Lack of Association Between Two Restriction Fragment Length Polymorphisms in the Genes for the Light and Heavy Neurofilament Proteins and Alzheimer's Disease

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ABSTRACT: The etiology of Alzheimer disease (AD) remains unknown. The hypothesis of genetic factors playing a role in the causation of the disease, at least in its familial form, has been borne out by results showing linkage in several early-onset AD families to a locus on the proximal part of the long arm of chromosome 21. Linkage was not detected in several other families using the same markers. The metabolism of neurofilaments is perturbed in AD, as indicated by the presence of neurofilament epitopes in neurofibrillary tangles, as well as by the severe reduction of the expression of the gene for the light neurofilament subunit in AD brain. To detect a possible anomaly that might relate to the disease, we have searched for an association between the genes for the light subunit and the heavy subunit of the neurofilament triplet, and AD. Genotypes for restriction fragment length polymorphisms (RFLP) at each of the two loci were determined for an AD group and a control group. Allelic frequencies at a TaqI-defined RFLP for the gene for the light neurofilament subunit were 0.70 for the 3.7 kb allele and 0.30 for the 2.9 kb allele. HincII detected an RFLP for the heavy neurofilament subunit gene with frequencies of 0.31 for the 18.0 kb allele and 0.69 for the 6.8 kb allele. Frequencies were found to be similar in the two groups for both light and heavy neurofilament subunit loci. Although it cannot be excluded that mutations at other sites of the neurofilament genes are relevant to AD, the data reported here do not support an association between these genes and the disease.

RÉSUMÉ: Absence d'association entre deux polymorphismes détectés par des fragments de restriction dans les gènes codant pour les protéines neurofilamentaires légère et lourde et la maladie d'Alzheimer La cause de la maladie d'Alzheimer (MA) demeure inconnue. L'hypothèse que des facteurs génétiques jouent un rôle dans la maladie, du moins dans sa forme familiale, a été confirmée dans un ensemble de quatre familles MA à début précoce, où un linkage a été démontré entre la maladie et un locus situé sur la partie proximale du bras long du chromosome 21. Il n'a pas été possible de déceler ce linkage dans plusieurs autres familles MA, en utilisant les mêmes marqueurs. Le métabolisme des neurofilaments est perturbé dans la MA, comme en témoignent la présence d'épitopes de neurofilaments dans les enchevêtrements neurofibrillaires, et la baisse substantielle d'expression du gène pour la petite sous-unité des neurofilaments dans le cerveau affecté par la maladie. Afin de détecter une anomalie possiblement reliée à la MA, nous avons cherché s'il y avait une association entre les gènes pour la petite et la grosse sour-unités des neurofilaments et la MA. Les génotypes pour des polymorphismes dans la taille des fragments de restriction à chacun des deux loci ont été déterminés dans un groupe contrôle et un groupe MA. Les fréquences alléliques pour un polymorphisme TaqI dans le gène de la petite sous-unité des neurofilaments sont de 0.7 pour l'allèle de 3.7 kb et 0.3 pour l'allèle de 2.9 kb. HincII détecte un polymorphisme dans le gène de la grosse sous-unité des neurofilaments, avec des fréquences de 0.31 pour l'allèle de 18.0 kb et 0.69 pour l'allèle de 6.8 kb. Les fréquences alléliques sont similaires dans les deux groupes pour les deux loci examinés. Ces résultats ne supportent pas une association entre les gènes des neurofilaments et la MA, quoiqu'on ne puisse exclure une mutation à un autre site.

