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Genetics

A mitochondrial DNA clone is associated with increased risk for Alzheimer disease

(mitochondria/genetics/evolution/risk for disease)

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ABSTRACT **Severe mitochondrial genetic mutations lead to early degeneration of specific human tissues; milder mitochondrial mutations may cause degeneration at a later point in life. A mutation at position 4336 was reported to occur at increased frequency in individuals with Alzheimer disease (AD) and Parkinson disease [Shoffner, J. M., Brown, M. D., Torroni, A., Lott, M. T., Cabell, M. F., Mirra, S. S., Beal, M. F., Yang, C.-C., Gearing, M., Salvo, R., Watts, R. L., Juncos, J. L., Hansen, L. A., Crain, B. J., Fayad, M., Reckord, C. L. & Wallace, D. C. (1993) *Genomics* 17, 171–184]. We have investigated the notion that this mutation leads to excess risk of AD by using a case-control study design of 72 AD autopsies and 296 race- and age-matched controls. The 4336G mutation occurred at higher frequency in AD autopsies than age-matched controls, a statistically significant difference. Evolutionary analysis of mtDNAs bearing the 4336G mutation indicated they were more closely related to each other than to other mtDNAs, consistent with the model of a single origin for this mutation. The tight evolutionary relatedness and homoplasmy of mtDNAs that confer elevated risk for a late-onset disease contrast strikingly with the distant relatedness and heteroplasmy of mitochondrial genomes that cause early-onset disease. The dichotomy can be explained by a lack of selection against mutations that confer a phenotype at advanced age during most of the evolution of humans. We estimate that ≈1.5 million Caucasians in the United States bear the 4336G mutation and are at significantly increased risk of developing mitochondrial AD in their lifetime. A mechanism for 4336G-mediated cell death is proposed.**

Alzheimer disease (AD) results from early death of cholinergic neurons in the brain (1). The genetic causes of AD appear to be heterogeneous (2–4), as early-onset AD can result from mutations of loci on chromosomes 14 and 21 (5–9). Late-onset AD appears to be genetically distinct from early-onset AD; one identified risk factor for late-onset AD is high incidence of the apolipoprotein E type 4 allele (10–13).

Individuals with mutations of the mitochondrial genome that cause serious defects of the electron transport chain experience several severely deleterious degenerative phenotypes including sensorineural deafness, ataxia, cardiomyopathy, and an Alzheimer-like dementia (for review, see ref. 14). It was recently reported that an A → G mutation of the mitochondrial $tRNA^{Gln}$ gene at position 4336 occurs at increased frequency in Caucasian persons who died of AD and/or Parkinson disease (15). We have investigated this mutation in a number of pure AD patients and age-matched controls sufficient to significantly resolve three issues: (*i*) Do aged persons with the 4336G mutation have an increased risk for AD? (*ii*) Do persons with the 4336G mutation normally survive to the age at which AD occurs? (*iii*) What is the relative risk for AD of persons in the at-risk age group (>50 years) given the presence of the mutation? We have investigated these issues by studying the frequency of the 4336G mutation in an independent group of 72 AD patients and 296 age-matched controls and by evolutionary analysis of their D-loop sequences.

MATERIALS AND METHODS

Sources of DNA Samples. AD samples were obtained from the Alzheimer's Disease Research Consortium of Los Angeles, University of Southern California Medical Center. All samples had undergone neuropathological analysis to confirm AD diagnosis. Age-matched and race-matched controls with no diagnosis of AD (as indicated by neuropathology reports) were obtained from the above; the National Neurological Research Specimen Bank, Veterans Administration Medical Center, Los Angeles; the Brain Tissue Resource Center, Boston; and the Los Angeles County/University of Southern California Medical Center (150 age-matched controls). Other controls were from cancer patients (143 age-matched controls) and 3 other patients. The mean age at death of AD patients was 81.3 years (range, 66–94 years); for age-matched controls (50 years or older) the mean age at death was 69.2 years (range, 50–104 years).

