

## Molecular genetics of inherited normal tension glaucoma

Yang Pan, Takeshi Iwata

Normal tension glaucoma (NTG) is a complex optic neuropathy characterized by progressive retinal ganglion cell death and glaucomatous visual field loss, despite normal intraocular pressure (IOP). This condition poses a unique clinical challenge due to the absence of elevated IOP, a major risk factor in typical glaucoma. Recent research indicates that up to 21% of NTG patients have a family history of glaucoma, suggesting a genetic predisposition. In this comprehensive review using PubMed studies from January 1990 to December 2023, our focus delves into the genetic basis of autosomal dominant NTG, the only known form of inheritance for glaucoma. Specifically exploring optineurin (*OPTN*), TANK binding kinase 1 (*TBK1*), methyltransferase-like 23 (*METTL23*), and myocilin (*MYOC*) mutations, we summarize their clinical manifestations, mutant protein behaviors, relevant animal models, and potential therapeutic pathways. This exploration aims to illuminate the intricate pathogenesis of NTG, unraveling the contribution of these genetic components to its complex development.

**Key words:** *METTL23*, *MYOC*, normal tension glaucoma, *OPTN*, *TBK1*

Glaucoma is a major optic neuropathy characterized by optic nerve damage and notable retinal ganglion cell (RGC) loss and is the leading cause of irreversible blindness worldwide.<sup>[1]</sup> Primary open-angle glaucoma (POAG) represents the most common type of glaucoma.<sup>[2]</sup> The Baltimore Eye Survey reported that more than half of POAG patients do not exhibit elevated intraocular pressure (IOP), defining the case for normal tension glaucoma (NTG).<sup>[3]</sup> NTG displays optic nerve head (ONH) damage, progressive retinal nerve fiber layer (RNFL) thinning, distinct visual field defects, open anterior chamber angles upon gonioscopy, and maintains an IOP below 21 mmHg without affecting the rest of the retina.<sup>[4]</sup>

Epidemiologic studies have identified several risk factors associated with NTG, including age, gender, ethnicity, and family history.<sup>[5]</sup> First, the average age of NTG patients is in their 60s, generally older than individuals with POAG.<sup>[6]</sup> Second, some studies have indicated a higher prevalence of NTG among females than males.<sup>[7]</sup> However, this gender difference may be attributed to the longer life expectancy of females. Third, the incidence of NTG varies among different populations. Notably, research has shown a higher NTG prevalence in Asians (52%–92%), notably Japanese individuals (92%), compared to those of White ethnicity (30%–39%).<sup>[8–11]</sup> In addition, Asian Americans have been found to have a 159% increased risk of NTG compared to non-Hispanic White individuals,<sup>[10]</sup> possibly reflecting an increased demographic association and genetic susceptibility. Moreover, approximately 21% of NTG patients have a family history of glaucoma,<sup>[12]</sup> further suggesting a genetic predisposition.

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While most forms of glaucoma have a hereditary component, they exhibit different patterns of inheritance. Glaucoma can occur at all ages, with Mendelian inheritance typical for the early-onset familial NTG and juvenile open-angle glaucoma (JOAG) and complex traits evident in adult-onset POAG and primary angle-closure glaucoma.<sup>[11]</sup> Notably, gene mutations associated with early-onset glaucoma have significant biological effects, while variants contributing to adult-onset glaucoma typically have more minor effects.<sup>[13]</sup>

To date, genome-wide association studies (GWAS) have successfully identified the genomic loci for POAG.<sup>[11]</sup> A recent multiethnic meta-analysis, encompassing 34,179 cases and 349,321 controls (NTG: 3247 cases, 47,997 controls; High-tension glaucoma (HTG): 5144 cases, 47,997 controls), identified 127 loci.<sup>[14]</sup> All NTG loci, except rs1812974 near *ARHGEF12*, showed nominal associations with HTG.<sup>[14]</sup> In contrast to GWAS, which generally focuses on the common variants with smaller effects (minor allele frequency >1%), NTG pedigree studies have revealed rare mutations in four genes, most of which have been validated through *in vitro* and animal models.<sup>[2,15]</sup> Numerous large NTG pedigrees have been described, typically demonstrating an autosomal dominant inheritance pattern, suggesting a Mendelian form of glaucoma caused by mutation in a single gene. This review examines the genetic role in NTG, drawing insights from PubMed studies published between January 1990 and December 2023. It delves into rare mutations identified through NTG pedigree studies, exploring pedigree characteristics, clinical

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features, protein function, mutation effects, and relevant animal models. The insights gained from these discoveries provide new perspectives on the fundamental molecular mechanisms underlying glaucoma, potentially paving the way for novel gene-based therapies and neuroprotection strategies. Furthermore, gene-based diagnostic testing for mutations responsible for NTG promises to detect individuals in the earliest stages of the disease when therapeutic interventions are more effective.

## Optineurin (OPTN)

The human optineurin (*OPTN*) gene is located on the short arm of chromosome 10 (10p13) and comprises 15 exons, including two noncoding exons and 13 coding exons [Fig. 1].<sup>[16]</sup> This chromosomal region was previously designated as the *GLC1E* locus for POAG.<sup>[17]</sup> Predictions of *OPTN* promoter sequence reveal the presence of multiple nuclear factor kappa B (NF-κB) binding sites within 600 base pairs (bp) of the transcriptional regulatory region.<sup>[16]</sup> The *OPTN* gene encodes a 577-amino acid (aa) protein, with two coiled-coil (CC) domains, highly conserved among species.<sup>[18]</sup> Furthermore, alternative splicing in the 5'-untranslated region produces at least three distinct isoforms, all sharing the same reading frame.<sup>[16]</sup>

