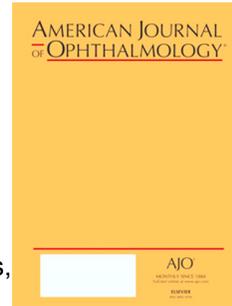


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Copy number variations of *TBK1* in Australian patients with primary open angle glaucoma

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Abstract

Purpose

To investigate the presence of *TBK1* copy number variations in a large well characterised Australian cohort of patients with glaucoma comprising both normal-tension glaucoma and high tension glaucoma cases.

Design

A retrospective cohort study

Methods

DNA samples from patients with normal-tension glaucoma, high-tension glaucoma and unaffected controls were screened for *TBK1* copy number variations using real-time quantitative polymerase chain reaction. Samples with additional copies of the *TBK1* gene were further tested using custom comparative genomic hybridization arrays.

Results

Four out of 334 normal-tension glaucoma cases (1.2%) were found to carry *TBK1* copy number variations using quantitative polymerase-chain reaction. One extra dose of the *TBK1* gene (duplication), was detected in three normal-tension glaucoma patients, while two extra doses of the gene (triplication), was detected in a fourth normal-tension glaucoma patient. The results were further confirmed by custom comparative genomic hybridization arrays. Further the *TBK1* copy number variation segregated with normal-tension glaucoma in the family members of the probands, showing an autosomal dominant pattern of inheritance. No *TBK1* copy number variations were detected in 1045 Australian patients with high-tension glaucoma or in 254 unaffected controls.

Conclusion

We report the presence of *TBK1* copy number variations in our Australian normal-tension glaucoma cohort, including the first example of more than one extra copy of this gene in glaucoma patients (gene triplication). These results confirm *TBK1* to be an important cause of normal-tension glaucoma, but do not suggest common involvement in high-tension glaucoma.

Copy number variations of *TBK1* in Australian patients with primary open angle glaucoma.

Short title; *TANK binding kinase 1* and primary open-angle glaucoma

Mona S Awadalla,¹ John H Fingert,^{2,3} Benjamin E Roos,^{2,3} Simon Chen,⁴ Richard Holmes,⁵ Stuart L Graham,⁶ Mark Chehade,⁷ Anna Galanopolous,⁷ Bronwyn Ridge,¹ Emmanuelle Souzeau,¹ Tiger Zhou,¹ Owen M Siggs,¹ Alex W Hewitt,⁸ David A Mackey,⁸ Kathryn P Burdon,^{1,9} Jamie E Craig¹

¹ Department of Ophthalmology, Flinders University, Flinders Medical Centre, Adelaide, Australia

² Department of Ophthalmology and Visual Sciences, Carver College of Medicine, University of Iowa, Iowa, IA

³ Stephen A. Wynn Institute for Vision Research, University of Iowa, Iowa, IA

⁴ Vitreoretinal Unit; Vision Eye Institute; Sydney, Australia

⁵ Palmerston North Hospital, New Zealand

⁶ Australian School of Advanced Medicine, Macquarie University, New South Wales, Australia

⁷ South Australian Institute of Ophthalmology, Royal Adelaide hospitals, Adelaide, Australia

⁸ Lions Eye Institute, Perth, Western Australia, Australia

⁹ Menzies Research Institute Tasmania, University of Tasmania, Hobart, Australia

Corresponding Authors

Dr Mona S Awadalla

Department of Ophthalmology, Flinders Medical Centre, Adelaide, Australia

Email mona.awadalla@flinders.edu.au

Phone +61 8 8204 5062 Fax +61 8 8277 0899

Introduction

Glaucomas are a group of eye diseases with a common feature of progressive irreversible degeneration of the optic nerve with corresponding loss of the peripheral visual field.¹ Glaucomas are the leading cause of irreversible blindness worldwide, and primary open-angle glaucoma is the most prevalent subtype worldwide.² The main risk factor for glaucoma is elevated intra-ocular pressure, however approximately 20-50% of all primary open-angle glaucoma cases present with normal intra-ocular pressure range (10-21 mmHg) and are termed normal-tension glaucoma.³

