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PAX6 missense mutations associated in patients with optic nerve malformation

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Purpose: *PAX6* missense mutations are likely to cause a spectrum of ocular, neurological, and systemic developmental defects and have been reported in various ethnic groups. The purpose of this study was to investigate the clinical features of optic nerve malformation caused by *PAX6* mutations in Indian patients.
Methods: Total genomic DNA was isolated from peripheral blood of 27 sporadic probands affected with congenital optic nerve malformation, unaffected family members, and 50 unrelated age-matched controls. Informed consent was obtained from all study subjects. Polymerase chain reaction was carried out to explore *PAX6* defective alleles using single-strand conformation analysis (PCR-SSCA) followed by automated bidirectional sequencing.
Results: We identified two novel *PAX6* missense mutations in two unrelated sporadic probands. The mutation analysis revealed variation at position c.469G>C, codon 36 in proband ONH 4-1 with optic nerve hypoplasia. The other de novo mutation was observed at c.514G>C, codon 51 in proband ODC 5-1 with optic disc coloboma. Both G>C base substitutions cause a relatively conservative amino acid change, altering glycine to alanine residues within the paired DNA-binding domain.
Conclusions: In this study, we have been able to identify two sequence variations in the *PAX6* gene. These missense mutations may uniquely alter the structure and expression of PAX6 protein, resulting in distinct clinical phenotypes. Mutation analysis of 27 probands for *PAX6* has resulted in only two significant variants. This finding demonstrated that the frequency of *PAX6* mutations associated with optic nerve malformation is low, requiring the elucidation of other candidate genes in other patients.

PAX6, a paired-box transcriptional factor, is considered to be the master control gene for eye morphogenesis. It is expressed in ocular and nonocular tissues, such as the olfactory epithelium, central nervous system, brain, and pancreatic islets [1,2]. *PAX6* mutations are associated with autosomal dominant aniridia and a variety of congenital eye abnormalities involving the anterior chamber and retina [3,4]. Coding mutations in *PAX6* may impair retinal ganglion cell development, leading to optic nerve hypoplasia/aplasia with persistent hyaloid vessels (PHPV). Recent genetic studies show that *PAX6* deficiency affects early progenitor cell proliferation and later neuronal differentiation, leading to brain abnormalities in mice and humans [5,6]. The majority of PAX6 ocular defects are caused by haploinsufficiency. Most congenital optic nerve anomalies are sporadic, but in some cases, optic nerve coloboma (OMIM 120430) or optic nerve hypoplasia (OMIM 165550) may be inherited in an autosomal dominant manner. The 22 kb human *PAX6* gene that encodes a 422-amino acid protein with paired and homeobox DNA-binding domains and a PST transcriptional activation domain were identified by positional cloning in 11p13 [7]. *PAX6* contains an alternative exon that encodes a 14-amino acid insertion (5a isoform) in the paired domain [8]. *PAX6* variants and nonaniridic phenotypes associated with missense mutations are documented at the *PAX6* allelic variation database (*PAX6*). Despite the low number of cases, *PAX6* missense mutations are clinically important, since they are likely to cause unique ocular and neurological disorders. Here we correlate genetic and clinical features for optic nerve malformations with *PAX6* gene mutations in Indian patients.

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METHODS

Patients: All studies were conducted in accordance with the tenets of the Declaration of Helsinki. Pediatric and neuro ophthalmologists recruited 27 unrelated patients associated with optic nerve malformation (their available family members) and 50 age-matched controls for this study. Informed consent was obtained from all individuals. Ophthalmic examinations included slit lamp biomicroscopy, measurement of intraocular pressure (IOP) by applanation tonometry, gonioscopic evaluation of the anterior chamber angle, and perimetry by automated field analyzer. Table 1 summaries the clinical diagnosis of sporadic probands affected with various congenital optic nerve malformation.

DNA preparation: Total genomic DNA was isolated from blood by the salt precipitation method [9] and suspended in Tris-EDTA buffer (pH 8) before being stored at -20 °C for further analysis.

