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A novel mutation (F71L) in αA-Crystallin with defective chaperone-like function associated with age-related cataract☆

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ABSTRACT

Age-related cataract (ARC) is a multifactorial disease and the leading cause of blindness worldwide. Genetic predisposition in association with other etiological factors may contribute to ARC. However, gene mutation studies on ARC are scanty. In the present work, we identified a genetic variation (F71L) in the exon-2 of *CRYAA* (αA-crystallin) gene in three unrelated female sporadic cases among 711 ARC patients but not in 265 normal non-cataractous controls by SSCP and RFLP analysis. By comparing human recombinant wild-type and F71L-αA-crystallin, we characterized the functional significance of this missense mutation. Chromatography, fluorescence and far- and near-UV CD studies indicated that F71L missense mutation did not significantly affect the apparent molecular mass, secondary and tertiary structures and hydrophobicity of αA-crystallin. While the mutant αA-crystallin displayed significant (35–90%) loss of chaperone-like activity (CLA) in thermal aggregation of carbonic anhydrase, βL- and γ-crystallins, it showed moderate (10–50%) loss in CLA in DTT-induced aggregation of insulin and lysozyme. This is the first report of an αA-F71L mutation being associated with ARC and suggests that ARC in individuals carrying this mutation (F71L) might be due to the overall loss of in vivo chaperone activity due to interaction with other environmental factors.

1. Introduction

Cataract results from loss of transparency of the normal crystalline eye lens mainly due to the disruption of micro architecture of lens. Human cataract is a debilitating eye disease that afflicts millions worldwide. Cataract accounts for an estimated 16 million cases of blindness worldwide, with approximately half of all cases originating from Africa and Asia [1,2]. Despite the availability of treatment, cataracts still comprise a significant risk for visual impairment all over the world, particularly in older individuals. Approximately 80% of cataracts are age-related, the cause of which is unknown but, generally age-related cataracts (ARC) are considered as a multifactorial disease [1,2]. In the developing countries prevalence of cataracts is higher and onset is earlier, implicating contribution of additional socio-demographic and medical factors including genetic to the development of ARC.

Although, epidemiological research has been focused mostly on the role of environmental risk factors in ARC, recent studies provided the evidence for the contribution of genetic factors in the pathogenesis of ARC [3,4]. The Lens Opacity Case Control Study indicated that a positive family history was a risk factor for mixed nuclear and cortical cataracts [5] and the Italian American Cataract Study Group supported a similar role for family history as a risk factor in cortical, mixed nuclear and cortical, and posterior subcapsular cataracts [6]. The Framingham Offspring Eye Study showed that individuals with an affected sibling had three times the likelihood of having a cataract [7]. Results of the Beaver Dam Eye Study suggested that a single major gene could account for as much as 35% of nuclear and up to 75% of cortical cataract variability [8]. The twin eye study recognized importance of genetic component with heritability accounting for

Abbreviations: ANS, 8-anilino-1-naphthalene sulfonic acid; ARC, age-related cataracts; CA, carbonic anhydrase; CD, circular dichroism; CLA, chaperone-like activity; RFLP, restriction fragment length polymorphism; SSCP, single strand conformation polymorphism

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53–58% of the liability for age-related cortical cataract and indicated that hereditary tendency was consistent with a combination of additive and dominant genes [9]. Similarly, the genetic factors were found to account for approximately 48% of the risk for nuclear cataract [10].

A recent study identified several loci through the genome scan on age-related cortical cataracts [11]. Although, these loci did not coincide with the map coordinates of known crystallin genes, the study revealed the weaker loci are critical for age-related cortical cataract or act as modifiers, and suggested that the pathways and genes that are less crucial to the development of the lens fibers may play a role in ARC. Over the past few years, attempts are being made for the identification of new loci for ARC. Identifying the genes and characterizing the proteins they encode will provide better comprehension of the molecular processes that are involved in the cataract development.

Although, evidence for the genetic component to the development of ARC is increasing, mutation(s) in gene(s) that are associated with ARC are very few compared to those for congenital and hereditary cataracts. α-Crystallin, a member of the small heat shock protein family constitutes a major portion of the eye lens cytoplasm [12]. Eye lens α-crystallin is composed of two subunits, αA and αB, encoded by *CRYAA* and *CRYAB* genes respectively. In humans, the *CRYAA* gene is found on chromosome 21 and codes for a 173-residue protein, while the *CRYAB* gene is found on chromosome 11, coding for a 175-residue protein [13]. It is now recognized that α-crystallin functions like a chaperone and plays a decisive role in the maintenance of eye lens transparency (reviewed in [13,14]). Conversely, it is well established that certain point mutations in αA and αB-crystallin genes are linked with non-syndromic, hereditary human cataracts (reviewed in [13,14]). In αA-crystallin, R49C and W9X mutations in the N-terminal domain, and the R116C, R116H, and G98R in the C-terminal domain are known to cause human cataracts. In αB-crystallin, the C-terminal R120G, 450delA, D140N mutations, and the N-terminal P20S mutation cause human cataracts. Many of these mutations are inherited by autosomal dominant mechanisms expressing congenitally or early in life. We have investigated the genetic variations in the three exons of *CRYAA* gene among patients with different types of ARC and report the mutations detected in exon-2 of *CRYAA*, which was further analyzed for its functional role in causing opacity of the lens.

2. Subjects, materials and methods

2.1. Patients

We conducted a case–control study of different types of ARC during the period of "2003–2009". The Ethics Committee of Department of Genetics, Osmania University, approved this study. About 711 cases with different types of ARC were screened for mutations in the exon-2 of *CRYAA* gene. The patients studied were referred to Sarojini Devi Eye Hospital (SDEH) and Institute of Ophthalmology, Hyderabad, India. Diagnosis of different types of cataracts was done based on the silt-lamp examination by an ophthalmologist following LOC-III Classification.

