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Initial Investigation Indicating Association of Indoleamine 2,3-Dioxygenase Gene Variants with Age Related Cataracts

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Abstract

Exposure to UV is one of the major risk factor for the development of age related cataracts (ARCs). UV filters present in lens protect the lens and retina from UV induced photo-damage and obstruction of normal vision. UV filters are produced by Kynurenine pathway, wherein Indoleamine 2,3-dioxygenase (*IDO*) is the first rate limiting enzyme. So, the present study was conducted for the first time to screen for the genetic variants of *IDO* gene causing different types of ARCs.

331 cases [110- Nuclear (NC); 110- Cortical (CC) and 111- Posterior subcapsular cataracts (PSC)] and 210 normal individuals were screened by SSCP, Sequencing and RFLP techniques. *In silico* analysis was carried out using different bioinformatic tools.

The study revealed 2 novel (c.596_597delinsTT in exon 7; c.822C>T in exon 9) and 2 known (c.422+90G>A in intron 4; -/CAA del in intron 8) variants in exon and exon-intron boundaries of *IDO* gene. Further, 2 novel (c.-979 G>A& c.-471 T>G) and 1 Known (c.-738 A>G) variants were detected in promoter region, affecting the transcription factor binding sites *in-silico*. The known c.422+90G>A variant having destructive effect on enhancer sites was found in 6 patients (3 NC and 3 PSC) and novel variant (c.596_597delinsTT) in exon 7 of *IDO* was found in 2 cases (1NC and 1CC), with a probable damaging effect on protein *in-silico*. CAA deletion in intron 8 was found to be polymorphic, showing 2 fold risk for developing PSC. Exon 9 c.822C>T variant detected only in controls was a silent mutation.

The variants associated with ARCresultsin the alteration of the structure and function of *IDO* protein–a vital component required for normal synthesis and action of UV filters involved in maintaining lens transparency and clear vision. Further studies in this line would confirm the role of *IDO* gene in developing ARCs.

INTRODUCTION

Indoleamine 2, 3–dioxygenase (*IDO*), a heme containing enzyme catalyzes the first and rate limiting step of tryptophan (trp) catabolism along the kynurenine (kyn) pathway. *IDO* plays a role in peripheral immune tolerance, and also regulates homeostasis by preventing autoimmunity or immunopathology that occurs due to uncontrolled and overreacting immune responses. *IDO* is also involved in the protection of fetal allograft, engraftment of skin and organ transplantation [1-4]. Pathophysiological role of *IDO* has been identified in cancers [5,6] and also in some neurological disorders like Alzheimer's and Parkinson's diseases [7-10].

The presence of UV filters in the lens of human eye establishes the role of *IDO* in the development of age-related cataracts (ARCs). Kyn and its derivatives like 3OHKyn and 3OHKG (3-hydroxy kynuranine glucoside) generated in kyn pathway by the catabolism of trpact as UV filters [11-13], that are capable of protecting lens from UV damage when they are free in solution. With ageing, the levels of free UV filters get decreased and they can covalently bind to lenticular proteins leading to the accumulation of protein adducts in the lens, resulting in lens opacification [11,15,16].

IDO enzyme is encoded by a gene *IDO,* which has been evolved due to gene duplication of a more ancient proto-*IDO* gene before the divergence of marsupial and eutherian mammals. *IDO* (OMIM No.147435) is a single copy gene located on chromosome 8p12-11 comprising 10 exons spanning 15 kb and codes for a protein of 403 amino acids with a molecular weight of 45,332 daltons. Sequence variations of *IDO* gene are recorded in NCBI data base. About 18 variants were identified in exons and exon-intronic boundaries of human *IDO* gene have not shown clinical significance except those variants identified in the study conducted by us which highlighted the association of *IDO* variants with Age Related Cataracts (ARCs). The 1.3kb upstream region of *IDO* gene denotes its promoter which harbors different regulatory sequences which include three gamma activation sequences (GAS) and two interferon stimulated response elements (ISRE) [17,18]. So far, very few studies are carried out on *IDO* genetic variants and their functional significance [19-23], but did not reveal association with any condition.

