

## ORIGINAL ARTICLE

# POMT2 mutations cause $\alpha$ -dystroglycan hypoglycosylation and Walker-Warburg syndrome

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**Background:** Walker-Warburg syndrome (WWS) is an autosomal recessive condition characterised by congenital muscular dystrophy, structural brain defects, and eye malformations. Typical brain abnormalities are hydrocephalus, lissencephaly, agenesis of the corpus callosum, fusion of the hemispheres, cerebellar hypoplasia, and neuronal overmigration, which causes a cobblestone cortex. Ocular abnormalities include cataract, microphthalmia, buphthalmos, and Peters anomaly. WWS patients show defective O-glycosylation of  $\alpha$ -dystroglycan ( $\alpha$ -DG), which plays a key role in bridging the cytoskeleton of muscle and CNS cells with extracellular matrix proteins, important for muscle integrity and neuronal migration. In 20% of the WWS patients, hypoglycosylation results from mutations in either the protein O-mannosyltransferase 1 (*POMT1*), *fukutin*, or *fukutin related protein (FKRP)* genes. The other genes for this highly heterogeneous disorder remain to be identified.

**Objective:** To look for mutations in *POMT2* as a cause of WWS, as both *POMT1* and *POMT2* are required to achieve protein O-mannosyltransferase activity.

**Methods:** A candidate gene approach combined with homozygosity mapping.

**Results:** Homozygosity was found for the *POMT2* locus at 14q24.3 in four of 11 consanguineous WWS families. Homozygous *POMT2* mutations were present in two of these families as well as in one patient from another cohort of six WWS families. Immunohistochemistry in muscle showed severely reduced levels of glycosylated  $\alpha$ -DG, which is consistent with the postulated role for *POMT2* in the O-mannosylation pathway.

**Conclusions:** A fourth causative gene for WWS was uncovered. These genes account for approximately one third of the WWS cases. Several more genes are anticipated, which are likely to play a role in glycosylation of  $\alpha$ -DG.

Walker-Warburg syndrome (WWS) is a congenital disorder characterised by multiple anomalies of the brain, muscle, and eye. This combination of malformations is also found in muscle-eye-brain disease (MEB) and in Fukuyama congenital muscular dystrophy (FCMD). The most severe anomalies are seen in WWS patients, especially with regard to the brain malformations. Brain malformations typical of WWS are agyria, agenesis of the corpus callosum, cerebellar vermis and septum, and occasional occipital encephalocele.<sup>1–3</sup> The pathogenesis underlying WWS, MEB, FCMD, and other congenital muscular dystrophies (CMD) such as congenital muscular dystrophy 1C (MDC1C) and 1D (MDC1D) involves functional disruption of  $\alpha$ -dystroglycan ( $\alpha$ -DG) by mutations in genes that are involved in O-mannosylation of this protein.<sup>4–10</sup> The O-linked carbohydrate chains of  $\alpha$ -DG are an important component of the dystrophin glycoprotein complex which mediates the interaction between the extracellular matrix and the cytoskeleton of muscle cells and neurones.<sup>11–15</sup> A common O-mannosyl glycan structure found on  $\alpha$ -DG is NeuAc $\alpha$ 2-3Gal $\beta$ 1-4GlcNAc $\beta$ 1-2Man-Ser/Thr.<sup>16, 17</sup> The first step in the synthesis of this glycan structure takes place in the endoplasmic reticulum by one or more proteins from a family of well conserved protein O-mannosyltransferases (PMTs). These proteins catalyse the transfer of a mannose from dolichyl phosphate activated mannose to serine or threonine residues of secretory proteins. In yeast, a family of seven PMTs (Pmt1p to Pmt7p) are known. Double and triple mutants of the PMTs in yeast indicate that proper

