Pseudomitochondrial genome haunts disease studies

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ABSTRACT

The accidental amplification of nuclear mitochondrial pseudogenes (NUMTs) can pose a serious problem for mitochondrial disease studies. This report shows that the mutation spectrum left by spurious amplification of a NUMT can be detected because it usually differs considerably from the authentic natural spectrum. This study examined the problem introduced by an *ND5* gene NUMT that was recorded in a proband with hearing loss and reviews other disease studies erroneously reporting NUMT variation as genuine mutations in their patients. NUMTs can emerge in population genetic studies, as exemplified here by cases in this study and from published sources. Appropriate database searches and a phylogenetic approach can prevent hasty claims for novelty of mitochondrial DNA (mtDNA) variants inadvertently derived from NUMTs and help to direct investigators to the real source.

Mitochondrial (mt)DNA sequences in the nuclear genome (nuclear mitochondrial pseudogenes; NUMTs) have been found in virtually all species and to date, a large number of NUMTs has been recorded in humans.[1–4] The existence of NUMTs that are sufficiently long to cover a targeted fragment of human mtDNA has long posed a problem in studies of mitochondrial diseases.[5–11] Although this should be widely known in principle, a combination of unfortunate laboratory conditions and false expectations has led to a low but steady flow of publications in which NUMTs were mistaken as authentic mtDNA. A classic case constitutes the 5842 bp NUMT on chromosome 1, which was clarified by Herrnstadt *et al*.[12] The same NUMT was later misinterpreted as sperm mtDNA by Thangaraj *et al*[13] (see Bravi *et al*[14] for a clarification). Conversely, when targeting nuclear "mtDNA", it may easily happen that a fragment of mtDNA is mistaken for an authentic part of some NUMT. For instance, Biswas *et al*[15] discovered that the HapMap data for the region covering this 5842 bp NUMT in chromosome 1 did not report the nuclear DNA variation but rather the corresponding paralogous mtDNA variation. The diploid genome sequence of J Craig Venter (Levy *et al*[16] contained some mtDNA fragments that were introduced by the whole-genome shotgun sequencing technique used (authors' unpublished data).

Recently, Goios *et al*[17 18] conducted some experiments in which NUMTs would co-amplify and found that the probability of mtDNA co-amplifying when NUMT DNA is the target is higher than the probability of the reverse contamination occurring. There is no risk of contamination with NUMT DNA with routine techniques for mtDNA amplification.[17 18] However, it is not clear whether in the field of medical genetics routine techniques are always executed correctly,[19 20] and moreover, whether some routinely used primers may fail to amplify the targeted mtDNA sequence in some specific sample because of some accidental mutations in the recognition region of a primer or some other reason. Thus, the warning of Parr *et al*[11] that apparently somatic mutations could in fact appear as co-amplified NUMTs under some circumstances cannot be set at zero.

In this paper, we revisit some bizarre mtDNA sequence variations identified in published sources and our unpublished data, which were caused by accidental amplification of NUMTs. We used internet-based search engines in order to achieve a nearly perfect coverage of the published mtDNA variation and referred to published views and snapshots of the worldwide mtDNA phylogeny. Our results show that researchers may still ignore the possibility of a NUMT contribution when a seemingly novel "mtDNA" sequence is encountered, even though this is a well-documented problem that has haunted the investigation of pathogenic mtDNA mutations for several years.

NOVEL CASE, OLD-ESTABLISHED NUCLEAR MITOCHONDRIAL PSEUDOGENE

In a recent study, the clinical and molecular characterisation of an Argentinean family with aminoglycoside-induced impairment was reported.[21] The authors determined the entire mtDNA sequence variation in one proband and claimed that the variant A827G located in the 12S rRNA gene was pathogenic, based on two lines of evidence: (1) the evolutionary conservation of position 827 in several vertebrate species and (2) the already reported putatively pathogenic role of A827G in families with hearing impairment.[22–24] The conclusion of Chaig *et al*[21] was apparently misled by these previous studies claiming a pathogenic role of A827G in aminoglycoside induced and non-syndromic hearing loss, notwithstanding the caveat that the variant A827G is a common polymorphism in East Asian which (together with C15535T) defines haplogroup B4b'd as a whole and therefore is unlikely to contribute (as a high-penetrance risk factor) in rare disorders such as hearing loss.[25]

An audit of the complete mtDNA sequence variation in the proband as reported in table 1 by Chaig *et al*[21] revealed more serious problems. First, the presence of several indicators for Native American haplogroup B2 status (G499A, A827G, A3547G, G4820A, T4977C, C6473T, 8281–8289del (commonly known as 9bp del), T9950C and C15535T) and the absence of a handful of expected variants (C11177T (specific for haplogroup B2), G13590A (specific for the larger haplogroup B4b in which B2 is nested), A16183C, T16189C and

