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Association study of polymorphisms in LRP1, tau and 5-HTT genes and Alzheimer's disease in a sample of Colombian patients

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Summary. Analysis of genetic susceptibility factors for Alzheimer's disease (AD) in populations with different genetic and environmental background may be useful to understand AD etiology. There are few genetic association studies of AD in Latin America. In the present work, we analyzed polymorphisms in 3 candidate genes; the LDL receptor related protein-1, the microtubule-associated protein Tau and the serotonin transporter genes in a sample of 106 Colombian AD patients and 97 control subjects. We did not find a significant allelic or genotypic association with any of the three polymorphisms analyzed using different statistical analysis, including a neural network model or different sample stratifications. To date, APOE polymorphisms are the only genetic risk factors identified for AD in the Colombian population. It may be factible that future combination of high-throughput genotyping platforms and multivariate analysis models may lead to the identification of other genetic susceptibility factors for AD in the Colombian population.

Keywords: Alzheimer's disease, genetic association, Colombia.

Introduction

Alzheimer's disease (AD) is the main type of dementia in the world. Although the medical and social impact of AD is projected to be greater in developing countries (Wimo et al., 2003), the specific etiologic factors in these populations with diverse genetic and environmental background remain largely unexplored.

Considering that the study of genetic susceptibility factors may be a key approach to understand the etiology of AD, there are only a few genetic association studies of AD in Latin American populations; we have found previously a strong association with coding and promoter APOE polymorphisms in our Colombian AD sample (Arboleda et al., 2001; Parra-Bonilla et al., 2003; Camelo et al., 2004). However, variations in the Apolipoprotein E (APOE) gene only explain a fraction of the genetics risk associated with

AD, and it is possible that polymorphisms in additional genes may confer additional risk to develop AD (Warwick-Daw et al., 2000). Based on the potential biological contribution to the pathophysiology of AD, several studies have analyzed potential candidate genes for AD in diverse populations, including the LDL receptor related protein-1 (LRP1), the microtubule-associated protein Tau and the serotonine transporter (5-HTT) genes (Selkoe, 2002; Bertram and Tanzi, 2004).

LRP is a member of the LDL superfamily and one of the main receptors for apoE and also a receptor for alpha-2-microglobulin (A2M) in the adult brain. LRP1 gene is located in chromosome 12, has 89 exons and spans 85 kb (Beffert et al., 2004), and encodes a large transmembrane protein of 4544 aminoacids. The main polymorphism in LRP analyzed to date is a silent C766T change in exon 3 (single nucleotide polymorphism database identifier *dbSNP ID*: rs1799986) showing conflicting results in relation to its contribution to AD risk (Sanchez-Guerra et al., 2001; Panza et al., 2004).

Tau has a key function in controlling the microtubule cytoskeleton in the axon and is also the main component of the neurofibrillary tangles (NFT's) characteristic of AD. The Tau gene is located in chromosome 17 spanning around 100 kb, has 14 exons and encodes 6 tau protein isoforms by alternative splicing, the longest of which is 441 aminoacids (Schraen-Maschke et al., 2004). Several genetic changes in Tau have been studied in diverse neurodegenerative diseases. A dinucleotide repeat polymorphism in intron 10 of tau gene, which is in complete linkage disequilibrium with several SNPs that constitutes two extended haplotypes, was the first marker studied (Schraen-Maschke et al., 2004). The association of tau polymorphisms with AD is also controversial (Russ et al., 2001; Seripa et al., 2004).

5-HTT is a protein of 630 amino acids. It functions as a key player in the control of the serotoninergic transmission, in the regulation of mood states and in the regulation of the hippocampal function. 5-HTT gene is located in chromosome 17, has 14 exons and a length of 31 kb (Lesch, 2004). Although a functional polymorphism in the promoter region has been identified as a risk factor for several neuropsychiatric diseases, its role in AD etiology remains to be clarified (Li et al., 1997; Tsai et al., 2001).

In the present study, we have analyzed the association of common polymorphisms in LRP1 (C766T polymorphism), Tau (dinucleotide repeat polymorphism in intron 10) and 5-HTT (a 44 bp VNTR polymorphism in the promoter region) genes with AD in a sample of the Colombian population.

