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RESEARCH ARTICLE

Genetic Analysis of ‘PAX6-Negative’ Individuals with Aniridia or Gillespie Syndrome

Morad Ansari1, Jacqueline Rainger1, Isabel M. Hanson1, Kathleen A. Williamson1, Freddie Sharkey1, Louise harewood1, Angela Sandilands1, Clayton-Smith2, Helene Dollfus5, Pierre Bitoun5, Françoise Meire5, Judy Fant5, Brunello Franco6,7, Birgit Lorenz8, David S. Taylor8, Fiona Stewart10, Colin E. Willoughby11, Meriel McEntagart12, Peng Tee Khaw13, Carol Clericuzio14, Lionel Van Maldergem15, Denise Williams16, Ruth Newbury-Ecob17, Elisia I. Traboulsi18, Eduardo D. Silva19, Mukhlis M. Madlom20, David R. Goudie21, Brian W. Fleck22, Dagmar Wieczorek23,24, Juergen Kohlhase25, Alice D. McTrusty26, Carol Gardiner27, Christopher Yale28, Anthony T. Moore19, Isabelle Russell-Eggitt9, Lily Islam9, Melissa Lees29, Philip L. Beales9, Stephen J. Tuft13, Juan B. Solano30, Miranda Splitt31, Jens Michael Hertz32, Trine E. Prescott33, Deborah J. Shears34, Ken K. Nischal35, Marline Doco-Fenzy36, Fabienne Prieur37, I. Karen Temple38, Katherine L. Lachlan39, Giuseppe Damante40, Danny A. Morrison41, Veronica van Heyningen1, David R. FitzPatrick42

1 MRC Human Genetics Unit, Institute of Genetics and Molecular Medicine, University of Edinburgh, Western General Hospital, Edinburgh, United Kingdom, 2 Faculty of Medical and Human Sciences, Manchester Centre for Genomic Medicine, Institute of Human Development, University of Manchester, Manchester Academic Health Science Centre (MAHSC), Manchester, United Kingdom, 3 Service de Génétique Médicale, Hôpital de Haute-Pierre, Strasbourg, France, 4 Medical Genetics Departments, University Hospital Jean Verdier, Bondy, France, 5 Department of ophthalmopediatrics, Hôpital Universitaire des Enfants Reine Fabiola, Bruxelles, Belgium, 6 Medical Genetics, Department of Medical Translational Sciences, Federico II University, Naples, Italy, 7 Telethon Institute of Genetics and Medicine (TIGEM), Pozzuoli, Italy, 8 Department of Ophthalmology, Justus-Liebig-University Giessen, Universitätsklinikum Giessen and Marburg UKGM, Giessen, Germany, 9 Institute of Child Health, University College London, UK and Great Ormond Street Hospital for Children, London, United Kingdom, 10 Northern Ireland Regional Genetics Service (NIRGS), Belfast City Hospital, Belfast, United Kingdom, 11 Department of Eye and Vision Science, Institute of Ageing and Chronic Disease, University of Liverpool, Liverpool, United Kingdom, 12 Medical Genetics Unit, St George’s University of London, London, United Kingdom, 13 Moorfields Eye Hospital, London, UK and University College London Institute, Ophthalmology, London, United Kingdom, 14 Department of Pediatric Genetics, University of New Mexico Health Sciences Center, Albuquerque, New Mexico, United States of America, 15 Centre de Génétique Humaine, Université de Franche-Comté, Besançon, France, 16 Clinical Genetics Unit, Birmingham Women’s Hospital, Birmingham, United Kingdom, 17 Department of Clinical Genetics, University Hospitals Bristol, Bristol, United Kingdom, 18 Center for Genetic Eye Diseases, Cole Eye Institute, Cleveland Clinic Foundation, Cleveland, OH, United States of America, 19 Department Ophthalmology, University Hospital of Coimbra, Coimbra, Portugal, 20 Children’s Hospital, Doncaster Royal Infirmary, Doncaster, United Kingdom, 21 Human Genetics Unit, University of Dundee College of Medicine, Dentistry and Nursing, Ninewells Hospital, Dundee, United Kingdom, 22 Department of Ophthalmology, Princess Alexandra Eye Pavilion, Chalmers Street, Edinburgh, United Kingdom, 23 Institut für Humangenetik, Universitätssklinikum Essen, Universität Duisburg-Essen, Essen, Germany, 24 Institut für Humangenetik, Universitätssklinikum Düsseldorf, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany, 25 Center for Human Genetics, Freiburg, Germany, 26 Department of Life Sciences, Glasgow Caledonian University, Glasgow, United Kingdom, 27 Clinical Genetics, Southern General Hospital, Glasgow, United Kingdom, 28 Department of Paediatrics and Child Health, Ipswich Hospital, Ipswich, United Kingdom, 29 North East Thames Regional Genetics Service, Great Ormond Street Hospital for Children NHS Foundation Trust, Great Ormond Street Hospital, London, United Kingdom, 30 Ruber International Hospital, Medical Genetics Unit, Mirasierra, Madrid, Spain, 31 Northern Genetics Service, Institute of Genetic Medicine, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle Upon Tyne, United Kingdom, 32 Department of Clinical Genetics, Odense University Hospital, Odense C, Denmark, 33 Department of Medical Genetics, Oslo University Hospital, Oslo, Norway, 34 Department of Clinical Genetics, Churchill Hospital, Oxford University Hospitals NHS Trust, Oxford, United Kingdom, 35 UPMC Eye
Abstract

