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AIFM1-associated X-linked spondylometaphyseal dysplasia with cerebral hypomyelination

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Abstract
Spondylometaphyseal dysplasia with cerebral hypomyelination (SMD-H) is a very rare but distinctive phenotype, unusually combining spondylometaphyseal dysplasia with hypomyelinating leukodystrophy. Recently, SMD-H has been associated with variants confined to a specific intra-genic locus involving exon 7, suggesting that AIFM1 plays an important role in bone development and metabolism as well as cerebral myelination. Here we describe two further affected boys, one with a novel intronic variant associated with skipping of exon 7 of *AIFM1* and the other a synonymous variant within exon 7 of *AIFM1.* We describe their clinical course and radiological and genetic findings, providing further insight into the natural history of this condition.

Introduction

The term spondylometaphyseal dysplasia describes a genetically heterogeneous group of generalised disorders of the skeleton which predominantly involve the metaphyses of long bones and the spine. They share some clinical and radiological features in common, which do not in most cases include primary neurological symptoms [Mortier et al., 2019]. Likewise, central hypomyelinating conditions are a diverse group of conditions, without skeletal dysplasia. The most renowned, Pelizaeus-Merzbacher disease (PMD) is caused by variants in *PLP1*, which encodes proteolipid protein 1, the most common structural myelin protein.

The rare and unique combination of a skeletal dysplasia with a PMD-like syndrome, now known as X-linked spondylometaphyseal dysplasia with cerebral hypomyelination (SMD-H), was first recognised as a novel entity in 1999, when a family with three affected boys was described [Bieganski et al.,1999]. The family structure suggested the likelihood of X-linked recessive inheritance. Further cases were subsequently reported from different parts of the world, [Neubauer et al., 2006; Kimura-Ohba et al., 2012], but the causative gene remained elusive. SMD-H has to date been reported in a total of 19 patients from 8 families [Bieganski et al., 1999; Neubauer et al., 2006; Kimura-Obha et al., 2013; Mierzewska et al., 2017; Miyake et al., 2017] and recently associated with variants in the Apoptosis Inducing Factor- Mitochondrion 1 gene (*AIFM1)* located at chromosome Xq26.1 [Mierzewska et al., 2017; Miyake et al., 2017]. This gene is a known cause of several neurological presentations such as ataxia, neuropathy, cognitive delay, hearing loss and seizures [Kettwig et al., 2015], but prior to 2017 had not been implicated in skeletal and cartilage development. In all SMD-H patients from 8 families who have been analysed to date, 5 different *AIFM1* variants have been identified in a very specific region of the gene, including and close to the boundary between intron 6 and exon 7 (Table 1).

Here we describe 2 affected British boys with this condition, one with a synonymous variant within exon 7 and the other with a novel intron 6 variant, adding to accumulating knowledge of the phenotype and natural history and expanding the spectrum of causative variants in *AIFM1.* Awareness of this rare phenotype is important, particularly that the skeletal dysplasia emerges after the neurological symptoms. Therefore, it is worth considering SMD-H in hypotonic male infants with significant cerebral hypomyelination who remain without a diagnosis following neurological investigations.

Methods

Both families were identified through Clinical Genetics Services in the UK. For Patient 1, after the recognition of the genetic basis of SMD-H, Sanger sequencing of intron 6 and exon 7 of the *AIFM1*

gene was undertaken in our NHS Genomic Laboratory Hub. For Patient 2, we reviewed the data from a previous clinical diagnostic exome to search for an *AIFM1* variant in the intron 6/exon 7 boundary target region (NM_004208.3) and confirmed the variant using Sanger sequencing, as for Patient 1.

DNA Extraction and Sanger Sequencing

Genomic DNA was isolated from peripheral blood leukocytes using a Gentra Puregene cell kit (QIAGEN Ltd). Target regions *AIFM1* (intron 6 and exon 7) were amplified using a MegaMix (MicroZone). Primers were designed to GenBank Reference Sequence NM_004208.3 using Primer3 software. Primer sequences are available on request.

M13 tagged bidirectional sequencing was undertaken using a BigDye Terminator v3.1 Cycle Sequencing Kit and 3730 DNA Genetic Analyzer (Applied Biosystems) with Mutation Surveyor DNA Variant Analysis Software v3.97 (Softgenetics). Alamut software v2.3.1 (Interactive Biosoftware, Rouen, France) was used to predict the effect of genetic variation. The software integrates PolyPhen-2, Align GVGD and SIFT and five splice site prediction programs: SpliceSiteFinder, MaxEntScan, Human Splice Finder, NNSPLICE and GeneSplicer. To further evaluate the variant found in Patient 1, we undertook semi- quantitative analysis of *AIFM1* mRNA.

