SHORT REPORT

Polymorphisms in neprilysin gene affect the risk of Alzheimer's disease in Finnish patients

S Helisalmi, M Hiltunen, S Vepsäläinen, S Iivonen, A Mannermaa, M Lehtovirta, A M Koivisto, I Alafuzoff, H Soininen

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Objectives: Neprilysin (NEP) is an amyloid β -peptide (A β) degrading enzyme expressed in the brain, and accumulation of A β is the neuropathological hallmark in Alzheimer's disease (AD). In this study we investigated whether polymorphisms in the NEP gene have an effect on the risk for AD.

Methods: The frequencies of seven single nucleotide polymorphisms (SNPs) and apolipoprotein E (APOE) were assessed in 390 AD patients and 468 cognitively healthy controls. Genotypes of the study groups were compared using binary logistic regression analysis. Haplotype frequencies of the SNPs were estimated from genotype data.

Results: Two SNPs, rs989692 and rs3736187, had significantly different allelic and genotypic frequencies (uncorrected $p=0.01$) between the AD and the control subjects and haplotype analysis showed significant association between AD and NEP polymorphisms.

Conclusion: Taken together, these findings suggest that polymorphisms in the NEP gene increase risk for AD and support a potential role for NEP in AD.

Alzheimer's disease (AD) is the commonest progressive neurodegenerative disease leading to dementia. Deposition of amyloid β -peptide (A β) as senile plaques in brain is one of the neuropathological hallmarks of AD, and neprilysin (NEP; EC 3.4.24.11) is a major A β degrading enzyme.¹ The NEP gene is located on chromosome 3q25.1–q25.2, and expression of the gene is transcriptionally regulated in a tissue specific manner.² Since NEP enzyme levels are the lowest in those AD brain areas that are most vulnerable to senile plaque development compared with other brain regions and in peripheral organs, it is speculated that mutations in the NEP gene may cause downregulation or upregulation of NEP expression and thus act as risk or protective factors, respectively, for AD.^{1,3} Since results from genetic association studies are ambiguous, but promising^{4–7} we evaluated whether allelic variations across the NEP gene modify the risk for AD in a Finnish population.

SUBJECTS AND METHODS

The subjects were examined in the Department of Neurology, Kuopio University Hospital.^{8,9} The hospital ethical committee approved the study. The study subjects included 390 patients with AD (mean age of onset was 72 (SD 7) years; 273 (70%) women) and 468 controls (mean age at examination or death 70 (5) years; 283 (61%) women), who had no signs of dementia on interview and neuropsychological testing. Thirty six per cent of patients with AD had a positive family history of AD, but inconclusive evidence of autosomal dominant transmission.⁸ All subjects with AD underwent a comprehensive clinical evaluation during which the clinical

diagnosis of probable AD was made according to the NINCDS-ADRDA criteria.¹⁰ This study included 63 AD cases (16% of all AD cases) with age of onset 65 years or below, and they were screened for known mutations in APP, PSEN-1 or PSEN-2 genes. Although none were found, it is still possible that some of these patients may carry rare variants of these genes.

Seven intronic single nucleotide polymorphisms (SNPs), at ~15 kb intervals, were selected for screening and amplification assays were designed for each SNP with the primers as described in table 1. Assays for the SNPs were typed in two multiplex polymerase chain reaction (PCR) reactions, subsequently mixed together to perform a single SNaPshot reaction. SNaPshot multiplex reactions were performed according to the manufacturer's instructions. Samples were analysed and allele peaks were determined with ABI3100 Genetic Analyzer and Genotyper 3.7 (Applied Biosystems). Apolipoprotein E (APOE) genotyping was determined by a standard method.⁸

In the statistical analyses two-sided Fisher's exact test and an unbiased estimate of exact p test were used for comparing allele and genotype frequencies, respectively. Odds ratios, as the estimates of relative risk of disease, were calculated using binary logistic regression with 95% confidence intervals (CI). To account for multiple testing, we used the Bonferroni method and corrected for 14 tests (seven SNPs and APOE $\epsilon 4$ status). All SNPs were in Hardy-Weinberg equilibrium in cases and controls ($p>0.05$). Statistical analyses were carried out with SPSS version 10.0 and estimation of haplotype frequencies was performed with Arlequin version 2.0 (<http://lgb.unige.ch/arlequin/>). Pairwise linkage disequilibrium (LD) of SNPs was determined using the 2LD program and Arlequin (<http://www.iop.kcl.ac.uk/IoP/Departments/PsychMed/GepiBSt/software.shtml>). Haplotype frequencies were compared for case and control subjects using the RxC program employing the metropolis algorithm to obtain unbiased estimates for exact p values with standard errors.¹¹ The level of statistical significance was set at $p=0.05$.