### *OPTN* mutations in NTG

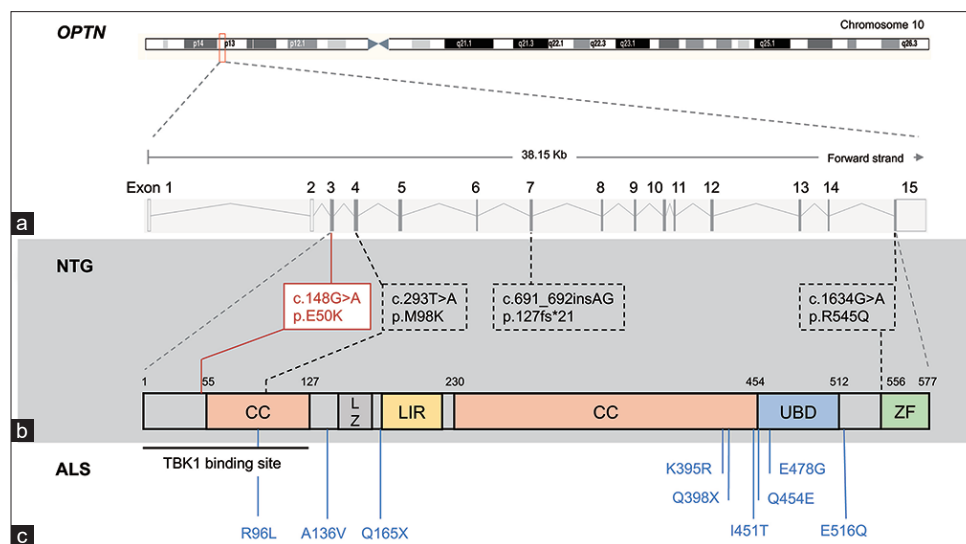
In 1998, Sarfarazi *et al.*<sup>[17]</sup> reported a four-generation NTG pedigree, comprising 16 affected individuals. Through linkage analysis, a subset of genetic markers within *GLC1E* locus consistently co-segregated with NTG in the pedigree.<sup>[17]</sup> Considering the expression in the retina, *OPTN* was identified as the prime candidate gene for further investigation.<sup>[18]</sup> Genetic analysis revealed that all family members with NTG carried *OPTN* (c.458G>A, p.E50K) mutation, conclusively establishing that *OPTN*<sup>E50K</sup> mutation causes NTG.<sup>[17]</sup> *OPTN* was marked as the first gene associated with NTG.<sup>[18]</sup> The *OPTN*<sup>E50K</sup> mutation was detected in approximately 1%–2% of NTG patients across various cohorts and was notably absent in matched control populations assembled from clinical settings in the USA, Europe, Japan, and Australia.<sup>[19–21]</sup>

Two additional mutations, *OPTN* c.691-692insAG (resulting in a premature stop) and *OPTN*<sup>R545Q</sup> (c.1634G > A), were initially linked to NTG.<sup>[18]</sup> However, regarding the role of *OPTN*<sup>R545Q</sup> in NTG, subsequent analyses have generated conflicting data, as it was frequently found in both NTG patients and controls.<sup>[16,19–24]</sup> In addition, a fourth *OPTN* variant, *OPTN*<sup>M98K</sup> (c.293T>A), was also identified in both NTG-affected cases and controls. Interestingly, the frequency of *OPTN*<sup>M98K</sup> is statistically higher in NTG patients compared to controls in certain populations,<sup>[21–25]</sup> and due to its location within the CC domain, it may potentially contribute as a risk for NTG in specific populations.

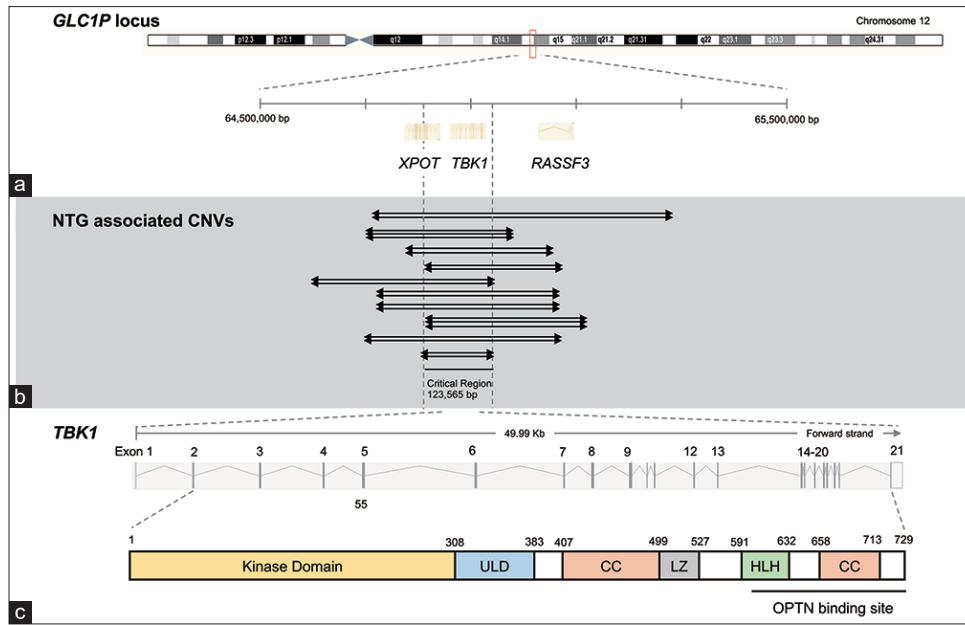
Genetic studies have also linked different sets of *OPTN* mutations with amyotrophic lateral sclerosis (ALS), a motor neuron disease.<sup>[26]</sup> Several loss-of-function *OPTN* mutations, including R96L, A136V, Q165X, K395R, Q398X, I451T, Q454E, E478G, and E516Q, have been associated with ALS [Fig. 1].<sup>[26]</sup> *OPTN* c.691-692insAG, initially found in an NTG patient, was later identified as a founder mutation in Moroccan and Ashkenazi Jews, significantly increasing the risk of developing ALS in heterozygous carriers.<sup>[27]</sup>

