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## Implication of Chromosome 18 in Hypertension by Sibling Pair and Association Analyses Putative Involvement of the *RKHD2* Gene

Blanca Guzmán, Bru Cormand, Marta Ribasés, Daniel González-Núñez, Albert Botey, Esteban Poch

**Abstract**—This study aims to test the implication of regions on chromosomes 9, 17, and 18 in essential hypertension (EH) by combining sibling-pair linkage analysis and case-control association studies. The selection of these chromosomal regions is based on previous evidence of their implication in EH or in related phenotypes by comparative genomics in several rat models and from genome-wide linkage studies in humans. For the affected sibling-pair linkage analysis, 27 microsatellite markers were genotyped in 56 pedigrees from Spain with hypertensive sibling pairs. Linkage analysis showed significant excess allele sharing at the D18S474 marker on 18q21.1, as shown by maximum likelihood of allele sharing methods (logarithm of odds=3.24;  $P=0.00011$ ) and nonparametric linkage calculations (nonparametric linkage=3.32;  $P=0.00044$ ). On the contrary, no significant results with any of the markers analyzed on chromosomes 9 and 17 were obtained. We further focused on the Ring finger and KH domain containing 2 (*RKHD2*) gene located 6 Kb distal from D18S474 and performed a case-control association study based on linkage disequilibrium in 112 hypertensive patients and 156 control subjects. We selected 2 *RKHD2*-tagged single nucleotide polymorphisms, rs1941958 and rs1893379, covering, in terms of linkage disequilibrium, the entire gene, and observed a significant overrepresentation of the rs1941958G-rs1893379T *RKHD2* haplotype in the group of hypertensive patients in comparison with controls ( $2P=0.0004$ ; odds ratio: 2.32). We also detected epistatic effects between the 2 *RKHD2* single nucleotide polymorphisms ( $2P=0.002$ ; odds ratio: 2.48). Our data confirm the implication of chromosome 18 in EH and support a contribution of *RKHD2* to the genetic susceptibility of this complex phenotype. (*Hypertension*. 2006; 48:883-891.)

**Key Words:** chromosome 18 ■ hypertension ■ linkage ■ sibling-pair analysis ■ association studies  
■ polymorphisms ■ *RKHD2* gene

Essential hypertension (EH) is a common risk factor for cardiovascular diseases, end stage renal disease, stroke, and peripheral vascular diseases. The physiological determinants of blood pressure are the product of cardiac output and systemic vascular resistances, and the regulation of these factors is inherently complex and influenced by both genetic and environmental factors.

Several research groups have focused their investigations on the genetic basis of hypertension. The first and most successful approach was based on linkage analysis and led to the discovery of genes involved in monogenic forms of hypertension, as well as arterial hypotension.<sup>1</sup> Although this strategy represented a huge advance in the knowledge of blood pressure regulation, monogenic forms only represent a tiny minority of the hypertensive population. The second approach consisted of testing the role of several candidate genes for EH, chosen on the basis of their involvement in

monogenic forms of hypertension and the molecular analysis of animal models, by both linkage and association studies. However, no relevant susceptibility genes with a major effect on hypertension have been identified yet, suggesting that those genes involved in monogenic hypertension may not be significantly involved in EH. On the other hand, association studies have shown, in general, inconsistent results, and only 2 candidate genes, angiotensinogen and  $\alpha$ -adducin, have been replicated in multiple studies.<sup>2,3</sup> Finally, genome-wide linkage analyses in hypertensive sibling pairs have shown evidence for different loci with a potential involvement in the etiology of EH. Between 1999 and 2003, >20 articles have reported genome scans in hypertensive sibling pairs.<sup>4</sup> However, although some of them have shown significant evidence for linkage, mainly on chromosomes 18q,<sup>5</sup> 17q,<sup>6</sup> 2p,<sup>7</sup> and 4p,<sup>8</sup> most of the studies have reported nominal or suggestive results in almost every chromosome,<sup>4</sup> making it difficult to

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prioritize the strongest candidate genes or regions for replication. Stoll et al<sup>9</sup> systematically evaluated hypertension-related QTLs from different animal models and translated them to the human genome by a comparative genomics mapping approach, thus defining high-priority candidate regions for human EH. This systematic approach predicted 26 chromosomal regions in the human genome potentially involved in the genetic predisposition to EH. All of these approaches help researchers to focus on candidate regions for positional cloning and fine mapping of hypertension genes.

The aim of the present study was to test the involvement of regions on chromosomes 9, 17, and 18 by linkage analysis in 105 Spanish sibling pairs from 56 nuclear families with EH and to evaluate the implication of specific genes by means of population-based association studies in 112 patients and 156 normotensive controls. The selection of chromosomes 17 and 18 was based on evidences from genome-wide studies,<sup>5,6</sup> linkage analysis, and the comparative genomics strategy,<sup>5,6,10–13</sup> whereas focus on chromosome 9 derived from comparative genomics.<sup>9</sup>

