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Quantitative Evaluation of the Mitochondrial DNA Depletion Syndrome

David Dimmock,[1,2] Lin-Ya Tang,[1] Eric S. Schmitt,[1] and Lee-Jun C. Wong[1*]

BACKGROUND: The mitochondrial DNA (mtDNA) depletion syndromes (MDDSs) are autosomal recessive disorders characterized by a reduction in cellular mtDNA content. Mutations in at least 9 genes [*POLG*, polymerase (DNA directed), gamma; *DGUOK*, deoxyguanosine kinase; *TK2*, thymidine kinase, mitochondrial; *TYMP*, thymidine phosphorylase; *MPV17*, MpV17 mitochondrial inner membrane protein; *SUCLA2*, succinate-CoA ligase, ADP-forming, beta subunit; *SUCLG1*, succinate-CoA ligase, alpha subunit; *RRM2B*, *RRM2B*, ribonucleotide reductase M2 B (TP53 inducible); and *C10orf2*, chromosome 10 open reading frame 2 (also known as *TWINKLE*)] have been reported to cause mtDNA depletion. In the clinical setting, a simple method to quantify mtDNA depletion would be useful before undertaking gene sequence analysis.

METHODS: Real-time quantitative PCR (qPCR) was used to measure the mtDNA content in blood, muscle, and liver samples and in skin fibroblast cultures from individuals suspected of mitochondrial disorders, with or without deleterious mutations in genes responsible for MDDS.

RESULTS: The mtDNA content was quantified in 776 tissue samples (blood, n = 341; muscle, n = 325; liver, n = 63; skin fibroblasts, n = 47) from control individuals. mtDNA content increased with age in muscle tissue, decreased with age in blood samples, and appeared to be unaffected by age in liver samples. In 165 samples (blood, n = 122; muscle, n = 21; liver, n = 15; skin fibroblasts, n = 7) from patients with molecularly proven MDDSs, severe mtDNA depletion was detected in liver and muscle tissue with high specificity and sensitivity. Blood samples were specific but not sensitive for detecting mtDNA depletion, and skin fibroblasts were not valuable for evaluating mtDNA depletion. Mutations in the *POLG*, *RRM2B*, and *MPV17* genes were prospectively identified in 1 blood, 1 liver, and 3 muscle samples.

CONCLUSIONS: Muscle and liver tissues, but not blood or skin fibroblasts, are potentially useful for rapid screening for mtDNA depletion with real-time qPCR.

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Mitochondria are ubiquitous in eukaryotic cells and are essential for their survival. Their major function is to generate ATP, the energy currency for supporting cellular activities. Mitochondria contain their own DNA and their own machinery for transcription and translation. Human mitochondrial DNA (mtDNA)[3] is a circular double-stranded 16.6-kb molecule that encodes 13 protein subunits of the respiratory chain, as well as 22 tRNAs and 2 rRNAs essential for mitochondrial protein synthesis. Nevertheless, the replication, repair, transcription, and translation of mtDNA depend on the nuclear genome *(1)*.

The mtDNA depletion syndromes (MDDSs) are a clinically heterogeneous group of autosomal recessive disorders caused by molecular defects in the nuclear genes involved in mtDNA biosynthesis and the maintenance of the deoxynucleotide pools. MDDSs cause a reduction in cellular mtDNA content *(2)*. At the present time, mutations in at least 9 genes [*POLG*,[4] polymerase (DNA directed), gamma; *DGUOK*, deoxyguanosine kinase; *TK2*, thymidine kinase, mitochondrial; *TYMP*, thymidine phosphorylase; *MPV17*, MpV17 mitochondrial inner membrane protein; *SUCLA2*, succinate-CoA ligase, ADP-forming, beta subunit; *SUCLG1*, succinate-CoA ligase, alpha sub-

[1] Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX; [2] Department of Pediatrics, Medical College of Wisconsin, Milwaukee, WI.
* Address correspondence to this author at: Department of Molecular and Human Genetics, Director, Mitochondrial Diagnostic Laboratory, Baylor College of Medicine, One Baylor Plaza, NAB 2015, Houston, TX 77030. Fax 713-798-8937; e-mail ljwong@bcm.edu.
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[3] Nonstandard abbreviations: mtDNA, mitochondrial DNA; MDDS, mitochondrial DNA depletion syndrome; MNGIE, mitochondrial neurogastrointestinal encephalomyopathy; qPCR, quantitative PCR; Cq, threshold cycle.
[4] Human genes: *POLG*, polymerase (DNA directed), gamma; *DGUOK*, deoxyguanosine kinase; *TK2*, thymidine kinase, mitochondrial; *TYMP*, thymidine phosphorylase; *MPV17*, MpV17 mitochondrial inner membrane protein; *SUCLA2*, succinate-CoA ligase, ADP-forming, beta subunit; *SUCLG1*, succinate-CoA ligase, alpha subunit; *RRM2B*, ribonucleotide reductase M2 B (TP53 inducible); *C10orf2*, chromosome 10 open reading frame 2 (also known as *TWINKLE*); *MT-TL1*, mitochondrially encoded tRNA leucine 1 (UUA/G); *B2M*, beta-2-microglobulin; *SLC25A4*, solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4.

unit; *RRM2B*, ribonucleotide reductase M2 B (TP53 inducible); *C10orf2*, chromosome 10 open reading frame 2 (also known as *TWINKLE*)] have been found to cause mtDNA depletion *(3–17)*.

