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Heterozygous deletion of a 2-megabase region including the dystroglycan gene in a patient with mild myopathy, facial hypotonia, oral-motor dyspraxia and white matter abnormalities

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**Running Title:** Heterozygous deletion of the dystroglycan gene

**Key Words:** Dystroglycan, muscular dystrophy, learning difficulties, white matter, oral-motor dyspraxia

## ABSTRACT

Dystroglycan is a protein which binds directly to two proteins defective in muscular dystrophies (dystrophin and laminin  $\alpha 2$ ) and whose own aberrant post-translational modification is the common aetiological route of neuromuscular diseases associated with mutations in genes encoding at least six other proteins (POMT1, POMT2, POMGnT1, LARGE, FKTN, FKRFP). It is surprising, therefore, that to our knowledge no mutations of the human dystroglycan gene itself have yet been reported. Here we describe a patient with a heterozygous *de novo* deletion of a ~2-megabase region of chromosome 3 which includes the dystroglycan gene (*DAG1*). The patient is a 16-year old female with learning difficulties, white matter abnormalities, elevated serum creatine kinase (CK), oral-motor dyspraxia and facial hypotonia but minimal clinically significant involvement of other muscles. As these symptoms are a subset of those seen in disorders of dystroglycan glycosylation (muscle-eye-brain disease and Warker-Warburg syndrome), we assess the likely contribution to her phenotype of her heterozygosity for a null mutation of *DAG1*. We also show that the transcriptional compensation seen in the *Dag1*<sup>+/-</sup> mouse is not seen in the patient. Although we cannot demonstrate that haploinsufficiency of *DAG1* is the sole cause of this patient's myopathy and white matter changes, this case serves to constrain our ideas of the severity of the phenotypic consequences of heterozygosity for null *DAG1* mutations.

## INTRODUCTION

Dystroglycan is an intriguing molecule with a fascinatingly complex biogenesis, a large number of interacting partners, and a wide-reaching biological involvement in a broad range of processes<sup>1</sup>. Although transcribed and translated from a single gene, *DAG1*, dystroglycan is cleaved post-translationally to generate two polypeptides; a highly

glycosylated extracellular subunit,  $\alpha$ -dystroglycan, and a transmembrane subunit,  $\beta$ -dystroglycan<sup>2</sup>. This cleavage is essential<sup>3</sup> and may occur by autoproteolysis<sup>4</sup>. Glycosylation appears to be of a highly specialised nature, such that the enzymatic pathway involved has been conserved for 500 million years of metazoan evolution, apparently for the sole purpose of modifying this single protein<sup>5</sup>. This post-translational modification pathway involves at least six proteins<sup>6</sup>, namely four glycosyltransferases – protein-*O*-mannosyltransferases 1 and 2 (POMT1, POMT2), protein-*O*-mannose  $\beta$ -1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1), acetylglucosaminyltransferase-like protein (LARGE) – and two probable phosphotransferases – fukutin and fukutin-related protein (FKTN, FKR). Mutations in the genes encoding these proteins can give rise to disorders ranging from a late-onset limb-girdle muscular dystrophy (LGMD2I, LGMD2K, LGMD2M-2O)<sup>7</sup> via congenital muscular dystrophies (MDC1C, MDC1D and FCMD) through to muscle-eye-brain disease (MEB) and Walker-Warburg syndrome (WWS)<sup>7</sup>. While the milder disorders result in an apparently simple myopathy reminiscent of the DMD-BMD spectrum<sup>8</sup>, FCMD, MEB and WWS also have a variety of ocular and brain defects (including cobblestone lissencephaly, white matter abnormalities, learning difficulties, agenesis of the corpus callosum, myopia, cataracts and microphthalmia)<sup>9,10</sup>. The relationship between degree of dystroglycan hypoglycosylation and phenotypic severity is significant but complex<sup>11</sup>.

Dystroglycan is known to bind to a number of intracellular and extracellular partners. Within the cell, the C-terminal tail of  $\beta$ -dystroglycan interacts with dystrophin, utrophin, DRP2 and other signalling molecules<sup>1</sup> while, outside the cell, the glycosyl residues of  $\alpha$ -dystroglycan bind to the laminin globular (LG) domains of molecules such as laminin  $\alpha$ 2 (merosin)<sup>2</sup>, neuexin<sup>12</sup>, perlecan<sup>13</sup>, and agrin<sup>14</sup>. Dystroglycan (via its sugar moieties) is also an important cell-surface receptor for several pathogens (*Mycobacterium leprae*<sup>15</sup>, several Old World arenaviruses<sup>16</sup>). Most of our ideas regarding the function of dystroglycan, based on the

phenotypes of mouse<sup>17-21</sup> and fruit fly dystroglycan mutants and human glycosylation defect patients, involve a role in the establishment and/or maintenance of basement membranes and their interaction with cell surfaces.

