Lack of association of p53 polymorphisms and haplotypes in high and normal tension open angle glaucoma

D P Dimasi, A W Hewitt, C M Green, D A Mackey, J E Craig


Background: The final common pathway for open angle glaucoma (OAG) is retinal ganglion cell apoptosis. Polymorphisms in p53, a major regulator of apoptosis, affect the efficiency of cell death induction. Association studies of p53 haplotypes and OAG have had conflicting results.

Objective: To examine the association between p53 haplotypes and OAG in a larger white population than in previous reports, and extend the analysis to normal tension glaucoma.

Methods: 345 unrelated people with OAG were recruited (283 subjects with high tension glaucoma and 62 with normal tension glaucoma) and compared with 178 age matched controls. Genomic DNA was analysed for the p53 codon 72 Arg/Pro polymorphism as well as for the presence or absence of a 16 bp intron 3 insertion.

Results: In this white cohort no association was found between glaucoma (high or normal tension) and either sequence variant or haplotype.

Conclusions: The p53 codon 72 Arg/Pro polymorphism is not associated with age of onset or severity of glaucoma.

Open angle glaucoma (OAG)—a complex, heterogeneous optic neuropathy—is estimated to affect 70 million people worldwide, making it the second most common cause of blindness in the developed world. OAG is characterised by loss of retinal ganglion cells, cupping of the optic nerve head, and irreversible visual field loss. The most prevalent type of OAG, high tension glaucoma (HTG), is associated with raised intraocular pressure. However, approximately one third of OAG cases have normal intraocular pressure and are designated normal tension glaucoma (NTG). Mutations in three genes—myocilin, optineurin, and WDR36—have both been implicated in HTG and NTG. Collectively, mutations in these genes account for a small proportion of cases. Functional genomic investigations have shown that myocilin and optineurin have fundamentally different pathogenic roles. Nonetheless, regardless of the primary pathological mechanism, retinal ganglion cell apoptosis is the final common pathway for all forms of OAG.

Apoptosis is a genetically regulated form of automated cell death. The primary regulating gene in apoptosis, tumour protein p53, has been shown to cause the transcriptional induction of redox related genes, the formation of reactive oxygen species, and the oxidative degradation of mitochondrial components, which together culminate in cell death. Deregulation of this process can lead to the onset of disease, and aberrant regulation of apoptosis has been associated with cancer, autoimmune disease, and neurodegenerative disorders. Recently, it was shown that a specific functional polymorphism in exon 4 of the p53 gene (Arg72Pro) alters the ability to induce apoptosis in vitro, with the Arg72 variant having enhanced apoptotic potential. This finding, together with the observation that apoptosis is a hallmark of OAG, provides enough biological plausibility to examine whether common sequence variations in the p53 gene are associated with OAG.

In two separate populations, Lin et al and Ressiniotis et al have both suggested that different polymorphic variants in the p53 gene are associated with OAG. In a small Chinese cohort, Lin et al found that the Pro72 polymorphism was significantly more common in OAG patients than in controls. Ressiniotis et al studied this same polymorphism, as well as a 16 base pair (bp) insertion within intron 3, in a British population. The p53 haplotype which had the bp insertion in conjunction with the Arg72 polymorphism was commoner in OAG patients than in controls. However, this positive association conflicted with earlier work that was conducted in an Indian population. In the Indian cohort studied, Acharya et al found no association between any p53 allele or haplotype and OAG.

To investigate the association between p53 haplotypes and OAG further, we studied the occurrence of both the codon 72 polymorphism and the 16 bp intron insertion in a large, well characterised white Australian cohort of OAG patients, compared with a carefully matched population based control group. Unlike the previous investigations of p53 polymorphisms and OAG, we specifically separated NTG patients. Refinement of the OAG phenotype, through delineating the NTG and HTG subgroups, permitted scrutiny for subtle modifier effects. The prevalence of the individual alleles and the overall haplotype were analysed to allow comparison with the previous p53 association studies. We also wished to examine whether any of the p53 sequence variants could act as modifiers of phenotypic severity.

METHODS

Sample population and case definition

We recruited 345 unrelated people with OAG through the glaucoma inheritance study in Tasmania (GIST). GIST is derived from a white population in southeastern Australia. The clinical features for diagnosis have been described previously. In summary, OAG was defined by the presence of the following:

- in at least one eye, optic disc cupping (cup:disc ratio ≥ 0.7); or
- a 0.2 intereye disparity in cup:disc ratio; or
- focal rim notching, with corresponding visual field loss.

Visual field assessments were conducted using a Humphrey visual field analyser (Humphrey Instruments, San Leandro, California, USA), threshold 24-2, and graded as abnormal if the mean deviation or pattern standard deviation was abnormal if the mean deviation or pattern standard deviation was abnormal.