Materials and methods

Subjects

We determined allele and genotype frequencies of LRP1, Tau and 5-HTT polymorphisms in a sample of 106 probable AD patients and 97 sex and age matched control subjects. Patients were evaluated by an interdisciplinary group in a memory clinic in Bogotá, Colombia, following the NINCDS-ADRDA criteria for the clinical diagnosis of AD (Arboleda et al., 2001). The mean age ($\pm$SD) of the entire sample was 73.3 years ($\pm$8.8) for the patients and 72.2 years ($\pm$8.7) for the controls. The age at onset for the entire AD sample was 68.8 years ($\pm$8.7). 34 patients had early-onset AD (EOAD) with mean age of 64.0 years ($\pm$5.2) and a mean age at onset of 63.4 years ($\pm$4.7); 72 patients had late-onset AD (LOAD) with an age of 77.9 years ($\pm$6.0) and a mean age at onset of 76.3 years ($\pm$6.0). 71% of the patients were female and 40% had a positive family history of dementia. Patients had a mean of 7.1 years ($\pm$4.2) of education. Control subjects were free of cognitive impairment based in clinical evaluation and mini mental state examination status (MMSE: score >27). The study was approved by the institutional ethics committee and each subject or its closest relative signed an informed consent.

Genotyping of polymorphisms

Genomic DNA was extracted from peripheral blood using the salting out method as described (Lahiri and Nurnberger, 1991). Two microliters of DNA solution (100–200 ng) were used for each PCR amplification reaction in a 25 µl final volume using 20 pmol of each primer, 200 µM of dNTPs and 0.6–1.0 U of Taq polymerase (Promega, Madison, WI) in a PTC100 Thermal Cycler (MJ Research, Watertown, MA).

Genotyping of LRP1 C766T polymorphism was carried out as described by Beffert et al. (1999). Briefly, a PCR amplification was carried out with primers 5′ GGG GTC CAG GAC TGC ATG TA 3′ and 5′ CCA GGA CAG TAC TCG GAA GGT 3′ (2.0 mM of $MgCl_2$, Ta of 60°C, 32 cycles) and digested with RsaI restriction enzyme (Promega, Madison, WI). An aliquot of the amplified product was run on a 12% polyacrylamide gel at 200 V, followed by silver staining. Alleles and genotypes were scored accordingly to: C allele as a 96 bp band and T allele as a 115 bp band.

Tau intron 10 dinucleotide repeat polymorphism analysis was performed as described by Oliva et al. (1998). Briefly, a PCR amplification was carried with primers 5′ GCC TCG CAA ATT GCT GGG AT 3′ and 5′ AGG TGA CTG GGT AGA GAC AGA GC 3′ (2.0 mM of $MgCl_2$, Ta of 58°C, 32 cycles). An aliquot of the amplified product was run in a 30 cm denaturing 8% polyacrylamide gel at 1600 V, followed by silver staining. A0, A1, A2 and A3 alleles gave bands of 140, 142, 144 and 146 bp, respectively. External controls of heterozygote and homozygote DNA samples for the H1 and H2 haplotypes were kindly provided by Dr. C. Zekanowski (Laboratory of Neurodegeneration, International Institute of Molecular and Cellular Biology, Poland) (Peplonska et al., 2003).

5-HTTLPR polymorphism genotyping was carried out as described by Caspi et al. (2003). A PCR amplification was carried out with primers 5′ ATG CCA GCA CCT AAC CCC TAA TGT 3′ and 5′ GGA CCG CAA GGT GGG CGG GA 3′ (1.5 mM of $MgCl_2$, Ta of 66C, 35 cycles) and an aliquot of the amplified product was run on a 12% polyacrylamide gel at 300 V, followed by silver staining. Alleles and genotypes were scored as follows: the short allele as a 375 bp band and the long allele as a 419 bp band. A random subsample was re-analyzed to assure consistency in the genetic results.

