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## Mitochondrion





# Homoplasmy of the m. 8993 T>G variant in a patient without MRI findings of Leigh syndrome, ataxia or retinal abnormalities



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## ABSTRACT

Leigh syndrome is a progressive neurodegenerative syndrome caused by multiple mitochondrial DNA and nuclear DNA pathological variants. Patients with Leigh syndrome consistently have distinct brain lesions found on MRI scanning involving abnormal signal in the basal ganglia, brainstem and/or cerebellum. Other clinical findings vary depending on the genetic etiology and epigenetic factors. Mitochondrial DNA-derived Leigh syndrome phenotype is thought to be modulated by heteroplasmy level. The classic example is the clinical expression of the pathological variant, m. 8993 T>G. At heteroplasmy levels above 90%, the resulting phenotype is Leigh syndrome, but at levels 70-90% patients present with a syndrome of neuropathy, ataxia and retinitis pigmentosa. We describe a 15-year old girl with homoplasmic variant in m.8993 T>G and clinical and biochemical findings consistent with Leigh syndrome but with normal brain MRI findings and without retinal abnormalities or ataxia.

#### 1. Introduction

Leigh syndrome is a progressive neurodegenerative disorder, defined neuropathologically by spongiform basal ganglia and brainstem lesions (Leigh, 1951; Rahman et al., 1996). Brain MRI, particularly T2 and FLAIR (fluid attenuated inverse recovery) sequences, can identify abnormal signal in the deep gray matter indicating spongiform and vacuolation changes (Saneto et al., 2008). Progression is rapid and death occurs usually within two years of initial presentation (Sofou et al., 2018). In addition to brain findings, patients usually present with a range multisystemic phenotypes (Sl et al., 1999). The phenotypic heterogeneity of Leigh syndrome is due in part to the range of the pathological variants in nuclear or mitochondrial DNA (mtDNA) genes.

Multiple pathological variants in mtDNA can give rise to Leigh syndrome (Lake et al., 2016). Variation in phenotype expression can be modulated by mtDNA copy number. There are multiple mtDNA copies within each mitochondrion. The copies of mtDNA in each mitochondrion may not be genetic homogeneous, with some copies having one variant and other copies with the unaltered nucleotide change. This mixture of normal and variant genes within the mtDNA genome is termed heteroplasmy, while homoplasmy is the description of identical genes within mtDNA. For the most part, the expression of the clinical phenotype is related to the percentage of heteroplasmy and the energy demands of a given tissue.

Two prototypical mitochondrial syndromes, directly related to heteroplasmy at the m. 8993 site are Leigh syndrome and neuropathy, ataxia, and retinitis pigmentosa (NARP). These two syndromes can be due to a result of a single pathological variant, m. 8993 T>G, in the mitochondrial adenosine triphosphate synthase subunit 6 gene, MT-ATP6. At high heteroplasmy (>90%), patients present with Leigh syndrome demonstrating MRI changes in the basal ganglia, brainstem and/or cerebellum combined with progressive developmental delay, failure to gain weight, hypertrophic cardiac myopathy, lactic acidosis, leukodystrophy, myopathy, optic atrophy and seizures (Sl et al., 1999; Lake et al., 2016). In a group of 7 patients with Leigh syndrome, the age of onset was 6-months, suggesting a more involved phenotype (Sofou et al., 2018). Lower heteroplasmy (70%-90%) produces NARP. Individuals with heteroplasmy below 60% are usually asymptomatic or oligosymptomatic and are without brain MRI changes on MRI (Lake et al., 2016). Higher heteroplasmy of this genetic variant is thought to consistently result in Leigh syndrome (Sl et al., 1999; Tatuch et al., 1992; Uziel et al., 1997).

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#### 2. Case report

A 12-year old girl presented with intractable generalized seizures, significant intellectual disability, autistic behaviors, and referred to mitochondrial clinic due to the diagnosis of Leigh syndrome. Pregnancy was normal but caesarian delivery occurred due to breech presentation. At birth, she had mild jaundice. She did not require bilirubin-light therapy and treatment was fluids with sun light exposure. Hypotonia was noticed soon after delivery. She had congenital hip dysplasia with surgical correction at 11-months of age. She walked at 22 months of age, which is late but in part related to her history of hip surgery and casting. She had single words at 2 years and simple sentences beginning several months later. Due to language delay and hypotonia, MRI scan of the brain occurred at just over 2 years of age (Fig. 1) and was interpreted as normal.

