Mini Review

Glyc-O-genetics of Walker–Warburg syndrome


Walker–Warburg syndrome (WWS) is the most severe of a group of multiple congenital anomaly disorders known as the cobblestone lissencephalies. These are characterized by congenital muscular dystrophy in conjunction with severe brain malformation and ocular abnormalities. In the last 3 years, important progress has been made towards the elucidation of the genetic causes of these disorders. Mutations in three genes, POMT1, fukutin and FKRP, have been described for WWS, which together account for approximately 20% of patients with Walker–Warburg. It has become evident that some of the underlying genes may cause a broad spectrum of phenotypes, ranging from limb girdle muscular dystrophy type 2I to WWS. In some cases, a genotype–phenotype correlation can be recognized. In line with the known or proposed functions of the resolved genes, all patients with cobblestone lissencephaly show defects in the O-linked glycosylation of the glycoprotein α-dystroglycan. Perhaps, the missing genes underlying the remainder of the unexplained WWS patients have also to be sought in the pathways involved in O-linked protein glycosylation.

Walker–Warburg syndrome (WWS; MIM 236670) was named after Walker, who reported the first patient with lissencephaly (smooth brain) in 1942 (1), and after Warburg who proposed its autosomal recessive inheritance (2, 3). Patients with this rare developmental disorder have a life expectancy of less than 3 years (average 0.8 years). Numerous congenital anomalies have been reported, but the most consistent are malformations of three structures: muscle, eye and brain. This triad distribution of developmental defects is also found in muscle-eye-brain disease (MEB; MIM 253280) and Fukuyama congenital muscular dystrophy (FCMD; MIM 253800), which together with WWS comprise the group of the cobblestone lissencephalies. The clinical features of WWS are most severe, especially with regard to the brain phenotype, which may be diagnosed prenatally (4). Typical brain anomalies include hydrocephalus, neuronal over-migration, causing a cobblestone cortex, lissencephaly, agenesis of the corpus callosum, fusion of the hemispheres, dilatation of the fourth ventricle, cerebellar hypoplasia and occasionally occipital encephalocele (Fig. 1) (5). In addition to the brain phenotype, patients can have a number of congenital ocular abnormalities, such as cataract, microphthalmia, buphthalmus, persistent hyperplastic primary vitreous and Peters anomaly. WWS patients have little motor activity due to the severe congenital muscular dystrophy (2–4). Serum creatine kinase levels are usually well above 1000 U/l. Differentiation between WWS, MEB and FCMD can be made by examination of the brain architecture using imaging techniques like MRI and CT-scanning or by post-mortem examination (Fig. 1). The structural eye defects in FCMD patients are generally mild if present at all, which distinguishes these patients from WWS and MEB patients (5, 6). On the basis of familial co-occurrence, it was initially suggested that WWS and FCMD are allelic disorders (7), but subsequent genetic analyses contradicted that hypothesis (5). It was demonstrated that the three types of cobblestone lissencephaly have a different genetic causation, because a number of
WWS families did not map to the known loci for FCMD at 9q31 and MEB at 1p34-p33 (8, 9). Ironically, now that we know more about the causative genes for cobblestone lissencephaly, it becomes evident that allelism between these three syndromes does occur, albeit rarely.

**WWS genetics**

It is typical for rare autosomal recessive disorders that most cases originate from populations with a high consanguinity rate. One clear conclusion from attempts at homozygosity mapping for WWS is that the disease is genetically heterogeneous (10). This suggests that WWS is the result of malfunctioning of a molecular complex or pathway and not of a single gene disruption. The heterogeneity has precluded the identification of WWS genes by a positional cloning strategy. For this reason, a functional candidate gene approach combined with directed linkage mapping seems to be the only good alternative to identify one or more genes underlying WWS. Clinical similarity with MEB, and the notion that the POMGnT1 gene encodes a glycosylation enzyme, led to the identification of the first causative gene in WWS (10), the POMT1 gene on chromosome 9q34. Mutations in this gene account for 6/30 WWS patients (20%). Further evidence for genetic heterogeneity in WWS came with the identification of mutations in two other genes. Homozygous nonsense mutations were identified in the fukutin gene in two unrelated Turkish families (11, 12). The fukutin gene was tested in these families, because it is the causative gene in FCMD. The vast majority of FCMD patients are homozygous for a mild founder mutation in the Japanese population that arose about 3000 years ago (13, 14). In contrast, the WWS fukutin mutations appear to be more severe. Fukutin mutations probably contribute very little to the prevalence of WWS. In our series, only three of 30 consanguineous WWS families mapped to this locus and a fukutin mutation was identified in only one of them (12). The third known WWS gene is FKRP, which encodes the fukutin-related protein. Mutations in FKRP have also been detected in a number of congenital muscular dystrophies with variable severity (15–17). The clinical spectrum associated with FKRP mutations was broadened by the identification of a homozygous missense mutation in a WWS patient of Pakistani origin (18). The patient presented typical WWS features and was previously included in a clinical review of MEB and WWS by Cormand et al. (5). Again, mutations in the FKRP gene account for only a marginal number of WWS patients as only two of 29 consanguineous patients in our series showed homozygosity at the FKRP locus and mutations were found in only one of those two (18).
In our experience, causative mutations in the three known WWS genes are found in eight of 40 (20%) patients (10, 12, 18) (van Reeuwijk, unpublished data). Genome-wide linkage studies in the unexplained families are still in progress, and the number of remaining loci remains unclear. Given the lack of overlapping linkage intervals between individual families, it is entirely possible that three or more genes remain to be identified in WWS (van Reeuwijk, unpublished data).

