

Original Article

Genetic variation in MME in relation to neprilysin protein and enzyme activity, A β levels, and Alzheimer's disease risk

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Abstract: Neprilysin (NEP), also known as membrane metalloendopeptidase (MME), is considered amongst the most important β -amyloid (A β)-degrading enzymes with regard to prevention of Alzheimer's disease (AD) pathology. Variation in the NEP gene (MME) has been suggested as a risk factor for AD. We conducted a genetic association study of 7MME SNPs – rs1836914, rs989692, rs9827586, rs6797911, rs61760379, rs3736187, rs701109 - with respect to AD risk in a cohort of 1057 probable and confirmed AD cases and 424 age-matched non-demented controls from the United Kingdom, Italy and Sweden. We also examined the association of these MME SNPs with NEP protein level and enzyme activity, and on biochemical measures of A β accumulation in frontal cortex – levels of total soluble A β , oligomeric A β ₁₋₄₂, and guanidine-extractable (insoluble) A β – in a sub-group of AD and control cases with post-mortem brain tissue. On multivariate logistic regression analysis one of the MME variants (rs6797911) was associated with AD risk (P = 0.00052, Odds Ratio (O.R.) = 1.40, 95% confidence interval (1.16-1.70)). None of the SNPs had any association with A β levels; however, rs9827586 was significantly associated with NEP protein level (p=0.014) and enzyme activity (p=0.006). Association was also found between rs701109 and NEP protein level (p=0.026) and a marginally non-significant association was found for rs989692 (p=0.055). These data suggest that MME variation may be associated with AD risk but we have not found evidence that this is mediated through modification of NEP protein level or activity.

Keywords: Neprilysin, MME, gene, association, β -Amyloid, alzheimer disease, polymorphism

Introduction

Neprilysin (NEP, EC3.4.24.11; also known as neutral endopeptidase, enkephalinase, common acute lymphocytic leukaemia antigen, and CD10) is a type II metalloproteinase widely expressed in many tissues [1]. Within the brain, NEP is expressed by pyramidal neurons within the neocortex and by vascular smooth muscle cells in the cerebral vasculature [2, 3]. Several lines of evidence suggest a role for NEP in the pathobiology of AD and cerebral amyloid angiopathy (CAA) [3-5]. NEP cleaves A β *in vitro* [6] and protects against A β deposition *in vivo* in

human APP transgenic mice [7-9]. NEP mRNA and protein levels were reported to be reduced in post-mortem human tissue in association with AD and CAA [3, 4].

The gene which encodes NEP (MME) is located on chromosome 3q25.1-q25.2 (MIM:120520) and has previously been reported as a putative linkage region (3q12.3-q25.31) in familial forms of AD [10, 11]. Four different MME mRNA transcripts with varying 5' sequences have been identified which result from alternative transcription sites, the type 1 isoform being expressed predominantly in neurons [12]. To date,

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Table 1. Demographic information for each of the four starting cohorts

Cohort	Diagnosis	No. of cases	No. of females	Age range (yrs)*	Mean (\pm SD) age (yrs)*
Total	AD	1057	634	-	-
	Control	424	232	-	-
Bristol	AD	289	165	57-99	80.5 \pm 8.0
	Control	90	36	62-96	79.3 \pm 8.2
Belfast	AD	638	398 ^a	54-95 ^b	76.4 \pm 7.8
	Control	215	146 ^c	40-100 ^d	74.4 \pm 9.0
Italy I	AD	15	6	53-84	70.5 \pm 10.4
	Control	24	7	59-85	77.1 \pm 5.3
Italy II	AD	115	65 ^e	51-82 ^f	69.9 \pm 7.1
	Control	95	43	60-100 ^g	73.2 \pm 8.6

*For the Bristol cohort the ages indicated are the age at death. For the three clinical cohorts the age is age at disease onset for the AD cases and age that blood was taken for analysis for control cases. ^aGender information missing from 13 cases, ^bAge information missing from 127 cases, ^cGender information missing from 1 control, ^dAge information missing from 3 controls, ^eGender information missing from 18 cases, ^fAge information missing from 21 cases, ^gAge information missing from 2 controls. The absence of data in relation to age-range and mean age for the total cohort is because it combines both age at death and age at disease onset.

numerous single nucleotide polymorphisms (SNPs) and other variants within MME have been reported to have varying degrees of positive or negative association with risk of AD [2, 5, 13-24]. Ongoing meta-analysis of SNP-based association studies in the online AlzGene (www.Alzgene.org) database has shown little to no overall association between MME variants and AD. However it remains unclear whether such variants might alter the protein levels or activity of neprilysin, potentially modifying the disease by affecting A β degradation.

