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Estimation of the genetic contribution of presenilin-1 and -2 mutations in a population-based study of presenile Alzheimer disease

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Two closely related genes, the presenilins (*PS*), located at chromosomes 14q24.3 and 1q42.1, have been identified for autosomal dominant Alzheimer disease (AD) with onset age below 65 years (presenile AD). We performed a systematic mutation analysis of all coding and 5′-non-coding exons of *PS*-1 and *PS*-2 in a population-based epidemiological series of 101 unrelated familial and sporadic presenile AD cases. The familial cases included 10 patients of autosomal dominant AD families sampled for linkage analysis studies. In all patients mutations in the amyloid precursor protein gene (*APP*) had previously been excluded. Four different *PS*-1 missense mutations were identified in six familial cases, two of which where autosomal dominant cases. Three mutations resulted in onset ages above 55 years, with one segregating in an autosomal dominant family with mean onset age 64 years (range 50–78 years). One *PS*-2 mutation was identified in a sporadic case with onset age 62 years. Our mutation data provided estimates for *PS*-1 and *PS*-2 mutation frequencies in presenile AD of 6 and 1% respectively. When family history was accounted for mutation frequencies for *PS*-1 were 9% in familial cases and 18% in autosomal dominant cases. Further, polymorphisms were detected in the promoter and the 5′-non-coding region of *PS*-1 and in intronic and exonic sequences of *PS*-2 that will be useful in genetic association studies.

INTRODUCTION

Alzheimer disease (AD) is the most common cause of senile dementia and the fourth leading cause of death in western societies. AD is a neurodegenerative disorder of the central nervous system characterized by progressive loss of memory and intellectual functioning due to the appearance in the brain of two major lesions: senile plaques and neurofibrillary tangles. The exact biochemical pathway leading to neurodegeneration is still unknown. In most AD cases the first symptoms of memory dysfunction or behavior changes become apparent after age 65 years (late-onset or senile AD); however, in many cases the disease starts earlier in life (early-onset or presenile AD). There are no indications that the disease in presenile AD cases is different from that observed in senile AD cases, apart from a more severe pathology and more rapid clinical progression. Both senile and presenile AD have a genetic etiology; however, genetic cases are more frequent among presenile AD cases (for a review see 1). Also, several AD families have been documented that segregate presenile AD in an autosomal dominant manner. In these families a positional cloning approach has been employed to identify AD genes (for a review see 1). To date, three AD genes are known that, when mutated, lead to presenile AD: the amyloid precursor protein gene (*APP*) on chromosome 21 at 21q21.1 (2); the presenilin-1 gene (*PS*-1) on chromosome 14 at 14q24.3(3); the presenilin-2 gene (*PS*-2) on chromosome 1 at 1q42.1 (4,5). Although the normal function of the amyloid precursor protein (app) is unknown, mutations have been demonstrated to alter endoproteolysis of app such that more of a 42 amino acid long form of amyloid β (Aβ42) is produced (6). Rapid deposition of Aβ42 in AD brains is an early morphological event in AD pathology. Also, the normal and pathological functions of the presenilin proteins (ps-1 and ps-2) are unknown (for a review see 7). Since they both constitute integral membrane proteins with six to eight transmembrane domains (TM) and one large hydrophilic loop (HL) (8,9), similar functions of ps-1 and ps-2 were predicted. Remarkably, mutations in *PS*-1 and *PS*-2 also produce more Aβ42, suggesting that *PS* mutations and *APP* mutations lead to AD pathology through a common biochemical pathway.

In *APP* seven different mutations have been identified in autosomal dominant families with presenile AD or AD-related

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phenotypes (6). All mutations occur in exons 16 and 17, encoding the Aβ proteolysis product of app. However, extensive mutation analyses have shown that mutations in *APP* are rare, since they segregate in only 5% of the presenile AD families (1). Since the *PS* genes were identified more recently (for a review see 7), extensive mutation analyses have not yet been performed. However, initial estimates based on linkage analysis studies suggested that 70% of the presenile AD families were linked to chromosome 14 (10). Later, a mutation in *PS*-1 was found in each chromosome 14-linked AD family (3,11,12). To date 42 different missense mutations and one in-frame splice site mutation in *PS*-1 have been reported in 82 families world wide (13). Mutation screenings of *PS*-1 in presenile AD families not selected on the basis of linkage to chromosome 14 indicated that *PS*-1 mutations contribute less (30–50%) than initially estimated (14,15). A possible explanation for the lower mutation frequency of *PS*-1 may be that the initial mutation data were biased towards finding *PS*-1 mutations, since most families analyzed had been included in a genome-wide search for AD genes and were known to be linked to chromosome 14. Only one mutation has been reported in a proven non-familial AD case (16); however, only few mutation studies of sporadic AD cases have been performed.

Initial linkage studies indicated that *PS*-2 mutations are far less frequent than *PS*-1 mutations (5). Apart from the one *PS*-2 mutation identified in the chromosome 1-linked group of Volga German AD families, only one other *PS*-2 mutation has been identified to date (5,17). However, in contrast to *PS*-1 families onset ages in *PS*-2 families are highly variable and usually later in life (13). Therefore, it cannot be excluded that *PS*-2 families may have been missed, since presenile AD families with earlier onset ages are more likely to be ascertained for linkage analysis studies since they have more clear inheritance patterns. To date no mutation reports of *PS*-2 in non-familial presenile AD cases have been published; however, PS-2 has been less intensively investigated for mutations than *PS*-1.

