Variations in *NTF4, VAV2*, and *VAV3* Genes Are Not Involved with Primary Open-Angle and Primary Angle-Closure Glaucomas in an Indian Population

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Purpose. Recently, the neurotrophin-4 (*NTF4*), *VAV2* and *VAV3* genes have been implicated in primary open-angle glaucoma (POAG) in the European and Japanese populations, respectively. This study was conducted to determine their involvement in an Indian population with POAG and primary angle-closure glaucoma (PACG).

Methods. The entire *NTF4* gene and the POAG-associated SNPs rs2156323 (*VAV2*) and rs2801219 (*VAV3*) and their flanking regions were screened by resequencing in a clinically well-characterized cohort of 537 subjects that included cases of POAG (n = 141), PACG (n = 111), and ethnically matched normal controls (n = 285). The data were analyzed by using appropriate statistical software.

Results. Resequencing of *NTF4* revealed a nonsynonymous (A88V), silent (P151P) and two changes in the 3′UTR region, along with a known polymorphism (rs11669977) in cases of POAG; the PACG cases exhibited only the A88V variation. Of interest, the A88V mutation observed in Europeans was more prevalent in our normal control subjects (4.91%, 95% CI, 2.95–8.07) than in the POAG (2.14%, 95% CI, 0.73–6.11; P = 0.200) and PACG (2.85%, 95% CI, 0.97–8.06; P = 0.577) cases. There were no major differences in the presenting intraocular pressure, cup-to-disc ratio, and visual field defects among patients harboring the A88V variation. The other variations in *NTF4* were not associated with the cases. The risk alleles of rs2156323 and rs2801219 in the Japanese were not associated with POAG (P = 0.533 and 0.133, respectively) and PACG (P = 0.223 and 0.394, respectively) in the Indian cohort.

Conclusions. The present data indicate a lack of involvement of variations in *NTF4*, *VAV2*, and *VAV3* with glaucoma pathogenesis in an Indian population. (*Invest Ophthalmol Vis Sci.* 2010; 51:4937–4941) DOI:10.1167/iovs.10-5553

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The glaucomas comprise a group of clinically and genetically heterogeneous optic neuropathies characterized by a gradual and progressive loss of vision.[1] Globally, glaucoma is the second leading cause of irreversible blindness,[2] and it is estimated that it will affect ~80 million people by the year 2020.[3] Primary open-angle glaucoma (POAG; OMIM 137760; Online Mendelian Inheritance in Man/ http://www.ncbi.nlm.nih.gov/Omim/ provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD), presents a complex etiology characterized by elevated intraocular pressure (IOP), optic nerve head changes, degeneration of retinal ganglion cells, and visual field loss[1,4] and is attributed to multiple genes with various magnitudes of effect.[5] Among the several loci mapped in POAG, only three genes, *MYOC* (OMIM 601652),[6] *OPTN* (OMIM 602432),[7] and *WDR36* (OMIM 609669),[8] have been characterized that exhibit high levels of allelic heterogeneity with a diverse mutation spectrum worldwide.

Recently, it was demonstrated that heterozygous mutations in a novel gene neurotrophin-4 (*NTF4* [OMIM 162662]) that harbored the *GLC1O* (19q13.3) locus (OMIM 613100), was involved in POAG.[9] Using three large case–control cohorts of POAG patients (n = 892) and control subjects (n = 895) from the European population comprising a discovery and two replication cohorts, the authors identified 15 mutations accounting for an overall frequency of 1.68% (95% CI, 1.02–2.76) of POAG. The patients harboring mutations in the replication groups had normal-tension glaucoma (NTG), whereas there was a mixture of intraocular pressure (IOP)–related juvenile open-angle glaucoma (JOAG) and POAG cases in the discovery group. Despite the phenotypic variability, the frequency of *NTF4* mutations in the discovery group (2.25%; 95% CI, 1.19–4.23) and the replication groups I (1.42%; 95% CI, 0.55–3.58) and II (0.95%; 95% CI, 0.26–3.38) were not significantly different. Two mutations (A88V and R206W) were prevalent across these three cohorts.[9] However, Liu et al.[10] tried to replicate the involvement of *NTF4* in a Caucasian population from the Southeastern United States and observed a total lack of involvement of this gene in POAG.

