

Biparental inheritance of mitochondrial DNA revisited

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Standfirst

Evidence for a biparental mode of mitochondrial DNA (mtDNA) inheritance has been sparse and remains controversial. Recent studies using a range of complementary techniques do not support paternal transmission of mtDNA, and highlight the co-amplification of rare, concatenated nuclear mtDNA segments as a technical artefact that may explain previous observations.

First demonstrated in humans in 1980¹, strict maternal inheritance of mitochondrial DNA (mtDNA) has stood the test of time, with the only counterexample², a single case report suggesting paternal mtDNA inheritance, never having been convincingly replicated. Uniparental inheritance enables rapid cytoplasmic genome evolution, contributing to extensive polymorphism in the extant population. This has been exploited by population geneticists to map human migration throughout history, and to match biological samples in forensic science. Most human cells contain >100 copies of mtDNA, and a mixed mtDNA population in the same individual is termed ‘heteroplasmy’. Precise heteroplasmy levels change rapidly upon transmission to offspring owing to a genetic bottleneck during oogenesis. Thus, novel variants are either lost or progress to fixation within a few generations, explaining

why high heteroplasmy levels are uncommon in the population. In 2018, a controversial study describing high levels of heteroplasmy in three multigenerational families proposed a previously unrecognized biparental form of mtDNA inheritance³. However, newly published data obtained using a range of complementary techniques do not support paternal transmission and suggest a more parsimonious explanation (Supplementary Table 1).

Challenging the maternal paradigm

Using a combination of long-range PCR amplification and next generation sequencing, Luo et al. identified three large families where multiple individuals apparently harboured an unusually high number of heteroplasmic mtDNA variants³ (referred to by others as ‘multiHets’). MtDNA haplotype segregation studies persuaded the authors that biparental inheritance of mtDNA had occurred in these families. Unlike most previously published pedigrees, remarkably consistent levels of heteroplasmy were seen across multiple generations, and the same ‘heteroplasmic haplotype’ was consistently transmitted at high levels (24–76% haplotype frequency) within each ostensibly unrelated family. These findings were widely discussed in social and mainstream media, prompting commentaries either extolling the new discovery⁴ or expressing sceptical viewpoints, pointing out the need for orthogonal proof before rejecting an established dogma⁵.

Several technical explanations were proposed to explain the apparent ‘biparental transmission’, including that the study had detected segments of mtDNA embedded within the nuclear genome, so-called Nuclear-Mitochondrial DNA segments (NUMTs). Luo et al. subsequently counter-argued that the linearized nature of these sequences would have rendered them undetectable to long-range PCR using outward-facing primers. For single-copy NUMTs, the contaminating haplotype would also be diluted by genuine mtDNA to extremely low levels, well below reported values. However, if the sequence integrated into a nuclear chromosome involved a number of concatenated mtDNA repeats (termed a “Mega-NUMT”⁶), this could both allow PCR-amplification and also lead to high levels of a mixed haplotype.

Prevalence of multiHet individuals

An initial attempt to replicate Luo et al.’s findings using whole-genome sequencing (WGS) did not detect any signatures of biparental mtDNA inheritance in 41 families⁷. A larger WGS study in 11,035 parent–child trios excluded sample contamination and validated familial

relationships using autosomal SNPs⁸. A signature resembling biparental mtDNA inheritance was seen in 7 families, with the paternal haplotype at allelic fractions of 5–25%. However, the paternally inherited haplotypes were transmitted only to half of the offspring, and an equal number were maternally transmitted, suggesting nuclear DNA transmission. Wei et al. used split-read analyses to identify rare or unique NUMTs transmitted from the father in all 7 families with the biparental inheritance signature⁸. Five different integration sites were detected, all at non-coding loci. Split-reads mapping to different segments of mtDNA were consistent with Mega-NUMTs, and two integration sites shared between unrelated families were thought to represent ancestral Mega-NUMTs.

Subsequently, Lutz-Bonengel et al. reported similar, albeit coincidental, findings based on a single individual who was a forensics laboratory trainee⁹, mapping a Mega-NUMT integration site to 14q31 using fluorescence *in situ* hybridization. DNA analysis of 19 other family members using a variety of tissues (bone DNA extraction using grave site samples were even obtained for deceased grandparents) showed mixed haplotypes in several individuals, again consistent with nuclear DNA transmission.

