Association of Apolipoprotein E (*APOE*) gene in Primary Open- Angle Glaucoma (POAG).

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Aim : To investigate the association between Apolipoprotein E (*APOE*) gene and primary open-angle glaucoma (POAG) in a cross sectional study of eastern Uttar Pradesh and eastern Bihar subjects.
Methods: 23 cases (17 men, 6 women) and 27 control (21 men , 6 women) were undergone systematic examination of optic disc, visual field examination with automated static perimetry , Intraocular pressure (IOP) measurement with Goldmann applanation tonometry. Spectral domain HD OCT used to measure RNFL thickness. Cases and control were genotyped with polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

Results: The mean ages were 54.00±14.190 and 52.26±12.424 years in POAG and control groups, respectively . No Polymorphism in cases affected with POAG was observed. Intraocular pressure (IOP), cup/disc ratio (C/D) and RNFL thickness were compared among cases and control . p value <0.05 was considered as statistically significant.

Conclusions: Our study found no link between polymorphisms in *APOE* gene and POAG in eastern Uttar Pradesh and eastern Bihar patients, although a larger sample is required to elucidate the association of APOE gene polymorphisms in the pathogenesis and course of primary open-angle glaucoma (POAG) .

Introduction

Glaucoma is chronic progressive optic neuropathy, characterized by optic nerve head (ONH) changes and visual field loss. Elevated intraocular pressure (IOP) is generally accepted as the major modifiable risk factor for glaucoma, however, factors other than IOP also play role in the pathogenesis and progression of glaucoma, particularly in subjects with normal tension glaucoma (NTG).

Glaucoma is the leading cause of irreversible blindness worldwide and has become one of the most challenging health issues currently being confronted by mankind[1]. It is the second leading cause of blindness worldwide, estimated to affect about 70 million people, with 6.7 million of these being bilaterally blind [2]. *It is the third leading cause of blindness in India .12 million people are affected accounting for 12.3% of the countries blindness due to glaucoma* [3]. Primary open-angle glaucoma (POAG) is the major type of primary glaucoma in most populations. POAG is a genetically heterogeneous disorder and at least 22 genetic loci have been mapped for POAG of which only *GLC1A* (myocilin, *MYOC*), *GLC1E* (optineurin, *OPTN*), *GLC1G* (WD repeat domain 36, *WDR36*), and *GLC3A* (*cytochrome P4501B1, CYP1B1*) have been characterized[4,5]. However, mutations in these genes account for less than 10% of POAG cases. It appears that POAG is a complex trait and multiple genes, each with allelic variations, and environmental factors contribute to the pathogenesis and phenotype and increase individual's susceptibility to glaucomatous optic neuropathy, with no particular gene having a single dominant effect . Currently, several genes have been reported to be associated with POAG, and the apolipoprotein E (APOE) gene has received increasing attention[6,7].

Apolipoprotein E (*APOE*), which is the major apolipoprotein in the central nervous system, plays an important role in neural function and repair after injury. APOE is up-regulated in response to oxidative stress and is endowed with antioxidant properties[8]. The APOE gene has been mapped to the 19q13 region, and its common polymorphism has three alleles in exon 4, namely, ε2, ε3, and ε4. These three alleles define the

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following six APOE phenotypes:ε2/ε2, ε3/ε3, ε4/ε4, ε2/ε3, ε2/ε4, and ε3/ε4[9]. ApoE isoforms may have different effects on defective arterial constriction or dilation in vascular dysregulation because of their differential roles in lipid transport in the blood circulation. Atherosclerosis may restrict blood supply to the retina, and ApoE4 is associated with atherosclerosis. Lipid oxidation associated with atherosclerosis can be protected by the anti-oxidation properties of ApoE[10]. ApoE2, followed by ApoE3, has been shown to be more effective than ApoE4 in inhibiting hydrogen peroxide-induced cytotoxicity in cultured B12 cells[11]. Oxidative stress, due to reactive oxygen species, is a cause of retinal ganglion cell death, thus leading to neurodegeneration in glaucoma[12]. It is likely that the *ApoE* genotype is associated with protective properties against oxidative neurogeneration in glaucoma, E4 more susceptible to oxidative damages than E2 or E3.

In the rat eye, it has been shown to be synthesized by Müller cells, secreted in the vitreous, absorbed by the retinal ganglion cells (RGC), and transported down the optic nerve[13]. Its possible role in RGC metabolism, together with its documented effect on neuronal survival following ischemic and traumatic insults, has led to the hypothesis that particular APOE isoforms could be related to neuronal damage in glaucoma patients[14]. Given the potential similarities between the cellular events leading to degeneration in both Alzheimer's disease and glaucoma, the higher incidence of glaucoma in Alzheimer's disease[15,16]. And APOE ε4 allele as a risk factor for Alzheimer's disease. APOE seems to be a pliable candidate for glaucoma susceptibility.

