Identification of Mutations in *TMEM5* and *ISPD* as a Cause of Severe Cobblestone Lissencephaly

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Cobblestone lissencephaly is a peculiar brain malformation with characteristic radiological anomalies. It is defined as cortical dysplasia that results when neuroglial overmigration into the arachnoid space forms an extracortical layer that produces agyria and/or a "cobblestone" brain surface and ventricular enlargement. Cobblestone lissencephaly is pathognomonic of a continuum of autosomal-recessive diseases characterized by cerebral, ocular, and muscular deficits. These include Walker-Warburg syndrome, muscle-eye-brain disease, and Fukuyama muscular dystrophy. Mutations in *POMT1*, *POMT2*, *POMGNT1*, *LARGE*, *FKTN*, and *FKRP* identified these diseases as alpha-dystroglycanopathies. Our exhaustive screening of these six genes, in a cohort of 90 fetal cases, led to the identification of a mutation in only 53% of the families, suggesting that other genes might also be involved. We therefore decided to perform a genome-wide study in two multiplex families. This allowed us to identify two additional genes: *TMEM5* and *ISPD*. Because TMEM has a glycosyltransferase domain and ISPD has an isoprenoid synthase domain characteristic of nucleotide diP-sugar transferases, these two proteins are thought to be involved in the glycosylation of dystroglycan. Further screening of 40 families with cobblestone lissencephaly identified nonsense and frameshift mutations in another four unrelated cases for each gene, increasing the mutational rate to 64% in our cohort. All these cases displayed a severe phenotype of cobblestone lissencephaly A. *TMEM5* mutations were frequently associated with gonadal dysgenesis and neural tube defects, and *ISPD* mutations were frequently associated with brain vascular anomalies.

Cobblestone lissencephaly (cobblestone-LIS) is a peculiar brain malformation with characteristic radiological anomalies. It is defined as cerebral cortical dysplasia with an irregular limit between the white and gray matter, dysmyelination, severe dysplastic cerebellum with cysts, and brainstem hypoplasia.[1] Cortical malformations are due to neuroglial overmigration into the arachnoid space, resulting in the formation of an extracortical neuroglial layer responsible for the agyria and/or an irregular, "cobblestone" surface of the brain and ventriculomegaly. The underlying mechanism is a disruption of the glia limitans, the outermost layer of the brain.[2–4] Cobblestone-LIS is considered to be pathognomonic of a continuum of autosomal-recessive disorders resulting in ocular and muscular deficits. These disorders include Walker-Warburg syndrome (WWS, [MIM 236670]), muscle-eye-brain disease (MIM 253280), and Fukuyama muscular and cerebral dystrophy (MIM 253800). Walker-Warburg syndrome, also known by the HARD ± E eponym (hydrocephalus, agyria, retinal dysplasia, ± encephalocele), is characterized by major neurological deficit, visual and muscular impairment, and a rapidly fatal outcome.[5] Less severe features within the same spectrum include subtle eye abnormalities, a milder neurological deficit, and muscular dystrophy and can be observed in muscle-eye-brain disease, which was first described in a Finnish population,[6] and in Fukuyama muscular and cerebral dystrophy, which is common in Japan.[7,8] Classically, these syndromes with cerebral ocular and muscular dystrophy are attributed to abnormal glycosylation of alpha-dystroglycan and are now designated

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as "muscular congenital alpha-dystroglycanopathy with brain and eye anomalies" (MDDGA). Aberrant post-translational modification of alpha-dystroglycan has been associated with mutations in at least six genes: *POMT1* (MIM 607423), *POMT2* (MIM 607439), *POMGNT1* (MIM 606822), *FKTN* (MIM 607440), *FKRP* (MIM 606596), and *LARGE* (MIM 603590).[9–13] Another group designated as having congenital muscular dystrophy-dystroglycanopathy with mental retardation (MDDGB) has been also associated with mutations of these six genes (MIM 613155, 613156, 613151, 613152, and 606612). Except for *LARGE*, these genes have also been implicated in the autosomal-recessive limb-girdle muscular dystrophies (LGMD, renamed MDDGC [MIM 609308, 613158, 613157, 611588, and 607155, respectively) characterized by variable age of onset, normal cognition, and no structural brain changes.[14–18]

Our molecular and neurohistopathological study of fetal cases of cobblestone-LIS demonstrated genetic heterogeneity and morphological diversity, along with some genotype-phenotype correlations.[19,20] In our growing cohort of 90 cobblestone-LIS families, 42 (47%) unrelated cases remained without any mutations in any of the six genes known to be implicated, suggesting that other genes might also be involved.

