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Report

The Gene Encoding Nicastrin, a Major γ-Secretase Component, Modifies Risk for Familial Early-Onset Alzheimer Disease in a Dutch Population-Based Sample

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Nicastrin regulates γ-secretase cleavage of the amyloid precursor protein by forming complexes with presenilins, in which most mutations causing familial early-onset Alzheimer disease (EOAD) have been found. The gene encoding nicastrin (NCSTN) maps to 1q23, a region that has been linked and associated with late-onset Alzheimer disease (LOAD) in various genome screens. In 78 familial EOAD cases, we found 14 NCSTN single-nucleotide polymorphisms (SNPs): 10 intronic SNPs, 3 silent mutations, and 1 missense mutation (N417Y). N417Y is unlikely to be pathogenic, since it did not alter amyloid β secretion in an in vitro assay and its frequency was similar in case and control subjects. However, SNP haplotype estimation in two population-based series of Dutch patients with EOAD ($n = 116$) and LOAD ($n = 240$) indicated that the frequency of one SNP haplotype (HapB) was higher in the group with familial EOAD (7%), compared with the LOAD group (3%) and control group (3%). In patients with familial EOAD without the APOE ε4 allele, the HapB frequency further increased, to 14%, resulting in a fourfold increased risk (odds ratio = 4.1; 95% confidence interval 1.2–13.3; $P = .01$). These results are compatible with an important role of γ-secretase dysfunction in the etiology of familial EOAD.

The amyloid β peptide (Aβ) is the main component of the neuritic plaque, the histological hallmark lesion in the brains of patients with Alzheimer disease (AD [MIM 104300]). Aβ is generated by β- and subsequent γ-secretase cleavage of the amyloid precursor protein (APP [MIM 104760]). Mutations in APP and the presenilins (PSEN1 [MIM 104311] and PSEN2 [MIM 600759]) cause the rare familial early-onset type of AD (EOAD) (AD Mutation Database). Since the large majority of causative EOAD mutations directly influence γ-secretase activity, at the level of either the substrate (APP mutations at the γ cleavage site) or the enzyme (all PSEN mutations), γ-secretase dysfunction is crucial in familial EOAD pathogenesis. Recent findings by us (van Duijn et al. 1999; Theuns et al. 2000) and others (Lambert et al. 2001), of genetic association between the promoter region of PSEN1 and EOAD, further emphasize the importance of altered γ-secretase activity in EOAD. Recently, a novel type 1 transmembrane glycoprotein, designated "nicastrin" (NCSTN [MIM 605254]), was identified as a component of the presenilin-containing γ-secretase complex that is involved in the cleaving of APP and Notch in their transmembrane domains (Yu et al. 2000; Chen et al. 2001). Like all pathogenic PSEN mutations, missense mutations in a conserved hydrophilic domain (DYIGS→AAIGS) in NCSTN strongly in-

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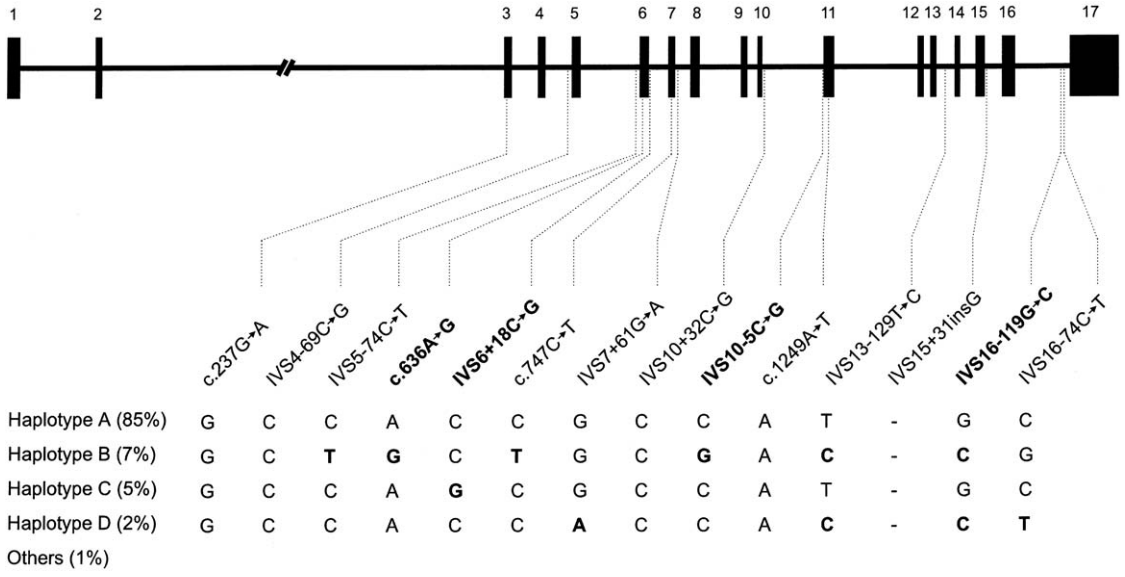

