# A silent mutation in human alpha-A crystallin gene in patients with age-related nuclear or cortical cataract

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## ABSTRACT

A cataract is a complex multifactorial disease that results from alterations in the cellular architecture, i.e. lens proteins. Genes associated with the development of lens include crystallin genes. Although crystallins are highly conserved proteins among vertebrates, a significant number of polymorphisms exist in human population. In this study, we screened for polymorphisms in crystallin alpha A (*CRYAA*) and alpha B (*CRYAB*) genes in 200 patients over 40 years of age, diagnosed with age-related cataract (ARC; nuclear and cortical cataracts). Genomic DNA was extracted from the peripheral blood. The coding regions of the *CRYAA* and *CRYAB* gene were amplified using polymerase chain reaction and subjected to restriction digestion. Restriction fragment length polymorphism (RFLP) was performed using known restriction enzymes for *CRYAA* and *CRYAB* genes. Denaturing high performance liquid chromatography and direct sequencing were performed to detect sequence variation in *CRYAA* gene. *In silico* analysis of secondary *CRYAA* mRNA structure was performed using CLC RNA Workbench. RFLP analysis did not show any changes in the restriction sites of *CRYAA* and *CRYAB* genes. In 6 patients (4 patients with nuclear cataract and 2 with cortical cataract), sequence analysis of the exon 1 in the *CRYAA* gene showed a silent single nucleotide polymorphism [D2D] (*CRYAA*: C to T transition). One of the patients with nuclear cataract was homozygous for this allele. The *in silico* analysis revealed that D2D mutation results in a compact *CRYAA* mRNA secondary structure, while the wild type *CRYAA* mRNA has a weak or loose secondary structure. D2D mutation in the *CRYAA* gene may be an additional risk factor for progression of ARC.

KEY WORDS: Single nucleotide polymorphism; SNP; restriction fragment length polymorphism; crystallin; age-related cataract; nuclear cataract; cortical cataract; crystallin alpha A; crystallin alpha B; CRYAA; CRYAB; silent mutation

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## INTRODUCTION

A cataract is the result of alterations in the molecular architecture of the lens, specifically in the lens proteins [1]. Cataract is the leading cause of blindness, with 17.7 million people affected around the world [2]. Moreover, it is an irreversible age-related process for which there is no effective pharmacological treatment [3].

Crystallins are the predominant lens proteins, and they include alpha-, beta-, and gamma-crystallins. Alpha-crystallin is a large multimeric protein composed of two types of related subunits, alpha-A and alpha-B. Both of these subunits share sequence homology with other members of the small heat shock protein family [4] and exhibit chaperone-like activity in preventing the aggregation of other proteins [5].

Age-related cataracts (ARCs) include nuclear cataract, cortical cataract, and posterior subcapsular cataract (PSC). Nuclear cataracts are characterized by an increase in light scattering, often accompanied by yellow or brown coloration [6]. Cortical cataracts occur in mature fibre cells in the outer third of the lens, resulting in damage to the cytoplasm. They increase in severity by extending along the length of the affected fibre cells towards the optic axis. PSC cataracts usually amount to less than 10% of ARCs [7].

The association between different mutations in the crystallin alpha genes and cataracts has been identified in several studies. R21W mutation in crystallin alpha A (*CRYAA*) gene is associated with dominant cataract and microcornea [8]. R12C mutation in the *CRYAA* gene is associated with posterior polar, dense nuclear, and lamellar cataract with involvement of the anterior and posterior poles. R116H mutation in

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the *CRYAA* gene is also associated with nuclear cataract [8]. In the exon 2 of the *CRYAA* gene, F71L mutation resulted in defective chaperone-like function, associated with ARC [9]. Vicart et al. reported dominant myopathy associated with cataract, caused by R120G missense mutation. Further *in vitro* studies showed that R120G mutation causes defective chaperone-like functions in alpha-B protein [10]. Beta-crystallins are expressed from the early developmental stages in the eye lens; their expression continues and increases after birth, so that the highest concentrations are found in the lens cortex [11]. The molecular basis of crystallin alpha B (*CRYAB*) gene expression has not been completely understood. In mammals, the majority of the studies has been performed on the promoters of crystallin beta B1 (*CRYBB1*) and *CRYBB2* genes [12].