Can. J. Neurol. Sci. 1990; 17:302-305

Alzheimer's disease (AD) is a neurodegenerative disease of unknown etiology. A clustering of cases has been shown in several pedigrees, with a pattern of autosomal dominant transmission.[1-4] This familial form of AD, as well as the observation of

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Received November 7, 1989. Accepted February 20, 1990

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an increased risk of developing the disease for first-order relatives of affected patients in both the early-onset and the late-onset forms,[5-10] have indicated that a genetic component is involved in the etiology of the disease. Familial cases are thought to represent from 15%[6] to 40%[11,12] of all AD cases, although Breitner and colleagues have argued that these figures are underestimated because many relatives of AD patients die from competing causes before the onset of symptoms.[13] Even in sporadic cases where a genetic transmission is not apparent, a genetic basis may still be responsible for a susceptibility to putative non-genetic etiological factors such as viral infection or environmental exposure.[14]

Linkage analysis with four extended AD pedigrees has shown that AD is linked in these families to a locus on the long arm of chromosome 21, between 21q11.2 and 21q21.1;[15] these findings were confirmed in a series of six early-onset AD families.[16] Additional studies using the same probes failed to establish linkage to this locus for other families, with either the early-onset or the late-onset forms of AD.[17-19]

Since there is a severe perturbation of the cytoskeleton in the areas of the brain affected by AD, the genes for cytoskeletal proteins are obvious candidates for molecular genetic analysis in this disease. The involvement of the cytoskeleton in AD is documented by the presence in neurons of intracellular abnormal filamentous structures, or neurofibrillary tangles;[20] the detection in tangles of cytoskeletal protein epitopes, namely tau,[21-23] and the middle and heavy members of the neurofilament triplet NF-M and NF-H;[24-26] and the immunodetection of axonal cytoskeletal markers in the cell perikaryon.[27] The presence of the light subunit of the neurofilament triplet (NF-L) has not been detected in tangles. However, the expression of the NF-L gene has been found to be reduced to approximately 30% of normal in the cortex of patients affected by the disease;[28,29] this is lower than can be accounted for by the neuronal loss that accompanies AD. An alteration in the genes for neurofilament proteins could lead to abnormal neurofilament assembly and result in the typical accumulation of filamentous structures observed in AD.

The human gene for NF-L has been cloned and sequenced; it maps on chromosome 8.[30] The gene for NF-H has also been characterized[31] and assigned to chromosome 22.[32,33] To determine whether a mutation in the NF-L or the NF-H gene could account for the variations in neurofilament metabolism observed in AD, we looked for linkage disequilibrium between the disease and restriction fragment length polymorphisms (RFLP) for these two genes by comparing allelic frequencies between a control group and a group of unrelated patients.

Table 1. Characteristics of the Subjects Genotyped in this Study. Neuropathological criteria for a diagnosis of AD were satisfied for all AD brain samples and in the case of one patient who had a biopsy. The other living patients had a diagnosis of probable AD. Description of control subjects is in the section Material and Methods.

	Control	Alzheimer
N	55	36
Age		
mean	52	75
range	30-87	57-90
Sex		
male	21	20
female	34	16
Source of DNA		
brain	18	32
blood	37	4

Material and Methods

Subjects

The association study included 91 unrelated subjects: 55 controls and 36 AD cases (Table 1). Living AD cases (4) were selected from an AD clinic using current NINCDS-ADRDA diagnostic criteria;[34] in one case, biopsy material allowed a definite diagnosis. Other cases (33) were obtained from the Douglas Hospital Research Center Brain Bank; all of these had neuropathological diagnosis of AD. The group of controls consisted of 37 living subjects and 18 autopsied brain samples. None of these individuals had a known history of neurological disease. In addition, plaque and tangle counts had been performed on 9 of the brain samples. All the subjects were Caucasian.