## Methods

### Study Participants

The hypertensive probands were recruited in the Nephrology Department and Hypertension Unit of the Hospital Clinic of Barcelona on the basis of the following criteria: established EH diagnosed before the age of 60 years and a family history of established hypertension in  $\geq 1$  sibling and 1 parent. The criteria for established hypertension consisted of antihypertensive treatment for  $\geq 3$  months with registered diastolic blood pressure (DBP) values  $\geq 95$  mm Hg or DBP  $\geq 95$  mm Hg in 2 consecutive visits in untreated patients. Patients with diabetes mellitus, body mass index (BMI)  $>35$  kg/m<sup>2</sup> (weight/height<sup>2</sup>), alcohol consumption  $>50$  g per day, or on oral contraceptives were excluded from the study. Blood pressure was measured in all of the subjects with a sphygmomanometer. Systolic blood pressure (SBP) was taken to be the pressure at which the first Korotkoff sound was first heard, and the DBP was taken to be the pressure at the fifth Korotkoff phase. The blood pressure considered was the mean of 3 determinations 2 minutes apart after an initial 5-minute rest. The diagnosis of EH was considered on the basis that no known causes of high blood pressure could be detected after complete clinical, biochemical, and radiological examination. None of the patients had familial history of premature cardiovascular disease or diabetes nor had renal impairment (serum creatinine  $>132$   $\mu$ mol/L), cardiac failure, or evidence of coronary heart disease, stroke, or peripheral vascular disease. All of the participants completed a questionnaire to determine ancestry and to exclude those with a family history of diabetes, renal, heart, and thyroid disease. The local ethics committee approved the study, and an informed consent was obtained from all of the patients and their relatives who participated in the sibling-pair analysis or the association study. The procedures of the study followed the principles of the Declaration of Helsinki.

For the sibling-pair analysis, the selected population provided linkage information for a total of 105 sibling-pair comparisons from 56 nuclear families, composed of 39 pairs, 12 trios, and 5 quartets of affected siblings, with the following distribution: 35 female/female, 17 male/male, and 53 female/male affected pairs. The average age at assessment of the hypertensive sibling pairs was (mean  $\pm$  SD)  $60 \pm 10.7$  years, the mean BMI was  $23 \pm 3.2$  kg/m<sup>2</sup>, and the average SBP and DBP were  $160 \pm 20$  mm Hg and  $92 \pm 13$  mm Hg, respectively.

For the association study, we selected 112 white unrelated hypertensive patients with similar clinical characteristics as the sibling pairs and 156 frequency sex-matched white normotensive subjects (60% men and 40% women). The inclusion criteria for the control individuals were SBP  $<130$  mm Hg and DBP  $<80$  mm Hg in 3

separate occasions and no personal or familiar history of hypertension or premature cardiovascular disease (diabetes mellitus, ischemic heart disease, stroke, or peripheral vascular disease). Most of the patients with EH were men (N=63; 56.25%). The average age at assessment was  $55 \pm 12$  years for EH patients and  $53 \pm 17$  years for control subjects. The average BMI was  $28 \pm 4.9$  kg/m<sup>2</sup> for case subjects and  $24 \pm 4$  kg/m<sup>2</sup> for control subjects. The mean SBP was  $168 \pm 19$  mm Hg for EH patients and  $117 \pm 11$  mm Hg for control subjects, and the average mean DBP was  $104 \pm 9$  mm Hg for patients and  $69 \pm 7$  mm Hg for normotensive control subjects.

### Selection of Candidate Regions and Genetic Markers

Candidate chromosomal regions for human hypertension were selected based on genome-wide scans, linkage studies, and the comparative genomics strategy reported by Stoll et al.<sup>9</sup> We explored 2 candidate regions on chromosomes 9 and 18 on the basis of their synteny with QTLs identified in experimental animal models of salt-sensitive hypertension.<sup>9</sup> The 18q21 to 18q23 region contains the ubiquitin protein ligase *NEDD4-like* gene (*NEDD4L*), with a putative role in renal Na<sup>+</sup> regulation, and the 9q34.3 to q32 region contains also several genes with a potential involvement in EH, including *prostaglandin-endoperoxide synthase 1* (*PTSG1*), *tenascin C* (*TNC*), and the *ATP-binding cassette subfamily A, member 1* (*ABCA1*). In addition, candidate regions on chromosome 17 were explored according to previous studies that reported suggestive linkage in both human and animal forms of hypertension<sup>6,13–16</sup> and the presence of several candidate genes on the 17p11.2 to 17q21.3 genomic region, such as the *adenosine A2B receptor* (*ADORA2B*) and the *nitric oxide synthase 2* (*NOS2*), among others.<sup>9</sup>

### Genotyping

Genomic DNA was extracted from peripheral blood lymphocytes by standard procedures. Markers D18S58, D18S38, D18S483, D18S484, D18S1009, D18S1144, D18S1091, D18S1092, D18S1103, and D18S1145 were taken from Stoll et al.,<sup>9</sup> whereas markers D17S949, D18S450, and D18S59 were selected as suggested by Rutherford et al.<sup>13</sup> The rest of the markers (D9S159, D9S279, D9S1881, D9S1798, D9S298, D9S1821, D17S1799, D17S1795, D18S1127, D18S64, D18S70, D18S465, D18S474, and D18S1161) were chosen to span suggestive regions by using the Human Genome Sequence of the University of California Santa Cruz (UCSC) Genome Bioinformatics web site (genome.ucsc.edu). Genotyping was based on PCR amplification of genomic DNA and capillary electrophoresis. Each reaction contained 1 to 10 ng of DNA, 0.9  $\times$  Ecogen buffer, 0.35  $\mu$ mol/L of each primer, 0.1 U of AmpliTaq EcoTaq (Ecogen), 0.25 mmol/L of each dNTP, and 2.5 mmol/L of MgCl<sub>2</sub> in a total volume of 10  $\mu$ L. The forward primer was fluorescently labeled at the 5' end, with 6-carboxy-fluoresceine, tetrachloro-6-carboxy-fluoresceine, or hexachloro-6-carboxy-fluoresceine (Applied Biosystems). The cycling reaction was performed on a thermal cycler (MJ Research Inc). PCR conditions consisted of an initial denaturation step at 96°C for 5 minutes followed by 35 cycles of denaturation at 96°C for 30 sec, annealing at 58°C (D9S298, D9S1881, D9S1821, D9S159, D18S38, D18S64, D18S1009, D18S58, D18S484, D18S450, D18S59, and D18S1161), at 60°C (D9S279, D9S1798, D18S1092, D18S1091, D18S1103, D18S70, D18S1127, D18S465, D18S474, D18S1144, D18S483, and D18S1145), or at 62°C (D17S949, D17S1799, and D17S1795), and extension at 72°C for 30 sec. The amplification products were tested by electrophoresis on a 3% agarose gel and ethidium bromide staining and visualized under a UV (245 nm) transilluminator. The fragments were analyzed with the semiautomated ABI Prism 310 Genetic Analyser and GENOTYPER software (Applied Biosystems).

