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The nuclear background influences the penetrance of the near-homoplasmic m.1630 A>G MELAS variant in a symptomatic proband and asymptomatic mother

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Abstract

In this study, we report the metabolic consequences of the m.1630 A>G variant in fibroblasts from the symptomatic proband affected with the mitochondrial encephalomyopathy lactic acidosis and stroke-like episode Syndrome and her asymptomatic mother. By long-range PCR followed by massively parallel sequencing of the mitochondrial genome, we accurately measured heteroplasmy in fibroblasts from the proband (89.6%) and her mother (94.8%). Using complementary experimental approaches, we show a functional correlation between manifestation of clinical symptoms and bioenergetic potential. Our mitochondrial morphometric analysis reveals a link between defects of mitochondrial cristae ultrastructure and symptomatic status. Despite near-homoplasmic level of the m.1630A>G variant, the mother's fibroblasts have a normal OXPHOS metabolism, which stands in contrast to the severely impaired OXPHOS response of the proband's fibroblasts. The proband's fibroblasts also exhibit glycolysis at near constitutive levels resulting in a stunted compensatory glycolytic response to offset the severe OXPHOS defect. Whole exome sequencing reveals the presence of a heterozygous nonsense *VAR2* variant (p.R334X) exclusively in the proband, which removes two thirds of the *VAR2* protein containing key domains interacting with the mt-tRNA^{val} and may play a role in modulating the penetrance of the m.1630A>G variant despite similar near homoplasmic levels. Our transmission electron microscopy study also shows unexpected ultrastructural changes of chromatin suggestive of

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Conflict of interest

There are no conflict of interest to disclose

differential epigenomic regulation between the proband and her mother that may explain the differential OXPHOS response between the proband and her mother. Future study will decipher by which molecular mechanisms the nuclear background influences the penetrance of the m.1630 A>G variant causing MELAS.

Keywords

MELAS; mitochondrial tRNA^{Val}; metabolic adaptability; OXPHOS; Glycolysis; Whole exome sequencing

1. Introduction

The mitochondrial encephalopathy lactic acidosis and stroke-like episode syndrome (MELAS, OMIM 540000) is a progressively neurodegenerative disease with an early onset of heterogeneous clinical symptoms, stroke-like episodes and chronic lactic acidosis being the cardinal clinical features [1-3]. MELAS symptoms include encephalopathy, seizures, dementia, recurrent migraine-like headaches, cyclical vomiting, hearing loss, optic atrophy, peripheral neuropathy, diabetes, nephropathy, short stature, myopathy and cardiomyopathy [4,5]. They are caused by a chronic energy deficit that affects the oxidative phosphorylation (OXPHOS) pathway responsible for ATP synthesis [6,7]. ATP is produced upon electron transfer through the first four OXPHOS complexes, with complexes I and II being the two points of entry for electrons and ATP synthesis occurring at complex V.

Most MELAS are caused by mtDNA variants. Phenotypic variability among MELAS patients results from the heteroplasmic load of the pathogenic mitochondrial DNA (mtDNA) variant, its tissue distribution, and the threshold for OXPHOS defects specific for each tissue or variant [15]. MELAS variants may affect a subset of the multi-copy mitochondrial genome, causing heteroplasmy, which stems from the variable ratios of mutant mitochondrial DNAs (mtDNAs) and wild type (WT) mtDNAs co-existing within mitochondria. Most MELAS patients harbor the m.3243 A>G variant, which maps in the mitochondrial *MT-TL1* gene encoding tRNA^{Leu(UUR)} [8]. However, MELAS can be caused by other rare pathogenic mitochondrial variants mapping in various mitochondrial tRNA genes, such as *MT-TV* encoding tRNA^{Val}, *MT-TK* encoding tRNA^{Lys}, *MT-TH* encoding tRNA^{His}, *MT-TQ* encoding tRNA^{Gln}, and *MT-TF* encoding tRNA^{Phe} [9-11].

Over the last decade, the rare pathogenic mitochondrial variant m.1630 A>G has been reported in only two published studies, one on a patient with MELAS [10] and the other on a patient with mitochondrial neurogastrointestinal encephalopathy (MNGIE) [12]. It maps in the mitochondrial *MT-TV* gene and substitutes the residue adenine for guanine in the anticodon-stem of the tRNA^{Val} thereby compromising its secondary structure [10]. Pathogenicity of this variant was demonstrated using a cybrid model, which revealed reduced levels of the mitochondrial-encoded subunit COXI and oxygen consumption by the first four OXPHOS complexes [10].

In this study, we aimed to address two questions: first does the m.1630 A>G variant impact the global metabolic phenotype by modulating the interplay between OXPHOS and

glycolysis and altering the mitochondrial functional ultrastructure; and second, does the nuclear background influence the metabolic penetrance of the m.1630A>G variant? Thus, we derived dermal fibroblasts from the symptomatic proband and her asymptomatic mother, both harboring the m.1630 A>G variant at similar near homoplasmic levels.

