RESEARCH PAPER

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Genetic variability of the gene cluster *CALHM*1–3 in sporadic Creutzfeldt-Jakob disease

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Perturbations of calcium homeostasis have been associated with several neurodegenerative disorders. A common polymorphism (rs2986017) in the *CALHM1* gene, coding for a regulator of calcium homeostasis, is a genetic risk factor for the development of Alzheimer disease (AD). Although some authors failed to confirm these results, a meta-analysis has shown that this polymorphism modulates the age at disease onset. Furthermore, a recent association study has explored the genetic variability of *CALHM1* gene and two adjacent paralog genes (*CALHM3* and *CALHM2)* in an Asian population. Since several lines of evidence suggest that AD and prion diseases share pathophysiologic mechanisms, we investigated for the first time the genetic variability of the gene cluster formed by *CALHM1* and its paralogs in a series of 235 sporadic Creutzfeldt-Jakob disease (sCJD) patients, and compared the genotypic and allelic frequencies with those presented in 329 controls from the same ancestry. As such, this work also represents the first association analysis of *CALHM* genes in sCJD. Sequencing analysis of the complete coding regions of the genes demonstrated the presence of 10 single nucleotide polymorphisms (SNP) within the *CALHM* genes. We observed that rs4918016-rs2986017-rs2986018 and rs41287502-rs41287500 polymorphic sites at *CALHM1* were in linkage disequilibrium. We found marginal associations for sCJD risk at *CALHM1* polymorphic sites rs41287502 and rs41287500 [coding for two linked missense mutations p.(Met323Ile); (Gly282Cys)], and rs2986017 [p.(Leu86Pro)]. Interestingly, a TGG haplotype defined by the rs4918016-rs2986017-rs2986018 block was associated with sCJD. These findings underscore the need of future multinational collaborative initiatives in order to corroborate these seminal data.

Introduction

Calcium homeostasis has key roles in different intracellular and extracellular processes, and therefore is essential for cell physiology. Perturbations of calcium regulation has been described in several neurodegenerative disorders, such as Alzheimer (AD), amyotrophic lateral sclerosis, Parkinson, Huntington and prion diseases.[1-11]

In 2008, Dreses-Werringloer and collaborators identified a polymorphism (rs2986017) associated with AD in the gene *CALHM1* coding for a novel regulator of calcium homeostasis called CALHM1, which appears to be involved in the metabolism of amyloid β precursor protein (APP).[12] Although some authors have failed to confirm these results, a meta-analysis has shown that this polymorphism modulates the age at disease onset.[13] Additionally, a recent association study explored the genetic variability of *CALHM1* gene and two adjacent paralog genes *CALHM3* and *CALHM2* (coding for CALHM3 and CALHM2, respectively*)* in an Asian population; although this study failed to find an association of these genes with AD.[14]

Several lines of evidence suggest that AD and prion diseases share pathophysiologic mechanisms.[15-23] In this paper, we investigated the genetic variability of the gene cluster formed by *CALHM1* and its paralogs in a Caucasian population of Spanish origin, and explored the potential association of these genes with sporadic Creutzfeldt-Jakob disease (sCJD).

Results

This study included 235 sCJD patients (50.6% women) and 329 control subjects (60.2% women). Age at study inclusion for control individuals followed a non-normal distribution with a mean

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Table 1. Polymorphisms found in *CALHM1* and *CALHM3* genes coding regions

Gene	SNP	Coding triplet	HGVS reference	Protein position	Function	Amino acid change
CALHM1	rs2986016	NA	NG_016855.1:g.8633T > C	NA	UTR-3	NA
	rs41287500	ATG → ATA	NG_016855.1:g.8558G > A	323	missense	Met → Ile
	rs41287502	GGT→ TGT	NG_016855.1:g.8433G > T	282	missense	Gly → Cys
	rs41317256	GTC → ATC	NG_016855.1:g.5489G > A	117	missense	Val → Ile
	rs2986017	CTG → CCG	NG_016855.1:g.5397T > C	86	missense	Leu → Pro
	rs4918016	CCG → CCA	NG_016855.1:g.5395G > A	85	synonymous	Pro → Pro
	rs2986018	GCA → GCG	NG_016855.1:g.5290A > G	50	synonymous	Ala → Ala
	rs146465393	GCC → ACC	NG_016855.1:g.5228G > A	30	missense	Ala → Thr
CALHM3	rs2986035	GAC → AAC	NC_000010.10:g.105233110C > T	299	missense	Asp → Asn
	rs3014199	NA	NC_000010.10:g.105235925T > C	NA	intron	NA

of 73.2 y and standard deviation of 11.0 y. Age at onset for the sCJD group followed a non-normal distribution with a mean of 67.2 ± 10.8 y.

