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TBK1 and Flanking Genes in Human Retina

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Abstract

Purpose—The gene that causes normal tension glaucoma (NTG) in a large pedigree was recently mapped to a region of chromosome 12q14 (*GLC1P*) that contains the genes *TBK1*, *XPOT*, *RASSF3*, and *GNS*. We sought to investigate the structure of the chromosome 12q14 duplication and explore the ocular expression of *GLC1P* locus genes.

Methods—The location of the chromosome 12q14 duplication in this pedigree was examined with fluorescent *in situ* hybridization (FISH) using probes for *TBK1* and *GNS*. The expression pattern of *XPOT*, *TBK1*, *RASSF3*, and *GNS* was investigated with immunohistochemistry of human eyes.

Results—The karyotype of an NTG patient from pedigree GGO-414 was normal and FISH studies demonstrated that the duplicated DNA is organized as a tandem repeat on chromosome 12q14. Of the genes in or near the chromosome 12q14 duplication, *TBK1* showed expression in the retina that is specific to the retinal ganglion cells and the retinal nerve fiber layer. Expression of *RASSF3* and *XPOT* was relatively uniform throughout the retina, while *GNS* expression was expressed in a pattern consistent with Müller cells.

Conclusions—Previous studies demonstrated that chromosome 12q14 duplications are associated with NTG inherited as an autosomal dominant trait. FISH studies now demonstrate that the duplicated segments are tandemly organized on chromosome 12q14 in close proximity. The specific expression of *TBK1* in human retinal ganglion cells compared to the widespread pattern of expression of neighboring genes provides additional evidence that *TBK1* is the glaucoma gene in the chromosome 12q14 duplication within the *GLC1P* locus.

Keywords

Fluorescent in situ hybridization; normal tension glaucoma; *TBK1*

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

INTRODUCTION

A significant fraction of primary open angle glaucoma (POAG) is heritable and many cases of POAG have a clear autosomal dominant inheritance pattern. These Mendelian forms of POAG cases are caused primarily by the actions of individual genes and have been successfully studied using classic positional cloning strategies. Seventeen POAG loci, designated *GLC1A* – *GLC1Q*, have been mapped with linkage analysis.^{1–4} The gene at the *GLC1A* locus, myocilin (*MYOC*), is associated with glaucoma that occurs with markedly high intraocular pressure.^{5,6} Conversely, the gene at the *GLC1E* locus, optineurin (*OPTN*) is associated with glaucoma that occurs without elevated intraocular pressure,⁷ often termed normal tension glaucoma (NTG). Genes at the *GLC1F*, *GLC1G*, and *GLC1O* loci have also been reported, but the association of these genes with glaucoma is controversial.^{8–16}

Recently, a new NTG gene was mapped to a chromosome 12q14 locus (*GLC1P*). Duplication of a gene within this locus, TANK binding kinase 1 (*TBK1*), has been implicated as the cause of NTG in families with *GLC1P*-related glaucoma.³ Unique but overlapping duplications of chromosome 12q14 have been identified in Caucasian, African American, and Asian NTG pedigrees.^{3,17} The minimum overlap of the chromosome 12q14 duplications in these NTG pedigrees spans 300 kb and encompasses *TBK1* and exportin (*XPOT*). The borders of this minimal critical region intersect the Ras association domain 3 (*RASSF3*) gene and are within 50 kb of the glucosamine N-acetyl-6-sulfatase (*GNS*) gene.³ Of these genes in or around the chromosome 12q14 region, compelling evidence has been gathered that suggests *TBK1* is the glaucoma-causing gene in the *GLC1P* locus. RNA expression of *TBK1* has been shown to be significantly increased by the duplication.³ Preliminary studies also suggested that *TBK1* is expressed specifically in the retinal ganglion cells and nerve fiber layer of the human retina, which is consistent with a role in glaucoma pathogenesis.³ Finally, *TBK1* is known to interact with the only other known NTG gene, optineurin (*OPTN*).^{18,19} Interaction with a known NTG gene further implicates *TBK1* as the glaucoma gene in the *GLC1P* locus.