2.2. Inclusion and exclusion criteria

Only primary cases of cataracts were included in the present study. Patients with secondary cataracts like acquired cataracts that are caused due to trauma, toxins, etc., and the complicated cataracts that occur due to inflammatory and degenerative ocular diseases have been excluded from the study. In addition, patients with associated conditions like diabetes, hypertension, myopia, glaucoma, any syndrome and those who were under medication like steroids etc., were also not considered.

2.3. Controls

A total of 265 randomly selected age and sex matched normal healthy individuals without the history of cataract, diabetes, hypertension, and other ocular diseases were used as controls to compare with patients group.

2.4. Collection of data and blood samples

From all the patients and controls included in the study, information was collected using a specified proforma. The information recorded included—sex, age, age at onset, duration of disease, type of cataract, occupation, body mass index (BMI), menarcheal and menopausal history for female patients, any other associated conditions, information on habits, diet and detailed four generation family history. The information collected was cross-matched with the accompanying relatives who were mostly first-degree relatives. Five to 10 ml of venous blood was collected in EDTA vaccutainers from ARC patients and control subjects. The samples were collected with informed consent of the subjects after explaining to them the purpose of the study. Samples were collected only from those who voluntarily co-operated to participate in the study.

2.5. DNA isolation

DNA was isolated from blood using salting out method [15].

2.6. SSCP analysis

Mutation screening was carried out using single strand conformation polymorphism (SSCP) analysis. PCR amplification (Applied Biosystems-9800 Fast Thermal Cycler) was performed with 50 ng of genomic DNA, in 100 μl PCR reaction mixture containing 10 μl 10× PCR buffer 200 μM each dATP, dGTP, dTTP, dCTP; 5.0 pmol of each primer and 2.5 U taq polymerase. The primers used were forward primer—5′-CTGTCTCTGCCAACCCCAGCAG-3′ and reverse primer—5′-CCCTGTCCC-ACCTCTCAGTGCC-3′. The PCR conditions were, initial denaturation at 95 °C for 2 min, denaturation at 94 °C for 45 s, annealing at 65 °C for 45 s, and extension at 72 °C for 45 s, for 30 cycles followed by final extension at 72 °C for 5 min. The PCR products (223 bp) were run on 0.8% agarose gel using ethidium bromide stain and monitored under UV transilluminator. The PCR products were denatured and snap cooled immediately. The denatured PCR products were analyzed by non-denaturing 12% polyacrylamide gel electrophoresis at 100 V for 16–18 h at constant temperature using an Amersham (Pharmacia Biotech SE600-15-1.5) electrophoresis unit. Silver staining was done to visualize the bands. With the detection of a mobility shift, the exact nature of mutation was confirmed by sequencing on an automated DNA sequencer (Applied Biosystems).

2.7. Restriction fragment length polymorphism (RFLP) analysis

For RFLP analysis the primers were specifically designed by introducing 2 mismatches within the forward primer (forward primer—5′-AGGTTCAATCCGACCGGGACAAGAT-3′ and reverse primer—5′-CCCTGTCCCACCTCTCAGTGCC-3′) which generated two recognition sites for Dpn II restriction enzyme one at 22 bp and another at 75 bp of amplified products. The digested products produce three fragments of 75, 60 and 22 bp for wild-type and only two fragments of 97 and 60 bp for mutant due to loss of site at 22 bp. PCR amplification was performed with 50 ng of genomic DNA in 100 μl PCR reaction mixture containing 10 μl 10× PCR buffer (100 mM Tris–HCl, pH 8.8, 500 mM KCl, 15 mM $MgCl_2$, 0.01% w/v gelatin), 200 μM each dATP, dGTP, dTTP, dCTP, 5.0 pmol of each primer and 2.5 U taq polymerase. The PCR conditions were: initial denaturation 95 °C for 2 min, denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min, for

30 cycles followed by final extension at 72 °C for 5 min. The PCR products (157 bp) were checked for amplification on 0.8% agarose gel using ethidium bromide stain under UV transilluminator. The PCR products were restriction digested with 2 U of Dpn II restriction enzyme (*5′↓GATC restriction site*) (New England Biolabs) at 37 °C for 12 h. The restriction fragments were separated on 15% acrylamide gels by electrophoresis for 3 h at 150 V. The staining of the PAGE gels was done using ethidium bromide and the bands were visualized under UV transilluminator.

2.8. Cloning and expression of wild-type and mutant αA-crystallin

Human αA-crystallin cDNA was cloned into pET-23d (+) vector (Novagen, Gibbstown, NJ) [16,17]. This cloned cDNA was used as a template to generate the F71L mutation in the αA-crystallin gene using a quick-change site directed mutagenesis kit (Stratagene, La Jolla, CA) and the mutagenic primer (5′-CCGACCGGGACAAGTTAGTCATCTTCCTCG-3′). The F71L mutation was confirmed by automated DNA sequencing. Expression plasmids were introduced into *Escherichia coli* strain BL21 and cultured as previously described for overexpression of recombinant proteins [16,17].

2.9. Purification of the wild-type and mutant recombinant αA-crystallin

The recombinant wild-type and F71L αA-crystallins were overexpressed in *E. coli* using IPTG, the proteins were purified to homogeneity essentially according to previously reported methods [17]. The homogeneity of purified F71L and wild-type αA-crystallin was assessed by SDS-PAGE and simultaneously with immunodetection using polyclonal anti-αA-crystallin antibodies [18]. The concentrations of wild-type and mutant proteins were estimated by Lowry method.