O-mannosylation is required for its cell wall rigidity and cell integrity.<sup>18</sup> In *Drosophila* and human there are only two orthologues: *POMT1* and *POMT2*.<sup>16</sup> RNAi knockdown of either *POMT1* or *POMT2* in *Drosophila* causes a rotation of the abdomen by 30–60°, demonstrating the requirement of both *POMT1* and *POMT2* for normal muscle development in the fly.<sup>19</sup> No embryonic nervous system or eye abnormalities were observed for *POMT1* knockouts.<sup>20</sup> In addition, it has been shown that in humans *POMT1* and *POMT2* are also both required for O-mannosyltransferase activity.<sup>19, 21</sup> Previously we have shown that mutations in either *POMT1*, *FCMD*, or *FKRP* result in hypoglycosylation of  $\alpha$ -DG, giving rise to the autosomal recessive disorder WWS.<sup>22–24</sup> The phenotypic similarity seen in the fly knockdown of *POMT1* and *POMT2* and the simultaneous requirement for both proteins to obtain O-mannosyltransferase in fly as well as human made us reconsider *POMT2* as candidate gene for WWS. In this study we show that *POMT2* mutations also cause WWS.

## METHODS

### Genetic analysis

Using standard methods we extracted DNA from peripheral blood lymphocytes. Genome-wide homozygosity mapping was carried out at our linkage facility using the 10 cM spaced

**Abbreviations:**  $\alpha$ -DG,  $\alpha$ -dystroglycan; CMD, congenital muscular dystrophy; FCMD, Fukuyama congenital muscular dystrophy; MEB, muscle-eye-brain disease; PMT, protein O-mannosyltransferase; WWS, Walker-Warburg syndrome

**Table 1** Primer sequences and conditions for PCR reaction

Exon	Forward primer	Reverse primer	Product length (bp)	PCR annealing temperature (°C)
1	ggagttgcagttccctgagc	taccctcgggccaatcagag	609	58
2	ccatgctttatgaaggcatttg	tggctccagcccttaggaac	224	58
3	gcagctggaggggaagttcag	ttagttggccccaggggttc	235	58
4	tcatcaggctccctgtcttaaatg	gggcccctgaatgtactgatttc	294	58
5	gtttcctactactgggtgcttg	acagaatfttggagttgccacag	250	58
6	aagacagggcacagcacagc	ccagaacacagccactctgc	384	58
7	ggctggcccatgttalcctg	aggggtgtggccttctgag	502	58
8	tcctaccaccactctgtcc	ctttccaccctgtccatc	339	58
9	ttagtgagccctgtgcttc	tgtcatggcgaacagcattg	255	58
10	tggctgggattctgaaattg	tcagcaaacccatctcag	304	58
11	ggggctttcctttgtgtctc	tgtggcctgtccattgac	353	58
12	ggtttgggtcactctctcc	tcctgtgcagcctctatcc	403	58
13	gccatttcccttctgacacg	gcagacagcagggaacacag	334	58
14	ggaaaagagaaggagcctgtgg	cggaggagtgatgagaag	386	58
15	ctgggtgggaatgtggacacc	attcatggctgcccaaaagc	214	58
16	ctgggcccacaattctgtc	cggctcctctctgttctc	203	58
17	tctccccctaatgggtgtg	ggaatgggcagatgagaacg	207	58
18	gcgttctcatctgccattc	gggtgtaaaccaaggaatgg	246	58
19	acagcaaggaaggccagag	tgtctgtctcccaagtcag	263	58
20	cctggctgactccagggttc	acactgggagggcatgtgag	185	58
21	agctccagcaggaggaatgg	ttcagctgcactccagag	342	58

microsatellite marker set from Applied Biosystems (ABI Prism linkage mapping set version 2, Applied Biosystems, Foster City, California, USA) and at MRC geneservice in Cambridge with use of the GeneChip Mapping 10K 2.0 array from Affymetrix (Santa Clara, California, USA). Additional homozygosity mapping for *POMT2* was undertaken by polymerase chain reaction (PCR) of the flanking microsatellite markers D14S279, D14S983, and D14S59, which were subsequently resolved on 8% polyacrylamide sequencing gels and developed by silver staining.

