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DETECTION OF POINT MUTATIONS IN CODON 331 OF MITOCHONDRIAL NADH DEHYDROGENASE SUBUNIT 2 IN ALZHEIMER'S BRAINS

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SUMMARY: Point mutations in codon 331 of mitochondrial NADH dehydrogenase subunit 2 (ND2) were detected in 10 of 19 Alzheimer's brains but not in 11 normal brains. The same mutations were also detected in 2 of 6 patients with amyotrophic lateral sclerosis (ALS). However, neurofibrillary tangles and neuritic plaques characteristic of Alzheimer's disease were found histologically in the brain of one ALS patient who was positive of the mutation. The finding suggests that a point mutation in ND2 is a potential risk factor for Alzheimer's disease.

Alzheimer's disease (AD) is characterized by the presence of numerous neurofibrillary tangles (NFT) and neuritic (senile) plaques in the neocortex, especially in the hippocampus. The principal component in NFT is paired helical filaments (PHF). During the course of our study of the biogenesis of PHF by reverse genetics, we isolated two cDNA clones from two Alzheimer brain libraries using antibodies to PHF as the probes. One of the antibodies was polyclonal (1) and the other a monoclonal (2). Nucleotide sequence analysis of the two clones revealed that they were nearly identical to an open reading frame coding for the carboxyl terminal region of NADH dehydrogenase subunit 2 (ND2) in the human mitochondrial DNA (mtDNA) (3). By comparing the nucleotide sequence of the two clones with the published sequence (3), we found a base substitution at the first position of codon 331 in both clones resulting in a change of one amino acid in this region of ND2. The data suggest that point mutations in the ND2 gene may be involved in AD. To test this hypothesis, we examined the target region of the mtDNA isolated from the brains of 19

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Abbreviations: ND2, ND4= NADH dehydrogenase (EC 1.6.99.3) subunit 2,4; PCR= polymerase chain reaction; NFT= neurofibrillary tangles; PHF= paired helical filaments; SNuPE= single nucleotide primer extension; pfu= plaques forming unit; ALS= amyotrophic lateral sclerosis.

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Alzheimer patients, 11 normal individuals and 6 patients with amyotrophic lateral sclerosis (ALS). We now report that 10 of the 19 Alzheimer brains and two of the six ALS had point mutations in codon 331. The mutations were not detected in the 11 normal controls.

MATERIALS AND METHODS

Frozen brain tissues were provided by the Brain Bank of this Institute and the New York University Medical Center. The Alzheimer cases were histologically confirmed. Sequenase kit was purchased from United States Biochemical, [^{32}P]dNTP (3000 Ci/mmole) and [^{35}S]dATP (1000 Ci/mmole) from Amersham, Taq DNA polymerase from Cetus or Boehringer Mannheim, NuSiew from FMC, Centricon-30 from Amicon.

ISOLATION OF MITOCHONDRIA: One gram of a frozen frontal cortex was homogenized in 10 ml of an isolation solution (0.25 M sucrose, 0.5 mM EDTA, 10 mM Tris-HCl, pH 7.5) and the homogenate was centrifuged at 2,000 x g for 3 min. The supernatant was re-centrifuged at 12,500 x g for 8 min and the pellet was suspended in 10 ml of 3% Ficoll medium (3% Ficoll, 0.12 M mannitol, 30 mM sucrose, 50 μM EDTA, 10 mM Tris-HCl, pH 7.5). Five ml of the suspension was layered on 20 ml of a 6% Ficoll medium (2 x 3% Ficoll medium) and centrifuged at 11,500 x g for 30 min. The pellet was re-suspended in 5 ml of 3% Ficoll medium and centrifuged at 11,500 x g for 10 min. Mitochondria in the pellet were suspended in 2 ml of the isolation solution and stored at -80°C.

