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Association Study of the Serotoninergic System in Migraine in the Spanish Population

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In order to evaluate the contribution of 19 serotonin-related genes to the susceptibility to migraine in a Spanish population we performed a case–control association study of 122 single nucleotide polymorphisms (SNPs), selected according to genetic coverage parameters, in 528 migraine patients —308 with migraine without aura (MO) and 220 with migraine with aura (MA)— and 528 sex-matched migraine-free controls. The single-marker analysis identified nominal associations with the migraine phenotype or with the MO or MA subtypes. The multiple-marker analysis revealed risk haplotypes in three genes that remained significantly associated with migraine after correction by permutations. Two-marker risk haplotypes were identified in the *HTR2B* (rs16827801T-rs10194776G) and *MAOA* (rs3027400G-rs2072743C) genes conferring susceptibility to MO, and a four-marker haplotype in *DDC* was specific of MA (rs2329340A-rs11974297C-rs2044859T-rs11761683G). The present study supports the involvement of *HTR2B* and *MAOA* genes in the genetic predisposition to MO, while *DDC* might confer susceptibility to MA. These results suggest a differential involvement of serotonin-related genes in the genetic background of MO and MA. © 2009 Wiley-Liss, Inc.

Key words: serotonin; SNP; *HTR2B*; *DDC*; *MAOA*

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INTRODUCTION

Migraine is a highly prevalent neurological disorder characterized by recurrent episodes of headache and autonomic nervous system dysfunction (migraine without aura, MO), which are accompanied in some patients by transient neurological symptoms constitutive of migraine aura (migraine with aura, MA) [Headache Classification Subcommittee of the IHS, 2004]. Migraine is considered a polygenic multifactorial disease with several genes participating in its pathogenesis through interaction with environmental factors [Montagna, 2000; Wessman et al., 2007].

Several evidences suggest a role of the serotoninergic system in migraine pathophysiology. First, pharmacological agents interacting with serotonin (5-HT) and/or its receptors are highly

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effective in acute and prophylactic migraine treatment [The Subcutaneous Sumatriptan International Study Group, 1991; Hargreaves, 2007]. In addition, drugs known to facilitate 5-HT release are able to induce migraine attacks [Panconesi and Sicuteri, 1997] and low 5-HT blood levels predispose to cortical spreading depression (CSD) in rats [Supornsilpchai et al., 2006], the suggested upstream event of a migraine attack, particularly in MA [Moskowitz, 2007]. Although there is controversy, most studies point toward altered 5-HT levels in migraine patients both during attacks and interictally [Ferrari and Saxena, 1993; Hamel, 2007] and suggest that reduced 5-HT availability could be a fundamental event in migraine pathophysiology.

Studies in different populations have focused on the serotoninergic pathway while seeking for genetic factors contributing to migraine susceptibility. The analysis of a few polymorphisms within genes encoding the 5-HT transporter (*SCL6A4*), several 5-HT receptors (*HTR1A*, *HTR1B*, *HTR1D*, *HTR2A*, *HTR2C*), and the enzymes involved in 5-HT synthesis or degradation tryptophan hydroxylase (*TPH*), monoamine oxidase A and B (*MAOA*, *MAOB*), and L-dopa decarboxylase (*DDC*), have provided negative or conflicting results in different populations [Hamel, 2007].

The aim of the present study was to evaluate the participation of 19 genes involved in serotoninergic function in the susceptibility to migraine through a case–control association study.

