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ASSOCIATION BETWEEN EXON 5 OF PAX6 GENE POLYMORPHISM AND CONGENITAL CATARACT IN NORTH INDIA

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Abstract - Background: Congenital cataract is among most common treatable causes of visual impairment and blindness during infancy amounting to 10% of all vision loss worldwide.

Aim: Our aim was to determine association of PAX-6 gene in congenital cataract patients and their normal parents in North India.

Setting and Design: It was clinical prospective hospital based study done at a Tertiary care centre.

Material and Method: Forty five patients were enrolled as cases and 89 parents as controls. Ethical clearance was obtained and written and informed consent was taken from all parents. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was done in both groups to find out PAX 6 gene polymorphisms. All cases underwent cataract extraction bilaterally in different sittings followed by intraocular lens implantation.

Results: A total of 45 patients were taken who suffered from bilateral congenital cataract. These comprised of 35 males (77.7%) and 10 (22.2%) females with sex ratio of 3.5:1. We screened exon5 of PAX6 gene for novel polymorphisms that could be used to detect an association of PAX6 gene with congenital cataract patients and their parents. Unfortunately, no polymorphism in patient affected with congenital cataract was observed in contrast to other studies which demonstrated positive association in South Asian population.

Conclusion: Further work in this study is required and may possibly let us a peep into the role of PAX6 in the development of eye and its association with the congenital cataract in this country.

Keywords: Congenital Cataract, PAX 6 Gene, Gene Polymorphism, Exon 5

INTRODUCTION

Congenital cataract stands as commonest cause of vision loss which can be treated easily during childhood and account for approximately 10% of all loss in visual acuity in world. Its prevalence is 0.02.-0.07% per live births in developed countries[1-3], and 5 to 15 per 10,000 in developing countries[4].

Age of presentation of congenital cataract is within one year of life. In India around 20,000 to 40,000 new cases of congenital cataract are diagnosed every year[4]. If the cataract is small, occupies the anterior part of the lens, or its periphery, it is usually visually insignificant [5].

On the basis of morphology congenital cataract can occur as zonular, complete, polar, cerulean, coronary, capsular, sutural, membranous, nuclear, and are usually occurs in a specific part of crystalline lens, and may be static and progressive both.[6] Generally, the more posterior location and denser the cataract, the more is the deprivation in visual function.[7]

Congenital cataract is heterogeneous both from clinical and genetic point of view; sporadic congenital cataract is usually passed on as an autosomal dominant inheritence. Other modes of inheritance like autosomal recessive and sex linked inheritance are less commonly seen in population.[8]

We already know that different types of mutations in the same gene can cause similar kind of cataract, whereas the different types of cataract in same family suggest that same type of mutation in a single gene can lead to different phenotypes[9].

More than 25 genes and loci on different number of chromosomes have been found to be associated with congenital cataract[10]which include genes encoding avian musculoaponeurotic fibrosarcoma (MAF)[11] , cytoskeletal structural proteins[12], aquaporine[13], transcription factor 3 (PITX3)[14], specific lens connexins (Cx43, Cx46, and Cx50)[15], and heat shock protein factor 4 (HSF4)[16] ,crystallins (CRY-A, CRY-B, and CRY-G)[17]

Paired box protein Pax-6 also called **oculorhombin** is a transcription factor that is encoded by *PAX6* gene in humans.[18] There are two different binding DNA sites present on encoded protein which function as transcription regulation process of gene. It is main regulatory gene involved in eye and brain development. Mutations of the Pax6 gene can lead to different developmental anomalies of the eyes including aniridia, and Peter's anomaly.[19-24]

The aniridia occurs due to one deficient haploid of PAX6 gene, which is a paired box DNA-binding protein. There are two binding domains of PAX6 gene the paired and the homeodomain which bind paired DNA. These domains function as separate unit by utilizing Pax6 to carry out processes of molecular signaling that monitors the functions of Pax6.[25] Some cancers like alveolar rhabdomyosarcoma can also be attributed to *PAX* mutations.[26]These findings suggest potential in therapeutic applications for research in PAX6 gene and thus may lead to a more detailed understanding of its role in development.[27]

MATERIAL AND METHODS-

The present study was undertaken to evaluate the association of PAX6 gene polymorphisms in congenital cataract patients during the period of July 2015 and June 2016. Ethical approval was obtained for the study.