She developed staring episodes at age 5–6 years, with generalized 4–5 Hertz spike/polyspike and wave complexes on the electroencephalogram. She has multiple medically intractable seizures per day, all short lasting less than 15 s and self-limited.

On neurological exam at 13-years of age; she lacked smooth pursuit and accommodation but there was no ophthalmoparesis, optic atrophy, or retinal involvement on eye exam. She was hypotonic throughout but more so in her axillary structures and could not heel-to-toe walk without falling to one side. She had genu valgum at the knees and wore anklefoot orthotics. She continued to have difficulty in keeping up with her peers in activities and when fatigued, she becomes clumsy.

She has completed several neuropsychological evaluations. By history, she developed single words at age 24 months with 2-word

sentences over the next several months, but for unclear reasons lost language at age 4. She slowly has regained some expressive language with intensive applied behavior analysis (ABA) treatment. At age 7 years, her WISC-IV overall Intelligence Quotient (IQ) was 59 (<1%); non-verbal IQ on the CTONI-2 was 92 (30%). She was retested at age 13vears and classified as mild intellectual disability (IQ = 69; adaptive composite = 70). On the WISC-V, her Verbal Comprehension Index (VCI), Visual Spatial Index (VSI), and Fluid Reasoning Index (FRI) were below average (7th, 3rd, and 2nd percentiles, respectively). Her Processing Speed Index (PSI) and Working Memory Index (WMI) were exceptionally low, below the 1st percentile. Screening of her academic skills, at age 13 years, suggested performance equivalent to the 1st grade level in reading, math calculation, and spelling. She had pronounced impairments in processing and motor speed, attention, and executive function. Her verbal/language skills were mildly delayed but improved over time. Memory skills were generally average. She was formally diagnosed with Autistic Spectrum Disorder at age 7 years.

Mother is Caucasian and Hispanic, and father is Hispanic. Family history revealed no relatives with features similar to their daughter. Mother's sister is healthy and has a normally developing daughter. The maternal grandmother died of a drug overdose at age 39 years. The mother had two subsequent pregnancies that resulted in spontaneous abortions. The desire to have more children led the parents to use a donor egg and in vitro fertilization. The mother delivered fraternal twins, a boy and a girl. Both met developmental landmarks on time without regression. However, the twin girl has been diagnosed with a learning disability and is now in special education and the boy diagnosed with attention deficit/hyperactivity disorder.



**Fig. 1.** Normal brain MRI at 2 years of age. Basal ganglia and midbrain MRI signal abnormalities are typical in Leigh syndrome but are not present in this case; (A–C). Axial T2, fluid-attenuated inversion recovery (FLAIR), and diffusion-weighted sequences (DWI), respectively, at the level of the basal ganglia (arrows) demonstrates no signal abnormality; (D–F). Axial T2, FLAIR, and DWI sequences of the brain stem also show normal signal including the midbrain and periaqueductal gray (arrowhead).

