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Brief Report: *VAX1* mutation associated with microphthalmia, corpus callosum agenesis and orofacial clefting – the first description of a *VAX1* phenotype in humans

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Abstract

Vax1 and *Vax2* have been implicated in eye development and the closure of the choroid fissure in mice and zebrafish. We sequenced the coding exons of *VAX1* and *VAX2* in 70 patients with anophthalmia/microphthalmia. In *VAX1*, we observed homozygosity for two successive nucleotide substitutions c.453G>A and c.454C>A, predicting p.Arg152Ser, in a proband of Egyptian origin with microphthalmia, small optic nerves, cleft lip/palate and corpus callosum agenesis. This mutation affects an invariant residue in the homeodomain of *VAX1* and was absent from 96 Egyptian controls. It is likely that the mutation results in a loss of function, as the mutation results in a phenotype similar to the *Vax1* homozygous null mouse. We did not identify any mutations in *VAX2*. This is the first description of a phenotype associated with a *VAX1* mutation in humans and establishes *VAX1* as a new causative gene for anophthalmia/microphthalmia.

Keywords

Anophthalmia/microphthalmia; *VAX1*; *VAX2*; coloboma

Malformations of the eye such as anophthalmia (absence of the eye) and microphthalmia (reduction in eye size) show a birth prevalence of approximately 1/5000 [Shaw et al. 2005]. Anophthalmia and microphthalmia (A/M) are characterized by absent or rudimentary eye formation despite the presence of adnexal ocular structures [Verma and FitzPatrick, 2007; Ragge et al., 2007]. Mutations in several transcription factors that are expressed during eye development have been implicated in both syndromic and non-syndromic A/M [for reviews, see Verma and FitzPatrick, 2007; Rainger et al., 2008] but it is clear from the frequency of mutations in each causative gene that novel causes of A/M remain to be discovered.

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During ocular development, *Shh* [MIM# 600725] induces ventralization of the optic vesicle and a mutation predicted to interfere with autocatalytic processing of SHH has caused microphthalmia and coloboma [Schimmenti et al., 2003]. *Shh* induces the expression of two homeobox-containing genes: ventral anterior homeobox 1 [*Vax1*; MIM# 604294] and ventral anterior homeobox 2 [*Vax2*; MIM# 604295] [Zhao et al., 2010]. *Vax1* and *Vax2* share high sequence homology with members of the *Emx* and *Not* homeodomain-containing gene families and likely originated from the duplication of a single ancestral gene due to the 100% conservation of the homeodomains [Ohsaki et al., 1999; Schulte et al., 1999]. *Vax1* and *Vax2* have distinct expression patterns in the developing eye [Ohsaki et al., 1999; Take-uchi et al., 2003]. At embryonic day (E) 8 in the mouse, *Vax1* mRNA is present in the anterior neural ridge and adjacent ectoderm and at E10.5, expression is present at site of the putative optic disc and optic stalks, the olfactory placodes and the rostral tip of the neural tube [Bertuzzi et al., 1999; Hallonet et al., 1999]. During mid-embryogenesis, *Vax1* is expressed in the diencephalon adjacent to lateral ventricles of the brain and in the septum that is ventral to the corpus callosum [Bertuzzi et al., 1999; Bharti et al., 2011]. At E18.5, *Vax1* expression is visible in the ventral optic stalk, the glial cells of the optic nerve, the optic chiasm and in the rostral diencephalon [Hallonet et al., 1999; Bertuzzi et al., 1999]. In contrast, *Vax2* expression is present almost exclusively in the ventral part of the developing optic vesicle from E9.0 onwards, although there is weak expression in the optic stalk at E12.5 [Barbieri et al., 1999]. In *Danio rerio*, *Vax1* and *Vax2* are both expressed in overlapping domains in the ventral portion of the developing eye [Take-uchi et al., 2003].

Animal models of loss of gene function also implicate the *Vax* genes in ocular development. *Vax1* homozygous null mice had colobomas detectable from the earliest stages of development that were fully penetrant and moderately severe [Hallonet et al., 1998; Bertuzzi et al., 1999]. Colobomas were rare and milder in *Vax2* homozygous null mutants, but *Vax1* and *Vax2* double mutant mice had severe colobomas that were fully penetrant [Barbieri et al., 2002; Mui et al., 2002]. In *Danio rerio*, injections of antisense morpholinos against *Vax1* or *Vax2* results in colobomas and reduced retinal pigment at the site of the choroid fissure [Take-uchi et al., 2003]. The severity of the colobomas was increased with loss of both *Vax1* and *Vax2*, thus indicating that the genes act synergistically in fish and mice [Take-uchi et al., 2003].

