Alzheimer's & Dementia
Manuscript Draft

Manuscript Number: ADJ-D-13-00131

Title: Calcium-sensing receptor gene polymorphisms and sporadic Alzheimer's disease.

Article Type: Research Article

Keywords: Alzheimer's disease; Genetics; Polymorphism; Calcium-sensing receptor; CASR

Abstract: Background: Calcium-sensing receptor (CASR) gene is widely expressed in central nervous system in which plays a role in several primary functions. CASR is activated by apolipoprotein E (APOE) and by amyloid β-peptide, suggesting a possible role in Alzheimer's disease (AD) pathogenesis.

Methods: 321 AD and 486 cognitive healthy patients were enrolled in a real-life case-control setting. Three CASR polymorphisms encoding A986S, R990G and Q1011E protein variants were investigated in blinded fashion. A propensity score 1:1 matched setting was derived from the baseline scenery to assess robustness of the observed results. Data analyses were made with univariate and multivariate logistic regression statistics, also hypothesizing different genetic models of inheritance.

Results: Diverse associations of CASR genotypes encoding 986 and 1011 protein variants with AD were observed.

Conclusion: Our results suggested that particular CASR isoforms might be diversely associated with AD. More studies are needed, however, to clarify the role of CASR protein in the biochemical pathways underling the pathogenesis of AD.

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1. Introduction

The worldwide rising of population size and life expectancy make sporadic Alzheimer's disease (AD) a major health problem. AD occurred predominantly in later life, with a prevalence spanning from 20% after 75 years to 30% after 85 years. Thus, about 5% of people aged 65 years or older have AD, and with about 3 - 4 million people affected and about 350,000 new cases per year AD is currently the most frequent cause of dementia in U.S. and in Western countries. According to future previsions, AD prevalence will nearly double every 20 years, to 65.7 million in 2030 and 115.4 million in 2050 [1,2,3].

The G-protein-coupled calcium-sensing receptor, encoded by the CASR gene at locus 3q21.1, is widely expressed in central nervous system (CNS), and recent data suggested a pivotal role in several primary CNS functions, including signal conduction and neuron-glia interactions [4]. First of all, activation of CASR with elevated calcium concentrations promoted axonal growth in late fetal sympathetic neurons, whereas CASR-deficient mice or knock-out mice failed to promoted axonal growth. Second, CASR plays an important role in postnatal dendritic arborization, with a marked effect on size and complexity of hippocampal pyramidal neurons. CASR responds to changes in synaptic activity in the pyramidal layers of hippocampus and to local changes in calcium concentrations and thus participates in modifying dendritic architecture in response to synaptic activity during learning and memory performance of hippocampal pyramidal neurons [5]. Third, neuronal migration is dependent on calcium concentrations. It has been demonstrated that expression of CASR in mice neurons synthesizing gonadotropin-releasing hormone (GnRH) and its regulation of chemotaxis impacts on GnRH migration *in vivo*. Thus, CASR-null mice have significantly reduced numbers of GnRH neurons in the preoptic hypothalamus compared with mice with both copies of CASR alleles [6]. Fourth, CASR plays a role in the regulation of ion channels and neuronal excitability. Using the patch-clamp cell-attached technique it has been shown that the activity of a calcium-permeable non-selective cation channels is modulated by CASR in rat hippocampal pyramidal neurons and neocortical

terminals [7,8]. Finally, CASR is activated by apolipoprotein E and amyloid β peptide through the involvement of a signal transduction cascade that results in downstream release of calcium and compounds that could further amplify the detrimental effects of CASR activation by a feedback mechanism of action. Each of these downstream products has been found altered in AD patients [9]. All this data strongly suggest that common alterations in the CASR protein may influence the overall sensitivity to calcium in the brain, thus influencing the pathological pathways underlying AD. This concept have been also remarked in two recent reviews [10,[11],[12]].