TBK1 encodes a kinase that has a key role in regulating autophagy, a cellular process by which accumulating proteins, defective organelles, and pathogens are eliminated. *TBK1* phosphorylates a protein encoded by another NTG gene, optineurin, that has a downstream function in autophagy.¹⁹ When phosphorylated by *TBK1*, optineurin brings together elements of the autophagosome (i.e. LC3) and proteins marked for degradation by ubiquitin, which ultimately leads to their clearance.^{20,21}

In our previous studies we have shown that chromosome 12q14 duplications in the *GLC1P* locus are associated with NTG. In these studies, *TBK1* duplications were detected using single nucleotide polymorphism analysis, comparative genome hybridization, and quantitative polymerase chain reaction experiments.³ In the current study, we confirm the presence of the 780 kb chromosome 12q14 duplication that was detected in the African American NTG pedigree GGO-414 using FISH. Moreover, we provide more evidence that *TBK1* is the NTG gene in the *GLC1P* locus by comparing its expression in human retina with the expression pattern of other genes encompassed by the duplication (*XPOT*, *RASSF3*, and *GNS*). With immunohistochemistry, we show that the *TBK1* is specifically expressed in the tissue most affected by NTG, the retinal ganglion cells, while *XPOT*, *RASSF3*, and *GNS* have a more diffuse non-specific retinal expression pattern. Of the proteins produced by genes in the chromosome 12q14 duplication, only *TBK1* is expressed most strongly in the retinal ganglion cells, the site of NTG pathogenesis. These data provide more support for the pathogenesis of *TBK1* duplication in human NTG.

MATERIALS AND METHODS

Patient Samples

Fibroblast cells were obtained from skin biopsies from patients with a *TBK1* mutation. Both donor eyes and fibroblast cells were obtained following the tenets of the Declaration of Helsinki. Informed consent was obtained from patients that contributed fibroblast cells.

Karyotype Analysis and FISH of the Chromosome 12q14 Duplication in an NTG Pedigree

Fibroblast cell cultures used in karyotype analysis and in FISH experiments were obtained from skin biopsies of the NTG pedigree members as previously described.³ Cells were arrested in metaphase by adding ethidium bromide (final concentration 12.5 µg/ml) for 40 min followed by colcemid (final concentration 6 µg/ml). After 1–2 h, the cells were incubated for 25 min at room temperature with hypotonic solution (3:1 mixture of 0.8% sodium citrate and 0.075 molar potassium chloride). Cells were then fixed three times with a 3:1 methanol/acetic acid. Chromosome spreading was performed on coverslips in a Thermotron chamber (Holland, MI). Coverslips were mounted on glass slides after the drying process. Ten to twenty G-banded metaphases were analyzed. Karyotype images were captured in CytoVision computerized imaging system (Applied Imaging, USA).

FISH studies were carried out using Empire Genomics probes (Buffalo, NY). The region of the *GNS* gene was probed with a BAC clone (RP11-9947614) that spans chromosome 12 from 63,398,666 to 63,571,921 bp (hg18). The region of the *TBK1* gene was probed with BAC clone (RP11-6008) which spans chromosome 12 from 63,110,864 to 63,280,603 bp (hg18). Probes were prepared with a Vysis-Abbott kit using the manufacturer's protocols (Abbott Laboratories, Abbott Park, IL). Hybridization was performed on a Hybrite system programmed for melting temperature at 75 °C for 1 minute. After overnight hybridization at 37 °C, the slides were washed in 0.4X SSC/0.3% NP-40 for 2 minutes at 72 °C and in 2X SSC/0.1% NP-40 for 1 minute at room temperature. Slides were then counterstained with DAPI for viewing and images were captured through CytoVision computerized imaging system (Applied Imaging, USA). A minimum of 100 interphase nuclei and 20 metaphase nuclei were examined to confirm the localization of the probes to chromosome 12q14. The criteria for identifying a duplication was detection of two independent, adjacent probe signals (three signals total) in more than 70% of interphase nuclei combined with confirmation of the chromosomal location in metaphase nuclei.

Immunohistochemistry

Eyes from five donors were evaluated in immunohistochemical studies. Donor characteristics are shown in Table 1. Eyes were preserved within 7 hours of death in 4% paraformaldehyde solution in phosphate buffered saline (PBS) for 2 hours. Superotemporal wedges spanning from the macula to the ciliary body were collected, cryoprotected in sucrose solution and embedded in optimal cutting temperature solution²² (Ted Pella, Redding, CA). Cryostat sections were collected and used for immunohistochemistry as described previously.²³ Briefly, sections were blocked for 20 minutes in 1% horse serum in PBS, and were then incubated for 1 hour in the appropriate primary antibody, followed by 3 × 10 min washes in PBS. Sections were then overlaid with biotinylated secondary antibody for 30 minutes (1:100 dilution from Vector Laboratories, Burlingame, CA). Following 3 × 20 minute washes, sections were treated with avidin biotin horseradish peroxidase complex, washed, and developed in peroxidase substrate (Vector VIP substrate kit, Vector Laboratories).