2. Materials and methods

2.1. Subjects

This study was approved by the Institutional Review Board of the George Washington University and Children's National Medical Center and was conducted in accordance with the ethical principles of the Declaration of Helsinki of 1975 (revised 1983). Patient skin biopsy was performed only after receiving written informed consent with permission to study the derived dermal fibroblasts.

2.2 Skin biopsy and fibroblast culture

Skin biopsy was performed on a 24-year-old proband and her 55-year-old mother. Dermal fibroblasts were derived from 3 mm skin biopsy in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 2 mM glutamine, 2.5 mM pyruvate, 0.2 mM uridine, FGF-2 (10 ng/ml) and 20% fetal bovine serum, as described [13]. Derived dermal fibroblasts were frozen at passage 2 and never used beyond passage 10. Human primary dermal fibroblasts from a healthy adult (Cat# GM03377E) were obtained from the Coriell Cell Repositories (Camden, NJ).

2.3 DNA purification and determination of heteroplasmy

DNA was extracted from dermal cultured fibroblasts at passage 3 using the QIAamp DNA mini kit according to the manufacturer's recommendations (Qiagen; Germantown, MD). Heteroplasmy was determined using a Long-Range PCR (LR-PCR)-based Next Generation Sequencing (NGS) approach as described [14,15]. We applied a very stringent detection method by choosing the very stringent cutoff of 1.33% heteroplasmy based on three S.D. above the mean error, which resulted in a 99.9 % confidence [14].

2.4 Whole exome sequencing

Total genomic DNA isolated from dermal fibroblasts to measure heteroplasmy was also subject for whole exome sequencing (WES). Briefly, genomic DNA was fragmented to be 350 base pair-long and library was constructed with Agilent Exome capture system (Agilent Technologies; Santa Clara, CA) following the manufacturer's instructions. Sequencing was performed using an Illumina HiSeq platform (Illumina; San Diego, CA) by synthesis chemistry with paired end read length of 150 bp. Reads were aligned to the human reference genome (UCSC hg19) with NextGENe software (SoftGenetics; State College, PA). Variants were identified and annotated using an in-house bioinformatic pipeline. Candidate variants were filtered using 1000 Genomes Project and ExAC. Computational analysis of variant's pathogenicity was performed using PolyPhen-2, SIFT, and the web-based application of Mutation Taster. Human Gene Mutation Database (HGMD), ClinVar and in house variant database were used to identify the reported mutations as described [19]. The pathogenicity

of variants was evaluated using the American College of Medical Genetics and Genomics (ACMG) guidelines by board certified molecular geneticists.

2.5 Transmission electron microscopy

Fibroblasts from the proband and her mother were fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences; Hatfield, PA), 1% paraformaldehyde in 0.12 M sodium cacodylate buffer (Electron Microscopy Sciences) for 20 minutes at room temperature followed by 40 minutes on ice, as described [13]. Samples were imaged with a FEI Talos F200X-transmission electron microscope (FEI Company, Hillsboro, OR).

2.6 Analysis of mitochondrial respiratory and glycolytic activities

Bioenergetic status was measured using the Seahorse Extracellular Flux XFp Analyzer (Agilent Technologies; Santa Clara, CA), as described [13]. Optimal cell density (5,000/well) and the uncoupler FCCP (fluoro 3-carbonyl cyanide-methoxyphenyl hydrazine; 2 μ M) were determined using the Cell Energy Phenotype Test kit. Skin fibroblasts were seeded in triplicate on poly-D lysine-coated plates and incubated for 24 hours at 37°C in 5% CO₂ atmosphere. Prior to the assay, the supplemented DMEM medium was changed to unbuffered Base Medium supplemented with 2 mM glutamine (Invitrogen), 2 mM pyruvate (Sigma; St Louis, MO), and 7.1 mM glucose (Sigma) depending on the assay and adjusted to pH 7.4 with NaOH. Using the XFp Mito Stress Test kit, OCR (oxygen consumption rate) and ECAR (extracellular acidification rate) were measured under basal conditions and after sequential injections of oligomycin (1 μ M), FCCP (2 μ M) and a mix of rotenone and antimycin A (1 μ M) following the manufacturer's recommendations. Using the XFp Glycolytic Stress Test assay, we measured the glycolytic functions under glucose starvation and after sequential exposure to saturating amount of glucose (10 mM), the inhibitor of ATP synthase oligomycin (1 μ M), and 2-deoxy-D-glucose (2DG; 50 mM), an inhibitor of glycolysis. We used the XFp Glycolytic Rate Assay to determine the total proton efflux and the glycolytic proton efflux. Prior to the assay, the supplemented DMEM medium was changed to the XF base medium without phenol red supplemented with 2 mM glutamine, 10 mM glucose, 1 mM pyruvate, and 5.0 mM HEPES. OCR and ECAR were measured under basal conditions and after sequential injections of rotenone/antimycin A (0.5 μ M) and 2-DG (50 mM). All the data from three independent experiments, each including three replicates, were normalized to cell numbers after the assay and plotted as OCR (pmol/min/cell $\pm$ S.E.M.) and ECAR (mpH/min/cell $\pm$ S.E.M.) as a function of time using the Seahorse MultiReport Generator software. Statistical analyses were performed using the unpaired student t-test with p-value of less than 0.05 considered statistically significant.