Extraction of mtDNA and PCR: To 1 ml of the mitochondrial suspension was added 0.1 ml of a 10% SDS and the mixture was incubated at 37°C for 10 min. The solution was extracted with equal volume of phenol saturated with TE. After centrifugation, the aqueous and organic phases were re-extracted with phenol and TE respectively. To the combined aqueous solution, sodium acetate (pH 5.2) was added to a final concentration of 0.3 M and mtDNA was precipitated with 2 volumes of ethanol at -20°C overnight.

The target region was amplified by PCR using the following oligonucleotides as the primers:
ND2-001: 5'-CTCGCACCTGAAACAAGCTAACA-3'.
ND2-003: 5'-GGGAAGCTTAGTATAAAAGGGGAGATAGGT-3'.
A Hind III site 5'-AAGCTT-3'was present in ND2-003 in order to generate fragments that can be subcloned into M13mp18 and M13mp19.
One hundred pmoles of both primers and 100 ng mtDNA were added to a 100 μl of a PCR reaction buffer (4) which also contained 2 units of a Taq DNA polymerase. Amplification was performed in an automatic thermocycler (Coy) for 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min. Initial denaturation of the template was done at 94°C for 3 min. cDNA inserts in lambda gt11 were also amplified by these procedures. About 5x10^5 pfu intact phages were substituted for mtDNA as the template. Lambda gt11 primers flanking the insert at the EcoRI site in the lacZ gene were included in the amplification solution instead of the ND2 primers. Phages were grown on an agarose plate (150 mm) and harvested in SM (0.1 M NaCl, 0.01 M $MgSO_4.7H_20$, 0.05 M Tris-HCl, pH 7.5, 0.1% gelatin). The fluid was centrifuged at 10,000 x g for 30 min and the supernatant was centrifuged at 63,000 x g for 30 min. Phages in the pellet were suspended in SM.

NUCLEOTIDE SEQUENCING: The PCR product was concentrated to 40 μl by centrifugation in a Centricon-30 and digested with EcoRI and Hind III and subcloned onto M13mp18 and M13mp19. The recombinant DNA was used to transform Escherichia coli MV1190 (IBI) and grown in LB broth at 37°C. Single-stranded DNA was extracted from the bacteriophages and the nucleotides were sequenced according the method of Sanger et al.(5)

using [^{35}S]dATP as the label. Both L- and H-strands were sequenced at least two times in both directions.

DETECTION OF POINT MUTATIONS BY SINGLE NUCLEOTIDE PRIMER EXTENSION: Single nucleotide primer extension (SNuPE) was performed according to the method described by Kuppuswamy et al.(6). The target fragment produced by PCR described above was purified by electrophoresis in a 1.2% agarose gel. The band containing the target fragment was excised from the gel, extracted by phenol and used as the template. A primer corresponding to nt 5436 to 5459 of the mtDNA (3) was synthesized with an ABI 380A DNA synthesizer. The sequence of the primer was 5'-ACCCCATTCCTCCCCACACTCATC-3' which locates immediately before codon 331. To 20 μl of a PCR reaction buffer (4), 1 pmole of the primer, 50 ng of the template, 1 unit of a Taq DNA polymerase and 1 μl of a [^{32}P]dNTP were added. The extension was carried out for 2 min each at 96^{0}C, 55^{0}C, and 72^{0}C and 5 μl of the reaction mixture was mixed with 5 μl of a stop solution (95% formamide, 0.05% each of bromophenol blue and xylene cyanol). The mixture was heated at 90^{0}C for 2 min and 5 μl of the solution was applied to a gel consisting of 3% NuSieve and 1% agarose. Electrophoresis was run at 200 V at room temperature until the blue dye migrated 3/4 of the gel. Autoradiography was done at -70^{0}C on a Kodak X-ray film.

