DNA methylation at the 9p21 glaucoma susceptibility locus is associated with normal-tension glaucoma

Kathryn P Burdon 1,2*

Mona S Awadalla 1*

Paul Mitchell 3

Jie Jin Wang 3

Andrew White 3

Miriam C Keane 1

Emmanuelle Souzeau 1

Stuart Graham 4

Ivan Goldberg 5

Paul R Healey 3, 5

John Landers 1

Richard Mills 1

Stephen Best 6

Alex W Hewitt 2,7

Shiwani Sharma 1

Jamie E Craig 1

[1] Department of Ophthalmology, Flinders University, Adelaide, South Australia, Australia

[2] Menzies Institute for Medical Research, University of Tasmania, Hobart, Australia

[3] Centre for Vision Research, Department of Ophthalmology and Westmead Institute for Medical Research, University of Sydney, Westmead, Australia.

[4] Department of Clinical Medicine, Faculty of Medicine and Health Sciences, Macquarie University, Sydney, New South Wales, Australia.

[5]Eye Associates, Glaucoma Unit, Sydney Eye Hospital, Australia; Discipline of Ophthalmology, The University of Sydney, Sydney, Australia

[6] Auckland Eye, Auckland, New Zealand; Auckland District Health Board, Greenlane Clinical Centre, Auckland, New Zealand; Discipline of Ophthalmology, University of Auckland, Auckland, New Zealand.

[7] Centre for Eye Research Australia, Royal Victorian Eye and Ear Hospital &Ophthalmology, Department of Surgery, the University of Melbourne, Melbourne, Australia.

*contributed equally

Corresponding Author

Prof Jamie E Craig

Department of Ophthalmology, Flinders University, Bedford Park, SA 5042, Australia

Phone +61 8 8204 5064. Email: Jamie.craig@flinders.edu.au

Abstract

Purpose

Recent genome-wide association studies reported strong association of genetic variation at the *CDKN2B/CDKN2B-AS1* locus on 9p21 with normal-tension glaucoma (NTG) in multiple populations. The mechanism by which this locus causes disease remains to be elucidated. We investigated the association of DNA methylation of CpG islands at this locus with NTG.

Methods

We conducted a retrospective case-control study of 178 NTG cases and 202 unaffected controls from Australia. *CDKN2B* and *CDKN2B-AS1* promoter methylation was measured quantitatively using the MassCleave assay, and assessed for association with the disease, and the genotype of the associated risk variants using IBM SPSS statistics 22. CpG sites at which methylation status was associated with NTG were validated using pyrosequencing.

Results

We identified one CpG site (F1:13-14) in *CDKN2B* promoter which showed significant association with NTG (p =0.001). The association was highly significant in female cases (p= 0.006) but not in male cases (p=0.054). The association was validated using an independent method confirming the likely association of DNA methylation with NTG in females (p=0.015), but not in males (p=0.497). In addition, methylation at CpG sites in *CDKN2B* was also associated with genotype at rs1063192, which is known to confer risk for NTG.

Conclusion

This study reveals an association of methylation status in the *CDKN2B* promoter with NTG, particularly in females. This suggests that the observed genetic association with the disease at this locus could be in part due to epigenetic mechanisms, and is likely to be independent to association of non-synonymous coding variation within the gene.

Introduction

Glaucoma is a heterogeneous group of optic neuropathies characterised by progressive loss of peripheral vision due to loss of retinal ganglion cells. [1] Collectively, they are the leading cause of irreversible, preventable blindness worldwide. As an age-related condition, the number of people with glaucoma is predicted to double over the next 20 years. [1] Primary open-angle glaucoma (POAG) is the most common subtype of the disease, particularly amongst Caucasian populations. While elevated intraocular pressure (IOP) is the leading risk factor, at least 20% of POAG patients have IOP measurements repeatedly within the normal range, a subgroup termed normal-tension glaucoma (NTG). [2]

Several genes have been found to contribute to rare Mendelian forms of POAG but these account for only a small proportion of POAG cases. [3-6] The majority of Primary open-angle glaucoma is a complex polygenic disease with both genetic and environmental risk factors. Common genetic variation at many candidate genes have been assessed for association with glaucoma risk but most findings have been difficult to replicate [7] until a genome-wide association study (GWAS) identified a novel locus for POAG, *CDKN2B/CDKN2B-AS1* on chromosome 9p21. [8] SNPs in the region have been associated with POAG in multiple studies including Caucasian, Afro-Caribbean and Japanese populations. [9-14] Stratification by type of POAG indicated that this locus is more significantly associated with NTG than with high tension glaucoma. [9, 10, 13-16] We recently reported that females have a stronger association signal at the 9p21 locus, and variants at this locus are considered a risk factor for developing advanced NTG. [17] Association of variants at the 9p21 locus has also been reported with non-ocular diseases

including cardiovascular disease, [18] diabetes, [19] intracranial aneurysm, [20] and glioma. [21] The pathogenic variants in this region associated with glaucoma are yet to be identified.