Polymerase chain reaction: Polymerase chain reaction (PCR) analysis was carried out to amplify *PAX6* coding exons 4-13 using primers previously described in the literature [8]. Each 20 μl reaction contains 100 ng of genomic DNA, 1X PCR buffer, 0.5 U of Taq (Promega, Madison, WI), 2 mM $MgCl_2$, 200 mM dNTPs, and 100 pM of primers. Modified thermocycling conditions were performed as reported elsewhere in the literature [10].

Table 1. Clinical features of study subjects affected with congenital optic nerve malformation

Study Cases	Age/ Sex	Best vision RE	Best vision LE	Diagnosis RE	Diagnosis LE	Systemic findings
ONH						
1-1	8/M	1/6	5/6	Nystagmus	Nystagmus	DMS
2-1	4/M	6/60	HM	Nystagmus	Micro cornea, Nystagmus	NA
3-1	11/M	6/60	4/60	Nystagmus	Nystagmus	NA
4-1*	4/M	3/60	3/60	Pupil-poor reactive in light	Pupil-poor reactive in light	DMS
5-1	7/M	1/60	6/6	Latent nystagmus	Normal	NA
6-1	6m/M	-	-	--	--	DMS
7-1	4/M	5/60	2/60	Myopia, horizontal jerky nystagmus	Myopia, horizontal jerky nystagmus	NA
8-1	17/F	3/60	3/60	--	--	NA
9-1	9/F	HM	HM	--	--	HT
ODC						
1-1	2/F	6/60	6/60	Microcornea	Microcornea	-
2-1	8m/M	-	-	Nystagmoid movements	Nystagmoid movements	-
3-1	15/M	2/60	2/60	Microphthalmos, Nystagmus	Microphthalmos, Nystagmus	-
4-1	18/M	3/60	PL	Microphthalmos, Nystagmus	Microphthalmos, Nystagmus	-
5-1*	6/M	HM	HM	--	--	-
6-1	4m/M	-	-	Anophthalmos	--	-
7-1	1/M	-	-	Microcornea/ microphthalmos	Microcornea/ microphthalmos	-
8-1	11/F	6/18	2/60	Optic disc pit	--	NA
9-1	10/M	NLP	NLP	Microphthalmos	Microphthalmos	-
10-1	10/M	6/60	-	Myopic fundus, Nystagmus	Anophthalmos	-
11-1	1/F	-	-	Normal	Microphthalmos	-
12-1	1/F	-	-	Microphthalmos, Microcornea	--	-
13-1	5/M	-	6/6	Severe microphthalmos	Normal	-
14-1	8m/M	-	-	Microphthalmos, Nystagmus, Telecanthus	Microphthalmos, Nystagmus, Telecanthus	-
MGS						
1-1	3/M	6/9	HM	Normal	MGS, ODC	-
2-1	18m/M	-	-	Normal	MGS, ONH	-
3-1	7/M	-	-	MGS, ODC, cataract	Severe microphthalmos	-
4-1	11/F	6/60	6/6	MGS, ODC	Normal	-

This table describes the clinical diagnosis of 27 sporadic probands affected by a variety of congenital optic nerve malformations. “ONH” refers to optic nerve hypoplasia, “ODC” and “MGS” indicates optic disc coloboma and morning glory syndrome, respectively. “DMS” designate De-Morsier’s syndrome. In the “best vision” column, “LP” refers to light perception; “NLP” indicates no light perception. In the “systemic findings” column, “NA” refers to not available and “HT” indicates hypothyroidism. The asterisk designates the cases with *PAX6* mutations identified in this study. “HM” refers to hand motions.

Single strand conformation analysis and heteroduplex analysis: PCR products were analyzed by combined heteroduplex (HD) and single-strand conformation analysis (SSCA) on 10% nondenaturing polyacrylamide gel [10].

