Candidate gene analysis of IP-10 gene in patients with Alzheimer’s disease

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Abstract

Interferon-γ-inducible Protein-10 (IP-10) is supposed to play a role in Alzheimer’s disease (AD) development, as demonstrated by increased levels in cerebrospinal fluid from patients with AD. A mutation scanning of IP-10 exonic region was carried out in 10 patients with AD and 10 age-matched controls, demonstrating the presence of two previously reported single nucleotide polymorphisms (SNPs) in exon 4 (G → C and T → C) as well as a novel SNP in exon 2 (C → T). Exon 4 G → C and T → C allelic variants were next evaluated in a population of 279 AD patients and 251 controls, in order to determine whether their presence could influence the susceptibility towards the development of the disease. These two SNPs were in complete linkage disequilibrium. No differences in haplotype frequencies were found in AD patients as compared with controls, even stratifying according to the presence of Apolipoprotein E 4 allele, gender or age at onset. A new protocol was developed to easily determine the C → T SNP in exon 2. A preliminary analysis revealed a very low frequency of this allelic variant (1%). Therefore, the complete association study was not carried out because the size of our population was not sufficient to draw reliable conclusions. According to these results, IP-10 does not seem to be a risk factor for AD. However, a novel rare polymorphism has been identified, which could exert a role in AD susceptibility. Thus, further studies on larger populations are needed before confidently excluding IP-10 as a susceptibility gene for AD.

Keywords: Alzheimer’s disease; Polymorphisms; Haplotypes; Risk factor; Interferon-γ-inducible Protein-10 (IP-10)

Amyloid beta (Aβ) deposition in the brain is likely to play a critical role during the pathogenesis of Alzheimer’s disease (AD) as it originates a chronic inflammatory response, which contributes to neuronal death [1]. Among inflammatory molecules demonstrated in senile plaques, several chemokines have been identified, and the upregulation of a number of them has been associated with AD pathologic changes [19]. In particular, Interferon-γ-inducible Protein-10 (IP-10) immunoreactivity has been shown to be markedly increased in reactive astrocyes in AD brains, together with its expression level. Moreover, astrocytes positive for IP-10 were found to be associated with senile plaques [18,20]. Chemokine levels, including IP-10, were evaluated in cerebrospinal fluid (CSF) from AD patients, divided in mild AD patients, who had a Mini Mental State Examination (MMSE) score >14, and severe AD (MMSE ≤ 14), and compared with age-matched controls as well as with patients with Frontotemporal Lobar Degeneration, demonstrating that CSF IP-10 levels are increased only in mild AD, whereas Monocyte Chemotactic Protein (MCP)-1 and Interleukin-8 (IL-8) are increased in all AD patients, independently of the degree of the cognitive decline, but with highest peaks in mild AD [6,8]. This evidence has been then demonstrated also in subjects with Mild Cognitive Impairment (MCI), which is considered the prodromal phase of AD [7]. These results strongly suggest that chemokine upregulation is a very early event during AD pathogenesis, even preceding the clinical manifestation of the disease, and that IP-10 is specifically increased in AD. However, in serum samples from AD patients, IP-10 levels seem to be increased [6], conversely to MCP-1, whose levels seem to parallel those found in CSF [5]. Given the previous considerations, it is conceivable that genetic variants in IP-10 gene sequence could have
a role in conferring increased susceptibility to AD, as well as in influencing serum levels of the protein. IP-10 gene is composed by four exons interrupted by three introns [10], and to date two SNPs have been described in exon 4, consisting in a \( G \rightarrow C \) and in a \( A \rightarrow C \) substitution in the untranslated 3' region of the gene (http://www.ensembl.org/).

A frequency of 46% for the rs3921 and of 39% for the rs8878 analysis of a 92-Caucasian individual population, resulting in a frequency of 46% for the rs3921 and of 39% for the rs8878 (http://www.ensembl.org/).

On the basis of these studies, underlying the possible importance of allelic variants in IP-10 in the pathogenesis of AD, a mutation scanning of IP-10 exons was carried out in a limited population of patients and controls, and allelic variants with a high (≥5%) frequency were subsequently evaluated in a larger population of AD patients as well as in a same-size control population of age-matched healthy subjects through allelic discrimination, in order to determine whether their presence could influence the susceptibility or exert a protective effect towards the development of the disease.

Two hundreds and seventy-nine AD patients (191 women and 88 men, mean age at disease onset \( \pm \) S.E.M. = 74.5 \( \pm \) 0.49 years, mean age at diagnosis = 75.8 \( \pm \) 0.60 years) were consecutively recruited at Alzheimer Units of Ospedale Maggiore Policlinico (Milan) and Ospedale L. Sacco (Milan). All patients underwent a standard 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 MMSE. Disease duration was defined as the time in years between the first symptoms (by history) and the clinical diagnosis. The diagnosis of probable AD was made by exclusion according to NINCDS-ADRDA criteria [11].