We report molecular genetic analysis of 42 affected individuals referred with a diagnosis of aniridia who previously screened as negative for intragenic PAX6 mutations. Of these 42, the diagnoses were 31 individuals with aniridia and 11 individuals referred with a diagnosis of Gillespie syndrome (iris hypoplasia, ataxia and mild to moderate developmental delay). Array-based comparative genomic hybridization identified six whole gene deletions: four encompassing PAX6 and two encompassing FOXC1. Six deletions with plausible cis-regulatory effects were identified: five that were 3’ (telomeric) to PAX6 and one within a gene desert 5’ (telomeric) to PITX2. Sequence analysis of the FOXC1 and PITX2 coding regions identified two plausibly pathogenic de novo FOXC1 missense mutations (p.Pro79Thr and p.Leu101Pro). No intragenic mutations were detected in PITX2. FISH mapping in an individual with Gillespie-like syndrome with an apparently balanced X;11 reciprocal translocation revealed disruption of a gene at each breakpoint: ARHGAP6 on the X chromosome and PHF21A on chromosome 11. In the other individuals with Gillespie syndrome no mutations were identified in either of these genes, or in HCCS which lies close to the Xp breakpoint. Disruption of PHF21A has previously been implicated in the causation of intellectual disability (but not aniridia). Plausibly causative mutations were identified in 15 out of 42 individuals (12/32 aniridia; 3/11 Gillespie syndrome). Fourteen of these mutations presented in the known aniridia genes; PAX6, FOXC1 and PITX2. The large number of individuals in the cohort with no mutation identified suggests greater locus heterogeneity may exist in both isolated and syndromic aniridia than was previously appreciated.

Introduction

Abnormal development of the iris is a feature of a variety of congenital human ocular anomalies, of which, the best characterized is complete aniridia (MIM 106210), a dominantly inherited condition with an incidence of less than 1 in 50,000 [1]. Aniridia presents as congenital absence of the iris, although a visible partial rim or sector of iris tissue strand is often present [2]. Foveal hypoplasia, cataract, keratopathy and glaucoma sometimes develop in second or third decade contributing to visual morbidity [3]. Non-ocular anomalies including hyposmia and structural brain changes are sometimes observed in individuals with complete aniridia [4].