In this study we aimed at evaluating the genetic contributions of *PS*-1 and *PS*-2 mutations to presenile AD in autosomal dominant, familial and sporadic cases. To overcome several of the biases mentioned above we used a unique sample of 101 unrelated familial and sporadic presenile AD cases ascertained in a population-based manner in The Netherlands (18,19). The sample contained 11 autosomal dominant, 56 familial and 34 sporadic presenile AD cases. The mean onset age was 56.7 ± 5.4 years (range 45–64 years), being lower than 60 years in 71 cases and lower than 50 years in 10 cases. In the autosomal dominant AD families all probands had an onset age before 65 years; however, in four families the mean onset age was above 65 years. In addition we analyzed the families of 10 autosomal dominant presenile AD cases included in this population-based study (20). In all cases mutations in exons 16 and 17 of the *APP* gene were previously excluded (19,20) while one family was shown to be linked to chromosome 14 (20).

Table 1. Intronic PCR primers flanking the coding and 5′-non-coding exons of *PS*-1 and *PS*-2

PS-1 Exon	Primer	Sequence	Size (bp)	*PS*-2 Exon	Primer	Sequence	Size (bp)
1A	S182ex1A-3	TTCTCCCCGCAATCGTTTCTCCAG	297	1	PS-2ex1-3	TGTTAGCAGCGGTGTTTG	248
	S182ex1A-4	GCCCATGTCCGCGGTGCCTTCC			PS-2ex1-4	TCTGCTCGGAGGGATGGAC	
1B	S182ex1B-3	AGGAGGGGCGGCCCGTTTCTCG	523	2	PS-2ex2-1	CAGGGCCAGGGGGAGGAA	303
	S182ex1B-4	AGCCTCTGCCACCACCGNAGGATC			PS-2ex2-2	AAAAGCAGGTTGGGAGTCAC	
2	S182ex2-3	TGGATGACCTGGTGAAATCCTATT	223	3	PS-2ex3-1	GTCCTCCACTGCCTTTGTCTCAC	328
	S182ex2–4	CAGAAAACAAAGCCTCTTGAGGTT			PS-2ex3–2	CTTCCCTTCTCCCTCCCGCATCAG	
3	S182ex3-1	ACAAAGTTCTGTTTTTCTTTCCC	247	4	52PS-2x4	AAAAATCCGTGCATTACAT	395
	S182ex3-2	CAGCATTTCTCAGAGGTGAGG			3PS-2x4	GCTGGTTGTGAGCTGCAGGTACAGTG	
4	S182ex4-1	CGTTACCTTGATTCTGCTGA	371	5	PS-2-ex5-1	AGCCTCGAGGAGCAGTCAG	241
	S182ex4-2	GACATGCTGTAAAGAAAAGCC			PS-2-ex5–2	GCAGACGGAGAGAAGCGT	
5	S182ex5-3	GATTGGTGAGTTGGGGAAAAGTG	335	6	5PS-2x6	GGTATCAGTCTCAGGATCATGGG	265
	S182ex5-4	ATACCCAACCATAAGAAGAACAGG			3PS-2x6	TGGGGAAGACTGGAGCTCGATG	
6	S182ex6-3	GGTTGTGGGACCTGTTAATT	149	7	PS-2-ex7-1	GTAAAGAGGGCCAGGTTGGG	387
	S182ex6-4	TTAATTCTGAAAGACAGACCC			PS-2-ex7-2	GTGCAGCACTGGGGACGATTT	
7	S182ex7-1	GGAGCCATCACATTATTCTAAA	326	8	52PS-2x8	GGGCAGGCTCTTCTTCAGGG	251
	S182ex7-2	AACAAATTATCAGTCTTGGGTTT			3PS-2x8	GAAAGCCACGGCCAGGAAG	
8	S182ex8-1	TTACAAGTTTAGCCCATACATTTT	215	9	PS-2ex9–3	ACCGCCTGAGACGTGAACCTT	235
	S182ex8-2	TCAAGTTCCCGATAAATTCTAC			PS-2ex9–4	TCCCTCTGCCCCTCCTGAACT	
9	S182ex9-1	TGTGTGTCCAGTGCTTACCTG	188	10	5PS-2x10	CTCTGACCAGCTGTTGTTTC	249
	S182ex9-2	TGTTAGCTTATAACAGTGACCCTG			3PS-2x10	AGCCTCCACCCTCTGTCT	
10	S182ex10-1	CCAGCTAGTTACAATGACAGC	345	11	5PS-2x11	TTCCATTCTGTGCACGCCTC	244
	S182ex10-2	TCAAAAAGGTTGATAATGTAGCT			3PS-2x11	ACCTGCCCCCACCACAATG	
11	S182ex11-1	GGTTGAGTAGGGCAGTGATA	275	12	PS-2ex12-3	ACACCAGGGATCACCACGCTCAC	344
	S182ex11-2	TTAAAGGGACTGTGTAATCAAAG			PS-2ex12–4	TGCCTCCTCCTCACCAAGTAAACA	
12	S182ex12-1	GTCTTTCCCATCTTCTCCAC	199				
	S182ex12-2	GGGATTCTAACCGCAAATAT					