METHODS

The present study was undertaken to evaluate the association of *APOE* gene polymorphism in Primary open angle glaucoma in Eastern Uttar Pradesh & Eastern Bihar patients. The study was done after approval from Departmental Research committee (DRC) and Ethical committee of Banaras Hindu University . Written informed consent was obtained from each patients. 50 patients (23 case and 27 control) were enrolled with age equal or greater than 35 and less than 80.

Before going through ocular examination a detailed history was taken. Personal interview was conducted to determine profile, exposure to the risk factors of glaucoma like family history of glaucoma, ocular trauma, past eye surgery, past treatment for glaucoma. History was taken and general checkup was done to rule out diabetes, anemia and hypertension.

All subjects underwent full clinical and ophthalmologic evaluation, IOP measurement by Goldman applantation tonometry, Slit lamp biomicroscopy, Zeiss 4 mirror Gonioscopy, Automated Perimetry (Humphrey 30-2), OCT & Pachymetry was used the measurement of central corneal thickness (CCT) .

Visual field examination with Humphrey field analyser using SITA standard 30-2 was performed within 3 months. Subjects were excluded If fixation loss greater than 20% and false positive and false negative errors greater than 33%. Patients were excluded who had history of Blunt ocular injury, severe Uveitis, Exofoliation Glaucoma, Diabetes Mellitus, intraocular surgery and laser treatment.

An additional exclusion criterion includes refractive error higher than ±4.00 D .

Best corrected visual acuity measured from 6 meter distance with Snellen's visual acuity chart. The visual acuity of each eye, both with and without corrections was noted. Refraction was carried out manually using Streak retinoscope followed by subjective corrections. Anterior segment was examined both by torch light and slit-lamp. A provisional diagnosis of suspected glaucoma was made when the subject had one or more of the following conditions: intralobular pressure (IOP) ≥ 21 mmHg in either eye, vertical cup-to-disc ratio (VCDR) ≥ 0.7 in either eye or cup-to disc ratio (CDR) asymmetry ≥ 0.2, and focal thinning, notching, or a splinter hemorrhage.

Genetic analysis: Venous blood was obtained from the subjects and stored at -20 °C for less than three months before DNA extraction. DNA isolation was done by "Salting Out" method and dissolved in Tris-

EDTA (TE) buffer. The genotypes of *APOE* polymorphisms were determined by the PCR-RFLP method. *APOE* gene polymorphisms were investigated using the primer sequences 5'-GAA CAA CTG ACC CCG GTGGCG-3' (forward) and 5'-GGA TGG CGC TGA GGC CGC GCT-3' (reverse). The amplified product for exon4 were subjected to 2% agarose gel electrophoresis and 3.5% Agarose Gel Electrophoresis for Restriction digestion of ApoE gene using HhaI overnight at 37 °C.

The statistical analysis was done using SPSS for Windows version 16.0 software. For comparing two groups of mean Student's 't' test was used. For categorical data Chi-square and Fischer's Exact test was used. The critical value of 'p' indicating the probability of significant difference was taken as <0.05 for comparison.

RESULTS-

During the period July 2015 to June 2016 total 50 samples were collected. Of this 50 samples, 23 were cases (46%) and 27 control (54%). These 23 cases comprises of 17 male & 6 female (sex ratio 2.83:1) where as in 27 controls 21were males & 6 females (sex ratio 3.5:1) . During the study we obtained 2 cases (8.6%) with family history .

The mean age of cases and control who came in OPD of SS Hospital was 54.00±14.190 and 52.26±12.424 respectively .All cases in both eye had higher IOP (>21 mm of Hg) as compared to controls. The level of IOP in right & left eye were statistically significant $p<0.05$. With a mean deviation found in cases right eye (24.61±3.100), left eye (23.13±4.742) and control right eye (14.07±1.796), left eye (14.81±1.594). All cases in both eye had higher C:D ratio as compared to control had in normal range (0.2-0.5) which was statistically significant $p<0.05$. With a mean deviation found in cases right eye (0.7152±0.13604), left eye (0.648±0.1904) and control right eye (0.4259±0.12586), left eye (0.433±0.1144) . Majority of the cases in both eye had lower RNFL (Retinal nerve fiber layer) Thickness as compared to control had in normal range (90±8 µm) which was statistically significant $p<0.05$. With a mean deviation found in cases right eye (57.65±17.809), left eye (64.52±23.245) and control right eye (91.19±6.697), left eye (90.63±4.617).Majority of the cases in both eye had lower CCT as compared to control had in normal range (550±10 µm) which was statistically significant $p<0.05$. With a mean deviation found in cases right eye (530.74±5.065), left eye (531.26±4.484) and control right eye (548.11±4.799), left eye (548.78±5.337).