Significant differences for gender distribution were found between sCJD patients and the control group (p = 0.05). Analysis of age distribution yielded statistically significant differences for sCJD patients (p < 0.001) compared with the control group (67.2 ± 10.8 vs. 73.2 ± 11.0). Potential effects of the differential gender and age distributions on the calculation of odds ratios were minimize by the use of logistic regression algorithms controlled age and gender. Homozygosis at polymorphic codon 129 in the *PRNP* gene was shown to be strongly associated with sCJD (OR = 4.87, 95% CIs = 3.21–7.39, p = 8.49 × 10^{-14}) in agreement with a previous report for this population.[22]

By sequencing analysis of the complete coding regions of the *CALHM* genes, we found eight single nucleotide polymorphisms (SNP) in *CALHM1*, 2 SNP in *CALHM3*, and none in *CALHM2* (**Table 1**). All of these SNPs were already identified in The Single Nucleotide Polymorphism Database (dbSNP) of Nucleotide Sequence Variation (www.ncbi.nlm.nih.gov/projects/SNP/index.html).

After tagging analysis of sequence data of cases and controls in Haploview, we opted for rs2986017 as tag SNP for rs2986018 (D′ = 1.0, r^2 = 0.80, LOD = 307.7) and rs41287502 as tag SNP for rs41287500 (D′ = 1.0, r^2 = 1.0, LOD = 134.61) (see **Fig. 1**). For further analysis, only those SNPs with a minor allele frequency (MAF) above of 1% were selected. Linkage disequilibrium plot indicated that rs4918016, rs2986017 and rs2986018 within *CALHM1* formed a haplotype block. The distribution of *CALHM* genotypes are shown in **Table 2**. All polymorphisms were in Hardy-Weinberg equilibrium, with the exception of rs41287502 in sCJD (p = 0.03). The association analysis with sCJD population of polymorphisms at *CALHM* genes cluster is shown in **Table 2**.

A Bonferroni correction for six comparisons yielded an adjusted significance level of p = 0.008. Based on this adjusted p value, we observed several non statistically significant trends for CALHM genes variants (**Table 2**). Thus, we found a dose-dependent risk tendency associated with *CALHM1* rs41287502 T-allele carriers (OR = 2.21, 95% CIs = 1.12–4.34, p = 0.02) that was mainly driven by *PRNP* M129M individuals (OR = 3.56, 95% CIs = 1.22–10.36, p = 0.02). Rs41287502 T-allele carriers presented also a longer disease duration (p = 0.04). While we could not observe a relevant tendency for rs2986017 minor allele T-carriers, we found a risk tendency in a recessive model associated with *CALHM1* rs2986017 TT-genotype (OR = 2.09, 95% CIs = 1.09–4.01, p = 0.03), which was mainly driven by individuals with earlier onset (OR = 6.66, 95% CIs = 1.38–32.15, p = 0.02).

At the *CALHM3* gene, we also observed a risk tendency for rs3014199 G-allele carriers (OR = 1.72, 95% CIs = 0.98–3.01, p = 0.06) that was driven by earlier onset subjects (OR = 3.05, 95% CIs = 1.18–7.84, p = 0.02).

Interestingly, we also found that the TGG haplotype at polymorphic sites rs2986017, rs4918016 and rs2986018 defining a haplotype block (**Fig. 1**) was associated with sCJD (OR = 1.88, χ^2 = 5.61, p = 0.018). We performed a permutation test for haplotypes to obtain a measure of significance corrected for multiple testing bias. After 10^5 permutations, we observed that only 4.5% permutations exceeded the χ^2 value yielding a corrected p value of 0.045. Moreover, a TG haplotype at the polymorphic sites of rs2986017 and rs2986018 within the defined haplotype block was clearly associated with the disease (OR = 1.94, χ^2 = 6.45, p = 0.011, corrected p = 0.025).