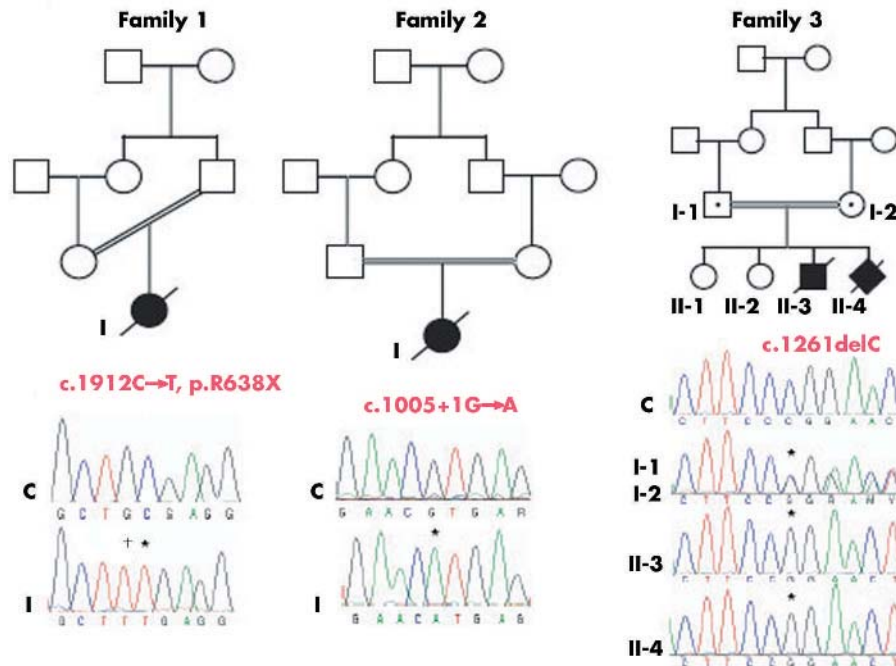
**Mutation analysis**

All 21 exons of the *POMT2* gene were amplified using specific primers for the 5'- and 3'-flanking intron sequences. Primers and PCR conditions are given in table 1. After purification from agarose gels, the PCR products were used for direct

sequencing using the BigDye terminator kit (Perkin Elmer Applied Biosystems, Norwalk, Connecticut, USA), which were analysed on an ABI3700 capillary sequencer. The presence of the identified mutations in the normal population was tested by restriction enzyme analysis in chromosomes from control individuals. For this, the relevant amplicon was digested with *TaqI* (c.1912C→T), *HpyCH4 IV* (c.1005+1G→A), and *NciI* (c.1261delC) (New England BioLabs, Beverly, Massachusetts, USA).

**Immunohistochemistry**

Muscle biopsies from control and WWS patients were obtained after informed consent of patients and approval of the ethics commission. Myoblast cell cultures were established by enzymatic and mechanical treatment of muscle biopsies and by plating in Dulbecco's modified Eagle's



**Figure 1** *POMT2* mutations in families with Walker-Warburg syndrome (WWS). For each of the families with *POMT2* mutations the pedigrees and DNA sequencing results are shown. Mutations are indicated with an asterisk (\*). Patient 1 is homozygous for a nonsense mutation in exon 19 (c.1912C→T, p.R638X). The mutation in this patient is preceded by a known polymorphism indicated by a dagger (†) sign. Patient 2 is homozygous for a mutation in intron 8 (c.1005+1G→A) which is predicted<sup>32</sup> to result in the disruption of the donor site for intron splicing of intron 8. This mutation also disrupts a restriction site for the *HpyCH4 IV* enzyme and was not present in 100 chromosomes of controls. Patient 3 is homozygous for a deletion of 1 bp in exon 12 (c.1261delC) which tends to a premature stop codon (p.T433X). The same mutation was found heterozygously in both parents and homozygously in an affected sibling in this family.