In humans, *VAX1* has two isoforms, the first containing 3 exons and encoding a 334 amino acid protein and the second containing 4 exons and encoding a 186 amino acid protein. *VAX2* has 3 exons and encodes a 290 amino acid protein. *VAX1* and *VAX2* have been little studied in human patients with eye disease. Homozygosity mapping in a consanguineous family excluded *VAX1* in two affected individuals diagnosed with Temtamy syndrome who manifested optic colobomas, agenesis of the corpus callosum, intractable seizures, craniofacial dysmorphism and skeletal anomalies [Li et al., 2007].

In view of the paucity of human data, we chose to sequence both genes in patients with A/M. Two patient groups were studied. In the first group, DNA samples from 70 patients with A/M were sequenced for *VAX1* and *VAX2* sequence alterations. From this first group, 35 samples were collected under an Institutional Review Board (IRB)-approved protocol for the Anophthalmia/Microphthalmia Registry and Gene-screening project (Albert Einstein Medical Center, Philadelphia, PA) and 35 samples were collected under an IRB-approved protocol from the Institut de Recherche en Ophtalmologie (Ecole Polytechnique Fédérale de Lausanne and Université de Lausanne, Sion, Switzerland). A second group of 10 A/M patients was then selected with at least one additional finding of cleft palate and/or agenesis of the corpus callosum. These patients were identified from the population-based California Birth Defects Monitoring Program with IRB approvals from the California Committee for the Protection of Human Subjects and the University of California, San Francisco (UCSF)

Committee for Human Research. Finally, control chromosomes were obtained using a protocol from the UCSF Committee for Human Research. We sequenced the coding exons and intron-exon boundaries of *VAX1* [NM_001112704.1 (long isoform) and NM_199131.2 (short isoform); http://www.ncbi.nlm.nih.gov/gene)] and *VAX2* [NM_012476.2] as previously described [Slavotinek et al., 2006]. Primers are provided in Supp. Table S1.

Sequencing of *VAX1* in the 70 unselected patients with A/M revealed one mutation, with homozygosity for two adjacent nucleotide substitutions, c.453G>A and c.454C>A, that predicted p.Arg152Ser in the long isoform of *VAX1* (Fig. 1A) in a single patient (Table 1). This patient was an Egyptian male with bilateral microphthalmia and small optic nerves, cleft lip and palate, corpus callosum agenesis, hippocampal malformations and absence of the pineal gland. Birth weight was 3750 g, but no head circumference measurement from the time of birth was available. Bilateral severe microphthalmia (Fig. 1B) and bilateral cleft lip and palate (Fig. 1C) were present. A TORCH screen at birth was negative and chromosome analysis showed an apparently normal male karyotype, 46,XY. At 4 months of age, his development was delayed and he had no head control. His weight was 5.5 kg (3rd percentile), height 57 cm (3rd percentile) and head circumference was 41 cm (25th percentile). His anterior fontanelle was open and measured 3cm x 3cm. Examinations of the chest, heart, abdomen, genitalia, skeletal system, skin and muscle tone were described as unremarkable. His hearing was also reported to be normal.

The child was reviewed at 3.5 years of age. His weight, height and head circumference were at the 3rd percentile. He had global developmental delays and was able to sit and to transfer objects from one hand to the other. His only words were ‘dada’ and ‘mama’ and his developmental level was assessed as that of a 6-month child. An echocardiogram and renal ultrasound scan were normal as were skeletal radiographs. A magnetic resonance imaging (MRI) scan of the brain was reported to show absence of the normal ocular globes bilaterally, with the right orbit showing a fluid-filled cyst. The corpus callosum was absent. There was a small nodule of soft tissue in the anterior hemispheric fissure of unknown origin. The pineal gland was absent and the hippocampus had a vertical orientation. No hormonal analyses have been done to test pituitary function. The parents have had no obvious eye malformations.