At least 90% of aniridia cases are caused by heterozygous loss-of-function mutations in PAX6 [5]. Almost all cases of classical aniridia associated with PAX6 haploinsufficiency present with foveal hypoplasia. Heterozygous, presumed hypomorphic, missense mutations in PAX6
have also been associated with other ocular diseases including anterior segment dysgenesis [6] and optic nerve malformations [7]. Rarely, isolated aniridia is caused by mutations in FOXC1 [8,9] or PITX2 [10]. Mutations in these genes are more commonly associated with juvenile-onset glaucoma [11] and anterior segment dysgenesis [12–14] presenting with syndromic features of rare cardiac anomalies for FOXC1 and hypodontia and umbilical anomalies for PITX2.

Several syndromic forms of iris developmental anomalies have been described. The best known is WAGR (Wilms’ tumour, aniridia, genital anomalies and mental retardation; MIM 194072), a contiguous deletion syndrome on 11p13 [15]. Gillespie syndrome (MIM 206700) is characterized by a pathognomonic iris anomaly; absence of the pars pupillaris of the iris and the pupillary border. Individuals with Gillespie syndrome are also distinguished from complete aniridia by having a normal fovea and no evidence of progressive opacification of the cornea and lens, nor development of glaucoma. The extraocular features are non-progressive cerebellar ataxia and psychomotor delay [16]. Several cases of Gillespie syndrome have been reported [17–39].

In the literature, Gillespie syndrome has been most commonly considered to be an autosomal recessive disorder [36–38]. Analysis of the PAX6 gene in six Gillespie syndrome patients revealed no intragenic mutations [20,26,40]. PAX6 mutations have been reported in two individuals [33,39] described as Gillespie syndrome but with significantly atypical features such as corectopia and ptosis (33). A single affected girl described as having a Gillespie syndrome-like phenotype has been reported with an apparently balanced X:autosome reciprocal translocation t(X;11)(p22.32;p12) [22] and atypical features of superior coloboma, foveal hypoplasia and vermis hypoplasia. This case is included in this study as individual 1371.

Here, we report genomic copy number and extended mutation analysis in 42 unrelated affected individuals all of whom had been scored as negative for intragenic PAX6 mutations. Eleven of these probands had been referred to us with a diagnosis of Gillespie syndrome and 31 with non-syndromic aniridia. One of the 11 Gillespie syndrome individuals was the case with the apparently balanced reciprocal translocation t(X;11)(p22.32;p12) [22]. In this case we used FISH to map both breakpoints. In total, 15 plausible disease-causing heterozygous loss-of-function mutations were identified: nine affecting PAX6, four affecting FOXC1, one affecting PITX2 and one affecting PHF21A. These data suggest that other disease loci or mutational mechanisms causing aniridia remain to be discovered.

**Materials and Methods**

**Patient samples**

All aspects of this study were performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from the participants and recorded. The study was approved by the UK Multicentre Regional Ethics Committee under the number 06/MRE00/76. All patients were phenotypically characterized by experienced ophthalmologists or geneticists. The study cohort consisted of 42 unrelated individuals with aniridia or Gillespie syndrome (S1 Table) each of whom had been previously screened for intragenic PAX6 mutations by single-strand conformation polymorphism (SSCP), denaturing high performance liquid chromatography (DHPLC) and direct sequencing (S2 Table).

**DNA preparation and quality control**

Genomic DNA was prepared from either lymphoblastoid cell lines (LCL) or saliva using a Nucleon DNA extraction kit (Tepnel Life Sciences, UK). DNA quality was checked by agarose gel electrophoresis and NanoDrop spectrophotometry (Thermo Scientific).
Array comparative genomic hybridization (aCGH)

Genome-wide analysis of DNA copy number was carried out using the Roche Nimblegen 12X135k whole-genome array (median probe spacing of approximately 12 kb) according to the manufacturer’s instructions with minor modifications, as described previously [41].

