Association of Common *SIX6* Polymorphisms With Peripapillary Retinal Nerve Fiber Layer Thickness: The Singapore Chinese Eye Study

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Purpose. Recently the common *SIX6* missense variant rs33912345 was found to be highly associated with glaucoma. The aim of this study was to investigate the association between this *SIX6* variant and peripapillary retinal nerve fiber layer (RNFL) thickness measured by spectral-domain optical coherence tomography (SD-OCT) in a population setting.

Methods. Study subjects were enrolled from the Singapore Chinese Eye Study (SCES), a population-based survey of Singaporean Chinese aged 40 years or older. Subjects underwent a comprehensive ocular examination. Spectral-domain OCT was used to measure RNFL thicknesses. Genotyping of *SIX6* rs33912345 (Asn141His) was performed using HumanExome BeadChip.

Results. A total of 2129 eyes from 1243 SCES subjects (mean age: 55.0 ± 7.4 years) with rs33912345 genotype data and SD-OCT images were included for the analysis. Of these, 26 eyes of 21 subjects had glaucoma. The frequency of rs33912345 risk variant C (His141) was 80% in the study subjects. Each rs33912345 C allele was associated with a decrease of 1.44 μm in RNFL thickness after adjusting for age, sex, genetic principal components, and axial length ($P = 0.001$). These associations remained similar in 2096 nonglaucoma eyes in which each C allele was associated with a decrease of 1.39 μm in RNFL thickness ($P = 0.001$). The strongest association was observed in the superior RNFL sector (a decrease of 2.83 μm per risk allele, $P < 0.001$) followed by the inferior RNFL sector (a decrease of 2.24 μm per risk allele, $P = 0.003$), while the association did not reach significance in the nasal and temporal sectors.

Conclusions. Nonglaucomatous individuals with the *SIX6* missense variant have reduced RNFL thickness in regions known to be particularly affected in those with glaucoma. This may be the primary mechanism for increased risk of POAG in individuals who carry the *SIX6* His141 risk variant.

Keywords: *SIX6*, genetic association, retinal nerve fiver layer thickness, glaucoma

Glaucoma, characterized by progressive loss of retinal ganglion cells, is one of the leading causes of global blindness and affects more than 64 million worldwide.[1] Positive family history of glaucoma has been recognized as an important risk factor for the disease.[2,3] Genetic factors have been thought to play an important role in the development of glaucoma. Recently, genome-wide association (GWA) studies identified multiple loci associated with the susceptibility to glaucoma, including the *SIX1–SIX6* locus.[4,5]

Variants in the *SIX–SIX6* locus (such as rs10483727) were first associated with quantitative optic nerve parameters, such as vertical cup–disc ratio (VCDR),[6] which is used clinically to evaluate the degree of glaucoma and monitor disease progression. Subsequently, case-control GWA studies on primary open-angle glaucoma (POAG) found a significant association between rs10483727 and the risk of POAG.[4,5] The association of the *SIX1–SIX6* locus with VCDR and POAG were independently confirmed by other GWA studies.[7,8]

The associated single nucleotide polymorphism (SNP) rs10483727 is located in an intergenic region between *SIX1* and *SIX6*, and the causal variants driving the observed association have remained unclear. Recently a common missense variant in *SIX6*, rs339122345 (Asn141His), which is in strong linkage disequilibrium (LD) with the originally identified rs10483727 (1000 Genomes data [available in the public domain at http://www.1000genomes.org]: $r^2 = 1.0$, D' =

1.0 in CEU; and $r^2 = 0.92$, D' = 0.96 in CHB and JPT), was discovered to alter the protein function of *SIX6*.[9] Furthermore, in a small study of 30 POAG patients, those homozygous for this *SIX6* risk variant (C, His141) had thinner retinal nerve fiber layer (RNFL) thickness ($P = 0.03$) compared to those homozygous for the nonrisk variant (A, Asn141).[9] It was therefore hypothesized that the *SIX6* risk variant disturbs the development of the neural retina, leading to loss of retinal ganglion cells and thus higher risk of glaucoma.[9]

The purpose of this study was to investigate the association between the *SIX6* missense variant rs33912345 and peripapillary RNFL thickness measured in a population setting in which the majority of individuals did not have glaucoma. Retinal nerve fiber layer loss may precede optic disc cupping and functional changes in the early stage of glaucoma. Therefore, studying the effect of *SIX6* variants on RNFL thickness may help in understanding the role of *SIX6* in glaucoma.

