CLINICAL INVESTIGATION

Mutation analysis of circadian clock gene *BMAL1* in 21 Pakistani congenital cataract families

Abstract

Background: Given the cataract phenotype in *Bmal1*-/- mice, we aimed to identify potential disease-causing variants in the human circadian clock candidate gene *BMAL1* in 21 probands of consanguineous Pakistani congenital cataract families.

Methods: Ophthalmic examinations were performed for the probands and available family members. Genomic DNA was isolated from a volume of 5 ml of peripheral blood. The entire coding region of the candidate gene *BMAL1* was analyzed in the probands of 21 families with targeted Sanger sequencing.

Results: A heterozygous missense variant c.41A>T; p.(Asp14Val) was detected in 1 of the 21 patients, which has a rare allele frequency of 0.000065 (2/30576 individuals) exclusively in the South-Asian population. The variant did not co-segregate with the disease phenotype in the family. A non-synonymous variant (rs2290037) in the heterozygous state was also identified in 5 out of 21 probands with a higher allele frequency of 0.1190 as compared to the global population (0.06626; 15570/234984 individuals).

Conclusion: Our study is the first to investigate the core circadian clock gene *BMAL1* in humans for their association with congenital cataract. Unfortunately, no clear association between human genetic *BMAL1* variants and cataract was found. Compared with targeted NGS technologies, traditional Sanger sequencing remains an indispensable cost-effective tool especially to report mutation profiles in small study cohorts. Our study may act to guide further studies in the molecular clockwork pathway from other (disease) populations.

Keywords: congenital cataract • consanguineous • circadian • clockwork • infantile • mutation

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Background

Congenital or infantile cataract is defined by the opacity of the crystalline lens resulting in the (predisposition for) partial to complete pediatric visual disability. The World Health Organization (WHO) describes cataract as the primary cause of blindness throughout the world affecting 16 million people worldwide [1]. The incidence of congenital cataract (CC) is estimated to be 1-6 cases per 10,000 live births in developed countries, and 5-15 cases per 10,000 in the underdeveloped countries [2]. Approximately 200,000 children every year are affected by lifelong vision impairment due to congenital cataract [3]. Inherited cataracts represent a significant contribution to CC [4-6]. Currently, over 48 genes have been delineated in the Cat-Map database [7] underlying the pathogenesis of congenital cataract. Nearly 50% of the disease is accounted for by mutations in the crystalline genes [8-10]. Connexin genes comprise approximately a share of 25% [11-13] along with the causative gene mutations described in other structural proteins, namely beaded filament structural protein 2 (*BFSP2*) [14], lens intrinsic membrane protein (*LIM2*) [15,16], aquaporin0 (*MIP*) [17,18],

enzymes like glucosaminyl (N-acetyl) transferase 2 (*GCNT2*) [16] and in transcription factors such as paired-like homeodomain 3 (*PITX3*) [19], avian musculoaponeurotic fibrosarcoma (*MAF*) [20], heat shock transcription factor 4 gene (*HSF4*) [21].

Identification of new genetic mutations in cataract patients will improve our understanding of cataractogenesis during childhood and could provide further insights into lens biology. There is mounting experimental evidence that suggests a direct or indirect involvement of the circadian clock in cataract [22-27]. Circadian rhythms are 24-hour temporal programmes, widely distributed in mammalian tissues and synchronized by a master-hypothalamic clock [28]. In mammals, the core clock genes, including *Bmal1*, *Clock*, *Cry*, and *Per*, are rhythmically expressed in the Suprachiasmatic Nuclei (SCN)-the master clock in the hypothalamus and also in almost all peripheral cells/tissues, including the lens and retina of the eyes [28]. The clock transcription factors control the expression of numerous target genes in a circadian manner, influencing many physiological and biochemical processes [29], including those in the eye [30]. Clock factors act upstream of or in cooperation with tissue-specific transcription factors to temporally modulate *RNA polymerase* II loading, histone modification or three-dimensional conformation of the chromatin [31]. Thus, cycling transcriptomes are rather tissue-specific [32]. In addition, the clock orchestrates the temporal expression of genes during the development of the eye *per se* [33-36]. Notably, circadian clock genes *Bmal1* and *Clock* have been observed to be involved in the pathophysiology of cataract in mice [37-39]. It has been reported that genes implicated in cataract development in humans, may also be the key players in animal models like rodents and vice versa [40,41]. Daily variations in levels of crystalline mRNAs and proteins in the retinal photoreceptor cells of rats highlight the role of circadian processes in retinal crystalline synthesis [42]. In humans, decreased potential for circadian photo-entrainment is known to be associated with cataract development [43]. Moreover, visual acuity and circadian photoreception via the photosensitive retinal ganglion cells is profoundly impaired in cataract patients [44-46]. Finally, progressive loss of vision leading to blindness fails to render the required input signals to the biological clock [47,48]. These data prompted us to investigate the possible association between cataract development and the clock.