Targeted analysis of genomic deletions/duplications was performed using a customized oligonucleotide microarray (Agilent Technologies) consisting of 44,000 60-mer oligonucleotide probes (4X44k), designed using eArray (Agilent Technologies). The design consisted of a 3 Mb genomic region (chr11:30,262,916–33,296,085; hg18) containing the PAX6 gene with an average probe spacing of 76 bp. ‘Dye-swap’ experiments were performed followed by copy number analysis, as previously described [42].

Polymerase chain reaction (PCR) and mutation analysis

Primer sequences and PCR conditions used for amplification and sequencing of the FOXC1, PITX2, PHF21A and ARHGAP6 genes are provided in S2 Table. PCR reactions were performed in 12μl volumes containing 1μl of 1-in-20 diluted, whole-genome amplified DNA (Genomiphi, GE Healthcare), 6μl of 2 X ReddyMix PCR Mastermix (Abgene), 833 nM of each oligonucleotide primer and 2.4μl of 5 X GC-mix (where appropriate). PCR conditions generally consisted of an initial denaturation at 95°C for 5 minutes, followed by 32 cycles of 94°C for 60 seconds, primer annealing for 60 seconds, and 72°C for 60 seconds, and a final cycle of 72°C for 10 minutes. The products were visualized using agarose gel electrophoresis to ensure adequate yield and proper sizing of each exon fragment. Sequencing of PCR products was performed in both directions as described elsewhere [43]. Sequence traces were analyzed using Mutation Surveyor sequence analysis software version 3.30.

Fluorescence in situ hybridization (FISH)

Metaphase spreads for FISH were prepared from patient lymphocytes as described elsewhere [44]. BAC clones were selected from the Ensembl database (http://www.ensembl.org) or the UCSC Human Genome Browser (http://genome.ucsc.edu) and ordered from the BACPAC resources centre (Children’s Hospital Oakland Institute). For the initial mapping of the clones, DNA was isolated using a rapid alkaline lysis miniprep method (Qiagen mini/midi plasmid kit). Probes were labeled with biotin-16-dUTP or digoxigenin-11-dUTP (Roche) by nick translation. Probe labelling, DNA hybridization and antibody detection were carried out as described previously [45]. Following hybridization, slides were mounted with a drop of Vectorshield antifadent containing DAPI (Sigma). Antibody detection was carried out by fluorescent microscopy using a Zeiss Axioscop microscope. Images were collected using a cooled CCD (charged coupled device) camera and analyzed using SmartCapture software (Digital Scientific).

Results

Patient cohort

Our study cohort consisted of 42 unrelated individuals (14 male, 28 female) with iris developmental anomalies (Table 1, S1 Table). Eleven of these individuals (2 male, 9 female) had been referred to us with a diagnosis of Gillespie syndrome including individual 1371 who had been previously reported with an apparently balanced reciprocal translocation: t(X;11)(p22.32;p12) [22]. Each proband had been scored negative for intragenic PAX6 mutations by SSCP, DHPLC and/or direct sequencing in our lab.
DNA copy number analysis of the PAX6 locus

To identify causative segmental aneuploidy, two array-based comparative genomic hybridization (aCGH) approaches were used: a 135k whole-genome array and a custom-designed targeted array covering a contiguous 3 Mb genomic region (chr11:30,262,916–33,296,085; hg18) encompassing PAX6. This identified four individuals with heterozygous deletions, all encompassing PAX6 and ranging in size from 96 kb to 650 kb: individual 2193 (chr11:31,199,000–31,849,000), individual 377 (chr11:31,394,000–31,914,000), individual 1510 (chr11:31,779,000–31,933,000) and individual 1977 (chr11:31,698,271–31,794,414) (Table 1, Fig 1, S1 Fig). Five individuals had deletions with breakpoints immediately telomeric.

