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Mitochondrial DNA deletions in Alzheimer's brains: A review

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Abstract

Mitochondrial dysfunction and increased oxidative stress have been associated with normal aging and possibly implicated in the etiology of late onset Alzheimer's disease. DNA deletions, as well as other alterations, can result from oxidative damage to nucleic acids. Many studies in the last two decades have investigated the incidence of mitochondrial DNA deletions in post-mortem brain tissues of late onset Alzheimer's disease patients as compared to age-matched normal controls. Published studies are not entirely concordant, but their differences might shed light on the heterogeneity of Alzheimer's disease itself. Our understanding the role that mitochondrial DNA deletions plays in disease progression may provide valuable information that could someday lead to a treatment.

Keywords

Alzheimer's disease; mitochondrial DNA deletion; DNA damage; oxidative stress; neurodegeneration

1. Introduction

1.1 Basic overview of mitochondrial biology

Mitochondria have conventionally been referred to as the cellular powerhouses, but it has become abundantly clear that mitochondria are also critical to a host of homeostatic and signaling processes which extend well beyond ATP production. The number of

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mitochondria varies widely by cell type. Modulation of mitochondrial number occurs through mitochondrial biogenesis, mitophagy, mitochondrial fission, and mitochondrial fusion (1–4); regulation of these processes differs vastly both within cells and between cell types, resulting in varying numbers, sizes and shapes of mitochondrial populations (5). Some cell types have as few as four mitochondria, appearing as isolated bean-shaped organelles, while cell types with high energy requirements (e.g., brain, muscle, liver) can have over one thousand mitochondria, appearing as a dynamic network (6, 7). According to the endosymbiotic theory of mitochondrial evolution, a topic long discussed in the molecular evolutionary literature, mitochondria are bacterial in origin and arose from a symbiotic relationship between a eubacterial and archaeal ancestor; this hybrid evolved into the current day eukaryote (8). One of the main lines of evidence supporting this theory lies in the fact that mitochondria have their own DNA. Mitochondrial DNA (mtDNA) is a multi-copy, extrachromosomal genome which is transcribed and replicated independently of cell cycle. Most mitochondria contain between one and ten copies of mtDNA, the number of which is regulated in a cell-specific manner by mechanisms that are not completely understood (9, 10). Fission and fusion are critical for long-term maintenance of mitochondrial function; when deficient, increased mtDNA damage is observed. Hypothetically, this is due to the lack of functional complementation that results when mtGenomes are redistributed through fission and fusion (11).

Mitochondrial DNA is inherited maternally due to the higher level structuring of the spermatozoa and the selective elimination of male mitochondria during early embryogenesis (12, 13). The mitochondrial genome is double-stranded, circular and approximately 16.6kb. The coding region contains 13 genes essential to the complexes of the electron transport chain, 22 tRNAs, two rRNAs, and a non-coding control region (CR) which contains the promoters and the origin of heavy strand replication (Figure 1). The mtDNA contains some of the genes required for the oxidative phosphorylation complexes (Table 1): seven subunits of Complex I (ND1, ND2, ND3, ND4, ND4L, ND5, ND6), one subunit of Complex III (Cyt b), three subunits of Complex IV (COX1, COX2, COX3), and two of the subunits of Complex V (ATPs6, ATPs8). These proteins represent only a fraction of the total mitochondrial proteome, estimated to contain greater than 1000 proteins (14). The remaining proteins are nuclear DNA (nDNA) gene products required for mitochondrial function; they are transcribed, processed, and translated prior to mitochondrial import and compartment targeting. There is some recent evidence for RNA import into the mitochondria (15). The implications of this finding are currently under further investigation.