CONSTRUCTION AND SCREENING OF cDNA LIBRARIES: Poly(A)+RNA was extracted from the frontal cortex according the guanidinium thiocyanate/cesium chloride method (7). cDNA was synthesized by the method described by Watson and Jackson (8) and inserted into the EcoRI site in lambda gt11. The libraries were screened with antibodies to PHF as described by Huynh et al.(9).

RESULTS AND DISCUSSION

A lambda cDNA library derived from an Alzheimer brain (case 443) was screened with a rabbit antiserum (82g) against PHF (1) and 14 positive clones were isolated from 3 x 10^4 pfu. Another cDNA library (case 986) was screened with a monoclonal antibody (3-39) (2) and 3 clones were picked up from 3 x 10^4 pfu. One clone was randomly selected from each library for nucleotide sequencing and the results are shown in Fig. 1. The clone recognized by antiserum 82g was designated 82-2 whereas that reacted with MAb 3-39 was named 39-1. It is seen that the nucleotide sequences of both clones were nearly identical with that corresponding to the 3'-end of an open reading frame coding for ND2 (3). Further analysis of the nucleotide sequences revealed that a base substitution at the first position of codon 331 occurred in both cDNA clones as compared with the corresponding position in the published sequence (3). The base substitution was a change from G to A in clone 82-2 and a G to T in clone 39-1. The result of the base substitution was a replacement of alanine by threonine in clone 82-2 and a change of alanine to serine in clone 39-1 (Fig 1). The data seem to indicate that point mutations are implicated in AD.

To test whether this is the case, nucleotide sequence of cDNA clones isolated from normal brain libraries were analyzed. Results from these studies revealed that the target