### Table 1. Details of the clinical diagnoses and genetic pathology identified in individuals in this study.

<table>
<thead>
<tr>
<th>Individual ID</th>
<th>DECIPHER ID</th>
<th>Clinical feature</th>
<th>Genetic pathology</th>
<th>Genomic coordinates (hg18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1851 (control)</td>
<td>323119</td>
<td>Aniridia</td>
<td>PAX6 deletion (previously identified by FISH)</td>
<td>chr11:21,254,000–32,564,000</td>
</tr>
<tr>
<td>2193</td>
<td>323118</td>
<td>Aniridia</td>
<td>PAX6 whole-gene deletion</td>
<td>chr11:31,199,000–31,849,000</td>
</tr>
<tr>
<td>377</td>
<td>323104</td>
<td>Aniridia</td>
<td>PAX6 whole-gene deletion</td>
<td>chr11:31,394,000–31,914,000</td>
</tr>
<tr>
<td>1510</td>
<td>323113</td>
<td>Aniridia</td>
<td>PAX6 whole-gene deletion</td>
<td>chr11:31,779,000–31,933,000</td>
</tr>
<tr>
<td>1514</td>
<td>323114</td>
<td>Aniridia</td>
<td>PAX6 telomeric deletion</td>
<td>chr11:30,874,642–31,654,833</td>
</tr>
<tr>
<td>753</td>
<td>323108</td>
<td>Aniridia</td>
<td>PAX6 telomeric deletion</td>
<td>chr11:30,967,000–31,704,000</td>
</tr>
<tr>
<td>659</td>
<td>323107</td>
<td>Aniridia</td>
<td>PAX6 telomeric deletion</td>
<td>chr11:31,379,000–31,708,000</td>
</tr>
<tr>
<td>1449</td>
<td>323112</td>
<td>Gillespie syndrome</td>
<td>FOXC1 whole-gene deletion</td>
<td>chr6:1,543,591–1,675,085</td>
</tr>
<tr>
<td>1246</td>
<td>323110</td>
<td>Aniridia</td>
<td>FOXC1 whole-gene deletion</td>
<td>chr6:1,543,591–1,675,085</td>
</tr>
<tr>
<td>1839</td>
<td></td>
<td>Aniridia</td>
<td>FOXC1 c.235C&gt;A p.(Pro79Thr) de novo</td>
<td>Not applicable</td>
</tr>
<tr>
<td>1634</td>
<td></td>
<td>Aniridia</td>
<td>FOXC1 c.302T&gt;C p.(Leu101Pro) de novo</td>
<td>Not applicable</td>
</tr>
<tr>
<td>1194</td>
<td>323109</td>
<td>Aniridia</td>
<td>PITX2 telomeric deletion</td>
<td>chr4:111,994,000–115,504,000</td>
</tr>
<tr>
<td>1371</td>
<td></td>
<td>Gillespie syndrome</td>
<td>Translocation t(X;11)(p22.32;p12)</td>
<td>See Fig 5</td>
</tr>
</tbody>
</table>

DNA copy number analysis of the PAX6 locus.

Fig 1. Identification of PAX6 whole-gene deletions. Genome-wide array CGH analysis identified a 650 kb deletion in individual 2193 (chr11:31,199,000–31,849,000), a 520 kb deletion in individual 377 (chr11:31,394,000–31,914,000), a 96 kb deletion in individual 1977 (chr11:31,698,271–31,794,414), all involving PAX6. Red bars show the position of the deletions. Genes transcribed on the forward strand are in blue and those transcribed on the reverse strand are in green, also indicated by arrows. Genomic coordinates are based on the Human Genome Assembly hg18.