Southern analysis

Blood was drawn (20-25 ml) and used for leucocyte preparation on Ficoll-Paque (Pharmacia), which was followed by DNA extraction. Alternatively, DNA was extracted from brain tissue. Cortex (approximately 2 g) was allowed to thaw before DNA extraction. Brain tissue was homogenized in 0.15M NaCl, 0.1M EDTA pH 8.0, and SDS added to a final concentration of 2%. Prior to phenol extraction, sodium perchlorate was added to the homogenate to a concentration of 1M. High molecular weight DNA was isolated from peripheral blood leucocytes or brain homogenate according to standard protocols.[35]

DNA (5 µg) was digested to completion with TaqI or HincII (Pharmacia) and the fragments separated on 0.8% agarose gels. DNA was tranferred to nylon membranes (Nytran, Schleicher Schuell)[36] and UV-fixed for 4 minutes. The probes used were a genomic clone encoding the entire NF-L human gene in a pT718 vector,[30] and a 1.4 kb HindIII-BamHI fragment encompassing most of the first intron of the NF-H gene.[31] The probes were oligo-labelled[37] with α-^{32}P-dCTP to a specific activity of 8×10^8 cpm/µg. Hybridization was carried out at 65°C for 18 hours in 10% dextran sulphate, 3XSSC, 5X Denhardt's, 150 µg/ml herring sperm DNA, 0.2% SDS, and 5×10^7 cpm of probe. Filters were washed at 65°C with 3XSSC and 0.2% SDS, and then with 0.3XSSC and 0.1% SDS. For autoradiography, filters were exposed on Kodak XAR-5 films at −70°C with a Dupont Lighting Plus intensifying screen.

Results

TaqI defines an RFLP at the NF-L locus with 2 alleles of 3.7 kb and 2.9 kb. The location of the polymorphic site is shown in Figure 1A. The 3.7 kb and 2.9 kb alleles were found to have a frequency of 0.70 and 0.30 respectively in the general population[38] (Table 2); another study has reported frequencies of 0.62 and 0.38 for the two alleles.[39] A probe for the NF-H gene detects a 2-allele HincII polymorphism with alleles of 18.0 kb and 6.8 kb (Figure 1B). The frequency of these two alleles in the population is respectively 0.31 and 0.69[40] (Table 3). The TaqI and HincII RFLPs have polymorphic information contents (PIC) of 0.33 and 0.34 respectively, and constitute reasonably informative markers, according to the criteria set by Botstein et al.[41]

https://doi.org/10.1017/S0317167100030614 Published online by Cambridge University Press

Table 2. Frequencies of NF-L Alleles Defined by TaqI in the Control and the AD Groups. The values in parentheses refer to the number of alleles examined. A x^2 test indicates that the frequencies are not significantly different ($x^2 = 0.39$; $x^2 = 0.52$ with controls older than 50).

Allele	Control (n = 39)	Alzheimer (n = 35)
3.7 kb	0.70 (55)	0.66 (46)
2.9 kb	0.30 (23)	0.34 (24)

Table 3. Frequencies of NF-H Alleles Defined by HincII in the Control and the AD Groups. The values in parentheses refer to the number of alleles examined. A x^2 test indicates that the frequencies are not significantly different ($x^2 = 0.02$; $x^2 = 0.62$ with controls older than 50).

Allele	Control (n = 34)	Alzheimer (n = 32)
18.0 kb	0.31 (21)	0.30 (19)
6.8 kb	0.69 (47)	0.70 (45)

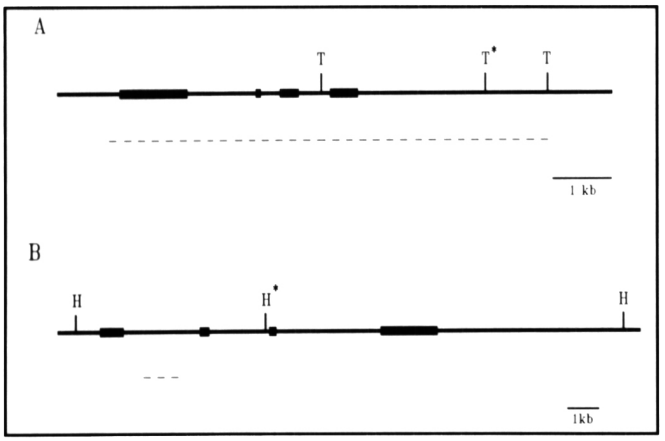

Figure 1 — Location of the polymorphic sites with respect to the gene structure of NF-L and NF-H. Boxes represent exons. Only sites involved in the polymorphic system are shown. The probe is represented by a dashed line. An asterisk indicates the variable site.
A: Map of NF-L (adapted from [30]); T: TaqI.
B: Map of NF-H (adapted from [31]); H: HincII.