Sequencing: PCR products that showed altered mobility on SSCA were gel-eluted and purified using Perfect-prep gel

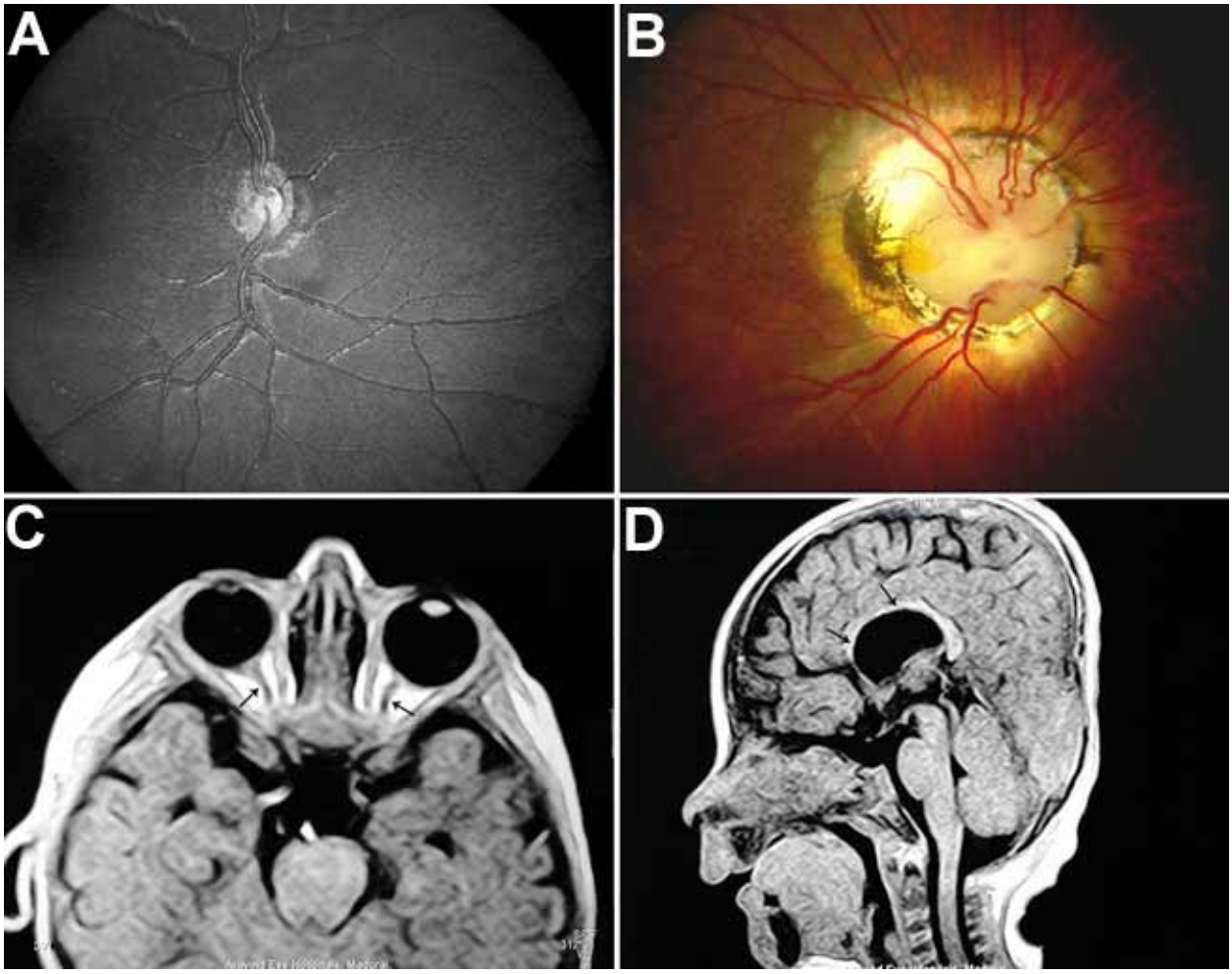


Figure 1. Fundus photography and MRI scan report of the affected probands. **A**: Ophthalmoscopic appearance of a 4-year-old male showing an abnormally small optic nerve head that is surrounded by a yellowish mottled peripapillary halo boarded by a dark pigment ring (double ring sign). **B**: Ophthalmic exam of a 6-year-old boy showed the fundus picture of optic disc coloboma with large wafer-like defect, enlarged disc funnel shaped excavation surrounded by an elevated annulus of chorioretinal pigmentary disturbance. Blood vessels emerge from the rim of excavation like the spokes of a wheel. **C**: MRI scan of axial view (T_1 weighted image [WI]) shows thinning of optic nerves in proband ONH 4-1 (arrow). **D**: MRI scan (saggital view, T_1 WI) shows the congenital absence of septum pellucidum with hypogenesis of corpus callosum (arrow).

TABLE 2. CLINICAL FINDINGS OF PROBANDS WITH *PAX6* MUTATION

