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Interpretation of mitochondrial tRNA variants

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Purpose: To develop criteria to interpret mitochondrial transfer RNA (mt-tRNA) variants based on unique characteristics of mitochondrial genetics and conserved structural/functional properties of tRNA.

Methods: We developed rules on a set of established pathogenic/benign variants by examining heteroplasmy correlations with phenotype, tissue distribution, family members, and among unrelated families from published literature. We validated these deduced rules using our new cases and applied them to classify novel variants.

Results: Evaluation of previously reported pathogenic variants found that 80.6% had sufficient evidence to support phenotypic correlation with heteroplasmy levels among and within families. The remaining variants were downgraded due to the lack of similar evidence. Application of the verified criteria resulted in rescoring 80.8% of reported variants of uncertain significance (VUS) to benign and likely benign. Among 97 novel variants, none met pathogenic criteria. A large proportion of novel variants (84.5%) remained as VUS, while only 10.3% were likely pathogenic. Detection of these novel variants in additional individuals would facilitate their classification.

Conclusion: Proper interpretation of mt-tRNA variants is crucial for accurate clinical diagnosis and genetic counseling. Correlations with tissue distribution, heteroplasmy levels, predicted perturbations to tRNA structure, and phenotypes provide important evidence for determining the clinical significance of mt-tRNA variants.

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INTRODUCTION

Mitochondrial diseases are clinically and genetically heterogeneous due to mitochondrial dysfunctions that may be caused by defects in either mitochondrial DNA (mtDNA) or nuclear genes (nDNA).[1] Thus, making a definitive molecular diagnosis of a mitochondrial disorder is challenging.[2] Up to date, defects in approximately 250–300 nuclear genes known to encode mitochondrial structural/functional proteins result in patients with mitochondrial disorders.[3] Such nuclear gene defects are amenable to variant classification using American College of Medical Genetics and Genomics (ACMG) guidelines.[4] However, these guidelines require modification for the interpretation of mtDNA variants owing to the characteristics of mtDNA, which include multiple and variable numbers of mtDNA per cell and among different cell types, random segregation, heteroplasmy, tissue differences in energy requirements, fission/fusion balances, thresholds, maternal inheritance, meiotic bottleneck, and preferential elimination of defective mitochondrial genomes in rapidly dividing cells resulting in tissue and age related heteroplasmy discrepancies.[1,5,6]

Mitochondrial transfer RNA (mt-tRNA) genes account for only ~8% of the entire mitochondrial genome.[7] However, the frequency of pathogenic variants in mt-tRNAs is significantly higher (~8.5×) than that of mitochondrial messenger RNA (mt-mRNA) based on the total length of corresponding coding regions.[7] Thus, mt-tRNA variants are a major cause of mtDNA disorders. The classical secondary structure of the canonical mt-tRNA resembles a cloverleaf, containing four stems and three loops.[8] Mt-tRNAs are transcribed from the double-stranded mtDNA in two polycistronic precursor transcripts followed by cleavage and post-transcriptional modification that occurs at specific nucleotide positions.[9] Each mt-tRNA undergoes aminoacylation by the corresponding specific aminoacyl-tRNA synthetase (mt-ARS).[10] Any variant that damages the mt-tRNA structure or impairs processing, post-transcriptional modification, aminoacylation, or codon recognition may disrupt mitochondrial function. Therefore, interpretation of mt-tRNA variants must take tRNA structure/function and mitochondrial characteristics into consideration.

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In this study, we review previously characterized mt-tRNA variants to extract classification heuristics. We then apply these rules to classify reported variants of uncertain significance (VUS) and novel mt-tRNA variants.

MATERIALS AND METHODS

Development and validation of criteria for the interpretation of mt-tRNA variants

Approximately 10,000 mtDNA variants were identified in our Molecular Diagnostic Laboratory at Baylor College of Medicine and Baylor Genetics from 2005 to 2016. Removal of high frequency mitochondrial single-nucleotide polymorphisms (mtSNPs) (>5%) resulted in a total of 550 mt-tRNA variants with 97 novel and 453 previously reported. The latter were divided into three groups: pathogenic (P), VUS, and benign (B) variants, according to published reports (Fig. 1 and Supplemental Table 1; "reported" column). We reviewed literature of all 453 reported variants for evidence of pathogenicity. We deduced classification rules from definitely P and B variants with sufficient evidence ("re-evaluation before addition of new cases" column). We then validated and refined these criteria using our new cases ("after addition of new cases" column). We subsequently applied such fine-tuned, verified criteria to re-evaluate reported variants with insufficient evidence, followed by the application of the established criteria (Tables 1, 2) to reclassify reported VUS and interpret novel variants (Fig. 1). The rules for combining criteria to classify sequence variants are the same as those of the ACMG guidelines.[4]

Application of MitoTIP scores

MitoTIP scores are included as an alternative computational supporting criterion. MitoTIP scores were originally interpreted within quartiles.[11] We explored the distribution of MitoTIP scores in 41 confirmed pathogenic (P) variants in MITOMAP (https://www.mitomap.org) and 122 probable benign (B) variants in the Human Mitochondrial Genome Database (mtDB) (http://www.mtdb.igp.uu.se/). Supplemental Fig. 1A shows that 76% (31/41) of P variants had MitoTIP scores >16, while 17% (7/41) of P variants and 4% (5/122) of B variants had MitoTIP scores within the range of 12.5–16. Supplemental Fig. 1B shows that 86% (105/122) of SNPs had MitoTIP scores <10. Based on this observation, we set a PM7 (Table 1) for variants with MitoTIP scores >16, PP3 for variants between 12.5 and 16, and BP4 (Table 2) for MitoTIP scores <10.

