Vol. 186, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1992 Pages 491-497

# IS A POINT MUTATION IN THE MITOCHONDRIAL ND2 GENE ASSOCIATED WITH ALZHEIMER'S DISEASE?

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Received June 8, 1992

A specific mitochondrial DNA mutation at position 5460 in the ND2 gene of the human mitochondrial genome was recently reported to exist in 10 of 19 patients with Alzheimer's disease, implying an association between this mtDNA mutation and the occurrence of the disease. We have analyzed tissues from 15 patients with Alzheimer's disease for the presence of the ND2 mutation, and have not been able to confirm these findings. We believe that this mutation is not specifically associated with Alzheimer's disease, but rather, is a neutral polymorphism present in the population. 

In recent years, both point mutations and large-scale rearrangements in human mitochondrial DNA (mtDNA) have been associated with specific mitochondrial encephalomyopathies (1). In addition, deletions of mtDNA, observable only by the polymerase chain reaction, or PCR (2), have now been detected in human tissues in both normal aging (3-5) and in age-related disorders, including Parkinson's disease (3,6), and in cardiomyopathies and ischemic heart disease (7-9).

To date, however, there is little evidence for the accumulation of point mutations in tissues during aging, even though such mutations, presumably resulting from oxidative damage to mtDNA, are likely to occur (10-14). It is for this reason that we were interested in a recent report indicating that a specific mtDNA point mutation was associated with Alzheimer's disease (AD) in 10 of 19 AD patients studied (15). These workers found two types of mutation, both at mtDNA position 5460 (16) in codon 331 of ND2 (i.e. subunit 2 of NADH dehydrogenase-ubiquinone oxidoreductase, or complex I of the respiratory chain): a G->A transition converting Ala (GCC)->

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Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; MERRF, myoclonus epilepsy with ragged-red fibers; mt, mitochondria; ND2, subunit 2 of mitochondrial NADH dehydrogenase-ubiquinone oxidoreductase (complex I of the respiratory chain; EC 1.6.99.3); PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SNuPE, single-nucleotide primer extension.

0006-291X/92 $4.00

Thr (ACC) in 3 AD patients, and a G->T transversion converting Ala (GCC)->Ser (TCC) in 5 other AD patients; both mutations coexisted in yet 2 other AD patients. The mutation was heteroplasmic in 2 of the 3 patients in the first group and in 4 of the 5 patients in the second group, that is, it coexisted with a subpopulation of wild-type mtDNAs; no wild-type mtDNA was found in the last 2 patients. No mutations were detected in all 11 normal brains studied, but the G->A mutation (homoplasmic) was found in 2 of 6 patients with amyotrophic lateral sclerosis. The initial mutations were first found in clones isolated from a λgt11 library of AD brain cDNA, and were then demonstrated by dideoxy sequencing of PCR products subcloned into M13 vectors. Further screening for these mutations was performed mainly by a single-nucleotide primer extension (SNuPE) method (17). This method is based on the ability of Taq polymerase to use only 1 of the 4 dNTPs to extend a primer whose 3' end, when hybridized to a PCR template, terminates immediately prior to nt-5460; the extended base should be complementary to the nucleotide on the template PCR strand.

We report here the use of mispairing PCR (18) followed by restriction fragment length polymorphism analysis (RFLP) to search for mutations at nt-5460 in tissues from 14 sporadic AD patients, 1 familial AD patient, and 5 controls. In none of them were any of the described (15) nucleotide changes detected. This result leads us to conclude that the base substitution at the first position of codon 331 of ND2 gene might be a neutral polymorphism not specifically related with Alzheimer's disease.

## MATERIALS AND METHODS

### Patients

Frozen brain tissues from patients #1, #2 - #12, and #15 - #20 (Table I) were kindly provided by Drs. T. Wisniewski (New York University), P. Gambetti (CaseWestern Reserve University) and J. Powers (Columbia University), respectively. All patients with diagnosed AD were confirmed at autopsy by evidence of neuritic plaques neurofibrillary tangles in brain. Fibroblasts from patients #13 and #14 were obtained from the NIA Aging Cell Depository of the National Institute of Aging (lines AG06844-B and AG07603-A, respectively). In addition to the above samples, frozen mitochondria from patients #21 (control), #22 (G->T mutation, heteroplasmic) and #23 (G->A mutation, homoplasmic) (15) were kindly provided by Dr. K. Iqbal (New York Institute for Basic Research in Developmental Disabilities).

### Preparation of DNA

Total DNA was extracted from 100-200 mg of frozen tissue and from frozen mitochondria as reported (19).

