Association of a NOS1 promoter repeat with Alzheimer’s disease

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Received 17 August 2006; received in revised form 16 February 2007; accepted 4 March 2007
Available online 6 April 2007

Abstract
The gene encoding NOS-I (NOS1) displays a complex transcriptional regulation, with nine alternative first exons. Exon 1c and 1f are the most abundant forms in the brain. A functional single nucleotide polymorphism (SNP) in exon 1c and a polymorphism in exon 1f, consisting of a variable number of tandem repeats (VNTR) originating short (S) and long (L) alleles, were studied in 184 patients with Alzheimer’s disease (AD) and 144 gender- and age-matched controls. No differences were found for the Ex1c G-84A. The Ex1f-VNTR S allele was significantly more common in AD (55% versus 44%, P = 0.009, OR = 1.52) as was the S/S genotype (28% versus 14%, P = 0.008; OR = 2.37). The S allele showed a highly significant interaction with the ApoE ε4 allele (OR: 10.83). Therefore, short alleles of the NOS1 exon 1f-VNTR are likely to be susceptibility factors for AD, and interact with the ε4 allele to markedly increase the AD risk.

Keywords: Alzheimer’s disease; Genetics; Polymorphism; Neuronal nitric oxide synthase (NOS1); Risk factor

1. Introduction
Alzheimer’s disease (AD) is the most common form of dementia in the elderly, and is supposed to be a multifactorial disease caused by both genetic and environmental factors. At present, among the former, only the ε4 allele of the Apolipoprotein E (ApoE) gene has been demonstrated to be a risk factor for sporadic AD (Corder et al., 1993), although other candidate genes are under investigation. Many of them are related to oxidative damage in AD pathogenesis, a process which is considered to play a key-role during the development of the disease (Akiyama et al., 2000), β-Amyloid (Aβ) deposits in AD brains can lead to the production of superoxide radicals, reacting with nitric oxide (NO) to peroxynitrite, which in turn induces cellular injury. In this regard, several hallmarks of oxidative damage have been demonstrated directly in AD brains (Akiyama et al., 2000) and NO has repeatedly suggested to be involved in the pathogenesis of the disease (Law et al., 2001). NO is produced by three isoforms of nitric oxide synthase (NOS). The so-called “endothelial isoform” (NOS3), is localized in endothelium in proximity to hippocampal neurons, a principal site of AD pathology (Dinerman et al., 1994) thereby allowing NO flux from the vessels to hippocampal neurons (Moreno-Lopez et al., 2000; Reif et al., 2004). The corresponding gene, NOS3, has thus been proposed as candidate gene for AD. A common polymorphic variant in NOS3 gene, consisting in a single base change (G894T) and resulting in an amino acid substitution at position 298 of NOS3 protein (Glu298Asp), has been widely studied in several AD populations (Crawford et al., 2000; Dahiyat et al., 1999; Guidi et al., 2005; Higuchi et al., 2000; Monastero et al., 2003; Sánchez-Guerra et al., 2001; Singleton et al., 2001; Tedde et al., 2002). A recent
meta-analysis demonstrated an association of the SNP with AD in African American, but not Caucasian populations (Akomolafe et al., 2006). Elevated levels of inducible NOS (NOS2) isoform have been also observed in the brains of AD patients (Rupniak et al., 2000), and, conversely, NOS2 knock-out mice are protected from AD-like pathologies (Nathan et al., 2005). NOS-I however is the most abundantly isoform expressed in the brain. A remarkable loss of neurons expressing the neuronal form (NOSI) has been found in the entorhinal cortex layer II and in the hippocampus of patients with AD, suggesting that NOSI expressing neurons are highly susceptible to neurodegeneration (Thorns et al., 1998), although also a selective sparing of nitrinergic neurons in AD has been demonstrated (Hyman et al., 1992). An upregulation of NOS-I was observed immunohistochemically in astrocytes surrounding Aβ plaques present at places of clear neuronal loss (Simić et al., 2000), which is especially intriguing given the fact that NOS-I cannot be physiologically found in astrocytes. Accordingly, a dysregulation in the expression of NOSI was shown in neurons (Luth et al., 2002), which was correlated with nitrotyrosin formation in the hippocampus (Hensley et al., 1998; Luth et al., 2002). Intriguingly, linkage of late onset AD with 12q22, the chromosomal region harboring the NOSI gene, was shown in families with no evidence of linkage to D9S741 or α-catenin (Li et al., 2006). Recent genetic analyses demonstrated that the synonymous C276T polymorphism in exon 29 of the NOSI gene represents a risk factor for the development of AD (Galimberti et al., 2005), whereas the dinucleotide polymorphism in the 3′UTR of NOSI is not associated with the disease (Liou et al., 2002). To date, the promoter region of NOSI, located approximately 200 kb upstream of this polymorphism, has not been investigated for susceptibility to AD. NOSI shows a complex transcriptional regulation with the presence of nine alternative first exons (termed 1a-1i) resulting in NOSI transcripts with different 5′ untranslated regions (Wang et al., 1999). The use of multiple alternative promoters allows cell-, tissue-, and site-specific transcriptional regulation of NOSI in different physiological and pathophysiological stages (Saur et al., 2002). The alternative first exons 1c and 1f are the most abundant forms detected in the brain (Bros et al., 2006; Saur et al., 2002). Three single nucleotide polymorphisms (SNPs) have been identified in exon 1c, but only the G-84A variant displays a functional effect, as the A allele decreases transcription levels by 30% in in vitro models (Saur et al., 2004). Regarding exon 1f, a variable number of tandem repeats (VNTR) polymorphism has been recently reported in its putative promoter region, termed NOSI Ex1f-VNTR. This VNTR is highly polymorphic and consists of different numbers of dinucleotides (B–Q), which, according to their bimodal distribution, have been dichotomized in short (B–J) and long (K–Q) alleles for association studies (Reif et al., 2006). Both Ex1c G-84A and Ex1f-VNTR are associated with psychosis and prefrontal functioning in a population of patients with schizophrenia (Reif et al., 2006). Notably, both Ex1c and Ex1f transcripts are found in the hippocampus and the frontal cerebral cortex (Bros et al., 2006; Reif et al., 2006), i.e. brain regions implicated in the pathogenesis of schizophrenia as well as AD.