We therefore decided to use an SNP chip-based, genome-wide linkage approach combined with an exome study in two multiplex families (Figure S1 in the Supplemental Data available online). All fetal cases were included in the study on the basis of our diagnostic criteria of cobblestone-LIS as well as (1) neuroglial ectopia within the arachnoid space, (2) abnormal cortical lamination, and (3) brain stem and cerebellar dysplasia as described in Devisme et al.[20] Informed consent was obtained from parents in accordance with French law and with the ethical standards of the national committee on human experimentation. Molecular screening was performed on genomic DNA (gDNA) extracted from frozen fetal tissues according to standard protocols. Genome-wide linkage studies were performed by Affymetrix Genome-wide Human SNP-Array 250K in two multiplex cobblestone-LIS families (F1 and F2) via Genotyping Console software (Affymetrix). Family F1 is consanguineous and includes four affected fetuses. Five runs of homozygosity (ROH) higher than 1 Mb were shared by three of the affected fetuses studied and were identified with the software PLINK[21] in chromosomes 4 (two regions), 12, 15, and 20 (Table S1). The second multiplex family F2 is nonconsanguineous and includes three affected fetuses. Multipoint linkage analysis was performed with the software Merlin.[22] Ten linkage regions that were more than 2 Mb and had an LOD score >1 were found on chromosomes 1, 6, 7 (two regions each), 10, 11, 17, 20, and 21 (Table S1). Illumina exome capture and next-generation sequencing on an Illumina platform were performed on samples from two affected fetuses of each of the F1 and F2 families. After regions were filtered on the basis of known variants, homozygosity for F1, two alleles mutated for F2, and location in the region of ROH (F1) or linkage regions (F2), a mutation in only one gene was found to segregate in the two siblings for each family (Table S2). In family F1, one homozygous variant, c.795delG (p.Arg266Glyfs*8), was found in exon 5 of *TMEM5* (NM_014254.1 [MIM 605862]) in the ROH region of chromosome 12. In family F2 an apparently homozygous c.638T>G p.Met213Arg missense mutation was found in exon 3 of *ISPD* (NM_001101426.3 MIM 614631]) in the largest linkage region on chromosome 7 (Table S1). Primers were designed in the flanking intronic region of both genes (Table S3) for Sanger sequencing with fluorescent di-deoxy capillary sequencing on an ABIPRISM 3130XL sequencer (Applied Biosystems). Sequence analysis was performed with Seqscape (Applied Biosystems) software. This analysis confirmed that all the F1 fetuses had inherited the homozygous c.795delG *TMEM5* frameshift mutation from heterozygous parents. In the F2 family, the *ISPD* missense mutation was confirmed in siblings and was found in the heterozygous state in the mother only. Further molecular investigation by qPCR in exon 3 (data not shown) identified a paternally inherited deletion, confirming compound heterozygosity for a mutation and an intragenic deletion in family F2. Data from the SNP array in this family indicated the absence of any paternal contribution to the genotype of the affected fetuses for three intragenic SNPs (SNP_A-1826269, SNP_A-1948943, and SNP_A-1922943), which was consistent with hemizygosity from hg19: chromosome7:16,306,467 (intron 6) to 16,344,268 (intron 4). We therefore concluded that the size of the deletion in this family encompasses at least exon 3 to exon 6.

In order to confirm the involvement of *TMEM5* and *ISPD* in cobblestone-LIS, we tested each coding exon and intron/exon junction of the two genes for mutations by direct gDNA SANGER sequencing in 40 cobblestone-LIS families that were part of our cohort but that did not have any mutation in the six genes known to be involved in alpha-dystroglycanopathies. We found pathogenic mutations in *TMEM5* or *ISPD* in another eight families (Table 1). We identified a total of five distinct *TMEM5* mutations, including three frameshift (c.795delG [p.Glu265fs*8], c.1064_1091del [p.Asp355Valfs*33], and c.279delA [p.Gly94Glufs*33]) and two missense (c.1016A>G [p.Tyr339Cys] and c.1019_1020delinsTT [p. Arg340Leu]) mutations, in five families (Figure 1A). In *ISPD*, we identified nine distinct mutations, including two intragenic deletions, two nonsense mutations (c.256A>T [p.Arg86*] and c.773C>A [p.Ser258*]), one splice mutation (c.257+2T>G), and four missense mutations (c.466G>A [p.Asp156Asn], c.638T>G [p.Met213Arg], c.676T>C [p.Tyr226His], and c.713C>T [p.Thr238Ile]), in five families (Figure 1B). None of the missense variations were present in the DbSNP, 1000 Genomes, or Exome Variant Server data banks. All the amino acids concerned were conserved from humans to zebrafish. Furthermore, we used four different in silico prediction software packages

Table 1. Summary of the Pathogenic *TMEM5* (NM_014254.1) and *ISPD* (NM_001101426.3) Mutations Detected in This Study

Family Number, Number of Sibs	Consanguinity	Gene	Zygosity	Nucleotide Variant	Amino Acid Alteration	Exon(s) Affected	Type of Mutation	Panther Prediction $p_{deleterious}$	Align GVGD Class	PolyPhen-2	SIFT
F1, 4 fetuses	yes	*TMEM5*	homozygous	c.795 del G	p.Arg266Glyfs*8	exon 5	frameshift	NA	NA	NA	NA
F7, 2 fetuses	no	*TMEM5*	homozygous	c.1016A>G	p.Tyr339Cys	exon 6	missense	0.82	C65	probably damaging	affected protein (0.00)
F8, 1 fetus	no	*TMEM5*	heterozygous	c. 1019_1020del insTT	p.Arg340Leu	exon 6	missense	0.57	C65	probably damaging	affected protein (0.00)
				c.1064_1091del	p.Asp355Valfs*33	exon 6	nonsense	NA	NA	NA	NA
F9, 1 fetus	no	*TMEM5*	heterozygous	c.1016A>G	p.Tyr339Cys	exon 6	missense	0.82	C65	probably damaging	affected protein (0.00)
				c.1064_1091del	p.Asp355Valfs*33	exon 6	frameshift	NA	NA	NA	NA
F10, 1 fetus	?	*TMEM5*	homozygous	c.279 delA	p.Gly94Glufs*33	exon 2	frameshift	NA	NA	NA	NA
F2, 3 fetuses	no	*ISPD*	heterozygous	c.638T>G	p.Met213Arg	exon 3	missense	0.67	C65	probably damaging	affected protein (0.03)
			heterozygous	deletion	deletion	exons 3 to 6 at least	gene deletion	NA	NA	NA	NA
F3, 2 fetuses	yes	*ISPD*	homozygous	c.466G>A	p.Asp156Asn	exon2	Missense	0.64	C15	probably damaging	affected protein (0.00)
F4, 1 fetus	no	*ISPD*	heterozygous	c.713C>T	p.Thr238Ile	exon4	Missense	0.22	C65	probably damaging	affected protein (0.01)
				c.256A>T	p.Arg86*	exon1	Nonsense	NA	NA	NA	NA
F5, 1 fetus	no	*ISPD*	heterozygous	c.257+2T>G	*	exon1	Splicing defect	NA	NA	NA	NA
				c.773C>A	p.Ser258	exon4	Nonsense	NA	NA	NA	NA
F6, 1 fetus	no	*ISPD*	heterozygous	c.676T>C	p.Tyr226His	exon3	Missense	0.45	C65	probably damaging	tolerated (0.43)
				deletion	deletion	Ex4 to ex6	Gene deletion	NA	NA	NA	NA

Panther: the probability that a given variant will cause a deleterious effect is estimated by $p_{deleterious}$ such that a subPSEC score (substitution position-specific evolutionary conservation score based on an alignment of evolutionarily related proteins) of -3 corresponds to a $p_{deleterious}$ value of 0.5. Phenotype prediction for Align GVGD class C65 corresponds to "most likely interferes with function." PolyPhen-2 extracts various sequences and structure-based features of the substitution site and feeds them into a probabilistic classifier. For the SIFT score, the amino acid substitution is predicted to be harmful if the score is ≤ 0.05 and is predicted to be tolerated if the score is >0.05. NA = not applicable.

