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Matthew-Wood syndrome is caused by truncating mutations in the retinol binding protein receptor gene *STRA6*

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Running title: *STRA6* mutations in Matthew-Wood syndrome

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Abstract

Retinoic acid (RA) is a potent teratogen in all vertebrates when tight homeostatic controls on its endogenous dose, location or timing are perturbed during early embryogenesis. *STRA6* encodes an integral cell membrane protein that favors RA uptake from soluble retinol-binding protein; its transcription is directly regulated by RA levels. Molecular analysis of *STRA6* was undertaken in two consanguineous human fetuses we previously described with the Matthew-Wood syndrome [MIM 601186] in a context of severe microphthalmia, pulmonary agenesis, bilateral diaphragmatic eventration, duodenal stenosis, pancreatic malformations and intrauterine growth retardation. The fetuses had either a homozygous insertion/deletion in exon 2 or a homozygous insertion in exon 7, respectively, predicting a premature stop codon in *STRA6* transcripts. Five other fetuses presenting at least one of the two major signs of clinical anophthalmia or pulmonary hypoplasia with at least one of the two associated signs of diaphragmatic closure defect or cardiopathy had no *STRA6* mutations. These findings suggest a molecular basis for the prenatal manifestations of Matthew-Wood syndrome and that phenotypic overlap with other associations may be due to genetic heterogeneity of elements common to the RA and fibroblast growth factor signaling cascades.

Microphthalmia refers to a clinical spectrum that is characterized by a congenital reduction in the size of the optic globe(s), which may be reduced to a vestige only visible on histological analysis. This most severe form of microphthalmia is sometimes called secondary or clinical anophthalmia, and occurs later in development than primary anophthalmia, due to a lack of optic vesicle formation from the embryonic prosencephalon. Isolated severe microphthalmia/anophthalmia demonstrates both genetic and phenotypic heterogeneity in humans, currently implicating genes coding for transcription factors. *CHX10* mutations lead to microphthalmia, coloboma and cataracts [1, 2]; mutations in the *RAX* gene have been identified in an individual with unilateral anophthalmia and sclerocornea in the other eye [3]. *PAX6* mutations lead to diverse congenital ocular malformations, the most common of which is aniridia, but a few genotypes have been described to date that engender primary anopthalmia [4] or microphthalmia [5-7], as documented in the *PAX6* Allelic Variant Database.

Syndromic microphthalmias can be associated with craniofacial dysmorphic features, heart and vascular malformations, skeletal and limb anomalies, skin or gut defects, mental retardation and hydrocephalus or combinations thereof [MIM 164180, 206900, 206920, 248450, 300166, 301590, 309801, 600776, 605856, 607932, 610125, 610126, 601349]. Although rare, the association of severe microphthalmia and pulmonary hypoplasia [MIM 601186] is a distinct entity known as Matthew-Wood syndrome [8]. Most authors have reported further associations of MWS with cardiac and/or diaphragmatic malformations and intrauterine growth retardation (IUGR) [9-13].

We have excluded mutations in genes encoding the fibroblast growth factor *FGF10* and its receptor *FGFR2IIIb* respectively in two familial cases of MWS [14]. These proteins are essential for the development of all affected organs in MWS [15-17]. Meanwhile, *STRA6* gene

mutations were recently implicated in heterogeneous postnatal associations of clinical anophthalmia, pulmonary hypoplasia, diaphragmatic hernia, and cardiac defects [18]. A molecular analysis of the *STRA6* gene was undertaken in the two MWS families we had described as well as in five other fetuses presenting at least one of the two major signs of clinical anophthalmia or pulmonary hypoplasia and at least one of the two associated signs of diaphragmatic closure defect or cardiopathy.

In all seven fetuses examined, the presence of severe malformations was noted on ultrasound examination and after genetic counseling, pregnancies were interrupted. Clinical data are summarized in Table 1. Chromosome and molecular analyses and pathological examinations were carried out in all cases with full parental consent. Genomic DNA was extracted from frozen tissue in fetal cases and from peripheral blood samples for parents according to standard protocols.