### Synthesis of oligonucleotides

The ND2 mutations at position 5460 creates no "convenient" polymorphic restriction site for RFLP analysis. This problem can be overcome, however, by "mispairing PCR" (18). Briefly, a PCR primer is synthesized which, in conjunction with a known nucleotide in the target DNA (allele A), introduces a restriction site at the locus of interest, but does not create the restriction site when the target nucleotide is absent (allele B); when the resulting PCR products are cleaved by the appropriate restriction enzyme and the digestion products are electrophoresed and visualized on appropriate gels, the two alleles can be distinguished as an RFLP. We have used this procedure successfully (20) to search for the known mtDNA A->G transition at position 8344 in the $tRNA^{Lys}$ gene associated with MERRF (21), a maternally-inherited mitochondrial disorder.

To search for the ND2 mutation, we synthesized two mispairing oligonucleotide primers (Operon Technologies, Alameda, CA), both of which introduced a *BglI* site (5'-

**Table I.** DNA samples used in the present study

| Sample | Code | Age | Sex | Diagnosis | Tissue |
|---|---|---|---|---|---|
| 1 | | | | AD | Frontal cortex |
| 2 | 0-86326 | 76 | M | AD | Temporal cortex |
| 3 | 0-86397 | 90 | F | AD | Temporal cortex |
| 4 | 0-86330 | 64 | M | Normal | Temporal cortex |
| 5 | 0-88239 | 78 | F | AD | Temporal cortex |
| 6 | 0-86329 | 63 | F | AD | Temporal cortex |
| 7 | 0-86319 | 63 | F | AD | Temporal cortex |
| 8 | 0-86108 | 68 | F | Normal | Temporal cortex |
| 9 | 0-86350 | 81 | M | AD | Temporal cortex |
| 10 | 0-86402 | 64 | M | Normal | Temporal cortex |
| 11 | 0-86372 | 88 | F | AD | Temporal cortex |
| 12 | 0-80331 | 70 | M | AD | Temporal cortex |
| 13 | AG06844-B | 59 | M | AD (familial) | |
| 14 | AG07603-A | 61 | F | Normal | |
| 15 | OC441 | 87 | F | AD | Frontal cortex |
| 16 | OC442 | 93 | F | AD | Frontal cortex |
| 17 | OC416 | 68 | M | AD | Frontal cortex |
| 18 | OC388 | 78 | F | AD | Frontal cortex |
| 19 | OC409 | 88 | M | AD | Frontal cortex |
| 20 | 90-184 | 75 | F | Normal | Frontal cortex |
| 21 | #90 | 79 | | AD | |
| 22 | #3925 | 76 | | AD | |
| 23 | #28095 | 87 | | AD | |

GCCNNNN|NGGC-3'; nucleotides in bold) into the 214-nt PCR products. The forward (F) primer introduced a GG doublet instead of AT at positions 5301-5302 of the published sequence (16); the backward (B) primer introduced GG instead of CA at positions 5468-5469 (heavy-strand orientation; mispairing dinucleotides are indicated in lower case letters):

BglI-5282F (nt 5282-5311): 5'-AAAAAACAATA**GCCTCATCgg**CCCCACCAT-3'
BglI-5495B (nt 5495-5461): 5'-AAAAGGGGAGATAGGTAGGAGTAGC**GccGTAAGGG**-3'

The backward primer will introduce a *BglI* site only into those mtDNAs that contain the wild-type G at position 5460; any mutation (and specifically, either G->A or G->T) at this location will destroy this restriction site, and the PCR fragment will not be cleaved at position nt-5466 (the *BglI* cut site on the heavy strand). On the other hand, the forward primer will introduce a *BglI* site into *all* molecules (whether mutated at nt-5460 or not). *BglI* should digest all molecules at position 5299 (the cut site on the heavy strand); this site, therefore, serves as an internal control for completeness of digestion by the enzyme.

**Polymerase Chain reaction**

Amplification of mtDNA by mispairing PCR was performed for 25 cycles in a 100 µl reaction containing about 100 ng of total DNA, 100 pmoles of each primer, 12.5 nmoles of each dNTP, 2.5 U Taq DNA polymerase (Amplitaq , Cetus) in 10 mM Tris·HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 1 drop of mineral oil. Amplification was performed using a Perkin Elmer Cetus thermal cycler: denaturation was at 94°C for 1 min, annealing was at 55°C for 1 min, and extension was at 72°C for 45 sec. After checking the PCR products on 1% agarose gel, a final PCR cycle was performed in the presence of 10 µCi of [$\alpha$-$^{32}$P]dCTP (3000 Ci/mmol; New England Nuclear) and 2.5 U of added Taq polymerase, to avoid underestimation of the possible mutant population after restriction-enzyme digestion (21) ("last-cycle-hot" PCR [1]).

