Polymorphisms in the Oxidized Low-Density Lipoprotein Receptor-1 Gene and Risk of Alzheimer’s Disease

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The +1073 C/T polymorphism of the oxidized low-density lipoprotein receptor-1 (OLR1) gene has been reported to be associated with late-onset Alzheimer’s disease, whereas for the +1071 T/A polymorphism no association was found. We genotyped 169 sporadic Alzheimer’s disease patients and 264 sex- and age-matched nondemented controls from Southern Italy for OLR1 +1073 C/T and +1071 T/A polymorphisms and for apolipoprotein E and LBP-1c/CP2/LSF. We also performed haplotype analysis. For the +1073 C/T polymorphism, the C allele and the CC genotype have been associated with a higher risk for Alzheimer’s disease without apolipoprotein E or CP2 interaction. The two polymorphisms were in linkage disequilibrium, with the haplotype T-C at significant increased risk of developing Alzheimer’s disease in the whole sample and in elderly persons 70 years or older. In our population, the +1073 C/T OLR1 polymorphism exhibited a significant association with Alzheimer’s disease, further supporting the role of OLR1 as a candidate risk gene for sporadic Alzheimer’s disease.

Recent progress implicates vascular factors and inflammatory mechanisms as playing a major part in the pathogenesis of Alzheimer’s disease, leading to the hypothesis that genes involved in vascular or inflammatory pathways may be good candidates for Alzheimer’s disease (1). Oxidized low-density lipoprotein receptor-1 (OLR1) is a major endothelial receptor for oxidized low-density lipoprotein (oxLDL) (2). Expression of OLR1 is induced by inflammatory cytokines, oxidative stress, and oxLDL in vitro and by proatherogenic stimuli in vivo, such as hypertension, hyperlipidemia, and diabetes mellitus (3–5). Indeed an involvement of OLR1 in atherosclerosis and vascular disease (6,7), as well as in inflammatory processes (8), has been suggested.

Furthermore, the OLR1 gene maps to chromosome 12p, and different genome screen studies have strengthened the argument that at least one Alzheimer’s disease susceptibility gene exists on chromosome 12 (9–13). In fact, some genes within the Alzheimer’s disease linkage region on chromosome 12 [the transcription factor LBP-1c/CP2/LSF (for brevity, CP2), LDL receptor-related protein 1, and alpha-2-macroglobulin] have been associated with the disease, although the findings were equivocal (14–16). It has also been shown that OLR1 is widely expressed in the central nervous system, including several regions of the brain and spinal cord (17).

With this background, Luedecking-Zimmer and colleagues screened the OLR1 gene and found a polymorphism in the 3’ untranslated region (UTR), +1073 C/T, which was reported to be associated with late-onset Alzheimer’s disease after stratification by apolipoprotein E (APOE) genotypes (18). More recently, Lambert and associates supported the association of the 3’ UTR polymorphism with the risk of Alzheimer’s disease in a cohort of French sporadic and American familial Alzheimer’s disease patients, but in an APOE genotype-independent fashion. Further, the authors reported in the same cohorts a new polymorphism, +1071 T/A, located two bases upstream of the original one, which, however, was not associated with the disease (19). In contrast, a new report on the association of the 3’ UTR +1073 C/T polymorphism with Alzheimer’s disease in a large family sample did not reveal any evidence of interaction between the polymorphism and the disease, even after stratification based on onset age and APOE status (20). Finally, in a UK cohort, no difference was found between 356 Alzheimer’s disease patients and 358 matched controls for both OLR1 +1071 and +1073 polymorphisms, also after stratification based on onset age and APOE ε4 allele presence (21).

We examined the potential relationship between the OLR1 +1073 C/T polymorphism and the risk of Alzheimer’s disease in a sample of sporadic patients and age- and sex-matched controls from Southern Italy. We also described in our population the new polymorphism +1071 T/A and evaluated its relationship with Alzheimer’s disease alone or in synergistic association with the +1073 C/T polymorphism. Finally, we examined the putative interaction between the +1073 polymorphism and APOE or CP2 allele strata. This last gene is, in fact, a plausible candidate gene for influencing Alzheimer’s disease risk (22), and in a recent study we reported that the A allele of the G/A polymorphism in the 3’ UTR of the CP2 gene increases the risk of sporadic Alzheimer’s disease, without interaction with APOE alleles (14).