The genotyping of the diallelic single nucleotide polymorphisms (SNPs) rs1941958 and rs1893379 was performed with TaqMan PCR (Applied Biosystems). The amplification reactions were performed in a total volume of 50  $\mu$ L. Thermal cycling was performed using a 2-step PCR protocol: 50°C for 2 minutes, 95°C for 10 minutes, and

40 cycles of 95° for 15 s and 60°C for 1 minute. The PCR reaction mix contained 2.5 μL of TaqMan universal PCR master mix, 60 ng of template DNA, and 0.8 μmol/L of each primer in a total volume of 50 μL. The genotyping primers were designed using the Applied Biosystems support. The intensities of the fluorescent dyes in each reaction were read automatically during PCR cycling in an ABI PRISM 7900 HT sequence detector (Applied Biosystems). The real-time amplification data were analyzed using an ABI PRISM 7900 SDS software. The fluorescent signal was normalized by dividing the emission intensity of the reporter dye (FAM) by the emission intensity of a reference dye (6-carboxy-X-rhodamine) present in the TaqMan Universal PCR Master Mix to obtain a ratio defined as “R<sub>n</sub>” (normalized reporter) for a given reaction tube.

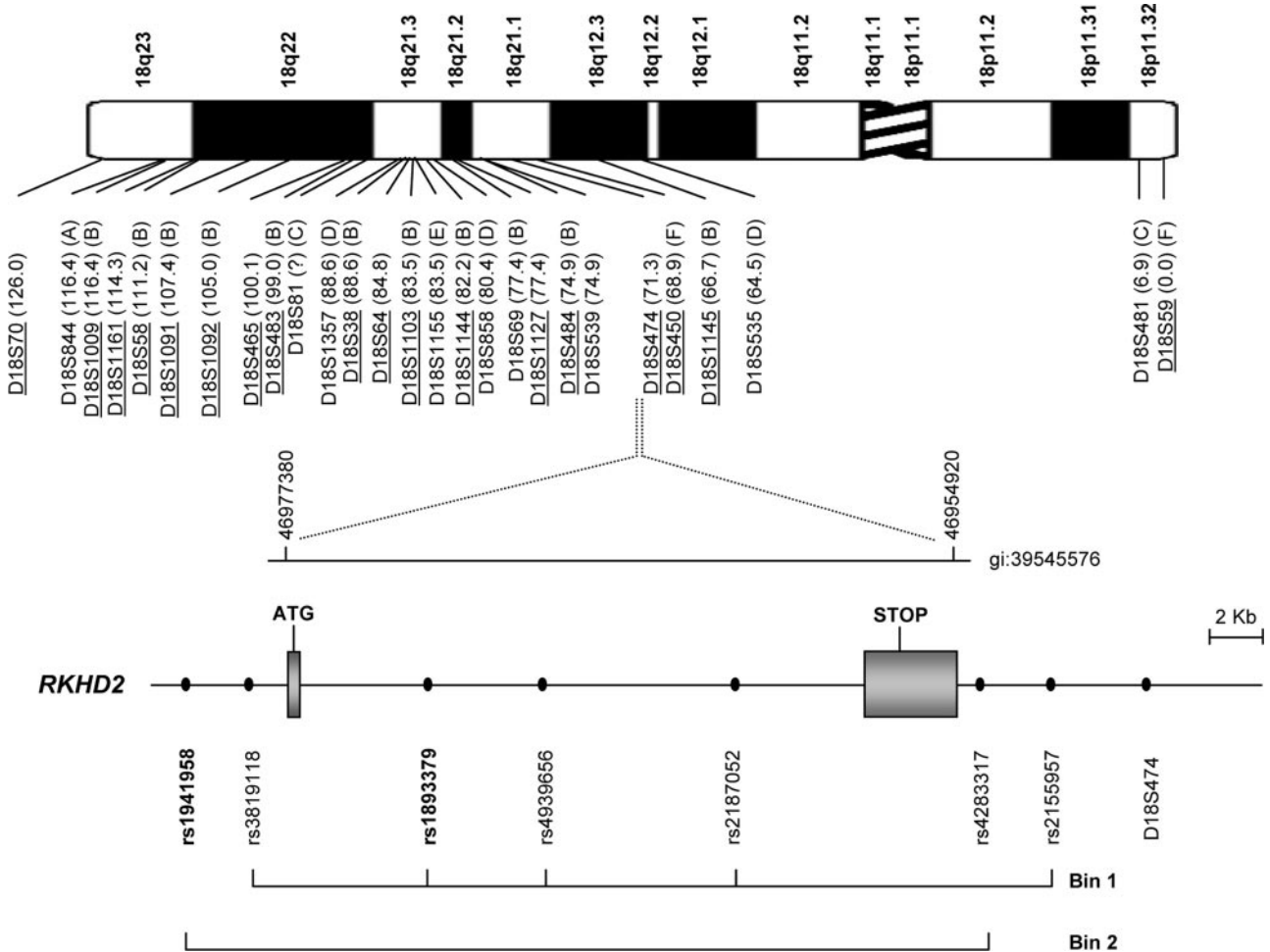
**Statistical Analysis**

**Sibling-Pair Analysis**

Sibling-pair analysis of 56 pedigrees was performed with the GENEHUNTER 2.1 linkage software.<sup>17</sup> The program provides nonparametric linkage analysis by only using data on affected sibling pairs, thereby avoiding problems of incomplete penetrance or variable age of onset. Regions of significant excess allele sharing were searched across 3 candidate regions on chromosomes 9, 17, and 18 by estimating the maximum likelihood values (MLS) of the proportion of siblings sharing 0, 1, and 2 alleles identical by descent and