The p.Arg152Ser mutation affects an invariant residue in the third helix of the homeodomain (Supp. Table S2) and was not present in 96 Egyptian controls; the phenotypically normal, but consanguineous parents were heterozygotes and an unaffected sibling was wildtype for these two nucleotides (data not shown). Polyphen-2 (Prediction of Functional Effects of Human Non-synonymous Single Nucleotide Polymorphisms) predicted that the mutation was ‘probably damaging’, with a score of 1.0 (sensitivity 0.0 and specificity 1.0) and it was not present in either the Database of Single Nucleotide Polymorphisms (dbSNP; http://www.ncbi.nlm.nih.gov/projects/SNP/; date of search 9.21.11) or the 1000 Genomes Project website (http://www.1000genomes.org/; date of search 9.21.11).

It has recently been shown that the Vax proteins function as activators of a potent dominant-negative isoform of the canonical Wnt signaling mediator Tcf7l2, designated dnTcf7l2 [Vacik et al., 2011]. They achieve this by binding to and activating an internal promoter located in the fifth intron of the *Tcf7l2* gene. DnTcf7l2 lacks the activating beta-catenin domain for Tcf7l2 and therefore functions as a strong repressor of canonical Wnt target genes. This dominant-negative antagonist is expressed throughout the developing forebrain and its morpholino-mediated loss in *Xenopus* leads to embryos lacking the anterior head region [Vacik et al., 2011]. To assess the effect of the *VAX1* mutation on its ability to activate *dnTCF7L2*, we used quantitative RT-PCR (Q-RT-PCR) to measure expression of

the most abundant *dnTCF7L2* mRNA, the exon 1b isoform [Vacik et al., 2011], in HEK293 cells following transfection with either wild-type or mutated *VAX1* expression constructs. Wild-type *VAX1* and mutated *VAX1*/pArg152Ser expression constructs were purchased from Origene (Rockville, MD). 1 μg of the expression construct was transfected into HEK293 cells in a 24-well plate using Fugene6 (Roche, Indianapolis, IN). Total RNA was isolated 40 hours after transfection using TRIZOL (Invitrogen, Carlsbad, CA) and reverse transcription was performed using Superscript III (Invitrogen, Carlsbad, CA). Quantitative RT-PCR reactions were performed in triplicate using SYBRGreen PCR master mix (Applied Biosystems, Foster City, CA) and normalized against expression of *GAPDH*. The Q-RT-PCR primers have been provided in Supp. Table S1.

As expected, neither wild-type nor mutant *VAX1*/p.Arg152Ser induced the expression of full-length *TCF7L2* mRNAs (Fig. 1D). However, we found that wild-type *VAX1* stimulates the expression of *dnTCF7L2* mRNA 1b, while the mutant form does not (Fig. 1D). These results suggest that the *VAX1* mutation prevents this homeodomain transcription factor from activating *dnTCF7L2*, and that this in turn leads to de-repression of *TCF7L2* target genes and hyperactivation of Wnt signaling. It is possible that this mechanism explains at least part of the observed phenotype found with the *VAX1* p.Arg152Ser mutation.

Similar to the phenotype that was observed in the patient with p.Arg152Ser, *Vax1* homozygous null mice had clefting of the secondary palate with 100% penetrance and agenesis of the corpus callosum [Bertuzzi et al., 1999]. We therefore sequenced *VAX1* in 10 A/M patients selected to have additional phenotypic findings of orofacial clefting and/or agenesis of the corpus callosum (Supp. Table S3). In a patient with bilateral anophthalmia and bilateral cleft lip and palate, we detected one heterozygous sequence alteration, c. 945C>T, in exon 3 (Supp. Table S3; data not shown). This alteration is not listed in dbSNP or 1000 genomes and was absent in the DNA from 160 control chromosomes obtained from patients with diaphragmatic hernia; the parents were unavailable for testing. However, c. 945C>T is a silent substitution of unclear significance as it does not alter an amino acid residue (p.Ala315Ala) and was not predicted to alter splicing [Automated Splice Site Analysis; Rogan et al., 1998; Nalla and Rogan, 2005]. Furthermore, we did not find a second alteration in this patient that would have been consistent with the autosomal recessive inheritance pattern found in the patient with p.Arg152Ser.