Proband	Age/ Sex	Best vision RE	Best vision LE	Pupil	Iris	Glaucoma RE	Glaucoma LE	Optic nerve hypoplasia RE	Optic nerve hypoplasia LE	Optic disc coloboma RE	Optic disc coloboma LE
ONH4-1	4/M	3/60	3/60	PRL	N	-	-	+	+	-	-
ODC5-1	6/M	HM	HM	RL	N	-	-	-	-	+	+

This table summarizes the clinical data and phenotypes of subjects with *PAX6* mutations. Neither patient had a refractive error, nystagmus, cataract, or macular hypoplasia. "HM" denotes hand movements. The terms "RE" and "LE" indicates the right eye and the left eye, respectively, "PRL" refers to poor reactive in light. "RL" refers to reactive in light and "N" indicates normal. The symbols "+" and "-" represent present and absent, respectively.

clean up kit (Eppendorf, Hamburg, Germany). They were sequenced bidirectionally on an Applied Biosystems (ABI) model 3730 automated sequencer at Microsynth (Balgachi, Switzerland).

PAX6 paired domain-DNA complex model: The paired domain of PAX6 (PDBID 6PAX) protein structure [11] was used to generate the structure with the mutated residues (G36A, G51A) incorporated using the Homology module of the In-

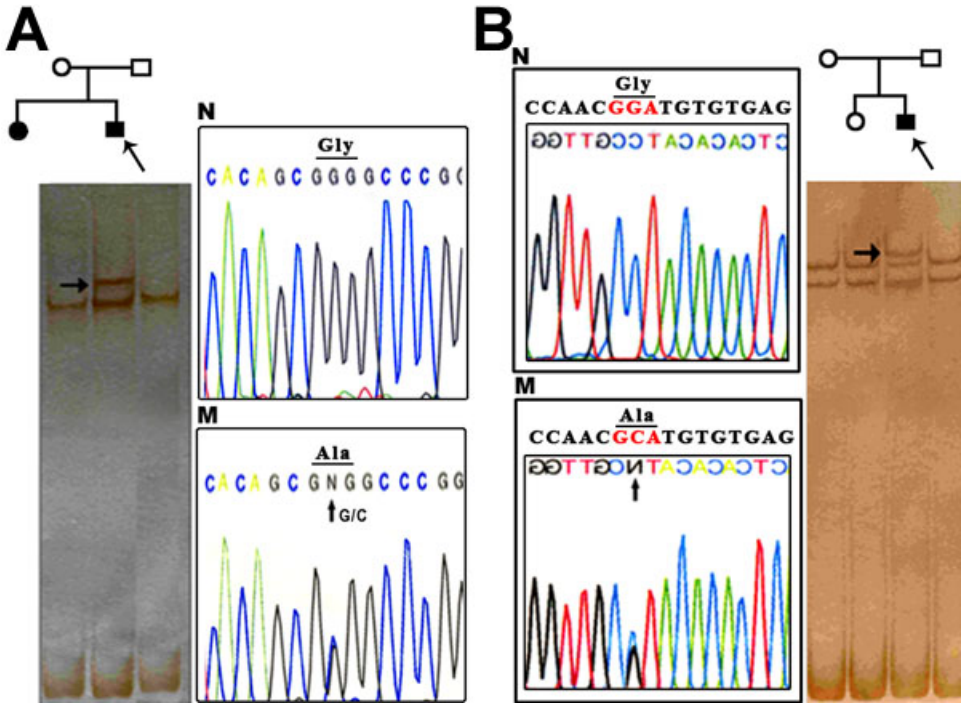
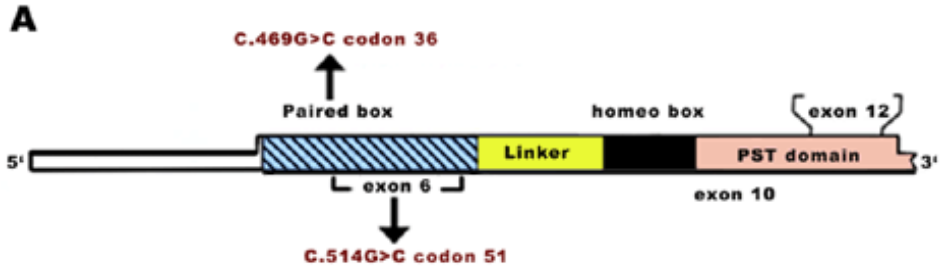