MATERIALS AND METHODS

Subjects

The clinical sample consists of 528 Caucasian patients with migraine recruited in three centers of Spain (Hospital Universitari Vall d'Hebron, Barcelona, Catalonia; Hospital Sant Joan de Déu, Manresa, Catalonia; and Fundación Pública Galega de Medicina Xenómica, Santiago de Compostela, Galicia) between 2002 and 2007. All subjects fulfilled the International Criteria for Headache Disorder, 2nd edition (ICHD-II [Headache Classification Subcommittee of the IHS, 2004]) for migraine and were diagnosed by specialized neurologists with MO (n = 308, 58.3%) or MA (n = 220, 41.7%). Patients who received both diagnoses were classified into the MA group. Individuals suffering from migraine with hemiplegic aura, a severe MA variant usually showing monogenic inheritance, were excluded. Seventy-eight percent of patients were females. Diagnosis was blind to genotype. The control sample consists of 528 Caucasian Spanish unrelated adult subjects (blood donors, individuals that underwent surgery unrelated to migraine, or unaffected partners of migraine patients), matched for sex with patients recruited in the same areas (Hospital Universitari Vall d'Hebron, Barcelona, Catalonia; Fundación Pública Galega de Medicina Xenómica, Santiago de Compostela, Galicia), and in whom migraine and positive family history of migraine in the first degree relatives was excluded by personal interview of the subject. The average age at assessment was 37.2 years (SD = 16.3; range 5–86) for patients and 55.4 years (SD = 17.6; range 20–96) for controls. This study was approved by the local Ethics Committees and informed consent was obtained from all adult subjects, children, and their parents according to the Helsinki declaration.

DNA Isolation and Quantification

Genomic DNA was isolated from peripheral blood lymphocytes by a salting-out procedure [Miller et al., 1988], the QIAGEN maxikit or the QUIAamp DNA Minim Kit (QIAGEN, Hilden; Germany) or with the Chemagic Magnetic Separation Module I, and Chemagic DNA kit (Chamgen AG, Baesweiler, Germany). Otherwise, DNA was obtained from saliva using the Oragene extraction kit (DNA Genotek, Ottawa, Canada). The DNA concentrations of all samples were determined on a NanoDrop spectrophotometer (NanoDrop Technologies, LLC, Wilmington, DE).

SNP Selection, SNPlex Design, Genotyping, and Quality Control

Nineteen candidate genes involved in the serotoninergic neurotransmission pathway that encode the 14 serotonin receptors (*HTR1A*, *HTR1B*, *HTR1D*, *HTR1E*, *HTR1F*, *HTR2A*, *HTR2B*, *HTR2C*, *HTR3A*, *HTR3B*, *HTR4*, *HTR5A*, *HTR6*, and *HTR7*), the serotonin transporter (*SLC6A4*), and four enzymes involved in serotonin synthesis (*TPH1* and *DDC*) or degradation (*MAOA* and *MAOB*) were selected.

The SNPs selection and SNPlex design have been described previously [Ribasés et al., 2009]. Briefly, SNPs covering each candidate gene plus 3–5 kb flanking sequences were picked from the CEU panel of HapMap database (www.hapmap.org). TagSNPs included were at an r^2 threshold <0.85 from all SNPs with minor allele frequency (MAF) >0.15 or 0.25 (for those genes with more than 15 tagSNPs) according to HapMap. A total of 122 SNPs were included in the SNPlex assay. After genotyping, minimal allele frequencies were determined in our control population, and although three SNPs displayed MAF values between 0.12 and 014, they were not excluded from the analysis (Supplementary Table I).

To detect population stratification, 48 unlinked anonymous SNPs located at least 100 kb distant from known genes were also genotyped [Sánchez et al., 2006].

Genotyping was performed using the SNPlex platform (Applied Biosystems, Foster City, CA) in the Barcelona node of the National Genotyping Center (CeGen) as described [Tobler et al., 2005]. Two CEPH samples were included in all genotyping assays and a 100% concordance with HapMap data was obtained.

Statistical Analyses

Considering that serotoninergic system may confer susceptibility to MO and MA phenotypes by different mechanisms and to reduce heterogeneity, these clinical samples were first analyzed separately. Only when a gene was found to be associated with both MO and MA, the two data sets were studied together.

The analysis of minimal statistical power for the χ^2 test was performed post hoc using the Genetic Power Calculator software [Purcell et al., 2003], assuming odds ratio (OR) values of 1.3, 1.5, and 1.7, a prevalence of 0.14 [Jensen and Stovner, 2008], a significance level (α) of 0.05 and the mean MAF value as calculated in our control population (0.317), and showed statistical powers of 46%, 81%, or 95% in the MO group, 39%, 72%, or 90% in MA, and 58%, 91%, or 98% when all patients were considered.