### Clinical features of *OPTN*-associated NTG

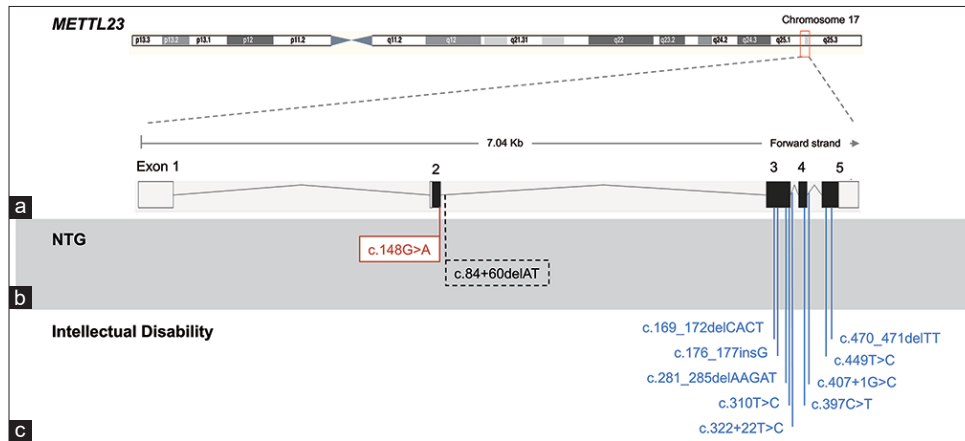
*OPTN*-associated NTG patients typically present with early-onset disease following an autosomal dominant inheritance pattern. Aung *et al.*<sup>[28]</sup> conducted a comparative analysis, assessing the clinical features of NTG patients with *OPTN*<sup>E50K</sup> mutation ( $n = 11$ ) in contrast to NTG patients without glaucoma-causing mutations ( $n = 87$ ). It was observed that patients carrying *OPTN*<sup>E50K</sup> mutation were diagnosed at a younger age (average of  $40.8 \pm 15$ ) and exhibited larger cup-to-disc (C/D) ratios (mean of  $0.86 \pm 0.1$ ) compared to NTG controls.<sup>[28]</sup> Similar clinical findings have been consistently reported in subsequent studies. For instance, Minegishi *et al.*<sup>[16]</sup> described a three-generation Japanese pedigree with six family members carrying *OPTN*<sup>E50K</sup>, diagnosed at an early age (mean of 36.8 years) and displayed low IOP (mean of 13 mmHg). In addition, Hauser *et al.*<sup>[19]</sup> reported an *OPTN*<sup>E50K</sup> NTG patient diagnosed at 38, with normal IOP (15 mmHg) in



**Figure 1:** Disease-associated mutations in *OPTN* gene. (a) *OPTN* gene encodes a 577-amino acid protein with coiled-coil (CC), leucine zipper (LZ), LC3-interacting region (LIR), ubiquitin-binding (UBD), and zinc finger (ZF) domains. (b) Normal tension glaucoma (NTG)-linked mutations (red box: causative; dotted box: risk variants). (c) Amyotrophic lateral sclerosis (ALS)-associated mutations



**Figure 2:** Disease-associated CNVs spanning *TBK1* gene. (a) *GLC1P* locus includes *TBK1* with flanking *XPOT* and *RASSF3* genes. (b) CNVs duplicating or triplicating *TBK1* in NTG patients (indicated by arrows). (c) *TBK1* gene encodes a 729-amino acid protein with kinase, ubiquitin-like (ULD), coiled-coil (CC), leucine zipper (LZ), and helix-loop-helix (HLH) domains. CNV = copy number variation, NTG = normal tension glaucoma



**Figure 3:** Disease-associated variants in *METTL23* gene. (a) *METTL23* consists of five exons; exon 1 and part 2 are noncoding. Several pathogenic variants in *METTL23* have been reported to be associated with normal tension glaucoma (NTG) (b) and familial intellectual disability (c). Red box: causal mutation; dotted box: risk variant

the right eye and 14 mmHg in the left eye) and a C/D ratio of 0.9. Ayala-Lugo *et al.*<sup>[29]</sup> also reported an OPTN<sup>E50K</sup> NTG patient diagnosed at 42 years old with a maximum IOP of 18 mmHg and a C/D ratio of 1.0. More recently, Fox and Fingert<sup>[13]</sup> documented a four-generation NTG pedigree with six family members carrying OPTN<sup>E50K</sup>, all exhibiting low IOPs (mean of 14.6 ± 1.2 mmHg).

**OPTN protein function in NTG**

The human OPTN protein is a 64-kDa scaffold protein consisting of 577 aas, while the mouse *Optn* gene encodes a 584-aa protein (67 kDa) that shares a 78% identity with human OPTN.<sup>[30]</sup> OPTN is a highly conserved protein expressed ubiquitously in all human organs, including the retina, heart, brain, liver, kidney, placenta, and pancreas.<sup>[18]</sup> It exhibits exceptionally high expression in the skeletal muscle.<sup>[18]</sup> OPTN encompasses several structural domains, including two CC domains, a

leucine zipper (LZ) domain, an LC3-interacting region (LIR), a ubiquitin-binding domain (UBD), and a zinc finger (ZF) domain [Fig. 1].<sup>[31]</sup> Normally, OPTN undergoes posttranslational modifications that significantly affect its function, including phosphorylation and ubiquitination.<sup>[26]</sup> One of the major kinases capable of phosphorylating OPTN on at least nine serine and two threonine residues is TANK binding kinase-1 (TBK1).<sup>[32]</sup>

OPTN is a multifunctional protein involved in several cellular processes. First, it acts as an autophagy receptor or adaptor, playing a role in several forms of selective autophagy, including xenophagy,<sup>[33]</sup> mitophagy,<sup>[34]</sup> and aggrephagy.<sup>[35]</sup> Second, it participates in vesicular trafficking, Golgi maintenance,<sup>[37]</sup> secretion,<sup>[38]</sup> and receptor recycling.<sup>[39,40]</sup> Third, OPTN significantly impacts the innate immune response by negatively regulating the NF-κB pathway.<sup>[41]</sup> This diversity in the functions of OPTN can be attributed to its ability to