Table 2. Missense mutations detected in *PS*-1 and *PS*-2

Gene	Exon	Location	Mutation	Family	Onset age (years)	Family history[a]	Restriction site change
PS-1	4	236C→T	Ala79Val	1005	53	AD	*Aci*I, *Bbe*I, *Ban*I, *Ehe*I, *Hae*II, *Hin*P1I, ***Hha*I**, *Hsp*92I, *Kas*I, *Nar*I, *Nla*IV
				1087	55	F	
				1061	58	F	
	5	344A→G	Tyr115Cys	1066	45	AD	*Csp*6I, ***Rsa*I**
	7	692C→T	Ala231Val	1072	58	F	***Bgl*I**, *Bsp*1286I, *Mwo*I
	9	953A→G	Glu318Gly	1069	57	F	
PS-2	4	185G→A	Arg62His	1121	62	S	***Aci*I**

[a]AD, autosomal dominant; F, familial; S, sporadic.
Nucleotide positions are relative to the translation start site in the *PS*-1 or *PS*-2 cDNA. Restriction enzymes used in this study to test for the presence of the mutation are denoted in bold (Fig. 1A). The pedigrees of families with a *PS*-1 mutation are depicted in Figure 2.

Table 3. Polymorphisms detected in *PS*-1 and *PS*-2

Gene	Exon	Location	Codon	Allele frequencies	Restriction site change
PS-1	1A	(–48)C→T		0.88/0.12	*Hga*I, *Hsp*96I, *Sfa*NI
	1B	–364C→A		0.18/0.82	***Bst*XI**
	8	(+16)A→C		0.54/0.46	*Alw*26I
PS-2	3	69C→T	Ala23	0.79/0.21	***Dde*I**
	3	129C→T	Asn43	n.d.	***Tsp*RI**
	4	(–42)A→G		0.54/0.46	***Nco*I**, *Nla*III, *Sty*I
	4	261C→T	His87	0.46/0.54	***Bbr*PI**, *Bsa*AI, *Eco*72I, *Mae*II, *Nla*III, *Nsp*I, *Tai*I
	5	366G→A	Thr122	n.d.	
	7	708T→C	Ser236	n.d.	***Bss*HII**, *Bst*UI, *Cag*8I, *Hin*PI, *Tsp*RI
	11	(+24)A→G		0.55/0.45	*Alu*I, *Bst*71I, *Bst*F5I, *Cvi*JI, *Msp*A1I, ***Pvu*II**

The nucleotide position of exonic polymorphisms are relative to the translation start site in the *PS*-1 or *PS*-2 cDNA. The nucleotide positions of intronic polymorphisms are relative to the start (+) or end (–) of the intron. Allele frequencies were determined in the 118 control individuals using the restriction enzymes denoted in bold (Fig. 1B). n.d., not determined.

RESULTS

Mutation analysis of *PS*-1 and *PS*-2

Previously, the complete genomic structure of *PS*-1 and *PS*-2 was determined identifying 10 coding exons in each gene (21,22). In order to avoid confusion we used the exon numbering 3–12 for *PS*-1 in this study, as introduced by Clark *et al.* (11). Intronic primer pairs were designed allowing PCR amplification of the 10 coding exons of *PS*-1 and *PS*-2 (Table 1). All 101 AD patients were examined for mutations in *PS*-1 and *PS*-2 by SSCP analysis and PCR cycle sequencing.

In *PS*-1 we identified four missense mutations in exons 4, 5, 7 and 9 respectively and one intronic polymorphism in intron 8 (Tables 2 and 3 and Fig. 1). The Ala79Val mutation was identified in three patients (1005, 1061 and 1087) (Table 2). To test whether the mutation in the three patients has the same ancestral origin we genotyped three simple tandem repeat (STR) markers flanking *PS*-1 (23,24). For D14S1028, D14S77 and D14S1004 all patients shared one common allele with allele frequencies calculated among the cases of respectively 0.27, 0.05 and 0.12. These data suggested that the three patients carrying the Ala79Val mutation might be related, although not closely, since genealogy studies had not indicated a familial relationship. The other three mutations occurred only once and none of the mutations were present in the 118 control individuals. The polymorphism observed in intron 8 is identical to that reported by Wragg *et al.* (25) and allele frequencies were determined by the primer mismatch PCR assay described by those authors (Table 3). Since the SSCP pattern of the intron 8 polymorphism could have masked the presence of mutations, we sequenced exon 8 in all cases. No other mutations were found.

In one patient a missense mutation in *PS*-2 was detected, resulting in an Arg→His substitution at codon 62 in exon 4 (Table 2 and Fig. 1A). Restriction digestion analysis showed that the mutation was absent in the other patients and controls. In addition, SSCP and sequence analysis of exon 4 identified two different polymorphisms in intron 3 and at codon His87 respectively (Table 3). Also, the SSCP patterns observed for exons 3, 5, 7 and 11 were due to polymorphisms, since the nucleotide changes involved intronic variations or exonic silent mutations (Table 3 and Fig. 1B). The polymorphism in exon 5 (Thr122) was seen in only one patient and represents a very rare polymorphism. Also, the polymorphism in exon 7 (Ser236) is rare, since it was seen in only two patients. The allele frequencies of the other more frequent polymorphisms were estimated in the 118 control