WWS and related disorders: is there a genotype–phenotype correlation?

The clinical spectrum arising from mutations in genes involved in cobblestone lissencephaly is much wider than previously envisaged. There are several factors that may account for this: (i) the function or step in O-linked glycosylation that is catalyzed by the corresponding protein; (ii) the presence of complementing genes; (iii) the severity of the mutation. Information about the first two possibilities is provided in the next section; in this paragraph, we elaborate on the third possibility by providing some examples of a genotype–phenotype correlation.

The clearest genotype–phenotype correlations are seen for fukutin gene mutations. The Japanese founder mutation is a 3-kb retrotransposon insertion in the 3′ UTR, which results in reduced levels of fukutin mRNA, yet with the capacity to produce a normal protein (19). Patients who are homozygous for this retrotransposon mutation have the typical FCMD phenotype. The phenotype is significantly more severe in compound heterozygotes carrying the insertion in combination with a nonsense or missense mutation in the other allele (13, 19, 20). WWS is at the most severe end of the phenotypic spectrum and is caused by homozygous nonsense mutations in the fukutin gene (11, 12).

A much wider phenotypic spectrum is observed for mutations in the FKRP gene. Mutations in FKRP were first described by Brockington and coworkers in patients with a severe form of congenital muscular dystrophy, denoted MDC1C (15), and in the much milder limb girdle muscular dystrophy type 2I (LGMD2I) (16). The clinical manifestation of the muscular dystrophy in LGMD2I patients is quite variable, and homozygous carriers of FKRP mutations can be asymptomatic at older age (21). Clinical variability is also seen at the severe end of the spectrum. A patient was reported with severe congenital muscular dystrophy and mental retardation, cerebellar cysts and other cerebellar abnormalities (17). Subsequently, FKRP mutations were identified in the gradually more severe MEB and WWS phenotypes. A genotype–phenotype correlation is not obvious for the FKRP mutations that are found in the various disorders. Most of the FKRP mutations create amino acid substitutions and their functional consequences are hard to predict owing to a lack of knowledge about the function of FKRP protein. In some instances, the effects of missense mutations can be inferred from the associated phenotypes. For example, the homozygous mutation in FKRP (Tyr307Asn) that was found in an MEB patient was previously found in heterozygosity in patients suffering from LGMD2I. The less severe mutation in the second allele, Leu276Ile, is a common mutation in patients with LGMD2I and often found in homozygosity in them causing a relatively mild form of LGMD2I. In contrast, the patient with a compound heterozygous mutation of Tyr307Asn and Leu276Ile suffered from quite severe LGMD2I and died in his late teens, suggesting that the Tyr307Asn mutation is more detrimental for FKRP function than is the Leu276Ile (18).

The final example of a genotype–phenotype correlation is provided by mutations in the POMGnTI gene. MEB is most prevalent in the Finnish population due to a founder mutation in the POMGnTI gene (22). Recently, Toda and coworkers reported a much wider clinical spectrum associated with POMGnTI gene mutations, which were identified in patients from all over the world. The mutant POMGnTI phenotype varied from severe FCMD to MEB to a WWS-like phenotype, which was less severe than classical WWS. A slight correlation was observed between the clinical severity of the brain phenotype and the location of the mutations in this gene. Mutations near the 5′-terminus of the POMGnTI-coding region tend to show more severe brain abnormalities, including hydrocephalus, compared to patients with mutations more downstream who do not have hydrocephalus (Fig. 3). No differences were observed for the skeletal muscle in which (in both cases) staining of the α-dystroglycan (α-DG) was highly reduced (23).