Till date studies are conducted only explaining the pathological role of quantitative levels of *IDO* and its catabolites in developing different conditions like cancers, Alzheimer's and Parkinson's diseases but are not extended to correlate with

molecular alterations. We have analyzed for the first time the association of *IDO* gene and its promoter variants with different types of ARCs viz. Nuclear Cataract (NC); Cortical Cataract (CC) and Posterior Sub- capsular cataract (PSC).

MATERIALS AND METHODS

In the present study, 331 age related cataract patients registered for surgery at Sarojini Devi Eye Hospital and institute of ophthalmology, Hyderabad and 210 healthy normal individuals for comparison with patients were investigated to evaluate the contribution of *IDO* gene variation for developing lens opacity. All the patients studied had primary cataract cases without any associated conditions like diabetes, hypertension, myopia, thyroid disorder, trauma etc. They were categorized into 3 groups based on LOC-III classification system [24], as Nuclear cataract (NC), Cortical cataract (CC) and Posterior Polar cataract (PSC). Control subjects were selected at random by personal contacts, of the employees of government and private organizations with the provision for annual health check up. All the controls were without the history of cataract, diabetes, hypertension and other ocular diseases. Clinical and family details etc., were recorded from patients and controls in a specified proforma and approval for the study was obtained from institutional ethical committee following the Helsinki declaration.

EDTA/whole blood samples were collected to isolate DNA by rapid non-enzymatic method [23], from 331 patients (110-NC; 110- CC and 111- PSC) and 210 healthy normal individuals for conducting Polymerase Chain Reaction (PCR), Single-Strand Conformation Polymorphism (SSCP), Sequencing and Restriction Fragment Length Polymorphism (RFLP).

17 sets of primers were designed for amplifying 10 exons along with intronic boundaries of *IDO* gene and their annealing temperatures used for PCR amplification are given in Table 1. SSCP analysis was carried out by running the samples on 10–12% Polyacrylamide denaturing gels prepared by using 40% acrylamide solution (37.5g acrylamide, 1g bis-acrylamide), and the run was carried out at 100 V for 12-18 h. Later the gels were stained by silver staining and bands were visualized by Systronics gel documentation. Samples showing mobility shift in banding pattern was sequenced to identify the nucleotide variation through the commercial source (Vimta Labs Pvt Ltd, Hyderabad) using ABI 3100 genetic analyzer.

Later, RFLP analysis was planned to genotype the sequence variants identified by sequencing. Restriction enzymes for RFLP were selected using the web-based program NEB cutter v.2.0 from New England Biolabs (http://tools.neb.com /NEBcutter2/). The enzymes used for RFLP genotyping were HhaI for the variants identified in exon 7 and intron 4, AatII for exon 9, BseMI and AciI for promoter variants viz..c.-979 G>A and c.-471T>G respectively (Table 2). For RFLP genotyping 5µl of amplified PCR products were digested with 1-3 units of the restriction enzyme and incubated overnight at 37°C. Then Electrophoresis (2%Agarose/ 8-10%PAGE) was used to separate the PCR products digested by the restriction enzymes.

Different bioinformatic tools like CLUSTAL X (http://www.softpedia.com/get/Science-CAD/Clustal-X.shtml), Polymorphism Phenotyping (Polyphen; http://genetics.bwh.harvard.edu/pph2/), Sorting Intolerant From Tolerant (SIFT; http://sift.jcvi.org/), and Human splicing finder (HSF; http://www.umd.be/HSF/) were employed to study the importance of variants associated with ACRs.

For protein modeling Rasmol and Swiss probe dbv viewer were used for mutational analysis to analyze the effect of mutation on the structure of the protein. The mutants were modeled by using the Triton v.4.0.0 package. Triton uses the methodology of computational site-directed mutagenesis and the *in silico* testing of mutant properties. The 3D structure of mutant was generated by the Modeller V9v3 program. This program uses the method of comparative protein modeling by satisfaction of spatial restrains to model structures of homology proteins based on the 3D structure of template protein.