Figure 2. Genetic analysis of *PAX6* in two unrelated families with optic nerve malformation. **A**: The exon 5 PCR product of *PAX6* showed an extra band (arrow) on the 10% polyacrylamide gel, and sequencing of the normal (N) and mutant (M) DNA revealed the G>C change that replaces Gly by Ala in proband ONH 4-1. **B**: The unusual banding pattern was observed in exon 6 of *PAX6*, and the antisense strand sequencing of normal (N) and mutant (M) DNA showed the nucleotide change G>C (GGA [Gly51] to GCA [Ala]) in probands ODC 3-1.



B:

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                36                                                  51
                *                                                   *
PAX6_HUMAN      HSGVNQLGGVFNGRPLPDSTRQKIVELAHSGARPCDISRILQVSNGCVSKIL
PAX6_XENLA      HSGVNQLGGVFNGRPLPDSTRQKIVELAHSGARPCDISRILQVSNGCVSKIL
PAX6_MOUSE      HSGVNQLGGVFNGRPLPDSTRQKIVELAHSGARPCDISRILQVSNGCVSKIL
PAX6_DROME      HSGVNQLGGVFGGRPLPDSTRQKIVELAHSGARPCDISRILQVSNGCVSKIL
PAX6_toy        HSGINQLGGVYNGRPLPDSTRQKIVELAHSGARPCDISRILQVSNGCVSKIL
PAX6_RAT        HSGVNQLGGVFNGRPLPDSTRQKIVELAHSGARPCDISRILQVSNGCVSKIL
PAX6_COTJA      HSGVNQLGGVFNGRPLPDSTRQKIVELAHSGARPCDISRILQVSNGCVSKIL
PAX6_BRARE      HSGVNQLGGVFNGRPLPDSTRQKIVELAHSGARPCDISRILQVSNGCVSKIL
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Figure 3. Schematic of *PAX6* and amino acid conservation analysis of PAX6 protein. **A**: A schematic of *PAX6* shows the location of nucleotide changes in the paired box that were found in this study. **B**: Amino acid sequence alignment of the human PAX6 paired domain (amino acids 34-57) homologs were compared with other species (PAX6_DROME is the Drosophila Pax-6 eyeless homolog and PAX6_toy is the toy-twin of eyeless homolog). The Gly36 and Gly51 shown in red are highly conserved.

sight II software (Accelrys Software, Bangalore, India). The mutated structure was energy minimized until convergence of the energy gradient to rmsd of less than 1 kcal/mole/A and Consistent Valence Force Field was used. The PAX6 5a isoform region was inserted at residue 48 (GenBank M93650) along with the mutated residues, using the PAX6 structure as template. The loop search algorithm of the homology module was used with default parameters to identify region 607 from pdb structure 141 as the most optimal structure for the 5a insertion. The PAX6-5a isoform structure was subjected to molecular dynamics optimization, with the best conformation chosen for energy minimization to convergence of the energy gradient.

