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Clinical Report

A Novel Mutation In *GMPPA* In Siblings With Apparent Intellectual Disability, Epilepsy, Dysmorphism And Autonomic Dysfunction¹.

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ABSTRACT

GMPPA encodes the GDP-mannose pyrophosphorylase A protein (GMPPA). The function of GMPPA is not well defined, however it is a homologue of GMPPB which catalyzes the reaction that converts mannose-1-phosphate and guanosine-5'-triphosphate to GDP-mannose. Previously, biallelic mutations in *GMPPA* were reported to cause a disorder characterized by achalasia, alacrima, neurological deficits and intellectual disability. In this study, we report a female proband with achalasia, alacrima, hypohydrosis, apparent intellectual disability, seizures, microcephaly, esotropia, and craniofacial dysmorphism. Exome sequencing identified a previously unreported homozygous c.853+1G>A variant in *GMPPA* in the proband and her affected sister. Their unaffected parents were heterozygous, and unaffected brother homozygous wild type for this variant. Lymphoblast cells from the affected sisters showed complete loss of the GMPPA protein by western blotting, and increased levels of GDP-mannose in lymphoblasts on high performance liquid chromatography. Based on our findings and the previous report describing patients with an overlapping phenotype, we conclude that this novel variant in *GMPPA*, identified by exome sequencing in the proband and her affected sister, is the genetic cause of their phenotype and may expand the known phenotype of this recently described glycosylation disorder.

KEY WORDS

GMPPA, GDP-mannose, intellectual disability, exome sequencing, Triple-A syndrome

INTRODUCTION

Congenital disorders of glycosylation represent a group of rare genetic, metabolic disorders due to defects in glycosylation that manifest as multisystem diseases [Freeze et al., 2012]. Two main types of protein glycosylation exist, namely N-glycosylation and O-glycosylation. N-glycosylation involves the attachment of N-glycans to an amino group of asparagine residues whereas O-glycosylation involves the attachment of O-glycans to the hydroxyl group of threonine or serine residues. Congenital disorders of glycosylation are a rapidly growing family of genetic disorders with over one hundred disorders being identified in the past thirty years [Jaeken, 2010]. The GDP-mannose molecule is an essential cellular requirement for a number of glycosylation pathways, including O-mannosylation of membrane and secretory glycoproteins, such as alpha- dystroglycan [Ning et al., 2000]. Recently, mutations in *GMPPA* were associated with a novel disorder known as AAMR (alacrima, achalasia, and “mental-retardation syndrome”, OMIM 615510) characterized by intellectual disability and autonomic dysfunction [Koehler et al., 2013]. Koehler et al. [2013] identified *GMPPA* mutations in 13 individuals from nine unrelated families with achalasia, alacrima and neurological deficits. Although the phenotype of these patients partially overlaps with Triple-A syndrome (OMIM 231550), characterized by achalasia, congenital alacrima, adrenal insufficiency and progressive neurologic dysfunction [Milenkovic et al., 2010], they were mutation negative for AAAS (OMIM 605378), the only gene known to be mutated in Triple-A syndrome.

In this study, we clinically and molecularly investigated two affected sisters from a consanguineous Lebanese family with an overlapping phenotype.

MATERIALS AND METHODS

All scientific methods and ethics information can be found in the supplemental material.