RNA extraction and RT-PCR protocol for cDNA preparation

Blood samples were collected into PAXgene RNA collection tubes (Qiagen, UK) and RNA was extracted using the QIAcube Connect extraction machine (Qiagen, UK) and the standard protocol of the RNeasy kit (Qiagen, UK). cDNA preparation was then carried out using 1.5µl of 10mM dNTP mix (Promega, UK), 2µl of 0.1M Dithiothreitol (Invitrogen, UK), 1µl of 40 U/µl Moloney Murine Leukemia Virus *Reverse Transcriptase* (Invitrogen, UK), 1µl of 40 U/µl RNAaseOut ribonuclease inhibitor (Invitrogen, UK), 4µl of *Reverse Transcriptase buffer* (Invitrogen, UK), 1µl of 10ng/ul random hexamer primers (Thermo Fisher, UK) and 10µl of RNA. Samples were incubated for one hour at 37^{o}C followed by 10mins at 65^{o}C.

RNA splicing analysis

RNA analysis was carried out by amplification of cDNA using a forward PCR primer (situated in exons 5 or 6) and a reverse primer (situated in exons 8 or 9). A bespoke reverse primer was also designed to span the exon 6 – exon 8 splice junction; this was used in conjunction with the exon 5 forward primer to generate a product specific for the abnormal transcript lacking exon 7. This reaction was carried out as a duplex with a second larger PCR control product (amplifying *BRCA1* exons 17 to 19). PCR reactions were carried out in a 20µl volume containing 1.5µl of cDNA, 10nM of each primer (Promega, UK), 2µl of 10x Platinum Taq buffer (Invitrogen, UK), 0.2mM of each dNTP, 1.5mM $MgCl_2$ and 0.5 units of Platinum Taq polymerase (Invitrogen, UK). Cycling parameters were 94^{o}C for 12 minutes followed by 35 cycles of 94^{o}C for 30 seconds, 60^{o}C for 30 seconds and 72^{o}C for 30 seconds. PCR products were checked by gel electrophoresis and then bi-directionally sequenced using the standard protocol of the Big-Dye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, USA) and separated on an ABI 3130*xl* Genetic Analyzer (Applied Biosystems, USA). Subsequent data were analysed using the Mutation Surveyor (version 3.1) software (SoftGenetics, USA).

Case reports

Family history

Patient 1 is the third of four boys of non-consanguineous healthy parents. One of his elder brothers had congenital dislocation of the hips treated with surgery. This brother was later found to have developmental delay, caused by an unrelated *de novo* unbalanced chromosome translocation. Patient 2 is the first of two children of non-consanguineous healthy parents. He has one older sister. Of note, his maternal uncle died aged 14 years with a developmental disorder characterised by progressive ataxia and a post-mortem examination demonstrated central nervous system features consistent with PMD. Unfortunately, there is no information on the uncle's stature or skeletal features, although his death was from respiratory disease. The mother of Patient 2 showed slowly progressive spastic diplegia from adolescence onwards and subsequently died from metastatic breast cancer.

Phenotype

Both patients had unremarkable gestational and perinatal histories with normal vaginal delivery at term and normal birth weight. Both showed initial symptoms during infancy (Patient 1 at 3-6 months and Patient 2 at 18 months) with developmental delay, particularly of motor milestones. Patient 2 also developed nystagmus from 20 months alongside the presentation of motor delay. Later, additional symptoms emerged including short stature, relative macrocephaly, truncal ataxia, and nystagmus. Their facial features are subtly different from family members, with a depressed nasal bridge and flat maxillary region (Figure 1).

Skeletal features

Both Patients 1 and 2 have marked short stature (Patient 1 height SDS -5.2, Patient 2 height SDS -5.7) with kyphoscoliosis and generalised progressive enlargement of both large and small joints of upper and lower limbs, resulting in marked joint contractures. Patient 1 has abnormal dentition and had pectus carinatum. Problems with joints and growth were apparent in Patient 2 from around 5 years of age.