***BglI* digestion and gel electrophoresis**

Ten µl of the PCR reaction were digested with 5 U of *BglI* (Boehringer Mannheim) in 40 µl of final volume of 50 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl , and 1 mM

dithiothreitol for 3 hours at 37°C. Twenty µl of each sample was electrophoresed through a 10% polyacrylamide non-denaturing gel (29:1 acrylamide:bis) at 400 volts until the bromophenol blue dye migrated 3/4 of the gel length. Autoradiography of the gel was done at 4°C for 3 hours on Kodak XAR X-ray film.

**DNA sequencing**

The PCR products were purified with the Magic PCR Prep kit (Promega), and were subjected to direct DNA sequencing using the dsDNA Cycle Sequencing System (BRL).

## RESULTS

We analyzed mtDNA from 15 AD patients and 5 normal controls by mispairing PCR+RFLP analysis. In each amplification, the uncut PCR product was a 214-bp fragment, as expected; the presence of 2 mismatched bases in both primers did not appear to affect the efficiency of amplification. As noted above, each PCR fragment containes either 1 or 2 *BglI* sites: the site created by the backward-primer is diagnostic for the mutation, whereas the site created by the forward primer checks for the functionality of the enzyme (see Fig. 1A).

Digestion of the wild-type 214-bp PCR fragment with *BglI* results in 3 fragments of 167, 30, and 17 bp (based on H-strand distances). A mutation at nt-5460, either from G->A or from G ->T (15), would cause the loss of the *BglI* cut site at position 5466, resulting in the shift of the 167-bp band to 197-bp (i.e. 167-bp +30-bp; Fig. 1A). As shown in Fig. 1B, the only sample in our analysis which was positive for the 197-bp fragment was from patient #23, which was the positive control for a homoplasmic G->A mutation (15). The other positive control, patient #22, was described as heteroplasmic for the G->T mutation (15); however, in our study we were not able to detect this mutation. In order to mimic the heteroplasmic condition of wild-type and mutated DNAs, and to assess whether or not our method could resolve a heteroplasmic population of PCR fragments, we mixed DNA from patient #21 (wild-type G at position 5640; Fig. 1B) with DNA from patient #23 (mutated A at position 5640; Fig. 1B) in different proportions, namely 0%, 10%, 30%, 50% and 80%, and 100% #23-derived mutated DNA. We were able to detect the RFLP in a mixture containing at least a 10-fold dilution of mutated molecules, using the same conditions of amplification and digestion as was used for the probands (Fig. 1B, right-most 6 lanes).

As a final confirmation of our results, we subjected the uncut PCR products from 7 samples (5 AD and 2 control subjects) to direct DNA sequencing. In each case, the nucleotide at position 5640 was confirmed unambiguously to be a G in all 7 sequences examined.

## DISCUSSION

Involvment of mitochondria in Alzheimer's disease was considered by Parker (22), who showed a defect of cytochrome *c* oxidase in platelets. However, a possible role of mitochondria in the pathogenesis of AD may exist as a sequel to derangement of other cellular functions or to loss of specific cell types, but might not be etiologic. In fact, although the etiology of AD is still not clear, the genetic defect(s) underlying the disorder seems to be nuclear and not mitochondrial (23).

Compared to both single-nucleotide primer extension and direct sequencing of PCR products, we consider our experimental approach (i.e. RFLP analysis of radiolabelled PCR

