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review

Disease-related versus polymorphic mutations in human mitochondrial tRNAs

Where is the difference?

Catherine Florentz+ & Marie Sissler

UPR 9002 du CNRS, Département Mécanismes et Macromolécules de la Synthèse Protéique et Cristallogenèse, Institut de Biologie Moléculaire et Cellulaire, 15 rue René Descartes, F-67084 Strasbourg Cedex, France

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A number of point mutations in human mitochondrial (mt) tRNA genes are correlated with a variety of neuromuscular and other severe disorders including encephalopathies, myopathies, cardiopathies and diabetes. The complexity of the genotype/phenotype relationships, the diversity of possible molecular impacts of the different mutations at the tRNA structure/function levels, and the exponential discovery of new mutations call for the search for unifying features. Here, the basic features (at the levels of primary and secondary structure) of 68 'pathogenic' mutations are compared with those of 64 'polymorphic' neutral mutations, revealing that these standard parameters for mutant analysis are not sufficient to predict the pathogenicity of mt tRNA mutations. Thus, case by case molecular investigation remains the only means of assessing the growing family of pathogenic mutations in mt tRNAs. New lines of research are suggested.

Introduction

Human mitochondria (mt) have evolved to maintain a very small and compact circular DNA encoding only 13 proteins, two ribosomal RNAs and 22 transfer RNAs (tRNAs) (Anderson *et al.*, 1981). This genome undergoes a far higher mutation rate (5 to 100 times) than does nuclear DNA, due to an oxidative environment (e.g. Allen and Raven, 1996; Pesole *et al.*, 1999). Sequence comparisons within and among large human populations have led to the recognition of population-specific neutral polymorphisms, and have made possible the reconstruction of human prehistory and population movements (e.g. Allen and Raven, 1996; Krings *et al.*, 1997; Wallace *et al.*, 1999; Ingman *et al.*, 2000). However, in addition to the polymorphic mutations, there exist a number of other mutations that have been correlated with a wide range of moderate to very severe human neurological disorders and other pathologies (e.g. Schon *et al.*, 1997; Wallace, 1999). More than 115 different disease-related mutations, referred to as 'pathogenic', have been found throughout the mt genome (Kogelnik *et al.*, 1998 and references therein), with 38% occurring in protein, 4% in ribosomal RNA and 58% in tRNA encoding genes.

The large prevalence of mutations in tRNA genes and their exponential rate of discovery (three mutations known in 1990, aprroximately 70 in 2001), together with the key role of tRNA in mt protein synthesis, call for urgent clarification of the molecular mechanisms for their pathogenicity. However, this task, which has been tackled in a number of cases (e.g. Enriquez *et al.*, 1995; Hao and Moraes 1997; El Meziane *et al.*, 1998; Helm *et al.*, 1999; Chomyn *et al.*, 2000; Kelley *et al.*, 2000; Yasukawa *et al.*, 2000), is rather complex due to the variety of molecular impacts these mutations might potentially have on tRNA biology [at the levels of biosynthesis, maturation, folding, stability, aminoacylation, interaction with translation factors and ribosomal components (Söll and RajBhandary, 1995)]. Moreover, the genotype/phenotype relationship of each mutation is dependent on additional features such as heteroplasmy (presence of both wild-type and mutated mt DNA) and organ specificity. Here, the primary features of pathogenic and polymorphic mutations within human mt tRNA genes are reviewed in an attempt to find straightforward distinctive features. Such an analysis is especially valuable for tRNA genes, since it is possible to estimate directly the theoretical structural impact of mutations at the level of the

+Corresponding author. Tel: +33 3 88 41 70 59; Fax: +33 3 88 60 22 18; E-mail: C.Florentz@ibmc.u-strasbg.fr

Table I. Disease-related ('pathogenic') and polymorphic mutations in human mitochondrial tRNA genes and gene products