CLINICAL REPORT

The parents were Lebanese and first cousins (Figure 1A). The mother had one well son from a previous union. The couple had two early spontaneous miscarriages, one unaffected son and two affected daughters. A fetal scan of the proband at 18 weeks showed a left diaphragmatic hernia, polyhydramnios and an amniotic leak. She was born at 36 weeks gestation by normal vaginal delivery and intubated. Her birth weight was 2.6 kg (25th - 50th centile), length 50 cm (90th centile), and OFC 33 cm (10th -50th centile). She had complete agenesis of the left hemi-diaphragm at surgical repair on day 2. She was discharged home at age 10 weeks on tube feeds, and then had a fundoplication and gastrostomy tube placement for recurrent aspiration pneumonia and gastro-oesophageal reflux, surgical correction of a left esotropia, and a laparotomy for a duodenal perforation. Her vision and hearing were normal. She had global developmental delay, chronic constipation, reduced tears and hypohydrosis. When examined at age 2 years and 10 months, she had microcephaly (OFC 45 cm; <2nd centile), constant writhing movements and stereotypic hand movements. Craniofacial features included a low anterior hairline, dolichocephaly, a prominent metopic ridge, bitemporal narrowing, telecanthus, prominent earlobes, a broad nasal bridge, a broad, square nasal tip, an overhanging columella, hypoplastic alae nasae, a high arched palate, a Cupid's bow upper lip, downturned corners of the mouth, prognathism, a long pointed chin and generalized hirsutism. At age 5 years,

she could only sit independently, she remained in diapers and had no words. She developed pseudomembranous colitis at age 5½ years which was unresponsive to vancomycin. Biopsy of the descending and sigmoid colon and rectum showed active chronic colitis. She was diagnosed with inflammatory bowel disease, and treated with prednisolone, azathioprine and salazopyrin. She had osteopenic tibial and vertebral thoracic crush fractures, treated with zoledronate. At age 8 years, she was microcephalic (OFC 48.5 cm, <2nd centile). She remained non-verbal, non-ambulatory and fully dependent. She had hand stereotypies and bruxism. She developed seizures, with an abnormal almost continuous high amplitude posterior spike and wave EEG pattern, and was treated with anticonvulsants. At age 10 years, she still could not walk, was not toilet trained, was non-verbal and attended a school for the disabled. She never fed orally. By age 12 years, her OFC was 49.5 cm (<2nd centile) her height (80 cm) and weight (19 kg) were << 3rd centile. Normal investigations included karyotype, sub-telomere FISH analysis, serum transferrin isoforms, serum very long chain fatty acids, serum lactate, pyruvate and ammonia, serum 7-dehydrocholesterol, urine metabolic screen, amino acid profile, organic acid profile, glycosaminoglycan screen, plasma amino acids, acylcarnitine profile, MLPA microdeletion screen, DNA array (Agilent aCGH 44K array), EEG, *MECP2* sequencing and deletion/duplication analysis and methylation studies for Angelman syndrome. Cerebral MRI was normal apart from ventricular prominence. Radiographs showed bilateral coxa valga, a right subluxed hip, vertebral crush fractures and osteopenia. Bone mineral density was low for age.

Her affected sister was born after a normal pregnancy. Delivery was induced at 41 weeks gestation by emergency cesarean for fetal distress. The birth weight was 3360 g (25th

centile), length 56 cm (97th centile) and OFC 34 cm (25th centile). She was examined at 4 months of age and had similar dysmorphism to her sister with the added features of arched eyebrows, a right anteverted ear and failure to thrive. By age 18 months, she had developmental delay and seizures, constant writhing movements, repetitive sucking of her fingers, congenital severe chronic constipation, alacrima, absent saliva, xerostomia, hypohydrosis, strabismus, a fine horizontal nystagmus, a pale left optic disc and generalized hirsutism. Recurrent upper respiratory tract infections improved after tonsillectomy and adenoidectomy. At age 2 years, she was non-verbal and could only sit with support. Her OFC was 48cm (63rd centile), and weight 10.3 kg (4th centile). At age 4½ years, her OFC was 51 cm (80th centile) and weight 13 kg (1st centile). Her length at age 4 years 9 months was 108 cm (24th centile). Unlike her sister, she was not microcephalic, she had xerostomia and absent saliva, was able to eat pureed food and has not developed inflammatory bowel disease. Investigations included a karyotype, 60K CGH array and cerebral MRI, with normal results. Her EEG was abnormal, and very similar to her sister's.