Potential genetic stratification was tested by analyzing 45 SNPs (three failed) in Hardy–Weinberg equilibrium (HWE) from the anonymous 48-SNPs set using three different approaches: the STRUCTURE software, Fst coefficient, and the method by Pritchard and Rosenberg, as described [Ribasés et al., 2009].

The evaluation of linkage disequilibrium (LD) patterns from the genotype data of controls was performed using Haploview 3.32 [Barrett et al., 2005] to detect redundancies ($r^2 > 0.85$).

Single-Marker Analysis

The analysis of HWE (threshold set at $P < 0.01$) and the comparison of both genotype and allele frequencies between cases and controls were performed with the SNPassoc R library [González et al., 2007]. Dominant (11 vs. 12 + 22) and recessive (11 + 12 vs. 22) models were also analyzed when an initial association was identified under a codominant genotype model or in the alleles comparison. All tests were adjusted for geographical area (Catalonia or Galicia) and sex. For the X-linked genes we considered only females in the comparison of genotype frequencies and both males and females in the allele comparisons. After the multiple comparison correction of Bonferroni, considering two clinical groups (MO and MA) and the analysis of genotypes (under a codominant model) and alleles at the 101 SNPs that fulfilled inclusion criteria for MAF (>0.10), LD ($r^2 < 0.85$ with any of the other SNPs), HWE ($P > 0.01$) and call rate (>90%), the nominal significant threshold set at $P < 0.05$ was lowered to $P < 1.24E{-}04$ ($=0.05/(2 \times 2 \times 101)$).

Multiple-Marker Analysis

The haplotype-based association study was restricted to those genes showing nominal association in the single-marker analyses. The best haplotype, up to four markers, from all possible combinations was identified in the relevant group as previously described with the UNPHASED software [Dudbridge, 2003]. Significance was estimated by a 10,000 permutations procedure.

The specific individual haplotype estimation was performed using the PHASE 2.1 software [Stephens et al., 2001] considering cases and control subjects separately. The frequency of the risk haplotype carriers was compared between cases and controls adjusting by sex and geographical origin using the SNPassoc software. For those haplotypes where a significant association was observed, frequencies were also compared between the two clinical subgroups of migraine (MO vs. MA).

Potential additive or epistatic effects between the risk haplotypes identified within a group were implemented using a stepwise logistic regression procedure with the SPSS 12.0 software, as previously described [Ribasés et al., 2009].

RESULTS

To investigate the possible involvement of the serotonin neurotransmission system in migraine susceptibility, we analyzed tagSNPs in 19-candidate genes encoding proteins involved in synthesis, degradation, signaling, or transport of serotonin in 528 migraine cases (308 MO and 220 MA) and 528 controls.

From the 122 SNPs included in the SNPlex assay, 21 were discarded for several reasons (see Supplementary Table I).

Analysis of Single Markers

Population admixture was excluded in our sample using the STRUCTURE software (Supplementary Table II), the Fst coefficient ($\theta = 0$ with a 99% confidence interval (CI) of 0.000–0.001), and the Pritchard and Rosenberg method ($P = 0.268$).

As there is controversy as to whether MO and MA share etiological factors [Goadsby et al., 2002; Wessman et al., 2007] and in order to detect possible specific susceptibility genes, the MO and MA groups were first considered separately. The MO group displayed nominally significant differences in 9 SNPs within 7 genes (*HTR1E*, *HTR2A*, *HTR2B*, *HTR2C*, *HTR3A*, *HTR4*, and *MAOA*), while in the MA group our analysis revealed differential frequency distributions for 11 SNPs within 6 genes (*HTR1E*, *HTR2A*, *HTR2C*, *HTR3A*, *HTR7*, and *DDC*). To gain statistical power, the analysis of the *HTR1E*, *HTR2A*, and *HTR2C* genes, showing nominal association with both MO and MA, was also performed considering both clinical subtypes together and, as expected, differences between the migraine and control groups remained statistically significant. These data are summarized in Table I. However, after applying the correction of Bonferroni for multiple comparisons setting the significant threshold at 1.24E−04 none of the obtained *P*-values remained significant.

