

Identification of a Non-Stop Mutation in *PAX6* Causing a Unique Presentation of Aniridia in an Iranian Family Trial

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Abstract- Non-syndromic aniridia (iris hypoplasia) as an autosomal dominant eye disorder results from the chromosomal abnormalities and mutations within the paired box gene 6 (*PAX6*). The aim of this study was to investigate on the clinical and the underlying genetic alteration in *PAX6* gene in a large pedigree with five generations of Iranian family with an autosomal dominant aniridia. Here, we reported unique clinical features in terms of presenting nystagmus, ptosis, minimal iris abnormality, foveal hypoplasia and late-onset clinical limbal stem cell deficiency. Genomic DNA was extracted from the affected members and polymerase chain reaction (PCR) was conducted using specific primers to amplify coding sequence of *PAX6*. Then, PCR products were subjected to bidirectional dye terminator sequencing. A heterozygous transversion mutation A→T (c.1268A>T, p.*423Lext*15) in exon 13 of *PAX6* was identified in all affected individuals, but not in the healthy members. This is the first report of non-stop mutation in *PAX6* gene in an Iranian family accompanied with an isolated form of unusual congenital aniridia running within this family.

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Introduction

Aniridia is a rare panocular genetic eye disorder that is present at birth and is almost always bilateral. Also, it is characterized as either partial or complete absence of the iris accompanied with other ocular abnormalities, leading to severe visual impairment. The prevalence of aniridia varies from 1:47,000 in Sweden to 1:100,000 infants born worldwide (1-3).

About two-thirds of aniridia cases are inheritable with an autosomal dominant transmission, high penetrance and variable expressivity, while the remaining (one-third) regarded as a sporadic condition with a low risk of recurrence. Sporadic cases are often associated with other ocular anomalies, such as Wilm's tumor-aniridia syndrome and Gillespie syndrome (4). Genetically, aniridia is mainly caused by mutations in one copy of human *PAX6*, whereas the homozygous

mutations lead to lethal phenotype (5,6).

The *PAX6* (NG_008679) located on 11p13, consists of 14 exons which encode a regulatory protein of 436 amino acids. This protein is composed of three distinct domains, two DNA binding domains, a paired box (PD) and a paired-type homeodomain (HD), and a 153-amino acid carboxyl-terminal transactivation domain rich of proline/serine/threonine (PST) (7).

It has been thought that during the embryonic stage, *PAX6* is contributed to the development of forebrain, pancreas and ocular tissues, including corneal epithelium, lens, and retina by activating specific genes (8,9). Since the majority of non-syndromic aniridia patients with *PAX6* genetic mutations appear to carry a normal allele, it is understandable that the disease caused by the dominant negative effect of mutated protein. In addition, several studies reported that a mutation in the *PAX6* gene could cause diverse eye

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abnormalities accounting for 40%–80% of non-syndromic aniridia (2,10-12). The *PAX6* heterozygous loss of function due to point mutations and chromosomal abnormalities have been identified in 90% of aniridia cases. In this regard, more than 900 mutations reported in *PAX6* associated with a wide spectrum of aniridia in the different ethnic group (http://lsdb.hgu.mrc.ac.uk/home.php?select_db=PAX6).

Accumulation of genetic alterations in *PAX6* is commonly believed to promote eye defects. If this concept provides our knowledge of aniridia pathogenesis worldwide, it has not been reported in Iran. In the present study, we report unusual clinical features in a kindred pedigree of an Iranian family with an aniridia.

Materials and Methods

Ascertaining family members

This study was approved by Institutional Review Board (IRB) of Tehran University of Medical Sciences (TUMS) and informed consent was obtained from all affected individuals. The affected patients with aniridia underwent a complete ophthalmologic examination comprising visual acuity measurement, slit lamp examination and fundoscopy (Farabi Eye Hospital in Tehran, Iran).

Mutation screening

Five ml of peripheral whole blood was obtained from participating family members. Genomic DNA was isolated using an AccuPrep® Genomic DNA Extraction Kit (Bioneer, Korea) according to the manufacturer's protocol. DNA was examined in terms of quality and quantity using the measurement of A260/280 nm and gel agarose electrophoresis, respectively, and stored at -20°C until required. PCR amplification of the coding sequence of *PAX6* gene was carried out using primers reported previously (2). PCR was typically performed using 1 unit Taq DNA polymerase (SinaClon, Iran), 10 µM of each primer, 200 µM of each dNTPs, 1.5 mM MgCl₂, 50 ng DNA, 1X PCR buffer and 5% DMSO in 25 µl of PCR reaction volume. Thermal cycling was performed using a Touch-down amplification program on an ABI 2720 thermocycler (Applied Biosystems). The PCR conditions included an initial denaturation step for 5 min at 96°C, followed by 30 cycles at 95°C for 30 s, primer annealing 30 s at 68°C with a 1°C decrement every second cycle down to 53°C for 40 s and 72°C for extension, then 16 cycles of 95°C for 30 s, 53°C for 40 s and 72°C for 60 s and 7 min final extension at 72°C.