This study investigated the potential genetic association between 7 MME variants (rs1836914, rs989692, rs9827586, rs6797911, rs61760379, rs3736187, rs701109) and AD. From this cohort of 1057 AD and 424 control cases (derived from 4 contributing AD research centres in the UK, Northern Ireland and Southern Italy), a sub-group of post-mortem samples was also assessed for association between the MME variants and NEP protein level and enzyme activity, previously found to be altered in AD [3], and for association with previously obtained quantitative measures of A β accumulation: levels of total soluble A β , oligomeric A β ₁₋₄₂ and total guanidine-extracted (insoluble) A β [25, 26].

Materials and methods

Study cohort

DNA was extracted from brain tissue obtained from the South West Dementia Brain Bank

(SWDBB) in Bristol (Human Tissue Authority license 12273) from cases with a post-mortem diagnosis of probable or definite AD according to CERAD criteria [27] or from normal control brains (Table 1). The neuropathological assessment of these cases was previously reported [26]. The remaining samples of the cohort (Table 1) comprised DNA that was extracted from blood taken from living patients attending four clinical research centres (Belfast, Northern Ireland; Bari, Italy (Italy I) and San Giovanni Rotondo, Italy (Italy II)) constituting a further 334 controls and 768 AD samples (Table 1) with an NINCDS-ADRDA diagnosis of probable AD [28]. Together 424 controls and 1057 cases with probable or definite AD were investigated for MME variation and AD risk.

Other data retrieved for each case included gender, age and APOE genotype. The manner in which age was recorded differed for the post-mortem and clinical cohorts. Age at death was the value recorded for the post-mortem cohort whilst that used for the three clinical cohorts (Belfast and Italy I and II) was age at assessment (i.e. when blood was taken) for non-demented controls and the age of disease onset for the AD cases.

Genetic analysis

Genomic DNA was extracted from brain tissue and blood by use of commercial DNA extraction kits (Nucleon ST Extraction kit, Nucleon Biosciences, Manchester, UK) and diluted to 10 ng/ μ l in Sigma® water (Sigma Aldrich, St Louis, USA)

as described elsewhere [1]. Following the provision of sequence information relevant to our SNPs of choice, the SNP genotyping was conducted at KBiosciences (www.kbiosciences.co.uk) using their proprietary in-house KASPar genotyping technology, a PCR-based genotyping method that is combined with allele-specific amplification followed by fluorescence detection. All assays were performed according to the manufacturers' quality controls and protocols.

Seven SNPs from across MME were selected for genotyping based either on their previous reported associations with AD (see Alzgene: www.alzgene.org for data summaries of all studies) or which served as 'tag-SNPs' according to HAPMAP (build 35) (www.hapmap.org) to give some coverage across the gene. All reported SNPs can be found in the dbSNP database (www.ncbi.nlm.nih.gov/SNP/) under their respective ID numbers.

All cases had previously been APOE genotyped, following an established protocol [29].

NEP protein and enzyme activity measurements

NEP protein level was measured by sandwich ELISA as reported previously [30] and NEP enzyme activity level was measured by an immunocapture-based fluorogenic activity assay [31]. Both parameters had previously been determined in brain tissue homogenates within a subset of the SWDBB cases (N=95 AD, 52 controls) examined here. NEP protein level and enzyme activity were adjusted for neuronal content on the basis of measurements of the neuronal marker (neuron-specific enolase (NSE)), as previously described [30], to allow for variation in neuronal number between samples.