computing a maximum logarithm of odds (LOD) score by comparing the likelihood of the observed data under the estimated allele-sharing proportions to the likelihood under mendelian segregation. These MLS values were estimated under the assumptions of no dominance and dominance variance using the “estimate” command. The “pairs used” command was activated to accommodate sibships with >2 affected siblings by weighting the inflation of significance because of statistical dependence among all of the possible pairs. The siblings were considered as part of a whole family for the determination of inheritance vectors, and then each pair was treated as a separate pedigree for the purposes of analysis. The 3 chromosomal regions were studied by both single-point and multipoint analyses. In addition, single-point and multipoint nonparametric LOD scores (NPLs) and the corresponding *P* values were calculated using GENEHUNTER 2.1 on the 56 pedigrees subjected previously to sibling-pair analysis.<sup>17</sup>

The markers allele frequencies were calculated from the genotypes of all of the unrelated subjects included in the analysis. Marker order and genetic distances were obtained from the genetic map of the Marshfield Medical Research Foundation (research.marshfieldclinic.org/genetics) and were the following: 17p-D17S799-36.5 cM-D17S1795-24.8 cM-D17S949-17q, 9p-D9S279-0 cM-D9S298-15.8 cM-D9S1881-0.62 cM-D9S1798-0.88 cM-D9S1821-5.2 cM-D9S159-9q. The 18 markers analyzed on chromosome 18 are shown in Figure 1.



**Figure 1.** Ideogram of chromosome 18 with the location of microsatellite markers used in the present linkage study (underlined) together with others used by other authors in several hypertension genomic scans: (A) Reference,<sup>22</sup> (B) Reference,<sup>9</sup> (C) Reference,<sup>6</sup> (D) Reference,<sup>12</sup> (E) Reference,<sup>5</sup> and (F) Reference.<sup>13</sup> The sex-averaged genetic distances from the short arm telomere are indicated in centimorgans as defined in the Marshfield Genetic Map (research.marshfieldclinic.org/genetics). Below, diagram of the human *RKHD2* gene and relative position of different sequence variants. The exons are indicated as ■. The *RKHD2* SNPs were organized in 2 haplotype bins according to the LD Select software. Those in bold represent tagged SNPs considered in the linkage and association studies.

### Case-Control Analysis

The power analysis was performed post hoc with the Power Calculator software ([calculators.stat.ucla.edu/powercalc](http://calculators.stat.ucla.edu/powercalc)) assuming a significance level of 0.05, a lifetime risk of 2, and the frequency of the alleles associated with hypertension as the frequency of the risk factor (0.455 and 0.202 for the rs1941958 and rs1893379 SNPs, respectively). Hardy-Weinberg equilibrium was tested for each SNP by  $\chi^2$  tests using the Hardy-Weinberg equilibrium software.<sup>18</sup> To minimize the number of markers selected for genotyping and to ensure full genetic coverage of the *Ring finger and KH Domain containing 2 (RKHD2)* gene, we used the LD select software to evaluate linkage disequilibrium (LD) patterns, define haplotype bins, and identify tagged SNPs from the HapMap project data (release 16c, June 2005).<sup>19</sup> Haplotype estimations from the population genotype data were assessed by the PHASE 2.0 software.<sup>20</sup> Because no direction in the association for the different SNPs was expected a priori, the comparison of genotype, allele, and haplotype frequencies was performed by 2-tailed Fisher's exact test using the statistical package SPSS 12.0 (SPSS). Gender was evaluated as a covariate by a likelihood ratio (LR) test comparing 2 logistic regression models: in the first model we considered the affection status as a dependent variable and the 2 *RKHD2* SNPs genotypes as independent variables; in the second model, the individual's gender was included as an additional independent variable. Because all of the subjects were white of Spanish origin, ethnicity was not considered in the logistic model. To determine epistatic effects between the rs1941958 and rs1893379 SNPs, a stepwise logistic regression procedure was implemented to compare 2 different regression models by a LR test using the statistical package SPSS 12.0. In the first model, we considered the affection status as a dependent variable and the rs1941958 and rs1893379 SNPs as predictive variables. In the second model, we included the interaction rs1941958\*rs1893379 as an independent variable in the logistic regression model. In the population-based association analysis, the significance threshold was set at  $2P < 0.025$  after the multiple comparison correction of Bonferroni considering 2 different SNPs.

### Results

A total of 18 microsatellite markers on chromosome 18 (Figure 1), 3 markers on chromosome 17, and 6 markers on chromo-

some 9 were genotyped in 56 families with  $\geq 2$  affected siblings. The results of the single point sibling pair and NPL analyses for selected markers are shown in Table 1. Sibling-pair linkage analysis showed an excess allele sharing at marker D18S474, with a maximum likelihood of identical by descent sharing (MLS) LOD score of 3.24 both under dominance variance ( $P=0.00011$ ) and nondominance variance ( $P=0.00005$ ). Nonparametric linkage analysis using all of the available family members produced an NPL of 3.32 ( $P=0.00044$ ) on the same marker. Multipoint linkage analysis spanning this chromosome 18 region produced a linkage peak at D18S474, with an NPL value of 1.95 ( $P=0.02$ ; Figure 2A) and an MLS LOD score of 2.00 ( $P=0.0001$ ). The MLS proportions around this marker deviated significantly from Mendelian expectations (Figure 2B). In contrast, no significant linkage results were obtained at the neighboring microsatellite markers on chromosome 18 or at any of the markers analyzed on chromosomes 9 and 17 (Table 1).