Due to the patient's cognitive defects, epilepsy, muscle endurance difficulties, and neurological exam, she underwent a full work-up for possible etiology at the age of 14 years. Her biochemical labs demonstrated multiple elevations of lactate greater than 3 (normal range < 2.1mmole/L), elevation of plasma alanine 576 (normal range < 560 mcmol/L), low levels of citrulline 2 (normal range, 10-50 mcmol/L, and normal urine acids, CK, and ammonia. The findings on multiple investigations (n = 4) of acyl carnitines demonstrated elevations of C3, mean = 1.63 (propionyl carnitine; normal range, 0-0.9 mcmole/L) and C-5-hydroxycartinine, mean = 0.17 (normal range, 0-0.14 mcmole/L). She underwent next Next Generation Sequencing (NGS) of the entire mtDNA and nuclear DNA (also referred as Whole Exome Sequencing (WES) or massive parallel gene sequencing) using blood cells (GeneDx, Gaitherbury, MA). The testing revealed a homoplasmic variant in the mtDNA genome, m. 8993 T>G and no pathological variants of concern in the nuclear DNA. An MRI of the brain was performed and was interpreted as normal (Fig. 2). There were no signal changes in the basal ganglia, brainstem, or cerebellum suggestive of Leigh syndrome. Although tissue variation is not thought to occur with the m. 8993 T>G variant, we performed NGS of mtDNA from urine sediment cells and 100% homoplasmy was confirmed (GeneDx, Gaithersbury, MA). Mother underwent NGS of blood mtDNA and was found to have approximately 25% heteroplasmy. The non-synonymous base changes reported in the three patients that have been hypothesized to possibly cause ATPase enzymatic changes were not found in our patient (D'Aurelio et al., 2010). The maternal haplogroup was A2g1 on sequencing (GeneDx, Gaithersbury, MA) (Fig. 2).

#### 3. Discussion

Leigh syndrome is a progressive devastating neurodegenerative disease. There are multiple genetic pathways, both nuclear and mtDNA pathological variants giving rise to Leigh syndrome (Sofou et al., 2018; Ruhoy and Saneto, 2014). Diagnosis depends on classical changes on the MRI scan of the brain; laboratory values; and clinical features including motor and intellectual delays, signs and symptoms of brain stem and/or basal ganglia disease, progressive neurological disease and seizures (D'Aurelio et al., 2010). Laboratory abnormalities of elevated lactate in blood and/or CSF, increased alanine and acyl carnitines C3 and C5-OH, and decreased plasma citrulline concentrations are consistently found in a small series of patients with ATP6 induced Leigh syndrome (Larson et al., 2019). In multiple small studies, high heteroplasmy (>90%) of the m. 8993 T>G pathological variant gives rise to Leigh syndrome (Sofou et al., 2018; Sl et al., 1999; Tatuch et al., 1992). These small series are in line with two large studies describing ATP6-associated disease. One of the studies, patients were from several international databases (n = 132)and the other study was derived from a large literature review (n = 218). Both studies described various nucleotide variants within the ATP6 gene, including the m. 8993 T>G pathological variant. The overall conclusion was that a wide range of ATP6 phenotypes exist and phenotypes are not reliably related to heteroplasmy (Stendel et al., 2020; Ganetzky et al., 2019).

The possibility of modification or risk variants has been shown in other diseases. D'Aurelio et al. investigated whether hypomorphic background variants within mtDNA might alter other mitochondrial functions and play a role in phenotypic expression of ATPase disease (D'Aurelio et al., 2010). They looked at three patients with m. 8993 T>G



Fig. 2. Normal brain MRI at 13 years of age; (A–C). Axial T2, fluid-attenuated inversion recovery (FLAIR), and diffusion-weighted sequences (DWI), respectively, at the level of the basal ganglia (arrows) demonstrates no signal abnormality; (D–F). Axial T2, FLAIR, and DWI sequences of the brain stem also show normal signal including the midbrain and periaqueductal gray (arrowhead).

and one patient with m. 8993 T>C, and one patient with m. 9176 T>G. Bioenergetics of homoplasmic cybrids from two patients; one patient harboring the m. 8993 T>G variant had non-synonymous base changes m. 6300 G>A (COX1), m. 12,092 C>T (ND4) and in one patient harboring the m. 9176 T>G variant and non-synonymous base changes in m. 4917 A>G (ND2) and m. 12,092 C>T (ND4) were compared to homoplasmic cybrids of identical m. 8993 T>G and m. 9176 T>G without these variants. Cybrids containing the non-synonymous variants were demonstrated to compromise assembly and reduced enzymatic activities of ETC complexes I and IV, while other m. 8993 T>G and m. 9176 T>G cybrids did not. The results of this study suggested that mtDNA background can play a role in modulating biochemical defects. Our patient did not express any of these non-synonymous nucleotide changes. Whether other modifying mtDNA variants exist is unknown. There have not been any negatively phenotypic altering mtDNA variants published in the literature. NGS variants in nuclear genes that might alter mitochondrial function were not reported in our patient's NGS sequence report.