The primary transcriptional regulator of the circadian clock, the brain, and muscle aryl hydrocarbon receptor nuclear translocator-like protein BMAL1 is implicated in the regulation of early to premature ocular aging [37,38,49]. More than 50% of *Bmal1* deficient mice developed cataract before the 40th week of life [37]. Also, deletion of *Bmal1* disrupts clock-dependent oscillatory gene expression and behavioral rhythmicity coincident with eye pathologies, reduced body weight, impaired hair growth, abnormal bone calcification, neurodegeneration, and a shortened lifespan [49-52]. Recently, the conditional deletion of *Bmal1* in endothelial and hematopoietic cells of the murine retina [53] demonstrated pathologic hallmarks of diabetic retinopathy, thereby expanding on the ocular pathology as a consequence of molecular *Bmal1* defects. It has been identified that BMAL1-mediated activation of the DNA repair system can render remedial mechanisms for treating photodamage, including photoaging [54].

Due to the heterogeneous nature of congenital cataracts, the involvement of additional genetic and environmental factors cannot be dismissed. In this study, we aimed to identify the disease-causing variants in the *BMAL1* gene associated with the CC phenotype in the consanguineous Pakistani families to explore any existing links between the circadian clock and ocular abnormalities.

Materials and Methods

Subjects

The patients were recruited at the pediatric ophthalmology department of Al-Shifa Eye Trust Hospital, Rawalpindi, Pakistan. The study was approved by the Institutional Review Board of the Al-Shifa Eye Trust Hospital (Rawalpindi, Pakistan), and adhered to the tenets of the Declaration of Helsinki with the approval code PK2014:102. Written informed consent was obtained for study participation from the participants and/or their parents, as appropriate. Comprehensive, ocular, medical, and family histories were obtained from the parents/available family member. A detailed ophthalmic examination was performed for both affected and unaffected individuals of families. Blood samples were collected from affected and unaffected siblings, and from the parents. Genomic DNA was extracted using QIAGEN DNA Blood Midi Kit (QIAGEN, Germantown, Maryland, USA).

PCR and sanger sequencing

PCR amplification of the 16 coding exons of the *BMAL1* gene was performed in the (n=21) probands of the consanguineous cataract families using a PE 9700 thermocycler (Applied Biosystems, Foster City, CA).

Primers for the *BMAL1* gene (NM_001351814.1) were designed using Primer 3 [55] to cover exon/intron boundaries up to 100 base pairs into introns and are presented in Table 1. All amplicons were subjected to the following cycling conditions: initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30s, 64°C for 30s, and 72°C for 30s. PCR products were analyzed on 2% agarose gels followed by Sanger sequencing using ABI BigDye chemistry (Applied Biosystems Inc., Foster City, CA, USA), and were processed through an automated ABI 3730 Sequencer (Applied Biosystems, Inc.) using standard protocols.

Data processing

The obtained sequences were aligned with the reference sequence (NM_001351814.1) using CodonCode Aligner (version 6.1) (CodonCode Co., Centerville, MA, USA). Intra-familial segregation analysis was also performed upon the identification of variant in the exon 5 of the *BMAL1* gene in the respective family.

Potential pathogenicity of the identified DNA variants was evaluated by publicly available tools including PhyloP, Grantham and polymorphism phenotyping v-2 (PolyPhen-2) (version 2.1.0 r367) [56] MutationTaster [57], and Sorting Intolerant From Tolerant (SIFT, [58]) to predict the functional impact of the sequence variants on the encoded protein. To determine the amino acid conservation among different species, protein sequences were obtained from UniProt [59] database. Kalign (2.0) was used for the multiple nucleotides and amino acid sequence alignment.

Table 1: Primer sequences of *BMAL1 (ARNTL)*.