Four genes have been located in the close vicinity of marker D18S474 (<300 Kb), both at the proximal side (*ME2*, *ELAC1*, and *SMAD4*) and at the distal side (*RKHD2*). We focused on the *RKHD2* gene, putatively involved in protein ubiquitination and located only 6 Kb distal from D18S474 (Figure 1). We investigated its possible involvement in EH through linkage and case-control analyses. To minimize the number of markers selected for genotyping by ensuring an appropriate coverage of the *RKHD2* gene, we considered the HapMap project data ([www.hapmap.org](http://www.hapmap.org), release 16c), consisting of 15 SNPs spanning 32.5 kb (*RKHD2* gene plus 5 kb upstream and downstream from the gene) and located within the single *RKHD2* intron ( $n=6$ ), and the 5' ( $n=4$ ) and 3' regions ( $n=5$ ). Seven SNPs showed a minor allele frequency

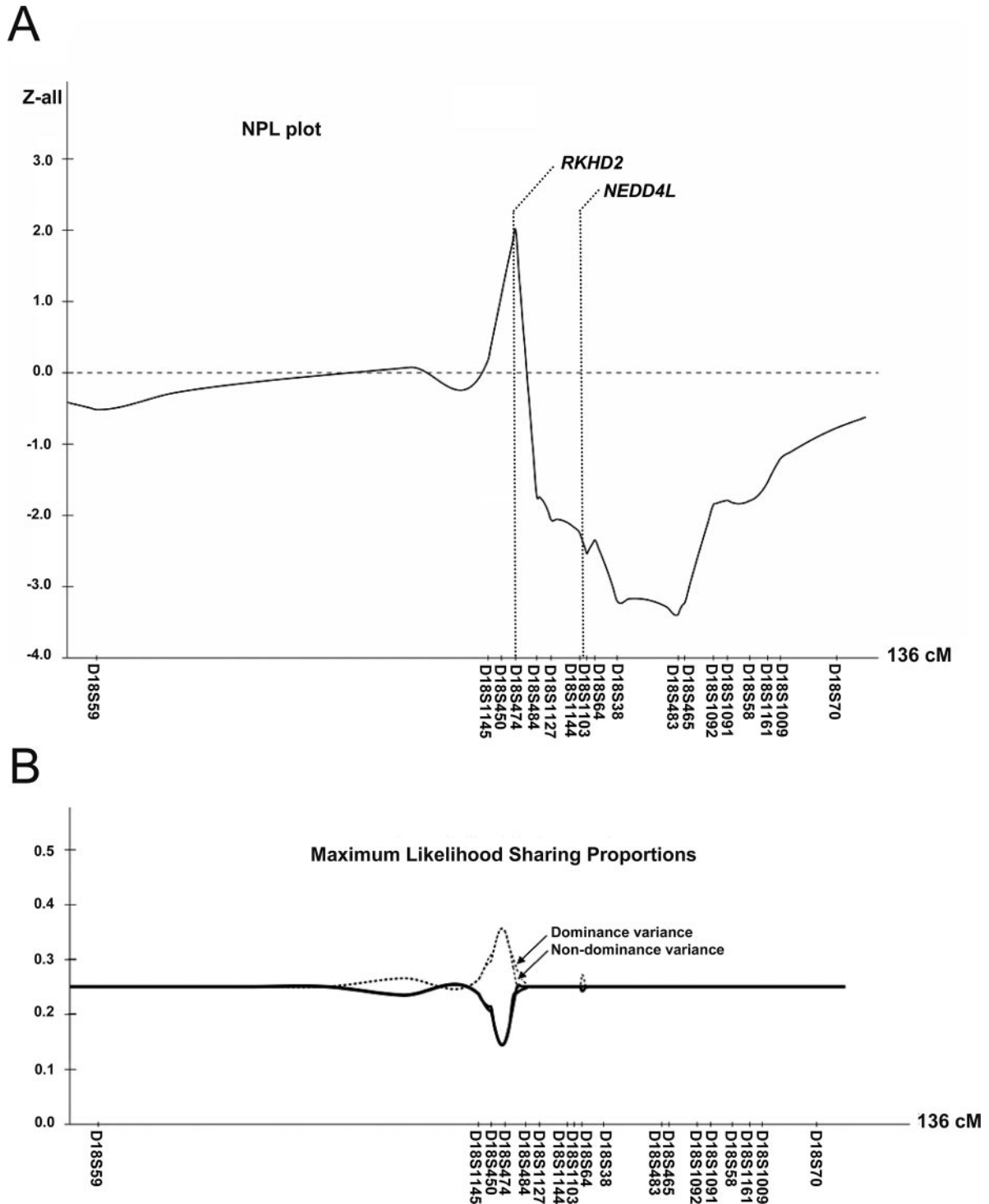
**TABLE 1. Nonparametric LOD Score (NPL) and Maximum Likelihood of Identical by Descent Sharing LOD Score (MLS) Analyses for Microsatellite Markers on Chromosomes 18, 9, and 17 (GENEHUNTER v2.1)**

Marker	Position (cM)*	NPL	P	MLS LOD (D)	P	MLS LOD (non-D)	P
Chr 18							
D18S1145	66.7	-0.06	0.521	0.01	0.513	0.01	0.415
D18S450	68.9	1.32	0.09	1.33	0.053	0.65	0.042
D18S474	71.3	3.32	0.00044†	3.24	0.00011†	3.24	0.00005†
D18S484	74.9	-1.43	0.928	0.01	0.542	0.01	0.438
D18S1127	77.4	-1.68	0.957	0.01	0.554	0.01	0.448
Chr 9							
D9S279	120.0	-1.11	0.87	0	0.60	0	0.50
D9S1881	135.8	0.64	0.26	0.08	0.35	0	0.50
D9S1798	136.5	-1.33	0.91	0.12	0.30	0.08	0.27
D9S159	142.5	-1.34	0.92	0	0.60	0	0.50
Chr 17							
D17S1795	68.4	-1.12	0.87	0	0.60	0	0.50
D17S1799	75.0	-1.14	0.88	0	0.60	0	0.50
D17S949	93.3	-0.67	0.75	0	0.60	0	0.50

D indicates dominance; non-D, nondominance; Chr, chromosome.