RESULTS

Evaluation of reported mt-tRNA variants

We identified 550 rare (frequency <5% in general populations) mt-tRNA variants (https://www.ncbi.nlm.nih.gov/clinvar/) in our laboratory. Among them, 97 are novel, and 453 have been previously reported, with 72 classified as P, 261 VUS, and 120 B (Fig. 1). After a careful review of published literature, we found that only 40 of the 72 reported P variants had sufficient evidence to support pathogenicity. The determining factors include functional studies correlated with heteroplasmy (PS3), multiple reports supporting pathogenicity (PS5), MitoTIP score >16 (PM7), >5% heteroplasmy in the affected with correlation of phenotypes in multiple tissues (PM8), and matrilineal family members or independent families (PM9). The remaining variants lack one or more of these findings (Supplemental Table 1).

Validation and confirmation of previously classified P variants with our new cases

The main evidence for pathogenicity is functional study results (PS3) and the correlation between heteroplasmy and biochemical, cellular, or clinical phenotypes (PM8, PM9). For all novel variants, PS3 alone will not qualify a variant as P. For example, m.5540G>A (*MT-TW*) and m.8362T>G (*MT-TK*) have each only been reported in one simplex case with functional studies, without any clinical correlation. The m.5540G>A variant was reported as heteroplasmy in a 36-year-old female with progressive encephalopathy (PP4, PP6).[12] Single-fiber analysis correlated mitochondrial dysfunction with variant heteroplasmy (PS3). After reviewing the report, it was scored as likely pathogenic (LP, under column "before addition of new cases") (PS3 + PM7 + 2PP) (Table 3). We identified a new patient with encephalomyopathy carrying this variant, with heteroplasmy at 25% in blood and 51% in muscle (PM8). This variant was absent in the asymptomatic mother (PM9). This new case adds two PMs to the original reported case and upgrades m.5540G>A to P.

Each of the two variants (m.616T>C [*MT-TF*] and m.15915G>A [*MT-TT*]) have been reported twice, but the heteroplasmy did not correlate with clinical phenotypes (lacking PM8). For example, m.616T>C was reported in a patient with severe epilepsy.[13] It was nearly homoplasmic in several tissues from the proband, yet heteroplasmic in blood and buccal swabs from asymptomatic matrilineal relatives. Additional two pedigrees with m.616T>C had tubulointerstitial kidney disease.[14] All affected individuals were homoplasmic in blood. Functional studies confirmed mitochondria dysfunction in these patients but correlations with heteroplasmy levels were not evident (PM10). Due to the discordant phenotype, this variant can only qualify as LP (PM7, PM9, and PM10). We detected this variant as homoplasmic in the blood of a patient with seizures and renal tubulopathy as well as at 89% (blood) and 86% (muscle) in the patient's asymptomatic mother. This new case suggests that this variant may have high phenotypic thresholds in different tissues and that the apparent homoplasmy may need to be carefully quantified using accurate methodology.[15] The addition of PS5 strengthens the pathogenicity of this variant (Table 3).

Downgrade of previous P variants to LP

Most of the downgraded reported P variants lack functional evidence (PS3), the most important determining factor for pathogenicity (Table 3). For example, the m.4296G>A (*MT-TI*) variant was previously reported in a patient with

Table 1 Criteria for the classification of pathogenic mt-tRNA variants.