Pathogenic mutations in tRNA stems

aa specificity	Gene (a)	Gene product (b)	(c)	(d)	(e)
Phe	G583A	G — C	A•C	acc.	7—66
	A606G	A — U	G•U	ant.	29—41
	T618C	A — U	A•C	ant.	29—41
Val	G1606A	G — C	A•C	acc.	5—68
	G1642A	C — G	C•A	ant.	27—43
Leu(UUR)	C3254G	A • C	A•G	D	12—23
	C3256T	G — C	G•U	D	10—25
	A3260G	A — U	G•U	ant.	29—41
	T3271C	A — U	A•C	ant.	30—40
	delT3272	A — U	delU	ant.	29—41*
	A3302G	U — A	U•G	acc.	2—71
	C3303T	G — C	G•U	acc.	1—72
Ile	A4269G	A — U	G•U	acc.	7—66
	T4274C	U — A	C•A	D	13—22
	T4285C	U — A	C•A	ant.	27—43
	G4298A	C — G	C•A	ant.	30—40
	G4309A	G — C	A•C	T	51—63
	C4320T	A • C	A-U	T	52—62
Gln	A4336G	G • A	G•G	acc.	7—66
Met	G4450A	G — C	A•C	T	53—61
Trp	G5521A	G — C	A•C	D	10—25
	insT5537		insU	ant.	after 27
	G5540A	G — C	A•C	ant.	30—40
	G5549A	C — G	C•A	ant.	31—39
Asn	C5703T	C — G	U•G	ant.	27—43
Cys	A5814G	A — U	G•U	D	13—22*
Ser(UCN)	C7497T	G — C	G•U	D	13—22
	A7511G	A — U	G•U	acc.	4—69
	A7512G	A — U	G•U	acc.	3—70
Asp	A7543G	A — U	G•U	ant.	29—41
Lys	A8296G	A — U	G•U	acc.	2—71
	G8313A	C — G	C•A	D	12—24
	G8328A	C — G	C•A	ant.	31—39
	G8342A	G — C	A•C	T	53—61
	T8356C	A — U	A•C	T	49—65
	G8363A	C — G	C•A	acc.	1—72
Gly	T9997C	U — A	C•A	acc.	7—66
	T10010C	A — U	A•C	D	12—23
Ser(AGY)	C12258A	G — C	G•A	acc.	7—66
Leu(CUN)	G12315A	G — C	A•C	T	52—62
Thr	G15915A	G — C	A•C	ant.	30—40
	G15950A	C — G	C•A	acc.	3—70
Pro	T15965C	G • U	G-C	T	50—64

Polymorphic mutations in tRNA stems

aa specificity	Gene (a)	Gene product (b)	(c)	(d)	(e)
Val	A1603G	A — U	G•U	acc.	2—71
	G1664A	U • G	U-A	acc.	6—67
Leu(UUR)	C3254T	A • C	A-U	D	12—23
Gln	T4388C	U • G	C-G	D	13—22
Ala	A5655G	A — U	G•U	acc.	1—72
Cys	C5821T	C — G	U•G	acc.	5—68
	C5824T	C — G	U•G	acc.	3—70
	A5826G	A — U	G•U	acc.	1—72
Ser(UCN)	G7453A	G • U	A-U	ant.	26—44
	G7476A	U • G	U-A	ant.	28—42
Asp	G7521A	G • U	A-U	acc.	4—69
	T7579C	A — U	A•C	acc.	6—67
	T7581C	G • U	G-C	acc.	4—69
Gly	T10001C	U — A	C•A	D	11—24
	G10014A	G — C	A•C	ant.	27—43
Arg	T10410C	U • U	C•U	acc.	6—67
	G10427A	G • A	A•A	ant.	26—44
	T10457C	A — U	A•C	T	53—61*
	T10463C	U • U	U•C	acc.	6—67
His	T12175C	A — U	A•C	ant.	29—41
Ser(AGY)	A12234G	A • A	A•G	ant.	28—42
	G12236A	C — G	C•A	ant.	26—44
Thr	A15907G	U — A	U•G	D	13—22
	C15913T	C — G	U•G	ant.	28—42
	A15924G	U — A	U•G	ant.	31—39
	G15927A	C — G	C•A	ant.	28—42
	G15928A	C — G	C•A	ant.	27—43
	T15942C	A — U	A•C	T	52—62
	del15944T	A — U	delU	T	50—64
	C15946T	G — C	G•U	acc.	7—66
	A15951G	U — A	U•G	acc.	2—71
Pro	A16017G	A — U	G•U	acc.	7—66