A written consent was taken from the parents of children mentioning the pros and cons of the treatment. The study is based on data having 33 sporadic cases and 12 familial cases of congenital cataract. During the study 45 children with congenital cataract and their parents (89 in number as one child had only one parent) were recruited after written consent.

INCLUSION CRITERIA

All cases of congenital cataract as per history and clinical evaluation.

EXCLUSION CRITERIA

Unwilling guardians. Patients with history of trauma. Patients with history of use of steroids. Patients with history of previous radiation therapy. Patients with history of previous laser therapy. Patients with posterior segment pathology. Patient receiving treatment for some disease.

Low birth weight child or child suffering from protein energy malnutrition.

Patients' age, gender, duration of symptoms, age at onset of disease, presence or absence of any other associated symptoms, use of any medication, antenatal, natal and perinatal history, developmental history and immunization history were recorded from hospital data. All the cases in our study suffered from bilateral disease.

All patients underwent biological workup including complete blood profile, renal function test, serum electrolytes, liver function profile, blood sugar levels. All the patients underwent the following tests on very first day of visit and then at regular follow up at 7th post operative day , 4 weeks and 8 weeks: Visual acuity using Snellen's chart (if possible), Vision with pin hole (if possible), Retinoscopy under mydriasis, Refraction by autorefractor and best corrected visual acuity, Slit lamp examination, Distant direct ophthalmoscopy, Indirect ophthalmoscopy and Ultrasonography B Scan of both eyes.

GENETIC ANALYSIS

3 to 5 ml of peripheral venous blood was collected from patient along with parents in EDTA coated vials from the subjects and stored at -20 degrees Celsius for less than 3 months before DNA extraction.

DNA isolation was done by ''Salting Out'' method and dissolved in tris- EDTA (TE) buffer. Primers used for amplification of PCR were designed using Primer3 software version 0.4.0 (http://frodo.wi.mit.edu/primer3/) (Rozen S et al., 2000) for PAX6 gene **exon5** using sequences from the NCBI Gene (Reference GRCh38.p2 Primary Assembly NC_000002.12) and they were amplified by Thermocycler (Applied Bio system). *In silico* PCR analysis and Blast searches were performed using the UCSC Genome Bioinformatics website (website http://genome.ucsc.edu/).

PCR was used to amplify DNA with candidate gene primers for sequencing. Primers were purchased from NEB and prepared from dry oligonucleotides to make up a working concentration of 5pmol/µl. Gel electrophoresis was used to enable us to find out if the desired region of the DNA was amplified during PCR reaction. By separating PCR products by size, it allowed us to estimate the size of the amplified product.

DNA Sequencing

Sequencing was used to screen the candidate gene in the relevant affected individuals. PCR products were first purified using the EXOSAP protocol. Purified PCR products were then added to the reaction mixture for sequencing amplification. Sequencing reactions were analysed using 3130*xL* Genetic Analyzer (Applied Biosystems®). Sequencing files obtained from the 3130*xL* Genetic Analyzer (Applied Biosystems®) were analysed using FinchTv viewer.

RESULTS

A total of 135 children underwent cataract surgery in single unit in the Ophthalmology department in a medical college.

Of this 135, 45 were affected with congenital cataracts. These comprise of 35 males (77.77%) and 10 (22.22%) females, a sex ratio of 3.5:1.The mean age of male and female patients affected with congenital cataract was 4.3±0.888194 and 4.75±1.258306 years respectively. During the study according to religion the patients were categorized into two categories Hindu (14) and Muslims (3). We observed that maximum patient suffering from congenital cataract were Hindu in this part of country.

The study also includes the type of cataract during one year period. The type of cataract included zonular, nuclear and total. During the period zonular (37 or 82.22%), nuclear (5 or 11.11%) and total (3 or 6.67%) were the major type of cataract encountered in our study. As the percentage of zonular cataract was maximum in our study; hence we also checked the distribution pattern of zonular cataract according to sex. We observed that the percentage of male candidates were maximum in zonular cataract.