Figure 1 Genomic structure of NCSTN and major SNP haplotypes, with their estimated frequencies in 78 patients with familial EOAD. SNPs used for case-control association analysis are in boldface.

creased Aβ production, whereas deletions in this domain inhibited Aβ production (Yu et al. 2000). NCSTN maps to 1q23, a region that has shown evidence for linkage to (Kehoe et al. 1999) and association with LOAD (Hiltunen et al. 2001).

We evaluated the contribution of genetic variations in NCSTN in two large series of patients with EOAD (onset before or at age 65 years) and LOAD (onset after age 65 years). Patients with EOAD were derived from a Dutch population-based study of EOAD in the four northern provinces of the Netherlands and metropolitan Rotterdam (van Duijn et al. 1994*a*). The patients were sampled during two study periods. The original sample was collected between 1980 and 1987 and has been described elsewhere (van Duijn et al. 1994*b*). The initial study was extended between 1997 and 2000, in a genetically isolated part of the previously described area, with the same sampling criteria. The current sample comprises 96 patients from the original group, with an additional 20 patients from the extended study. Mean age at onset was 56.4 ± 5.5 years, and 23% of patients in the sample were male. The diagnosis was independently confirmed by a member of the research team and by a neurologist using a standardized protocol consistent with the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria for AD (McKhann et al. 1984). The disease in 38 patients was classified as sporadic, and the disease in 78 patients was classified as familial (i.e., at least one first-degree relative with dementia), 10 cases of which fulfilled our criteria of autosomal dominant inheritance (Cruts et al. 1998). In this sample, seven patients were identified who carried a causative PSEN mutation (five in PSEN1 and two in PSEN2) (Cruts et al. 1998; authors' unpublished data). Patients with EOAD were compared with a control group of 114 individuals in the same age range (mean age at examination 60.6 ± 3.3 years; 46% male). Patients with LOAD were drawn from the Rotterdam study, a population-based prospective study on residents of age $\geqslant$55 years from a Rotterdam suburb in the Netherlands (Hofman et al. 1991). Dementia was diagnosed by a three-step examination, as detailed elsewhere (Ott et al. 1995). The diagnosis of probable AD was based on NINCDS-ADRDA criteria (McKhann et al. 1984): 339 patients with AD were ascertained at the start of the study, and 116 patients with incident AD were diagnosed at the first follow-up period. DNA was available for 240 patients with probable LOAD cases (mean age at onset 84.1 ± 6.9 years; 20% male). Patients with LOAD were compared to 239 control individuals (mean age at examination 72.1 ± 3.7 years; 15% male). All control individuals were taken from the Rotterdam study and were screened for cognitive dysfunction. Potential control subjects with dementia were excluded.

We determined the genomic organization of NCSTN (gDNA: 15.9 kb; cDNA: 2.9 kb) by alignment of the