Pathogenic mutations in tRNA loops

aa specificity	Gene (a)	Gene product (b)	(c)	(d)	(e)
Val	G1644T	G	U	var.	45
Leu(UUR)	A3243G	A	G	D	14
	A3243T	A	U	D	14
	T3250C	U	C	D	20
	A3251G	A	G	D	20:01
	A3252G	A	G	D	21
	T3264C	U	C	ant.	33
	C3275A	C	A	var.	44
	A3288G	A	G	T	57
	T3291C	U	C	T	60
Ile	A4317G	A	G	T	59
Asn	A5692G	A	G	ant.	38
Ser(UCN)	insG7472		insG	var.	46
Lys	A8344G	A	G	T	55
Gly	A10006G	A	G	D	18
	A10044G	A	G	T	59
Ser(AGY)	C12246A	C	A	T	55
Leu(CUN)	T12297C	U	C	ant.	33
	G12301A	G	A	ant.	37
	A12320G	A	G	T	57
Glu	A14709G	A	G	ant.	37
Thr	A15923G	A	G	ant.	38
	delT15940	delU		T	60
Pro	G15990A	G	A	ant.	36
Met	T4409C	U	C	con. 1	8

Polymorphic mutations in tRNA loops

aa specificity	Gene (a)	Gene product (b)	(c)	(d)	(e)
Phe	T593C	U	C	D	17
Leu(UUR)	T3290C	U	C	T	59
Ile	C4312T	C	U	T	54
	C4318G	C	G	T	60
Gln	T4343C	U	C	T	59
	A4386G	A	G	D	16
Met	T4454A	U	A	T	58
Trp	C5554T	C	U	var.	44
Ala	G5601A	G	A	T	59
	G5603A	G	A	T	57
Ala	G5633A	G	A	con. 2	26
Asn	G5704A	G	A	con. 2	26
	T5711C	T	C	D	20
Cys	C5780T	C	U	T	54
	T5811C	U	C	D	21
Tyr	T5843C	T	C	T	54
	A5826G	A	G	disc.	73
Asp	T7547C	U	C	ant.	33
	ins7561C		C	var.	48
	A7571G	A	G	T	59
Lys	A8348G	A	G	T	57
Gly	T10034C	U	C	var.	48
	A10042G	A	G	T	56
His	C12153T	C	U	D	16
	A12171G	A	G	ant.	37
Ser(AGY)	C12239T	C	U	var.	48
	A12248G	A	G	T	57
Leu(CUN)	T12285C	U	C	D	21
	T12298C	U	C	ant.	34
Glu	A14674G	A	G	disc.	73
Thr	C15904T	C	U	D	19
	T15932C	U	C	var.	48

Mutations have been retrieved from updated databases (Kogelnik *et al.*, 1998; Ingman *et al.*, 2000) (http://infinity.gen.emory.edu/mitomap.html and http://www.genpat.uu.se/mtDB) and are classified according to their location in the mt genome and in tRNA structural domains (see Figure 1 for nomenclature and numbering). Mutations with ambigous status (described both as pathogenic and polymorphic in the literature) are not listed. They correspond to Leu(CUN)T12311C, Leu(CUN)A12308G, LysA8308G, TyrG5877A, IleA4295G, IleA4300G and ThrA15924G.

(*a*) Numbering according to the standard sequence (Anderson *et al.*, 1981). The letter on the left corresponds to the wild-type sequence and the letter on the right to the mutated sequence. These sequences are those of the coding DNA strand for each tRNA, i.e. the light DNA strand for tRNAs Phe, Val, Leu(UUR), Ile, Met, Trp, Asp, Lys, Gly, Arg, His, Ser(AGY), Leu(CUN), and Thr genes, and the heavy DNA strand for tRNAs Pro, Glu, Ser(UCN), Tyr, Cys, Asn, Ala, Gln. This is contrary to conventional assignments, where the sequence information for all tRNA genes is given with respect to the light DNA strand sequence.