Evidence	ACMG criteria	Criteria for mt-tRNA variants
Very strong	**PVS1** Null variant in a gene where loss of function is a known mechanism of disease.	**PVS1** Not applicable.
Strong	**PS1** Same amino acid change as a previously established pathogenic variant regardless of nucleotide change.	**PS1** Not applicable.
	PS2 De novo in a patient with disease and no family history.	**PS2** Present at ≥5% heteroplasmy, in ≥2 different tissues of the affected individual but not detected in asymptomatic mother. If mother's sample is unavailable, and not detected in other asymptomatic matrilineal relatives (such as proband's siblings, proband's maternal grandmother), it will be downgraded to PM9. Note: The percentage of heteroplasmy should be analyzed by dependable, clinically validated method, such as deep NGS using one-piece long-range PCR product of the circular mtDNA template. The methodology must be able to distinguish very low heteroplasmy (e.g., 0.1%) from true zero at a particular nucleotide position.
	PS3 Well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product.	**PS3** Well-established in vitro or in vivo functional studies supportive of a damaging effect on the mitochondrial function. These could be transmitochondrial cybrid assays, ETC enzymology, OCR, ATP synthesis rate, mtDNA copy number, COX deficient fibers, single-fiber assay, morphology, and functional measurements correlated with heteroplasmy levels. Downgrade to PM10 if result is not correlated with heteroplasmy.
	PS4 The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls.	**PS4** The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls.
		PS5 Rare variants previously reported as pathogenic. Note: Not all reports are reliable, especially studies published before the application of deep NGS. Review the literature cautiously before using this criterion.
Moderate	**PM1** Located in a mutational hot spot and/or critical and well-established functional domain without benign variants.	**PM1** Variants that alter the anticodon.
	PM2 Absent from controls in Exome Sequencing Project, 1000 Genomes, or ExAC.	**PM2** Absent from databases, e.g., mtDB and MITOMAP, and absent or low heteroplasmy (<5%) in the asymptomatic mother of a proband exhibiting ≥5% heteroplasmy. If mother's sample is unavailable, it will be downgraded to PP7. Note: The percentage of heteroplasmy should be analyzed by a dependable, clinically validated method, such as deep NGS, using one-piece long-range PCR product of the circular mtDNA template.
	PM3 For recessive disorders, detected in *trans* with a pathogenic variant.	**PM3** Not applicable.
	PM4 Protein length changes due to in-frame deletion/insertions in a nonrepeat region or stop-loss variants.	**PM4** Not applicable.
	PM5 Novel missense change at amino acid residue where a different missense change determined to be pathogenic has been seen before.	**PM5** Change occurs at the same nucleotide position as a previously well-established pathogenic variant attributed to a different nucleotide. Example: m.3243A>T vs. m.3243A>G.
	PM6 Assumed de novo, but without confirmation of paternity and maternity.	**PM6** Not applicable (see PS2).
		PM7 MitoTIP prediction score >16.0.
		PM8 Heteroplasmy (≥5%) among different tissues of an affected individual correlates with tissue clinical or biochemical phenotypes. Example: The muscle heteroplasmy in a myopathic patient is at 10%, while the heteroplasmy in unaffected tissues like blood is at 3%. If correlation with tissue clinical or biochemical phenotypes is not available then downgrade to PP6.
		PM9 At least two independent families, or two matrilineal family members from one family demonstrate correlation of heteroplasmy (≥5%) with clinical or biochemical phenotypes.
		PM10 Well-established in vitro or in vivo functional studies supportive of a damaging effect on the mitochondrial function. These could be transmitochondrial cybrid assays, ETC enzymology, OCR, ATP synthesis rate, mtDNA copy number, COX deficient fibers, single-fiber assays, morphology consistent with mitochondrial disease, and functional measurements not correlated with heteroplasmy.
Supporting	**PP1** Cosegregation with disease in multiple affected family members in a gene definitively known to cause the disease.	**PP1** Replaced by PM8 or PM9 when cosegregate with disease and ≥ 5% heteroplasmy.
	PP2 Missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease.	**PP2** Not applicable.
	PP3 Multiple lines of computational evidence support a deleterious effect on the gene or gene product.	**PP3** The range of MitoTIP prediction score is within [12.5–16].
	PP4 Patient's phenotype or family history is highly specific for a disease with a single genetic etiology.	**PP4** Patient's phenotype or family history is highly specific for a mitochondrial disease with a single genetic etiology.
	PP5 Reputable source recently reports variant as pathogenic but the evidence is not available to the laboratory to perform an independent evaluation.	**PP5** Not applicable.
		PP6 Heteroplasmic in an affected proband with a degree ≥ 5%.
		PP7 Absent from databases, e.g., mtDB and MITOMAP, and is heteroplasmic; mother is unavailable.

The ACMG criteria are listed in the left column and the new criteria for mt-tRNA variants are on the right.
ACMG American College of Medical Genetics and Genomics, *ATP* adenosine triphosphate, *COX* cytochrome C oxidase, *ETC* electron transport chain, *mt-tRNA* mitochondrial transfer RNA, *NGS* next-generation sequencing, *OCR* oxygen consumption rate, *PCR* polymerase chain reaction.

Table 2 Criteria for the classification of benign variants.

Evidence	ACMG criteria	Criteria for mt-tRNA variants
Standalone	**BA1** Allele frequency is above 5% in Exome Sequencing Project, 1000 Genomes, or ExAC.	**BA1** Top-level haplogroup defining variants.
Strong	**BS1** Allele frequency is greater than expected for disorder.	**BS1** Reported in public databases (e.g., MITOMAP or mtDB) or literature as polymorphism.
	BS2 Observed in healthy adult individual for a recessive (homozygous), dominant (heterozygous), or X-linked (hemizygous) disorder with full penetrance expected at an early age.	**BS2** Found homoplasmic in more than three unrelated healthy adults.
	BS3 Well-established in vitro or in vivo functional studies shows no damaging effect on protein function or splicing.	**BS3** Not applicable.
	BS4 Lack of segregation in affected members of a family.	**BS4** Homoplasmy in both probands and at least two asymptomatic matrilineal family members.
Supporting	**BP1** Missense variant in a gene for which primarily truncating variants are known to cause disease.	**BP1** Not applicable.
	BP2 Observed in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder; or observed in *cis* with a pathogenic variant in any inheritance pattern.	**BP2** Not applicable.
	BP3 In-frame deletions/insertions in a repetitive region without a known function.	**BP3** Not applicable.
	BP4 Multiple lines of computational evidence suggest no impact on gene or gene product.	**BP4** The MitoTIP prediction score is ≤ 10.
	BP5 Variant found in a case with an alternate molecular basis for disease.	**BP5** In the presence of a known pathogenic genetic cause unless there is evidence of more than one disease and clinically explained.
	BP6 Reputable source recently reports variant as benign but the evidence is not available to the laboratory to perform an independent evaluation.	**BP6** Found to be homoplasmic more than once in asymptomatic adults in private reputable laboratory databases.
	BP7 A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved.	**BP7** Not applicable.

The ACMG criteria are listed in the left column and the new criteria for mitochondrial transfer RNA (mt-tRNA) variants are on the right.
ACMG American College of Medical Genetics and Genomics, *mt-mRNA* mitochondrial messenger RNA.

Leigh syndrome.[16] This variant was found at 78% and 85% in the proband's blood and fibroblasts, respectively, and exhibited less than 5% heteroplasmy in asymptomatic matrilineal relatives. However, at the time of the first report, the method for heteroplasmy quantification was allele refractory mutation system-based quantitative polymerase chain reaction (ARMS qPCR), which is not as accurate as deep next-generation sequencing (NGS) on full-length PCR amplified mtDNA due to the difference in amplification efficiency in the presence of the discriminating variant in the primer. The electron transport chain complex (ETC) activities of the proband's cultured fibroblasts showed impaired activities of complex I + III (33%) and complex IV (30%). However, results of ETC activity alone without correlation with phenotype or different tissues were insufficient for PS3, as mentioned above. More recently, we have identified the m.4296G>A at 2% heteroplasmy in the blood of another patient who in addition had a 64% heteroplasmic mtDNA single large deletion, which most likely explained the mitochondrial disease of this individual. This new observation disputes the pathogenicity of m.4296G>A. Therefore, current evidences are insufficient to support the pathogenicity of this variant, and justify downgrading to LP (3PM). This case prompted the creation of PM10 and modification of BP5 (a second variant explains the disease).