The clinical presentation of a MDDS depends on which gene is involved and may be tissue specific or multisystemic *(2–6)*. Patients with autosomal recessive mutations in *MPV17* predominantly present with hepatic failure during infancy *(5)*, whereas individuals with *POLG* mutations can present with MDDS at any age, primarily with encephalopathy and, to a lesser extent, hepatic impairment *(6)*. Mutations in *DGUOK* are associated with hepatic and neurologic symptoms during infancy and may be the most common cause of mitochondrial hepatoencephalopathy *(3, 7)*. More recently, autosomal recessive mutations in *C10orf2* have been found in patients with early-onset hepatoencephalopathy *(7, 8)*. Mutations in *TK2* and *SUCLA2* are responsible for the myopathic form of MDDS *(4, 9)*. *SUCLA2* mutations have also been causally associated with Leigh-like encephalopathy, dystonia, and deafness *(9–11)*. Mutations in *SUCLG1* cause severe lactic acidosis and encephalomyopathy *(12)*. MDDS due to defects in the *SUCLA2* or *SUCLG1* gene can be distinguished from other forms of MDDS by a persistent increase in the methylmalonic acid concentration *(9–12)*. The predominantly gastrointestinal form of MDDS, mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), is caused by a thymidine phosphorylase deficiency due to *TYMP* mutations and primarily affects smooth muscle and the brain *(13)*. Mutations in *RRM2B* cause mtDNA depletion in multiple organs that produces broad clinical manifestations, ranging from infantile encephalopathy to MNGIE-like disease *(14, 16, 17)*.

A definitive diagnosis of MDDS is difficult because of its extreme genetic and clinical heterogeneity. Diagnosis is usually based on finding a gene with 2 deleterious mutations in trans that are responsible for the patient's clinical symptoms. A gene or a group of genes can be selected for sequence analysis on the basis of family history and clinical, biochemical, histopathologic, and imaging results. If there were a simple, reliable way to evaluate mtDNA depletion, specific genes could be selected for sequence-based testing. In addition, given that the MDDSs demonstrate an autosomal recessive inheritance, establishing a diagnosis would permit more accurate genetic counseling for recurrence risks. Furthermore, an efficient screening method that excludes MDDS would allow alternative diagnoses to be considered, avoiding unnecessary and costly sequencing.

Historically, the standard approach to measuring mtDNA content is Southern blot analysis with radioactive probes for both mtDNA and nuclear DNA, followed by densitometric analysis of the autoradiogram or phosphoimage *(18)*. This method is tedious and requires a large amount of DNA. Furthermore, it allows only an estimate of the mtDNA content. The more recent development of real-time quantitative PCR (qPCR) holds promise *(14)*; however, its adoption into clinical practice has been hindered by a lack of data from appropriate control populations.

Therefore, the purpose of this study was to establish reference values for and to assess the utility of qPCR for evaluating mtDNA content in various tissues by studying both individuals without MDDS and patients with molecularly proven MDDS.

Materials and Methods

DNA SAMPLES

Control DNA samples were obtained from individuals without identifiable mutations known to cause MDDS. We arbitrarily chose 281 blood samples, 325 muscle tissue samples, and 47 skin samples from those submitted to the Mitochondrial Diagnostic Laboratory of the Medical Genetics Laboratories at Baylor College of Medicine for that molecular evaluation of mitochondrial disorders. These samples had tested negative for common point mutations or deletions in mtDNA. Some of these samples were also negative for point mutations in selected genes responsible for MDDS and complex assembly (i.e., patients suspected of having mitochondrial disease but without identifiable mutations). An additional 60 blood samples were obtained from healthy children through a separate institutional review board–approved protocol. The reference value for the mtDNA content of liver tissue was established with 63 liver tissue samples (excess tissue from transplant donors) obtained through the Liver Tissue Cell Distribution System for Minneapolis, MN, and Pittsburgh, PA.

We also analyzed 165 samples from patients with molecularly proven MDDS (i.e., 2 deleterious mutations): 122 blood, 21 muscle, 15 liver, and 7 skin fibroblast samples from patients with 2 proven mutations in 1 of 8 nuclear genes (*POLG*, *DGUOK*, *TK2*, *TYMP*, *MPV17*, *SUCLA2*, *SUCLG1*, and *RRM2B*) known to cause mtDNA depletion. No samples from patients with *C10orf2* mutations were available.