The advent of gene sequencing analysis began with Sanger sequencing technology over four decades ago. The Sanger sequencing technique relies on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during in vitro DNA replication. Sanger methodology remains the "gold standard" for gene sequencing but is unable to detect mosaic nucleotide changes below a threshold of 15%-20% and can miss a significant proportion of low-level mosaic mutations (Rohlin et al., 2009; Jamuar et al., 2014). Insensitivity of heteroplasmy detection with Sanger sequencing was described in 29 patient samples of apparent homoplasmy previously detected by Sanger methodology, but were found to be heteroplasmic,  $\pm$  12.5% by NGS sequencing (Tang et al., 2013). Accurate percentage of heteroplasmy requires quantification by other methodologies, including PCR-RFLP analysis, real-time amplification refractory mutation system quantitative PCR or pyrosequencing (Bai and Wong, 2004; Tang et al., 2012; White et al., 2005). Unfortunately, commercial use of these methodologies is not universally available and many reported papers describing heteroplasmy do not mention which of these techniques were used or not used. However, NGS coupled with the small size of the mitochondrial genome results in high coverage of each nucleotide position and extremely accurate quantification of low-level heteroplasmy (Tang et al., 2013; Vasta et al., 2012). NGS reproducibility and low experimental error rates give rise to reliable estimates of heteroplasmy of 1%-2% (Wong, 2013).

There have been other patients with high heteroplasmy, based on less sensitive testing (non-NGS testing), who had NARP but not Leigh syndrome. Claeys et al. described a patient with 94% heteroplasmy in urine and 92% in buccal swab and presumed homoplasmy in blood in a patient with NARP (Claeys et al., 2016). Enns et al. reported that using PCR and restriction fragment length polymorphism analysis, levels of heteroplasmy within a single family had various heteroplasmy levels, 40% - 95%, between blood, buccal, skin, and pooled hair bulbs (Enns et al., 2006). These small case reports/series are in line with two large studies describing ATP6-associated disease. One of the studies was from groups of patients from several international databases (n = 132) and the other was large literature review (n = 218) that described various nucleotide variants within the ATP6 gene, including the m. 8993 T>G pathological variant. Both studies conclude that a wide range of phenotypes exists and based on the various methodologies used to measure heteroplamsy, phenotypes are not strictly related to heteroplamsy (Stendel et al., 2020; Ganetzky et al., 2019). However, those patients who had m. 8993 T>G variants with apparent homoplasmy were not defined by sequencing methodology and hence variation in heteroplasmy/homoplasmy was not well described by NGS standards.

It is difficult to explain our patient. By NGS sequencing she had homoplasmy of m. 8993 T>G in multiple tissues; elevated blood lactates, serum alanine, acyl carnitines C3 and C5-OH; reduced plasma citrulline levels; seizures, intellectual delay, and motor delays. There was a described regression of language at age 4-years. Yet, multiple MRI scans were normal. Eye exam did not reveal any signs or NARP, such as ophthalmoplegia or retinitis pigmentosa. Clinically, she did not express ataxia or neuropathy on exam.

Her mitochondrial haplotype is not common in the Leigh syndrome literature. Our patient would conform biochemically to the eight patients reported by Larson et al. (Larson et al., 2019). Although the literature is convincing that heteroplasmy between tissues of this variant is rare, we cannot rule out that brain tissue might have lower heteroplasmy. Given the large literature of non-variant heteroplasmy between tissues, this does not seem likely. Unfortunately, there are no ATPase 6 enzymatic tests that accurately measure forward enzymatic activity. The relationship of phenotype variation with variants in ATP6 needs to be evaluated before better phenotype to genotype can be made. Having ATP6 enzymatic test would help investigate possible modifying variants. We cannot rule out that our patient has a different enzymatic activity compared to other m. 8993 T>G cells. What also remains unknown is whether there are modifying factors affecting degree of CNS involvement with the m. 8993 T>G pathological variant. Rare patients, such as our patient, may reveal potential CNS regulators of CNS changes and disease progression.

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