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ARTICLE

PITX2 and *FOXC1* spectrum of mutations in ocular syndromes

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Anterior segment dysgenesis (ASD) encompasses a broad spectrum of developmental conditions affecting anterior ocular structures and associated with an increased risk for glaucoma. Various systemic anomalies are often observed in ASD conditions such as Axenfeld-Rieger syndrome (ARS) and De Hauwere syndrome. We report DNA sequencing and copy number analysis of *PITX2* and *FOXC1* in 76 patients with syndromic or isolated ASD and related conditions. *PITX2* mutations and deletions were found in 24 patients with dental and/or umbilical anomalies seen in all. Seven *PITX2*-mutant alleles were novel including c.708_730del, the most C-terminal mutation reported to date. A second case of deletion of the distant upstream but not coding region of *PITX2* was identified, highlighting the importance of this recently discovered mechanism for ARS. *FOXC1* deletions were observed in four cases, three of which demonstrated hearing and/or heart defects, including a patient with De Hauwere syndrome; no nucleotide mutations in *FOXC1* were identified. Review of the literature identified several other patients with 6p25 deletions and features of De Hauwere syndrome. The 1.3-Mb deletion of 6p25 presented here defines the critical region for this phenotype and includes the *FOXC1*, *FOXF2,* and *FOXQ1* genes. In summary, *PITX2* or *FOXC1* disruptions explained 63% of ARS and 6% of other ASD in our cohort; all affected patients demonstrated additional systemic defects with *PITX2* mutations showing a strong association with dental and/or umbilical anomalies and FOXC1 with heart and hearing defects. *FOXC1* deletion was also found to be associated with De Hauwere syndrome.
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INTRODUCTION

Anterior segment dysgenesis (ASD) disorders encompass a broad spectrum of conditions associated with abnormal development of structures located in the anterior segment of the eye and an increased risk for glaucoma.[1,2] One common form of ASD is Axenfeld-Rieger syndrome (ARS), characterized by specific ocular anomalies with or without systemic abnormalities. Ocular findings include posterior embryotoxon, iris malformation, corectopia/polycoria, irido-corneal adhesions, and ~50% risk for glaucoma.[3,4] Common systemic abnormalities include craniofacial dysmorphism with maxillary hypoplasia, hypodontia, and umbilical anomalies; hearing loss, heart anomalies, developmental delay, and other variable features have also been reported.[3,5]

The homeodomain-containing transcription factor *PITX2* located at 4q25 was the first ARS gene to be identified.[6] A second gene, the forkhead transcription factor *FOXC1* located at 6p25, has also been linked to ARS.[7,8] Mutations in these two genes, *PITX2* and *FOXC1*, are estimated to explain ~40% of ARS.[5,9,10] In general, mutations in the *PITX2* gene appear more likely to be associated with ocular, dental, and umbilical anomalies, whereas mutations in *FOXC1* appear to be associated with isolated ocular or ocular, heart, and/or hearing defects.[5,10,11] The phenotype associated with mutations in both of these genes is variable; even within a single family there is often variation in the specific combination of features that are seen.[5,10,11]

The human *PITX2* mutations identified to date cluster in the homeodomain and C-terminal region,[5,9,10] and mainly result in a complete or partial loss of function, with mutant proteins that retain some wild-type activity producing milder phenotypes.[12–14] Dominant-negative and gain-of-function mutations have also been reported but represent an apparent minority.[15–17] Other types of *PITX2* mutations include deletions of coding exons and chromosomal translocations.[5,6,18] A novel mechanism, deletion of an upstream regulatory region of *PITX2*, was recently reported in one patient with ARS.[19]

Human *FOXC1* mutations associated with ARS include missense mutations in the forkhead domain, nonsense and frameshift mutations throughout the gene, and whole gene deletions.[5,10] As with *PITX2*, these mutations primarily result in complete or partial loss of function, particularly affecting the protein's transactivational function.[5] Duplications of *FOXC1* have also been reported in various types of anterior segment disorders.[5,11,20]

Another condition associated with ARS is De Hauwere syndrome, characterized by anterior chamber eye defects, hypertelorism, psychomotor retardation, hypotonia, hearing loss, femoral head anomalies, and hydrocephalus/enlarged ventricles.[21,22] The first familial occurrence

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was described by De Hauwere *et al*[21] in 1973. Previous screening of the *PITX2*-, *FOXC1*-, and *BARX1*-coding regions in a patient with De Hauwere syndrome did not identify any mutations.[22]

In this manuscript, we present *PITX2* and *FOXC1* DNA sequencing and copy number analysis of 71 patients affected with ASD (including 38 with ARS) with or without systemic defects, 4 patients with related conditions, and 1 patient with De Hauwere syndrome.

METHODS

Human subjects

This human study was approved by the Institutional Review Boards of the Children's Hospital of Wisconsin and the University of Iowa. Signed informed consent was provided by all participants and/or their legal guardians, as appropriate. We present DNA sequencing and copy number analysis of *PITX2* and *FOXC1* in 38 probands with ARS and 33 with other ASD (primarily Peters anomaly), with or without non-ocular defects, 4 probands with overlapping non-ocular features but other eye phenotypes, and 1 proband with De Hauwere syndrome whose clinical diagnosis, features, and photographs were presented previously;[22] 19 of the probands had an affected family member (16 ARS, 2 other ASD, and 1 other).

Gene sequencing

The *PITX2* and *FOXC1* genes were analyzed by direct DNA sequencing of PCR products encompassing all coding exons and exon/intron junctions. For *PITX2*, sequence was obtained for *PITX2A*, *PITX2B*, and *PITX2C* isoforms, using the previously described primers and conditions;[23] for *FOXC1*, *FOXC1ii*, *FOXC1iii*, and *FOXC1iv* primers and conditions described by Kaur *et al*[24] were utilized in combination with the following new primer set to include the 5′ region of the gene: *FOXC1*-5′F, GAGACCGAGAAAAGGTGACG, and *FOXC1*-5′R, AAGCGGTCCATGATGAACTGG. The PCR product of 814 bp was amplified using the following conditions: initial denaturation at 98° for 5 min followed by 35 cycles of 98 (1 min), 61 (1 min), and 72°C (1 min 30 s) and then 7-min final extension at 72°C. All DNA sequences were analyzed manually and using Mutation Surveyor (SoftGenetics, State College, PA, USA). All mutations were confirmed in an independent sequencing reaction. Mutations were named according to the most commonly used reference sequences, NM_153427.1 (*PITX2A*) and NM_001453.2 (*FOXC1*). In addition, 180 Caucasian controls were screened for variation in *PITX2*, and 76 Caucasian and 74 African American controls were screened for variation in *FOXC1*; identified variations were also compared with the ~5000 exomes included in the Exome Variant Server (http://evs.gs.washington.edu/EVS/).[25]

