The apolipoprotein ε4 gene is associated with elevated risk of normal tension glaucoma


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Purpose: Inheritance of a particular apolipoprotein E gene polymorphism, the ε4 allele, has been associated with elevated risk for Alzheimer’s disease and a poor outcome following head injury. The neuronal injury associated with Alzheimer’s disease and brain injury may have a number of similarities with the nerve cell changes associated with glaucoma. Thus, we have investigated the association of inheritance of apolipoprotein E allelic isoforms (ε2, ε3, and ε4) with relative risk for different forms of glaucoma.

Methods: Apolipoprotein E genotype was examined in a Tasmanian population sample comprised of glaucoma sufferers with elevated or normal intraocular pressure and compared to a control sample of elderly Tasmanians without glaucoma.

Results: Approximately twice as many normal tension (38.0%) and high tension (34.2%) glaucoma cases possessed an ε4 allele compared to control cases (18.9%). The odds of ε4 carriers having normal tension glaucoma were significantly greater than for ε3 homozygotes (odds ratio 2.45, 95% confidence interval [1.02-5.91]) even after adjusting for age and gender (odds ratio 2.87 [1.02-8.05]). The increased odds of high tension glaucoma among ε4 allele carriers were not significant (adjusted odds ratio 1.53 [0.64-3.68]).

Conclusions: The data indicate that, in the Tasmanian population, inheritance of the ε4 allele is associated with elevated risk for glaucomatous changes that are not related to increased intraocular pressure.

The apolipoprotein E protein has a number of lipid transport related roles in various tissues. The gene encoding apolipoprotein E on chromosome 19q13.2 has three polymorphic variants in humans designated as ε2, ε3, and ε4. Inheritance of ε4 and ε2 alleles has been associated with higher and lower risk, respectively, of Alzheimer’s disease [1,2], although this may not be the case for all ethnic populations [3,4]. The precise involvement of apolipoprotein E in Alzheimer’s disease pathology has not been settled [5]. For example, there is evidence that the apolipoprotein E protein may act to promote the development of β-amyloid deposits [6-9]. Alternatively, apolipoprotein E may have a more generalized role in neural repair cellular pathways, particularly with respect to the mobilization of cholesterol and other lipids following neuronal damage [10]. In this respect, possession of ε4 alleles has been associated with a poor outcome following various forms of traumatic head injury [11-13] as well as more severe disease progression in multiple sclerosis [14,15] and reduced survival time in amyotrophic lateral sclerosis [16].

We have presented evidence that the neuronal pathology of Alzheimer’s disease may be attributed to an aberrant regenerative response of nerve cells triggered by the gradual compression and physical damage to axons within β-amyloid plaques that form in the brain [5]. Thus, the apolipoprotein E genotype may affect the response of neurons to injury by plaque formation [5]. Similarly, the selective injury to retinal ganglion cells observed in glaucoma may be caused by axonal injury, perhaps by compression and restriction of normal axoplasmic flow, at the level of the lamina cribrosa [17,18]. For the majority of glaucoma cases, prolonged increased intraocular pressure may exert physical force on the structural framework of the lamina cribrosa leading to axonal injury. However, there are also a significant proportion of cases designated normal tension glaucoma (NTG) in which a glaucoma-like pattern of degeneration is present in the absence of elevated intraocular pressure. The underlying cause of retinal neuron degeneration in NTG has yet to be determined. A further point of similarity between Alzheimer’s disease and glaucoma is reflected in the pattern of cell vulnerability. In the neocortex, it is the subgroup of cortical nerve cells containing the neurofilament triplet proteins that are susceptible to neurofibrillary tangle formation [5] and it is the subgroup of retinal ganglion cells selectively containing these proteins that are highly susceptible to degeneration in glaucoma [19].

Given the potential similarities between the cellular events leading to degeneration in both Alzheimer’s disease and glaucoma, we proposed that the apolipoprotein E isoforms are a plausible candidate for glaucoma susceptibility. With respect to eye diseases, there has been a reported negative association of risk for age related macular degeneration with inheritance of the ε4 allele [20]. We therefore sought to investigate whether
the apolipoprotein E genotype is associated with either NTG or high tension glaucoma (HTG) in the Tasmanian population.