Although crystallins are recognized as highly conserved proteins among the vertebrate species, a significant number of polymorphisms exist in human population and the process of lens opacification remains unclear. Hence, the present study evaluated possible causative mutations in the alpha crystallin genes in the blood samples of patients with ARC.

# MATERIALS AND METHODS

## Patients and samples

The prospective study was conducted on patients with visually significant ARC who underwent extracapsular cataract extraction (ECCE) surgery in a tertiary care center in North India. The study was approved by the Institutional Ethical Committee. Written informed consent was obtained from patients, according to the institute's guidelines. The study adhered to the tenets of the declaration of Helsinki.

All patients underwent detailed ocular examination, including determining the type of cataract and fundus evaluation. In each case, the pupil was dilated with topical cyclopentolate (1%) and tropicamide (1%). The density of cataract was graded by slit-lamp biomicroscope, according to the Lens Opacity Classification System III [13].

The blood samples from the patients with nuclear or cortical cataract were obtained. Normal, healthy individuals having no cataract (as confirmed by the examination with slit-lamp biomicroscope) served as a control. The exclusion criteria were patients with a history of diabetes mellitus, traumatic cataract, or history of using systemic or topical steroids.

The mean age of patients with nuclear cataract (n = 100) was 68.25 ± 9.94 years and of those with cortical cataract (n = 100) was 58.65 ± 1.04 years. The mean age of the healthy controls (n = 100) was 43.4 ± 9.62 years. Because it was difficult to find healthy subjects of higher age without cataract, healthy controls with a wide age range were included in the study.

## DNA extraction

Genomic DNA was extracted from whole blood samples of the patients and controls using QiAamp DNA blood mini kit (Qiagen, USA).

## Restriction fragment length polymorphism (RFLP)

Each of the alpha-crystallin genes (*CRYAA* located on 21 and *CRYAB* located on 11 chromosome) consists of 3 exons. Most of the polymorphisms associated with cataract have been reported in the exon 1 of the *CRYAA* gene. In addition, polymorphisms detected by restriction enzymes HinfI, HhaI, and MSPI in the exon 1 of the *CRYAA* and by BsaJI enzyme in the exon 3 of the *CRYAB* gene have been previously described [7]. Ten µl of PCR products were digested with restriction enzymes in a total volume of 30 µl, containing 1X restriction buffer (Fermentas USA, USA and New England Biolabs, UK). The list of primers, restriction enzymes, and the size of RFLP products are described in Table 1.

Twelve percent polyacrylamide gel electrophoresis (PAGE) with 0.5 X TBE (45mM Tris/Borate buffer [pH 8.0]/1mM EDTA) was used to separate the RFLP products.

**TABLE 1.** A list of primers used for the amplification of the *CRYAA* and *CRYAB* genes and restriction enzymes used in this study

| Primers | Exon/Restriction enzyme | Normal allele/Mutated allele (bp) | Product size (bp) | Conditions |
|---|---|---|---|---|
| *CRYAA*<br>Forward-<br>5'-ctccagggtccccgtggta-3'<br>Reverse –<br>5'-aggagaggccagcaccac-3' | Exon 1/HinfI<br><br>Exon 1/MSPI | 202 and 52/146, 51, and 56 bp<br><br>116, 90, and 48/254 bp | 254 | 37°C, 10 min<br><br>37°C, 10 min |
| *CRYAA*<br>Forward-<br>5'-cacgcctttccagagaatc-3'<br>Reverse-<br>5'-ctctgcaaggggatgaagtg-3' | Exon 1/HhaI | 286, 96, and 84/382, 84 bp | 486 | 37°C, 16 hours |
| *CRYAB*<br>Forward-<br>5'-ttggtctcacctaagggt-3'<br>Reverse-<br>5'-ccattcacagtgaggacc-3' | Exon 3/BSaJI | 121 and 96/121, 86, and 10 bp | 217 | 55°C, 16 hours |