PCR products were electrophoretically separated on 1.5% agarose gels and visualized with ethidium bromide. PCR amplification products were purified by the PCR and Gel Purification Kit (Bioneer, Korea) and used as template for DNA sequencing, which was performed at the DNA sequencing center (Macrogen, Korea). The sequencing data for each fragment was analyzed using Chromas Version 2.32 software, and similarity search was performed using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>).

Results

Ocular phenotypes

Twenty-two individuals were affected with aniridia and other ocular abnormalities in this Iranian family. Patients' age was ranging from 6 to 42. Ophthalmologic examinations revealed nystagmus, ptosis, scleral show, iris abnormality, foveal hypoplasia and peripheral limbal stem cell deficiency. All the patients were complaining about decreased vision since childhood with no specific systemic disease. The BCVA was below 5/10 in all family members. The affected patients showed unique clinical features. Proband who is 74-year old was only cased with frank central corneal conjunctivalization. Other patients' examination showed the ill-defined anatomy of limbus (lack of palisade of Vogt) and the peripheral area of conjunctivalization with normal epithelium in the central cornea. The iris abnormality findings including mild corectopia and subtle iris stromal abnormality (lack of normal crypt and fold in iris stromal) were seen in all patients except one who had frank iris involvement with partial loss of iris tissue. The pupil had normal contour in the rest of the patients, but it did not dilate adequately with the cycloplegic drop. Moreover, signs of aqueous deficiency dry eye including abnormal Schirmer test, tear break up time (TBUT), and corneal staining was common in young patients without having any symptom of dry eye. Another unusual finding in this family was bulbar conjunctival keratinization (Figure 1). It was observed in both young and middle-aged patients not compatible with the severity of dry eye. Intraocular pressure (IOP) amongst all patients was normal. In spite of the fact that there was no congenital cataract and glaucoma, senile cataract was developed earlier in these patients than the general population. The thin anterior capsule was detected during phacoemulsification for one patient. Table 1 shows clinical features of the affected patients.

The proband (III:3) was a 74-year-old man who presented with decreased vision and congenital

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nystagmus in both eyes (Figure 2). Best corrected visual acuity (BCVA) was finger counting in both eye. Eye examination showed diffuse corneal epithelium opacity with superficial vascularization and dens cataract. The left eye underwent superficial keratectomy and extra-

capsular cataract surgery. The vision was improved, and epithelium was smooth and clear for 3 months. Foveal hypoplasia could be seen after surgery. Subsequently, the epithelium gradually became cloudy.

Table 1. Clinical summary of affected individuals in this study

No.	Gender F/M	Age	Schirmer test >10 mm	TBU T >10	Ptosis	Scleral show	IOP	Corneal staining (PEE)	Peripheral corneal conjunctivalization	Conjunctival Keratinization
IV:1	M	42	Yes	No	Yes	Yes	NL	Scant	No	No
IV:3	M	38	Yes	No	No	Yes	NL	No	No	No
IV:11	M	36	OD: YES OS: NO	Yes	No	No	NL	Diffuse	No	Temporal & Nasal
IV:17	F	27	No	No	No	Yes	NL	NO	360	Temporal
IV:25	M	30	No	No	Yes	Yes	NL	Inferior	Inferior	Inferior & Temporal
IV:27	M	25	No	No	No	Yes	NL	Yes	Inferior	No
V:1	F	17	No	No	No	No	NL	No	360	No
V:2	F	16	Yes	No	No	Yes	NL	No	Inferior	Temporal
V:8	M	9	Yes	Yes	No	Yes	NL	Diffuse	Inferior	Temporal
V:9	M	6	Yes	Yes	Yes	Yes	NL	No	Inferior & Superior	No

OD: Oculus Dexter (right eye), OS: Oculus Sinister (left eye), TBUT: Tear Breakup Time, IOP: Intraocular Pressure, PEE: Punctate Epithelial Erosion, NL: Normal.