Neurological features

Both patients have nystagmus with associated visual impairment in Patient 1. Both have developed progressive ataxia and spasticity, associated with truncal hypotonia, areflexia in lower limbs, dysarthria and head titubation. Both patients have retained intellectual ability, despite the severity of neurological symptoms. Neither patient has seizures. Ophthalmological investigation showed that Patient 1 has retinal dystrophy and hypermetropia.

Respiratory features

As a consequence of the chest deformity and muscular weakness, both patients have respiratory compromise. At the age of 10 years, Patient 1 was admitted to intensive care unit for ventilation for several weeks during an episode of pneumonia, narrowly avoiding a tracheostomy. Patient 2 is now 17 years old and already has a tracheostomy. He has had several admissions presenting with upper airway obstruction resulting in a need for intubation from around 10 years of age and has required a permanent tracheostomy for subglottic stenosis from about 15 years of age.

Radiological findings

The radiological findings on skeletal surveys and MRI brain scans of Patients 1 and 2 were similar to each other and to those of previously reported cases (Figure 1). The skeletal findings are striking and are present throughout the skeleton. Shared features include: mild shortening of the long bones, irregular metaphyses showing flaring, cupping and irregular ossification with some striations, markedly small and irregular epiphyses, small laterally-placed capital femoral epiphyses, apparent widening of the hip joints and triradiate cartilages, generalised brachydactyly involving both metacarpals and phalanges, thoraco-lumbar hyperkyphosis, mild platyspondyly and vertebral endplate irregularity with variable ossification defects of the anterior vertebral body margins.
The brain scans for both boys revealed diffuse cerebral hypomyelination with relative sparing of the brain stem and cerebellum. An additional finding in Patient 2 was of global reduction in bulk of the cerebral white matter. Review of a second scan in Patient 1 at the age of 10 years (not shown) did not show progressive loss of myelination.

Other investigations

Prior to diagnosis, both patients underwent extensive metabolic investigations including analysis of white cell enzymes, very long chain fatty acids, serum lactate and PLP. Patient 1 had a normal muscle biopsy. Both patients had abnormal nerve conduction studies suggesting a mixed axonal and demyelinating large fibre sensorimotor peripheral neuropathy. Both patients had normal array CGH.

Genotype

In Patient 1, Sanger sequencing of the *AIFM1* intron 6-exon 7 region revealed the variant c.697-27T>G in intron 6 ((NM_004208.3). Investigation of the functional consequences of the variant by RNA splicing analysis of whole blood identified both a normal-sized transcript and a low-level transcript deleted for exon 7 (r.697_781del) (Figure 2A). The r.697_781del transcript causes downstream disruption of the transcriptional reading frame and was absent from normal controls (Figure 2). The *AIFM1* intronic variant was classified as likely pathogenic (ACGM codes PS3, PM2, PP4), and the mother of Patient 1 showed mosaicism for the variant in her peripheral leukocytes. There was no evidence of the r.697_781del transcript in the mother by standard RNA analysis (Figure 2A), however the abnormal transcript was identified by targeted RNA analysis using a PCR primer designed to span the novel exon 6/8 splice junction (ttggagtacctc/CTTCTTCC, exon 6 lower case and exon 8 upper case; Figure 2B). The low-level mosaicism for the variant was considered to be consistent with her normal phenotype.

In Patient 2 re-analysis of clinical exome data detected *AIFM1* c.720C>T, p.(Asp240Asp). The same variant was identified in maternal DNA confirming carrier status in his mother
This synonymous variant in exon 7, has previously been reported in association with H-SMD, together with functional studies confirming pathogenicity (PS2, PS3, PM2 PS4_moderate, Miyake et al., 2017). Importantly, these functional studies showed this synonymous variant to exhibit a similar loss of *AIFM1* expression as other mutation classes (missense and intronic). This was demonstrated by comparing *AIFM1* mRNA and protein levels in patient fibroblasts and osteoblasts with equivalent cells from unrelated controls.

Discussion

The patients described here have clinical, radiological and genetic characteristics consistent with the diagnosis of SMD-H, bringing the total number of reported cases, all males, to 21 from 10 families (Table 1). Boys with SMD-H are asymptomatic at birth and the presenting clinical feature is usually

motor developmental delay, seen in the first 2 years of life, with a few exceptions where onset was after 4 years of age [Miyake et al., 2017]. Over time, a complex neuropathic phenotype develops with a combination of upper and lower motor neuron signs. Alongside progressive muscle weakness, upper motor neurone features include spasticity from the leukoencephalopathy and cerebellar signs of truncal and limb ataxia, nystagmus, dysarthria and tremor. There is progressive functional difficulty and loss of mobility, requiring the use of a walking frame or wheelchair. Some children can stand but are never able to walk independently. Despite the severity of these problems, some boys retain good understanding and some cognitive function. Overall, the neurological course appears to be relatively mild compared to that of PMD with a slow decline which manifests predominantly as progressive ataxia and spasticity [Kimura-Ohba, 2013]. Likewise, previous literature has demonstrated that the MRI brain findings are fairly stable and do not progress rapidly. This appears to be the case in our patients so far.