Ninety-seven samples from symptomatic individuals suspected of having a mitochondrial disorder but with sequence analyses revealing only 1 mutation or an unclassified novel variant were also evaluated for mtDNA depletion (*POLG*, 59 blood and 6 muscle samples; *DGUOK*, 16 blood samples; *TK2*, 1 muscle and 5 blood samples; *MPV17*, 6 blood samples; *TYMP*, 4 blood samples) (see Table S4A in the Data Supplement that accompanies the online version of this article at

http://www.clinchem.org/content/vol56/issue7). We also measured the mtDNA content of blood samples from 49 asymptomatic, molecularly confirmed carrier parents or siblings of MDDS patients (see Table S4B in the online Data Supplement).

DNA was extracted from available tissues with a Gentra Puregene Blood Kit (Qiagen) according to manufacturer's instructions.

This study was performed according to protocols approved by the Institutional Review Board of the Baylor College of Medicine. The clinical details of most of the patients with genetically determined MDDS have been published elsewhere *(3, 5, 6, 16–22)*.

REAL-TIME qPCR ANALYSIS

Each 10-μL PCR reaction contained 5 μL iTaq SYBR Green Supermix with ROX (Bio-Rad Laboratories), 300 nmol/L of each primer, and 0.3 ng of total DNA quantified by either a NanoDrop 1000 (Thermo Fisher Scientific) or Quant-iT PicoGreen (Invitrogen) according to manufacturers' instructions. DNA purity was assessed by ensuring the A_{260}/A_{280} ratio was >1.8. The real-time PCR thermocycling conditions were 2 min at 50 °C, 10 min at 95 °C, and 45 cycles of 15 s of denaturation at 95 °C and 30 s of annealing/extension at 62 °C. The fluorescence signal intensities of PCR products were recorded and analyzed with a 7900HT Fast Real-Time PCR System (Applied Biosystems) and the manufacturer's Sequence Detection System software (version 2.2.2). mtDNA content was measured by real-time qPCR with primers specific for the regions of mitochondrial gene *MT-TL1* [mitochondrially encoded tRNA leucine 1 (UUA/G)] and nuclear gene *B2M* (beta-2-microglobulin), as previously described *(23)*. These primer pairs were chosen for regions for which polymorphic variants have not been reported. The *MT-TL1* gene region is deleted in <1.6% of described deletions, and our previous studies have demonstrated that these 2 loci produce the most consistent and reliable results *(23)*. Further details of qPCR validation are available in the online Data Supplement. The relative mtDNA copy number was calculated as the difference in the numbers of threshold cycles (Cq) between the nuclear gene and the mtDNA gene (ΔCq), in which the amount of mtDNA calculated per cell, $2(2^{-\Delta Cq})$, accounts for the 2 *B2M* copies in each cell nucleus. To obtain consistent results, we kept the amount of DNA in the qPCR reaction at approximately 0.2–0.5 ng in a 10-μL reaction mixture to maintain the Cq value for *B2M* at approximately 28. All samples were assayed in duplicate, and any detected mtDNA depletion was confirmed by repeat analysis in a second run. Results were accepted if the difference between the duplicate measurements was <30%; otherwise, the reactions were repeated. For depletion patients for whom sufficient DNA was available, we used dual genome array comparative genomic hybridization to evaluate the copy number to relative to that of a known calibrator, as previously described. In all cases, the 2 techniques produced concordant results *(19, 21)*.

TISSUE MISHANDLING

A representative liver sample was stored at 25 °C for 36 h, at room temperature for 2 weeks, or at 4 °C for 3 months to emulate extreme mishandling. In addition, we evaluated 8 liver samples in which the tissue had been flash-frozen immediately and 30 min after liver removal.

CONCURRENT CONTROL: POOLED CONTROL

DNA samples of patients with molecularly proven mutations were analyzed concurrently with an age- and tissue type–matched pooled sample of control DNA. The matched control DNA was pooled by mixing equal amounts of DNA from 10 age- and tissue-matched control samples. The pooled control sample was analyzed concurrently with each MDDS sample to ensure that control values remained within ±1 SD. The mtDNA content of the MDDS sample is presented in the figures as a percentage of that of the mean value of the age- and tissue-matched pooled controls.

INTRARUN VARIATION

A pooled DNA sample was repeatedly run on 39 occasions between November 24, 2008, and September 23, 2009. This single sample showed a mean (SD) Cq difference of 10.14 (0.26) cycles (SE, 0.042 cycles).