Three variants (m.1624C>T [*MT-TV*], m.14674T>C [*MT-TE*], and m.14709T>C [*MT-TE*]) are downgraded to LP due to the presence of apparent homoplasmy in both the affected proband and the asymptomatic mother. For example, the homoplasmic m.1624C>T (*MT-TV*) variant was reported in a family with profound mitochondrial disease (PP4), resulting in one child with Leigh syndrome and six neonatal deaths.[17] Steady state levels of mt-tRNA^{Val} were dramatically reduced in a tissue-specific manner (PS3).[18] The variant is located at a structurally important position as supported by MitoTIP score (PP3). However, the discordant phenotype of the homoplasmic variant in the phenotypically mild mother argues against the pathogenicity of m.1624 (combined criteria = 1PS + 2PPs = LP).

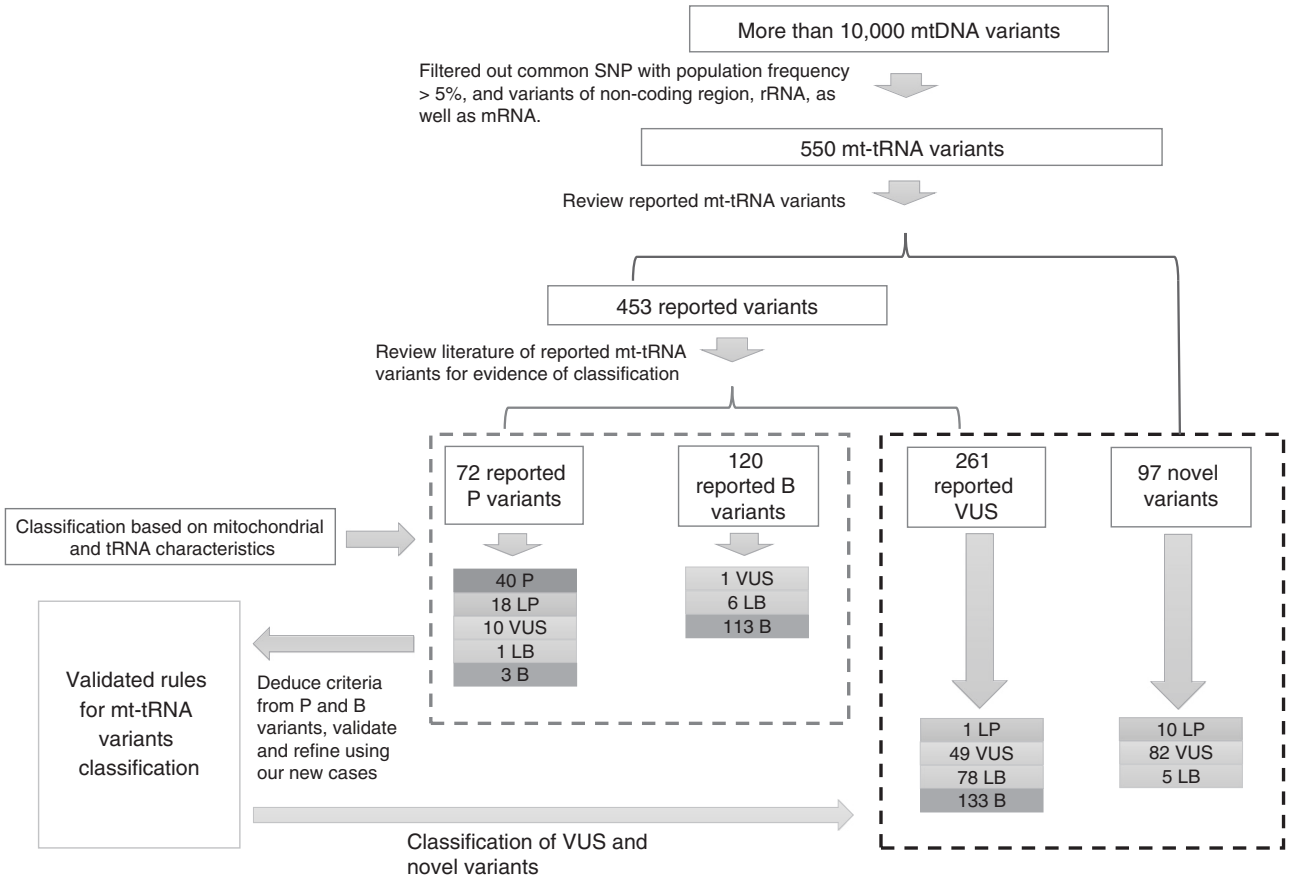

Fig. 1 Flowchart of mitochondrial transfer RNA (mt-tRNA) interpretation. *B* benign, *LB* likely benign, *LP* likely pathogenic, *mtDNA* mitochondrial DNA, *mRNA* messenger RNA, *rRNA* ribosomal RNA, *P* pathogenic, *SNP* single-nucleotide polymorphism, *VUS* variant of uncertain significance.

