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Original Article

Evaluation of neprilysin sequence variation in relation to CSF β-Amyloid levels and Alzheimer disease risk

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Abstract: Neprilysin (NEP) is a principal peptidase involved in the degradation of β-amyloid (Aβ), and as such its encoding gene (*MME*) has been the target of numerous genetic association studies on Alzheimer disease. Here, in order to attempt replication of previous findings we have investigated several single nucleotide polymorphisms (SNPs) that have been claimed to be associated with AD. A key feature of the present study is the complementary investigation of both AD risk and quantitative measures of AD severity, including cerebrospinal (CSF) fluid levels of $A\beta_{1-42}$. In contrast to the effects of *APOE*, none of these measures are detectably influenced by genetic polymorphism in the *MME* region. We thus, fail to find support for previous results suggesting that *MME* impacts AD.

Key words: Neprilysin, β-Amyloid, Alzheimer disease, MME, Metalloendopeptidase, Polymorphism

Introduction

Alzheimer disease (AD; MIM 104300) is the most common cause of dementia in ageing human populations, and numerous association studies have addressed its genetic etiology in recent years [1]. Among proposed candidate genes claimed to be involved in AD, the only widely accepted one showing consistent and strong evidence of association is apolipoprotein E (*APOE*) [2]. The most common neuropathological hallmarks of AD are senile plaques, the deposition of amyloid β-peptide (Aβ), and formation of neurofibrillary tangles. There are several proteins involved in the degradation of Aβ, among which insulin-degrading enzyme (IDE) (EC 3.4.24.56), neprilysin (NEP) (NEP; EC 3.4.24.11), endothelin-converting enzymes (ECE 1 and 2) (EC 3.4.24.71), and plasminogen activator urokinase (PLAU) (EC 3.4.21.73) play a central role. NEP, also known as neutral endopeptidase, enkephalinase, CD10, common acute lymphocytic leukemia antigen (CALLA), or membrane metalloendopeptidase (MME), is a type II membrane metalloendopeptidase, potentially involved in the degradation of Aβ in vivo [3].

NEP was originally proposed as an important Aβ-degrading protein [4] and is now, together with IDE, considered among the most important Aβ-degrading peptidases with regard to AD pathology [5]. One difference between the two is the capacity of degrading the oligomeric forms of Aβ; whilst NEP is able to degrade these forms [6], IDE is not [7, 8]. Iwata et al. (2005) [5] suggests a 2-fold $A\beta_{42}$-elevating effect in the brain of Neprilysin-KO (knock-out) (-/-) mice, compared with 1.3-fold for ECE 2-KO (-/-), 1.3-fold for ECE 1-KO (+/-), and 1.4-fold for IDE-KO (-/-) mice.

The hippocampus and temporal gyrus in the human brain are especially vulnerable to senile plaque formation. NEP mRNA levels are lower in these areas, compared to caudate and peripheral organs that are resistant to senile plaque formation [9]. Decreased levels of NEP protein in the brain of AD patients compared to healthy controls together with an

increase of Aβ deposition has previously been reported [10]. In addition, a down-regulation of neprilysin expression in pyramidal neurons was also observed [10], which might be consistent with neprilysin as a regulator of Aβ accumulation level and plaque formation in hippocampal areas.

The gene encoding neprilysin (*MME*) is located on chromosome 3q25.1-q25.2, spans approximately 80 kb and contains 24 exons. Transcriptional regulation of the gene occurs in a tissue specific manner [11, 12, 13]. Four known different mRNA transcripts with varying 5´- sequences are generated using alternative transcription start sites [11, 13]. Among these four transcripts, type 1 is predominantly expressed in neurons and potentially influenced by a dinucleotide repeat polymorphisms in the promoter region [5]. All the other major transcripts also vary in their 5´-untranslated (5´UTR) regions [11].