The 9p21 locus harbours two tumour suppressor genes *CDKN2A* and *CDKN2B* (cyclin dependent kinase inhibitor 2A and 2B) which regulate and inactivate the retinoblastoma tumour suppressor protein (pRb) pathway. Furthermore, *CDKN2B* has been implicated in regulating cellular apoptosis in response to stress stimuli. [22] *CDKN2A* and *CDKN2B* transcription is regulated by *CDKN2B-AS1* (previously known as ANRIL). [23] Both genes were reported to be significantly upregulated in the retina in a rat model of elevated IOP, suggesting a possible role in apoptosis in retinal ganglion cells, [23] and in POAG pathogenesis. [8] Consistently, the genetic variation across the region has been shown to alter expression levels of this long non-coding RNA and the protein coding genes, [24] although the mechanism by which this occurs is as yet unclear.

DNA methylation refers to the situation where a methyl group is transferred to cytosine residues in the context of CG dinucleotide (known as CpG sites). CpG sites are asymmetrically distributed throughout the genome and CpG dense regions are known as CpG islands. Methylation of CpG islands, particularly in the promoter region of genes, is associated with silencing of gene expression and is referred to as an epigenetic effect. [25, 26] DNA is almost completely demethylated during early embryogenesis, and during organogenesis large numbers of genes are methylated and their expression is suppressed throughout life. Whilst epigenetic effects are well known in cancer, their exploration in eye diseases is still in its infancy; though recent studies in age-related macular degeneration, [27-29] cataract, [30] pterygium [31] and retinoblastoma [32] have shown some effects relevant to eye diseases.

We hypothesized that in the absence of coding mutations accounting for disease, DNA methylation at CpG islands in the regulatory regions of *CDKN2B* and *CDKN2B-AS1* could account, in part, for the association with NTG risk. This could be mediated through genetic variations altering the methylation status, genetic factors independent of this locus having an effect, or acquired non-genetic changes in methylation status. As genotype has shown a possible correlation with methylation state, [33] this could provide a further insight in to the mechanism of the association with the SNPs in the region. We thus first screened coding exons in *CDKN2B* in our Australian Caucasian POAG cohort for variants to rule out their contribution then evaluated DNA methylation in these glaucoma-associated gene promoters.

Methods

Patients with normal-tension glaucoma and unaffected unrelated controls were recruited through the Australian and New Zealand Registry of Advanced Glaucoma (ANZRAG). [34] Additional controls were obtained from the Blue Mountains Eye Study (BMES). [35] All participants were described previously in a study that reported the association of genetic variants near CDKN2B-AS1 with POAG. [8] Approval was obtained from the Human Research Ethics Committee of the Southern Adelaide Health Service and the University of Sydney for investigation of the epidemiology and genetics of ocular disease. The study was conducted in accordance with the Declaration of Helsinki and its subsequent revisions. Written informed consent was obtained from each participant.

Each participant received a complete eye evaluation including slit lamp examination, measurement of the central corneal thickness with ultrasound pachymetry, visual acuity, intra-ocular pressure, fundus examination with special attention to optic disc parameters, and

visual field assessment using the Humphrey 24-2 SITA-standard. The diagnosis of NTG was based on the presence of glaucomatous optic disc rim thinning, with vertical cup-to-disc ratio (VCDR) ≥ 0.7 or asymmetry of VCDR ≥ 0.7, and corresponding glaucomatous visual field loss to the course of retinal nerve fibre thinning. Visual field loss secondary to glaucoma follows specific pattern ranging from arcuate defects, nasal steps, up to tunnel vision and total loss of field. [36, 37] . NTG was defined as highest recorded IOP for both eyes < 21 mmHg.