Results

CONTROL mtDNA CONTENT IN VARIOUS TISSUES

The mean mtDNA content (expressed as the relative number of mtDNA copies per nuclear genome) in the control samples was 330 in blood, 2700 in muscle, 2100 in liver, and 600 in skin fibroblasts. The mtDNA content in control blood and muscle tissue samples appeared to vary with age (see Table 1 in the online Data Supplement). There was a trend for the mtDNA content in blood to decrease with increasing age (Fig. 1A). In contrast, the mtDNA content in muscle tended to increase with increasing age (Fig. 1B). The mtDNA content in skin fibroblasts appeared to increase with age; however, the magnitude of the increase was not statistically significant (Fig. 1D). The mtDNA content in nonpathologic liver tissue did not vary with age (Fig. 1C). The mtDNA content in blood samples from individuals without mitochondrial disorders (see last 2 rows of Table S1A in the online Data Supplement) was similar to that in control blood samples from patients suspected of mitochondrial disease but without identi-

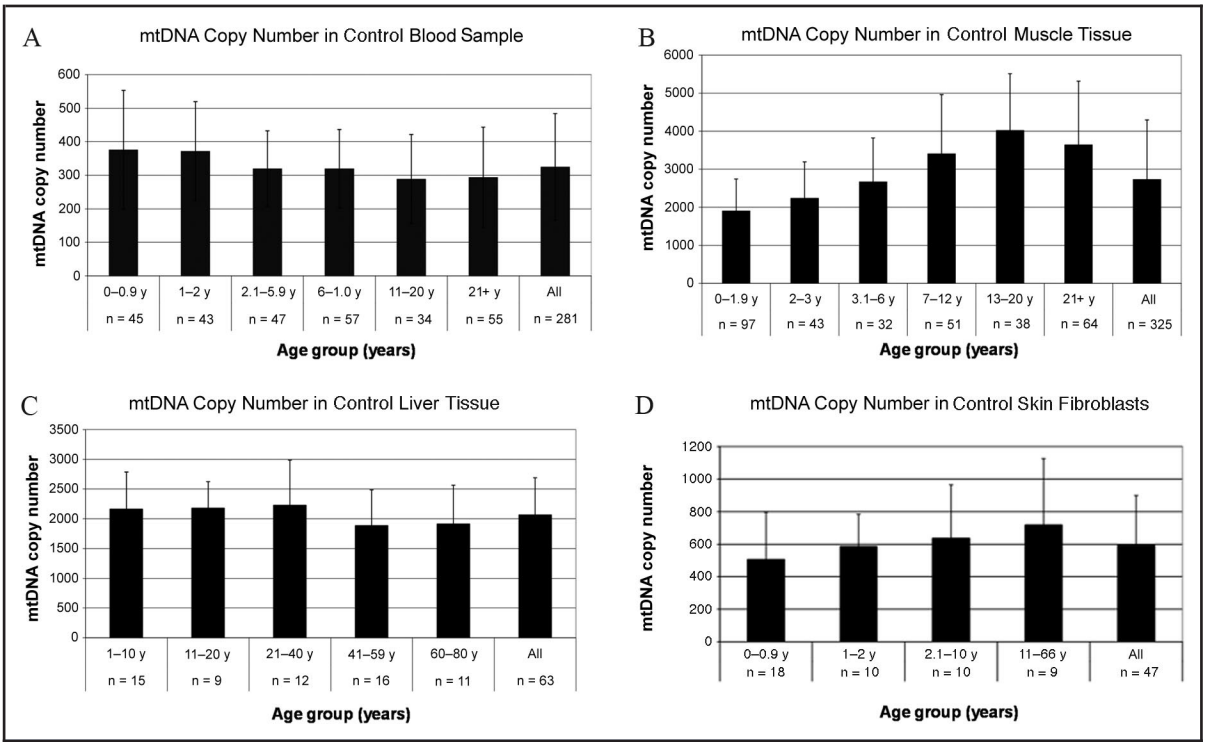

Fig. 1. Control mtDNA content across all samples by age, as measured by real-time qPCR.
mtDNA content in blood samples (n = 281) (A), muscle tissue (n = 325) (B), liver tissue (n = 63) (C), and skin fibroblasts (n = 47). y, years.

fiable mutations. Because mtDNA content varied with age and tissue type for typical data from healthy individuals (see Table 1 in the online Data Supplement), we used pooled age- and tissue-matched control DNA samples as concurrent controls for the analysis of samples with proven mtDNA depletion (see below). Mishandled liver tissue samples or samples that experienced a delay before freezing showed no differences in mitochondrial copy number. There was no correlation between copy number and the length of time in storage at −80 °C.

EVALUATION OF mtDNA CONTENT IN VARIOUS TISSUES FROM PATIENTS WITH MOLECULARLY PROVEN MDDS

The mtDNA content in various tissue samples from patients with proven mutations in nuclear genes known to cause MDDS were compared with an age- and tissue-matched pool of control DNA samples. Results for the mean mtDNA content are presented as a percentage of that of the pooled control sample.

