The tRNA^{Gln} 4336 mitochondrial DNA variant is not a high penetrance mutation which predisposes to dementia before the age of 75 years

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Abstract

The genetic factors that predispose to Alzheimer's disease (AD) are heterogeneous. Two recent reports have suggested that a mitochondrial DNA mutation within the tRNA^{Gln} gene, located at position 4336, may be a risk factor for AD, as it was found in 10/256 (3.9%) cases with AD confirmed by necropsy. Although low prevalences of this mutation were detected in non-demented subjects in both of these studies, the controls were not carefully matched with the AD cases. We have investigated the frequency of this mutation in two community based elderly cohorts in Cambridgeshire, who have participated in longitudinal studies of cognitive function. The 4336 mitochondrial mutation was detected in 8/443 people examined. These people were found to be non-demented at ages 74, 81, 84, 86, 89, 90, 91, and 102 years, in contrast to the previously described cases whose onset of dementia occurred between 60 and 76 years (mean 68). Accordingly, we believe that this mitochondrial variant is not a high penetrance mutation which predisposes to dementia before the age of 76 years.

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ataxia, and retinitis pigmentosa (NARP), myoclonic epilepsy and ragged red fibre disease (MERRF), or Kearns-Sayre syndrome (KSS) may sometimes develop dementia. Oxidative phosphorylation (OXPHOS) enzyme activity decreases with age in normal people and there is an age related accumulation of mitochondrial DNA damage in stable tissue. In addition, biochemical assays have shown that AD patients have OXPHOS defects in various tissues including platelets,¹⁴ neocortex,¹⁵ and fibroblasts.¹⁶ Since some of the OXPHOS enzymes are coded for by mitochondrial DNA, there are logical reasons to investigate this genome for mutations in AD.

This possibility was strengthened by two recent reports^{17,18} which indicated that a systemic A to G point mutation within the tRNA^{Gln} gene, at position 4336, occurred at a higher frequency in white, late onset Alzheimer patients confirmed at necropsy (3.2% and 4.1%, respectively) compared to controls (0.8% and 0.34%, respectively). It has therefore been suggested that this mutation may be causative for the development of AD pathology.

These findings were important to replicate, particularly since the AD cases in these studies were chosen from brain banks and were compared to controls that were not carefully matched for age and sex. Furthermore, if the mitochondrial 4336 variant were neutral, it could have been over-represented in the AD samples by chance, because it is rare.

Materials and methods

Two study populations were investigated.

(1) Subjects enrolled in the Cambridge Centre of the MRC Multicentre Study of Cognitive Function and Ageing (MRC CFA Study).¹⁹ In this study, cognitive function of a random sample of 2500 people aged 65 years and over was assessed on at least two occasions, separated by an interval of two years. In addition, a subset of the sample was assessed in more detail approximately one month after the initial screen. This subset included all those respondents with evidence of cognitive impairment and a comparable number of non-cognitively impaired people.

At each screening and assessment interview two composite measures of cognitive state were obtained. The AGE-CAT organicity score was derived from the Geriatric Mental State (GMS)

Alzheimer's disease (AD) is a neurodegenerative disorder resulting in progressive cognitive decline and dementia. The disorder may be broadly categorised into two groups according to the age of onset.¹ An age cut off of 60 or 65 years has been used to distinguish early onset from late onset cases, although these are indistinguishable neuropathologically. The causes of both early and late onset AD are heterogeneous. Mutations in candidate genes on chromosomes 21,²⁻⁴ 14,⁵⁻⁸ and 1^{9,10} have been identified for the majority of familial early onset cases, and the apo E4 allele is associated with increased risk for the late onset disease.^{11,12}

There is some evidence to suggest that abnormalities in mitochondrial metabolism may be associated with a spectrum of neurological symptoms, including dementia.¹³ Patients who are severely affected with mitochondrial DNA mutations causing neurogenic weakness,

examination, a standardised interview developed to detect psychiatric morbidity in epidemiological studies.^{20,21} The organicity score is obtained by applying a computerised algorithm to the answers the respondent gives to questions assessing memory and orientation. Observations of the respondent made by the interviewer are also incorporated. The range of possible scores is from 0 to 6; a score of 0 indicates a low probability of cognitive impairment, while increasing scores correspond to increasing likelihood of a diagnosis of dementia being made by a clinician. In the CFA study a score of 3 or above was taken to indicate the presence of dementia.