2.10. Size exclusion chromatography

The molecular size of the wild-type and mutant proteins was determined by the gel filtration chromatography on a 600×7.5 mm TSK-G4000 SW column (Tosoh Co., Japan) using a Shimadzu HPLC system [19]. The column was equilibrated with 0.1 M sodium phosphate buffer, pH 6.9, containing 0.1 M sodium sulfate at a flow rate of 1 ml/min. Column was calibrated using standard molecular weight markers (thyroglobulin—669, γ-globulin—160, BSA—67, ovalbumin—45 kDa).

2.11. Tryptophan fluorescence

The intrinsic tryptophan fluorescence spectra of wild-type and mutant αA-crystallin were analyzed using Jasco-FP-6500 spectrofluorometer (JASCO Corporation, Tokyo, Japan). Protein samples of 0.1 mg/ml in 20 mM sodium phosphate buffer pH 7.2 were excited at 280 nm and emission spectra were recorded between 300 and 400 nm.

2.12. ANS fluorescence

The relative surface hydrophobicity of wild-type and mutant αA-crystallin was measured using 8-anilino-1-naphthalene sulfonic acid (ANS). Protein samples of 0.1 mg/ml were added to 50 μM of ANS solution in 10 mM of sodium phosphate buffer. The samples were excited at 385 nm and the emission spectra were recorded between 400 and 600 nm using a Jasco-FP-6500 spectrofluorometer.

2.13. Circular dichroism (CD) studies

Secondary and tertiary structures of the protein were evaluated by far- and near-UV CD spectra in a Jasco-810 spectropolarimeter (JASCO Corporation, Tokyo, Japan). Protein concentrations used for far- and near-UV were 0.1 and 0.5 mg/ml, respectively in 0.1 mg/ml in 20 mM sodium phosphate buffer, pH 7.2. All the CD spectra were an average of six accumulations and corrected with respected blanks.

2.14. Chaperone assays

Chaperone-like activity (CLA) of wild-type and F71L mutant αA-crystallin was assessed by measuring the ability of these proteins to suppress the aggregation of different client proteins under different assay conditions. Heat-induced aggregation of βL-crystallin, γ-crystallin, carbonic anhydrase (CA), and the DTT-induced aggregation of insulin and lysozyme were monitored by measuring light scattering as a function of time at 360 nm using a Lamda-35 spectrophotometer (Perkin-Elmer) essentially according to the previously described methods [17,20,21].

3. Results

The baseline characteristic features of 711 cases studied showed a higher incidence in female as compared to males in almost all types of ARC (Table 1). The mean ages at onset of cataracts were 60.1 ± 9.69 in

Table 1
Baseline characteristic features found in different types of age-related cataract patients and control subjects studied.

	NC		CC		PSC		MT		Total cases		Controls	
	N	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
Total	205	28.8	182	25.5	178	25.0	146	20.5	711	–	265	–
Males	93	45.4	86	47.5	75	42.1	61	42.0	315	44.3	163	61.8
Females	112	54.6	96	52.7	103	57.9	85	58.0	396	55.7	102	38.5
Early onset	22	10.7	48	26.4	55	30.9	33	23.0	158	22.2	–	–
Late onset	183	89.2	134	73.6	123	69.1	113	77.0	553	77.8	–	–
Familial	34	16.5	30	16.4	28	15.7	04	03.0	96	13.5	18	6.7
Non-familial	171	83.4	152	83.5	150	84.3	142	97.0	615	86.5	247	93.2
Consanguineous	36	17.5	31	17.0	39	21.9	16	11.0	122	17.2	28	10.6
Non-consanguineous	169	82.4	151	82.9	139	78.1	130	89.0	589	82.8	237	89.4
Tobacco	61	29.7	55	30.2	48	26.9	48	33.0	212	29.8	–	–
Non-tobacco	144	70.2	127	69.8	130	73.0	98	67.0	499	70.2	–	–
Smokers	51	34.8	47	54.6	40	53.3	26	43.0	164	52.1	67	41.1
Non-smokers	42	43.7	39	15.3	35	46.7	35	57.0	151	47.9	96	58.9
Alcholics	46	22.4	30	16.5	33	18.5	36	25.0	145	20.4	89	33.6
Non-alcoholics	159	77.6	152	83.5	145	81.5	110	75.0	566	79.6	176	66.4
Vegetarians	10	4.9	19	10.4	05	2.8	11	08.0	45	6.3	37	14.0
Non-vegetarians	195	95.1	163	89.6	173	97.2	135	92.0	666	93.7	228	86.0

For smokers data, only male subjects of cases and controls were reported to be smokers; NC—nuclear cataracts, CC—cortical cataracts, PSC—posterior subcapsular cataracts, MT—mixed type cataracts.

nuclear (NC); 56.6 ± 10.5 in cortical (CC); 54.9 ± 10.8 in posterior subcapsular (PSC) and 58.0 ± 9.91 in mixed type (MT) cataract patients. The incidence of early onset cases of ARC (age-at-onset ≤50 years) was high in PSC (31%) and CC (26%) as compared to NC (11%). The low frequency of NC cases among early onset of ARC is expected as the mean age at expression for the condition in the present study was 60.1 ± 9.69. Table 1 also provides further information such as the frequency of smoking, alcohol consumption, family history and consanguinity of control subjects and cataract patients.