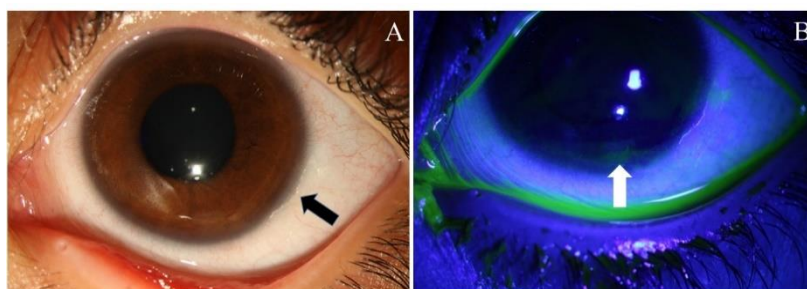


Figure 1. Ophthalmological examinations of affected individuals. Slit-lamp photographs of the anirida patients show A: Temporal conjunctival keratinization and minimal iris abnormality (black arrow). B: Inferior peripheral conjunctivalization of cornea after fluorescein staining of cornea (white arrow)

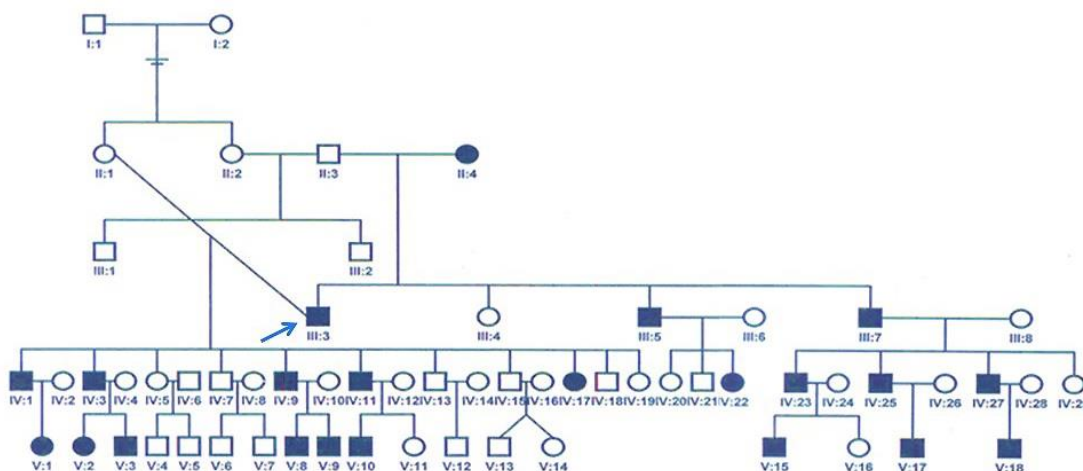


Figure 2. Pedigree of an Iranian family of five generations with aniridia. Filled symbols represent subjects with aniridia, and open symbols represent normal subjects

Mutation analysis of *PAX6* gene

Mutation analysis of *PAX6* in the affected family members has revealed a heterozygous non-stop mutation (c.1268A>T, p.*423Lext*15) in *PAX6*. This mutation substitutes the stop codon with a leucine residue, potentially adding 15 amino acids to the C-terminus of *PAX6* in exon 14 (Figure 3). This mutation was detected in the affected members, but not in other unaffected

members. Co-segregation of this genetic change among affected individuals suggested that it represents an inheritable mutation-causing aniridia. The identified mutation has not been previously reported in the Iranian patients, but it has been reported 8 times in Human *PAX6* Allelic Variant Database http://lsdb.hgu.mrc.ac.uk/home.php?select_db=PAX6.

