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The Frequency of Point Mutations in Mitochondrial DNA Is Elevated in the Alzheimer's Brain

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Using a PCR-based strategy, we found that point mutation frequencies in mitochondrial DNA (mtDNA) were 2- to 3-fold higher in the parietal gyrus, hippocampus, and cerebellum from subjects with Alzheimer's disease (AD) compared to normal controls. In contrast, levels of a commonly studied deletion mutation, mtDNA^{4977}, were not elevated in AD. The frequency of point mutations did not vary significantly among the three brain areas, whereas the frequency of mtDNA^{4977} was 15- to 25-fold lower in the cerebellum in comparison to the cortex; this regional variation was seen in both the normal and Alzheimer's brain. In blood mtDNA, point mutation frequencies were not elevated in AD patients. The elevated frequency of point mutations in all three brain regions is consistent with the idea that increased oxidant stress is associated with AD.

Key Words: **Alzheimer's disease; mitochondrial DNA; mutations; brain.**

There is widespread evidence for oxidative stress in the Alzheimer's brain (1). One measure of such damage is the DNA oxidative adduct, oxo^{8}dG. In Alzheimer's disease (AD), elevations in the level of oxo^{8}dG in mitochondrial DNA (mtDNA) from the brain are observed, suggesting that mitochondria are subject to excess oxidative stress (2). MtDNA may be an especially useful cellular macromolecule for the analysis of oxidative damage. Free radical attack on DNA results in oxidative adducts which, if not repaired, are highly mutagenic upon DNA replication (3). Since mtDNA replicates continuously throughout life (4, 5), the frequency of point mutations in mtDNA is predicted to rise upon oxidative damage to mitochondria. Although the etiology of oxidant stress is not identified by mtDNA analysis, there is increasing evidence that oxidative phosphorylation is compromised in AD (6), and that disturbances in mitochondrial respiration lead to increased generation of reactive oxygen species (7). These observations have prompted numerous studies to determine the relationship of sporadic or maternally inherited mtDNA mutations with AD (6). Nevertheless, a strong association of mtDNA mutations with AD in a majority of patients remains controversial.

We report here on the application of a PCR-based technique that detects point mutations at a restriction site with frequencies as low as 10^{-5}. We found that the frequency of point mutations in mtDNA from the Alzheimer's brain was two- to threefold higher than in the normal brain. In contrast, the level of a commonly found deletion mutation, mtDNA^{4977} (8), was not significantly elevated. Furthermore, whereas regional variation in the frequency of mtDNA^{4977} was observed, no such variation was found for the frequency of point mutations. We suggest that these results support the hypothesis that AD is associated with disturbances in oxidative metabolism in the brain.

MATERIALS AND METHODS

Tissue samples. Brain samples were obtained from autopsies performed at St. Louis University-affiliated hospitals. All brains were histologically examined to confirm AD neuropathologically in the demented patients according to NIH criteria (9). In the control group, there was no evidence of neurodegenerative disease. In all subjects, no evidence of old or recent hypoxemic/ischemic changes were noted. Blood samples were obtained from AD patients—diagnosed according to established clinical criteria (9)—and normal caregivers living together in a nursing home. The study protocol and informed consent procedures were approved by the St. Louis University Institutional Review Board.

Internal standards. These were made by PCR from tissue DNA as described (10). A linking primer, 5′AGTACACCGAGCCCGTATTTACCCTATAGCGAGAACCAACACCTC, was used for construction of the wild-type internal standard (together with primer H8738, 5′-ATAAGAGATCAGGTTCGTCC) and the deletion internal standard (together with H13650, 5′-GGGGAAGCGAGGTTGACCTG), whereas the internal standard for measuring point mutations was made using the linking primer 5′-GGAGGTAAGCCTGGTTAGGCTGGTG together with L15978, 5′-CACCATTAGCACCCAAAGC. The internal standards were synthesized by PCR in the presence of [α-^{32}P]dATP, gel purified, and stored at −20°C in TE buffer (10 mM