Statistical analysis

Genotype and allele frequencies were calculated using direct gene counting method and the significance of the differences between patients and controls were evaluated using Fisher's test, with an alpha level of 0.05 (two-sided P value). Hardy-Weinberg equilibrium of genotype frequencies in all subsamples was evaluated. Analysis was also carried out in subgroups stratificated by age at onset, sex, family history of dementia, and APOE4 carrier status (Arboleda et al., 2001). Statistical analysis of gene frequencies was done with Graphpad Instat program. SAS 8.0 software was used for logistic regression analysis and the NNPERM program was used for neural network analysis of genetic association (North et al., 2003). NNPERM calculations are based in an artificial neural network (ANN) (in which input neurons or units corresponds to genetic polymorphisms, output units to affection status and connections and *hidden units* to interrelations between genes). The proposed main advantage of the use of ANNs in genetic associations is the possibility that complex non-linear relationships between multiple genes and affection status can be analyzed in a case-control dataset of moderate size using a Monte-Carlo simulation (North et al., 2003; Serretti and Smeraldi, 2004). Analysis of statistical power was carried out using the *Genetic Power Calculator* (Purcell et al., 2003) available at *http://statgen.iop.kcl.ac.uk/gpc/* and the *Power Calculator Program* available at *http://calculators.stat.ucla.edu/powercalc/binomial/case-control/index.php.*

Results

The allele and genotype frequencies for the LRP1, Tau and 5-HTT polymorphisms in the

Table 1. Genotype and allele frequencies for LRP1 polymorphism in a sample of Colombian patients with AD stratified by age of onset and APOE4 carrier status

	Total AD	Total controls	LOAD	LOAD controls	APOE4+ AD	APOE4+ controls
Genotype						
CC	84 (79.2)	78 (80.4)	56 (77.8)	55 (85.9)	44 (78.6)	11 (78.6)
CT	22 (20.8)	18 (18.6)	16 (22.2)	8 (12.5)	12 (21.4)	3 (21.4)
TT	0 (0)	1 (1.0)	0 (0)	1 (1.6)	0 (0)	0 (0)
Total	106 (100)	97 (100)	72 (100)	64 (100)	56 (100)	14 (100)
Allele						
C	190 (89.6)	174 (89.7)	128 (88.9)	118 (92.2)	100 (89.3)	25 (89.3)
T	22 (10.4)	20 (10.3)	16 (11.1)	10 (7.8)	12 (10.7)	3 (10.7)
Total	212 (100)	194 (100)	144 (100)	128 (100)	112 (100)	28 (100)

Table 2. Genotype and allele frequencies for Tau polymorphism in a sample of Colombian patients with AD stratified by age of onset and APOE4 carrier status

	Total AD	Total controls	LOAD	LOAD controls	APOE4+ AD	APOE4+ controls
Genotype						
A0/A0	76 (71.7)	65 (67.7)	50 (69.4)	44 (69.8)	39 (69.6)	10 (71.4)
A0/A1	0 (0)	4 (4.2)	0 (0)	4 (6.3)	0 (0)	1 (7.1)
A0/A2	1 (0.9)	0 (0)	1 (1.4)	0 (0.0)	1 (1.8)	0 (0)
A0/A3	28 (26.4)	26 (27.1)	20 (27.8)	14 (22.2)	16 (28.6)	3 (21.4)
A3/A3	1 (0.9)	1 (1.0)	1 (1.4)	1 (1.6)	0 (0)	0 (0)
Total	106 (100)	96 (100)	72 (100)	63 (100)	56 (100)	14 (100)
Allele						
A0	181 (85.4)	160 (83.3)	121 (84.0)	106 (84.1)	95 (84.8)	24 (85.7)
A1	0 (0)	4 (2.1)	0 (0)	4 (3.2)	0 (0)	1 (3.6)
A2	1 (0.5)	0 (0)	1 (0.7)	0 (0)	1 (0.9)	0 (0)
A3	30 (14.2)	28 (14.6)	22 (15.3)	16 (12.7)	16 (14.3)	3 (10.7)
Total	212 (100)	192 (100)	144 (100)	126 (100)	112 (100)	28 (100)

complete sample and stratified by age of onset and APOE4 carrier status are shown in Tables 1, 2 and 3. Genotype frequencies for the three polymorphisms were in Hardy-Weinberg equilibrium ($P > 0.05$).