Polymorphic markers D15S188, D15S160, D15S991, D15S114, flanking the *STRA6* gene, were chosen using the UCSC Genome Browser and examined in fetal cases 1 and 2 (Figure 1). The parents of case 1 are a consanguineous couple of Romanian origin, and the parents of case 2 are a consanguineous couple of Portuguese origin [14]. Homozygous haplotypes were demonstrated in each fetus, while the clinically unaffected parents of case 1 had a heterozygous haplotype with an allele presumably inherited from a common ancestor (DNA was unavailable from the other family members of case 2).

Primers were subsequently designed to cover the 20 exons and exon-intron junctions of the *STRA6* gene (NM_022369), including exon 1A and 1B (the first non-coding exon may be alternatively spliced), using Primer3 software [19] (Table 2). PCR reactions were treated with the ExoSAP enzyme mix as per manufacturer's instructions (GE-Amersham). Sequencing was

performed for all seven fetal DNAs using Big Dye v3.1 Terminator Cycle Sequencing Reactions on an ABI 3130 (Applied Biosystems). Both the sense and antisense strands of the PCR-amplified fragments were analyzed with Sequence Analysis software (Applied Biosystems).

Cases 1 and 2 both presented homozygous mutations in the coding sequence of *STRA6* (Figure 1). A homozygous insertion/deletion in exon 2 (c.50_52delACTinsCC) for fetus 1 causes a frameshift and the appearance of a premature stop codon (p.Asp17Ala fsX55). An older brother with isolated bilateral coloboma of the retina and iris was heterozygous for this mutation, as were the clinically unaffected parents. Case 2 presented a homozygous single base insertion in exon 7 (c.527_528insG) that also predicts a premature stop codon (p.Gly176Gly fsX59).

Case 4 had six intronic variations and one conservative amino acid substitution (Table 3), all of which were homozygous and documented single nucleotide polymorphisms in the general population (dbSNP). Parental samples for fetus 4 were not available for analysis**.** As the fetus was not known to come from a consanguineous background and had a normal karyotype, the hypothesis of a small, heterozygous deletion was considered. QMPSF (quantitative multiplex PCR of small fluorescent fragments) [20], was undertaken to measure the number of genomic *STRA6* copies for case 4. The results indicated that this fetus did not present a deletion of the *STRA6* gene that would explain the observed homozygosity of the SNPs (data not shown).

A single heterozygous variation located in intron 13 (c.1407+32G>A) was observed in case 5, that has not been identified to date in dbSNP (Table 3). We screened 260 control chromosomes without observing the c.1407+32G>A variation. The only tissue available from

fetus 5 for expression analysis was a frozen lung sample. *STRA6* transcripts were not observed in either total lung RNA extracted from an age-matched fetus affected with an unrelated disorder or from the case 5 tissue sample (data not shown). Therefore, the consequence of this variation on *STRA6* transcription remains to be determined.

We report homozygous mutations in the *STRA6* gene in two fetuses presenting the principal features of MWS, including bilateral severe microphthalmia and pulmonary agenesis. Both also had bilateral diaphragmatic eventration and one had a cardiac malformation. The observation that both fetuses came from consanguineous families and, moreover, that one family demonstrated sibling recurrence, had already evoked a recessive model of inheritance for MWS [14]. By finding the molecular anomaly, it is now possible to affirm that MWS is indeed an autosomal recessive disorder that can be ascribed to mutations in the *STRA6* gene.