TaqMan assays and Affymetrix copy number analyses

Patients were screened as previously described using TaqMan assays (Applied Biosystems/Life Technologies, Carlsbad, CA, USA) for the *PITX2* and *FOXC1* regions and/or Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA, USA);[19,26] clinical Agilent 105K oligonucleotide array (Agilent Technologies, Santa Clara, CA, USA) and Affymetrix 6.0 array data were used for one patient each (cases 21 and 27, respectively). The following *PITX2* probes were used for TaqMan assays: Hs00452261_cn (P1, located in the last exon of *PITX2*), Hs00958157_cn (P2, *PITX2C* promoter), Hs01402614_cn (P3, most 5' *PITX2* exon, exon 1A), Hs06705585_cn (P3B, located at ~50 kb upstream of *PITX2*), Hs04822300_cn (P4, located 110 366 kb 5' of *PITX2*), Hs04838001_cn (P5, located 284 481 kb 5' of *PITX2*), and Hs04811562_cn (P6, located 649 476 upstream of *PITX2*). The following 6p25 region TaqMan probes were used: Hs03037749_cn, located in the middle of *FOXC1* (single exon) and Hs00919636_cn, targeting exon 2 of *GMDS*.

RESULTS

A total of 28 mutations in *PITX2* or *FOXC1* were identified in probands, explaining 37% (26/71) of all ASD (including ARS) and 63% (24/38) of ARS specifically; the final two mutations were found in one patient without ASD (1/4; 25%) and the patient with De Hauwere syndrome. Among these mutations, 18 are nucleotide changes in exons (13) or introns (5), 9 are deletions of the coding region, and 1 is a deletion of the distant upstream region. The race/ethnicity of the probands included 7 Caucasian, 2 Hispanic, 2 of mixed race, 1 other, and 16 unknown/unreported. None of the identified mutations were seen in the controls screened here or in the ~5000 exomes included in the Exome Variant Server.[25] Mutations identified in this study were submitted to Leiden Open Variation Database (https://grenada.lumc.nl/LOVD2/mendelian_genes/home.php).

PITX2 mutations: phenotypes and genotypes

PITX2 mutations and deletions were identified in 24 unrelated cases (Table 1, Figure 1), all of whom demonstrated dental and/or umbilical anomalies. In addition, several previously reported polymorphisms were observed in both patient and control populations. Mutations/deletions were identified in 18/18 (100%) probands with ARS and dental and umbilical anomalies, 3/9 (33%) with ARS and either dental or umbilical anomalies, 2/7 (29%) with other ASD and either dental or umbilical anomalies, and 1/4 (25%) with umbilical and/or dental anomalies without ASD (Table 1). Overall, *PITX2* disruption was identified in 21/38 (55%) of all ARS and 21/27 (78%) of ARS with dental and/or umbilical defects.

Other less frequently observed systemic defects in probands with *PITX2* abnormalities included developmental delay (5/24; 21%), growth disorder (short stature or failure to thrive) (3/24; 13%), Meckel's diverticulum (3/24; 13%), anal anomalies (2/24; 8%), heart defects (2/24; 8%), hearing loss (1/24; 4%), omphalocele (1/24; 4%), and hypospadias (1/24; 4%). Notable ocular observations were congenital glaucoma in two individuals (2/24; 8%; in contrast to the later onset developmental glaucoma usually described) and the lack of characteristic ARS ocular features in three patients (3/24; 13%). Our overall frequency of glaucoma (7/24; 29%) was lower than expected and likely reflects the young age of many participants; among patients >20-years-old, 5/9 (56%) had glaucoma. Finally, a natal tooth was reported in one patient (case 21), which is the first observation of this feature in conjunction with ARS/*PITX2* mutation to our knowledge.

A total of 14 different nucleotide mutations were observed (3 mutations were seen in more than one case) (Table 1). Seven of these mutations (seen in nine cases) are novel and are consistent with previously reported mechanisms. The remaining nine cases represent new occurrences of previously reported changes.[5]

The previously reported c.253-11A>G mutation represented the most frequent mutation in our sample, and was associated with variation in phenotype in the three affected families (cases 7–9; Table 1). Case 7 is affected with ARS, dental, and umbilical anomalies. Case 8 has dental and umbilical anomalies along with strabismus and amblyopia, but no structural eye anomalies. Case 9 has Peters anomaly with umbilical anomalies, but no dental defects.

A novel intronic mutation c.253-1G>A, was identified in two independent families with ocular, dental, and umbilical anomalies (cases 10 and 11), and is predicted to disrupt the 3′ splice site. This is expected to lead to cryptic splice site activation or exon skipping, and to result in the production of an aberrant protein product or decreased protein production. Analysis by the CRYP-SKIP program[27] (http://cryp-skip.img.cas.cz/) identifies three 3′ splice sites with a predicted strength of ≥0.5 (out of 1.0). Utilization of any one of these three sites would be predicted to cause deletion of 233, 430, or 866 nucleotides, respectively, all resulting in frameshift and truncation of the *PITX2* protein.