Our study also recorded preoperative visual acuity and retinoscopy values of all the cases. We found that most of our cases had vision of range between 6/60 to 6/18 (37, 82.22%) followed by 5 cases having vision of < 6/60 (11.11%) and 3 cases having vision of > 6/18 (6.67%). In retinoscopy done at one arm distance we found that 40 (88.88%) of them had values between range of + 2.50 DS to + 5.00 DS in both meridians while one each had value < +2.50 DS and > +5.00 DS values in both meridians respectively.

The study also recorded preoperative fundus details of patients among which in 27 (60.0%) of them we could easily see all the fundus details very clearly through cataract while in 8 (17.77%) of them we had

some difficulty in visualizing fundus details. We could barely see fundus in remaining 10 (22.22%) of them. Subsequently, these 10 cases underwent Ultrasound B Scan of their eyes which were found to be absolutely normal.

Our study also looked for preoperative axial length of cases for planning of surgery and prediction of postoperative visual outcomes. We found majority of our cases had axial length between 20 – 22 mm (88.2%), two had axial length less than 20 and remaining three had greater than 22 mm.

All our 45 cases underwent cataract extraction bilaterally in different sittings followed by intraocular lens implantation. Subsequently, post-operative visual acuity was also calculated after one month of surgery. Majority (37, 82.22%) of our cases developed visual acuity ranging between 6/9 – 6/18 and one of them obtained visual acuity of 6/9. 7 cases obtained visual acuity of < 6/18 after surgery.

PAX6 Genotyping

We screened exon5 of PAX6 gene for novel polymorphisms that could be used to detect an association of PAX6 gene with congenital cataract patients and their parents. The amplified products for exon5 were subjected to gel electrophoresis done on 2 percent agarose gel polymer (Fig.1). Our present Study could not find any polymorphism in patient affected with congenital cataract.

DISCUSSION

A prospective study was done involving patients that were referred for treatment in tertiary eye care centre. In our study, it was observed that during the last 1 year the male to female ratio was 3.5:1. However, the ratio is very high this may be due to an underestimation of the true situation since; in India many people do not come to hospital if a girl child has this type of disease. This needs a door to door study of a location. In our study we did observe that the occurrence of zonular cataract was most prevalent and that of nuclear, total, sutural were least. We conclude that zonular or lamellar cataract is the most common type of congenital cataract as also demonstrated in other studies.

The distribution according to religion showed maximum number of affected patients belong to Hindu religion as in Indian scenario majority belongs to Hindu community. Study involving more cases from the general population may confirm the same.

Majority of our cases had preoperative visual acuity between 6/18 – 6/60. This is due to the fact that zonular cataract demonstrates different grades of opacities in different areas of lens with possibly clear area in between zones. One of them who had visual acuity of < 6/60 may have been due to total cataract in which whole lens was opaque thus did not allow any light to pass through.

Majority of the cases who underwent retinoscopy with eye ointment atropine at one arm distance revealed a hypermetropic fundus. This finding is consistent with age of presentation of disease. As majority of these patients are children their eye ball is in continuous phase of development hence have a hypermetropic fundus till age of 8 years. One of them who demonstrated a myopic fundus may have been due to lenticular myopia induced by cataract. Axial length also followed the same rule of hypermetropia as majority of fundus demonstrated axial length ranging from 20-22 mm.

Fundus details could be deciphered in majority of cases as zonular cataract usually allow for fundus examination through clear portion of lens. In cases where details could not be appreciated were possibly due to greater density of cataract and involvement of all the zones of lens in cataract. Following surgery majority of our patients developed visual acuity of 6/9-6/12 with two of them having visual acuity of 6/6. One patient developed 6/18 vision which may have been due to central nuclear nature of cataract which did not allow any light to pass through thereby producing stimulus deprivation amblyopia.