We have described a new homozygous sequence alteration in *VAX1*, p.Arg152Ser, in a male with a phenotype of severe microphthalmia, agenesis of the corpus callosum and cleft palate that is highly reminiscent of the mouse model for loss of function of this gene. We consider that this mutation is pathogenic on the basis of the homozygosity observed in the patient and the segregation of the mutation with the disease phenotype in the family, the *in-silico* predictions of likely pathogenicity and the absence of the sequence alteration in control individuals of the same ethnicity. The mutation is likely to cause microphthalmia because of loss of function. Our functional studies suggest that one mechanism whereby the mutation exerts it phenotypic effects is through failure to produce dnTCF7L2 and consequent hyperactivation of Wnt signaling. However, the involvement of other pathways has not been excluded. In the chick, *cVAX* has been shown to interact with *Paired box gene 6* [*PAX6*; MIM# 607108] and this interaction may be important in delineating the boundary between the dorsal and ventral retina [Leconte et al., 2004]. At the stage of optic cup development, *PAX6* and *cVAX* are expressed in a gradient from the dorsal retina (primarily *PAX6* expression) to the ventral retina (primarily c *VAX* expression) [Leconte et al., 2004]. c *VAX* can bind to both the paired domain and the homeodomain of *PAX6*, although it is unknown if the c *VAX*/*PAX6* interaction occurs via the paired domain, homeodomain, or both [Leconte et al., 2004]. During normal eye development, *Pax6* represses *VAX1*; in turn, c *VAX* can repress the activity of the intronic, *PAX6* α-enhancer in the retina, thus

repressing *PAX6* transactivation and contributing to *PAX6* downregulation [Leconte et al., 2004]. As the p.Arg152Ser amino acid substitution occurs in the VAX1 homeodomain, we reasoned that it could alter the ability of VAX1 to repress *PAX6*. In addition, mutations causing loss of function in *PAX6* have also been associated with agenesis of the corpus callosum [Abouzeid et al., 2009].

It is also still possible that the mutation in *VAX1* has altered the function or regulation of another gene or genes besides *Tcf7l2* and *Pax6*. In a double-knockout mouse for Chicken Ovalbumin Upstream Promoter Transcription Factor 1 [*COUP-TFI*; MIM# 132890] and Chicken Ovalbumin Upstream Promoter Transcription Factor 2 [*COUP-TFII*; MIM# 107773] targeted specifically to the eye, *COUP-TFI* and *COUP-TFII* mutants had greatly reduced *Vax1* and *Vax2* expression and manifested microphthalmia and colobomas [Tang et al., 2010] and a possible interaction between *VAX1* and a *COUP-TF* gene was not investigated.

The finding of a corpus callosum defect and other brain malformations in the patient with the p.Arg152Ser mutation was strongly suggestive for a role for *VAX1* in human brain development. This has been supported by animal models of loss of *Vax1* function, as *Vax1* homozygous null mutant mice have previously been reported to have agenesis of the corpus callosum [Bertuzzi et al., 1999]. More recently, *Vax1* mutant mice have been found to have a second, rostrally located Rathke's pouch that has the potential to develop into a pituitary gland with hormone secreting capabilities [Bharti et al., 2011]. The pituitary duplication was attributed to loss of *Vax1*, with failure to repress Fibroblast growth factor 10 [*Fgf10*; MIM# 602115] in the neuroectoderm rostral to the infundibulum of the pituitary gland [Bharti et al., 2011].

The corpus callosum abnormalities associated with loss of *Vax1* function in the mouse may also be due to altered *Pax6* signaling. *Vax1* is expressed in the ventral telencephalon [Ellison-Wright et al., 2004] and *Pax6* in the dorsal telencephalon [Haubst et al., 2004], suggesting that *Pax6* and *Vax1* share adjacent expression domains in the developing cortex, similar to the situation in the developing eye. Mice homozygous for mutations or null alleles of the *Pax6* gene also harbor a wide variety of neurodevelopmental abnormalities including absence of the pineal gland and corpus callosum [Schmahl et al., 1993; Estivill-Torrus et al., 2001]. Brain imaging studies in patients with *PAX6* loss of function mutations have also revealed a wide range of malformations, including absence of the pineal gland, hypogenesis of the corpus callosum, significantly smaller corpus callosum volumes and Probst bundles [Ellison-Wright et al., 2004; Bamiou et al., 2007; Abouzeid et al., 2009]. However, there are other known *Vax1* target genes that have a role in axon guidance, such as *Netrin-1* and the receptor tyrosine kinase *EphB3* [Mui et al., 2005]. In contrast to its involvement in brain development, little is known about the role of *Vax1* in palate development. The cleft palate observed in *Vax1* homozygous null mice may be associated with the transient expression of *Vax1* in the first branchial arch [Hallonet et al. 1999]. It is interesting to note that several SNPs in *PAX6* showed significant evidence of linkage disequilibrium in cleft lip and/or cleft palate case-parent trios from four populations [Sull et al., 2009].