In a parallel study, the *VAV2* (OMIM 600428) and *VAV3* (OMIM 605541) genes were implicated in POAG in the Japanese population.[11] The authors provided functional evidence suggesting that *Vav2* ($Vav2^{-/-}$)- and *Vav2/Vav3* ($Vav2^{-/-}$ $Vav3^{-/-}$)-deficient mice exhibit a spontaneous glaucoma phenotype resulting in progressive iridocorneal changes and elevated IOP. Based on a genome-wide association study (GWAS), it was further demonstrated that intronic SNPs in *VAV2* (rs2156323) and *VAV3* (rs2801219) are significantly associated with susceptibility to POAG in the Japanese.[11]

The mutation spectrum of *NTF4* and the significantly strong association of SNPs in *VAV2* and *VAV3*, prompted us to understand their involvement in an ethnically different population

TABLE 1. Details of Primers Used for Screening Regions Harboring the POAG-Associated SNPs in the *VAV2* and *VAV3* Genes

Primer	Primer Sequence (5′–3′)	Strand	Annealing Temperature	Product Size (in bp)
VAV2F	TGTGACGGCTGCAAGGCAGG	Sense		
VAV2R	TTCCAGTGCTCTCCCGGCCA	Antisense	58°C	401
VAV3F	AAGCAATAGCAGGGCAGATG	Sense		
VAV3R	GAGAACAGGCATGCTCCAAT	Antisense	58°C	434

(Indian) with POAG. For the sake of comparison, we also screened another cohort of primary glaucoma, namely, primary angle-closure glaucoma (PACG; $n = 111$).

METHODS

Clinical Diagnosis of the Subjects

The study protocol was approved by the Institutional Review Board and adhered to the guidelines of the Declaration of Helsinki. We enrolled a clinically well-characterized cohort of 537 subjects that included cases of POAG ($n = 141$), PACG ($n = 111$), and ethnically matched normal control subjects ($n = 285$). All the subjects presented at the L. V. Prasad Eye Institute, Hyderabad, India, between January 2002 and March 2008. Clinical diagnoses of all our consecutive POAG and PACG cases and normal control subjects were independently made by at least two glaucoma specialists, after application of stringent inclusion and exclusion criteria published elsewhere.[12] There was good interobserver agreement (kappa statistics, $\kappa = 0.92$) between the clinicians. All the cases and controls were matched with respect to age and ethnicity and were recruited from the same geographic regions in Southern India.

Screening of the *NTF4* Gene

Screening of *NTF4* was accomplished by bi-directional sequencing on an automated DNA sequencer (ABI 3100, Applied Biosystems, Inc. [ABI], Foster City, CA). The entire gene was PCR amplified with the primers cited by the authors in the previous study.[9] A 25-μL PCR reaction was set up with 100 ng of genomic DNA, 2.5 μL 10× of PCR buffer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 5 picomoles of each primer, and 1 unit of *Taq* polymerase (Bangalore Genei, Bangalore, India) in a 96-well thermal cycler (Veriti; ABI) at an annealing temperature of 58°C. The amplicons were purified with PCR cleanup columns (Nucleospin; Macherey-Nagel, Düren, Germany) and sequenced with dye termination chemistry, according to the manufacturer's guidelines (BigDye; ABI). The trace files were analyzed with the help of sequencing analysis software (ABI).

Validation of an *NTF4* Variant

All the subjects exhibiting the A88V change variation along with a subset of subjects without this variation were further confirmed by PCR-based restriction digestion of the amplicons with *Bse*NI enzyme (MBI Fermentas, Hanover, MD) at 65°C for 14 hours. The digested amplicons were electrophoresed on 10% nondenaturing polyacrylamide gels and the band patterns, and sizes of the corresponding fragments were visualized with the help of a 100-bp DNA ladder (MBI Fermentas). The genotypes were directly scored from the gels and correlated with the sequencing data.

Screening of *VAV2* and *VAV3* Genes

The region harboring the associated SNPs rs2156323 in *VAV2* and rs2801219 in *VAV3* were screened by resequencing with predesigned primers (Table 1). To screen an extended region harboring the associated SNPs, we designed primers to screen two flanking SNPs in *VAV2* (rs3819500 and rs2073930) and five flanking SNPs in *VAV3* (rs6689477, rs6689476, rs6697852, rs6686831, and rs59614404). The sequencing protocol was as described for *NTF4*. Subsets of these sequencing experiments were repeated independently by a second investigator for further confirmation of the genotypes.