Identification of coding variants

Genomic DNA was extracted from 8 ml of venous blood using the QiaAmp Blood Maxi Kit (Qiagen, Valencia, California) according to the manufacturer's protocol. Coding variants in *CDKN2B* were retrieved from whole exome sequences of 100 unrelated cases with NTG and 104 unaffected unrelated Australian controls. As *CDKN2B-AS1* is a non-protein coding gene, analyses were only conducted on *CDKN*2B represented in the exome capture platform. Exome capture was performed using the Agilent SureSelect system and paired-end libraries were sequenced on an Illumina HiSeq 2000 at Macrogen Inc. (Seoul, South Korea). Reads were mapped to the human reference genome (hg19) using BWA (bio-bwa.sourceforge.net/), and duplicates were marked and removed using Picard (http://broadinstitute.github.io/picard/). Variants were called using SAMtools, [38] and annotated with ANNOVAR (www.openbioinformatics.org/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/), 1000 Genomes [39] and dbSNP v138 [40] databases, all accessed February2017. The potential functional significance of missense variants was analysed using Polyphen2 and SIFT programmes. [41, 42]

Determining methylation status of CpG islands

Total of 178 unrelated patients were recruited to the study, including the 100 participants (56.2%) who underwent exome sequencing. 68 patients were carrying either the homozygous risk haplotypes at the 9p21 region (consisting of SNPs rs3217992, rs1063192, rs7049105, rs10120688 and rs4977756, as defined previously [8]), and 20 cases with homozygous protective haplotypes. An additional 90 cases were included regardless of the genotype. A total of 202 controls were selected from the ANZRAG and BMES to match as closely as possible to the cases for age, sex and genotype (risk haplotype n=54, protective haplotype n=57, other genotypes n=91). We used the same samples for the discovery and the validation analysis. The samples were spread over 6 plates. Plate to plate variations were assessed as a covariate using multivariate logistic regression, as it has a potential to cause variation and bias to the DNA methylation analyses.

Genomic DNA (200ng) was subjected to bisulfite conversion, using the Methyl Easy Xceed kit (Human Genetic Signatures, NSW, Australia). Methylation assays for assessing the methylation status of CpG dinucleotides upstream of *CDKN2B* and *CDKN2B-AS1* were designed using the EpiDesigner primer design tool (www.epidesigner.com). These two genes were selected as their promoter regions are closest to the peak association signal in this region. [8, 13]

Four fragments were selected to cover the CpG islands upstream of *CDKN2B* and *CDKN2B-AS1*. Primer sequences and the number of CpG sites for each fragment are shown in **Supplementary Table 1**. Not all sites were resolvable with this methodology, and the numbering of each CpG dinucleotide is depicted in **Supplementary Figure 1**. Specific CpG

sites are denoted as Fragment:site number (e.g. F1:13-14 denotes CpG sites 13 and 14 in fragment 1).

Amplicons were generated from the bisulfite converted DNA, subjected to the MassCleave assay (Sequenom Inc. San Diego, CA) and analysed by MALDI-TOF mass spectrometry (Sequenom Inc.) at the Australian Genome Research Facility, Brisbane, Australia. Individual methylation ratios for each sample at each CpG dinucleotide were estimated with the Sequenom EpiTYPER® software (Sequenom Inc.). [43]

Confirming the findings at the *CDKN2B* promoter using pyrosequencing

Pyromark AssayDesign 2.0 (Qiagen, Australia) software was used to design a single assay covering the region of interest from the EpiTyper analysis (correlating to CpG 13 to CpG 17 in fragment 1). The forward primer sequence was 5'-GGGGATTAGTGGAGAAGGT-3' and the reverse primer 5'-CCCTAAAACCCCAACTACCTA-3'. DNA samples were subjected to bisulfite conversion and clean-up using the Qiagen Epitect Bisulfite conversion kit. A well characterised control DNA was included to monitor bisufite conversion efficiency. PCR amplifications of bisulfite converted DNA were conducted using the Qiagen Pyromark PCR kits with appropriate positive and negative control reactions. All PCR amplifications were assessed for performance by resolution on agarose gels. Pyrosequencing was performed with the Qiagen PyroMark Gold pyrosequencing reagents on the PyroMark Q24 system at the Australian Genome Research Facility, Perth, Australia. A positive control was sequenced in parallel with all samples. The pyrosequencing data was analysed using the PyroMark Q24 v2.0.6 analysis software (Qiagen) to give a percentage of methylated reads at each CpG dinucleotide.

