Доклади на Българската академия на науките
Comptes rendus de l'Académie bulgare des Sciences
Tome 77, No 10, 2024

MEDICINE
Medical genetics

MUTATIONAL AND CLINICAL SPECTRUM OF THE *PAX6* GENE IN BULGARIAN PATIENTS WITH ANIRIDIA

Maya Atanasoska[1,2]✉, Radoslava Vazharova[1,4]✉, Anna Popova[3], Veselin Kukenski[4], Lubomir Balabanski[1], Silvia Andonova[5], Alexey Savov[5], Draga Toncheva[1,6]

Received on November 2, 2021
Accepted on February 24, 2022

Abstract

The *PAX6* gene has highly conservative structure and developmentally essential function and is expressed in structurally different organs and systems. Haploinsufficiency of the *PAX6* gene product leads to aniridia and aniridia-like phenotypes. A total of 9 patients and 115 controls were examined with the use of a variety of molecular-genetics methods (SNP array, Sanger sequencing, NGS, WES) and in collaboration with different clinicians. Two pathogenic CNVs, three potentially pathogenic variants and two nonsynonymous SNVs in the *PAX6* gene were identified: two out of the three potentially pathogenic variants have not been previously reported. The heterogeneous nature of aniridia and the variable spectrum of the *PAX6* mutations were confirmed by our study. Approximately 75% of the registered cases in the Bulgarian population were studied and five disease-related variants were discovered. The small number of polymorphic variants in the controls supports the highly conserved role and nature of the *PAX6* and its product.

Key words: aniridia, the *PAX6* gene, SNP array, NGS, WES

Introduction. The *PAX6* gene (OMIM 607108) is highly conserved, developmentally important gene. Isolated through positional cloning in 1991 [1, 2], the gene is located on chromosome 11p13 and contains 14 exons including one alternatively spliced exon 5a. Transcribed as a 2.7 Kb mRNA the *PAX6* gene encodes a

https://doi.org/10.7546/CRABS.2024.10.12

422 (436 when including exon 5a) amino acids protein [3]. The gene has a crucial role in the eye development, in differentiation and tissue maintenance on different parts of the brain, ventral spinal cord and the pancreas [4, 5]. It is expressed in many eye structures: the optic sulcus, the eye vesicle, the lens placode, the differentiating retina and in the future cornea [6]. The tissue expression cascade of the gene is identified in the several structures of the brain (frontal cortex, ventral thalamus, pituitary gland, epiphysis, fovea isthmi, lateral ventricle, olfactory bulb and epithelium and cerebellum), ventral spinal cord and the endocrine pancreas [6, 7]. Its major role in the morphogenesis of ocular tissues is supported by the widespread expression in different tissues and organs [6]. Impaired structure and function on the *PAX6* leads to classical aniridia with variable phenotypic expression. According to Tzoulaki et al. [8], 2/3 of the missense mutations in *PAX6* located in the paired domain (exons 5, 5a, 6 and 7) are associated with non-aniridia phenotypes. Pathogenic mutations in the gene can also result with an aniridia-like phenotype such as Peter's anomaly, autosomal dominant form of keratitis, nystagmus and foveal hypoplasia, coloboma of the iris and optic nerve defects [1, 6, 9].

The worldwide incidence of aniridia is between 1/40 000 and 1/100 000. About 2/3 of the cases are familial with autosomal-dominant inheritance, the remaining 1/3 are sporadic as a result of de novo mutation.

Materials and methods. ***Clinical overview of the patients and controls.*** A total of nine patients with aniridia and 115 healthy controls were studied. The group of aniridia patients includes five females and four males from 3 to 42 years at the time of the sample collection. Three of the studied patients are family related with clear dominant mode of inheritance of the trait. No patients exhibit aniridia as part of a syndrome. Eye changes in all nine patients with congenital aniridia – sporadic or familial, do not differ from those described in the available literature. The controls with age range from 3 to 40 years were asymptomatic for aniridia or aniridia-like phenotypes.

Methods. DNA was extracted from K2EDTA blood samples according to the manufacturer protocols (QIAamp Blood kit manual) from nine patients after receiving a signed informed consent. Controls' DNA samples were collected from the DNA bank of Gynecology and Assisted Reproduction Hospital "Malinov MD". A SNP array and Sanger Sequencing analysis were performed on patients with aniridia to confirm the diagnosis and determine the potential risk of Wilms tumour. Controls were examined through TruSight One gene panel on an Illumina MiSeq platform and whole exome sequencing (WES) on a BGISEQ-500/MGI-2000 platform.