Sixty patients had an early disease onset (LOAD; ≤ 65 years), whereas remainders had a late onset of the disease (LOAD; > 65 years). In order to get the best diagnostic uniformity for an accurate phenotypic characterization of AD patients, the above-mentioned guidelines were carefully discussed among participants from the centers involved in this collaborative study.

Then, in each center, cases were discussed by a team composed by clinicians, neuropsychologists, neuroimaging and laboratory experts. After the recruitment period, an accurate follow-up of patients was done, to further confirm clinical diagnoses. The control group consisted of 251 healthy volunteers matched for ethnic background and age (147 women and 104 men, mean age \( \pm \) S.E.M. = 73.3 \( \pm \) 0.70 years). One hundred and five of them were seen at the Alzheimer Unit of the Ospedale Maggiore Policlinico (Milan) for subjective memory complaints (Group 1). These subjects underwent the standard battery of examinations, and the diagnosis of AD, as well as of other dementias, was excluded. Patients with Mild Cognitive Impairment, diagnosed according to the criteria of Petersen et al. [12], were not included in this group. All these control subjects did not develop dementia after a follow-up period of 9–12 months. The remaining control subjects (Group 2) were healthy age-matched volunteers recruited either at nursing homes or at the Policlinico Hospital (non-consanguineous patients' kindreds). MMSE scores of all controls were ranged between 28 and 30. No significant age-related differences were found between the groups (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 presence of the ApoE e4 allele, which is considered a known risk factor for AD [3]. All the information about patients and controls are summarized in Table 1.

<table>
<thead>
<tr>
<th>Characteristics of subjects</th>
<th>Controls (n = 251)</th>
<th>AD patients (n = 279)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (m/f)</td>
<td>104:147</td>
<td>88:191</td>
</tr>
<tr>
<td>Mean age at sampling (year)</td>
<td>73.3 ( \pm ) 0.70</td>
<td>75.8 ( \pm ) 0.60</td>
</tr>
<tr>
<td>Mean age at onset (year)</td>
<td>74.5 ( \pm ) 0.49</td>
<td>4.8 ( \pm ) 0.15</td>
</tr>
<tr>
<td>Mean disease duration (year)</td>
<td>( \geq ) 20</td>
<td>( \geq ) 20</td>
</tr>
<tr>
<td>ApoE e4 carriers (n (%))</td>
<td>33 (13.2)</td>
<td>113 (40.5)</td>
</tr>
</tbody>
</table>

* Blood collected at time of diagnosis.

** Time in years between the first symptoms (by history) and the clinical diagnosis.

\( \leq 65 \) years, \( \geq 65 \) years.

\( P \leq 0.02 \), AD vs. controls. OR (95% CI): 4.48 (2.95–6.81).

### Table 1

Table 2.

<table>
<thead>
<tr>
<th>Characteristics of subjects</th>
<th>Controls (n = 251)</th>
<th>AD patients (n = 279)</th>
</tr>
</thead>
</table>
| ApoE genotype determined by Polymerase Chain Reaction-Restriction Fragment Length Polymorphisms (PCR-RFLP) assay. DNA was amplified using specific primers and then digested with BglI [9].

Exons 1 through 4 were amplified using specific primers (Table 2).

DHIPLC analysis was performed by a WAVE DNA 3500-Fragment Analysis System (Transgenicom, NE, USA) running samples on a DNAsep cartridge, as previously described [13]. Briefly, 10 \( \mu \)l of each PCR reaction was loaded on the column, previously equilibrated with two blank injections. Due to the prediction of at least three different melting profiles (software Navigator version 1.6, Transgenicom) each amplicon was run at three different temperature (53.5, 55.7 and 58 °C) to ensure a thorough search of any DNA sequence variation. Any sample that eluted with a symmetrical peak was mixed with an equal amount of a known wild-type homozygote sample, denatured and reannealed, and run again on the DHIPLC column in order evaluate the presence of homogenous DNA variations. When the elution profile of the sample was asymmetrical, the DHIPLC analysis was repeated on a new amplification to exclude the
chance of PCR-dependent misincorporation. When confirmed by the second DHPLC run, the nature of the DNA variations was obtained by direct automated sequencing of the same amplicon on the column.

For direct sequencing, fragments were purified using ExoSAP-IT® Kit (usb, USA). Sequencing was performed with an ABI PRISM 3100 gene analyzer (ABI).

A mutation was introduced by a primer containing the SNP. A mutation was introduced by a primer with a mismatch, which results in a restriction site for an enzyme (AciI). The restriction site was determined using the Webcutter software program available from the web (Webcutter 2.0, http://www.firstmarket.com/cutter/cut2.html). PCR product fragments were visualized on a 4% agarose gel stained with ethidium bromide. For the exon 2 A→C SNP, a novel PCR-RFLP protocol was developed to easily detect this allelic mutation. It consists of a PCR amplification carried out using a forward primer with a mismatch, which results in a restriction site for AciI when mutation does not occur, yielding 26 and 112 bp fragments. One hundred patients as well as 100 controls were screened by using this technique, but the SNP was found in 2 patients only. Therefore, given the very low frequency of this SNP (1%), the complete association study was not carried out because the size of our populations was not sufficient to draw reliable conclusions.