For the LRP1 polymorphism there was no significant association for the CC genotype (CC vs CT + TT) or C allele (C vs T) in the total sample ($P = 0.48$ and $P = 0.55$), in LOAD group ($P = 0.15$ and $P = 0.23$), or in APOE4 carrier patients ($P = 0.65$ and $P = 0.65$). For the Tau polymorphism there was no significant association for the A0/A0 genotype (A0/A0 vs A0/A0 + A0/A1 + A0/A2 + A0/A3 + A3/A3) or A0 allele (A0 vs A0 + A1 + A2 + A3) in the total sample ($P = 0.32$ and $P = 0.33$), in LOAD group ($P = 0.55$ and $P = 0.55$), or in APOE4 carrier patients ($P = 0.58$ and $P = 0.58$). For the 5-HTT polymorphism there was no significant association for the ss genotype (ss vs sl + ll) or s allele (s vs l) in the total sample

Table 3. Genotype and allele frequencies for 5-HTT polymorphism in a sample of Colombian patients with AD stratified by age of onset and APOE4 carrier status

	Total AD	Total controls	LOAD	LOAD controls	APOE4+ AD	APOE4+ controls
Genotype						
ss	51 (49.0)	45 (53.6)	32 (44.4)	28 (54.9)	30 (54.5)	9 (69.2)
sl	32 (30.8)	25 (29.8)	24 (33.3)	14 (27.5)	16 (29.1)	2 (15.4)
ll	21 (20.2)	14 (16.7)	16 (22.2)	9 (17.6)	9 (16.4)	2 (15.4)
Total	104 (100)	84 (100)	72 (100)	51 (100)	55 (100)	13 (100)
Allele						
s	134 (64.4)	115 (68.5)	88 (61.1)	70 (68.6)	76 (69.1)	20 (76.9)
l	74 (35.6)	53 (31.5)	56 (38.9)	32 (31.4)	34 (30.9)	6 (23.1)
Total	208 (100)	168 (100)	144 (100)	102 (100)	110 (100)	26 (100)

($P = 0.31$ and $P = 0.23$), in EOAD group ($P = 0.34$ and $P = 0.34$), in LOAD group ($P = 0.16$ and $P = 0.14$), or in APOE4 carrier patients ($P = 0.26$ and $P = 0.29$). There was no association for LRP1, Tau and 5-HTT polymorphisms in male, female or APOE4− patients and in patients with or without family history of AD (data not shown).

Logistic regression analysis was performed evaluating age and sex as covariates. There was no association or interaction with any of the three polymorphisms analyzed. Genotype association analysis based in a neural network simulation, carried out as described previously (North et al., 2003), did not show association with LRP1, Tau and 5-HTT polymorphisms ($P = 0.39$, 0.61 and 0.61, respectively).

Analysis of statistical power showed that with the sample sizes and gene frequencies for the control subjects reported here, the power estimates are 70% (alpha value of 0.05) to detect associations with odds ratios (OR) of 3.5, 2.8 and 2.0 for LRP1, Tau and 5-HTT polymorphisms, respectively.

Discussion

Recent studies have shown that the prevalence of dementia in Colombia is near 1.3% among individuals older than 50 years (Pradilla et al., 2003) and that Colombia has a higher burden of mental disorders than other countries (Demyttenaere et al., 2004). This is probably due to a combination of specific genetic and environmental susceptibility factors (Uhl and Grow, 2004). The population living in Bogota (the Capital city) is composed of an European genetic background with some historical admixture with Amerindians (Yunis et al., 2000a, b).

Although the usefulness of genetic association studies have been challenged, they remain as a very interesting analytical tool for understanding complex brain disorders (Lohmueller et al., 2003; Uhl and Grow, 2004). In addition to genetic association studies in AD performed by us previously (markers in the coding and promoter regions of APOE, ACE and A2M genes) (Arboleda et al., 2001; Parra-Bonilla et al., 2003; Camelo et al., 2004), in the present report we analyzed the possibility that common polymorphisms in LRP1, Tau and 5-HTT genes could contribute to the development of AD in our sample. Although we considered the fact that previous positive associations were found in specific subsamples of AD, we did not find association of AD with any of the three genetic polymorphisms analyzed using different statistical analysis and sample stratifications.

This is the first report analyzing the association of LRP1 with AD in a Latin American population. There are 22 previous studies examining the C766T marker and AD in Caucasian, African-American and Asian populations (Kang et al., 1997; Baum et al., 1998; Hollenbach et al., 1998; Kamboh et al., 1998; Lambert et al., 1998; Woodward et al., 1998; Beffert et al., 1999; Bullido et al., 2000b; Hatanaka et al., 2000; Hu et al., 2000; Bi et al., 2001; Emahazion et al., 2001; McIlroy et al., 2001; Prince et al., 2001; Sanchez-Guerra et al., 2001; Perry et al., 2001; Verpillat et al., 2001; Causevic et al., 2003; Kolsch et al., 2003; Luedecking-Zimmer et al., 2003; Panza et al., 2004; Pritchard et al., 2005). In nine of these studies an association was described in the overall sample or in specific AD subgroups (male patients, APOE4 carriers, among others). Our frequencies are in an intermediate point between European and Asian populations and the present population seems to have one of the smallest difference between cases and controls (Comparative Tables and graphics not shown).