Mitochondria are the production site of a significant proportion of cellular reactive oxygen species (ROS), the degree of which varies by cell type (16, 17). MtDNA is thought to be more highly susceptible to oxidative damage due to 1) its close proximity to the high concentration of ROS, 2) the lack of efficient DNA repair mechanisms in the mitochondria (18, 19), and 3) the lack of DNA-protective histones (20), although the latter two views have been recently questioned (21, 22). Oxidative damage to DNA results in strand breaks, abasic sites (apurinic/apyrimidinic), base changes, and deletions. These processes have been extensively studied and reviewed in the literature, specifically in reference to diseases such as cancer (23–25). Since there are multiple mtGenomes per cell, it is possible to have a heterogeneous population of mtGenomes in one cell or individual, a condition known as

heteroplasmy. While heteroplasmy can be inherited at the germline level (26), it often arises as the result of somatic, *de novo* mutations (27). Variations in mtDNA molecules, due either to damage or natural variability, can result in nucleic acid changes, defective or altered proteostasis, and altered mtDNA replication and transcription efficiency (28, 29).

1.2 Mitochondrial DNA and Alzheimer's disease

The brain is heavily dependent on oxidative metabolism; mitochondrial function is required for proper neuronal activity, as is indicated by the extremely high number of mitochondria and high mtDNA content in neurons (30, 31). Mitochondrial dysfunction is implicated in normal aging and Alzheimer's disease processes. Late onset Alzheimer's disease (LOAD) brains display a significant reduction in oxidative phosphorylation complex protein content, complex activity and energy production; these hallmarks of reduced energetic metabolism have long been associated with LOAD and neurodegeneration (32, 33). Mitochondrial dysfunction occurs very early in disease progression, if not precursory to LOAD (34), which has formed the basis of the "mitochondrial cascade hypothesis": mitochondrial malfunction results in increased levels of reactive oxygen species (ROS), causing damage to mitochondrial components (specifically mtDNA), which, in turn, further increases malfunction and cellular oxidative stress. Consequently, the processing of amyloid beta is altered, causing its cleaved products to accumulate into plaques; this cycle ultimately ends with cell death (35–37). The mitochondrial cascade hypothesis of LOAD has sparked studies of mtDNA alterations that may result from excessive oxidative stress (38). Of particular interest are large scale mtDNA deletions which can result from oxidative damage to DNA. A 4977bp deletion (mtDNA₄₉₇₇) has repeatedly been associated with both normal aging and age-related disease states, such as Alzheimer's disease. This deletion is the most frequently associated with age-related mitochondrial DNA changes; however, other deletions of varying size have also been reported as well (39). Here we review the natural causes of mtDNA deletions, mtDNA deletions associated with LOAD, and the consequences of mtDNA deletions with respect to research design, results, and possible explanations. Early studies which first associated deletions with LOAD as well as contemporary studies using recent advances in technology are discussed.

1.3. Deletions in mtDNA

Mitochondrial DNA deletions have been classified into three groups. Class I deletions are the most common, occurring between two perfect repeat motifs in the mtGenome. Class II deletions, which are the least common, occur when the excised segment falls between two imperfect repeat motifs in the mtGenome. Class III deletions occur sporadically and are not associated with any particular DNA motifs. One particular Class I deletion in which a 4977bp excision occurs between the directly repeated sequence ACCTCCCTCACCA, at positions 8470–8482 and 13447–13459 (Figure 1), was initially discovered and well-characterized in Kearns-Sayre syndrome patients (40) and has since been reported to increase in an age-dependent fashion on various tissue types, including neurons (41, 42). The exact mechanism for the formation of such deletions in the mtGenome is not entirely clear. The primary hypothesis has been that deletions occur between direct repeats as a result of faulty replication (43–45); this logic was founded on the fact that most deletions occur in the major arc of the mtGenome, and multiple mechanisms have been proposed. However,

recent evidence suggests that deletions may arise primarily through faulty repair of double strand breaks (46, 47). The mechanism may vary depending on the life-stage of the deletion event, whether germline or somatic (48–50).