Recent data indicates that three single nucleotide polymorphisms (SNPs) clustered in exon 7 of CASR gene may be predictive of alteration in serum calcium concentrations [13,14] and associated disorders [15]. These SNPs (rs1801725, rs1042636 and rs1081726) encoded missense variations (A986S, R990G, Q1011E) lying in the intracellular -COOH tail of CASR protein, potentially influencing CASR intracellular signaling, cell-surface exposition and desensitization [16].

In the present study we investigate these SNPs to assess a possible role of CASR gene in the pathogenesis of sporadic AD.

2. Materials and Methods

2.1. Study design

This was a multicenter real-life case-control study with a post propensity-score (PS) case-control matching validation.

2.2. Study guidelines

This study fulfill the Declaration of Helsinki [17], the guidelines for Good Clinical Practice [18] and the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines [19]. The approval of the study for experiments using human subjects was obtained

from the local Ethics Committees on human experimentation. Written informed consent for research was obtained from each patient or from relatives/legal guardian in the case of critically disabled demented patients prior to participation in the study.

2.3. Patient recruitment

The present study included all unrelated patients with clinically ascertained AD or ascertained free from any sign of cognitive impairment, who in the real-life consecutively attended (January 2010 to December 2011) the Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Casa Sollievo della Sofferenza in San Giovanni Rotondo (Geriatric Unit), the Catholic University School of Medicine in Rome (Department of Neurology) and the University of Perugia (Geriatric Unit). All patients included in the study were Caucasian mostly living in Central and Southern Italy for at least two generations. Patients with Jewish, Eastern Europe or Northern Africa descent were not included in the study.

2.4. Clinical evaluation and inclusion/exclusion criteria

To evaluate medical, cognitive and functional status, the comprehensive geriatric assessment (CGA) [20] was carried out at admission in all patients. Medical status was collected by a structured interview, a clinical evaluation, and a review of records from the patient's general practitioners. Cognitive status was screened by means of the Short Portable Mental Status Questionnaire (SPMSQ) [21]. Functional status was evaluated using the Activities of Daily Living (ADL) index [22], and the Instrumental Activities of Daily Living (IADL) scale [23]. Depression was diagnosed by means of the Geriatric Depression Scale-15 (GDS-15) [24,25]. Symptoms of depression, obsessive-compulsive disorder and anxiety, also in presence of diagnosis of AD caused the exclusion from the study.

The Mini Mental State Examination (MMSE) [26] and the Clinical Dementia Rating scale

(CDR) [27,28] were used to define the diagnosis of AD or to definitively exclude the presence of cognitive impairment. Briefly, dementia (CDR 1+) was confirmed and diagnosed by the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DMS-IV), whereas diagnosis of questionable dementia (QD) was made according to a CDR value of 0.5+ [29]. Diagnosis of mild cognitive impairment (MCI) was made, according to the Petersen criteria [30,31], in subjects with CDR 0.5+ and an MMSE value from 24 to 27. MCI subsets amnestic, with multiple impaired cognitive domains, and single non-memory domain were diagnosed as already described [31,32]. Diagnosis of QD or MCI caused the exclusion from the study. Patients with an SPMSQ score > 7, MMSE 28-30 and a CDR 0 were considered as cognitively healthy (CH) and were enrolled in the study. Patients with an SPMSQ ≤ 7, an MMSE < 24 and CDR 1+ were further investigated for a diagnosis of dementia.