The mtDNA content in blood samples from patients with molecularly proven MDDS was reduced to a mean of 72% (31%) of that of the pooled control (see Table 2A in the online Data Supplement). Most patients, however, had an mtDNA content within the reference interval, and some of the patients with *POLG* mutations had an mtDNA content greater than the mean control value (Fig. 2A). The mean mtDNA content in blood samples was reduced to 78% of the control value in samples from individuals with *POLG* mutations, 52% in those with *DGUOK* or *MPV17* mutations, 79% in those with *TK2* mutations, 70% in those with *TYMP* mutations, and 39% in those with *RRM2B* mutations (see Table 2A in the online Data Supplement).

On average, the mtDNA content in muscle and liver samples of patients with proven MDDS was reduced to 33% and 8%, respectively, of the values for the pooled control (Fig. 2, B and C; see Table 2, B and C, in the online Data Supplement). The mtDNA depletion in skin fibroblasts was not consistent (Fig. 2D; see Table 2D in the online Data Supplement).

We calculated the sensitivity and specificity of qPCR to measure the mtDNA content in blood, muscle, and liver samples at various cutoffs (i.e., percentage of the age- and tissue-matched pooled control values) (Table 1). Of the tissue types evaluated by qPCR for mtDNA depletion, it is clear that muscle and liver are the most appropriate.

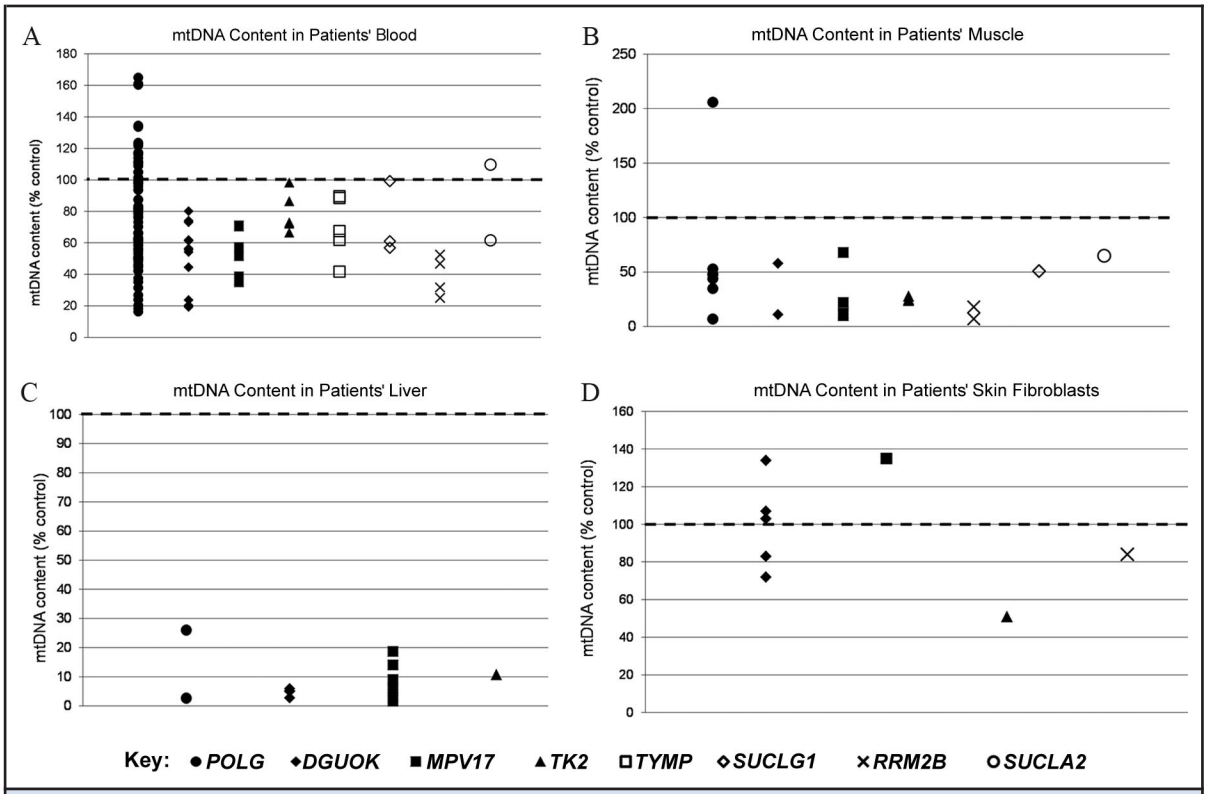

Fig. 2. mtDNA content in patients with molecularly proven MDDS caused by the affected gene.
mtDNA content is expressed as a percentage of that of a pooled control sample, which was prepared by pooling equal amounts of DNA from 10 age- and tissue-matched control samples. Blood, n = 122 (A); muscle tissue, n = 21 (B); liver tissue, n = 15 (C); skin fibroblasts, n = 7 (D).