PCR and Restriction Enzyme Analysis. DNA was prepared from paraffin blocks first treated with xylenes or from tissues by phenol/chloroform extraction. A short fragment of the mitochondrial genome was amplified by PCR from DNA prepared above or directly on cerebrospinal fluid samples by using Genereleaser (Bio-Ventures, Murphreesboro, TN) as described (16). Some samples required nested PCR, which was first achieved by using outer primers 5′-CTCCCCTGAACTACACAAC (H4056) and 5′-TATTAGAAGGATTATGGATGC (H4665) with PCR conditions of 94°C for 30 sec, 52°C for 30 sec, and 72°C for 30 sec. The inner primer CCCCCTCAATAAGACAT (H4268) and H4665 with the same PCR conditions were used to generate the DNA fragment for restriction enzyme analysis. This DNA fragment contained an *Nla* III site at position 4580 and the A → G transition at position 4336 created a second site. DNA fragments were separated on a 3% NuSieve agarose gel.

Evolutionary Analysis of D-Loops. D-loops were amplified with the primers ACTCTTACACCAGTCTTGTAAACC (H1592) and CCTGAAGTAGGAACCAGATG (H1649), by using conditions of 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min. The nucleotide sequence of the PCR product was determined with the inner primer GAAAAAGTCTTTAACTCCACC (H1598) by using an automated sequencing apparatus (Applied Biosystems). Phylogenetic analysis of the first 370 nucleotides of D-loops was carried out by transforming the sequence differences into a distance matrix (17), by using DNADIST of the PHYLIP package (18) and a phylogenetic

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Abbreviation: AD, Alzheimer disease.

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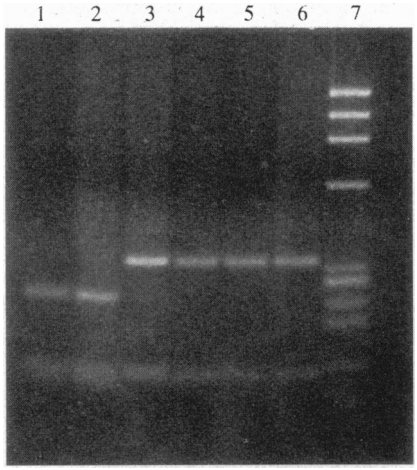

FIG. 1. Detection of 4336G mutation in AD patients. A fragment of mtDNA was amplified by using primers H4268 and H4665 and the resulting PCR product was digested with *Nla* III prior to separation on a 3% NuSieve agarose gel. The normal samples gave fragments of 333 and 106 bp; the presence of G4336 created a pattern of 244, 106, and 89 bp. Lanes: 1 and 2, AD patients with 4336G; 3 and 4, AD patients with 4336A; 5 and 6, aged-matched controls; 7, ϕX174 *Hae* III marker.

tree constructed by using the NEIGHBOR-JOINING program (19), with an African pygmy mtDNA as an outgroup.

RESULTS

Occurrence of the 4336G Mutation in AD Patients and Controls. The 4336G mutation creates an *Nla* III site that can be detected by restriction digestion. Typical results of PCR amplification and restriction enzyme digestion with *Nla* III appear in Fig. 1. Of 72 AD patients we surveyed in this manner, the 4336G mutation occurred in 6 individuals (8.3%). The frequency of the 4336G mutation was also determined in 480 controls, 296 of which were between the ages of 50 and 104 years. In 480 controls, 3 individuals (0.63%) bore the 4336G mutation. In age-matched controls, 1 of 296 (0.34%) bore the 4336G mutation (Table 1 and Fig. 2). This individual was 79 years old with no history of dementia.

The mean age at onset of dementia for those with the 4336G mutation was 67.8 years (range, 60–76 years), whereas for all other AD patients studied it was 71.0 years (range, 50–90 years). The mean age at death of AD patients in which 4336G occurred was 79.0 years (range, 68–88 years) vs. 81.3 years (range, 66–94 years) for all other AD patients studied.

Frequency of the 4336G Mutation Is Statistically Separable in AD Patients and Age-Matched Controls. The frequency of the 4336G mutation was 0.34% and 0.63% in age-matched and total controls, respectively, whereas the 4336G mutation occurred in 8.3% of AD patients. These frequencies are statistically significantly separated from each other at the $P < 0.005$ level (age-matched controls vs. AD patients, $G = 14.6$; all controls vs. AD patients, $G = 14.9$ using the G test, as in ref. 20). Thus there is statistically significant support that the frequency of the 4336G mutation is elevated in AD patients compared to age-matched controls.