Abbreviations: GIST, glaucoma inheritance study, Tasmania; HTG, high tension glaucoma; NTG, normal tension glaucoma; OAG, open angle glaucoma.
had a probability of normality of <5%, or if the glaucoma fields test was abnormal. HTG was diagnosed in 283 subjects who had an untreated intraocular pressure greater than 21 mm Hg. Sixty two subjects were diagnosed with NTG and (at different diurnal periods) had never been found to have an applanation intraocular pressure measurement greater than 21 mm Hg. The treating ophthalmologists made the diagnosis, and patients were reviewed as part of GIST to verify the diagnostic subclassification.

We recruited 178 age matched control subjects from the same population as the OAG cases. The control cohort comprised 124 people who resided in local retirement homes and 54 who were recruited through an adjuvant genetic study. Control subjects were included if after examination they were free of ocular hypertension or OAG.

The tenets of Helsinki were adhered to and ethics approval was obtained from the ethics committees of the Royal Hobart Hospital and the Royal Victorian Eye and Ear Hospital.

### Genetic analysis

Total genomic DNA was isolated from blood, using standard techniques. Analysis of the p53 haplotype followed previously described techniques. In brief, polymerase chain reaction (PCR) was undertaken using 50 ng of genomic DNA, 0.5 U HotStarTaq DNA polymerase (Qiagen, Valencia, California, USA), 10 mM Tris-HCl pH 8.4, 0.1% Tween-20, 1 mM dNTPs, 5 mM of each oligonucleotide primer, and water to a total volume of 20 μl.

The digest reaction consisted of 10 μl of PCR product, 2.5 U BstUI (New England BioLabs, Beverly, Massachusetts, USA). DNA fragments with a C nucleotide at codon 72 (Pro residue) remained undigested following incubation with BstUI, and were 448 bp or 432 bp in length depending on whether the 16 bp insertion was present or not. DNA fragments harbouring the G nucleotide at codon 72 (Arg residue) were digested with BstUI, and were 248 or 232 bp in length depending whether the 16 bp insertion was present or not.

#### Statistical and data analysis

The severity of the disease phenotype was determined in the OAG group by the age at diagnosis, severity of optic disc cupping, and GIST severity score. In brief the GIST score is a combined assessment of visual field severity, optic disc cupping, and the degree of intracocular pressure elevation. Scores of 0.7, 0.8, 0.9, and 1.0 infer mild, moderate, severe, or very severe disease, respectively. For a more detailed description of the derivation of the GIST score the reader is referred to Coote et al.

Allele and haplotype frequencies in the control group and the overall OAG group, as well as in the HTG and NTG subgroups, were expressed in terms of Hardy–Weinberg equilibrium, and evaluated using the χ² test through Intercooled Stata 7.0 (Stata Corporation, USA). The Kruskal–Wallis test was used to examine the equality of phenotypic markers in OAG subjects with different p53 Pro72Arg genotypes. Student’s t test was used to compare the clinical findings between the NTG and HTG subgroups.

### RESULTS

In the OAG group overall, there were 137 male subjects (39.7%), of whom 20 were classified as having NTG. The mean (SD) age of controls was 65.7 (22.3) years, and 53 (29.8%) were male. Accurate data on the age at diagnosis were available for 248 HTG and 41 NTG subjects. Subjects with NTG were diagnosed later (p = 0.0136) and had greater cup:disc ratios than those with HTG (p = 0.0053). However, there was no significant difference in the age at review between the NTG and HTG groups (p = 0.71). The mean (SD) maximum

### Table 1

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Arg-Ang</th>
<th>Arg-Pro</th>
<th>Pro-Pro</th>
<th>p Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>109 (61.2%)</td>
<td>57 (32.0%)</td>
<td>12 (6.7%)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>HTG</td>
<td>158 (55.8%)</td>
<td>101 (35.7%)</td>
<td>24 (8.5%)</td>
<td>0.496</td>
</tr>
<tr>
<td>NTG</td>
<td>29 (46.8%)</td>
<td>28 (45.2%)</td>
<td>5 (8.1%)</td>
<td>0.133</td>
</tr>
<tr>
<td>Total</td>
<td>296 (56.6%)</td>
<td>186 (35.6%)</td>
<td>41 (7.8%)</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>

Values are n (%).

*χ² test between disease subgroup and control cohort.

Arg, arginine; HTG, high tension glaucoma; NTG, normal tension glaucoma; OAG, open angle glaucoma; Pro, proline.