**METHODS**

**Case selection:** The Glaucoma Inheritance Study in Tasmania (GIST) project is a large study based in Tasmania and other states in Australia [21]. The primary aim has been to recruit all cases of glaucoma in the Tasmanian population, with a particular emphasis on identifying pedigrees with inherited forms of primary open angle glaucoma (POAG). All subjects recruited for the GIST project have undergone systematic examination of optic disc, visual field and intraocular pressure (IOP) [22,23]. The cases used in this investigation were unrelated to each other and were derived exclusively from the Tasmanian population. A series of elderly population controls were also screened for the presence of glaucoma, and those without glaucoma were subsequently utilized as control subjects (n=51). The mean age of these control subjects was 83.2 (±7.0, standard deviation) and that of the NTG (n=70) and HTG (n=72) cases was 73.0 (±10.3) and 75.6 (±9.1), respectively. Written informed consent was obtained from patients involved in the GIST (as well as control subjects), and the study was approved by the ethics committees of the Royal Victorian Eye and Ear Hospital (Melbourne), the University of Tasmania (Hobart) and the Royal Hobart Hospital (Hobart). All procedures were conducted in accordance with the declaration of Helsinki and subsequent revisions.

Subjects utilized were classified as normal (normal disc with full Humphrey visual field [Humphrey Instruments, San Leandro, CA], IOP less than 22 mm Hg), high tension glaucoma (HTG), or POAG (glaucomatous disc and/or field defect, IOP greater than 21 mm Hg) and normal tension glaucoma (NTG; glaucomatous disc and/or glaucomatous field defect in patients with no documented IOP greater than 21 mm Hg). Gonioscopy was utilized to confirm that all cases had open angles.

**Genomic DNA isolation and genotyping:** Genomic DNA was extracted from blood samples and apolipoprotein E genotyping conducted using protocols provided by QIAGEN Corp. (Bothell, WA). Briefly, 10 ml of blood was pelleted and the red/white nuclei were disrupted by shaking in the presence of 0.32 M sucrose, 10 mM Tris-HCl (pH 7.5), 5 mM MgCl2, and 1% Triton-X-100. The subsequent pellet was then digested with 1.3 mg Proteinase K in 1.15% SDS, 10 mM Tris-HCl (pH 7.5), 400 mM NaCl and 2 mM EDTA (pH 8.0) for 16 h at 50 °C. Saturated NaCl (6 M) was added to the digest. Protein and salts were precipitated with shaking and centrifugation, and the DNA containing supernatant was then ethanol precipitated. Genomic DNA was spooled out and washed in 70% ethanol after which the ethanol was evaporated. The DNA pellet was then re-suspended in 10 mM Tris-HCl, 1 mM Na2EDTA pH 8.0. Genomic DNA was PCR amplified in the presence of 200 µM each dNTP, 1X Q-Solution (QIAGEN), 1X QIAGEN® PCR Buffer, 1.5 U QIAGEN Taq DNA polymerase and 0.25 µM of each primer. The two primers used were: E2mut (5’ACT GAC CCC GGT GGC GGA GGA GAC GCG TGC) upstream primer and E3 (5’TGT CCC ACC AGG GGC CCC AGG CGC TCG CGG) downstream primer. The upstream primer differs from the genomic sequence at the red position to create an additional Afl111 recognition site in the PCR product. Reactions were treated to incubation at 94 °C for 3 min followed by 40 cycles of: 94 °C, 10 s; 65 °C, 30 s; 72 °C, 30 s. A final incubation at 72 °C for 7 min was carried out. The PCR product was then digested in two separate reactions at 37 °C for 48 h. One reaction used 10 µl PCR product, 2.5 units Afl111 (NEB), 25 µg BSA, 100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl2, 1 mM DTT pH 7.9 (NEB buffer 3). The other reaction used 10 µl PCR product, 1.5 units Hae11 (NEB), 25 µg BSA, 50 mM potassium acetate, 20 mM Tris acetate, 10 mM magnesium acetate, 1 mM DTT pH 7.9 (NEB buffer 4). Electrophoresis of both digests was carried out on a 4% agarose in 1X TAE gel. The gel was then stained with SYBR Gold and imaged with a digital camera. Apolipoprotein E alleles were identified by a unique pattern of digested fragments as provided by QIAGEN Corp.

Fisher’s exact test [24] was used to determine the significances of differences in allele and genotype frequencies among the groups. Logistic regression was used to estimate the odds of glaucoma in e4 and e2 allele carriers compared to e3/e3 homozygotes. Both unadjusted and adjusted (for sex and age) odds ratios were estimated.