It is thought that somatically accumulated, age-related deletions are the result of faulty DNA damage repair. The process hypothetically entails homologous annealing at direct repeats within the damaged genome, followed by excision of the non-recombined ends (46). *In vivo* evidence supports this hypothesis (42). A mouse model was developed with inducible endonuclease expression which selectively introduces mtDNA double strand breaks. The resulting mtDNA population exhibited the common deletion, as well as other well-characterized deletion patterns that occur between repeated motifs in natural systems. This study also demonstrated the first *in vivo* evidence that mitochondrial genomes with large deletions (assuming the origins of replication are not affected) accumulate faster than those with smaller deletions. This is presumably due to a replicative advantage, as is often observed when amplifying small targets by PCR. Recently the replicative advantage of mtGenomes with large deletions, referred to as clonal expansion, was shown to occur after anti-retroviral therapy in HIV patients ((51). Upon treatment, replication fails and mtDNA content is dramatically reduced. Upon resuming mtDNA replication (*i.e.*, when the treatments are stopped), pre-existing mtGenomes which contain age-related deletions are preferentially expanded clonally and result in deficiencies in mitochondrial function, resembling the accumulation of mtDNA deletions seen in the various tissues of much older individuals. This research indicates that clonal expansion of deleted mtGenomes is a plausible mechanism for the accelerated aging often seen with such treatments of HIV patients, also suggesting that expansion of mtDNA deletions may contribute to the “normal” aging processes and resulting phenotypes as well.

2. Early studies of mtDNA ₄₉₇₇ in Alzheimer's disease patients

Assessing the accumulation of mtDNA deletions in Alzheimer's disease particularly has been of interest given that 1) age is the number one risk factor for late onset Alzheimer's disease, and 2) mitochondrial dysfunction is a prominent feature of disease progression.

An early study by Corral-Debrinski et al. (52) was one of the first to report significant differences between LOAD patients and normal control group tissues (n = 20 and 19, respectively) in the prevalence of the mtDNA ₄₉₇₇ in various regions of the brain. The group developed a PCR based protocol for assessing the proportion of mtGenomes that contain the deletion compared to the number of genomes that do not have the deletion. Briefly, two PCR reactions are carried out in parallel for each sample: one using a primer set that flanks the mtDNA ₄₉₇₇ region, yielding a 593bp product if the deletion is present and an approximately 5.5kb product if the deletion is not present; another primer set that amplifies a region of the mtDNA thought to be rarely deleted (ND1/16S), mtDNA_{WT}, yielding a 609bp product. These reactions are driven towards short PCR product generation by minimizing the extension time and quantified by using a serial dilution standard curve of control DNA with a known mtDNA ₄₉₇₇ to mtDNA_{WT} proportion. In this study, mtDNA from tissue of the cortex (sub-classified as frontal, temporal, parietal, and occipital lobes), the putamen, and the cerebellum were tested using this PCR protocol, termed dilution PCR.

Several interesting results were reported. All regions of the brain showed age-related accumulation of mtDNA ₄₉₇₇ except the cerebellum, which showed a very low incidence of the deletion for all ages. The putamen harbored the highest incidence of mtDNA ₄₉₇₇. Overall, the LOAD group exhibited a 15-fold increase in the prevalence of deletions in cortical neurons. Also of interest, the age-related pattern of deletion incidence was markedly different in LOAD cases compared to the age-matched normal controls. The normal control group showed a higher incidence of mtDNA ₄₉₇₇ in the older members, whereas the trend was opposite with the LOAD group (i.e., the older LOAD subjects had a decreased incidence of mtDNA ₄₉₇₇). These results contradicted previous studies using Southern methods which failed to detect group differences in deletion rates, likely due to the increased sensitivity of PCR-based methodology.

