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OPTN gene: Profile of patients with glaucoma from India

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Purpose: Optineurin gene (*OPTN*) mutations are reported in primary open angle glaucoma patients (POAG) from different populations. The coding and noncoding regions of *OPTN* were screened for mutations in 100 Indian high tension glaucoma patients (HTG). The frequency of the *OPTN* M98K mutation in an additional 120 patients (70 HTG and 50 normal tension glaucoma [NTG]) was analyzed by restriction enzyme digestion.

Methods: The HTG patients (about 40 years of age) were characterized by open angles on gonioscopy, with raised intraocular pressure (IOP) more than 21 mmHg (<21 mmHg on office diurnal phasing for NTG), and typical glaucomatous disc changes with corresponding visual field defects in the absence of any secondary cause. One hundred HTG patients and controls were screened for *OPTN* mutations by direct sequencing using an ABI prism 310/3100 Avant genetic analyzer. The M98K status was analyzed by restriction enzyme digestion with *Stu*I. A genotype/phenotype correlation was also attempted for *OPTN* sequence alterations with clinical parameters such as age at diagnosis, intraocular pressure, cup:disc ratio, etc. The putative change in the transcription factor binding site for the IVS7 +24G>A polymorphism was attempted with AliBaba software (version 2.1).

Results: Six sequence alterations were observed in the 100 POAG patients by direct sequencing. The M98K substitution was observed in a total of 10 patients (7/170 HTG and 3/50 NTG) contributing to 4.1% in HTG and 6% in the NTG group and not in the controls. The IVS7+24G>A nucleotide change showed a significant difference in the HTG group (7/100) when compared to the control group (0/100) and found to be associated with increased IOP at diagnosis (p=0.03). The IVS7+24G>A polymorphism resulted in the creation of binding sites for transcription factors NF-1 and CPE that were not present in the wild type.

Conclusions: The current study suggests a possible role of SNPs rather than mutations in *OPTN* in POAG pathology in the Indian population.

Primary open angle glaucoma (POAG) is genetically a heterogeneous disease, where 20 chromosomal loci have been mapped to date, out of which three genes (*MYOC*, *OPTN*, and *WDR35*) have been well studied. The optic neuropathy-inducing gene (*OPTN*; OMIM 602432) located on the chromosomal region 10p15-14 (GLC1E) is the second characterized loci for POAG. Initial analysis by Rezaie et al. [1] has shown that *OPTN* mutations are critically responsible for 16.7% of normal tension glaucoma (NTG) patients with intraocular pressure (IOP) <22 mmHg. However, subsequent reports from different populations have shown that *OPTN* mutations are rare causes of glaucoma. The *OPTN* gene has 16 exons of which the first three are noncoding, but the remaining 13 code for an about 66 kda molecular weight protein with 577 amino acids. The gene has four transcript variants that differ in the 5'-UTR and is expressed in nonocular tissues such as the heart, brain, placenta, liver, skeletal muscle, kidney, and pancreas. In the eye it is expressed in the trabecular meshwork, nonpigmented ciliary epithelium, retina, Schlemm's canal, and the aqueous humor [2]. The OPTN protein interacts with different proteins that are involved in apoptosis, inflammation, and vasoconstriction. A hypothetical model for the OPTN protein interaction suggests that it functions through the TNFα and Fas ligand pathway [2]. The OPTN protein was proposed to render a neuroprotective role in optic neuropathies through downregulating TNFα production in the signaling pathway [2]. In a study by Funayama et al. [3], genetic statistical analysis of the TNF promoter polymorphisms and *OPTN* variants in Japanese POAG patients have shown the possible interaction between single nucleotide polymorphisms (SNPs) and the gene increases the risk for development and progression of glaucoma. Predominant nucleotide variations observed in the *OPTN* gene are five mutations and about 12 polymorphisms in different populations. However, distribution of these nucleotide changes between the patients and controls show distinctive ethnic differences. The R545Q mutation, reported initially as pathogenic by Rezaie et al. [1] is equally distributed between POAG patients and controls in the Chinese population [4]. In the Chinese population [4], 1.6% of sporadic POAG cases show *OPTN* mutations, while in the Japanese population [5] the same clinical association has not been shown. Another cohort study from the Japanese population has shown positive correlation with *OPTN* sequence changes and NTG [6]. Alward et al. [7] screened a total of 1,048 POAG patients (Japanese and Caucasians) and showed that the R545Q amino