In the present study, three single nucleotide polymorphisms (SNPs) located in the vicinity of the 3´UTR (rs989692 and rs701109), and the 5´UTR (rs3736187) of *MME* were genotyped, each previously being reported to be associated with risk for AD [14, 15]. Given the difficulties of using solely end-point diagnoses in genetic association studies, our primary goal here has been to test for potential genetic effects upon a quantitative measure of Aβ metabolism, specifically cerebrospinal fluid (CSF) levels of $A\beta_{1\text{-}42}$. Additional phenotypes, including age-at-onset (AAO), CSF tau levels, and AD risk have also been evaluated.

Materials and methods

Human Samples

A detailed description of the AD case and control population used in this study has been provided previously [16]. The maximum sample in which genotypes were obtained included 668 AD cases (245 males, 423 females) and 261 controls (121 males, 140 females). The samples were not age and sex matched. Average age in cases was 76.2±7.1 years (SD) and 73.2±9.2 years in controls. Average debut age for AD cases was 72.4±7.7 years and was established by a structured examination performed by a psychiatrist. This sample size permits approximately 80% power to detect an allele with an effect size (OR) of 1.5 at α = 0.05 for an allele with 25% population frequency.

CSF Biomarkers

CSF samples were obtained in the AD case-control study by lumbar puncture in the L3/L4 or L4/L5 inter-space. CSF Aβ42 was determined using a sandwich enzyme-linked immunesorbent assay (ELISA) (Innotest b-amyloid (1-42), Innogenetics, Ghent, Belgium) constructed to specifically measure Aβ42. The microtubule-associated protein tau, a CSF marker of neuronal degeneration, was determined using a sandwich ELISA (Innotest hTAU-Ag, Innogenetics, Ghent, Belgium) constructed to measure total tau, i.e., all isoforms of tau irrespective of phosphorylation state.

Genotyping

Genotyping was performed using hybridization with TaqMan® technology (ABI PRISM® 7700 Sequence Detection System; Applied Biosystems). The three markers used in the genotyping process were previously reported for positive association with AD [14, 15]; rs701109, rs989692, and rs3736187. Additional information on genotyped SNPs is available in dbSNP (http://www.ncbi.nlm.nih.gov/SNP/).

Statistical analysis

Deviations from Hardy-Weinberg equilibrium (HWE) for genotypes at individual loci, as well as differences in genotype and allele distributions between groups, were assessed using the chi-square test. Fisher's exact test was used where appropriate. Tests for association between single marker genotypes and quantitative traits were performed using analysis of variance (ANOVA) and significance of pair-wise comparisons assessed using Fisher's PLSD post hoc test. Logistic regression was used to adjust for the effects of age, gender, and APOE e4 status in case-control models. Multivariate tests for association of markers with disease risk were also conducted using logistic regression in an additive model, taking the form $\text{logit}(p) = \beta_0 + \beta_1 x_1$, where β_0 is the intercept and β_1 is the effect of the SNP to be estimated. For these tests, genotypes at all bi-allelic loci were re-coded as 0, 1 and 2 for allele '1' homozygous, heterozygous, and allele '2' homozygous genotypes. All of the above

statistical analyses were performed using StatView version 5.0 (Abacus Concepts). Linkage disequilibrium (LD) between *MME* marker pairs was estimated using the r^2 metric [17]. Haplotypes were estimated using PHASE v2.1 which implements a Bayesian method of haplotype estimation for unrelated individuals [18, 19]. For estimation of empirical P-values in case-control tests, 1000 permutations were used with all other settings at default.