*CRYAA*: crystallin alpha A; *CRYAB*: crystallin alpha B; bp: base pair

## Denaturing high performance liquid chromatography (DHPLC)

The search of the National Center for Biotechnology Information (NCBI) (www.pubmed.com) database showed that the highest number of mutations resulting in cataract formation is associated with the exon 1 of the *CRYAA* gene. Thus, we focused on screening the entire exon 1 of the *CRYAA* for polymorphisms, and DHPLC and direct sequencing were performed to check the presence of any unknown mutations.

Variants in the exon 1 of the *CRYAA* gene, in the control and test DNA samples, were identified by ion-pair reversed-phase HPLC. The stationary phase is composed of polystyrene beads coated with alkyl groups. The mobiles phase contains triethylammonium acetate (TEAA) and acetonitrile. The negatively charged phosphate backbone of partially denatured DNA fragments are attracted to the positively charged ammonium groups of TEAA. At increasing concentrations of acetonitrile, the TEAA/DNA attraction is reduced and the fragments begin to elute from the cartridge. The variants were detected using a mutation detection and Navigator software (Model 3500 HT; Transgenomic, CT, USA).

## DNA sequencing

The PCR products were purified using Microspin S400 columns (Amersham Pharmacia, Little Chalfont, UK) prior to sequencing. Sequencing was performed using specific primers and ABI PRISM Big Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA). The DNA fragments were analyzed on an ABI PRISM 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA). The sequencing results were compared with the gene reference sequence (UCSC browser; http://genome.ucsc.edu/). Sequence alignments and analysis were performed using Clustal-X version 1.8 (http://www.molbiol.ox.ac.uk/documentation/clustalx/clustalx.html) and Finch TV (www.geospiza.com/finchtv) software, and compared to the sequences in the database using Basic Local Alignment Search Tool (BLAST).

## Secondary mRNA structure prediction

The secondary mRNA structures of wild type and mutant *CRYAA* alleles were analyzed using CLC RNA Workbench software (CLC bio, Cambridge, MA).

# RESULTS

The RFLP analysis of the coding regions of the *CRYAA* and *CRYAB* genes showed no change in the band pattern, in the patient and control samples.

In ten samples, variants in the exon 1 of the *CRYAA* were detected by DHPLC. Figure 1 shows a representative DHPLC profile of the patient and control samples. The variants detected by DHPLC were subjected to direct sequencing and compared to the sequences in the database, using BLAST. The sequencing results revealed GAC→GAT (5075 C > T) transition in the exon 1 of the *CRYAA* gene in six patients. Out of these six patients one was homozygous having both variant alleles (5075 C > T) of the *CRYAA* gene, whereas the rest of the patients were heterozygous having one wild type and one variant allele (Figure 2).

The single nucleotide polymorphism (SNP) [D2D] identified in the present study [GAC→GAT (5075 C > T)] in the coding region of the *CRYAA* gene is "silent" and does not alter the amino acid sequence (aspartic acid) at the second position. The frequency of this polymorphism in the nuclear cataract patients was 4% as compared to 2% in the cortical cataract patients. The genotypes and phenotypes of the affected individuals are summarized in Table 2.

To investigate the effect of the synonymous mutation on the secondary structure of *CRYAA* mRNA, we analyzed the mRNA sequence of the wild type *CRYAA* allele (GAC) and the mRNA of the variant *CRYAA* allele (GAT), with the mutation located near the initiation codon. The calculated free energy ($G^{o}$) for the wild type *CRYAA* mRNA was $\Delta G^{o}$ = -6.0 Kcal, compared to $\Delta G^{o}$ = -5.9 Kcal for the mutant *CRYAA* mRNA. The mutant *CRYAA* mRNA had a compact secondary structure around the initiation codon (Figure 3B), while the wild type mRNA had a loose secondary structure around ATG (Figure 3A).

