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*Clinical Report*

# CDG-IL: An Infant With a Novel Mutation in the *ALG9* Gene and Additional Phenotypic Features

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**We describe the second case of congenital disorder of glycosylation type IL (CDG-IL) caused by deficiency of the ALG9 a1,2 mannosyltransferase enzyme. The female infant's features included psychomotor retardation, seizures, hypotonia, diffuse brain atrophy with delayed myelination, failure to thrive, pericardial effusion, cystic renal disease, hepatosplenomegaly, esotropia, and inverted nipples. Lipodystrophy and dysmorphic facial features were absent. Magnetic resonance imaging of the brain showed volume loss in the cerebral hemispheres and cerebellum and delayed myelination. Laboratory investigations revealed low levels of multiple serum proteins including antithrombin III, factor XI, and cholesterol. Hypoglycosylation was confirmed by the typical CDG type 1 pattern of serum transferrin analyzed by isoelectric focusing. A defect in the ALG9 enzyme was suggested by the accumulation of the DolPP-GlcNAc2Man6 and DolPP-GlcNAc2Man8 in the patient's fibroblasts and confirmed by mutation analysis: the patient is homozygous for the *ALG9* mutation p.Y286C. The causal effect of the mutation was shown by complementation assays in alg9 deficient yeast cells. The child described here further delineates the clinical spectrum of CDG-IL and confirms the significant clinical overlap amongst CDG subtypes.** 

**KEY WORDS: congenital disorders of glycosylation; CDG-IL**

## INTRODUCTION

Congenital disorders of glycosylation type I (CDG-I) are an increasingly recognized and expanding group of genetic conditions, caused by abnormalities in the synthesis of the lipid linked glycan precursor or in the attachment of glycans to proteins [Jaeken and Carchon, 2004]. Most affected children have multi-system involvement demonstrating the importance of normal protein glycosylation in cellular function, but more limited types with predominantly liver or bowel involvement have been described [Damen et al., 2004]. In 2004, a novel form of CDG, CDG-IL, caused by a deficiency in the enzyme α1,2-mannosyltransferase due to a defect in the *ALG9* gene was described in an infant with neuro-developmental impairment, seizures and hepatomegaly [Frank et al., 2004]. We describe the clinical, biochemical, and molecular features of second case of CDG-IL. The loss of function of the mutant α1,2-mannosyltransferase was shown in a yeast complementation assay.

## CLINICAL REPORT

A 7-month-old girl was admitted for evaluation of failure to thrive and hypotonia. She was the second child born to unrelated Caucasian parents at 37 weeks gestation with a birth weight of 2,600 g. The antenatal history was significant for the finding of a pericardial effusion. A persistent small-moderate pericardial effusion without structural heart disease persisted in the neonatal period, and ultrasonography revealed the presence of multiple small renal cysts bilaterally. Poor weight gain and delayed gross motor development was identified at her 2-month follow up visit.

On physical examination, the infant appeared pale, irritable, and wasted. Weight was 4.36 kg (<3rd centile), length 57 cm (<3rd centile), and head circumference 40 cm (3rd centile). She had a right-sided torticollis and intermittent esotropia. Inverted nipples were present. Her liver was felt 3 cm below the right costal margin and spleen tip 2 cm below the left costal margin. Muscle bulk and tone were markedly reduced with prominent head lag. Deep tendon reflexes were normal. Two months after admission (at 9 months), an increase in the pericardial effusion with tamponade necessitated a pericardiocentesis. Recurrent seizures treated with phenobarbital developed at 10 months of age. A routine electroencephalogram was normal.

At admission, serum albumin was 16 g/L (normal 32–48 g/L). Proteinuria was absent. Alpha-1-antitrypsin clearance in the stool was normal. Hemoglobin was 99 g/L (normal 110–140 g/L), white blood cell count $13.5 \times 10^9$/L with a normal differential count and the platelet count was normal. International normalized ratio and partial thromboplastin time were normal, but the antithrombin III (0.54 IU/ml, normal 0.85–1.25 IU/ml) and Factor XI (0.42 IU/ml, normal 0.5–1.5 IU/ml) levels were low. Factor VIII, Factor IX, protein C, and protein S levels were normal. The serum cholesterol level was low

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(2.08 mmol/ml, normal 3.20–4.91 mmol/L); haptoglobin, thyroxine, cortisol, and transferrin levels were normal. Endoscopic small bowel biopsies showed focal partial villous atrophy.