Together, the above studies suggest a critical role of NOSI in AD. To further explore a possible association of NOSI polymorphisms with AD, the distribution of Ex1c G-84A SNP and Ex1f-VNTR was analyzed in 184 patients with probable AD as well as in 144 gender- and age-matched healthy subjects. As there are several reports showing that the AD risk allele ApoE ε4 leads to increased NO production (Colton et al., 2004) we further tested for a gene × gene interaction with the ApoE ε4 allele.

2. Materials and methods

2.1. Subjects

One-hundred eighty-four Italian AD patients (123 women and 61 men, mean age at disease onset ±S.D.: 72.2 ± 10.1 years) were consecutively recruited at the Alzheimer Unit of the Ospedale Maggiore Policlinico (Milan). All patients underwent a standardized battery of examinations, including medical history, physical and neurological examination, screening laboratory tests, neurocognitive evaluation, brain Magnetic Resonance Imaging (MRI) or Computed Tomography (CT) and, if indicated, Positron Emission Computed Tomography (PET). Dementia severity was assessed by the Clinical Dementia Rating (CDR) and the Mini Mental Scale Examination (MMSE). Disease duration was defined as the time interval in between first symptoms and the establishment of the clinical diagnosis. The diagnosis of probable AD was made by exclusion according to NINCDS-ADRDA criteria (McKhann et al., 1984). The control group consisted of 144 healthy volunteers, including non-consanguineous patients’ kindred as well as non-demented subjects recruited in nursing homes, matched for ethnic background and age (82 women and 62 men, mean age ± S.D.: 69.8 ± 11.8 years). No significant differences in age or gender were found between cases and controls (P > 0.05). An informed consent to participate in this study was given by all individuals or their caregivers. Both patients and controls were genotyped for the ApoE status. Table 1 provides an overview on the sociodemographic variables of the sample.