# DISCUSSION

In the present study, we evaluated whether polymorphisms in the *CRYAA* and *CRYAB* genes are associated with ARC in patients with nuclear or cortical cataract, from the northern parts of India, compared to healthy controls. Based on the published data, we first screened for polymorphisms detected by restriction enzymes HinfI, MspI, and HhaI in

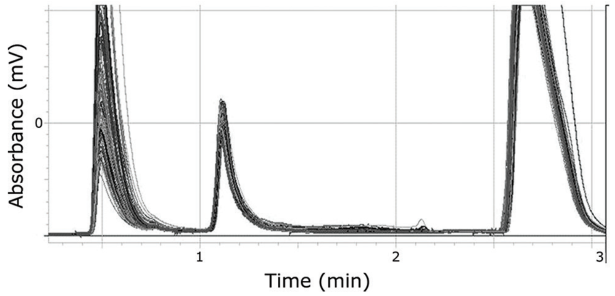


**FIGURE 1.** A representative denaturing high performance liquid chromatography chromatogram of the exon 1 of crystallin alpha A (*CRYAA*) gene.

the exon 1 of the *CRYAA* gene [8,14,15]and detected by BsaJI enzyme in the exon 3 of the *CRYAB* gene [10]. According to our RFLP results, there was no mutation in the cleavage sites of the *CRYAA* and *CRYAB* genes in the samples of nuclear and cortical cataract patients, nor in the control samples. Next, the exon 1 of the *CRYAA* gene was screened for the presence of mutations using DHPLC, followed by DNA sequencing, since the exon 1 of the *CRYAA* gene is known to be highly variable. Our analysis revealed the presence of a silent polymorphism, D2D SNP, in six patients.

D2D mutation is known to be associated with congenital cataract. In this study, four patients with nuclear cataract and two with cortical cataract had D2D silent mutation and one of the patients with nuclear cataract was homozygous for this allele. The affected patients were from different parts of North India, and were not closely related individuals. This C to T transition resulted in a codon change from GAC to GAT (aspartic acid) near the initiation codon ATG (methionine), and led to "silent variation" with no amino acid change.