Statistical Analysis

The variations observed in *NTF4* were compared across the POAG and PACG cases with normal control subjects using χ^2 tests. In the *VAV2* and *VAV3* SNPs, the maximum-likelihood estimates of allele frequencies, Hardy-Weinberg equilibrium, and haplotype frequencies were estimated from the genotype data at these loci by using the Haploview software (version 4.1) that uses the EM algorithm.[13] Pairwise linkage disequilibrium (LD) between the individual SNPs was calculated with the LD-plot function of this software. The χ^2 analysis and Fisher's exact test was used to assess significance, and odds ratios were calculated for the alleles and genotypes.

RESULTS

Screening of the *NTF4* Gene

Resequencing of the entire *NTF4* gene did not reveal any disease-associated mutations in the Indian cohort (Table 2) in POAG and PACG. We observed five variations that also included the most prevalent variant (A88V) in the previous study,[9] a silent change (P151P), two changes in the 3′UTR region, and a known polymorphism (rs11669977) in cases of POAG; the PACG cases exhibited only the A88V variation. Of interest, the heterozygous A88V variant was more prevalent in the normal control subjects (4.91%) than in the POAG (2.14%;

TABLE 2. Distribution of Heterozygous Variations in *NTF4* Observed in POAG and PACG Cases and Controls in the Indian Cohort

Nucleotide Change	Location in the Gene	Amino Acid Change	Number of Variations Observed*		
			POAG *n* (%)	PACG *n* (%)	Controls *n* (%)
c.263C>T	Exon 2	A88V	3/140 (2.14)	3/105 (2.85)	14/285 (4.91)
c.453G>A	Exon 2	P151P	1/141 (0.71)	0/111	0/285
c.790T>G	3′UTR	—	1/141 (0.71)	0/111	1/227 (0.44)
c.811G>A	3′UTR	—	1/141 (0.71)	0/111	0/285

* Actual number of individuals genotyped for a particular variation.

TABLE 3. The Clinical Phenotype of Subjects with the A88V Variation at Presentation in the Indian Cohort

Subject ID	Phenotype	Age at Onset/ Examination (y)	Sex	IOP at Presentation (RE, LE)	Cup-to-Disc Ratio at Presentation (RE, LE)	Visual Field Defects at Presentation* (RE, LE)
PO038	POAG	37	M	30, 22	0.7, 0.5	Severe, moderate
PO083	POAG	40	M	34, 40	0.9, 0.9	Severe, severe
PO113	POAG	62	F	28, 22	0.9, 0.9	Severe, severe
PA083	PACG	50	F	40, 24	0.9, 0.5	Severe, severe
PA139	PACG	65	M	22, 24	0.8, 0.7	Severe, severe
PA143	PACG	52	M	22, 44	0.6, 0.9	Early, severe
CO025	Control	66	F	17, 12	0.3, 0.3	—
CO030	Control	70	F	14, 16	0.3, 0.3	—
CO045	Control	68	M	14, 14	0.3, 0.3	—
CO124	Control	65	F	16, 16	0.2, 0.2	—
AO042	Control	68	M	14, 14	0.3, 0.3	—
AO049	Control	65	F	12, 12	0.3, 0.3	—
AO059	Control	67	F	12, 14	0.2, 0.1	—
AO102	Control	65	F	15, 15	0.2, 0.2	—
AC002	Control	68	M	12, 13	0.3, 0.3	—
AC006	Control	66	M	16, 16	0.3, 0.3	—
AC011	Control	65	F	16, 12	0.3, 0.3	—
AC035	Control	65	M	17, 16	0.3, 0.2	—
AC036	Control	69	F	17, 15	0.3, 0.1	—
AC051	Control	65	M	10, 12	0.2, 0.2	—

RE, right eye; LE, left eye.

* The visual field defects were characterized as early, moderate, and advanced glaucomatous loss based on the criteria of Hodapp et al.[14]

P_{exact} = 0.200) and PACG (2.85%; P_{exact} = 0.577) cases. The other variations were rarely seen in the controls (Table 2). The rs11669977 SNP was not associated with either POAG or PACG (P = 0.174 and 0.451 for the minor allele, respectively) similar to the previous study.[9] All the genotype data were independently validated by a second investigator who was masked to the phenotype of the subjects.

We analyzed for clinical differences at presentation with respect to IOP, cup-to-disc ratio, and visual field defects[14] among the POAG and PACG cases harboring the A88V variation (Table 3). As is evident in the table, there were no significant differences in the clinical profile between the POAG and PACG cases bearing this variation.