complete cDNA sequence (NCSTN cDNA [GenBank accession number AF240468]) against the human working draft sequence (Human Genome Project Working Draft Web site). NCSTN consists of 17 exons (fig. 1), all of which contain coding sequence. Using PCR primers flanking each exon, we screened NCSTN exons 1–16 and the coding part of exon 17 by denaturing high-performance liquid chromatography (DHPLC) in 78 patients with familial AD, including 10 patients with autosomal dominant EOAD. Direct sequencing of PCR fragments with aberrant DHPLC patterns revealed 14 single nucleotide polymorphisms (SNPs), of which 10 were located in introns and 4 in coding regions (table 1). Direct sequencing of NCSTN in the probands of the 10 autosomal dominant families revealed no causative mutations or additional variations. Only c.1249A→T predicts an amino acid substitution (N→Y at codon 417) and was found in a patient with familial EOAD (age at onset 57 years). No other family members were available for segregation analysis. NCSTN N417Y is a conservative amino acid substitution affecting a residue that is not evolutionarily conserved in mouse (A), *Drosophila* (E), or *Caenorhabditis elegans* (G) (Yu et al. 2000) but is located within a putative glycosylation site (N-Q-S). To test the potential contribution of N417Y to AD, we used a pyrosequencing assay (Alderborn et al. 2000; Theuns et al. 2001) to screen all patients with EOAD, as well as 235 patients with probable LOAD and a total of 474 control subjects taken from the Rotterdam study. In addition to the patient with familial EOAD, N417Y was also found in one patient with LOAD (familial; age at onset 87 years) and in three control subjects without dementia (ages 84, 87, and 95 years), resulting in identical N417Y carrier frequencies in patients with AD (2/354 = 0.6%) and controls (3/474 = 0.6%). To assess whether NCSTN N417Y influences APP processing, we measured Aβ40 and Aβ42 levels in conditioned media of HEK-293 cells transfected with the mutant and wild-type NCSTN cDNA. Results were compared with PSEN1 L392V, a clinical mutation causing familial EOAD that is known to increase Aβ42 levels (Sherrington et al. 1995; Citron et al. 1997), and PSEN1 D385A, an artificial mutation that substantially reduces Aβ production (Wolfe et al. 1999). Although both PSEN1 mutations showed the expected effects on Aβ production, Aβ40 and Aβ42 levels were similar for N417Y and wild-type NCSTN (data not shown).

To further evaluate the possibility that variations in NCSTN affect susceptibility to EOAD or LOAD, case-control association analysis was performed. Because of strong linkage disequilibrium between the SNPs in the 78 familial EOAD samples, we selected four informative SNPs (c.636A→G, IVS6+18C→G, IVS10−5C→G, and IVS16−119G→C) that allowed definition of four major haplotypes: A, B, C, and D (hereafter referred to as "HapA," "HapB," "HapC," and "HapD") (fig. 1). Through use of a pyrosequencing assay, genotypes for each of these four SNPs were determined in patients with EOAD, patients with LOAD, and with their age-matched control groups (table 2). Genotype frequencies for each SNP were in Hardy-Weinberg equilibrium (HWE) in all case and control groups ($P > .23$). No statistically significant differences in genotype frequencies were observed between patients with EOAD and LOAD and control subjects, at any of the four SNP loci tested. We then estimated four-locus haplotype frequencies from genotype data with a maximum-likelihood method using the expectation-maximization algorithm under the assumption of HWE (Excoffier and Slatkin 1995). Estimated haplotype distributions in the different groups were compared using CLUMP (Sham and Curtis 1995). CLUMP generates empirical *P* values that are based on Monte Carlo simulations and is useful for case-control comparisons of multiallelic genetic markers. To test for interaction between NCSTN and APOE, the case and control series were stratified for the presence of an APOE ϵ4 allele. Haplotypes were estimated separately in the patients with familial EOAD. Because haplotype frequencies were not statistically different between the two control groups ($P = .48$), and in order to obtain reliable haplotype frequency estimates in the different APOE strata, both control groups were pooled. In all groups studied, haplotype estimation revealed one frequent haplotype (HapA) and three rarer haplotypes (HapB, HapC, and HapD) (table 3). We found no significant differences in NCSTN haplotype distributions between patients with LOAD and control subjects (table 3). Moreover, haplotype frequencies remained highly stable across APOE strata in both the LOAD and control

Table 1

NCSTN Sequence Variations and Their Allele Frequencies in 78 Familial EOAD Cases

Location	Polymorphism	No. (Frequency) of Mutant Allele[a]
Exon 3	c.237G→A (Glu79)	2 (.01)
Intron 4	IVS4−69C→G	1 (.01)
Intron 5	IVS5−74C→T	13 (.08)
Exon 6	c.636A→G (Leu212)	13 (.08)
Intron 6	IVS6+18C→G	8 (.05)
Exon 7	c.747C→T (Asp249)	13 (.08)
Intron 7	IVS7+61G→A	2 (.01)
Intron 10	IVS10+32C→G	1 (.01)
Intron 10	IVS10−5C→G	13 (.08)
Exon 11	c.1249A→T (Asn417Tyr)	1 (.01)
Intron 13	IVS13−129T→C	16 (.10)
Intron 15	IVS15+31insG	1 (.01)
Intron 16	IVS16−119G→C	16 (.10)
Intron 16	IVS16−74C→T	3 (.02)

[a] Frequencies are given as proportion of $N = 156$ total alleles.