Methods

Study Population

The subjects for this study were enrolled from the Singapore Chinese Eye Study (SCES), a population-based cross-sectional study of eye diseases in Chinese adults residing in Singapore aged 40 years and older. The methodology of the SCES has been described in detail elsewhere.[10] The study adhered to the tenets of the Declaration of Helsinki, and ethics committee approval was obtained from the Institutional Review Board of the Singapore Eye Research Institute. Written informed consent was obtained from all participants.

Ocular Examination

All participants underwent a standardized and comprehensive ocular examination.[10,11] In brief, IOP was measured using the Goldmann applanation tonometer (GAT; Haag-Streit, Bern, Switzerland) before pupil dilation. One reading was taken from each eye. If the IOP reading was greater than 21 mm Hg, a repeat reading was taken, and the second reading was used for analysis. Central corneal thickness (CCT) was measured using an ultrasound pachymeter (Advent; Mentor O & O, Inc., Norwell, MA, USA); the mean of five measurements was used for analysis. Axial length was measured using noncontact partial coherence interferometry (IOL Master V3.01; Carl Zeiss Meditec AG, Jena, Germany); the mean of five measurements was used for analysis.

Static automated perimetry (Swedish Interactive Threshold Algorithm standard 24-2, Humphrey Field Analyzer II; Carl Zeiss Meditec, Dublin, CA, USA) was performed on one in five participants and in all glaucoma suspects (defined as below) before pupil dilation. A visual field was defined as reliable when fixation losses were less than 20% and false-positive and false-negative rates were less than 33%. Visual field test was repeated if the test result was unreliable. A glaucomatous visual field defect was defined as the presence of three or more significant ($P < 0.05$) nonedge contiguous points with at least one at the $P < 0.01$ level on the same side of the horizontal meridian in the pattern deviation plot, and classified as "outside normal limits" on the Glaucoma Hemifield Test, confirmed on two consecutive visual field examinations.

After pupil dilation using tropicamide 1% and phenylephrine hydrochloride 2.5%, the optic disc was evaluated using a +78-diopter (D) lens at 16 times magnification during slit-lamp fundoscopy (Haag-Streit model BQ-900), and VCDR was measured with an eyepiece measuring graticule.

Spectral-Domain OCT Imaging

In a substudy of SCES, conducted between June 2009 and June 2011, optic disc and RNFL imaging was performed using spectral-domain optical coherence tomography (SD-OCT) (Cirrus HD-OCT; Carl Zeiss Meditec) after pupil dilation. Optic nerve head and RNFL scan acquisitions were performed for each participant using an optic disc cube 200 × 200 scan protocol, which generates a cube of data in a 6- × 6-mm^2 grid with 200 × 200 axial measurements. Details of the Cirrus HD-OCT optic disc scan protocol have been described in detail elsewhere.[12] In brief, the subject's pupil was first centered and focused in an iris viewing camera on acquisition screen, and the lines scanning ophthalmoscope (LSO) with "auto focus" mode was then used to optimize the view of the retina. The "center" and "enhance" modes were used to optimize the Z-offset and scan polarization, respectively, for the OCT scan to maximize the OCT signal. The peripapillary measurement circle was centered on the optic disc before capturing the image. After each capture, motion artifact was checked with the LSO image with the OCT en face overlaid. Rescanning was performed if motion artifacts (indicated by discontinuity of blood vessels) or saccades through the peripapillary measurement circle (3.46-mm diameter around the optic nerve head [ONH]) were detected.