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Table 1

Sociodemographic variables of the sample studied

<table>
<thead>
<tr>
<th></th>
<th>AD (N = 184)</th>
<th>Controls (N = 144)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age ± S.D. (years)</td>
<td>72.2 ± 10.1^a</td>
<td>69.8 ± 11.8</td>
</tr>
<tr>
<td>Mean disease duration ± S.D. (years)</td>
<td>4.8 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>Gender ratio m:f (%)</td>
<td>33:67</td>
<td>43:57</td>
</tr>
<tr>
<td>ApoE ε4 ratio ε4−εε̅+ (%)</td>
<td>54:46^b</td>
<td>74:26</td>
</tr>
</tbody>
</table>

^a Age at onset of disease; P > 0.05 as compared with controls.
^b AD vs. controls: χ^2 = 14.53, P = 0.0001, OR:2.48, 95% CI: 1.55–3.99.

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2.2. Laboratory methods

2.2.1. DNA extraction

High-molecular weight DNA was isolated from whole blood using a Flexigene Kit (Qiagen, Hildren, Gemany) as described by the manufacturer.

2.2.2. ApoE genotyping

ApoE genotype was determined by Polymerase Chain Reaction-Restriction Fragment Length Polymorphisms (PCR-RFLP) assay. DNA was amplified using specific primers and then digested with HhaI, as previously described (Del Bo et al., 1997).

2.2.3. NOS1 genotyping

For detecting the Ex1c G-84A SNP, a novel PCR-RFLP protocol was developed. The following primers were used to amplify a region of 150 bp: 5′-CTGACTGCCCTTGCTCTCC-3′ and 5′-GGCAGCTGGGGTTAATGGAC-3′. PCR product was digested with Fnu4HI (New England Biolabs, Beverly, MA) at 37 °C. Digestion yields two fragments (92 and 58 bp) if the allele is wild type. Fragments were visualized on an agarose gel stained with ethidium bromide.

The Ex1f-VNTR polymorphism was determined by PCR amplification and product size determination by means of fragment analysis on a CEQ8000 DNA-sequencer (Beckman-Coulter, Krefeld, Germany), as previously described (Reif et al., 2006). Alleles have been dichotomized in short and long alleles as described (Reif et al., 2006), with up to 10 repeats (i.e. the J allele) being designated as short alleles.

2.2.4. Statistical analysis

Statistical analysis was performed using the SPSS for Windows 12.0.1 (SPSS Inc. Chicago, IL, USA). Hardy-Weinberg equilibrium was tested for using a $\chi^2$ goodness of fit test. Chi-square tests were performed to test for group differences in allele and genotype frequencies, with Fisher’s exact test being used to determine $P$-values for the allele comparisons. The odds ratio (OR) was calculated along with its 95% confidence interval (CI).

3. Results

Table 2 gives the allele and genotype frequencies of AD patients and controls in the total sample and after stratification for gender. Both AD and control populations were in Hardy-Weinberg equilibrium with regards to Ex1c-SNP and Ex1f-VNTR. Allele and genotype comparisons showed association between the NOS1 VNTR and AD in the total sample (Table 2). The S allele was significantly more common in AD patients (55% versus 44%, $P = 0.009$, OR = 1.52, 95% CI: 1.12–2.08) as was the S/S genotype (28% versus 14%, $P = 0.008$; OR = 2.37, 95% CI: 1.34–4.21). Conversely, no
Table 3

<table>
<thead>
<tr>
<th>NOS1 VNTR</th>
<th>ApoE</th>
<th>Total n (%)</th>
<th>Females n (%)</th>
<th>Males n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AD</td>
<td>Controls</td>
<td>AD</td>
<td>Controls</td>
</tr>
<tr>
<td>L</td>
<td>113 (0.31)</td>
<td>167 (0.45)</td>
<td>134 (0.50)</td>
<td>137 (0.51)</td>
</tr>
<tr>
<td>S</td>
<td>54 (0.15)</td>
<td>71 (0.40)</td>
<td>40 (0.40)</td>
<td>26 (0.40)</td>
</tr>
<tr>
<td>L/ε4 −</td>
<td>107 (0.31)</td>
<td>131 (0.89)</td>
<td>134 (0.50)</td>
<td>137 (0.51)</td>
</tr>
<tr>
<td>S/ε4 −</td>
<td>54 (0.15)</td>
<td>71 (0.40)</td>
<td>40 (0.40)</td>
<td>26 (0.40)</td>
</tr>
</tbody>
</table>

A chi-square test was used to test for the respective groups against the L/ε4− low risk group, as described in the text.