Screening of *VAV2* and *VAV3* Genes

Similar to *NTF4*, screening of the *VAV2* and *VAV3* SNPs did not indicate any association to POAG and PACG. There was no departure from Hardy-Weinberg equilibrium for any of these SNPs among the normal control subjects ($P > 0.05$). The risk alleles rs2156323 (*VAV2*) and rs2801219 (*VAV3*) in the previous study,[11] were not associated with POAG (P = 0.533 and P = 0.133) and PACG (P = 0.223 and P = 0.394), respectively (Table 4).

Similarly, the genotype frequencies at these loci were not significantly different among the POAG, PACG, and controls subjects (data not shown). The heterozygote and homozygote odds ratios (OR) for the minor alleles of rs2156323 (OR_{Het} = 0.84; 95% CI, 0.45–1.56 and OR_{Hom} = 0.48; 95% CI, 0.08–2.94) and rs2801219 (OR_{Het} = 1.18; 95% CI, 0.68–2.05 and OR_{Hom} = 1.54, 95% CI, 0.56–4.26) were lower than in the Japanese cohort.[11] Although there was a tight LD between the three SNPs of *VAV2* and six SNPs of *VAV3* (both D' = 1.0), the haplotypes generated with these SNPs (frequency >5%) did not exhibit any association with POAG and PACG (Table 5).

DISCUSSION

POAG is a complex disease that is attributed to the interplay of several genetic and nongenetic variables.[4] Globally, several chromosomal loci have been mapped in POAG, but the frequency and spectrum of mutations in the characterized genes do not explain the genetic contribution in a large proportion of cases.[1,5] The associations of certain candidate gene variants that were screened across multiple populations could not explain their potential involvement in POAG.[15] The involvement

TABLE 4. Distribution of Allele Frequencies of Different SNPs at the *VAV2* and *VAV3* Loci in POAG and PACG Cases and Normal Controls in the Indian Cohort

Gene	SNPs	Associated Allele	Freq. POAG Cases	Freq. Controls	χ^2	*P* (POAG vs. Controls)	Freq. PACG Cases	Freq. Controls	χ^2	*P* (PACG vs. Controls)
VAV2	rs2073930	C	0.885	0.851	1.172	0.279	0.884	0.851	0.833	0.361
	rs3819500	G	0.866	0.847	0.369	0.543	0.878	0.847	0.75	0.387
	rs2156323	G	0.885	0.886	0.389	0.533	0.907	0.866	1.486	0.223
VAV3	rs59614404	G	0.039	0.015	2.254	0.133	0.028	0.015	0.726	0.394
	rs2801219	G	0.039	0.015	2.254	0.133	0.028	0.015	0.726	0.394
	rs6686831	G	0.039	0.015	2.254	0.133	0.028	0.015	0.726	0.394
	rs6697852	C	0.039	0.015	2.254	0.133	0.028	0.015	0.726	0.394
	rs6689476	G	0.351	0.313	0.702	0.402	0.397	0.313	2.825	0.092
	rs6689477	A	0.039	0.015	2.254	0.133	0.028	0.015	0.726	0.394

TABLE 5. Distribution of Haplotype Frequencies at the *VAV2* and *VAV3* Loci in POAG and PACG Cases and Normal Controls in the Indian Cohort

Gene	Haplotypes	Freq. POAG Cases	Freq. Controls	*P* (POAG vs. Controls)	Freq. PACG Cases	Freq. Controls	*P* (PACG vs. Controls)
VAV2	C-C-G	0.866	0.836	0.365	0.854	0.836	0.643
	T-A-T	0.115	0.134	0.533	0.091	0.134	0.206
VAV3	A-A-C-T-T-C	0.651	0.687	0.416	0.605	0.687	0.098
	A-A-C-T-G-C	0.311	0.298	0.771	0.367	0.298	0.155

of novel candidate gene(s) and gene variant(s) are biologically more meaningful when they are replicated in different populations, thereby elucidating their potential role in the disease pathogenesis.[16] It is in this context that we attempted to validate the involvement of *NTF4*, *VAV2*, and *VAV3* in an ethnically different population.