The genetic contribution to primary open-angle glaucoma is well documented.⁴ Around half of all primary open-angle glaucoma patients have a positive family history,⁵ and first degree relatives of primary open-angle glaucoma patients have an approximately 9-fold increased risk of developing glaucoma.^{6,7} The first gene identified to be associated with familial normal-tension glaucoma was *Optineurin* (*OPTN*) in the GLC1E region on chromosome 10p15-14.⁸ Subsequent studies reported that mutations in *OPTN* cause 1% to 2% of primary open-angle glaucoma or normal-tension glaucoma.⁹⁻¹² Despite several studies on the *OPTN* gene, its exact role in causing primary open-angle glaucoma remains elusive.^{3, 13, 14}

Recently, a novel genetic locus (GLC1P) on chromosome 12q14 was reported to be linked to normal-tension glaucoma in an African American pedigree.¹⁵ A duplication that spans the *TANK binding kinase 1* (*TBK1*) gene was subsequently detected in this pedigree as well as in 2 out of 153 (1.5%) unrelated normal-tension glaucoma subjects from Iowa, 1 out of 252 (0.4 %) unrelated Japanese normal-tension glaucoma patients, and 1 out of 96 (1.0%) unrelated patients from New York.¹⁵⁻¹⁷ These data suggest that abnormal *TBK1* dosage (duplication) causes normal-tension glaucoma in these patients. The association between copy number variations of the *TBK1* gene and normal-tension glaucoma is supported by several additional observations. First, copy number variations are known to be involved in influencing gene expression and are risk factors for primary open-angle glaucoma (*GALC* gene)^{17, 18} and Axenfeld-Rieger syndrome (*FOXC1* gene)¹⁹ as well as a number of diseases such as HIV dementia complex²⁰ autism²¹ and Alzheimer's disease.²² Second, *TBK1* is specifically expressed in the ganglion cells and the nerve fiber layer of the human retina, which are involved in the pathogenesis of glaucoma.^{15, 23} Third, *OPTN* binds the *TBK1* protein, particularly in the presence of the recurrent severe glaucoma-causing mutation E50K in the *OPTN* gene.²⁴ Interestingly, three known normal-tension glaucoma genes (*TBK1*, *OPTN*, and *TLR4*) each encode proteins that directly interact with each other in a biological pathway that activates autophagy,^{25, 26} a process by which intracellular materials (e.g. proteins, organelles, or pathogens) are degraded. Together these data further implicate the role of *TBK1* gene in the pathogenesis of normal-tension glaucoma.

In this study, we aimed to investigate the presence of copy number variations of the *TBK1* gene in unrelated normal-tension glaucoma cases and unaffected controls recruited from the Australian population. We also explored the presence of the gene copy number variations in patients with high-tension glaucoma, thus attempting to define an overall contribution of *TBK1* copy number variations to glaucoma blindness.

Methods

Approval of this retrospective cohort study was obtained from the Southern Adelaide Clinical Human Research Ethics Committee. This study has been conducted in accordance with the Declaration of Helsinki and its subsequent revisions. The committee prospectively approved the recruitment of individuals and family members with primary open-angle glaucoma and its subtypes, the collection of blood or saliva samples for deoxyribonucleic acid extraction, the screening for genetic mutations, the data analysis and the making of genotype and phenotype correlations. Written informed consent was obtained from each individual to participate in this study. Recruitment was conducted through the Australian and New Zealand Registry of Advanced Glaucoma.⁵ The unaffected control cohort was collected from retirement villages in Adelaide, South Australia as previously described²⁷

Each participant was examined by their specialist, and received a complete eye examination including slit lamp examination of the anterior chamber, gonioscopy, measurement of central corneal thickness (CCT), visual acuity, intraocular pressure, fundus examination with special attention to optic disc health and size, and automated perimetry. The diagnosis of glaucoma followed the definition of the International Society of Geographical and Epidemiological Ophthalmology (ISGEO) described by Foster and colleagues,²⁸ with optic nerve damage and corresponding visual loss detected in at least one eye. Patients recruited in the study and identified as having normal-tension glaucoma followed the same criteria described by Fingert and colleagues¹⁵ (intra-ocular pressure less than or equal to 21mmHg in both eyes, unadjusted for CCT). High-tension glaucoma patients were diagnosed with intra-ocular pressure greater than 21 mmHg in at least one eye, along with glaucomatous optic nerve and visual field damage. Cases diagnosed with advanced glaucoma presented with either fixation involving visual field loss (at least 2 of the 4 central fixation squares having a pattern standard deviation of less than 0.5% on a reliable Humphrey 24-2 field), or severe global field loss at baseline (Mean Deviation of less than -22dB) in at least one eye.⁵ Family members of *TBK1* copy number variations carriers were recruited when available. The controls had no evidence of glaucomatous optic nerve damage, intra-ocular pressure of less than or equal to 21 mmHg, no family history of glaucoma, and were slightly older than cases by design for this aging disease. The study was first conducted using a total of 334 unrelated cases with normal-tension glaucoma and 254 unaffected controls. Sixty-three percent (n=212) of patients had advanced normal-tension glaucoma, while the remainder (n=122) had less severe (non-advanced) normal-tension glaucoma. A positive family history of glaucoma was present in 133 (40%) cases.