As we have seen, mutations in three of the genes involved in cobblestone lissencephaly give rise to a wide phenotypic spectrum, with or without a clear genotype–phenotype correlation (Figs 2 and 3). We also hypothesize that mild mutations in POMT1 might result in the clinically milder disorders such as MEB or FCMD. Indeed, there are still several such patients without a mutation in POMGnTI, fukutin or FKRP. Clinical variability remains to be explored.
for mutations in the LARGE gene. Compound heterozygous mutations in the human LARGE gene have been identified in a single patient with a novel type of congenital muscular dystrophy (MDC1D) and severe mental retardation (24). Interestingly, the Large\textsuperscript{myd} mouse, which carries an intragenic deletion of exon 4–7 in the LARGE gene, has a phenotype that is strikingly similar to the FCMD, MEB and WWS phenotypes in humans (25–27).

Abnormal glycosylation of ς-DG is a key feature of the cobblestone lissencephalies

The POMT1 gene was selected as a functional candidate for WWS, because of its anticipated

Fig. 2. Overview of the genotype–phenotype correlation seen in the cobblestone lissencephalies. The mutations in the genes resulting in the different disorders are depicted by + (rare) and ++ (common). The Large\textsuperscript{myd} mouse phenotype resembles a muscle-eye-brain disease (MEB)/Fukuyama congenital muscular dystrophy (FCMD) phenotype in humans represented by an asterisk (*). \textit{POMGnT1} mutations have been found in patients diagnosed as mild Walker–Warburg syndrome (WWS) and severe FCMD, because their phenotypic characteristics precluded an unequivocal classification into either of these categories.

Fig. 3. Schematic representation of the proteins involved in cobblestone lissencephalies. The type and position of the mutations found for each of the disorders is indicated: the colours represent the different disorders and the symbols represent the different kind of mutations following the code given in the box at the right. Compound heterozygosity (ch) is only indicated when associated with an intermediate phenotype. Note that the same missense mutation in POMGnT1 (E223K) in combination with a truncating mutation at the 5′ end (F149fs) gives rise to a more severe phenotype than in combination with a truncating mutation at the 3′ end (IVS17+1G>T/A). Protein domains are: PMT, protein mannosyl transferase domain; GnT-1, N-acetylgalcosaminyltransferase domain; MIR, mannosyltransferase-IP3R-RyR domain; SS, signal sequence; TM, transmembrane domain; DxD, Asp, Xaa, Asp motif.
role in the post-translational O-linked glycosylation of α-DG (10). Currently, there is compelling evidence that shows that the lack of O-linked glycosylation of α-DG is the key defect in WWS and other cobblestone lissencephalies (28–33). The first line of evidence is genetics. POMT1 is predicted to catalyze the first step in the O-linked protein mannosylation, the transfer of a mannosyl residue from dolichyl phosphate mannose to a serine or threonine residue in the target protein (34, 35). POMGnT1 takes care of the second step by adding an N-acetylglucosamine residue to a pre-existing protein O-linked mannose (36). The α-DG protein is the only known substrate for this type of glycosylation in mammals. The second line of evidence is provided by immunohistochemical and functional analyses of α-DG in material obtained from patients and animal models. There are many reports of strongly reduced or complete lack of glycosylation of α-DG in material from patients with WWS, MEB, FCMD and related congenital muscular dystrophies (10, 15–17, 24, 27, 37–42). In addition, it was demonstrated that the brain-specific deletion of α-DG gives rise to many of the brain malformations that are also seen in WWS, including disorganization of the cortical layers, fusion of cerebral hemispheres, discontinuous pial surface and abnormal migration of post-mitotic neurons (26). Finally, also the Large<sub>myd</sub> mouse has altered glycosylation of α-DG (25, 27, 43).