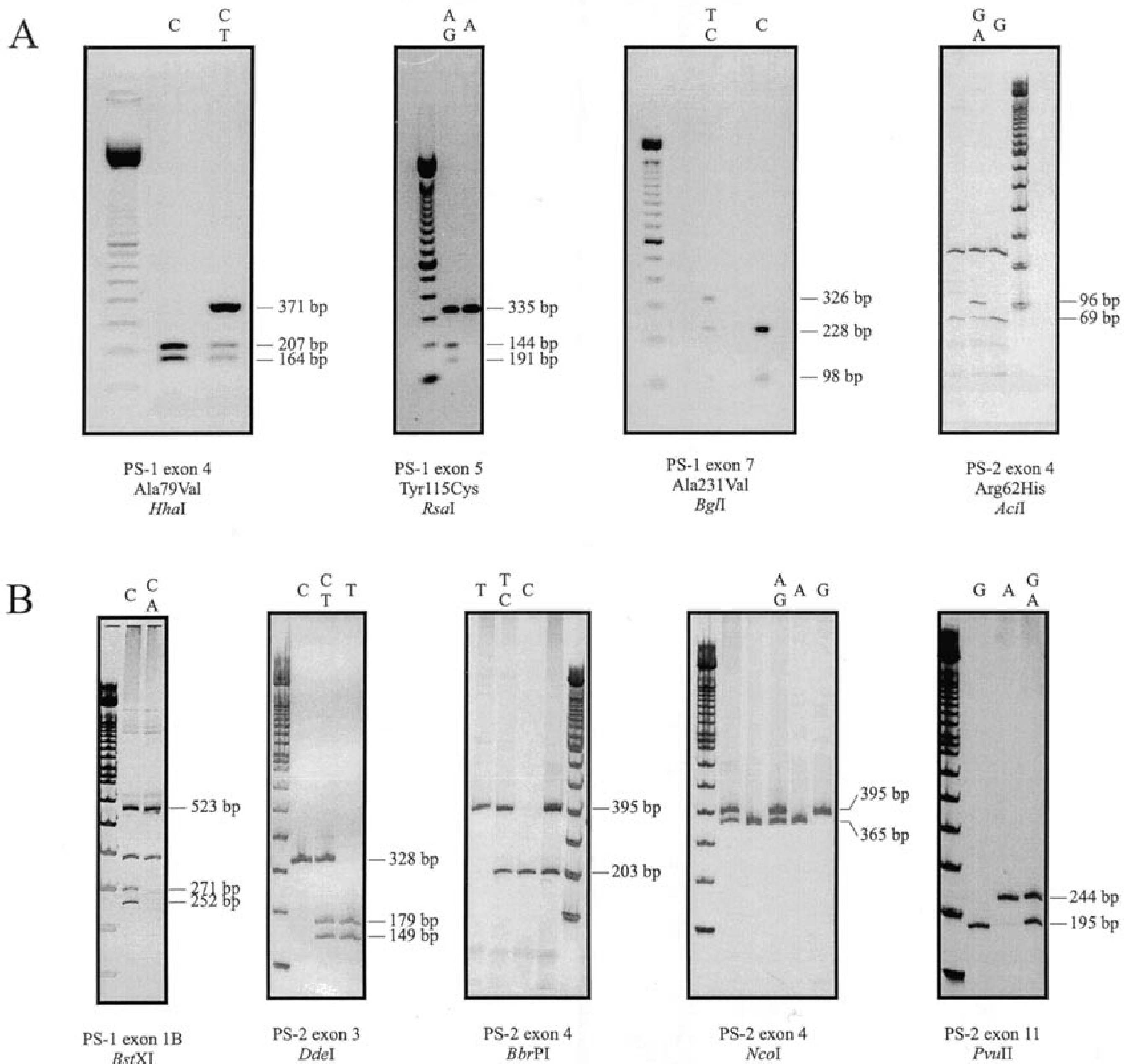

Figure 1. Restriction enzyme analyses of mutations and polymorphisms in *PS*-1 and *PS*-2. PCR amplified exons were digested with the restriction enzymes indicated. (**A**) Missense mutations in *PS*-1 and *PS*-2. (**B**) Polymorphisms in *PS*-1 and *PS*-2.

individuals by restriction enzyme digestion (Table 3). Comparison of the allele distribution in 15 control individuals showed that the two exon 3 polymorphisms are in linkage disequilibrium. Also, the two exon 4 polymorphisms are in linkage disequilibrium. Further, we demonstrated that the *PS*-2 polymorphisms in exons 3 (Ala23) and 4 (His87) and intron 11 show Mendelian inheritance.

To complete the mutation analysis of *PS*-1 and *PS*-2 we developed flanking primers for the three exons located in the 5′-untranslated region (5′-UTR) of *PS*-1 (numbered exons 1A, 1B and 2) and the two 5′-UTR of *PS*-2 (21,26). SSCP analysis followed by PCR cycle sequencing revealed two polymorphisms in the 5′-UTR of *PS*-1, but no mutations (Table 3). Allele frequencies of both polymorphisms were estimated by SSCP analysis of exon 1A and restriction digestion of exon 1B (Table 3 and Fig. 1B). Mendelian inheritance of both polymorphisms was demonstrated. No mutations or polymorphisms were detected in the 5′-UTR of *PS*-2.