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to PAX6: individual 1514 (chr11:30,874,642–31,654,833; hg18), individual 753 (chr11:30,967,000–31,704,000; hg18), individual 555 (chr11:31,086,579–31,649–842; hg18), individual 2014 (chr11:31,234,395–31,751,815) and individual 659 (chr11:31,379,000–31,708,000; hg18) (Table 1, Fig 2). Combining these with published data, we suggested a 243.9 kb critical region for PAX6 transcriptional activation between chr11:31,379,000 (hg18) and chr11:31,708,000 (hg18) (Fig 2).

**Mutation analysis of the FOXC1 locus**

An apparently identical 131 kb deletion (chr6:1,543,591–1,675,085; hg18) encompassing FOXC1 was identified as a de novo occurrence in two unrelated individuals 1449 and 1246 (Table 1, Fig 3). Each of these deletions had been confirmed in an independent UK laboratory using an alternative method. Furthermore, the two individuals were shown to be distinct based on their aCGH profile of genome-wide copy number variants (data not shown). We then screened FOXC1 in our cohort by direct sequencing. Two individuals were found to carry missense mutations in the FOXC1 fork-head domain (Table 1, Fig 3). Individual 1839 had a C>A transversion in codon 79 (c.235C>A, p.(Pro79Thr)) and individual 1634 had a novel T>C transition in codon 101 (c.302T>C, p.(Leu101Pro)). In both individuals, the mutations were absent from the unaffected parents and had most likely occurred de novo (Fig 3). The amino acid substitution p.(Pro79Thr) has been reported previously in a family with classical Axenfeld-Rieger syndrome and the mutant protein has impaired nuclear localization and transactivation activity [46]. The novel p.(Leu101Pro) mutation is predicted to disrupt the second alpha helix of the fork-head domain.
Mutation analysis of the PITX2 locus

Array CGH identified a 3.5 Mb deletion of 4q25-q26 (chr4:111,994,000–115,504,000; hg18) in individual 1194 (Table 1, Fig 4). This deletion encompasses 8 genes (Fig 4). The centromeric breakpoint is located in a gene desert 230 kb telomeric (5‘) to PITX2 encompassing several conserved PITX2 enhancer elements [47]. Subsequent screening of the PITX2 coding sequence in our cohort revealed no plausible disease-causing mutations.
Breakpoint mapping of a translocation in an individual with Gillespie syndrome

FISH was used to map the previously reported t(X;11)(p22.32;p12) reciprocal translocation in individual 1371 (Fig 5). The breakpoint on chromosome 11 (now 11p11.2) lay within a single BAC, RP11-618K13 [48], which contains 5 known genes, CRY2, MAPK8IP1, PEX16, GYLT1B, and PHF21A (also known as BHC80), located approximately 14.1 Mb centromeric to PAX6. The breakpoint was shown to lie within PHF21A using probes generated by long-range PCR from exons 14–16 (telomeric to the breakpoint) and exons 4–11 (centromeric to the breakpoint) (data not shown). The X chromosome breakpoint (now Xp22.2) was spanned by two overlapping BACs (RP11-121K9 and RP11-311A17) [48] covering two genes, AMELX and ARHGAP6 (Fig 5). Using a probe generated by long-range PCR, the breakpoint was localized within a large intron of ARHGAP6 (Fig 5).

Mutation analysis of breakpoint genes in Gillespie syndrome patients

Direct sequencing of the coding exons and essential splice sites of PHF21A and ARHGAP6 revealed only polymorphic variants in the 10 individuals with Gillespie syndrome who lacked a detectable chromosomal abnormality at these loci. HCCS is located approximately 150 kb telomeric to the X chromosome breakpoint in individual 1371. Mutations in this gene have been associated with microphthalmia with linear skin defects (MIM 309801). Direct sequencing of HCCS revealed no mutations in the 10 non-translocation Gillespie cases.