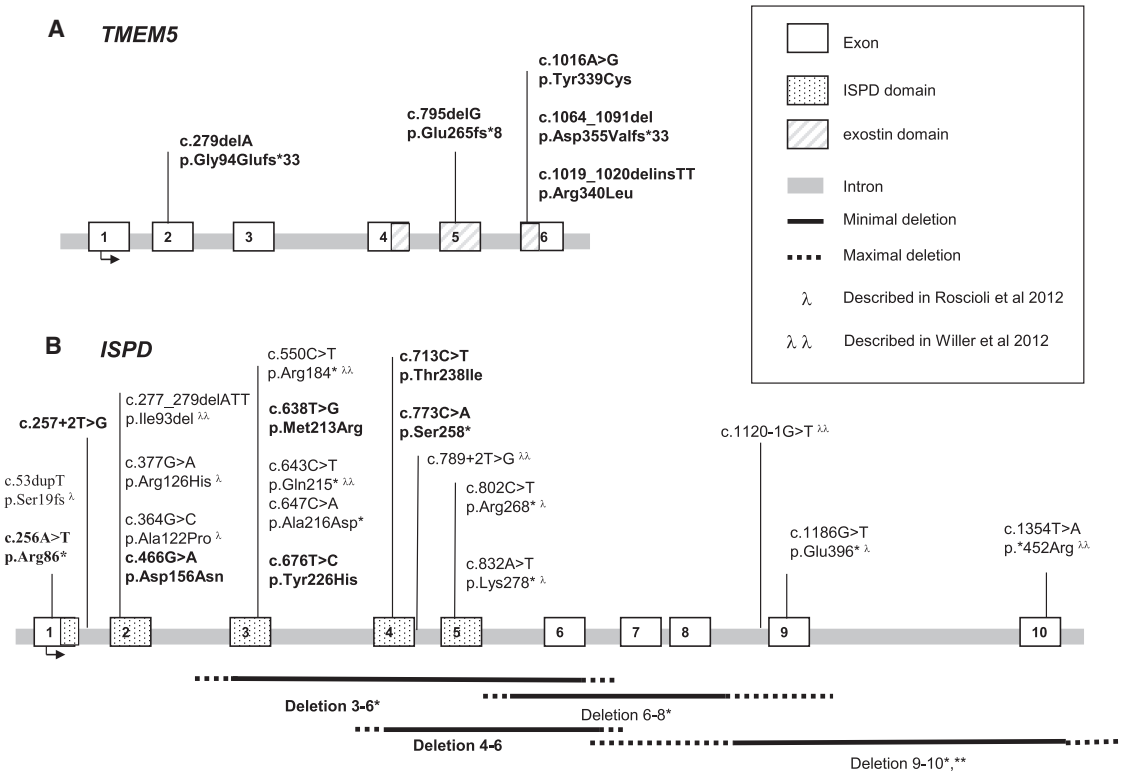

Figure 1. Diagram of the *ISPD* and *TMEM5* Exon-Intron Gene Structure
All the pathogenic changes identified in this study (bold) and in two previous *ISPD* studies, Roscioli et al., 2012[30] (λ) and Willer et al., 2012[31] (λ λ) are included.

(Polyphen, Panther, SIFT2, and Align GVGD) to evaluate the potential effect on protein function. All missense changes were predicted as being pathogenic by at least two out of four software packages (Table 1). Segregation of identified potentially disease-causing variants was performed in each available family member, and autosomal-recessive transmission was confirmed. In family F5, c.257+2T>G in *ISPD* appeared to be inherited de novo because it was not found in either parent's DNA. Paternity was confirmed by the Power Plex 16 system (Promega, Ca, USA), and allele-specific amplification was ruled out by PCR with two different pairs of primers. Two mutations in *TMEM5* were found, in each case in two unrelated families: c.1016A>G (p.Tyr339Cys) in F7 and F9 and c.1064_1091del (p.Asp355Valfs*33) in F8 and F9. We looked for a founder effect for these families by haplotype analysis with four microsatellite markers in the vicinity of *TMEM5* and found a common haplotype for one allele for each of the two unrelated families, suggesting that these individuals could share the same ancestral chromosome segment. In the nonconsanguineous family F7, in which affected individuals had a homozygous *TMEM5* mutation, parental DNAs were not available. We therefore confirmed homozygosity by qPCR, which excluded a heterozygous deletion.

Both the genes we identified in this study seem to be functionally related to glycosylation. *TMEM5* encodes a 443 amino acid, type II transmembrane protein of unknown function. The protein contains a signal anchor for a type II membrane protein encoded by amino acids 10–30, a cytoplasmic domain encoded by amino acids 1–9, and an extracellular domain encoded by amino acids 31–443.[23] Interestingly, TMEM5 contains a domain that has some similarities to exostosin (amino acids 218–353), which is a domain found in *EXT1*. *EXT1* codes for a glycosyltransferase required for the biosynthesis of heparan sulfate[24] and is a putative tumor suppressor gene involved in multiple type-I exostoses (MIM 133700).[25] A BlastP analysis identified a region of 19% identity with EXT1, which contains an aspartic acid-any amino acid-aspartic acid (DXD) motif that is present in many glycosyltransferases and is critical for catalytic function thought to be essential for binding the UDP-sugar donor through the coordination of a divalent cation, Mn^{2+} [26,27] (Figure S2). Furthermore, EXT1 contains three other conserved DXD motifs, but substitutions (Arg340Ser, Arg340His, or Arg340Leu; and Arg280Gly or Arg280Ser) that have been shown to result in defective heparan sulfate biosynthesis cluster around the second DXD motif in this region of homology with TMEM5[28,29] (Figure S2). Interestingly, three out of the five *TMEM5* mutations identified in our study are also located in the exostosin domain (Figures 1A and 2). TMEM5 is therefore thought to be a transmembrane protein with a glycosyltransferase function.

