

**Mutations of a human homologue of the *Drosophila eyes absent* gene (*EYAI*) detected in patients with congenital cataracts and ocular anterior segment anomalies.**

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## ABSTRACT

The *Drosophila eyes absent* gene (*eya*) is involved in the formation of compound eyes. Flies with loss-of-function mutations of this gene develop no eyes and form the ectopic eye in the antennae and the ventral zone of the head on target expression. A highly conserved homologous gene in various invertebrates and vertebrates has been shown to function in the formation of the eye. In contrast, a human homologue, *EYAI*, has been identified by positional cloning as a candidate gene for Branchio-Oto-Renal (BOR) syndrome, in which phenotypic manifestations are restricted to the areas of branchial arch, ear and kidney, with usually no anomalies in the eye. We have examined genomic DNA isolated from patients with various types of developmental eye anomaly for *EYAI* mutations by the use of polymerase chain reaction-single –strand conformation polymorphism and sequencing. We identified three novel missense mutations in patients who had congenital cataracts and ocular anterior segment anomalies. One of the patients had clinical features of BOR syndrome as well. This result implies that the human *EYAI* gene is also involved in the eye morphogenesis, and that a wide variety of clinical manifestations may be caused by *EYAI* mutations.

## INTRODUCTION

The *Drosophila eya* gene was isolated from a breakpoint region of flies with an eyeless phenotype (1). The gene has been shown to have an ability to restore the phenotype on injection of the cDNA expression construct and also to form the eye ectopically in the antennae and the ventral zone of the head on target expression (2). It has been suggested that the gene product may suppress programmed cell death in eye progenitor cells at a critical stage in eye morphogenesis (1). One of the human homologues has been isolated by positional cloning of the gene responsible for branchio-oto-renal (BOR) syndrome (3), and subsequent studies have revealed that the homologues designated as *Eya1*, *Eya2* and *Eya3* form a novel gene family in human beings and mice (4, 5). The original report demonstrated by *in situ* hybridization that murine *Eya1* was expressed in the otic, olfactory and renal primordia, but not in the branchial arch and eye (3). However a subsequent report showed that the *EYA* family members were also expressed in the developing eye; *Eya1* was expressed in the ocular anterior segment, lens and optic nerve sheath, and *Eya2* in the posterior segment (5). Thus, we speculated that the *EYA* family might be a potential candidate for eye anomalies, however, no anomalies in the eye have been described in patients with BOR syndrome. In this study, we have detected three novel missense mutations of the *EYAI* gene in patients with developmental ocular anomalies. The mutations provide an interesting genotype-phenotype correlation.

## RESULTS

### Clinical phenotypes of patients carrying the mutation

We have screened for *EYAI* mutations in genomic DNA isolated from patients with various types of eye anomaly (a total of 317 samples as described in Materials and Methods), and detected three mutations in the following patients.

Patient 1 is a 4-year-old girl who was introduced to our hospital with visual impairment. Ocular examinations revealed central corneal opacity, adhesion to the iris (Peters' anomaly)

and slight cataracts in both eyes, whereas the fundus was normal (Fig. 1a). Her visual acuity was 0.08 bilaterally. (The score is a fraction of the visual angle with which two points can be recognized separately. Visual acuity is usually measured using a chart with the Landolt rings and a normal score ranges from 1.0 to 2.0.) Her mother, aged 32, had nuclear-type congenital cataracts with 0.3 visual acuity. The patient and her mother were otherwise normal in appearance, intelligence and karyotype (46, XX). After finding the mutation, we asked our colleagues in the Departments of Otolaryngology and Pediatrics (National Children's Hospital, Tokyo, Japan) to conduct careful examinations of the patient, but no clinical findings suggesting BOR syndrome were obtained except for a slight elevation of the auditory brain stem response (ABR) threshold in hearing.

Patient 2 is a 3-year-old boy who presented at our hospital with iris anomaly. Eye examinations revealed bilateral persistence of the pupillary membrane, but a normal lens and fundus (Fig. 1b). His visual acuity was 0.5 in the right eye and 0.8 in the left eye with correction. He had no other systemic abnormalities, and was found normal in growth and intelligence for his age, even after careful re-examinations following the finding of the mutation as described for patient 1.