RESULTS

We describe two novel *PAX6* missense mutations in Indian patients with optic nerve hypoplasia and optic disc coloboma. Complete anterior and posterior segment findings of the two sporadic probands are given in (Table 2).

Proband ONH4-1 is a 4-year-old male with bilateral optic nerve hypoplasia (Figure 1A). His MRI scan revealed congenital absence of the septum pellucidum with hypoplasia of the corpus callosum, an absence of genu and rostrum, and thinning of the body splenium (Figure 1C,D). These findings were consistent with DeMorsier syndrome (OMIM 182230). SSCA and bidirectional sequencing analysis of *PAX6* showed a heterozygous c.469G>C mutation, GGG (Gly36) to GCG (Ala36) in exon 5 (Figure 2A). Proband ODC 5-1 is a 6-year-old male with congenital optic disc coloboma (Figure 1B). The amplified PCR product from exon 6 showed an unusual band shift on a nondenaturing SSCA gel (Figure 2B). Bidirectional sequencing revealed a heterozygous c.514G>C base-pair substitution, GGA (Gly51) to GCA (Ala51) in exon 6. Both variations (GenBank AY956820 and AY956821) replace a highly conserved glycine residue with an alanine residue (Figure 3B). These substitution mutations in the second α helix may disrupt the function of the NH_2-terminal subdomain of PAX6 (Figure 3A). Genetic analysis of unaffected parents and siblings in both families and 50 normal age-matched controls revealed only wild type *PAX6* alleles. The 5a insertion interferes with the DNA binding by the NH_2-terminal subdomain due to steric hindrance [12]. Mutations identified in our study and reported elsewhere have been mapped onto a three-dimensional model of PAX6 and PAX6-5a structure (Figure 4). Our PAX6 paired domain-DNA complex model shows that Gly51 and Gly36 are close to the DNA-binding surface, and suggest that G51A is likely to directly interfere with the DNA binding of the PAX6 protein. Analysis of amino acid mutation stability for G36A and G51A mutations identified in this study using the Amino Acid Mutation Stability Prediction Server MUpro (version 1.0) suggests a decrease in the stability of PAX6 protein structure.

DISCUSSION

A spectrum of phenotypes associated with *PAX6* missense mutations has recently been extended to include a variety of optic nerve malformations, such as morning glory disc anomaly and optic nerve hypoplasia in Japanese and European ethnic groups [4]. Hence we carried out a *PAX6* genetic study in Indian patients with a variety of optic nerve malformation phenotypes. Two novel *PAX6* missense mutations were identified in two unrelated probands with different clinical manifestations of optic nerve malformations.

Proband ONH 4-1 was identified to have a de novo heterozygous c.469G>C mutation in the *PAX6* paired domain and was diagnosed for bilateral optic nerve hypoplasia. Ocular con-