SNP array. Samples were prepared according to the Infinium HD Assay Protocol guide. DNA was hybridized onto HumanCoreExome-12V1-0 BeadChip (Illumina). The BeadChip contains 500 000 tag SNPs and exome-focused markers presented with high feature redundancy. Coordinates of the markers are based

on the GRCh37/hg19. The bead chips were scanned on an Illumina iScan system and analyzed through KaryoStudio 1.4.3.0.

Sequencing. Sanger sequencing was performed on patients with normal molecular karyotype. Initial amplification was carried out with a custom made set of primers, covering 100% of protein coding sequence and splice junctions in 12 amplicons at individually optimized reaction conditions. Sequencing reaction was performed with a Big Dye Terminator Mix 3.1 (Thermo Fisher). The products were resolved on an ABI Prism 3130xl genetic analyzer (Applied Biosciences).

WES and NGS. DNA samples from the DNA bank control group were used for library preparation according to the manufacturer protocol. Fifty controls were examined using TruSight One panel (Illumina), the prepared libraries were sequenced on an Illumina MiSeq system and 65 controls were examined through WES on a BGISEQ-500/MGI-2000 platform.

Results. Our analysis discovered a potential molecular cause for five out of nine patients – two deletions of the *PAX6* gene, one duplication (*PAX6*, NC_0000 11.9:g.31815297_31815318dup) a single base deletion and splice site mutations were detected (Table 1). We identified two pathological heterozygous deletions by SNP-array technique. The first one was with length of 167 108 Kb in chromosome 11p13 (31,724,195- 31,891,303) including the genes *PAX6* and *ELP4*. The second one was 1,589,219 Mb length in 11p14.1p13 (30554404 - 32143623) – the region contains the genes *MPPED*, *DCDC1*, *DNAJC24*, *IMMP1L*, *ELP4*, *PAX6*, and *RCN1*. Both deletions cover the *PAX6* gene and the *ELP4* which results with haploinsufficiency of these genes (Fig. 1). In the investigated patients with aniridia 11 CNVs with unknown significance were also detected. Sanger sequencing revealed potentially pathogenic variants in the *PAX6* gene in three patients, all sporadic cases of aniridia. Two of these variants have not been reported before in the databases (Fig. 1). A 22 base duplication in exon ten was identified in heterozygous state (*PAX6*, NC_000011.9:g.31815297_31815318dup, NM_001127612.1:c.798_819dup (p.Gln274Lysfs*17)) in one patient, resulting to a frameshift and predicted to lead in a premature termination of protein synthesis within 17 codons. The second mutation was a single base deletion in exon 6 in a heterozygous state (*PAX6*, NC_000011.9:g.31823260del, NM_001127612.1: c.208delG (p.Ala70Glnfs*9)). It is predicted to result in a premature stop codon within 8 codons. The predicted truncating effect on the protein makes these two gene variants strong candidates for the molecular cause of aniridia in the corresponding patients. We also identified a single base substitution in the exon5/ intron5 junction, 4 bases downstream of the splice site (*PAX6*, NC_000011.9: g.31824248T>A, NM_001368918.1:c.145A>T (p:Ile49Phe)). This mutation occurs at fourth position of the splice donor sequence and leads to substitution of adenine with thymine in sequence GTG**A**T into GTG**T**T. This mutation was already reported in the patients with the aniridia phenotype [9].

In the group of controls without clinical signs for aniridia we have identified two nonsynonymous SNVs in exon 9 of the *PAX6*. The first one (NC_000011.9:

Table 1

Characteristics of the variants in the *PAX6* gene identified in our study

	Mutated genes	In/Ex	Size of the mutation	Nomenclature according to 1SCN/HGVS	Predicted results	Prediction/ Condition	Status/ References
Pathogenic CNV	*PAX6: ELP4*	N/A	167.108 kb del	arr[hg 19] 11 p 13(31724195-31891303)x 1	Whole gene deletion/ Haploinsufficiency	Pathogenic/ Aniridia	
	MPPED2; *DCDC1*; *DNAJC24*; *IMMP1L*; *ELP4*; *PAX6*; *RCN1*	N/A	1.589 Mbp del	arr[hg 19] 11 p 14.1 p 13(30554404-32143623)xl	Whole gene deletion/ Haploinsufficiency	Pathogenic/ Aniridia	
Pathogenic variants in the *PAX6* gene	*PAX6*	Ex5/ln5	Nonsynonymous SNV	NC_000011.9:g.31824248T>A; NM_001368918.1:c.145A>T; NP_001355847.1:p.Ile49Phe; rs1131692290	Disrupt mRNA splicing	Pathogenic/ Aniridia	Reported [9]
	PAX6	Exon 6	1 bp del	NC_000011.9:g.31823260del; NM_001127612.1:c.208delG NP_001121084.1:p.Ala70Glnfs*9	Franteshift and premature termination	Pathogenic/ Aniridia	Novel
	PAX6	Exon 10	22 bp dup	NC_000011.9:g.3l815297_31815318 dup NM_001127612.l:c.798_819dup NP_001121084.1:p.Gln274Lysfs*17	Frameshift and premature termination	Pathogenic/ Aniridia	Novel
PAX6 variants in the controls	*PAX6*	Exon 9	Nonsynonymous SNV	NC_000011.9:g.31816319C>T; NM_001368892.2:c.583G>A NP_001355843.1:p.Glu195Lys; rs138803897		VUS/Aniridia, Coloboma, Foveal hypoplasia. Keratitis, Cataract with late-onset	Unreported
	PAX6	Exon 9	Nonsynonymous SNV	NC_000011.9:g.31814879G>A NM_001310159.l:c.1139C>T NP_001297088.1:p.Ser380Leu; rs3026384		Polymorphic, Benign	Novel (detected in 3 controls)

