ELECTRONIC LETTER

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

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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.¹ 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).²

Mutations in three genes—*myocilin*, *optineurin*, and *WDR36*—have been implicated in HTG and NTG.^{3–5} Collectively, mutations in these genes account for a small proportion of cases.^{6–7} Functional genomic investigations have shown that *myocilin* and *optineurin* have fundamentally different pathogenic roles.^{8–9} Nonetheless, regardless of the primary pathological mechanism, retinal ganglion cell apoptosis is the final common pathway for all forms of OAG.^{10–11}

Apoptosis is a genetically regulated form of automated cell death.¹² 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.¹³ Deregulation of this process can lead to the onset of disease, and aberrant regulation of apoptosis has been associated with cancer,^{14–15} autoimmune disease,¹⁶ and neurodegenerative disorders.¹⁷ 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,¹⁸ 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*¹⁹ and Ressiniotis *et al*²⁰ 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.²⁰ The *p53* haplotype which had the bp insertion in conjunction with the Arg72 polymorphism was commoner in OAG patients than in controls.²⁰ However, this positive association conflicted with earlier work that was conducted in an Indian population.²¹ In the Indian cohort studied, Acharya *et al* found no association between any *p53* allele or haplotype and OAG.²¹

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.²² 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

Abbreviations: GIST, glaucoma inheritance study, Tasmania; HTG, high tension glaucoma; NTG, normal tension glaucoma; OAG, open angle glaucoma

Table 1 Frequency distributions of *p53* codon 72 polymorphic genotype in control and OAG subjects

	Arg-Arg	Arg-Pro	Pro-Pro	p Value*
Control	109 (61.2%)	57 (32.0%)	12 (6.7%)	
HTG	158 (55.8%)	101 (35.7%)	24 (8.5%)	0.496
NTG	29 (46.8%)	28 (45.2%)	5 (8.1%)	0.133
Total	296 (56.6%)	186 (35.6%)	41 (7.8%)	

Values are n (%).

* χ^2 test between disease subgroup and control cohort.

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

had a probability of normality of <5%, or if the glaucoma hemifield 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.^{20, 21} 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× Qiagen PCR buffer, 2 mM dNTPs, 5pM of each oligonucleotide primer, and water to a total volume of 20 µl. Primer sequences flanked the two polymorphic sites (intron 3, 16 bp insertion; exon 4, C/G single nucleotide polymorphism (SNP) at codon 72).²¹ The forward primer was fluorescently labelled at the 5' end with FAM (Geneworks, Adelaide, Australia). The resulting full length PCR product was 448 bp in the presence of the 16 bp insertion or 432 bp in the absence of the insertion.

PCR products were digested with the restriction enzyme *Bst*U1 (New England Biolabs, Beverly, Massachusetts, USA). The digest reaction consisted of 10 µl of PCR product, 2.5 U of enzyme, 10× buffer, and water to a total volume of 20 µl. Following incubation at 60°C for two hours, the digested products were analysed with the ABI 3100 genetic analyser and GeneScan V3.7 software (Applied Biosystems, Foster

City, California, USA). DNA fragments with a C nucleotide at codon 72 (Pro residue) remained undigested following incubation with *Bst*U1, 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 *Bst*U1, 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 intraocular pressure elevation.²² 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.*²²

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 χ^2 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 2 Frequency distributions of *p53* haplotypes (intron 3 insertion and codon 72 polymorphism) in control and OAG subjects

	Del-Arg	Del-Pro	Ins-Arg	Ins-Pro	p Value*
Control	268 (75.3%)	38 (10.7%)	7 (2.0%)	43 (12.1%)	
HTG	412 (72.8%)	66 (11.7%)	5 (0.9%)	83 (14.7%)	0.333
NTG	85 (68.6%)	19 (15.3%)	1 (0.8%)	19 (15.3%)	0.292
Total	765 (73.1%)	123 (11.8%)	13 (1.2%)	145 (13.9%)	

Values are n (%).

* χ^2 test between disease subgroup and control cohort.