DG plays a key role in bridging the cytoskeleton of cells with extracellular matrix (ECM) proteins. DG consists of two subunits, α-DG and β-DG, which are obtained upon proteolytic cleavage of a precursor protein encoded by a single gene, DAG1. DG connects the cytoskeleton to the ECM in a number of tissues. In muscle, dystrophin is linked to the actin cytoskeleton and binds the transmembranous β-DG. The extracellular domain of β-DG connects the ECM by binding to α-DG, which in turn binds to Laminin-2 (44)(Fig. 4). Hypoglycosylation of α-DG in muscle results in a loss of interaction of this protein with the ECM protein laminin-2 and thereby disrupts the link between the sarcolemma of the muscle and the ECM (45–47). The predicted molecular weight of the α-DG protein is about 72 kDa. However, on immunoblots, the α-DG protein migrates as a diffuse band of 150–200 kDa, reflecting the extensive post-translational modification of the protein. This increase in molecular weight is largely explained by extensive glycosylation. Both N-linked and O-linked glycosylations occur. The level and types of glycosylation are likely tissue dependent as judged by different molecular weights. A common glycan structure –Sialylα2,3Galβ1,4GlcNAcβ1,2Manα2-O-Ser/Thr– is present at the central mucin region of α-DG coming from different sources (48–50). The Sialylα2,3Galβ1,4GlcNAc part of the sugar chain is required for the binding of α-DG to laminin (49, 51, 52).

**Fig. 4.** Schematic representation of the crucial role of dystroglycan (DG) in connecting the cytoskeleton of muscle cells with the extracellular matrix. This binding is disrupted by defects in the post-translational modification of α-DG, causing congenital muscular dystrophy. The O-mannosylation is represented by small orange spheres. The composition of these carbohydrate side chains and the enzymes that catalyze the addition of these sugar groups are shown in the right panel. Disrupted O-mannosylation of α-DG is seen in Walker–Warburg syndrome (WWS) and muscle-eye-brain disease (MEB) patients with mutations in the POMT1 and POMGnT1 genes, respectively, which encode the enzymes for the first two steps which cause MEB and WWS when malfunctioning. Similar glycosylation defects are seen in patients with mutations in the Fukutin, FKRP and LARGE gene, but the role of the corresponding proteins is not known.
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Using antibodies that recognize a carbohydrate epitope, it was demonstrated that cobblestone lissencephaly patients of various types (WWS, MEB and FCMD) have undetectable levels of normally glycosylated α-DG in skeletal muscle. In contrast, antibodies against the core peptide can detect residual amounts of α-DG protein. This apparent glycosylation defect of α-DG is strikingly similar for the various syndromes. Muscular α-DG shows a reduction of approximately 50 kDa in FCMD and MEB patients as well as in the Large<sup>myd</sup> mouse. Possibly, failure of glycosylation at any level has the same global effects on the total glycosylation of α-DG. In agreement with this is the notion that in yeast O-mannosylation may be a prerequisite before N-glycosylation can occur (53).

Very recent work demonstrated that overexpression of endogenous LARGE in the Large<sup>myd</sup> mouse diminishes muscular dystrophy by restoring the hypoglycosylation of α-DG. More strikingly, overexpression of LARGE in cells from MEB, FCMD and WWS patients also led to the restoration of functionally glycosylated α-DG, indicating that LARGE can compensate for the genetically distinct defects in these cells. These data show a possible regulatory role for LARGE in glycosylation of α-DG (54). It has been established that the molecular interaction between LARGE and the N-terminal domain of α-DG is crucial for O-linked glycosylation of the α-DG mucin domain (55). However, it is as yet unclear how overexpression of LARGE can functionally compensate for the genetic defects in cells from MEB, FCMD and WWS patients (54).

**WWS candidate genes**

Our genome-wide linkage analyses conducted on 15 consanguineous WWS families point to the existence of at least three further WWS loci (10) (van Reeuwijk, unpublished data). Therefore, the functional candidate gene approach in conjunction with homozygosity mapping remains a valid strategy to identify other WWS genes. Our prime targets are other components of the O-mannosylation pathway and genes known to result in hypoglycosylation of α-DG. One excellent candidate gene is the LARGE gene. As discussed above, Large<sup>myd</sup> mice have an altered glycosylation of α-DG and a phenotype that is strikingly similar to the MEB and WWS in humans (25–27, 43). POMT2 is another obvious candidate gene, sharing 36% amino acid identity with POMT1. Both genes are expressed in most human tissues, but POMT1 is expressed mainly in fetal brain, skeletal muscle and testis whereas POMT2 has its highest expression in testis (34, 56). In Drosophila melanogaster, orthologues of the human POMT1 and POMT2 genes cause the rt (rotated abdomen) and tw (twisted) phenotypes. These two fly mutants show a similar phenotype with up to 90% rotation of the abdomen resulting from defects in muscle development. In addition, mutant flies have reduced fertility and viability (57, 58). Another argument for the possible role for POMT2 in WWS is that coexpression of both POMT1 and POMT2 is required to obtain POMT enzymatic activity (59). This result suggests that a heterocomplex is formed between the two proteins during their synthesis, similar to what has been observed for the homologous PMT1 and PMT2 proteins (60, 61). Despite these theoretical considerations, no POMT2 mutations were identified in 30 unrelated WWS families (10). The human genome contains two other genes with some homology to POMT1: SDF2 and SDF2L1. Like POMT1/2, these two genes encode MIR domains typical of mannosyltransferases, but not the catalytic mannosyltransferase domain.