The ONH and RNFL algorithms native to Cirrus HD-OCT (software version 6.0.2) were used to measure disc area and peripapillary RNFL thicknesses (average and quadrants) automatically. Detailed descriptions of the ONH and RNFL algorithms have been previously published.[13] Study eyes with OCT scans showing retinal layer segmentation errors, signal strength of less than 6, or artifacts due to eye movements or blinking were further excluded from the analysis.

Definition of Glaucoma

A glaucoma suspect was defined as meeting any of the following criteria: (1) IOP > 21 mm Hg, (2) VCDR > 0.6 or VCDR asymmetry > 0.2, (3) signs consistent with pseudoexfoliation or pigment dispersion syndrome, (4) narrow angles (posterior trabecular meshwork visible for <180° during static gonioscopy), (5) peripheral anterior synechiae, (6) other findings consistent with secondary glaucoma, and (7) known history of glaucoma.

Glaucoma was defined according to the International Society of Geographical and Epidemiological Ophthalmology (ISGEO) criteria based on three categories.[14] In brief, category 1 cases were defined as optic disc abnormality (VCDR or VCDR asymmetry ≥ 97.5th percentile) with a corresponding glaucomatous visual field defect. Category 2 cases were defined as having a severely damaged optic disc (VCDR or VCDR asymmetry ≥ 99.5th percentile) in the absence of reliable visual field test results. Category 3 cases were defined for subjects who were blind (corrected visual acuity < 3/60), without visual field or optic disc data, and previous glaucoma surgery or IOP > 99.5th percentile.

Genotyping

Genotyping was performed using the HumanExome BeadChips (v1.0; Illumina, Inc., San Diego, CA, USA), which included the *SIX6* missense SNP rs33912345. Stringent quality control filters were used to remove poorly performing samples and SNP markers. Single nucleotide polymorphisms with a call rate < 99%, minor allele frequency (MAF) < 0.1%, or showing deviation from equilibrium ($P < 10^{-6}$) were removed. Routine quality control criteria on a per sample basis were carried out, and poorly performing samples were removed from further

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Table 1. Demographic Characteristics of the Study Population

	Total, $N = 1243$	Nonglaucoma, $N = 1222$	Glaucoma, $N = 21$	*P* Value*
Age, y	55.0 (7.4)	54.9 (7.3)	61.5 (8.3)	<0.001
Sex, female	614 (49.4)	608 (49.8)	6 (28.6)	0.054
SIX6 rs33912345				
Genotype AA	50 (4.0)	49 (4.0)	1 (4.8)	0.677
Genotype AC	399 (32.1)	394 (32.2)	5 (23.8)	
Genotype CC	794 (63.9)	779 (63.8)	15 (71.4)	

Data are presented as mean (standard deviation) for age and as number (%) for sex and genotype. *N*, number of subjects.
* Comparison between nonglaucoma and glaucoma subjects by unpaired *t*-tests for age, and χ^2 tests for sex and genotype.

analysis. The remaining samples were then subjected to biological relationship verification by using the principle of variability in allele sharing according to the degree of relationship. Identity-by-state information was derived using the PLINK software.[15] For those pairs of individuals who showed evidence of cryptic relatedness, we removed the sample with the lower call rate before performing principal component (PC) analysis. Principal component analysis was undertaken using EIGENSTRAT (available in the public domain at http://genepath.med.harvard.edu/~reich/EIGENSTRAT.htm) to account for spurious associations resulting from ancestral differences of individual SNPs.

Statistical Analysis

An allele-dosage regression model was conducted to determine the association between rs33912345 and peripapillary RNFL thickness as a quantitative trait. We assumed an additive genetic model where the dosage of the SNP is a variable varying from 0, 1, or 2, representing the number of copies of the risk allele (the C allele of rs33912345) carried. Primary analysis for RNFL was adjusted for age, sex, and the first three PCs. Further adjustment for potential confounders, including axial length, disc area, IOP, and CCT, was conducted.

Data from two eyes of the individuals were included in the analysis when data from both eyes were available. If one eye was excluded, data from the other eye were used. Generalized estimating equations with exchangeable correlation structures were applied to account for the correlation between pairs of eyes for each individual. *P* values < 0.05 were considered statistically significant. The data were analyzed with Stata 12.1 (StataCorp LP, College Station, TX, USA).