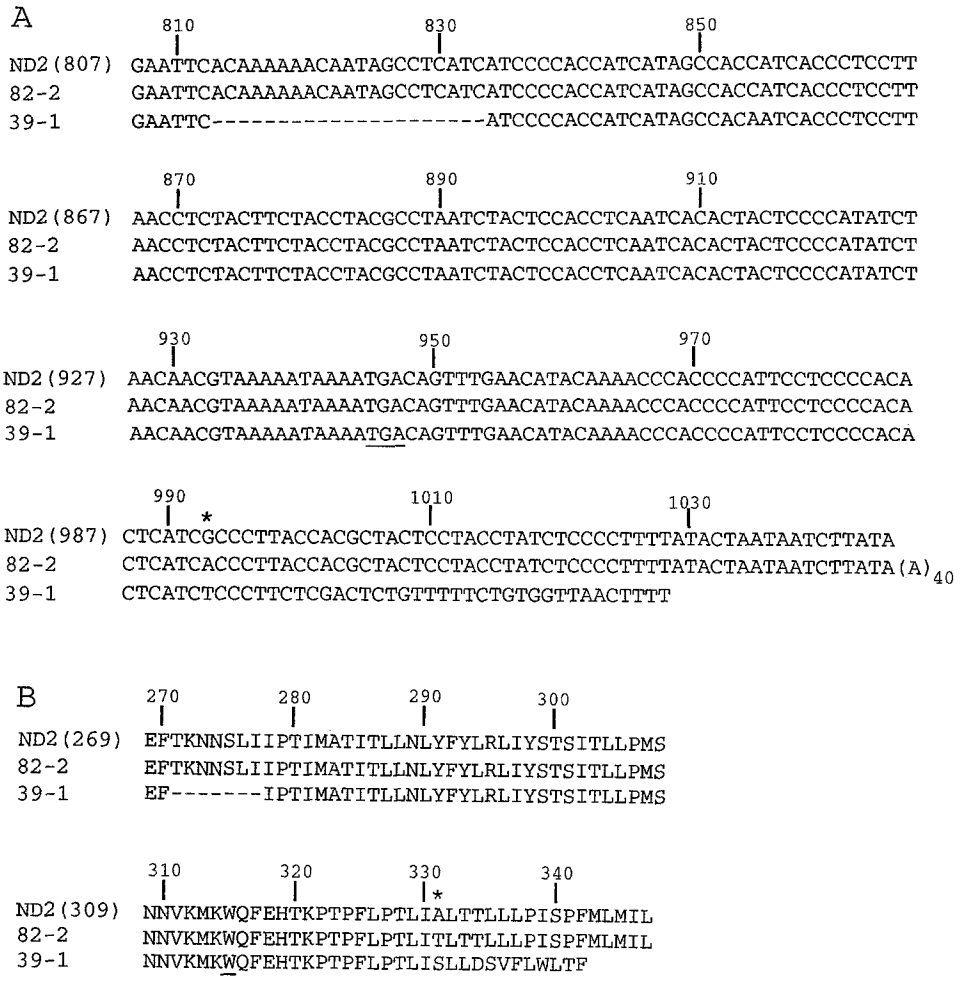

Figure 1. Nucleotide and predicted amino acid sequences of cDNA clones.

A. Nucleotide sequence. The cDNA inserts in lambda gt11 were subcloned in M13mp18 and M13mp19 and sequenced by the didoxynucleotide termination method (5). Both H- and L-strands were sequenced at least two times. ND2 is the published sequence (3) and is included here for comparison.

B. Amino acid sequence deduced from the nucleotide sequence. Note that TGA in the mitochondrion codes for tryptophane.

sequence of the cDNA clones examined was identical to that of published sequence (3) (see cases 9 and 713 in Table 1). Meanwhile, a base substitution at the third position of codon 331 was detected in a cDNA clone derived from an Alzheimer brain (case 226, Table 1). This was a silent mutation. To test whether the mutations occur in mtDNA instead of cDNA, the target region containing a 350 bp was amplified by PCR and the nucleotdides were sequenced. A small portion of a sequencing gel illustrated in Fig 2 shows that an A was found in an Alzheimer brain (case 28095) whereas a G appeared at the corresponding position of a normal control (case 15).

Table 1. Mutations in codon 331 of ND2 in human brain mtDNA

Case	Age	Diagnosis	Codon 331	AA	Method
226	85	AD	GCT/GCC*	Ala	S/PE†
337	80	AD	GCC	Ala	PE
986	70	AD	TCC	Ser	S/PE
443	71	AD	ACC/GCC	Thr/Ala	S/PE
90	79	AD	GCC	Ala	PE
329	66	AD	GCC/ACC	Ala/Thr	PE
353	86	AD	GCC/TCC	Ala/Ser	PE
28095	87	AD	ACC	Thr	S/PE
382	53	AD	GCC	Ala	PE
264	76	AD	GCC	Ala	PE
171	NA	AD	GCC	Ala	PE
189	91	AD	GCC	Ala	PE
214	73	AD	GCC	Ala	PE
274	85	AD	GCC	Ala	PE
3925	76	AD	TCC/GCC	Ser/Ala	PE
3890	84	AD	TCC/GCC	Ser/Ala	PE
85-50	85	AD	TCC/GCC	Ser/Ala	PE
196	68	AD	ACC/TCC	Thr/Ser	PE
1086	70	AD	ACC/TCC	Thr/Ser	PE
15	67	N‡	GCC	Ala	S
9	58	N	GCC	Ala	S
A19	17	N	GCC	Ala	PE
252	76	N	GCC	Ala	S/PE
713	20	N	GCC	Ala	S
71	83	N	GCC	Ala	PE
79	90	N	GCC	Ala	PE
149	65	N	GCC	Ala	PE
269	93	N	GCC	Ala	PE
272	93	N	GCC	Ala	PE
387	81	N	GCC	Ala	PE
27976	67	ALS	GCC	Ala	PE
ALS6	62	ALS	GCC	Ala	PE
3917	66	ALS	GCC	Ala	PE
3894	63	ALS	GCC	Ala	PE
28034	62	ALS	ACC	Thr	PE
313	64	ALS	ACC	Thr	S/PE