RESULTS

The distributions of APOE $\epsilon 2/\epsilon 3/\epsilon 4$ alleles differed as expected for the 390 AD cases compared with the 468 control subjects ($p<0.001$) and were consistent with those previously described in the same population.⁸ The odds ratio for the APOE $\epsilon 4$ allele was 4.7 (95% CI 3.8 to 5.9) and age at onset was approximately three years earlier for $\epsilon 4+$ versus $\epsilon 4-$ cases (71 v 74 years, $p<0.001$).

Allelic and genotypic frequencies for the SNPs 1 and 7 were significantly different between the study groups, but no significant differences were observed for SNPs 2–6 (table 2).

Abbreviations: A β , amyloid β -peptide; AD, Alzheimer's disease; APOE, apolipoprotein E; LD, linkage disequilibrium; NEP, neprilysin; SNP, single nucleotide polymorphism

Table 1 Details of the single nucleotide polymorphisms (SNPs)

SNP (location)	NCBI SNP Cluster ID*	Allele 1/ allele 2	Primers	T (°C)
SNP 1 (intron 1)	rs989692	C/T	F: TCCCCCTTACTCCCCTCTTA R: GCAAAACGAATGAACACATGA SS1 F: T ₃₇ AATAGATACTGGTATCAGTAG F: AAAGCCTACCATCAGGGACA R: CATGGACCACGCATTGAATA SS2 R: T ₁₉ CTCACCTCTGATTAATCCCTT F: CACTCCATTTTCCAATGTGCT R: TCCACTGTCGACTTATCACC SS3 F: C ₂₆ TCCACTATAAAAAATTCATC F: GTAGAGACGGGGTTTCACCA R: AACCAACACCTTTCCCAAT SS4 R: C ₄₃ AAACTAATAATCTTACAC F: CTTTACACACATTTCCATTTTGC R: CCTAGCATAATTAGCCAACCAAT SS5 R: T ₅₉ AAGCTTAAAGGCTTTGTTTTTC F: GCTCCAAGGGGTTTAGAAAGTT R: GCCTTCCCAAGAAGAAGAAAAT SS6 R: C ₃₀ ATCAAAAAAGAAAAAATTTTC F: TCTGTGATGAGGAGGGATGAC R: GATACACCATGCACTGGGATT SS7 R: C ₅₄ TTTACTTAAATAATATATTA	61.8
SNP 2 (intron 4)	rs2196521	C/T		58
SNP 3 (intron 4)	rs1025192	C/T		58
SNP 4 (intron 9)	rs1816558	C/T		58
SNP 5 (intron 11)	rs3773885	C/T		58
SNP 6 (intron 18)	rs3773882	C/T		61.8
SNP 7 (intron 19)	rs3736187	G/A		58

*From the clone gi:22044576 (<http://www.ncbi.nlm.nih.gov/>). Markers are ordered from pter to qter along chromosome 3.

Details of the SNPs may be found in the dbSNP database under their respective IDs.

F, Forward primer sequence (5'-3'); R, Reverse primer sequence (5'-3'); SS, SNaPshot primer with the length of tail described in subscript; T, annealing temperature (°C) for the polymerase chain reaction.

The genotype frequencies of SNP 1 TT and SNP 7 AA were higher in patients with AD. The age and sex adjusted odds ratio for the risk of AD in homozygous carriers of the SNP 1 T-allele and the SNP 7 A-allele was 1.85 (95% CI 1.17 to 2.94; $p < 0.01$) and 1.46 (95% CI 1.08 to 1.97; $p = 0.01$), respectively. In carriers of the SNP 1 T-allele and the SNP 7 A-allele, an age and sex adjusted odds ratio for the risk of AD was 1.32 (95% CI 1.00 to 1.74; $p = 0.05$) and 4.06 (95% CI 1.14 to 14.4; $p = 0.03$). The sample was stratified for the APOE genotype. A borderline significance was found in the APOE $\epsilon 4$ -negative subjects with homozygous carriers of the SNP 1 T-allele ($p = 0.06$) and the SNP 7 A-allele ($p = 0.05$). No significant differences were found in the APOE $\epsilon 4$ -positive group.

A strong pairwise LD between SNPs (all samples; 1716 alleles) was found across the gene suggesting the existence of a risk haplotype. Using a threshold D' value of 0.30, 62% of SNP pairs demonstrated LD, and with the likelihood ratio test of LD, 67% of SNP pairs had $p < 0.01$ (data not shown).