Homozygous null mutation of the dystroglycan gene (*Dag1*) in mouse is incompatible with life, resulting in resorption of mutant embryos by embryonic day 10.5, probably due to the developmental failure of the extraembryonic Reichert's membrane<sup>21</sup>; similar problems underlie the lethality of the FKTN-null mouse<sup>22</sup>. Conditional ablation of dystroglycan in differentiated skeletal muscle gives rise to a very mild myopathy (as dystroglycan is still expressed in satellite cells and at the neuromuscular and myotendinous junctions)<sup>17</sup>. Ablation of dystroglycan in the brain recapitulates the lissencephaly seen in the severe human dystroglycanopathy patients, and reveals that these may arise through failure of the glia limitans<sup>18</sup>; other dystroglycanopathy-like structural abnormalities are also seen, and hippocampal long-term potentiation (a correlate of learning and memory) is impaired. Ablation in Schwann cells causes loss of DRP2 and dystrophin Dp116 complexes, abnormal myelination and node of Ranvier structure, and an evident peripheral neuropathy<sup>19</sup>. Ablation of dystroglycan in all embryonic tissues combines all features of the above to give a severe WWS-like phenotype (lissencephaly, microphthalmia, tremor, small size, congenital muscular dystrophy, high frequency of neonatal death)<sup>20</sup>. Constitutive heterozygosity for the *Dag1* null mutation seems to have no gross phenotypic impact on the mouse<sup>21</sup>.

We here describe a patient with a phenotype which is a subset of the symptoms of the WWS/MEB spectrum. She has a heterozygous *de novo* deletion which includes the *DAG1* gene. We discuss the implications of this case for the spectrum of dystroglycanopathic phenotypes.

## MATERIALS & METHODS

## **Cytogenetics**

DNA from the proband and her parents was tested for genome imbalance using oligonucleotide arrays with 44,000 probes across the genome (Agilent). Hybridization was carried out according to the manufacturer's recommendations, and the commercial analysis software was used, incorporating a 3-probe cut-off for imbalance flagging. The Database of Genomic Variants (DGV) (<http://projects.tcag.ca/variation/>)<sup>23</sup> was used to identify putatively benign copy number variants (CNVs).

## **Quantitative RT-PCR.**

Patient muscle RNA was prepared from 100 mg of vastus lateralis biopsy material by homogenising in 1 ml of QIAzol (RNA extraction kit, QIAGEN) and subjecting to RNA extraction according to the manufacturers' instructions. Human muscle RNA purchased from Ambion was used as a normal control sample. RNA was quantified using an RNA Nano Chip on an Agilent 2100 Bioanalyser (Agilent Technologies). The RNA samples were used in a quantitative nested RT-PCR using SYBR green detection in an ABI Prism 7000<sup>®</sup> Sequence Detection System (see Supplementary Information for primer sequences and detailed method). For each amplicon, measurements of threshold cycle (Ct) were used to infer initial template copy number when compared to Ct measurements of the respective serially diluted standard. Each datum plotted is the mean of three measurements ( $\pm$ SD) normalised to  $\alpha$ -dystrobrevin and with the normal muscle expression level set to 100%.

## **RESULTS**

We describe here a 16-year-old female patient with severe learning difficulties, facial hypotonia (Figure 1A), raised serum CK levels, oral-motor dyspraxia and subcortical

white matter anomalies (Figure 1B – see Supplementary Information for a comprehensive description). A vastus lateralis muscle biopsy taken at the age of seven years showed non-specifically abnormal findings with some atrophic fibres and type 1 fibre predominance; occasional staining for neonatal myosin suggested a small amount of fibre regeneration (Supplementary Figure 1). Immunohistochemical staining for  $\alpha$ -dystroglycan, laminins  $\alpha 2$  and  $\alpha 5$ , dystrophin, utrophin and  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -sarcoglycans appeared normal. No evidence of inflammation or necrosis was reported, and mitochondrial staining was normal.