 METHODS

Study Participants

A total sample of 433 participants from Southern Italy was studied: 169 Alzheimer’s disease patients, and 264 middle-
aged, unrelated caregivers (spouses, friends, or neighbors) or volunteers who presented themselves to the Centre for Aging Brain, Memory Unit, Department of Geriatrics, Bari University Hospital, Italy, between June 1998 and March 2004. The Alzheimer’s disease group included 99 patients with sporadic late-onset Alzheimer’s disease (age at onset ≥70 years; mean actual age (±SD) = 76.4 ± 4.46; 57 women and 42 men) and 70 patients with sporadic early-onset Alzheimer’s disease (age at onset <70 years; mean actual age (±SD) = 59.7 ± 6.18; 50 women and 20 men). We used 70 years as cutoff age to ensure homogeneous age groups, and we also adopted this convention in other recent studies (14,15).

The age at onset of Alzheimer’s disease symptoms was estimated by semistructured interviews with the patients’ caregivers (23). Clinical diagnosis of probable Alzheimer’s disease was made according to the National Institute for Neurological and Communicative Disorders and Stroke/Alzheimer’s Disease and Related Disorders Association criteria (24). The nondemented sex- and age-matched control group was composed of 264 persons, 118 men and 146 women, and the mean age at collection (±SD) was 71.7 ± 7.09. The group of healthy participants included 177 individuals ≥70 years (99 women and 78 men) and 87 individuals <70 years (47 women and 40 men). The ascertainment, diagnosis, and collection of patients and controls have been described in detail elsewhere (24). All participants included in the study were Caucasians residing in Southern Italy. The study protocol received the approval from the Ethical Committee of the University of Bari. Informed written consent was obtained from all participants or their relatives prior to the collection of blood samples.

APOE, CP2, and OLR1 Genotyping

Genomic DNA was extracted from peripheral blood samples using the High Pure Polymerase Chain Reaction (PCR) Template Preparation Kit (Roche Diagnostics, Monza, Italy). CP2 and APOE genotypes were determined as previously described (14,25). The OLR1 genotyping was initially performed on a LightCycler system (Roche, Mannheim, Germany) by a melting curve analysis using specifically designed hybridization probes with fluorescent dyes (sensor probe, 5′-ATTCTAgCTACCTgTATTATTT CACCTAgC-fluorescein; anchor probe, red 640 – gTCCCA AgCTTCCCTgCCAgCCT-phosphate; TIB Molbiol, Berlin, Germany). The primers used in the amplification were forward primer, 5′-TTgAATTTTgAaggCTCTTggAagA-3′, and reverse primer, 5′-ggTgATATAATgAaggTAAAgaAA gACTgAg-3′. The following 20-μl reaction mixture was used for PCR amplification and subsequent detection of fluorescence from hybridization probes: 50 ng of genomic DNA, 10 pmol each primer, 3 pmol each probe, 2.5 mM MgCl₂, 1× DNA master hybridization probes (Roche Diagnostics). The amplification program consisted of initial denaturation for 2 minutes at 95°C, then 40 cycles with three temperature segments. The first segment was 94°C for 5 seconds at 20°C/s of temperature transition rate for denaturation; a second segment was of 15 seconds at 54°C and 20°C/s for both primer and probe annealing. A third temperature segment for primer extension was 72°C for 10 seconds at 20°C/s. After amplification, the temperature was raised to 94°C for 30 seconds, lowered to 40°C at 20°C/s, and held at 40°C for 1 minute. A melting curve analysis profile was obtained by raising the temperature to 80°C at 0.10°C/s while collecting fluorescence data continuously. The melting temperatures were 59°C for the 3′-UTR T allele and 64°C for the 3′-UTR C allele. In some cases, the melting curve graph showed peaks with unusual melting temperatures which were between the two characteristic melting temperatures. All these samples were sequenced, and a new genetic variation was shown only two bases upstream of the original mutation (+1071 T/A). In ongoing work, this latter mutation was reported by Lambert and colleagues as a new polymorphism in French and American populations, corroborating our initial findings. Indeed, we continued the OLR1 genotype analysis by sequencing the samples for both genetic variations (+1071 T/A and +1073 C/T).