The skeletal dysplasia in SMD-H, like other examples of SMD, also seems to be progressive and compounds the disability brought about by the neurological phenotype. In these cases, the neurological phenotype emerges first, with evidence of skeletal abnormalities emerging later in the clinical timeline. This is important for clinicians to be aware of so they can be mindful of the possibility of subsequent skeletal dysplasia in male patients with cerebral hypomyelination, hypotonia and an unknown diagnosis. Consequently, they can monitor for skeletal abnormalities and initiate radiological investigations where appropriate. Review of the data from the published cases and ours, suggests that it may be difficult to distinguish SMD-H on the basis of skeletal radiology alone. The epiphyseal changes are quite striking, and the disorder should perhaps be considered a spondyloepimetaphyseal dysplasia (SEMD). The skeletal features quite strongly resemble those of pseudoachondroplasia (PSACH), a more common disorder. Distinction from PSACH on radiological grounds alone may be difficult, but PSACH does not demonstrate the neurological features present in SMD-H. PSACH is caused by variants in Cartilage Oligomeric Matrix Protein (COMP) and is associated with endoplasmic reticulum stress in chondrocytes, inflammation and disordered growth of cartilage (Suleman et al., 2012). The prognosis of SMD-H depends on individual factors, especially the respiratory complications of the disorder. Some of the reported patients and one of ours have a tracheostomy and it seems likely that the upper airway is vulnerable to collapse, perhaps through qualitative changes in cartilage in the trachea and related structures.

In all relevant cases, pedigree analysis is consistent with X-linked recessive inheritance of SMD-H. SMD-H is a condition only expressed in boys so far, although family studies, as in our second case, suggest that carrier females may have an attenuated neurological phenotype. In our second family, it is likely that the mother's brother was affected with SMD-H, but there is insufficient evidence to confirm this. The genetic basis of SMD-H is intriguing. The variants in *AIFM1* associated with SMD-H to date are summarised in Table 1. As the phenotype is so consistent and the mutational spectrum is very narrow, no meaningful genotype-phenotype relationships can be observed. All causative *AIFM1* variants reported thus far affect expression of exon 7. They occur in a very specific intra-genic region around the boundary of intron 6 and exon 7, which is significant as there are very few examples where variants in specific regions of a gene cause such a distinct phenotype. Intron 6 splicing variants have been reported twice. In Family 6 reported by Miyake et al., in 2017 (and previously by Neubauer et al., 2006), the c697-44T>G variant resulted in similar reduction in *AIFM1* mRNA and AIFM1 protein levels in fibroblasts and osteoblasts as exon 7 variants. In our study, the c.697-27T>G variant resulted in an *AIFM1* mRNA transcript deleted for exon 7 (r.697_781del),

predicted to cause downstream disruption of the transcriptional reading frame. We estimate that only approximately 5-10% of c.697-27T>G alleles produce an *AIFM1* RNA transcript lacking exon 7. This is difficult to quantify precisely and is based on a comparison of the strength of the normal and mutant bands following separation by agarose gel electrophoresis (Figure 2A, P1 lanes), and the latter may be subject to preferential amplification due to its smaller size. The mother of the proband is a low-level heterozygous mosaic for the c.697-27T>G variant; consequently the *AIFM1* RNA transcript lacking exon 7 was not detectable using conventional RNA analysis (Figure 2A) and the abnormal RNA transcript was only detectable in her using a targeted RNA primer that spans exons 6 and 8 (Figure 2B). The Genotype-Tissue Expression (GTEx) Portal (GTEx Consortium, 2015) shows that while there is some AIFM1 expression in blood, it is much higher in other tissues and there is evidence of alternate transcription (https://www.gtexportal.org/home/gene/AIFM1). It is therefore possible but not proven, that expression of the mutant transcript follows the same pattern.