Since this pioneering report, many similar studies were published, some of which validate the results of the Corral-Debrinski study, while others contradict. Blanchard et al. (41) studied the mtDNA ₄₉₇₇ rate in frontal cortex tissue of elderly (71–95 years old) LOAD and age-matched normal controls (n = 6 for each group). As Corral-Debrinski reported, an age-related increase was observed; however, this study failed to detect a significant difference between the LOAD group and controls. Hamblet et al. (53) published results on a similar study using temporal cortex tissue of LOAD and age-matched normal controls (n = 9 for each group). The dilution PCR methodology previously described was used to quantify the percentage of mtDNA ₄₉₇₇. A general age-related increase was observed; additionally, the LOAD group exhibited a 6.5 fold increase over the normal control group. While this difference is less than that reported in the Corral-Debrinski study, the Hamblet cohort had a mean age of 10 years younger, which may account for the discrepancy. Lezza et al. (54) used a different PCR-based method to investigate the incidence of mtDNA ₄₉₇₇ in frontal and parietal cortex tissues of LOAD and age-matched normal controls (n = 7 and n = 6, respectively). DNA from mitochondrial isolates was assayed using a semi-quantitative PCR protocol, where amplification is assessed at intermediate stages during the PCR to deduce the amount of starting DNA template. The results indicate that the LOAD group has a significantly smaller percentage of mtDNA ₄₉₇₇ when compared to the controls. Interestingly, Lezza et al. also assessed the percentage of oxidized guanine bases (8-Oxoguanine, 8-oxoG) in the mitochondrial DNA, an indicator of oxidative damage to the mtDNA. In normal controls, this measure of oxidative damage correlated with the age-related increase in mtDNA ₄₉₇₇; however, in the LOAD group, the age-related decrease in mtDNA ₄₉₇₇ observed was accompanied by an increase in 8-oxoG. Hirai et al. (55) used *in situ* hybridization to assess the mtDNA ₄₉₇₇ incidence in hippocampal neurons of LOAD and normal controls (n = 10 and n = 8, respectively). The LOAD group exhibited a marked increase in mtDNA ₄₉₇₇ labeling in the large pyramidal neurons as compared to the control group. The investigators also used *in situ* methods to quantify total mtDNA content and 8-oxoG. Both total mtDNA content and 8-oxoG was elevated in cells with deletion accumulation. Notably, neurons with neurofibrillary tangles had decreased overall mitochondrial DNA content, both with and without the deletion.

These early studies do not provide a clear picture of the prevalence or nature of the common mtDNA ₄₉₇₇ in LOAD. There may be several confounding factors in these studies. First, the

sample sizes are relatively small considering that the expected effect size is small (*i.e.*, the ratio of mtGenomes with deletions is relatively small compared to the number of intact mtGenomes). It is quite possible that these studies are excessively underpowered to consistently detect, or fail to detect, significant group differences. Secondly, while the PCR is extremely robust and the PCR-based studies discussed here all used derivative protocols, slight differences in approach, including extraction procedures (56), can increase the variability. This is especially problematic when effect size is small. Also, these brain homogenate DNA extracts do not contain cell-specific mitochondrial DNA. MtDNA from the neurons as well as glial cells and endothelial cells of the brain vasculature are co-extracted. The extraneous cell types may not only dilute the mtDNAs of interest, but also introduce another source of variability. The more contemporary studies in this area employ more sensitive and specific methodologies.

3. Recent studies of mtDNA deletions in Alzheimer's disease patients

Several studies in the past decade have further investigated mtDNA deletions in LOAD. Employing methods such as *in situ* hybridization and laser capture microdissection, these studies investigate the incidence of the mtDNA ₄₉₇₇ in a cell-specific manner.

Hirai et al. (57) used *in situ* hybridization to probe for the mtDNA ₄₉₇₇ in various cell types of the hippocampus, frontal cortex, temporal cortex, and cerebellum. Their study included LOAD tissue (n = 27) and normal controls, classified as either young or old (n = 12, n = 8, respectively). The common deletion was more prominent in LOAD neurons of all areas except the cerebellum, where no difference was detected. The most dramatic difference was reported in the large hippocampal neurons, where a four-fold increase was observed. No differences in the prevalence of mtDNA ₄₉₇₇ were observed between controls (either old or young) and LOAD tissues in other cell types such as glial and granule cells. The authors specifically note that when the mtDNA ₄₉₇₇ was quantified in a tissue homogenate using real-time PCR, significant group differences were not seen; this supports the notion that multiple cell types may be the root of inconsistent results from the earlier PCR-based studies.

Chronic hypoperfusion of the brain causes elevated ROS production in the endothelial cells of the brain vasculature walls and has been proposed as a possible initiating factor of LOAD. Two very similar publications, Aliyev et al. (58) and Aliev et al. (59), investigated the ultrastructure of mitochondria/lysosomes and the occurrence of mtDNA ₄₉₇₇ in the endothelial cells of the brain microvessels, implicating mitochondria in the excessive ROS production and resulting oxidative stress. Using *in situ* hybridization, the mtDNA ₄₉₇₇ deletion was quantified in LOAD group and compared to normal controls (number of subjects not specified). The authors report a 3 fold elevation of mtDNA ₄₉₇₇ in the capillary endothelial cells of LOAD group, which also corresponds to a significant increase in gross abnormalities, 8-oxoG and APP accumulation.