The second measure of cognitive state was the Mini-Mental State Examination test score.²² This is a widely used instrument which covers orientation, memory, and a number of other higher cortical functions. The maximum score is 30 and lower scores indicate some degree of cognitive impairment. Various cut offs have been used to indicate dementia.

In the Cambridge component of the CFA study, the second wave of screening was modified to incorporate a nested case-control study. All respondents who had an AGE CAT organicity score of 3 or above at baseline or follow up were entered into the case-control study. Each case was paired with a single age and sex matched control selected from the non-impaired respondents. These respondents were interviewed at home by an experienced psychiatrist using the full GMS examination, augmented with CAMCOG, a neuropsychological battery which forms part of the CAMDEX interview^{23,24} and additional cognitive items. Subjects were also given a brief physical examination (including tests for Parkinsonism) and mouthwash samples were collected in normal saline for genetic analysis. The History and Aetiology Schedule of the GMS was given to a carer or relative and was modified to include the Hachinski Ischemia scale²⁵ and the Blessed Dementia Scale²⁶ to determine if there were any changes in the respondents' memory or self-care ability. In addition to obtaining the AGE CAT organicity score and MMSE score, the psychiatrist made a clinical diagnosis of dementia using DSM-III-R criteria.²⁷ For our study, both AGE CAT and DSM-III-R criteria for dementia were used to classify people as cases and controls.

Cases and controls were age matched within a maximum of five years of each other. A total of 346 respondents were eligible for entry into the case-control study, from whom 277 interviews were completed and 174 mouthwash samples were collected. Some respondents died before mouthwash samples were obtained and some cases with severe dementia could not provide samples; thus only 130 samples from 65 age and sex matched pairs were collected. A further 44 samples were obtained from the control group but not from their matched case. All cases and controls investigated in this study were older than 70 years.

(2) The second, distinct, study group came from a 10 year longitudinal study of cognitive function and ageing.²⁸ Participants were ori-

ginally aged 75 and over, identified from population registers for family practitioners in the city of Cambridge. The participants had taken part in at least three further screening interviews since baseline: 2.4 years, 6 years, and 10 years. These interviews included Mini-Mental State Examinations. In this 10 year follow up, of the original 1968, 546 who had taken part in the previous follow ups were available for interview. Of these, 83% (446) agreed to be seen and successful venepuncture was carried out on 63% of these, 182 females and 100 males. These represent a sample of people who were all aged 84 or older at the time of venepuncture.

These studies have been approved by the ethics committee at Addenbrooke's Hospital. Informed consent was given by all cases who were investigated. These people were all formally evaluated before DNA testing.

PCR AND RESTRICTION ENZYME ANALYSIS

DNA was extracted from mouthwash cell pellets by phenol extraction (population 1) or from venous blood (population 2). A short fragment of 288 bp was amplified by PCR, with primers which were designed using OLIGO Primer Analysis Software (National Biosciences, Inc). The primers were located at positions 4230 to 4250 bp (4230F: 5' CAT TAC AAT CTC CAG CAT TCC 3') and 4517 to 4497 bp (4517R: 5' GCC TGC AAA GAT GGT AGA GTA 3'), according to the designation used by Anderson *et al.*²⁹ The PCR reaction contained 50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 8.0, at room temperature, 2.5 mmol/l MgCl₂, 0.01% gelatin, 30 ng of each primer, 250 mol/l of each dNTP, and 0.3 U of *Taq* polymerase (Gibco BRL). The cycle parameters, performed on a Hybaid Thermocycler, were three minutes initial denaturation at 94°C followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for one minute. Restriction digestion was carried out by the direct addition of 1.7 U of *Ava*II (New England Biolabs), diluted in 1 × PCR buffer, to each PCR reaction, followed by overnight digestion at 37°C. DNA fragments were separated on a 1% agarose gel.

