Distribution of p53 codon 72 polymorphism in Indian primary open angle glaucoma patients

Moulinath Acharya,¹ Sayan Mitra,¹ Arijit Mukhopadhyay,¹ Mita Khan,² Susanta Roychoudhury,¹ Kunal Ray¹

(The first two authors contributed equally to this publication)

¹Human Genetics & Genomics Division, Indian Institute of Chemical Biology, Jadavpur, Kolkata - 700 032, India; ²Regional Institute of Ophthalmology, Medical College, Kolkata - 700 073, India

Purpose: Glaucoma is a complex neurodegenerative disorder of the eye. Primary Open Angle Glaucoma (POAG) is the most common type, accounting for over half of the total cases. Recently, a significant difference in the distribution of the codon 72 polymorphism of the tumor suppressor gene p53 between control subjects and POAG patients of Chinese origin (p=0.00782) was demonstrated. The proline residue at codon 72 of the p53 gene was significantly over represented in the POAG patients relative to healthy controls. The purpose of this study was to investigate whether the reported association between the p53 polymorphism and POAG is a common phenomenon irrespective of geographical location or ethnicity of the population.

Methods: Sixty seven unrelated POAG patients, ranging from 10-65 years of age (mean±SD of 41.16±18.52 years), and 112 control subjects having a similar age range of 18-63 years (mean±SD of 36.64±14.65 years) were enrolled in this study. A region of the p53 gene encompassing two polymorphic sites, a 16 bp duplication in intron 3 and a BsuUI RFLP in exon 4, were amplified by polymerase chain reaction from Indian POAG patients and normal healthy controls. A single base change (G to C) in codon 72 alters the amino acid residue from arginine to proline and removes the polymorphic BsuUI site mentioned above. The amplified DNA fragments were digested with the restriction enzyme and the digestion patterns of the fragments were used to identify the alleles for both the polymorphic sites.

Results: No significant association between p53 alleles and Indian POAG patients were observed by analyzing either codon 72 polymorphism (p=0.5627) or the intronic 16 bp duplication polymorphism (p=0.059). Haplotype analysis, reported to be a better predictor of association of the p53 gene with different types of cancer, was also performed and no association of any haplotype was detected with POAG (p=0.1831).

Conclusions: Association between the p53 gene encoding for proline at codon 72 and POAG presumably exists in some ethnic populations but cannot be used as a predictor for the role of the gene as a common regulator of cell death of retinal ganglions leading to POAG.

Glucoma is a complex neurodegenerative disorder of the eye and can affect all ages. It is estimated that approximately 70 million people worldwide suffer from glaucoma. Untreated, glaucoma is a leading cause of irreversible blindness. Some of the ‘risk factors’ may be inherited to a variable extent. Primary Open Angle Glaucoma (POAG) is the most common type of glaucoma, accounting for over half of the total cases. A positive family history of glaucoma is a significant risk factor for the disease. At least two genes, MYOC and optineurin [1,2], and 4 other chromosomal loci has been linked to POAG [3-6]. In addition, intraocular pressure, ethnicity, some aspects of vascular function, and high refractive error are major risk factors [7].

It has been proposed that the death of ganglion cells by apoptosis represents a common pathway that leads to glaucomatous vision loss [8-10]. However, the mechanism of ganglion cell apoptosis is not well understood. The suggestion that ganglion cell death in glaucoma is apoptotic comes from a lack of any demonstration of cell necrosis rather than evidence of apoptosis [11]. Lin and colleagues have argued that genomic instability of p53 in other disease processes suggests its possible role in the glaucomatous neuropathy as well [12]. High levels of expression of p53 in normal rodent and murine ocular tissues have also been reported [13-16]. To resolve the suspected role of p53 in glaucomatous neuropathy Lin et al. [12] investigated the role of p53 codon 72 polymorphism which changes arginine (Arg) to proline (Pro) due to a G to C transition, and observed significant difference in the distribution of the polymorphism between the control subjects and the Chinese POAG patients. It has been reported that the frequency of the Pro allele is significantly higher in the POAG group and the Pro form of p53 codon 72 increases the risk of developing POAG in Chinese people.