A mobility shift was observed following SSCP analysis of exon-2 of the *CRYAA* gene in 3 female patients (2 with nuclear and 1 with mixed type cataract) out of 711 subjects diagnosed with ARC. Such change was not observed among 265 age-matched controls (Fig. 1A). The mobility shift indicated that the mutation could be homozygous. Direct sequencing of amplified products from these patients identified a novel single base alteration of C > A transversion at position 213 (counting the A of the start codon as no. 1) in exon-2 of *CRYAA* gene. This change predicts a substitution of leucine (TTA; L) for phenylalanine (TTC; F) at amino acid position 71 (F71L) (Fig. 1B). The chromatogram confirmed the mutation was homozygous for the transversion C > A. The age-at-onset of cataract in these patients ranged between 48 and 50 years and hence was considered as early onset of ARC in view of the mean age of NC cases (60 years) found in the present study. This genetic change was further confirmed by RFLP analysis by designing primers with mismatch. Restriction fragment length polymorphism analysis of ARC patients was done using Dpn II restriction enzyme that recognizes *5′↓GATC* site. The analysis showed three fragments of 75, 60 and 22 bp for wild-type and two fragments of 97 and 60 bp for mutant forms as expected (Fig. 1C). This confirmed the homozygosity of the mutation F71L in all the 3 patients. All the 3 patients with the mutations were unrelated and did not report any family history of congenital or senile cataracts at the time of investigation.

We then cloned both wild-type and mutant genes and expressed in *E. coli* to understand the effect of F71L conversion on structural and the chaperone properties. Fig. 2A shows the SDS-PAGE profile of wild-type and mutant proteins before and after induction as well as homogeneity of purified recombinant αA-crystallins. Polyclonal anti-αA-crystallin antibodies [18] were able to recognize the F71L-αA-crystallin (Fig. 2B). Size exclusion chromatography analysis revealed similar elution time both for the mutant and wild-type proteins suggesting insignificant differences in the molecular masses of their oligomeric complexes (Fig. 3). Dynamic light scattering (DLS) data also suggest insignificant change in hydrodynamic volume of the

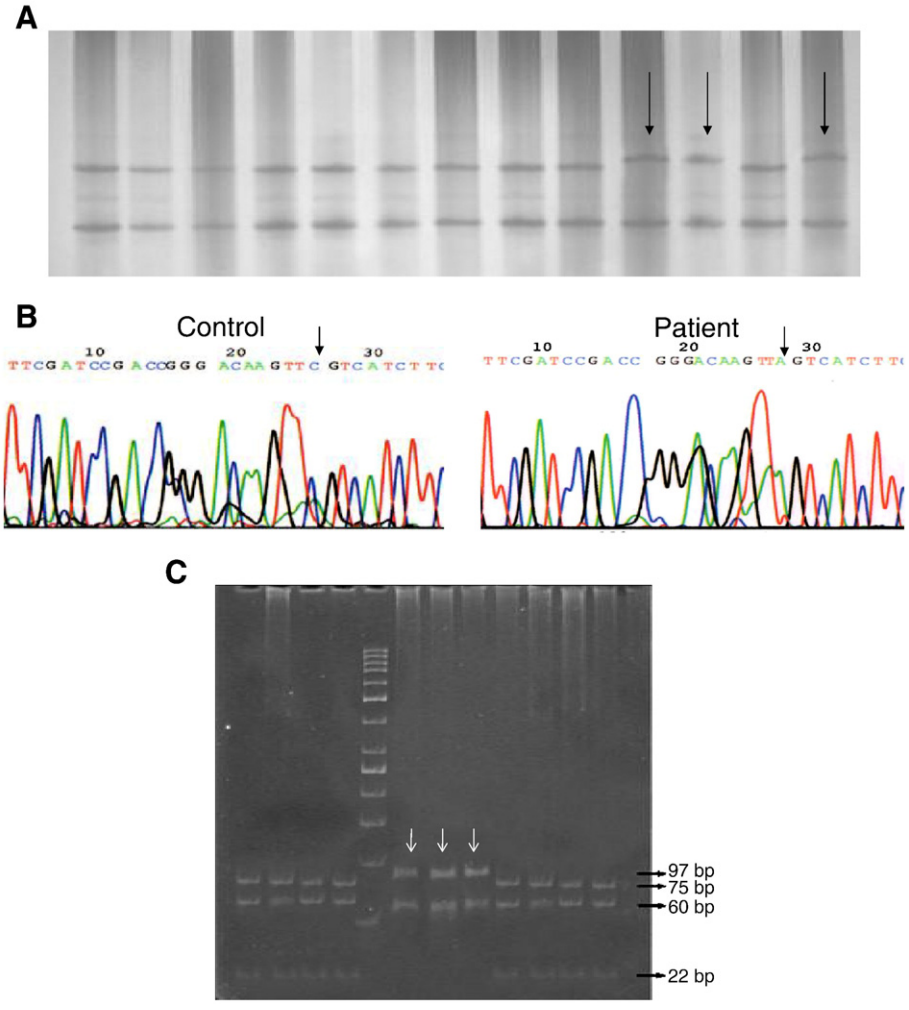

Fig. 1. Screening of mutations in *CRYAA* exon-2 in normal subjects and ARC patients. *Panel A:* SSCP gel showing the mobility shift pattern in the 3 patient samples of ARC (indicated by an arrow). Lanes with mobility shift (shown with arrows) represent the samples from two nuclear cataract and one mixed type cataract patients with F71L mutation on 12% non-denaturing PAGE gels. Rest of the lanes (without mobility shift) represents samples of non-cataractous healthy control subjects and samples from ARC patients without mutation. *Panel B:* Sequence analysis of a partial fragment of exon-2 of the *CRYAA* gene. Arrows indicate substitution of C > A at position 213 which resulted in the substitution of phenylalanine (TTC) to leucine (TTA) at amino acid position 71 (F71L). *Panel C:* RFLP analysis of PCR products of exon-2 of the *CRYAA* gene upon restriction digestion with Dpn II restriction enzyme generating fragments of 75, 60 and 22 bp for wild-type (without mobility shift) and fragments of 97 and 60 bp for mutant form (mobility shift shown with arrows).