\*Sex averaged genetic distances from the short arm telomere, as defined in the Marshfield Genetic Map ([research.marshfieldclinic.org/genetics](http://research.marshfieldclinic.org/genetics)).

†Significant P values in both the NPL and the MLS analysis.



**Figure 2.** Nonparametric linkage analysis on chromosome 18 for hypertension in the whole sibling-pairs set. (A) Multipoint NPLs. LOD scores are along the Y axis, and relative positions of the markers are indicated on the x axis. The positions of the *RKHD2* and *NEDD4L* genes are also shown. (B) Maximum likelihood of identical by descent sharing (MLS) proportions on chromosome 18 for hypertension. The continuous line indicates the proportion of siblings sharing 0 alleles, and the dashed line indicates proportion of siblings sharing 2 alleles. The calculations were made under the assumption of nondominance variance and dominance variance, as indicated on the graph. A significant deviation from the 0.25 Mendelian expectations was observed around marker D18S474 under the 2 models.

>10% and were considered for the study. Using the LD Select software,<sup>19</sup> we evaluated the LD pattern for these 7 *RKHD2* SNPs, identified 2 haplotype bins (B1: rs3819118, rs1893379, rs4939656, rs2187052, rs2155957 and B2: rs1941958, rs4283317), and selected 1 tagged SNP from each

bin for the genotyping (rs1893379 for B1 and rs1941958 for B2; Figure 1).

Single-point and multipoint MLS and NPL analysis carried out with the 2 *RKHD2* polymorphisms showed no significant linkage results (data not shown). For the population-based

**TABLE 2. Distribution of Genotypes and Alleles for the *RKHD2* SNPs in 112 Hypertensive Patients and 156 Normotensive Control Subjects**

SNPs	Genotypes N (%)			Alleles N (%)		Genotype 11 vs 12+22		Genotype 11+12 vs 22		Allele 1 vs Allele 2	
	11	12	22	1	2	OR (95% CI)	2P*	OR (95% CI)	2P*	OR (95% CI)	2P*
rs1941958†						1.77 (1.02 to 3.07)	0.048	1.36 (0.78 to 2.36)	0.33	1.40 (0.99 to 1.98)	0.054
Hypertension	36 (32.1)	49 (43.8)	27 (24.1)	121 (54.0)	103 (46.0)						
Controls	33 (21.1)	76 (48.7)	47 (30.1)	142 (45.5)	170 (54.5)						
rs1893379†						1.96 (0.80 to 4.83)	0.17	1.70 (1.03 to 2.79)	0.043	1.62 (1.08 to 2.4)	0.024
Hypertension	12 (10.7)	41 (36.6)	59 (52.7)	65 (29.0)	159 (71.0)						
Controls	9 (5.8)	45 (28.8)	102 (65.4)	63 (20.2)	249 (79.8)						

\*P values after Bonferroni correction ( $2P < 0.025$ ).

†Alleles 1=rs1941958G and rs1893379T; Alleles 2=rs1941958A and rs1893379C.

association approach, we genotyped 45 unrelated patients from the sibling pairs set and extended the sample to a total of 112 hypertensive individuals. This group and 156 unrelated controls matched for ethnicity and sex were genotyped for the 2 *RKHD2* SNPs. As expected from the HapMap data, the LD analysis showed that rs1941958 and rs1893379, located upstream and in the *RKHD2* intron, respectively, were not in LD in our set of individuals ( $D' = 0.21$  and  $r^2 = 0.008$ ). Both polymorphisms were in Hardy–Weinberg equilibrium and showed statistical powers of 79.2% and 69.1%, respectively. The comparison of genotype frequencies of the 2 tagged SNPs in cases and controls showed significant differences (rs1941958: GG versus GA+AA,  $2P = 0.048$ , odds ratio: 1.77; rs1893379: TT+TC versus CC,  $2P = 0.043$ , OR: 1.7; Table 2). Moreover, allelic frequencies of the rs1893379 SNP in the hypertensive group significantly differed from normotensive controls ( $2P = 0.024$ ; OR: 1.62; Table 2), and a suggestive but not significant overrepresentation of the G allele of the rs1941958 SNP was observed in hypertensive patients ( $2P = 0.054$ ; Table 2). After the multiple comparison correction of Bonferroni, considering 2 different sequence variants, only the association between the rs1893379 SNP and EH remained positive. Inclusion of gender as a covariate in a logistic regression model where the affection status was the dependent variable and the *RKHD2* SNPs genotypes were the dependent variables did not improve the goodness-of-fit to data. Thus, gender was not further considered in the subsequent analyses of haplotypes or epistatic phenomena.

We then performed a haplotype-based analysis, a strategy that sometimes can increase statistical power with respect to the

**TABLE 3. Haplotype Distribution of the rs1941958 and rs1893379 *RKHD2* SNPs in 112 Hypertensive Patients and 156 Normotensive Control Subjects**

Haplotype		Hypertension	Controls
rs1941958	rs1893379	N (%)	N (%)
G	C	69 (30.8)	106 (34.0)
A	C	90 (40.2)	143 (45.8)
G	T	52 (23.2)	36 (11.5)
A	T	13 (5.8)	27 (8.7)
2P		0.0035	

comparison of individual markers, considering the 2 *RKHD2* sequence variants. The estimation of haplotypes from the genotypic data showed 4 possible allelic combinations, 3 of which consisted of common haplotypes covering >90% of the total *RKHD2* haplotype diversity (Table 3). Consistent with the analysis of individual markers, we observed that haplotype frequencies of the *RKHD2* gene in patients significantly differed from controls ( $2P = 0.0035$ ; Table 3) because of an overrepresentation of the rs1941958G–rs1893379T allelic combination in the group of hypertensive patients in comparison with controls ( $2P = 0.0004$ ; OR: 2.32; Table 4).