Discussion

CALHM1 gene encodes for a cerebral calcium channel component controlling cytosolic calcium homeostasis, CALHM1. The *CALHM1* rs2986017 polymorphism [p.(Leu86Pro)] has been associated with increased risk for late-onset AD in some, but not all reports.[12,25-34] Other studies had described its association with an earlier age at onset of AD.[12,13,26,30] Shibata and collaborators have recently extended the AD risk association analysis to other adjacent *CALHM1* paralogs, *CALHM2* and *CALHM3* with negative results for an Asian population.[14] We have investigated the genetic variability of the coding regions of the gene cluster formed by *CALHM1*, *CALHM2* and *CALHM3* in a Caucasian population of Spanish origin of sCJD cases and healthy controls, representing the first association analysis of *CALHM* genes in sCJD.

Of the 10 SNP in the *CALHM* genes here described (**Table 1**), two of them (rs2986017 and rs4918016) have been studied on several populations with minor allele frequencies (MAF) similar

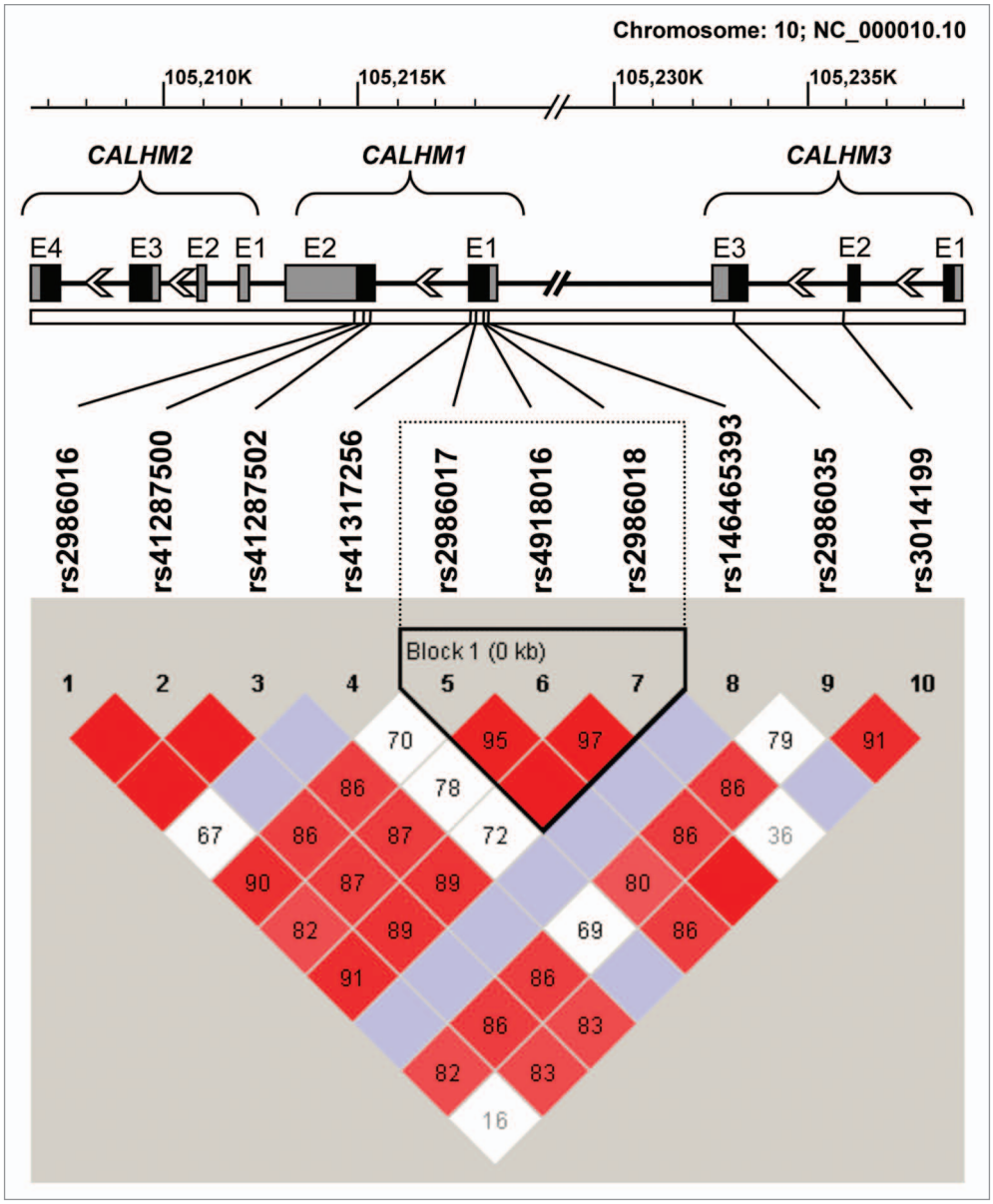

Figure 1. Standard (D′/LOD) linkage disequilibrium plot of the coding regions of *CALHM1*, *CALHM2* and *CALHM3*. Haplotype blocks were generated using the confidence interval method by Haploview v4.2 software.[24] Exon (E) structure of these genes is depicted above the linkage disequilibrium plot. Coding regions are indicated by black boxes, while untranslated 5' and 3' exonic regions are represented by gray boxes.

to those found in our population in Caucasians (for a review, see refs. 13 and 25), and relatively different MAF in Asian populations.[14,32] The rs2986018 and rs2986035 SNPs have only been described in Asian populations, showing lower MAF for rs2986018 on these populations compared with ours.[14,32]

Based on an adjusted significance level for multiple comparisons, we observed several marginal association findings that may be of relevance. We found that the polymorphic sites rs41287502 and rs41287500 at *CALHM1* [both resulting in an amino acid change (p.[Met323Ile]; [Gly282Cys])] were in linkage disequilibrium (D′ = 1.0, r^2 = 1.0, LOD = 134.61) and associated with a sCJD risk trend (OR = 2.21, p = 0.02 for all individuals; OR = 3.56, 95%, p = 0.02 for M129M individuals), and longer disease duration (p = 0.04). Whether these two linked missense mutations at *CALHM1* gene alters the functionality of the protein remains to be explored.

