

## Analysis of European mitochondrial haplogroups with Alzheimer disease risk

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### Abstract

We examined the association of mtDNA variation with Alzheimer disease (AD) risk in Caucasians (989 cases and 328 controls) testing the effect of individual haplogroups and single nucleotide polymorphisms (SNPs). Logistic regression analyses were used to assess risk of haplogroups and SNPs with AD in both main effects and interaction models. Males classified as haplogroup U showed an increase in risk (OR = 2.30; 95% CI, 1.03–5.11;  $P = 0.04$ ) of AD relative to the most common haplogroup H, while females demonstrated a significant decrease in risk with haplogroup U (OR = 0.44; 95% CI, 0.24–0.80;  $P = 0.007$ ). Our results were independent of APOE genotype, demonstrating that the effect of mt variation is not confounded by APOE4 carrier status. We suggest that variations within haplogroup U may be involved in AD expression in combination with environmental exposures or nuclear proteins other than APOE.

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Progressive dementia is a characteristic sign of the neurodegeneration suffered by Alzheimer disease patients. The slow atrophy of the frontal, parietal and temporal lobes usually develops in the sixth decade and is accompanied by senile plaques and neurofibrillary tangles [17]. Mitochondrial (mt) dysfunction has been proposed as an underlying mechanism of AD pathogenesis [2].

A significant decrease in energy metabolism within mitochondria is found restricted to the brain regions that are affected in AD including the hippocampus and temporal cortex [11]. Specifically, biochemical studies have

shown that there is a selective malfunction of complex IV (cytochrome c oxidase, COX) in brain and platelets of AD patients [6]. Complex IV is the terminal enzyme complex of the electron transport chain. The catalytic core of the enzyme is composed of mitochondrially encoded subunits COI, COII, COIII plus 10 subunits that are encoded by the nuclear genome.

Although cybrid (cytoplasmic hybrid) evidence strongly supports the role of mt genes in AD pathology [18], no causative mt mutations have been revealed. However, it has been suggested that inherited European mt haplogroups K and U may influence AD risk in Caucasians by neutralizing the effect of the major AD risk factor polymorphism apolipoprotein (APOE) 4 allele [3]. Haplogroups are defined by ancestral polymorphisms that are continent-specific nine

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Table 1  
Characterization of European haplogroups

| Haplogroup | 1719 | 4580 | 7028 | 8251 | 9055 | 10398 | 12308 | 13368 | 13708 | 16391 |
|------------|------|------|------|------|------|-------|-------|-------|-------|-------|
| H          |      |      | C    |      |      | A     |       |       |       |       |
| I          | A    |      | T    | A    |      | G     |       |       |       | A     |
| J          |      |      | T    |      |      | G     |       |       | A     |       |
| K          |      |      | T    |      | A    | G     | G     |       |       |       |
| T          |      |      | T    |      |      | A     |       | A     |       |       |
| U          |      |      | T    |      |      | A     | G     |       |       |       |
| V          |      | A    | T    |      |      | A     |       |       |       |       |
| W          |      |      | T    | A    |      | A     |       |       |       |       |
| X          | A    |      | T    |      |      | A     |       |       |       |       |

primary mt haplogroups have been identified in European populations (H, I, J, K, T, U, V, W, X) [20].

To test the association of individual haplogroups with AD risk, we genotyped 10 single nucleotide polymorphisms (SNPs) that define the nine pre-defined European haplogroups (Table 1). Since AD risk is strongly influenced by sex and APOE genotype, we tested interaction models for each risk factor. In one model we tested for interaction or confounding between APOE4 carrier status (APOE4+, APOE4–) and mitochondrial haplogroups and SNPs. A second interaction model was tested similarly with sex.