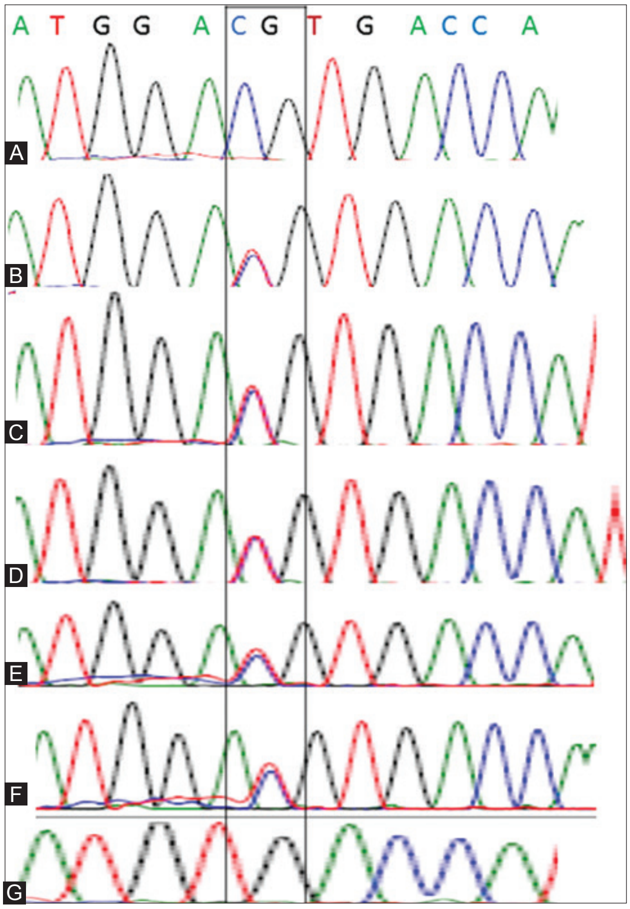


**FIGURE 2.** DNA sequence chromatograms of C to T transition in crystallin alpha A (*CRYAA*) gene. (A) Control sample. (B-F) Heterozygous transition (5075 C > T) in a cataract patient (one wild type [C] and one variant allele [T]). (G) Homozygous transition in a cataract patient (two variant alleles [T]).

D2D mutation in the exon 1 of the *CRYAA* gene was also reported in a Brazilian population [16]. Out of 10 patients with D2D polymorphism in their study, six had nuclear cataract and four patients had lamellar cataract. Among the affected patients, one patient also had R12C mutation in the *CRYAA* gene and another patient had a silent polymorphism S119S in the crystallin gamma D (*CRYGD*) gene, located on chromosome 2. In addition, among the 10 affected individuals, 9 had a silent polymorphism Y117Y or R95R in the *CRYGD* gene [16].

Generally, it was assumed that "silent" mutations are inconsequential to health, because such changes in DNA would not alter the protein makeup, encoded by genes. Hence, if a protein composition is correct, any small glitches in the process leading to its construction would not affect the health of an individual [17]. D2D mutation found in the present study resulted in the creation of a second initiation codon AUG, immediately following the original one. But if the second AUG was used as the initiation codon, it would result in a frameshift mutation and a truncated polypeptide. However, previous studies have shown that eukaryotic mRNA usually adhere to the first-AUG rule, which states that, in most cases, the triplet AUG nearest to the 5' end is the important site of translation initiation [18-20]. This led to the development of the scanning model, which highlights that 40S ribosomal subunit enters at the 5' end of mRNA and moves linearly, stopping when it encounters the first AUG codon [21]. Two mechanisms makes exceptions to the first-AUG rule. Reinitiation at a downstream AUG may be possible when the AUG codon that is nearest to the 5' end is followed shortly by a stop codon [19]. The second mechanism leading to the access to a downstream AUG codon is leaky scanning. Leaky scanning model highlights that 40S ribosomal subunit stops at the first AUG codon if it is present in the context of optimal Kozak consensus sequence (gcc) gccRccAUGG where R is a purine (adenine or guanine). The small subunit of the ribosome recognizes the AUG sequence on mRNA molecule as a translational start site from which a protein is encoded by the mRNA molecule [18,21]. If

**TABLE 2.** Genotype and phenotype characteristics of cataract patients

| Number | Sex | Age (years) | Phenotype | Genotype | Nucleotide substitution | Type of variant |
|---|---|---|---|---|---|---|
| 1 | M | 70 | Nuclear cataract | Heterozygous | C→T | Silent (D2D) |
| 2 | F | 76 | Nuclear cataract | Heterozygous | C→T | Silent (D2D) |
| 3 | M | 64 | Nuclear cataract | Homozygous | C→T | Silent (D2D) |
| 4 | M | 62 | Cortical cataract | Heterozygous | C→T | Silent (D2D) |
| 5 | M | 68 | Cortical cataract | Heterozygous | C→T | Silent (D2D) |
| 6 | F | 55 | Nuclear cataract | Heterozygous | C→T | Silent (D2D) |

*CRYAA* Human (wild type): MD*VTIQHPWFKRTLG, *CRYAA* Human (silent mutation): MD*VTIQHPWFKRTLG, *CRYAA*: crystallin alpha A; M: male; F: female

