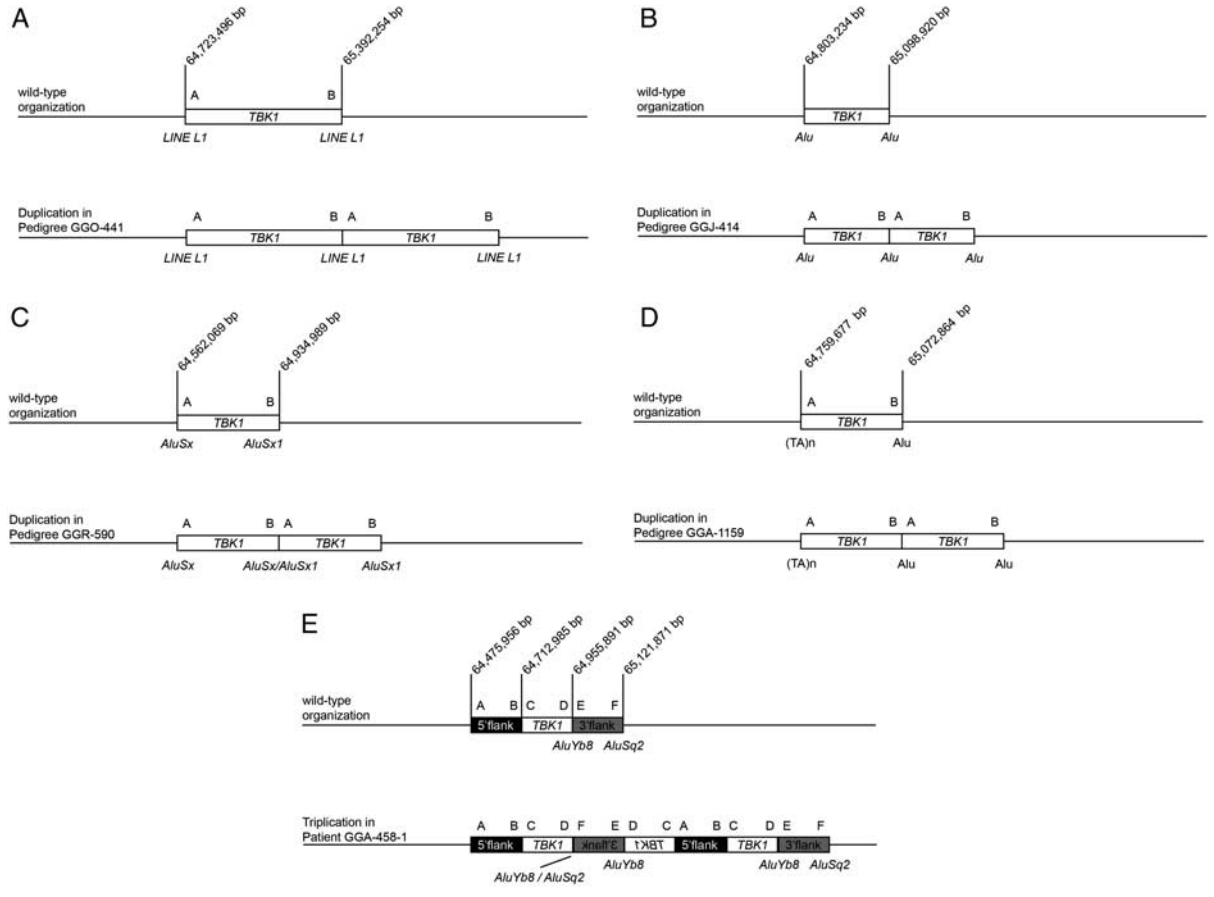

FIGURE 1. Genomic organization of CNVs spanning TBK1 in 5 glaucoma patients. Five different chromosome 12q CNVs encompassing the *TBK1* gene were detected in 5 normal tension glaucoma patients. A, A tandem duplication of 668,758 bp was detected in patient GGO-441-4 with a border that lies within an *LINE L1* repetitive DNA sequence element. B, A second, different tandem repeat of 295,686 bp was identified in normal tension glaucoma patient GGJ-414-1. The ends of the duplicated DNA segment lie within *Alu* repetitive DNA sequence elements. C, A third tandem repeat of 372,920 bp that is bounded by *Alu* repetitive DNA sequence elements was detected in normal tension glaucoma patient GGR-590-1. D, A fourth *Alu*-mediated tandem repeat of 313,187 bp was detected in normal tension glaucoma patient GGA-1159-1. E, A complex CNV containing duplicated, triplicated, and inverted segments was detected in normal tension glaucoma patient GGA-458-1. CNV indicates copy number variation; LINE, long interspersed nuclear element; TBK1, TANK-binding kinase 1.

segment of chromosome 12 that is tandemly duplicated (Table 1). The borders of the duplicated sequence are located within a simple dinucleotide $(TA)_n$ repeat and an *Alu* element respectively (Fig. 1D).

