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 *et al* 2018**[2] **and related correspondance**[3,4]	**Rius *et al* 2019**[5]	**Wei *et al* 2020**[6]	**Lutz-Bonengel *et al* 2021**[7]	**Bai *et al* 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

	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 *MT-ND2* (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

						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	Only approx. position by FISH whereas precise BPs determined by WGS. 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]
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	Identical insertion sites in apparently unrelated families suggests that some Mega-NUMTs are old ancestral events. Identity by Descent analysis could help confirm single mutational origin and give approximate age. Same insertion site and mtDNA haplotype but not necessarily the same copy

							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 - 5.73×10^{-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	ρ0 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

				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	Paternally transmitted apparent large-scale mtDNA deletions/duplications did not appear to be associated with a disease phenotype. 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. 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. 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.	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.

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]

References

1 Schwartz, M. & Vissing, J. Paternal inheritance of mitochondrial DNA. *N Engl J Med* **347**, 576-580, doi:10.1056/NEJMoa020350 (2002).

2 Luo, S. *et al.* Biparental Inheritance of Mitochondrial DNA in Humans. *Proc Natl Acad Sci U S A* **115**, 13039-13044, doi:10.1073/pnas.1810946115 (2018).

3 Luo, S. *et al.* Reply to Lutz-Bonengel et al.: Biparental mtDNA transmission is unlikely to be the result of nuclear mitochondrial DNA segments. *Proc Natl Acad Sci U S A* **116**, 1823-1824, doi:10.1073/pnas.1821357116 (2019).

4 Lutz-Bonengel, S. & Parson, W. No further evidence for paternal leakage of mitochondrial DNA in humans yet. *Proc Natl Acad Sci U S A* **116**, 1821-1822, doi:10.1073/pnas.1820533116 (2019).

5 Rius, R. *et al.* Biparental inheritance of mitochondrial DNA in humans is not a common phenomenon. *Genet Med* **21**, 2823-2826, doi:10.1038/s41436-019-0568-0 (2019).

6 Wei, W. *et al.* Nuclear-mitochondrial DNA segments resemble paternally inherited mitochondrial DNA in humans. *Nat Commun* **11**, 1740, doi:10.1038/s41467-020-15336-3 (2020).

7 Lutz-Bonengel, S. *et al.* Evidence for multi-copy Mega-NUMTs in the human genome. *Nucleic Acids Res* **49**, 1517-1531, doi:10.1093/nar/gkaa1271 (2021).

8 Bai, R. *et al.* Interference of nuclear mitochondrial DNA segments in mitochondrial DNA testing resembles biparental transmission of mitochondrial DNA in humans. *Genet Med*, doi:10.1038/s41436-021-01166-1 (2021).

9 Turnbull, C. *et al.* The 100 000 Genomes Project: bringing whole genome sequencing to the NHS. *BMJ* **361**, k1687, doi:10.1136/bmj.k1687 (2018).

10 Wimmer, K., Callens, T., Wernstedt, A. & Messiaen, L. The NF1 gene contains hotspots for L1 endonuclease-dependent de novo insertion. *PLoS Genet* **7**, e1002371, doi:10.1371/journal.pgen.1002371 (2011).

11 Schaap, M. *et al.* Genome-wide analysis of macrosatellite repeat copy number variation in worldwide populations: evidence for differences and commonalities in size distributions and size restrictions. *BMC Genomics* **14**, 143, doi:10.1186/1471-2164-14-143 (2013).

12 Mitsuhashi, S. *et al.* Nanopore-based single molecule sequencing of the D4Z4 array responsible for facioscapulohumeral muscular dystrophy. *Sci Rep* **7**, 14789, doi:10.1038/s41598-017-13712-6 (2017).