Results

Of the 1691 SCES subjects who completed Cirrus HD-OCT in at least one eye, 1252 consented to genetic studies, were successfully genotyped, and passed genotyping quality control filters. An additional 32 eyes were further excluded due to OCT signal strength of less than 6 or RNFL segmentation error, leaving a final sample size of 2129 eyes from 1243 subjects for analysis.

Demographic and clinical characteristics of the 1243 subjects included in this study are shown in Table 1. The mean age was 55.0 ± 7.4 years (range, 44–83 years); 614 subjects (49.4%) were females, and 21 subjects (26 eyes) had glaucoma. The mean global peripapillary RNFL thickness was 96.2 ± 9.9 μm (Table 2). As expected, the glaucoma subjects were older ($P < 0.001$), and glaucoma eyes had thinner global RNFL thickness than nonglaucoma eyes (77.0 ± 14.3 vs. 96.5 ± 9.6 μm, $P < 0.001$).

The genotype frequencies of *SIX6* rs33912345 among the 1243 SCES Singapore Chinese were 50 (4.0%) for genotype AA, 399 (32.1%) for AC, and 794 (63.9%) for CC (Table 1). The allele frequency of the C allele (His141) was 80% in the study subjects. There was strong LD between rs33912345 and the previously identified SNP rs10483727 ($r^2 = 0.98$ and D' = 0.99) in our Chinese study population. The exome chips included three other coding variants within the *SIX6* gene (rs45549246, rs114981075, and rs146737847). However, none of them had a MAF > 0.2% in our study population.

The RNFL thickness by rs33912345 genotypes and sectors is summarized in Table 3. In the multivariate regression model adjusted for age, sex, and the first three PCs, each rs33912345 risk allele C was significantly associated with a decrease of 1.37 μm in RNFL thickness ($P < 0.003$; model 1 in Table 4). The strength of the association became slightly greater after

Table 2. Clinical Characteristics of the Study Eyes

	Total Eyes, $n = 2129$	Nonglaucoma Eyes, $n = 2103$	Glaucoma Eyes, $n = 26$	*P* Value*
Intraocular pressure, mm Hg	14.3 (3.0)	14.3 (3.0)	16.7 (4.2)	<0.001
Central corneal thickness, μm	552.8 (34.0)	552.9 (34.0)	542.2 (33.2)	0.109
Vertical cup-to-disc ratio	0.40 (0.12)	0.39 (0.12)	0.67 (0.14)	<0.001
Disc area, mm^2	1.97 (0.38)	1.97 (0.38)	2.30 (0.50)	<0.001
Axial length, mm	24.0 (1.3)	24.0 (1.3)	24.1 (1.7)	0.594
Peripapillary RNFL thickness, μm				
Global	96.2 (9.9)	96.5 (9.6)	77.0 (14.3)	<0.001
Temporal sector	71.5 (12.3)	71.7 (12.2)	62.4 (16.2)	<0.001
Superior sector	120.9 (17.2)	121.3 (16.9)	92.0 (19.5)	<0.001
Nasal sector	68.1 (10.8)	68.2 (10.7)	64.6 (13.1)	0.094
Inferior sector	124.3 (17.3)	124.8 (16.8)	89.1 (19.6)	<0.001

Data are presented as mean (standard deviation) and are eye specific. *n*, number of eyes.
* Comparison between nonglaucoma and glaucoma eyes using logistic regression models with generalized estimating equations.