Finally, Bai et al. searched for multiHet individuals with ≥ 5 heteroplasmic mtDNA variants that appeared to have heteroplasmy levels of 10–90%; this study investigated only individuals with no previous blood transfusions or organ transplants, and validated their findings in an independent blood sample¹⁰. Exome and PCR-amplified mtDNA sequencing identified the multiHet phenomenon in 104 out of 27,388 individuals. The difference in incidence likely reflects differences in ascertainment, sequencing techniques and bioinformatic pipelines. Their findings confirmed that the phenomenon is rare – and likely discounted in many laboratories as suspected sample contamination.

Inverse correlation with mtDNA content

In tissues with high energy demands, such as muscle, cells generally have a greater ratio of mtDNA to nuclear DNA. If the heteroplasmy signature were due to Mega-NUMTs rather than bona fide biparental inheritance, an inverse correlation between the Mega-NUMT haplotype fraction and mtDNA content would be expected. Comparing DNA from buccal cells and muscle, the anticipated dilution effect due to higher mtDNA content in muscle was clearly observed^{9,10}. A similar signature was also seen in blood samples, where mtDNA levels reflect the composition of different cell types in whole blood⁸. Hair shaft analysis is often used in

forensic sciences; DNA from this source contains only minute amounts of highly fragmented nuclear DNA. Similarly, thrombocytes are a type of blood cell known to lack a nucleus. Lutz-Bonengel et al. demonstrated loss of the Mega-NUMT heteroplasmy signature in both of these sources of DNA⁹. Conversely, progressively depleting mtDNA from cells led to a predominance of the Mega-NUMT associated haplotype⁹.

Clinical relevance and future perspectives

Rare variants that arise in a Mega-NUMT will be at very low allelic fractions unless propagated into multiple copies of the 16.6 kb repeat. However, deletions and inversions may be more problematic: tandem repeats are prone to rearrangement and such events were noted in two of the studies^{8,10}. In clinical genetics and other disciplines where it is important to clarify whether a multiHet pattern is due to rare Mega-NUMT or genuine mtDNA variants, muscle DNA should be used if possible. This procedure is relatively routine in adult mitochondrial disease clinics where biopsies are frequently performed for respiratory chain enzyme assays. In other situations, a cost-effective way to resolve unexpectedly high levels of heteroplasmy is to analyse DNA from hair shafts. Establishing a Mega-NUMT database that contains genomic positions and the mtDNA haplotype involved will also be of great use to disentangle future cases of multiHet individuals. Additional information should also include the range of repeat numbers seen, secondary rearrangements associated with the Mega-NUMT and validated PCR primers that can be used to test for such integrations.

Further studies on Mega-NUMTs will determine how often such rearrangements occur *de novo* and whether they can lead to disease, most likely via disruption of a constrained nuclear gene. The precise structure of Mega-NUMTs can be determined using a combination of Southern blotting and ultra-long-read sequencing methodologies. Published data regarding the number of repeat units, ranging from 2–56^{8,9}, are indirect estimates rather than direct measurements. More precise comparisons of Mega-NUMTs with identical insertion sites will shed light on the evolution and approximate ages of these complex structural rearrangements. Other unanswered questions include how Mega-NUMTs occur and whether there is an RNA intermediate. Is there a two-stage process involving integration of a single-copy NUMT and subsequent amplification, or a single insertion event? The circular nature of mtDNA makes the latter option combined with a rolling-circle mechanism seem attractive. Further work will help improve the understanding of replicative and transcriptional dynamics

within mitochondria, and whether there are any common sequence motifs found at insertions sites akin to L1-mediated insertions, which are enriched for AATTTT.

The provocative study by Luo et al. has prompted detailed follow-up studies from scientists working in the fields of genomics, forensics and commercial clinical genetics testing. This has led to a diverse set of experimental data supporting the existence of mega-NUMTs more quickly than might have occurred otherwise, providing an alternative explanation that does not challenge established dogma, and advances our understanding of the evolving human genome.

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Competing interests

The authors declare no competing interests.

Supplementary information

Supplementary information is available for this paper at <https://doi.org/10.1038/s415XX-XXX-XXXX-X>

Biparental inheritance of mitochondrial DNA revisited

Supplemental Table 1: Notes comparing experimental data from studies addressing the phenomenon of “apparent” paternal/biparental mtDNA inheritance in humans.