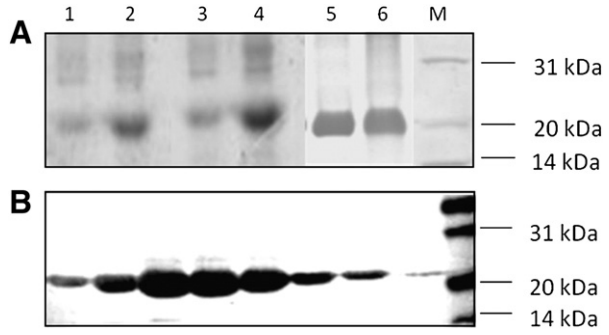

Fig. 2. *Panel A:* SDS-PAGE profile of wild-type and F71L-αA-crystallin at various stages of expression and purification. Lanes 1 and 2 represent before and after induction of wild-type, lanes 3 and 4 represent before and after induction of F71L αA-crystallin, lanes 5 and 6 represent purified wild-type and F71L αA-crystallin and lane M represents molecular weight marker. *Panel B:* Immunoblot analysis of fractions of F71L-αA-crystallin obtained from final gel filtration step using polyclonal αA-crystallin specific antibody.

protein due to this mutation (data not shown). Both the far- and near-UV CD spectra (Fig. 4) of αAF71L mutant protein were similar to wild-type αA-crystallin suggesting no major difference in their secondary and tertiary structures, respectively.

Intrinsic tryptophan fluorescence of the proteins showed that the emission maxima for both proteins were similar, indicating no change in the tryptophan environment of the F71L mutant protein when compared to wild-type protein (Fig. 5A). Further, tryptophan fluorescence data corroborate near-UV CD spectra supporting the findings that F71L mutation is unlikely to affect the tertiary structure. As it is understood, ANS is a hydrophobic site-responsive probe, which has negligible fluorescence in aqueous media. Binding of this probe to hydrophobic sites in proteins results in a several fold enhancement in its florescence with a shift in the emission maximum to a lower wavelength. The ANS fluorescence emission spectra of both the mutant and wild-type protein were similar suggesting unaltered surface hydrophobicity due to this mutation (Fig. 5B).

In heat-induced aggregation of βL-crystallin, the F71L mutant showed about 50% less CLA as compared to wild-type αA-crystallin (Fig. 6). Similarly, the CLA of F71L mutant was decreased by approximately 35% in heat-induced aggregation of γ-crystallin when compared to wild-type of αA-crystallin (Fig. 7A). F71L mutant displayed almost no CLA (90% loss) in heat-induced aggregation of CA as compared to wild-type αA-crystallin (Fig. 7B). Although, CLA measurements were routinely carried out at high temperature (60 °C), the physiological relevance of these assays is in question as the human

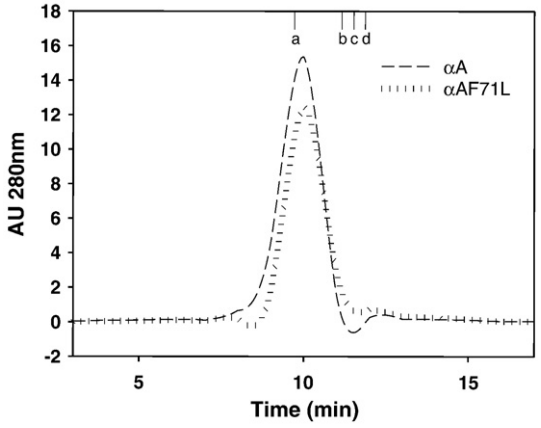

Fig. 3. Size exclusion chromatography profile of wild-type and F71L-αA-crystallin on a TSK G-4000 SW size exclusion column. Position of molecular weight markers is shown on top, (a) thyroglobulin—669, (b) γ-globulin—160, (c) BSA—67 and (d) ovalbumin—45 kDa.

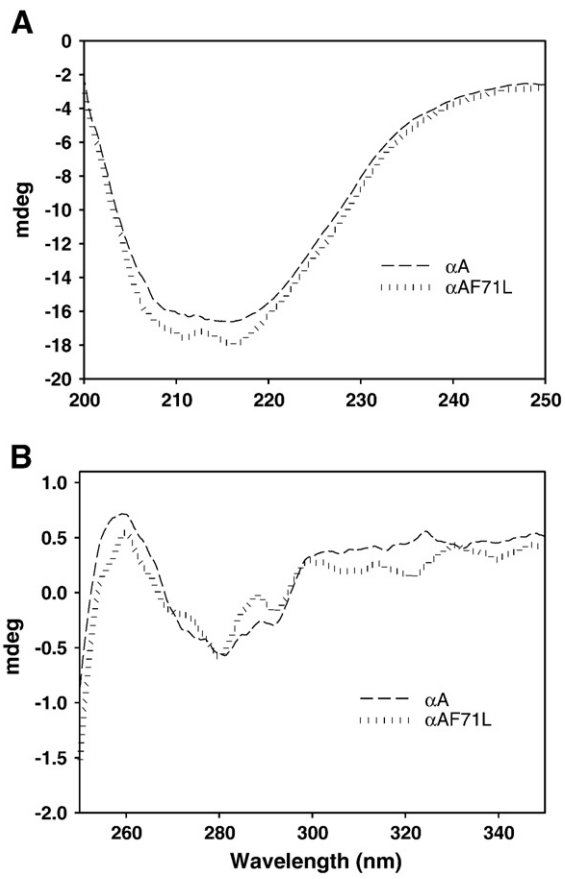

Fig. 4. Far- (*Panel A*) and near-UV CD spectra (*Panel B*) of wild-type and F71L-αA-crystallin.

lenses seldom expose to that high temperature. Therefore, we have measured CLA of native and mutant αA-crystallins at physiological temperatures. Similar to heat assays, in DTT-induced aggregation of lysozyme assay, the F71L mutant showed about 50% less CLA as compared to wild-type αA-crystallin (Fig. 8B). However, decrease in the CLA of F71L mutant was marginal (10–15% loss) in DTT-induced aggregation of insulin when compared to wild-type of αA-crystallin (Fig. 8A).