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Table 3. Peripapillary Retinal Nerve Fiber Thickness by *SIX6* rs33912345 Genotypes

	All Eyes			Nonglaucoma Eyes		
RNFL	**AA, *n* = 79**	**AC, *n* = 674**	**CC, *n* = 1376**	**AA, *n* = 79**	**AC, *n* = 667**	**CC, *n* = 1357**
Global	98.8 (11.1)	96.9 (9.7)	95.8 (10.0)	98.8 (11.1)	97.1 (9.4)	96.0 (9.6)
Temporal sector	71.1 (11.1)	71.8 (12.4)	71.4 (12.4)	71.1 (11.1)	71.9 (12.2)	71.6 (12.3)
Superior sector	128.2 (17.9)	121.8 (17.6)	120.0 (16.9)	128.2 (17.9)	122.1 (17.3)	120.4 (16.5)
Nasal sector	68.9 (11.3)	68.5 (10.9)	67.9 (10.7)	68.9 (11.3)	68.5 (10.8)	68.0 (10.7)
Inferior sector	126.9 (18.1)	125.6 (16.8)	123.6 (17.4)	126.9 (18.1)	126.0 (16.4)	124.0 (16.9)

Data are presented as mean (standard deviation). *n*, number of eyes.

adjusting for axial length (a decrease of 1.44 μm per copy of the risk allele, $P = 0.001$; model 2), and remained similar after further adjusting for disc area, IOP, and CCT (a decrease of 1.31 μm per copy of the risk allele, $P = 0.003$; model 3). These associations remained similar and statistically significant in nonglaucoma eyes. Each risk allele C was significantly associated with a decrease of 1.25 to 1.39 μm in RNFL thickness (all $P < 0.01$; Table 4).

In investigation of the four sectors, the association of RNFL thickness with rs33912345 was significant in the superior and inferior sectors. The direction of effect was similar in the nasal and temporal sectors but was not statistically significant. In the model adjusted for age, sex, first three PCs, and axial length (Table 5), each rs33912345 risk allele C was associated with a decrease of 2.83 μm ($P < 0.001$) and 2.24 μm ($P = 0.003$) in the superior and inferior RNFL thickness, respectively. Similar patterns were observed when only nonglaucoma eyes were included in the analysis, with the strongest association seen in the superior sector (a decrease of 2.76 μm per copy of the risk allele, $P < 0.001$), followed by the inferior sector (a decrease of 2.16 μm per copy of the risk allele, $P = 0.004$, Table 5). The results were similar when the models were further adjusted for disc area, IOP, and CCT (data not shown).

Discussion

Our study showed that the *SIX6* missense variant rs33912345 (C, His141) was significantly associated with reduced RNFL thickness in a population-based Singaporean Chinese dataset. Furthermore, this finding was the same in nonglaucomatous subjects. To our knowledge, this is the first study to report the association of *SIX6* missense variant and RNFL thickness in a population-based sample. Our findings are consistent with the results from a previous clinic-based study of 30 POAG patients that found that the global, superior, and inferior RNFL were reduced in those with the His141 risk variant.[9] We provided further evidence to support the hypothesis that *SIX6* risk variants affect retinal ganglion cells either in development, in adulthood, or in both, thus increasing POAG susceptibility.[9]

Table 4. Association Between *SIX6* rs33912345 and Peripapillary Retinal Nerve Fiber Thickness Measured by Cirrus HD-OCT

	All Eyes, *n* = 2129			Nonglaucoma Eyes, *n* = 2103		
	β*	**SE**	***P* Value**	***β****	**SE**	***P* Value**
Model 1†	−1.37	0.47	0.003	−1.34	0.46	0.003
Model 2‡	−1.44	0.45	0.001	−1.39	0.44	0.001
Model 3§	−1.31	0.45	0.003	−1.25	0.43	0.004

n, number of eyes; SE, standard error.

* *β*, changes in RNFL thickness (in μm) per copy of the rs33912345 C allele.

† Model 1: adjusted for age, sex, and principal components.

‡ Model 2: adjusted for age, sex, principal components, and axial length.

§ Model 3: adjusted for age, sex, principal components, axial length, disc area, central corneal thickness, and intraocular pressure.