N/A, not applicable. AF, allelic fraction.

| Reference | Schwartz and Vissing 2002 ¹ | Luo <i>et al</i> 2018 ² and related correspondance ^{3,4} | Rius <i>et al</i> 2019 ⁵ | Wei <i>et al</i> 2020 ⁶ | Lutz-Bonengel <i>et al</i> 2021 ⁷ | Bai <i>et al</i> 2021 ⁸ | Take home messages |
|---|---|---|-------------------------------------|---|---|--|---|
| Initial screening method (details and primer positions) | mtDNA amplified from multiple tissues (blood, muscle, hair roots and skin fibroblasts) using two overlapping long-range PCRs, with the primers OLA (5756–5781) + D1B (282–255) and D1A (336–363) + OLB (5745–5721). | Two independent long-range PCR reactions to amplify whole mtDNA (using primer pairs F2120 - R2119 and mt16426F - mt16425R respectively) followed by next generation sequencing. | Genome Sequencing | Genome Sequencing, as part of the 100K Genomes Project ⁹ | D-loop analysis (primers F15900-R599 and F15851-R639 and Sanger sequencing). Also two overlapping long-range PCRs (2480–10858 and 10653–16569 1–2688) and next generation sequencing. | Exome sequencing (commercial genetic testing laboratory) and long-range PCR of mitochondrial genome using primers F16561-R16560 or F16428- R16427. | Complementary methods – genomic and molecular. Genomic research, clinical genetic testing (commercial) and forensics settings. |
| Repeat sequencing | Samples mix-up excluded by analyses of repeated blood and muscle samples. Repeated muscle biopsies were from right and left vastus lateralis muscles. Genotyping of all samples for | Performed independently at two different CLIA-accredited laboratories. | N/A – no positive cases identified | No - repeat sequencing, however contamination ruled out by checking nuclear | Analysis by two independent forensic laboratories with independent samples | Repeat mitochondrial genome analysis on a new blood sample | Suitable levels of QC checks and/or repeat sequencing performed in all studies |

NRG – biparental mtDNA

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| | five microsatellites indicated that all samples came from the same person. | | | genome and relatedness checks also performed. | | | |
| Incidence | N/A | N/A | 0/41 | 7/11,035 in unrelated parent-child trios | N/A | The “multiHet” phenomenon (5 or more SNVs with AF of 10-90% in mRNA, rRNA or tRNA regions mapping to the mitochondrial genome) was seen in 104/27,388 (approximately 1/263) unrelated individuals. | 1/1576 to 1/263 Depends on whether specifically looking for paternal transmission or just screening for multiHets. Also depends on AF cutoff, coverage and other aspects of analysis pipeline. Any other reasons for big difference? Unlikely due to recruitment criteria. |
| Cohort type | Single patient with mitochondrial myopathy attributed to a 2bp deletion in <i>MT-ND2</i> (no nuclear gene testing performed) | Cohort size not specified: “a set of patients initially referred for clinical evaluation for mitochondrial disease” | Paediatric patients with suspected mitochondrial disease and their parents | Patients and unaffected family members with a range of rare genetic conditions. | Single large kindred of unaffected individuals (index case is laboratory trainee who provided buccal sample for exclusion purposes) | Individuals referred for mtDNA genome analysis | Does not really matter as unlinked to disease (with exception of Schwartz and Vissing). |
| Segregation pattern seen | Sister had maternal haplotype. Patient’s mtDNA haplotype in muscle matched that seen in the father and paternal uncle. | Autosomal dominant inheritance seen in families | N/A | | Mostly maternal transmissions of Mega-NUMT but consistent with AD inheritance. | 4 families chosen for more in depth analysis – contaminating haplotype can be inherited from both parents (e.g. paternal grandmother in family 1) and so consistent with autosomal | Segregation consistent with nuclear DNA transmission and not linked to disease |