In *VAX2*, we identified four coding SNPs in the 70 A/M patients: c.43C>A, predicting p.Pro15Pro; c.70C>G, predicting p.Arg24Gly; c.761C>G, predicting p.Pro254Arg and c. 825A>G, predicting p.Leu275Leu (Table 1). Two of these SNPs were non-synonymous - p.Pro254Arg, which was predicted to be ‘probably damaging’ by Polyphen-2 with a score of 0.995 (sensitivity 0.67 and specificity 0.97) and p.Arg24Gly, which was predicted to be ‘possibly damaging’, with a score of 0.444 (sensitivity 0.89 and specificity: 0.90). However, the allele frequencies for these SNPs in the 70 A/M patients were not significantly different from allele frequencies reported for control chromosomes in dbSNP (Table 1). We therefore

did not find evidence of a direct involvement of *VAX2* in the pathogenesis of human A/M in the small cohort sequenced.

In conclusion, we present the first description of a homozygous *VAX1* mutation, c.453G>A and c.454C>A, that predicted p.Arg152Ser, in a male with severe bilateral microphthalmia and small optic nerves, cleft lip and palate, corpus callosum agenesis, hippocampal malformations and absence of the pineal gland. A similar phenotype to the patient of microphthalmia, agenesis of the corpus callosum and cleft palate has also been observed in mice with loss of *Vax1* function, making this the most likely mechanism. The mutation may act by preventing the formation of *dnTCF7L2*, resulting in hyperactivation of the Wnt pathway. This is the first description of a patient with a *VAX1* mutation and establishes *VAX1* as a new causative gene for A/M in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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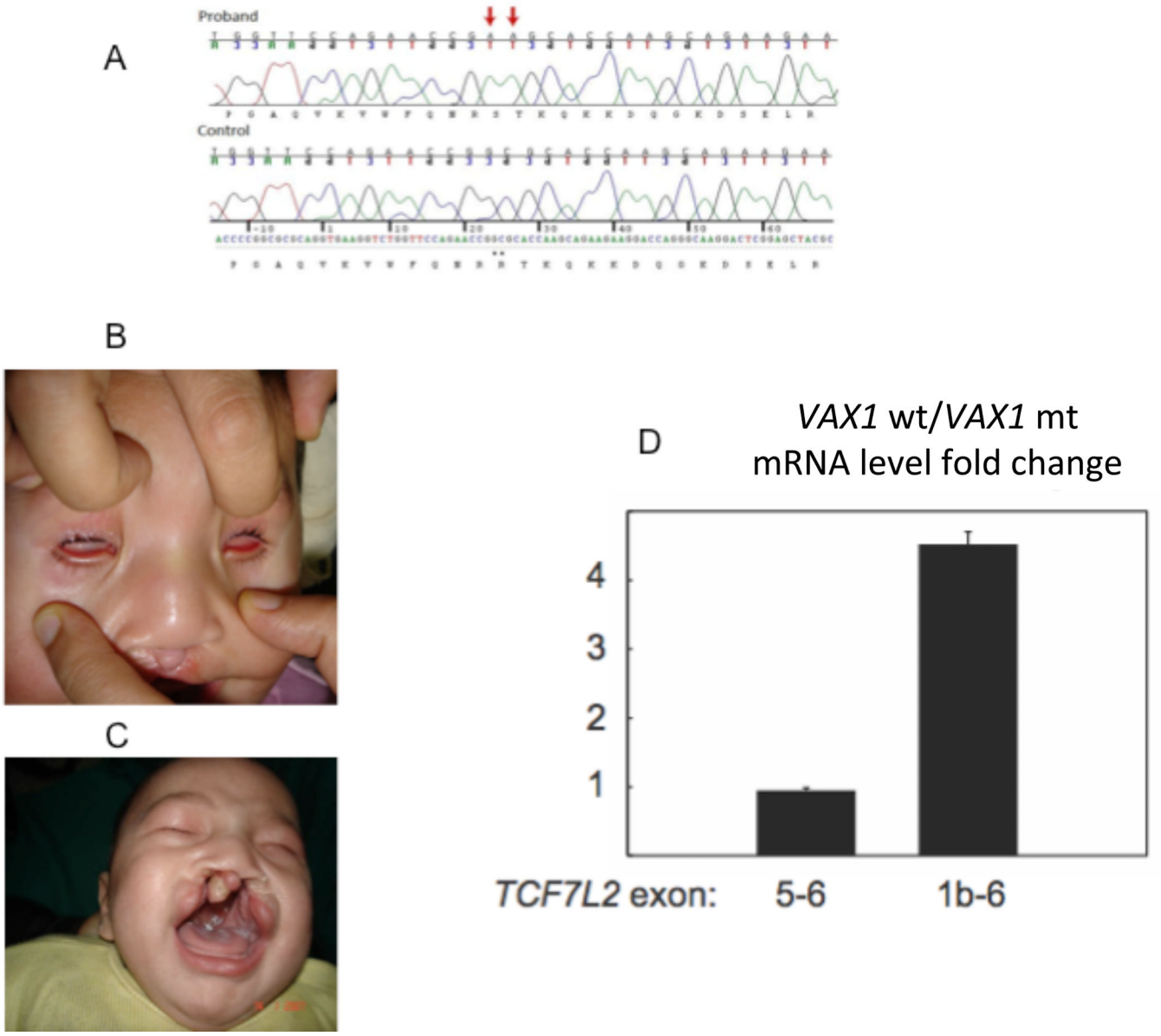