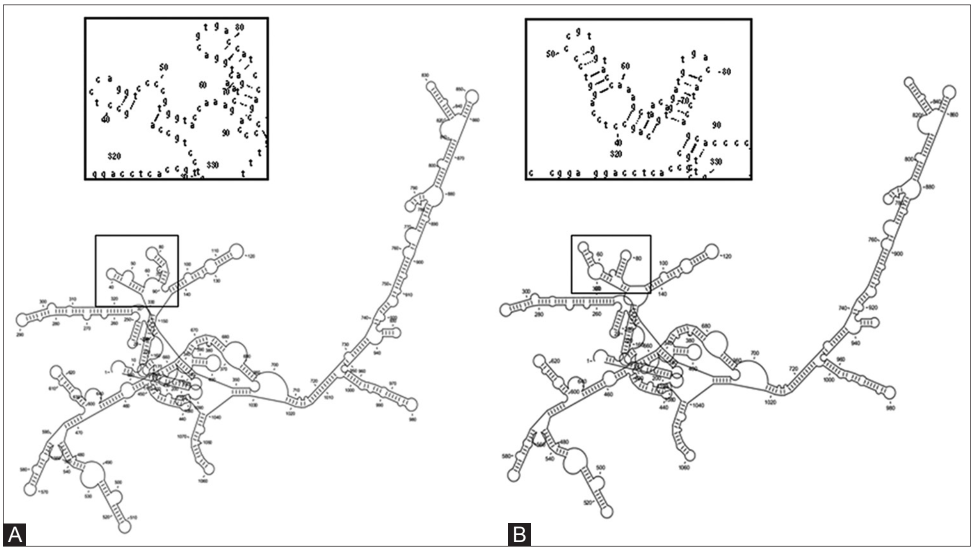

**FIGURE 3.** The alterations in the secondary structure of crystallin alpha A (*CRYAA*) mRNA, caused by a single base variation, are shown magnified in the box. (A) A loose secondary structure of wild type (GAC) *CRYAA* mRNA predicted by CLC RNA Workbench software. (B) A compact secondary structure of mutant type (GAT) *CRYAA* mRNA predicted by CLC RNA Workbench software.

the first AUG occurs in a suboptimal context during the translation process, that is, in the absence of purine in position -3 or G in position +4, some 40S subunits will pass this first AUG codon and initiate instead at the downstream site, leading to a formation of two proteins that were independently started from a single mRNA [22,23]. This type of dual translational initiation could be ruled out in the context of silent mutation found in the present study, since the newly introduced AUG is not surrounded by the Kozak sequence.

Using bioinformatic analysis, we predicted the secondary structures of the wild type and variant allele mRNA of the *CRYAA* gene. D2D silent mutation resulted in a compact mRNA secondary structure surrounding the initiation codon ATG of the variant allele, while the wild type mRNA had loose secondary structure in this region. This result suggests that D2D polymorphism could potentially affect the recognition of the initiation codon by the ribosome for protein synthesis. This in turn could alter the translation kinetics resulting in reduced synthesis of alpha-crystallin by the lens epithelial cells, leading to a deficiency of alpha-crystallin in the eye lens. Silent mutations resulting in changes of mRNA secondary structure as well as mRNA splicing site and altered translational kinetics have been reported previously [24].

Lens proteins, which play an important role in maintaining the transparency of eye, are synthesized throughout the life. The presence of D2D silent mutation could result in reduced deposition of alpha-crystallin in the eye lens. As *CRYAA* is known to have an important role in maintaining the proteins in soluble form, its deficiency could potentially lead to opacification of the lens. Thus, the D2D mutation could be an important risk factor for ARC. In addition, families of patients positive for D2D silent mutation should be informed about the possible consequences of this mutation.

The limitations of the present study include the difference in the average age between the cortical and nuclear cataract groups and the control group, which was mainly due to the unavailability of age-matched controls. In addition, it might be more beneficial to screen the complete *CRYAA* and *CRYAB* gene sequences for polymorphisms, that are directly or indirectly associated with development of nuclear or cortical cataract.

The important outcome of this study is the detection of D2D polymorphism in the cataract patients, which might have a role in ARC in north Indian populations and, thus, could be used as a risk predictor for ARC in this population.

## DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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