Genetic Variations in the Promoter Region of *IDO* Gene Associated with Age-Related Cataracts

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Abstract

Background: IDO is the first rate limiting enzyme involved in the synthesis of UV filters of the lens via tryptophan degrading kynurenine path way. With ageing, accumulation of UV filter modified lens proteins lead cataract formation. The present study is planned to screen for promoter variations of Indoleamine 2, 3-dioxygenase *(IDO)* gene in age-related cataracts (ARCs).

Methods: 331 ARCs [110-nuclear, 110-cortical and 111-posterior sub capsular cataract] and 210 healthy controls were screened by Single Stranded Conformation polymorphism followed by sequencing the samples showing mobility shift. For variant samples the putative Transcription Factor Binding sites (TFBs) were predicted using JASPAR software

Results: SSCP followed by sequencing revealed two novel variations c.-979G>A in two cases and c.-471T>G in three cases and one known variation c.-738A>G in three cases and one control. Variation c.-979G>A showed loss of GATA2 site for variant allele 'A' while c.-471T>G created SPIB and ETS1 TFBs for variant allele 'G'. The variant c.-738A>G showed loss of SOX10 TFBs for allele 'G'.

Conclusion: These promoter variants may be affecting the IDO expression leading to the development of ARCs. The study appears to be the first of its kind reporting the involvement of *IDO* promoter variations in the development of ARCs.

KEYWORDS: Age related Cataract, Indoleamine 2,3-dioxygenase, Promoter, Variants

Introduction

Indoleamine 2, 3 dioxygenase (IDO) is a first rate limiting enzyme involved in tryptophan (Trp) catabolism via kynurenine pathway. About 95% of tryptophan, the essential amino acid present in the body is converted to kynurenine derivatives. IDO is expressed in a wide range of cells constitutively or in inducible manner. The tissues where IDO is expressed include kidney, liver, lung, placenta, intestine and brain. IDO is induced by a cytokine IFN-gamma in myeloid lineage cells, epithelial cells, fibroblasts and endothelial cells (Mellor and Munn, 2004 and Takikawa, 2005). IDO plays an important role in several infectious conditions by depleting the Trp concentration which leads to the production of immunomodulatory kynurenine metabolites. This reduction in Trp levels results in the inhibition of growth of various pathogens (Takikawa, 2005).

Further, over expression of IDO was shown to be involved in different human cancer types (Uyttenhove et al, 2003) and depletion of Trp due to over expression of IDO inhibits the growth of tumor. Thus the constitutive expression of IDO in tumors can be considered as predictive marker for tumor progression. IDO is also implicated in the pathophysiology of neurodegenerative diseases like Alzheimers, Parkinson and Huntington disease (Ogawa et al, 1992; Pearsons et al, 1995; Okuda et al, 1998; Cerstiaens et al, 2003; Sas et al, 2007).

Involvement of IDO was reported to play a role in the development of nuclear cataract (Bova et al, 2001). IDO present in the lens epithelium produces low molecular weight compounds by kynurenine path way. These low molecular compounds viz Kynurenine, 3-OHkynurenine and 3-OHkynurenine glucoside in the lens act as UV filters protecting the lens from UV induced photo damage (Vanheyningen, 1973; Wood and Truscott, 1994). With ageing, the levels of free UV filters in the lens were found to decrease at a rate of 12% per decade accompanied by increase in the levels of UV filter modified proteins leading to lens opacification (Bova et al, 2001). This suggests that the accumulation of modified lens proteins during ageing acts as an important factor causing loss of lens transparency and cataract formation (Vazquez et al, 2002; Korlimbinis 2007).

IDO enzyme is encoded by a gene designated as *IDO* or *INDO*. It is a single copy gene located on chromosome 8, comprising 10 exons coding for 403 amino acids. The 5' upstream of the ATG start codon of *IDO* gene constitutes promoter that contains numerous well defined regulatory elements involved in the regulation of *IDO* expression. Most of the regulatory sequences confer responsiveness to interferons, specifically to IFN-gamma. Transcriptional induction of *IDO* by IFN-gamma is mediated through the JAK/STAT pathway. Promoter of *IDO* contains three gamma activation sequences (GAS) and two interferon stimulated response elements (ISRE; Chon et al, 1996; Robinson et al, 2006). Very few studies are available on genetic variants of *IDO* promoter. An unpublished data on resequencing of the promoter region of *IDO* gene among the population of 48 African Americans (AA) and 48 Caucasians (CA) revealed the presence of 6 genetic variants, of which 5 variants were identified in AA and 3 variants in CA populations "(Personal communication, Arefayene M, Department of Pharmacology and Toxicology, Indiana University, USA)". A Study conducted by Soichot et al, (2011) in the population of 41 healthy unrelated Caucasians revealed the presence of a variable number of tandem repeats (VNTR) in the 1.3KB promoter region of the *IDO* gene. These observations prompted the present study to screen for the sequence variations in the promoter of *IDO* gene and look for putative alterations in the transcription factor binding sites leading to different types of age related cataract (ARC).