The *SIX* gene family in humans is composed of six members (*SIX1*–*SIX6*). Members of this conserved gene family were originally identified through homology to the *Drosophila* sine oculis gene, which is required for normal eye development and visual system.[16,17] During embryonic development, *SIX6* is expressed in neural retina, optic nerve, and the pituitary.[16,18,19] In humans, interstitial deletion on chromosome 14q22.3-q23, which includes *SIX6*, causes bilateral anophthalmia and absence of the optic nerve.[20–22] This supports the importance of *SIX6* in eye development and ocular diseases. Missense *SIX6* variants, including rs33912345, have been shown to be functional, reducing eye size and optic nerve volume in a zebrafish model, which further supports the role of *SIX6* in glaucoma risk.[9,23]

Interestingly, the frequency of the C risk allele at *SIX6* rs33912345 varies greatly among ethnic groups. In our Singaporean Chinese population, the frequency of the C allele is 80%, which is close to the frequency of 70.8% in the Beijing Chinese and Japanese panel ($n = 120$) in the 1000 Genomes project,[24] but much higher than that in people of European ancestry (37% in a recent report [$n = 256$][9] and 40% in the 1000 Genomes CEU population [$n = 120$]).[24] In people of West African ancestry, the frequency of the C risk allele is 99% in both glaucoma cases and controls.[25] It has been hypothesized that increased risk for POAG may be based in part on the very high rs33912345 risk allele *SIX6* prevalence in West Africans.[9] However, persons of European ancestry generally have thinner RNFL thickness than Asians, Hispanics, and persons of African ancestry.[26,27] Therefore, the difference in the risk allele frequency of rs33912345 does not explain the observed ethnic disparity in the optic nerve structure and RNFL thickness. It is certainly possible, if not likely, that other genes contribute to observed population differences. Further studies are needed to elucidate the role of *SIX6* variants on the differential risk of POAG among ethnic groups.

In this study, we found that the association of RNFL thickness with rs33912345 is much stronger in the superior and inferior sectors compared to the nasal and temporal sectors. In healthy eyes, the RNFL is thickest in the inferior and superior sectors, where ganglion cells are known to be more susceptible to glaucomatous damage. Our results suggest that the superior and inferior RNFL thickness may have stronger genetic determinants, at least for *SIX6*. Although the association was not significant in the nasal and temporal sectors, the direction of effect was consistent with that observed in the superior and inferior sectors. This region-specific effect of *SIX6* variant rs33912345 on RNFL thinning is of particular interest clinically. Loss of superior and inferior RNFL is what would

Table 5. Association Between *SIX6* rs33912345 and Peripapillary Retinal Nerve Fiber Thickness by Sectors

	All Eyes, $n = 2129$			Nonglaucoma Eyes, $n = 2103$		
RNFL Sector	**β***	**SE**	***P* Value**	**β***	**SE**	***P* Value**
Temporal RNFL	−0.18	0.56	0.746	−0.18	0.56	0.746
Superior RNFL	−2.83	0.75	<0.001	−2.76	0.73	<0.001
Nasal RNFL	−0.52	0.49	0.287	−0.49	0.49	0.317
Inferior RNFL	−2.24	0.77	0.003	−2.16	0.75	0.004

SE, standard error; *n*, number of eyes.

* β, changes in RNFL thickness (in μm) per copy of the rs33912345 C allele, adjusted for age, sex, principal components, and axial length.

naturally lead to increased CVDR, a hallmark of glaucomatous optic neuropathy, and coincides with progression of visual field loss to central and temporal visual islands, a classic manifestation of end-stage glaucoma. It is particularly interesting to see this pattern occurring in a population-wide manner for nonglaucomatous subjects.

The strengths of our study include a large sample size with standardized clinical examination and detailed scrutiny of OCT scan quality. Furthermore, common risk factors for glaucoma such as IOP, CCT, axial length, and disc area were systematically measured and included as covariates in our analysis. This further substantiates the validity of our findings. However, our study was limited by its cross-sectional study design. Longitudinal studies are needed to assess the impact of *SIX6* risk variants on the rate of ganglion cell and axon loss. Additionally, since our subjects were older than 40 years, it is not possible to determine the age at which RNFL changes occur.

In summary, we found that the common *SIX6* missense variant rs33912345 is associated with reduced regional and global RNFL thickness in the Singapore Chinese population without glaucoma. The effect of *SIX6* variants on glaucoma susceptibility may be mediated through their effect on the RNFL thickness and ganglion cells.

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