Abnormal Glycosylation Profile and High Alpha-Fetoprotein in a Patient with *Twinkle* Variants

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Abstract The *C10orf2* gene encodes Twinkle, a protein involved in mitochondrial DNA (mtDNA) replication. *Twinkle* mutations cause mtDNA deletion or depletion and are associated with a large spectrum of clinical symptoms including dominant progressive external ophthalmoplegia (adPEO), infantile-onset spinocerebellar ataxia (IOSCA), and early-onset encephalopathy. The diagnosis remains difficult because of the wide range of symptoms and lack of association with specific metabolic changes. We report

herein a child with early-onset encephalopathy, unusual abnormal movements, deafness, and axonal neuropathy. All laboratory investigations were normal with the exceptions of high alpha-fetoprotein levels and an abnormal glycosylation profile. These abnormal parameters resulted in misdiagnosis as a previously unidentified congenital disorder of glycosylation (CDG) type I syndrome. Whole exome sequencing revealed two point mutations in *C10orf2* that were confirmed by Sanger sequencing; neither had been previously reported. This report enlarges the clinical phenotype of *Twinkle* mutations and suggests that an abnormal glycosylation profile suggestive of CDG type I associated with high blood alpha-fetoprotein levels without obvious cause should prompt *Twinkle* sequencing.

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Introduction

Mitochondrial DNA (mtDNA) depletion syndromes (MDS) are disorders with varied genetic origins and clinical symptoms, characterized by a severe reduction in mtDNA content. Genes associated with MDS encode proteins involved in maintenance of the mitochondrial nucleotide pool or in mtDNA replication (Copeland 2012; El-Hattab and Scaglia 2013), as observed for chromosome 10 open reading frame 2 (*C10orf2*), also called *Twinkle*.

Twinkle mutations were initially described in patients who exhibited dominant progressive external ophthalmoplegia (adPEO) (Spelbrink et al. 2001) and infantile-onset spinocerebellar ataxia (IOSCA) (Nikali et al. 2005). The clinical symptoms of IOSCA are progressive; symptoms initially include ataxia, athetosis, and muscle hypotonia with loss of deep tendon reflexes, then ophthalmoplegia

and hearing loss, and finally sensory neuropathy and epilepsy. The onset of the disease is between 1 and 2 years of age (Koskinen et al. 1994). Recently, case studies of two children with early-onset encephalopathy and liver failure associated with *Twinkle* mutations were reported (Hakonen et al. 2007). The phenotype was close to Alpers hepatocerebral syndrome (Alpers 1931), which is caused by mutations in *POLG*. Thus, *Twinkle* mutations result in a highly nonspecific clinical spectrum, and no suggestive biochemical changes have thus far been identified, explaining in part why the diagnosis remains difficult.

Here we report on a patient who presented with early-onset encephalopathy, abnormal eye and limb movements, deafness, and axonal neuropathy very likely related to *Twinkle* mutations. Interestingly, an abnormal glycosylation profile of serum glycoproteins was observed, evoking a congenital disorder of glycosylation (CDG).

Case Description

This male infant was born from non-consanguineous parents at 38 weeks of gestation, after an uneventful delivery with a birth weight of 3,560 g (P50), a height of 49 cm (P50), and a head circumference of 35 cm (P50). The mother presented with a dengue infection during the first trimester of pregnancy. There was no significant family history, except for an early miscarriage. The first months of the infant's life were marked by regular vomiting. The child was hospitalized at 20 days of life for malaise ascribed to a severe gastroesophageal reflux. Concerns about neurological development were noted first at the age of 5 months, when the child presented with progressive hypotonia and psychomotor regression. The first assessment was carried out at the age of 7 months. At that time, generalized hypotonia, persistence of archaic reflexes, and motor incoordination were observed. Episodes of abnormal eye movements, sometimes with tongue protrusion, were also noted. Feeding difficulties became progressively more prominent with recurrent vomiting episodes. At the age of 11 months, the child had intermittent chorea, especially in the upper limbs and face, non-systematized eye movements with normal eye tracking and ophthalmoscopy, and absence of deep tendon reflexes. The head circumference growth was regular on -1 standard deviation, whereas growth of weight and height faltered from 7 months of age. There was no dysmorphic feature. The patient exhibited a progressive loss of contact, recurrent episodes of abnormal movements, and died at the age of 21 months.