Haplotypes with frequencies above 5% (CTTTTCA, 12.8% in AD ν 17% in controls; CTCCCCG, 7% ν 12%; CTCCCCA, 10.2% ν 9.1%; CTCCCTA, 6.1% ν 6.6%; CTTTCCA, 6.7% ν 6.5%; TTCCCCA, 9.2% ν 5.5%) were included into statistical tests. Haplotype analysis with 780 alleles in all AD cases and 936 alleles in all controls showed that one haplotype with SNP 1 T-allele and SNP 7 A-allele was commoner for the AD cases compared with the controls (TTCCCCA: 9.2% ν 5.5%). The

Table 2 Allele and genotype frequencies of polymorphisms

Locus	Allele	Allele frequency			Genotype	Genotype frequency		
		Control (n = 936)	AD (n = 780)	p value for allele*		Control (n = 468)	AD (n = 390)	p value for genotype†
SNP 1	C	0.70	0.64	0.01 (0.14)	CC	0.48	0.41	0.01 (0.14)
	T	0.30	0.36		CT	0.45	0.46	
					TT	0.07	0.13	
SNP 2	C	0.12	0.14	0.36	CC	0.03	0.03	0.54
	T	0.88	0.86		CT	0.19	0.22	
					TT	0.78	0.75	
SNP 3	C	0.52	0.53	0.92	CC	0.28	0.28	0.98
	T	0.48	0.47		CT	0.49	0.49	
					TT	0.23	0.23	
SNP 4	C	0.41	0.39	0.50	CC	0.18	0.17	0.73
	T	0.59	0.61		CT	0.46	0.44	
					TT	0.36	0.39	
SNP 5	C	0.68	0.68	1.00	CC	0.46	0.47	0.62
	T	0.32	0.32		CT	0.44	0.41	
					TT	0.10	0.12	
SNP 6	C	0.78	0.78	1.00	CC	0.59	0.61	0.62
	T	0.22	0.22		CT	0.37	0.34	
					TT	0.04	0.05	
SNP 7	G	0.19	0.14	0.01 (0.14)	GG	0.03	0.01	0.01 (0.14)
	A	0.81	0.86		GA	0.31	0.25	
					AA	0.66	0.74	

*Fisher's exact test and †unbiased estimate of exact p value. The Bonferroni corrected p values for SNP 1 and SNP 7 are given in parenthesis.

twofold overrepresentation of **TTCCCCA** for AD cases was significantly overrepresented among cases compared with the remaining haplotypes (OR 1.77, 95% CI 1.22 to 2.56; $p < 0.01$; data not shown).

DISCUSSION

The candidate gene based association study with the NEP gene in a large clinic based series of AD and control subjects implicated NEP as a risk gene for AD. Primarily, we explored AD and control subjects as a whole to extract the maximum statistical power that the data from genotypes can provide and in order to obtain reliable haplotype frequency estimates. Previously, one SNP (rs701109; ~12 kb from the SNP 7 to the 3'UTR) has been associated with AD in Spanish patients under 75 years.⁷ This is in line with our finding that SNP 7 AA genotype was associated with increased AD risk in the whole AD group and also in cases under 75 years. These associated polymorphisms may be in LD with a pathogenic variation either in NEP itself or in a nearby gene. The plausible explanation for significant associations with AD and polymorphic sites (SNP 1, SNP 7, and rs701109) could be that these SNPs are in LD with each other harbouring the common haplotype block(s) across the NEP gene as described in the case of the CYP19 gene.¹² Our finding of considerable LD (D') between SNP pairs across the NEP gene showing a strong intermarker association even in long distances (>70 kb) also supports this idea. Additionally, the finding that the SNP 1 T-allele and SNP 7 A-allele containing haplotype with a high odds ratio is relevant and reinforces the hypothesis.

While the evidence of the importance of NEP polymorphisms in AD is now described from two genetically distant populations, the results of this study should be interpreted with caution, in particular those of the APOE modified tests owing to the smaller sample sizes of the study groups, and independent replication studies are needed to verify the finding in other populations.

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Authors' affiliations

S Helisalmi, M Hiltunen, S Vepsäläinen, S Iivonen, M Lehtovirta, A M Koivisto, I Alafuzoff, H Soininen, Department of Neuroscience and Neurology, University Hospital and University of Kuopio, Kuopio, Finland

S Helisalmi, M Hiltunen, S Vepsäläinen, S Iivonen, A M Koivisto, H Soininen, Brain Research Unit, Clinical Research Centre/Mediteknia, University of Kuopio, Kuopio, Finland

A Mannerman, I Alafuzoff, Department of Pathology and Forensic Medicine, University Hospital and University of Kuopio, Kuopio, Finland

M Lehtovirta, Department of Neurology, Jorvi Hospital, Espoo, Finland

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Correspondence to: S Helisalmi, PhD, Brain Research Unit, Clinical Research Centre/Mediteknia, Department of Neuroscience and Neurology, Kuopio University, Harjulantie 1, PO Box 1627, 70211 Kuopio, Finland; seppo.helisalmi@uku.fi

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