Venous blood samples were obtained from the participants for the study. Genomic DNA was extracted from peripheral whole blood using the QiaAmp Blood Maxi Kit (Qiagen, Valencia, CA, USA). DNA from each subject was tested for *TBK1* duplications using TaqMan® Copy Number Assays (Life Technologies™, Carlsbad, CA, USA). The segment of the *TBK1* gene was amplified in four replicates for each DNA sample. The experiment was conducted using the StepOne Plus® real-time PCR instrument, which quantitates the gene of interest, normalized it to an endogenous reference gene (RNase P) known to be present in two copies in a diploid genome. Evaluation of the copy number of genomic DNA targets was performed using the CopyCaller 2.0 software (Life Technologies™, Carlsbad, CA, USA) with default settings. For detailed mapping of duplication events, patients with

detected *TBK1* duplications were analysed using custom 8x60K SurePrint G3 Human custom comparative genomic hybridization arrays microarrays (Agilent, Santa Clara, CA, USA) that interrogated over 55,000 probes in the *GLC1P* locus that spans 9.5 Mbp between rs12227270 and rs7488555 on chromosome 12q14 using the manufacturer's protocol.¹⁵

To further explore the relationship between two apparently unrelated individuals with an identical duplication, we analysed the haplotypes surrounding the duplication region. The three carriers with primary open-angle glaucoma were also part of a previously reported genome-wide association scan.²⁹ Along with 590 other participants with primary open-angle glaucoma, they were genotyped on the Omni1 array (Illumina, San Diego, CA, USA). The most likely haplotype pair across the duplication region (chr12:64173733-65613733, hg19) in each participant in the GWAS were estimated using Beagle3.3.2 (<http://faculty.washington.edu/browning/beagle/beagle.html>).³⁰ The haplotypes across the whole region were visually compared between patients AG624 and AG724. The haplotypes for AG604 with a different duplication were also compared.

Mutation screening of *TBK1* was performed on 95 unrelated cases with high-tension glaucoma, 100 unrelated cases with normal-tension glaucoma and 104 unaffected unrelated controls from Australia. Exome capture was performed using the Agilent SureSelect system and paired-end libraries were sequenced on an Illumina HiSeq 2000 by Macrogen Inc. (Seoul, South Korea). Reads were mapped to the human reference genome (hg19) using BWA, and duplicates were marked and removed using picard. Variants were called using SAMtools, and annotated with ANNOVAR. Variants were described according to the recommendations of the Human Genome Variation Society (<http://www.hgvs.org/>) and referenced against the NHLBI Exome Variant Server (<http://evs.gs.washington.edu/EVS/> [July 2014]), 1000 Genomes,³¹ and dbSNP v138 databases (<http://www.ncbi.nlm.nih.gov/snp>).

Results

TBK1 copy number variations were detected in four (1.2%) of 334 Australian cases with normal-tension glaucoma using quantitative polymerase-chain reaction assays (Figure 1). Three unrelated probands, GFMC524, AG604, and AG624, were found to have three copies of the gene (1 extra dose), while AG724 participant was found to carry four total copies of *TBK1* (2 extra doses). No copy number variations were detected in any of the unaffected controls. This rate is similar to previously published data where overlapping copy number variations were found in 1.3% of Caucasian normal-tension glaucoma subjects from Iowa and in 1% of normal-tension glaucoma patients from New York.^{15, 17} Affected siblings of the probands AG724 and GFMC524 (AG724.1 and GFMC524.1, respectively) were also shown to carry *TBK1* duplications using the quantitative polymerase-chain reaction assay. The inheritance of *TBK1* copy number variations and normal-tension glaucoma is shown in Figure 2 for these pedigrees. Interestingly, all of the members of one pedigree that were diagnosed with normal-tension glaucoma (AG604, AG604.1, and AG604.2) had two extra copies of *TBK1* (triplication), while previously reported cases had one extra copy (Figure 1). All families display an autosomal dominant inheritance pattern of *TBK1* copy number variations and normal-tension glaucoma, providing further evidence that these copy number variations are pathogenic. Moreover, these data also suggest that the extra copies of the *TBK1* gene are tandem repeats on the same allele, that is, a gene duplication in pedigrees with one extra copy of *TBK1* and a gene triplication in pedigrees with two extra copies.