Concerning *ISPD*, two recent independent studies simultaneously reported mutations in *ISPD* in postnatal WWS

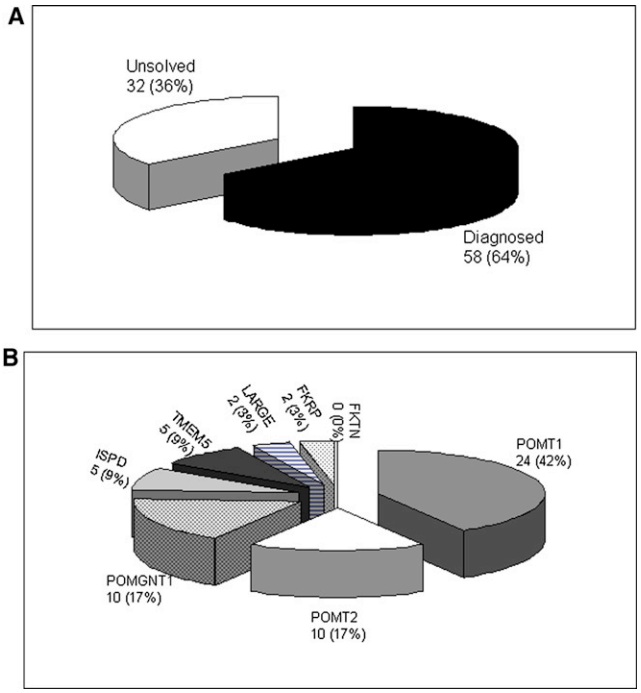

Figure 2. Results of Molecular Diagnosis of Cobblestone-LIS Fetuses
(A) Proportion of cases diagnosed after this study in our cohort of 90 cobblestone-LIS fetuses.
(B) Contribution of alpha-dystroglycanopathy gene mutations in our 58 diagnosed cobblestone-LIS fetuses.

cases (MDDGA7 [MIM 614631]).[30,31] *ISPD* encodes an isoprenoid-synthase-domain-containing protein and belongs to the super-family of nucleotide diP-sugar transferases (amino acids 47–278). In plants, protozoa, and some bacteria, ISPD is part of the methylerythritol phosphate (MEP) pathway that leads to the polyisoprenoid alcohols (dolichols and polyphenols).[32] This MEP pathway involving ISPD is missing in animals, which use an alternative ISPD-independent mevalonate pathway for isoprenoid synthesis.[33,34] The specific role of ISPD in humans is therefore unclear, but it is likely that ISPD synthesizes a new nucleotide sugar that could be incorporated into alpha-dystroglycan O-glycan.[30] Assays on fibroblasts derived from *ISPD*-mutated WWS subjects complemented with wild-type *ISPD* restored alpha-dystroglycan glycosylation.[31] Furthermore, the knockdown presentation in zebrafish reflected the WWS semiology, including hydrocephalus, reduced eye size, and muscle degeneration.[30] Glycosylation of alpha-dystroglycan was reduced in zebrafish embryos injected with an dose of *ISPD* antisense morpholino oligonucleotides (MOs) sufficient to knock down *ISPD* isoforms. Coinjection with subeffective doses of *FKTN* or *FKRP* MO reduced glycosylation more than injecting subeffective doses of *ISPD, FKTN*, or *FKRP* MO alone.[30] These findings support a cooperative interaction between *ISPD* and both *FKTN* and *FKRP* in alpha-dystroglycan glycosylation.

The *ISPD* mutations identified here are newly described, apart from the deletion of exons 3 to 6, which was previously described by Roscioli et al..[30] Figure 1B shows a recapitulation including previously published mutations. Interestingly, the deletion of exons 3 to 6 in *ISPD* is located in a known CNV (hapMap variation_2683: four observed losses in 270 control samples, corresponding to a carrier frequency of 1.5%). The frequency of the CNV does not exclude its having a role in a rare disease, but it is possible that homozygosity leads to embryonic lethality, similar to that known for another glycosylation-related disease, *PMM2*-CDG (MIM 212065), and to frequent p.Arg141His mutation,[35] which could explain the discrepancy between the relatively high frequency of this variation and the low frequency of cobblestone-LIS.

So far, only three out of the six genes mutated in alpha-dystroglycanopathies have a clearly established function; these are *POMT1*, *POMT2*, and *POMGNT1*, the genes encoding the three glycosyltransferases involved in early steps of alpha-dystroglycan *O*-mannosylation. The function of Fukutin-related protein (FKRP) and fukutin (FKTN) remains unknown. Both are Golgi-resident proteins, and FKRP is required for the posttranslational modification of alpha-dystroglycan in the Golgi apparatus.[36] Fukutin-related protein resides in the Golgi cisternae of skeletal muscle fibers and forms disulfide-linked homodimers via an N-terminal interaction.[37] Fukutin seems to bind the core area of alpha-dystroglycan during glycosylation.[38] The exact function of LARGE is also unknown, but the overexpression of *LARGE* restores *O*-glycosylation of alpha-dystroglycan in cells of individuals with *POMT1, POMGNT1, LARGE*, or *FKTN* mutations.[39] LARGE is involved in a phosphorylation process of the O-glycosyl alpha-dystroglycan, which is required for laminin binding and occurs on a specific Thr317 or Thr319 of the mucin-like domain of alpha-dystroglycan.[40,41] It has also been shown that LARGE induces the functional glycans in a DPM2- and *O*-mannosylation-dependent manner.[42] Like the other genes involved in alpha-dystroglycanopathies, the two genes identified in this study are thought to be glycosyl or nucleotide diP-sugar transferases, on the basis of their similarities with known related domains.