RESULTS

Bioinformatic analysis of the exome data identified homozygous variants in three candidate genes; *LRFN2* (leucine rich repeat and fibronectin type III domain containing 2), *RESP18* (Regulated Endocrine-Specific Protein 18) and GDP-mannose pyrophosphorylase A (*GMPPA*) in both affected girls. The unaffected brother did not have any variants in these genes. As there was a close clinical resemblance between these girls and those with *GMPPA* mutations [Koehler et al., 2013], *GMPPA* was selected as the most likely candidate. We identified a novel homozygous splice site variant c.853+1G>A (g.220370278; RefSeqGene NG_033833.1; NM_013335) in intron 9 of the *GMPPA* gene

in the proband and her sister. Sanger sequencing confirmed the presence of this variant in both girls (III:3 and III:4), while their parents (II:6 and II:7) were heterozygous and unaffected brother (III:2) homozygous for the normal allele (Figure 2).

In silico analyses were used to determine the potential effect of the *GMPPA* variant on the structure of *GMPPA*. The splice site predictor Berkeley Drosophila Genome Project predicted the variant to ablate the splice site, and the splice site mutation predictor Cryp Skip program predicted this to result in an alternate splice site half way through exon 9, most likely disrupting the structure of the protein. Further, Alamut v2.4 (Interactive Biosoftware) analysis predicted that the variant occurs in the donor splice site of intron 9 and is likely to result in skipping of exon 9. If exon 9 is skipped then the mRNA may be targeted for nonsense mediated mRNA decay thus resulting in no protein formation. To our knowledge, this variant has not been reported previously and is not present in ClinVar [Landrum et al., 2016]. It has also not been reported in the dbSNP, ExAC or the 1000G databases.

In order to assess whether the variation resulted in a reduction in *GMPPA*, western blotting was conducted using whole cell lysates from lymphoblasts isolated from both sisters (III:3 and III:4), the mother (II:6), father (II:7), and a wild-type control. *GMPPA* was found to be completely absent in the two affected girls. (Figure 1B). As the variation alters a normal splice of *GMPPA*, the transcript and/or protein is probably unstable.

GMPPA mutations have been previously reported to cause raised levels of GDP-mannose [Koehler et al., 2013]. We measured GDP-mannose by HPLC and observed a significant increase in the proband (III:3) and her sister (III:4), whereas the mother (II:6) and father (II:7) had levels comparable to that of a wild type control (Figure 1C).

DISCUSSION

Here, we describe two sisters from a consanguineous union with a novel splice site variation in *GMPPA* presenting collectively with achalasia, alacrima, xerostomia, hypohydrosis, developmental delay, seizures, microcephaly, constant writhing movements and stereotypies, congenital chronic constipation, inflammatory bowel disease, congenital diaphragmatic hernia due to complete agenesis of the left hemi- diaphragm, esotropia, nystagmus, and craniofacial dysmorphism. We believe that at least some of their features, namely the achalasia, alacrima, xerostomia, hypohydrosis, apparent intellectual disability, ophthalmological manifestations and possibly also the chronic constipation are due to the *GMPPA* variant and consistent with the *GMPPA* variant glycosylation disorder described previously by Koehler et al. [2013]. We identified some additional features in these two sisters, which perhaps should be evaluated in newly identified patients. We cannot be sure which of those additional features might be attributable to the *GMPPA* variant because of the complex consanguinity in the kindred. Through careful literature searches on the candidate variants returned from the exome sequencing analysis, we identified that the sisters in this study had a phenotype overlapping with patients with mutations in *GMPPA* and presenting with achalasia, previously reported to be associated with a glycosylation disorder characterized by intellectual disability and autonomic dysfunction [Koehler et al., 2013]. They found a homozygous nonsense mutation that co-segregated with achalasia and alacrima, developmental delay and gait abnormalities in a consanguineous Pakistani kindred. They also identified *GMPPA* mutations in ten additional individuals from eight unrelated families with achalasia, alacrima and neurological deficits and concluded that a Triple-A-like syndrome could be added to the growing list of congenital disorders of