The borders of the copy number variations detected in normal-tension glaucoma probands GFMC524, AG604, AG624, and AG724 were assessed using comparative genomic hybridization (Figure 3). The copy number variations in these Australian normal-tension glaucoma patients are all novel and differ from previously reported copy number variations in the extent of chromosome 12q14 that is involved. Both probands AG624 and AG724 had a duplication, extending from approximately 64,68 Mbp to 65,09 Mbp on chromosome 12. These probands were not known to be related, however, detection of identical copy number variation borders suggested a founder effect. This hypothesis was investigated by comparing haplotypes spanning the *TBK1* locus using genotypes obtained from a prior genome-wide association study. These two patients were found to share a common haplotype over a greater than 1.4 Mbp segment of chromosome 12q14 (between rs10506464 and rs1909340), which further supports a founder effect in these two individuals. A 300 Kbp duplication was detected in normal-tension glaucoma proband AG604 that has similar borders as a previously reported copy number variation in a Japanese normal-tension glaucoma patient GGJ-414 (Figure 3).¹⁵ Genotype data were not available to explore a possible founder effect between these two patients. When the copy number variations from the current report and those from prior reports were analysed,¹⁵⁻¹⁷ the overlap defined a critical region (~131 kbp), which harbours the *TBK1* gene and part of the *XPOT* gene (Figure 3).

Table 1 shows the clinical features of patients carrying the *TBK1* copy number variations. All of them presented with a family history of glaucoma, large cup to disc ratio (ranges from 0.80 to 0.95) and intra-ocular pressure in the normal range (the maximum recorded untreated intra-ocular pressure ranged from 12 mmHg to 17 mmHg). However, the central corneal thickness varied between the affected probands. GFMC524 had thin CCT (496 microns OD, 505 microns OS), and AG624 had thick CCT (622 microns OD, 621 microns OS). Most of the patients who carry

the *TBK1* copy number variations were diagnosed at a relatively young age except for case AG604 who was diagnosed at age 60. However, the onset of the disease in patient AG604 is likely to have been much earlier given the advanced visual field loss that was observed at the time of diagnosis.

To further explore the role of *TBK1* copy number variations in primary open-angle glaucoma in general, 1045 patients with high-tension glaucoma were screened by quantitative polymerase-chain reaction. No *TBK1* copy number variations were identified, indicating that in our dataset *TBK1* duplications were found only in normal-tension glaucoma cases. The demographic feature and clinical differences between the two subtypes of primary open-angle glaucoma and normal controls are illustrated in Table 2.

A cohort of 195 Australian cases with primary-open angle glaucoma (including 100 normal-tension glaucoma cases) and 104 unrelated unaffected controls were screened for disease-causing variants in the coding sequence of *TBK1*. A total of three single nucleotide variants were identified. Two synonymous variants were detected in 87 unaffected controls (p.N22N, p.I326I). One, previously published¹⁵, non-synonymous variant (p.V464A) was found in three normal-tension and five high-tension glaucoma cases and seven unaffected controls. None of these variants are likely to account for disease.

Discussion

Primary open-angle glaucoma is known to be a genetically heterogeneous disease. Recently, Fingert and colleagues identified a large duplication within a novel locus (GLC1P) to be associated with primary open-angle glaucoma and its subtype normal-tension glaucoma located on chromosome 12q14.¹⁵ Although the overlapping duplication encompassed four genes (*TBK1*, *XPOT*, *RASSF3*, and *GNS*), *TBK1* was considered the strongest candidate gene for normal-tension glaucoma by virtue of its biology and the critical region defined by duplications in multiple patients. *TBK1* is expressed in cells affected by glaucoma (human retina),¹⁵ with a clearly documented direct interaction with the *OPTN*, another gene known to cause normal-tension glaucoma.^{24, 32} *TBK1* encodes a protein kinase that participates in both autophagy and NF- κ B signalling pathways,^{25, 26} The specific mechanism by which *TBK1* duplication causes normal-tension glaucoma is still undetermined, however there is a plausible hypothesis that copy number variations of *TBK1* cause either a dysregulation of autophagy or of NF- κ B signalling pathways that ultimately leads to apoptosis of retinal ganglion cells and the development of normal-tension glaucoma.⁷