Analysis of Multiple Markers

Only the genes showing single-marker associations were considered for the multiple-marker analysis in the relevant migraine group. The haplotype-based association study of *HTR3B* and *HTR4* in the MO group, *HTR7* and *HTR3A* in the MA sample, and *HTR1E*, *HTR2C*, and *HTR2A* in the MO–MA group showed no differential distributions between cases and controls. In contrast, risk haplotypes were identified in *HTR2B*, *MAOA*, and *DDC*, which remained significant after applying a multiple comparison correction by permutation (see adjusted *P*-values in Table II).

The analysis of all possible SNP combinations within the *HTR2B* gene revealed a two-marker haplotype (rs16827801-rs10194776) associated with MO (best adjusted *P*-value = 0.0017; Table II), with an over-representation of the T-G allelic combination in MO patients (OR = 1.43, 95% CI = 1.16–1.76; Table III). In this regard, T-G haplotype carriers showed a 1.92-fold risk of suffering MO when compared to non-carriers (95% CI = 1.26–2.91; $P = 0.0016$; data not shown).

We also identified a positive association between MO and a two-marker haplotype in the *MAOA* gene (rs3027400-rs2072743; best adjusted *P*-value = 0.006; Table II) due to an over-representation of the G-C haplotype in cases (OR = 1.41, 95% CI = 1.10–1.80) and the T-T allele combination in controls (OR = 1.49, 95% CI = 1.14–1.95; Table III). As a result, G-C haplotype carriers displayed an OR of 1.78 (95% CI = 1.09–2.89; $P = 0.0177$).

And, finally, the haplotype-based study of *DDC* revealed an association between MA and a four-marker haplotype (rs2329340-rs11974297-rs2044859-rs11761683; best adjusted *P*-value = 0.0019; Table II). The allelic combination A-C-T-G was significantly

TABLE I. Nominally Significant Results of the Association Study of 101 SNPs from 19 Serotonine-Related Genes in 528 Migraine Patients (308 Migraine without Aura, 220 Migraine with Aura) and 528 Controls