Diagnosis of possible/probable AD was made according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke - Alzheimer's Disease and Related Disorders Association Work Group (NINCDS-ADRDA) [33]. Diagnosis of possible/probable vascular dementia (VaD) was made according to the criteria of the National Institute of Neurological Disorders and Stroke - Association Internationale pour la Recherche et l'Enseignement en Neurosciences Work Group (NINDS-AIREN) [34]. Diagnosis of AD or VaD was always supported by neuroimaging evidence (computed tomography scan and/or nuclear magnetic resonance). In particular, the presence of multiple cortical/subcortical infarcts or an infarct in a strategic area such as the thalamus or temporal lobe and/or lesions of the white matter indicated probable VaD. The absence of the above mentioned cerebrovascular lesions indicated AD. Differential diagnosis between AD and VaD was also based on the Hachinski Ischemic Score [35] to address unclear AD/VaD diagnoses. In particular scores ≤ 4 were considered as probable AD, scores ≥ 7 were included into the VaD group that was excluded from the study. Scores between 5 and 6 were diagnosed as mixed dementia (MxD), and also excluded from the study. Diagnosis of dementia with Lewy body (DLB) was made according to

the criteria of DLB Consortium [36]. Diagnosis of FTLD, and in particular of the clinical subtypes bvFTD and PPA, was made according to therevised criteria for bvFTD (37,38) or PPA (39,40). All these patients were excluded from the study. To rule out the possibility of cognitive impairment due to medical or psychiatric conditions, subjects who had a present or past medical or psychiatric condition, or psychoactive substance use that can cause cerebral dysfunction, were excluded from the study. From this study we also excluded: 1) all subjects with serious comorbidity, tumors and other diseases that could be causally related to cognitive impairment, 2) subjects with a history of alcohol or drug abuse, 3) subjects with head trauma, 4) subjects with recent behavioral and personality changes and 5) all subjects with ascertained blood infections and/or other conditions (vitamin B_{12} deficiency, anemia, high medication levels, disorders of the thyroid, kidneys or liver) that can cause memory impairment.

At the end of the screening, a total of 321 patients with a clinical diagnosis of sporadic AD and 486 CH patients were enrolled in the study.

2.5. Genetic analysis

From each patient, a 3ml blood sample was collected in a BD Vacutainer K2E 5.4 mg (ref. 368856) (Becton-Dikinson, Franklin Lake, NJ, USA). All samples were encoded and stored at -20°C before processing. Genomic DNA was purified from frozen blood samples following salting-out method [41].

The three single-nucleotide polymorphism (SNPs) of CASR gene rs1801725 (122,003,757 bases from pter) ($G^{2956}\rightarrow T$) (A986S), rs1042636 (122,003,769 bases from pter) ($G^{2968}\rightarrow A$) (R990G) and rs1081726 (122,003,832 bases from pter) ($G^{3031}\rightarrow T$) (Q1011E) were investigated in blinded fashion. SNP genotypes were determined by DNA sequencing. Briefly, a 320bp fragment of the exon 7 of the CASR gene encompassing all the three SNPs was PCR amplified with the primers forward 5' > CGA AGA CCC ATT CCC ACA GC > 3' and reverse 5' > CGG TCA GAT CTA AGT CCG TT > 3'. PCR product was purified (ExoSAP-IT, GE HealthCare, Life

Science, Piscataway, NJ, USA), directly sequenced (BigDye Terminator v1.1 Cycle Sequencing Kit, Life Technologies Ltd, Paisley, UK) using the reverse primer, and loaded onto an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

The two SNPs rs429358 (50,103,781 bases from pter) (C^{3937}→T) (Arg^{112}→Cys) and rs7412 (50,103,919 bases from pter) (C^{4075}→T) (Arg^{158}→Cys), composing the common APOE polymorphism, were determined as previously described [42].

2.6. Statistical analysis

Patient characteristics in the baseline setting (real-life) were compared according to gender and to cognitive status by means of the Pearson chi-square test (categorical variables) and Mann-Whitney U test (continuous variables). The value of standardized mean difference (SMD) was also reported. Pearson chi-square test was also used to verify the Hardy-Weinberg equilibrium (HWE) for the genotype distributions. To assess the robustness of the results observed from the analysis in the baseline setting, a propensity score (PS) matched case-control setting was derived from the baseline scenery [43,44]. In particular, PS logistic regression statistics were built to predict the probability of AD onset according to age at baseline, sex, APOE (genotypes 3/4 + 4/4 vs. genotypes 2/2 + 2/3 + 2/4 +3/3) plus a set of possible interactions. Based on its propensity score, a 5 to 1 digits greedy 1:1 matching algorithm [44] was used to identify a unique matched CH for each AD patient. This allowed an unbiased comparison between AD and HC patients. Adequacy of covariate balance in the matched sample was eventually assessed with McNemar or Wilcoxon's signed rank test.