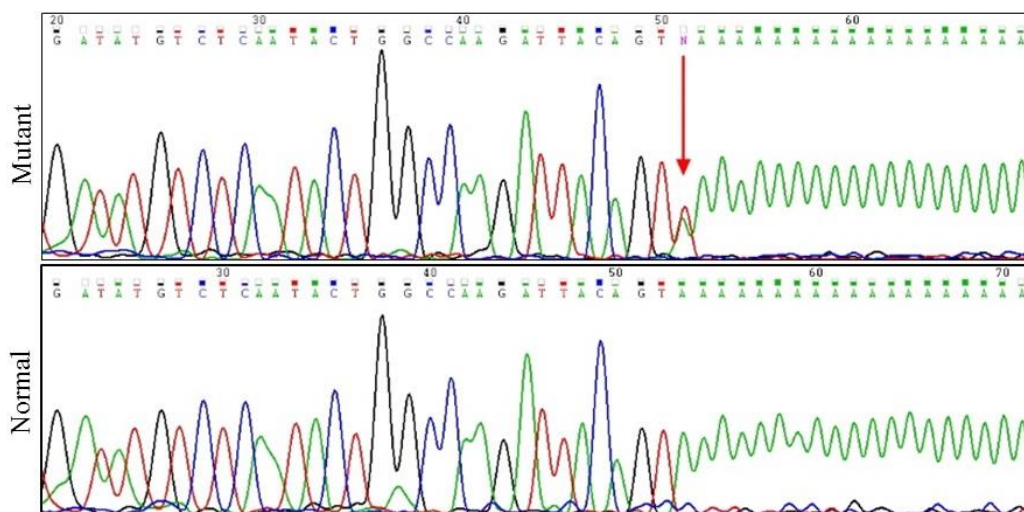


Figure 3. Mutation analysis of *PAX6* gene. Detection of stop codon (TAA) change to leucine amino acid code (TTA) in mRNA encoding the *PAX6* by PCR direct DNA sequencing (A) Sequence chromatogram derived from a heterozygote patient (c.1268A>T, p.*423Lext*15) (B) Normal chromatogram. Arrow indicates the position

Discussion

Non-syndromic aniridia is a severe, congenital ocular malformation transmitted in an autosomal-dominant mode of inheritance with high penetrance and variable expression. In this study, we considered this family due to unique ocular presentations. The most characteristic finding was a late presentation of clinical limbal stem cell deficiency. Although abnormal limbal appearance (absence of palisade of Vogt) or small peripheral islands of conjunctivalization were seen in all patients, prominent central stem cell deficiency was detected after the sixties in one patient. Another unique finding in this family was bulbar conjunctival keratinization. It may be due to the effect of the *PAX6* mutation on conjunctival stem cell and goblet cells that causes keratinization of the conjunctiva and worsen dry eye (4). Limbal stem cells are exposed to the environmental factors (e.g., UV and air pollution), and cumulative effects of these factors can deteriorate

genetically compromised cells. Moreover, we detected the high prevalence of dry eye in this family. Dry eye can accelerate central conjunctivalization of the cornea by deteriorating limbal stem cell environment. Early diagnosis and treatment of dry eye could delay significantly clinical stem cell deficiency. Minimal iris abnormalities were seen in all affected patients except one. It is important for ophthalmologists to be aware of these fine iris changes to correctly diagnosis the disease, Skeens *et al.*, (2011) previously reported a case series of aniridia with minimal iris changes (13).

In our study, the screening in five-generations of twenty-two aniridia patients showed a non-stop heterozygous mutation in *PAX6* gene, but not in unaffected family members. Our findings revealed that the first alternative in-frame stop codon was 45 bases after the mutated stop codon, suggesting that the *PAX6* mutated protein would be elongated by an extra 15 amino acids into the 3'-UTR.

So far, over 119 non-stop mutations have been

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reported in 87 different genes. This kind of genetic alterations approximately accounts for 2% of codon-changing mutations (14). According to published data by now, only a few aniridia cases associated with non-stop mutations in *PAX6* have been reported (13). Also, a few non-stop mutations causing human inherited disorders have been reported such as in the *F10* and *FOXE* genes, respectively causing factor X deficiency (15) and anterior segment dysgenesis (16).

Although it is not quite understandable how nonsense mutation in the stop codon of *PAX6* could be associated with a wide range of clinical presentations, a long enough abnormally extended sequence of this protein could support causal association with clinical phenotypes in aniridia. Several factors such as gene-gene and gene-environment interactions and mRNA stability could modulate the expression of the genetic defect. It has been determined that cells remove mRNAs that contain premature translational stop codons or that lack in-frame stop codons via a specific RNA-degradation process (17,18). A meta-analysis study using nonstop mutations in different genes causing human inherited disease suggested an alternative stop codon at greater distance from the mutated stop codon could lead to decrease of the amount of translated protein and ultimately might cause distinct clinical phenotypes (19). In addition, experimental and bioinformatics studies postulated that the C-terminal region of *PAX6* is involved in transcriptional activation of the *PAX6* target genes through interaction with its protein partners (6,20).

In conclusion, it seems that the nonstop mutation in *PAX6* gene plays critical roles in the pathogenesis of an unusual aniridia phenotype in our studied family. Since the present study was conducted in order to screen genetic alteration in *PAX6* coding sequence in a family with a unique aniridia condition, further studies using animal model will provide opportunities, previously and currently unavailable, for the biologic significance of the observation to address critical question that arises from the present work (21). Such animal model affected with aniridia due to non-stop mutation in *PAX6* gene could thus provide eye tissues which are useful for functional, transcriptomic and proteomic studies.

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