A total of 989 unrelated late-onset AD patients and 328 controls were examined in this study. Diagnosis of AD was consistent with NINDS-ADRDA consensus diagnostic criteria [14]. One proband from each of the family-history positive families ( $N = 579$ ) was chosen for inclusion into the case group plus 410 sporadic cases. These samples were derived from three sources previously described [16]. Spouses of AD patients were used as controls if individuals had no obvious signs of cognitive or neurological impairment when enrolled in the study as determined by personal interview by clinical personnel of Duke CHG, the Joseph and Kathleen Bryan ADRC or Vanderbilt PHG. Mean age-at-onset (AAO) in affected individuals in the overall sample was  $69.8 \pm 8.4$  years while the mean age-at examination (AAE) was  $76.0 \pm 9.6$  years. AAO was recorded as that age at which the first symptoms were noted by the participant or a family member. Control mean AAE was  $68.1 \pm 8.9$  years. The AD case group was composed of 34.9% males and 65.1% females while the control group consisted of 45.1% males and 54.9% females. All participants were Caucasian Americans. Written consent was obtained from all participants in agreement with protocols approved by the institutional review board at each contributing center.

Genomic DNA was isolated from whole blood samples by the DCHG DNA banking Core using Puregene (Gentra Systems, Minneapolis, MN). APOE PCR amplification of 30 ng dried genomic DNA was performed in 10  $\mu$ l reactions containing 1 $\times$  PCR reaction buffer, 0.24 mM dNTP mix, 1.5 mM  $Mg^{2+}$ , 10% dimethyl sulfoxide, 0.5 U Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA) and 0.08  $\mu$ g of each forward and reverse APOE primer. Thermocycling consisted of a 94 °C hold for 3 min for denaturation and

polymerase activation, 25 cycles of 94 °C for 15 s, 65 °C for 20 s and 70 °C for 50 s, and a final extension step at 70 °C for 10 min. The PCR product was treated with 5 U of *Hha*I enzyme (Invitrogen, Carlsbad, CA) in 0.5 $\times$  *Hha*I reaction buffer and digested for 90 min at 37 °C. The reaction product was loaded onto a 6% nondenaturing acrylamide gel and run at 56 mA for 30 min. The gel was treated with 1 $\times$  SYBR<sup>®</sup> Gold nucleic acid gel stain (Molecular Probes, Eugene, OR) for 5 min. DNA fragments were visualized with a Hitachi FMBio II fluorescent scanner. Fragment sizes were analyzed with Bioimage software to generate an APOE genotype for each sample. Methods for mitochondrial SNP genotyping and classification of haplogroups are described elsewhere [19].

Data were stored and managed using the Pedigene<sup>®</sup> system [10]. All statistical analyses were performed using SAS software release 8.1 (SAS Institute Inc, Cary, NC). Statistical significance was declared at  $\alpha = 0.05$ . A Student's *t*-test was conducted to test for differences in AAE between cases and controls, with a significant difference found ( $P$ -value < 0.0001). To assess differences in distribution of sex between cases and controls we used a chi-square test, and found a significant difference in distribution ( $P$ -value = 0.0004). Therefore, to adjust for potential confounding we used AAE and sex as covariates in the analyses.

Logistic regression analyses were used to assess risk of mitochondrial haplogroups and SNPs with AD in both main effects and interaction models. All analyses were carried out with AAE, sex, and APOE4 status as covariates. The APOE4 carrier status (+, –) was coded as one if an individual has at least one APOE4 allele and zero otherwise. Two different interaction models, one including interaction terms for APOE4 carrier status and mt haplogroups/SNPs and one including terms for sex and mt haplogroups/SNPs, were used to examine if risk of AD conferred by haplogroups or SNPs differed significantly by sex or APOE4 status. We performed unconditional logistic regression to generate odds ratios and their associated 95% confidence intervals to assess risk of AD among members of different haplogroups relative to the most common European haplogroup, H. In addition, we used unconditional logistic regression to assess risk of AD among carriers of each mitochondrial SNP versus those not carrying the change.  $P$ -values reported for haplogroups and