A β measurements

Tissue preparation and ELISA methods used to measure total soluble and guanidine-extractable (insoluble) A β levels in the same SWDBB cases as those for NEP assays were also reported previously [25]. In summary, soluble and insoluble fractions were prepared from fresh frozen human brain tissue and measured by sandwich ELISA in which monoclonal anti-A β (clone 4G8, raised against amino acids 18-22; Millipore, Durham, UK) was used for the capture step and biotinylated anti-human β -amyloid monoclonal antibody (clone 10H3; Thermo Fisher Scientific, Northumberland, UK) for the

detection step [25]. The development and validation of the oligomeric A β ₁₋₄₂ ELISA, and the tissue preparation methods and measurement of oligomeric A β ₁₋₄₂ levels in the SWDBB subjects included in this study were reported previously [25]. A sandwich ELISA was designed in which a rabbit polyclonal pan-A β ₁₋₄₂ antibody (Millipore, Durham, UK) was used for the capture step and monoclonal mouse anti-oligomeric A β antibody (clone 7A1a; New England Rare Reagents, ME, USA) for the detection step [25].

Statistical analysis

The chi-squared statistic (χ^2) was used to assess deviation from Hardy-Weinberg equilibrium (HWE) for genotypes at individual loci as well as to assess differences in genotype distributions between demented and non-demented groups. Multivariate tests factoring in age, gender, APOE ϵ 4 status and cohort for the association of markers with disease risk were also conducted by logistic regression in an additive model. Tests for association between single marker genotypes and quantitative traits were performed by analysis of variance (ANOVA). STATA v11.1 (www.stata.com) was used to perform all of the above statistical analyses. Sample size calculations were performed a priori to ensure a suitable sample size was present to detect a 20% shift in allele frequency at a significance level of $p < 0.05$. Haplotype frequencies in the MME region were estimated after linkage disequilibrium (LD) block definition in individual blocks, by use of Haploview v4.1 [32]. LD blocks were defined by solid spines. LD between marker pairs within MME was estimated using the r^2 metric [33]. The calculation of empirical p-values for haplotypes in case-control tests was performed using 1000 permutations.

Results

Genetic association analysis

All of the polymorphisms examined were in Hardy-Weinberg equilibrium when assessed in cases and controls separately. Genotype frequencies for each MME SNP are displayed in **Table 2**. All of the genotypes except for rs6797911 were not seen to differ between AD and control. For rs6797911, a p-value of 0.035 was found. After further logistic regression analysis in which gender, age and APOE ϵ 4 were used as co-variants, as well as identifiers for the different population, rs6797911 frequency re-

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Table 2. Genotype frequencies for MME SNPs in AD

rsID/sample Chromosome location	Case/control (N)	Polymorphism			Genotypes	Logistic
rs1836914 Chr3:154 796 360	Controls (404)	AA (.95)	AG (.05)	GG 0	P = 0.943	P = 0.993
	AD (1040)	992 (.95)	48 (.05)	0		
rs989692 Chr3:154 801 365	Controls (398)	CC (.27)	CT (.46)	TT (.27)	P = 0.127	P = 0.572
	AD (1018)	254 (.25)	528 (.52)	236 (.23)		
rs9827586 Chr3:154 825 385	Controls (390)	GG (.39)	AG (.46)	AA (.15)	P = 0.494	P = 0.987
	AD (1005)	355 (.35)	482 (.48)	168 (.17)		
rs6797911 Chr3:154 862 390	Controls (394)	TT (.44)	AT (.43)	AA (.13)	P = 0.035	P = 0.00052*
	AD (998)	362 (.36)	474 (.48)	162 (.16)		
rs61760379 (IVS17-294) Chr3:154 877 885	Controls (372)	CC (.98)	CT (.02)	TT 0	P = 0.876	P = 0.444
	AD (992)	972 (.98)	20 (.02)	0		
rs3736187 Chr3:154 886 278	Controls (375)	AA (.85)	AG (.14)	GG (.01)	P = 0.802	P = 0.764
	AD (984)	860 (.87)	126 (.12)	4 (.01)		
rs701109 Chr3:154 898 407	Controls (402)	AA (.18)	AG (.43)	GG (.39)	P = 0.406	P = 0.192
	AD (1040)	154 (.15)	466 (.45)	420 (.40)		