In our previous study, we proposed a new classification of cobblestone-LIS in three subtypes depending on the severity of cortical malformations. In our classification, the most severe form, called cobblestone-LIS(A), is usually linked to mutations in *POMT1* (34%) and *POMT2* (8%); the least severe, cobblestone-LIS(C), is linked to *POMGNT1* mutations; and an intermediate type, cobblestone-LIS(B), is linked to *LARGE* mutations.[20] *TMEM5* and *ISPD* mutations were found in cobblestone-LIS(A), associated with occipital neural-tube defects, facial clefts, visceral malformations, and gonadal dysplasia (Table 2). It is noteworthy that neural-tube defects, visceral malformations, and gonadal dysplasia were frequently observed in *TMEM5*-mutated cases (5/7 cases) but were absent in *ISPD*-mutated fetuses. In our previous study, we showed that visceral malformations were observed in almost half of the cases with *POMT1* mutations (9/22) and in most of cases with

Table 2. Clinical Data of *ISPD*- and *TMEM5*-Mutated Cobblestone-Lissencephaly Fetal Cases Identified in This Study

									Cerebellar Dysplasia					Retinal Dysplasia			
		Devisme et al., 2012[20]								Extension		Severity		Extension		Severity	
Gene	Fetus	Family Number	Fetus Number	NTD	Limb Deformations	Gonadal Dysgenesis	Visceral Malformation	Brain Vascular Anomalies	ECL/CP	Diffuse	Focal	Major	Minor	Diffuse	Focal	Major	Minor
ISPD	F2-1	F26	35	+	-	-	-		>1	+		+		+		+	
ISPD	F2-2	F26	67	+	+	+	+		>1	+			+		+		+
ISPD	F2-3	F26	68	+	-	-	+		>1	+			+		+		+
ISPD	F3-1	F23	29	-	-	-	-	+	>1	+			+	+			+
ISPD	F3-2	F23	30	-	+	-	-		>1	+			+	NE	NE	NE	NE
ISPD	F4	F25	34	-	+	-	-		>1	+		+		+		+	
ISPD	F5	F55	69	+	-	-	-	+	>1	+		+		NE	NE	NE	NE
ISPD	F6	/	/	-	-	-	-		NE	NE		NE		NE	NE	NE	NE
TMEM5	F1-1	F1	1	+	-	+	+		>1	+			+	NE	NE	NE	NE
TMEM5	F1-2	F1	2	-	-	+	+		>1	+			+	+			+
TMEM5	F1-3	F1	3	+	-	-	-		>1	+			+	+			+
TMEM5	F1-4	/	/	NE	NE	NE	NE		NE	NE	NE	NE	NE	NE	NE	NE	NE
TMEM5	F7-1	F24	31	+	-	+	+		>1	+		+		+		+	
TMEM5	F7-2	F24	32	+	-	-	-		>1	+			+	+		+	
TMEM5	F8	F27	36	-	-	+	-		>1	+		+		+			+
TMEM5	F9	F50	62	+	+	+	+		>1	+		+		+			+
TMEM5	F10	F54	66	-	-	-	-		>1	+		+		NE	NE	NE	NE

Abbreviations are as follows: NTD = neural-tube defect; ECL/CP = extracortical layer/cortical plate; and NE = not evaluated.

POMT2 mutations (4/5).[20] *POMT1* was frequently associated with cleft lip and cleft palate.[20,43] *POMT2* and *TMEM5* were frequently associated with gonadal dysgenesis, found in 3/5 of the *POMT2* cases[20] and 5/8 of the *TMEM5* cases. It is noteworthy that in two cases with *ISPD* mutations, the neuropathological study revealed severe vascular dysplasia and perfusion impairment, leading to focal brain parenchyma necrosis.

An overview of mutated alpha-dystroglycanopathy genes in our cohort of 90 cobblestone-LIS families is provided in Figure 2. Altogether, mutations in *POMT1* (n = 24, 42%), *POMT2* (n = 10, 17%), *POMGNT1* (n = 10, 17%), *TMEM5* (n = 5, 9%), *ISPD* (n = 5, 9%), *LARGE* (n = 2, 3%), and FKRP (n = 2, 3%) (Figure 2B) have now been identified in 58 cobblestone-LIS families. *TMEM5* and *ISPD* each accounted for almost 10% of the mutated cases, which is consistent with the previously published data for *ISPD* in postnatal WWS cases.[30,31] Both genes were involved three times more frequently than *LARGE* in our series of cobblestone-LIS families and may therefore be considered to be two additional important genes with regard to this pathology. Our study raises the mutational rate to 64% in our cohort and once more highlights the importance of fetal pathological examination, not only for accurate diagnosis and subsequent genetic counseling but also for the classification of homogeneous groups and genotype-phenotype correlations that considerably accelerate molecular screening.

More recently, four other genes related to alpha-dystroglycanopathies were reported, although they were found in only a few individuals with mild myopathy. Three of these genes are related to glycosylation, and the fourth is the dystroglycan gene *DAG1* itself (MIM 128239). One individual with dilated cardiomyopathy and a low but normal IQ was found to have a mutation in *DPM3* (MIM 605951);[44] two siblings with CMD and mental retardation were found to harbor mutations in *DPM2* (MIM 603564);[45,46] and four families with dilated cardiomyopathy were reported to have *DOLK* (MIM 610746) mutations.[47] Finally, two individuals were found to carry a mutation in *DAG1*; one carried a heterozygous deletion of a 2 MB region including *DAG1*,[48] and the other, with LGMD and cognitive impairment, had a homozygous missense mutation.[49] Considering the numbers of individuals reported to date, these four genes should be considered to be minor alpha-dystroglycanopathy genes.

The molecular basis of cobblestone-LIS remains unexplained in more than 36% (32/90) of our families (Figure 2A), suggesting either that some of the mutations of these genes were not detected or that other genes remain to be discovered. We studied all the genes by sequencing and genomic assays with CGH array analysis or qPCR to exclude intragenic rearrangements. Large-scale DNA rearrangements have now been identified in five genes associated with dystroglycanopathies (these genes are *LARGE, POMT1, POMT2, POMGnT1*, and *ISPD*).[30,50] In some cases, such as a recent LGMD case in which an alteration in the *POMGNT1* promoter was reported,[51] mutations might be located in noncoding sequences; in other cases, there might be a deep intronic mutation, and so cDNA analysis by RT-PCR will need to be performed.