3. Results

3.1 Clinical manifestations

At the age of 15, the proband presented clinical symptoms associated with MELAS, among them stroke-like episodes, seizures, and high lactate levels (Table 1). The diagnosis of MELAS was confirmed by genetic testing that revealed the presence of the rare m.1630 A>G variant [10]. Though the proband's mother is asymptomatic, she harbors the same variant as her daughter. However, the maternal heteroplasmy in blood (93%) and urine

(98%) was more elevated than that of the daughter with 75% and 95% in blood and urine samples, as quantified by restriction fragment length polymorphism [10].

At the age of 24, the proband was referred to us to seek other opinions. The proband exhibits additional clinical symptoms, including two additional infarcts, in keeping with the progressive nature of MELAS (Table 1). She has a well-controlled epilepsy with seizures triggered by her illness. She has developed a mild ataxia, occasional headaches and tinnitus, as well as an unsteady gait. She uses a wheelchair for long distances. Neurological examination revealed a normal trural tone with mild bilateral weakness in the upper and lower extremities in proximal and distal muscles. Fine motor skills were normal. However, the proband has developed a positive tremor, which worsens with movement. Even though her deep tendon reflexes are intact (2+), the proband displays a positive ankle clonus and a mild contracture to the right lower extremity. Examination of her cranial nerves revealed loss of peripheral vision, decreased upgaze and nystagmus on extreme right gaze. The proband appears cognitively younger than her age. However, she is oriented to person, place and time. Her speech is difficult to understand due to articulation problems. The proband suffered from chronic renal failure and underwent a kidney transplant.

3.2 Determination of the heteroplasmy levels and whole exome sequencing of fibroblasts from the proband and her mother

We accurately measured the heteroplasmic load of the m.1630A>G variant from dermal cultured fibroblasts of the proband and his mother using the accurate and sensitive LR-PCR-based NGS approach [14,15,16]. This analysis revealed a higher heteroplasmy load in the asymptomatic mother (94.8%) than that in the symptomatic proband (89.6%) (Fig. 1).

Due to the discordant phenotype between the asymptomatic mother and affected proband based on similar genotype, we performed whole exome sequencing (WES) to investigate whether nuclear encoded mitochondrial-related gene variants could alter the penetrance of the m.1630A>G variant. After filtering steps were applied to 30,000 variants (Fig. 2), our analysis revealed only one heterozygous pathogenic variant in the mother's nuclear genome mapping in the *C8B* (NM_000066.2) and *PRB3* (NM_006249.4) genes (Table 2). The c.336delC (p.Asn112Thrfs) variant causes a frameshift in exon 3 of the *C8B* gene resulting in a truncated protein [17]. Since the *C8B* gene encodes the beta subunit of the complement 8 (C8) protein, it is highly unlikely to contribute to the MELAS asymptomatic phenotype of the proband's mother. In contrast, the proband's nuclear genome had ten heterozygous variants, three of them being likely pathogenic and mapping in genes encoding mitochondrial proteins involved in amino acid metabolism and protein translation (Table 2). The c.2T>C (p.Met1Thr) missense variant maps in the *IVD* gene encoding the mitochondrial isovaleryl-CoA dehydrogenase enzyme involved in leucine metabolism. The c.683C>T (p.Pro228Leu) missense variant maps in the *KARS* gene encoding lysyl-tRNA synthetase, which catalyzes the aminoacylation of both cytoplasmic and mitochondrial tRNA^{Lys} to attach the amino acid lysine [18]. The c.1000C>T (p.Arg334Ter) nonsense variant, maps in the *VARS2* gene encoding the mitochondrial valyl-tRNA synthetase 2 enzyme responsible for aminoacylation of the mitochondrial-specific tRNA^{Val}, which is affected by the m.1630A>G variant. This nonsense mutation introduces a termination codon in the tRNA

synthetase domain removing two-third of the *VAR2* protein containing enzymatic domains necessary to interact with mt-tRNA^{Val} molecules. Sequencing of the mitochondrial genome revealed two additional missense mtDNA variants with disease association in the proband's mitochondrial genome and nine in the mother's mitochondrial genome (Table 3). Given their very low heteroplasmy, they are highly unlikely to contribute to the disease phenotype. The older age of the mother may account for the higher number of low heteroplasmic variants due to lifetime accumulation of somatic oxidative DNA damages. Thus, the nonsense heterozygous *VAR2* variant may be the only identified nuclear variant with the potential to exacerbate the penetrance of the m.1630A>G variant, as both variants affect mitochondrial tRNA^{Val} and subsequently translation of mitochondrial-encoded subunits of OXPHOS complexes.