medium plus fetal calf serum (FCS), penicillin, streptomycin, and amphotericin B (Sigma, Poole, Dorset, UK).<sup>25</sup> Myotubes were obtained by confluent myoblast cultures allowed to differentiate for seven days in cultures medium. Samples for immunohistochemical analysis were fixed in 2% paraformaldehyde in phosphate buffer saline (PBS) and incubated overnight with an anti- $\alpha$ -dystroglycan monoclonal antibody (VIA4-1, Upstate Biotechnology, Lake Placid, New York, USA) diluted 1/50, washed with PBS and then with an anti-mouse IgG TRITC conjugated antibody (Dako, Glostrup, Denmark). The same sample was then incubated with a polyclonal anti-caveolin 3 antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) diluted 1/50 followed by FITC conjugated anti-goat antibody. The sample was washed in PBS, mounted in Pro-long anti-fade reagent (Molecular Probes, Eugene, Oregon, USA), and examined with a Nikon epifluorescence microscope at a magnification of  $\times 100$ .

Immunohistochemistry on muscle biopsies was carried out as described by Jimenez-Mallebrera *et al.*<sup>26</sup> In Brief, frozen 8  $\mu$ m sections were incubated with primary monoclonal antibodies to  $\beta$ -DG (NCL-b-DG, Novocastra Laboratories, Newcastle upon Tyne, UK),  $\alpha$ -DG (IIH6, Upstate Biotechnology), and sheep polyclonal antibody recognising the core protein of  $\alpha$ -DG.<sup>27</sup> These were then revealed with an appropriate biotinylated secondary antibody (Amersham 1:200; Amersham Life Sciences, Amersham, UK) followed incubation with streptavidin conjugated to Alexa 594 (Molecular Probes) and visualised by epifluorescence microscopy.