Another novel mutation, c.708_730del (p.S237Afs*48), seen in case 18 with ocular and umbilical defects but unknown dental status (<1

Table 1 Summary of *PITX2* mutations and phenotypes identified in this study

Case	Mutation[a]/ deletion	Predicted effect	Eye	Dental	Umbilicus	Heart	Hearing	Other	Age (years)	Family hx
Intragenic PITX2 mutations										
1	**c.134dupA**	**p.H45Qfs*154**	ARS, microcornea	MD, DA	RU, UH	–	–	Ankyloglossia	1.5	Yes
2A	**c.143_144delGC**	**p.S48Tfs*150**	ARS	HD	RU	–		–	30	Yes
2B	**c.143_144delGC**	**p.S48Tfs*150**	ARS	HD	RU	–	–	DD, hypertelorism, prominent forehead	2..5	Child of 2A
2C	**c.143_144delGC**	**p.S48Tfs*150**	ARS	Unk	Omphalocele	–	Hearing loss	–	<1	Child of 2A
3	c.185G>A	p.R62H	ARS	HD	UH	–	–	–	40	Yes
4A	**c.225G>A**	**p.W75***	ARS, GL, vascular loops	HD	RU	–	–	Hypospadias	37	Yes
4B	**c.225G>A**	**p.W75***	ARS	Unk	RU, UH	–	–	MH, small mouth, hair whorl ankyloglossia, sacral dimple,	<1	Child of 4A
5	**c.225G>A**	**p.W75***	ARS, aniso-metropia	HD, MD	RU	–	–	MH, Imperforate anus, thin upper lip	13	Yes
6	p.247G>T	p.V83F	ARS	HD	RU	–	–	Mild DD, GD, abnormal head size	3	No
7	c.253-11A>G	Truncated HD due to splicing defect[b]	ARS	HD, MD	RU	–	–	MH, small mouth, high arched palate, hyperextensible joints; clinodactyly; narrow nails, lopped ears, prominent occiput	9	No
8	c.253-11A>G	Truncated HD due to splicing defect[b]	Strabismus, amblyopia	HD	RU	Anomalous pulmonary venous return	–	DD, GD	10	No
9	c.253-11A>G	Truncated HD due to splicing defect[b]	PA, nystagmus, esotropia	–	UH	–	–	GD, nasal bridge anomaly, high arched palate, prognathia, smooth philtrum, long thin face, posteriorly rotated ears, microcephaly	14	No
10	**c.253-1G>A**	**Truncated HD due to splicing defect**	ARS	HD, delayed eruption	UH	–	–	Hypertelorism, Meckel's diverticulum	2	No
11	**c.253-1G>A**	**Truncated HD due to splicing defect**	ARS, myopia	HD, MD	RU	–	–	MH, microcephaly, thin upper lip, short philtrum, mild DD, asymmetric ocular position	16	No
12	**c.257G>C**	**p.W86S**	ARS	DA	UA	–	–	–	43	Yes
13	c.258G>T	p.W86C	ARS, microcornea	HD, MD	RU	–	–	MH, prognathia, Meckel's diverticulum	3	No
14	c.269G>C	p.R90P	ARS, GL	HD	UH	–	–	Prognathia, Meckel's diverticulum, broad forehead	32	No
15	**c.289_290delAG**	**p.R97Gfs*101**	PA	Unk	RU	–	–	–	<1	No
16	c.366delC	p.D122Efs*33	ARS, GL	HD, persistent primary teeth	–	–	–	MH	29	Yes
17	c.398G>A	p.W133*	ARS, CA, CGL, PA nystagmus	HD	RU	–	–	Inguinal hernia	1.5	Yes
18	**c.708_730del**	**p.S237Afs*48**	ARS	Unk	RU, UH	–	–	Small mouth, nasal bridge anomaly, anteriorly placed anus	<1	No
PITX2 deletions										
19	4q25-q26 deletion (6.4 Mb)	Deletion of ***PITX2*** *ENPEP, C4orf32, C4orf16, TIFA, ALPK1, NEUROG2, LOC91431, C4orf21, LARP7,*	ARS, GL	HD, MD	RU	–	–	MH, nasal bridge anomaly, low thyroid	21	No

Table 1 (Continued)

Case	Mutation[a]/ deletion	Predicted effect	Eye	Dental	Umbilicus	Heart	Hearing	Other	Age (years)	Family hx
		ANK2, CAMK2D, ARSJ, UGT8, AND NDST4								
20	4q25 deletion (1.1 Mb)	Deletion of ***PITX2*** *EGF, ELOVL6, ENPEP*	ARS, myopia (−12; −8), missing eye muscles (L), astigmatism	HD, persistent primary teeth	RU, UH	–	–	Micrognathia, abnormal ocular position, inguinal hernia, ulcerative colitis, diverticulitis, attention deficit hyperactivity disorder, micropenis, sleep disorder, transient ischemic attack, hypercholesterolinemia	54	Uncertain
21	4q25-q28.2 deletion (19.2 Mb)	Deletion of ***PITX2*** and 65 genes from *ELOVL6* to *SCLT1*	ARS, CGL, CA, severe IH/aniridia,	Natal tooth	RU	Ventricular septal defect	Hearing loss	Mild DD, MH, thin upper lip, high arched palate, low-set ears	<1	No
22	Gene deletion (extent ND)	Deletion of ***PITX2*** Other genes ND	ARS	HD, MD	Omphalocele	–	–	MH, Ridged palate	3	No
23	Gene deletion (extent ND)	Deletion of ***PITX2*** Other genes ND	ARS, GL, retinal detachment	DA	–	–	–	–	65	Yes
24	Distant upstream region deletion (extent ND)	Deletion of regulatory region of ***PITX2*** Other genes ND	ARS, blue sclerae	MD	UH, RU	–	–	Low hair line, DD, autism, digit anomaly, thin upper lip	2	No

Abbreviations: ARS, Axenfeld-Rieger syndrome (any combination of posterior embryotoxon, irido-corneal adhesions, iris hypoplasia, and pupillary anomalies); CA, cataract; CGL, congenital glaucoma; DA, dental anomaly; DD, developmental delay; GD, growth disorder (short stature, failure to thrive); GL, glaucoma; HD, hypodontia; IH, iris hypoplasia; MD, microdontia; MH, maxillary hypoplasia; PA, Peters anomaly; RU, redundant umbilical skin; UA, umbilical anomaly; UH, umbilical hernia; Unk, unknown.
[a]Numbering is relative to reference sequence NM_153427.1, where +1 is the A of the ATG initiation codon; novel mutations are shown in bold.
[b]Confirmed by analysis presented by Maciolek *et al*.[14]

year of age), is the most 3′ mutation reported to date (case 18). This mutation causes frameshift/truncation and was not seen in the180 controls screened here or in the ~5000 exomes included in the Exome Variant Server.[25] As this mutation is located in the final exon of the gene, it is not expected to be subject to nonsense-mediated decay,[28] suggesting that disruption of the 14-amino-acid motif is sufficient to impair gene function.