3.3. Functional outcome of the m.1630 A>G variant on mitochondrial respiration

In light of the previously reported reduction in oxygen consumption using cybrid cells harboring the m.1630A>G variant [10], we investigated its metabolic consequences on the OXPHOS metabolism within the context of the nuclear background of fibroblasts from the proband and the mother. As a control subject, we used commercially available dermal fibroblasts from a healthy adult whose metabolic profile has already been characterized and comparable to two other healthy subjects as described [13]. We utilized the Seahorse Extracellular Flux XFp analyzer for live-cell measurement of oxygen consumption rate (OCR), a key functional indicator of the mitochondrial ATP-coupled respiration. Using the real-time live cell energy phenotype assay, we found that the baseline metabolic phenotype of the proband's fibroblasts is half of that of the mother's fibroblasts (Fig. 3). The proband's fibroblasts had a decreased overall metabolic potential upon FCCP exposure, indicative of a stunted response to energy demand. Thus, the proband's fibroblasts are more metabolically quiescent with diminished bioenergetic adaptability than the mother's fibroblasts.

We next investigated the functional impact of the m.1630 A>G variant on key OXPHOS parameters using the Mitochondrial Stress Test assay (Fig. 4A, B). We found that the proband's fibroblasts exhibited a severe decline of the basal OCR by 70% when compared to that of the mother and healthy adult (Fig. 4C). Exposure to oligomycin, an inhibitor of the ATP synthase, caused a similar drop (72%) of ATP-linked respiration in the proband's fibroblasts (Fig. 4C). Next, we measured the maximal respiratory capacity evoked by exposure to the protonophore FCCP and found that the proband's fibroblasts exhibited a 78% loss of maximal respiration capacity when compared to that of the mother and healthy subject (Fig. 4C). Similarly, the proband's fibroblasts displayed a 87% loss of spare respiratory capacity, which severely stunted its bioenergetic capacity to adapt and sustain an energy crisis (Fig. 4C). This profile stands in contrast with the comprehensive OXPHOS signature of the fibroblasts derived from the proband's mother that is comparable to that of a healthy adult (Fig. 4C). Collectively, our bioenergetic results indicates that the nuclear background influences the functional impact of the m.1630 A>G variant on the OXPHOS signature.

3.4 Mitochondrial morphometric analysis of fibroblasts derived from the proband and her mother

Since the ultrastructure and morphology of cristae control the mitochondrial bioenergetic functions [19], we investigated by transmission electron microscopy (TEM) whether the m.1630A>G variant could alter mitochondrial ultrastructures. The mother's fibroblasts had mitochondria with a normal ultrastructural morphology characterized with numerous and closely apposed cristae and a highly electron dense mitochondrial matrix (Fig. 5A). In contrast, most mitochondria from the proband's fibroblasts exhibited rare and short cristae that appeared disorganized, while some mitochondria were devoid of cristae (Fig. 5B). All the proband's mitochondria displayed a weak electron dense mitochondrial matrix, indicative of diminished metabolic activities. Thus, the overall abnormal mitochondrial ultrastructural morphology concurs with the symptomatic status and the altered OXPHOS phenotype of the proband, while the normal mitochondrial ultrastructure of the mother's fibroblasts is consistent with her asymptomatic status and normal OXPHOS metabolism. Finally, our TEM study consistently highlighted ultrastructural changes of chromatin between the proband and her mother. The proband's fibroblasts displayed increased aggregation of densely packed chromatin at the nuclear periphery and inside of the nuclear space, when compared to the mother's fibroblasts (Fig. 5). Thus, our TEM results are consistent with increased heterochromatin arrangement in the proband's fibroblasts, implying a difference in chromatin organization between the proband and her mother.

3.5. Functional outcome of the m.1630 A>G variant on glycolysis

We next examined the bioenergetic consequences of the m.1630 A>G variant on the glycolytic pathway to investigate whether energy reprogramming could occur to sustain an energy crisis provoked by limited mitochondrial ATP production. We performed a Glycolysis Rate assay, since it directly and accurately assesses glycolytic activity by correlating one-to-one with lactate accumulation (Fig. 6A). The total Proton Efflux Rate (PER) and the Glycolytic Proton Efflux Rate (GlycoPER) were measured using both OCR and ECAR values in order to account for mitochondrial (CO₂) acidification from the mitochondrial TCA cycle making up for some of the acidification of the medium [20].