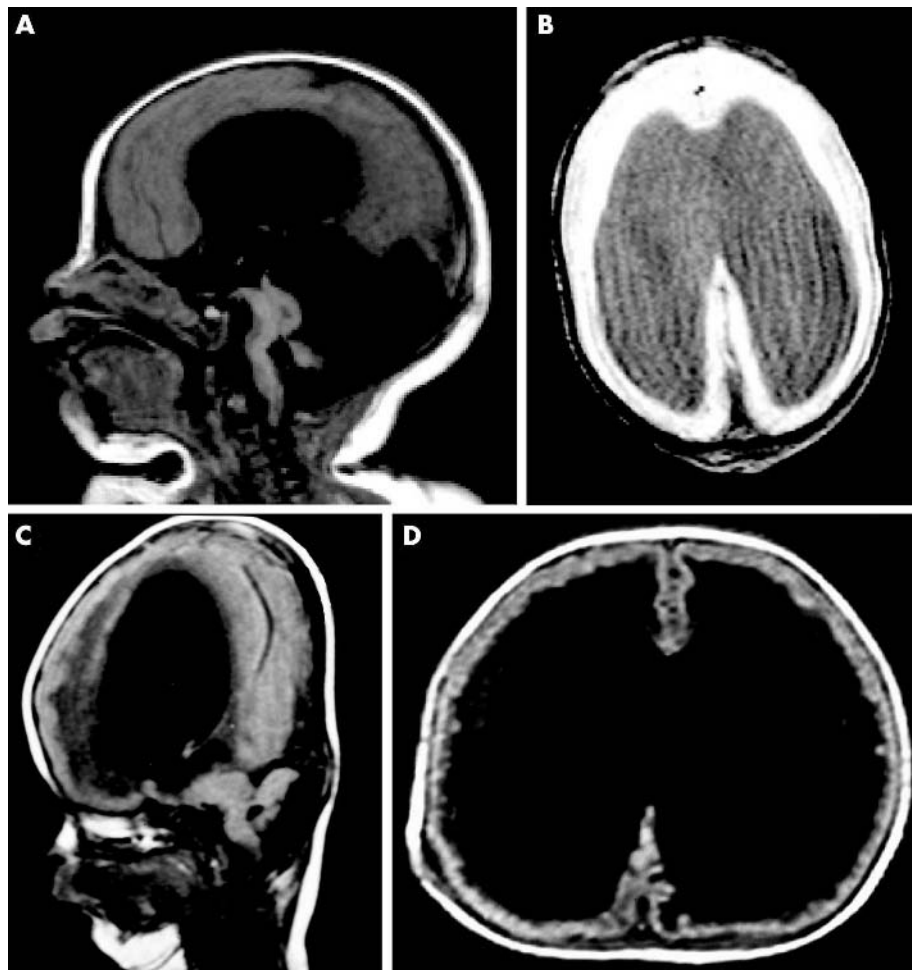
## RESULTS

### Direct linkage mapping of candidate genes using genome-wide homozygosity data

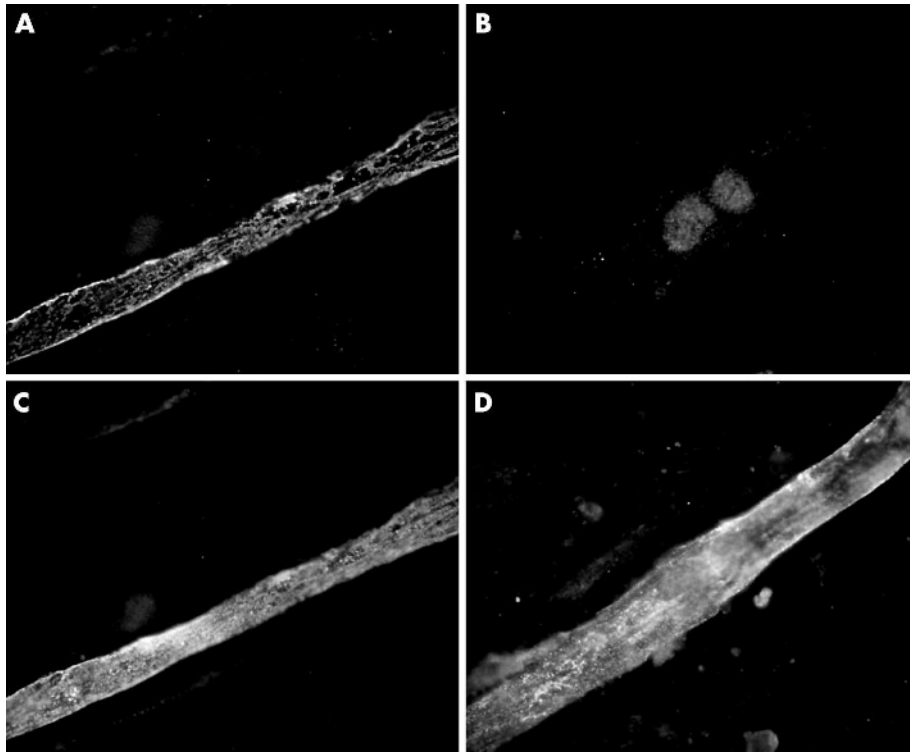
We carried out genome-wide homozygosity mapping of 12 WWS patients from 11 unrelated consanguineous families in which linkage to the known WWS loci (*POMT1*, *Fukutin*, and *FKRP*) has been excluded. Depending on the amount of DNA available we used the 10 cM spaced microsatellite marker set (ABI Prism linkage mapping set version 2) or the GeneChip Mapping 10K 2.0 Array (Affymetrix). As can be expected from the genetic heterogeneity of WWS, these homozygosity mapping results of mostly singletons failed to point to a single WWS locus and indeed indicated further genetic heterogeneity. Although we failed to detect *POMT2* mutations in an earlier study of 24 WWS families,<sup>22</sup> we targeted *POMT2* as a candidate gene based on the premise that all three previously identified WWS genes—*POMT1*, *fukutin* and *FKRP*—are involved in O-mannosyl glycan synthesis. A similar function is likely for *POMT2*, based on the overlapping expression profiles and homologous amino acid composition compared with *POMT1*. In addition, recent reports indicate that *POMT2* is required for the enzymatic activity of *POMT1* in human as well as *Drosophila*.<sup>19–21</sup> Our new mapping data indicated possible linkage to the *POMT2* locus in four of 11 unrelated WWS families.