**Table 1: Overview of OPTN<sup>E50K</sup> mouse models for NTG**

Mouse lines	Promotor	Main results	Reference
<b>Transgenic mice</b>			
Mouse Optn <sup>E50K</sup>	CAG (CMV immediate enhancer/ $\beta$ -actin)	Progressive retinal degeneration is characterized by loss of RGCs and connecting synapses in the peripheral retina, leading to a thinning of the nerve fiber layer at ONH at normal IOP in 16-month-old mice	[47]
Human OPTN <sup>E50K</sup>	c-kit	-	[48]
BAC-hOPTN <sup>E50K</sup>	BCA with <i>OPTN</i> endogenous regulatory sequences	More stressed or degenerating optic nerve axons in BAC-hOPTN <sup>E50K</sup> mice than in 18-month-old control mice at normal IOP	[49]
<b>Knock-in mice</b>			
Optn <sup>E50K/E50K</sup>	-	The thinning of the RGC fiber layer around ONH from 6 months at normal IOP. At 12 months, the thickness of RGC fiber layer around ONH reduction was 20% compared to WT mice	[16]
Optn <sup>E50K/E50K</sup>	-	Aging-related RGC loss and visual dysfunction without elevated IOP	[50]

BAC=bacterial artificial chromosome, IOP=intraocular pressure, NTG=normal tension glaucoma, ONH=optic nerve head, OPTN=optineurin, RGCs=retinal ganglion cells, WT=wild type

**Table 2: Summary of TBK1 CNV detected in NTG pedigrees**

Pedigree (ancestry)	CNV (kb)	Diagnosed age (years)	Max IOP (mmHg, OD/OS)/ Cup-to-disc ratio (first examination, OD/OS)	Reference
GGO-441 (African-American)	Duplication (690–780)	36 $\pm$ 8.2	18.2 $\pm$ 4.1/16.7 $\pm$ 3.6/ 0.95 $\pm$ 0.083/0.93 $\pm$ 0.10	[53]
GGA-458 (German)	Triplication (646)	29 $\pm$ 6.7	19.0 $\pm$ 4.3/18.8 $\pm$ 3.8/ 0.85 $\pm$ 0.071/0.85 $\pm$ 0.071	[55]
GGJ-414 (Japanese)	Duplication (296)	42 (II-4)	18/17 (II-4)/ 0.75/0.7 (II-4)	[54]
AG604 (Australian)	Triplication (347)	60 (AG604)	12/12 (AG604)/ 0.95/0.95 (AG604)	[56]
AG624 (Australian)	Duplication (406)	44 (AG624)	17/17 (AG624)/ 0.95/0.90 (AG624)	[56]
AG724 (Australian)	Duplication (406)	43 (AG724)	14/14 (AG724)/ 0.80/0.90 (AG724)	[56]
GFMC524 (Australian)	Duplication (442)	32 (GFMC524)	13/13 (GFMC524)/ 0.85/0.85 (GFMC524)	[56]
S227 (Korean)	Duplication (124)	48 $\pm$ 16 (39–68)	15.0 $\pm$ 1.7/15.0 $\pm$ 2/ -	[57]

CNV=copy number variation, IOP=intraocular pressure, NTG=normal tension glaucoma, OD/OS=oculus dexter/oculus sinister, TBK1=TANK binding kinase 1

interact with numerous potential partners and to be involved with multiple pathways.<sup>[16]</sup>

The effect of OPTN<sup>E50K</sup> on cell survival and other functions in glaucoma has been investigated using cultured cells and transgenic mouse models, as discussed in the following section. Studies utilizing various cell lines have revealed that the OPTN<sup>E50K</sup> mutant induces apoptosis-like cell death in retinal cells, such as RGC-5, 661W, and human primary retinal cells.<sup>[42–44]</sup> However, this effect is not observed in several other cell lines tested, including IMR-32, HeLa, Cos-1, and NSC34.<sup>[42–44]</sup> These findings suggest that the OPTN<sup>E50K</sup> mutant may contribute to glaucoma by directly inducing the death of RGCs. Notably, Minegishi *et al.*<sup>[30]</sup> have reported an accumulation of insoluble overexpressed OPTN<sup>E50K</sup> in the endoplasmic reticulum (ER) of HEK293 cells, which aligns with the results obtained from neurons derived from induced pluripotent stem cells from E50K NTG patients (OPTN<sup>E50K</sup> iPSCs). The OPTN<sup>E50K</sup> mutant exhibits a strong interaction with TBK1, hindering the proper oligomerization and solubility of OPTN, both of which are crucial for OPTN's intracellular transition.<sup>[30]</sup> Treatment with BX795, a TBK1 inhibitor, effectively reverses the abnormal insolubility of the OPTN<sup>E50K</sup> mutant, which delineates the intracellular dynamics

of the endogenous mutant protein, which causes insolubility associated with TBK1, resulting in POAG.<sup>[30]</sup>

In 2020, VanderWall *et al.*<sup>[45]</sup> generated OPTN<sup>E50K</sup> iPSCs and observed that RGCs differentiated from OPTN<sup>E50K</sup> iPSCs exhibited numerous neurodegenerative deficits, including neurite retraction, autophagy dysfunction, apoptosis, and increased excitability. Recently, Gomes *et al.*<sup>[46]</sup> differentiated RGCs and astrocytes from OPTN<sup>E50K</sup> iPSCs and identified significant changes in OPTN<sup>E50K</sup> astrocytes, including autophagy dysfunction. Co-culture experiments demonstrated that astrocytes induce neurodegenerative properties in otherwise healthy RGCs, while healthy astrocytes alleviate some neurodegenerative features in OPTN (E50K) RGCs.<sup>[46]</sup>

#### OPTN mouse models

Several mouse models of NTG have been developed to mimic human NTG, primarily caused by the well-characterized OPTN<sup>E50K</sup> mutation [Table 1]. In 2010, Chi *et al.*<sup>[47]</sup> generated the first transgenic mouse that highly overexpressed mouse Optn<sup>E50K</sup>, equivalent to human OPTN<sup>E50K</sup>, using the CMV immediate enhancer/ $\beta$ -actin (CAG) promoter. The overexpression of mouse Optn<sup>E50K</sup> led to progressive retinal degeneration, characterized by loss of RGCs and connecting



synapses in the peripheral retina. This resulted in a thinning of RNFL at ONH at normal IOP in 16-month-old mice, thus confirming the association between *OPTN*<sup>E50K</sup> and NTG. Furthermore, *Optn*<sup>E50K</sup> mice exhibited massive apoptosis and degeneration of the entire retina, leading to approximately 28% reduction in the retina thickness.