Discussion

A high proportion of cases of aniridia is caused by loss-of-function mutations in a single gene, PAX6. Here we studied individuals with aniridia and Gillespie syndrome, who had previously
scored negative for intragenic PAX6 mutations, using a variety of molecular approaches to identify causative mutations. The rationale for the analysis was that we had a strong prior expectation that this cohort would be heavily enriched for causative structural chromosomal anomalies involving PAX6 itself, but also for possible new disease loci and/or novel mutational mechanisms. In the event, we identified deletions that result in PAX6 haploinsufficiency in

Fig 5. Fluorescence in situ hybridization (FISH) was used to map the translocation breakpoints on chromosomes 11 and X in individual 1371. The breakpoint-spanning BAC clones RP11-311A17 (Xp22.2; left panel) and RP11-618K13 (11p11.2; right panel) show signals on both the derivative 11 and derivative X. The schematic diagram demonstrates the position of the BAC clones and the genes involved, to scale. Breakpoint-spanning BACs are coloured in red, with the approximate position of the breakpoints shown by orange bars, as determined by long-range PCR. Genes transcribed on the forward strand are in blue and those transcribed on the reverse strand are in green. Genomic coordinates are shown on the x-axis and are based on the Human Genome Assembly hg18.

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only 9/42 probands: four encompassing PAX6 itself and five removing 3’ (telomeric) cis-regulatory elements that are essential for PAX6 function. A wealth of evidence exists from animal models [49–52] and human translocation breakpoint mapping [53,54] showing that genomic elements located in a region ~120kb 3’ to the transcription unit are essential for the transcriptional activation of PAX6. For chromosomal deletions the most convincing evidence is from somatic cell hybrid analysis of two deletions that were shown to abolish PAX6 transcription [55]. The deletions studied in this somatic cell hybrid analysis both overlap with the 3’ deletions identified here (Fig 2) and by combining our data with the published data we suggest a new 244 kb ‘critical region’ which contains essential cis-regulatory elements (Fig 2). The patient cohort in the present study is part of a larger cohort of iris developmental anomalies patients in which one individual with aniridia was recently found to have a plausibly causative de novo single nucleotide variant (SNV) in a conserved non-coding element within the ‘critical region’ [56]. While it is possible that similar mutations may exist in other cis-regulatory elements, it is significant that most of the individuals in the present study were included in the cohort of 60 individuals screened for PAX6 regulatory mutations by Bhatia et al. [56] and no further mutations were identified in the regions analyzed.

Four individuals had deletions or intragenic mutations which are likely to result in FOXC1 haploinsufficiency. One individual had a large deletion upstream of PITX2 that plausibly impairs developmental expression of this gene by removing known enhancer elements. Deletions of FOXC1 were previously shown to account for a considerable proportion of individuals with anterior segment dysgenesis, who also presented with extraocular features such as hearing defects and mental retardation [57]. FOXC1 and PITX2 encode transcriptional regulators that physically interact with each other and are co-expressed in a number of tissues during development including the periacular mesenchyme [58]. Mutations in these genes have most commonly been associated with Axenfeld-Rieger syndrome [59], but aniridia has been reported for both [8,9,60]. Of note, 3 of the 4 individuals reported here with FOXC1 haploinsufficiency, and the individual with the PITX2 cis-regulatory mutation have congenital glaucoma associated with their aniridia phenotype. However, none of the nine individuals with PAX6 mutations had congenital glaucoma. Digenic inheritance of FOXC1 and PITX2 mutations was reported in a severely affected individual in a family with several affected members presenting with variable ocular phenotypes associated with Axenfeld-Rieger syndrome [13]. The presence of both FOXC1 and PITX2 mutations impaired the transactivation activity of these proteins in vitro significantly more than when only one mutation was present. The cellular and developmental interactions between PAX6, FOXC1 and PITX2, and physical co-binding at regulatory elements in the developing iris are as yet poorly understood. This is presumably due to the difficulty in obtaining sufficient tissue, although the available human genetic data suggest that this would be an informative area of study.