Additionally, we observed a risk trend in a recessive model associated with *CALHM1* rs2986017 TT-homozygous genotype, which was mainly driven by individuals with earlier sCJD onset, similarly to previous studies in AD.[12,13] Interestingly, this SNP together with rs4918016 and rs2986018 define a haplotype block; and the haplotype TGG (formed by the lower frequency alleles) was found clearly associated with sCJD even after correction for multiple comparison bias.

Although our analysis comprises a big proportion of cases diagnosed in Spain in the last years, the number of cases studied

Table 2. Genotypic frequencies and odds ratios for the association of *CALHM1* and *CALHM3* SNPs with sCJD (minor allele carriers vs. non-carriers)

Gene	SNP	Genotypes	Genotypic frequency, n(%)		OR [95% CI]	p value*
			CONTROL	sCJD		
CALHM1	**rs2986016**	CC	172 (58.5)	95 (56.5)	1.08 [0.72–1.62]	0.72
		TC	113 (38.4)	66 (39.3)		
		TT	9 (3.1)	7 (4.2)		
		Total	294 (100)	168 (100)		
	rs41287502	GG	297 (94.6)	208 (89.7)	2.21 [1.12–4.34]	0.02
		TG	16 (5.1)	21 (9.0)		
		TT	1 (0.3)	3 (1.3)		
		Total	314 (100)	232 (100)		
	rs2986017	CC	181 (55.0)	115 (49.6)	1.26 [0.88–1.79]	0.21
		TC	128 (38.9)	94 (40.5)		
		TT	20 (6.1)	23 (9.9)		
		Total	329 (100)	232 (100)		
	rs4918016	GG	158 (48.0)	132 (56.9)	0.71 [0.50–1.00]	0.05
		AG	152 (46.2)	81 (34.9)		
		AA	19 (5.8)	19 (8.2)		
		Total	329 (100)	232 (100)		
CALHM3	**rs2986035**	AA	183 (59.6)	133 (56.6)	1.07 [0.74–1.53]	0.72
		AG	109 (35.5)	97 (41.3)		
		GG	15 (4.9)	5 (2.1)		
		Total	307 (100)	235 (100)		
	rs3014199	AA	294 (91.3)	202 (86.3)	1.72 [0.98–3.01]	0.06
		AG	28 (8.7)	32 (13.7)		
		GG	0 (0.0)	0 (0.0)		
		Total	322 (100)	234 (100)		

*Adjusted significance level for six multiple comparisons p = 0.008.

is necessarily limited due to the rarity of the disease. Thus, international joint initiatives will be required to ensure universal validity to the primary data here presented.