At 1 year of age, brain and spinal cord MRI were normal. An MIBG scan (metaiodobenzylguanidine scintigraphy) was also performed to rule out neuroblastoma

and did not reveal abnormalities. Echocardiography and abdominal ultrasounds were normal. Electroencephalogram showed a poorly organized spontaneous electric activity, without physiological background rhythm, but no focal or generalized discharges. Auditory evoked potentials showed a deep and bilateral sensorineural hearing loss.

Electromyography confirmed sensorimotor neuropathy of the four limbs, with neuropathy more pronounced on the lower limbs. A neuromuscular biopsy disclosed neurogenic muscular atrophy in conjunction with a moderate but progressive axonal neuropathy.

Extensive laboratory investigations (including urine and blood amino acids, urine organic acids, very long chain fatty acids, ceruloplasmin and copper, amino acids, glucose, and neurotransmitters in the cerebrospinal fluid [CSF]) were normal. Repeated plasma lactate measurements were normal. CSF lactate was normal (1.62 mmol/L) as was the CSF lactate to pyruvate ratio.

Alpha-fetoprotein (AFP) was measured as early-onset ataxia-telangiectasia was suspected. AFP levels were consistently high ($>4,000$ ng/mL, normal value: <100 ng/mL) in the absence of any liver failure or liver tumor. Molecular analyses of the *ataxia-telangiectasia* (*ATM*) and the aprataxin (*APTX*) genes were negative. Western blots of serum glycoproteins (Seta et al. 1996), were performed to rule out a CDG syndrome, and showed an abnormal glycosylation profile suggestive of type I CDG (Fig. 1). Both the *O*-glycosylation pattern and the apoC3 pattern were normal. PMM2-CDG, the most frequent type of CDG, was excluded because of a normal fibroblast activity of phosphomannomutase, and metabolic labeling on fibroblasts did not show accumulation of intermediate sugars. Sequencing of 16 genes known or possibly involved in CDG I (*MPI*, *ALG6*, *DPM1*, *ALG2*, *ALG7*, *ALG9*, *DK1*, *RFT1*, *ALG11*, *SRD5A3*, *ALG13*, *ALG14*, *ALG5*, *ALG10*, *DOLPPI*, and *DHDDS*) did not reveal any mutations, and there were no gene rearrangements in 94 genes involved in glycosylation on a custom CGH array. Taken together these results led to the diagnosis of CDG Ix. Unfortunately, transferrin isoelectric focusing was not performed at that time and remaining serum is not sufficient to perform this analysis.

Finally, a whole exome sequencing (WES) was performed, which revealed two heterozygous mutations in the gene *c10orf2*, encoding the mtDNA helicase Twinkle. These mutations were confirmed by Sanger sequencing. Each mutation was inherited from one parent. These two missense mutations in exon 1 (c.316G>A (p.Lys106Glu) and c.1181G>A (p.Arg394His)) were predicted to be deleterious by three of four functional effect prediction algorithms (PolyPhen-2, SIFT, Align GVGD, Mutation Taster). Neither mutation had been previously reported.

Fig. 1 For CDG I screening, an SDS-PAGE electrophoresis followed by western blotting for four serum *N*-glycan proteins (transferrin, TRF; alpha-1 antitrypsin, AAT; haptoglobin, HPT; orosomucoid, ORO) was performed. The profile from a CDG I patient (lane CDG Ia, the previous name for PMM2-CDG) revealed several bands

corresponding to glycoforms missing one or more *N*-glycans (arrows). Proteins from the patient sample (lane P) presented abnormal profiles with a main normal band (N) and additional bands with lower molecular weight in comparison to the non-CDG I control (lane C)

Retrospectively, severe mtDNA depletion in skeletal muscle (90% depletion) was found. Respiratory chain activities measurements showed inconclusive results in the muscle (due to a long period of storage) and were normal in cultured skin fibroblasts.

Discussion

We report for the first time a patient with secondary glycosylation disorder and elevated AFP caused by previously unreported missense mutations in *Twinkle*. Despite this is a single case with newly reported mutations, the pathogenicity of these latter is highly probable, as (1) they were predicted as pathogenic by three different algorithms, (2) the clinical phenotype is very similar to early-onset *Twinkle*-related disorders, and (3) severe mtDNA depletion in skeletal muscle was found. In addition, we looked for mutations in all genes involved in CDG but did not find any. Given the delay since the patient's death, further functional validation was not possible.