Genotype counts (frequencies) are shown for various markers in MME in AD cases and controls. Percentages given were expressed relative to the total number of samples successfully assayed for each SNP (denoted in parentheses). Chi-square statistics and associated P values were obtained from 2x3 (genotype) contingency tables. Additionally, logistic regression statistics are presented for additive (linear) genetic models and include gender, age, and APOE-e4 status as co-variables, as well as a cohort identifier (italian, english, swedish) to control for population stratification. The Odds Ratio (OR) for rs6797911 was 1.40 with a 95% confidence interval of 1.16-1.70.

mained significantly different between AD and controls ($p=0.00052$). This significant association generated an OR = 1.40 (95% CI = 1.16-1.70). However, and perhaps not surprisingly, haplotype analysis did not reveal any evidence of association with any of the resultant haplotypes and AD risk (data not shown).

Association of MME variants with A β concentration and neprilysin level and activity

To evaluate effects of MME sequence variants on A β levels, multi-locus ANOVA models were fit

using four SNP markers (rs989692, rs9827586, rs6797911, rs701109). All of these were intronic except for rs701109 which is mapped to the 3'UTR of MME. These SNPs were selected for a number of reasons. rs6797911 had previously been demonstrated to be genetically associated with AD; rs989692 had been reported to be associated with age at onset of AD [13]; rs701109 is one of the more commonly studied SNPs to date (see Alzgene: <http://www.alzgene.org/meta.asp?genelD=73>); and rs9827586 is a HapMap defined tag SNP that is located between rs989692 and

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Table 3. Quantitative trait associations for MME

rsID/sample	Trait	Genotypes			Significance
rs989692		C/C	C/T	T/T	P = 0.058
	NEP protein	126.2 \pm 18.11 (12)	220.3 \pm 17.72 (35)	164.0 \pm 29.32 (17)	
	NEP enzyme activity	77.4 \pm 9.23 (12)	76.87 \pm 5.76 (35)	70.63 \pm 7.61 (17)	NS
rs9827586		GG	G/A	A/A	P = 0.014
	NEP protein	178.75 \pm 19.96 (20)	221.21 \pm 22.97 (33)	150.17 \pm 18.95 (14)	
	NEP enzyme activity	67.48 \pm 4.92 (21)	80.45 \pm 6.64 (33)	74.63 \pm 8.10 (14)	P = 0.006
rs6797911		T/T	T/A	A/A	NS
	NEP protein	187.02 \pm 22.56 (26)	229.15 \pm 21.52 (32)	188.72 \pm 34.12 (11)	
	NEP enzyme activity	72.11 \pm 6.56 (28)	79.89 \pm 5.97 (31)	64.24 \pm 5.28 (12)	NS
rs701109		A/A	G/A	G/G	P = 0.026
	NEP protein	322.45 \pm 65.69 (7)	194.22 \pm 18.90 (31)	190.35 \pm 21.93 (32)	
	NEP enzyme activity	67.99 \pm 11.86 (8)	70.41 \pm 4.45 (31)	79.63 \pm 6.52 (38)	NS

Genotypic means \pm SEM of NEP protein levels and NEP enzyme activity levels (P values <0.05 considered significant). Genetic association was observed between MME rs9827586 and both NEP protein level (p = 0.014) and strongly significant with enzyme activity (p = 0.0006). There was also some evidence of association between rs701109 and NEP protein levels p=0.026. The fact that both rs9827586 and rs701109 have similar patterns of effect is perhaps reflected by the fact that only rs701109 was in weak linkage disequilibrium with rs9827586 ($r^2 < 0.5$). There was also suggestive although non-significant evidence of association between rs989692 and NEP protein levels p=0.058.

rs6797911. All analyses were adjusted for post-mortem delay, age, gender and disease status.