Table 1. Contingency table of 4336G association with Caucasian AD

Samples tested	AD +, no.	AD −, no.	Total no.
4336 +	6	1	7
4336 −	66	295	361
Total	72	296	368

Presence (4336 +) or absence (4336 −) of the 4336G mutation in AD patients (AD +) and age-matched controls (AD −) from this study is shown.

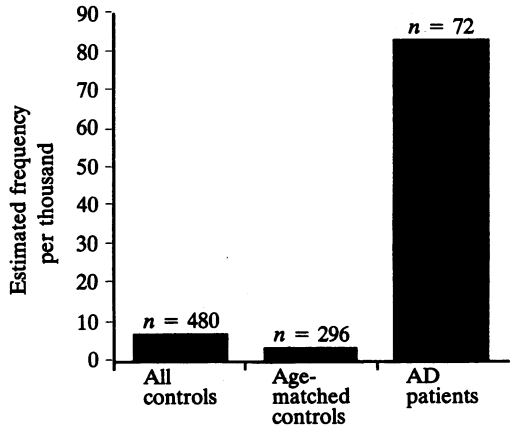

FIG. 2. Mean estimated frequency of the 4336G mutation in the Caucasian population and AD patients.

Analysis of Risk for AD Given the Occurrence of 4336G. By using the frequency of the 4336G mutation in AD cases and age-matched controls obtained in this study, it is possible to calculate an odds ratio to determine the excess risk of developing AD for persons carrying the mutation. The occurrence of 4336G in AD patients and age-matched controls from this study is shown in Table 1. The odds ratio was 26.8 (95% confidence interval 3.17–226.4) by using Woolf's method (as in ref. 21), indicating that persons aged >50 years with the 4336G mutation have a statistically significantly higher mean risk of developing AD than Caucasians without 4336G.

Our best estimate of the increased risk for developing "mitochondrial AD" as a result of the 4336G mutation is 27-fold for persons aged >50, all other factors being equal.

mtDNAs Bearing the 4336G Mutation Are Members of a Single Clone. The D-loops of the mitochondrial genomes from the 6 unrelated AD patients with the 4336G mutation and 7 other patients without this mutation were sequenced. Phylogenetic analysis by the neighbor-joining method was performed on the sequences from these 13 AD patients along with 81 random Caucasians, Africans, and Asians (from ref. 22–24). D-loops of 4336G bearing mtDNAs were more closely related to each other than any other D-loop, with all 6 patients clustering tightly into one node of the phylogenetic tree (Fig. 3). Each of these mtDNAs carrying the 4336G mutation also harbored a T → C polymorphism at position 16304 in the noncoding D-loop, as observed earlier (15). Other AD patients without the 4336G mutation were scattered throughout the Caucasian population on the tree, exhibiting no clustering (Fig. 3).

DISCUSSION

There Is No Excess of the 4336G Mutation in Aged Populations. AD has a striking age dependence, which makes age-matching critical for testing the hypothesis that a specific genetic variation leads to increased risk. Less than 0.01% of people aged 40 or younger exhibits AD; but >20% of persons 85 years or older exhibit AD symptoms (25–28). Two disparate causes could result in an increased frequency of a particular genetic variant in AD patients. One explanation (model 1) is that the variant causes increased risk for AD. An alternative explanation (model 2), however, is that the particular variant is simply overrepresented in older individuals. We observe 4336G at low frequencies in young and old control groups, much lower than in the AD group, lending no support to model