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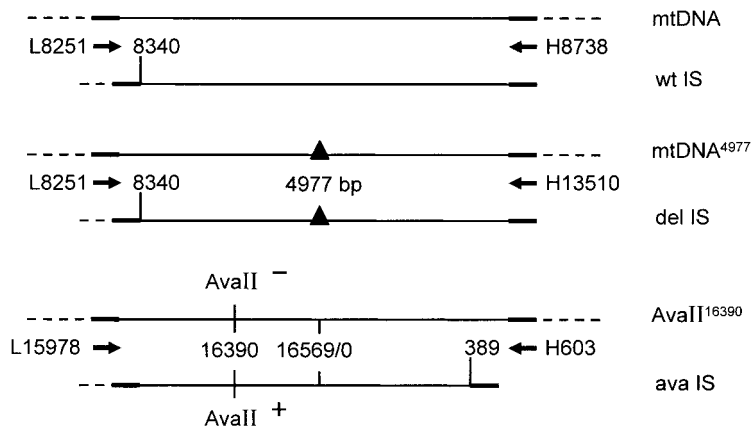

FIG. 1. Internal standards for competitive PCR. Diagrammed are the structures of the internal standards relative to their corresponding mtDNA templates. Primers (numbered according to the genomic map position of their 5′ ends) are indicated by the arrows; their homologous sequences in the templates are designated by the thickened bar; dashed lines indicate flanking DNA sequences. The wild-type (wt) and mtDNA^{4977} (del) internal standards (IS) were constructed so that the L8251 sequence was appended to mtDNA at nt 8340. The AvaII^{16390} internal standard (Ava IS) had the H603 sequence appended at nt 389. The map position of the AvaII site is designated, as is the map origin (16569/0) for the circular mtDNA genome.

Tris–HCl, pH 7.5, 1 mM EDTA). The number of internal standard molecules was calculated from their measured radioactivity, the known specific activity of the labeling isotope, and their adenine content.

PCR analysis. MtDNA^{4977} was selectively amplified by "short-cycle" PCR (8) using the primers L8251 and H13510. Wild-type mtDNA was amplified using the primers L8251 and H8738. The point mutation analyzed (termed AvaII^{16390}) was the loss of an AvaII restriction site located at nucleotide (nt) 16390 of the mitochondrial genome. AvaII^{16390} mutant molecules were selectively amplified by using tissue DNA first exhaustively digested with AvaII before PCR and primers (L15978 and H603) flanking the AvaII site.

Brain DNA was prepared from 30–100 mg frozen (−70°C) tissue and blood DNA from 2- to 5-ml samples (11). DNA was digested overnight with AvaII and then a further 3 h in the morning with additional enzyme. DNA was purified by phenol/chloroform extraction and ethanol precipitation, and then dissolved in TE buffer and stored frozen at −20°C. This DNA was also used for the determination of the number of wild-type and mtDNA^{4977} molecules since there are no AvaII sites in the regions amplified.

DNAs were boiled for 5 min before addition to 25-μl PCR mixtures containing [α-^{32}P]dATP (0.2–1.0 Ci/mmol), 10 mM Tris–HCl (pH 9.0 at 25°C), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM oligonucleotide primers (diagramed in Fig. 1), and 0.5 units *Taq* DNA polymerase. All amplification reactions were performed for 30 cycles of 1 min 94°C, 1 min 54°C, and either 1 min 72°C (for the wild-type and mtDNA^{4977} reactions) or 2 min 72°C (for the AvaII^{16390} reactions). The DNA sequence of primers employed, in addition to those described above, were L8251, 5′-GCCCGTATTTACCCTATAGC; H13510, 5′-TGGAGTAGAAACCTGTGAGG; and H603, 5′-TGCTTTGAGGAGGTAAGC.

Following amplification, the wild-type and mtDNA^{4977} reaction mixtures were applied to a 1% agarose gel and electrophoretically fractionated. After electrophoresis, the gel was soaked in 95% ethanol, compressed to ~1/5th its original thickness, and then subjected to PhosphorImage analysis. The AvaII^{16390} reaction mixtures after amplification were extracted with phenol/chloroform, made to 3 M ammonium acetate, and the DNA precipitated with 2 vol ethanol. After solubilization, DNA was digested for 3 h with AvaII, electrophoretically fractionated, and the gel was then processed as above.