As expected, the frequency of the ApoE ε4 allele was significantly increased in AD as compared with controls (46% versus 26%; χ² = 14.53, d.f. = 1, P = 0.0001, OR: 2.48, 95% CI: 1.55–3.99; Table 1). To examine the combined influence of the NOS1 VNTR S allele and the ApoE ε4 allele on the risk to develop AD, we stratified our sample according to the risk of possessing no, either one, or both risk alleles as inferred from the OR of the above association tests (S allele: OR = 1.52; ε4 allele: OR = 2.48). Non-carriers of risk alleles (risk = 1) were compared with carriers of the NOS1 S allele without the ε4 allele (risk = 2), with carriers of the ε4 allele without the S allele (risk = 3), and with carriers of both risk alleles (risk = 4). Table 3 summarizes the results of the respective chi-square tests. All pairwise comparisons yielded highly significant results (all χ² ≥ 10, all P ≤ 0.001), as illustrated by comparing the odds ratios: whereas comparing risk groups 2 versus 1 gave an OR = 1.73, OR was more than doubled for the comparison 3 versus 1 (OR = 4.05), and a more than six times higher ratio was obtained for the
comparison 4 versus 1 (OR = 10.83). This increase in OR was similar in female and male subgroups (Table 3). However, the even higher OR of the risk 4 versus 1 comparison in males (OR = 15.39) must be viewed cautiously, as adding just one control male to the risk = 4 group would yield an OR of about 7.5. Fig. 1 summarizes the interaction between Ex1f-VNTR and ApoE in the whole sample as well as after stratifying for gender. Unlike the finding for the NOS1 VNTR main effect previously described, there were no gender-specific effects for the gene × gene interaction test results.

4. Discussion

According to our results, the presence of the S allele of NOS1 Ex1f-VNTR represents a risk factor for the development of AD. Most interestingly, the effect of this allele is likely to be gender specific, as it was found in females only. In addition, the S allele was shown to interact synergistically with the ApoE ε4 allele both in males and females, increasing the risk to develop AD more than 10-fold.

The distribution of Ex1c-SNP in Italian healthy subjects was similar to the previously reported observations in other Caucasian populations (Reif et al., 2006; Saur et al., 2004). The frequency of the exon1f-VNTR S/S genotype in 644 unselected, middle-aged controls from Germany and Sweden is 19.6% (Reif, unpublished data), which is in-between Italian controls and cases. As those subjects are still at the population-based risk to develop AD later on, this finding is expected.

AD is a multifactorial disease, and genetic factors play a primary role in orchestrating pathological events and in changing the disease phenotype from patient to patient. In this scenario, NOS1 seems to be a risk factor for AD, which was significant only in the female population after stratification for gender. Given the small size of the male subgroup, this however might be due to a type II error, as the direction of allelic differences was in the same direction as females. Thus, replication studies in larger populations are desirable; also testing for possible interactions with other genes or additional environmental factors present in females but not males is worthwhile. In fact, epidemiological data indicate that the prevalence of AD in Italy is increased in females compared with males (The Italian Longitudinal Study on Aging, 1997). Therefore, it is conceivable that different factors contribute to the development of the pathology in females rather than males, including genetic ones. In this regard, several association studies demonstrated a gender-specific effect of several genes in the development of AD, such as the ATP-binding cassette transporter 1 polymorphism (Sundar et al., 2006) or the estrogen receptor α (Porrello et al., 2006). On a molecular basis, it is noteworthy that the NOS1 exon 1f promoter region harboring the VNTR features the estrogen receptor binding motif, providing a rationale of how sex hormones may interact with genetic variation at the NOS1 locus. To further elucidate this, we are at present investigating estrogen effects on gene expression in correlation with the Ex1f-VNTR genotype in reporter gene assays.