DOES THE mtDNA CONTENT IN BLOOD SAMPLES FROM PATIENTS WITH *POLG* MUTATIONS CORRELATE WITH AGE OR DISEASE?

Mutations in the *POLG* gene do not always cause mtDNA depletion in blood (Fig. 2A), unlike mutations in other genes, such as *DGUOK* or *MPV17*. Indeed, about a quarter of the patients with POLG deficiency had an mtDNA content greater than that of the age- and tissue-matched pooled control value. Patients with mutations in the *POLG* gene present with a wide spectrum of disease phenotypes, including Alpers syndrome, autosomal recessive progressive external ophthalmoplegia, sensory ataxic neuropathy with dysarthria and ophthalmoparesis, and spinocerebellar ataxia

Table 1. Sensitivity and specificity of real-time qPCR screening for MDDS in various tissues.[a]

	Sample type																	
	Blood (n = 403)					Muscle (n = 346)					Liver (n = 78)				Skin (n = 55)			
Controls/patients, n	281/122					325/21					63/15				47/7			
Cutoff, %	30	50	60	**70**	80	30	50	**60**	70	80	**50**	60	70	80	50	60	70	80
Sensitivity, %	7	23	37	**51**	62	57	71	**90**	100	100	**100**	100	100	100	0	14	14	25
Specificity, %	98	96	91	**80**	70	93	85	**80**	74	63	**97**	95	89	79	93	90	83	71

[a] mtDNA content cutoff values are expressed as a percentage of the age- and tissue-matched controls. Data in bold reflect the cutoffs used in our diagnostic laboratory.

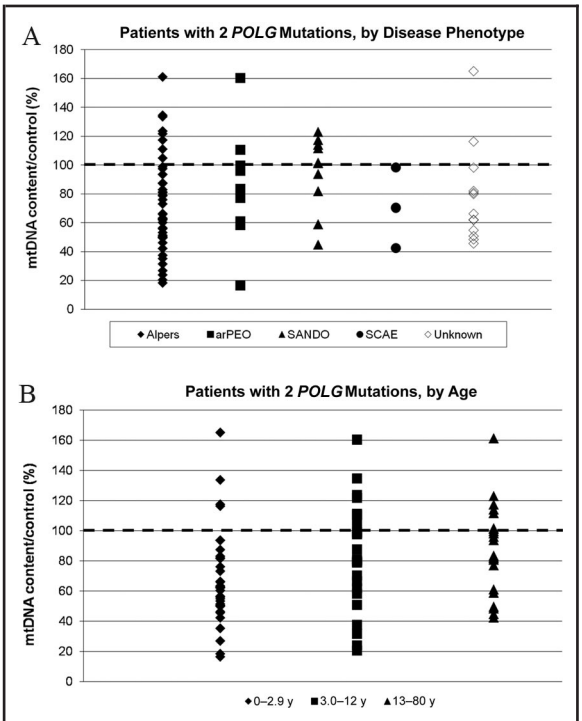

Fig. 3. mtDNA content in blood samples from 81 patients with 2 mutant alleles in the *POLG* gene.

mtDNA content is expressed as a percentage of that of a pooled control sample, which was prepared by pooling equal amounts of DNA from 10 age- and tissue-matched control samples. Data stratified by disease phenotype (A) and by age (B). Alpers, Alpers syndrome; arPEO, autosomal recessive progressive external ophthalmoplegia; SANDO, sensory ataxic neuropathy with dysarthria and ophthalmoparesis; SCAE, spinocerebellar ataxia with epilepsy; y, years.

with epilepsy. We investigated whether age of disease onset or disease phenotype affected the relative mtDNA content in blood samples from patients with 2 *POLG* mutations. The mean mtDNA content in patients 0–2 years of age with 2 *POLG* mutations was lower than that of patients 3–12 years of age and patients 13–60 years of age (67%, 83%, and 88% of the matched control value, respectively; Fig. 3A). The linear regression, however, was not statistically significant, however ($R^2 = 0.0352$, and $t = 1.70$; $P = 0.093$; see Table 3A in the online Data Supplement). Furthermore, the mtDNA content in blood samples did not correlate with disease phenotype (Fig. 3B; see Table 3B in the online Data Supplement).

mtDNA CONTENT IN SYMPTOMATIC INDIVIDUALS WITH ONLY 1 MUTATION IN A GENE KNOWN TO CAUSE MDDS

The mtDNA content was not reduced in blood samples from 97 symptomatic individuals with a single mutation or unclassified variant in *POLG*, *DGUOK*, *TK2*, or *TYMP* (mean mtDNA content 105%, 98%, 118%, and 141% of the age-matched pooled control value, respectively) (Fig. 4; see Table 4A in the online Data Supplement). Similarly, no mtDNA depletion was apparent in muscle samples (Fig. 4).