Immunohistochemistry was performed using antibodies directed against proteins encoded by genes in the *GLC1P* locus. Specificity of the labeling was validated by either using multiple

antibodies (RASSF3, XPOT), or blocking with a 5–10× excess of recombinant protein (RASSF3). Antibodies were directed against: GNS (Sigma Chemical, St. Louis, MO); and TBK1 (Calbiochem, La Jolla, CA; 2.5 µg/mL) as described previously.³

RESULTS

FISH Analysis of the Chromosome 12q14 Duplication in an NTG Pedigree

Chromosomal analysis of fibroblast cells from an affected member (subject III-1) of the previously described NTG pedigree GGO-441³ produced a grossly normal karyotype (Figure 1). There are no balanced translocations, large inversions, or any other large-scale structural abnormalities.

FISH was used to confirm the duplication of a chromosome 12q14 DNA segment using probes for two genes in the duplication, *TBK1* and *GNS*, in nuclei from fibroblast cells from patient III-1. The *TBK1* probe labeled homologous regions of each chromosome 12. However, labeling of one chromosome was much more intense than the other suggesting a tandem repeat of *TBK1* on one chromosome 12 that produced a stronger fluorescent signal (Figure 2). The *GNS* cDNA probe produced a similar fluorescence pattern (Figure 2) also indicating that the chromosome 12q14 duplication in this patient is a tandem repeat. However, given the resolution of FISH and the size of our probe, we were unable to determine whether or not the tandem duplication involves an inversion.

Immunohistochemical Localization was Performed for GNS, XPOT, RASSF3, and TBK1

Antibodies directed against both XPOT and RASSF3 showed diffuse labeling of multiple retinal cell types throughout the retina. RASSF3 was localized to the ganglion cells, inner nuclear layer, outer nuclear layer nuclei, and especially in rod and cone inner segments (Figure 3). Endothelium of some large vessels was also immunoreactive. Labeling was not found to be remarkably enriched in any cell type. Similarly, antibodies directed against XPOT showed weak, diffuse labeling of multiple cell types, including all three nuclear layers and photoreceptor cell inner segments. This pattern was observed in all five donors. In one case, nerve fiber labeling was also apparent. Consistent patterns of labeling were observed for both anti-RASSF3 and anti-XPOT antibodies from different sources and raised in different species.

Labeling with anti-GNS antibodies revealed a pattern consistent with retinal glial cells, with labeling of rare nuclei in the inner nuclear layer and radial fibers that spanned nuclear layers. External limiting membrane labeling was also observed, consistent with Müller cell reactivity. In some regions, radial fibers could be traced from near the internal limiting membrane to the external limiting membrane in all five cases. In the choroid, vascular endothelial labeling of large vessels was observed with only minor labeling of the choriocapillaris.

In our experiments, the distribution of TBK1 protein appeared to be more restricted than the other tested antigens (XPOT, RASSF3, and GNS). In four of the five tested donors, TBK1 immunoreactivity was greater in the ganglion cell layer and nerve fiber layer than in the rest of the retina, either in individual ganglion cell bodies or in axons. In one of five cases, an eye with signs of mild diabetic retinopathy, no reactivity was observed. Of the proteins encoded by genes in the chromosome 12q14 duplication, only TBK1 was specifically expressed in the retinal ganglion cells and the retinal nerve fiber layer.

DISCUSSION

The heredity of glaucoma has been successfully investigated using a variety of approaches including twin studies, population-based association studies, and positional cloning.²⁴ *TBK1* has been identified and studied as a glaucoma gene using the positional cloning approach. Linkage analysis of the large African American NTG pedigree mapped a glaucoma-causing gene to chromosome 12q14, *GLC1P*.³ The *GLC1P* locus was further evaluated for the presence of copy number variations associated with NTG in the pedigree and a 780 kb duplication was detected using microarray-based SNP analysis, quantitative PCR, and comparative genome hybridization.³ This duplication spanned two genes, (*XPOT*, *TBK1*), interrupted *RASSF3*, and was near *GNS*. Our current report confirms the presence of this chromosome 12q14 duplication using FISH and indicates that the duplicated genomic segment is organized as a tandem duplication. The hybridization pattern shows the molecular basis of the co-inheritance of the duplication and glaucoma in an autosomal dominant pattern. Two tandemly-duplicated copies of the chromosome 12q14 segment (and two copies of *TBK1*) are located within the *GLC1P* locus and are linked with inheritance of glaucoma.