Bender et al. (60) employed laser capture microdissection followed by a multiplex real-time qPCR method to quantify the ratio of mtDNA ₄₉₇₇ to normal mtDNA in specific cell types. This method is superior to previously mentioned PCR approaches since the multiplex design

minimizes well-to-well variability due to pipetting error and cell-specific conditions can be investigated in the absence of background signal. Thirty neurons were captured from three regions of LOAD and age-matched normal control brain specimens (n = 9 and n = 8, respectively): the putamen, the frontal cortex, and the substantia nigra. The authors detected the mtDNA ₄₉₇₇ in all three tissue types, with the highest occurrence being in the dopaminergic neurons of the substantia nigra, although no group differences between LOAD and the control group were observed, even in the frontal cortex as one would expect. A previous study by these same authors, in which similar methods were used, investigated the regional common deletion rate in Parkinson's disease patients (61). Unlike in their Alzheimer's study, a significant difference in mtDNA ₄₉₇₇ was observed in the substantia nigra neurons of the Parkinson's disease group compared to the controls.

Blokhin et al. (62) present a study of the mtDNA ₄₉₇₇ in the lesions of multiple sclerosis. Three groups were analyzed: multiple sclerosis, age-matched normal controls, elderly normal controls (>60 years of age) and neurodegenerative positive controls (cortex tissue from two LOAD subjects). The group used laser microdissection to isolate specific cells based on mitochondrial function (COX positive or COX negative) and quantified the mtDNA deletion ratio by analyzing the real-time amplification profiles of ND4 compared to ND1. The two LOAD subjects exhibited a marked increase in both prevalence of COX negative neurons and in the ratio of mtDNA ₄₉₇₇ in the COX negative neurons. The elderly normal controls demonstrated a similar trend, however not to the extent of the LOAD controls. No significant difference in prevalence of COX negative cells or mtDNA deletion ratio was seen between the multiple sclerosis group and the age-matched controls. No significant difference in mtDNA deletion ratio was observed in the COX positive cells of all groups.

Krishnan et al. (63) recently reported their study investigating the correlation of mtDNA ₄₉₇₇ with pyramidal neurons that are COX negative. Hippocampal sections from LOAD and age-matched normal control groups (n = 10 and n = 6, respectively) were assessed for mitochondrial function, followed by mtDNA ₄₉₇₇ quantification and identification. The deletion ratio was quantified in COX negative and COX positive neurons by real-time qPCR, and the exact break points were identified by long PCR followed by Sanger sequencing. Their findings indicate that the LOAD group exhibited a larger percentage of COX negative neurons than the control group. Additionally, the COX negative neurons for both LOAD cases and controls contained a markedly increased ratio of mtDNA ₄₉₇₇ compared to normal mtDNA. When sequenced, several different breakpoints were observed, with deletion sizes ranging from 3670bp to 6088bp. Six different breakpoints were observed, all in the vicinity of the common 4977 deletion and all associated with flanking repeats.

Recent developments in technology and approach offer the advantage of increased sensitivity and specificity of results. Single cell assessments of mtDNA state provide more accurate assessment of the occurrence of mtDNA deletions as well as more insight into the potential implications of mtDNA deletions in LOAD progression. This area of research has not been very active in the last decade, likely due to the contradicting results from the earlier studies; however, the methods employed in the recent studies are promising.

4. Gaps in the knowledge and future directions

The contemporary studies discussed here indicate that mitochondrial deletions are associated with the biochemical deficit observed in late onset Alzheimer's disease (*i.e.*, increased proportion of COX negative neurons). However, a causal relationship has not been directly established. Experimentally drawing that conclusion would be a natural transition from these studies. This review focuses on the occurrence of deletions in studies of human brain tissues. Although most animal models of Alzheimer's disease do not truly mimic the complex pathogenesis associated with the late-onset form of the disease, further experimentation with animal models may provide some insight into the potential role that mtDNA deletions play in LOAD pathogenesis. Studies using transgenic mouse models of human neurodegenerative diseases, including Alzheimer's disease, have investigated oxidative damage to mtDNA and mtDNA repair mechanisms (64, 65), but the occurrence and timing of deletions in the mtGenome have not been thoroughly investigated in this context. An interesting study by Scheffler et al. (66) recently created a congenic mouse model of Alzheimer's disease to provide the first *in vivo* evidence that mtDNA variants can have specific phenotypic effects on mitochondrial function, A β load/clearance, as well as cellular function (with regard to microglial activation). The presence of large mtDNA deletions was not investigated in this model, but would have been an interesting experiment.