T16217C (specific for B4)) is puzzling.[26] The recorded mtDNA control region mutation pair (C16142T, T16356C) in this patient occurs simultaneously in samples with haplogroup status H1b, seen in samples from Latvia,[27] although at a very low frequency. Moreover, an inappropriate consensus sequence instead of the revised Cambridge reference sequence (rCRS)[28] might have been used for comparison, as all variants (12 polymorphisms in total) between the rCRS and the root of the super-haplogroup R were absent.

Second and most strikingly, among the 43 variants reported in the proband, as many as 31 were atypically located in a single gene, *ND5*. A translation of *ND5* would stop prematurely at amino acid position 120. If the proband really did have this truncated *ND5* gene, she would probably not have had hearing loss only. This indicates that searches should be made for a homologous sequence elsewhere (eg, in the nucleus). Indeed, a BLAST search in GenBank (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) using the reported suspicious *ND5* sequence showed a reasonable match to a contig located at chromosome 5, along with matches to mtDNA genome and contigs on several chromosomes, including 2, 4, 7 and 10. Finally, 7 of the 12 mutations (C12792T, T12892C, T13174C, C13272T, G13466A, C16142T and T16356C) claimed as novel in the table in the study of Chaig *et al*1[21] could have been found as authentic mtDNA mutations at the end of 2007 through an internet search (using the search engines Google and Yahoo!).

It turns out that all variant nucleotides between positions 11590 and 13488 in the *ND4* and *ND5* genes in the proband reported by Chaig *et al*[21] match the variants in the NUMT (chromosome 5, 134289898 to 134295116), except for C12696G instead of the probably intended T12696C. Conversely, most NUMT mutations were recorded in the proband between positions 12696 to 13488, leaving out only 11 from a total of 42 mutations relative to the rCRS (see www.ianlogan.co.uk/numts/numt_chr_5.htm for the list of the mutations (relative to the rCRS) from the NUMT on chromosome 5). It thus seems that the sole mutation A11590G in *ND4* might have arisen in parallel as an authentic mtDNA mutation in the proband's matriline. This variant is one of the characteristic mutations for the African haplogroup L3h1 but also occurs independently within haplogroups H, U6a andZ1, although it has not been found in the reported B2 mtDNAs.[29]

In order to explain the predominant amplification of the NUMT, we took a closer look at the primer pairs used by Chaig *et al*,[21] which covered the fragment with numerous mutations as reported in their table 1. One primer pair comprised the 24 bp forward primer and 17 bp reverse primer with recognition sequences 5′-ACT CAA ACT ACG AAC GAA CGC ACT-3′ (L strand 11751–11774) and 5′-CTC AGC CGA TGA ACA GT-3′ (H strand 12769–12753), respectively. The forward primer, however, seems to have a superfluous quartet repeat, AACG, which would have led to three mismatches with the rCRS, including one mismatch at site 11774, which was located at the 3′-end of the L-strand primer. More importantly, this 4 bp mismatch could have affected the correct amplification of the authentic sequence and thus favoured the accidental amplification of the NUMT. In addition, the reported A→G change at site 12753 would introduce a mismatch at the 3′ end of the H-strand primer. Therefore, this primer pair will have problems in amplifying an authentic sequence from mtDNA, but not in the NUMT, where it would perfectly match.

UNPUBLISHED CASE, BIZARRE NUCLEAR MITOCHONDRIAL PSEUDOGENES

In our previous analyses of complete mtDNA genomes,[26 30 31] we amplified the entire mtDNA sequence in 15 overlapping fragments. The whole sets of primers performed well in our studies, with one exception, in sample YN170 (first studied for partial mtDNA variation in Yao *et al*[32]), where we encountered NUMT amplification on chromosome 5. Most fragments in this sample gave unsuspicious variation (table 1; sequences were deposited in GenBank under accession numbers EU822919 to EU822920), confirming the expected haplogroup R9 status and indicating a new sister branch to haplogroup F within R9 (based on variant 249delA).[26 30] Using the forward and reverse primers L394/H1782, binding at 375–394 and 1801–1782, respectively, we obtained a fragment of about the expected length. However, direct sequencing of this fragment using these two primers and additional inner sequencing primers yielded numerous nucleotide variants relative to the rCRS in region 402–1756 (table 1). Repeated amplification and sequencing gave identical results for this fragment.