To evaluate the statistical association between the presence of the AD clinical phenotype and the CASR rs1801725, rs1042636, rs1801726 SNPs, univariate and multivariate logistic regression statistics were estimated under free, dominant or recessive models of inheritance in both baseline and PS-matched settings. Results were expressed as odds ratios (OR) along with their 95%

confidence intervals (95%CI). P-values < 0.05 were considered statistically significant. All the analyses were performed using SAS Release 9.1.3 (SAS Institute, Cary, NC).

3. Results

3.1. Baseline setting

Patient characteristics according to gender are summarized in Table 1. AD prevalence was higher in females than in males (44.91% vs. 32.21%; p<0.001). No significant difference was observed in the mean age as well as in the distribution of APOE and CASR genotypes.

Patient characteristics according to the observed clinical phenotype are summarized in Table 2. AD was older (74.40 ± 8.62 vs. 46.88 ± 18.20; p < 0.001) and more frequent females (67.29% vs. 54.53%; p < 0.001) than CH patients. Also, a higher frequency of APOE-ε4 carriers in AD than in CH patients was observed (46.11% vs. 18.52%; p < 0.001). No differences in the distribution of CASR genotypes were detected.

3.2. PS-matched setting

The propensity score-based greedy matching algorithm successfully matched 238 (119 AD and 119 CH) of the patients of the baseline sample. After PS matching patient's age, sex, and APOE genotypes were balanced. Patient characteristics according to the observed clinical phenotype are summarized in Table 3. A significant difference was observed in the distribution of rs1801726 genotypes (p = 0.045), meanly due to an underrepresentation of the C/G genotype in AD (3.36% vs. 10.08%). Notably, the statistical trend observed in the baseline setting (p = 0.082) (Table 2) was predictive of this association.

3.3. Logistic regression models

Results of the analysis investigating the association between AD phenotype and CASR genotypes by means of logistic regression models, also using different models of inheritance, are

summarized in Table 4.

The univariate model confirmed the underrepresentation of the rs1801726-C/G genotype in AD (p = 0.048) observed in the PS-matched sample. Thus, the rs1801726-C/G genotype appeared a genetic factor protective against AD (OR = 0.310, 95% CI 0.097 – 0.991). Notably, the statistical trend observed in the univariate analysis of the baseline sample (p = 0.087) was predictive of this association.

The multivariate model confirmed in the baseline sample the results observed in the PS-matched sample. The rs1801726-C/G genotype was confirmed underrepresented in AD (p = 0.007), thus acting as a protective factor (OR = 0.253, 95% CI 0.093 - 0.687). This multivariate model also showed significant differences in the distribution of the rs181725 genotype (p = 0.028), meanly due to an overrepresentation of the T/T genotype in AD (4.98% vs 3.09%), thus acting as a risk factor (OR = 3.888, 95% CI 1.162 – 13.009) meanly in a recessive fashion (p = 0.029; OR = 3.806, 95% CI 1.150 – 12.602). This association, however, was not confirmed in the PS-matched setting.

3.4. Haplotype and linkage disequilibrium analysis

Estimated haplotype frequencies in the PS-matched setting are summarized in Table 5. In both AD and CH patients haplotypes -G-G were the most frequent, accounting more than 90% of the overall haplotype frequencies. All the other haplotypes showed an overall frequency ≤ 5%. No differences were observed in the distribution of the two main haplotypes between the study groups.

