Possible Influence of a Non-Synonymous Polymorphism Located in the NGF Precursor on Susceptibility to Late-Onset Alzheimer's Disease and Mild Cognitive Impairment.

Possible influence of a non-synonymous polymorphism located in the NGF precursor on susceptibility to late-onset Alzheimer's disease and mild cognitive impairment.

Emilio Di Maria,[1,2]* Elisa Giorgio,[2§] Vera Uliana,[2] Cristian Bonvicini,[3] Francesca Faravelli,[2] Sergio Cammarata,[4] Daniela Galimberti,[5] Elio Scarpini,[5] Orazio Zanetti,[6] Massimo Gennarelli,[3,7] Massimo Tabaton.[8]

[1] Department of Health Sciences, University of Genova, Genova, Italy

[2] Division of Medical Genetics, Galliera Hospital, Genova, Italy

[3] Genetic Unit, IRCCS Centro S. Giovanni di Dio-Fatebenefratelli, Brescia, Italy

[4] Division of Neurology, Galliera Hospital, Genova, Italy

[5] Dino Ferrari Centre, Department of Neurological Sciences, University of Milan, IRCCS Foundation Ospedale Maggiore Policlinico Mangiagalli and Regina Elena, Milan, Italy

[6] Alzheimer Unit, IRCCS Centro S. Giovanni di Dio-Fatebenefratelli, Brescia, Italy

[7] Department of Biomedical Sciences and Biotechnologies, University of Brescia, Italy

[8] Department of Internal Medicine, University of Genova, Genova, Italy

[§] current address: Department of Genetics, Biology and Biochemistry, University of Torino, Italy.

***** to whom correspondence should be addressed.

Corresponding Author:
Emilio Di Maria, M.D., Ph.D.
Dept. of Health Sciences, University of Genova
c/o Genetica Medica, Galliera Hospital
Via Volta, 6 - 16128 Genova – Italy
e-mail: emilio.dimaria@unige.it - phone +39 0105634368 - fax +39 01057481222

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Abstract

The complex network of neurotrophic factors is supposed to play a role in neurodegeneration, but the effect of variations in their coding genes on susceptibility to sporadic Alzheimer's disease was not established. The mature form of nerve growth factor (NGF) derives from a precursor, proNGF, which was recently discovered to exert crucial functions in brain.

We designed a case-control association study to test the hypothesis as to whether polymorphisms located in the proNGF genomic region influence the liability to Alzheimer's disease and its prodromal form, mild cognitive impairment. Three independent case-control samples, with individuals aged >60 years, were collected in Italian Alzheimer Units.

One polymorphism located in the proNGF region, rs6330, demonstrated a minor allele frequency >5% and was used in the association study. The minor allele of rs6330 was more frequent in patients from the three sample series as compared to respective normal controls. Multivariate logistic regression showed a significant association under the dominant model in one cohort (OR 1.83, 95% CI 1.00-3.54) and in the pooled case-control sample (OR 1.47, 95% CI 1.03-2.08).

These findings further suggest that proNGF may play a role in Alzheimer-type neurodegeneration and that genetic variations in the *NGF* locus may influence the occurrence of sporadic, late-onset Alzheimer's disease.

Introduction

Alzheimer's disease (AD), the most common form of dementia, typically presents at disease onset with prominent memory loss, variably associated with deficits in other cognitive domains and with an impairment in functional and instrumental daily life abilities [1]. A pathological condition characterised by impairment in memory with otherwise normal performance has been named amnestic mild cognitive impairment (aMCI). Patients with aMCI convert to AD at a rate of about 16% per year, whereas the conversion rate may be as high as 80% after 6 years [2]. Multiple lines of evidence suggest that aMCI is prodromal to AD, and that the two clinical entities share the same genetic background [3-5].