Previous tests had revealed the patient to have a grossly normal 46, XX karyotype, and tests for subtelomeric deletion and the FRAXA, FRAXE, and DMPK expansions proved negative. Array CGH analysis of the proband's DNA, however, identified a heterozygous  $\sim 1.9$ -Mb deletion of material in the short arm of chromosome 3, band p21.31 as her only unusual copy number variation. The minimum extent of the deletion (as defined by the last deleted probes) is chr3:48286183-50219661 and the maximum extent chr3:48260361-50252191 (as defined by the first non-deleted probes; NCBI Build 36). The telomeric breakpoint therefore lies within the ZNF589 gene and the centromeric breakpoint in either SLC38A3 or GNAI2. A custom multiplex ligation-dependent probe amplification (MLPA) assay was used to independently confirm the deletion in the patient (probe site - 3:49126936); the MLPA assay also showed that the deletion was not present in either parent and has therefore occurred *de novo* in the proband. No clearly overlapping deletion was found on the DECIPHER database (<https://decipher.sanger.ac.uk>) or ECARUCA (<http://agserver01.azn.nl:8080/ecaruca/ecaruca.jsp>). The patient's details have been submitted to DECIPHER (patient number 251232).

Bioinformatic analysis of the maximally deleted region showed it to contain 62 protein-coding genes and three miRNA genes (see Supplementary Table 4 for a detailed assessment of each gene). Of these, six cause recessive disease in humans (with no reported

heterozygous null phenotype), a further six have normal or almost normal phenotypes in homozygous null mice, and a further ten have normal phenotypes in heterozygous null mice. Thus for 22 of the genes there is reason not to expect haploinsufficient phenotype in humans. For most of the remainder, a broad biochemical function is either known or inferred, but the specific organ system(s) in which they act is unknown. In addition, eight of the genes are deleted in putatively benign CNVs found in the normal population and can therefore also be discounted. However, in the approximate centre of the deleted region lies *DAG1*, whose conditional knockout in mouse and aberrant post-translational modification in humans causes symptoms strikingly reminiscent of our patient.

In order to assess the impact of a heterozygous deletion of the entire *DAG1* gene on *DAG1* transcript levels (especially given the evidence from *Dag1*<sup>+/-</sup> mice for a degree of transcriptional compensation), we performed quantitative RT-PCR on RNA extracted from the muscle biopsy material. This shows that normalised levels of DAG1 mRNA in the patient's muscle are approximately 60% of those from wild-type muscle (Figure 1D; p<0.01, two-tailed t-test). We were not able to accurately assess protein levels; immunohistochemistry using antibodies against  $\alpha$ -dystroglycan core protein,  $\alpha$ -dystroglycan glycosyl moieties (IIH6) and  $\beta$ -dystroglycan showed normal staining (see Supplementary Figure 2), consistent with the limited quantitative properties of this technique. There was insufficient material to perform a western blot, which would also be unlikely to distinguish between 100% and 60% of wild-type protein levels. **We cannot therefore ascertain the levels of dystroglycan protein in the patient.** In an attempt to rule out a recessive effect on the *DAG1* gene, we amplified and sequenced *DAG1* cDNA corresponding to the patient's remaining, intact, allele (see Supplementary Information). We found this to be normal in sequence and structure.

1

## DISCUSSION

To our knowledge this is the first description of a potentially pathogenic mutation of the *DAG1* gene in humans. The patient presented here is essentially heterozygous for a null mutation of *DAG1*, and this gives us an opportunity to assess the consequences of this for humans. Although the mouse *Dag1* null homozygote dies before implantation in the uterus, the corresponding heterozygote is described as phenotypically normal. Notably, analysis of

2 the heterozygote mouse shows that muscle dystroglycan protein levels are indistinguishable from normal (although western blots may have poor discrimination in the 1-to-2-fold range)

2 and that muscle *Dag1* transcript levels are ~80-90% of wild-type levels (by northern blot), suggesting some form of compensation<sup>21</sup>. In our human heterozygote we see a much lesser degree of compensation (~60% of normal), which may account for the observed phenotypic difference from the mouse. In addition, we note that compensation in the mouse may be

2 tissue-specific, that the relationship between transcript levels and protein levels is in turn likely to be tissue-specific and complex, and that a neurological phenotype of the type seen in our patient may not be so evident in the cursory analysis of the *Dag1*<sup>+/-</sup> mouse<sup>21</sup>.