These two *STRA6*-mutated fetuses would not have survived postnatally. In both cases, the mutations would have led to a truncated protein if translated. Homozygous *STRA6* mutations have also been observed in peri- and postnatal patients from two other families as well as in three sporadic cases with a similar phenotypic spectrum [18]. However, four missense mutations were found to be associated with a severe clinical phenotype while two cases with a truncating mutation had milder clinical signs, with no growth retardation or apparent pulmonary anomalies. Indeed, one of those patients has survived into his teens. Comparison of all reported patients with *STRA6* mutations (Table 1) thus demonstrates that there is no correlation to date between the nature of a coding mutation and the severity of the phenotype.

The recent functional study of fifty random missense mutations introduced into bovine *Stra6* has shown that a few of these are sufficient to prevent cell surface expression and one, although allowing protein insertion into the membrane, abrogates vitamin A entry into the cell

[21]. Similar studies will now need to be conducted with documented human mutations in order to draw conclusions, but it is probable that phenotypic severity is a result of the reduction in perceived retinoic acid (RA) dose within sensitive target tissues, rather than a simple distinction between missense and nonsense mutations.

We also undertook molecular analysis of *STRA6* in five other fetuses with pulmonary and ocular or cardiac malformations, but no other patent mutations were identified, despite some intriguing variations (Table 3). The clinical diversity of patients with *STRA6* mutations, and the large phenotypic overlap with those who are not mutated, strongly suggests that MWS and related syndromes are not only clinically but genetically heterogeneous.

The only necessary diagnostic criterion predicting the involvement of *STRA6*, based on the patients currently reported here and in the previous study [18], is severe microphthalmia (clinical anophthalmia). Microphthalmia with any macroscopically residual presence of the ocular globe does not correlate with *STRA6* mutations in either series (Table 1). Obviously, since many genes have previously been identified in both isolated and syndromic microphthalmia, this feature is not sufficient to orient molecular testing. The severe eye malformations subsequent to *STRA6* mutations are always observed in association with at least one other sign, among those of pulmonary defects, congenital diaphragmatic eventration/hernia or cardiovascular malformation involving the common aorticopulmonary trunk or pulmonary arteries. Furthermore, according to our two MWS cases and descriptions of MWS in the literature, pancreatic malformations and IUGR may also be secondary diagnostic criteria.

Pulmonary defects range from agenesis (this report) to hypoplasia or unilobar lung among mutated MWS families, to no obvious lung problems in either member of family 2 examined by Pasutto *et al*. [18]. Pulmonary and diaphragmatic malformations

(eventration/hernia) are not always associated, occurring separately or in combination even among members of the same family [18]. This observation leads us to conclude that in the context of *STRA6* mutations, the pulmonary phenotype of mutated patients is a primary malformation and not a consequence of diaphragmatic hernia. However, the joint presence of clinical anophthalmia and pulmonary and/or diaphragmatic anomalies is still not sufficient to guarantee *STRA6* involvement, because other cases with bilateral anophthalmia and hypoplastic lungs (MWS patients GM23728 and CD50396 from Pasutto *et al.* [18] and our case 4) do not present coding sequence mutations (Table 1).

Cardiovascular involvement is frequent, but inconstant. Case 2 had a ventricular septal defect and pulmonary trunk agenesis, while case 1 presented isolated agenesis of the pulmonary arteries. Furthermore, *STRA6* mutations described by Pasutto *et al.* also give rise to conotruncal or great artery malformations (truncus arteriosus, tetralogy of Fallot, pulmonary valve or arterial stenosis, right aortic arch) in at least some family members [18]. Other affected members with identical mutations had no cardiovascular signs (cf. MWS4-BE). Previously described cases of MWS [9, 12] also show a preponderance of pulmonary artery absence, ductus arteriosus or ventricular septal defects.