We were unable to detect any disease-associated mutation in *NTF4* in our POAG and PACG patients (Table 2). Similar findings have been observed in a Caucasian POAG cohort from the Southeastern United States that exhibited nine nonsynonymous variations in *NTF4,* including two common variants observed in the European cohort (A88V and R206W); but none of these was significantly associated with POAG.[10]

Of interest, the A88V was the common variation observed across the Indian, European,[9] and American[10] cohorts. It is intriguing to note that the A88V was one of the most prevalent (5/15) mutations (33.33%; 95% CI, 15.18–58.29) among the European POAG patients in their discovery and replication groups but not in the normal control subjects.[9] Four of their five patients harboring the heterozygous A88V change had NTG. In contrast, the A88V was observed among the Indian POAG and PACG patients with raised IOP and also among the control subjects who had no signs and symptoms of glaucoma or any family history of the disease at presentation. All the normal control subjects presented with IOP <21 mm Hg, cup-to-disc ratio of <0.4:1, and no changes in the optic nerve head and visual fields suggestive of glaucoma in either eye (Table 3). Similar to the Indian cohort, most of the control subjects in the American cohort exhibited the A88V variation (Table 6). It was also noted that the mean ages at the time of diagnosis among the control subjects in the Indian (66.6 ± 1.7 years) and American (67 years) cohorts were similar.

The discordant results could be due to the clinical heterogeneity between the Indian and European[9] cohorts, as none of our cases had NTG. This discrepancy also was not attributable to the screening techniques, since our primers and sequencing methods were identical with those in the previous study.[9] We also observed a higher frequency of the V88 allele (~5%) in the general population, which could be due to a different genetic profile between the Indian and European populations. It may be worthwhile to mention that the overall frequency of the V88 allele was also higher in the Indian subjects than in the American subjects (Table 6). It has also been demonstrated through molecular modeling that the A88V variation may affect the residues of the TrkB binding site,[9] but the observation of this variant in a large number of control subjects both in the Indian and American[10] cohorts does not suggest that it has a major functional role in vivo.

In regard to *VAV2* and *VAV3*, the risk alleles in the Japanese POAG patients[11] did not exhibit any association in the Indian cohort (Table 4). Lack of association at these loci in our POAG cases could be due to clinical heterogeneity (all the Indian POAG cases had raised IOP) or because of a different genetic profile in these two populations. The relative risks for the heterozygote genotypes of rs2156323 (0.92; 95% CI, 0.70–1.22) and rs2801219 (1.07; 95% CI, 0.84–1.39) were also lower in the Indian cohort compared with the Japanese.[11] Now that we have performed a customized genotyping with respect to the POAG-associated alleles within a defined region, an extensive screening of the entirety of both genes may be warranted, to generate a complete picture. A posthoc power calculation indicated that our POAG cohort had an 82% power to detect an association ($P = 0.05$) for the risk alleles of these SNPs under a one-stage design. Functionally deficient *Vav2* and *Vav3* mice exhibit buphthalmos, along with iridocorneal changes, elevated IOP, and optic nerve head cupping that may mimic developmental glaucomas. Thus, it may be speculated that *VAV2* and *VAV3* is major candidate genes for primary congenital glaucoma in humans.

In summary, variations in the *NTF4* gene were not associated with primary glaucomas in an Indian population; a similar finding from an American cohort further points to the lack of involvement of *NTF4* in POAG. We could not replicate the association of risk alleles in *VAV2* and *VAV3* in our cohort. Based on our nonreplication of an earlier GWAS data in the Japanese population with POAG,[17] it may seem that the risk alleles in the Japanese are not shared among the Indian patients for reasons described earlier. Although it would be interesting to see the involvement of these genes

TABLE 6. Distribution of the Frequencies of A88V Variation in POAG Cases and Normal Controls in the Different Populations

Population	Frequency of A88V in POAG Cases	Frequency of A88V in Normal Controls	*P**
European[9]	5/892 (0.56%, 95% CI, 0.24–1.30)	0/895 0 (95% CI, 0–0.43)	—
American[10]	1/443 (0.22%, 95% CI, 0.04–1.27)	5/533 (0.94%, 95% CI, 0.40–2.18)	0.097
Indian (present Study)	3/140 (2.14%, 95% CI, 0.73–6.11)	14/285 (4.91%, 95% CI, 2.95–8.07)	0.200

Data are expressed as the number affected (percentage, 95% CI).

* Fisher's exact test.

across different populations worldwide, but unlike the universal involvements of *MYOC*,[18] *OPTN*,[19] *WDR36*,[20] and *CYP1B1*[21] in POAG, variations in *NTF4*, *VAV2*, and *VAV3* genes do not seem to be major risk factors in the pathogenesis of glaucoma.

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