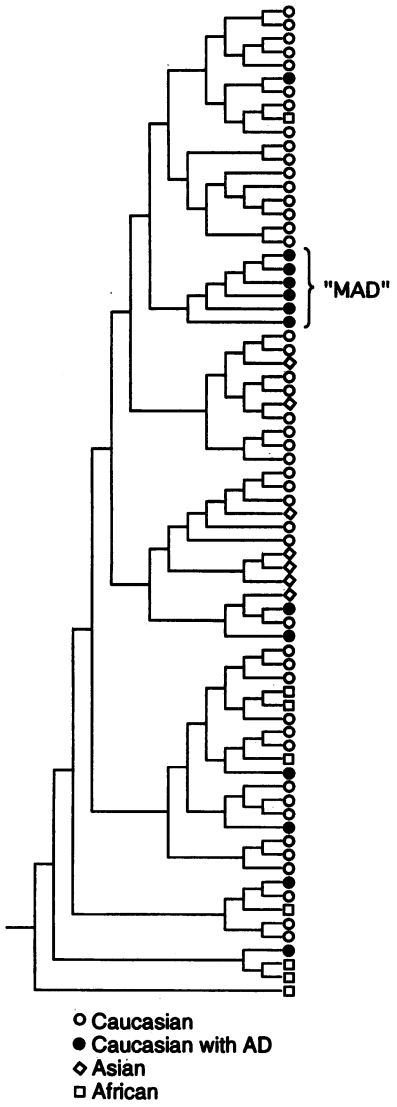

FIG. 3. Phylogenetic analysis of AD patients with the 4336G mutation. Mitochondrial D-loop sequences were used to generate a phylogenetic tree by using the PHYLIP package (18) and printed out via MACCLADE (24). Caucasians are represented by open circles; those with AD are represented by solid circles. Africans are represented by squares and Asians are represented by diamonds. MAD, mitochondrial AD.

2. We conclude that the occurrence of 4336G leads to increased incidence of AD.

The 4336G Mutation Is Associated with Elevated Risk for Late-Onset AD. We have observed that the 4336G mutation occurs at statistically significantly higher frequency in late-onset AD patients than both age-matched and total controls. Our best estimate of the mean increased risk for AD given the 4336G mutation is 27-fold. The mean ages of onset and death of 4336G—bearing AD patients were 3.2 and 2.25 years earlier than those for all AD cases, respectively; but these differences were not statistically significant.

The simplest interpretation of these data is that the 4336G mutation leads to excess AD. It has been proposed that late-onset AD may be the result of multiple genetic and environmental causes (3, 4). Data presented here are consistent with a specific mitochondrial genetic cause (the 4336G mutation) in about 8% of AD patients. If we assume that there are 210 million Caucasians in the United States, ≈1.5 million (i.e., 0.7%) of them are at excess risk for developing AD as a result of bearing the 4336G mutation.

Table 2. Possible dichotomy of mitochondrial genetic disease

Disease	Mutation removal	Characteristics	Examples
Early onset	Strong negative selection	Heteroplasmy Distant evolutionary relationship	LHON MELAS FICM MERRF
Late onset	Intermittent negative selection	Homoplasmy Clonal evolutionary relationship	MAD MID

LHON, Leber hereditary optic neuropathy; MELAS, mitochondrial encephalomyopathy, lactic acidosis, and stroke-like symptoms; FICM, fatal infantile cardiomyopathy; MERRF, myoclonic epilepsy and ragged-red fiber disease; MAD, mitochondrial AD; MID, maternally inherited deafness.

How a Deleterious mtDNA Clone May Survive Natural Selection. Mitochondrial genomes bearing the 4336G mutation appear to be more closely related to one another than to other mitochondrial genomes present in Caucasians. This observation contrasts with the distant relatedness of mitochondrial genomes that cause early-onset mitochondrial disease such as Leber hereditary optic neuropathy (29, 30), as shown in Table 2. The striking disparity in relatedness of mitochondrial genomes that cause early- and late-onset disease is most easily explained by the differential activity of negative selection to extinguish specific lineages. Recombination of human mitochondrial genes is absent or extremely rare. Therefore, once a deleterious change occurs, it is forever linked to the clonal background on which it arose until its extinction (Fig. 4). Deleterious mtDNA clones that lead to early-onset disease are likely to be rapidly eliminated by negative selection. Indeed, a likely reason they are only observed heteroplasmically is that homoplasmy is lethal. In contrast, the 4336G mutation occurs homoplasmically and leads to a disease whose mean age of onset is 67.8 years, and whose full phenotypic expression (death with AD) occurs at 79 years. Since these ages are well past the age of female reproduction, selective pressure