The first patients described with *Twinkle* mutations were from a Finnish cohort with IOSCA also called Perrault syndrome (Koskinen et al. 1994; Lönnqvist et al. 1998). The described phenotype is that of a slow loss of acquisitions, starting with hypotonia, ataxia and athetosis,

subsequent ophthalmoplegia, deafness and sensory neuropathy in adolescence, and very late epilepsy.

Hakonen et al. described a more severe disease associated with compound heterozygous *Twinkle* mutations in two brothers, who had very early-onset encephalopathy at around 6 months of age with abnormal eye, face, and limb movements, sensory neuropathy, and hypotonia (Hakonen et al. 2007). The evolution was marked by the emergence of intractable epilepsy and liver failure, mimicking Alpers syndrome described in patients with *POLG* mutations. The clinical story of our patient resembles that described by Hakonen et al. No seizures were reported for our patient, perhaps because of his death at 21 months of age; the reported patients presented their first seizures at 25 months and 3.5 years of age. AdPEO patients and IOSCA patients with *Twinkle* mutations were described with mtDNA deletions in skeletal muscle, but Hakonen's patients had mtDNA depletion instead of deletion, mainly in the brain and liver samples, but also in the muscle. Similarly, our patient exhibited severe mtDNA depletion in the muscle.

High AFP was the only liver abnormality in our patient. In other patients with *Twinkle* mutations reported to have liver failure, AFP levels were not mentioned. AFP may be elevated in non-mitochondrial disorders such as hepatocarcinoma or any cause of liver regeneration, ataxia-telangiectasia, and ataxia with oculomotor apraxia. All these

possibilities were investigated and eventually ruled out. There are no reported links between mitochondrial disease and elevated AFP, apart from situations of liver failure and/or liver regeneration, which was not the case for our patient. This elevated AFP could be an early sign of liver disease, before liver failure with elevated transaminases and protein synthesis decrease.

Secondary abnormalities of protein glycosylation have been reported in patients with liver disease, alcohol intake, hereditary fructose intolerance, galactosemia, and hemolytic uremic syndrome (Helander et al. 2001; Jaeken 2011). In addition, there are some nonspecific CDG presentations that could mimic mitochondrial disease. In contrast, this is the first description of abnormal CDG profile in mitochondrial disease. The lack of specificity of the clinical manifestations presented by our patient initially led to extensive diagnostic investigations. These investigations revealed no abnormalities suggestive of mitochondrial disease, specifically no lactate accumulation. The only biochemical abnormality was an atypical glycosylation defect, suggestive of CDG type I. The absence of mutations or micro-rearrangements in known CDG genes led us to perform WES. It has been shown that mitochondria play an important role in glycosylation, and *O*- and *N*-glycan chain sialyltransferases and glycosyltransferases are located in the outer mitochondrial membrane (Gasnier et al. 1991a, b). We speculate that mtDNA depletion could disorganize mitochondrial membranes and therefore disrupt glycosylation processes. Conversely, glycosylation abnormalities are known to affect mitochondrial function (Tan et al. 2014). Further experiments are necessary to study the interplay among *Twinkle* mutations, mtDNA depletion, and glycosylation abnormalities. A systematic investigation (even retrospectively) of glycosylation defects in *Twinkle* patients might reveal other patients with similar abnormal glycosylation profile.

In conclusion, these data further expand the phenotype of *Twinkle* mutations and suggest that high AFP along with abnormal CDG type I profile in absence of other cause should prompt *Twinkle* sequencing.

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Take-Home Message

Twinkle mutations can be associated with elevated alpha-fetoprotein and an abnormal CDG profile.

Compliance with Ethics Guidelines

Conflict of Interest

Juliette Bouchereau, Sandrine Vuillaumier Barrot, Thierry Dupré, Stuart E. H. Moore, Ruxandra Cardas, Yline Capri, Pauline Gaignard, Abdelhamid Slama, Hélène Ogier de Baulny, Nathalie Seta, Manuel Schiff, and Laurent Servais declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from the family of the patient for being included in the study.

Details of the Contributions of Individual Authors

Juliette Bouchereau analyzed the results and wrote the paper. Sandrine Vuillaumier Barrot performed the experiments. Thierry Dupré performed the experiments. Stuart E. H. Moore performed the experiments. Ruxandra Cardas performed the experiments. Yline Capri performed the experiments. Pauline Gaignard performed the experiments. Abdelhamid Slama performed the experiments. Hélène Ogier de Baulny performed the experiments. Nathalie Seta performed the experiments. Manuel Schiff wrote the paper. Laurent Servais wrote the paper. Manuel Schiff and Laurent Servais contributed equally to the manuscript.

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