Genomic analysis identified deletions involving the *PITX2* coding region in five patients and a deletion involving the distant upstream region of *PITX2* in one patient, which was located at least 50 kb upstream of *PITX2* and did not affect any *PITX2* exons (Table 1, Figure 2). The extent of the deletion was characterized by array data in three cases (cases 19–21) and ranged in size from at least 1.1 Mb to 19.2 Mb, deleting between 4 and 65 genes. Three cases (cases 22–24) were analyzed by *PITX2*-specific TaqMan assays only because of insufficient amounts of available DNA, and therefore the exact size of the deletion could not be determined.

Family members were available for testing for 13 cases. Unaffected family members were available for cases 2, 4, 6, 7, 9, 10, 11, 15, 18, 21, 22, 23, and 24; none of these family members carried the mutation/deletion identified in the proband. The mother of case 24 (with upstream deletion) has isolated posterior embryotoxon, but she did not show evidence of the deletion seen in her son. Affected family members were available for two families (cases 2B, 2C, and 4B), all of whom carried the same mutation seen in the proband (Table 1).

FOXC1 mutations: phenotypes and genotypes

No intragenic mutations in the *FOXC1* gene were identified. Several previously reported polymorphisms were observed in both patient and control populations. Genomic analysis identified deletions involving the *FOXC1*-coding region in three probands with ARS (cases 25–27) as well as the case of De Hauwere syndrome (case 28)[22] (Table 2, Figure 3). Deletion size ranged from at least 0.98 Mb to at least 1.5 Mb, deleting between 2 and 11 genes. For the three probands with ARS (cases 25–27), the deletion included both *FOXC1* and *GMDS* (cases 25–27) with nine additional 6p25.2 genes deleted in one case (case 27). The telomeric deletion break point was positioned 18–40 kb from *FOXC1*. Therefore *FOXF2*, the next gene telomeric to *FOXC1*, was not included in any of these deletions with a distance of 175–197 kb between the deletion start and the *FOXF2* gene. For the patient with De Hauwere syndrome (case 28), the telomeric deletion break point was localized 717 kb from *FOXC1*, and therefore included both the *FOXF2* and *FOXQ1* genes in addition to *FOXC1* and *GMDS* (Figure 3). This patient was also found to carry a 0.52-Mb duplication of 6p21.33–p21.33 including *HLA-A, HCG9, ZNRD1, PPP1R11, RNF39, TRIM31, TRIM40, TRIM10, TRIM15, TRIM26, FLJ45422, TRIM39*, and *RPP21*. Three of the four probands with *FOXC1* deletion (cases 25, 26A, and 28) demonstrated heart or hearing defects, and three (cases 25, 26A, and 27) had glaucoma (congenital/juvenile onset). All had normal dentition and umbilicus; one family member demonstrated mildly redundant periumbilical skin (case 26B). Family members were available for testing in one case; TaqMan assay in case 26B (the affected child of case 26A) identified deletion of *FOXC1*, as seen in his mother; Affymetrix array data was not available to determine the extent of the deletion in the child.

DISCUSSION

PITX2 or *FOXC1* disruption was found to explain 63% (24/38) of ARS specifically but only 6% (2/33) of other ASD (non-ARS), yielding 37% (26/71) overall mutation frequency in our ASD cohort.

Consistent with other reports, our data provide strong evidence that *PITX2* disruption is associated with ARS with dental and/or umbilical anomalies whereas *FOXC1* alterations are primarily seen in ARS with isolated ocular or ARS with heart and/or hearing defects.[5,11] This suggests that despite the significant overlap in ocular phenotypes, non-ocular features may provide distinction between the syndromes associated with these two genes. In addition, 6p25 deletion was identified in a patient with De Hauwere syndrome and a minimal ~1.3 Mb deletion region was shown to be strongly associated with this phenotype.

PITX2 mutations alone were found to explain 55% of ARS specifically (78% of ARS with dental and/or umbilical defects) and 6% of other ASD (non-ARS), yielding 33% overall *PITX2* mutation frequency in our ASD population. Two previous studies that screened for both nucleotide mutations and genomic deletions of *PITX2* reported a lower proportion of cases explained by disruption of

