BRIEF COMMUNICATION

Functional reassessment of *PAX6* single nucleotide variants by in vitro splicing assay

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Abstract

Nucleotide variants that disrupt normal splicing might be the cause of a large number of diseases. Nevertheless, because of the complexity of splicing regulation, it is not always possible to accurately predict the effect of nucleotide sequence changes on splicing events and mRNA structure. Thereby, a number of newly identified nucleotide variants are falsely classified as VUS (a variant of uncertain significance). In the present study we used the minigene assay to analyze the functional consequences of six intronic (c.142–5T>G, c.142–14C>G, c.142–64A>C, c.141+4A>G, c.1032+6T>G, c.682+4delA), one missense (c.140A>G) and one synonymous (c.174C>T) variants in the *PAX6* gene found in patients with congenital aniridia. We revealed that all except one (c.142–64A>C) variants lead to the disruption of normal splicing patterns resulting in premature termination codon formation followed by mRNA degradation through the nonsense mediated decay pathway. This produces a null allele of the *PAX6* gene. That allowed us to reclassify the analyzed variants as loss-of-function and to establish their functional role.

Introduction

Congenital aniridia is a rare autosomal dominant panocular disorder. Loss-of-function nucleotide variants and haploinsufficiency of the *PAX6* gene is thought to be the main mechanism of congenital aniridia etiopathogenesis [1]. Nevertheless, true functional consequences of different types of *PAX6* variants, which could include effects on splicing, are still in question. This is a reason why some

aniridia associated *PAX6* nucleotide variants remain outside the databases.

A previously conducted molecular genetics study of a cohort of 110 patients from 84 unrelated Russian families with a clinical diagnosis of congenital aniridia revealed six single nucleotide variants out of canonical splicing site dinucleotides, which may affect splicing: five intronic (c.142–5T>G, c.142–14C>G, c.141+4A>G, c.1032+6T>G, c.682+4delA) and one missense variants (c.140A>G) [2]. Two more *PAX6* sequence variants were newly identified later: one synonymous (c.174C>T) and one deep-intronic (c.142–64A>C) (unpublished data). Among those, three variants were previously described in aniridia patients (c.140A>G [3], c.141+4A>G [4], c.1032+6T>G [5]), but without any experimental validation of the effect on the function. According to the recommendations of the American College of Medical Genetics and Genomics [6], almost all detected nucleotide variants were predicted to be pathogenic or likely pathogenic, except for the c.174C>T, c.682+4delA, and c.142–64A>C which were classified as variants of uncertain significance. In the present study, we perform functional analysis of eight *PAX6* single nucleotide variants (SNVs) to evaluate their influence on the splicing pattern, which is not obvious.

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Subjects and methods

A clinical and molecular genetic study was performed in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of the Federal State Budgetary Institution “Research Center for Medical Genetics,” Moscow, Russia, with written informed consent obtained from each participant and/or their legal representative, as appropriate. DNA samples for analysis were obtained from eight patients with a diagnosis of congenital aniridia. The clinical picture of the patients is described in Table 1. All nucleotide variants were named in accordance with the HGVS recommendations [7]. Nucleotide annotation and exons numbering were based on reference sequences NM_000280.4 and NG_008679.1. All *PAX6* variants were submitted to the Leiden Open Variation Database (LOVD) (<http://pax6.hgu.mrc.ac.uk/>).