*GCC is wild-type; †S = Sanger, PE = SNuPE; ‡N = normal; NA = not available

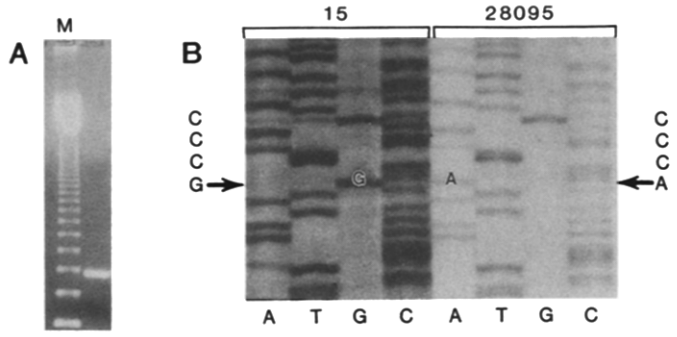

Figure 2. Nucleotide sequence of a target region of mtDNA. The target region between nt5161 and 5500 of the mtDNA (3) was amplified by PCR and sequenced as described in Methods.

A. A PCR product showing a 350 bp fragment. M= a 123 bp DNA ladder (BRL).

B. A small portion of a sequencing gel showing a base substitution as indicated. Note that a G was present in a normal brain (case 15) whereas an A was detected at the corresponding position in an Alzheimer brain (28095).

To evaluate the significance of the preliminary finding, we then adopted the SNuPE method (6) to screen mtDNA isolated from more Alzheimer and normal brains. To test the specificity of the method, we used the cDNA of clone 39-1 which is known as a T mutant (Fig 1), as the template and demonstrated that only T was added to the 3'-end of the primer (case 986 in Fig 3). It is also seen in Fig 3 that the primer was extended by an A in an Alzheimer case (28095). This was in agreement with the finding in nucleotide sequencing (Fig 2). As shown in Fig 3, nine of 13 AD contained either an A or a T mutant. A few patients even harbored both A and T mutants. It is interesting to note that most of the AD mutation in the ND2 gene were heteroplasmic (a mixture of wild and mutant genotypes) and the degree of heteroplasmy varied from patient to patient.

Mutations in codon 331 of the ND2 gene is summarized in Table 1. It shows that mutations occurred in 10 of 19 AD brains resulting in a change of one amino acid. In contrast, mutations in the target region in the 11 "normal" brains were not detected. However, an A mutant was detected in two of the six ALS patients. It should be pointed out that neurofibrillary tangles and neuritic plaques were observed histologically in the ALS patient 313. Patient 28034 was initially diagnosed as ALS with subsequent additional diagnosis of Hodgkin's disease and progressive multifocal leukoencephalopathy.

In this paper, we report detection of point mutations in codon 331 of the ND2 gene in a significant number of Alzheimer brains. We had performed nucleotide sequencing of 6 mtDNAs and 6 cDNA clones containing 350 bp in length and found that mutations occurs only in codon 331 in this region which represents about 30% of the ND2 gene (3). The