A BLAST search showed that the bizarre variation in the fragment 402–1756 completely matches (except for the heteroplasmic position 1389) an NUMT on chromosome 5 (chromosome 5, 79982694–79983880). Internet searches (on Google) with "mtDNA" plus one of the above mutations, such as

Table 1 Partial mtDNA sequencing results for sample YN170 with intrusive NUMT

Region	Nucleotide variants relative to rCRS
1–407	A73G, T236C, 249delA*, A263G, 315+C
404–1756	C420T, C437T, T450C, T453C, T454C, 459delC, C462A, 463delC, C468A, A472T, T480C, T482C, A490G, 494+T, C498T, T504C, C506T, C510T, G513A, C516T, C518T, 523–524delAC, A533G, C572A, C573T, A576G, C592T, A621C, G625A, C632T, T634C, C661G, 666–669delCTTT, C708T, T710C, T711C, A714G, 721delT, C756T, T773C, A813G, T825A, C870T, G877C, T883G, G930A, C956A, C957T, C958T, 961delT, C964T, C979T, C1009T, G1018A, A1039G, T1040C, C1106T, C1120T, T1284C, A1292G, C1322T, G1348C, C1376T, C1377T, G1389R, G1393A, C1405T, T1451G, A1536G, C1556T, C1619T, T1654C, G1664A, C1693T, G1709A, G1719A, C1733T
1473–8538	A2706G, C3970T*, A4769G, A6437G, G6446A, C7028T, T7861C, A8440G, A8489T
8539–8596	ND
8597–9410	T8610C, A8860G
9411–9812	ND
9813–14564	A11002G, C11308T, G11719A, T13215C, G13928C*, C14227T
14565–14598	ND
14599–16569	C14766T, A15326G, T15479C, G15734A, T16157C, C16256T, T16304C*, A16335G

ND, not determined.
*Variants specific for haplogroup R9 or F.
Insertions (+) and deletions (del) are scored at the last possible site.
R denotes heteroplasmy for both A and G.
The mtDNA sequence and the NUMT sequence are deposited in GenBank under accession numbers EU822919–EU822920.

Box 1 How to avoid preferential a priori amplification of nuclear mitochondrial pseudogenes

- Use BLAST to check the mtDNA primer sequence in GenBank to test whether it has sequence identity to some nuclear genomic sequence.
- Ensure the template DNA was extracted using the standard DNA extraction technique.
- If possible, use DNA extract from ρ0 (rho zero) cells lacking mtDNA as a positive control during the PCR amplification.
- Amplify the mtDNA sequence in a large fragment—that is, size >2.0 kb.
- Check using an appropriate allelic ladder and e.g. electrophoresis, whether the size of the amplicon corresponds with the expected size. If available, a positive control can be used for reference.

"C572A", immediately direct one to the previously mentioned website (www.ianlogan.co.uk/numts/numt_chr_5.htm) with reference to an NUMT on chromosome 5 (79981597–79983766) or chromosome 11 (104886010–10488459, with fewer total matches). However, the same search performed a couple of weeks later (26 May 2008) returned only the latter reference to the chromosome 11 NUMT. This demonstrates that the success of searches using certain search engines (in this case, Google) is time-dependent (notably, entering the same query "mtDNA C572A" into the internet engine of Yahoo! (US version) still retrieved both references).

Enigmatically, in the sequences covering the recognition sites of primers L394/H1782, which were amplified by primer pairs L1466 (located at 1445–1466)/H3054 (located at 3074–3054) and L15996 (located at 15975–15996)/H408 (located at 429–408), respectively, we did not find any variant in authentic sequences. Moreover, in the fragment that was amplified by using primers L1466/H3054, we did not find the last nine NUMT mutations in the overlapping segment (region 1473–1756) either (table 1). The exact reason why primers L394/H1782 failed to amplify the authentic sequence multiple times remains unknown. For a possible explanation, one could speculate that the specific NUMT fragment in that person accidentally showed an extremely high copy number variation,[11 33] so that the nuclear fragment could win over the paralogous mitochondrial fragment in the PCR.

DIFFERENT CASES, SAME NUCLEAR MITOCHONDRIAL PSEUDOGENES

Although NUMTs are abundant in the human genome,[1–4] it may not necessarily mean that different laboratories could easily retrieve the same NUMT in different samples, especially with different primer pairs and conditions for amplification. Under some favourable conditions, the same NUMT could accidentally be obtained in different samples. As mentioned above, Thangaraj *et al*[13] sampled the 5842 bp NUMT on chromosome 1 that was described previously by Herrnstadt *et al*.[12] Parts of this long NUMT have also invaded some other studies.