Although SSCP analysis of *PS*-1 and *PS*-2 was negative in eight of the 10 autosomal dominant probands included in our sample, we could not rule out that mutations may have been missed, since the sensitivity of SSCP is not 100%. Therefore, we performed a mutation analysis of *PS*-1 and *PS*-2 cDNA synthesized from RNA isolated from cultured lymphoblasts of a

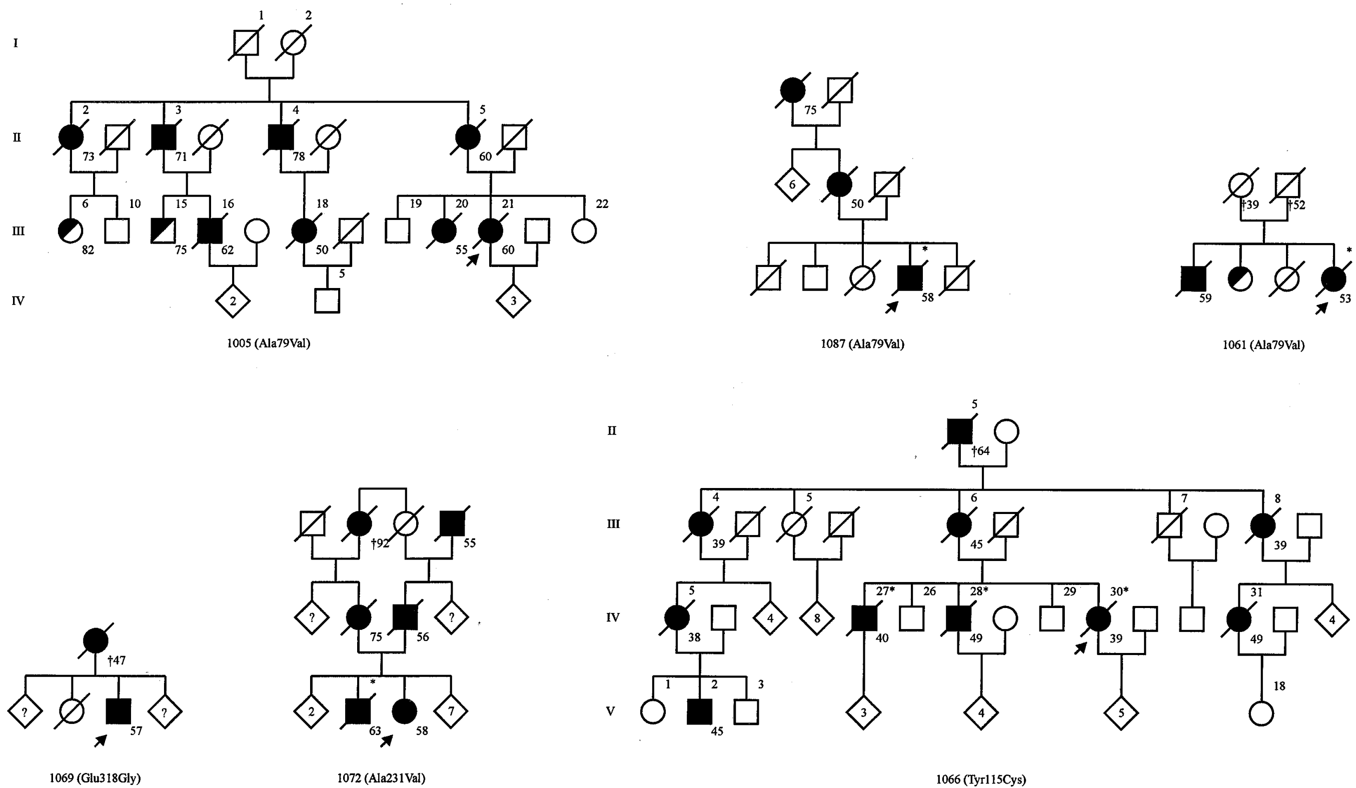

Figure 2. Pedigrees of the families of the autosomal dominant AD cases and the familial cases in which a *PS*-1 mutation was observed. Probands were included in the mutation analysis, except for families 1005 (III-20) and 1066 (V-2). Families 1061, 1069, 1072 and 1087 had not been included in our previous linkage studies (20). Symbols: circles depict females, squares depict males; open symbols: unaffected individuals; filled symbols: AD patients; partly filled symbols: patients diagnosed with CVA. Roman numbers to the left of the pedigree denote generations. Numbers below the patient symbols denote age at onset or age at death (†). Arrows indicate the probands. Patients that had autopsy confirmation of AD are indicated by an asterisk.

patient and an escapee. No other than the Ala79Val and Tyr115Cys mutations in *PS*-1 were detected.

Segregation analysis

All six *PS*-1 mutations occurred in patients with a positive family history of presenile AD, while the *PS*-2 mutation was observed in a sporadic case (Table 2). Two *PS*-1 mutations were present in autosomal dominant cases (1005 and 1066), while four *PS*-1 mutations were detected in familial cases (1061, 1069, 1072 and 1087) (Fig. 2). In the latter families the inheritance pattern was consistent with autosomal dominant transmission (Fig. 2); however, these families had not been selected for linkage studies since they did not fulfil our rigid criteria for autosomal dominant AD (1061, 1069 and 1087) or showed bilineal transmission of AD (1072) (20). Only in family 1072 were additional family members available and the *PS*-1 mutation Ala231Val was shown to be present in at-risk individuals.