Fryns syndrome [MIM 229850] has a clinical spectrum that includes diaphragmatic hernia and, less frequently, microphthalmia, facial dysmorphy and distal limb anomalies. Fetal case 3, presenting with bilateral microphthalmia, pulmonary hypoplasia, diaphragmatic hernia and cardiac involvement and cleft palate was diagnosed with Fryns syndrome. Despite the implication of the same organ systems as in MWS and absence of a digital phenotype, no mutations in the *STRA6* coding sequence were found. Patients GM23728 and CD50396 from Pasutto *et al*. [18] also had a similar phenotype (Table 1); the latter was diagnosed with MWS,

presented true clinical anophthalmia and had a cleft palate. Palate involvement might therefore be suggestive of Fryns syndrome rather than MWS. Phenotypic overlap between these two disorders indicates that similar cases diagnosed with Fryns or MWS have either a non-coding mutation in *STRA6* or involve another gene necessary for the cellular interpretation of RA levels. For some authors, animal models of retinoid deficiency also evoke the PAGOD syndrome [MIM 202660] (pulmonary tract and pulmonary artery, agonadism, omphalocele, diaphragmatic defect, and dextrocardia) which shares features with Fryns and MWS [22].

RA, a small lipophilic hormone derived from retinol (vitamin A), is a ligand for nuclear receptors (RARα, β and γ), that act in homodimers or in heterodimers with RXR partners to bind DNA and regulate the expression of many genes, including the *Stra* (stimulated by retinoic acid) targets [23, 24]. The functionally identified *Stra* genes have different roles and structurally unrelated products. For example, *Stra1* encodes ephrin B1, a bidirectional, membrane-bound signalling molecule highly expressed in the embryonic neural crest [25]; *Stra7*, later identified as the evolutionarily conserved transcription factor Gbx2 [26], partners with the homeobox transcription factor *Otx2* in the specification of the isthmic organizer (midbrain/hindbrain junction) [27].

Otx2 was also subsequently identified as a transcriptional target of RA, which leads to derepression of *Pax6* transcription in the optic cup [28]. Interestingly, both *OTX2* [29] and *PAX6* [4] are responsible for human anophthalmias [MIM 610125 and MIM 607108.0005 respectively], through heterozygous loss-of-function with incomplete penetrance for the former and compound heterozygous loss-of-function engendering a primary anophthalmia for the latter. Mutations in *EFNB1* (encoding human ephrin B1) induce craniofrontonasal syndrome [MIM 304110], sometimes in association with congenital diaphragmatic hernia (CDH) [30, 31]. We note

that *CRABP1* (cellular retinoic acid binding protein 1), another transcriptional target and effector of cytoplasmic RA levels [32], is located close to reported CDH loci in the long arm of chromosome 15. Experimental or teratogenic reductions in RA levels also lead to CDH in both animals and humans [33, 34].

The murine *Stra6* gene encodes an integral transmembrane protein that is expressed in the developing eye, lung, other endodermal gut derivatives, limbs and somites [23]. In addition to being stimulated by RA, *Stra6* encodes a receptor for soluble retinol binding protein, efficiently mediating retinol uptake from the circulation into target cells [21].

Signaling by RA within the caudal pharyngeal endoderm of the vertebrate embryo is critical for the organization of the adjacent aortic arch vessels and heart. Sensitivity of only the most posterior aortic arches, which persist in direct continuity with the outflow tract of the heart, may be a result of the localized mesodermal production of retinaldehyde dehydrogenase 2 (Raldh2), a major enzyme for RA synthesis from retinol during development [35]. *Raldh2*$^{-/-}$ mice demonstrate 3rd and 4th arch artery malformations with agenesis of the 6th arch [36] in addition to cardiac septation defects [37] and partial pancreatic agenesis [38]. The variable implication of the cardiac outflow tract and vascular derivatives of the embryonic 4th (definitive aorta) and 6th (ductus arteriosus and proximal pulmonary artery) aortic arches in our patients is consistent with an underlying field defect affecting the perception of RA dose by the endoderm.

Indeed, murine *Stra6* is highly expressed in the pharyngeal endoderm and mesenchyme along the embryonic gut [23]. Our two severely affected, mutated patients had duodenal stenosis and pancreatic malformations, in addition to lung agenesis. These organs are among the many

derivatives of the embryonic endoderm, produced by localized outpocketings into the mesoderm that will consolidate into the definitive structure.