Downgrade of previous P variants to VUS

We have downgraded ten reported P variants to VUS because they were reported for the first time without functional studies and correlation of heteroplasmy with phenotypes (lack of PS3 and PM8/PM9). Our new cases did not provide evidence to support pathogenicity. Thus, they remain as VUS (Table 3). For example, the m.1643A>G (*MT-TV*) variant was previously reported in a patient with encephalopathy.[19] This variant has a MitoTIP score of 11.9, which does not meet pathogenic criteria. This variant was initially described as nearly homoplasmic in the proband and heteroplasmic in her asymptomatic mother. However, further analysis of heteroplasmy revealed near homoplasmy in blood samples of both the proband and her asymptomatic mother. Thus, there was no correlation with phenotype. We identified this variant in one patient with 1.8% heteroplasmy in blood, which does not explain the phenotype. Without correlation between the degree of heteroplasmy and phenotype, we cannot confirm the pathogenicity of this variant, thus it is downgraded to VUS (PM10 + PP6).

Downgrade of previous P variants to B or LB

Three previously reported P variants, m.3236A>G (*MT-TL1*), m.4381A>G (*MT-TQ*), and m.15926C>T (*MT-TT*), were reported in large cohorts without any functional or heteroplasmy studies, and found in multiple healthy individuals in public databases.[20–22] We thus downgraded these variants to B. Similarly, we found m.3275C>A (*MT-TL1*) in an asymptomatic mother, hence when combined with one report in MITOMAP, this variant was classified as likely benign (LB).[23]

Re-evaluation of previous VUS

We have re-evaluated 261 reported VUS (Supplemental Table 1). The majority (81%) of them were scored as LB (78/261 = 30%) or B (133/261 = 51%), while 49 remained as VUS. We upgraded the m.12148T>C (*MT-TH*) VUS to LP based on an additional case with clinical features supporting pathogenicity. The MitoTIP score is 16.2. This variant was heteroplasmic by Sanger sequencing in the proband and not present in the blood of the proband's mother and sister, but at 10.2% heteroplasmy in the mother's urine. Thus, m.12148T>C is classified as LP (PM7, PM9, PP4, PP6). The majority of VUS become LB or B because they have been reported in public databases as polymorphisms (BS1), or are homoplasmic in multiple healthy adults (BS2) and/or asymptomatic matrilineal relatives (BS4) in our database.

Table 3 Reported pathogenic mt-tRNA variants with discordant evaluation.

Position	Gene	Nucleotide change	MitoTIP score	Location	Reported	Re-evaluation		Added criteria by our cases	Criteria
						Before addition of new cases	After addition of new cases		
616	*MT-TF*	T>C	17.5	ACS	P	LP	P	PS5	PS5, PM7, PM9, PM10
5540	*MT-TW*	G>A	16.1	ACS	P	LP	P	PM8, PM9	PS3, PM7, PM8, PM9, PP4, PP6
8362	*MT-TK*	T>G	18.6	AS	P	LP	P	PM9	PS3, PM7, PM9, PP4, PP6
15915	*MT-TT*	G>A	16.1	ACS	P	LP	P	PS5	PS5, PM7, PM8, PM9, PM10
618	*MT-TF*	T>C	14.8	ACS	P	LP	LP		PM5, PM8, PM9, PP3
1624	*MT-TV*	C>T	15.3	DS	P	LP	LP		PS3, PP3, PP4
1630	*MT-TV*	A>G	16.7	ACS	P	LP	LP		PS3, PM7, PP4, PP6
1659	*MT-TV*	T>C	15.5	TS	P	LP	LP	PM8	PM8, PM9, PP3, PP6
3242	*MT-TL1*	G>A	7	DS	P	LP	LP		PS5, PM9, PM10
3252	*MT-TL1*	A>G	11.5	DL	P	LP	LP	PM9	PM8, PM9, PM10
3288	*MT-TL1*	A>G	10.9	TL	P	LP	LP	PM9	PM8, PM9, PP4, PP6
4284	*MT-TI*	G>A	10.7	C2	P	LP	LP	PM9	PM8, PM9, PM10
4296	*MT-TI*	G>A	12.4	ACL	P	LP	LP	PM8, PM10	PM8, PM9, PM10
4308	*MT-TI*	G>A	17.3	TS	P	LP	LP		PM7, PM8, PM9, PM10, PP6
8299	*MT-TK*	G>A	14.4	AS	P	LP	LP		PM8, PM10, PP3, PP6
9997	*MT-TG*	T>A	19.6	AS	P	LP	LP	PM9	PM5, PM7, PM9, PM10
10406	*MT-TR*	G>A	16	AS	P	LP	LP		PM7, PM8, PM9, PM10
12264	*MT-TS2*	C>T	16.7	AS	P	LP	LP		PM7, PM9, PM10
14674	*MT-TE*	T>C	9.6	DB	P	LP	LP		PS5, PP4, PP6
14709	*MT-TE*	T>C	13	ACL	P	LP	LP		PS5, PP3, PP4, PP6
15995	*MT-TP*	G>A	16.9	ACS	P	LP	LP		PM7, PM9, PP4, PP6
16002	*MT-TP*	T>C	16.3	DS	P	LP	LP		PM7, PM8, PM9, PP6
1643	*MT-TV*	A>G	11.9	VR	P	VUS	VUS		PM10, PP6
3250	*MT-TL1*	T>C	10.3	DL	P	VUS	VUS		PM8, PP6, BS4
4290	*MT-TI*	T>C	12.5	ACL	P	VUS	VUS	BS4	PP3, BS4
4320	*MT-TI*	C>T	17.9	TS	P	VUS	VUS	BP6	PM7, BP6
5559	*MT-TW*	A>G	15.5	TS	P	VUS	VUS	PM9	PM9, PP3, PP6
5693	*MT-TN*	T>C	9.9	ACL	P	VUS	VUS		PS3, BP4
7520	*MT-TD*	G>A	13	AS	P	VUS	VUS		PP3
12293	*MT-TL2*	G>A	15	ACS	P	VUS	VUS	BP5	PP3, PP6, BP5
15950	*MT-TT*	G>A	12.9	AS	P	VUS	VUS	BP6	PP3, BP6
16018	*MT-TP*	ins15		AS	P	VUS	VUS	BP6	BP6
3275	*MT-TL1*	C>A	2	VR	P	VUS	LB	BS4	BS4, BP4
3236	*MT-TL1*	A>G	11.2	AS	P	VUS	B	BS2	BS1, BS2
4381	*MT-TQ*	A>G	5.4	DL	P	LB	B	BS4	BS1, BS4, BP4
15926	*MT-TT*	C>T	1.1	ACS	P	LB	B	BS2	BS1, BS2, BP4
12148[a]	*MT-TH*	T>C	16.2	DS	VUS	VUS	LP	PM9, PP4, PP6	PM7, PM9, PP4, PP6