Materials and methods

A total of 331 ARC cases [110- Nuclear cataract (NC); 110- Cortical cataract (CC) and 111- Posterior sub capsular cataract (PSC)] reported at Sarojini Devi Eye Hospital and Institute of Ophthalmology, Hyderabad, India were investigated for sequence variations in the promoter region of *IDO* gene. Diagnosis of cataract was done according to lens opacities classification system III (LOCIII; Chylack et al, 1993). Only primary cataract cases were included in this study. Patients with secondary cataract arising due to trauma, inflammation and other associated conditions like diabetes, hypertension, myopia,

glaucoma, thyroid dysfunction and other ocular diseases were excluded from the study for comparison with patients data, 210 healthy normal control subjects were selected randomly by house visits, and from government and private organizations that have the provision of regular health checkup including ophthalmic examination. The present study was approved by institutional ethical committee following the Helsinki declaration. All the subjects were explained about the nature and outcome of the study before taking their informed consent.

From all the cases and control subjects 5ml of venous blood samples were collected in EDTA vaccutainers. Genomic DNA was extracted from peripheral blood leukocytes using non enzymatic salting out method (Lahiri and Nurnberger, 1991). 7 overlapping primer sets (Table 1) were used to amplify 1.3Kb promoter of *IDO* gene by polymerase chain reaction (PCR). PCRs were performed using 10µl PCR mix containing 1X PCR buffer, 200µM dNTPs (Sigma Aldrich, Schnelldorf, Germany), 0.25 units of Taq polymerase (Sigma Aldrich) and 2.5 pmols of forward and reverse primers (Ocimum Biosolutions (India) Ltd, Hyderabad, India). The PCR conditions followed to amplify the 7 regions were of initial denaturation at 95°C for 5m, followed by 30 cycles of denaturation at 95°C for 1m, annealing at 53°C - 60°C for 40s, extension at72°C for 1m and a final extension at 72°C for 5m. PCR products were denatured with low ionic strength (LIS) buffer (10% sucrose, 0.01% bromophenol blue and 0.01% xylene cyanol) at 95°C for 10m followed by snap cooling in ice. Single strand conformation polymorphism (SSCP) analysis was carried out by running the samples in 10%-12% denaturing gels prepared by using 40% acrylamide solution (37.5g acrylamide and 1g bisacrylamide of Sigma aldrich) and the run was carried out at 100V for 18hrs. Bands were visualized by silver staining (Mamata et al, 2011) and recorded using Systronics gel documentation (Spectronics, Westbury, NY). Samples showing mobility variations were sequenced (Vimta labs Ltd, Hyderabad, India) on ABI 3100 DNA sequence analyzer to identify the nucleotide changes.

Sequencing results were analyzed using National centre for biotechnology information (NCBI) blast. JASPAR bioinformatic tool was used to predict the consensus sequences of putative transcription factor binding sites (TFBS). For this study TFBS were predicted with a high relative score threshold value of 90%, with default threshold value of 80%.

Restriction fragment length polymorphism (RFLP) was designed for the two novel variants (c.-979G>A; c.-471T>G) identified in the present study. Genotyping was done by separating the digested fragments on 3% agarose gels. The presence of G>A transition at position -979 created a cutting site for BseMI restriction enzyme, resulting in three different fragments of 106bp, 139bp and 245bp in heterozygotes and only a single fragment of 245bp in wild type homozygotes. The second substitution T to G occurring at position c.-471 created a restriction site for the enzyme AciI resulting in fragments of 250bp, 62bp and 188bp in heterozygotes and single fragment of 250bp in the wild type homozygotes.

Results

Screening for variations in the promoter region of *IDO* gene by SSCP analysis using 7 overlapping primer sets showed the presence of heterozygous banding patter in three of

the 7 regions studied (Table-2). Sequencing of these samples revealed the presence of variations c. -979G>A, c.-738A>G and c.-471T>G. Of these, two were novel (c.-979G>A; c.-471T>G) and one is already reported in NCBI database with reference sequence number rs118067147 (c.-738A>G). The two novel variations c.-979G>A and c.-471T>G, identified in this study have been registered by the first author MM in NCBI SNP database as rs267606591 and rs267606592 respectively.