RA is particularly necessary for normal growth and form of the lung. *Fgf10*$^{-/-}$ mice demonstrate complete lung agenesis [15, 16] while in knockout mice for the appropriate Fgf10-binding isoform of *Fgfr2*, the tracheal bifurcation at the origin of the bronchi is absent [17]. In *Raldh2*$^{-/-}$ mouse embryos, *Fgf10* is no longer expressed in the lung bud and complete agenesis results [39]. It appears likely that *Stra6*, expressed among other places in the *Raldh2*$^{+}$ bronchial mesenchyme of the early lung [23], mediates retinol entry into the mesoderm and a subsequent effect on Fgfr2 signaling in the endoderm. Indeed, supplying exogenous RA for short periods can partially rescue both *Fgf10* expression and lung agenesis, leading to unilobar or unilateral right-sided lung development; longer rescue periods lead to better recovery and more subtle alveolar malformations [40].

Stra6 is also expressed at all stages of eye development, within the optic vesicle initially and later, within the periocular mesenchyme, the choroid and the optic nerve (and forebrain) meninges. Expression in the retinal pigment epithelium persists throughout adult life in both mouse and humans [18, 23], indicative of the continued need for RA for ocular function. The consistency of clinical anophthalmia in *STRA6*-mutated patients argues for the need for vitamin A uptake to further all stages of eye development following initial optic specification.

Stra6 transcripts are also detected in several other sites including the forebrain, the isthmic organizer and the neurohypophysis. However, no *STRA6*-mutated patients present central nervous system (CNS) malformations or pituitary anomalies, although IUGR or short stature may indicate a more subtle effect (Table 1). Murine expression patterns do not always suffice to explain clinical outcome [41]. Despite the strong, localized brain expression of the RA

target *Gbx2* (*Stra7*), its absence in mice gives rise only to posterior branchial arch anomalies and cardiac malformations, reminiscent of those observed in patients with *STRA6* mutations or in *Raldh2*$^{-/-}$ mice [42]. There may also be species-specific differences in the RA sensitivity of the developing brain; the clinical spectrum of human vitamin A deficiency syndrome does not include the exencephaly observed in mouse models [43].

In conclusion, *STRA6* mutations are responsible for a large spectrum of congenital malformations with no current evidence of a genotype-phenotype correlation. Different transcriptional targets of RA signaling in humans appear to effect subset phenotypes of those observed in more generalized deficiencies [43]. MWS is thus part of a growing family of human syndromes due to mutations in genes encoding effectors of the powerful developmental morphogen, retinoic acid.

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Web resources

***PAX6* Allelic Variant Database**, http://pax6.hgu.mrc.ac.uk/

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/

University of California Santa Cruz (UCSC) **Genome Browser**, http://genome.ucsc.edu/cgi-bin/hgTracks (reference sequence NM_022369)