Results

Genotype counts for rs3736187 and rs989692 were found to be in HWE across both case and control samples. However, rs701109 deviated marginally from HWE (p = 0.014). No significant differences for the three studied markers were observed between cases and controls in allelic or genotypic models (**Table 1**). Results presented are uncorrected for multiple testing. We estimated that approximately 20 independent tests were conducted across the study (not including both genotype and allele tests as those are highly correlated, and not including HWE tests). There was no evidence that haplotypes estimated from the three markers had significantly different frequencies between cases and controls (global p-value from 1000 permutations = 0.432). We also used logistic regression to model the potential independent effects of each of the three markers. In contrast to the well-documented effect of *APOE*, none of the three markers exhibited significant effects (not shown). In these multivariate models, the best independent significance was for rs989692 (p = 0.078). The borderline significance from this model as well as from the chi-square analysis (**Table 1**) is in opposition to previous findings [15] where the T allele of rs989692 was shown to be enriched in AD.

To evaluate the possible effects of *MME* sequence variation on AD-related phenotypes, we used ANOVA to explore for both individual marker effects and multi-locus effects (in order to capture possible allelic heterogeneity). We note that for multi-locus models using all three markers colinearity is not a factor since LD is relatively weak between sites (all pair-wise r^2 values are below 0.3). Our primary focus was upon CSF $A\beta_{42}$, but we also considered CSF tau, mini-mental state examination (MMSE) scores (in cases only), and AAO of cases. Multi-locus ANOVA models were fit using all three markers, and included *APOE* genotype (rs429358), a disease status identifier (since both cases and controls were analyzed together for some phenotypes), age, and gender as covariates. Individual marker tests in ANOVA models were performed using the same covariate strategy as above. The results of these analyses were broadly negative, with the only strong evidence of an effect being for *APOE*. We show the analysis for all three markers and CSF $A\beta_{42}$ in **Table 2**.

Table 1. Genotype frequencies for markers rs3736187, rs701109, and rs989692 in *MME*

rsID/sample	Case/control	Polymorphism			Genotypes	Alleles
rs3736187		G/G (0)	A/G (1)	A/A (2)		
	AD	0 (0.00)	80 (0.12)	570 (0.88)	*P* = 0.28	*P* = 0.85
	Controls	1 (0.01)	23 (0.12)	171 (0.87)	OR 1.05, 0.65-1.68	OR 1.04, 0.66-1.66
rs701109		G/G (0)	A/G (1)	A/A (2)		
	AD	110 (0.16)	298 (0.45)	260 (0.39)	*P* = 0.38	*P* = 0.57
	Controls	38 (0.20)	75 (0.40)	74 (0.40)	OR 1.06, 0.85-1.33	OR 1.07, 0.85-1.35
rs989692		T/T (0)	C/T (1)	C/C (2)		
	AD	140 (0.21)	343 (0.52)	182 (0.27)	*P* = 0.068	*P* = 0.070
	Controls	51 (0.28)	90 (0.49)	43 (0.23)	OR 1.24, 0.98-1.57	OR 1.24, 0.98-1.56

Genotype counts and frequencies for markers rs3736187, rs701109, and rs989692 in *MME* are presented. Significance has been estimated by means of chi-square analysis and the respective two-tailed p-values are shown from both 3x2 contingency tables for genotypes, and 2x2 tables for alleles. Odd ratios with 95% confidence intervals are shown from both genotype (logistic regression) and allele models.

Table 2. Quantitative trait associations for *MME*

rsID	Trait	Genotypes			Significance
rs3736187		G/G	A/G	A/A	
	Aβ42	0±0 (0)	579±26 (80)	543±8.5 (579)	NS
	Tau	0±0 (0)	658±40.5 (80)	619±13.1 (593)	NS
	AAO	0±0 (0)	71.1±0.8 (63)	73.3±0.3 (508)	NS
	MMSE	0±0 (0)	22.0±0.6 (61)	21.6±0.2 (490)	NS
rs701109		C/C	C/T	T/T	
	Aβ42	532±19.8 (116)	554±12.3 (298)	518±11.9 (254)	NS
	Tau	647±31.9 (119)	613±17.7 (304)	635±19.5 (261)	NS
	AAO	72.5±0.8 (100)	72.5± 0.5 (257)	73.1±0.5 (234)	NS
	MMSE	21.2±0.6 (96)	21.5±0.3 (247)	21.2±0.4 (229)	NS
rs989692		T/T	C/T	C/C	
	Aβ42	565±18.3 (149)	535±10.4 (360)	534±16.2 (173)	NS
	Tau	637±28.9 (150)	622±16.1 (367)	627±23.8 (181)	NS
	AAO	73.6±0.6 (126)	73.4±0.4 (307)	71.5±0.7 (157)	F(2,573) = 3.3, P = 0.036
	MMSE	21.8±0.5 (113)	21.5±0.3 (304)	21.8±0.4 (156)	NS