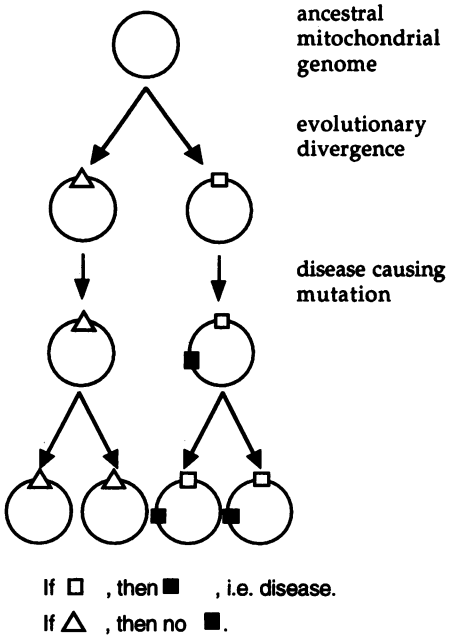

FIG. 4. Clonal linkage of pathogenic 4336G mutation to D-loop polymorphisms. Mutations, such as 4336G represented by a black box, arose only once on a distinct mitochondrial background and remain linked to that lineage. Thus the occurrence of 4336G or any other clonal mitochondrial disease can be assayed by D-loop sequencing.

on such a mutation may be minimal. Thus, mutations that solely cause late-onset phenotypes may be evolutionarily neutral.

Other Homoplasmic mtDNA Mutations Undergo Weak Negative Selection. A similar situation to the 4336G mutation exists with another "mildly deleterious" mtDNA mutation, the 1555G maternally inherited deafness mutation (16, 31). This particular mutation is deleterious in the context of either exposure to aminoglycoside antibiotics (16, 31) or a particular nuclear genetic defect (31). Aminoglycosides are an environmental exposure that is intermittent and evolutionarily recent. Phylogenetic analysis of D-loops from 1555G deaf cases shows a clonal relationship (32). The 4336G and 1555G mutations may define a class of mitochondrial disorders that undergo intermittent negative selection. Three observations are unique to these two mutations that set them apart from most mitochondrial genetic diseases that have been described: (*i*) The mutations occur homoplasmically, (*ii*) the mutations cause disease that is likely to undergo intermittent negative selection, and (*iii*) mutant genomes are clonally related. Since the disease-causing mutations occur clonally, the likelihood of occurrence of the 4336G or 1555G mutations can be indicated by analysis of the D-loop. Thus D-loop sequencing can be informative about two disparate mitochondrial diseases. It is possible that mitochondrial D-loop analysis could be used to identify mitochondrial lineages at increased risk for other degenerative disease.

A Hypothesis: The 4336G Mutation May Lead to Defects in Complex I and Increased Free-Radical Damage to Neurons. Mutations in tRNAs have been observed in multiple mitochondrial genetic diseases, but no general model for why these mutations are deleterious has been proven. It was proposed that since most of the mitochondrial genome encodes complex I subunits, defects in mitochondrial protein synthesis are more likely to impair complex I (NADH dehydrogenase) than any other mitochondrial gene product (33). Drugs that inhibit complex I induce production of mitochondrial superoxide and induce cell death (34–39). The brain toxicity of a complex I inhibitor is suppressed by MnSOD, the mitochondrial superoxide dismutase (33), supporting the notion that mitochondrial superoxide could be a significant neurotoxin, and we suggest that excess superoxide generation may be an important outcome of the 4336G mutation.

Note Added in Proof. Our further studies of the 4336G mutation indicate that it may not be evenly distributed throughout the Caucasian AD population. The 4336G mutation occurred only twice in 122 AD cases from the National Neurological Research Specimen Bank and Boston Brain Bank, a frequency of 1.6%. Our revised frequency data, combined with those of ref. 15, include 17 4336G occurrences in 367 patients with neurological disease (4.6%) but only 1 occurrence in 322 age-matched controls (0.3%), still consistent with an increased risk for AD of 15.6 (95% confidence interval 2.1–117.8), but lower than the estimate that appears in the text.

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