		Cases				Controls					Genotype 11 vs. 12 + 22		Genotype 11 + 12 vs. 22		Allele 2 vs. allele 1	
Gene	SNP	11	12	22	Sum[a]	11	12	22	Sum[a]	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Migraine without aura																
HTR1E	rs828358	165 (55.0)	121 (40.3)	14 (4.7)	300	327 (63.4)	160 (31.0)	29 (5.6)	516	0.012	1.54 (1.14–2.08)	0.0049	1.06[b] (0.54–2.08)	0.86	1.33[b] (1.04–1.69)	0.024
HTR2A	rs7984966	169 (55.6)	116 (38.2)	19 (6.2)	304	251 (48.3)	221 (42.5)	48 (9.2)	520	0.037	1.42[b] (1.06–1.92)	0.016	1,56[b] (0.88–2.78)	0.12	1.35 (1.07–1.71)	0.011
	rs7322347	81 (26.9)	161 (53.5)	59 (19.6)	301	120 (23.5)	260 (50.9)	131 (25.6)	511	0.070	1.27[b] (0.91–1.76)	0.16	1.49[b] (1.04–2.13)	0.029	1.26 (1.02–1.55)	0.032
HTR2B	rs10194776	121 (39.6)	149 (48.9)	35 (11.5)	305	171 (32.6)	241 (46.0)	112 (21.4)	524	0.0048	1.35[b] (0.99–1.82)	0.055	1.92[b] (1.25–2.94)	0.0017	1.39[b] (1.11–1.69)	0.0027
HTR2C	rs4911871	149 (64.8)	76 (33.0)	5 (2.2)	230	273 (67.4)	113 (27.9)	19 (4.7)	405	0.036	1.20 (0.84–1.72)	0.31	2.70[b] (0.95–7.69)	0.045	1.03 (0.77–1.37)	0.85
HTR3B	rs11214775	158 (53.7)	109 (37.1)	27 (9.2)	294	253 (49.5)	227 (44.4)	31 (6.1)	511	0.025	1.19[b] (0.88–1.59)	0.26	1.79 (1.03–3.12)	0.042	1.01 (0.8–1.27)	0.96
HTR4	rs7721747	107 (35.4)	135 (44.7)	60 (19.9)	302	146 (28.1)	277 (53.4)	96 (18.5)	519	0.034	1.45[b] (1.06–2.00)	0.021	1.07 (0.74–1.56)	0.71	1.15[b] (0.93–1.43)	0.19
MAOA	rs3027400	131 (62.4)	74 (35.2)	5 (2.4)	210	212 (53.7)	150 (38.0)	33 (8.3)	395	0.0093	1.41[b] (0.99–2.00)	0.057	3.45[b] (1.32–9.09)	0.0047	1.45[b] (1.10–1.89)	0.0070
	rs2072743	114 (49.8)	104 (45.4)	11 (4.8)	229	179 (44.9)	176 (44.1)	44 (11.0)	399	0.043	1.14[b] (0.81–1.61)	0.45	2.33[b] (1.16–4.76)	0.012	1.23 (0.96–1.57)	0.097
Migraine with aura																
HTR1E	rs828358	124 (57.9)	85 (39.7)	5 (2.4)	214	327 (63.4)	160 (31.0)	29 (5.6)	516	0.0064	1.37 (0.98–1.93)	0.068	2.56[b] (0.95–6.66)	0.042	1.12[b] (0.85–1.49)	0.41
	rs1581774	147 (70.3)	61 (29.2)	1 (0.5)	209	371 (72.2)	125 (24.3)	18 (3.5)	514	0.016	1.15 (0.80–1.66)	0.46	7.14[b] (0.95–50)	0.011	1.01[b] (0.72–1.39)	0.98
HTR2A	rs9534511	68 (31.5)	120 (55.6)	28 (12.9)	216	173 (33.3)	242 (46.5)	105 (20.2)	520	0.012	1.18 (0.83–1.68)	0.36	1.72[b] (1.09–2.78)	0.017	1.09[b] (0.85–1.37)	0.51
	rs6561332	56 (26.3)	129 (60.6)	28 (13.1)	213	165 (31.9)	252 (48.8)	100 (19.3)	517	0.016	1.24 (0.86–1.79)	0.25	1.66[b] (1.04–2.63)	0.028	1.05[b] (0.83–1.33)	0.68
HTR2C	rs475717	114 (66.3)	49 (28.5)	9 (5.2)	172	292 (71.9)	103 (25.4)	11 (2.7)	406	0.13	1.42 (0.95–2.11)	0.090	2.12 (0.83–5.43)	0.12	1.42 (1.03–1.95)	0.035
	rs6318	117 (70.1)	43 (25.7)	7 (4.2)	167	298 (74.3)	96 (23.9)	7 (1.8)	401	0.10	1.40 (0.92–2.13)	0.12	2.88 (0.95–8.71)	0.066	1.42[b] (1.02–2.00)	0.038
	rs2428721	102 (59.3)	59 (34.3)	11 (6.4)	172	269 (66.6)	121 (30.0)	14 (3.4)	404	0.036	1.53 (1.04–2.25)	0.032	2.40 (1.03–5.60)	0.048	1.52 (1.13–2.05)	0.0068
HTR3A	rs1176717	129 (59.4)	71 (32.7)	17 (7.9)	217	330 (63.7)	170 (32.8)	18 (3.5)	518	0.042	1.25 (0.89–1.75)	0.19	2.46 (1.21–5.01)	0.015	1.33 (1.01–1.76)	0.044
HTR7	rs1298056	164 (76.3)	51 (23.7)	0 (0)	215	376 (73.3)	122 (23.8)	15 (2.9)	513	0.0058	1.19[b] (0.81–1.75)	0.36	1.03[b] (1.01–1.04)	0.0014	1.31 (0.92–1.87)	0.12
DDC	rs1982406	90 (42.9)	105 (50.0)	15 (7.1)	210	286 (55.3)	185 (35.8)	46 (8.9)	517	0.0035	1.70 (1.22–2.38)	0.0018	1.09[b] (0.58–2.04)	0.79	1.35[b] (1.05–1.75)	0.020
	rs6944090	106 (50.7)	93 (44.5)	10 (4.8)	209	311 (59.7)	181 (34.7)	29 (5.6)	521	0.021	1.60 (1.14–2.24)	0.0062	1.07 (0.50–2.29)	0.86	1.37[b] (1.05–1.82)	0.022
All migraine patients																
HTR1E	rs828358	289 (56.2)	206 (40.1)	19 (3.7)	514	327 (63.4)	160 (31.0)	29 (5.6)	516	0.0018	1.48 (1.14–1.92)	0.0031	1.41[b] (0.76–2.56)	0.27	1.25[b] (1.01–1.56)	0.040
HTR2A	rs7984966	279 (53.6)	203 (39.0)	39 (7.4)	521	251 (48.3)	221 (42.5)	48 (9.2)	520	0.11	1.30[b] (1.01–1.67)	0.039	1.23[b] (0.79–1.96)	0.35	1.22 (1.01–1.49)	0.044
HTR2C	rs4911871	262 (65.3)	130 (32.4)	9 (2.3)	401	273 (67.4)	113 (27.9)	19 (4.7)	405	0.029	1.14 (0.84–1.55)	0.39	2.50[b] (1.08–5.88)	0.028	1.01[b] (0.78–1.28)	0.96