Aging and family history of dementia are the major risk factors for AD. Whereas mutations of the presenilin 1 (*PS1*), presenilin 2 (*PS2*), and amyloid precursor protein (*APP*) genes account for early-onset AD cases with autosomal dominant inheritance, the ε4 allele of the apolipoprotein E (*APOE*) gene is the only established genetic susceptibility factor for sporadic late-onset AD (LOAD) which was validated in numerous genetic association studies (see: the AlzGene website, www.alzforum.org; reference [6] for a recent review). Genome-wide association studies (GWAS) and meta-analyses discovered novel loci (namely *BIN1*, *CLU*, *CR1*, *PICALM*) [7-9]. Whereas it was established that *APOE* ε4 confers a substantial risk, the additional risk due to other AD-associated genes is low, typically 10-20% [10]. Based on this findings, the current model postulates that multiple risk alleles with small effect underlie the genetic liability to LOAD.

Neurotrophic factors may play a key role in determining part of the genetic susceptibility to LOAD. Nerve growth factor (NGF) is known to play important roles in brain function, by regulating neuronal survival and differentiation, neurite growth and synapse plasticity. Several lines of evidence suggested that NGF is involved in AD type neurodegeneration, including cholinergic deficit, tau hyperphosphorylation and amyloid plaques deposition [11;12]. NGF derives from its precursor, proNGF, which is proteolytically cleaved to release the C-terminal mature form (mNGF) [13]. Recent studies highlighted the role for proNGF in normal neuronal function as well as in neurodegenerative processes. In contrast with previous knowledge, it was shown that proNGF is the predominant form in the brain and increases in patients with AD and MCI [14;15]. The susceptibility of forebrain cholinergic neurons, which is a hallmark in AD, may be mediated by the respective effect of NGF and proNGF [16]. *NGF*, the human gene coding for proNGF (chromosome 1p13.1), consists of three exons spanning 52.3 Kb. The entire coding sequence is comprised in exon 3 and codes for the 241-amino acid polypeptide.

Despite its putative relevance in brain development and function, the role for *NGF* variants was not deeply investigated in patients with neurodegenerative disorders. One previous study investigated the association between AD and the neurotrophin system, and found a trend for association with one polymorphism (rs6330) located in the *NGF* coding region [17].

Based on this rationale, we designed a study aimed at testing the hypothesis that genetic variations located in the genomic region coding for proNGF may influence the occurrence of AD. We analysed a data set derived from a cross sectional investigation in three cohorts of Italian patients who were diagnosed as having AD or aMCI. We report positive findings that need to be replicated in large independent cohorts.

Materials and methods

Patients

Consecutive outpatients asking for a consultation and referred to as having cognitive dysfunction were invited to participate into the study. Three case-control cohorts, named 1-3 from herein, were collected at four Alzheimer units, located in Genova (2 units, cohort 1), Brescia (cohort 2) and Milan (cohort 3), Italy. Patients from the cohort 1 were diagnosed as having amnestic MCI. Cohorts 2 and 3 comprised AD patients only.

A panel of structured clinical and neuropsychological tools was administered to assess the diagnosis. Neuropsychological tests, with cut-off scores adjusted for age and education of the Italian population, were used to evaluate multiple cognitive functions, including the immediate and delayed recall of a short story and word list memory test, two tests specific for episodic memory [18-20]. The study protocol was detailed elsewhere, as a subset of the study cohort was previously investigated [4;18]. Subjects with a score of both memory tests 1.5 SD below the mean of the proper age, a Clinical Dementia Rating (CDR) score of 0.5, and a Global Deterioration Scale (GDS) score below 14, were diagnosed with MCI according to international criteria [21]. Patients

who fulfilled the following criteria were classified as to AD: i) alteration of another cognitive domain beside memory; ii) CDR scale ≥1; iii) alteration of activity (IADL scale); iv) NINCDS criteria of probable AD [18].