Primer 3 software, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi

Single Nucleotide Polymorphism Database, http://www.ncbi.nlm.nih.gov/SNP/

Mouse Genome Informatics, http://www.informatics.jax.org

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Cases	*STRA6* mutation	Eyes	Lungs	Diaphragm	Cardiovascular	Face	Other	Growth	Age at death	Consanguinity
Fetus 1*	p.D17A fsX55	bi AO	bi agenesis	bi eventr	bi absence of PA branches	mild dysmorphism	duodenal stenosis, annular pancreas	IUGR	31 wg	Yes, recurrence
Fetus 2*	p.G176G fsX59	bi AO	bi agenesis	bi eventr	pulmonary trunk and PA absence, VSD	mild dysmorphism	duodenal stenosis, absent pancreas, polylobed spleen	IUGR	28 wg	Yes
Fam2-IV:1	p.G50A fsX22	bi AO	-	CDH	ASD, VSD	mild dysmorphism	MR	SS	alive at 14 years	Yes, recurrence
Fam2-IV:3 (sib)	p.G50A fsX22	bi AO	n/a	CDH	n/a	mild dysmorphism	n/a	-	23 wg	
MWS4-BE	p.T644M	bi AO	hypo	CDH	-	-	bi hydronephrosis	-	alive at 3 months	No, recurrence
Brother MWS4-BE	n/a	n/a	hypo unilobar	-	Fallot, PDA	-	horseshoe kidney undescended testes	-	1 day	
Sister MWS4-BE	n/a	bi AO	hypo unilobar	-	PDA, CoA	-	uterine dysplasia	-	1 day	
MWS1-EE	p.R655C	bi AO	hypo	uni eventr	-	-	hypotonia uni inguinal hernia	-	3 months	Yes, recurrence
Brother MWS1-EE	n/a	bi AO	-	-	TA, RAA, PDA, PA atresia	-		SS	22 months	
MWS6-BK	p.P90L p.T321P	bi AO	hypo	CDH uni eventr	PDA	-	hypo kidneys bicornuate uterus	PTB (36 wg)	1 day	Yes, recurrence?
Fam1-IV:2	p.P293L	bi AO	ACD	-	PSt, PDA	mild dysmorphism	ectopic kidney DD	PTB (33 wg)	6 months	Yes, recurrence
Fam1-IV:4 (cousin)	n/a	bi AO	n/a	-	single ventricle PA atresia	n/a	-	-	2 days	
CD50396*	-	bi AO	hypo	uni eventr	VSD	CP hypo alæ nasi	hypo bicornuate uterus hypo spleen	-	1 day	No
Fetus 3**	-	bi AO	hypo	bi CDH	hypo L ventricle and aorta, mitral valve atresia, VSD	CP	CC agenesis arhinencephaly Dandy-Walker	-	16 wg	No
Fetus 4	-	uni AO	-	-	single ventricle tricuspid valve atresia, ASD	-	arhinencephaly	-	22 wg	No
MWS3-KH	-	bi MO/AO	-	CDH	-	-	-	-	n/a	No

RHP006.070	-	bi MO/AO	-	bi eventr	-	-	MR	-	n/a	No
PB-E03_053	-	bi MO/AO	-	CDH	-	brachycephaly	MR, sparse hair bi inguinal hernia	-	alive at 10 years	No
GM23728	-	bi MO abnormal cornea, iris	hypo unilobar	hypo, uni eventr	hypo PA CoA	-	renal dysplasia	-	neonatal	No
AS20861-FF264	-	uni MO	-	CDH	-	-	ocular cyst, DD	-	alive at 13 months	No
MWS2-FA	-	bi coloboma	-	CDH	-	-	skin patches, brittle hair	-	n/a	Yes
MWS5-LR	-	coloboma	-	CDH	-	-	-	-	n/a	No
Fetus 5	-	-	hypo	uni CDH	dextroposed aorta over VSD	-	SUA	-	32 wg	No
AvdW22260	-	-	hypo	CDH	-	-	-	PTB (28 wg)	1 day	No
Twin 2 AvdW22260	-	-	hypo	CDH	-	CP	-	PTB (28 wg)	1 day	
PM22479***	-	-	-	CDH	-	hypertelorism	hypo CC omphalocoele	-	neonatal	Yes, recurrence
Brother*** PM22479	n/a	-	-	CDH	ASD	bi CLP hypertelorism	hypo CC	-	neonatal	
Fetus 6	-	-	bi hypo	L agenesis, R eventr	hypo L heart	-	polysplenia renal dysplasia SUA	IUGR	30 wg	No
Fetus 7	-	-	bi agenesis	-	L atrial isomerism, R ventricular anomaly	-	polysplenia renal agenesis	-	24 wg	No