(*b*) Wild-type sequence of the affected position. For mutations in stems, the sequence of both nucleotides forming the base pair is indicated as well as the type of interaction between the two nucleotides. Hyphens correspond to WC interactions and centered dots to mismatches (all non-classical interactions). The degree of conservation of each wild-type nucleotide and of the secondary interaction is according to a sequence compilation of 31 mammalian mitochondrial genomes (Helm *et al.*, 2000) and indicated by the background colour (100% conservation, dark gray background; 90% = conservation <100%, light gray; 50% = conservation <90%, white background). Conservation refers always to the specific tRNA family.

(*c*) Mutated sequence in the affected tRNA. 'del' and 'ins' stand for 'deletion of' and 'insertion of', respectively.

(*d*) tRNA structural domain affected by the mutation. Nomenclature of domains is as in Figure 1 with the following abreviations: acc., acceptor stem; ant., anticodon domain; D, D-domain; T, T-domain; var., variable region; disc., discriminator base; con. 1, connector 1; con. 2, connector 2.

(*e*) Nucleotide positions according to conventional tRNA numbering. Affected positions are in bold characters. *Considered here as base pair at the end of a stem, but could also belong to the neighboring loop (Helm *et al.*, 2000).

gene product, the tRNA. The structural knowledge of classical tRNA (Söll and RajBhandary, 1995) and mammalian mt tRNA (Helm *et al.*, 2000) makes it possible to consider the (i) positions of mutated nucleotides relative to the positions of tRNA structural determinants, (ii) degree of conservation of the affected position, (iii) nature of the primary sequence changes, and (iv) potential secondary structure perturbations they may induce.

Random distribution

As shown in Table I, which lists the 68 pathogenic and 64 polymorphic mutations affecting mt tRNA genes that are currently described in the literature, both types of mutations are found in nearly all tRNA genes. This table confirms that the $tRNA^{Leu(UUR)}$ gene is a hot spot with regard to pathogenic mutations (16 cases)