In the absence of mitochondrial OXPHOS inhibitors, the proband's fibroblasts exhibit a 57% increase in basal glycolysis when compared to that of the mother and a healthy subject, indicating glycolysis as the main pathway for ATP production (Fig. 6B, C). This is further corroborated by the proband's fibroblasts having 94% PER derived from glycolysis, unlike fibroblasts from the mother and healthy subject being more oxidative with only 69% PER (Fig. 6D). Furthermore, the proband's fibroblasts showed a severe decrease of the parametric ratio basal mitoOCR/glycoPER when compared to that of fibroblasts from the mother and healthy subject, indicating that the m.1630A>G variant in the nuclear context of the proband make the fibroblasts more glycolytic than oxidative (Fig. 6E).

We next assessed the compensatory glycolytic response of the proband's fibroblasts upon full inhibition of mitochondrial ATP production by rotenone and antimycin A (Fig. 6A, B). We found that the proband's fibroblasts exhibited a negligible compensatory glycolysis response with only a 1.19 fold increase in compensatory glycolysis, unlike fibroblasts from

the mother and a healthy subject displaying a 2.69 fold increase (Fig. 6F). Thus, only the proband's fibroblasts display an insufficient glycolytic response to overcome an acute OXPHOS metabolic crisis, which may result from their very high glycolytic status (94%) preceding the provoked energy crisis.

We further analyzed the impact of the m.1630 A>G variant on the glycolytic capacity and reserve using the Glycolysis Stress Test assay. It assesses glycolysis by quantifying the ECAR rate after having starved the cells for glucose followed by injection of saturating amounts of glucose to fuel both glycolysis and mitochondrial OXPHOS, followed by injection of saturated amount of oligomycin to inhibit mitochondrial ATP synthase and a final injection of 2-deoxyglucose to inhibit glucose catabolism (Fig. 7A, B). As expected, fibroblasts from the mother and a healthy subject displayed similar levels of glycolysis, while the proband's fibroblasts were 57% more glycolytic (Fig. 7C). Fibroblasts from the mother and a healthy subject showed increased glycolysis by 54% following inhibition of mitochondrial ATP synthesis, indicative of a substantial glycolytic capacity (Fig. 7C). In contrast, the proband's fibroblasts had a severely stunted glycolytic capacity with only a 13% increase in ECAR (Fig. 7C). Concordantly, the proband's fibroblasts displayed a sharply curtailed glycolytic reserve of only 28% of that of the mother's fibroblasts and control fibroblasts (Fig. 7C). Thus, our results show that the proband's fibroblasts possess a limited glycolytic reserve and a defective metabolic plasticity.

4. Discussion

In this study, we report a novel and comprehensive perspective of the metabolic consequences of the m.1630 A>G variant associated with MELAS in the unique case of a symptomatic proband and her asymptomatic mother, both harboring similar near-homoplasmic levels. Our collective findings from fibroblasts of the symptomatic proband and her asymptomatic mother support the idea that the nuclear background influences the penetrance of the near-homoplasmic m.1630 A>G variant resulting in differential clinical and metabolic phenotype.

Little is known about the m.1630 A>G variant, as it is scarcely identified in patients with a mitochondrial disease. Over the last decade, only two patients were reported to harbor this variant, one with MELAS and the other one with MNGIE [10,12]. This variant maps in the mitochondrial-encoded *MT-TV* gene, which is a hot spot for other pathogenic variants causing distinct clinical phenotypes [21-30]. It is predicted to disrupt the secondary structure of the anticodon stem of the mt-tRNA^{Val} at a position recently assigned with a high secondary structure score, a key component of the overall pathogenicity score, using the interface Mitochondrial tRNA Informatics Predictor [31]. Pathogenicity was confirmed by cybrid studies derived from the proband [10]. Furthermore, the m1630A>G variant harbored by the proband and her mother results in a severe decrease of mt-tRNA^{Val} levels as a result of instability rather than reduced transcription levels [10].