RESULTS

The Epidemiological data of the present study showed high preponderance of female patients (NC:50.9%; CC:54.5%; PSC:60.4%) as compared to males (NC:49.1%; CC:45.5%; PSC:39.6%) indicating high risk for females for developing age related cataracts. The ages of the patients selected for this study were falling within the range of 40 to 85 yrs and the controls were within 40 to 80 yrs. The mean ages were recorded as 61.3±0.23 for NC, 58.1±0.23 for CC, and 56.6±0.22 for PSC patients and 49.1±0.10 for controls. The mean age at onset of cataract revealed early onset for the subjects with PSC (55.6±0.22) followed by CC (57.5±0.23) and NC (60.4± 0.23) cases.

Sequencing of variant samples identified by SSCP screening of all the 10 exons and intronic boundaries of *IDO* gene revealed the presence of 4 genetic variants (two Novel: c.596_597delinsTT in exon 7; c.822C>T in exon 9 and two known: c.422+90G>A in intron 4; (-/CAA) del in intron 8) (Table 3).

Novel variants in exon 7 and 9 of *IDO* gene

The novel variant c.596_597delinsTT identified in exon 7 has been registered in NCBI as rs267606590. It was found in heterozygous state among two of the cases (one with NC and one with CC) studied and none among controls. The two probands with this variation were males with an age at onset of 59 and 45 years respectively. The deletion (CG) and insertion (TT) of two successive nucleotides at c.596 and c.597 positions causes codon change leading to substitution of alanine (A) at position 199 by valine (V) in variants. This variation p.A199V showed loss of site for HhaI enzyme.

Alignment of *IDO* amino acid sequence from several species using CLUSTAL X showed high conservation for amino acid Alanine at 199 position. SIFT and PolyPhen tools predicted "probable damaging effect" with PSIC score of 1.53 by the variant on protein function and with a significant SIFT score of 0.00. Superimposition of *IDO* mutant and wild type proteins (2DOT) by Triton package showed RMSD value of 1.19, which indicated wide variation between the wild type and mutant protein structure. The functional study of this variant is understudy for its further characterization *in-vitro*.

The other novel variant identified in exon 9 showed C to T transition in heterozygous condition at c.822 of *IDO* gene. It was

Table 1: List of Primers and annealing temperatures used to amplify all the 10exons and 1.3kb promoter sequence of *IDO* gene.

***IDO* gene exonic regions**				
Region	**Sequence (5'-3')**	**Oligo (bp)**	**Product (bp)**	**Annealing**
Exon 1	F- CAAAAGTGGAAGTAATTTCTCAC	23	285	55°C
	R- GAAGTTAACTTGGCCAGGTAAG	22		
Exon 2-3	F- GAAGGCAAGGCATACTATCAG	21	365	55°C
	R- GGAAAGTTAAATGTAAATTAGATG	24		
Exon 4	F- CAGGAGCAAGACTCCATCTC	20	378	62°C
	R- GTAGTGGTAGACACAGCAGTC	21		
Exon 5	F- GCTTTTTCTTTTTACCTATGTCTTACC	27	228	53°C
	R- TGGAGTCTATTGATAAACCTACATTCA	27		
Exon 6	F- GATAGTAAGGCCTGCCACAC	20	297	56°C
	R- GTTTAGGCTCCGAAGTGATTG	21		
Exon 7	F- CTGGACAACTGAGCGAGACTC	21	273	62°C
	R- CTATTCTACACCTGGAACATTTG	23		
Exon 8	F- CATTATCAGTTGTACACAACACC	23	215	55°C
	R- GGATATTAGGGACCAACCAAG	21		
Exon 9	F- GGATCATGAAATCCATCTCTTG	22	339	58.3°C
	R- GTGCTTTGTAGATATCCAAATAC	23		
Exon 10a	F- CAGTGAATGCTATATTGGTGATC	23	298	55°C
	R- GCAGATGGTAGCTCCTCAGG	20		
Exon 10b	F- CCTGAGGAGCTACCATCTGC	20	252	56°C
	R- GTAATGACAGGAATGCATACAG	22		
***IDO* gene promoter region**				
Region 1	F- TTCCTTGAACTGATTCCCAAA	21	218	53°C
	R- GCATATGGCTTTCGTTACAGTC	22		
Region 2	F- AGTAGAGAATAGCGCGAGAGC	21	245	50°C
	R- GCATGCAAGTCTGTGGTTCA	20		
Region 3	F- AACGGGCAACTTGGTTTCTT	20	297	50°C
	R- AGCATTTGCCCTTCTCACAT	20		
Region 4	F- CCCGCAGTCAGGTACAGTTAG	21	250	62°C
	R- AAAATATTAGTGTCATGTTTCAGCA	25		
Region 5	F- TTTCCTACTTCAGAGCCATTGAC	23	250	60°C
	R- CAGAAAGGCCTGAAGGAAAAC	21		
Region 6	F- TTTCCATAAAGTAAAATGTTCTTCTCC	27	234	50°C
	R- TCCACTTTTGGAATGGTTTCA	21		
Region 7	F- GCACAGAGATGCTTTTGTGG	20	207	52°C
	R- TGTGCCATTCTTGTAGTCTGCT	22		