In conclusion, *TMEM5* and *ISPD* are two additional important genes involved in cobblestone-LIS. They are thought to be glycosyl or nucleotide diP-sugar transferases and are responsible for the severe type A. Genotype-phenotype correlation improves the efficiency of genetic counseling through better orientation of molecular screening on the basis of the clinical severity of the cobblestone-LIS type. Our results do indeed suggest a molecular diagnostic strategy pointing through *POMT1, POMT2, TMEM5*, and *ISPD* in severe cobblestone-LIS type A. Because six other genes have been shown to be involved in the wide clinical spectrum of alpha-dystroglycanopathies, *ISPD* and *TMEM5* will also be studied in less severe clinical cases presenting a spectrum of defects ranging from congenital muscular dystrophy (CMD) associated with mental retardation and cerebellar malformation to mild limb girdle muscular dystrophy (LGMD) with or without mental retardation. The same combination of an SNP array and exome sequencing in multiplex or consanguineous families is now underway in the remaining unexplained cases and can be expected to lead to the identification of so-far-unknown genes that contribute to cobblestone-LIS.

Supplemental Data

Supplemental data include two figures and three tables and can be found with this article online at http://www.cell.com/AJHG.

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Web Resources

The URLs for data presented herein are as follows:

1000 Genomes Project, http://www.1000genomes.org/
Align GVGD, http://agvgd.iarc.fr/agvgd_input.php
BlastP (protein-protein BLAST), http://blast.ncbi.nlm.nih.gov/
dbSNP build 131, http://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi?build_id%BC131
Exome Variant Server, evs.gs.washington.edu/EVS/

ExonPrimer, http://ihg.helmholtz-muenchen.de/cgi-bin/primer/ExonPrimerUCSC.pl?db=hg19&acc=uc010ktx.2 (ISPD) and uc001srq.1 (TMEM5)
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/
PolyPhen, http://genetics.bwh.harvard.edu/pph2/
Panther, http://www.pantherdb.org/tools/csnpScoreForm.jsp
SIFT, http://blocks.fhcrc.org/sift/SIFT.html

References

1. van der Knaap, M.S., Smit, L.M., Barth, P.G., Catsman-Berrevoets, C.E., Brouwer, O.F., Begeer, J.H., de Coo, I.F., and Valk, J. (1997). Magnetic resonance imaging in classification of congenital muscular dystrophies with brain abnormalities. Ann. Neurol. *42*, 50–59.
2. Nakano, I., Funahashi, M., Takada, K., and Toda, T. (1996). Are breaches in the glia limitans the primary cause of the micropolygyria in Fukuyama-type congenital muscular dystrophy (FCMD)? Pathological study of the cerebral cortex of an FCMD fetus. Acta Neuropathol. *91*, 313–321.
3. Barkovich, A.J., Kuzniecky, R.I., Jackson, G.D., Guerrini, R., and Dobyns, W.B. (2001). Classification system for malformations of cortical development: Update 2001. Neurology *57*, 2168–2178.
4. Barkovich, A.J., Guerrini, R., Kuzniecky, R.I., Jackson, G.D., and Dobyns, W.B. (2012). A developmental and genetic classification for malformations of cortical development: Update 2012. Brain *135*, 1348–1369.
5. Dobyns, W.B., Pagon, R.A., Armstrong, D., Curry, C.J., Greenberg, F., Grix, A., Holmes, L.B., Laxova, R., Michels, V.V., Robinow, M., et al. (1989). Diagnostic criteria for Walker-Warburg syndrome. Am. J. Med. Genet. *32*, 195–210.
6. Santavuori, P., Somer, H., Sainio, K., Rapola, J., Kruus, S., Nikitin, T., Ketonen, L., and Leisti, J. (1989). Muscle-eye-brain disease (MEB). Brain Dev. *11*, 147–153.
7. Fukuyama, Y., Osawa, M., and Suzuki, H. (1981). Congenital progressive muscular dystrophy of the Fukuyama type—Clinical, genetic and pathological considerations. Brain Dev. *3*, 1–29.
8. Kobayashi, K., Nakahori, Y., Miyake, M., Matsumura, K., Kondo-Iida, E., Nomura, Y., Segawa, M., Yoshioka, M., Saito, K., Osawa, M., et al. (1998). An ancient retrotransposal insertion causes Fukuyama-type congenital muscular dystrophy. Nature *394*, 388–392.
9. Beltrán-Valero de Bernabé, D., Currier, S., Steinbrecher, A., Celli, J., van Beusekom, E., van der Zwaag, B., Kayserili, H., Merlini, L., Chitayat, D., Dobyns, W.B., et al. (2002). Mutations in the O-mannosyltransferase gene POMT1 give rise to the severe neuronal migration disorder Walker-Warburg syndrome. Am. J. Hum. Genet. *71*, 1033–1043.