APO E Genotyping

Exon4 of APO E gene for novel polymorphisms/mutations case and control used to detect an association of APO E gene with POAG cases and control. The amplified product for exon4 were subjected to 2% agarose gel electrophoresis and 3.5% Agarose Gel Electrophoresis for Restriction digestion of ApoE gene using HhaI .

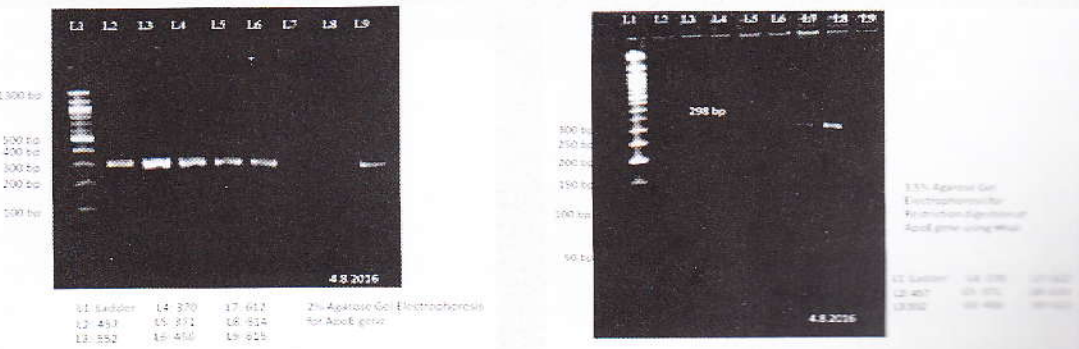

Figure 1,2: 2% Agarose Gel Electrophoresis (AGE) amplified product & 3.5% Agarose Gel Electrophoresis (AGE) for restriction digestion of APO E gene using Hhal amplified product of POAG cases sample (370, 371, 456, 457, 552, 612, 614, 615) of APO E Exon4

We were unable to detect any polymorphism in patient affected with Primary open angle glaucoma.

Discussion

Genetic factors are receiving increasing attention for their role in many forms of glaucoma[17,18]. It is also well known that patients with POAG or their family members have a much higher tendency toward a rise in intraocular pressure (IOP) with use of steroids, indicating a possible hereditary association between steroid response and glaucoma. In addition, the prevalence and severity of POAG, particularly in older age groups, is greater in black and Hispanic Americans compared to whites, which may indicate an increased genetic susceptibility to POAG in these population[19,20].

The high prevalence of POAG, variability in age of onset, and variable penetrance (variable phenotypic expression of a disease despite carrying the genetic mutation) in some pedigrees that have been reported argue strongly that in most cases POAG is inherited as a "complex" trait that does not demonstrate simple Mendelian inheritance. It appears likely that there is interplay between various environmental and genetic factors, or between multiple genes, resulting in a high degree of variability in phenotypic expression and severity of disease. The most frequently mentioned genes with regard to open-angle glaucomas (OAGs) are myocilin (MYOC) (1q23-q24)[21] and optineurin (OPTN) (10p13)[22]. The pathophysiolology of POAG is not precisely known but is felt to be multifactorial[23,24] and polygenetic in etiology. A positive family history, especially among first degree relatives, is a well-known risk factor for POAG.

Our understanding regarding the genetics of POAG is incomplete, and the molecular biology of glaucoma in general is currently a subject of intense investigation. Our study have investigated the APO E polymorphisms involved in oxidative stress, neurotrophic mechanism, and cell morphogenesis in Indian patients with POAG. Single-nucleotide polymorphisms (SNPs) have important implications in human genetic studies, as the presence of a specific SNP allele can be implicated as a causative factor of a genetic disorder. Identification of SNPs allow location and identification of genes of functional importance, which can be used as genetic markers in genetic mapping studies. In addition, understanding the associated polymorphisms may provide an increased understanding of the molecular mechanism of a disease.