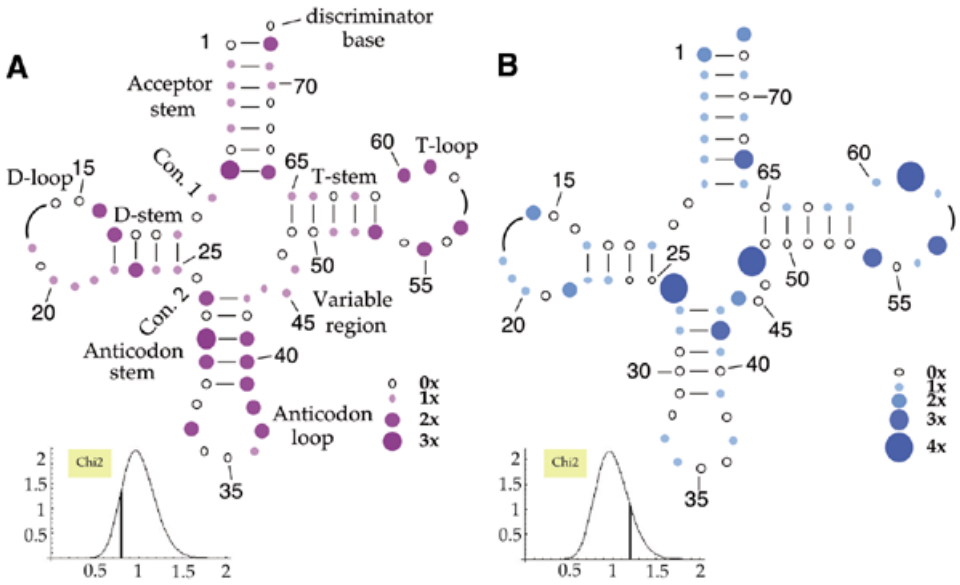

Fig. 1. Distribution of mutations in human mitochondrial tRNA within a common cloverleaf representation. Distribution of (**A**) pathogenic and (**B**) polymorphic mutations. Since the sizes of D- and T-loops of the affected mt tRNAs vary considerably (indicated by curved lines), mutations in these loops have been depicted as being closest to the last bp of the stems. Con. 1 and 2 represent Connector 1 and 2, respectively. Insets: the statistical character of the distribution of each type of mutation has been assessed by comparing the number of mutations for the 57 positions retained in the cloverleaf (all except those in the D- and T-loops, where size variation prohibits a meaningful comparison) with the number that would result from a purely random distribution. A normalized χ^2 value has been calculated for the difference, providing a measure of the 'distance' between the observed and hypothetical values. The numbers obtained were 1.20 and 0.80 for the polymorphic and pathogenic distributions, respectively. Both values (shown as vertical bars in the insets) indicate randomness unambiguously when compared with the theoretical χ^2 distribution obtained assuming 57 degrees of freedom (bell-shaped curves in the insets).

(Schon *et al.*, 1992), and highlights a similar status for the $tRNA^{Thr}$ gene with regard to polymorphic mutations (seven cases). Statistical analysis of the distribution of pathogenic and polymorphic mutations over the different structural domains of a typical mt tRNA reveals a random distribution (Figure 1), suggesting that all of the domains are equally susceptible to both types of mutation. However, within an individual tRNA, pathogenic and polymorphic mutations generally do not affect the same position (there is a single known exception). The random distribution of mutations throughout the cloverleaf is striking, considering that in the case of classical tRNAs (i.e. non-mt tRNAs), specific domains, and even individual nucleotides, have been recognized as being crucial for particular structural and functional properties (Giegé *et al.*, 1998). For example, the anticodon loop and the end of the acceptor-stem are important specifically for aminoacylation identity. It might have been anticipated that such rules hold true for mt tRNA, with some domains prone to mutations that have no, or only little impact on structure and function, and conversely, other domains being structurally and/or functionally sensitive to changes.

A more detailed analysis that determines the evolutionary conservation of each affected position was undertaken. The degree of conservation of each nucleotide within each of the 22 specific tRNA families in mammalian mt has been established previously by comparing tRNA gene sequences of 31 mammalian genomes (Helm *et al.*, 2000). Most pathogenic mutations described in the literature have been shown previously to hit conserved elements most often (Schon *et al.*, 1997). This is summarized here, with 47/68 (70%) mutations affecting highly conserved nucleotides and 38/43 (90%) affecting conserved base-pairing in stems (Figure 2). Reciprocally, 53/64 (83%) polymorphic mutations occur at poorly conserved nucleotides. These mutations, however, affect conserved and poorly conserved secondary structure features equally (16/32)

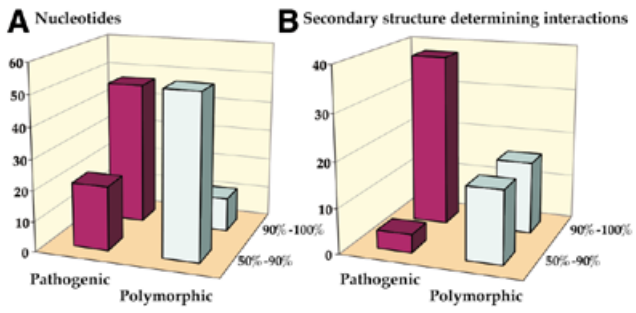

Fig. 2. Analysis of the degree of conservation of the positions affected by pathogenic or polymorphic mutations. (**A**) Degree of conservation of the affected individual nucleotides. (**B**) Degree of conservation of the secondary structure-determining interactions affected by the mutation (e.g. conserved WC bp, conserved mismatch, …). In both parts of the figure, the percentages of conservation refer to the situation in specific mammalian mt tRNA as calculated in Helm *et al.* (2000). For simplicity, only two categories are considered here, namely conservation within 50–90% of sequences and conservation within 90–100% of sequences. No nucleotide or base pair is conserved to <50%. The vertical axes of the diagrams indicate the number of mutations in each category.