4. Discussion

In this study, we identified a novel mutation in the exon-2 of *CRYAA* gene in three unrelated female sporadic cases among 711 ARC patients and characterized the functional significance of this missense mutation. The data revealed that despite insignificant structural changes, the F71L mutant αA-crystallin displayed significant loss of CLA in thermal and chemical aggregation assays.

Previous epidemiological studies have established that cataract development is affected by various factors such as diabetes, female gender, sunlight or UV light exposure, myopia, nutritional deficiencies, trauma and steroid use [1,2,22,23]. Although, epidemiological studies reported the involvement of genetic factors in the pathogenesis of age-related cataracts [3–11], the roles of specific genes causing susceptibility to the condition are not established and only a beginning is made with focus on unraveling underlying genetic basis and genome wide scanning for ARC. However, there are now several reported mutations in congenital and hereditary cataracts involving genes that encode proteins with structural, metabolic, transport functions [24–26]. It is plausible that some of these genes may be involved in adult cataract with late expression of phenotype due to interaction with several noxious factors along with aging process.

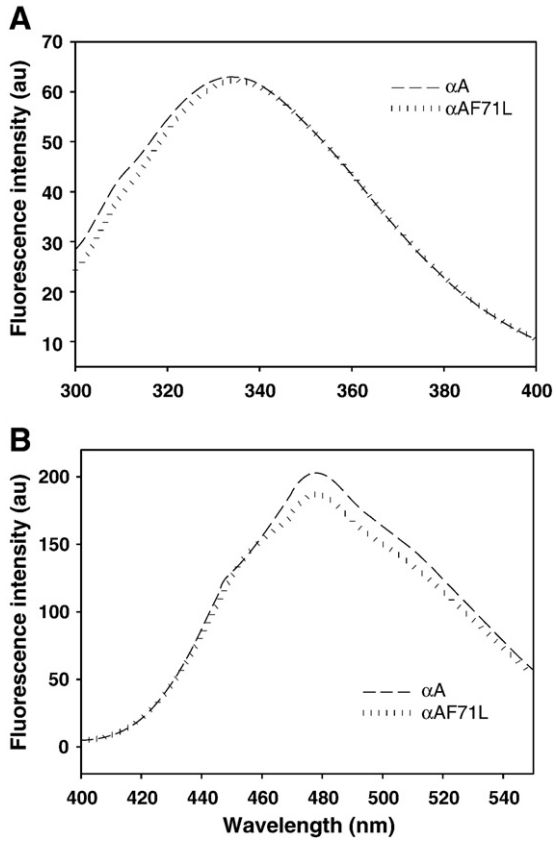

Fig. 5. Tryptophan (*Panel A*) and ANS fluorescence spectra (*Panel B*) of wild-type and F71L-αA-crystallin.

Crystallins are the major structural proteins in the lens accounting for up to 90% of total soluble protein [12,13,27]. There are three distinct families of crystallins: α-, β- and γ-crystallins, whose structure, stability and short-range interactions are thought to contribute to the lens transparency [12,13,27]. The human lens is also susceptible to age-related degenerative changes such as accumulation of insoluble proteins and oxidative damage and hence senile cataract has become the most common form of blindness [27]. In humans, lenticular α-crystallin exists as a hetero-oligomer of approximately 800 kDa with two subunits, αA and αB occurring in a stoichiometry of 3:1 [12,14,20]. αA-Crystallin appears to be largely lens-specific, whereas αB-crystallin is also expressed in many other tissues. Both of these proteins are known for their chaperone activity as evident from suppression of protein aggregation [13,14]. Thus they presumably protect other lens proteins from the adverse effects of heat, chemicals, and UV light irradiation. Hence, α-crystallins are instrumental in maintaining transparency of the lens with their CLA [13,14].

Recently, several mutations in α-crystallins have been associated with cataract in humans [3,13,14, 24–26,28]. But there are no reports on *CRYAA* gene mutations in ARC. This is the first report of an F71L mutation in αA-crystallin being associated with ARC in Indian patients. Nevertheless, the reasons for the time lag shown by this mutation in the expression of a cataract phenotype around 50 years of age are not clear. Certain mutations may have delayed expression, mostly because of interaction with several triggering factors including aging, cross-linking of DNA and proteins resulting in the formation of adducts. Therefore, we have investigated the underlying molecular basis of F71L mutation in causing cataract by biochemical and biophysical approaches.

It is observed that this mutation did not lead the gross structural changes of the protein in terms of apparent molecular mass of oligomeric complex, the secondary and tertiary structures and the surface hydrophobicity of αA-crystallin under native conditions. Interestingly, the CLA of F71L mutant αA-crystallin varied with different client proteins under different assay conditions. For example, it showed loss (15–90%) of CLA in thermal and chemical aggregation assays compared to native recombinant αA-crystallin. A previous study also reported that Phe71 is essential for CLA of αA-crystallin [29]. Replacement of Phe71 with Gly (F71G) did not result in significant structural changes, but had severely impaired CLA of αA-crystallin [29]. Likewise, there are many instances where alterations in oligomeric size, structure and hydrophobicty are not uniformly associated with loss or gain of CLA of α-crystallin.