This polymorphism has been subject to many epidemiological studies that explore the possible association of this polymorphism with risk of different cancer types [17]. However, the results of the epidemiological studies remained inconclusive; some exhibited strong association while others did not.
On the other hand, the major risk factors for glaucoma include the ethnicity of the population. We were, therefore, interested in investigating whether the results of our study using Indian POAG patients supports the observation made by Lin et al. [12] and whether the two studies are compatible with the concept of p53 as one of the primary regulators of retinal ganglion cell death during POAG by apoptosis.

Lin et al. [12] have observed significant differences in the distribution of the polymorphism between the control subjects and Chinese POAG patients. To investigate whether this observation represents a common mechanism for glaucomatous cell death, we examined Indian POAG patients for association of specific allele of p53 (if any) with Indian POAG patients using both codon 72 and an intron 3 16-bp duplication polymorphisms.

**METHODS**

**Study population:** Blood samples were collected from 67 unrelated POAG patients ranging from 10-65 years of age (mean±SD of 41.16±18.52 years) from the Regional Institute of Ophthalmology, Medical College, Kolkata between 2000 to 2002. All patients of the study received an ophthalmic examination, which included intraocular pressure, visual acuity, automated perimetry, gonioscopy, optic disc examination and retinal examination. Only those patients affected with POAG were included in the study. As a control, 112 normal healthy individuals from eastern India were selected with ages ranging from 18-63 years, with mean±SD of 36.64±14.65 years. Patients and controls were age-matched (t=1.75, df=177, p=0.0819) and there was no sex bias (χ²=0.17, df=1, p=0.6801). Healthy individuals with no history of POAG were taken from the same population group of eastern India from which the POAG patients were enrolled in the study. It has been estimated that 1.8% of the population of India is blind and 0.15% suffers from glaucoma [18]. Hence, unlike similar studies done on common disorders (e.g., cardio-vascular disease, asthma) the possibility of the data being skewed due to predisposed individuals in the control population would be extremely low in the present study. Prior to the sample collection, written consent was taken from all the study individuals. The internal review committee on research using human subjects cleared the project after due deliberations.

**Polymerase chain reaction:** DNA extraction from blood was done using standard protocols [19]. Polymerase chain reaction (PCR) was performed in 25 µl reaction volume using standard buffer, MgCl₂ (1.5 mM), DMSO (10%), nucleotides (200 µM), Taq polymerase supplied by Life Technologies (USA) and primers 1 (5'-CCT GAA AAC AAC GTT CTG GTAA-3'); and 2 (5'-GCA TTG AAG TCT CA T GGA AG-3', purchased from MWG biotech, Germany) which flanked the two polymorphic sites (intron 3 16 bp duplication and exon 4 BstU I RFLP). Cycling was performed as follows; a pre PCR step of 5 min denaturation at 94 °C followed by 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 55 °C, 30 s extension at 72 °C, and finally a 7 min incubation at 72 °C.

The resulting PCR product consisted of a 432 or 448 bp DNA fragment depending on the absence or presence of the intron 3 16 bp duplication in the template genomic DNA. The length of the polymorphism was observed using 6% polyacrylamide gel analysis. After electrophoresis, the gels were stained with ethidium bromide and photographed under UV light.

**Restriction enzyme digestion:** PCR products (5 µl) were subjected to restriction digestion with BstU I (New England Biolabs Inc. Beverly, MA, USA) in a 20 µl digestion mixture at 60 °C for 4 h. Digested products were separated in 6% polyacrylamide gels, stained with ethidium bromide and photographed under UV light. Amplified DNA fragments with the 16 bp duplication (448 bp) or without it (432 bp) and harboring Arg at codon 72 would contain the polymorphic BstU I site and would be digested to an invariable 202 bp DNA frag-