In addition to confirming the association of the *TBK1* gene copy number variations with normal-tension glaucoma, we also provide the first report of a *TBK1* gene triplication in a family with normal-tension glaucoma. After making this discovery, we retested our American pedigrees that were previously reported to have *TBK1* gene duplications, and we found compelling evidence that one of these pedigrees (GGA-458)¹⁵ in fact has a *TBK1* triplication (data not shown). It is tempting to hypothesize that patients with two extra doses of the *TBK1* gene may have a more severe phenotype than those patients with one extra dose, i.e. earlier onset of disease. Moreover, such a genotype – phenotype relationship might be mediated by increased *TBK1* gene expression. A previous study reported a 1.60-fold increase in the expression level of *TBK1* in patients carrying the duplication than in controls.¹⁵ It would be interesting to examine the expression level in our patients with a *TBK1* triplication.

The absence of *TBK1* duplication in the Australian high-tension glaucoma cohort provides confirmation that *TBK1* duplications appear to occur specifically associated with the normal-tension glaucoma subtype. Nonetheless, it is interesting to speculate that the likely phenotype if a *TBK1* copy number variation carrier had elevated intra-ocular pressure by chance, could be significantly more severe. The Australian cohort shows a similar rate of mutation in normal-tension glaucoma as other Caucasian studies. *TBK1* copy number variations are responsible for 0.4% to 1.3% of normal-tension glaucoma cases in different populations.¹⁵⁻¹⁷ As such it is a rare, but easily detectable marker for significant disease which appears to be highly penetrant within families. Analysis of coding variants in our Australian cohort did not show any mutations likely to cause disease. Considering these data in conjunction with the previously published data by Fingert and colleagues¹⁵ indicates that coding variants in *TBK1* are not a common cause of normal-tension glaucoma.

As no mutations have been reported in unaffected controls, this assay may be an important predictor of normal-tension glaucoma risk in select patient populations (i.e. strongly familial normal-tension glaucoma or in relatives of patients with known *TBK1* copy number variations) leading to regular clinical screening of carriers of *TBK1* copy

number variants. Identifying the genetic risk(s) will facilitate early diagnosis and treatment of any complications arising from this condition and prevent the advanced vision loss seen in three of our four *TBK1*-associated normal-tension glaucoma cases.

ACCEPTED MANUSCRIPT

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c. Contributions to Authors in each of these areas:

Conception and design (MSA, JHF, KPB, JEC); Analysis and interpretation (MSA, JHF, OMS, Be R, KPB); final approval of the article (MSA, OMS, JHF, KPB, JEC); data collection (MSA, Be R, TZ, ES, Br R, SC, RH, SG, MC, AG, AWH, DAM); provision of material, patients or resources (MSA, ES, Br R, KPB, JEC); statistical expertise (MSA, Be R, KPB); writing the article and literature search (MSA); administrative, technical, or logistic support (Be R, ES, Br R).

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Figure 1. Assessment of *TBK1* gene dosage by quantitative polymerase-chain reaction in Australian patients with primary open-angle glaucoma. The x-axis shows the number of copies of the *TBK1* gene that were detected in each subject. The normal dosage of two copies of *TBK1* was detected in the control (Subject 1466). Three probands from unrelated pedigrees (AG624, AG724, and GFMC524) were found to have one extra copy of *TBK1* (3 total copies) while the proband AG604 was found to have two extra copies of *TBK1* (4 total copies). AG; advanced glaucoma, GFMC; non-advanced glaucoma; the positive control was from a normal-tension glaucoma patient previously reported to carry a *TBK1* gene duplication.¹⁶

Figure 2. Pedigrees of the Australian probands with normal-tension glaucoma carrying the *TBK1* copy number variations. Black symbols indicate individuals with normal-tension glaucoma. The proband is indicated by an arrow. Participants carrying a *TBK1* duplication or triplication are indicated by a (+), and tested wild-type individuals are denoted with a (-).