Genotypes for the two RFLPs were determined for the control group and the AD groups. The frequencies of the 3.7 kb and of the 2.9 kb NF-L alleles in 35 unrelated AD cases were compared with those of 39 controls (Table 2). The values differ slightly but the differences are not statistically significant. Whereas the age of AD cases varies from 57 to 90 with a mean of 75 years, the group of controls is younger, ranging from 30 to 87 years, with a mean of 52 years. We analysed the data using only controls older than 50 (13 subjects). The difference in the proportions of the 2 alleles between the 2 groups was still not significant. The distribution of the two HincII-defined NF-H alleles was also found to be similar in the control group and in the AD group (Table 3).

Discussion

Analysis with RFLP markers can be exploited to detect an association between a given gene and a disease. Several studies have demonstrated the usefulness of candidate gene RFLP analysis in providing markers of genetic risk, for instance in the case of the ß-globin gene for sickle-cell anemia,[42] or in the case of the apolipoprotein genes for hyperlipidemia.[43]

Neurofilament perturbation in AD is severe enough to warrant an examination of the genes coding for these proteins. A decrease in NF-L expression was reported in AD brain;[28,29] in addition, a reduction in the translation of the mRNA for NF-H has been observed in polysomes prepared from AD cortex.[44] The other members of the neurofilament triplet, NF-M and especially NF-H, have long, heavily phosphorylated C-termini.[45] The deduced amino acid sequence of the C-terminal region of the NF-H gene in mouse[46] and man[31] contains serine-rich repeats that probably correspond to these phosphorylation sites in the proteins. The C-terminal parts of neurofilament proteins constitute the side-arms protruding from the filament structure and are thought to be responsible for interactions between neurofilaments and other cytoskeletal proteins.[47] Anti-neurofilament monoclonal antibodies cross-reactive with neurofibrillary tangles recognize determinants in the multi-phosphorylation domain.[26] A mutation in one of the neurofilament genes, giving rise to an RFLP, might result in a change in one amino acid, or might alter the sequences involved in the regulation of its expression. Both variable sites tested here could lead to the latter type of change. An abnormal protein, or abnormal levels of protein, could ultimately result in filament build-up.

In this study, we used a TaqI RFLP at the NF-L locus and a HincII RFLP at the NF-H locus to determine whether a particular allele of one of these two genes is associated with AD. We have found that there is no difference between the allelic frequencies of our normal and affected groups. Some of the younger control subjects possibly possess the putative genetic factor associated with AD and they will develop the disease if they live long enough. Analysis of the data with a subset of control subjects older than 50 years yielded the same results, for both NF-L and NF-H. Genetic heterogeneity may be present in AD, but it has not been possible to prove it conclusively yet. Heterogeneity will hamper attempts to detect an association in small population samples. Due to this potential problem, it may be a good strategy to conduct both linkage analysis and association studies with candidate gene markers. Although we cannot exclude a mutation at some other site in the neurofilament genes, our results do not support an association between the genes for neurofilaments and AD.

Acknowledgements

The authors wish to thank Dr. Robert Lazzarini for the use of his human NF-H clone, the Douglas Hospital Research Center Brain Bank for a generous supply of brain samples, Ms. Manon Léger for secretarial assistance, and Ms. Diane Lacoste for graphic work. This work was supported by the Medical Research Council of Canada (grant no. 10027) and by the Fondation de l'Âge d'Or du Québec.

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