Table 1. Overview of clinical features in cases undergoing *STRA6* molecular analysis from our series (bold; fetuses 1-7) and Pasutto *et al*. 2007. * = diagnosed Matthew-Wood syndrome; ** = diagnosed Fryns syndrome; *** = suspected Donnai-Barrow syndrome. (*Abbr*. : ACD = alveolar capillary dysplasia; AO = clinical anophthalmia; ASD = atrial septal defect; bi = bilateral; CDH = congenital diaphragmatic hernia; CC = corpus callosum; CoA = coarctation of aorta; C(L)P = cleft (lip and) palate; DD = developmental delay; eventr = deventration; Fallot = tetralogy of Fallot; hypo = hypoplasia; IUGR = intrauterine growth retardation; L = left; MO = microphthalmia; MR = mental retardation; PA = pulmonary artery; PDA = persistent ductus arteriosus; PSt = pulmonary valve stenosis; PTB = preterm birth; R = right; RAA = right aortic arch; SS = postnatal short stature; SUA = single umbilical artery; TA = truncus arteriosus; uni = unilateral; VSD = ventricular septal defect; wg = weeks gestation).

	Oligonucleotide sequences (5'→3')	
Exon	**Forward**	**Reverse**
1a	GGGGTGGGTTCCTCTGAT	CACCCCAGGTCTCCAAACT
1b	GCTGAAGGCAGGTATGTGTG	CCTCTCGTGTCCCCTCCT
2	AAGCCTCTTTTCACATCTGTAGTG	CAGTTGCAACCTCTGCCATC
3	TGGGTAAAGCCTCAGTGTGA	GTTGGACTTGCATCCTGGTT
4	CAAGCCCTCAAACTCAGACC	TGGGGGTCCTGACTAAACCT
5	CCACCTCCTTGATTTATGGAA	GCATCGTTGTAAAGACTGGATG
6-7	ACCTTCTCATTTTGCCCTTG	CTCAAAGGAGGCACTGTGGT
8	GCAACGGATTCTGGTTCTTG	GGAGTAGGGCTGTCTTGGG
9-10	ACGAATGGGTCGAGGCAG	TCTGTGCAAGGGAGGGTAAC
11	CTTGGGAGGGAGGAGGG	GGTTGAGGGCAGGGCTC
12	CCAGCGTCTCCCCTGTTAG	CATAGACCTTGGGTCTCCCC
13	TGGCAGGGGTTCTGAGG	CACAGGACTCCCACTCCTTC
14	TGGCCCAGAGGAGGATTTAG	CCAACTGAGGCCAGTGTCTG
15-16	AAAGCCCTTGGTTCTGGG	ACACCGAAGAAGAGGCGAG
17	AGGTCTGACACTGACCCTGG	GATGCCTTCCTCACTGCTTG
18	TGGATGCCTCCAGTGTGG	AGGGGCACACATCCTTCC
19	GATCAGGTCTGAGGGCCAG	GAGGAGGATGGTAGGCAGG

Table 2. *STRA6* oligonucleotides used for sequencing. The annealing temperature for PCR was 60°C for all primers. For QMPSF, fluorescent primers corresponding to *STRA6* exon 13 were used, and *MLH1* was chosen as a reference (GTAGTCTGTGATCTCCGTTT, 5'; ATGTATGAGGTCCTGTCCT, 3'). Co-amplification was carried out for 21 cycles, the peaks integrated and proportional DNA copy numbers estimated using Genotyper 3.7 software (Applied Biosystems).