A schematic representation of the estimated values of the linkage disequilibrium (LD) coefficient D' in the PS-matched setting is presented in Figure 1. In AD we observed a weak LD between rs1801725 and rs1042636 (D' = 0.02) in respect to CH patients (D' = 1.00). No differences were observed between rs1042636 and rs1801726 and between rs1801725 and rs1801726.

4. Discussion

In the present study we attempt to investigate the possible role of CASR gene in the pathogenesis of Alzheimer's disease throughout the analysis of three functional polymorphisms predictive of alteration in serum calcium concentrations [13,14] and associated disorders [15]. Several experimental data in mice, strongly suggested an involvement of CASR calcium regulation in several biochemical mechanisms underlying AD pathogenesis [4,5,6,7,8,9,10].

To our knowledge this is the first study that attempted to investigate the CASR locus for its possible implications in AD pathogenesis. To this aim we recruited in a real-life case-control setting all consecutive unrelated patients attending the study centres that were selected only for their cognitive phenotypes of sporadic AD or CH, without any a priori selection. Surely such study design undoubtedly presented some limitations, such as the presence of a myriad of environmental and non-environmental factors introducing background noise in the analysis. On the other side, however, such study design strongly reflected the characteristics of the general population of patients attending clinical care, including their genetic background. In such study we expected that only risk factors with a major role emerge from the background, whereas those with a minor role remains occulted. To fill this potential limitation and to confirm the results observed in the analysis of the baseline sample, we derived a case-control setting matched for the main parameters showing differences in the real-life recruitment, i.e. age and sex. In fact as for all observational sources of data, when addressing any comparison between exposure levels, one major issue is the lack of randomization. Thus, a multivariate adjustment of the analysis on the baseline setting might be not sufficient to appropriately reduce potential selection bias. For this reason we also included the APOE genotype in the matching process.

To derive the case-controls matched setting we used the PS methodology. PS methodology is a useful tool to reduce selection bias in observational studies [36,37] and has already been used in many therapeutic fields. Here PS 1:1 matching was used in a confirmatory fashion to assess the robustness of our findings. Accordingly, the analysis on the PS matched setting that result

balanced for sex, age and APOE genotypes, confirmed the suggestions observed in the analysis of the baseline setting, i.e. a potential role of the rs1081726-C/G genotype in AD ($p = 0.082$ in the baseline setting vs. 0.045 in the PS matched setting). The further confirmation of this association in the logistic regression models ($p = 0.007$ in the multivariate analysis of the baseline sample vs. $p = 0.048$ in the univariate analysis of the PS matched sample) warrants the robustness of the OR estimates and the bona fide of our results. Thus, the 1011 Gln/Glu variant of CASR protein appeared to be protective against AD.

Indeed, in the multivariate analysis we also observed an association of the rs1801725-T/T variant (encoding the protein isoforms Ser/Ser), that was overrepresented in AD, thus acting as a risk factor for AD. The presence of genetic variant in the same genetic background balancing the risk for a clinical phenotype is expected from a genetic investigation, and further warrants the bona fide of our study. This association, however, was not confirmed in the analyses of the PS-matched setting. This may be a major limitation of the present study.

A further separated discussion must be dedicated to the association observed for rs1081726-C/G. First of all, this is the association of a heterozygote. Second, in the analysis the rare homozygotes are missing.

With regard to the association of a heterozygote genotype with a phenotype, we extensively discuss this point in previous studies [45,46]. The association of a heterozygote genotype with a given diseases is not a common one. However, it could be explained by molecular heterosis. Subjects heterozygous for a genetic polymorphism may show significantly greater (positive heterosis) or minor (negative heterosis) effects for a quantitative or dichotomous trait than homozygotes for either allele [47].