Patient GGA-458-1 (*TBK1* Gene Triplication)

Bennett et al[24] previously reported clinical features of a large, 4-generation pedigree (GGA-458-1) with severe NTG. This pedigree was subsequently studied with genetic approaches and found to harbor a CNV spanning the *TBK1* gene. Initially the CNV was characterized as a 233,000 bp duplication using quantitative PCR and microarray analyses.[10] However, subsequent reanalysis with additional quantitative PCR and custom microarray assays demonstrated that the CNV included triplication of *TBK1* gene sequences.[13] In this study, we confirm that patient GGA-458-1 has a triplication of *TBK1* coding sequences using genome sequencing approaches. Moreover, our analysis reveals the complex structural organization of the CNV in this pedigree in which some segments of DNA are duplicated while others are triplicated (Fig. 1E). This rearrangement involves 645,914 bp of DNA from 64,475,956 to 65,121,870 bp which can be divided into 3 parts: a 5′flanking segment (64,475,956 to 64,712,84 bp) which is upstream of *TBK1*; a central segment (64,712,985 to 64,955,890) which contains the entire *TBK1* gene; and a 3′ flanking segment (64,955,891 to 65,121,870) which is downstream of *TBK1* and is bordered on both sides with an *Alu* element. The CNV in patient GGA-458 includes duplication of both flanking segments and a triplication of the central segment that encompasses the *TBK1* gene. The organization of these segments in the CNV is complex (Fig. 1E) and includes 2 contiguous inverted segments (central segment containing TBK1 and the 3′ flanking segment). Sanger DNA sequencing was used to confirm one of the borders detected in patient GGA-458-1 by genomic sequencing.

We also compared several clinical features of glaucoma between patients with a *TBK1* gene duplication (n = 11) and patients with a *TBK1* gene triplication (n = 7) (Supplemental Tables 1 to 3, Supplemental Digital Content 1, http://links.lww.com/IJG/A139; Supplemental Digital Content 2, http://links.lww.com/IJG/A140; and Supplemental Digital Content 3, http://links.lww.com/IJG/A141).[10–13] There was no statistical difference in the age at diagnosis of glaucoma when patients with a *TBK1* duplication (mean age of 38.7 ± 7.4 y) were compared with patients with a *TBK1* triplication (mean age of 33.8 ± 14.1 years), *P*-value = 0.36. Similarly, the maximum recorded IOPs in patients with a *TBK1* duplication (mean of 16.5 ± 3.1 mm Hg) and in patients with a *TBK1* triplication (mean of 17.9 ± 4.4 mm Hg) were not statistically different (*P*-value = 0.28). Central corneal thicknesses of patients with a *TBK1* duplication ranged from 484 to 622 μm (mean, 529.4 ± 44.5 μm) and were not available from patients with *TBK1* triplications.

DISCUSSION

CNV mutations have been identified as a cause of several inherited eye diseases including Axenfeld-Rieger syndrome,[25–27] primary congenital glaucoma,[28] and primary open-angle glaucoma.[29] Here we investigated the structure and cause of CNVs of *TBK1* in NTG patients using genome sequencing. Four NTG patients with chromosome 12 duplications were each shown to have tandemly-oriented, duplicated DNA segments. The borders of each of the duplicated DNA segments lie within highly repetitive DNA sequences (*L1*, *Alu,* and dinucleotide repeats). A fifth NTG patient (GGA-458-1) had a complex rearrangement including duplicated, triplicated, and inverted segments. This patient also had repetitive DNA sequence elements flanking the borders of some of the rearranged elements (*Alu*). CNVs with an analogous complex organization as patient GGA-458-1 have been previously attributed to a single error in recombination.[30] As a result, it is likely that the same type of recombination abnormality led to the CNV that we discovered in patient GGA-458-1. In all 5 NTG cases in this report, genome sequencing identified the precise extent and location of CNVs spanning the *TBK1* gene.

It is a plausible hypothesis that glaucoma patients with *TBK1* gene duplication have disease due to increased *TBK1* expression from the extra copy of the gene in their genomes. Similarly, patients with a triplication of the *TBK1* gene might have more severe disease than patients with a *TBK1* gene duplication, due to even higher *TBK1* transcription. However, we were unable to detect a difference in some key clinical features of glaucoma between these patient groups. Although our sample size is relatively small (n = 18), we detected no statistical difference in age at diagnosis or maximum IOP when we compared patients with a *TBK1* gene duplication and patients with a *TBK1* gene triplication.

Our initial assessment of the extent of the duplicated DNA segments in 4 patients with different duplications spanning the *TBK1* gene was remarkably accurate. The sizes of these duplicated DNA segments were first estimated to the nearest 1 kbp using custom microarrays of over 74,000 hybridization probes for chromosome 12q.[13] Later these chromosome 12q duplications were more precisely mapped with genome sequencing. In each case, the initial microarray estimates of the size of the duplicated DNA sequence were <5% different than the more exact measurements from genome sequencing experiments. Moreover, the locations of the ends of the duplicated DNA were mapped with microarray studies to within 15 kbp of their precise position as identified with genome sequencing. We found that both microarray-based analyses and genome sequencing accurately measured the size of the large duplicated chromosome 12q segments that encompass the *TBK1* gene in our glaucoma patients. Genome sequencing was superior to microarray-based measures of the extent of more complex CNVs like the one detected in patient GGA-458-1 that included duplicated, triplicated, and inverted segments.

When homologous chromosomes pair during meiosis, misalignment of repetitive sequences may occur between sister chromatids. Subsequent nonallelic homologous recombination may create duplications or deletions of the DNA segment between misaligned repetitive segments. Such nonallelic homologous recombination is frequently driven by *Alu* elements due to their high frequency in the human genome.[31]

Our initial studies of chromosome 12q CNVs with microarrays mapped the ends of the duplicated sequences to the nearest kbp. These early studies lacked the precision necessary to map the borders of the duplicated sequences within known repetitive elements that may be short in length, that is ~280 bp for *Alu* elements. However, in this study, genome sequencing successfully placed the borders of the CNVs within *Alu* elements in 4 of 5 NTG patients. An *L1*-element was similarly found at a breakpoint of the CNVs in the final patient. In summary, genome sequencing has advantages over microarray mapping in determining the

structure and precise location of CNVs. Genomic sequencing has demonstrated that NTG may be added to the long list of diseases that are caused by repetitive sequence-mediated CNVs.

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