Primer Name	Sequence 5′-3′	Product size
ARNTL_E5_Forward	GCTCTTTCCATTCTATCACATGC	476
ARNTL_E5_Reverse	TGTCGCCACCTAGAGTTGG	
ARNTL_E6-7_Forward	TGGCTGTTCGAACTTTATGA	567
ARNTL_E6-7_Reverse	ACAGGACCAAACATGCAGAG	
ARNTL_E8_Forward	TGCCTTGTCAGATGAACATTGA	568
ARNTL_E8_Reverse	GGCATACTACTGAAGGCTACAT	
ARNTL_E9_Forward	AGAGACTAGGCCACTTACAGA	345
ARNTL_E9_Reverse	AGAAATGTGAAGCCTGTCCA	
ARNTL_E10_Forward	TCCTGTGCTTTGGATGCTT	442
ARNTL_E10_Reverse	TGCAGCAATAGAAGAAAGCCA	
ARNTL_E11_Forward	AACCTCCAGATGCCTCCTTC	456
ARNTL_E11_Reverse	GCCAAAGATAGCTCTGGTGC	
ARNTL_E12_Forward	AGTGAGGCAGGCAAGAAAAG	390
ARNTL_E12_Reverse	AGCCAGAAACCATGGAACC	
ARNTL_E13_Forward	TCCCTACCTACATCCCATCC	487
ARNTL_E13_Reverse	TTCTTAGAAAAGCCAGCTGATG	
ARNTL_E14_Forward	GCAGCTTTGACCTTGCTCTC	347
ARNTL_E14_Reverse	GGCTGGCTGACTCTACATCC	
ARNTL_E15_Forward	CTAAAGAGCGATGTCGTTGG	415
ARNTL_E15_Reverse	AGCTTCTGCCAGTCCTGAG	
ARNTL_E16_Forward	ACCTCTGCTGAACTGTGTCC	470
ARNTL_E16_Reverse	GAAATCCGCACATCATCC	
ARNTL_E17_Forward	ACTGCAAATGGATCATGGGA	383
ARNTL_E17_Reverse	TGTTTAACAAGCAGCATCCCT	
ARNTL_E18_Forward	GCTTGCCAAACCCTAATCTAGAT	349
ARNTL_E18_Reverse	CCTCACACAGATGCATTTACTTC	
ARNTL_E19_Forward	AGAAAACTGAAGCCATTTGAAGC	399
ARNTL_E19_Reverse	CTCCACCAAAACTCAAATACTGG	
ARNTL_E20_Forward	AAGCAGCATCTCACCCTACC	423
ARNTL_E20_Reverse	TCAATGGCTCTGAGATGGCT	

Results

Mutation detection

All coding sequences of *BMAL1* of 21 cataract probands were screened. In one proband, we identified a c.41A>T; p.(Asp14Val) variant; in five probands, we detected a non-synonymous variant (rs2290037). The remainder of the sequences were wild-type.

In one proband, a *BMAL1* c.41A>T; p.(Asp14Val) a missense variant in exon 5 was present in a heterozygous form (Figure 1B). This particular variant c.41A>T; p.(Asp14Val) was bioinformatically predicted to be deleterious by SIFT, damaging by PolyPhen-2 and disease-causing by Mutation Taster. The wildtype nucleotide and amino acid residues were highly conserved with a PhyloP score of 4.73, and a grantham score 152 respectively. The nucleotide and amino acid residues were found to be highly conserved among different orthologous species (Figure 1C and 1D). The p.Asp14Val variant was present with a rare allele frequency of 0.000065 (2/30576 individuals) exclusively in the South-Asian population [60]. Segregation analysis of the variant was performed, but the c.41A>T; p.(Asp14Val) variant did not co-segregate with the disease phenotype in the family (Figure 1A).

In addition, we detected a non-synonymous variant (rs2290037) in *BMAL1* intron 7 in 5 out of 21 probands. In our patient set, the variant occurred with a much higher allele frequency of 0.1190 than the general population.

Discussion and Conclusion

Circadian clock genes influence disease susceptibility due to their pleiotropic activities on gene expression by involvement in multiple pathways or via direct involvement with circadian clock function [61]. Earlier studies have reported *BMAL1* gene variants in humans contributing to fertility and seasonality [62] as well as in hypertension and type-2 diabetes [63]. Given the occurrence of cataract in *Bmal1-/-* and *Clock-/-* mice, we tested the hypothesis that human *BMAL1* mutations could be involved in human cataract.

In this study, we showed that genetic variations in *BMAL1* in 21 patients with CC disease did not account for the disease phenotype. Although the identified missense variant c.41A>T; p.(Asp14Val) altered the wild-type amino acid sequence, occurred in a highly evolutionary conserved residue, and was also determined to be "probably pathogenic" by bioinformatics, it happened in the heterozygous state in a single patient and did not segregate in the family. Thus, we could not correlate the Asp14Val variant with the occurrence of congenital cataract. We did not exclude potential additional pathogenic mutations in our CC probands occurring outside of the coding exons and the flanking intron splice sites. A non-synonymous heterozygous variant (rs2290037) with a higher allele frequency was detected in