RESULTS

Experimental strategy. The frequency of deletion and point mutations in mtDNA was calculated by quantification of the number of mutant molecules relative to wild-types using competitive PCR (12). The deletion mutation analyzed was mtDNA^{4977}, whereas the point mutation was AvaII^{16390}—*i.e.*, the loss of an AvaII restriction site located at nt 16390 of the mitochondrial genome. Each type of template (*i.e.*, wild-type, mtDNA^{4977}, or AvaII^{16390}) was separately quantified relative to a known amount of an internal standard that was included in the PCR. Figure 1 diagrams the structure of each internal standard relative to the mtDNA template for which it served as a measure. Internal standards were constructed so as to yield a smaller amplification products compared to those from their corresponding mtDNA templates. After amplification in the presence of [α-^{32}P]dATP, the labeled products were electrophoretically fractionated and quantified using a Molecular Dynamics PhosphorImager. The number of mtDNA templates was calculated from the ratio of radioactivity in the amplification products from the tissue DNA relative to the internal standard, after correction for their relative adenine content.

Validation of the quantification strategy. To verify that competitive PCR provided consistent and reliable quantification, we performed trial reactions containing the same amount of tissue mtDNA but differing amounts of internal standard. The data in Fig. 2A show that quantification of the number of mtDNA^{4977} molecules in this trial (mean ± SD: 35 ± 2 × 10^4) varied little with respect to either the amount of internal standard or the cycles of PCR. Similar trial reac-

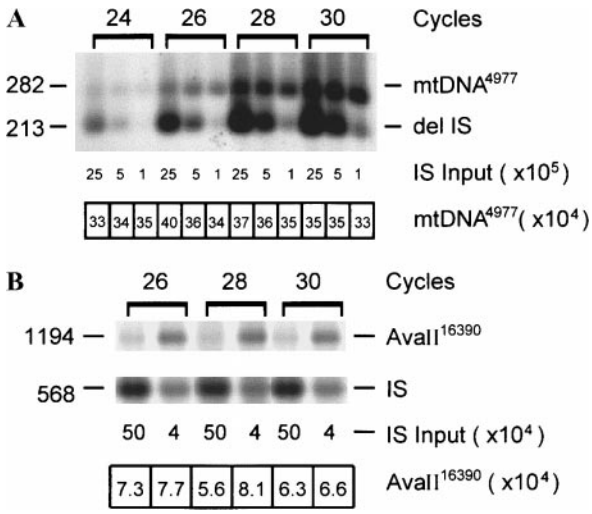

FIG. 2. Validation of quantification strategy. (A) Quantification of mtDNA^{4977}. Mixtures of the identical amount of tissue DNA and 1, 5, or 25 $\times$ 10^5 molecules of the deletion internal standard (del IS) were amplified for 24, 26, 28, and 30 cycles. Shown is a PhosphorImage scan of the electrophoretically fractionated reaction products with the size (bp) and origin of the labeled DNA bands indicated. The calculated number of mtDNA^{4977} molecules per reaction in the tissue sample is indicated below. (B) Quantification of AvaII^{16390}. Mixtures of the identical amount of tissue DNA and 4 or 50 $\times$ 10^4 molecules of point mutation internal standard (IS) were amplified for 26, 28, and 30 cycles. Shown is a PhosphorImage scan of the PCR AvaII^{16390} and IS product bands. The calculated number of AvaII^{16390} molecules per reaction is shown below.

tions to ascertain the variability in measuring AvaII^{16390} molecules showed that here too quantification was consistent with respect to either changes in the ratio of mutant to internal standard molecules or the cycles of PCR (Fig. 2B; mean $\pm$ SD: 7.3 $\pm$ 1.5 $\times$ 10^4). Reconstruction experiments demonstrated that mtDNA^{4977} could be detected at frequencies as low as 1×10^{-6}, whereas for AvaII^{16390} the sensitivity of detection was approximately 1×10^{-5} (data not shown).