### Mutation analysis of *POMT2*

Mutation analysis in all four families led to the identification of mutations in two of these, a nonsense mutation (c.1912C $\rightarrow$ T, p.R638X) in patient 1 and a splice site mutation



**Figure 2** Brain magnetic resonance imaging of patient 1 at two days of age (A and B) and patient 2 at 16 months of age (C and D). Both patients show hydrocephalus, dilatation of the ventricles, agyria, cerebellar hypoplasia, and absence of the corpus callosum.



**Figure 3** Immunofluorescence analysis of cultured myotubes obtained from a control individual (A and C) and patient 2 (B and D). A double labelling was carried out with anti- $\alpha$ -DG glycoepitope antibody (VIA4-1) (A and B), and anti-caveolin3, a marker of muscle differentiation (C and D). These were visualised using an anti-mouse TRITC conjugated secondary antibody and an anti-goat FITC conjugated antibody, respectively. In control myotube,  $\alpha$ -DG labelling co-localises with caveolin3 at the sarcolemma. In the WWS patient myotube, caveolin 3 was expressed with a pattern similar to the control myotube while  $\alpha$ -DG staining was severely reduced. Magnification  $\times 500$ .  $\alpha$ -DG,  $\alpha$ -dystroglycan; WWS, Walker-Warburg syndrome.

(c.1005+1G→A) in patient 2, both found in homozygosity. Both mutations result in the disruption of a restriction site for *Taq1* and *HpyCH4 IV*, in exon 19 and exon 8, respectively. These disruptions were not observed in control individuals digested with *Taq1* (170 chromosomes) and *HpyCH4 IV* (290 chromosomes). No mutations were detected in the *POMT2* exons in the other two families that showed linkage to the *POMT2* locus. Reasons for this could be that their linkage was a fortuitous finding or that a mutation resides in parts of the *POMT2* gene such as an intron or regulatory elements that have not been analysed. An additional six consanguineous WWS families were then tested for homozygosity at the *POMT2* locus. In two of these WWS families the data were consistent with linkage to *POMT2*. We identified a homozygous 1 base pair (bp) deletion (c.1261delC) in one of these families. This mutation introduces a premature stop codon (p.T433X). The mutation was also homozygously present in an affected sibling and disrupts one of the two restriction sites for *NciI* in exon 12, which was used to verify the absence of this mutation in 140 control chromosomes from the normal population. Pedigrees and DNA sequencing results of the three *POMT2* mutated families are shown in fig 1.

#### Clinical description WWS patients with *POMT2* mutations

Patient 1 is a girl born to uncle-niece consanguineous Moroccan parents. A prenatal ultrasound indicated hydrocephalus which was confirmed by magnetic resonance imaging, which also showed type II lissencephaly (fig 2, panels A and B). At birth at 37 weeks gestation, weight was 3150 grams, and head circumference 36 cm. The child was severely hypotonic. Ophthalmological examination showed bilateral Peters anomaly with cataracts, left sided microphthalmia, and right sided buphthalmos. The creatine kinase was greatly raised at 12 144 U/l. MRI of the brain documented hydrocephalus, cobblestone lissencephaly, and aplasia of the corpus callosum.

Patient 2 is a male child born to first cousin Pakistani parents. Severe hydrocephalus was diagnosed prenatally on ultrasound and the child was delivered by caesarean section. A left sided cleft lip and palate was diagnosed. Imaging of the brain by MRI revealed aplasia of the posterior vermis, hypoplasia of the pons and cerebellum, and severe hydrocephalus and cobblestone cortex (fig 2, panels C and D). Ophthalmological investigation documented bilateral cataracts and persistent pupillary membrane. The retina could not be visualised. A muscle biopsy was consistent with a diagnosis of congenital muscular dystrophy. There was increased variability of fibre diameter, increased endomysial fibrosis, and basophilic regeneration. External genitalia were normal. The child died at the age of six months.