Inclusion criteria for the association study were: i) diagnosis of either MCI (cases from cohort 1) or AD (cases from cohorts 1-3); ii) age >60 years; iii) informed consent to clinical and neuropsychological assessments, as well as to genetic analysis for research purposes. Individuals with no personal history of cognitive impairment aged >60 years were included as controls; all were recruited from the same geographical area of the three patients series, respectively. All individuals were unrelated and of Italian ancestry. The study protocols were approved by the local Ethical Review Boards. Age and gender distribution of the sample series are reported in table 1.

Genotyping

The genomic fragment (chr1: 115630039-115631020, NCBI36/hg18 assembly) encompassing the *NGF* coding sequence and including the region which encodes proNGF was examined by direct sequencing on an ABI-3130 platform (Applied Biosystems, Foster City, CA, USA), according to standard procedures. Genotype assignment was performed using the SeqScape program (Applied Biosystems, Foster City, CA, USA), eventually checked by visual inspection of electropherograms. The *APOE* genotypes were assessed by restriction fragment length analysis, following the current protocols. For the statistical analysis, the ε2 and ε3 alleles were grouped, i.e. genotypes were encoded based on the number of ε4 alleles. Stringent quality control procedures were applied. Details of laboratory protocols are available on request. Requisites for inclusion of SNPs in the association study were: i) completion rate for genotyping >95%; ii) minor allele frequency (MAF) >5%; iii) no deviation from Hardy-Weinberg equilibrium in controls (exact test).

Statistical analysis

Multivariate logistic regression models were applied to test the hypothesis of association, using the disease status as dependent variables and the genotype as predictor. *APOE* genotype, age and gender were included as covariates in the regression models. The best fit model was identified comparing the estimates by the likelihood-ratio test. Differences in genotype frequencies between cohorts and deviations from HWE were tested by Fisher's exact test. Alpha value was set at 0.05. The three cohorts were examined individually and subsequently pooled in one case-control sample. Assuming 10% as disease prevalence and the observed rs6330 allelic frequency, the Genetic Power Calculator [22] estimated more than 80% probability to detect an odds ratio (OR) ≥1.5 in the pooled analysis. All calculations were performed using Stata 9 (StataCorp LP, College Station, TX, USA) if not differently stated. The linkage disequilibrium (LD) profile of the NGF genomic region was analysed using the solid spine of LD algorithm as implemented in HaploView 4.2 [23]; genotype data were extracted through the HapMap interface (www.hapmap.org), phaseIII/rel#2, NCBIB36/hg18 assembly, population of European ancestry from the CEPH collection.

Table 1

Results

The genomic region encoding proNGF was examined by direct sequencing. Three coding SNPs (rs6330, rs11466110 and rs11466111) located in this region and two SNPs (rs6325 and rs35941329) in the surrounding boundaries were listed in the dbSNP database. Only rs6330 displayed a MAF >5% in cohort 1, which was used as pilot series to assess genotype frequencies. Thus rs6330 was subsequently genotyped in cohorts 2 and 3 and included in the association study. No deviation from HWE was detected in patients and controls from the three cohorts.

APOE, age and gender were tested as covariates in logistic regression models. The best fit was obtained by the model which included *APOE* and age as covariates. APOE ε4 was significantly over-represented in all patients series (table 1). Considering the pooled case-control sample (n=828), the OR associated to carrying at least one ε4 allele was 5.41 (95% C.I. 3.60-8.13; z=8.13, p<0.001).

We firstly tested the hypothesis of association of proNGF with AD on each independent cohort. The T allele of rs6330 was more frequent in cases as compared to normal controls in all sample series (table 1). The same trend was observed in the three cohorts, but multivariate logistic regression demonstrated a significant difference only in cohort 1 (OR=1.83, 95% C.I. 1.003-3.355; n=266; z=1.97, p=0.049). As no significant difference in rs6330 genotype frequency distribution was observed between the three cohorts, the test was applied also on the pooled case-control sample, allowing to accept the hypothesis of association under the dominant model (OR=1.47, 95% C.I. 1.035-2.075; n=828; z=2.15, p=0.031). Finally, significant association was detected in the pooled sample also under the codominant model (OR=1.33, 95% C.I. 1.052-1.682; n=828; z=2.38, p=0.017).