With regard to the missing identification of the recessive genotypes G/G of rs1042636 and in particular of the G/G of rs1801726, this missing identification may lead to the simplistic conclusion that our sample size is not suitable for such an analysis, and that the association of rs1801726 that we observed may be the results of this limitation (false positive). Indeed, we

decided this investigation with the known that these recessive variants were rare in the Caucasian population [48]. Protein variants R990G and Q1011E are more common in Asian [49,50] and Afro-American populations [49]. However, a total sample of 807 subjects (where 321 had AD and 486 were healthy subjects) and the observed distribution of rs1801725 genotypes allowed to detect an OR of at least 2.78, under a recessive model of inheritance, with an alpha level of 0.05 and a statistical power of 0.80. Thus, the power of our analysis is sufficient to warrants the quality of our results. It must be noted that all the observed genotype frequencies in the baseline and PS-matched samples, including those of rs1042636 and rs1801726 fitted the HWE equilibrium. This was a further warrants of the quality of our study.

Since different haplotypes of CASR have been related to disorder of calcium regulation and associated diseases, in the present study we also estimated the haplotype frequencies and LD involving A986S, R990G and Q1011E polymorphisms in the PS-matched setting. In the analysis, only a significant difference for a minor haplotype was observed. Conversely, the estimation of LD indicates that different LD strength guide the composition of the –COOH tail between AD and CH, suggesting that different genetic background drive rs1801725 and rs1042636 troughout AD pathogenesis

Finally, it must be noted that CASR gene locus (3q21.1) lies in the chromosomal region 3q12.3 – q25.31 that is suggested as a putative liked region for AD [51]. This region also includes dopamine receptor D3 (DRD3) and glycogen synthase kinase 3 beta (GSK3B) genes at locus 3q13.3, and transferrin (TF) gene at locus 3q12. Clearly, we cannot exclude possible linkage disequilibrium of our CASR polymorphism with genetic variants in these genes already associated with AD. Such as analysis is not the aim of the present study.

In conclusion, our results suggested that particular CASR isoforms might be diversely associated with AD. More studies, however, are needed to clarify the role of CASR protein in biochemical pathways underling the pathogenesis of AD.

Acknowledgements

This work was supported by "Ministero della Salute", IRCCS Research Program, Ricerca Corrente 2012-2014, Linea n. 2 "Malattie complesse", by the "5 x 1000" voluntary contribution, and partially by Italian Ministry of Education, University and Research (MIUR), grant no. 7020430 to CM.

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Table 1

Baseline setting: characteristic of patients according to gender.

			Male		Female		p	All
Number of subjects			326		481			807
Age (years)*	Mean ± SD		58.73 ± 19.76		57.22 ± 20.59		0.329	57.83 ± 20.26
	Range (min-max)		25 - 92		25 - 100			25 - 100
Clinical phenotype†	AD		105	(32.21%)	216	(44.91%)	< 0.001	321
	CH		221	(67.79%)	265	(55.09%)		486
APOE†	2/2		--	--	2	(0.42%)	0.238	2
	2/3		36	(11.04%)	38	(7.90%)		74
	2/4		8	(2.45%)	8	(1.66%)		16
	3/3		195	(59.82%)	282	(58.63%)		477
	3/4		82	(25.15%)	136	(28.27%)		218
	4/4		5	(1.53%)	15	(3.12%)		20
rs1801725†	G/G	A/A	203	(62.27%)	306	(63.62%)	0.517	509
	G/T	A/S	113	(34.66%)	154	(32.02%)		267
	T/T	S/S	10	(3.07%)	21	(4.37%)		31
rs1042636†	A/A	R/R	297	(91.10%)	436	(90.64%)	0.824	733
	A/G	R/G	29	(8.90%)	45	(9.36%)		74
	G/G	G/G	--	--	--	--		--
rs1801726†	C/C	Q/Q	306	(93.87%)	460	(95.63%)	0.262	766
	C/G	Q/E	20	(6.13%)	21	(4.37%)		41
	G/G	E/E	--	--	--	--		--

*Mann-Whitney U test.