Figure 3. Relative positions of copy number variations detected in Australian cases (AG604, AG624, AG724, and GFMC524) with normal-tension glaucoma in the current report and copy number variations in pedigrees GGO-441, GGA-458, GGJ-414, and GGR-590 were previously reported.¹⁶⁻¹⁸ The extent of each copy number variation in base pairs is in parentheses (hg19 build) and is also depicted by black boxes while the genes encompassed by duplications are depicted as grey boxes.

Table 1. Clinical features of normal-tension glaucoma patients carrying *TBK1* copy number variations.

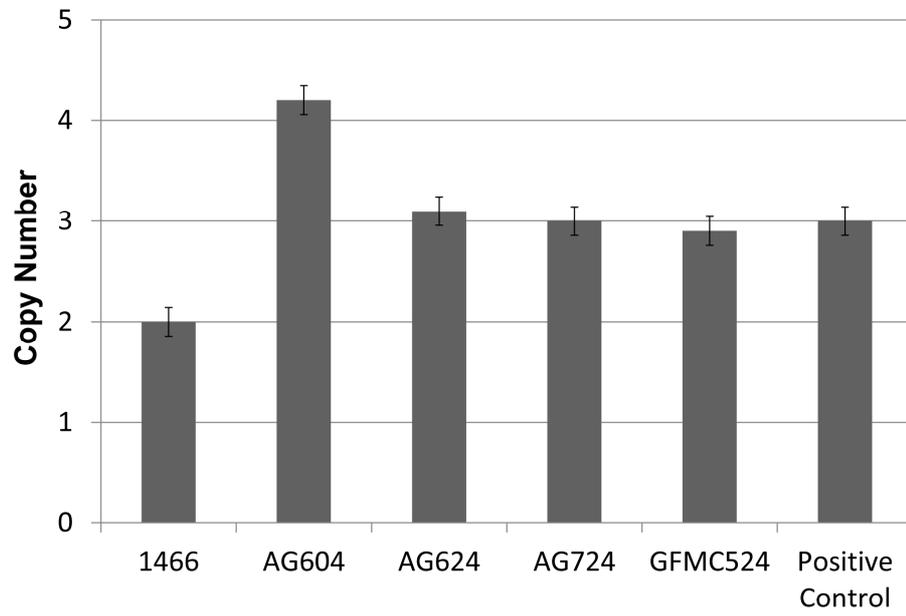
ID	Age of Diagnosis (years)	Highest IOP_OD (mmHg)	Highest IOP_OS (mmHg)	CCT OD (microns)	CCT OS (microns)	CDR OD	CDR OS
GFMC524	32	13	13	496	505	0.85	0.85
AG604	60	12	12	N/A	N/A	0.95	0.95
AG624	44	17	17	622	621	0.95	0.90
AG724	43	14	14	560	550	0.80	0.90

AG= Advanced glaucoma, GFMC= non-advanced glaucoma, IOP= intra-ocular pressure, CCT= central corneal thickness, CDR= cup to disc ratio, OD= right eye, OS= left eye, N/A= not available

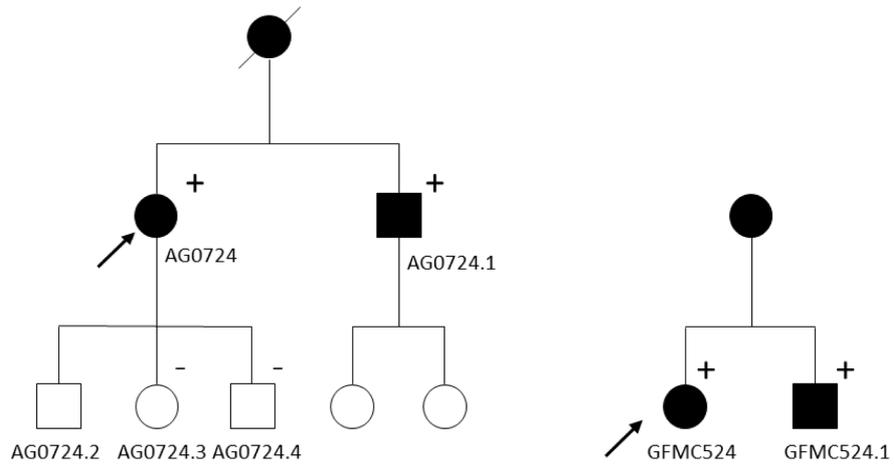
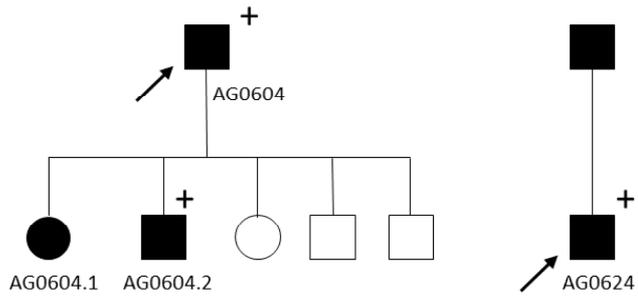
Table 2: Demographic and clinical characteristics of the Australian cohort including normal-tension glaucoma patients, high-tension glaucoma patients and normal unaffected controls