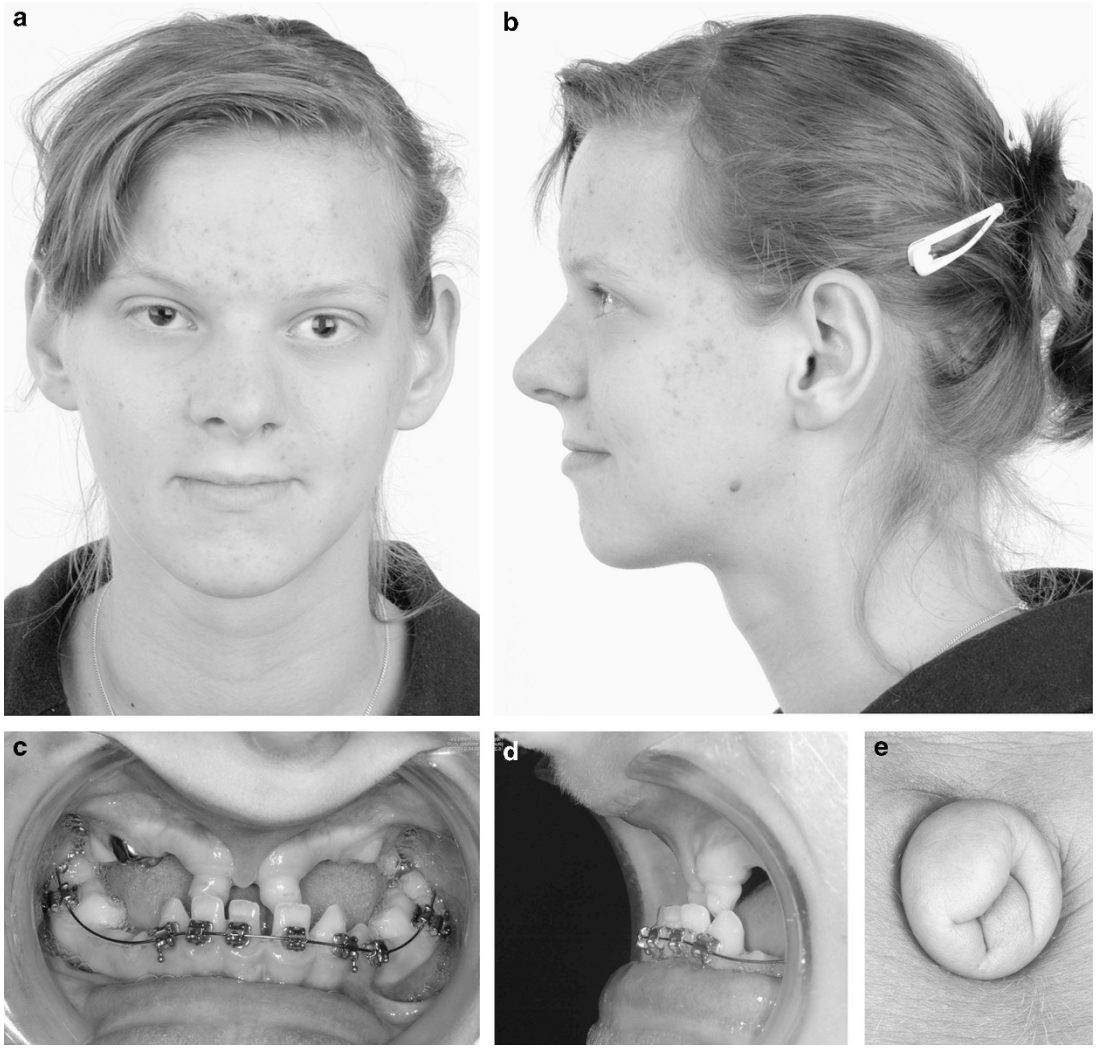

Figure 1 Photograph of a patient (case 11) with ARS, demonstrating the characteristic facial features (**a**, **b**), dental defects (**c, d**), and umbilical anomaly (**e**). The color reproduction of this figure is available at the *European Journal of Human Genetics* online.

Figure 2 Summary of *PITX2* mutations identified in this study. (**a**) Schematic drawing of *PITX2* gene and upstream region. Identified point mutations are shown with red numbered arrows (1–18) whereas identified deletions are shown as red lines (19–24); the solid line shows the minimum deletion size, with the dashed line representing the maximum extent of the deletions. *PITX2* exons are shown as numbered boxes, regions corresponding to the homeobox and 14-amino-acid-conserved domains are indicated as black and light gray boxes, respectively. Initiation codons corresponding to the *PITX2* isoforms are shown with black arrows. The stop codon is indicated with a black arrow and asterisk. The positions of the TaqMan probes are shown and labeled P1–P6; for upstream probes P3B and P4–P6, the distance (in base pairs) from the beginning of the gene is indicated in parenthesis. The positions of probes that demonstrated haploid state are shown as black diamonds, whereas probes that showed diploid state are shown as gray diamonds. (**b**) DNA sequencing chromatograms for normal and mutants alleles; mutation position is indicated with an arrow. (**c**) Copy number analysis (TaqMan assay and Affymetrix array) data. For *PITX2* TaqMan assays, probes P1–P3 correspond to *PITX2* exons and probes P3B and P4–P6 correspond to the upstream region, patient sample values were compared with control (C) results for every assay. Probes that showed half of the normal value are noted in red. For Affymetrix array analysis, Genotyping Console representation of chromosome 4 marker data is shown with heterozygous deletions indicated with arrows. The color reproduction of this figure is available at the *European Journal of Human Genetics* online.

PITX2: 4.7%[18] and 16%[10] of ASD (neither study provided the proportion of ARS specifically). The variation is most likely due to differences in sample composition as our cohort is enriched with ARS cases with additional systemic defects, particularly dental and umbilical anomalies that appear to be strongly associated with *PITX2* mutations. In the study reported by Dr D'haene *et al*,[10] 11 out of 13 families with *PITX2* mutations had both dental and umbilical defects (six with ARS, three with other ASD, and two with no data on ocular features), one family demonstrated ARS and dental anomalies whereas the umbilicus was not examined and no records were available for the last *PITX2*-positive family; heart and hearing defects were each seen in one family with *PITX2* mutation.[10]