**RESULTS**

Frequencies of apolipoprotein E genotypes and alleles in controls, NTG cases and HTG cases are presented in Table 1. Table 1 shows that the e4 allele was more common in NTG cases and HTG cases than controls. Overall, approximately twice as many NTG (38.0%) and HTG (34.2%) cases possessed at least one apolipoprotein e4 allele in comparison to the control group (18.9%). In particular, the relative proportion of the e3/e4 genotype was higher in both the NTG and HTG groups relative to controls (p=0.026 and p=0.027, re-

**Table I. APOLIPOPROTEIN E GENOTYPE AND ALLELIC FREQUENCIES**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control (n=51)</th>
<th>Normal tension glaucoma (n=70)</th>
<th>High tension glaucoma (n=72)</th>
</tr>
</thead>
<tbody>
<tr>
<td>r2/r2</td>
<td>2 (3.9%)</td>
<td>5 (7.1%)</td>
<td>1 (1.4%)</td>
</tr>
<tr>
<td>r2/r3</td>
<td>9 (17.7%)</td>
<td>5 (7.1%)</td>
<td>3 (4.7%)*</td>
</tr>
<tr>
<td>r3/r3</td>
<td>30 (58.8%)</td>
<td>33 (47.1%)</td>
<td>45 (62.5%)</td>
</tr>
<tr>
<td>r3/r4</td>
<td>6 (11.8%)</td>
<td>21 (30.0%)*</td>
<td>21 (29.2%)*</td>
</tr>
<tr>
<td>r2/r4</td>
<td>2 (3.9%)</td>
<td>5 (7.1%)</td>
<td>2 (2.8%)</td>
</tr>
<tr>
<td>r4/r4</td>
<td>2 (3.9%)</td>
<td>1 (1.4%)</td>
<td>0 (0.0%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Allele frequency</th>
<th>Control (n=51)</th>
<th>Normal tension glaucoma (n=70)</th>
<th>High tension glaucoma (n=72)</th>
</tr>
</thead>
<tbody>
<tr>
<td>r2</td>
<td>15 (14.7%)</td>
<td>20 (14.3%)</td>
<td>7 (4.9%)*</td>
</tr>
<tr>
<td>r3</td>
<td>75 (73.5%)</td>
<td>92 (65.7%)</td>
<td>114 (79.2%)</td>
</tr>
<tr>
<td>r4</td>
<td>12 (11.8%)</td>
<td>28 (20.0%)</td>
<td>23 (16.0%)</td>
</tr>
</tbody>
</table>

Distribution of genotypes and allelic frequencies in normal tension glaucoma, high tension glaucoma, and cases lacking glaucoma (controls). For each row of the table, differences in proportions between cases and controls were assessed using Fisher’s exact test. Asterisks (“*”) indicate significant differences (a=0.05 for each test).
respectively; Fisher’s exact test for a difference of proportions). In addition, there were fewer individuals with the ε2/ε3 genotype in both the NTG and HTG groups, but this reached significance only for the HTG group (p=0.027). The only significant difference in allele frequencies was the lower frequency of the ε2 allele in HTG cases (4.9%) relative to the control group (14.7%, p=0.011).

Table 2 shows odds ratios for the presence of NTG and HTG in ε4 carriers and ε2 carriers compared to the reference group of ε3/ε3 homozygotes. Unadjusted for age and sex, presence of an ε4 allele was associated with a significant increase in the odds of NTG (p=0.045). This odds ratio increased marginally after adjustment for age and sex, with a corresponding p value of 0.046. There was also an increase in the odds of HTG amongst ε4 carriers but it was not significant (p=0.26). The observed protective effect of the ε2 allele for HTG was not significant after adjustment (p=0.30).

**DISCUSSION**

The analysis of apolipoprotein E genotypes in this Tasmanian population sample indicates that inheritance of the ε4 allele may represent a risk factor for glaucoma, particularly for cases associated with normal intraocular pressures. Thus, in addition to gene mutations linked with familial forms of glaucoma (e.g., myocilin [25], optineurin [26]), there are likely to be specific gene polymorphisms that increase or decrease risk for heritable and sporadic forms of the disease (e.g., apolipoprotein E and OPA1 [27]).

It is important to note that the frequencies of the three apolipoprotein E alleles in the Tasmanian control population differ significantly from those reported in a large sample of Australian subjects aged over 70 drawn from the electoral roll in Canberra and Queanbeyan [28]. In this sample of 1276 alleles [28], the frequencies of the ε2, ε3, and ε4 alleles were 6.4%, 80.8%, and 12.9% respectively (p=0.02 for overall difference in frequencies compared to the Tasmanian controls). This suggests that the population distribution of apolipoprotein E alleles in Tasmania differs from the distributions in other Australian states. Tasmania is an island with a relatively stable and homogenous population. Compared to the mainland states of Australia, levels of immigration have been low subsequent to the initial colonization by Europeans in the early 1800s.