Variables	Mean age years (SD)	Sex (% female)	Mean IOP mmHg (SD)	Mean CCT microns (SD)	Mean CDR (SD)
NTG (N=334)	62.4 (11.4)	61%	16.9 (2.4)	510.7 (40.2)	0.8 (0.1)
HTG (N=1045)	53.0 (14.6)	50%	25.8 (8.9)	519.8 (43.3)	0.8 (0.2)
Normal controls (N=254)	75.9 (8.9)	58%	12.8 (2.3)	544.7 (7.2)	0.2 (0.12)

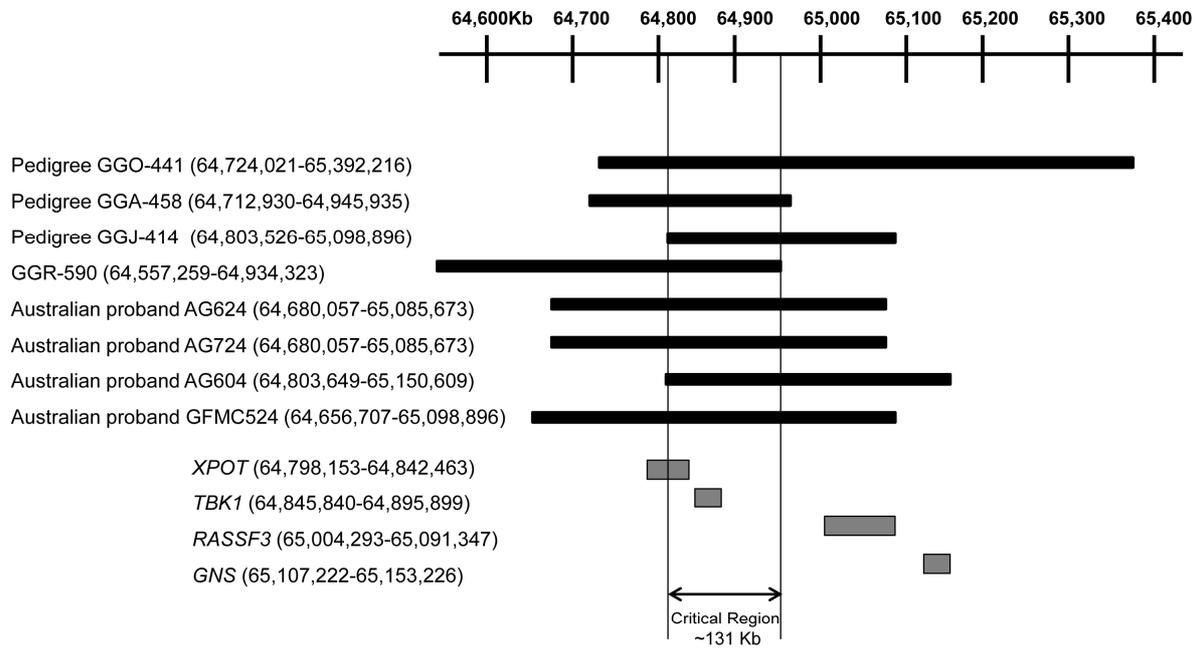
SD=standard deviation, NTG= normal-tension glaucoma, HTG=high-tension glaucoma, IOP=intra-ocular pressure, CCT=central corneal thickness, CDR=cup to disc ratio



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Dr Mona S Awadalla is a Postdoctoral Fellow at Flinders University, Australia. She earned her MBBS degree in 2007 from Alexandria University, Egypt, and her PhD degree in 2013 from Flinders University. Dr Awadalla main areas of scientific interest are genetics, nanophthalmos and glaucoma.

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