10. van Reeuwijk, J., Janssen, M., van den Elzen, C., Beltran-Valero de Bernabé, D., Sabatelli, P., Merlini, L., Boon, M., Scheffer, H., Brockington, M., Muntoni, F., et al. (2005). POMT2 mutations cause alpha-dystroglycan hypoglycosylation and Walker-Warburg syndrome. J. Med. Genet. *42*, 907–912.
11. Godfrey, C., Clement, E., Mein, R., Brockington, M., Smith, J., Talim, B., Straub, V., Robb, S., Quinlivan, R., Feng, L., et al. (2007). Refining genotype phenotype correlations in muscular dystrophies with defective glycosylation of dystroglycan. Brain *130*, 2725–2735.
12. Taniguchi, K., Kobayashi, K., Saito, K., Yamanouchi, H., Ohnuma, A., Hayashi, Y.K., Manya, H., Jin, D.K., Lee, M., Parano, E., et al. (2003). Worldwide distribution and broader clinical spectrum of muscle-eye-brain disease. Hum. Mol. Genet. *12*, 527–534.
13. Clement, E., Mercuri, E., Godfrey, C., Smith, J., Robb, S., Kinali, M., Straub, V., Bushby, K., Manzur, A., Talim, B., et al. (2008). Brain involvement in muscular dystrophies with defective dystroglycan glycosylation. Ann. Neurol. *64*, 573–582.
14. Balci, B., Uyanik, G., Dincer, P., Gross, C., Willer, T., Talim, B., Haliloglu, G., Kale, G., Hehr, U., Winkler, J., and Topaloğlu, H. (2005). An autosomal recessive limb girdle muscular dystrophy (LGMD2) with mild mental retardation is allelic to Walker-Warburg syndrome (WWS) caused by a mutation in the POMT1 gene. Neuromuscul. Disord. *15*, 271–275.
15. Biancheri, R., Falace, A., Tessa, A., Pedemonte, M., Scapolan, S., Cassandrini, D., Aiello, C., Rossi, A., Broda, P., Zara, F., et al. (2007). POMT2 gene mutation in limb-girdle muscular dystrophy with inflammatory changes. Biochem. Biophys. Res. Commun. *363*, 1033–1037.
16. Clement, E.M., Godfrey, C., Tan, J., Brockington, M., Torelli, S., Feng, L., Brown, S.C., Jimenez-Mallebrera, C., Sewry, C.A., Longman, C., et al. (2008). Mild POMGnT1 mutations underlie a novel limb-girdle muscular dystrophy variant. Arch. Neurol. *65*, 137–141.
17. Brockington, M., Yuva, Y., Prandini, P., Brown, S.C., Torelli, S., Benson, M.A., Herrmann, R., Anderson, L.V., Bashir, R., Burgunder, J.M., et al. (2001). Mutations in the fukutin-related protein gene (FKRP) identify limb girdle muscular dystrophy 2I as a milder allelic variant of congenital muscular dystrophy MDC1C. Hum. Mol. Genet. *10*, 2851–2859.
18. Vuillaumier-Barrot, S., Quijano-Roy, S., Bouchet-Seraphin, C., Maugenre, S., Peudenier, S., Van den Bergh, P., Marcorelles, P., Avila-Smirnow, D., Chelbi, M., Romero, N.B., et al. (2009). Four Caucasian patients with mutations in the fukutin gene and variable clinical phenotype. Neuromuscul. Disord. *19*, 182–188.
19. Bouchet, C., Gonzales, M., Vuillaumier-Barrot, S., Devisme, L., Lebizec, C., Alanio, E., Bazin, A., Bessières-Grattagliano, B., Bigi, N., Blanchet, P., et al. (2007). Molecular heterogeneity in fetal forms of type II lissencephaly. Hum. Mutat. *28*, 1020–1027.
20. Devisme, L., Bouchet, C., Gonzalès, M., Alanio, E., Bazin, A., Bessières, B., Bigi, N., Blanchet, P., Bonneau, D., Bonnières, M., et al. (2012). Cobblestone lissencephaly: Neuropathological subtypes and correlations with genes of dystroglycanopathies. Brain *135*, 469–482.
21. Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M.A., Bender, D., Maller, J., Sklar, P., de Bakker, P.I., Daly, M.J., and Sham, P.C. (2007). PLINK: a tool set for whole-genome association and population-based linkage analyses. Am. J. Hum. Genet. *81*, 559–575.
22. Abecasis, G.R., Cherny, S.S., Cookson, W.O., and Cardon, L.R. (2002). Merlin—Rapid analysis of dense genetic maps using sparse gene flow trees. Nat. Genet. *30*, 97–101.
23. Yokoyama-Kobayashi, M., Yamaguchi, T., Sekine, S., and Kato, S. (1999). Selection of cDNAs encoding putative type II membrane proteins on the cell surface from a human full-length cDNA bank. Gene *228*, 161–167.
24. Lind, T., Tufaro, F., McCormick, C., Lindahl, U., and Lidholt, K. (1998). The putative tumor suppressors EXT1 and EXT2 are glycosyltransferases required for the biosynthesis of heparan sulfate. J. Biol. Chem. *273*, 26265–26268.