In autosomal dominant families 1005 and 1066 co-segregation of the mutation with presenile AD was confirmed by restriction enzyme digestion (Table 2 and Fig. 1A). Previously we had used the informative families of the 10 autosomal dominant cases included among the 101 cases in genetic linkage studies (20). Family 1066 (mean onset age 42 years) was linked to D14S43 located at chromosome 14q24.3 [multipoint LOD score ($Z = 3.71$) at zero recombination] (20). Family 1005 was not informative for the chromosome 14 STR markers used.

DISCUSSION

Mutation analysis of all 10 coding exons as well as the 5′-non-coding exons of *PS*-1 and *PS*-2 by SSCP analysis and PCR cycle sequencing identified four different missense mutations in *PS*-1 in six patients (Ala79Val, Tyr115Cys, Ala231Val and Glu318Gly) and one missense mutation in *PS*-2 in one other patient (Arg62His) among 101 unrelated presenile AD cases in our study. All but one of the mutations (7,27) are novel mutations that were absent in 118 control individuals. Based on our mutation data we calculated a mutation frequency of 6% for *PS*-1 and 1% for *PS*-2 in presenile AD. Since all *PS*-1 mutations occurred in familial cases, i.e. cases with at least one first degree relative with AD, the mutation frequency is estimated at 9% (six out of 67 cases) in familial presenile AD cases. Among the familial cases in our study there were 11 cases belonging to families fulfilling our rigid criteria for autosomal dominant AD, i.e. three patients in two generations with a clinical diagnosis of AD in at least two cases (20). In two autosomal dominant families a *PS*-1 mutation was found resulting in a mutation frequency of 18% (two out of 11 cases) in autosomal dominant presenile AD.

Since the mutation screening of *PS*-1 and *PS*-2 was performed by SSCP analysis, we cannot exclude that our estimates of

mutation frequencies of *PS*-1 and *PS*-2 are underestimates, as the sensitivity of SSCP is not 100% (28). However, we systematically analyzed each PCR-amplified exon of *PS*-1 and *PS*-2 together with positive controls of known mutations in the presence and absence of glycerol as denaturant. Mutations in *PS*-1 and *PS*-2 may also have been masked by polymorphisms in exonic and flanking intronic sequences. When polymorphic SSCP patterns were observed we sequenced the PCR products in five to 10 cases showing homozygous and heterozygous SSCP patterns. In each case the underlying polymorphism was detected and confirmed by restriction enzyme digestion of the amplified product. The efficiency of our mutation analysis strategy is demonstrated by detection of a mutation in exon 4 of *PS*-2 in one patient that had a unique SSCP pattern different from that of the polymorphic SSCP patterns observed for exon 4. Also, sequence analysis of the polymorphic PCR products of exon 8 of *PS*-1 identified no mutations.

The onset age in the *PS*-1 mutation cases varied from 45 to 58 years, with three mutations (Ala79Val, Ala231Val and Glu318Gly) having onset ages above 55 years (Table 2). Most *PS*-1 mutations reported so far had onset ages between 35 and 55 years (29). However, the majority of these mutations were located in exons 5 and 8, coding in part for TM II and the large HL after TM VI (13). Here the mutations are predicted to interfere with the α-helical structure of TM II or the proteolytic processing of ps-1 occurring in HL VI (7). The ps-1 mutations identified in this study are located in the N-terminal region (Ala79Val in exon 4), in TM V (Ala231Val in exon 7) and in the middle part of HL VI (Glu318Gly in exon 9). Most likely these mutations have a milder effect on ps-1 functioning, possibly because the amino acid changes are semi-conserved (Ala79Val and Ala231Val) or they are located in a functionally less important region (Glu318Gly). The Glu318 mutation is located in a region of ps-1 that is less conserved in ps-2 and ps of other species (13). Also, co-segregation of the Glu318Gly mutation with AD could not be demonstrated, since no other relatives in family 1069 were available. High variability in onset age was observed in *PS*-1 family 1005 segregating the Ala79Val mutation with a mean onset age in the family of 64 years, ranging from 55 to 78 years. In one mutation carrier the disease was not yet fully penetrant at age 76 years. Possibly, the onset age in this family is modulated by other genetic and/or environmental factors. In this respect it is important to note that the non-penetrant case had an APOE ε3ε3 genotype, which may have delayed his onset age (30). However, APOE studies in larger samples of *PS*-1 cases and families are needed to conclude that the APOE genotype modulates expression of *PS*-1 mutations. Also, no effect of the APOE genotype on onset age was observed in chromosome 14-linked AD families with *PS*-1 mutations, leading to very early onset ages and severe phenotypes (31). Another possibility is that the *PS*-1-linked phenotype is modulated by a polymorphism in ps-2 or that mutations in ps-1 and ps-2 act together to express the disease phenotype (digenic effect). However, SSCP analysis as well as RT-PCR analysis of *PS*-2 cDNA did not detect sequence alterations in ps-2. Family 1005 is also of particular interest since in this family several patients had been identified with cerebrovascular accidents (CVA) (Fig. 2). However, none of them carried the *PS*-1 mutation, indicating that the CVAs in this family are not related to presenile AD. In contrast to the previous three *PS*-1 mutations, the Tyr115Cys mutation in exon 5, corresponding to HL I, was detected in chromosome 14-linked family 1066, with mean onset age of 42 years (range 39–49 years) (20). The latter provides evidence that some of the earlier mutation studies may indeed have been biased towards finding *PS*-1 mutations resulting in earlier onset ages and more severe phenotypes.