Figure 1.

A. Chromatogram from the proband and a control, showing homozygosity for c.453G>A and c.454C>A (c.453_454delinsAA) in the proband (marked by arrows). **B.** Frontal view of the proband, showing bilateral severe microphthalmia. **C.** Frontal view of the proband, showing bilateral cleft lip. **D.** Mutated *VAX1* cannot activate endogenous *dnTCF7L2*. As determined by Q-RTPCR, the expression of full-length *TCF7L2* mRNA (exons 5-6) is not affected by overexpression of the human wild-type *VAX1* in HEK293 cells, while the expression of *dnTCF7L2* (exons 1b-6) is induced. This induction is not seen with mutated *VAX1*. Fold change is the ratio between expression normalized to *GAPDH* in cells transfected with the wild-type *VAX1* expression construct versus cells transfected with the mutated *VAX1* expression construct. Error bars are mean ± standard deviation (n = 3).

Table 1

Sequence Alterations in *VAX1* and *VAX2* in 70 Anophthalmia/Microphthalmia Patients

Gene/Location	Nucleotide Alteration	Amino Acid Alteration	Allele Frequency (n = 140 chromosomes)	dbSNP[a]	Allele Frequency from dbSNP	Polyphen-2 Score[b]
VAX1						
Exon 3	c.453_454delinsAA	p.Arg152Ser	GC = 0.993 AA = 0.007	-	-	1.0; probably damaging
VAX2						
Exon 1	c.43C>A	p.Arg15Arg	C = 0.864 A = 0.136	rs2234495	C = 0.875 (120)[c] A = 0.125	-
Exon 1	c.70C>G	p.Arg24Gly	C = 0.993 G = 0.007	rs2234496	C = 0.924 (118)[d] G = 0.076	0.444; possibly damaging
Exon 3	c.761C>G	p.Pro254Arg	C = 0.907 G = 0.093	rs2234500	C = 0.933 (120)[e] G = 0.067	0.995; probably damaging
Exon 3	c.825A>G	p.Leu275Leu	A = 0.993 G = 0.007	rs2234501	A = 0.983 (120)[f] G = 0.017	-

Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen) for both genes. The initiation codon is codon 1.

The *VAX1* exon 3 sequence alteration is in isoform 1 of *VAX1.*

[a] dbSNP = Database of Single Nucleotide Polymorphisms; http://www.ncbi.nlm.nih.gov/proiects/SNP/

[b] Polyphen-2 Score = http://genetics.bwh.harvard.edu/pph2/

[c] =CEU Allele frequency = ss231220873_pilot_1_CEU_low_coverage_panel; number of chromosomes in brackets

[d] = YRI Allele frequency = ss219317729_piot_1_YRI_low_coverage_panel; number of chromosomes in brackets

[e] = CEU Allele frequency = ss231220980_pilot_1_CEU_low_coverage_panel; number of chromosomes in brackets

[f] = CEU Allele frequency = ss231220981_pilot_1_CEU_low_coverage_panel; number of chromosomes in brackets