Our collective findings extend those of the cybrid-based study by investigating the metabolic OXPHOS phenotype of the proband's mother. Our live-cell mitochondrial respiratory results demonstrate that the m.1630 A>G variant exclusively induces a severe decline of all the

bioenergetic OXPHOS parameters in the proband's fibroblasts. Though the mother's fibroblasts harbor a higher heteroplasmy than the proband, she exhibits a normal OXPHOS response, congruent with her asymptomatic status. In contrast, the proband's fibroblasts display a decreased basal respiration and ATP-linked respiration as a result of a curtailed substrate oxidation. Most notably is the attrition of the maximum respiration and spare respiratory capacity, two key bioenergetic parameters for preventing energy exhaustion upon a sudden surge of energy demand leading to enhanced cellular survival [32,33]. These two parameters are principally relevant to the proband's clinical manifestations given their implications in neuronal and muscular pathologies [34]. Thus, the difference in amplitude of these two parameters may explain the difference in MELAS symptomology between the proband and her mother. This is further supported by our morphometric findings of normal mitochondrial cristae ultrastructure and number in the mother's fibroblasts, known to correlate with respiratory efficiency due to the embedding of the respiratory chain supercomplexes in the membrane of the cristae [19,35,36]. In contrast, the proband's mitochondria have an abnormal ultrastructural morphology, congruent with the severely impaired OXPHOS metabolism.

Our compelling results on glycolysis convey a novel perspective on the metabolic phenotypic variability between the proband and her mother, which was not investigated in a cybrid-based model. The proband's fibroblasts have a limited compensatory glycolytic capacity due to a nearly constitutive glycolytic state for ATP production, which is congruent with one of the proband's clinical symptoms, lactic acidosis. The negligible glycolytic reserve further exacerbates the detrimental metabolic phenotype of the proband's fibroblasts. Such dysregulated metabolic flexibility prevents the proband's fibroblasts to maintain ATP production during an acute ATP crisis, which may contribute to the progressing nature of MELAS symptomology [1-4]. In contrast, the mother's fibroblasts do not chiefly rely on glycolytic ATP production as a result of normal OXPHOS metabolism, and exhibit a normal glycolytic reserve and capacity, which may in part explain her asymptomatic status.

The discordant metabolic profile between the proband and her mother lends credence to the nuclear background influencing the phenotypic expression of the near homoplasmic m.1630 A>G variant associated with MELAS. Given that the mother's fibroblasts exhibit mitochondrial respiratory parameters similar to those of healthy fibroblasts despite the near homoplasmic levels of the m.1630A>G variant, our functional mitochondrial results support the hypothesis of "protective" nuclear factor(s) present in the fibroblasts of the proband's mother. This is congruent with the findings of the original 2011 study on the m.1630A>G variant demonstrating OXPHOS dysfunction caused by homoplasmic and heteroplasmic (67%) levels of the m.1630 A>G variant in the context of a neutral nuclear background of transmitochondrial cybrid lines created with the 143p^o osteosarcoma immortalized cell line [10]. A similar phenotypic discordance was reported between the mildly symptomatic mother and her ten deceased children, all harboring a homoplasmic m.1624C>T variant mapping in the *MT-TV* gene encoding the mitochondrial tRNA^{val}, in keeping with the nuclear background influencing the phenotypic signature of the homoplasmic m.1624C>T variant [37].

We can envision three possible explanations for the differential metabolic phenotype between the proband and her mother despite harboring similar heteroplasmy. First, our WES analysis reveals the presence of a heterozygous nonsense *VARs2* variant (p.R334X) exclusively in the proband's nuclear genome, which maps in the gene encoding the mitochondrial valyl-tRNA synthetase. In the absence of the father's DNA sample, we cannot determine whether this *VARs2* variant is due to a *de novo* mutation or of paternal inheritance. Given that post-transcriptional processing of mitochondrial tRNAs is critical for accurate and effective translation of mitochondrial-encoded OXPHOS proteins, enzymes involved in these processes have been proposed to function as potential nuclear modifier genes [38]. This nonsense *VARs2* variant removes two-third of the entire protein, which includes most of the synthetase domain along with the entire editing, anticodon binding and tRNA binding domains, all involved in charging valine to mt-tRNA^{Val} molecules [39]. Thus, this *VARs2* loss-of-function variant results in *VARs2* haploinsufficiency in the proband. This is particularly relevant, as increase in levels of *VARs2* expression has been shown to be critical to attenuate the OXPHOS phenotype caused by a homoplasmic m.1624C>T variant mapping in the *MT-TV* gene in patient-derived myoblasts and cybrid cells attenuates [40]. Although this nonsense *VARs2* variant may in part underlie the discordant metabolic phenotypes between the proband and her mother, we cannot exclude other nuclear variant(s) could further compromise the mitochondrial OXPHOS response. Recent WES studies have uncovered an association between the *VARs2* variant and early-onset mitochondrial encephalopathies [41-43]. More specifically, a recessive *VARs2* variant was described in a patient sharing key clinical features with the proband, such as ataxia, short stature, seizures, chair-bound, speech and cognitive impairment [44]. Second, we cannot rule out that epigenetic regulation of the mother's nuclear genome might lessen the functional impact of the m.1630A>G variant in the absence of nuclear pathogenic variants. Given the well-established reciprocal interplay between mitochondrial functions and chromatin landscape, epigenetic regulation might also act as a determinant of phenotypic expression of the m.1630 A>G variant in the mother. Third, the differential phenotype between the mother and the proband might also be due to epigenetic dysregulation of the proband's nuclear genome. The last two scenarios are supported by our TEM results showing differential heterochromatin content between the proband and her mother and in agreement with our recent study demonstrating epigenetic regulation of mitochondrial biogenesis and bioenergetics in neuroprogenitor cells [45].