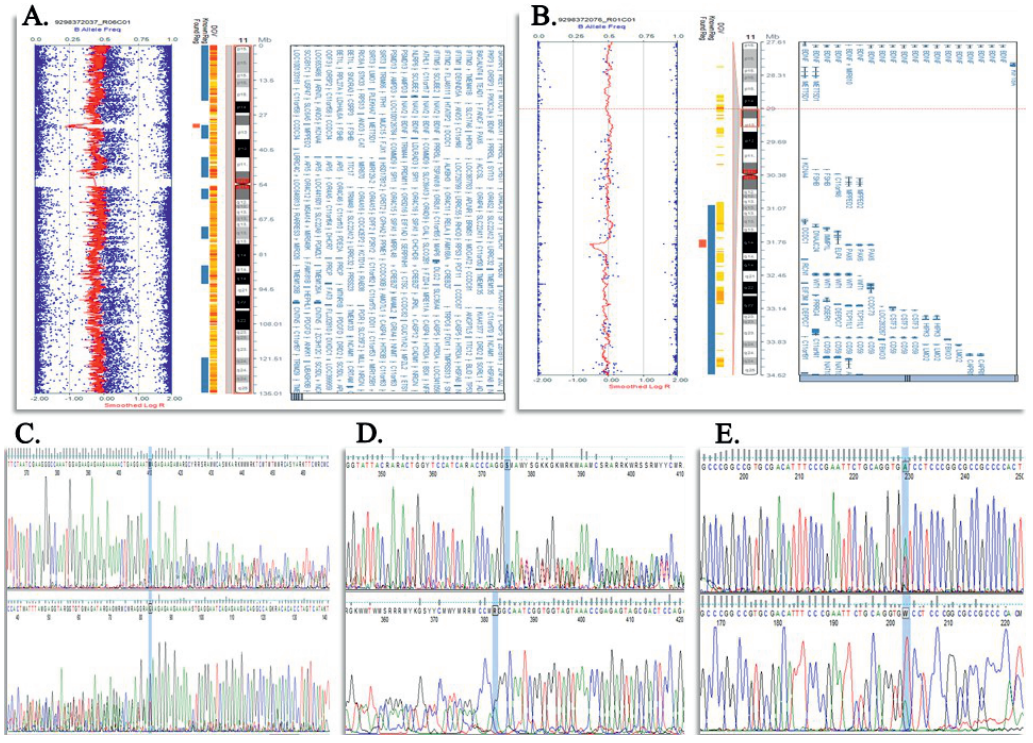


Fig. 1. The detected pathogenic variants in the patients included in our study. Two heterozygous deletions – arr[hg19] 11p13(31724195–31891303)x1 (A) and arr[hg19] 11p14.1p13(30554404–32143623)x1 (B) were identified through SNP-array. CNVs are shown on the left side: red marked deletion. Pathogenic variants determined via Sanger Sequencing are presented on panels (C) (NM_001127612.1:c.798_819dup (p.Gln274Lysfs*17)), (D) (NM_001127612.1:c.208delG (p.Ala70Glnfs*9)), and (E) (NM_001368918.1:c.145A>T (p:Ile49Phe)). The highlighted region shows the localization of the mutation. Variants in C and D sections are unreported in the databases

g.31814879G>A; NM_000280.5:c.C1139T; NP_001297088.1:p.Ser380Leu; rs3026384) was previously reported in ClinVar as benign, with high population frequency (gnomAD v3.1.1: 0.2251) not consistent with disease and detected in homozygous state in healthy controls. The other nonsynonymous variant is a missense mutation (NC_000011.9:g.31816319C>T; NM_001258462:c.G583A; NP_001355823.1:p.Glu195Lys; rs138803897) with an extremely low population frequency (gnomAD v2.1.1: 0.000007954). The variant was classified as a variant with unknown significance (VUS) and was detected only in one person from our controls.