In this study, we could not show an association between APOE genotypes/alleles and POAG. Althrough, APOE is a 36-kDa glycoprotein that plays an essential role in lipid and cholesterol transport[25,26]. There is strong evidence that the prevalence of POAG is greater in Alzheimer's disease (AD) patients, and an association between POAG and Alzheimer's disease exists[27,28]. It has also been reported that AD and glaucoma share some common features and that AD patients exhibit widespread axonal degeneration of the optic nerves and the loss of retinal cells, especially ganglion cells[29,30]. Previous studies have shown that the *ε4* allele has been linked to central nervous diseases, such as Parkinson disease, Alzheimer disease, and amyotrophic lateral sclerosis[31,32,33]. In fact, POAG can be considered a neurodegenerative disease as wel[34].

Ressiniotis et al[35]., Lake et al[36]., and Zetterberg et al[37]. have shown that the APOE genotype or alleles do not constitute a risk factor for POAG and NTG, comparable with our results. In the study of Ressiniotis et al[35]. in English population, the frequency of the ε3 allele was 72.6% in POAG group and 76% in control group and the frequency of the APOE ε4 allele in their control population was 13.3%, which was not different than the glaucoma group (14.6%). In their study, Lake et al[195] found no significant difference in frequency of APOE ε3 and ε4 alleles between the normal tension glaucoma group (73.9% and 17.1%, respectively) and the control population (76.5% and 15.5%, respectively). In addition, comparing those patients with progressive NTG disease to the controls revealed no association between APOE genotype and

the disease progression. In the study of Jia et al[38]., ε2,ε3, and ε4 frequencies were found to be 8.75%, 82.25% and 9%, respectively, in Northern Chinese, which were not statistically different between POAG patients and control group. In contrast to these studies, Junemann et al[39] have shown a significant association between the level of IOP and the APOE ε2 allele in German patients, and Vickers et al[40] showed that the APOE ε4 allele was associated with elevated risk for NTG in the Tasmanian population. In a recent study[41], the frequency of the APOE ε4 allele in POAG group was significantly higher, whereas the frequency of the APOE ε2 allele was found to be significantly lower than those in control group in Chinese population. In contrary, Mabuchi et al[42] found a significantly lower frequency of the APOE ε2 and ε4 alleles in Japanese patients with OAG, and Lam et al[43] found lower frequency of the ε4 allele in patients with NTG, but not with high tension glaucoma in Chinese, indicating a protective effect of the ε4 allele against glaucoma. In a study by Fan et al[44] APOE ε4 carriers were found to have a decreased NTG risk (p=0.007).

Song et al[45]conducted a meta-analysis based on nine case-control studies to evaluate the association between the APOE gene ε2/ε3/ε4 polymorphism and the risk of POAG. Corder et al., who claimed that the effects of the ε4 allele dose are associated with increased risk for Alzheimer Disease[46]. Similarly, Schmechel et al. also noted that patients with two ε4 alleles exhibited a distinct neuropathological phenotype compared with other patients[47]. Copin et al. reported that the *APOE* promoter gene polymorphism affected visual field loss and optic nerve damage[48].

Wang et al[49] evaluated only the genetic models of the allele ε2 versus allele ε3, allele ε4 versus allele ε3, ε2 carriers versus allele ε3, and e4 carriers versus allele ε3, and ignored the functions of the genotypes of the APOE gene & indicated no association between the APOE gene and the POAG risk. Yaun et al[50] reported that the ε4 allele may be a latent risk factor in developing primary glaucoma in the Chinese population. Liew et al[51] found a weak association between *APOE* ε4 and retinal microvascular degeneration.

As shown above, there is no consensus whether APOE alleles constitute a risk factor or are protective against glaucoma. There are several possible explanations for these discrepancies. APOE might have a more obvious effect in populations exposed to different environmental factors or with a different genetic background. The pathogenesis and genetic risk factors for glaucoma are not fully understood yet. Genetic polymorphisms in APOE have been investigated in several studies in different populations. Polymorphisms have important implications in human genetic studies and screening for such alleles helps in the detection of a genetic predisposition to disease. However, there are conflicting results about the association of these polymorphisms with glaucoma development and phenotype. The main problem in identifying the gene variants associated with susceptibility to common diseases is that the observed results are not replicated in subsequent studies that used different populations and/or larger numbers of cases versus controls. This discrepancy in the literature may reflect sampling bias, as some of the studies have small number of subjects or it could be attributed to ethnic disparity. Also in glaucoma studies, the inclusion of a normotensive glaucoma group, which has risk factors other than elevated IOP and therefore has a different pathogenesis, may make a study more sensitive to underlying neurodegenerative risk factors.

This is the first population based study in India POAG patients for APOE gene polymorphisms that might be associated with POAG. Our study found no link between polymorphisms in APOE gene and POAG in eastern Uttar Pradesh & eastern Bihar subjects, although a larger sample is required to identify the role of these polymorphisms in the pathogenesis and course of glaucoma if their effects are mild.

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