Mutation frequencies in the normal and Alzheimer's brain. We measured the frequency of mtDNA^{4977} and AvaII^{16390} in the parietal gyrus, hippocampus, and cerebellum from some 20 subjects with AD and an approximately equal number of normal subjects of similar ages. The frequencies of point and deletion mutations in the parietal gyrus and hippocampus were separately averaged for each subject and are presented as cortex values. This simplification was appropriate since (i) the mean frequency of each mutation was nearly the same in the parietal gyrus and hippocampus, and (ii) mutation frequencies in those brain areas showed moderate to high correlation among subjects ($r \geq 0.5$ for either mutation in both the normal and Alzheimer's group). Four subjects (2 normals, 2 Alzheimer's) were homoplasmic for AvaII^{16390}, presumably reflecting naturally occurring sequence polymorphisms.

The results from this study are presented in Fig. 3, where the frequencies of mtDNA^{4977} and AvaII^{16390} in the cortex and cerebellum of each subject are plotted versus the subject's age. The main finding was that the frequency of AvaII^{16390} was significantly elevated in the Alzheimer's compared to the normal brain (Figs. 3A and 3B). Considering only subjects above age 60, the mean frequency ($\times 10^{-4}$) of AvaII^{16390} in the cortex was nearly 3-fold higher in the Alzheimer's compared to the normal brain: 3.5 $\pm$ 0.9 (SEM) versus 1.3 $\pm$ 0.5, respectively. A nearly 2.5-fold increase was observed in the cerebellum (Alzheimer: 2.8 $\pm$ 0.5; normal: 1.2 $\pm$ 0.5). For both brain areas, these differences were statistically significant ($P < 0.05$) using the Mann–Whitney U test since mutation frequencies within groups were

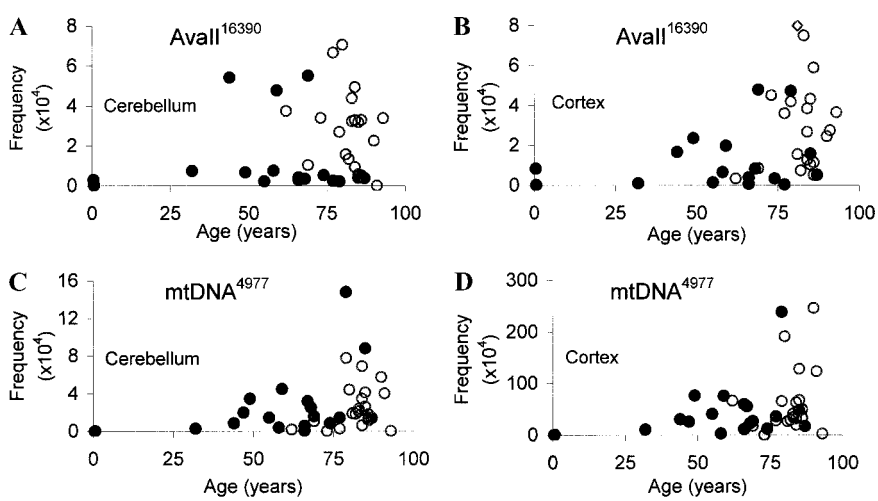

FIG. 3. Mutation frequencies in Alzheimer's and normal brains. Plotted are the frequencies of AvaII^{16390} versus the subject's age for the cerebellum (A) and cortex (B) and the frequencies of mtDNA^{4977} versus the subject's age for the cerebellum (C) and cortex (D). Filled circles, normal subjects; open circles, Alzheimer; the open diamond in B is the datum from an Alzheimer subject with a frequency of 16.5 $\times$ 10^{-4}.

not normally distributed. Other characteristics of the subjects (sex, race, time interval between death and freezing of the brain samples, and coexisting disease) did not correlate with the frequency of AvaII^{16390}.

The average age of the subjects in the Alzheimer's group (82 years) was, however, higher than that for the group of normals above age 60 (71 years). Because some studies indicate that the frequency of point mutations in mtDNA rises with age (13), the data were further analyzed to determine whether age differences might have contributed to the apparent association of elevated point mutation frequencies with AD. In this study, we found no significant correlation with age for the frequency of AvaII^{16390} (averaged over all three brain areas for each subject) in either the Alzheimer's group ($r = 0.04$) or in the group of normal subjects of all ages ($r = 0.18$). Furthermore, the five youngest Alzheimer's subjects (average age, 72 years) had a higher mean frequency of the AvaII^{16390} mutation than the five oldest normals (average age, 80 years): 3.8×10^{-4} versus 1.1×10^{-4}, respectively. Both values were not significantly different from the mean of the corresponding groups as a whole. Thus, it appeared that age was not a significant confounding factor for the correlation of AD with the frequency of the AvaII^{16390} mutation.