The OR in Ex1f in females was almost as large as the risk conveyed by ApoE ε4 allele, but notably the risk resulting from the interaction of Ex1f-VNTR S allele and ε4 is dramatically increased in the overall population. An influence of the ε4 allele on nitric oxide production has been recently reported. In particular, NO production by peritoneal macrophages as well as by microglia from transgenic APOE4 mice, which express exclusively the ε4 allele, was increased as compared with that of cells isolated from APOE3 mice, which conversely only express the ε3 isoform (Colton et al., 2002; Xu et al., 1996). Importantly, although the mechanism for these isoform-specific differences in NO production is not understood yet, expression of iNOS mRNA and protein were not different between APOE4 and APOE3 mice, suggesting that other NOS isoforms could be responsible of the observed effect (Colton et al., 2002). Similar results have been demonstrated in human-derived macrophages from patients with Alzheimer’s disease carrying the ε4 allele as compared either with patients carrying the APOE ε3/3 genotype or with normal aged controls (Colton et al., 2004).

To date, both the VNTR in exon 1f promoter and the G-8A4a allele in exon 1c promoter were suggested to be functional, thus contributing to the regulation of the transcription of different alternative exons (Zhang et al., 2004). In the human brain, NOS1 exons 1c and 1f are expressed in several brain regions involved in the pathogenesis of AD: the former mainly in the putamen and the hippocampus, and the latter preferentially in the basal ganglia (Reif et al., 2006). The detailed mechanism of this differential spatial expression however is still unknown. Concerning the Ex1c G-8A4a SNP, reporter gene assays revealed a 30% decrease for the A allele or ε3/3 genotype or with normal aged controls (Saur et al., 2004). Despite these in vitro results, no association was found between the exon 1c G-8A4a SNP and AD. However, it has to be considered that the G-8A4a association study could be underpowered, given the small minor allele frequency further arguing for a replication study in a larger sample. Besides, it should be taken into account that the in vivo system is of great complexity as compared with in vitro reporter assays. For instance, several regulatory factors are involved in transcription regulation in humans, including Sp and ZNF, demonstrated to play a critical role in the regulation of exon 1c basal promoter region (Saur et al., 2002). An expression analysis in human cells would be of help to clarify the functional implications of this polymorphism in humans.

Regarding the Ex 1f-VNTR, its function to date is unknown. VNTR alleles are distributed in a bimodal fashion, with one peak for long and two peaks for short alleles, thus suggesting a functional relevance of this polymorphism (Reif et al., 2006). The VNTR polymorphism has been recently studied in a population of schizophrenic patients. Although no association was found with the risk of developing the disease, the NOS1 Ex1f-VNTR was associated with prefrontal brain functioning and disease severity, according to neuropsychological testing and several psychopathological
measures (Reif et al., 2006). Therefore, a possible role in pathologies affecting behavior and cognition, including AD, could be conceivable. Further studies on larger samples of subjects with different neurodegenerative disorders are surely needed to clarify the role of NOS1 in dementia.

In conclusion, this is the first association study of NOS1 and AD demonstrating that the S allele of the VNTR polymorphism in exon 1f of the NOS1 gene acts as a susceptibility factor for the development of AD gender-specifically in females. Moreover, the interaction with the ApoE ε4 allele illustrates the synergistic effect of risk-factor genes in increasing the susceptibility towards AD. However, to confirm these results, further studies in larger populations are certainly needed, together with a functional analysis.

Disclosure statement

Authors do not have conflicts of interest, including financial, personal or other relationships with other people or organizations within 3 years of beginning this work that could inappropriately influence the work.

Acknowledgements

We are indebted to T. Töpner for excellent technical assistance. This work was supported by grants from Associazione “Amici del Centro Dino Ferrari”, Monzino Foundation, IRCCS Ospedale Maggiore Milano, Centre of Excellence for Neurodegenerative Diseases of the University of Milan, Ing. Cesare Cusan, Deutsche Forschungsgemeinschaft (Grant RE1632/1-1 and 1-3 to A.R., KFO 125/1-1 to A.R. and K.P.L. and SFB581 to K.P.L.), Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (IZKF Würzburg, 01KS9603, to K.P.L) and the European Commission (NEW-MOOD LSHM-CT-2003-503474, to K.P.L.).

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