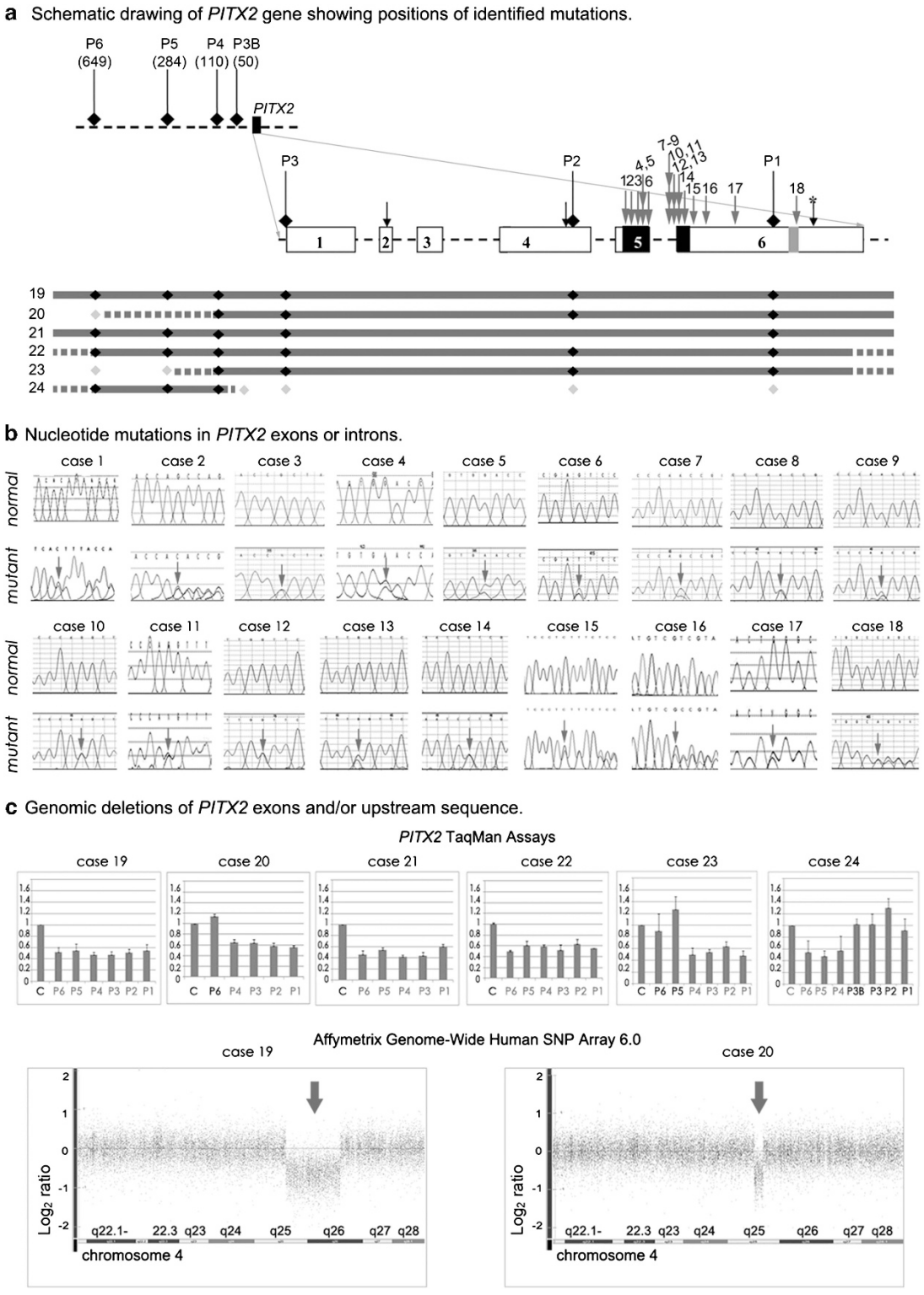

Table 2 Summary of *FOXC1* deletions and phenotypes identified in this study

Case	Mutation/deletion	Predicted effect	Eye	Dental	Umbilicus	Heart	Hearing	Other	Age (years)	Family hx
Intragenic FOXC1 mutations										
None identified										
FOXC1 deletions										
25	6p25.3–p25.2 deletion (0.98 Mb)	Deletion of *FOXC1 GMDS*	ARS, CGL, entropion with trichiasis	–	–	Atrial septal defect	Hearing loss	Large, low-set ears	18 months	No
26A	6p25 deletion (1.10 Mb)	Deletion of *FOXC1 GMDS, LOC340156 (partial)*	ARS, GL	–	–	Heart murmur	Hearing loss	Small mouth	30	Yes
26B	6p25 deletion (extent ND)	Deletion of *FOXC1* Other genes ND	ARS, CGL	Unk	Mild RU	– (echo WNL)	–		<1	Child of 26A
27	6p25.3–p25.2 deletion (1.5 Mb)	Deletion of *FOXC1 GMDS, C6orf195, MYLK4, WRNIp1, SERPINB1, MIR4645, MGC39372, SERPINB9, SERPINB6, NQO2*	ARS, CGL, severe IH/ aniridia,	Unk	–	–	–	Maxillary hypoplasia, broad nasal bridge	<1	Yes
28	6p25.3–p25.3 deletion (1.3 Mb) 6p21.33–p21.33 duplication (0.52 Mb)	Deletion of *FOXC1 FOXQ1, FOXF2, GMDS (partial)* Duplication of *HLA-A, HCG9, ZNRD1, PPP1R11, RNF39, TRIM31, TRIM40, TRIM10, TRIM15, TRIM26, FLJ45422, TRIM39, RPP21*	ARS, CA	–	–	–	hearing loss	De Hauwere syndrome: dislocated hips, flattened femoral heads, dural ectasia/meningoceles, short stature, hydrocephalus, joint laxity/pain, skeletal anomalies, hypertelorism hyperthyroidism[a]	49	No

Abbreviations: ARS, Axenfeld-Rieger syndrome (any combination of posterior embryotoxon, irido-corneal adhesions, iris hypoplasia, and pupillary anomalies); CA, cataract; CGL, congenital glaucoma; GL, glaucoma; IH, iris hypoplasia; RU, redundant umbilical skin; Unk, unknown; –, no anomaly noted.
[a]Full phenotypic information can be found in Lowry *et al*[22] or in Table 3.

Another interesting phenotypic observation is that three patients with intragenic *PITX2* mutations in our study were found to display a growth disorder (13%). This finding provides further support for a potential role of *PITX2* in SHORT syndrome and similar conditions.[18,29]

Of particular note are three *PITX2* mutations reported in our study. First, the deletion we identified involving the distant upstream region (but not the coding region) of *PITX2* in one ARS patient is similar to our previously reported ARS case,[19] and thus our report highlights the importance of this recently discovered mechanism, loss of the upstream regulatory region, as a cause of ARS. It will be crucial to screen for this type of mutation in additional patients with ARS in order to determine its frequency. Disease-causing mutations outside of the coding region have been identified in other genes; for example, deletion of the downstream regulatory region of *PAX6* was found in a number of families affected with aniridia.[2,30] Disruption of the regulatory regions of genes should be considered as a mechanism for other conditions not fully explained by intragenic mutations.

Second, the novel c.708_730del (p.S237Afs*48) mutation represents the most C-terminal alteration identified to date. This mutation occurs in the middle of a 14- amino-acid-conserved domain of yet unknown function[6] providing further support for the functional significance of this motif. Finally, the c.253-11A>G mutation in *PITX2* in three of our cases appears to represent a 'hot spot' as it has now been reported in six independent families (cases 7–9, our study;[6,10,31]). Two of our three probands carrying this mutation have atypical ocular phenotypes. This mutation was studied extensively by Maciolek *et al*[14] and was shown to shift splicing exclusively to a new AG acceptor site created 11 nucleotide upstream. The resulting *PITX2* protein is truncated at 138 residues followed by 117 frame-shifted amino acids.[14] Owing to its distance from the splice site, this mutation may be easily missed in routine genetic screening.