The following year, Meng *et al.*<sup>[48]</sup> generated another transgenic mouse that overexpressed human *OPTN*<sup>E50K</sup> using the c-kit promoter. However, they only confirmed the expression of human *OPTN*<sup>E50K</sup> in the retina of this transgenic mouse model and did not measure IOP or histological abnormalities. In 2015, Tseng *et al.*<sup>[49]</sup> created the third *OPTN* transgenic mouse (BAC-h*OPTN*<sup>E50K</sup>) with low expression of *OPTN*<sup>E50K</sup> using a bacterial artificial chromosome (BAC) that contained the *OPTN* promoter region and human *OPTN* gene. Histological analysis revealed a 40% RGC loss with normal IOP. In addition, the study of optic nerve axons revealed stressed and damaged optic nerve axons in BAC-h*OPTN*<sup>E50K</sup> mice compared to 18-month-old control mice.

In 2016, Minegishi *et al.*<sup>[16]</sup> generated an *Optn*<sup>E50K</sup> knock-in (KI) mouse model using CRISPR/Cas9 editing. Thinning of the RGC fiber layer around ONH in *Optn*<sup>E50K</sup> KI homozygous (*Optn*<sup>E50K/E50K</sup>) mice was observed from 6 months, despite normal IOP. At 12 months, the thickness of the RGC fiber layer around ONH was reduced by 20% compared to wild-type (WT) mice. Furthermore, the optic cup deepened in *Optn*<sup>E50K/E50K</sup> mice, similar to the changes seen in NTG patients. The administration of TBK1 inhibitor amlexanox proved effective in treating these *Optn*<sup>E50K</sup> KI mice. Presently, amlexanox holds approvals from both the Japanese Pharmaceuticals and Medical Devices Agency (PMDA) and the US Food and Drug Administration (FDA) for clinical trials. In addition, Hou *et al.*<sup>[50]</sup> established another *Optn*<sup>E50K</sup> KI mouse model and identified aging-related RGCs loss and visual dysfunction in *Optn*<sup>E50K/E50K</sup> mice without elevated IOP. Electron microscopy results also demonstrated significant morphological anomalies in the mitochondria of RGCs axons in 3-month-old *Optn*<sup>E50K/E50K</sup> mice, with these changes worsening with age, suggesting that the damaged mitochondria-associated dysfunction of RGCs axon may play an etiological role in NTG.

## TANK Binding Kinase-1 (TBK1)

*TBK1* is located at 12q14.1 and contains 21 exons, of which exon 1, parts of exon 2, and exon 21 are noncoding [Fig. 2].<sup>[51]</sup> Mutation-associated haploinsufficiency of *TBK1* has been implicated as a causal mutation in several types of neuroinflammatory diseases, including ALS, frontotemporal dementia (FTD), Alzheimer's disease (AD) and related tauopathies, herpes simplex virus-1 (HSV-1) encephalitis (HSE), and atypical parkinsonian syndrome.<sup>[52]</sup> Copy number gains of *TBK1* have been associated with NTG, but *TBK1* does not appear to be involved in the pathogenesis of HTG; additional data suggest that *TBK1* is not associated with JOAG, pigmentary glaucoma, or steroid-induced glaucoma.<sup>[15]</sup> In this section, we will focus on the role of *TBK1* in NTG.

### *TBK1* mutations in NTG

In 2011, linkage analysis of an African-American NTG pedigree mapped a new glaucoma-causing gene to a 780-kbp duplication within the *GLC1P* locus on chromosome 12q14.<sup>[53]</sup> Subsequent copy number variation (CNV) analysis and expression analysis

of the *GLC1P* locus in retina suggested that *TBK1* duplication is likely responsible for NTG.<sup>[53]</sup> Both duplication and triplication of *TBK1* have been detected in NTG pedigrees from Europe, Japan, Australia, and Korea [Table 2 and Fig. 2].<sup>[53–57]</sup> These *TBK1* CNVs have been detected and confirmed by various molecular genetic techniques, including quantitative polymerase chain reaction (PCR), chromosomal microarray analysis, and genomic sequencing.<sup>[15]</sup> The cohort of unrelated NTG patients was also evaluated for *TBK1* mutations. *TBK1* CNV mutations were detected in two (1.3%) of 152 NTG patients from Iowa,<sup>[53]</sup> one (0.4%) of 252 Japanese NTG patients,<sup>[54]</sup> one (1.0%) of 96 NTG patients from New York,<sup>[58]</sup> four (1.2%) of 334 NTG patients from Australia,<sup>[56]</sup> two (1.4%) of 143 NTG patients from India,<sup>[59]</sup> and one (0.2%) of 143 Korean NTG patients.<sup>[57]</sup>

### Clinical features of *TBK1*-associated NTG

Patients with NTG associated with *TBK1* duplication or triplication have typical clinical findings of glaucoma. The presenting features of NTG in seven members of the first *TBK1* pedigree from African-Americans (GGO-441) include: 1) an early age at diagnosis with a mean of  $36 \pm 8.2$  years; 2) low IOP with a mean of  $18.2 \pm 4.1/16.7 \pm 3.6$  mmHg (oculus dexter/oculus sinister [OD/OS]); and 3) large C/D ratios with a mean of  $0.95 \pm 0.083/0.93 \pm 0.10$  (OD/OS).<sup>[53]</sup> Similar clinical findings were observed in other reported *TBK1*-associated NTG pedigrees, including GGA-458, GGJ-414, AG624, AG724, and GFMC524.<sup>[53–57]</sup> These family members also had an early onset of NTG (diagnosed before age 45) and a large C/D ratio, as summarized in Table 2.