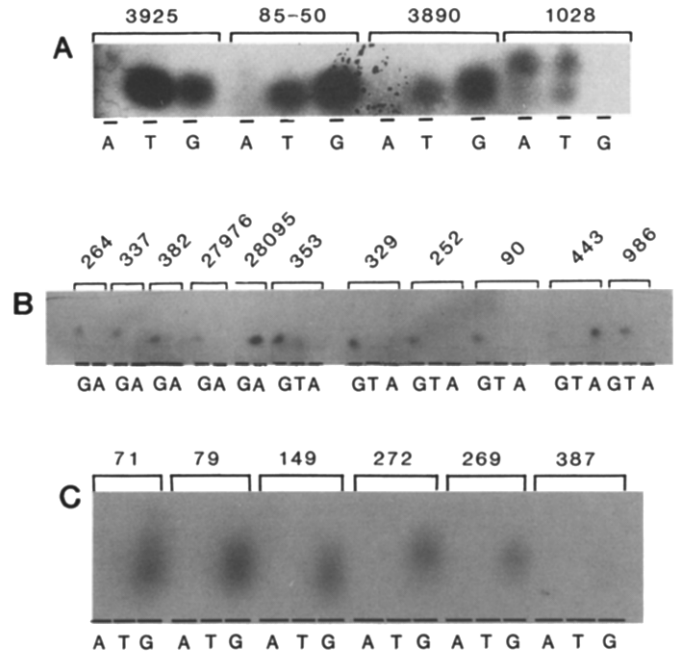

Figure 3. Detection of point mutations by SNuPE. The procedures were detailed in Methods. The autoradiograph shows the extension of the primer 5'-ACCCCATTCCTCCCCACACTCATC-3' by adding the indicated nucleotide to the 3'-end.

A. All four cases were AD patients.

B. 264, 337, 382, 28095, 353, 329, 90, 443 and 986 were AD; 252 was normal; 27976 was an ALS case.

C. All six cases were normal individuals.

mutations were not detected in normal individuals in the age range between 17 and 93. (Table 1). The significance of the mutations with regard to the pathogenesis of AD is not yet understood. However, it is known that a point mutation in codon 340 of the ND4 gene was found to be the cause of Leber's hereditary optic neuropathy (10,11). Like ND4, ND2 is a subunit of complex I which is a key enzyme in the respiratory chain. Mitochondrial mutation has not been linked to AD and this can be tested whether maternal inheritance is involved in the disease. However, alteration in metabolism of glucose and glutamine in Alzheimer skin fibroblasts have been reported by Peterson and Goldman (12) and by Sims et al. (13). Their data suggest that the alteration of metabolism is a consequence of an uncoupling of oxidation to phosphorylation in AD mitochondria. Blass et al. (14) showed that Alzheimer antigens recognized by anti-PHF antibodies were induced in cultured skin fibroblasts by an uncoupling reagent. Furthermore, an uncoupler of oxidative phosphorylation is capable of disrupting microtubules in HeLa cell (15) and it was suggested that the process is brought about by the rapid dissipation of the mitochondrial electrochemical gradient. It is possible that the disruption of microtubules could free the

associated tau protein for abnormal phosphorylation leading to the formation of PHF-tau (16-18). This hypothesis is supported by the finding of a protein kinase of 60 kDa highly active in Alzheimer brains (19). This kinase is probably the same enzyme that is dependent on calcium/calmodulin (CaM) for activity (20). The calcium/CaM dependent kinase is capable of converting the normal tau isoforms to PHF-tau in high efficiency and phosphorylation is modulated by membrane phospholipids. It is conceivable that a mutation in the ND2 gene could have the same effect of an uncoupler in oxidative phosphorylation on Alzheimer brains. It is of interest to note that optic nerve degeneration was found in association with AD (21). It is possible that a defective ND2 may be involved in this optic neuropathy.

The two cDNA clones were selected by two antibodies raised against PHF-enriched fractions isolated from AD brains (1,2). However, the nucleotide sequences of the two clones did not reveal any sequence similarity to those of PHF proteins. A possible explanation is that they were picked up by cross reaction.

In the resent study, we found that most of the AD brains in which mutations occurred, were heteroplasmic (Table 1). Manifestation of mitochondrial genetic disease is reflected on the extent of the defect in oxidative phosphorylation and the age of patients (22). Normal mtDNA can ameliorate the effect of mutations and the disease exacerbates as the age increases. The same protective function of wild-type mtDNA is probably in operation in AD. However, since the central nervous system is highly dependent on mitochondrial energy (23), a defective ATP-generating system could have a greater impact on brain function than on other organs. This may explain why dementia manifests in Alzheimer patients even though they may have only a minor fraction of a mutant mtDNA in the brain (Fig 3).

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