Table 2  
Odds ratios of mt haplogroups stratified by sex

| Haplogroup | Males <sup>a</sup> |                 |          |          | Females <sup>b</sup> |                 |          |          |
|------------|--------------------|-----------------|----------|----------|----------------------|-----------------|----------|----------|
|            | OR                 | <i>P</i> -value | Lower CI | Upper CI | OR                   | <i>P</i> -value | Lower CI | Upper CI |
| I          | 0.39               | 0.19            | 0.10     | 1.58     | 0.43                 | 0.16            | 0.14     | 1.39     |
| J          | 0.84               | 0.64            | 0.41     | 1.71     | 0.85                 | 0.64            | 0.42     | 1.70     |
| K          | 0.66               | 0.31            | 0.30     | 1.47     | 0.53                 | 0.10            | 0.25     | 1.12     |
| T          | 0.99               | 0.98            | 0.46     | 2.14     | 0.70                 | 0.33            | 0.35     | 1.43     |
| U          | 2.30               | 0.04            | 1.03     | 5.13     | 0.44                 | 0.007           | 0.24     | 0.80     |
| V          | 0.68               | 0.54            | 0.20     | 2.30     | 0.90                 | 0.88            | 0.23     | 3.57     |
| W          | 2.40               | 0.29            | 0.47     | 12.18    | 1.53                 | 0.66            | 0.22     | 10.56    |
| X          | 0.61               | 0.54            | 0.13     | 2.91     | 1.29                 | 0.80            | 0.17     | 9.50     |

<sup>a</sup> *n* cases = 345, *n* controls = 148.

<sup>b</sup> *n* cases = 644, *n* controls = 180.

SNPs parameter estimates are from the Wald chi-square associated with that particular haplogroup or SNP, and are not adjusted for multiple testing.

Results from the unconditional logistic regression analyses adjusted for sex, AAE and APOE4 carrier status (+, –) were performed for both sporadic cases (*N* = 410) and family history positive cases (*N* = 579) versus controls. Variables of sex, AAE, and APOE4 status were significantly associated with increased risk of AD (*P*-value = 0.01, *P*-value < 0.0001, *P*-value < 0.0001, respectively). Since the results and mt haplogroup frequencies for both the sporadic and family history positive data sets were similar, overall results (*N* = 989 cases) are presented here.

Because the APOE4 allele is associated with a known higher risk of AD [5], we assessed both interaction and confounding effects between APOE4 carrier status and mitochondrial haplogroups and SNPs using logistic regression models. Employing the likelihood ratio test statistic, the general model was superior to the interaction model in terms of overall fit (*P*-values all >0.05) (data not shown). However, the interaction model with sex and mt haplogroup revealed a significant interaction between haplogroup U and sex (*P*-value = 0.009). Since the interaction model with sex showed significant results, we conducted logistic regression analyses separately for males and females.

Stratification by sex demonstrated a prominent difference in the direction of association when sexes were considered separately (Table 2). Males classified as haplogroup U showed a significant increase in risk (OR = 2.30; 95% CI, 1.03–5.13; *P* = 0.04) of AD relative to the most common haplogroup H, while females demonstrated a significant decrease in risk with the U haplogroup versus females classified as haplogroup H (OR = 0.44; 95% CI, 0.24–0.80; *P* = 0.007).

Analysis of individual SNPs revealed that risk of AD was increased in males who carried the 10398A allele (OR = 1.74; 95% CI, 1.08–2.82; *P* = 0.02) versus males that carried the G allele. In addition, females carrying SNP 7028T showed a reduction of AD risk, (OR = 0.66; 95% CI, 0.44–1.00; *P*-value = 0.05). Finally, allele 12308G appeared to be protective in the female stratum (OR = 0.56;

95% CI, 0.35–0.90; *P* = 0.02). All three variants (10398A, 12308G, and 7028T) define haplogroup U and demonstrate sex-specific effects that are consistent with the haplogroup analysis. Increased risk of AD in males classified as U appears to be driven by the 10398A polymorphism while the presence or absence of 10398A was not statistically significant in females. Additionally, both polymorphisms 12308G and 7028T showed a decrease in risk of AD in females most likely contributing to the protective effect of haplogroup U in this group. Results for these two SNPs independently were not statistically significant in males.