Patient 3 is an 8-year-old boy who had first presented at our hospital with nystagmus and systemic edema at 20 days-of-age. Examinations revealed bilateral nuclear-type congenital cataracts with the normal fundus, and multicystic dysplasia in his right kidney, which did not function and caused hypocalcemia (Fig.1 c, d). The cataracts were operated on at 1-month-of-age, and the right kidney was removed at 2 months. He was later diagnosed as having conductive deafness with the malleus anomaly. He also has cervical fistula that occluded spontaneously. These clinical findings, besides the cataracts, coincide with typical features of BOR syndrome. He now has 0.2 visual acuity with esotropia, and slight mental retardation.

### **Mutations of the *EYAI* gene**

Patient 1 and her mother had an A to G nucleotide substitution at position 1688 of a cDNA form of the *EYAI* gene (accession No. Y10260, in exon 15), which is expected to result in R514G (Fig. 2a). Patient 2 had a G to A substitution at position 1136 (exon 10), which resulted in E330K (Fig. 2b). The mutation was not detected in his parents who are apparently normal, thus indicating it was sporadic. Patient 3, but not his normal parents, had a G to A substitution at position 1325 (exon 12), which results in G393S (Fig. 2c).

All the mutations detected above occurred on one of the alleles (heterozygous) and were not detected in unaffected members of the immediate family nor in more than 100 normal individuals. The relationship of biological paternity and maternity in the above pedigrees was confirmed with multiple microsatellite markers.

## **DISCUSSION**

BOR syndrome (OMIM 113650) is an autosomal dominant disorder with incomplete penetrance and variable expressivity. Anomalies are usually detected in the latero-cervical fistulas, in the outer, middle and inner ears and in the kidney. However, BOR patients show different combinations of these symptoms with varying degrees of severity even within the same family. Branchio-otic (BO) syndrome (OMIM 602588), which had been considered a

different medical entity because of the absence of renal anomaly, has been demonstrated to be allelic with the finding of mutations of the *EYAI* gene (6). Most mutations identified to date in the BOR and BO syndromes are situated in the C-terminal region (271 aa. residues encoded by the last 8 exons, the *eyaHR* region), and cause translational termination by nonsense and frameshift mutations as well as by splice errors (3, 4, 6, 7). These mutations occur on one of the alleles, thus haploinsufficiency of the gene product has been suggested to develop the BOR phenotype. A recent study on heterozygous *Eya1* mice generated with knockout technology supports this (8). In contrast, only two missense mutations, S454P and L472R, have been identified (3, 4).

There has been no description of unusual eyes for BOR patients, although anomalies of the anterior segments of the eye are easily recognized. Anomalies of the lacrimal ducts are sometimes associated with BOR syndrome (9), but the lacrimal ducts differ from the structure of the eye in terms of the morphogenic pathway (10). We have detected three novel missense mutations in patients with anomalies in the anterior segments of the eye. The mutations detected occurred in the *eyaHR* region, which is relatively conserved among *EYA* family members of different species (3-5, 11, 12). Notably, the glutamic acid residue at 330, and the glycine residue at 393 are conserved among all the *EYA* family members identified to date, while the arginine residue at 514 is conserved in *EYAI* and *EYA2* of both human and mice, and lysine occurs in *EYA3* (1, 3-5). These amino acid residues may play an important role in its reaction or maintenance of the protein structure, but molecular studies in experimental systems are required to reveal the function. Currently there are no experimental systems with which to investigate its function, although the *EYAI* product is suggested to be involved in apoptosis.

Patient 3 had cataracts in addition to renal and otic anomalies, thus he is atypical BOR syndrome patients. In contrast, the affected areas are almost limited to the eye in the other two patients, which indicates that some forms of the *EYAI* mutations seem to affect only the morphogenesis of the eye and not the branchial arch or ear. It is interesting that the three novel mutations detected in this study are missense mutations and do not result in premature termination of translation. Therefore, the mutated protein with an amino acid substitution may have a dominant effect especially in the process of the eye morphogenesis. Peters' anomaly derives from insufficient separation of the lens vesicle from the surface ectoderm, and persistent pupillary membrane represents an incomplete involution of anterior tunica vasculosa lentis (13). Thus, these diseases may be accounted for by suppression of programmed cell death in progenitor cells of the anterior segment of the eye at an early stage, and probably also in lens fiber cells in cataract.