Statistical analysis

All analyses were performed using IBM SPSS statistics 22.0. The distribution of the degree of methylation at each CpG site deviated from normality as assessed by the Smirnov-Kolomogorov test, therefore the nonparametric Mann-Whitney U test was used to test association between methylation levels and NTG or SNP genotype. Bonferroni correction for the number of CpG sites analysed in each promoter was employed. The threshold for determining significance was therefore $p < 0.002$ for *CDKN2B* (28 sites) and $p < 0.003$ for *CDKN2B-AS1* (15 sites). Multivariate logistic regression was used to adjust for relevant covariates (age, sex, genotype, and plate to plate variation) to explore the association of methylation with NTG.

Results

Demographic and clinical characteristics of cases and controls are shown in **Table 1**. There was a significant difference in cup-to-disc ratio and intraocular pressure (IOP) between NTG cases and normal controls, as expected (p-value < 0.01). No age or sex differences were found due to the selection of age- and sex-matched controls.

Table 1: Clinical characteristics of NTG cases and unaffected controls

Variables	Cases	Controls	p-value
Number	178	202	-
Sex (% female)	56%	52%	0.41
Mean age in years (SD)	77.8 (9.9)	78.1(7.4)	0.73
IOP in mmHg (SD)	17.0 (3.0)	12.8 (2.3)	<0.01
Cup/disc ratio (SD)	0.9 (0.1)	0.2 (0.1)	<0.01

We first screened the Australian participants for potential disease-causing variants in the exons of *CDKN2B*. A single synonymous variant in *CDKN2B* (p.G130G) was found in an unaffected control. No predicted pathogenic variants were found in NTG cases.

Association of *CDKN2B* and *CDKN2B-AS1* promoter methylation status with NTG

Overall, CpG islands at the 9p21 locus showed relatively low levels of DNA methylation (**Supplementary Figure 2)**. In the *CDKN2B* promoter, methylation levels at three sites were associated with NTG ($p < 0.05$). Only one Fragment, fragment 1 sites 13-14 (F1:13-14), survived Bonferonni correction ($p < 0.001$, **Table 2**). This location which contained two CpG dinucleotides could not be resolved further by the EpiTyper assay. The methylation level was observed to be higher in controls than in cases (**Table 2**). In the *CDKN2B-AS1* promoter, one CpG site in fragment 4, F4:25-27, was associated with NTG but did not survive correction for multiple testing ($p = 0.003$) (**Table 2)**.

Table 2: Association of methylation of CpG dinucleotides in *CDKN2B* and *CDKN2B-AS1* promoters with NTG. P-values from Mann-Whiteny U test are indicated. Values in bold indicates significant association with NTG following Bonferonni correction. (full data presented in Supplementary table 2).

Gene	Fragment:CpG site(s)	Glaucoma status	Mean rank	p-value
CDKN2B	F1:13-14	Controls	197.16	**0.001**
		Cases	162.49	
CDKN2B	F1:15-17	Controls	176.08	0.012
		Cases	150.12	
CDKN2B	F2:35-37	Controls	171.75	0.031
		Cases	195.49	
CDKN2B-AS1	F4:25-27	Controls	201.45	0.003
		Cases	169.85	

As a secondary analysis, we stratified the cohort by sex. In this analysis, F1:13-14 (*CDKN2B*), F2:35-37 (*CDKN2B*) and F4:25-27 (*CDKN2B-AS1*) showed association with NTG among the female group with p-values of 0.006, 0.006 and 0.003, respectively. A trend of association between F1:15-17 (*CDKN2B*) and the disease was found in the male group with p-value of 0.011 **(Table 3)**. None of these associations survived correction for multiple testing.

Table 3: Association of methylation status of the CpG sites in *CDKN2B* and *CDKN2B-AS1* promoters with NTG, stratified by sex.

(full data presented in Supplementary table 3).