Statistical Analysis

The statistical analysis was performed by using STATA software (release 8.0; STATA Corp., College Station, TX). The Pearson chi-square test was performed to make genotype and allele comparisons as well as to test for agreement of data with Hardy–Weinberg principles. Allele frequencies were determined by allele counting. To express variances of genotype and allele frequencies we used 95% confidence intervals (CIs), the upper and lower values of which were calculated according to Wilson’s formulas. The differences among age at onset of Alzheimer’s disease symptoms in relation to different OLR1 alleles were calculated with the Mann–Whitney U test. To evaluate whether the association between +1073 and +1071 OLR1 genotypes and Alzheimer’s disease was homogeneous in all APOE and CP2 strata, separately evaluated, we used a logistic model. In comparing OLR1 genotypes we used the chi-square value corresponding to 0.05/3 = 1.7% for each of the individual comparisons, adjusted for Bonferroni inequality. Finally, to evaluate the possible risk or protective role for Alzheimer’s disease of the OLR1 alleles, we calculated the odds ratios (OR) and the 95% CI between participants with and without at least one C or T allele. The chi-square or z tests were calculated by asymptotic and exact p values. No statistical analysis was performed for the AA genotype, because it is not possible to compute any calculation when the number of nonempty rows or columns in 2 × 2 contingency tables is one. Haplotype analysis was performed by command “hapipf”, within the STATA statistical package (26). The association between the OLR1 +1071 and +1073 marker loci was evaluated by likelihood ratio test to verify if linkage disequilibrium was present between the loci of interest. Then we tested for the association between one locus and Alzheimer’s disease conditional on the alleles at the other locus. The threshold of significance was set at p < .05.

Results

The OLR1 genotype and allele frequencies of the 3′ UTR polymorphisms in the whole Alzheimer’s disease sample, early-onset and late-onset Alzheimer’s disease patients, and age-matched and sex-matched nondemented controls are shown in Table 1. The genotype distributions for both polymorphisms were in Hardy–Weinberg equilibrium (+1073 C/T patients: Pearson χ² = 0.01, df = 1, p = 92;
+1073 C/T controls: Pearson $\chi^2 = 3.7$, df = 1, $p = .06$; +1071 T/A patients: Pearson $\chi^2 = 3.2$, exact $p$ value = .14; +1071 T/A controls: Pearson $\chi^2 = 3.4$, exact $p$ value = .09.

Significant differences were found in OLR1 - +1073 C/T genotype frequencies between Alzheimer’s disease patients and controls (CC vs CT and TT: Pearson $\chi^2 = 7.35$, df = 1, Bonferroni $p < .05$) (Table 1). A significant increase in C allele frequency was also observed in the Alzheimer’s disease sample compared to the healthy controls (Pearson $\chi^2 = 6.40$, $p = .01$). The presence of the C allele was associated to Alzheimer’s disease by an OR of 1.24 (95% CI: 1.05-1.46), whereas the CC genotype was associated with the disease by an OR of 1.48 (95% CI: 1.24-1.78). When the whole Alzheimer’s disease sample was subdivided into early-onset and late-onset Alzheimer’s disease patients, significant differences were found only for the +1073 CC genotype frequency between the late-onset patients and controls (Pearson $\chi^2 = 6.38$, df = 1, Bonferroni $p < .05$). In contrast, no differences in +1071 allele and genotype frequencies were observed either in total Alzheimer’s disease patients or in the two subtypes (late-onset and early-onset) compared to control participants (Table 1). Furthermore, Alzheimer’s disease patients bearing the +1073 C allele showed a mean age at onset very similar to that in those who were carriers of the T allele ($z = 0.09, p > .93$). Finally, no interaction was found between OLR1 3'-UTR polymorphisms and APOE allele strata, as well as between the +1073 polymorphism and CP2 allele strata (data not shown).