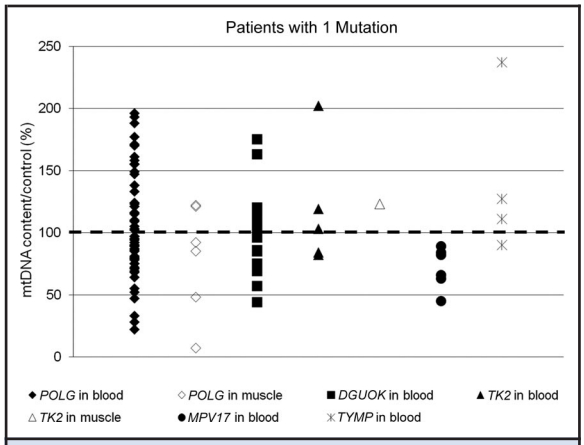

Fig. 4. mtDNA content in patients with only 1 mutant allele.

mtDNA content is expressed as a percentage of that of a pooled control sample, which was prepared by pooling equal amounts of DNA from 10 age- and tissue-matched control samples.

mtDNA CONTENT IN CARRIER PARENTS AND SIBLINGS OF MDDS PATIENTS

The mtDNA content of blood samples from 49 asymptomatic carrier parents or siblings of MDDS patients was similar to that of the age-matched pooled control value (see Table 4B in the online Data Supplement).

PROSPECTIVE SEQUENCE ANALYSIS OF SAMPLES WITH AN mtDNA CONTENT <30% OF THE MATCHED POOLED CONTROL

We sequenced DNA from 1 liver, 9 blood, and 10 muscle samples with an mtDNA content <30% of the matched pooled control by the Sanger method for mutations in the *POLG*, *DGUOK*, *MPV17*, and *TK2* genes. One blood sample bearing compound heterozygous mutations (p.251I and p.P587L, in cis) and p.R853Q in the *POLG* gene was detected. Two muscle samples with *POLG* mutations, one homozygous for p.G848S and the other compound heterozygous for p.A467T and p.G848S, were found.

Subsequent examples of patients for prospective sequence analysis include a 42-year-old woman who presented with a MNGIE-like syndrome *(16)*. The mtDNA content in a muscle sample was 12% of the matched pooled control. Sequencing of the *POLG*, *SLC25A4* [solute carrier family 25 (mitochondrial car-

rier; adenine nucleotide translocator), member 4], *C10orf2*, *SUCLG1*, and *SUCLA2* genes identified no deleterious mutations. Sequencing of the *RRM2B* gene, however, revealed 2 compound heterozygous missense mutations. The mtDNA content in a liver sample from an infant who presented with liver failure was 16% of the control. A sequencing analysis of the *POLG*, *TYMP*, *TK2*, *RRM2B*, *DGUOK*, and *SUCLA2* genes from a blood sample revealed no deleterious mutations. Although sequencing of the *MPV17* gene identified no mutations, exon 7 failed to amplify. Oligonucleotide array comparative genomic hybridization analysis *(20)* detected a homozygous 1.8-kb deletion encompassing exon 7 of the *MPV17* gene.

These results support the assertion that the real-time qPCR method has the potential to be useful for screening for mtDNA depletion if a sample of the affected tissue is available. If mtDNA depletion is confirmed by qPCR, sequencing analysis of the genes associated with the clinical presentation is warranted.

Discussion

A quantitative evaluation of mtDNA content in affected tissues can be very useful as the first step toward definitive identification of the disease-causing mutations in candidate genes *(24)*. This study has established the range of mtDNA content in various tissues of control individuals. The mtDNA content varies not only among different tissues but also with the age of the individual. Tissues with high energy demands, such as muscle and liver, have a higher mtDNA content than blood and skin fibroblasts.

It is interesting that the relative mtDNA content decreases with age in blood but increases with age in muscle. The increase in mtDNA content in muscle with increasing age may reflect increased muscle energy demands or a compensatory amplification to compensate for the loss of mitochondrial function *(25)*. The extent of the decrease in blood is not as substantial as the extent of the increase in muscle. The mechanism behind the decrease in mtDNA content in blood with increasing age is not clear. Because rapidly dividing cells (such as blood cells) with large mtDNA deletions have been shown to be preferentially eliminated, the difference between dividing and nondividing cells in the accrual of mutations may account for the divergent trends in blood and muscle tissues.

In addition to the age-dependent changes in mtDNA content in blood and muscle tissues, healthy individuals show wide variation in the mtDNA content of the same tissue. This variation may reflect differences in activity levels *(26)* or genetic differences in mtDNA replication.