The novel variant c.-979G>A (rs267606591) showed the presence of transition G to A at position -979 in heterozygous state in two of the cases studied (one with NC and other with PSC). Both the probands with NC and PSC were females of age 62 and 55yrs respectively. Frequency of heterozygotes for this variation accounts for 0.6%.

The second variant c.-471T>G (rs267606592), showed the presence of T to G transversion at position -471. Three probands with PSC showed this variation, of whom, two were females 60 and 56yrs old and one was male 47yrs old. The male proband showed early onset of cataract as compared to females. Frequency of this heterozygote accounts for 0.9%.

None of the controls showed these two novel variants. These two variants may be causally related with development of the respective cataract types.

c.-738A>G (rs118067147) variant reported in the population of Caucasians (Arefayene, 2008) showed the substitution of A to G at position -738 from that of first nucleotide of start codon. Three out of 331 cataract cases (2NC and 1PSC) and one out of 210 controls subjects of the present study showed this variation. Among the two probands with NC one was female and other was male who were 62yrs and 50yrs old respectively. The third proband with PSC was a female of 58yrs age. The control subject with this variation is a male, 42 yrs old and the possibility of him developing cataract at later age cannot be ruled out. Heterozygous frequency of this variant works out to 0.9% in cases and 0.5% in controls.

Further, it is interesting to note that the two variants c. -738 A>G already reported and c.-979 G>A novel variant found in this study were present in the same proband with NC. This patient is a female of 62yrs had positive family history of cataract.

The variants identified were analysed for changes caused in the Transcription binding sites that are likely to result in variation in the *IDO* gene expression. Application of JASPAR software predicted 180 putative TFBs within 1.3KB promoter of wild type with a relative score of 90%. The transition of c. -979G>A found in the present study showed loss of GATA2 transcription site in carriers of mutant allele A, which was found in two patients (one with NC and one with PSC). In case of c.-471T>G, the presence of mutant allele ‘G’ created two TFB Sites for SPIB and ETS1 with a relative score of 95% in three patients with PSC. These TFBS were located on sense (+) and antisense (-) strands respectively. No TFBs were predicted for normal allele ‘T’.

Two TFBs, namely FOXC1 and SOX10 were predicted with a relative score threshold value of 96% for wild type allele A at position c.-738 in 3 patients (2NC, 1PSC) and 1 control subject. FOXC1 and SOX10 were present on sense and antisense strands of DNA

respectively. The presence of variant allele 'G' at this position showed loss of site for SOX10. No change is predicted for FOXC1 site in the subjects with variant allele 'G' which was also present in the wild type allele 'A'. The risk for cataract development caused by this variant has to be further evaluated with large sample study or evaluation of the function of this variant allele.

Discussion

Age related cataract is now described as a multifactorial disease. Recent survey by the World Health Organization (WHO) revealed that ARC is responsible for 51% of world blindness, which represents about 20 million people (Pascolini and Mariotti, 2010). As per CAT-MAP database about 19 genes have been implicated in the development of ARCs, of which 10 genes are linked with inherited cataracts (Shiels et al, 2010).

Indoleamine 2, 3 dioxygenase the rate limiting enzyme in tryptophan catabolism is shown to be associated with several diseases including autoimmune disorders, cancers, depression, neurological diseases like Alzheimers, Parkinson and Huntington, altered maternal tolerance of paternal antigens and apoptosis. The enzyme catalyses the production of UV filter molecules like kynurenine and 3OH kynurenine that protect lens from losing its transparency. When these filters are degraded and modify the lenticular proteins, it results in cataract formation.

Large inter-individual variations were observed in the activity of IDO in different clinical conditions reflecting the possible involvement of inherited genetic variability. Few studies describing the frequencies of the variants of *IDO* gene in some normal populations are registered in NCBI SNP database, and reported by Arefayene (2009) on the African American and Caucasians samples. Only two reports are published on the association of Indoleamine 2,3 dioxygenase polymorphisms in pre-eclapsia patients and with recurrent spontaneous abortions in Iranian women focusing negative results (Nishizawa et al,2010; Amani et al, 2011). No studies till date are available on the genetic variation of *IDO* gene causing cataracts except for our report showing the association of c.422+90G>A; rs4613984 present in the intron down stream to the exon 4 of *IDO* gene (Mamata et al, 2011). Subsequently we conducted the present study to look into the variations in thepromoter of *IDO* gene which could be related with the cataract formation.