### Table 2

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Del-Ang</th>
<th>Del-Pro</th>
<th>Ins-Ang</th>
<th>Ins-Pro</th>
<th>p Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>268 (75.3%)</td>
<td>38 (10.7%)</td>
<td>7 (2.0%)</td>
<td>43 (12.1%)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>HTG</td>
<td>412 (72.8%)</td>
<td>66 (11.7%)</td>
<td>5 (0.9%)</td>
<td>83 (14.7%)</td>
<td>0.333</td>
</tr>
<tr>
<td>NTG</td>
<td>85 (68.6%)</td>
<td>19 (15.3%)</td>
<td>1 (0.8%)</td>
<td>19 (15.3%)</td>
<td>0.292</td>
</tr>
<tr>
<td>Total</td>
<td>765 (73.1%)</td>
<td>123 (11.8%)</td>
<td>13 (1.2%)</td>
<td>145 (13.9%)</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>

Values are n (%).

*χ² test between disease subgroup and control cohort.

Arg, arginine; HTG, high tension glaucoma; NTG, normal tension glaucoma; OAG, open angle glaucoma; Del, absence of the 16 bp insertion on intron 3; Pro, proline.
DISCUSSION

OAG is a complex trait and its inheritance has been shown to follow both Mendelian and non-Mendelian models. Given the prevalence of OAG and the fact that it is amenable to treatment when detected early, genetic predisposition screening could reduce morbidity. However, such screening programmes are currently limited by the paucity of identified causative genes. Genetic association studies defining susceptibility to OAG may provide important insights into the pathogenesis of OAG. More recently, Ressiniotis et al found that the Arg72 variant, was a significant risk factor in the development of OAG. Thus, biologically, the Arg72 polymorphism may be expected to confer pro-apoptotic susceptibility. Interestingly, Lin et al concluded that the Pro72 variant was a significant risk factor in the development of OAG. More recently, Ressiniotis et al found that the p53 intron 3 insertion combined with an Arg72 polymorphism was significantly overrepresented in OAG (no association was found for the Arg72Pro variant alone). Neither finding was corroborated in our white study cohort. We subanalysed the glaucoma cases by markers of severity (age of diagnosis, GIST severity score, severity of disc cupping, and peak intraocular pressure) to test for any association with the Arg72Pro variant and found no significant differences.

Research into the genetics of complex traits is often confounded by a poor description of the phenotype. HTG and NTG may represent a polarised phenotypic spectrum of the same genetic disorder; alternatively they may be genetically distinct entities. Nonetheless, whole disease group analysis (OAG) as well as subgroup analysis (HTG and NTG) of our population did not reveal any p53 association. In considering the proposed mechanism of p53 involvement in OAG it would seem more likely that a genetic mechanism favouring apoptosis would have a greater role in NTG in which raised intraocular pressure is not present. We examined this for the first time by separating our OAG cases into HTG and NTG. No p53 haplotype was significantly overrepresented in NTG cases. The major limitation of this study is that there were a relatively few patients subcategorised into the NTG group. Further investigation in larger well characterised cohort of NTG cases may be required to fully elucidate minor phenotypic modification by p53 sequence variants. We did not seek information on a past history of cancer. Investigations of the association between the sequence variants. We did not seek information on a past history of cancer. Investigations of the association between the sequence

**Table 3** Phenotypic markers in combined OAG subjects for p53 Pro72Arg genotype subcategories.

<table>
<thead>
<tr>
<th></th>
<th>Arg-Arg (n = 187)</th>
<th>Arg-Pro (n = 129)</th>
<th>Pro-Pro (n = 29)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at review (years)</td>
<td>72.3 (0.86)</td>
<td>73.1 (1.03)</td>
<td>73.7 (2.14)</td>
<td>0.610</td>
</tr>
<tr>
<td>Age at diagnosis (years)</td>
<td>62.9 (0.98)</td>
<td>63.7 (1.16)</td>
<td>63.7 (2.23)</td>
<td>0.924</td>
</tr>
<tr>
<td>GIST severity score</td>
<td>0.85 (0.01)</td>
<td>0.84 (0.01)</td>
<td>0.86 (0.02)</td>
<td>0.527</td>
</tr>
<tr>
<td>Worst eye CDR</td>
<td>0.8 (0.05)</td>
<td>0.8 (0.01)</td>
<td>0.78 (0.03)</td>
<td>0.299</td>
</tr>
<tr>
<td>Maximum recorded IOP (mm Hg)</td>
<td>28.5 (0.72)</td>
<td>26.7 (0.73)</td>
<td>29.3 (1.97)</td>
<td>0.582</td>
</tr>
</tbody>
</table>

Values are mean [SE].

Arg, arginine; CDR, cup:disc ratio; GIST, glaucoma inheritance study in Tasmania; IOP, intraocular pressure; OAG, open angle glaucoma; Pro, proline.
consideration of the phenotypic severity to examine possible mild modifier effects.

ACKNOWLEDGEMENTS

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