Two vectors were used for creating the minigene reporter plasmids. Six out of eight SNVs were investigated using minigenes in pSpIPiG vector kindly provided by Dr. K.A. Lukyanov. For this aim, two different genomic regions containing the exon-of-interest with the complete sequence of adjacent introns and parts of neighboring exons were cloned into the linker sequence as previously described [8] (Table S1). To introduce mutations into the wild-type minigene QuickChange Site-Directed Mutagenesis Kit was used (Agilent Technologies). Two other SNVs were investigated using the pSpl3-Flu vector, which was created by the insertion of the HIV *tat* intron sequence from the pSPL3 vector (a kind gift from Dr Thomas v. O. Hansen) into the linker sequence between GFP and TurboFP635 reporter genes of the pSpIPiG. In each of these cases, exons-of-interest were amplified with at least 150 bp of flanking intronic sequences and cloned into the multiple cloning site within the HIV *tat* intron sequence as previously described [9, 10] (Table S1). Nucleotide sequences of all primers used for genomic DNA amplification and plasmid mutagenesis are presented in Table S1.

Splicing reporter plasmids were transfected into HEK293T and A375 human cell lines using Metafectene (Biontex) according to the manufacturer’s protocol. Forty-eight hours after transfection, total RNA was isolated using guanidine thiocyanate-phenol-chloroform extraction. RNA was treated with DNaseI (Thermo Scientific) and reverse transcribed using the ImProm-II™ Reverse Transcription System (Promega). To detect splicing alterations, minigene-specific cDNA was amplified with plasmid-specific primers. PCR products were separated by 12% denaturing urea polyacrylamide gel electrophoresis (urea-PAGE). Each DNA band was gel purified and sequenced with primers used for amplification.

Results

We performed a functional analysis of two novel, three recently identified and three previously well-known SNVs of the *PAX6* gene (Table 1). According to the *in silico* analysis with Human Splicing Finder (HSF) and IntSplice on-line tools [11, 12], seven out of eight investigated variants could influence five different splice-sites (SSs) of *PAX6* pre-mRNA: (i) intron 5 donor SS (c.141+4A>G, c.140A>G); (ii) intron 5 acceptor SS (c.142–5T>G, c.142–14C>G), (iii) intron 6 donor SS (c.174C>T); (iv) intron 8 donor SS (c.682+4delA); (v) intron 11 donor SS (c.1032+6T>G). SNV c.142–64A>C could lead to the creation of an intronic ESE (exonic splicing enhancer) site and probably could not affect splicing. To determine the effects of each sequence variant on *PAX6* pre-mRNA splicing we used the minigene assay, which was carried out in HEK293T and A375. The results for both cell lines were equal, indicating that observed changes were not cell-type-specific (Fig. 1).

Two nucleotide variants in intron 5, c.142–5T>G and c.142–14C>G, were found to lead to the creation of a novel acceptor AG dinucleotides within intron 5 and exon 6 extension by 4 and 13 nucleotides, respectively (Fig. 1a). Synonymous SNV c.174C>T resulted in the appearance of a novel intron 6 donor SS within the exon 6 and led to its shortening by 185 nucleotides (Fig. 1a). Surprisingly, we observed an additional isoform transcribed from the WT reporter construct with a shortened exon 6 (15 nt in length) (Fig. 1a). In Ensemble database release 91, we found six different isoforms of human *PAX6* transcripts, which use this alternative shortened exon 6. That gives the evidence for the natural occurrence of this minor *PAX6* isoform.

Analysis of sequence variants affecting intron 5 donor SS revealed that missense (c.140A>G) and intron (c.141+4A>G) variants disrupt a donor SS and lead to exon 5 skipping (Fig. 1b). Another intron variant, c.682+4delA, leads to a damage of natural donor SS of intron 8 and activation of downstream cryptic SSs. Major transcript isoform, in that case, contained exon 8 elongated by 38 nucleotides (Fig. 1c). Another major PCR-product from mutant construction corresponds to plasmid-derived cryptic SS which is not present in *PAX6* pre-mRNA being an experimental artifact (Fig. 1c). Another investigated variant, c.1032+6T>G, leads to the disruption of intron 11 donor SS and exon 11 skipping (Fig. 1d).