Comparisons of genotype frequencies were performed only in women for *MAOA* and *HTR2C* genes located on chromosome X. CI: confidence interval; OR: odds ratio; SNP: single nucleotide polymorphism.
[a]Sum differences between SNPs are due to genotyping failure.
[b]When odds ratio < 1, the inverted score is shown.

TABLE II. Haplotype Analysis of *HTR2B*, *MAOA*, and *DDC* SNPs in 308 Migraine Without Aura (MO) or 220 Migraine with Aura (MA) Patients and Controls Subjects Using the UNPHASED Software

Gene	Phenotype	Marker[a] haplotype	Global *P*	Best haplotype *P* (adjusted *P* Value)	Risk haplotype OR (95% CI)
HTR2B	MO	2 3[b]	0.000599	0.000521 (0.0017)	1.43 (1.16–1.76)
		1 2 3	0.001901	0.00306 (0.0133)	1.36 (1.11–1.67)
MAOA	MO	1 2	0.0107	0.00180 (0.0061)	1.41 (1.10–1.80)
DDC	MA	4 5	0.0192	0.00748 (0.0288)	1.70 (1.25–2.31)
		2 4 5	0.0146	0.00674 (0.034)	2.01 (1.35–2.98)
		2 4 5 10[b]	0.00977	0.00109 (0.0019)	2.31 (1.48–3.59)

CI: confidence interval; OR: odds ratio; SNP: single nucleotide polymorphism.
[a]*HTR2B*: 1-rs4973377; 2-rs16827801; 3-rs10194776; *MAOA*: 1-rs3027400; 2-rs2072743; *DDC*: 2-rs2329340; 4-rs11974297; 5-rs2044859; 10-rs11761683.
[b]Best allelic combination (higher OR).

more frequent in MA cases than in controls (OR = 2.31, 95% CI = 1.48–3.59; Table III), with a risk to develop MA for this haplotype carrier of 1.95 (95% CI = 1.22–3.11; $P = 0.0057$).

Analysis of Additive and Epistatic Effects

Since risk haplotypes in both the *HTR2B* and *MAOA* genes were identified in the same phenotype group (MO), we evaluated the potential additive effect of the rs16827801T-rs10194776G and rs3027400G-rs2072743C haplotypes in this clinical subgroup.

Assuming a simple additive model, the combined effect of the two risk haplotypes in the MO phenotypic variance in our Spanish sample was estimated to be 3.1%. We also evaluated possible interactions between these allelic combinations, but we found no evidence supporting the existence of epistatic effects between both genes in the risk to develop MO (data not shown).

DISCUSSION

This study investigates the role of 19 genes from the serotonin neurotransmission system in migraine using a genetic coverage approach.

Whether MO and MA share susceptibility factors is still a matter of debate [Goadsby et al., 2002; Wessman et al., 2007]. Therefore, we initially analyzed the MO and MA groups separately and performed analysis in the whole migraine group only in those genes that had shown evidence of association in both clinical subgroups. Risk haplotypes for MO were identified in the *HTR2B* and *MAOA* genes, and for MA in *DDC*.