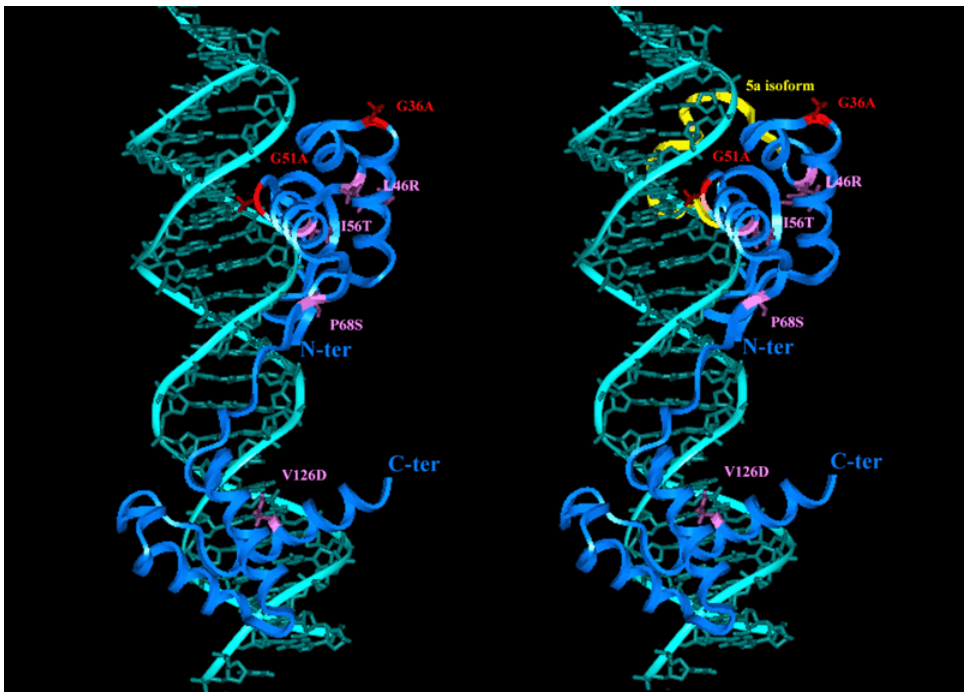

Figure 4. Overview of PAX6 paired domain DNA complex modeled structure. The PAX6 mutated structure and the PAX6 5a isoform (yellow) are shown as a ribbon model with the mutated residues from other studies (pink) depicted as sticks. The mutations identified in this study are shown in red. The DNA bases (light green) and the ribbon representation of the PAX 6 protein are represented in blue.

ditions and brain MRI findings were consistent with DeMorsier syndrome. The boy had poor vision and his pupils reacted abnormally to light. Irides were normal and he showed no evidence of cataract or glaucoma. Another novel c.514G>C missense mutation was identified in proband ODC 5-1, who exhibited optic disc coloboma that resulted in poor visual acuity (hand motion only). Anterior segment findings were normal whereas fundus examination showed enlarged discs bilaterally, with funnel-shaped excavation and blood vessels emerging from the rim like spokes on a wheel. In both pedigrees there was no history of consanguineous marriage. Changes were not detected in the unaffected immediate family members and in 50 age-matched normal controls analyzed.

As both mutations are sporadic, it is difficult to evaluate whether these mutant alleles would be associated with purely optic nerve phenotypes in these cases. The mutations will generate full-length proteins with single abnormal amino acids that might interrupt the formation of the boundary between the optic cup and optic stalk [13,14]. Such proteins could display partial loss of function or even a gain of function, which in turn could account for their association with phenotypes that are distinct from classical aniridia [15,16]. Two-thirds of the missense mutations previously reported affected crucial paired domain residues, and may affect DNA-binding activity [4,15,16]. It is interesting to note that both of the mutations identified in this study result in the introduction of a missense change that leads to the replacement of highly conserved glycine by alanine in the paired domain of PAX6, highlighting the functional importance of this region of protein.

The PAX6 paired domain model studies suggest that G51A lies within a DNA-binding helix, whereas G36A may affect DNA binding indirectly via its effect on protein folding (Figure 4). Other missense mutations located in the paired domain (L46R, I56T, and V126D) have been correlated with optic nerve hypoplasia and coloboma phenotypes (*PAX6*). The exact pathological consequences are likely to depend upon the precise amino acid substitution and its effects on the folding properties of PAX6.

Mutations in *HESX1* (OMIM 182230) have also been reported to be associated with septo-optic dysplasia and optic nerve malformation [17], and mutations in *PAX2* (OMIM 167409) have been reported to cause optic nerve coloboma [18]. *PAX6* mutation frequency in the study subjects is relatively low. Determining the etiology of disease in the remaining patients requires screening for other gene mutations associated with various optic nerve malformations. Our genetic study of various optic nerve malformations will enable the understanding of the significance of the candidate genes involved in pathogenesis and contribute to genetic counseling, especially for sporadic probands affected with congenital optic nerve anomalies.

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