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acid change initially shown to be disease causing is a polymorphism. *OPTN* mutations were also studied in juvenile open angle glaucoma (JOAG) patients. In a cohort of 66 JOAG patients, Willoughby et al. [8] have demonstrated that *OPTN* mutations H486R and SNP in exon 4 (L42L) cause JOAG. Thus there exists a vast difference in observations on the potential role of *OPTN* in the pathology of glaucoma. The literature has only one report on *OPTN* involvement in POAG patients from India [9]. Hence, more screening studies of the *OPTN* gene for mutations in the Indian population is necessary before deciding on the possibility of *OPTN* changes as a diagnostic marker for glaucoma.

METHODS

Clinical material: Patients diagnosed with POAG (high tension glaucoma [HTG] and NTG) were recruited from the glaucoma clinic of Sankara Nethralaya and from the Chennai Glaucoma Study [10], a population-based prevalence study. The project had ethics approval, and informed consent was given by the controls and patients for both the clinical and genetic examination.

Inclusion criteria: All subjects >40 years of age were enrolled and categorized as patients and controls after a detailed clinical examination. The comprehensive ophthalmic examination included measurement of best corrected visual acuity, slit lamp biomicroscopy, applanation tonometry, gonioscopy, pachymetry, dilated fundus examination that included stereobiomicroscopic evaluation of the optic disc and macula with a 78 D lens, and examination of the retina using the indirect ophthalmoscope, Humphrey visual fields, and optic disc documentation of patients >40 years of age were included in the study. Standardized inclusion criteria for NTG were used, which was the presence of glaucomatous optic neuropathy (defined as loss of neuroretinal rim with a cup:disc ratio of 0.6 or greater) with compatible visual field loss and open angles on gonioscopy, and a mean IOP without treatment that was consistently <21 mmHg on diurnal testing. The control samples were age matched and selected after complete ophthalmic examination similar to that of the glaucoma cases.

Laboratory methods: Genomic DNA was extracted from 5 ml of heparinized blood samples of 220 patients (170 POAG and 50 NTG) and 100 controls by the standard phenol chloroform and ethanol precipitation method [11]. The intron exon boundaries of the coding region of *OPTN* were amplified by 13 sets of primers according to Leung et al. [4]. In order to screen for mutations, the amplified products from 100 POAG subjects were electrophoresed and directly sequenced using an ABI Prism 3100 Avant genetic analyzer. PCR-based restriction endonuclease digestion (RED) for the rapid detection of M98K change was performed in an additional 70 HTG patients and 50 NTG and 100 unrelated healthy controls with the enzyme *Stu*I as the nucleotide change results in creation of the restriction site. Restriction enzyme (5 units; as per table) was incubated with 5 µl of the respective PCR product at 37 °C for 16 h and the digested material was analyzed using agarose gel electrophoresis.

RNA secondary structure modeling for M98K: Bioinformatic analysis was performed for the possible structural change in the M98K mRNA by using the MFOLD Web browser, which predicts the energetically most stable RNA secondary structures. MFOLD is a dynamic programming al-

Table 1.

Location	Sequence change	Codon change	Genotype frequency POAG	Genotype frequency Control
Substitution				
Exon 5	c.603T>A	M98K	0/ 3/97	0/ 0/100
Synonymous change				
Exon 4	c.412G>A	T34T	6/11/83	8/15/ 77
Exon 7	c.907T>C	P199P	0/ 1/99	0/ 0/100
Noncoding change				
Intron 6	IVS6-5T>C	-	0/ 9/98	0/12/ 88
Intron 7	IVS7+24G>A	-	1/ 6/93	0/ 0/100*
Intron 15	IVS15+10G>A	-	0/ 1/99	0/ 0/100

OPTN sequence alterations in Indian primary open angle glaucoma (POAG) patients. The table shows the distribution of *OPTN* nucleotide changes in the *OPTN* gene in 100 POAG patients and controls. "Genotype frequency" is represented as mutant homozygotes/heterozygotes/wildtype homozygotes. The asterisk indicates a p=0.013 by Fisher's exact test.

Table 2.