Two of the detected mutations do not exist in the biological parents. This high incidence of sporadic mutation may be accounted for by our sample collection. Our pooled specimens are derived from an affected individual and are not intended to cover large affected families. Although we hear of the family history from the patient in clinical practice, we only examine other family members directly when a mutation is detected. The other mutation is associated with affected individuals in two generations, but the evidence is not so strong that the mutation causes the disease. However, as *EYA* family members of other species are clearly involved in eye morphogenesis, our study recaptures the attention paid to ocular anomalies caused by the *EYAI* gene, which was once thought not to relate to the eye of human beings. Extensive surveys for mutations, especially in large affected families, and functional analyses of the gene product would validate the significance of the mutation we detected.

## **MATERIALS AND METHODS**

### **DNA Samples**

This study was conducted in accordance with the World Medical Association Declaration of Helsinki. Our use of human subjects was conducted under the program approved by the National Children's Hospital Experimental Review Board, and deemed exempt from human subject regulations. We collected DNA samples from individuals with various types of congenital eye anomaly. The samples include 53 patients with ocular anterior segment anomalies including Peter's anomaly, 43 patients with aniridia, 71 patients with congenital cataracts, 22 patients with isolated foveal hypoplasia and 128 patients with optic nerve anomalies, and were used in analyses for *PAX6* mutations (14-18). DNA samples of the family members were also collected when a mutation was detected. All the subjects in our study are apparently Japanese. After obtaining informed consent, blood samples were collected from peripheral veins into lithium heparin tubes. Genomic DNA was prepared from isolated leukocytes using a standard phenol-chloroform procedure. The DNA samples used for normal controls have been described previously (19-20).

### **Polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) assay and sequencing**

PCR primers used for amplification of exons 1-16 of the *EYAI* gene were synthesized using a DNA/RNA synthesizer (Applied Biosystems, model 392, Foster City, CA) based on previous reports (3, 4). PCR was performed with 100 ng of genomic DNA and rTaq DNA polymerase (Takara, Shiga, Japan) in PCR buffer containing 1.5 mM of MgCl<sub>2</sub> and [ $\alpha$ -<sup>32</sup>P] dATP for 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min. The annealing temperature was adjusted to 45 °C for exon 10. Products were denatured at 94 °C for 5 min and loaded on 5% non-denatured polyacrylamide gels. The following four running buffers were used: 0.5 x TBE with or without 10% glycerol and 1.0 x TBE with or without 10% glycerol. The running condition was either at 4°C or at room temperature. Thus, we analyzed the products in 8 different conditions for SSCP. After running for 12-18 hrs at 200-300V, gels were dried and exposed to an X-ray film for 6-24 hrs. PCR products showing an aberrant mobility compared with those of healthy donors were sequenced after subcloning on pUC18 using a DNA sequencing kit (Amersham, Cleveland, OH) and an automatic DNA sequencer (A373, Applied Biosystems). Sequence variations were confirmed in at least 6 independent colonies, and also with direct sequencing of the amplified products.

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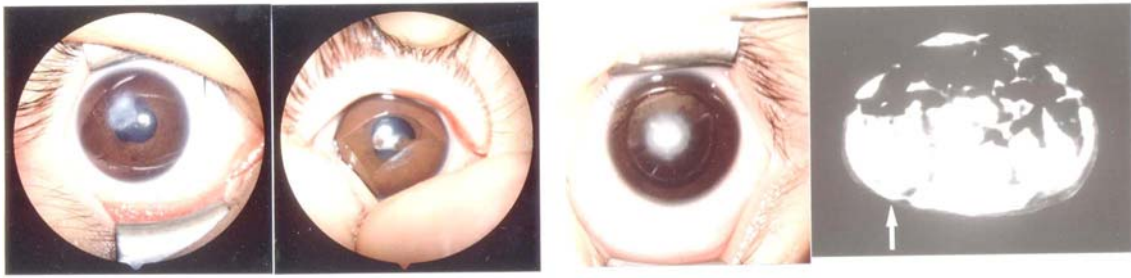
### **ABBREVIATIONS**