As patients and controls were recruited from two different geographical areas of Spain (Catalonia and Galicia), we first ruled out population stratification, which can lead to false positive results, using a panel of 48 unlinked SNPs and three different analytical approaches. Additionally, several considerations were taken into account in order to reduce potential sources of heterogeneity or stratification: cases and controls were carefully matched by sex, all of them were Caucasian, we selected controls without personal or familiar history of recurrent or severe headaches, and inclusion criteria for patients were restricted to ICHD-II guidelines. However, some contention around the latter point exists; some authors believe that ICHD-II classification favors clinical heterogeneity and suggest the need for alternative phenotyping strategies in migraine association studies in order to stratify samples into less heterogeneous groups [Wessman et al., 2007].

TABLE III. Haplotype Distributions of *HTR2B* and *MAOA* in 308 Migraine Without Aura (MO) Patients and of *DDC* in 220 Migraine with Aura (MA) Patients, and 528 Controls

Gene	Phenotype	Marker[a] haplotype	Cases	Controls	Haplotype-specific p; OR (95% CI)
HTR2B	MO	2 3			
		T G	391 (64.3)	582 (55.7)	0.000521; 1.43 (1.16–1.76)
MAOA	MO	1 2			
		G T	26 (5.4)	47 (5.3)	—
		G C	353 (73.9)	592 (66.7)	0.00412; 1.41 (1.10–1.80)
		T T	99 (20.7)	249 (28.0)	0.00180; 1.49 (1.14–1.95)[b]
DDC	MA	2 4 5 10			
		A C C G	60 (20.7)	172 (22.2)	—
		A C T G	39 (13.4)	48 (6.3)	0.00109; 2.31 (1.48–3.59)
		G T C A	49 (16.9)	115 (14.9)	—
		G T T A	142 (49.0)	439 (56.6)	—

CI: confidence interval; OR: odds ratio.
[a]*HTR2B*: 2-rs16827801; 3-rs10194776; *MAOA*: 1-rs3027400; 2-rs2072743; *DDC*: 2-rs2329340; 4-rs11974297; 5-rs2044859; 10-rs11761683.
[b]Underrepresented in MA patients in comparison with control subjects.

Our single-marker analysis showed associations with several genes that did not withstand Bonferroni correction for multiple testing. However, adjusting for the number of independent tests may be too conservative to control for false positives, as the 101 SNPs analyzed were not always independent in terms of LD and the clinical subgroups are related. For this reason, in the multiple-marker analysis we considered all genes displaying nominal associations, to ensure that combinations that might modulate the migraine phenotype were not missed. The results provided evidence of risk-conferring haplotypes in *HTR2B*, *MAOA*, and *DDC*, which remained statistically significant after applying a correction by permutations.

A two-SNP risk haplotype, rs16827801T-rs10194776G, conferring susceptibility to MO, was identified in the *HTR2B* gene. The involvement of this receptor in migraine has been previously suggested, although no case–control association studies have been performed [Panconesi and Sicuteri, 1997]. It was proposed that 5-HT2B receptors, located on endothelial cells of meningeal blood vessels may trigger migraine headache through nitric oxide (NO)-dependent mechanism [Schmuck et al., 1996; Manivet et al., 2000]. A study described that persistent treatment with dihydroergotamine (DHE), an antimigraine drug which interacts with multiple 5-HT receptors [Silberstein, 1997], caused 5-HT2B receptor desensitization inhibiting NO production, and suggested that it may contribute to its therapeutic efficacy [Schaerlinger et al., 2003].

The SNPs of the identified risk haplotype rs3027400G-rs2072743C in MO are located between *MAOA* and *MAOB*, within the 3′ region of both genes, so they could have functional effects on either of them. Three independent association studies have analyzed the involvement of a functional VNTR polymorphism in the promoter region of *MAOA* in migraine, two of which did not find evidence of association, whereas the third identified a trend in a small subset of males with MO ($P = 0.042$) [Marziniak et al., 2004; Johnson and Griffiths, 2005; Filic et al., 2005]. However, the LD relation between this VNTR and the SNPs considered in the present study is not known and could explain the discrepancies. In another study, the exonic c.1460T>C variant, was not found to be associated with MO [Cevoli et al., 2006]. Although MAO inhibitors are not frequently used in the migraine treatment, improvement has been reported by some migraine patients after treatment with MAO inhibitors [Rapoport, 2008], which may support the participation of *MAOA* in migraine susceptibility.