Only one *PS*-2 mutation, Arg62His, was observed in a sporadic AD case with an onset age of 62 years. The two published *PS*-2 mutations had been identified in autosomal dominant families with presenile AD in TM II and TM V of ps-2 (4,5). The mutated Arg62 codon is not conserved in human ps-1 and ps of other mammalian species and is located in a region of the N-terminal domain that is generally not conserved between ps-1, ps-2 and the *Caenorhabditis elegans* homolog sel-12 (32). Also, the mutation itself is a conserved amino acid substitution. Therefore, it cannot be excluded that this ps-2 mutation is a rare polymorphism not related to AD pathogenesis, since the patient also had an APOE ε3ε4 genotype which may have increased her risk of developing AD (30).

In conclusion, our mutation data showed that *PS*-1 and *PS*-2 mutations are rare genetic causes of presenile AD in general. Also, the frequency of *PS*-1 mutations in autosomal dominant AD families (18%) is less frequent than initially estimated. No mutations were identified in exons 16 and 17 of *APP* of any of the cases (33), suggesting that other AD genes must exist. It is possible that a fraction of the cases may be attributed to the presence of an APOE ε4 allele. In a previous study of this population-based sample we demonstrated that the risk for developing presenile AD is significantly increased in APOE ε4 homozygotes independent of family history and in APOE ε4 heterozygotes in which the family history is positive (33). In the sample of 101 cases there were 19 APOE ε4 homozygotes and 31 familial APOE ε4 heterozygotes.

In contrast to previous reports (29), we observed several *PS*-1 mutations leading to AD with onset ages above 55 years. Also, one of these *PS*-1 mutations was identified in an autosomal dominant family with a mean onset age of 64 years (range 55–78 years). The identification of these *PS*-1 mutations predicts that *PS*-1 mutations with even milder effects on ps-1 functioning may be present in senile AD. This is of particular importance since a genetic association between an intronic *PS*-1 polymorphism and senile AD has been reported (25), although this association could not be replicated in all studies. Possibly, the association is the result of a functionally more relevant sequence variation elsewhere in the *PS*-1 gene. Preliminary data obtained by sequence analysis failed to demonstrate sequence variations in the coding region of *PS*-1 (25). However, the promoter and 5′-non-coding region of *PS*-1 had not been examined, since these sequences became available only recently. In this respect the identification by us of two polymorphisms in the 5′-non-coding region of *PS*-1 is of interest, since these polymorphisms may be used in genetic association studies to test whether *PS*-1 is also a susceptibility gene for senile and/or presenile AD. Also, the intronic and exonic polymorphisms identified in *PS*-2 are useful to test the role of this gene in AD, an analysis that has not yet been performed.

MATERIALS AND METHODS

Subjects

All patients with a clinical diagnosis of AD and onset at or before age 65 years, made in the period January 1980 and July 1987, living in metropolitan Rotterdam and the four northern provinces

were ascertained (18). The clinical diagnosis of AD was independently confirmed by two neurologists using a standardized protocol according to NINCDS-ADRDA criteria for AD (34). A total of 198 patients participated in the study (18). Onset age was defined as the age at which memory problems or behavior changes were first noted. Cases were considered familial when at least one first degree relative suffered from dementia. The percentage of familial cases in the total sample of presenile AD patients was 48% (18,33). Of familial cases the pedigree was considered to segregate with autosomal dominant AD if at least three patients with dementia were reported in two generations and if there were at least two patients with detailed medical records on the clinical diagnosis of AD (18,33).

Blood samples were drawn from 100 randomly selected AD patients (33) and detailed data on family history of disease were collected (18). Affected and unaffected relatives of 17 families were visited at home, where blood was drawn. All relatives were assessed for family history of disease, risk factors for AD and memory performance (20). Blood samples were obtained from 118 control individuals matched for age within 5 years and place of residence. The controls were drawn randomly from the population register of the municipality of the patient (33). Cognitive status of the control individuals was tested and none of them showed symptoms of dementia at the time of the study. Leukocytes were collected from total blood of the patients and relatives and permanent lymphoblast cell lines were obtained by transformation using Ebstein–Barr virus. DNA was extracted from total blood or cultured lymphoblasts using a standard phenol/chloroform DNA extraction procedure.

In our initial genetic analyses of the 100 cases we had included nine autosomal dominant cases. However, recently an at-risk individual in 1066 (V-2, Fig. 2) was diagnosed with probable AD and included in this study, bringing the total number of cases to 101. Mutation analysis of exons 16 and 17 of *APP* excluded the presence of *APP* mutations in all cases (33). The informative families of 10 autosomal dominant cases were used in linkage analysis studies with chromosome 14, 19 and 21 markers (20). Family 1066 (mean onset age 42 years) was conclusively linked to chromosome 14, while two others were excluded. The other results were not informative. Since the linkage analysis studies were performed before the presenile AD locus on chromosome 1 was identified, the families had not been analyzed for linkage with chromosome 1 markers.