BOR syndrome, Branchio-Oto-Renal syndrome;

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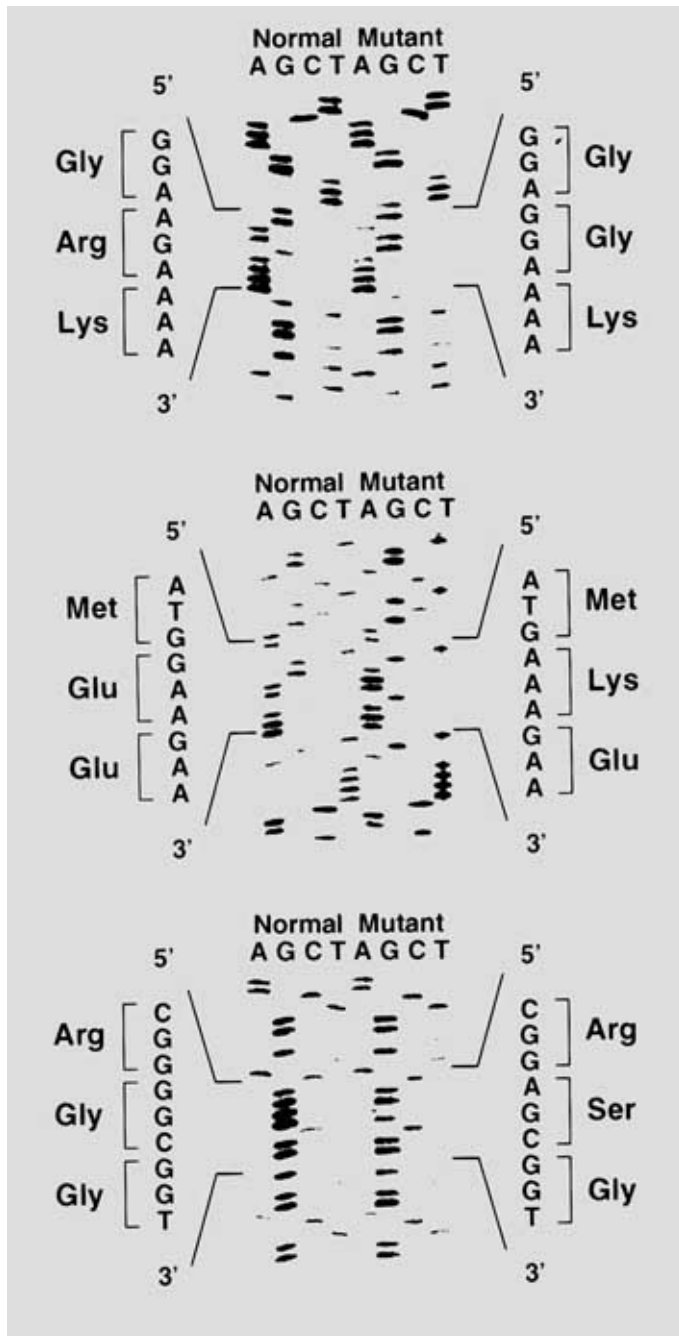
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**Figure 1**

Anomalies of anterior segments of the patients, including (a) central corneal opacity and adhesion to the iris (Peters' anomaly) in patient 1, (b) insufficient opening of the pupil in patient 2, and (c) congenital cataracts with the iris dilated by mydriatics in patient 3. These anomalies occurred bilaterally and only the appearance of the right eye of each patient is illustrated. (d) an ultrasonograph of the right multicystic dysplastic kidney (arrow) of patient 3.



**Figure 2**

Sequencing of the normal and mutant alleles from the patients and normal controls showing the mutations: (a) an A to G substitution at position 1688 in exon 15 detected in patient 1, which resulted in R514G, (b) a G to A nucleotide substitution at position 1136 in exon 10 detected in patient 2, which resulted in E330K, and (c) a G to A substitution at position 1325 in exon 12 detected in patient 3, which resulted in G393S.