### *TBK1* function in NTG

*TBK1* is an 82-kDa protein consisting of 729 aa and comprises a kinase domain, a ubiquitin-like domain, and CC domains 1 and 2.<sup>[16]</sup> It is conserved across eukaryotes, with human paralogs identified in zebrafish, mice, primates, and amphibians. Mouse and human *TBK1* exhibit a significant level of sequence similarity, sharing at least 94% homology.<sup>[52]</sup> *TBK1* belongs to the non-canonical I $\kappa$ B kinase (IKK) family and displays 64% homology to IKK $\epsilon$  (also known as IKKi), which plays a critical role in regulating the balance between interferon-1 (IFN-1) and IFN-II signaling pathways.<sup>[60]</sup>

*TBK1*, also known as NF- $\kappa$ B activating kinase (NAK) and T2K, is a serine/threonine protein kinase that plays a key role in the regulation of various cellular processes, including innate immunity, inflammatory cytokine production, autophagy, mitochondrial metabolism, and cell survival/proliferation.<sup>[52]</sup> *TBK1* can be activated by pathogen-associated molecular patterns, damage-associated molecular patterns, inflammatory cytokines, and oncogenic kinases.<sup>[60]</sup> Its biological activity was initially recognized in innate defenses against pathogens, where it plays a crucial role in regulating the production of type I IFN, including IFN- $\alpha$  and IFN- $\beta$ .<sup>[52]</sup> Recent studies have shown that *TBK1* connects the pathogen-stimulated processes of inflammation/immunity, metabolism, and proliferation in diseases, including inflammatory diseases, type II diabetes, obesity, neurovegetative diseases, and some cancers.<sup>[60]</sup>

*TBK1* gene duplications lead to increased *TBK1* mRNA transcription in human fibroblast cells.<sup>[60]</sup> However, the details of how the excess *TBK1* can cause RGC death and NTG remain unknown. There are three hypotheses that warrant further investigation: 1) studies of RGCs with a *TBK1* duplication derived from induced pluripotent stem cells (iPSCs) have

suggested that this mutation is associated with changes in autophagy;<sup>[61]</sup> 2) stimulation of inflammation and the innate immune system by *TBK1* might contribute to RGC death and NTG by production of proinflammatory cytokines and glial cell activation, which have been observed in studies of other neurodegenerative diseases;<sup>[15]</sup> and 3) *TBK1* may influence NF- $\kappa$ B signaling upstream of IKK complex.<sup>[52]</sup> Further research is needed to clarify these potential mechanisms related to excess *TBK1* expression in NTG.

#### **TBK1 mouse model**

The *TBK1* mouse model (Tg-*TBK1*) was generated using a BAC vector to integrate the natural human *TBK1* gene into the mouse genome.<sup>[62]</sup> This transgene provides the mouse line with an additional copy of *TBK1* gene to mimic the *TBK1* duplications that cause NTG in humans. Increased *TBK1* expression was detected in RGCs of Tg-*TBK1* mice. Furthermore, Tg-*TBK1* mice developed RGC loss that increased with age at normal IOP.<sup>[62]</sup> Heterozygous Tg-*TBK1* mice exhibited a 13% RGC loss at 18 months of age compared to controls, while homozygous Tg-*TBK1* mice had a 20% loss at 6 months.

### **Methyltransferase-like 23 (METTL23)**

Human methyltransferase-like 23 (*METTL23*) is located at 17q25.1 and comprises five exons, with exon 1 and a part of exon 2 being noncoding [Fig. 3]. *METTL23* functions as a histone arginine methyltransferase. Several pathogenic variants of *METTL23* are associated with familial NTG and familial intellectual disability [Fig. 3],<sup>[2,63]</sup> suggesting potential etiologic links among arginine methylation, nervous system disorders, and glaucoma.<sup>[1]</sup>

#### **METTL23 mutations**

Recently, in a whole exon data analysis involving 11 samples from three-generation Japanese NTG pedigree (comprising six affected and five unaffected), *METTL23* and *CEP290* were identified as the putative NTG-causing genes.<sup>[2]</sup> The *in silico* predictive tool (Human splicing Finder) indicated that *METTL23* c.A83G causes abnormal splicing, resulting in functionally null proteins, whereas the *CEP290* variant was excluded due to its low pathogenicity score. The *METTL23* c.A83G mutation was heterozygous in all NTG patients. This allele was transmitted vertically from the affected parents to the affected children, indicating an autosomal dominant inheritance pattern.<sup>[2]</sup> No additional exon-spanning duplications or deletions were detected in any of the patients, and no *TBK1* CNVs were detected.<sup>[2]</sup> In addition, the *METTL23* c.A83G mutation is not found in any other glaucoma patients enrolled in the Japan Whole Exome Project.<sup>[2]</sup>

A second *METTL23* variant, specifically *METTL23* c.84+60delAT, was identified in 14 individuals from 1029 unrelated Japanese NTG cases and found in eight of 1402 age-matched Japanese controls.<sup>[2]</sup> No *TBK1* CNVs or *OPTN*<sup>E50K</sup> mutations were detected in any NTG patient with the *METTL23* c.84+60delAT variant. The distribution of this allele between the patients and controls suggests a potential contribution to NTG, as indicated by statistical tests ( $P = 0.03$ , Fisher's exact test or  $\chi^2$  with Yates' correction;  $P = 0.038$ , logistic regression adjusting for age and sex).<sup>[2]</sup> However, further work, including analyses of additional data sets, will be necessary to confirm and further explore this finding.

#### **Clinical features of METTL23-associated NTG**

Patients carrying *METTL23* c.A83G have typical clinical characteristics of NTG, including 1) early age at diagnosis, with a mean age of  $47 \pm 9.6$  years; 2) low IOP with a mean of  $15.4 \pm 3.5$  in the right eye and  $14.6 \pm 2.9$  mmHg in the left eye; and 3) large C/D ratios with a mean of  $0.88 \pm 0.05$  in the right eye and  $0.88 \pm 0.04$  in the left eye at the first examination.<sup>[2]</sup> None of the affected individuals with *METTL23* c.A83G had a history of developmental delay or retinal disease.