We also determined possible epistatic effects between the 2 *RKHD2* SNPs by a stepwise logistic regression analysis. Thus, we considered these 2 sequence variants as predictors of the affection status by comparing 2 logistic regression models by a LR test: in the first model we considered the rs1941958 and rs1893379 SNPs as independent variables, whereas in the second model we included the interaction rs1941958\*rs1893379 in the equation. The comparison of both models by the LR test supported the participation of the interaction between the rs1941958 and rs1893379 sequence variants in EH ( $P = 0.03$ ; Table 5). Thus, using subjects carrying none of the *RKHD2* susceptibility alleles as the reference group, the observed OR for subjects with both risk alleles is 3.45 ( $1.20 \times 1.16 \times 2.48$ ) compared with an expected OR of 1.39 ( $1.20 \times 1.16$ ) if there is no interaction (Table 5).

## Discussion

In the present study, we have performed a targeted genomic search aimed to test several candidate chromosomal regions involved in human EH by linkage analysis in hypertensive sibling pairs and found an excess allele sharing at the D18S474 marker on chromosome 18q21.1. Consistent with these results, the case–control analysis of 2 SNPs covering

**TABLE 4. Comparison of the *RKHD2* Haplotype Frequencies Considering the rs1941958 and rs1893379 SNPs in 112 Hypertensive Patients and 156 Normotensive Control Subjects**

Haplotype	Hypertension	Controls	2P; OR (95% CI)
	N (%)	N (%)	
rs1941958G+rs1893379T	52 (23.2)	36 (11.5)	0.0004; 2.32 (1.5 to 3.7)
Others	172 (76.8)	276 (88.5)	

**TABLE 5. LR Test to Compare 2 Different Logistic Models and Determine Possible Interactions Between the rs1941958 and rs1893379 SNPs of the *RKHD2* Gene**

Variable*	Log Likelihood	$\chi^2$	<i>P</i>	OR (95% CI)†
Null Model	−361.5			
Model 1‡	−359.0	10.4	0.005	
rs1941958				1.48 (1.04 to 2.10)
rs1893379				1.70 (1.14 to 2.55)
Model 2§	−356.7	15.0	0.002	
rs1941958				1.20 (0.80 to 1.79)
rs1893379				1.16 (0.68 to 1.99)
rs1941958×rs1893379				2.48 (1.07 to 5.73)
LR test	−2 (LogV Model 1−LogV Model 2)	$\chi^2$ (degrees of freedom)	<i>P</i>	
	4.6	4.6 (1)	0.03	

\*Alleles of reference: rs1941958A and rs1893379C.

†OR is the allele-based ORs.

‡Model 1: case/control versus rs1941958+rs1893379.

§Model 2: case/control versus rs1941958+rs1893379+rs1941958\*rs1893379.

the *RKHD2* gene, which maps in the vicinity of the D18S474 marker (Figure 1), provided evidence for an association between this positional candidate gene and the hypertensive phenotype.

With respect to human disease, several studies have supported the role of chromosome 18 in hypertension (depicted in Figure 1). First, a study focused on autosomal dominant orthostatic hypotension reported linkage to 18q in 2 of 3 families studied.<sup>21</sup> Moreover, a genome scan for QTLs influencing blood pressure responses to a postural challenge in whites revealed suggestive evidence for linkage of SBP response to chromosome 18q.<sup>12</sup> Similarly, a genome-wide scan performed in Mexican Americans revealed linkage of SBPs and DBPs to chromosome 2; suggestive linkage of SBP to chromosomes 8, 21, and 18;<sup>22</sup> and suggestive linkage of pulse pressure to chromosomes 7, 8, 18, and 21.<sup>23</sup> In addition, genome scans in white subjects from the Framingham Heart Study found significant linkage of SBP to chromosome 17 and suggestive linkage of DBP to chromosomes 17 and 18.<sup>6</sup> These studies support a possible involvement of loci on chromosome 18q in the normal regulation of blood pressure. However, an unresolved question is whether the locus or loci influencing normal blood pressure variation may contribute to the EH phenotype.

Our study provides evidence for the participation of chromosome 18q in EH in Spanish patients. It is of note that, to our knowledge, this is the first linkage study in essential polygenic hypertension performed in Spain, and the observed results are in agreement with a recent genome-wide scan in 120 Icelandic families with EH, which showed significant linkage to 18q with no suggestive linkage to other regions.<sup>5</sup> Moreover, a recent study performed in Australian hypertensive sibling pairs focused specifically on chromosome 18<sup>13</sup> and, whereas multipoint linkage analysis produced no relevant peaks, significant single-point linkage results were found with 2 markers located on 18p and 18q. The 18p marker (D18S59), however, has also been analyzed in the present study and has shown no linkage to EH by sibling-pair analysis. Although the broad area of chromosome 18 showing

linkage in the different populations suggests that multiple genes may be implicated in the genesis of EH (Figure 1), these results encourage further mapping efforts to localize the chromosome 18 loci involved in both the regulation of blood pressure and the development of EH.