			Female		Male	
Genes	**Fragment CpG site**	**Glaucoma status**	**Mean rank**	**Mann-Whitney U**	**Mean rank**	**Mann-Whitney U**
CDKN2B	F1:13-14	Controls	95.99	0.006	85.55	0.054
		Cases	75.33		71.65	
	F1:15-17	Controls	83.26	0.323	77.28	0.011
		Cases	76.06		60.09	
	F2:35-37	Controls	77.44	0.006	77.95	0.639
		Cases	98.12		81.35	
CDKN2B-AS1	F4:25-27	Controls	112.08	0.003	89.44	0.375
		Cases	88.44		82.96	

To determine whether the observed DNA methylation is genotype-dependent, we compared the degree of methylation between homozygous carriers of the risk alleles for rs1063192 (representing the full risk haplotype) to carriers of the protective alleles. Interestingly, F1:13-14 in *CDKN2B* showed no association with the genotype at this SNP (**Table 4**) despite both the SNP and methylation status being associated with NTG. The results showed three additional sites in the *CDKN2B* promoter to be statistically significantly associated with genotype, F1:18-19, F2:24-27 and F2:35-37, and to be more methylated in participants

carrying the homozygous risk allele than in participants carrying the homozygous protective alleles (Table 4).

Table 4: Association between methylation of the associated CpG sites in *CDKN2B* gene promoter and rs1063192 genotype.

(full data presented in Supplementary table 4).

CpG site_fragment	rs1063192 genotype	Mean Rank	Mann-Whitney U
F1:13-14	Wild type	131.88	0.042
	Homozygous	112.23	
F1:18-19	Wild type	97.88	**0.002**
	Homozygous	126.70	
F2:24-27	Wild type	98.62	**0.001**
	Homozygous	129.59	
F2:35-37	Wild type	97.34	**0.001**
	Homozygous	130.16	

We then applied a logistic regression model to further explore the relationship between the most statistically significant CpG site in the overall analysis (F1:13-14) and a set of variables consisting of age, sex, genotype at rs1063192 and plate to plate variations. When all variables were considered, F1:13-14 in *CDKN2B* promoter was significantly associated with NTG status ($p < 0.001$, **Table 5**), independent of age, sex and genotype. However, borderline significance of the plate to plate variation ($p = 0.025$) observed in the logistic regression model could possibly account for the signal in the CpG site F1:13-14. To explore this, we conducted validation tests using a second method to target this CpG site in *CDKN2B* for further confirmation.

Table 5: Binary logistic regression of all relevant variables with *CDKN2B* methylation site associated with NTG.

Variables	p-value	OR	95% C.I.	
			Lower	Upper
Age	0.818	1.004	0.97	1.04
Sex	0.196	0.661	0.35	1.24
Plate	0.025	0.709	0.52	0.96
CpG F1:13-14	4.0×10^{-4}	1.061×10^{-8}	3.6×10^{-13}	3.0×10^{-4}
rs1063192 homozygotes	0.017	1.551	1.08	2.22
Constant	0.425	3.034		

OR; odds ratio. CI; confidence interval.

Validation of association of *CDKN2B* promoter methylation with NTG

To follow up the association of F1:13-14 with NTG, five CpG dinucleotides in fragment 1 including CpG sites 13 to 17 were assessed individually via pyrosequencing (**Table 6**). No significant signals were detected. However, when the analyses were stratified by sex, CpG13 in Fragment 1 was associated with NTG in the females (p = 0.015), with methylation degree lower in cases (5.24) than in controls (5.66), in agreement with the findings from the MassArray analyses. No association was found in the male group (p= 0.497).

Examination of the mean methylation detected using both methods and in each sex is shown in Supplementary table 5. These data show that CpG13 has on average, less methylation observed than CpG14, detectable by pyrosequencing. The degree of methylation and the differences are small, but statistically significant difference were detected using the appropriate non-parametric tests.

Table 6: Pyrosequencing results of the targeted CpG sites in *CDKN2B* promoter showing the mean rank for cases and controls and association with NTG in all participants and stratified by sex. Significant value ($p < 0.05$) is in bold.

	Combined			Female			Male		
CpG sites	Control	Case	Mann Whitney-U	Control	Case	Mann Whitney-U	Control	Case	Mann Whitney-U
CpG 13	197.63	182.42	0.178	112.82	92.69	**0.015**	85.66	90.90	0.497
CpG 14	198.77	181.12	0.118	109.33	96.35	0.117	90.13	85.35	0.535
CpG 15	198.85	181.02	0.114	110.58	95.04	0.061	89.18	86.54	0.732
CpG 16	195.05	185.33	0.389	109.99	95.67	0.084	86.14	90.31	0.588
CpG 17	199.01	180.84	0.108	110.9	94.71	0.051	89.45	86.20	0.637