Materials and Methods

Subjects. Patient populations included 235 sCJD cases [including 185 (78.7%) neuropathologically verified definite patients, and 50 (21.3%) probable cases] and 329 subjects with normal cognitive status measured by Mini Mental test. All subjects were Caucasians of Spanish origin. Samples for sCJD cases were obtained from patients with suspected prion diseases, submitted for diagnostic purposes under the guidelines of the Spanish National Referral and Surveillance system. Genetic cases of human prion diseases were excluded after complete DNA sequencing of *PRNP* coding region. Control samples were obtained with the adequate understanding and written consent of subjects, family members or legal guardians, as appropriate. The study was approved by the Bioethics and Animal Welfare Committee from the Instituto de Salud Carlos III, Madrid, Spain; and by the Ethical Research Committees from the CIEN Foundation, and Universidad Autónoma de Madrid, Madrid, Spain. All the data were analyzed anonymously, and clinical investigations have been conducted according to the principles expressed in the Declaration of Helsinki.

The sCJD group (n = 235) included 185 patients, and 50 probable sCJD cases.

DNA isolation and analysis. Total DNA was isolated from peripheral blood or cerebral tissue following standard procedures. The analysis of the polymorphism at codon 129 of the *PRNP* gene (rs1799990) was performed by DNA sequencing using specific primers.[35]

The complete coding sequences of *CALHM1*, *CALHM2* and *CALHM3* genes were analyzed by DNA sequencing using specific primers designed according to the corresponding human reference sequences (see **Table S1**). The amplification reactions were performed with 50 ng of genomic DNA and 1 unit of Taq DNA Polymerase (Applied Biosystems) in a volume of 25 μl. The final concentrations of other reactants were: 1x Taq DNA Polymerase Buffer, 0.1 mM dNTPs, 1.5 mM $MgCl_2$ and 0.1 μM of each primer. The PCR cycling conditions were as follows: initial denaturation at 96°C for 3 min followed by 35 cycles of 94°C for 30 sec, 63°C for 30 sec and 72°C for 1 min and a final extension at 72°C for 10 min. A 2 μl aliquot of the amplification

reaction was sequenced using 0.1 μM of the above primers and the BigDye Terminator v1.1 Cycle Sequencing Kit in an ABI PRISM 377 Analyzer (Applied Biosystems).

Haplotype analysis. Haplotypes assignation, linkage disequilibrium plot and haplotypes association analysis were performed by using Haploview v4.2 software. Tag SNPs were selected by Haploview Tagger tool by using the "aggressive tagging: 2-marker haplotypes" routine with an r-square threshold of 0.8. Haplotype blocks were generated using the confidence interval method.[24]

Statistical methods. Statistical analyses of nominal or categorical variables were performed by Fisher's exact text or Pearson's chi-square test. Quantitative variables (age at onset, disease duration) were analyzed by non-parametric statistical hypothesis contrast with Mann-Whitney U test. Additionally, logistic regression models controlled by age (as a linear variable) and gender were used to compare genotypic and allelic frequencies and to calculate association adjusted odds ratio (OR) and 95% confidence intervals (CIs). The Hardy-Weinberg test for genotype frequency distributions was performed on the observed genotype frequencies for population, with significance based on a standard observed-expected chi-square distribution with one degree of freedom. Deviations from normality of quantitative variables were checked by the Kolmogorov-Smirnov statistic with Lilliefors' significance. All statistical analyses were performed with the GraphPad 4 or PASWStatistics 18 softwares.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Conceived and designed the experiments: O.C., M.J.B. and M.C. Performed the experiments: O.C., M.J.B., R.H. and I.S. Analyzed the data: O.C. and M.C. Contributed materials: M.J.B., J.C., A.F.G., P.M.M., A.L., M.J.R., A.R., J.deP., I.F. and M.C. Wrote the manuscript: O.C., J.deP. and M.C. Critical revision of the manuscript: M.J.B., J.C., A.L. and I.F.

Supplemental Material

Supplemental materials may be found here:
http://www.landesbioscience.com/journals/prion/article/20785/

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