Many genetic studies have shown that genes involved in early onset cataract are also responsible for age-related cataract like mutations in *MIP* and *yC-crystalliri* gene which result in age related cataracts,[28,29] and familial adult onset pulverulent cataracts has also been shown association to the CAAR locus.[30] It may be suggested that mutations in certain genes can lead to detrimental effect on development of lens resulting in congenital cataract.

Glaser T et al., in 1994[31] found that PAX6 gene is found on short arm of human chromosome 11p13, and mutations in this gene leads to a variety of hereditary disorders related to eye, like aniridia, coloboma of the iris, keratitis, congenital cataracts, Peter's anomaly, and optic nerve defects. ***Fucheng Cai et al.***, 2010[32] identified a new deletion mutation of *PAX6* in a family with aniridia and congenital cataract in China. This finding expanded the varieties of mutation of *PAX6* and is useful for genetic counseling and prenatal diagnosis in families with aniridia and congenital cataract. Similar reports of another mutation in exon 6 of PAX6 gene family has been published by ***Dansault et al.*** [33]. All of them suffered from different congenital ocular disorders including congenital cataracts, diverse neurological disorders and variable cognitive impairments. Another PAX6 mutation in a patient with a positive family history suffering from cataract, aniridia, developmental delay and nystagmus has been identified by ***Chien et al.*** [34] recently. ***Manel Chograni et al.***[35] could not report any mutation in any of four genes of congenital cataract. He detected three intronic variations only in PAX6 gene: IVS4 -274insG (intron 4), IVS12 -174G>A (intron12) in the four studied families and IVS4 -195G>A (intron 4) in two families.

Manel Chograni et al.[36] found a new nonsense mutation (p.Q89X) in exon 6 of PAX6 gene in a Tunisian family with aniridia and congenital cataract. In addition, he also described the possible pathogenic effect of the reported nonsense mutation, p.R240X, in another Tunisian family with aniridia, congenital cataracts and other ocular anomalies. These two mutations lead to truncated proteins and added to the large group of nonsense mutations associated with aniridia. ***Li Wang et al.***[37] discovered a new missense mutation (c.1147A>T) in exon 12 of *PAX6* gene associated with autosomal dominant congenital aniridia and cataract in a family in China. It gave further proof of genotypic heterogeneity in congenital aniridia associated with *PAX6*. ***Noriyuki Azuma et al.***[38] identified a new missense mutation in four families with Peter's anomaly, congenital cataract, Axenfeld syndrome, and/or foveal hypoplasia, which is probably the first mutation identified in a region other than exon i.e. the region of splice variant. Two different PAX6 mutations in a family suffering from aniridia and congenital cataracts have been identified by ***Tom Glaser et al.***[39]

Very little work has been performed to correlate an association between PAX 6 gene and congenital cataract. PAX6 plays an important role in development of eye. It is emphasized that it may play an important role in congenital cataract too. We could not demonstrate any polymorphisms in exon 5 segment of PAX 6 gene in our subjects possibly due to smaller sample size and lesser familial cases. Other exons as well as introns have shown association between PAX 6 gene and congenital cataract as reported in previous studies. But we could not find any association in exon 5 segment and congenital cataract. Hence further work in this study is required and may possibly let us a peep into the role of PAX6 in the development of eye and its association with the congenital cataract in this country.