The annotation of the four genes in the chromosome 12q14 duplication strongly supports *TBK1* as the glaucoma gene in the *GLC1P* locus. *TBK1* functions as a kinase in autophagy and/or NF- κ B signaling pathways, which may influence the immune system and apoptosis. These functions are consistent with a role in glaucoma pathogenesis. Furthermore, two independent reports have shown that *TBK1* interacts with and phosphorylates optineurin,^{18,19} the protein encoded by the only other established NTG gene (*OPTN*).⁷ Initial functional studies have demonstrated that duplication of *TBK1* causes a significant increase in its transcription level *in vitro*.³ However, it remains possible that one of the other genes in or near the duplication (*XPOT*, *RASSF3*, or *GNS*) has a role in glaucoma pathogenesis. In this report, we explored this possibility by determining the ocular expression pattern of these other candidate genes. *TBK1* is known to have an expression pattern that is mostly restricted to the tissues most implicated in NTG pathogenesis, the retinal ganglion cells and their axons.³ Here we show that the other genes in the chromosome 12q14 duplication have a more diffuse expression in the retina (*XPOT* and *RASSF3*) and in Mueller cells (*GNS*). Compared with primary expression in retinal ganglion cells observed for *TBK1*, the expression of *XPOT*, *RASSF3* and *GNS* appears less consistent with known glaucoma disease processes.

Together these data provide additional evidence that mutations in *TBK1* cause glaucoma. Specifically, FISH studies confirmed the presence of tandemly-repeated *TBK1* duplications on chromosome 12q14 in NTG patients. Several genes are encompassed by the chromosome 12q14 duplication, however, our immunohistochemical studies show that *TBK1* has an expression pattern that is most consistent with having a role in glaucoma pathogenesis. While these data provide evidence that *TBK1* is a glaucoma gene, functional studies in transgenic mice will be necessary to more firmly establish how duplications of *TBK1* affect ganglion cells in the retina and cause glaucoma. These experiments are currently underway.

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FIGURE 1.

Karyotype for NTG patient in the GGO-441 pedigree. Patient III-1 is a member of the previously described GGO-441 glaucoma pedigree.³ No chromosomal abnormalities were detected in Patient III-1.

FIGURE 2.

FISH studies of chromosome 12q14. (A) Interphase FISH for *TBK1* (aqua) using cultured fibroblasts from patient III-1 (Figure 1). The *TBK1* probe shows three signals for *TBK1* confirming the duplication. (B) Metaphase FISH for *TBK1* (aqua) and downstream gene *GNS* (gold). Metaphase FISH shows one normal 12q14 allele with normal signal intensity for both *TBK1* and *GNS*. Also shown is one mutant 12q14 allele with stronger signals for both *TBK1* and *GNS* confirming that the duplication is in tandem and not inserted elsewhere in the genome.

FIGURE 3.

Immunohistochemical detection of proteins encoded by the *GLC1P* locus. (A) TBK1 labeling is enriched in the inner retina at the level of ganglion cells and axons. (B) XPOT showed weak, widespread distribution throughout the retina. (C) GNS expression was perivascular and in radial fibers and external limiting membrane. Walls of large choroidal vessels were also positive. (D) RASSF3 labeling was observed throughout the retina and the retinal pigment epithelium (RPE). (E) A section without exposure to primary antibody. Abbreviations: ganglion cell layer (GCL); inner nuclear layer (INL); outer nuclear layer (ONL); inner segments (IS); outer segments (OS); and choroid (CH). Scalebar =50 μ m.

TABLE 1

Characteristics of donors studied.

Donor	Age (years)	Sex	Cause of death	Eye disease	Death to preservation interval (hours)
1	85	F	Not noted	Not noted	6.5
2	85	M	Cardiac arrest	Not noted	7.0
3	65	F	Renal failure	Not noted	5.0
4	74	F	CPA, GI bleed	Not noted	5.5
5	88	M	None noted, DM	Diabetic retinopathy	<4.0