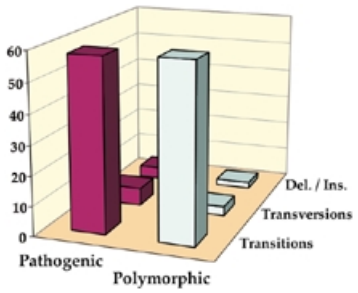

Fig. 3. Distribution of transitions, transversions and deletion/insertion in pathogenic and polymorphic mutations. The vertical axis of the diagram indicates the number of mutations in each category.

(Figure 2). Although this analysis reveals a dominant distinction between pathogenic and polymorphic mutations, a number of significant exceptions remain in both families (i.e. pathogenic mutations located at non-conserved nucleotides, and polymorphic mutations located at conserved nucleotides; Table I), so that the mere presence of a mutation at a conserved or non-conserved position is not sufficient to predict its pathogenicity. However, even this distinction needs to be made cautiously since base changes have sometimes been defined as being pathogenic based on the fact that they affect conserved positions.

Chemically 'mild' mutations predominate

At the level of primary sequence, the changes introduced by pathogenic mutations are known to be mainly transitions (replacement of a purine by a purine or of a pyrimidine by a pyrimidine) (DiMauro and Moraes, 1993; Schon *et al.*, 1997; Wallace *et al.*, 1999). Figure 3 depicts a distribution of 85% transitions, 9% transversions and 6% deletions/insertions. Unexpectedly, a similar trend is seen in the case of polymorphic mutations, with 92% transitions, 5% transversions and 2% deletions/insertions. Thus, both categories of mutation can be considered 'mild' in the sense that they conserve the puric or the pyrimidic architecture of the nucleotide. Notably, however, the more drastic changes (transversion, deletion/insertion) are found at a higher frequency in the pathogenic series of mutations (15%) than in the polymorphic series (7%) (Figure 3).

Consideration of the possible physical effects of mutations in double-stranded domains of the tRNA secondary structure provides a further comparison. The most frequently encountered effect for both types of mutations is the replacement of Watson–Crick base pairs (WC bp) by mismatches (Figure 4A). However, other changes (for example replacement of a mismatch by a WC, or of a mismatch by a mismatch) are more prevalent in polymorphic (37.5%) than in pathogenic mutations (14%) (Figure 4A), suggesting that polymorphism tolerates more variability.

Among the 12 theoretically possible conversions of the four types of WC bp to mismatches, only four dominant mismatches are found, namely: A·C, C·A, G·U and U·G (Figure 4B). This holds true for both pathogenic and polymorphic mutations, and reflects the restricted possibilities that result from a transition at a single nucleotide of a WC bp. These four mismatches are known to be the least disturbing changes that can be introduced naturally within RNA helices (Leontis and Westhof, 1998; Masquida and Westhof, 2000). A G·U interaction deviates the least from classical WC bp stereochemically; an A·C interaction is similar, due to isostericity. Thus, these four mismatches are able to substitute for each other while preserving the three-dimensional structure of the motif.

'Mild' structural mutations, 'strong' molecular effects

Given the analysis above, the presence of a restricted set of structurally mild mutations (transitions, weak mismatches) must result in molecular effects that can account for the differences between the neutral, polymorphic mutations and the deleterious pathogenic mutations. In fact, despite their apparent chemical and structural mildness, transitions as well as G·U and C·A mismatches can have strong negative effects on classical tRNA functions, especially on their aminoacylation properties (reviewed in Giegé *et al.*, 1998; Varani and McClain, 2000). Depending on their precise position within a given domain, or their impact on a particular nucleotide or base pair, these mutations may result in structural effects that range from very mild to dramatic. It is well established, for example, that G·U pairs introduce kinks into regular RNA helices, as well as bringing specific chemical groups into the minor groove of the helix and thereby (potentially) promoting either abnormal interactions with, or repulsion from, particular macromolecules (Varani and McClain, 2000). Severe molecular effects of chemically 'mild' pathogenic G·U or C·A mismatches on human mt tRNA have already been demonstrated experimentally. Examples include the $tRNA^{Asn}$ G5703A mutation, which replaces a C–G pair in the anticodon stem with a U·G mismatch (Hao and Moraes, 1997), and the $tRNA^{Ile}$ mutation A4269G, which leads to the replacement of a WC A–U pair in the acceptor stem with a G·U mismatch (Yasukawa *et al.*, 2000). Both mutations cause marked decreases in tRNA stability, and thus the reduction of their steady-state levels. Analysis of aminoacylation properties of wild-type $tRNA^{Ile}$, as well as of variants into which C·A pairs were introduced (T4274C, T4285C, G4298A), revealed large negative