glycosylation wherein the underlying pathophysiological mechanism is dysregulation rather than enzyme deficiency. In the current report, the sisters had many of the previously described neurological and autonomic nervous system abnormalities of this condition and neither had adrenal insufficiency, thus excluding a diagnosis of Triple-A syndrome. Additional features in both included craniofacial dysmorphism, hypohydrosis, generalized hirsutism, severe seizures, and chronic constipation, features not described previously by Koehler et al. [2013]. Furthermore, the proband had acquired microcephaly, inflammatory bowel disease and an atypical congenital diaphragmatic hernia, whereas her sister had xerostomia and absent salivation, all features not previously described. Xerostomia, hypohydrosis, absent salivation and chronic constipation, can be regarded as further features of autonomic dysfunction, and would be consistent with the condition described by Koehler et al. [2013]. We believe that these features expand the phenotype of this new disorder caused by variations in *GMPPA*. Given the multiple consanguineous loops within the family, it is possible that one or both sisters suffer from more than one condition, thus confounding which features may be truly attributable to the glycosylation disorder alone. Thus, we can draw no conclusions about the acquired microcephaly which is possibly related to the severity of seizures in the proband, nor to the craniofacial dysmorphism in both sisters in the context of a second possible separate autosomal recessive disorder. We believe it unlikely that the congenital diaphragmatic hernia or inflammatory bowel disease in the proband are related to the *GMPPA* variant. To date, there are no known human diseases associated with homozygous variants in either *LRFN2* or *RESP18*. Reports of further similarly affected individuals are needed to better define the phenotype of this relatively newly recognized disorder, which is distinct from the Triple-A syndrome.

The *in silico* results predicted this variant to disrupt the splice site and using western blotting, we showed that the splice site variation resulted in the total ablation of GMPPA expression and significantly elevated levels of GDP-mannose in the sisters' lymphoblast cells compared to their parents and healthy controls. GMPPA does not have catalytic activity, but it is able to bind to GDP-mannose [Szumilo et al., 1993], however, it is uncertain why the levels of GDP-mannose are elevated in GMPPA-deficient cells. Little is known about the function of GMPPA, however GMPPA may serve as a regulatory subunit and by allosteric actions, regulate the function of GMPPB [Koehler et al., 2013]. We conclude that the variation observed in *GMPPA* in the sisters in our study is the genetic cause of at least their autonomic and neurological phenotype and possibly also their poor growth. Whether or not the atypical congenital diaphragmatic hernia, inflammatory bowel disease and craniofacial dysmorphism are related to the glycosylation disorder is unknown. Our findings support the previous conclusion of Koehler et al. [2013] that variations in *GMPPA* should be added to the growing list of congenital disorders of glycosylation.

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INTERNET RESOURCES

Berkeley Drosophila Genome Project splice site prediction (http://www.fruitfly.org/seq_tools/splice.html), Cryp Skip splice site prediction (<http://crypskip.img.cas.cz/>), dbSNP browser (<http://www.ncbi.nlm.nih.gov/SNP/>), ExAC browser

(<http://exac.broadinstitute.org>).

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FIGURE CAPTIONS

Figure 1 (A) Pedigree of the family, where the affected girls are shown by the completely filled circles

(III:3 and III:4), the unaffected brother (III:2) by an open square and the parents (II:6 and II:7) by circles surrounding a dot. (B) Immunoblot showing complete absence of GMPPA protein in lymphoblast cells from the proband (III:3) and affected sister (III:4) compared to the unaffected mother (II:6) and unaffected father (II:7) and a wild-type control. (C) HPLC analysis showing elevated levels of GDP-mannose in lymphoblast cells of both GMPPA deficient patients (III:3 and III:4) relative to their parents (II:6 and II:7) and control.