When seen at 14 months of age, all growth parameters were below the 3rd centile, but her weight gain and linear growth velocity had increased. Her motor development was at a 4–5 months level. Magnetic resonance imaging showed increased cerebrospinal fluid spaces, widening of the sulci, delayed myelination, and enlargement of the lateral ventricles that, in the presence of microcephaly, suggested cerebral and cerebellar atrophy (Fig. 1). Seizure control was problematic despite multiple drug therapy. At 17 months, progressive microcephaly was evident with a head circumference of 43.7 cm (<3rd centile).

## MATERIALS AND METHODS

### CDG-I Diagnostic Work Up

The diagnosis of CDG was confirmed following the flow path as described by Grunewald et al. [2002]. The *ALG9* gene was screened for mutations by direct sequencing as described in Frank et al. [2004]. The carrier status of the parents was confirmed on genomic DNA.

### Yeast Strains and Media

Saccharomyces cerevisiae strains used in the study were derivates of YG414 (Mat ade2-101 ura3-52 his3 200 lys2-801 alg9::KanMX) and YG415 (Mat ade2-101 ura3-52 his3 200 lys2-801 alg9::KanMX wbp1-2) [Burda et al., 1996]. Standard yeast media and genetic techniques were used [Guthrie and Fink, 1991]. The YG414 strain and its transformants were propagated at 30°C; the YG415 strain and its transformants at 23°C.

### Cloning of Human *ALG9* (Y286C) cDNA Into a Yeast Expression Vector

To obtain a construct expressing the human *ALG9* (Y286C) cDNA in *S. cerevisiae* we cloned the mutated cDNA in a yeast expression vector (pCFZ41-GPD) under the control of the glyceraldehydes phosphate dehydrogenase promoter (GDP) [Mumberg et al., 1995]. The expression vector pCFZ41-GPD-Hs*ALG9* described by [Frank et al., 2004] was used as template for in vivo cloning by homologous recombination technique [Oldenburg et al., 1997]. Two overlapping PCR were amplified with primerset 1 (5′-GTGTGCAAGAAGTTTGGG and 5′-GGAGTAAAGATATTACAC AAAACAATGTTGAGTGG) and primerset 2 (5′-GAGAAGTCAGTGGTAGG and CCACTCAA-CATTGTTTTGTGTAATATCTTTACTCC). The primers introduce a transition of A to G at position c.860, resulting in a substitution of tyrosine (Tyr, Y) to cysteine (Cys, C) at amino acid position 286. Integration of the resulting PCR fragment into the linearized expression vector was achieved by homologous recombination in yeast cells during transformation. The transformants were plated on SD (-ura) plates to select for successful recombinants. The plasmid was re-isolated from yeast and amplified in *Escherichia coli*. The Y286C mutation was confirmed by sequencing.

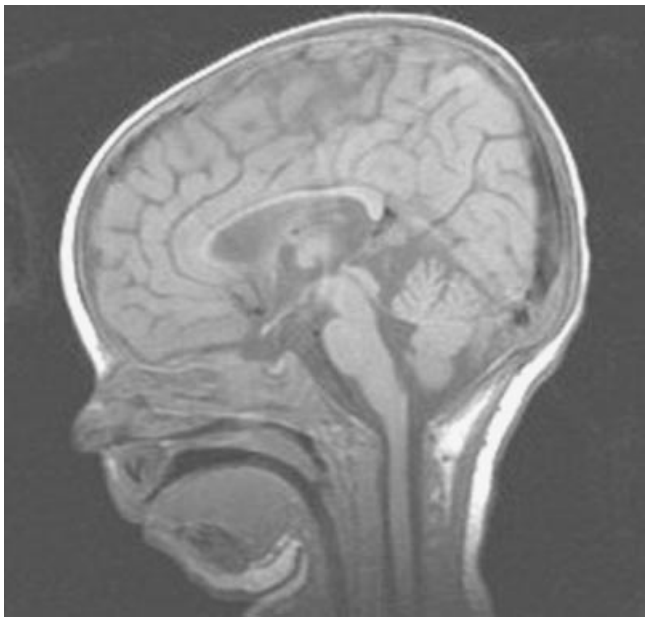

Fig. 1. Saggital MRI image demonstrating increased cerebrospinal fluid spaces, widening of the sulci, delayed myelination, and enlargement of the lateral ventricles.