In a study to compare the entire mtDNA genome of 45 pairs of mother and affected child, Kwon *et al*[34] identified seven nucleotide changes. Among them, five (T5580C, G5821A, C5840T, A8326G and G15995A) were identified in a woman with cystic fibrosis and suspected mitochondrial cytopathy compared with her mother.[35] All of these five mutations were heteroplasmic in the patient, based on their figure 1 and table 1.[34] An internet search (using Yahoo or Google) with "T5580C G5821A" immediately led to sources where these two mutations were already mentioned. Of these sources, two were particularly intriguing in that they refer to the Logan website for the NUMT on chromosome 1 (http://www.ianlogan.co.uk/numts/numt_chr_1.htm) that was reported by Herrnstadt *et al*[12] and to a sequence (GenBank accession number DQ112878) reported by Kivisild *et al*,[36] respectively. Note, however, that internet searches on Google that were performed at other times did not necessarily yield these two references. Based on the primer information (mtF5460/mtR6016) provided in Wong *et al*,[37] the three heteroplasmic mutations T5580C, G5821A and C5840T observed by Kwon *et al*[34] were apparently caused by amplifying both the authentic mtDNA (557 bp) and the NUMT in the patient. The two variants A8326G and G15995A (which were located in different PCR fragments) observed in this mother–child pair might be real, although the 5842 bp NUMT on chromosome 1[12] covered site 8326. Sequence DQ112878 reported by Kivisild *et al*[36] belonged to haplogroup N9a2c (following the most updated version of East Asian mtDNA phylogeny[26]) and contained a chunk of five private variants (G5471A, A5474G, A5498G, T5580C and G5821A) that were located in a 468 bp fragment amplified by primer pair L5419 (located at 5396–5419)/H5841 (located at 5863–5841). These five variants can actually be found in the NUMT on chromosome 1.

This NUMT on chromosome 1 might have invaded other cases also. For instance, the four NUMT mutations A5351G, C5387T, A5474G and C8203T occur in a haplogroup U5b1 sequence (GenBank accession number DQ156214) and the two NUMT mutations A5474G and A5498G in a haplogroup U5a sequence (GenBank accession number DQ156212), both reported in semen DNA by Montiel-Sosa *et al*.[38] All of these mutations (excluding A5351G, which is one of the characteristic mutations of the East Asian haplogroup M7b) constitute extremely rare events in authentic mtDNA.

Box 2 Quick guideline for a posteriori identification of nuclear mitochondrial pseudogenes

- Identify the fragment(s) showing a relatively high number of nucleotide changes or an unusual transversion:transition ratio or insertions/deletions.
- If the suspicious fragment is located in a protein-coding mtDNA gene, test whether it can be fully translated into an amino acid sequence.
- Perform a BLAST search using the suspicious fragment to identify sequence matches or near-matches and scrutinise the sources of matches or near-matches with high scores.
- Use mtDB (http://www.genpat.uu.se/mtDB/) and other mtDNA database resources to check which of the variant nucleotides in the suspicious fragment occur among the registered mtDNA sequences and in which combinations (haplotypes).
- Use internet search engines to seek further published sources for the variants in the suspicious fragment.
- In case a particular sequence pattern is suspicious, corroborate the same findings using alternative sets of primers.

CONCLUSION

The invasion of human NUMTs in mtDNA sequencing has been recognised as a potential source of errors in identifying pathogenic mutations more than ten years ago.[5–11] The recent cases of Chaig *et al*[21] and Kwon *et al*[34] document the omnipresent risk of false positive claims of association in mtDNA disease studies. Most of these erroneous claims arise from (1) deficient database searches and (2) "false" confirmation of a preconception that leads the researchers to take many apparent mutations at face value. Straightforward internet searches can prevent false claims about the novelty of mtDNA variants[39–41] and can even direct investigators to the real source (NUMT) of the presumed mtDNA variation. It is thus desirable that authors, reviewers and editors (especially of those journals or volumes that do not exercise a careful external reviewing process) would routinely perform pertinent database (eg, BLAST) or internet searches to test the novelty of mutations for major claims in a new manuscript.

Can we identify and eliminate all the NUMTs accidentally encountered in the studies? The novel case in our own laboratory and that of Kivisild *et al*[36] show that this may not be easy. Even if one is aware of potential artefacts, some may only show up later in retrospect with an improved resolution of the mtDNA phylogeny. However, the risk of NUMT invasion can be reduced if investigators follow some a priori and a posteriori guidelines to avoid and to identify NUMTs (see boxes).

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