NRG – biparental mtDNA

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| | | | | | | dominant inheritance. Segregation is not correlated with disease | |
| Insertion sites | N/A | N/A | N/A – no instances detected | Chr3 (x2), chr7 (x2), chr12, chr13 and chr17 | Chr14q31 – detected by FISH. Precise breakpoints not mapped | N/A | <p>Only approx. position by FISH whereas precise BPs determined by WGS.</p> <p>Further studies needed to see of any common signatures at insertion sites which could give idea of mechanism akin to retrotransposition events mediated by LINE1 endonuclease which often occur at AATTTT motifs.¹⁰</p> |
| Recurrent NUMTs identified | N/A | N/A | N/A | Families 4 and 6 have same insertion site (chr7:61095402-61095411) – as do families 5 and 7 (chr3:56128996-56128997) | N/A | N/A | <p>Identical insertion sites in apparently unrelated families suggests that some Mega-NUMTs are old ancestral events.</p> <p>Identity by Descent analysis could help confirm single mutational origin and give approximate age.</p> <p>Same insertion site and mtDNA haplotype but not necessarily the same copy</p> |

NRG – biparental mtDNA

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| | | | | | | | number – expansions and contractions would be expected, just as for microsatellites |
| Relative haplotype frequency vs mtDNA content | 90% paternal DNA in patient's muscle (both quadriceps) and 100% maternal in patient's blood, hair-roots and fibroblasts; estimated by solid-phase minisequencing using tritium labelled primer extension. | N/A | N/A | Intra-familial comparisons between individuals – but all blood samples so not very informative. Muscle DNA not available as families were not recruited with suspected mtDNA disease. | Comparison between individuals and between tissues (e.g. hair shafts, buccal, thrombocytes, bone) shows inversion correlation. | Heteroplasmy levels of paternally transmitted variants is highest in blood, lower in buccal, and absent in muscle or urine of the same individual, i.e. an inverse correlation with mtDNA content (R varying from 0.6797 - 0.9998 and P value from $0.0278 - 5.73 \times 10^{-10}$) – shown in figure 5. | Inverse correlation between mtDNA content and the Mega-NUMT haplotype frequency - entirely consistent with nuclear localisation. Pattern is opposite for Schwartz and Vissing case, suggesting in that instance it might be genuine rather than Mega-NUMT. Hair-shaft analysis might be effective way to confirm if pathogenic variant is really in mtDNA or in NUMT |
| Cell line studies performed | N/A | N/A | N/A | N/A | p0 cells prepared from skin biopsy yielded only the U mitotype | N/A | Complementary to the results from muscle/hair/thrombocytes |
| Estimation of number of copies in Mega-NUMT | N/A | N/A | N/A | 2-20 depending on which family – only | 45 (ddPCR data) or 56 (quadruplex real-time qPCR assay) | N/A | Indirect estimates – ultra-long read sequencing |

NRG – biparental mtDNA

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| | | | | blood samples available | | | needed to conclusively determine copy number. |
| Haplotype allelic fractions in blood | 0% (but 90% paternal haplotype in muscle DNA) | Ranged from 24-76% across 17 individuals | | 5-25% | 35% for IV-3 | NUMT haplotype in blood between 30-75% for the 4 families reported in detail (Figure 5). | 5-76% |
| Secondary rearrangements | N/A | N/A | N/A | Yes – deletions and inversions seen | N/A | <p>Paternally transmitted apparent large-scale mtDNA deletions/duplications did not appear to be associated with a disease phenotype.</p> <p>For instance Family B shows different allelic fractions for region 1 vs region 2. (Duplication of H2a haplotype for 1–9652 and single-primer PCR and junction sequencing also points to inversion with junction m.9652:109.</p> <p>In family D a 3.87kb (m.9921-13787) on paternal haplotype but still some copies of full length mtDNA from this haplotype suggesting Mega-NUMT contains at least a few full copies.</p> <p>Deletion not seen in muscle whereas true mtDNA deletions normally elevated in post-mitotic tissues – arguing it is not pathogenic and just present in subset of NUMT copies.</p> | Mega-NUMTs are prone to secondary rearrangements such as inversions and deletions – important to not report these as being of clinical relevance. Macrosatellites are known to be susceptible to genomic rearrangement and highly polymorphic ¹¹ so these findings are not surprising. |

NRG – biparental mtDNA

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| Long read sequencing | N/A | N/A | N/A | Nanopore sequencing used to validate NUMT detection method | N/A | PacBio sequencing to confirm phase of respective haplotypes in subset of families. | Nanopore sequencing read lengths >1Mb have been reported and this technology was used successfully to characterise a 13 copy version of the 3.3kb D4Z4 repeat array locus that is responsible for facioscapulohumeral muscular dystrophy. ¹² |
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