ACL anticodon loop, *ACS* anticodon stem, *AS* acceptor stem, *B* benign, *C1* connector 1, *C2* connector 2, *DB* discriminator base, *DL* D loop, *DS* D stem, *LB* likely benign, *LP* likely pathogenic, *mt-tRNA* mitochondrial transfer RNA, *P* pathogenic, *TL* T loop, *TS* T stem, *VR* variable region, *VUS* variant of uncertain significance.
[a]The only VUS upgraded to LP.

Re-evaluation of previously reported B variants

We re-evaluated 120 previously reported B variants. Only one (m.5793A>G [*MT-TC*]) was upgraded to VUS due to conflicting findings. This variant has been previously reported as a polymorphism.[22] However, its MitoTIP score is 14.9 (PP3). We found this variant at low heteroplasmy in the muscle of a proband with Leigh syndrome (PP4). Thus m.5793A>G was scored as a VUS. The majority of previously reported B variants remain as LB (6/120) or B (113/120) due to reported as polymorphism in public databases (BS1) and homoplasmy in healthy adults (BS2) or asymptomatic matrilineal relatives (BS4) in our database.

Interpretation of novel mt-tRNA variants

We identified 97 novel variants, 10 were classified as LP (Table 4), 82 as VUS, 5 as LB, and 0 as B (Supplemental Table 1). None of the novel variants are classified as P due to the lack of heteroplasmy correlation at the cellular (PS3) or pedigree (PS5) level. A majority of novel variants remain as VUS. Novel variants are usually classified as LP if their heteroplasmy levels correlate with phenotypes. The most commonly used criteria for classifying novel variants as LP include novel variant absence from public databases and asymptomatic mothers (PM2), correlation of heteroplasmy levels with phenotypes (PM8, PM9), high MitoTIP scores (PM7, PP3), and phenotypes or family history highly specific for a mitochondrial disease with a single genetic etiology (PP4). For example, a novel m.578T>C (*MT-TF*) variant was detected at 16% heteroplasmy in a proband's blood, and was not observed in the asymptomatic mother (PM2, PM9, PP6), with a MitoTIP score of 16.6 (PM7). Thus, it is predicted to be LP (3PM, 1PP). Another novel variant, m.4372C>T (*MT-TQ*), was detected at 22.8% heteroplasmy in a proband's blood, but not in the asymptomatic mother (PM2, PM9, PP6) with a MitoTIP score of 15.8 (PP3). Thus, we scored it as LP (2PM, 2PP).

Novel variants are LB when they are homoplasmic and have appeared more than three times in healthy adults (BS2) or more than twice in asymptomatic matrilineal relatives (BS4), as well as not exhibiting structural conservation (BP4). The number of LB variants is low in novel variants because most benign variants have already been identified and filtered out on the basis of frequency in large sequence data sets.

Table 4 Likely pathogenic novel mt-tRNA variants.

Position	Gene	Nucleotide change	MitoTIP score	Location	Previous classification	Added criteria by our case	Criteria
578	*MT-TF*	T>C	16.6	AS	Novel	PM2, PM9, PP6	PM2, PM7, PM9, PP6
4372	*MT-TQ*	C>T	15.8	ACS	Novel	PM2 PM9, PP6	PM2 PM9, PP3, PP6
4437	*MT-TM*	C>T	15	ACS	Novel	PM2, PM9, PM10, PP6	PM2, PM9, PM10, PP3, PP6
5889	*MT-TY*	A>G	14.9	AS	Novel	PM2, PM8, PM9, PP6	PM2, PM8, PM9, PP3, PP6
7566	*MT-TD*	G>A	14.8	TS	Novel	PM2, PM9, PP6	PM2, PM9, PP3, PP6
8319	*MT-TK*	A>G	15.4	ACS	Novel	PM2, PM9, PP6	PM2, PM9, PP3, PP6
10408	*MT-TR*	T>C	14.5	AS	Novel	PM8, PM10, PP6, PP7	PM8, PM10, PP3, PP6, PP7
10460	*MT-TR*	T>C	15.9	TS	Novel	PM2, PM9, PP4	PM2, PM9, PP3, PP4
12258	*MT-TS2*	C>T	17.2	AS	Novel	PM5, PP6, PP7	PM5, PM7, PP6, PP7
12335	*MT-TL2*	T>C	13.6	AS	Novel	PM8, PM9, PP7	PM8, PM9, PP3, PP7

ACL anticodon loop, *ACS* anticodon stem, *AS* acceptor stem, *B* benign, *C1* connector 1, *C2* connector 2, *DB* discriminator base, *DL* D loop, *DS* D stem, *LB* likely benign, *LP* likely pathogenic, *mt-tRNA* mitochondrial transfer RNA, *P* pathogenic, *TL* T loop, *TS* T stem, *VR* variable region, *VUS* variant of uncertain significance.