The presence of an *Ava*II restriction site within this PCR product implied the 4336 A to G mutation. The exact position and sequence of this variant was confirmed using an allele specific mutagenically separated PCR (MS PCR) assay.³⁰ Two allele specific primers were designed at positions 4356 to 4336 bp (MT4336WT: 5' GGG ATG GGT TCG ATT CTC AAA 3') and 4376 to 4336 bp (MT4336M 5' CGG AGA ATT TTG GAT TCT CAC CGA TGG GTT CGA TTC TGA TG 3'). Amplification using both of these primers in conjunction with primer 4230F resulted in PCR fragments of 127 bp or 147 bp respectively according to whether an A or G was present at position 4336. PCR products were sized on a 2% agarose/1% NuSieve gel.

Apo E genotypes were determined using the method of Hixson and Vernier³¹ and restriction fragments were visualised by silver staining.

Figure 1 Detection of the tRNA^{Gln} 4336 mitochondrial DNA mutation. A 288 bp fragment of the mitochondrial genome was amplified using the primers 4230F and 4517R. Following amplification, the PCR product was digested with *Ava*II and the resulting fragments were separated on a 1% agarose gel. The normal samples remained uncut following digestion (lanes 1 to 4); however, the presence of the homoplasmic tRNA^{Gln} 4336 A to G mutation created an additional *Ava*II restriction site, such that the five control samples (from cohort 1) which were found to harbour the mutation produced restriction fragments of 185 bp and 103 bp (lanes 5 to 9). Lane 10 contains an anonymous positive control (age not known); lane 11 shows a water only negative control. Lane 12 contains a 100 bp DNA ladder (Gibco BRL); fragment sizes are shown in base pairs (bp).

Figure 2 Mutagenically separated PCR (MS PCR) confirming the exact position and sequence of the tRNA^{Gln} 4336 mitochondrial DNA mutation. An MS PCR assay was carried out using the primers 4230F, MT4336M, and MT4336WT (see methods); PCR fragments of 147 bp or 127 bp were produced following amplification of the mutant or wild type target sequences, respectively. PCR products were separated on a 2% agarose/1% NuSieve gel. The position of the tRNA^{Gln} 4336 mutation was confirmed in all eight people with *Ava*II cutting sites; lanes 1 to 4 contain representative samples of people who had previously been found to harbour the mutation by restriction analysis; lane 5 contains an anonymous wild type control; lane 6 shows a water only negative control; lane 7 contains a 100 bp DNA ladder (Gibco BRL).

Results

SCREENING A CASE CONTROLLED ELDERLY

POPULATION FOR THE tRNA^{Gln} 4336 MUTATION The tRNA^{Gln} 4336 mitochondrial DNA mutation creates an *Ava*II restriction site,¹⁷ enabling detection by PCR and restriction digestion (see methods). Following *Ava*II digestion, a normal allele may be visualised as a single band of 288 bp, whereas a mutant allele appears as two fragments of 185 bp and 103 bp (fig 1). Allele specific mutagenically separated (MS PCR) analysis was used to confirm the exact position and sequence of the mutation at nucleotide 4336 in all people who were found to have the *Ava*II variant restriction site (see methods). This procedure uses a common upstream primer with two downstream primers, MT4336WT (wild type) and MT4336M (mutant). MT4336WT was designed to amplify

specifically when an A is present at position 4336, while MT4336M specifically amplifies when this is a G. Since MT4336M is 20 bp longer than MT4336WT, the 4336 G variant produces a PCR product that is 20 bp longer than the PCR product generated from the 4336 A wild type sequence (fig 2). Repeated experiments showed these primers to be specific and sensitive for both sequences. All eight people with variant *Ava*II restriction sites were shown to have a G at position 4336.