Besides, we did not find any confirmation that variant c.142–64A>C affects splicing. This SNV was found in a proband with congenital aniridia and his mother with subtle iris hypoplasia. Further analysis of segregation of this SNV in pedigree showed proband’s grandfather carried the variant and had no signs of aniridia. The deletion affecting

Table 1 A summary of reported *PAX6* single nucleotide variants (SNVs). Nucleotide annotation was based on reference sequences NM_000280.4 and NG_008679.1

SNV	c.140A>G	c.141+4A>G	c.142-14C>G	c.142-5T>G	c.174C>T	c.682+4delA	c.1032+6T>G	c.142-64A>C
LOVD ID	PAX6_00747	PAX6_00892	PAX6_00714	PAX6_00726	PAX6_00922	PAX6_00736	PAX6_00751	PAX6_00923
Novel/described	Prosser and van Heyningen [3]	Dubey et al. [4]	Vasilyeva et al. [2]	Vasilyeva et al. [2]	Novel	Vasilyeva et al. [2]	Skeens et al. [5]	Novel
Status	De novo	Familial (segregating)	De novo	Familial (segregating)	De novo	Familial (segregating)	Familial (segregating)	Familial (not segregating)
ACMG variant status classification (before minigene analysis)	Very strong evidence for pathogenic	Likely pathogenic	Likely pathogenic	Likely pathogenic	VUS	VUS	Likely pathogenic	VUS
In silico prediction (HSF)	Wild donor SS is broken, intron retention	Enhancer motif is broken, a wild type splicing has weakened	Activation of a new acceptor SS in intron 5 leading to exon extension by 13 nt	Activation of a new acceptor SS in intron 5 leading to exon extension by 4 nt	Activation of a new donor SS inside exon leading to exon shortening by 40 nt	Activation of a new donor SS leading to exon extension by 10 nt	Enhancer motif is broken, a wild type splicing has weakened	Probably no impact on splicing.
Consequences according to minigene assay	Exon skipping	Exon skipping	Exon extension	Exon extension	Exon shortening	Exon extension	Exon skipping	No effect
Effect	PTC/NMD	PTC/NMD	PTC/NMD	PTC/NMD	PTC/NMD	PTC/NMD	PTC/NMD	Benign
Clinical picture	Complete aniridia, nystagmus, macula hypoplasia, keratopathy, secondary glaucoma, strabismus, emotional lability	Probable: Complete aniridia, nystagmus, keratopathy, macula hypoplasia, pseudophakia, secondary glaucoma. Probable's brother: Complete aniridia, nystagmus, keratopathy, macula hypoplasia, secondary glaucoma. Probable's son: Complete aniridia, nystagmus, macula hypoplasia, partial optic atrophy, torticollis	Complete aniridia, nystagmus, macula hypoplasia, anterior chamber angle dysgenesis, keratopathy, partial optic atrophy, hyperactivity disorder, sleep disturbance	Probable: Iris hypoplasia, without nystagmus, macula hypoplasia, congenital cataract, strabismus, emotional lability. Probable's mother: Partial aniridia, without nystagmus, macula hypoplasia, cataract, keratopathy, ptosis, epicanthus, postoperative retinal detachment, vitreous body destruction	Complete aniridia, nystagmus, macula and optic disk hypoplasia, cataract	Probable: Complete aniridia, nystagmus, macula and optic disk hypoplasia, cataract	Probable: Complete iris coloboma, nystagmus, macula hypoplasia, cataract, renal pelvis doubling. Probable's father: Complete aniridia, nystagmus, macula hypoplasia, cataract	Probable has a complete aniridia, nystagmus and macula hypoplasia. (Probable also has a causative large chromosome deletion in 11p13: hg18: chr11:31285887-31628232)

LOVD Leiden Open Variation Database (<http://pax6.hgu.mrc.ac.uk/>), ACMG American College of Medical Genetics and Genomics, HSF Human Splicing Finder, PTC/NMD premature termination codon formation followed by mRNA degradation through the nonsense mediated decay pathway