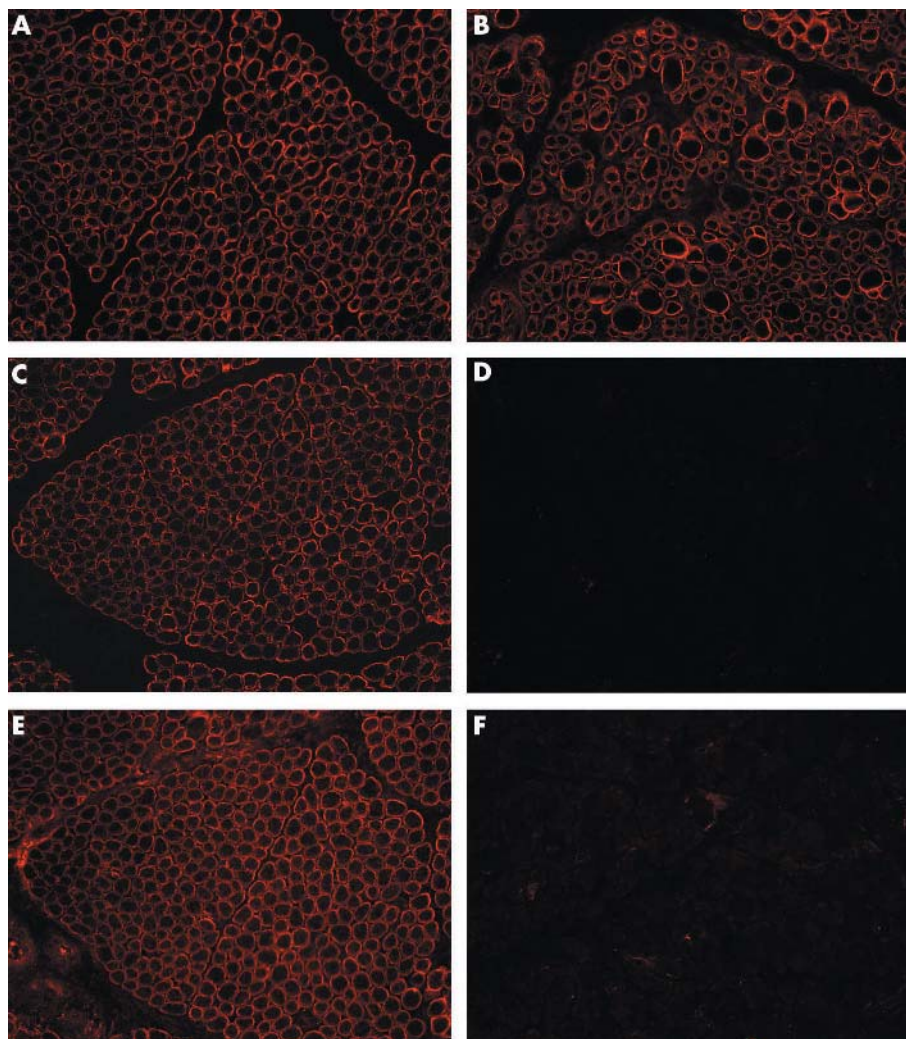
Patient 3 is a male infant referred at the age of two months and one week because of severe neonatal hypotonia, developmental delay, and poor visual behaviour, as previously reported.<sup>26</sup> He was the third child of a consanguineous Bengali family. His older siblings were healthy. Congenital hydrocephalus required a shunt at two weeks of age. Serum creatine kinase was markedly raised. Ophthalmological examination showed bilateral lamellar cataracts, and buphthalmos caused by anterior chamber anomalies. He had no head control and no apparent response to visuo-aoustical stimuli. Brain MRI at the age of five weeks showed severe hydrocephalus with a thin and smooth cortical mantle. The patient died at eight months of age during a respiratory infection.