Discussion

Common genetic variation at *CDKN2B/CDN2B-AS1* on chromosome 9p21 has been reported to be associated with larger cup-to-disc ratio and primary open-angle glaucoma, particularly the normal-tension subtype. [8, 15] Junemann et al [44] recently reported a significant elevation of global genomic DNA methylation in patients with POAG. Another study suggested that the hypoxic environment in glaucoma has a role in altering the epigenetic mechanisms, which can lead to fibrosis and accumulation of the extracellular matrix in the trabecular meshwork and lamina cribrosa at the optic nerve head. [45] Our initial analysis focused on sequence variation in the protein coding *CDKN2B* gene, however, the absence of likely disease-causing variants in 100 NTG patients prompted additional studies to explore the mechanism of association of this locus with POAG. As the involvement of epigenetics of specific glaucoma-causing genes had not been investigated, we aimed to identify the difference in methylation status of *CDKN2B* and *CDKN2B-AS1* CpG islands between NTG cases and controls in a larger sample and including patients known to carry the disease associated alleles. Such studies are important to understand the effect of the environment on genetic susceptibility for development of the disease.

Carriers of the risk alleles at *CDKN2B/CDKN2B-AS1* are reported to be three times more prone to develop NTG, the mechanism for which is likely to be increased vulnerability of the retinal ganglion cells in response to stress including change in intra-ocular pressure, even when measured pressures do not exceed the standard clinical definition of high pressure. [46] Wiggs et al [13] suggested that *CDKN2B-AS1* is involved in optic nerve degeneration in glaucoma through influencing the expression of *CDKN2B*, an inhibitor of cyclin-dependent kinase activity that is responsible for cell cycle maintenance. In addition, these loci play a

role in regulating the TGF-beta pathway, which is involved in regulating the intra-ocular pressure and protecting the optic nerve from degeneration in glaucoma.

The finding of this study suggests that the CpG islands in the promoters of *CDKN2B* and *CDKN2B-AS1* are not highly methylated in circulating lymphocytes. Differences in the methylation level between NTG cases and controls do appear to exist, particularly in females. The use of two methods to detect methylation status provides confidence in the result, however the limited size and the post-hoc nature of the sex stratified analysis does warrant some caution when interpreting the findings and replication in an independent cohort is required.

The association observed in females agrees with our recent study that reported a strong association between POAG relevant SNPs at 9p21 and females presenting with NTG [17] and may provide some of the explanation for these observed sex based differences if the genetic association is able to tag the methylation status. We also show that the degree of methylation at the most associated CpG site F1:13-14 in *CDKN2B* promoter, did not appear to be influenced by the genotype of NTG risk SNPs suggesting that it could have an independent role in pathogenesis of the disease. Additional associations were also noted between F1:18-19, F2:24-27 and F2:35-37, with the homozygous risk alleles in rs1063192. This could indicate that the homozygous genotype of the SNP is required for theses CpG sites to have an effect.

Estrogen is thought to have a protective effect against POAG risk.[47] Our female cases were predominantly of post-menopausal age which may explain the noticeable differences in the methylation status between the sexes. Additionally, the degree of methylation at the most associated CpG site F1:13-14 in *CDKN2B* promoter, did not appear to be influenced by the

genotype of at NTG risk SNPs, suggesting that it has an independent effect and could have played a role in causing pathogenesis of the disease, independent of the effect of genotype.

The main limitation of this study is that we examined the methylation status of the gene of interest using genomic DNA derived from lymphocytes, thus any acquired methylation changes in the retina may not be reflected in blood DNA. Lymphocyte DNA was used because of limited availability of eye-tissues (i.e. retina) for investigation, particularly from large numbers of patients, especially in the early stage of the disease. Furthermore, in the advanced blinding stages of the disease, the retinal ganglion cells would have undergone apoptosis and their methylation contribution to the overall genomic DNA of the tissue itself would be irreversibly lost.

To our knowledge, this is the first study to investigate the association between DNA methylation in specific glaucoma-associated genes and normal-tension glaucoma. We showed that alteration of the methylation pattern in the promoter region of *CDKN2B* could be associated with the risk of normal-tension glaucoma in females independent of the genotype effect of the associated SNPs. Females with NTG have lower levels of methylation than matched controls. Additional investigations in independent cohorts and in varied ethnic groups are needed to confirm these associations.

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