Arg, arginine 72 polymorphism; Del, absence of the 16 bp insertion on intron 3; HTG, high tension glaucoma; Ins, presence of the 16 bp insertion on intron 3; NTG, normal tension glaucoma; OAG, open angle glaucoma; Pro, proline 72 polymorphism.

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

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

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.

recorded intraocular pressure for HTG subjects was 30.4 (8.6) mm Hg, and 17.8 (2.3) mm Hg for the NTG group.

The Arg72 allele was more common than the Pro72 allele in each group. Frequency distributions of the *p53* codon 72 polymorphic genotype in control and OAG subjects are presented in table 1. There was no significant difference in the distribution of the codon 72 polymorphism between groups ($p = 0.35$).

The frequencies of the intronic insertion in the normal controls and the OAG subjects were also similar ($p = 0.95$). Ten subjects from the whole study group (three controls, six HTG subjects, one NTG subject) were homozygous for the intron 3, 16 bp insertion.

There was no statistical evidence for Hardy–Weinberg disequilibrium of *p53* haplotypes (intron 3 insertion and codon 72 polymorphism) between control and OAG subjects ($p = 0.22$). The haplotype frequency distributions were similar ($p = 0.44$) between the HTG, NTG and control subjects (table 2). In particular, the haplotype with Arg72 plus the intron 3 insertion, previously reported to be overrepresented in glaucoma cases,²⁰ was not more common in either HTG or NTG compared with the population control group ($n = 5$ (0.88%), $n = 1$ (0.81%), and $n = 7$ (1.97%) for the HTG, NTG, and control groups, respectively).

There was no difference in the age at diagnosis, GIST severity score, severity of disc cupping, or maximum recorded intraocular pressure for the OAG subjects with the Arg/Arg genotype compared with those with the Pro/Pro genotype (table 3).

DISCUSSION

OAG is a complex trait and its inheritance has been shown to follow both Mendelian and non-Mendelian models.²³ 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.^{3,4} Genetic association studies defining susceptibility to OAG may provide important insights into the pathogenesis of OAG, but should be treated with caution until independently replicated.

In our study population, there was no evidence for Hardy–Weinberg disequilibrium of *p53* gene haplotypes between OAG and control groups. This finding is in keeping with the previous work by Acharya *et al.*²¹ yet contrasts that of Lin *et al.*¹⁹ and Rassinotis *et al.*²⁰ It is also noteworthy that the studies by Lin *et al.*¹⁹ and Rassinotis *et al.*²⁰ reported glaucoma association with opposite alleles at the Arg72Pro polymorphism. Differences between the *p53* OAG association studies may reflect sampling bias, as all three previous studies were substantially smaller. Alternatively, population specific differences in the frequency of the genetic variants under consideration could account for the different findings. Clearly there are population differences in the frequency of these variants in normal individuals, and under these

circumstances the utmost care must be exercised in matching the disease and population control cohorts. One of the strengths of the current study is the larger size of the disease and control groups, in addition to lack of population admixture in the disease and control cohorts.

It is important to note that the Arg72 variant has been shown to be more efficient in inducing apoptosis, through enhanced localisation to mitochondria, than the Pro72 polymorphism.¹⁸ Thus, biologically, the Arg72 polymorphism may be expected to confer pro-apoptotic susceptibility. Interestingly, Lin *et al.* concluded that the Pro72, not the Arg72 variant, was a significant risk factor in the development of OAG.¹⁹ More recently, Rassinotis *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).²⁰ 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.¹⁸ 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 variations under study and cancer have yielded variable results.^{24–26} We are not aware of any data linking these polymorphisms with increased mortality.^{24–26}

When allelic heterogeneity is low, polymorphism association studies for the dissection of complex traits are likely to be informative. Population association studies of the same ethnic groups achieve strength when considered together, and empirical observations on a large scale are essential for reducing the risk of obtaining false positive results. The major strength of our study, compared with the previous investigations, is the larger numbers of subjects in both the disease and control groups, which were carefully matched from the same sample population. Additionally there was

consideration of the phenotypic severity to examine possible mild modifier effects.

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