Interestingly, the frequencies of carriers of the *HTR2B* or *MAOA* risk haplotypes were not different between MO and MA ($P = 0.061$ and 0.13, respectively). These results suggest that these haplotypes could also confer risk to MA, although the limited sample size may have prevented the identification of the association. Alternatively, the contribution of these genes to migraine may be specific to MO, but the fact that the MA sample is not completely MO-free, may have contributed to the lack of significant differences in haplotype frequency between the two groups.

Another finding arising from this study is the association of *DDC* with MA and the identification of a four-marker risk haplotype. A previous report, using a two-stage DNA pooling design, screened a 4-bp insertion/deletion (indel) in the 5′UTR region of *DDC* (rs3837091) and found no evidence of association with migraine [Johnson and Griffiths, 2005]. In that study, however, MO and MA patients were not analyzed separately. The indel polymorphism was not included in the present analysis, and no LD data between this sequence variant and SNPs in *DDC* is available. The distributions of risk haplotype carriers in the MA sample were significantly different not only from controls but also from MO (17.7% vs. 11.7%, $P = 0.046$), supporting that *DDC* is a MA-specific susceptibility factor. Indeed, results from previous association studies also suggest that there might be genetic factors that increase the risk for aura among migraineurs [Oterino et al., 2004].

Among previous migraine association studies looking into serotoninergic pathways, some have investigated the possible role of *SCL6A4*, the gene encoding the serotonin transporter, and reached conflicting results [Karwautz et al., 2008]. Most studies have focused on the two functional polymorphisms (5HTT-LPR and 5HTT-VNTR). Only one powered study focused on SNPs, and no evidence of association was found [Todt et al., 2005]. Our results add to those suggesting lack of association with *SCL6A4*. Among the five SNPs initially selected in our study, three were not successfully genotyped and therefore two tagged bins were not included in the final analysis. Furthermore, our set of SNPs is not comparable to the one used previously [Todt et al., 2005] with only one SNP in common (rs20209442) and the rest belonging to different LD bins. Further well-powered studies combining potentially functional polymorphisms and tagSNPs in *SLC6A4* are needed to elucidate its participation in the pathophysiology of migraine.

Splicing enhancers and silencers have been identified as splicing regulatory elements both in exons and introns [Black, 2003], and it is well known that aberrant splicing can be a deteminant in disease susceptibility [Wang and Cooper, 2007]. As the SNPs conforming the risk haplotypes identified here are all located in introns, it is plausible that they may have an effect on splicing efficiency. Alternatively, if these variants have no direct functional consequences, they could be in LD with functionally relevant polymorphisms.

Our case–control association study has some drawbacks and limitations.

First, the estimation of the statistical power of the sample varies considerably when different OR values are considered, and suggests that the study may have failed to identify variants with very subtle effects (OR 1.3, power around 50%). Second, the fact that the single-marker associations identified did not remain significant after multiple testing corrections indicates that some results could be due to type I errors, although subsequent multiple-marker analyses revealed significant risk haplotypes. And third, a significant feature of the present study is the SNP selection based on genetic coverage criteria. However, from the 133 SNPs initially selected, 11 could not be genotyped due to SNPlex design constraints and 9 additional ones that were successfully genotyped could not be included in the statistical analysis because of genotyping failure or Hardy–Weinberg disequilibrium (Supplementary Table I). These exclusions involved 10 different genes, including 2 SNPs in *DDC* and 1 in *HTR2B*. Furthermore, polymorphisms with MAF < 0.15 or not present in the HapMap database may have escaped our selection process but still confer susceptibility to migraine.

In spite of these limitations, common to most association studies, our results suggest a role of genes from the serotoninergic

pathway in the susceptibility to migraine. Thus, an even more exhaustive analysis of the *HTR2B*, *MAOA*, and *DDC* genes may identify additional functional susceptibility factors. Also, replication studies in different case–control data sets and family trios from other populations are warranted to confirm our findings.

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