A further sibling was diagnosed prenatally with severe hydrocephalus at 18 weeks' gestation. This pregnancy was subsequently terminated.

#### Histopathology of WWS muscle tissue

Immunolabelling of the glycoepitope of  $\alpha$ -DG with the VIA4-1 antibody<sup>28</sup> in cultured myotubes from patient 2 showed severely reduced staining compared with control myotubes (fig 3, panels A and B). Control labelling with anti-caveolin3, a marker of muscle differentiation, showed that  $\alpha$ -DG





**Figure 4** Muscle biopsy of control muscle (A, C, and E) and patient 3 (B, D, and F). Immunostaining with antibodies to  $\beta$ -dystroglycan ( $\beta$ -DG) was normal in both patient and control muscle (A and B). Immunostaining with antibodies that recognise core  $\alpha$ -dystroglycan ( $\alpha$ -DG) (C and D) and a glycosylated epitope (I1H6) of dystroglycan (E and F) showed normal expression in control but marked reduction of staining in the patient.

co-localises with caveolin3 at the sarcolemma of myotubes derived from a control individual (fig 3C). Similar normal staining was observed in myotubes derived from patient 2 (fig 3D). Reduced staining of  $\alpha$ -DG was also observed in patient 3 by immunolabelling of the core and glyco-epitope of  $\alpha$ -DG (fig 4, panels D and F). Normal staining was observed for  $\alpha$ -DG in control muscle (fig 4, panels C and E).  $\beta$ -DG staining was normal in the patient as well as in the control (fig 4, panels A and B). Reduction of immunolabelling of  $\alpha$ -DG and a mild reduction of laminin- $\alpha$ 2 in muscle tissue from patient 3 were described previously by Jimenez-Mallebrera *et al.*<sup>26</sup> No muscle tissue was available for patient 1.

## DISCUSSION

We have detected three homozygous mutations in *POMT2* in three families with typical WWS. The mutations included a nonsense mutation resulting in a premature stop codon, a splice site mutation, and a 1 bp deletion leading to a premature stop codon. All mutations were homozygous in the patients (fig 1). The phenotype seen in these WWS patients is indistinguishable from that of patients with *POMT1*, *FCMD*, or *FKRP* mutations. One of the sibpair from family 3 who carries a homozygous 1 bp deletion mutation was described earlier, and hypoglycosylation of  $\alpha$ -DG (fig 4) and a possible reduction of laminin- $\alpha$ 2 in muscle tissue was documented.<sup>26</sup> Absence of glycosylated  $\alpha$ -DG was also seen in a muscle biopsy from patient 2 in the present study (fig 3).

This is consistent with the postulated role of O-glycosylation in normal neuromuscular, brain, and eye development.<sup>11 29</sup>

Although previously no enzymatic activity of *POMT1* and *POMT2* was determined in vertebrates, the involvement of *POMT1* in WWS, the high amino acid identity between the two paralogues, and the overlapping expression pattern was reason to hypothesise that mutations in *POMT2* also give rise to WWS.<sup>30</sup> For this reason we previously undertook mutation analysis of *POMT2* in 24 unrelated patients but no causative mutations were detected.<sup>22</sup> In this study, 17 additional families were investigated, of which six showed possible linkage to the *POMT2* locus. We found *POMT2* mutations in three of these (fig 1), resulting in a frequency of 7% (3 of 41 families). Thus the incidence of *POMT2* mutations appears to be in the same range as that of *POMT1*. *POMT1* mutations were previously detected at a frequency of 20% and 7% in two large samples of WWS patients.<sup>22 31</sup> This is in accordance with the requirement of both proteins to obtain O-mannosyltransferase activity.<sup>19 21</sup> So far, mutations in *POMT1*, *POMT2*, *fukutin*, and *FKRP* together explain almost one third of the WWS patients in our cohort. The majority of WWS cases remain unexplained and further genetic heterogeneity is likely from our genome-wide homozygosity data.

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