Table 2: RFLP analysis for variants detected in *IDO* gene.

Variation detected	Restriction enzyme	Restriction site	Fragments generated (bp)
c.596_597 delins TT (Exon 7; Novel)	HhaI	5'...G C G C...3' 3'...C G C G...5'	AA - 150, 123 AV- 273, 150, 123 VV - 273
c.822 C>T (Exon 9; Novel)	AatII	5'...G A C G T C...3' 3'...C T G C A G...5'	CC - 234, 105 CT - 339, 234, 105 TT - 339
c.-979 G>A (Promoter; Novel)	BseMI	5'...G C A A T G N N...3' 3'...C G T T A C N N...5'	GG - 245 GA - 245, 139, 106 AA - 139, 106
c.-471T>G (Promoter; Novel)	AciI	5'...C C G C...3' 3'...G G C G...5'	TT - 250 TG - 62, 188, 250 GG – 62bp, 188
c.422+90 G>A (Intron 4; Known)	HhaI	5'...G C G C...3' 3'...C G C G...5'	GG - 295, 83 GA - 378, 295, 83 AA - 378

Table 3: Frequency of sequence variations detected in exons with intronic boundaries of *IDO* gene in probands with ARCs and controls.

	Variation	Subjects	Frequency						Observations
			W	%	H	%	V	%	
Intron4	c.422+90G>A	6 cases	325	98.1	5	1.5	1	0.3	Destructs 2 enhancer sites of splicing
Exon7	c.596_597delinsTT	2 cases	329	99.3	2	0.6			Possibly damaging effect on protein
Exon9	c.822C>T	1control	209	99.5	1	0.4			No effect on protein
Intron8	(-/CAA) del	Cases	303	91.5	26	7.9	2	0.6	CAA deletion showed risk for PSC
		Controls	198	94.3	12	5.7	-		

W: Homozygous wild type; H: Heterozygous; V: homozygous variant

Table 4: Frequency of sequence variations detected in promoter of *IDO* gene in probands with ARCs and controls.

	Variation	Subjects	Frequency				Bioinformatics
			W	%	H	%	
Region 2	c.-979G>A	2 cases	329	99.4	2	0.6	Loss of GATA2
Region 3	c.-738A>G	3 cases	328	99.1	3	0.9	Loss of SOX10
		1 control	209	99.5	1	0.5	
Region 4	c.-471T>G	3cases	328	99.1	3	0.9	Creates SPIB & ETS1

W: Homozygous wild type; H: Heterozygous

found in one of the control subjects as synonymous mutation coding for aspartic acid at 274th position of the protein. As per codon usage table the variant showed 46% of accessibility during protein synthesis. This variation resulted in the loss of restriction site for the enzyme Aat II. HSF predicted the break of potential branch point in variants, and ESE and EIE predicted destruction of enhancer site with a score of -100.

Known variants in intron 4 and 8 of *IDO* gene

c.422+90 G>A (rs4613984) transition was found in intronic region lying between exon 4 and 5 and is identified in six samples (3 with NC and 3 with PSC) that correlated with homo and heterozygous patterns. This transition caused loss of site of HhaI enzyme resulting in homozygosity in one patient (with NC) and heterozygosity in 5 patients (2 with NC and 3 with PSC). This known variation was not found in controls. HSF did not predict any effect on potential splice site/branch point with this variation, but showed destruction of two enhancer sites with a score of -100 and creation of a new silencer motif with a variation score of -16.23.