![Figure 1. Detection of the p53 polymorphism and construction of haplotype. A: Locations of the polymorphic sites within the p53 gene and the nature of the polymorphisms are shown. The allelic difference resulting from codon 72 polymorphism were determined by BstU I digestion of the PCR product obtained by using a pair of primers selected from exon 3 and intron 4 of the gene. B: All possible combinations of DNA fragments resulting from BstU I digestion of the PCR products and the haplotypes determined based on the digestion pattern are shown. C: Polyacrylamide gel (6%) analysis of PCR products from five representative samples, with (+) and without (-) BstU I digestion, is shown. The haplotypes determined based on the analysis are indicated at the bottom of the panel. The ins-Arg haplotype has not been detected in any sample. Lane M is a pBS/Hae III digested molecular weight marker. The sizes of the molecular weight marker and the BstU I digest are shown in the left and the right sides of the gel, respectively. DNA bands present only in samples 4 and 5 (four right lanes) above 458 bp size marker are due to heteroduplex formation between the 448 bp and 432 bp bands, which are retained in the native polyacrylamide gel.](image-url)
ment, together with a variable DNA fragment having a length of 246 bp or 230 bp, respectively. The amplified DNA fragment which contains Pro at codon 72 does not contain the polymorphic BstUI site. Thus the digestion patterns would represent four possible haplotypes as follows; 448 bp (ins-Pro); 432 bp (del-Pro); 202 bp and 246 bp (ins-Arg); and 202 bp and 232 bp (del-Arg) where ‘ins’ indicates insertion of an extra copy of 16 nucleotides in intron 3 and ‘del’ (for deletion) indicates lack of such insertion. However, we did not find the ins-Arg haplotype in any of the 172 samples analyzed (see Results section). The entire strategy for genotype and haplotype determination has been illustrated in Figure 1.

Genotypic distributions were examined for significant departure from the Hardy-Weinberg equilibrium by a goodness of fit \( \chi^2 \) test. Comparison of allelic and genotypic distributions was performed by \( \chi^2 \) statistics.

**RESULTS**

Unlike the studies undertaken by Lin et al. [12], instead of allele specific PCR we performed RFLP analysis to investigate the p53 codon 72 Pro/Arg polymorphism among the POAG patients and controls by amplifying a DNA fragment followed by digestion with BstUI as described in Materials and Methods. This strategy avoids any possibility of non-specific amplification of the non-targeted allele which is not uncommon in allele specific PCR. In addition, this strategy provided the opportunity to score another polymorphism (intron 3 16-bp duplication) in the p53 gene.

<table>
<thead>
<tr>
<th>Table 1. Genotype distribution and allelic frequencies of p53 codon 72 polymorphism in control subjects and POAG patients</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genotype</strong></td>
</tr>
<tr>
<td>---------------</td>
</tr>
<tr>
<td><strong>Test Group</strong></td>
</tr>
<tr>
<td>POAG</td>
</tr>
<tr>
<td>Control</td>
</tr>
</tbody>
</table>

The genotypic distribution of POAG patients were not significantly different from controls (\( \chi^2=1.150 \), degrees of freedom=2, \( p=0.5627 \)). Similarly, there was no significant difference between the two groups in allelic distribution (\( \chi^2=0.559 \), degrees of freedom=1, \( p=0.4547 \)). In the Chinese control group the genotype distribution is reported to be 42.3%, 44%, and 13.6% for Arg homozygote, Pro/Arg heterozygote and Pro homozygote, respectively. On the other hand, the distribution of the genotypes in the same order in Chinese POAG patients is 20.7%, 44.8%, and 34.5% [12].