Defects of energy metabolism are strongly apparent within brain regions of AD patients, however the cause(s) are not yet known. Previous studies of mt genes have failed to uncover conclusive evidence to support the pathogenic role of mt mutations in AD expression. In this study, we report a significant effect of inherited mt variation with disease susceptibility that is sex specific and independent of APOE genotype.

Initially, our overall results including both sporadic and family positive AD cases did not reveal significant overall association of any European haplogroup with increased risk of AD. Similar overall results were reported in two separate studies that assessed risk and mt haplogroup alone [3,4]. Since female sex is associated with a higher risk of AD, we assessed both interaction and confounding effects between sex and mitochondrial haplogroups. The analysis demonstrated a surprising sex-specific association of haplogroup U with AD risk where males carrying haplogroup U have a 2.3-fold increase in risk and females carrying U demonstrated approximately a half-fold decrease versus carriers of haplogroup H in both sexes.

The sex-specific result is intriguing since AD risk appears to be higher in females than males [1]. We suspect that this sex-specific risk result could reflect a functional difference of the mt SNPs in haplogroup U that is dependent on some factor associated with sex. A recent study investigating the association of longevity with mt haplogroups also showed a sex-specific effect of haplogroup U where U was observed less frequently in male centenarians (~4%) than in control males (23%) (*P*-value = 0.05). [7]. Our study also revealed

a lower frequency of the U haplogroup in age-matched controls (6.8%) compared to AD males (13.3%). This finding, taken together with our results, suggests that inheritance of U may have a negative effect on aging in Caucasian males.

A sex-specific effect of APOE4 has been reported in AD studies [8] and in a study of age-related cognitive decline where risk is significantly higher in women [15]. Since our results are independent of APOE genotype, we propose that the sex dependent risk conferred by haplogroup U is caused by an unrelated molecular mechanism involved in oxidative stress.

Our results contrast that of Carrieri and colleagues who reported a significant protective effect of haplogroups K and U in Italians carrying the APOE4 allele [3,4]. We demonstrated that the effect of mitochondrial haplogroups and SNPs on AD risk is independent of APOE status. Again, significant association of haplogroup U was only revealed when our data set was stratified by sex, which is not presented in the previous study.

Additionally, the frequency of haplogroup U was found to be greater in stroke patients and has been associated with elevated risk for migrainous occipital stroke [13]. Further analysis demonstrated that the association was due to a high frequency of subcluster U5 [9]. Given the diverse nature of the U haplogroup and its apparent association with a related neurologic disease, we suggest that variants within the different subclusters of U may predispose individuals to AD risk.

Recently, we reported an association of decrease risk of Parkinson disease with haplogroups J and K ( $P = 0.02$ ) in a Caucasian population [19]. Furthermore, we demonstrated that the frequency of SNP10398G was higher in controls than PD cases ( $P = 0.0001$ ). We also found a similar sex-specific association of this SNP in the PD study whereby the protective effect was stronger in females [12]. These findings indicate that 10398A may be a deleterious polymorphism thus predisposing carriers to both neurodegenerative diseases. Moreover, this effect appears to be modified by sex in AD and PD.

In summary, we have provided evidence for the contribution of mt variation in the risk of AD development in Caucasians. We do not support the previously reported association of haplogroup K with decrease risk of AD; instead we revealed a sex-specific association of haplogroup U with AD risk. Furthermore, our results were independent of APOE genotype demonstrating that the effect of mt variation is not confounded by APOE status. Evidence presented here supports our previous finding that the 10398 polymorphism appears to be important in risk of neurodegenerative diseases. The biological relevance of mt haplogroups and SNPs to specific loss of cholinergic neurons within AD brains remains to be elucidated; however, we suggest that variations within haplogroup U may be involved in pathogenesis alone, in combination with environmental exposure, or in synergy with nuclear proteins other than APOE.

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