### Complementation Assays

For the growth assay, YG415 (Δ*alg9 wbp1-2*) cells were transformed with yeast expression vector containing either wild-type or mutant human *ALG9* cDNAs and grown on selective medium lacking uracil. Cells were collected in their logarithmic phase, six times diluted 1/6 starting with 5 μl of $0.25 \times 10^7$ cells/ml and spotted on YPD plates by followed incubation at 23, 30, and 32°C, respectively for 96 hr. Analysis of carboxypeptidase Y (CPY) in *ALG9*-deficient cells (YG414) expressing different human *ALG9* cDNAs was detected via Western blot analysis. This was performed as described previously [Burda et al., 1996].

## RESULTS

The clinical presentation was suggestive of a congenital disorder of glycosylation, and transferrin isoelectric focusing revealed an abnormal, type 1 pattern with elevated disialo- and asialo-transferrin isoforms. Normal fibroblast phosphomannomutase and phosphomannoisomerase activities and the absence of mutations in the *PMM2* gene excluded CDG-Ia (MIM 212065) and CDG Ib (MIM 602579). To distinguish between the other CDG-I types (CDG-Ic to CDG-IL), lipid linked oligosaccharides from patients fibroblast were extracted and analyzed by DHPLC [Frank et al., 2004]. The lipid-linked oligosaccharide profile revealed an increase in *DolPP*-$GlcNAc_2$-$Man_6$ and *DolPP*-$GlcNAc_2Man_8$ structures suggesting a defect in the α1,2-mannosyltransferase (CDG-IL), a protein coded for by *ALG9*. Analysis of the *ALG9* cDNA identified a homozygous point mutation p. Y286C (c. 860A > G). The homozygous mutation was confirmed in the child's genomic DNA, and both parents were confirmed to be carriers.

Tyrosine (Tyr, Y) at position p.286 is conserved between human, *Saccharomyces cerevisiae*, *Macaca fascicularis*, *Schizosaccharomyces pombe*, *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Arabidopsis thaliana*, suggesting an essential role for this amino acid in the function of the enzyme [Baysal et al., 2002]. To verify the deleterious effect Y286C on the ALG9 function, we compared the complementation efficiency of the wild-type and mutant ALG9 cDNA in yeast cells deficient for alg9. In a first assay, the growth efficiency of the transformed yeast double mutant alg9 wbp1-2 was tested. Deficiency in lipid-linked oligosaccharide biosynthesis (alg9) in combination with reduced oligosaccharyltransferase activity (wbp1-2) results in a temperature-sensitive phenotype at 30°C [Stagljar et al., 1994]. At this restrictive temperature, both the normal and mutant ALG9 cDNAs were able to restore growth (Fig. 2). The HsALG9 transformants performed similar to the

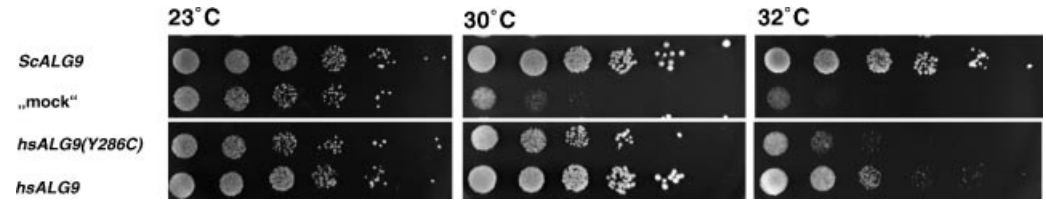


Fig. 2. Complementation of Δ*alg9 wbp1-2* yeast mutant strain with different human *ALG9* cDNAs. Yeast strain YG415 was transformed with either the vector alone (mock), the p*ALG9* plasmid expressing the *S. cerevisiae ALG9* gene (*ScALG9*), or plasmids expressing the wild-type human *ALG9* cDNA or the Y286C mutant *ALG9*, respectively. Transformants were spotted in 10-fold serial dilution starting at $5 \times 10^5$ cells on YPD plates and were incubated at 23, 30, and 32°C.

yeast alg9. The complementation with the mutant construct (HsALG9 (Y286C)) was less efficient, resulting is a reduced growth restoration. This difference became more prominent when the transformants were grown at 32°C.