<table>
<thead>
<tr>
<th>Table 2. Genotype distribution and allelic frequencies of p53 intron 3 16-bp duplication polymorphism in control subjects and POAG patients</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genotype</strong></td>
</tr>
<tr>
<td>---------------</td>
</tr>
<tr>
<td><strong>Test Group</strong></td>
</tr>
<tr>
<td>POAG</td>
</tr>
<tr>
<td>Control</td>
</tr>
</tbody>
</table>

The genotypic distribution of POAG patients were not significantly different from controls (\( \chi^2=5.66 \), degrees of freedom=2, \( p=0.0590 \)), but there was a difference in the allelic distributions (\( \chi^2=4.81 \), degrees of freedom=1, \( p=0.0283 \)). The smaller number of individuals/ chromosomes for control samples compared to Table 1 is due to repeated failure of PCR done for amplification of 16 bp duplication polymorphism for a few samples.
we decided to construct a haplotype using two polymorphisms (a 16 bp duplication in intron 3 and codon 72 as a BsuRI RFLP in exon 4), which could be scored by amplification of a single region of the p53 gene and restriction digestion as described in Materials and Methods. In that context, we examined the distribution of p53 haplotypes based on 16 bp duplication in intron 3 and codon 72 bi-allelic polymorphism (Pro/Arg) in exon 4 to identify any association of a specific haplotype with the POAG patients, relative to healthy controls (Table 3). However, we did not observe any significant association of any haplotype with the POAG patient group (p=0.1831). It is noteworthy that none of the 334 chromosomes analyzed contained the ins-Arg haplotype while the ins-Pro haplotype was observed (see Table 3). This observation suggests that the 16 bp duplication in intron 3 might have occurred originally in the “codon 72 Pro” allele of the p53 gene, and that the codon 72 Pro/Arg polymorphism is older than the polymorphism resulting from the 16 bp duplication in intron 3 of the gene.

**DISCUSSION**

Despite the lack of any significant association between p53 alleles and POAG patients in our study, these results are important in the context of observed variation of such association in different population groups and underscores the importance of further investigation for more comprehensive understanding of this subject. It is, however, not surprising that we did not find any association of p53 codon 72 polymorphism with the Indian POAG patients. While any such observation would have further supported the view that retinal ganglion cells die during POAG by p53-mediated apoptosis, the issue is far more complex. No evidence exists to suggest that ganglion cells die in true or experimental glaucoma by necrosis, a death process traditionally associated with ischaemia [27]. Thus, it has been argued that the basis for suggesting that ganglion cell death in glaucoma by apoptosis is not because of any conclusive evidence but more because of lack of any firm demonstration of cell necrosis [11]. Investigators studying other systems also have been confronted with the challenges for ascribing a specific form of cell death to a given situation in most pathological tissue sections. Accordingly, various authors have described cell death as occurring by secondary necrosis, inter-current apoptosis and necrosis, sequential apoptosis/necrosis, or necrosis superimposed over apoptosis [28]. It is possible that in reality apoptosis and necrosis form a continuum at the extremes of a biochemically overlapping death pathway rather than being totally separate modes with only limited molecular and cellular biological overlap [28]. Support for such a view comes from other studies which have shown that increased internal calcium, and reactive oxygen species play major roles in both necrosis and apoptosis [11].

As mentioned earlier the association of codon 72 polymorphism in p53 gene has not been unequivocal in most studies. It is possible that the polymorphism is associated with the Chinese POAG patients but not with other ethnic groups. However, both the original and this present study are based on relatively modest sample sizes to definitively include or exclude this gene from the list of candidate susceptibility genes. Form that perspective further studies using much larger sample sizes are needed to try to resolve whether or not the association is real.

**ACKNOWLEDGEMENTS**

We thank the staff members of RIO and Drs. Rajarshi Neogi and Arunima Banerjee of IICB for their help in collection of blood samples from the POAG patients and controls. We also acknowledge with thanks the help and support provided by the Directors of both RIO and IICB. Dr. Keya Chaudhuri’s help in statistical analysis is gratefully acknowledged. The financial help from Council of Scientific and Industrial Research, India for the study as well as for the pre-doctoral fellowship to MA, AM, and SM is gratefully acknowledged. The study has been partially supported by grants from Department of Science & Technology (to SRC), Department of Biotechnology (to SRC), Government of India, and Indian Council of Medical Research (to KR and SRC).

**REFERENCES**

7. Sommer A, Tielsch JM, Katz J, Quigley HA, Gottsch JD, Javitt J, Singh K. Relationship between intraocular pressure and primary

The print version of this article was created on 2 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.