Diagnosis	c.603G>A	Frequency	p value
POAG	G/A	4.1% (7/170)	0.049*
NTG	G/A	6.0% (3/ 50)	0.036*
Controls	G/G	0.0% (0/100)	

Comparison of the M98K distribution in primary open angle glaucoma (POAG) and normal tension glaucoma (NTG) compared to controls (Fisher's exact test). The asterisk indicates a p value <0.05.

Table 3.

Phenotype variable	IVS +24G>A polymorphism IVS7+24G>A (n=6)	IVS +24G>A polymorphism Wild-type G/G (n=69)	Mann-Whitney test (p value)
Age at diagnosis (years)	55.333±21.26	55.294 ±3.05	0.49
IOP at diagnosis (mm Hg)	27.0 ± 4.05	21.779 ±1.55	0.027*
Cup:disc ratio	0.715± 0.18	0.4937±0.0718	0.65

Genotype/phenotype correlations for IVS7+G>A polymorphism. Comparison of the clinical parameters cup:disc ratio, intraocular pressure (IOP) at diagnosis, and age at diagnosis between primary open angle glaucoma (POAG) patients with and without the IVS7+G>A polymorphism by the Mann-Whitney test. The asterisk indicates a significant comparison (p<0.05).

gorithm incorporating a nearest neighbor, thermodynamic, minimum free-energy method, and RNA sequence co-variation analysis. The wild-type and mutant mRNA secondary structures were modeled and compared.

Evaluation of putative transcription factor binding sites: The putative change in the transcription factor binding sites was predicted by AliBaba software for nucleotide alterations in the noncoding regions that were significantly higher in frequency in the patient group when compared to the controls.

Statistical analysis: Fisher's test was used to compare the allele frequencies for the detected nucleotide change between the patients and control groups. A p value of <0.05 was considered significant. The distribution of M98K between HTG, NTG, and controls were compared by the Fisher's exact test. The correlation between the clinical phenotypes and the nucleotide variation was analyzed by the Mann-Whitney test.

RESULTS

Direct mutation screening of *OPTN* in 100 POAG patients and controls showed six nucleotide changes both in the coding and noncoding region of the gene (Table 1).

M98K substitution: Out of 220 patients and 100 controls, heterozygous M98K was found in 4.1% of POAG and 6% of NTG patients and not in the controls (Table 2).

Distribution of the IVS 7+24G>A polymorphism: Seven of the total 100 HTG patients were positive for the IVS 7+24G>A polymorphism and none of the control subjects were