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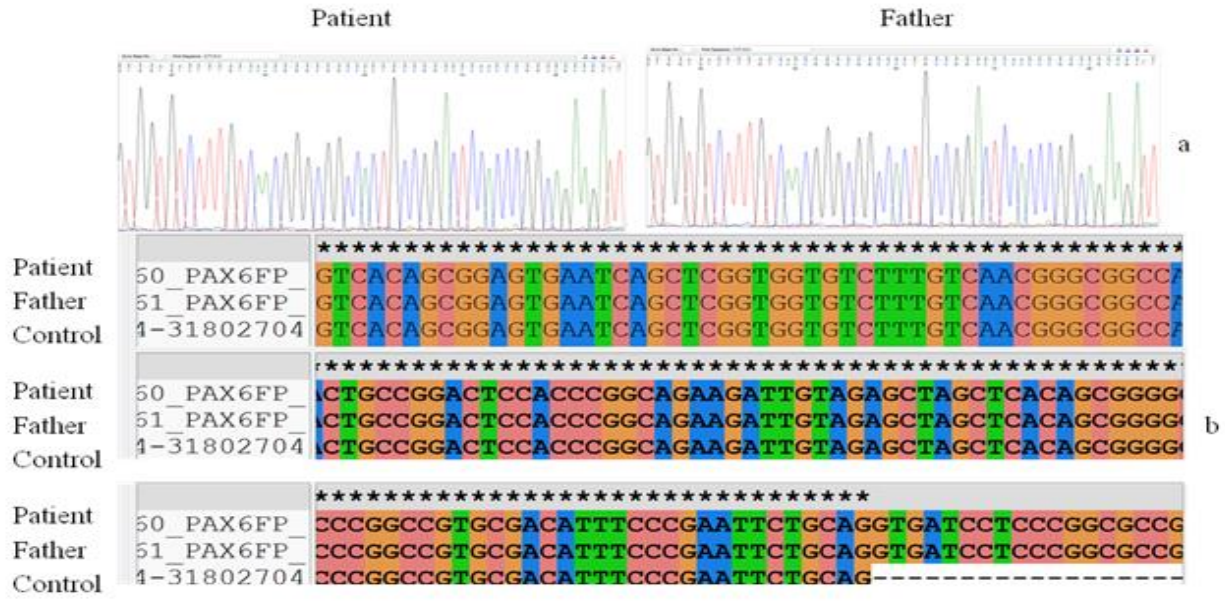
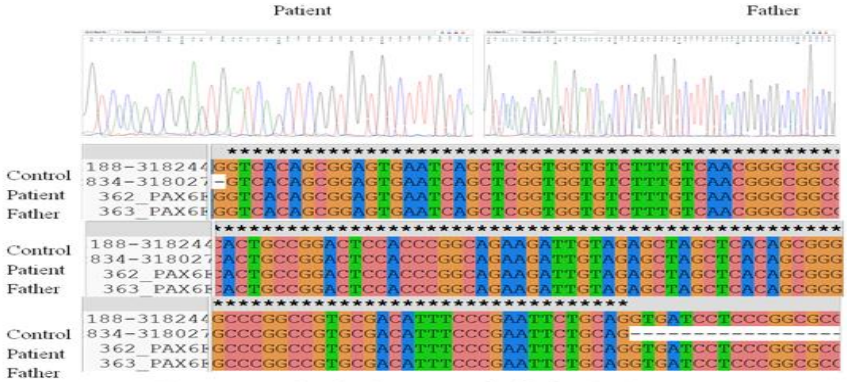


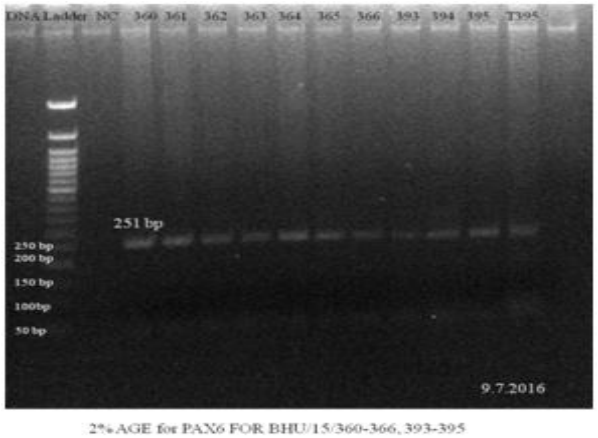
a. Figure representing the chromatogram for family 360-361.
b. Multiple sequence alignment show that no change was observed

SEQUENCING RESULTS FOR FAMILY 360-361



a. Figure representing the chromatogram for family 362-363.
b. Multiple sequence alignment show that no change was observed

SEQUENCING RESULTS FOR FAMILY 362-363.



2% AGE for PAX6 FOR BHU/15/360-366, 393-395

Figure 1: 2% Agarose Gel Electrophoresis (AGE) amplified product of congenital cataract family sample (360, 361, 362, 363, 364, 365, 366, 393, 394, 395) of PAX6 Exon5.