Our frequencies of Tau intronic polymorphism are similar to those found for Caucasian populations with a intermediate difference between cases and controls (Comparative Tables and graphics not shown), although not statistically significant. Previous reports in 25 studies from different origins

(including a cohort of AD patients living in New York, which are in great majority from Dominican Republic) (Crawford et al., 1999; Lilius et al., 1999; Roks et al., 1999; Baker et al., 2000; Bullido et al., 2000a; Kwon et al., 2000; Russ et al., 2001; Conrad et al., 2002, 2004; Cook et al., 2002; Green et al., 2002; Verpillat et al., 2002; Clark et al., 2003; Combarros et al., 2003; Oliveira et al., 2003; Streffer et al., 2003; Seripa et al., 2004; Tanahashi et al., 2004) have analyzed tau polymorphisms as part of the H1 and H2 haplotypes (Schraen-Maschke et al., 2004). In seven of these studies an association was found with some Tau markers mainly in APOE4 positive cases.

The 5-HTT polymorphism has been associated previously with AD (in seven reports) and with the presence of specific neuropsychiatric symptoms in AD (Li et al., 1997; Oliveira et al., 1998, 1999; Hu et al., 2000; Kunugi et al., 2000; Zill et al., 2000; Sweet et al., 2001; Tsai et al., 2001; Rocchi et al., 2003; Assal et al., 2004). The conceptual basis for the analysis of the 5-HTT polymorphism in AD was the correlation between the key role of serotonin transporter genetic variations in the control of mood states, the presence of depression and the risk for AD (Li et al., 1997; Mossner et al., 2000; Tsai et al., 2001). Only two initial reports (UK and Brazil) and one from Germany analyzing the 5-HTT promoter polymorphism and AD (Li et al., 1997; Oliveira et al., 1998, 1999; Hu et al., 2000) have shown a positive association in the overall sample. The 5-HTT frequencies in the present report are intermediate between those found for European and Asian populations (Comparative Tables and graphics not shown).

It is unlikely that our findings are false negative results. Statistical power estimates were 70% (alpha value of 0.05) to detect associations with ORs from 2.0 to 3.5 (considering the sample sizes and gene frequencies for the control subjects reported here); effect sizes which are in a range similar to the reported previously for these three polymorphisms (Li et al., 1997; Russ et al., 2001; Pritchard et al., 2005). In addition, the gene frequencies in cases and controls were very similar (several P values in the 0.4–0.6 range), making unlikely the possibility that with a greater sample size positive associations for the three polymorphisms may be found in this population. Previous reports analyzing the same polymorphisms studied by us have shown contradictory results. It is possible that, in addition to real differences in AD genetic risk between populations, methodological differences between association studies may account for the variability in the previous results for LRP1, Tau and 5-HTT and AD (Botstein and Risch, 2003; Lohmueller et al., 2003). For example, there are variations in the clinical characteristics of the samples, such as age of onset, percentage of familiar cases and coexistence of other neuropsychiatric alterations; there are also variations in statistical analyses and laboratory procedures.

Due to the genetic heterogeneity of some populations, including those from Latin America, it is very difficult to extrapolate from one country to another, or even within a specific country, specific genetic risks associated with a disease process (Tishkoff and Kidd, 2004). For example, the degree of linkage disequilibrium in specific genomic regions between possible ''causal'' genetic changes and a particular marker may vary between neighbor populations (The International HapMap Consortium, 2003). In agreement with other populations, in our AD sample from Colombia, the only consistently identified genetic risk factors for AD is the APOE gene (Arboleda et al., 2001; Parra-Bonilla et al., 2003).

The study of multiple markers using high-throughput technologies and its correlation with specific disease subgroups or QTLs would be a key approach for understanding the genetic contribution to the main neuropsychiatric diseases in diverse populations (Botstein and Risch, 2003).

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