Deletion of *FOXC1* was identified in four probands. No intragenic mutations were detected. The frequency of *FOXC1* deletion in ARS was 8% (3/38), which is lower than previously reported. D'haene *et al*[10] found that both mutations and deletions involving *FOXC1* accounted for 24% of patients with ARS or other ASD with and without non-ocular anomalies. The difference between their study and ours is most likely because of bias toward ARS with dental and umbilical defects in our cohort, and the higher proportion of *PITX2* mutations in those phenotypes. Three individuals with *FOXC1* deletion in our study demonstrated hearing loss and two had heart abnormalities; these results are consistent with previous reports of *FOXC1* mutations.[5,10] Interestingly, a higher proportion of *FOXC1* probands were affected with glaucoma (3/4; 75%) compared with *PITX2* probands (7/24; 29%). A similar increase in glaucoma was seen in a previous study with 12/16 (75%) *FOXC1* cases compared with 5/12 (42%) *PITX2* cases affected with glaucoma.[10] It is not clear

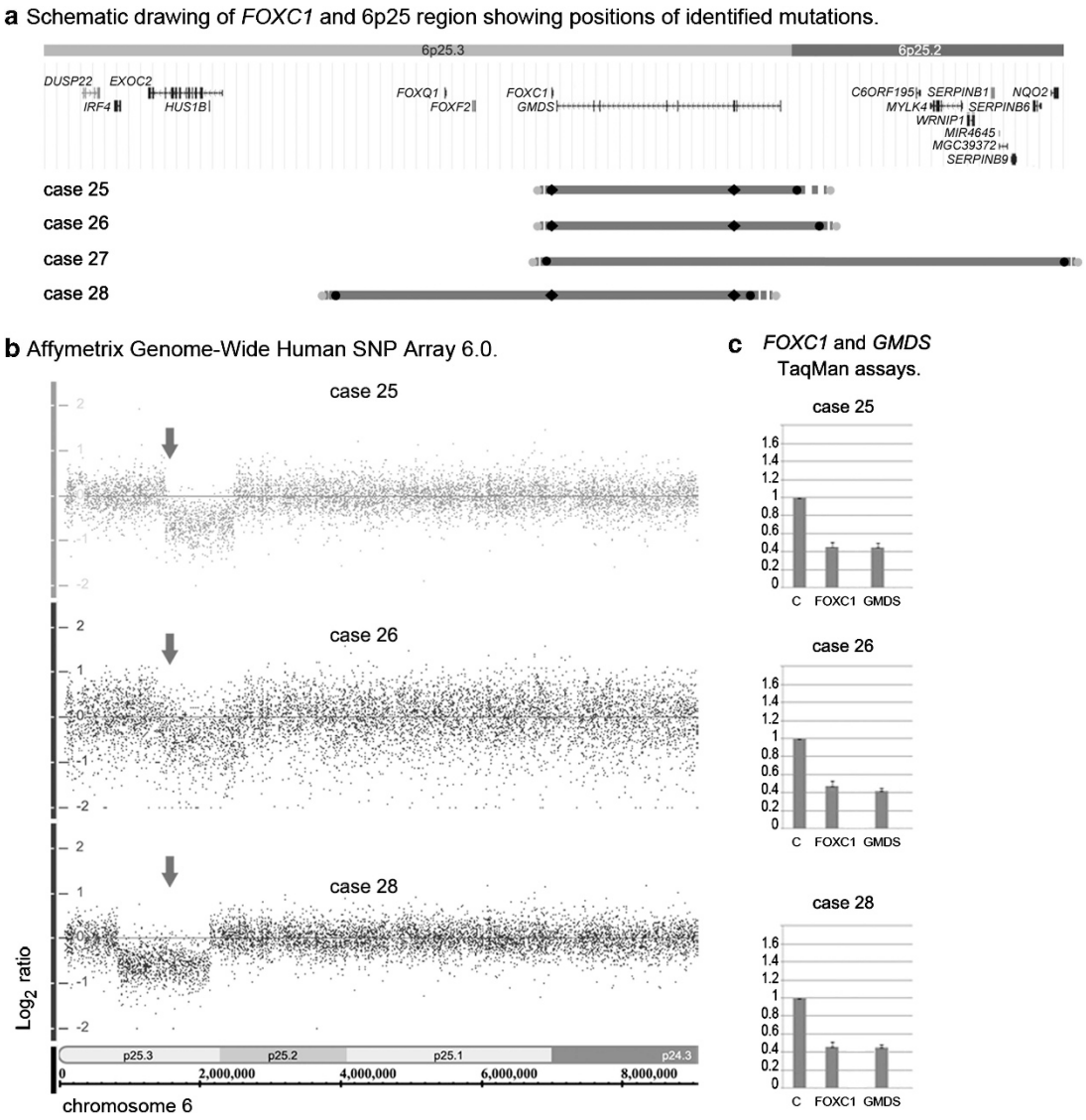

Figure 3 Summary of *FOXC1* deletions identified in this study. (**a**) Schematic drawing of the 6p25 region. All known human protein-coding and non-protein-coding genes from the NCBI RNA reference sequences collection (RefSeq) for the selected region are shown as seen in the UCSC Genome Browser (http://www.genome/ucsc.edu); GRCh37/hg19 assembly was utilized. Identified deletions are shown as red lines (25–28); the solid line shows the minimum deletion size, with the dashed line representing the maximum extent of the deletions. The positions of the TaqMan probes that demonstrated haploid state are shown as black diamonds; the positions of the Affymetrix probes that exhibited haploid state are shown as black circles and probes that displayed diploid state as gray circles. (**b**) Affymetrix array identification of *FOXC1*/6p25 deletions. Genotyping Console representation of chromosome 6 marker data for cases 25, 26 and 28 is shown with *FOXC1* position within the heterozygous deletions indicated with arrows. (**c**) Copy number analysis using TaqMan assays showing deletion of *FOXC1* and *GMDS* for cases 25, 26 and 28. Patient sample values were compared with control (C) results for every assay. The color reproduction of this figure is available at the *European Journal of Human Genetics* online.

whether *FOXC1* disruption is truly associated with an increased risk of glaucoma or perhaps an earlier onset.