A key question to be addressed in this case is the extent to which haploinsufficiency for *DAG1* is responsible for the patient's phenotype. The case for a major contribution by *DAG1* rests largely on the similarity between the patient's phenotype and a subset of the symptoms evinced by MEB/WWS patients. The myopathy, which in the latter is pronounced, congenital and global, is understandably milder in our patient, who has limited myopathic signs in her leg muscle (as shown by the histopathology of her muscle biopsy), frank muscle weakness only in her face and possibly postural muscles, and a chronically elevated serum CK level. Of the brain malformations seen in MEB/WWS, our patient has subcortical white matter abnormalities and ventricular dilatation<sup>9,10</sup>, but not the more severe structural anomalies such as lissencephaly. She also has considerable learning difficulties, with

significant speech delay compounded by oral-motor dyspraxia. Two other genes in the deleted region, namely *TREX1* and *LAMB2*, are linked to relevant phenotypes. Null mutations in *TREX1* give rise to recessive Aircardi-Goutière syndrome (OMIM 225750), which includes white matter anomalies; however these are invariably calcified, a feature excluded in our patient. Null mutations in *LAMB2* cause recessive Pierson syndrome (OMIM 609049), which includes severe muscle hypotonia associated with aberrant neuromuscular junctions; however none of the ocular or renal symptoms of Pierson syndrome are seen in our patient (although hemizyosity for *LAMB2* may exacerbate the consequences of hemizyosity for *DAG1*). Naturally we cannot exclude the possibility that the deletion may lead to haploinsufficiency or expression of a pre-existing recessive allele in one or several of the other hemizygous genes.

It is difficult to extrapolate one's expectations of a phenotype from a situation where a normal amount of core dystroglycan polypeptide is synthesised but it is all aberrantly glycosylated (WWS/MEB) to one where only 50-60% of the normal amount of dystroglycan is produced but it is all correctly glycosylated (this case). The two instances presumably reflect the consequences of qualitative and quantitative change, respectively (though aberrant glycosylation can also result in a quantitative reduction in the final steady-state levels of dystroglycan). Like other haploinsufficiency disorders, the detailed cell-autonomous outcome is also likely to be dependent on *cis* and *trans* genetic modifiers, environmental factors and local stochastic phenomena. In summary, the haploinsufficient phenotype seen here represents an interesting subset of the WWS/MEB pattern, in that learning difficulties, white matter anomalies and facial hypotonia are seen, but severe global myopathy, lissencephaly and eye defects are not. This is reminiscent of the respectively severe and mild effects of dystroglycan ablation in the brain and skeletal muscle of the mouse<sup>17,18</sup>, and a recent paper

suggests that similar combinations of severe dystroglycanopathy-like CNS features and mild skeletal myopathy are possible in humans<sup>24</sup>.

Although we cannot exclude effects from genes other than *DAG1* in this patient, this case places an upper bound on the severity of the phenotypic consequences of heterozygosity for null *DAG1* mutations in humans. We would suggest that the possibility of heterozygosity for null *DAG1* mutations should be investigated in other patients with this phenotype.

4

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## FIGURE LEGENDS

*Figure 1. A.* Facial features of the patient, aged 16 years, showing facial hypotonia with everted lower lip and protruding tongue. *B.* Axial magnetic resonance images of the patient's brain at age 5 years (upper panels) and 11 years (lower panels). T<sub>2</sub>-weighted (left hand panels) FLAIR (fluid-attenuated inversion recovery; right hand panels) images show mild ventricular dilatation and patchy, non-progressive high signal intensity changes in the subcortical white matter of both cerebral hemispheres (arrowheads), relatively symmetrical and slightly more marked in frontal regions. *C.* Overview of the deleted region, del(3)(p21.31p21.31)(48,286,183-50,219,661)dn (solid line – minimum deletion; dashed

lines – maximum deletion). The 65 genes (short horizontal lines) are listed in detail in Supplementary Table 4. The *DAG1* gene (bold), seven genes implicated in human genetic disease, and a putatively benign CNV are labelled. **D.** Levels of dystrophin and dystroglycan transcripts in normal and patient muscle. Columns show means of three measurements ( $\pm$ SD) normalised to  $\alpha$ -dystrobrevin and with the normal muscle expression level set to 100%.

Asterisk:  $p < 0.01$ .

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Figure 1