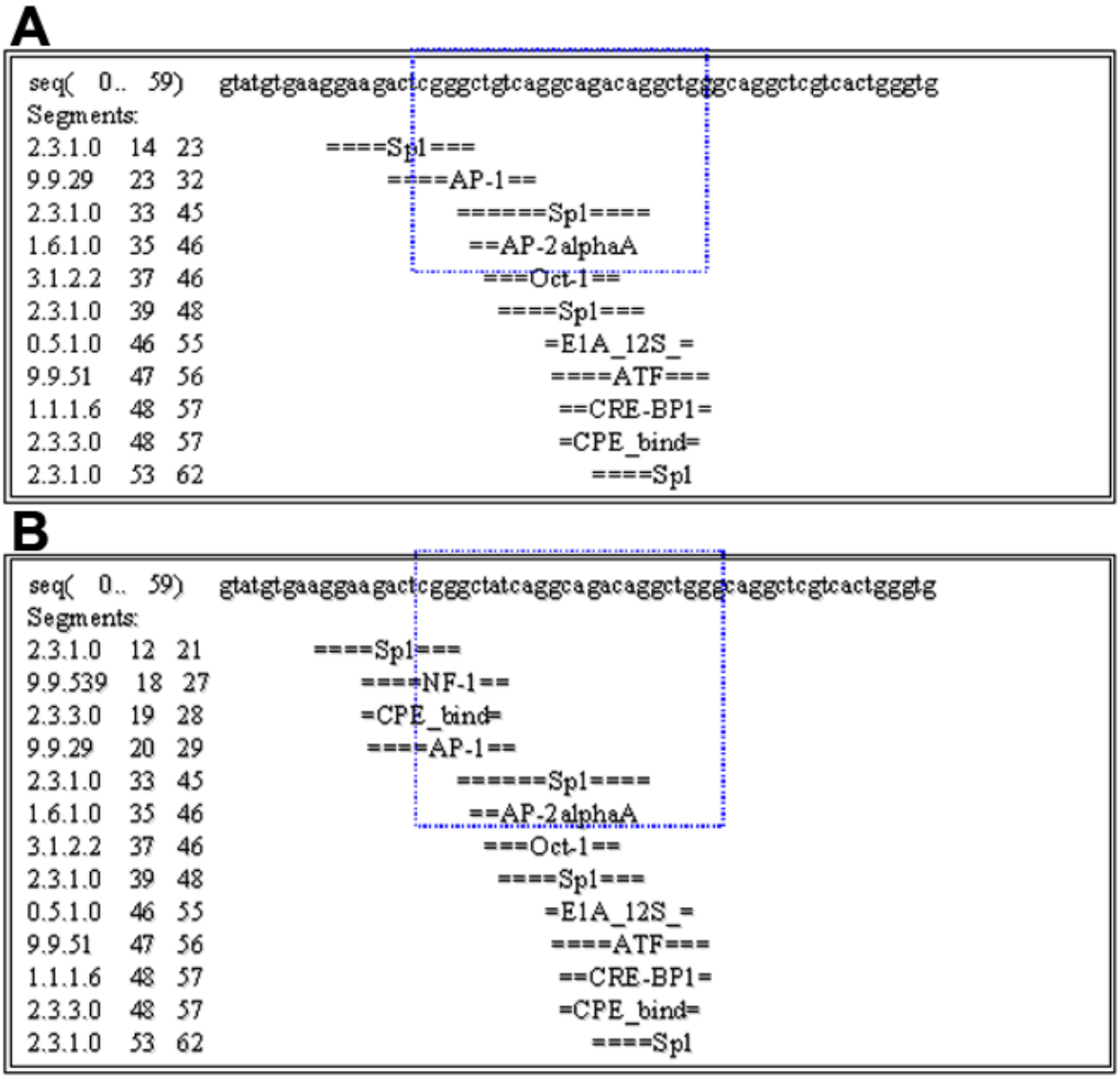

Figure 1. Effect of IVS7+24G>A on transcription factor binding sites. Comparison between the normal (**A**) and IVS7+24G>A (**B**) polymorphism for the putative transcription factor binding sites by AliBaba version 2.1 software. The blue dotted box shows the sites of alteration with the corresponding transcription factors.

positive for the change. Out of these seven patients, one was homozygous for the SNP. The distribution of the IVS 7+24G>A polymorphism within the patients group was compared and correlated with age of onset, IOP, and cup:disc ratio. It was found that the SNP strongly correlates with increased IOP (p=0.03; Table 3).

Evaluation of putative transcription binding sites: Seven percent of the patients were positive for the IVS7+24G>A nucleotide change that was not found in any of the 100 control subjects. The sequence was analyzed in the AliBaba software that scans for potential transcription factor binding sites. While the wild-type sequence has the binding site for transcription factor AP-1 (activating protein 1) the IVS 7 +24G>A polymorphism results in the creation of new binding site for the transcription factor NF-1 and CPE (Figure 1). The secondary structure of the M98K mutant mRNA did not show any difference with that of the wild-type mRNA as analyzed by MFOLD software.

DISCUSSION

In the current study, *OPTN* was analyzed for mutations in 100 HTG. Six nucleotide changes were observed (Table 1). Two of the six nucleotide changes (M98K and IVS7+24G>A) were significant in the patients compared to controls.