NTG patients carrying *METTL23* c.84+60delAT had distinct clinical characteristics, including 1) a mean age at diagnosis of  $62.3 \pm 12.0$  years, 2) maximum IOP with a mean of  $15.0 \pm 2.8/15.0 \pm 2.6$  (OD/OS), and 3) C/D ratio of  $0.76 \pm 0.07/0.82 \pm 0.11$  (OD/OS).<sup>[2]</sup> Interestingly, NTG patients with *METTL23* c.A83G had an earlier onset of disease and a larger C/D ratio at the initial examination than those with *METTL23* c.84+60delAT. Though the sample size is limited, these data suggest that *METTL23* c.A83G mutation may lead to more severe NTG than *METTL23* c.84+60delAT.

#### **METTL23 function in NTG**

*METTL23* is expressed at low levels, but is found ubiquitously in human tissues.<sup>[2]</sup> It belongs to a methyltransferase-like (*METTL*) family and shares homology with the conserved domains related to protein arginine methyltransferases (*PRMT*) activity, particularly *PRMT1-8*.<sup>[64,65]</sup> In 2017, Hatanaka *et al.*<sup>[64]</sup> demonstrated that *METTL23* possesses *PRMT* activity. It catalyzes the dimethylation of histone H3 at arginine 17 (H3R17). This enzymatic activity suggests that *METTL23* plays a role in histone arginine methylation, which may affect gene expression regulation and cellular processes.

The aberrant splicing by *METTL23* c.A83G mutation at the end of *METTL23* exon 2 was confirmed in various experimental models, including exon-trapping vector transfected HEK293T cells, iPSCs derived from NTG patients, and the retinas of *Mettl23* KI mice. NF- $\kappa$ B transcription factors play an important role in regulating the immune response, while PS2 (small secreted protein that belongs to the trefoil factor family) has been shown to suppress the NF- $\kappa$ B-activated expression of proinflammatory cytokines like tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin (IL)-1 $\beta$ .<sup>[66,67]</sup> Elevated expression of TNF- $\alpha$  and IL-1 $\beta$  by activated microglial cells in hypoxic neonatal retina has been linked to RGC apoptosis.<sup>[68,69]</sup> *METTL23* was expressed in RGCs and was found to be associated with NF- $\kappa$ B-mediated transcription of TNF- $\alpha$  and IL-1 $\beta$  through dimethylation of histone 3 arginine 17 (H3R17).<sup>[2]</sup>

Both *METTL23* c.A83G mutation and *METTL23* deficiency were associated with RGC loss and axonal degeneration at ONH in *Mettl23*-KI and -KO mice, similar to the findings in NTG patients. Similarly, *METTL23* c.84+60delAT mutation led to partially aberrant splicing in exon-trapping vector-transfected HEK293T cells, consistent with the milder clinical features observed in NTG patients with this mutation. These findings suggest a direct link among *METTL23*, histone methylation, immune response regulation, and RGC degeneration in NTG.

#### **METTL23 mouse model**

The mouse models of *METTL23* c.A83G-KI and *METTL23*-knockout (KO) generated by CRISPR/Cas9 demonstrated key pathological features relevant to glaucoma:

1) Ganglion cell complex (GCC) thickness reduction. In B-circular scans focused on ONH, both homozygous *Mettl23*-KI and -KO mice exhibited a reduction in GCC thickness starting at 2 months of age with normal IOP. This reduction was observed to progress in all *Mettl23*-KI and -KO mice by 6 months of age. 2) Optic nerve injury. Optic nerve fiber injury was evident in optic nerve cross sections, with statistically significant differences observed in both *Mettl23*-KI and -KO mice compared to control mice at 2 and 6 months. 3) RGC deficiency. Slight reductions in photopic negative scotopic threshold response (pSTR) amplitudes were noted in *Mettl23*-KI and -KO mice at 2 months of age. These reductions became more significant at 6 months of age, indicating a substantial loss of RGCs. 4) RGC loss. Examination of retinal whole mounts revealed a slight, though insignificant, decrease in RGCs in both *Mettl23*-KI and -KO mice compared to controls at 2 months, which developed significant RGC loss at 6 months. These findings support the conclusion that RGC degeneration in the *Mettl23*-KI and -KO mouse models is a progressive process, beginning with the axonal injury that precedes the loss of RGCs. This pattern of disease progression in the mouse models resembles the pathophysiological changes observed in NTG patients. Therefore, the *Mettl23*-KI and -KO mouse models provide valuable insights for studying specific aspects of glaucoma, particularly in cases with normal IOP.

## Myocilin (MYOC)

The human myocilin (*MYOC*) gene is located on chromosome 1q24 and spans approximately 17 kb. It comprises three exons and two introns.<sup>[70]</sup> Initially, *MYOC* was referred to as “the trabecular meshwork inducible glucocorticoid response” (*TIGR*) gene, but it was later renamed *MYOC*.<sup>[71]</sup> This gene was the first to be associated with POAG.<sup>[72]</sup>

### MYOC mutations in POAG

More than 100 POAG-associated mutations have been identified in *MYOC* gene, accounting for 3%–5% of POAG cases.<sup>[73]</sup> Specific mutations in *MYOC* gene can cause POAG with varying ages of onset and degrees of severity. One of the significant associations is with JOAG, characterized by autosomal dominant inheritance and highly penetrant severe phenotype with markedly elevated IOP (greater than 50 mmHg).<sup>[15]</sup> Another notable variant is the *MYOC* p.Q368T (rs74315329) variant, which is linked to a less-severe form (IOP <30 mmHg). This variant is more commonly observed in adult-onset POAG, the most prevalent form (approximately 1.6% of POAG patients).<sup>[15]</sup>

### MYOC mutations in NTG

Mutations in *MYOC*, including p.W286R and p.Q368T, have also been detected in NTG patients.<sup>[74–76]</sup> However, the presence of these mutations in NTG patients has been subjected to some controversy and variability in different studies. In 2019, a case–control study identified the p.Q368T mutation in 11 (0.8%) of 1333 NTG patients and 17 (0.4%) of 4758 controls. This finding suggested a potential association, with  $P = 0.04$  and an odds ratio of 2.3.<sup>[77]</sup> However, this association was not replicated in a more extensive study of 218,792 Finnish citizens.<sup>[78]</sup> In addition, the prevalence of the *MYOC* p.Q368T mutation is higher in POAG patients (1.6%), who typically have elevated IOP, compared to NTG patients (0.8%), indicating that this mutation is more strongly associated with POAG with elevated IOP.<sup>[77,79]</sup>