Table 2

Genotype Frequencies of Four Selected NCSTN SNPs in Patients with EOAD, Patients with LOAD, and Control Subjects

SNP and Genotype	EOAD versus Control Group			LOAD versus Control Group		
	No. (Frequency) in			No. (Frequency) in		
	EOAD Group	Control Group	P[a]	LOAD Group	Control Group	P[a]
c.636A→G:						
AA	103 (.89)	102 (.89)	.87	220 (.94)	175 (.93)	.85
AG	13 (.11)	12 (.11)		14 (.06)	12 (.07)	
Total	116	114		234	187	
IVS6+18C→G:						
CC	108 (.93)	102 (.89)	.33	217 (.92)	198 (.91)	.90
CG+GG	8 (.07)	12 (.11)		20 (.08)	19[b] (.09)	
Total	116	114		237	217	
IVS10−5C→G:						
CC	102 (.88)	102 (.89)	.71	215 (.92)	219 (.94)	.48
CG	14 (.12)	12 (.11)		19 (.08)	15 (.06)	
Total	116	114		234	234	
IVS16−119G→C:						
GG	99 (.85)	97 (.85)	.96	188 (.88)	167 (.92)	.15
GC+CC	17 (.15)	17 (.15)		26[c] (.12)	14 (.08)	
Total	116	114		214	181	

[a] Calculated using the χ^2 statistic with 1 df.
[b] Includes one subject with the GG genotype.
[c] Includes two subjects with the CC genotype.

groups, suggesting no interaction between NCSTN and APOE in the risk to develop LOAD. In the patients with EOAD, however, we found an increased frequency of HapB in those with familial EOAD (7%) and APOE $\epsilon4(-)$ EOAD (7%), compared with patients with LOAD (3%) and control subjects (3%). In patients with familial EOAD without APOE $\epsilon4$ alleles, the HapB frequency further increased, to 14%, resulting in a significantly different haplotype distribution ($P = .01$). When calculating risks by comparing the HapB frequencies in case subjects and their age-matched control subjects and by using the non-HapB haplotypes as reference, we found that the HapB frequency was significantly increased in patients with familial EOAD without APOE $\epsilon4$ alleles (odds ratio [OR] 4.1; 95% CI 1.2–13.3; $P = .01$). No homozygotes for HapB were observed. Assuming that individuals heterozygous at c.636A→G, IVS10−5C→G, and IVS16−119G→C are heterozygous HapB carriers, we found a significantly increased HapB/Hap* (where Hap* is any non-HapB haplotype) genotype frequency in patients with familial APOE $\epsilon4(-)$ EOAD (33%), compared with age-matched APOE $\epsilon4(-)$ control subjects (9%) (OR 4.9, 95% CI 1.5–16.3; $P = .005$). Since none of the apparent HapB carriers with EOAD had a PSEN mutation, the exclusion of the seven PSEN mutation carriers did not alter these results.

In the present study, we have investigated the contribution of genetic variations in NCSTN to the occurrence of AD, through use of a combined strategy of extensive mutation screening in patients with familial EOAD and subsequent case-control association analysis using four NCSTN polymorphisms in large population-based series of EOAD and LOAD. Although screening of all NCSTN coding exons and flanking intronic sequences in 78 patients with familial EOAD revealed a total of 14 NCSTN sequence variations, only NCSTN N417Y, detected in one patient with familial EOAD, was potentially pathogenic. However, its equal frequency in AD cases and controls (allele frequency 0.3%), its inability to alter Aβ production in an in vitro assay, and its low evolutionary conservation in different species strongly suggest that N417Y is a very rare but nonpathogenic variant. Although we can therefore conclude that mutation of NCSTN is not a frequent cause of EOAD, genetic association analysis did reveal that the risk of developing familial EOAD was modified by a significantly overrepresented NCSTN haplotype (HapB) in patients with familial EOAD (7%), particularly in those who lack APOE $\epsilon4$ alleles (14%), compared with control subjects (3%) and patients with LOAD (3%). Although we acknowledge the possibility of a false-positive finding, from a biological point of view the association between NCSTN and familial EOAD seems highly plausible, for several reasons. First, the association is caused by a specific overrepresentation of one haplotype in patients and is not due to frequency shifts in the control group, where NCSTN haplotypes remained highly stable even when stratified for APOE. Second, it is of interest that the most dramatic increase of HapB was

Table 3

Estimated NCSTN Four-Locus Haplotype Frequencies in Patients with EOAD, Patients with Familial EOAD, Patients with LOAD, and Control Subjects, Stratified for the Presence of an APOE ε4 Allele