M98K and risk for POAG: The M98K change was evident in 10 out of 220 cases but were not found in 100 controls (Table 1). Highly conserved amino acid M98K is located in the putative bZIP domain of the protein but it shows ethnic differences in its distribution between patients and controls. Initial studies have shown that the M98K was observed to be higher in the patients (13.6%) than controls (2.1%) [1]. Later studies from different populations have shown that the frequency distribution of the M98K variation and its association status with POAG varies between different cohorts. The M98K variant has also been found to be highly prevalent in high tension POAG of Caucasian ethnicity by a cross-sectional analysis in the Blue mountain eye study [12]. While most of the reports show a strong association of M98K variant with NTG, reports of no association of the variant with glaucoma exists in the literature. In a large study of 1,048 patients, Alward et al. [7], showed a significant association of M98K in Japanese NTG patients. While M98K was demonstrated to be a polymorphic variant in German [13], French, and Morocco populations [14], studies from British (London) and Japanese populations have shown this to be highly prevalent in NTG patients [7]. Our current observation shows that M98K is seen in 6% and 4.1% of NTG and HTG patients in the study population and statistical analysis did not show any significant correlation with the clinical parameters. While the cases are from all over India, and the controls are from Tamil Nadu, a southern state, the possible effect of the geographical distribution on the observed nil prevalence of M98K in the control group was queried. The data of 113 cases (108:HTG and 5:NTG) from south India were separated and analyzed for M98K out of the total 220 glaucoma cases against the controls. χ^2 analysis suggested a significant p value of 0.009 in the cases when compared to the controls. In their analysis of 200 unrelated *OPTN* patients, Mukopadhyaya et al. [9] observed that M98K was prevalent in both POAG (11%) and controls (5.5%) and thought that this might not be a risk-associated factor in the Indian population. In contrast to the current study, the M98K substitution was shown as a risk for HTG and NTG specifically in the southern Indian population represented from Tamil Nadu. While none of the NTG patients screened by Mukopadhyaya et al. [9] have the substitution, our study found 6% of the NTG patients had M98K. Such differences have been observed before. *OPTN* analysis by Fuse et al. [6] and Alward et al. [7] in a Japanese cohort revealed that the M98K variant was reported in approximately 17% of the patients, whereas a study by Tang et al. [5] in another Japanese cohort found a lack of association. The selection of controls is vital [15] while analyzing complex disorder association status to a sequence change. Proper clinical evaluation of controls similar to that of cases is mandatory to eliminate the bias due to improper phenotyping. This was not a problem in our study as the controls also underwent identical clinical examination as that of the patients by the same clinicians (RG and AH). Though the exact mechanism by which the substitution causes glaucoma is not well understood, it has been shown to affect the interaction with the RAB8 protein. The mRNA structure of both the wild type and M98K mutant sequence was analyzed by MFOLD software but did not show any difference in the RNA structure.

The IVS7+24G>A polymorphism was seen in 7% of HTG group (p=0.03; Table 2) compared to the control subjects. Clinical parameters namely age of onset, IOP at first diagnosis, and vertical cup:disc ratio were compared and the polymorphism strongly correlated with increased IOP (Table 3). Clinical correlation was, however, done in 75 patients for whom all the parameters were available. The IVS7+24G>A polymorphism was strongly associated with cup:disc ratio in the Chinese population [4]. The SNP was analyzed for putative transcription factor binding site through AliBaba software. While the results (Figure 1) showed that the wild type has a binding site for the transcription factor SP1, the G>A variation creates a binding site for AP1 (activator protein 1) transcription factor. Intronic sequences are reported to have a regulatory effect on transcription, splicing, etc. Enhancer elements required for gene expression are speculated to be located throughout the gene [16]. It is also observed that approximately the first and last 20 nucleotides of introns contain functional elements that are sensitive to disruption by single point mutations [16]. Since the *OPTN* gene has not been characterized well, our observation on the presence and alteration of intronic transcription factor binding sites is quite interesting but could yield clear information only by functional studies.

The other polymorphisms observed in the study were equally distributed between the cases and controls. It was interesting to note that a 65-year-old female patient with POAG from south India was positive for three nucleotide changes: *MYOC* Q48H, (a mutation specific for Indian population [17-19]), *OPTN* M98K, and IVS7+24G>A. The IOP at presentation was 21 and 23 mmHg in the left and right eyes, respectively. Optic disc examination revealed a cup:disc ratio of 0.6:1

with notching of the inferior rim of the right eye. She was noted to have corresponding visual field defects in the superior arcuate region on Humphrey visual field 24-2 testing. However, analysis of the other clinical parameters in this patient did not show any association. We do not know if collectively these genotypes produced the phenotype or if it was just a chance observation. Analysis in the Chinese population has shown that the P199P mutation was seen exclusively in controls (1/126) [4] but in the current study the change was seen in one of the patients. The IVS6-5T>C is distributed both in the cases (9%) and controls (12%). We did not observe the Asn303Lys and the Arg545Gln changes that was observed by Mukopadhyaya et al. [9]. Collectively, our findings suggest that *OPTN* nucleotide changes could play a minor role in causing glaucoma in the Indian population.

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