Polymerase chain reaction (PCR) analyses

About 200 ng DNA were amplified in a 25 µl reaction mixture containing 20 pmol each primer, 0.2 mM dNTPs, 0.2 U Taq DNA polymerase (Gibco-BRL, Gaithersburg, MD), 1.0 mM $MgCl_2$, 75 mM Tris–HCl, pH 9.0, 20 mM $(NH_4)_2SO_4$ and 0.01% Tween-20. The PCR amplification consisted of 30 cycles of 90 s at 94°C, 90 s at the empirically defined optimal annealing temperature and 90 s at 72°C.

In the PCR–SSCP analyses intronic PCR primers were used to amplify the exons and flanking intronic sequences of *PS*-1 and *PS*-2 (Table 1). The PCR amplification products were heat denatured, cooled on ice and separated using two different electrophoresis systems. The coding exons of *PS*-1 were analyzed on a 1× HydroLink MDE gel (J.T.Baker, Phillipsburg) with and without 10% glycerol. Electrophoresis was for 20 h at 800 V and at 4°C (with glycerol) or room temperature (without glycerol). The SSCP/heteroduplex patterns were visualized using silver staining. Alternatively, the non-coding exons of *PS*-1 and all exons of *PS*-2 were analyzed on a MultiPhorII electrophoresis system (Pharmacia Biotech, Uppsala, Sweden) using ExcellGel precast polyacrylamide gels and 1× HydroLink MDE gels containing 5% glycerol. Electrophoresis was at 600 V for 2–5 h, depending on the product sizes, and SSCP patterns were visualized using silver staining. SSCP analyses of exons 5–8, and 11 of *PS*-1 were performed in the presence of positive control samples of the Ile143Thr, Met146Leu, His163Arg, Ala246Glu, Leu286Val, Gly384Ala and Cys410Tyr mutations (3,23). When aberrant SSCP patterns were observed the sequences of the fragments were determined using cycle sequencing. The PCR amplification products were pretreated with 10 U exonuclease I and 2 U shrimp alkaline phosphatase to remove excess PCR primers and nucleotides. PCR amplification product (5 µl) was used as template in the cycle sequencing reaction using the ABI PRISM Dye Terminator Cycle Sequencing Core Kit (Applied Biosystems, Foster City, CA) according to the supplier's protocol, using the same primers as in the PCR amplification. The sequences were analyzed on an ABI 373A automated DNA sequencer.

Sequence variations in *PS*-1 and *PS*-2 were analyzed by restriction enzyme digestion of amplified products when they involved the creation or abolition of a restriction enzyme recognition site. Also, the intron 8 polymorphism in *PS*-1 was analyzed by *Bam*HI digestion as described (25). Genomic PCR amplification products were digested for 3 h using 5 U of the corresponding restriction enzyme (Tables 2 and 3) at the appropriate reaction temperature. The restriction fragments were separated on a 1.5–3% agarose gel, depending on the allele sizes, and visualized on an UV transilluminator after EtBr staining.

The APOE genotype was scored by PCR amplification of genomic DNA using published primers (35) in a standard PCR of 35 cycles. After digestion with 5 U *Hha*I for 3 h at 37°C the alleles were separated on ExcellGel precast polyacrylamide gels as described above and visualized using silver staining.

The STRs D14S1028, D14S77 and D14S1008 were amplified in a standard PCR using published primer sets, one of which was fluorescently labeled. The alleles were separated on a 6% polyacrylamide gel containing 8 M urea and analyzed on an ABI 373A automated DNA sequencer using GENESCAN 672 software (Applied Biosystems).

cDNA sequence analysis

Approximately 10^7 lymphoblast cells were homogenized in the presence of 1 ml TRIzol (Gibco-BRL). RNA was isolated by chloroform extraction and precipitated with isopropanol. After centrifugation for 10 min in a microfuge, the pellet was washed with 75% EtOH and dissolved in 20 µl DEPC-treated H_2O. First strand cDNA synthesis was performed with random primers using the SuperScript pre-amplification kit (Gibco-BRL) as described in the protocol supplied with the kit. The RNA was removed by adding 2 U RNase H and incubating for 20 min at 37°C. A standard PCR amplification of 30 cycles was performed using 100 ng first strand cDNA as template. Then *PS*-1 cDNA was PCR amplified using primer pairs 917/892, 901/111R and 1017/852 (3). The sequence of the amplification products was determined by cycle sequencing as described above. Sequencing primers were as published (3,23). *PS*-2 cDNA was PCR amplified using primer

pairs STM2-6/7 (5′-AACCAGCGCTGCCCTCTTTGAA-3′ and 5′-AGGATGACCCACGCGGACCACTC-3′) and STM2-8/9 (5′-CTACCCCACCCTCTTGCTGACTGT-3′ and 5′-CTCCCC-GCCCTAATCTGACCTTCT-3′). Cycle sequencing was performed using the same primers, primer 1021 (5) (5′-CAGAGGATGGA-GAGAACAC-3′), STM2-6 (5′-AACCAGCGCTGCCCTCTT-TGAA-3′), STM2-1 (5′-ATCGTGGTGGTAGCC-3′), STM2-2 (5′-GTTATGACCATCTTCTTGG-3′), STM2-4 (5′-TGTGCTGT-GTCCCAAAGG-3′) and PS2-1742AS (5′-TCCAATGAAAATT-CCCTGC-3′). The sequence of the *PS*-2 cDNA was determined by cycle sequencing as described above.

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