The blood, muscle, and skin samples used to determine the reference values for mtDNA content were from patients with suspected mitochondrial disorders but without common mutations in mtDNA. Most of these samples had been screened for mutations in the *POLG*, *DGUOK*, *MPV17*, and *TK2* genes and were negative for deleterious mutations. Although the individuals in this control pool may not reflect a truly healthy population, they are representative of individuals undergoing diagnostic testing for mitochondrial disease, for whom a distinction needs to be made between mtDNA depletion and other causes of illness, with all of the attendant concerns (fatty infiltration, for example). Therefore, the use of such samples to calculate relative mtDNA content is a reasonable approach. Furthermore, the mtDNA content in blood samples from these control individuals was indistinguishable from blood samples from 60 individuals who were younger than 5 years and with no suspicion of mitochondrial disease (unaffected healthy controls; see Table 1A in the online Data Supplement).

Our results clearly demonstrate that liver and muscle tissues are the best choice for detecting mtDNA depletion in patients with suspected MDDS with a high degree of specificity and sensitivity. The value of muscle tissue for diagnosing MDDS was confirmed by a prospective analysis in which *POLG* mutations were identified in 2 of 9 muscle samples with mtDNA depletion (i.e., $<$30% of the control). Furthermore, these approaches led us to the identification of a patient with mutations in *MPV17* and another with mutations in *RRM2B*. These results confirm that a real-time qPCR method maybe useful as a screening approach for detecting mtDNA depletion.

Liver appears to be the most specific and sensitive tissue for measuring mtDNA depletion in any MDDS patient, irrespective of the phenotype. At 50% of the mean control mtDNA value, mtDNA depletion was detected with a sensitivity of 100% and a specificity of 97% (Table 1). A severe reduction in mtDNA content was found in liver samples from patients with the hepatocerebral form of MDDS (*MPV17*, *DGUOK*, and *POLG* mutations; Fig. 2C). Notable is the mtDNA depletion (19% of control) we observed in both the liver sample and the muscle sample from a patient with myopathic MDDS caused by *TK2* mutations. This result suggests that liver tissue may have utility in the diagnosis of the myopathic forms of MDDS, as well as hepatocerebral forms. This result should also prompt an evaluation of the liver function in patients with *TK2* mutations.

Although muscle tissue is not quite as specific or as sensitive as liver tissue for detecting mtDNA depletion, it is still relatively specific and sensitive, and reductions in mtDNA were seen in muscle tissue from all patients.

With a cutoff mtDNA content of 60% of the control, a level commonly used clinically, mtDNA depletion was identified in muscle tissue with 90% sensitivity and 80% specificity (Table 1). The overlapping range in muscle tissue mtDNA content may be due to the increased variation stemming from differences in physical activity levels *(26)*.

The clinical utility of qPCR with blood samples is limited for assisting in MDDS diagnosis, because results obtained with blood samples are specific but not sensitive for detecting mtDNA depletion. Indeed, some of the blood samples from patients with *POLG* mutations had an mtDNA content greater than that of the age-matched pooled control. *POLG* mutations cause a broad spectrum of diseases, and mutations can cause multiple mtDNA deletions. Patients with mtDNA deletions may have an increased mtDNA content due to a compensatory amplification mechanism *(25)*. All patients with mutations in *DGUOK*, *MPV17*, *SUCLG1*, *RRM2B*, and *SUCLA2* had a reduced mtDNA content in blood samples, but the detection sensitivity and specificity were reduced compared with those for muscle and liver tissue. Because the mtDNA content of blood samples from patients with only 1 mutant allele and of samples from asymptomatic carrier parents and siblings of patients with autosomal recessive MDDS was similar to the mtDNA content of the age-matched pooled control, the blood mtDNA content cannot be used to identify carriers.

Conversely, despite the lack of sensitivity of blood, its specificity was demonstrated by the detection of pathogenic *POLG* mutations in 2 of 8 patients with mtDNA depletion in blood samples. Because of the limited number of genes sequenced in the prospective study, it is possible that the other individuals with mtDNA depletion in blood also had primary mtDNA depletion but that the molecular defects were in other genes that were not sequenced. Therefore, analysis of all genes responsible for mtDNA depletion is indicated when the presence of mtDNA depletion is detected in blood samples. The ability to detect *POLG* mutations prospectively in muscle samples from 2 patients further supports the importance of qPCR analysis, given that the results of studies of respiratory chain enzymes in muscle tissue from MDDS patients may be negative or inconclusive *(27, 28)*. The fact, however, that the mtDNA content of muscle may be close to typical in some individuals should prompt caution when results with muscle tissue are used to exclude a diagnosis of MDDS.

The mtDNA content in routine cultures of fibroblasts from MDDS patients was not consistent in this study, so the data do not support a utility for fibroblasts in screening for mtDNA depletion. This result is consistent with previously published experience *(3)*.

In conclusion, if the appropriate tissue is used, real-time qPCR can be an effective method for detecting mtDNA depletion.

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