The frequency of the tRNA^{Gln} 4336 mitochondrial DNA mutation was determined within two community based elderly populations in East Anglia. In cohort 1 (see methods), where all subjects were aged older than 70 years, 65 pairs of demented patients and age and sex matched non-demented controls and a further 44 non-demented people were investigated. The mutation was not detected in any of the 65 demented subjects but was found to be present in 3/65 of the paired control (non-demented) samples as well as in 2/44 of the additional unmatched control samples, and was homoplasmic in all cases (fig 1). All five control subjects harbouring the mutation were homozygous for the E3 allele.

In cohort 2, 282 people currently aged older than 84 were screened. A Mini-Mental State (MMSE) score of 21 or less was used to define the likely presence of dementia. The mitochondrial 4336 mutation was found in 2/59 people with MMSE scores of 21 or less and one person whose lowest MMSE score was 26. All three were homoplasmic for this variant and had apo E3/4 genotypes.

CLINICAL DESCRIPTION OF THE CONTROL SUBJECTS CARRYING THE tRNA^{Gln} 4336 MUTATION

Since risk of dementia is strongly age related, it is important to be able to relate cognitive state to age in those who have this variant. Serial tests of cognitive ability have been performed on the people we have investigated. AGE CAT organicity scores and MMSE scores are presented for the five people from cohort 1 who had this mutation (table 1). These cases were female and were aged 74, 81, 84, 86, and 91 years at the time of their most recent formal assessment. None of these cases showed clinical features of Parkinsonism.

All five had been enrolled as non-demented controls by virtue of having repeated AGE CAT organicity scores below 3. Indeed, only one of them (No 3) had an organicity score of more than 0, and this was on a single occasion and returned to 0 on the subsequent testing. Clinically, all five respondents were non-demented by DSM-III-R criteria. Subject 2, aged 91, showed some decline in MMSE scores. However, this lady had visual difficulties and hence had problems completing the visual tasks in the MMSE. She had some impairment of short term memory but lived alone and was, according to her informant, self-caring. Subject 5 had a number of MMSE scores below 20. However, she had a low educational level and her scores did not decline.

Table 1 Serial MMSE (mini-mental state) and AGE CAT scores in the eight people with the tRNA^{Gln} 4336 mitochondrial DNA mutation. Cases 1–5 were non-demented by DSM-III-R criteria. AGE CAT was not included in the study design for cohort 2

Subject	Age at test	MMSE	AGE CAT
1	78 y, 8 mth	28	0
	80 y, 7 mth	27	0
	80 y, 9 mth	29	0
	81 y, 9 mth	30	Not done
2	89 y, 4 mth	23	0
	91 y, 4 mth	18	0
	91 y, 5 mth	16	0
3	84 y, 1 mth	23	0
	86 y, 0 mth	21	1
	86 y, 4 mth	24	0
4	81 y, 10 mth	25	0
	83 y, 10 mth	23	0
	84 y, 2 mth	27	0
	84 y, 4 mth	23	0
5	84 y, 11 mth	23	Not done
	71 y, 2 mth	20	0
	71 y, 5 mth	19	0
	73 y, 4 mth	18	0
6	74 y, 2 mth	23	Not done
	79	22	
	86	20	
7	89	18	
	92	25	
	99	18	
8	102	Not done	
	80	29	
	87	27	
	90	26	

Subjects 1, 3, 4, and 5 were revisited in August–September 1995 in order to obtain repeat mouthwash samples, which confirmed the presence of the mitochondrial mutation. Although not formally examined, there was no evidence of cognitive decline. (Subject 2 declined to provide an additional mouthwash sample.)

MMSE scores of the three people from cohort 2 with this mutation are also shown (subjects 6, 7, and 8, table 1). Subject 6 was male and subjects 7 and 8 were females. These people had MMSE scores of more than 21 at ages 79, 92, and 90, respectively. Physician review of the most recent interview data from subjects 6, 7, and 8 suggest that all three were not demented (at ages 89, 102, and 90, respectively). The low MMSE scores of subject 6 can be accounted for by his very minimal education and subject 7 had visual impairment and refused some of the test items.

Discussion

We have investigated the frequency of the mitochondrial tRNA^{Gln} 4336 DNA mutation within two different elderly community based populations in Cambridgeshire. The mutation was detected in 8/334 (2.4%) of people who were not demented. This mutation was not detected in any person with dementia.