The *FOXC1* deletions identified in the three ARS probands (cases 25–27) in our study include one or more genes centromeric to *FOXC1*, which is consistent with the previous reports of patients with ARS.[10,20] Of special interest is the deletion identified in the patient with De Hauwere syndrome, which is the only one of the deletions reported here that extends in the telomeric direction. De Hauwere syndrome (OMIM: 109120), characterized by anterior segment eye defects, hypertelorism, psychomotor retardation, hypotonia, hearing loss, hydrocephalus/enlarged ventricles, and femoral head anomalies,[21,22] displays a significant overlap with 6p25 deletion syndrome (OMIM: 612582), defined as a combination of ocular anomalies (primarily anterior segment), hearing loss, congenital heart disease, hydrocephalus, developmental delay, and a characteristic facial appearance and typically associated with terminal deletions of 6p25.[32,33] Review of the literature identified that the skeletal features observed in De Hauwere syndrome, such as flattening of the femoral epiphyses and other femoral head anomalies sometimes diagnosed as Perthes disease, were reported in several previously described patients with 6p25 terminal deletions (Table 3),[32,34–38] suggesting that De Hauwere syndrome may be part of the 6p25 deletion syndrome spectrum. Case 28 in our study has the smallest and first interstitial deletion reported to date in association with the features of De Hauwere syndrome, thus defining a minimal deleted region for this phenotype.

Table 3 Summary of phenotypic and molecular characteristics of patients with De Hauwere syndrome and similar phenotypes

	De Hauwere *et al*[21a]	Gould *et al*[32] (patient 3)	Mirza *et al*[34] (patient 3)	Maclean *et al*[35]	Kannu *et al*[36]	Martinez-Glez *et al*[37]	Bedoyan *et al*[38]	This study (patient 28) Lowry *et al*[22]
Estimated deletion size	NT	~12 Mb (6p24-pter)	6.57 Mb (6p25.1-pter)	up to 7 Mb (6p25.1-pter)	2.2-2.4 Mb (6p25.2/3-pter)	2.7 Mb (6p25.2-pter)	2.21 Mb (6p25.3); 240 kb terminal	1.3 Mb (6p25.3)
Type of deletion	NT	Terminal	Terminal	Terminal	Terminal	Terminal	Complex (terminal)	Interstitial
Genes included	ND	Multiple (includes *FOXQ1, FOXF2, FOXC1, GMDS)*	Multiple (includes *FOXQ1,FOXF2, FOXC1, GMDS)*	Multiple (includes *FOXQ1, FOXF2, FOXC1, GMDS)*	Multiple (includes *FOXQ1, FOXF2, FOXC1, GMDS)*	Multiple (includes *FOXQ1, FOXF2, FOXC1, GMDS)*	Multiple (includes *FOXQ1, FOXF2, FOXC1, GMDS)*	Only *FOXQ1, FOXF2, FOXC1, GMDS* (partial)
Anterior eye defect	3/3	+	+	+	+	+	−	+
Hypertelorism	3/3	+	+	+	+	+	+	+
Developmental delay	3/3 (1 mild)	NR	+	+	Mild	+	+	−
Hypotonia	2/2	−	−	+	−	NR	−	−
Hearing loss	2/2	+	+	−	+	+	+	+
Heart defect	NR	+	NR	+	Heart palpitation	NR	+	−
Hydrocephalus/ enlarged ventricles	2/2	+	NR	+	−	+	−	+
Head circumference	10th centile	NR	>95th centile	50th centile	50–75th centile	>97th centile	25–50th centile	50–75th centile
Femoral head/ epiphyseal anomalies	2/2	+	+	−	+	+	+	+
Joint dislocations/ hyperlaxity	3/3	−	−	−	−	NR	+	+
Short stature	1/2	NR	NR	−	−	−	−	+
Other skeletal defects	Delayed bone age, large sella turcica	Club feet, scoliosis, delayed bone age, humeral head anomaly	NR	Tibial epiphyseal dysplasia and bowing, calcaneovalgus deformity, vertebral and skull anomalies, delayed ossification	Thoracic scoliosis; humeral epiphyseal, sella turcica, vertebral anomalies; hallux valgus	Club foot	Multiple epiphyseal dysplasia (long bones), vertebral anomalies	Vertebral anomalies, prominent dorsal sella, thoracic kyphosis, hallux valgus

Abbreviations: ND, not determined; NR, not reported; NT, not tested.
[a]Report of two affected siblings and affected mother; clinical evaluation of some features not provided for all patients.

The four genes affected in De Hauwere syndrome (case 28, this study), *FOXQ1*, *FOXF2*, *FOXC1*, and *GMDS*, are also deleted in the six previously described patients with features of De Hauwere syndrome (Table 3). A minimal deleted region in the 6p25 deletion syndrome was previously defined as ~2.1 Mb from the telomere to *FOXC1*, thus including *FOXQ1* and *FOXF2* but not *GMDS*.[39] *GMDS* is deleted in our other ARS cases and numerous other patients with interstitial 6p25 deletions lacking skeletal/other anomalies characteristic of De Hauwere syndrome (Table 2,[10]), suggesting that deletion of *GMDS* is not critical to the De Hauwere phenotype. The remaining deleted genes associated with features of De Hauwere syndrome, *FOXC1*, *FOXF2*, and *FOXQ1*, have been shown to have a role in the skeletal or craniofacial cartilage development in animal models. Mice homozygous for a null allele of the *FOXC1* homolog *Mf1* died shortly after birth and displayed an ASD, hydrocephalus, craniofacial defects, and skeletal abnormalities, including small, malformed, or missing bones and reduced ossification centers in the appendages;[40,41] evaluation of heterozygous mice revealed variable anterior segment ocular defects and some mild delays in ossification or reduction in the ossification centers of the sternum.[40] Mice homozygous for a *Foxf2* knockout allele exhibited cleft of the secondary palate and died within 12–18 h of birth with other

defects secondary to the cleft and no other skeletal anomalies;[42] a recent study of a *Foxf2* missense mutation suggested that the *Foxf2* gene also has a role in anterior segment ocular development in mice, whereas palatal defects were not observed.[43] Although no skeletal defects were reported in *Foxq1* deficient mice,[44] one recent study detected *Foxq1* expression in the developing craniofacial cartilage in zebrafish.[45] Further studies of patients with terminal and interstitial deletions will help to define which genes are critical for different aspects of 6p25 deletion phenotypes.

In summary, mutations in *PITX2* and *FOXC1* explain a significant portion of ARS phenotypes. Non-ocular features can be efficiently utilized to guide genetic testing strategy as dental and umbilical anomalies are strongly associated with *PITX2*, whereas heart and hearing defects show association with *FOXC1*. Identification of an interstitial 6p25 deletion encompassing *FOXC1* and other forkhead genes in the 6p25 region in a patient with De Hauwere syndrome, together with the previously reported cases, provides strong evidence for involvement of FOXC1 in this condition and defines the likely critical interval.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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