DISCUSSION

Key factors for the evaluation of pathogenicity

After careful review of reported pathogenic variants in mt-tRNA, we have observed that 19.4% did not have sufficient evidence for P or LP. The main reason is that early variant discoveries tended to be overcalled as pathogenic. Given the paucity of data, most newly discovered variants were observed once or rarely. Exacerbating this situation, previous sequencing and quantification technologies for heteroplasmy were less developed and unreliable. In addition, the large number of nuclear candidate genes and inefficient sequencing methodology contributed to misattributed etiologies. Advances of sequencing and quantification technologies now allow accurate detection and quantification of variant heteroplasmy,[15,24] thus providing a better correlation of molecular defects and heteroplasmy with clinical phenotype. In addition, mitochondrial characteristic muscle pathologies may be due to defects in previously unidentified nuclear genes rather than mtDNA. As a result, variants identified 10–20 years ago as novel or rare may no longer be recognized as such. Cumulative experience and additional cases allow a better understanding of the pathogenicity of previously reported variants.

An important factor for downgrading variants is high heteroplasmy (e.g., m.14709T>C) or homoplasmy (e.g., m.14674T>C, m.3250T>C, m.4290T>C) in asymptomatic mothers. Maternal inheritance, phenotypic thresholds, and heteroplasmy are major criteria unique to mitochondrial variants. Another common reason for a low pathogenicity score is the lack of functional studies showing correlation with heteroplasmy levels (PS3).

Absence from public databases is crucial for the evaluation of pathogenicity. Since mtDNA is highly polymorphic and mtDNA variants have been reported in almost every nucleotide position of the mitochondrial genome,[5,25] most benign variants have already been discovered. Thus, novel variants are by definition rare and more likely to be pathogenic. Consequently, we use PM2 for novel variants that were also not detected in the asymptomatic mother, and PP7 for novel variants regardless of the genotype of the mother. Another important factor is the structural conservation discussed below. Interestingly, we found that mtDNA content levels may also be an indicator of mt-tRNA defect. We have at least three families with mtDNA copy-number information. One had normal mtDNA content (m.4372C>T [*MT-TQ*], 97% in muscle). However, the other two (m.10460T>C [*MT-TR*] and m.12335T>C [*MT-TL2*]) had mtDNA proliferation to ~300% (threefold elevation), while ETC analyses in muscle showed complex deficiencies. Both were classified as LP due to "novel" variants that require a confirmatory case to upgrade to P (Table 4, Supplemental Table 1).

MitoTIP scores

MitoTIP is a newly developed in silico tool for predicting pathogenicity of novel mt-tRNA variants. The MitoTIP scores are calculated based on database frequencies, annotations of pathogenicity from MITOMAP, conservation among species, the position of the variant within the tRNA, and the nature of the nucleotide change (transversion/transition/deletion).[11] An important differentiation of mt-tRNAs versus mRNAs is their structural conservation, which makes the pathogenicity of mt-tRNA variants more amenable to in silico prediction. We developed moderate and supporting criteria for different levels of MitoTIP scores to fully incorporate the importance of structural conservation into the process of variant evaluation.

In most cases, MitoTIP scores are in accordance with our final classification; however, we also noticed that there are some outliers. For example, the MitoTIP score of m.3243A>G, the most common pathogenic variant, is only 13.3. This is because this variant is in the D-arm loop, which is not a common

location in secondary structures associated with disease. In addition, MitoTIP does not take post-transcriptional modification into consideration. More surprisingly, the critical nucleotides of anticodon triplets are of moderate to low MitoTIP scores, ranging from 6.6 to 13.1. This is because these sequences are neither well conserved across tRNA structures nor present in a conserved secondary structure commonly associated with disease (anticodon loop). The P variants with low MitoTIP score tend to cause milder disease and a higher phenotypic threshold. For example, m.3242G>A, although right next to the most common m.3243A>G variant, has a low MitoTIP score of 7.0, causing a mild mitochondrial myopathy at 94% heteroplasmy in muscle.[26]

Nuclear background may affect the expression of mt-tRNA variants

Similar to LHON, some mt-tRNA variants are homoplasmic in all types of tissues and in both affected probands and unaffected mothers.[10] The machineries of mtDNA replication, transcription, and translation all depend on the nuclear genome. In particular, for mt-tRNA, the post-transcriptional modification and function of mt-aminoacyl tRNA synthetases are regulated by the nuclear genome.[27] Accordingly, the nuclear background can influence the expression of mt-tRNA variants. For example, m.1630 A>G (MT-TV) is a rare variant only found in two families: one with mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) and the other with mitochondrial neurogastrointestinal encephalopathy (MNGIE) syndrome.[28–30] Although extensive functional studies have proved the pathogenicity of this variant, the presence of high heteroplasmy in both affected probands and asymptomatic mothers conflicts with the phenotype and pathogenicity of this variant. Our recent study[30] using exome sequencing analysis identified a nonsense variant, c.1000C>T (p.R334X) in the mitochondrial valyl-tRNA synthetase (*VARS2*), in the affected proband but not in the asymptomatic mother, although the mother carried higher variant heteroplasmy than the proband in blood. This variant results in a truncated protein, which lacks the C-terminal two-thirds of the VARS2 protein containing key domains interacting with the mt-tRNA^{Val}. The presence of the nuclear encoded *VARS2* variant may act synergistically with the *MT-TV* variant. Additional cases will further support the pathogenicity of this variant.