A likelihood ratio test confirmed there was strong evidence in support of linkage disequilibrium between the two loci (likelihood ratio test = 89.04, df = 1, $p < .001$). Haplotype analysis was carried out on the whole sample and on early- and late-onset Alzheimer’s disease patient subgroups (Table 2). Three common haplotypes, T-T, T-C, and A-C were found, whereas the fourth haplotype A-T was not observed in anyone in the study population. The estimated haplotype distribution was not significantly different between Alzheimer’s disease patients and controls, either in whole sample (likelihood ratio test $= 6.4$, df = 3, $p = .09$) or in late- (likelihood ratio test $= 4.5$, df = 3, $p = .22$) or early-onset Alzheimer’s disease patients (likelihood ratio test $= 4.0$, df = 3, $p = .26$) (Table 2A).

Assuming a T-T haplotype combination as reference, in comparison with haplotypes bearing the C allele (i.e., T-C and A-C), the T-C combined allele was at a significant increased risk of developing Alzheimer’s disease in the whole sample and in participants $\geq 70$ years (Table 2B). Furthermore, since the univariate analysis suggested that the allelic association to Alzheimer’s disease was predominately from the OLR1 +1073 polymorphism (in particular, the C allele), the +1071 and +1073 loci were tested for conditional independence. By this approach, the +1071 locus appeared to be conditionally independent of Alzheimer’s disease either in the total sample or in individuals $> 70$ years old, given +1073 locus [total sample: 1.03 (95% CI: 0.63-1.70); $< 70$ years: 1.54 (95% CI: 0.66-3.57); $\geq 70$ years: 0.81 (95% CI: 0.43-1.52)] (Table 2C).

### Discussion

In this study, we reported the association of the 3’ UTR +1073 C/T polymorphism in the OLR1 gene with sporadic Alzheimer’s disease without APOE or CP2 interaction, further supporting the findings of the previous studies on the functional role of OLR1 as a significant genetic risk factor for the development of the disease.

Luedecking-Zimmer and colleagues (18) initially reported the association of the 3’ UTR +1073 C/T polymorphism with the risk of late-onset Alzheimer’s disease in an APOE-dependent fashion. Support for this finding was provided by subsequent study in French sporadic and American familial Alzheimer’s disease samples. The authors also described a new genetic polymorphism, +1071 T/A, located in the 3’ UTR two bases upstream of the previously reported genetic variation, but no association with Alzheimer’s disease was observed (19). More recently, two independent studies (20,21) failed to replicate the initial findings in a large family sample and in a UK cohort of sporadic Alzheimer’s disease, respectively.

In the present study, we observed that the OLR1 +1073 C/T genotype and allele frequency differed between control and Alzheimer’s disease groups, with the C allele and the CC geno-
The association of the +1073 C allele with Alzheimer’s disease was maintained in the late-onset Alzheimer’s disease subgroup. However, the cutoff age of 70 years used in the present study may also result in reduced power in the early-onset sample due to the probable presence in this group of some late-onset Alzheimer’s disease patients. Thus, it appears likely that the OLR1 gene may be a credible susceptibility factor for Alzheimer’s disease, although it exerts only small effects in the general population which are detectable in some study samples (17,18) but not in others (19,20). It is possible that a moderate effect associated with the OLR1 +1073 polymorphism is due to its non-random association with a functional mutation present somewhere in the gene, or that there is linkage disequilibrium with another biologically relevant locus on chromosome 12, different to A2M. In fact, Bertram and colleagues (20) did not find any evidence of significant linkage disequilibrium between A2M and OLR1 polymorphisms, suggesting that, although A2M gene maps about 1 Mb pter of OLR1, the linkage disequilibrium is most likely not responsible for the observed independent Alzheimer’s disease association reported in different studies for these genes (18,19,28,29). However, confirmation of the observed association be-

Table 2. Haplotype Distribution of the Oxidized Low-Density Lipoprotein Receptor-1 (OLR1) 3'- Untranslated Region (UTR) Gene Polymorphisms in Alzheimer’s Disease (AD) Patients and Nondemented Age- and Sex-Matched Controls (A); Risk of Developing AD According to the Haplotype (B); Conditional Independence of AD for the OLR1 +1071 and +1073 Loci (C).