In summary, our study provides strong genetic and biochemical evidence that the nuclear background influences the metabolic phenotype caused by the m.1630A>G variant in this unique case of a symptomatic proband and her asymptomatic mother harboring similar near homoplasmic levels. Future studies will elucidate the pathogenic mechanisms by which the nuclear background influences the MELAS phenotypic signature caused by the m.1630 A>G variant.

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Figure 1:

Detection of the m.1630 A>G variant by LR-PCR-based next generation sequencing in fibroblasts of the proband and his mother. (A) The piled-up LR-PCR/massively parallel sequencing (MPS) result of the proband reveals a 89.6% heteroplasmy. (B) The piled-up LR-PCR/massively parallel sequencing (MPS) result of the proband's mother indicates 94.8% heteroplasmy.

Figure 2:

Filtering strategy for identifying variants. Whole exome sequencing was performed on total DNA isolated from fibroblasts derived from the symptomatic proband and her asymptomatic mother. Both harbor the m.1630A>G variant with similar levels of heteroplasmy. Reads were aligned to the human reference genome (UCSC hg19) with NextGENe software. Variants remaining after each filtering step are indicated on the right-end side of the corresponding box. The pathogenicity of variants was evaluated using the American College of Medical Genetics and Genomics guidelines with board certified molecular geneticist. HGMD = Human Gene Mutation Database; MAF = minor allele frequency; SIFT = Sorting Intolerant From Tolerant.

Figure 3:

The overall metabolic signature of the pathogenic m.1630 A>G variant in the fibroblasts of the proband and her mother. Basal oxygen consumption (OCR) and extracellular acidification rate (ECAR) were measured in fibroblasts from the mother (blue square) and the proband (red circle) and were normalized to the number of cells. Basal OCR is a measure of OXPHOS activity, while basal ECAR is a measure of glycolysis activity. The basal phenotype was determined in the absence of metabolic modulators and the stressed phenotype (full square or circle) was determined in the presence of the uncoupler FCCP (2 μ M). The metabolic potential represents the difference between stressed OCR or ECAR over baseline OCR or ECAR.

Figure 4:

Impaired mitochondrial respiratory functions in the fibroblasts of the proband. (A) Profile of the oxygen consumption rate (OCR) adapted from the Agilent Technologies brochure of the Mitochondrial Stress Test. (B) Compared OCR responses across the proband, her mother and healthy subject. (C) Quantitative data of basal respiration, ATP-linked respiration, maximal respiration, and spare respiratory capacity. Data are represented as means $\pm$ S.E.M., $n = 3$ of independent experiments, each with three replicates. * indicates statistically significant differences with a p value of 0.0001 between the proband and mother or healthy subject.

Figure 5:

The m.1630 A>G variant alters the morphology and abundance of cristae. (A) Mitochondrial morphometric analysis by transmission electron microscopy using dermal fibroblasts of the proband's mother. The scale bar is indicated at the bottom right corner of each micrograph. The last column of electromicrographs illustrates a low magnification of the nucleus (top row) with a high magnification of the nuclear periphery (bottom row). (B) Mitochondrial morphometric analysis by transmission electron microscopy using the proband's fibroblasts. The scale bar is indicated at the bottom right corner of each micrograph. The white arrows indicate mitochondria with abnormal cristae, while the white arrowhead indicate normal mitochondria. The last column of electromicrographs illustrates a low magnification of the nucleus (top row) with a high magnification of the nuclear periphery (bottom row).

Figure 6:

Altered proton efflux rate in the proband's fibroblasts. (A) Schematic representation of proton efflux from the Agilent Technologies brochure of the Glycolytic Rate assay. (B) Compared proton efflux rate across the proband, her mother and healthy subject. (C). Quantitative analysis of basal glycolysis. Data are represented as means $\pm$ S.E.M., $n=3$ of independent experiments, each with three replicates. * indicates statistically significant difference with a p value of 0.0001. (D). Quantitative analysis of %PER from glycolysis. Data are represented as means $\pm$ S.E.M., $n=3$ of independent experiments, each with three replicates. * indicates statistically significant difference with a p value of 0.0001. (E). Quantitative analysis of the ratio basal mito OCR versus GlycoPER. Data are represented as means $\pm$ S.E.M., $n=3$ of independent experiments, each with three replicates. * indicates statistically significant difference with a p value of 0.0001. (F) Quantitative of the ratio compensatory glycolysis versus basal glycolysis. Data are represented as means $\pm$ S.E.M., $n=3$ of independent experiments, each with three replicates. * and ** indicate statistically significant differences with a p value of 0.0067 and 0.0001.