AIFM1 seems intolerant of loss of function variants. As exon 7 is 85bp (including bases c.697 to c.781, corresponding to codons 233 to 261), the resulting transcript is predicted to be out of frame and to result in loss of function. We speculate that fully penetrant loss of function variants, which have so far not been reported, may be lethal in affected males. Mosaicism for the deleted transcript, observed in Patient 1, suggests that the co-existence of exon 7-deleted and normal transcript is sufficient to cause the SMD-H phenotype. Alternatively, the exon 7-deleted transcript may only be present in certain tissues, but our current observations have not determined how the effect is mediated. It is helpful to understand that the *AIFM1* variant spectrum includes mosaic, synonymous and deeper intronic splicing variants, as these may be missed or filtered out by conventional screening methods.

Mitochondrion Apoptosis Inducing Factor-1 (AIFM1) is a mitochondrial oxidoreductase that plays a role in oxidative phosphorylation in healthy cells and has been implicated in programmed cell death and mitochondrial metabolism [Joza et al., 2009]. Variants in this gene have previously been linked with a range of neurological disorders, severe X-linked mitochondrial encephalopathy, Cowchock syndrome (axonal sensorimotor neuropathy with deafness and intellectual disability) and hearing loss. These all satisfy a cohesive genotype-phenotype relationship (Kettwig et al., 2015), but SMD-H is the only AIFM1-related phenotype with skeletal dysplasia. It is not clear at present why variants located at such a specific intra-genic location within *AIFM1* result in SMD, whereas variants in other regions of the gene do not. Presumably, exon 7 of *AIFM1* is integral to its functional role in cells involved in cartilage and bone development and turnover. The similarity of the skeletal phenotype in SMD-H and PSACH, which has been associated with a novel form of chondrocyte stress characterised by reduced chondrocyte proliferation, retention of mutant COMP in the endoplasmic reticulum and increased and spatially dysregulated apoptosis in growth plates, raises the possibility that similar cellular pathology arises in SMH-H [Suleman et al., 2012].

As pointed out by Miyake et al., [2017], functional studies of an AIFM1-altered residue p.(Val243Leu) in a patient with SMD-H had essentially no impact on protein binding, folding or reduction-oxidation activity [Sevrioukova, 2016]. They suggested that SMD-H variants may result in a common specific disruption of mRNA splicing. Our data add to the hypothesis that skipping of exon 7 is the common underlying mechanism of SMD-H, through aberrant mRNA splicing, although further studies of this rare condition will be required to elucidate the pathology of SMD-H.

Figure Legends:

Figure 1: Clinical and radiological findings:

The clinical features of Patient 1 (left) and Patient 2 (right) include hypertelorism, a flat nasal bridge and malar region, kyphoscoliosis and pectus carinatum (patient 1). There were joint contractures at the elbows, knees and ankles.

Selected skeletal radiographs are shown with Patient 1 (left), age 4 years a-c and age 7 years d-k, and Patient 2 (right), age 6 years l-n and age 9 years o-u. Notable shared features include brachydactyly, irregular, flared and cupped metaphyses with metaphyseal striations, widening of the tri-radiate cartilages (asterisks), small, irregular epiphyses, laterally positioned capital femoral epiphyses and thoraco-lumbar hyperkyphosis with mild platyspondyly and vertebral end-plate irregularity. By 7 years, Patient 1 had developed a thoraco-lumbar scoliosis.

MRI brain, Patient 1 at 3 years. Axial T2 weighted section (1a) and Coronal T1 weighted section (1b) demonstrating diffuse supratentorial hypomyelination with relative preservation of the brain stem and cerebellum.

MRI brain, Patient 2 at 4.6 years. Axial T2 weighted section (2a) and Sagittal T1 weighted section (2b) also demonstrating diffuse supratentorial hypomyelination. Note also made of reduced cerebral white matter bulk with slender corpus callosum and a small mid brain.

Figure 2: RNA analysis

RNA analysis Patient 1 (P1), Patient 1's mother (M1) and normal controls (N). Gel image A shows the amplification of AIFM1 RNA using PCR primers in exons 5 and 9 where the upper band (arrowed) represents the full-length transcript and the lower band is the transcript which lacks exon 7. For gel image B, the upper band is produced from a control gene while the lower band is a targeted AIFM1 product using a forward primer in exon 5 and a reverse PCR primer which spans the 3' end of exon 6 and the 5' end of exon 8 (which will only detect transcripts lacking exon 7).

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