Figure 2. Sanger sequencing of genomic DNA validates segregation of the *GMPPA* mutation in all family members (III:3 = proband, III:4 = affected sister, III:2= unaffected brother, II:7 = unaffected father and II:6 = unaffected mother). The mutated and wild-type nucleotides are highlighted with an arrow. [Color figure can be viewed in the online issue, which is available at [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1552-4833](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1552-4833)].

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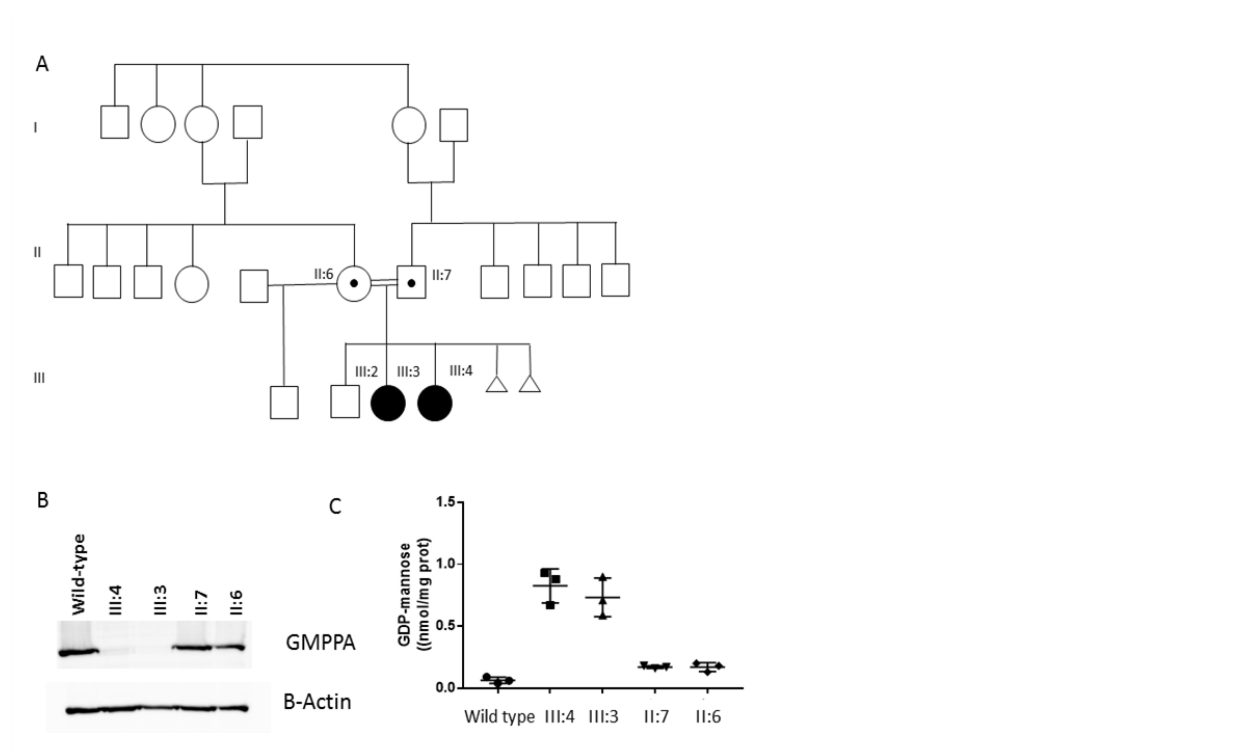


Figure 1 .

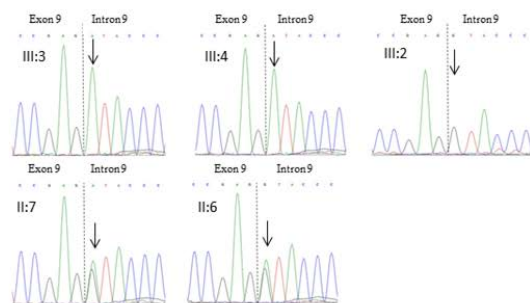


Figure 2 .