Discussion

In light of recent findings on the role for proNGF in brain development and function, we designed a cross-sectional study specifically focussed on variations located in the genomic region coding for proNGF. Multivariate analysis on a large sample comprising three independent cohorts suggested that the T allele of rs6330 is associated with a small additional risk to develop AD. The rs6330 polymorphism is located in the N-terminal region of proNGF, which is cleaved out to produce the mature NGF form.

The well established biological relevance of proNGF provided a reliable rationale for our candidate gene approach. Though the case-control design is intrinsically prone to type I error, given the prior probability of association suggested by functional studies the nominally significant result may be regarded as a positive finding.

The C/T rs6330 polymorphism corresponds to a change from alanine to valine at the amino acid 35 in the immature NGF peptide, in the portion which is cut during the proteolytic cleavage (figure 1).

The amino acid substitution may affect post-translational processing and consequently the respective level of both proNGF and NGF. An impairment in the maturation pathway from proNGF to NGF was proposed as a key factor to explain vulnerability of forebrain cholinergic neurons in AD [24]. Alternatively, or in addition to this indirect effect, the amino acid change may exert a direct effect on proNGF function. This hypothesis is supported by recent findings on the involvement of proNGF in AD pathophysiology [14-16], and is consistent with the dominant effect of the rs6330 variant found in this study. As the network of brain growth factors plays a key role in brain development and brain function along the whole life, it is conceivable that the neurodegenerative process is facilitated by a subtle unbalance of expressed levels or a slight perturbation in function of one factor. However, we would refrain from further speculating on possible models for the biological effect of rs6330, unless these speculations are supported by further experimental evidence.

We found an association of the rs6330 T allele with risk of AD, but in the opposite direction with respect to that reported previously [17]. In a study investigating the association of 21 SNPs within the neurotrophin system genes with AD, Cozza and coworkers found a nominally significant protective effect of the minor allele T of rs6330 on both familial and sporadic AD [17]. On the opposite, we found that the T allele is associated with an increased risk. In the present study we examined three different case-control samples of Italian ancestry with controls matched for geographical origin and observed the same trend in all cohorts. Genotypes were obtained by the mean of the most accurate method, i.e. direct sequencing, and results were checked by applying rigorous quality control. Moreover, the same trend for association was detected also under the codominant model in the pooled case-control sample. Though our findings appear statistically reliable and biologically plausible, we are aware of limitations of the study. Stringent inclusion criteria for normal individuals resulted in an unequal number of cases and controls and consequently in low statistical power to estimate genotype frequencies in single cohorts. Yet, we had sufficient power to detect a relatively weak association signal in the pooled case-control sample. However, though the risk of type I error was minimised because we avoided testing multiple genetic markers, the occurrence of spurious results cannot be ruled out.

Nevertheless, not only statistical artefacts, but also variations in extraneous factors may explain divergent results in genetic association studies. Differences in environmental exposures and ancestral composition between study populations may revert the effect of genetic factors. In particular, variations in local patterns of LD may result in genuine parameter differences between studies, and this happens more likely if genetic effect is small [25;26]. The inferred LD profile of the *NGF* locus that can be inspected through the HapMap interface showed that rs6330 lies in a small LD block (approximately 20 Kb) which encompasses *NGF* exon 3 and the surrounding non-coding regions (fig. 1). Two boundaries of frequent recombination, one extending in 5' direction across the *NGF* locus, define the LD block. Moreover, large variations in allele frequency were observed across populations of different ancestries, as reported by the HapMap project (last access Aug

2011), ranging from 0.129 in Chinese to 0.442 in Europeans from the CEPH collection. Taken together, these clues allow to infer that proNGF is coded by a genomic region subject to frequent recombination which may result in local variation of the LD structure across different samples. Therefore, all findings based on genomic polymorphisms in this region, though very intriguing, should be taken cautiously. The lack of positive signals pertaining to the *NGF* locus in genome-wide analyses [7-9] can be accounted for by the limited attributable effect size, as well as by the presence of interactions with other loci or variable LD structure which further limits efficacy of case-control GWAs. Family-based designs could be the most effective approach to dissect the LD profile across the *NGF* locus.