Genotypic means of quantitative traits ± SEM (N) for three markers in *MME*. Data for $A\beta_{42}$ and Tau are shown for all available materials (including cases and controls). Data for AAO and MMSE are for AD cases only. P-values < 0.05 are shown estimated using ANOVA and have been adjusted for disease status (AD, control), age, and gender, APOE-e4 status, as well as a sample identifier in statistical analyses (ANOVA). *$A\beta_{42}$* β-amyloid 42, *AAO* Age-at-onset, *MMSE* mini-mental state examination, NS not significant. We note that the same models with covariate adjustment indicate strong significance for an effect of APOE on *$A\beta_{42}$* levels (E4 carriers vs. non-carriers; P << 0.0001). The significance of the model for rs989692 and AAO is consistent with statistical noise.

Discussion

The importance of NEP in Aβ-degradation has been illuminated in several studies (e.g. [5]). NEP appears to be the only peptidase capable of degrading both soluble oligomeric, and monomeric Aβ. Studies in mice have shown an elevation of endogenous Aβ levels in brain in a gene-dose-dependent manner when NEP is disrupted. In vivo experiments of NEP-dependent degradation of exogenously administered synthetic $A\beta_{1\text{-}42}$ peptide have indicated that NEP plays a large role in post-secreted Aβ. Due to its axonal and presynaptic localization, it may play a role in the catabolism of Aβ at the neuronal synapses and surrounding areas; NEP activity could thus be a major determinant of Aβ concentration at these locations. Regions generally influenced in AD also show a decrease in NEP level [5].

Neprilysin is primarily expressed in kidney, where it appears to function in the inactivation of atrial natriuretic peptide [20], but is also expressed in prostate [21], in lung [22], and neutrophils [23]. It is also present in the mammalian brain where it appears to be localized to neuronal membranes [24].

MME is alternatively spliced at exons 1 to 3, the 5´-untranslated region, producing four separate mRNA transcripts. Transcript type 1 is predominantly expressed by neurons and contains exon 1, while type 2a and 2b mainly are expressed in peripheral tissues. Type 3 starts at exon 3 and is expressed by oligodendrocytes. The coding region has its start at exon 4 and is not affected by the alternative splicing. Two dinucleotide repeats have been reported, of which one is a GT repeat, and the other being a CA repeat. The GT repeat was reported to be positive for association with AD susceptibility in one study [25], but was simultaneously negative in three other studies [26, 27, 28]. Marker rs3736187 does occur immediately before the beginning of exon 20, but the site is not well conserved across species and there is no evidence that this exon is spliced. Neither of the other two markers plays any obvious role in alternative splicing.

Genetic association studies on *MME* and AD have led to both positive [14, 15, 25, 29] and negative [26, 27, 28] findings, reflecting the challenge of identifying genes potentially involved in complex diseases such as AD. In this study we were unable to replicate association between markers spanning *MME* and the AD. In addition, we failed to find any evidence of association with AD related quantitative traits, including CSF $A\beta_{42}$ levels, MMSE score, AAO, or CSF Tau. We can at present not exclude the possibility that functional genetic variation that may affect other AD-related phenotypes occurs in this genomic region, nor can we exclude the possibility that weak effects on the currently tested phenotypes may be present, but not detectable given present sample size and power.

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