In contrast, the linkage analysis enabled us to exclude the regions studied on chromosomes 9 and 17 in our sample of patients. With respect to chromosome 9q, our results are in disagreement with previous studies showing linkage, although only suggestive, in the British population.<sup>24</sup> We also detected no linkage of hypertension to 17q in our collection of sibling pairs. Since the identification of QTLs contributing to blood pressure variation in rat chromosome 10 in several experimental models of hypertension,<sup>14,15</sup> several studies have reported evidence of linkage of hypertension to the 17q syntenic region in humans.<sup>6,10,16</sup> Although the ACE gene, encoding the angiotensin I-converting enzyme, is located within the linked chromosomal region in rats, the human homologue is situated 18 cM proximal to the blood pressure QTL and, therefore, does not seem to overlap with the candidate region.<sup>16</sup> Close examination of the QTL interval at 17q12-21 discloses no obvious functional candidate genes for blood pressure variation.<sup>6</sup>

Because the *RKHD2* gene maps in the vicinity of the marker on chromosome 18 that displayed significant linkage in the sibling-pair analysis (Figure 1), we focused on this positional candidate gene to investigate its possible involvement in EH through a nonparametric linkage analysis and a case-control approach. Although single and multipoint MLS and NPL analysis showed no linkage of the trait to the 2 intragenic tagged SNPs, the case-control analysis revealed a strong association between the *RKHD2* gene and EH. The failure of the linkage analysis approach to detect a contribution of the *RKHD2* gene to the phenotype may be explained by the limited informativeness of SNPs as compared with microsatellite markers in a relatively small number of sibling pairs.

The single-marker association study revealed association between EH and the rs1893379 SNP located within the single

*RKHD2* intron. Consistently, the haplotype-based association study supported the single-marker results and revealed an overrepresentation of the rs1941958G–rs1893379T high-risk allelic combination in EH patients. According to the LD Select software, 2 haplotypic bins, tagged by rs1941958 and rs1893379, cover the entire *RKHD2* gene in terms of LD and do not extend distally or proximally to include any further gene (Figure 1). This probably excludes the fact that genes other than *RKHD2* account for the observed association. Moreover, the LD patterns across *RKHD2* as defined in the HapMap (www.hapmap.org, build 125) support this assessment. No other candidate genes were tested for association in the present study. However, from our data, we cannot totally exclude that other genes in the region may account for the linkage signal.

No experimental proofs of functional consequences of the rs1941958 or rs1893379 SNPs, located at the 5' and intronic regions of the *RKHD2* gene, respectively, have been described. Although the functional consequences of intronic variations have not been extensively studied, there are strong evidences of the existence of regulatory elements within these regions.<sup>25</sup> On the other hand, SNPs within the 5' region, such as rs1941958, could also have functional effects in the modulation of the gene transcription efficiency or the temporal and tissue-specific expression pattern. However, it remains uncertain whether the selected tagged SNPs, other SNPs within the gene that are in LD with them, or a combination of them are directly involved in the genetic susceptibility to EH. Moreover, we found evidence of epistatic effects between the 2 analyzed SNPs, which support that several sequence variants within the gene, rather than a single polymorphism, may contribute to this complex phenotype.

To our knowledge, there are no experimental data regarding the function of the *RKHD2* gene, except for its transcription pattern as determined by microarray techniques, which includes kidney and most of the tissues and cell lines analyzed (UCSC Genome Browser, genome.ucsc.edu). Comparative sequence analysis indicates the presence of RING and K homology (KH) domains in the protein (Gene Ontology Database, www.godatabase.org). The RING domain is a specialized Zn-finger domain probably involved in mediating protein–protein interactions<sup>26</sup> and has been implicated in a range of diverse biological processes, probably including E3 ubiquitin-protein ligase activity.<sup>27</sup> The ubiquitin-protein ligases contribute to the degradation of abnormal proteins in the cell through the ubiquitin-proteasome system by determining the substrate specificity for ubiquitination. On the other hand, the KH domain was first identified in the human heterogeneous nuclear ribonucleoprotein K. It is a domain of  $\approx 70$  amino acids that is present in a wide variety of nucleic acid-binding proteins,<sup>28</sup> and it has been shown to bind RNA.<sup>29,30</sup> Mechanistically, the possible involvement of the *RKHD2* gene in hypertension can only be speculative. Interestingly, mutations in the  $\beta$  (*SCNN1B*) or the  $\gamma$  (*SCNN1G*) subunits of the epithelial Na<sup>+</sup> channel of the distal nephron have been shown to be critical for renal Na<sup>+</sup> regulation by altering the ubiquitination process normally produced by interaction with NEDD4 and are the cause of Liddle syndrome, a rare form of salt-sensitive monogenic hyperten-

sion.<sup>31</sup> Also, SNP variation in the gene encoding the *NEDD4L* ubiquitin ligase, the putative regulator of the epithelial Na<sup>+</sup> channel,<sup>32</sup> has been associated with EH.<sup>33</sup> Although the *NEDD4L* gene is located within the 18q21 chromosomal region, its physical location 7 Mb distal from D18S474 and *RKHD2* probably excludes any contribution to the linkage and the association observed in the present study (Figure 2A).

### Perspectives

EH is a complex disorder that results from the interplay of multiple susceptibility genes and environmental factors. In recent years, a number of association studies with physiology-based candidate genes have given conflicting results. More recently, genome-wide linkage studies have disclosed candidate regions in several chromosomes, with most studies reporting weak or suggestive linkage and a few studies attaining significant linkage evidence. In any case, studies reporting genome-wide linkage results in hypertension have been followed only occasionally by a further inspection of positional candidate genes through association studies. In this study, we confirm the implication of chromosome 18 in EH by linkage analysis. Furthermore, we demonstrate that the positional candidate gene *RKHD2* is associated with EH. The product of this gene may play a role in the ubiquitination machinery of the cell, a process that had been implicated previously in monogenic hypertension (*SCNN1B* and *SCNN1G* genes) and in EH (*NEDD4L*). Because no studies have been performed to date about the distribution and organ-specific function of the *RKHD2* protein, further work is needed in this direction to disclose its possible participation in hypertension. Moreover, replication of our association study in other sets of patients should provide a better understanding about the involvement of the *RKHD2* gene in the predisposition to the hypertensive phenotype.

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### Disclosures

None.

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