We assessed the occurrence of particular descriptive phenotype terms (partial/variant aniridia, corneal anomalies, cataracts, glaucoma, microphthalmia/coloboma and extraocular features) in cases with and without a molecular diagnosis (S3 Table). The results showed an over-representation of individuals with partial/variant aniridia in whom no genetic defect was detected (approximately 60%) when compared to those with the same descriptive term but no genetic diagnosis (approximately 26%). This finding can be explained by the presence of 8/11 Gillespie syndrome patients in whom a genetic mutation is yet to be identified. The glaucoma feature appeared to be present in 26% of individuals with a molecular diagnosis (particularly in FOXC1 and PITX2 mutation-positive patients) compared to 7% of those without a diagnosis.

Finally, we report a more complex mutation associated with the breakpoints of a balanced X:autosome translocation in a single individual. On chromosome 11 the breakpoint disrupts PHF21A, which encodes a plant-homeodomain zinc finger protein and is highly expressed in...
brain tissue including the cerebellum [61]. The PHF12 protein is a component of the BRAF-histone deacetylase co-repressor complex, which mediates transcriptional repression of neuron-specific genes in non-neuronal cells [62]. Multiple translocation breakpoints disrupting PHF21A have been reported as causing intellectual disability [63] and alteration of PHF12 expression in the cerebellum might contribute to the ataxia seen in this case but we were unable to find any evidence that PHF21A could be causing the iris malformation. The breakpoint on the X chromosome disrupts ARHGAP6, which is highly expressed in kidney, heart, skeletal muscle, retina and fetal brain. ARHGAP6 encodes a guanine nucleotide exchange factor that activates Rho-GTPase to regulate signaling interactions within the actin cytoskeleton [64,65]. However, there is no human genetics evidence as yet that mutations in this gene are associated with any developmental disorder. We were also unable to find mutations in the neighbouring gene, HCCS, in the other Gillespie syndrome cases in our cohort. HCCS has been associated with syndromic microphthalmia [66]. It seems reasonable to consider individual 1371 as having a composite phenotype with PHF21A-disrupting breakpoint exacerbating the neurodevelopmental problems but the Gillespie syndrome being, as yet, unexplained.

Perhaps the most significant finding in this study is that we were unable to identify mutations in 27/42 individuals with aniridia and no detectable intragenic mutations in PAX6. Although there could be unidentified mechanisms for disrupting PAX6 function, our results also suggests that there may be as yet undiscovered genetic loci responsible for a considerable proportion of aniridia. Whole genome sequence analysis would be an attractive technique for the identification of novel causative mutations in the PAX6 region, and others involving new loci in PAX6-negative individuals with syndromic or isolated aniridia. The high frequency of cis-regulatory mutations that we have identified in this cohort highlight the importance of surveying the whole genome. This study has also confirmed that the majority of cases with Gillespie syndrome are not associated with detectable mutations at the PAX6 locus.

Supporting Information

S1 Fig. Array CGH data for PAX6 deletion individuals.
(DOCX)

S2 Fig. Array CGH data for PAX6 telomeric deletion individuals.
(DOCX)

S1 Table. Details of the clinical diagnoses of all the patients used in this study, including the genetic pathology (where applicable).
(DOCX)

S2 Table. Primer sequences and PCR conditions used for amplification and sequencing of the PAX6, FOXC1, PITX2, PHF21A and ARHGAP6
(DOCX)

S3 Table. Occurrence of particular descriptive phenotypes in cases with and without a molecular diagnosis.
(DOCX)

Author Contributions

Conceived and designed the experiments: DRF VvH. Performed the experiments: MA JR IMH KAW F. Sharkey LH JF. Analyzed the data: MA JR IMH KAW F. Sharkey LH JF. Contributed reagents/materials/analysis tools: AS JCS HD PB FM BF BL DST F. Stewart CEW MME PTK CC LVM DW RNE EIT EDS MMM DRG BWF DW JK ADM CG CY ATM IRE LI ML PLB
Reference