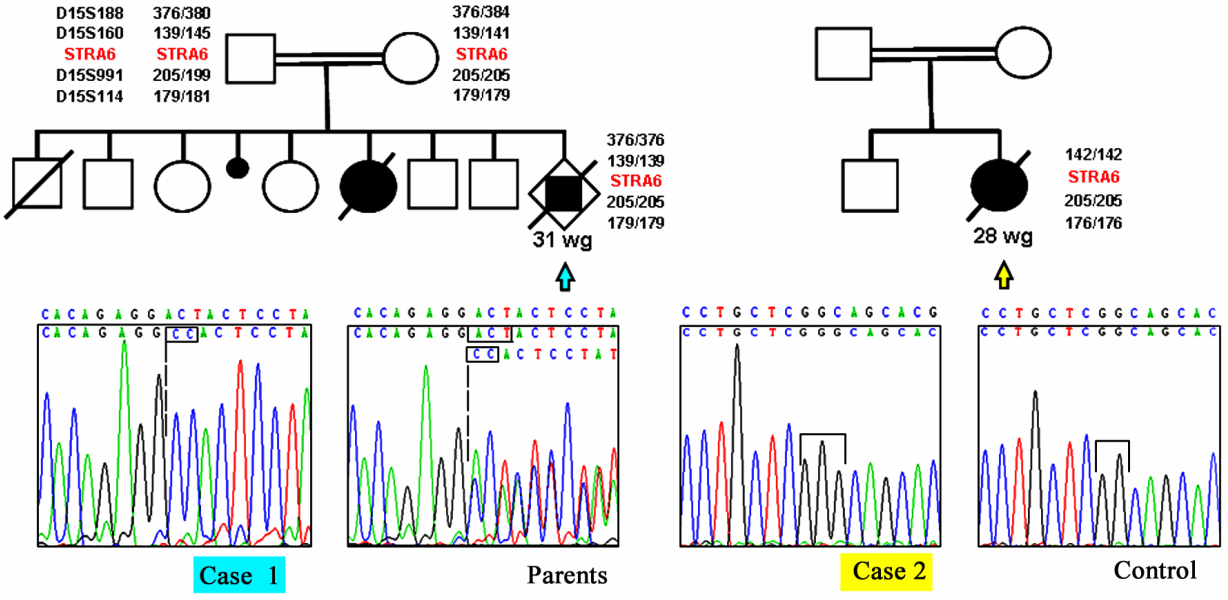
Fetal case	Nucleotide changes versus NM_022369	Predicted effect on ORF	Known SNP reference	Status
1	c.50_52delACTinsCC	p.Asp17Ala fsX55		Homozygous
2	c.527_528insG	p.Gly176Gly fsX59		Homozygous
3	none			
4	c.331C>T	p.Leu111Leu	rs11857410	Homozygous
	c.406+97A>G		rs34147822	Homozygous
	c.406+111A>G		rs35255788	Homozygous
	c.430+24T>A		rs971756	Homozygous
	c.431-37C>T		rs971757	Homozygous
	c.1685-24T>C		rs12913041	Homozygous
	c.1840+50T>C		rs12912578	Homozygous
5	c.596+9T>G		rs28541560	Heterozygous
	c.1301-43A>C		rs351240	Heterozygous
	c.1416G>A	p.Ser472Ser	rs351241	Heterozygous
6	c.1166+32G>A			Heterozygous
7	c.1167-10C>G		rs2277608	Heterozygous

Table 3. Sequence variations in *STRA6*.

Legend

Figure 1. Pedigrees of cases 1 and 2 with markers flanking the *STRA6* gene and electropherograms. Case 1 (blue arrow) had a homozygous insertion/deletion in exon 2 of *STRA6* (c.50_52delACTinsCC p.AspD17Ala fsX55). Case 2 (yellow arrow) had a homozygous insertion in exon 7 (c.527_528insG p.Gly176Gly fsX59). Markers D15S160, D15S991 and D15S114 were also homozygous; relatives' DNA is unavailable for further analysis. wg: weeks gestation

Figure 1.


D15S188
D15S160
STRA6
D15S991
D15S114
376/380
139/145
STRA6
205/199
179/181
376/384
139/141
STRA6
205/205
179/179
376/376
139/139
STRA6
205/205
179/179
31 wg
142/142
STRA6
205/205
176/176
28 wg
Case 1
Parents
Case 2
Control