In contrast to the frequency of the AvaII^{16390} mutation, the mean frequency of the deletion mutation, mtDNA^{4977}, was not significantly different in the Alzheimer's compared to the normal brain (Figs. 3C and 3D). Considering only subjects aged 60 and above, the mean frequency of mtDNA^{4977} ($\times 10^{-4}$) in the cortex of the Alzheimer's brain was 59.6 ± 14.4 (SEM) versus 51.0 ± 15.7 for normals. As has been reported previously for both normal and Alzheimer's subjects (14–16), a markedly lower frequency of mtDNA^{4977} was found in the cerebellum in comparison to the cortex (~15-fold for normal subjects, ~20-fold for Alzheimer's subjects). However, as in the cortex, there was no significant difference in the frequency ($\times 10^{-4}$) of mtDNA^{4977} in the AD versus the normal cerebellum: 2.54 ± 0.51 versus 3.26 ± 1.36, respectively.

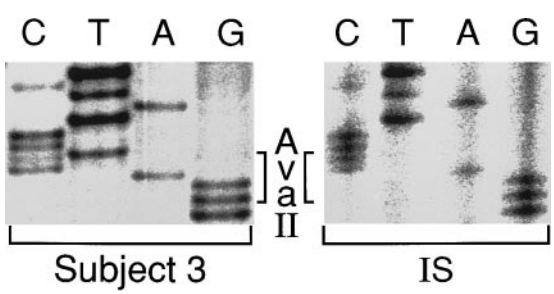

FIG. 4. DNA sequence of the AvaII^{16390} mutation. Shown are PhosphorImages of the sequencing gels for the region surrounding the AvaII site from an AvaII^{16390} mutant PCR product of an AD subject (#3) and the corresponding region from the internal standard (IS) which contains the wild-type sequence. The bases comprising the AvaII site are indicated.

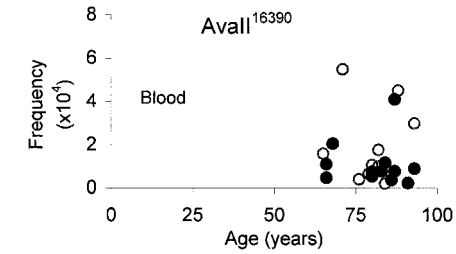

FIG. 5. Frequencies of AvaII^{16390} in the blood. Filled circles, normals; open circles, Alzheimer.

Several studies have shown that the frequency of mtDNA^{4977} rises with age in the brain (8, 14, 15, 17, 18) and one has reported that its frequency declines in AD subjects over the age of 75 (16). In this series of subjects we did not observe either phenomenon. The correlation of mtDNA^{4977} frequency with age was only slight in both the cortex ($r = 0.38$, 95% CI 0–0.7) and the cerebellum ($r = 0.46$, 95% CI 0–0.75). In the AD subjects a slight but nonsignificant positive, rather than negative, correlation was found for mtDNA^{4977} frequencies in the cortex and cerebellum ($r \sim 0.25$ for both brain areas).

Sequence analysis of the AvaII^{16390} mutations. Mutations within any one of the five nucleotides comprising the AvaII site are scored as a mutation by our analysis. DNA sequence analysis of the AvaII^{16390} PCR product from one AD subject revealed that the major sequence change was a C → T transition mutation occurring at position 5 of the AvaII site (Fig. 4). Because of the sequence context of the AvaII site, that mutation created a new HinfI site at that position (GGACCC [AvaII] → GGACTC [HinfI]). By digestion of AvaII^{16390} PCR products with HinfI we found that the predominant mutation in all AD and normal subjects was the same C → T transition mutation (data not shown).