## MYOC function

In humans and mice, *MYOC* is expressed in numerous ocular tissues, with its most prominent presence being in trabecular meshwork (TM).<sup>[80]</sup> This expression is known to be induced in cultured human TM cells by various factors, including glucocorticoids, oxidative stress, cell stress signaling pathways, and potentially unidentified factors in the aqueous humor.<sup>[81,82]</sup> Normally, TM cells secrete *MYOC*; however, mutations in *MYOC* can disrupt the secretion of both mutant and WT *MYOC*.<sup>[83]</sup> Notably, TM plays an important role in regulating IOP, with elevated IOP being the primary risk factor associated with glaucoma, but not for NTG. Despite extensive research, the precise function of *MYOC* in the retina remains unknown, but it appears to affect TM and the outflow of aqueous humor.<sup>[70]</sup> The genetic association and functional relevance to NTG are still under investigation.

### MYOC animal models

Several groups have used mouse genetics to gain insights into the normal and pathological functions of *MYOC* in retina. In 2001, Kim *et al.*<sup>[84]</sup> generated null mutations in mice, not resulting in a glaucoma phenotype. These findings helped elucidate genetic studies of glaucoma in human patients; a likely null mutation was identified as unrelated to glaucoma in humans.<sup>[85]</sup> Furthermore, in 2004, Gould *et al.*<sup>[86]</sup> observed that mice overexpressing mouse *Myoc* also did not develop elevated IOP or glaucoma. Conversely, more than 90% of glaucoma-related mutations affect the highly conserved functional domain of *MYOC*, known as the olfactomedin domain, likely affecting the structure.<sup>[87]</sup> The results from genetic models suggested that *MYOC* does not have a significant role in regulating IOP, and mutated *MYOC* with abnormal function (gain of function) was likely responsible for ocular hypertension.

Subsequently, several groups began testing disease-specific *MYOC*<sup>Y437H</sup> mutation in mice, a mutation responsible for a severe form of JOAG. Expression of human *MYOC*<sup>Y437H</sup> and mouse *Myoc* with a mutation orthologous to Y437H did not lead to RGC loss.<sup>[80]</sup> An important discovery was made when Shepard *et al.*<sup>[88]</sup> used the adenovirus to express human *MYOC*<sup>Y437H</sup> in the iridocorneal angle and observed elevated IOP. It is worth noting that human *MYOC* contains a targeting signal for peroxisomes that does not exist in mouse *Myoc*, which appears to be critical for the toxicity induced by mutant *MYOC* in TM cells.<sup>[88]</sup> Subsequently, three other groups have reported IOP elevation using different strategies to express human *MYOC*<sup>Y437H</sup> in mouse eyes.<sup>[80]</sup> Other *MYOC* mutations are also believed to involve alternative pathogenic mechanisms.<sup>[80]</sup> However, to date, there are still no animal models specifically linked between the *MYOC* gene and NTG.

## Conclusion

To date, only three genes (*OPTN*, *TBK1*, and *METTL23*) have been linked to familial NTG. Both *OPTN* and *TBK1* proteins play essential roles in NF- $\kappa$ B signaling and autophagy. Furthermore, they share common upstream signals and regulators and can mutually influence each other through positive or negative feedback loops.<sup>[11,31,32,60,67]</sup> Recently, *METTL23* has been shown to catalyze the dimethylation of H3R17 and upregulate SP2 transcription both *in vitro* and *in vivo*, along with TNF- $\alpha$  and IL-1 $\beta$  *in vivo*.<sup>[2]</sup> TNF- $\alpha$  and IL-1 $\beta$



are particularly significant for RGCs and optic nerve fibers. The loss of SP2 is associated with NF- $\kappa$ B-mediated activation of TNF- $\alpha$  and IL-1 $\beta$  *in vivo*.<sup>[66]</sup> Consequently, similar to OPTN and TBK1, METTL23 may also negatively regulate NF- $\kappa$ B-mediated TNF- $\alpha$  and IL-1 $\beta$  expression. These findings suggest that the other proteins involved in NF- $\kappa$ B signaling may also contribute to the development of NTG, and the modulation of NF- $\kappa$ B signaling could be a potential direction for the development of therapeutics.

Research into the role of histone arginine methylation in the development of neuronal diseases is rapidly advancing. The *METTL23* gene has recently been linked to histone arginine methylation in NTG.<sup>[2]</sup> In addition to NTG, mutations in *OPTN* and *TBK1* genes have been identified in patients with ALS.<sup>[15]</sup> Another gene associated with ALS, ataxin 2 (*ATXN2*), has also been linked to POAG.<sup>[89,90]</sup> Furthermore, ALS-linked mutations in the RNA-binding protein fused in sarcoma (*FUS*) result in the formation of insoluble intracytoplasmic protein aggregates.<sup>[91]</sup> This process was found to be alleviated by the depletion of PRMT1, which serves as a transcriptional coactivator by depositing dimethylarginines on histone H4R3.<sup>[92]</sup> Specifically, *METTL23* c.A83G mutation was shown to hinder the dimethylation of histone H3R17, leading to the onset of NTG.<sup>[2]</sup> Notably, several pathogenic variants of *METTL23* are also associated with familial intellectual disability,<sup>[2,63]</sup> indicating a possible connection among arginine methylation, nervous system disorders, and glaucoma.

NTG is an irreversible blinding disease, and its diagnosis is more complex compared to high-pressure forms of glaucoma. Understanding the role of NF- $\kappa$ B-mediated inflammation in NTG could serve as a catalyst for the development of novel diagnostic and therapeutic approaches. Considering that the optic nerve is part of the central nervous system, these findings may also have broader implications for understanding the pathogenesis of other neurodegenerative diseases.

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