The first two steps in the O-mannosyl glycan synthesis of α-DG are known to be disrupted in cobblestone lissencephalies by mutations in the POMT1 and POMGnT1 genes. It is well possible that disruption of other steps in this glycosylation process will give rise to a similar phenotype. The addition of β1,4-galactosyl (step 3) and the addition of α2,3-sialyl (step 4) are performed by the enzymes β1,4-galactosyltransferase (β4GalT) and α2,3-sialyltransferase (ST3Gal), respectively. A frameshift mutation in β4GalT1 has been implicated in congenital disorder of glycosylation type IIId (CDGIIId; MIM 607091). This patient had macrocephaly due to Dandy–Walker malformation, hypotonia, coagulopathy, myopathy with elevated creatine kinase, mild developmental delay, motor retardation and abnormal serum transferrin of which the sialic acid and galactose residues were lost (62). The enzyme GNE is known to catalyze a rate-limiting step in sialic acid biosynthesis and defects result in an improper sialylation of glycoproteins (63). Very recently, GNE mutations have been linked to hypoglycosylation of α-DG in patients with hereditary inclusion body myopathy (MIM 600737), an autosomal recessive neuromuscular disorder characterized by progressive muscle weakness (64, 65). Although further confirmation of this result is required, it indicates that all enzymes involved in O-glycosylation of α-DG...
are candidates for involvement in neuromuscular disorders.

The target proteins for O-mannosylation should also be considered for involvement in cobblestone lissencephalies. Currently, the only known O-mannosylated protein is \( \alpha \)-DG. We have sequenced the entire coding region of \( \alpha \)-DG in 11 linked WWS patients but found no mutations (van Reeuwijk, unpublished data). Other target proteins for O-mannosylation may exist, but could be hard to find because there is no recognizable motif in the proteins that are targets for O-mannosylation in yeast. Other proteins from the DGC complex (Fig. 4), connecting to or present in the ECM in muscle, the central nervous system and the neuromuscular junction are possible WWS candidate genes. An important ligand of \( \alpha \)-DG in the ECM of muscle is Laminin-2 (merosin), mutations of which cause MDC1A (MIM 607855), a common form of CMD and clinically related to FCMD.

**Conclusions and perspectives**

In the past few years, hypoglycosylation of \( \alpha \)-DG has been found to be the mechanism linking several neuromuscular diseases. This list includes WWS, MEB, FCMD, MDC1C, MDC1D, LGMD2I and some intermediate phenotypes (Fig. 2). Of these, WWS shows the most severe clinical features including CMD, severe brain and eye involvement. It has been shown that mutations in \( \text{POMT1, fukutin and FKRP} \) may each give rise to WWS. Knowing that mutations in most of the genes involved in WWS and related disorders show a wide clinical range, mutations in \( \text{POMGnT1 and LARGE} \) should also be sought in WWS patients. In view of the genotype–phenotype correlation, we also hypothesize that mild mutations in \( \text{POMT1} \) might cause MEB or even FCMD. It has been shown that POMT1 and POMGnT1 are responsible for the addition of an O-linked mannose and a GlcNAc\( \beta \)1,2 residue, respectively, to \( \alpha \)-DG (36, 59). Elucidation of the exact function of fukutin, FKRP and LARGE in O-mannosyl glycan synthesis could lead to further candidate genes for WWS. For this, a better understanding of the post-translational modification of \( \alpha \)-DG will be crucial. Genome-wide homozygosity mapping in consanguineous WWS families may lead to the identification of further genes involved in these disorders.

The genetic heterogeneity in WWS and related disorders imposes some difficulties for diagnostic studies and genetics counselling. Presently, mutation analysis for \( \text{POMT1 and POMGnT1} \) is available in a diagnostic setting (http://www.dnadiagnostiekknijmegen.nl), and we emphasize to include the other cobblestone lissencephaly genes in the future. Recently, enzymatic assays have been described to test the activity of POMT1 and POMGnT1 and to diagnose for the founder mutation in FCMD (59, 66, 67). These tests in combination with mutation analysis of the known cobblestone lissencephaly genes should allow prenatal diagnosis in selected families.

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