We were unable to obtain specimens for analysis from some respondents from the initial study populations who had severe dementia. Therefore, we may have failed to detect some demented cases with the mutation. However, unless the mutation causes very rapid decline in cognitive state once dementia becomes apparent, there is no reason to expect its presence to be under-represented in respondents with mild and moderate dementia.

These results conflict with two recent reports which suggested that the mitochondrial

tRNA^{Gln} 4336 mutation may be associated with an increased risk of AD.^{17,18} Shoffner *et al*¹⁷ detected the mutation in 2/62 (3.2%) brain samples obtained from patients who had pathological diagnoses of AD, in 5/73 (6.8%) brain samples of patients who were diagnosed as having both AD and Parkinson's disease (PD), and in 2/38 (5.3%) PD patients. These cases were aged between 68 and 83. Subsequently, Hutchin and Cortopassi¹⁸ reported the frequency of the mutation in brains of AD patients to be 8/194 (4.1%), compared to a frequency of 1/296 (0.34%) in controls aged 50 years or older.

The functional significance of a DNA variant can be analysed in a number of ways. First, one can show an excess of the variant genotype in AD cases *v* controls, as the two previous studies suggested.^{17,18} Although this mutation was rare in their control groups, the epidemiological methodology in these studies was prone to a number of potential biases. First, samples from brain banks do not necessarily reflect genotypes from the general population and should be compared with controls from other sources with caution (because of population stratification), particularly since there are suggestions that the frequency of this rare mutation may vary in different populations.^{17,18} Second, neither of the two previous studies used strict matched case-control pairs: Shoffner *et al*¹⁷ only examined 17 control brains of a similar age to their 62 AD cases and although Hutchin and Cortopassi¹⁸ genotyped 296 controls to match their 72 AD cases, these were clearly not matched one to one. The sex distribution of the cases and controls in these studies was not described.^{17,18} Furthermore, the mean age of death of the AD cases of Hutchin and Cortopassi¹⁸ was 81.3 compared to 69.2 for their controls. Although less than 1% of people aged under 70 have AD, this rises to about 15% in those aged 80 to 84 and more than 40% of those aged over 90.³² Thus, in this study, the cases had a much higher prior risk of dementia compared to the controls.

One also cannot ignore the possibility that allele frequencies at various loci (like apo E) may vary with age.³³ Unfortunately, the data that have been presented to show that there is no excess of the mitochondrial tRNA^{Gln} 4336 DNA mutation in aged populations¹⁸ lack power owing to the low frequency (generally <1%) of this variant^{17,18} (necessitating much larger sample sizes than 184 cases under 50 years and 296 cases over 50) and the use of a comparatively low age cut off.

A second way to show that a DNA variant affects AD risk is to show that it results in accelerated dementia. This trend is not borne out by our data; the ages of six of the AD cases with the mitochondrial tRNA^{Gln} 4336 DNA mutation have been documented and range from 60 to 76 (mean 67.8).¹⁸ Our subjects with the 4336 mutation were non-demented at ages 74, 81, 84, 86, 89, 90, 91, and 102 years and the non-demented aged control previously reported with this mutation was 79 years old.¹⁸ Thus, these people were non-demented at ages which are past the age of risk for this apparently

pathogenetic mutation. Indeed, five subjects with the 4336 variant were non-demented in the 81 to 89 year age range, where >15% of the general population are demented, and another three were non-demented aged older than 90, where about 40% of the population are demented.³² Thus, if this mutation is pathogenetic, it will have a low penetrance. Alternatively, the discrepancy between our results and those in the previous reports can be explained by postulating that tRNA^{Gln} 4336 variant does not confer an increased risk for AD but that a proportion of the mitochondrial molecules that harbour this variant harbour a pathogenetic mutation at another site.

In summary, our results suggest that the mitochondrial tRNA^{Gln} gene variant at position 4336 is not a high penetrance AD mutation. However, in view of the large body of data which implicates mitochondrial metabolism in the pathogenesis of AD, the possibility of other variants playing a role in this disease should not be discounted.

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