<table>
<thead>
<tr>
<th>A. Haplotype Combination</th>
<th>Whole Sample</th>
<th>Participants &lt;70 years</th>
<th>Participants ≥70 years</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients</td>
<td>Controls</td>
<td>Patients</td>
</tr>
<tr>
<td>TAGCTAATCTGTATTA</td>
<td>177 (52.4)</td>
<td>324 (61.4)</td>
<td>75 (53.6)</td>
</tr>
<tr>
<td>TAGCTAATCTGTATTA</td>
<td>120 (35.5)</td>
<td>150 (28.4)</td>
<td>45 (32.1)</td>
</tr>
<tr>
<td>TAGCAATCTGTATTA</td>
<td>41 (12.1)</td>
<td>54 (10.2)</td>
<td>20 (14.3)</td>
</tr>
<tr>
<td>TAGCAATCTGTATTA</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td></td>
<td>Ltest * = 6.4, df = 3, p = .09</td>
<td>Ltest * = 4.0, df = 3, p = .26</td>
<td>Ltest * = 4.5, df = 3, p = .22</td>
</tr>
</tbody>
</table>

B. Haplotype combination

<table>
<thead>
<tr>
<th>OR (95% CI)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>1.2</td>
<td>1.46 (1.1–2.0)</td>
</tr>
<tr>
<td>2.1</td>
<td>1.39 (0.9–2.2)</td>
</tr>
<tr>
<td>2.2</td>
<td>1.87 (0.9–3.86)</td>
</tr>
<tr>
<td>1.0</td>
<td>1.08 (1.02–2.0)</td>
</tr>
<tr>
<td>1.0</td>
<td>1.05 (0.64–2.06)</td>
</tr>
<tr>
<td>1.0</td>
<td>1.05 (0.64–2.06)</td>
</tr>
</tbody>
</table>

C. Conditional independence approach

<table>
<thead>
<tr>
<th>OR (95% CI)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.03 (0.63–1.70)</td>
<td>.90</td>
</tr>
<tr>
<td>1.54 (0.66–3.57)</td>
<td>.31</td>
</tr>
<tr>
<td>0.81 (0.43–1.52)</td>
<td>.51</td>
</tr>
</tbody>
</table>

Notes: *Ltest = likelihood ratio test; OR = odds ratio; CI = confidence interval.

1.1 = T-T; 1.2 = T-C; 2.1 = A-C; and 2.2 = A-T haplotype combined alleles.
tween the +1073 polymorphism and Alzheimer’s disease comes from the functional observations by Lambert and colleagues. It was found that the T allele binds with a weaker affinity to regulatory proteins as compared with the C allele. Moreover, the C allele is associated with a higher level of astrocyte activation in Alzheimer’s disease brain, indicating that OLR1 can act through inflammatory mechanisms (19). Finally, in reverse transcription-PCR experiments, Lambert and colleagues (19) observed a significantly reduced OLR1 expression in lymphocytes from Alzheimer’s disease patients bearing the C allele compared to controls, suggesting that the C allele may be functionally lowering the expression of the gene in Alzheimer’s disease patients.

The current data, in conjunction with the earlier association of the OLR1 with Alzheimer’s disease (18,19), provide credence to the hypothesis that the OLR1 gene may play a role in the development of the disease, in Southern Italy and in other populations. However, we must also acknowledge that the relatively small number of Alzheimer’s disease patients in the present study are limited in their statistical power, and further studies in large and different populations are required. Finally, more linkage disequilibrium-based studies involving the OLR1 gene and other nearby genes on chromosome 12, such as C-type lectin-like receptor-1 and -2 (CLEC1 and CLEC2), C-type lectin superfamily member 2 (CLECSF2) (all three of which, together with the OLR1 gene, belong to the lectin-like superfamily), and protein kinase STYK1 (a ubiquitously expressed protein involved in protein amino acid phosphorylation [www.ncbi.nlm.nih.gov]), are also needed to make definitive conclusions.

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