Another known variation rs3214412 showing (-/CAA) deletion in intron 8 was found both among cases and controls of the present study indicating the polymorphic nature of the variation found. The frequency of heterozygotes (ID) was high in PSC (10.8%) as compared to NC (7.3%), CC (5.5%) cases and controls (5.7%). Estimate of odds ratio showed protection for wild type allele 'I' (OR= 0.43; 95%CI =0.18-1.02; P=0.03) in PSC cases while variant allele 'D' showed 2 fold risk (OR= 2.28; 95%CI =1.01-5.38; P=0.03) for developing PSC. The bioinformatic tools, EIEs and PESE octomer from Zhang and Chasin predicted destruction of enhancer site with a variation score of -100.

Sequence variations detected in 1.3kb promoter of *IDO* gene

Screening for variations in promoter of *IDO* gene revealed the presence of 3 variants - two novel (c.-979 G>A & c.-471 T>G) and one known variant (c.-738 A>G) (Table 4).

Novel variants

The novel variant c.-979 G>A (NCBI reference number is rs267606591) was found in two of the cases (1 with NC & 1 with PSC), resulting in the loss of GATA2 transcription factor binding site. This variation created a restriction site for BseMI enzyme.

The presence of another novel variant c.-471 T>G in the promoter region was identified in 3 PSC cases. It has been registered in NCBI SNP database as rs267606592.The variantallele created two SPIB and ETS1 transcription factor binding sites. Wild type allele 'T' didn't show the presence of TFBs.

Known variant

A known variant rs118067147 (c.-738A>G) in heterozygous pattern was found in three of 331 cataract cases (2NC and 1PSC) and in one of 210 controls. Wild type allele 'A' showed the presence of 2 TFBs, FOXC1 and SOX10 sites. Variant allele 'G' showed the presence of binding site for FOXC1 only with a lesser threshold value of 94% and loss of SOX10 site when compared to normal allele 'A'.

DISCUSSION

Age-related cataract is a multi-factorial condition responsible for 51% of world blindness, which represents about 20 million people [25]. Nearly about 20 genes are indicated to be involved in the development of ARCs (http://cat-map.wustl.edu/) [26]. Recently, *IDO* gene has also been included in the database based on a preliminary study conducted by our group [27], which identified the *IDO* gene variations causing susceptibility to ARC specially revealing significant association of c.422+90G>A (rs4613984) variation with the disease - a first report.

Till now very few studies were available on *IDO* genetic variants. A general study by Arefayene et al., (2009) described the frequencies of the *IDO* gene variants which were identified among 48 African Americans and 48 Caucasian Americans. Functional studies revealed the reduced and loss of *IDO* activity in two of the variants studied by the authors. Two other clinical studies conducted with reference to pre-eclampsia and recurrent spontaneous abortions showed lack of their association with *IDO* gene variants [20,21]. Till date, studies are not available regarding the genetic variation of *IDO* gene causing cataracts except for our reports [22,27]. Of the variants identified by our study, c.596_597delinsTT variant showed adverse effect on *IDO* function by altering its structure. Due to this, the loss or reduced enzyme activity may further influence the rate of UV filter synthesis. The two variants identified in intronic region (c.422+90 G>A in intron 4 and -/CAA deletion in intron 8) though are not found to affect the potential splice site or branch point directly, they are likely to destruct the enhancer motifs of splicing. Thus these variations may be affecting splicing process leading to alteration of post translational modification of *IDO* protein. The creation or destruction of transcription factor binding sites by variants identified in the promoter of *IDO* gene, could possibly be affecting the regulation of *IDO* expression. This has to be further confirmed by *in vitro* functional studies.

Observations made by the present investigation indicate the role of *IDO* genetic variants in the formation of different types of cataracts due to alteration in the structure and function of *IDO* protein – a vital component required for the normal synthesis and action of UV filters that are responsible to maintain lens transparency and clear vision. We recommend further studies in this line among different populations will help in better understanding the role of *IDO* gene variants and its product in developing ARCs.

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