Sample and Haplotype[a]	Control Group Frequency	LOAD Group			EOAD Group			Familial EOAD Group		
		Frequency	χ^2	P[b]	Frequency	χ^2	P[b]	Frequency	χ^2	P[b]
Total Sample:[c]										
A (ACCG)	.89	.89			.89			.85		
B (GCGC)	.03	.03			.05			.07		
C (AGCG)	.04	.04	1.1	.91	.03	2.3	.69	.05	7.8	.10
D (ACCC)	.02	.02			.02			.02		
Other	.02	.02			.01			.01		
APOE ε4(+):[d]										
A (ACCG)	.92	.88			.92			.89		
B (GCGC)	.03	.01			.03			.05		
C (AGCG)	.04	.07	4.4	.35	.03	.1	1.00	.05	.4	.98
D (ACCC)	.01	.01			.01			.01		
Other	.01	.03			.01			.01		
APOE ε4(−):[e]										
A (ACCG)	.89	.89			.82			.74		
B (GCGC)	.03	.03			.07			.14		
C (AGCG)	.04	.02	4.3	.37	.01	8.9	.06	.05	15.1	.01
D (ACCC)	.02	.03			.05			.05		
Other	.02	.02			.04			.03		

[a] Underlined bases are those that differ from the wild-type haplotype.

[b] Generated using CLUMP, by random simulation of 1,000 2 × 5 tables with the same marginal totals as the one under consideration and by counting the number of times that a χ^2 value associated with the real table was achieved.

[c] Control group, $n = 472$; LOAD group, $n = 426$; EOAD group, $n = 232$; familial EOAD group, $n = 156$.

[d] Control group, $n = 130$; LOAD group, $n = 160$; EOAD group, $n = 152$; familial EOAD group, $n = 114$.

[e] Control group, $n = 342$; LOAD group, $n = 266$; EOAD group, $n = 80$; familial EOAD group, $n = 42$.

observed in patients with familial EOAD who lack all well-established genetic factors (i.e., APOE ε4 alleles and PSEN or APP mutations), suggesting that the familial occurrence within this more homogeneous subgroup might at least be partially attributed to the NCSTN locus. Our results also suggest that an association in the total EOAD group may have been masked by the high number of APOE ε4 carriers (65% of the sample), while the effect is only present in non-ε4 carriers. Third, our finding of an effect in EOAD but not in LOAD is in line with our previous studies showing an association of the PSEN1 regulatory region locus with EOAD (van Duijn et al. 1999; Theuns et al. 2000) but not LOAD (Dermaut et al. 2001) and is consistent with the concept that an increased production of Aβ by altered γ-secretase activity is the primary pathogenetic event in (familial) EOAD. In contrast, in LOAD, the relative importance of altered production versus degradation or clearance of Aβ is still a matter of debate. Although independent replication studies are needed to further validate our findings, it is presently unclear what the underlying biological mechanism of the association might be. Because we detected only intronic or silent variations, we are currently screening the upstream regulatory region of NCSTN, in order to assess the possibility that an undetected variation in LD with HapB that influences nicastrin expression levels might explain our association. In addition, it cannot be excluded that variations in a nearby gene in LD with NCSTN HapB may be responsible for the observed association. According to the human draft sequence database, NCSTN is flanked by the gene encoding the α subunit of the coatamer protein complex (COPA [MIM 601924]) at its 5′ side and by the gene encoding the nescient helix loop helix 1 protein (NHLH1 [MIM 601924]) at the 3′ side. However, there is currently no direct evidence that these genes are implicated in AD pathogenesis.

In conclusion, our data show that mutation of the NCSTN coding region is not a frequent cause of EOAD but suggest that a specific NCSTN haplotype modifies the risk of developing familial EOAD, particularly in those cases that lack an APOE ε4 allele. Contrary to our expectation that NCSTN might be responsible for the reported positional evidence of an LOAD gene on 1q23, variation in NCSTN does not confer risk of LOAD in our sample.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

AD Mutation Database, http://molgen-www.uia.ac.be/ADMutations/

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for human NCSTN [accession number AF240468])

Human Genome Project Working Draft, http://genome.ucsc.edu/

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for AD [MIM 104300], APP [MIM 104760], COPA [MIM 601924], PSEN1 [MIM 104311], PSEN2 [MIM 600759], NHLH1 [MIM 162360], and NCSTN [MIM 605254])

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