However, as the ε4 allele has similar frequencies in the large cohort (12.9%) and the Tasmanian control population (11.8%), estimates of the increased odds ratios for NTG and HTG among ε4 carrier did not change greatly when we conducted a further analysis using the larger Canberra cohort [28] as the control population. For example, the odds of NTG among ε4 carriers are still significantly higher than among ε3 homozygotes (2.20, 95% confidence interval {1.28-3.79}) and this result changes little if NTG cases younger than 70 are excluded to match the age restrictions of the Canberra cohort (2.31, {1.12-4.45}).

This finding is also interesting with respect to the reported linkage of the ε4 allele with lower risk for age related macular degeneration [20,29]. However, it has recently been suggested that this effect may be relatively modest and largely restricted to familial forms of this condition where the affected individual is of a younger age [30]. Apolipoprotein E immunoreactivity has been localized to basal laminar deposits and soft drusen in age related macular degeneration [20]. Apolipoprotein E has also been localized to the Müller cells (specialized retinal glia) [20,31] and this protein may be increased in Müller cells in glaucomatous eyes [32], indicating that this glial cell may have a role in the retinal response to glaucomatous injury.

Inheritance of the ε4 allele has also been associated with elevated risk to Alzheimer’s disease. In this regard, it is interesting that visual deficits have been reported in Alzheimer’s disease cases. However, there are conflicting reports as to whether visual field loss observed in a relatively high proportion of Alzheimer’s disease cases is associated with retinal or central damage [33-38]. It has recently been noted that both Alzheimer’s disease and Parkinson’s disease cases have increased glaucomatous retinal changes [39]. In the light of the current findings, there may be similar cellular processes involving apolipoprotein E related to neuronal damage that are relatively deficient in ε4 allele carriers. It has been argued that both Alzheimer’s disease and glaucoma are ultimately axon damaging conditions and it is how nerve cells respond to this injury that leads to overall neuronal degeneration and the clinical picture of progressive loss of function [19]. Müller

**TABLE 2. ODDS RATIOS BY GENOTYPE**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control (n=51)</th>
<th>Normal tension glaucoma (n=70)</th>
<th>High tension glaucoma (n=72)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Odds ratio (95% CI)</td>
<td>Odds ratio (95% CI)</td>
</tr>
<tr>
<td>ε3/ε3</td>
<td>30</td>
<td>1.05 {0.43-2.56}</td>
<td>2.01 {0.69-5.81}</td>
</tr>
<tr>
<td>ε2/ε−</td>
<td>13</td>
<td>1.54 {1.02-2.51}</td>
<td>2.45 {1.02-5.91}</td>
</tr>
<tr>
<td>ε4/ε−</td>
<td>10</td>
<td>2.87 {1.02-8.05}</td>
<td>2.20 {1.02-5.81}</td>
</tr>
</tbody>
</table>

Table of odds ratios (crude, adjusted for age and sex) for normal (NTG) and high (HTG) tension glaucoma, for ε2 carriers and ε4 carriers relative to ε3/ε3 homozygotes. Grouped genotypes contain at least one of the ε2 or ε4 alleles, respectively (i.e., ε2/ε4 cases are included in both) with the ε3/ε3 group serving as a reference.
cells that express particular apolipoprotein E isoforms may thus have an important role in regulating the response of retinal ganglion cells to injury. However, it cannot be ruled out that apolipoprotein E may be acting centrally to promote β-amyloid fibril formation in structures such as the lateral geniculate nucleus [40] and that these plaques are causing damage to retinal axons and visual pathways. In this regard, it would be intriguing to determine whether NTG cases may have a higher incidence of Alzheimer-type dementia.

Inheritance of the ε4 allele appears to be associated with elevated risk of glaucoma, particularly NTG, in our well-characterized Tasmanian glaucoma population. However, it will be important to replicate these results in populations from other geographical locations. In addition to identified genes with an autosomal dominant pattern of inheritance (myocilin, optineurin) there may be many gene variations that elevate or decrease risk for the retinal degeneration characteristic of glaucoma (OPA1, apolipoprotein E). The significance of inheritance of these apolipoprotein E allelic isoforms has yet to be established, as is the case for the potential role of this protein in many other neurodegenerative conditions, but it may be linked with associated hypertension, formation of central β-amyloid deposits or a more general role in the regulation of lipids following axonal injury. However, the current data points to a potential in overlap between the degenerative pathways underlying glaucoma, particularly NTG, and Alzheimer-type dementia and brain injury.

ACKNOWLEDGEMENTS

Fundened by the National Health and Medical Research Council, Clifford Craig Medical Research Trust, Ophthalmic Research Institute of Australia and Glaucoma Research Foundation. We would like to thank Marian Quilty and Jyoti Chuckowree for reading the manuscript.

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The print version of this article was created on 14 Oct 2002. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.