Although, there are no gross structural changes, αA-crystallin subunits encoded by the F71L mutant allele might be affected by minor but subtle alterations and may be less stable than wild-type subunits. Given that lens polypeptides normally have an extremely long half-life in lens fiber cells, it seems reasonable that loss of stability associated with the F71L mutation could result in a premature loss of overall CLA in the lens. Indeed we observed an increased light scattering by the mutant protein at 85 °C as compared to wild-type αA-crystallin (Fig. 9). Further, during DLS studies F71L mutant was found to precipitate on preheating (heating for 15 min at 60 °C and cooled back to room temperature) where as wild-type

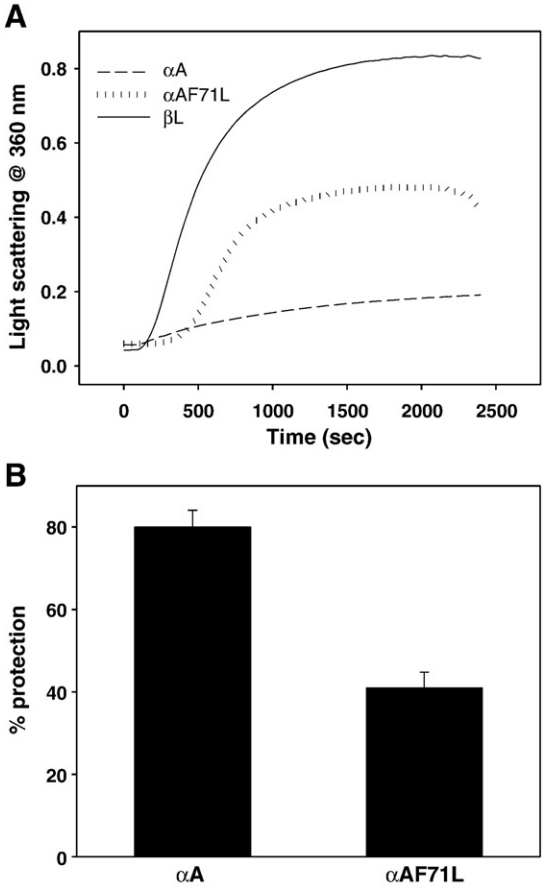

Fig. 6. Chaperone-like activity of wild-type and F71L-αA-crystallin in heat-induced aggregation of βL-crystallin assay. *Panel A:* Representative graph of chaperone activity of αA-crystallin in suppressing heat-induced aggregation of βL-crystallin at 60 °C. Solid curve—0.25 mg/ml of βL-crystallin; dashed curve—0.25 mg/ml of βL-crystallin with 0.05 mg/ml of wild-type αA-crystallin and dotted curve, 0.25 mg/ml of β-crystallin with 0.05 mg/ml of F71L-αA-crystallin. *Panel B:* Relative chaperone activity (percentage protection) of wild-type and F71L-αA-crystallin in βL-crystallin assay. Percentage protection was determined considering aggregation of βL-crystallin in the absence of α-crystallins as 100%. Data are mean ± SD ($n = 4$).

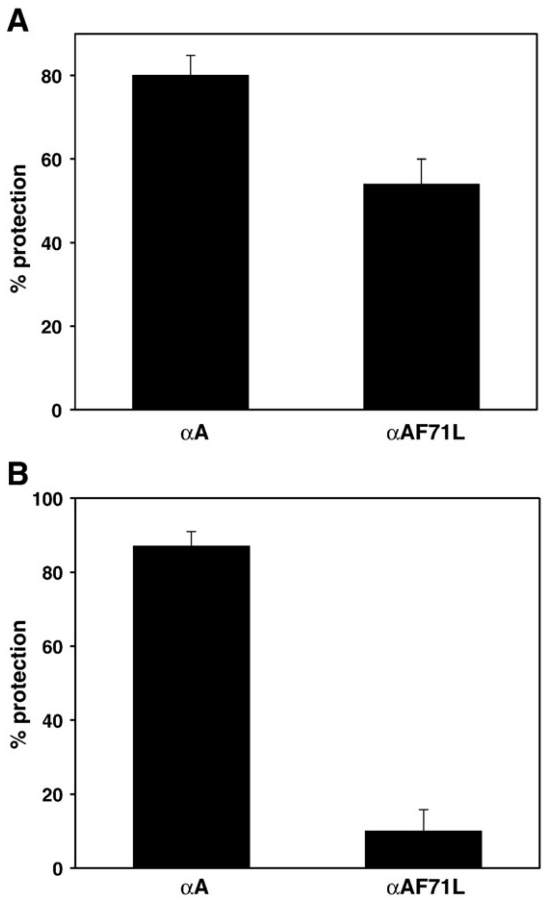

Fig. 7. *Panel A:* Relative chaperone activity of wild-type and F71L-αA-crystallin in heat-induced aggregation γ-crystallin assay. Percentage protection was determined considering aggregation of γ-crystallin (0.2 mg/ml) in the absence of α-crystallins as 100%. Concentration of wild-type and F71L αA-crystallin was 0.025 mg/ml. Data are mean ± SD ($n=4$). *Panel B:* Relative chaperone activity of wild-type and F71L-αA-crystallin in heat-induced aggregation carbonic anhydrase (CA) assay. Percentage protection was determined considering heat-induced aggregation of CA (0.2 mg/ml) in the absence of α-crystallins at 60 °C as 100%. Concentration of wild-type and F71L αA-crystallin was 0.2 mg/ml. Data are mean ± SD ($n=4$).