In light of current knowledge, genomic variations encompassing a larger genomic region including the entire *NGF* gene and its regulatory elements should be deeply explored. Replication studies on large independent samples are warranted to confirm the association with variants located in the proNGF genomic region. Investigating the biological effect of the rs6330 polymorphism by the mean of *in vitro* studies may add direct evidence to the statistical clue provided by association studies.

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Table 1. Description of the case-control samples, and results from association analysis (cohorts 1-3 and pooled cohort).

	Cohort (diagnosis)	n	Age, mean (SD)	Gender (M:F)	APOE[a]	n	%	rs6330 genotype	n	%	MAF (T)[b]	OR[c]	95% CI
1	Patients	172	75.7	95:107	ε4 null	107	62.21	CC	46	26.74	0.47	1.83*	1.00-3.35
	(MCI)		(5.5)		ε4 het	59	34.30	CT	91	52.91			
					ε4 hom	6	3.49	TT	35	20.35			
1	Controls	94	73.5	52:42	ε4 null	85	90.43	CC	32	34.04	0.45		
			(7.4)		ε4 het	9	9.57	CT	38	40.43			
					ε4 hom	0	0	TT	24	25.53			
2	Patients	174	80.1	56:118	ε4 null	101	58.05	CC	52	29.89	0.45	1.34	0.64-2.81
	(AD)		(6.5)		ε4 het	64	36.78	CT	87	50.00			
					ε4 hom	9	5.17	TT	35	20.11			
2	Controls	84	69.6	42:42	ε4 null	70	83.33	CC	31	36.90	0.39		
			(6.6)		ε4 het	11	13.10	CT	41	48.81			
					ε4 hom	3	3.57	TT	12	14.29			
3	Patients	209	76.7	75:134	ε4 null	117	55.98	CC	64	30.62	0.44	1.28	0.73-2.23
	(AD)		(6.9)		ε4 het	77	36.84	CT	104	49.76			
					ε4 hom	15	7.18	TT	41	19.62			
3	Controls	95	73.6	38:57	ε4 null	80	84.21	CC	32	33.68	0.39		
			(9.0)		ε4 het	14	14.74	CT	52	54.74			
					ε4 hom	1	1.05	TT	11	11.58			
Pooled													
	Patients	555	77.4	226:359	ε4 null	325	58.56	CC	162	29.19	0.45	1.47*	1.03-2.08
	(MCI and AD)		(6.6)		ε4 het	200	36.04	CT	282	50.81			
					ε4 hom	30	5.41	TT	111	20.00			
	Controls	273	72.3	132:141	ε4 null	235	86.08	CC	95	34.80	0.41		
			(7.9)		ε4 het	34	12.45	CT	131	47.99			
					ε4 hom	4	1.47	TT	47	17.22			

[a] APOE genotypes were grouped as ε4 homozygous carriers (ε4 hom), ε4 heterozygous carriers (ε4 het) and ε4 non-carriers (ε4 null).

[b] Minor allele frequency, i.e. frequency of the T allele.

[c] Odds ratios (OR) and 95% confidence intervals (CI) estimated for the rs6330 T allele (dominant model), including age and *APOE* genotype as covariates. Significant values are denoted with an asterisk.

Figure 1. Linkage disequilibrium (LD) profile and block partition of the *NGF* locus. Grey levels correspond to *D'* values. The upper panel depicts the *NGF* gene and proNGF polypeptide, coded by *NGF* exon 3; the region corresponding to the mature form is in grey. Chromosome 1 positions are reported, according to the NCBI B36/hg18 assembly. The dashed line indicates the rs6330 location.