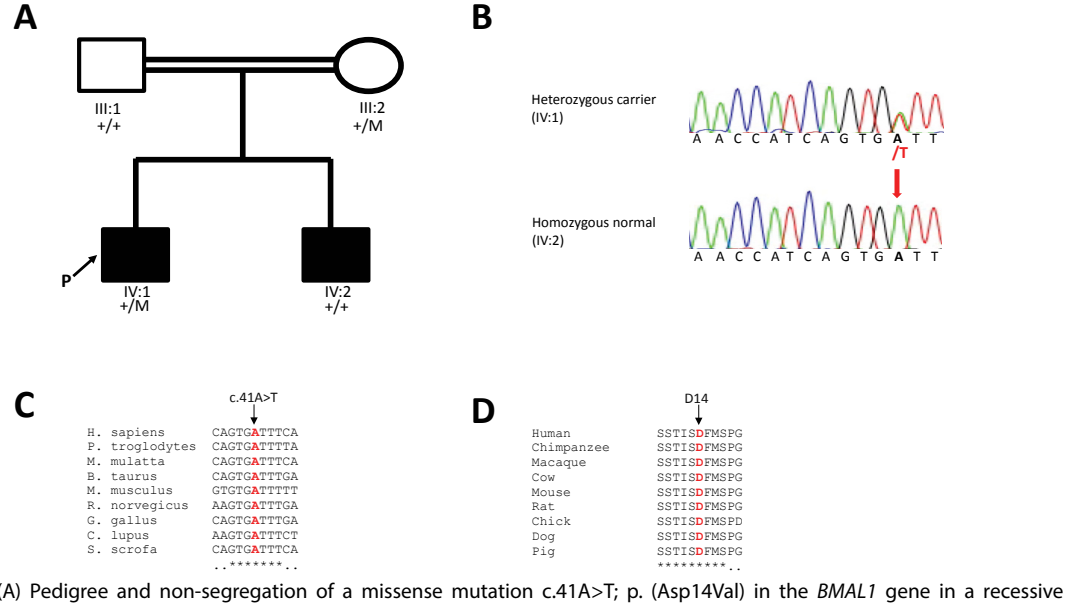

Figure 1: (A) Pedigree and non-segregation of a missense mutation c.41A>T; p. (Asp14Val) in the *BMAL1* gene in a recessive congenital cataract family; (B) DNA chromatogram of the *BMAL1* fragment for the affected individual (IV:1) carrying heterozygous genetic mutation; (C,D) Multiple sequence alignment showing the nucleotide and amino acid conservation in different species for the c.41A>T; p. (Asp14Val). The wild-type nucleotide (D) and amino acid (A) are indicated with an arrow and in red color.

the intronic region. It has been previously estimated that 5% of the rare non-synonymous heterozygous variants carry at least ~22 pathogenic derived alleles, which if turns out to be homozygous due to consanguineous marriages, can lead to recessive diseases [64].

To the best of our knowledge, our study is the first attempt to evaluate the presence of genetic variants in the *BMAL1* gene for congenital cataract. The samples were identified from a well-characterized epidemiological cohort, which has a high degree of genetic heterogeneity [65]. Based on results in the mice, it will be apt to state that the human circadian clock genes may not be such an attractive target for mutation analysis in cataract families. Yet, we cannot entirely exclude the involvement of *BMAL1* in human cataract. *BMAL1* gene is an intricate member of the clockwork web, and since *BMAL1* is not a sole member, *BMAL1-CLOCK* complex drives the clockwork machinery. Therefore, some more specific and additional screening of congenital cataract patients with (other) circadian clock genes (sequences) may be justified in subsequent studies.

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Conflict of Interest

The authors declare no competing conflict of interests.

Additional Funding

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Availability of Data and Materials

All relevant datasets are used in the manuscript. The analyzed data is available from the corresponding author upon request.

Authors' Contributions

U.B and A.A.B.B. conceived and designed the experiments; U.B. performed the experiments; S.M, M.I and S.N.S. recruited patients and collected samples; J.B, M.P.F.S., and A.A.B.B. contributed reagents/materials/analysis tools; and U.B. wrote the manuscript. All authors have read and approved the final manuscript.

Ethics Approval and Consent to Participate

The study was approved by the Institutional Review Board of the Al-Shifa Eye Trust Hospital (Rawalpindi, Pakistan), adhering to the tenets of the Declaration of Helsinki with the approval code PK2014:102.

Consent for Publication

The family members of the probands signed a written informed consent form for publication of all data.

Executive summary

Background: Given the cataract phenotype in *Bmal1-/-* mice, we aimed to identify potential disease-causing variants in the human circadian clock candidate gene *BMAL1* in 21 probands of consanguineous Pakistani congenital cataract families.

Methods: Ophthalmic examinations were performed for the probands and available family members. Genomic DNA was isolated from a volume of 5 ml of peripheral blood. The entire coding region of the candidate gene *BMAL1* was analyzed in the probands of 21 families with targeted Sanger sequencing.

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