A similar example is m.1624C>T, which was described in "Results." To upgrade this variant to P, it will require the identification of factors (most likely nuclear gene variants) that modify the phenotypic expression. Other variants that are possibly influenced by the nuclear genome may include m.3250T>C (*MT-TL1*), m.14674T>C (*MT-TE*) and m.14709T>C (*MT-TE*).

Variants affecting the critical nucleotides of anticodon triplets

Theoretically, variants causing anticodon substitutions interfere with the decoding process of a tRNA, thus, they are likely to be pathogenic. However, such variants are rarely reported, implying that they are incompatible with early developmental stages or lethal in embryogenesis. To date, only four such anticodon variants have been reported. Three of them were associated with a severe phenotype. For example, m.5545C>T (*MT-TW*, UGA for trp to stop codon UAA) causes severe multisystem disorder,[31] m.10437G>A (*MT-TR*, GCU to ACU for Thr) was observed in a 16-year-old boy with mitochondrial encephalomyopathy,[32] and m.14710G>A (*MT-TE*, CUU to UUU for Phe) was reported in a 41-year-old woman with mitochondrial myopathy and retinopathy.[33] However, the decoding process of tRNA is a critical and complex process, and anticodon nucleotides might not be the only determinant of codon recognition. For example, wobble modification may also affect the codon recognition.[34] In addition, since the discriminator base and the structural identity of the tRNA for the aminoacyl tRNA synthetases remain unaltered,[8,35] the mutant tRNA may still have partial ability to charge the correct amino acid. This may explain why m.15990C>T (*MT-TP*, UGG to UGA) at 85% heteroplasmy only causes myopathy[36] in the affected individual. Interestingly, the pathogenicity of m. 3267A>G (*MT-TL1*, UUA to CUA) and m.12300G>A (*MT-TL2*, CUA to UUA) may be low, because they do not alter the ability of tRNA binding with the codon for leucine.

Heteroplasmy detection

Accurate detection of the degree of heteroplasmy is crucial for the classification of mt-tRNA variants, and is important for establishing a clinical diagnosis and accurate genetic counseling. Previously, mtDNA variant heteroplasmy was typically analyzed by various PCR-based methods, including ARMS qPCR for common point variants[37] and Sanger sequencing for novel variants. However, these PCR-based methods cannot accurately quantify heteroplasmy because of the high frequency of mtDNA SNPs distributed along the entire mitochondrial genome and assay limitations[37] and because of PCR bias from the discriminator nucleotide in qPCR. In 2012, we developed the gold standard long-range PCR/massively parallel sequencing (LR-PCR/MPS) to evaluate every single base of the entire mitochondrial genome quantitatively and qualitatively by deep sequencing the amplified authentic circular mitochondrial genome without interference from nuclear homologs of mtDNA (NUMT).[15,38]

Criteria PS2 and PM2 are issued only for heteroplasmies quantified using this reliable and accurate gold standard NGS method. The 5% heteroplasmy cut-off of PM8, PM9, and PP6 is arbitrary, and is set based on numerous observations. More experience and supporting evidence may permit a more refined cut-off value.

Variant frequency and the use of control populations

The frequency of a variant in a control or general population is important for the assessment of its pathogenicity. We obtained the allele frequencies from public population and private laboratory databases. MITOMAP now displays

GenBank frequency data derived from 48,882 human mitochondrial DNA sequences with size greater than 15.4 kb, among which 47,248 are from neither cancer nor ancient DNA.[5] The mtDB contains mtDNA variants from over 2700 individuals.[25] Our clinical database contains more than 10,000 complete mtDNA sequences.

Disadvantages of public databases include (1) the sequences may not be of equal quality, (2) the quantification of heteroplasmy is not available, and (3) some sequences are derived from pathology samples or diseased patients. Therefore, it may not be accurate to use BS2 and BP9 based upon public databases. The MITOMAP database may be more reliable than mtDB due to the larger sample size. Our own database of >10,000 high-quality mitochondrial genome sequencing results serves as an invaluable resource for variant classification with clear clinical and sequencing information, which we are now sharing with the public. We have submitted all variants to ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), accession numbers SCV000992860 to SCV000993409.

Deletions and insertions

Unlike mRNA, small insertions and deletions (in/dels) in tRNA do not alter a reading frame. We used MitoTIP to predict the pathogenicity of single-nucleotide deletions. However, a prediction algorithm is not available for insertions. The pathogenicity of in/dels is structurally dependent, and is more likely to be pathogenic in the stems than in the loops of tRNA. However, this is not always true. In our database, there are only two pathogenic insertions: one in the anticodon stem (m.5537insT) and another in the variable loop (m.7471dupC). Five insertions are classified as B or LB, four in loops and one in a stem region. Eight deletions are re-evaluated as B or LB; five in loops and three in stem regions. Another example, m.16018ins15,[39] is an insertion of a 15-nucleotide duplication of m.16018_16032 at the end of the acceptor stem. Although previously reported as P, there was no functional or heteroplasmy support, thus, it was downgraded to VUS. We found this variant at near homoplasmy in an adult. Therefore, currently available reports do not provide sufficient evidence for the classification criteria for deletions and insertions.

In conclusion, proper interpretation of mt-tRNA variants is crucial for accurate clinical diagnosis and genetic counseling. We have developed criteria to specifically classify mt-tRNA variants. We used our unique large clinical database to validate the criteria. We found that testing of mtDNA in different tissues of the affected individuals and matrilineal relatives, quantification of heteroplasmy, and its correlation with function are essential variables in determining the clinical significance of mt-tRNA variants.

SUPPLEMENTARY INFORMATION

The online version of this article (https://doi.org/10.1038/s41436-019-0746-0) contains supplementary material, which is available to authorized users.

DISCLOSURE

The authors declare no conflicts of interest.

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