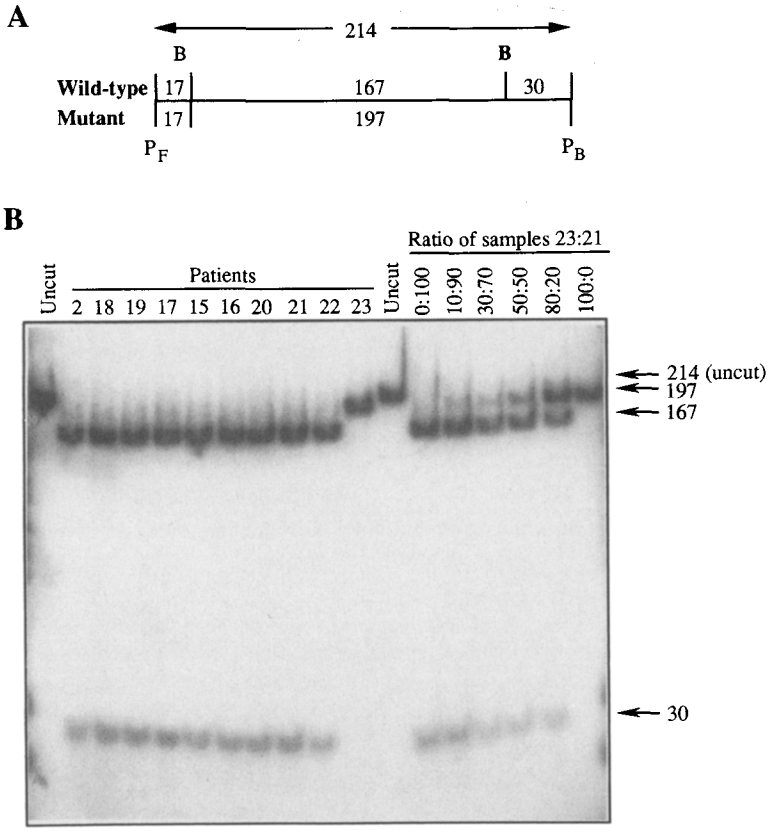


**Fig. 1.** PCR-RFLP analysis. **A.** Schematic map of the *Bgl*I cutting pattern of the 214-bp PCR product generated by using the forward ($P_F$) and backward ($P_B$) primers. The *Bgl*I site created by the backward mispairing primer when adjacent to the wild-type G at nt-5460 is shown in bold. Sizes of cleavage products, in bp, are shown. **B.** Typical autoradiogram of polyacrylamide gel electrophoresis of *Bgl*I-digested PCR products. Samples from other patients were analyzed in a similar manner, with similar results (data not shown). Lanes are noted by patient number (see Table 1). The rightmost 6 lanes show the indicated proportions of total DNA from subject #23(A at nt-5460) mixed with DNA from subject #21 (G at nt-5460). Sizes of expected fragments, in bp, are indicated at right. The predicted fragment sizes were in agreement with fragments generated by *Msp*I digestion of pBR322, which were used as size markers (not shown). The expected 17-bp fragment was electrophoresed off the bottom of the gel and is not visible.

products) to be both reliable and powerful in detecting even low levels of mutated mtDNAs. Nevertheless, we were unable to detect the mutation described by Lin et al. (15) in 14 sporadic cases and 1 familial case of AD, nor in 5 age-matched control subjects, even though we did detect the G->A mutation used as a positive control.

We believe that the described mutations at position 5460 (15) are not associated with, or diagnostic of, AD. Moreover, the finding of homoplasmic G->A mutations in 2 ALS patients implies that the mutation is also not specific for AD. Rather, we believe that these mutations are likely neutral mtDNA polymorphisms normally present in the population. Specifically, mutations at nt-5460 have been observed by others in individuals not affected by AD (N. Howell and D.C. Wallace, personal communication). Furthermore, the changes from Ala->Thr or Ala->Ser at this

site are not drastic amino acid substitutions, and are located in a region of the ND2 polypeptide which is not highly conserved during evolution. Although we have no information about the phylogenetic origin of the AD patients examined by Lin et al. (15), the presence of the mutation in 10 out of 19 patients might be explained if there were a common geographic, racial, or ethnic origin of the individuals harboring the ND2 mutations.

Our data do not confirm the association of AD and the nt-5460 mutation in a population size comparable to that analyzed by Lin et al. (15). We believe that the attribution of a pathogenetic role to point mutations of mtDNA may have to be demonstrated by more strict criteria of causality, especially as the data base of mtDNA polymorphisms grows. Specifically, a number of putatively pathogenic mtDNA point mutations have been reported which, in fact, are most likely neutral polymorphisms. For example, an A->G transition at nt-15924 in the $tRNA^{Thr}$ gene, which was reported to be etiologic in a patient with fatal infantile respiratory enzyme deficiency (24) was reported as an ostensible neutral polymorphism in an adult with Kearns-Sayre syndrome (25). We believe that criteria to assess the importance of a potentially pathogenic mutation in mtDNA should consider either: 1) the existence of maternal inheritance of the mutation, coupled with possible family history through the maternal lineage, or 2) the existence of a heteroplasmic population of mutated mtDNAs, especially if the mutation is sporadic.

## ACKNOWLEDGMENTS

We thank P. Gambetti (CaseWestern Reserve University, Cleveland, OH), T. Wisniewski (New York University Medical Center), J. Powers (Columbia University, New York, NY), and K. Iqbal (New York Institute for Basic Research in Developmental Disabilities, Staten Island, NY) for providing tissue samples, and J. Sadlock and J. Rogers for expert technical assistance. This work was supported by grants from the National Institutes of Health (P01-NS11766, RO1-NS28828 and P50-AG08702), the Muscular Dystrophy Association, the Aaron Diamond Foundation, and a donation from Libero and Graziella Danesi, Milano, Italy. V.P. was supported by the Sigma Tau Foundation.

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