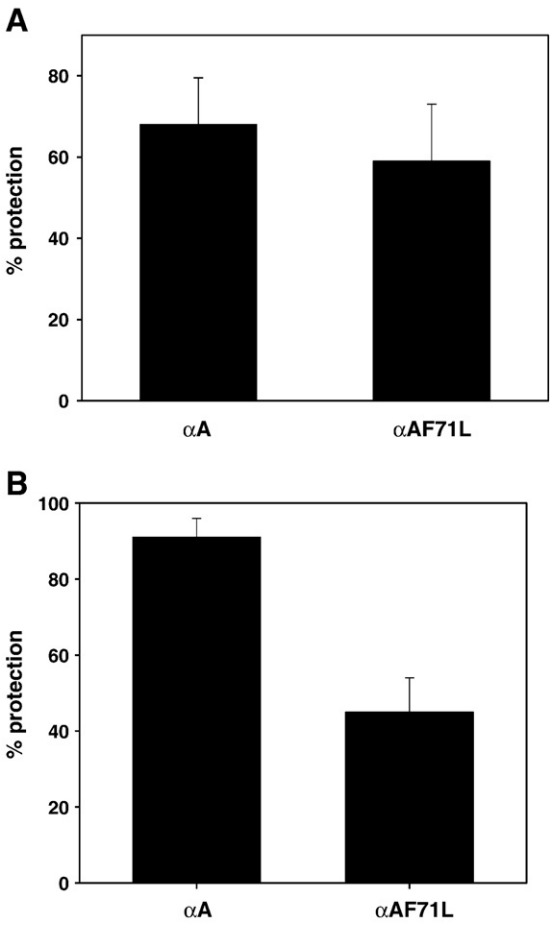

Fig. 8. *Panel A:* Relative chaperone activity of wild-type and F71L-αA-crystallin in DTT-induced aggregation of insulin assay. Percentage protection was determined considering DTT-induced aggregation of insulin (0.4 mg/ml) in the absence of α-crystallins at 60 °C as 100%. Concentration of wild-type and F71L αA-crystallin was 0.25 mg/ml. Data are mean ± SD ($n=4$). *Panel B:* Relative chaperone activity of wild-type and F71L-αA-crystallin in DTT-induced aggregation of lysozyme assay. Percentage protection was determined considering aggregation of lysozyme (0.5 mg/ml) in the absence of α-crystallins as 100%. Concentration of wild-type and F71L αA-crystallin was 0.5 mg/ml. Data are mean ± SD ($n=4$).

remained in solution.[1] These results reinforce the possibility that this mutation which can cause subtle structural alterations in association with other environmental factors, may predispose towards the cataract phenotype. This may explain the late age onset of cataract in individuals who are carriers of this mutation. Further, these results highlight the importance of αA-crystallin, which is the more abundant sub unit in the α-crystallin hetero-oligomer. Recently we reported that α-crystallin oligomers with αA and αB subunits in a 3:1 molar ratio could be a favored combination for lens to achieve optimal protection under both native and stress conditions [20]. Further, despite high sequence homology, αA- and αB-crystallins behave differently with increasing temperature. For example, at high temperatures recombinant αA-crystallin shows higher CLA than recombinant αB due to some structural transitions [17,30]. In addition, studies from our laboratory[2] and elsewhere indicate that αA-protects αB-crystallin from heat-induced aggregation/precipitation [13,31,32]. There is a good evidence to suggest that αA is more critical for lens than αB-crystallin. Hence, the preliminary data which indicate an altered stability for F71L mutant gain attention. Nevertheless, further investigations are necessary to understand the other possible factors that might predispose this genetic effect.

As reviewed [3], in addition to epidemiological evidence implicating genetic factors in ARC, a number of inherited cataracts due to mutations in various genes with post infantile age at onset or progression of cataract all through the life has been reported. These

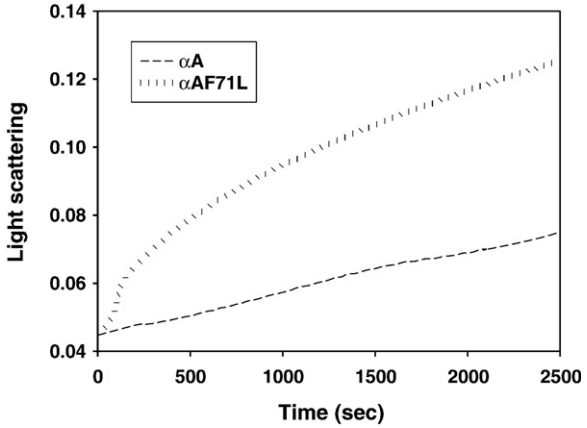

Fig. 9. Time-course of light scattering monitored for wild-type and F71L αA-crystallin (0.30 mg/ml) at 85 °C and pH 7.2.

[1] PNBS Srinivas and GB Reddy unpublished data.

studies indicate that a mutation in some genes that severely perturb the protein or affects its function might result in congenital cataracts, while a mutation that causes less significant damage to the same protein might lead to ARC in a more complex multifactorial fashion. For example, mutations in heat shock transcription factor 4 were observed in ARC, which account for only a small fraction of 150 sporadic, age-related cortical cataracts in a Chinese population [33]. Hence, it may be inferred that late expression of mutant allele in conjuncture with other triggering factors ultimately bringing down CLA in vivo may lead to the condition (NC).

In summary, we identified a novel mutation (F71L) in the exon-2 of *CRYAA* gene associated ARC. Further, we demonstrated that though F71L mutation did not cause major structural changes, the F71L mutant protein has substantially lowered CLA. Thus, as suggested by Murugesan et al. [34], the age-at-onset of cataract in individuals carrying this mutation may be due to the overall loss of in vivo chaperone activity of α-crystallin. However, in addition, there could be structural alterations due to F71L mutation with time due to environmental factors. For example, F71L mutant might be more susceptible to age-related posttranslational modifications. Since the patients with F71L αA-crystallin developed cataract during their elderly life i.e., around 50 years of age, decreased CLA and possibly along with some age-related modifications in patients carrying the mutation might be the possible underlying mechanism.

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