All detected variants are summarized in Table 1.

Discussion. Described as "the master regulator" the *PAX6* gene has a major impact in the ocular structure fate in a broad range of animals [7,10]. Many studies reported its conservative nature in different vertebrates and invertebrates and

its substantial role through phylogeny [10]. The gene was cloned in man, mice, zebrafish, chicken, axolotl and drosophila melanogaster [1, 11–15]. The human PAX6 protein is highly conserved, it shows 96% similarity with *Fugu* (400 million years of evolution) and 100% with mice (-5a isoform) [16]. The *PAX6* is a gene with low rate of benign missense mutations. The highly conserved nature and fundamental role of the gene and its product were supported by the limited number of polymorphic variants in our control group. The molecular basis and genetic cause for the phenotype in four out of nine of our patients remained unclear. No mutations in the *PAX6* gene were detected in these patients, nor genomic rearrangements that involve the gene. This reveals the possibility of novel pathogenic variants (for example deep intronic), variants in other undiscovered genes/genetic loci and unknown mechanisms that altered the function and/or structure of the gene. The possible causal candidate genes are: *ELP4*, *FOXC1*, *PITX2*, *TRIM44*, *Notch1*, *CYP1B1*, *DCDC5*, *DCDC1*, *DNAJC24* and *IMMP1L* [17].

It is presumed that about 8–20% of the aniridia cases worldwide are with unsolved genetic cause. Very often, when mutations in the *PAX6* gene, or rearrangements in the 11p13 region were not detected, mutations in the downstream targets of PAX6 could be speculated as the cause for aniridia phenotype [17, 18]. A more detailed study described by Wang et al. [19], established the downstream targets of PAX6 (the genes *Bmp4*, *Tgfβ2* and *Foxc1*).

The contemporary study of phenotype-genotype correlations in some of the inherited eye diseases leading to reduced vision or blindness is a current trend in ophthalmogenetics. Congenital aniridia is one of them. Congenital aniridia is a multistructural ocular pathology with a leading clinical finding partial or total absence of iris in the newborn. Other ocular changes in patients with congenital aniridia (eyelid ptosis, keratoconus, cornea plana, strabismus, etc.) have been described in the literature, but they are isolated. Each of the described ocular findings present in the newborn (more often) or identified later in the life (microcorneal, microphthalmia, increased intraocular pressure, macular hypoplasia, etc.). Therefore, in the absence of other, external manifestations (isolated aniridia), some authors describe it as "aniridia syndrome" [20].

Conclusion. Our study represents the largest documented series of aniridia patients examined for mutations in the *PAX6* gene in Bulgaria. The rarity of aniridia, the limited sample size and the clinical heterogeneity of phenotypes, demanded the application of a variety of molecular-genetics methods and collaboration with different clinicians. Our set of patients covers approximately 75% of the officially estimated cases in the Bulgarian population, yet it presents 5 unique disease-related variants. Two out of three variants, discovered by the Sanger sequencing, have not been previously reported. All cases with identified genetic variations in the *PAX6* have no familial history of the disease.

The present study enhances our understanding for genetic pathogenesis of aniridia. Establishing definitive molecular cause for aniridia and aniridia-like phe-

notype in our patients has a crucial clinical effect. It is a decision-making process for determining eligibility for future personal therapeutic approaches in both clinical and genetic level.

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[1]*Gynecology and Assisted Reproduction Hospital "Malinov M.D", Genetic Laboratory, 46 Gotse Delchev Blvd, 1620 Sofia, Bulgaria*
e-mails: majaatanasoska@gmail.com, radvazh@abv.bg, lubobal@yahoo.co.uk, dragatoncheva@gmail.com

[2]*Department of Genetics, Faculty of Biology, Sofia University "St. Kliment Ohridski", 8 Dragan Tsankov Blvd, 1164 Sofia, Bulgaria*

[3]*Medical Faculty, Diagnostic and Consulting Center, Pediatric Ophthalmology, University Alexandrovska Hospital, 1 St. Georgi Sofiiski St, 1431 Sofia, Bulgaria*
e-mail: annapopi@yahoo.com

[4]*Department of Biology, Medical Genetics and Microbiology, Faculty of Medicine, Sofia University "St. Kliment Ohridski", 1 Koziak St, 1407 Sofia, Bulgaria*
e-mail: bai.vesan@gmail.com

[5]*National Genetic Laboratory, UHOG "Maichin dom", Medical University of Sofia, 2 Zdrave St, 1431 Sofia, Bulgaria*
e-mails: siluchka@abv.bg, alexey.savov@abv.bg

[6]*Department of Medical Genetics, Medical University of Sofia, 2 Zdrave St, 1431 Sofia, Bulgaria*