25. Ahn, J., Lüdecke, H.J., Lindow, S., Horton, W.A., Lee, B., Wagner, M.J., Horsthemke, B., and Wells, D.E. (1995). Cloning of the putative tumour suppressor gene for hereditary multiple exostoses (EXT1). Nat. Genet. *11*, 137–143.
26. Wiggins, C.A., and Munro, S. (1998). Activity of the yeast MNN1 alpha-1,3-mannosyltransferase requires a motif conserved in many other families of glycosyltransferases. Proc. Natl. Acad. Sci. USA *95*, 7945–7950.
27. Pedersen, L.C., Tsuchida, K., Kitagawa, H., Sugahara, K., Darden, T.A., and Negishi, M. (2000). Heparan/chondroitin sulfate biosynthesis. Structure and mechanism of human glucuronyltransferase I. J. Biol. Chem. *275*, 34580–34585.
28. Cheung, P.K., McCormick, C., Crawford, B.E., Esko, J.D., Tufaro, F., and Duncan, G. (2001). Etiological point mutations in the hereditary multiple exostoses gene EXT1: A functional analysis of heparan sulfate polymerase activity. Am. J. Hum. Genet. *69*, 55–66.
29. Zak, B.M., Crawford, B.E., and Esko, J.D. (2002). Hereditary multiple exostoses and heparan sulfate polymerization. Biochim. Biophys. Acta *1573*, 346–355.
30. Roscioli, T., Kamsteeg, E.J., Buysse, K., Maystadt, I., van Reeuwijk, J., van den Elzen, C., van Beusekom, E., Riemersma, M., Pfundt, R., Vissers, L.E., et al. (2012). Mutations in ISPD cause Walker-Warburg syndrome and defective glycosylation of α-dystroglycan. Nat. Genet. *44*, 581–585.
31. Willer, T., Lee, H., Lommel, M., Yoshida-Moriguchi, T., de Bernabe, D.B., Venzke, D., Cirak, S., Schachter, H., Vajsar, J., Voit, T., et al. (2012). ISPD loss-of-function mutations disrupt dystroglycan O-mannosylation and cause Walker-Warburg syndrome. Nat. Genet. *44*, 575–580.
32. Richard, S.B., Bowman, M.E., Kwiatkowski, W., Kang, I., Chow, C., Lillo, A.M., Cane, D.E., and Noel, J.P. (2001). Structure of 4-diphosphocytidyl-2-C- methylerythritol synthetase involved in mevalonate- independent isoprenoid biosynthesis. Nat. Struct. Biol. *8*, 641–648.
33. Hunter, W.N. (2007). The non-mevalonate pathway of isoprenoid precursor biosynthesis. J. Biol. Chem. *282*, 21573–21577.
34. Miziorko, H.M. (2011). Enzymes of the mevalonate pathway of isoprenoid biosynthesis. Arch. Biochem. Biophys. *505*, 131–143.
35. Schollen, E., Kjaergaard, S., Legius, E., Schwartz, M., and Matthijs, G. (2000). Lack of Hardy-Weinberg equilibrium for the most prevalent PMM2 mutation in CDG-Ia (congenital disorders of glycosylation type Ia). Eur. J. Hum. Genet. *8*, 367–371.
36. Esapa, C.T., Benson, M.A., Schröder, J.E., Martin-Rendon, E., Brockington, M., Brown, S.C., Muntoni, F., Kröger, S., and Blake, D.J. (2002). Functional requirements for fukutin-related protein in the Golgi apparatus. Hum. Mol. Genet. *11*, 3319–3331.
37. Alhamidi, M., Kjeldsen Buvang, E., Fagerheim, T., Brox, V., Lindal, S., Van Ghelue, M., and Nilssen, O. (2011). Fukutin-related protein resides in the Golgi cisternae of skeletal muscle fibres and forms disulfide-linked homodimers via an N-terminal interaction. PLoS ONE *6*, e22968.
38. Yamamoto, T., Kawaguchi, M., Sakayori, N., Muramatsu, F., Morikawa, S., Kato, Y., Shibata, N., and Kobayashi, M. (2006). Intracellular binding of fukutin and alpha-dystroglycan: relation to glycosylation of alpha-dystroglycan. Neurosci. Res. *56*, 391–399.
39. Barresi, R., Michele, D.E., Kanagawa, M., Harper, H.A., Dovico, S.A., Satz, J.S., Moore, S.A., Zhang, W., Schachter, H., Dumanski, J.P., et al. (2004). LARGE can functionally bypass alpha-dystroglycan glycosylation defects in distinct congenital muscular dystrophies. Nat. Med. *10*, 696–703.
40. Yoshida-Moriguchi, T., Yu, L., Stalnaker, S.H., Davis, S., Kunz, S., Madson, M., Oldstone, M.B., Schachter, H., Wells, L., and Campbell, K.P. (2010). O-mannosyl phosphorylation of alpha-dystroglycan is required for laminin binding. Science *327*, 88–92.
41. Hara, Y., Kanagawa, M., Kunz, S., Yoshida-Moriguchi, T., Satz, J.S., Kobayashi, Y.M., Zhu, Z., Burden, S.J., Oldstone, M.B., and Campbell, K.P. (2011). Like-acetylglucosaminyltransferase (LARGE)-dependent modification of dystroglycan at Thr-317/319 is required for laminin binding and arenavirus infection. Proc. Natl. Acad. Sci. USA *108*, 17426–17431.
42. Hu, Y., Li, Z.F., Wu, X., and Lu, Q. (2011). Large induces functional glycans in an O-mannosylation dependent manner and targets GlcNAc terminals on alpha-dystroglycan. PLoS ONE *6*, e16866.
43. Vajsar, J., Baskin, B., Swoboda, K., Biggar, D.W., Schachter, H., and Ray, P.N. (2008). Walker-Warburg Syndrome with POMT1 mutations can be associated with cleft lip and cleft palate. Neuromuscul. Disord. *18*, 675–677.
44. Lefeber, D.J., Schönberger, J., Morava, E., Guillard, M., Huyben, K.M., Verrijp, K., Grafakou, O., Evangeliou, A., Preijers, F.W., Manta, P., et al. (2009). Deficiency of Dol-P-Man synthase subunit DPM3 bridges the congenital disorders of glycosylation with the dystroglycanopathies. Am. J. Hum. Genet. *85*, 76–86.
45. Godfrey, C., Foley, A.R., Clement, E., and Muntoni, F. (2011). Dystroglycanopathies: coming into focus. Curr. Opin. Genet. Dev. *21*, 278–285.
46. Messina, S., Tortorella, G., Concolino, D., Spanò, M., D'Amico, A., Bruno, C., Santorelli, F.M., Mercuri, E., and Bertini, E. (2009). Congenital muscular dystrophy with defective alpha-dystroglycan, cerebellar hypoplasia, and epilepsy. Neurology *73*, 1599–1601.
47. Lefeber, D.J., de Brouwer, A.P., Morava, E., Riemersma, M., Schuurs-Hoeijmakers, J.H., Absmanner, B., Verrijp, K., van den Akker, W.M., Huijben, K., Steenbergen, G., et al. (2011). Autosomal recessive dilated cardiomyopathy due to DOLK mutations results from abnormal dystroglycan O-mannosylation. PLoS Genet. *7*, e1002427.
48. Frost, A.R., Böhm, S.V., Sewduth, R.N., Josifova, D., Ogilvie, C.M., Izatt, L., and Roberts, R.G. (2010). Heterozygous deletion of a 2-Mb region including the dystroglycan gene in a patient with mild myopathy, facial hypotonia, oral-motor dyspraxia and white matter abnormalities. Eur. J. Hum. Genet. *18*, 852–855.
49. Hara, Y., Balci-Hayta, B., Yoshida-Moriguchi, T., Kanagawa, M., Beltrán-Valero de Bernabé, D., Gündeşli, H., Willer, T., Satz, J.S., Crawford, R.W., Burden, S.J., et al. (2011). A dystroglycan mutation associated with limb-girdle muscular dystrophy. N. Engl. J. Med. *364*, 939–946.
50. Vuillaumier-Barrot, S., Bouchet-Seraphin, C., Chelbi, M., Eude-Caye, A., Charluteau, E., Besson, C., Quentin, S., Devisme, L., Le Bizec, C., Landrieu, P., et al. (2011). Intragenic rearrangements in LARGE and POMGNT1 genes in severe dystroglycanopathies. Neuromuscul. Disord. *21*, 782–790.
51. Raducu, M., Baets, J., Fano, O., Van Coster, R., and Cruces, J. (2012). Promoter alteration causes transcriptional repression of the POMGNT1 gene in limb-girdle muscular dystrophy type 2O. Eur. J. Hum. Genet. *20*, 945–952.