Figure 7:

Impaired glycolysis activities in the proband's fibroblasts. (A) Profile of the extracellular acidification rate (ECAR) adapted from the Agilent Technologies brochure on the Glycolysis Stress Test assay. (B) Compared ECAR responses across the proband, her mother and a healthy subject. (C) Quantitative data of glycolysis, glycolytic capacity, and glycolytic reserve. Data are represented as means $\pm$ S.E.M., $n=3$ of independent experiments, each with three replicates. * indicates statistically significant differences with a p value of 0.05 between the proband and mother or healthy subject.

Table 1:

Chronology of key clinical features exhibited by the proband

Clinical features at 15 years of age	Clinical features at 24 years of age
Heteroplasmy in fibroblasts (RFLP): 60%	Heteroplasmy in fibroblasts (LP-PCR-NGS): 89.63%
Seizures at times of stroke-like episodes	Well controlled epilepsy, seizures triggered by illness
Lactic acidosis	Lactic acidosis
Acute right occipital lobe infarction	Two additional infarcts
Bilateral sensorineural hearing loss	Bilateral sensorineural hearing loss
Myopia	Myopia; visual field loss
Short stature	Short stature
Delayed puberty	Normal menses
Gastrointestinal problems	Gastrointestinal problems: GERD, bloating
Motor delay	Motor, Resting tremor
Small foci of mineralization bilaterally in the basal ganglia	Mild ataxia
Diffuse lactate peaks throughout the brain	Unsteady gait
	Occasional headaches and tinnitus
	Left ventricular hypertrophy
	Nephropathy

Abbreviations: RFLP: restriction fragment length polymorphism; LP-PCR-NGS: long-range PCR with next generation sequencing.

Table 2:

Nuclear variants identified by whole exome sequencing in the fibroblasts of the symptomatic proband and asymptomatic mother.

Patient	Gene	Deleterious variants	AA Change	Zygosity	Clinical significance
Mother	C8B	c.336delC	p.N112Tfs*22	Heterozygous	P
	PRB3	c.145C>C	p.R49C	Heterozygous	VUS
Proband	FKRP	c.1073C>T	p.P358L	Heterozygous	VUS
	IDUA	c.1225G>C	p.G409R	Heterozygous	LP
	IVD	c.2T>C	p.M1T	Heterozygous	VUS
	KARS	c.683C>T	p.P228L	Heterozygous	VUS
	PDX1	c.97C>A	p.P33T	Heterozygous	VUS/LP
	PIGN	c.1505A>G	p.Q502R	Heterozygous	VUS
	PRB3	c.145C>T	p.R49C	Heterozygous	VUS
	SERPINA1	c.863T>A	p.E288V	Heterozygous	LP
	UROS	c.217A>G	p.C73R	Heterozygous	P
	VARS2	c.1000C>T	p.R334X	Heterozygous	P

Highlighted in yellow is the only pathogenic variant identified in the mother's nuclear genome. Indicated in red is the nuclear variant with a likelihood to influence the penetrance of the pathogenic m.1630A>G mitochondrial variant harbored by the proband and her mother at similar heteroplasmy. Abbreviations: LP: likely pathogenic; P: pathogenic; VUS: variant of uncertain significance.

Table 3:

Mitochondrial variants revealed by sequencing of the mitochondrial genome of the proband and her mother

Patient	Mitochondrial genome position	Gene name	Genotype	Mutation call	Mutant allele frequency	Amino acid change
Mother	1630	TRNV	GA	A>G	94.78	-
	5894	Non-coding	AC	A>C	5.08	--
	6061	COX1	TG	T>G	3.25	p.I531S
	8743	ATP6	GA	G>A	2.25	p.V73M
	9247	COX3	GA	G>A	2.77	p.S14N
	9670	COX3	AG	A>G	3.05	p.N155S
	11711	ND4	GA	G>A	4.17	p.A318T
	12983	ND5	TC	T>C	4.39	p.L216P
	13025	ND5	AC	A>C	3.22	p.H230P
	13520	ND5	TC	T>C	3.57	p.I395T
Proband	1630	TRNV	GA	A>G	89.63	-
	7740	COX1	TG	T>G	9.41	p.S513A
	8701	ATP6	AG	A>G	1.69	p.T59A

The pathogenic MELAS m.1630A>G is highlighted in yellow to distinguish it from the other mitochondrial variants revealed by whole exome sequencing.