Conversely, an association analysis on the whole population was carried out for the G→C and T→C SNPs, known to have a frequency >5%. Haplotype frequencies for AD patients and controls are reported in Table 3. Both AD and control populations were in Hardy–Weinberg equilibrium. These two polymorphisms were in complete linkage disequilibrium ($D^2 = 1$). No differences in haplotype frequencies were found between AD patients and controls, even dividing healthy sub-

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Primer sequence</th>
<th>Annealing temperature (°C)</th>
<th>Amplification products' size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1</td>
<td>F: 5′-CAAGATGGACTTCCCCAGGACAG-3′</td>
<td>50</td>
<td>428</td>
</tr>
<tr>
<td>Exon 2</td>
<td>F: 5′-GATGATGATCATCTCTCTCTCAG-3′</td>
<td>57</td>
<td>448</td>
</tr>
<tr>
<td>Exon 3</td>
<td>F: 5′-ACAGCAAAACACACTGACAGTGA-3′</td>
<td>62</td>
<td>262</td>
</tr>
<tr>
<td>Exon 4</td>
<td>F: 5′-GCTGTCCTTCACATCTCCTCC-3′</td>
<td>61</td>
<td>228</td>
</tr>
</tbody>
</table>

Statistical analysis was performed using the Haploview 3.2 software [2]. Chi-square was used to test for differences in ApoE allele distribution between the groups. The odds ratio (OR) was calculated along with its 95% CI.

Table 3

<p>| IP-10 haplotype frequencies (%) in AD patients compared with age-matched healthy controls |
|-----------------------------------------------|-------------------------|----------------------------------|</p>
<table>
<thead>
<tr>
<th>Haplotype</th>
<th>AD patients (n = 219)</th>
<th>Healthy controls (n = 231)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG SNP</td>
<td>48 (16.7)</td>
<td>36 (14.3)</td>
</tr>
<tr>
<td>GC SNP</td>
<td>136 (48.7)</td>
<td>174 (71.6)</td>
</tr>
<tr>
<td>CC SNP</td>
<td>87 (31.6)</td>
<td>81 (34.1)</td>
</tr>
</tbody>
</table>

* Subjects recruited at the Alzheimer Unit.  
* Subjects recruited at nursing homes plus non-consanguineous patients' kindreds.
by gender, a further analysis was carried out separately either in males or females, confirming data obtained in the overall population (Table 4).

As observed in other populations [14,16], the frequency of the ApoE ε4 allele was significantly higher in AD patients than in controls (40.5% versus 13.2%, P < 0.001, Table 1). The presence of a single copy of this allele increases the risk of developing AD (OR: 4.48, 95% CI: 2.95–6.81). Stratifying AD patients according to the presence of the ApoE ε4 allele, no differences in IP-10 haplotype frequencies were found (P > 0.05, Table 4). Similarly, no differences were observed stratifying AD patients by age at onset (data not shown).

According to these results, IP-10 does not seem to be a risk factor for AD in our population. However, a novel polymorphism in exon 2 has been identified, which could exert a role in AD susceptibility. This new variant seems to be extremely rare, as in our population only two out of 200 subjects analyzed were heterozygous for the SNP (1% frequency). Interestingly, both these individuals were diagnosed as AD, whereas none of controls was a carrier of this allelic variant. These findings suggest a possible role of this SNP in AD susceptibility, but further studies on larger populations are certainly needed to clarify these preliminary results. Besides, a functional analysis should be considered, as this SNP results in a potential important aminoacid substitution (cysteine, a non-charged aminoacid instead of arginine, which is positively charged).

The exon 4 G → C and T → G SNPs are located in the 3′-untranslated region of IP-10 gene; thus, an effect on the stability of the related mRNA could be conceivable. In fact, mRNA for IP-10 is readily detectable within 2 h after treatment with inducers, suggesting that induction does not require new protein synthesis, although in part new transcription occurs [17].

AD is a multifactorial disease, and genetic factors play a primary role in orchestrating these pathological events and in changing the disease phenotype from patient to patient. At present, many efforts have been done to identify genetic variation having a potential role in human disease [15]. Despite IP-10 seems not to be a risk factor for AD, it could interact with other genes or with additional environmental factors to determine the overall risk to develop AD. The detailed knowledge of genetic factors involved in AD could modulate the severity of the pathogenic process or the response to drug treatment.

In conclusion, although we failed to find any evidence for association of AD with IP-10 variants considered, we described a new rare allelic variant in this gene, which could have a role in AD. Further association studies in larger populations, together with IP-10 functional analyses, should be carried out before this gene can be confidently excluded as candidate susceptibility factor.

Acknowledgements

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References