There was evidence of an association of rs9827586 with both NEP enzyme activity and protein expression (**Table 3**), particularly after adjustment for the level of neuronal specific enolase (NSE), a marker of neuronal content, allowing adjustment for neuronal loss in AD [30]. Genetic associations were also observed for rs989692 and rs701109 but these did not survive correction for multiple testing. We investigated the presence of genetic association between these variants and levels of total soluble and insoluble A β and soluble oligomeric A β 1-42, previously measured for the brain bank samples [26, 34-36]. For none of these variables was there evidence of association with any of the 4 selected SNPs in MME (data not shown).

Discussion

Excessive accumulation of A β is thought to initiate the development AD and to lead also to CAA. Levels of A β reflect the balance between A β production and clearance; age- and disease-related reduction in enzyme-mediated degrada-

tion of A β is one potential mechanism that may account for the abnormal accumulation of A β in AD and CAA [34].

Three MME SNPs located within the vicinity of the 3'UTR (rs989692 and rs701109) and the 5'UTR (rs373187) were previously been shown to be associated with AD in Spanish and Finnish populations [37, 38]. However these associations were not replicated in recent studies, including a large American Caucasian study [24], a recent Swedish population study [13] and our data from West-Central and Southern Europe. Furthermore, these three SNPs were not associated with biochemical measures of AD (CSF A β 42 and tau levels) or mini-mental state examination (MMSE) scores in the Swedish cohort, although there was a suggestion of association between AD age-at-onset and rs989692 [13]. Our present study has not provided evidence of an association between any of the selected 4 MME variants and total soluble A β , oligomeric A β 1-42 and total insoluble A β within the frontal cortex. A dinucleotide repeat polymorphism (GT) in the MME promoter region was reported to be associated with AD [20] and CAA risk [39] but this association has not since been

reproduced in most other studies [17, 18, 22, 24]. A large number of other SNPs within MME were not associated with AD [24].

Ours is the first study to report genetic association between AD and rs6797911. This same variant had a marginally significant association with AD in the Translational Genomics Research Institute (TGEN) Genome Wide Association Study (GWAS) [19, 40], the direction of effect being the same as in the present study. Further support for this association comes from one of the first large-scale GWAS meta-analyses by Lambert and colleagues who found association of SNP rs2016848 with AD, with a p-value of 0.0066; rs2016848 is a perfect proxy for rs6797911 [41]. The reason why we found no further associations within MME is likely to be because rs6797911 was in weak linkage disequilibrium (LD) with only rs101109 ($r^2 < 0.5$) with little LD with any of the others studied.

We have found that rs9827586 is strongly associated with NEP protein level and enzyme activity. One might have expected this to modulate AD risk given the function of NEP as an A β degrading enzyme, yet there was no evidence of association between this SNP and the post-mortem measures of A β load. It is possible that the observed association with NEP level and activity is simply a chance finding. However, we previously showed NEP enzyme activity to be increased in AD in relation to disease severity [30]. Variation at rs9827586 may influence the upregulation of NEP in AD in response to A β accumulation without having an effect on the development of the disease.

Elevated NEP activity may also help to limit the vasoconstrictive action of angiotensin II in the renin angiotensin system (RAS). There is now a growing body of evidence supporting the role of the RAS in the pathogenesis of AD [42]. We previously showed that the activity of angiotensin-converting enzyme, responsible for the synthesis of angiotensin II, is increased in the brain in AD [43, 44]. NEP cleaves angiotensin I (the precursor of angiotensin II) to generate angiotensin 1-7, which serves to counteract the function of angiotensin II. NEP, like ACE, is elevated in AD [30, 43, 44] - both perhaps upregulated by A β as a physiological response to prevent its continuing accumulation but both also affecting the regulation of arterial tone and cerebral blood flow and potentially thereby contributing to cognitive decline [45, 46].

In summary, we report genetic association between the MME variant rs6797911 and AD. We also provide evidence that other variants of MME, particularly rs9827586, or variants in linkage disequilibrium, may influence NEP level and activity and thereby have disease modifying effects in AD. Further research is needed to clarify the nature of the relationship between NEP activity, A β metabolism, angiotensin II and cerebral blood flow, and the role that genetic variation in MME plays in these interactions and in the progression of AD.

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