In a second experiment, the N-linked glycosylation efficiency in yeast was monitored using CPY, a vacuolar protein with four N-glycosylation sites, as a reporter glycoprotein. Deletion of the yeast *ALG9* locus leads to the accumulation of lipid-linked oligosaccharide intermediates which are not efficiently transferred onto proteins thus leading to hypoglycosylation [Burda et al., 1996]. The hypoglycosylation of the *alg9* yeast strain is reflected in the presence of CPY glycoforms lacking one or two N-linked oligosacharides (Fig. 3). The human *ALG9* cDNA complemented the yeast mutation partially, as shown by the improved glycosylation of CPY. The complementation efficiency of the Hs*ALG9* (Y286C) was less efficient and almost comparable to the empty vector.

## DISCUSSION

CDG are a heterogeneous group of autosomal recessive genetic conditions caused by the abnormal synthesis of glycan moieties or their attachment to glycoconjugates. Since the first report in 1980 by Jaeken et al. [1980], describing monozygotic twin girls with psychomotor retardation, unusual dysmorphic features and abnormal levels of serum and cerebrospinal fluid proteins the spectrum of presentation has continued to expand.

In 2004 a novel form of CDG, CDG-IL, was described in an infant who was microcephalic at birth who later exhibited progressive microcephaly, developmental delay, hypotonia, seizures, and hepatomegaly [Frank et al., 2004]. The defect was characterized by a deficiency of α1,2-mannosyltransferase which catalyzes the addition of the seventh and ninth mannose residues on growing lipid-linked oligosaccharides leading to an accumulation of *DolPP*-$GlcNAc_2Man_6$ and *DolPP*-$GlcNAc_2Man_8$ precursors. A homozygous point mutation c.1567G > A (amino acid substitution E523K) was detected in the *ALG9* gene, which codes for this transferase. Further proof of causation included the demonstration of functional homology between the human and yeast *ALG9* genes and confirmation of the deleterious effect of the mutation using a yeast complementation assay.

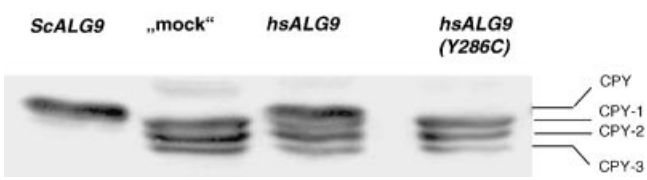


Fig. 3. Analysis of CPY in *alg9*-deficient strains expressing different human *ALG9* cDNAs. The Δ*alg9* strain YG414 transformed with either the vector alone (mock), the plasmid carrying the *S. cerevisiae ALG9* gene (*ScALG9*) and plasmids expressing the normal human *ALG9* cDNA, or the mutant *ALG9*(Y286C) cDNA. Glycosylation of CPY was visualized by Western blot analysis using CPY-specific antibodies. The position of mature CPY and the different glycoforms lacking up to four N-linked glycans are indicated.

The infant presented in this report is the second child described with CDG-IL. She had abnormal isoelectric focusing of transferrin isoforms, with increased asialo- and disialotransferrin, and an abnormal LLO profile with increased DolPP-GlcNAc2Man6 and DolPP-GlcNAc2Man8 precursors, low levels of antithrombin III, factor XI and cholesterol, and a novel mutation in the *ALG9* gene (Y286C). Similar to the infant first described by Frank et al. [2004], the infant described here exhibited progressive microcephaly, seizures, developmental delay, and hepatomegaly. Many of the infant's clinical features were not described in the original report but are common to other forms of CDG, including CDG-Ia [Krasnewich and Gahl, 1997]. These features include cystic renal disease, pericardial effusion and tamponade, partial villous atrophy, hypoproteinemia, esotropia, inverted nipples, coagulopathy, and brain imaging findings that suggested cerebral and cerebellar atrophy.

Importantly, the yeast complementation assays and functional analysis confirmed the causal effect of the Y286C mutation in our patient by demonstrating severely reduced enzyme activity. The clinical, biochemical, and genetic features described in this infant add to the spectrum of presentation of CDG-IL, which can be expected to be identified increasingly in the near future, and highlight the clinical overlap amongst the various types of CDG.

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