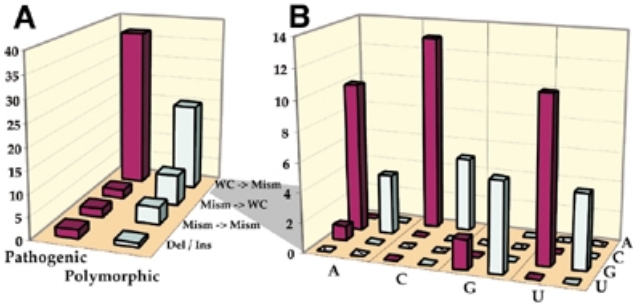

Fig. 4. Comparison of pathogenic and polymorphic sequence changes within double-stranded tRNA regions. (**A**) Distribution of the different types of secondary interaction changes observed. WC, Watson–Crick; mism, mismatch; del/ins, deletions/insertions. (**B**) Detailed view of sequence changes in WC→mismatch mutations. In both diagrams, the vertical axis indicates the number of mutations in each category.

effects on aminoacylation of the mutated tRNA, with up to a 1300-fold decrease in its rate (Kelley *et al.*, 2000).

Why are 'strong' mutations missing?

The 'mildness' common to most mutations analyzed here is rather striking. Whereas this is conceptually easy to explain in the case of polymorphic mutations with neutral phenotypic effects, stronger basic features might have been expected for pathogenic mutations, especially those that have particularly severe phenotypic consequences. The mutation of mt DNA is likely to be random, so that any nucleotide change should be observed within tRNA sequences, and any type of mismatch should be found within tRNA helical domains. Such mutations may well occur, but not become fixed due to drastic mt dysfunction that would prevent the survival of the cell, or even the organism (DiMauro and Moraes, 1993; Wallace *et al.*, 1999). Interestingly, evolutionary processes involving the replacement of one WC bp by another have been demonstrated to occur through G·U and C·A mismatches as transient intermediates (Rousset *et al.*, 1991). The restricted set of tolerated mutations in mt tRNA may reflect similar evolutionary rules.

The general absence of severe molecular perturbations (transversion, severe mismatches) within the category of pathogenic mutations remains an intriguing problem. Indeed, the heteroplasmic status of most pathogenic mutations is thought to allow for compensatory effects: threshold levels of the fully functional wild-type tRNA are expected to overcome the severe dysfunction of the mutated tRNA. Interestingly, it has recently been demonstrated that pathogenic versions of $tRNA^{Ile}$ not only have poor aminoacylation capacities, but also strongly inhibit aminoacylation of wild-type $tRNA^{Ile}$ (Kelley *et al.*, 2000). This is the first strong evidence in favour of cumulative negative effects controlling aminoacylation in a heteroplasmic environment. Thus, even under heteroplasmic conditions, strong mutations may not be tolerated.

Outlook

A correlation between genotype and phenotype in the case of mt tRNA mutations is a long-standing question that is difficult to handle. This review shows that, despite the accumulation of information about the positions of a large number of mutations within mt tRNAs, it is not possible to identify simple basic features that would make possible the prediction of pathogenicity of new mutations. Thus, systematic molecular investigations of pathogenic mutants need to be extended to a large number of cases in an attempt to find some unifying features at another level. As suggested by the work on $tRNA^{Ile}$, these investigations should examine not only the affected tRNA, but also its interactions, or relationships, with other mt components that may or may not be involved in protein synthesis. Such studies would make it possible to tackle cooperative features that might contribute, compensate or magnify the primary effects of a point mutation and thus lead to pathology.

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