Fig. 1 Results of minigene assay for eight *PAX6* SNVs in HEK293T cell line. pSplPIG (“V1”) based reporter constructions are presented at (a) and (b), pSpl3-Flu (“V2”) based reporter constructions are presented at (c) and (d). **a** RT-PCR-analysis of minigene splicing for Construction 1 is aimed at investigation *PAX6* nucleotide variants: c.142-5T>G, c.142-14C>G, c.174C>T, c.142-64A>C *PAX6* SNVs. **b** RT-PCR-analysis of minigene splicing for Construction 2 is aimed at investigation *PAX6* nucleotide variants: c.141+4A>G, c.140A>G. **c** RT-PCR-analysis of minigene splicing for Construction 3 is aimed at investigation *PAX6* nucleotide variant c.682+4delA. **d** RT-PCR-analysis of minigene splicing for Construction 4 is aimed at investigation *PAX6* nucleotide variant c.1032+6T>G. Schemes of the

minigene constructions are depicted above: white boxes = *PAX6* exons (with appropriate number); thick lines = *PAX6* introns (full-sized for (a) and (b), partial for (c) and (d)); thin lines = plasmid-specific HIV tat intron sequence of V2; grey boxes = vector specific exons; arrows = primers used for the cDNA amplification and sequencing; asterisks = position numbers of investigated nucleotide variants. Urea-PAGE of RT-PCR fragments generated from minigene spliced RNA of wild type (WT) and mutant (MUT) constructions are shown below. Splice products are shown schematically on the right (for (a): on the right — for mutant (MUT) constructions, on the left — for wild-type (WT) constructions), oblique shade areas represent exon extension by respective number of nucleotides (nt)

the *PAX6* distal 3′-*cis*-regulatory region was found in a proband [2].

Discussion

In the present study eight SNVs in the *PAX6* gene, which were found in patients with congenital aniridia, were functionally characterized. We showed that all investigated variants, except for the c.142-64A>C, lead to various alterations of splicing: (i) exon skipping (c.140A>G, c.141+4A>G, c.1032+6T>G); (ii) exon extension (c.142-5T>G, c.142-14C>G, c.682+4delA); (iii) exon shortening (c.174C>T). All these alterations disrupt the open reading frame, produce a premature termination codon (PTC) and probably trigger the mRNA degradation by the nonsense-mediated decay (NMD) mechanism [13] (Fig. 2). This results in the production of a null-allele of the *PAX6* gene and development of congenital aniridia through the

haploinsufficiency model of pathogenesis. The severity of the aniridia phenotype in this group of patients does not differ from that in a general cohort (Table 1).

One of the previously identified variants, c.140A>G, was described as a missense variant p.(Gln47Arg) located within the *PAX6* DNA-binding paired domain (PD) and affects its function [14, 15]. In the present study, we showed that the functional effect of this SNV is a consequence of splicing disruption rather than a dysfunctional protein.

Besides, the present study is a first description of a “synonymous” SNV (c.174C>T) as a cause of congenital aniridia.

The issue of the diverse phenotypic consequences of the different functional types of *PAX6* variants has been discussed for a long time, but no statistically significant correlations have been detected [16]. One reason for that might be the incorrect classification of nucleotide variants into functional categories. The functional analysis allowed us to classify seven *PAX6* SNVs as loss of function variants

Fig. 2 Scheme of the possible pathogenic action of investigated SNVs in the *PAX6* gene. Variants are listed on the left. Corresponding mRNA structures (with marked start codons, stop codons and premature stop codons (PTC)) are located next to each variant. Oblique shade areas represent exon extension by respective number of nucleotides (nt), dotted lines correspond to skipped part of mRNA. The number of nucleotides between PTC and last exon–exon junction is shown on the lower scheme (c.1032+6T>G). The possible consequences of aberrant splicing are listed on the right. NMD nonsense-mediated decay

which is very much in line with the severity of their carriers' phenotypes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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