Frequency of AvaII^{16390} in blood mtDNA. There is some evidence that disturbances in mitochondrial oxidative phosphorylation are present in formed blood elements in AD patients (19). Since we observed an increase in the frequency of AvaII^{16390} in the brain, we analyzed whole blood mtDNA obtained from AD patients and their normal caregivers. Figure 5 shows that, in contrast to the brain, the frequency of AvaII^{16390} ($\times 10^{-4}$) was not significantly elevated in blood mtDNA from AD patients (AD: 1.86 ± 0.52; normal, 1.13 ± 0.31).

DISCUSSION

Our studies indicate that the frequency of the point mutation, AvaII^{16390}, is two- to threefold higher in sub-

jects with AD compared to normals. This difference is observed in the parietal gyrus and hippocampus, both of which invariably show the pathology associated with AD, as well as in the cerebellum, which is usually spared severe neural degeneration (9, 20). The uniform elevation in point mutation frequencies across the three brain areas is consistent with the idea that AD has a systemic nature with manifestations not restricted to the cortical regions of the brain (20–24). However, we do not observe elevated point mutation frequencies in blood mtDNA from AD patients in whom platelet derived mitochondrial respiratory enzyme activity has been reported to be lower than age-matched normals (19). Although pathogenic mtDNA mutations are often much lower in blood cells compared to postmitotic tissues (25), we do not think that mitotic selection drove down frequencies of AvaII^{16390} mutation levels. The AvaII site is located in a non-coding region of the human mitochondrial genome. Indeed, it maps to a hypervariable segment of that region (26), which probably accounts for our observation that 4 of 40 subjects examined were homoplasmic for AvaII^{16390} in brain mtDNA.

The elevated frequencies of AvaII^{16390} in the Alzheimer's brain may reflect increased oxidative stress which is associated with AD (1). Oxidative damage to mtDNA, as indicated by measurements of oxo^{8}dG, is higher in the cortex and cerebellum of subjects with AD compared to age-matched controls (2, 27). Likewise, we find that point mutation frequencies are elevated in both the cortex and cerebellum of the AD brain. Furthermore, the two- to threefold elevation in mutation frequencies is on the same scale as the roughly 3 fold increase seen in oxo^{8}dG levels (2). Oxidative adducts like oxo^{8}dG are mutagenic upon DNA replication (28). In both the normal and AD subjects studied here, a C → T transition mutation located at the terminal position of the AvaII site was the predominant sequence change responsible for the formation of the AvaII^{16390} mutation. The fact that both normal and AD subjects showed the same mutation at the same site suggests that the same mutagenic process is operative in both groups, rather than a qualitatively different mechanism unique to the Alzheimer's group. Whether that mechanism is oxidative damage remains to be shown.

A unique aspect of this study is that both point and deletion mutations in mtDNA are measured from the same tissue samples. In contrast to the point mutation, AvaII^{16390}, we find that the mtDNA^{4977} deletion mutation is not elevated in the AD brain. Although one study reports increased levels of that mutation in the cerebral cortex of AD subjects (16), others show no elevation (29–31), and still another reports decreased levels (27). Two factors may help explain these differences. First, variable extents of neuronal loss during the course of the disease may skew mutation levels due to preferential cell death of neurons with high mutation frequencies. Second, mtDNA deletion mutations have been proposed to have a replicative advantage over full-length, wild-type molecules (32). This feature together with a stochastic element to the genesis of a deletion molecule relative to the course of the disease may combine to generate highly variable levels of mtDNA^{4977} among a group of AD subjects.

Among the group of normal subjects, we do not observe a marked increase with age of either AvaII^{16390} or mtDNNA^{4977} in the brain. Several reports show an age association of mtDNA^{4977} in the brain (8, 14, 15, 17, 18), and some (13, 33) but not all (34) studies show that point mutations in mtDNA rise with age. In those studies, the most pronounced increase in mutation levels is seen in subjects over the age of 75. Most of the tissue samples we examined derive from subjects younger than that—indeed, for the five subjects under the age of 50 the average frequencies of both AvaII^{16390} and mtDNA^{4977} are lower than the corresponding averages for those over the age of 50. These results underscore the importance of conducting further studies in order to characterize more fully the association of mtDNA mutations with age.

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