Regional differences in brain susceptibility to mtDNA deletions have been shown. For example, dopaminergic neurons were shown to be exceptionally susceptible to mtDNA deletions. Perhaps there are region-specific mechanisms by which deletions accumulate and/or expand within vulnerable neurons. Further investigating why certain regions of the brain show differential mtDNA damage may shed light on the specific pathology of LOAD. It is interesting that Parkinson's disease studies have been very consistent in describing mtDNA deletions (67) while studies of Alzheimer's disease brain tissue have not. There are several likely explanations for this. First, Parkinson's disease is caused by a focal degeneration of neurons in the substantia nigra and assessments of mtDNA deletions in this disease have focused on this brain region. In contrast, Alzheimer's disease affects the hippocampus, and entorhinal, frontal and parietal cortices; the extent of pathology in these regions varies from subject to subject (68–70). Further, in contrast to Parkinson's disease, Alzheimer's disease is believed to have multiple endophenotypes (71). Thus, Parkinson's disease is more likely to originate from one cause than Alzheimer's disease and, as a result, a study of Alzheimer's disease brains is more likely to show variability in mtDNA deletions. It would be interesting to specifically assess the vulnerable neuronal populations implicated in LOAD for mtDNA deletions using a large enough cohort in order to possibly classify LOAD subtypes based on differential mtDNA involvement.

Age is the number one risk factor for LOAD. The fact that mtDNA deletions have been shown to accumulate in brains with normal aging may be indicative of their involvement in the etiology and/or disease progression of LOAD. Further research on the mechanism and timing of mtDNA deletions that accumulate with age is ongoing, and future developments in this area of research may potentially provide valuable insight into this devastating disease.

Abbreviations used

mtDNA	mitochondrial DNA
LOAD	late onset Alzheimer's disease
mtDNA_{WT}	wild-type mitochondrial DNA
mtDNA₄₉₇₇	“common” 4977 base pair mitochondrial deletion

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1. **Systematic Review:** We used a combination of literature search engines (i.e., PubMed, Scopus, Google Scholar) to gather original English-language research investigating the occurrence of mtDNA deletions in LOAD brains.
2. **Interpretation:** MtDNA plays an uncertain role in the pathogenesis of Alzheimer's disease, but with advances in methodology, there is currently much interest in further understanding how deletions in particular may initiate and/or propagate disease progression. This review provides researchers with an important foundation for future work in this area.
3. **Future Directions:** A few specific future directions include (1) proving a causal relationship between mtDNA deletions and the bioenergetic deficit observed in cells of LOAD brains; (2) investigating the mechanism of mtDNA deletion formation, and if it differs regionally within the brains of those aging normally and those with neurodegenerative conditions; and (3) determining if genetic factors involved in mtDNA deletion formation account for the missing heritability of LOAD.

Figure 1.

Schematic of the mitochondrial genome, and the location of the “common” 4977bp deletion (mtDNA₄₉₇₇). Some slight variability in the exact break point for this deletion has been reported; therefore, the approximate positions have been indicated.

Table 1

Overview of the genes required for the oxidative phosphorylation complexes. The number of nuclear DNA genes (nDNA) is an estimate based on an advanced search using GeneCards (<http://www.genecards.org/>). The mtDNA only encodes a small fraction of the required subunits.

ETC complex	Complex name	nDNA genes	mtDNA genes
I	NADH dehydrogenase	46	7
II	Succinate dehydrogenase	5	0
III	Cytochrome bc ₁ complex	11	1
IV	Cytochrome c oxidase	10	3
V	ATP synthase	27	2