Only one study by Soichot et al, (2011) on the population of 41 unrelated healthy individuals has reported the presence of a VNTR polymorphism in the promoter region of IDO. Females with two VNTR (V2/V2) repeats showed significantly lower serum tryptophan concentrations when compared to wild (V1/V1) and heterozygous (V1/V2) condition. An unpublished data by Arefayene, (2008) showed the presence of c.-738G>A variation in heterozygous state in one of the 48 Caucasian samples and its functional studies showed no significant effect of FOXC1 site on IDO regulation.

The novel variants c.-979G>A and c.-471T>G detected in the present study had considerable effect on the TFBs. The presence of variant allele 'A' at c.-979 resulted in the loss of site for GATA2 which is a zinc finger transcription factor playing a critical role in regulation of gene expression especially in hematopoietic cells. GATA2 co-

occupies chromatin sites along with Scl/TAL1 complex and either activates or represses the transcription in a context-dependent manner (Bresnick et al, 2012).

The presence of other mutant allele 'G' at c.-471 position resulted in the creation of two sites for SPIB and ETS1. AS per NCBI data (www.ncbi.nlm.nih.gov/**gene**/) SPIB transcription factor belongs to the subfamily of ETS and acts as transcriptional activator by binding to the purine rich sequence on DNA i.e 5' GAGGAA3'. ETS1 transcription factor acts as a transcriptional activator or repressor for several genes which are involved in stem cell development, cell senescence and death. It also directly regulates the expression of cytokines and chemokines in a wide range of cells. Further, it is interesting to note the presence of two variants c. -738 A>G (already reported in data base) and c.-979 G>A (novel variant found in this study) in the same proband with NC which predicted loss of GATA2 and SOX10 TFB sites. SOX10 factor acts as a transcriptional activator by forming a protein complex with other proteins. Its function is strongly influenced by its co-existing proteins in the complex. However, further *in vivo* studies are required to deduce the exact role of altered transcription factors observed in the present study influencing the regulation of *IDO* gene and development of cataract.

It is inferred that the variations found in the present study may be affecting the levels of IDO and the synthesis of UV filters causing accumulation of UV filter modified proteins in the lens resulting finally in cataract formation. This appears to be the first finding demonstrating the involvement of *IDO* promoter variations in the development of cataracts. As far as the authors are aware no studies have been published on the role of genetic variants of *IDO* promoter linked to the pathogenesis of ocular diseases including cataract.

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Conflict of Interest

The authors declare no conflict of interest

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Table-1: Sequence of the primers and annealing temperatures used to amplify 1.3 Kb promoters

Region	Sequence (5'-3')	Oligo (bp)	Product (bp)	Annealing
Region 1	F- TTCCTTGAACTGATTCCCAAA	21	218	53^{o}C
	R- GCATATGGCTTTCGTTACAGTC	22		

Region 2	F- AGTAGAGAATAGCGCGAGAGC	21	245	50^{o}C
	R- GCATGCAAGTCTGTGGTTCA	20		
Region 3	F- AACGGGCAACTTGGTTTCTT	20	297	50^{o}C
	R- AGCATTTGCCCTTCTCACAT	20		
Region 4	F- CCCGCAGTCAGGTACAGTTAG	21	250	62^{o}C
	R- AAAATATTAGTGTCATGTTTCAGCA	25		
Region 5	F- TTTCCTACTTCAGAGCCATTGAC	23	250	60^{o}C
	R- CAGAAAGGCCTGAAGGAAAAC	21		
Region 6	F- TTTCCATAAAGTAAAATGTTCTTCTCC	27	234	50^{o}C
	R- TCCACTTTTGGAATGGTTTCA	21		
Region 7	F- GCACAGAGATGCTTTTGTGG	20	207	52^{o}C
	R- TGTGCCATTCTTGTAGTCTGCT	22		

Table-2: Variations detected in promoter regions of IDO gene in probands with ARCs and controls

IDO promoter	Variation	Subjects	Frequency						Predicted function of variant
			N	%	H	%	M	%	
Region 2	*c.-979G>A	2 cases	329	99.4	2	0.6	0	0.00	Loss of GATA2
Region 3	c.-738A>G	3 cases	328	99.1	3	0.9	0	0.00	Loss of SOX10
		1 control	209	99.5	1	0.5	0	0.00	
Region 4	*c.-471T>G	3cases	328	99.1	3	0.9	0	0.00	Creates SPIB & ETS1

* indicates novel variations found in the present study