†Pearson's chi-square test.

Table 2

Baseline setting: characteristic of patients according to the clinical phenotype.

			AD	CH	p	SMD
Number of subjects			321	486		
Age (years)*	Mean ± SD		74.40 ± 8.62	46.88 ± 18.20	< 0.001	193.299
	Range (min-max)		50 - 94	25 - 100		
Gender†	Male		105 (32.71%)	221 (45.47%)	< 0.001	26.383
	Female		216 (67.29%)	265 (54.53%)		
MMSE score			< 26	≥ 28		
CDR			0	1 +		
APOE†	3/4 + 4/4		148 (46.11%)	90 (18.52%)	< 0.001	-61.736
	Others		173 (53.89%)	396 (81.48%)		
rs1801725†,‡	G/G	A/A	201 (62.62%)	308 (63.37%)		1.569
	G/T	A/S	104 (32.40%)	163 (33.54%)	0.386	2.426
	T/T	S/S	16 (4.98%)	15 (3.09%)		-9.656
rs1042636†,§	A/A	R/R	289 (90.03%)	444 (91.36%)		
	A/G	R/G	32 (9.97%)	42 (8.64%)	0.523	-4.569
	G/G	G/G	--	--		
rs1801726†,¶	C/C	Q/Q	310 (96.57%)	456 (93.83%)		
	C/G	Q/E	11 (3.43%)	30 (6.17%)	0.082	12.873
	G/G	E/E	--	--	--	--

*Mann-Whitney U test

†Pearson chi-square test

‡HWE: p = 0.616 (AD), p = 0.317 (CH), p = 0.664 (All).

§HWE: p = 1.000 (AD), p = 1.000 (CH), p = 0.405 (All).

¶HWE: p = 1.000 (AD), p = 1.000 (CH), p = 1.000 (All).

Table 3

PS-matched setting: characteristic of patients according to the clinical phenotype.

			AD		CH		p	SMD
Number of subjects			119		119			
Age (years)*	Mean ± SD		69.78 ± 9.77		70.23 ± 10.78		0.434	- 4.331
	Range (min - max)		50 - 89		50 - 100			
Gender†	Male		70	(58.82%)	72	(60.50%)	0.593	3.426
	Female		49	(41.18%)	47	(39.50%)		
APOE†	3/4 + 4/4		34	(28.57%)	30	(25.21%)	0.371	- 7.586
	Others		85	(71.43%)	89	(74.79%)		
rs1801725†‡	G/G	A/A	83	(69.75%)	82	(68.91%)		- 1.822
	G/T	A/S	29	(24.37%)	35	(29.41%)	0.244	11.390
	T/T	S/S	7	(5.88%)	2	(1.68%)		- 22.162
rs1042636†,§	A/A	R/R	103	(86.55%)	107	(89.92%)		
	A/G	R/G	16	(13.45%)	12	(10.08%)	0.433	- 10.447
	G/G	G/G	--	--	--	--		
rs1801726†,¶	C/C	Q/Q	115	(96.64%)	107	(89.92%)		
	C/G	Q/E	4	(3.36%)	12	(10.08%)	0.045	27.091
	G/G	E/E	--	--	--	--		

*McNemar test.

†Wilcoxon signed rank test.

‡HWE: p = 0.062 (AD), p = 0.736 (CH), p = 0.365 (All).

§HWE: p = 1.000 (AD), p = 1.000 (CH), p = 1.000 (All).

¶HWE: p = 1.000 (AD), p = 1.000 (CH), p = 1.000 (All).

Table 4

Logistic regression models to evaluate the association between AD phenotype and CASR genotypes.

Genetic model		Univariate*						Multivariate†		
		Baseline sample			PS-matched sample			(only baseline sample)		
		p	OR	95%CI	p†	OR	95%CI	p	OR	95%CI
					rs1801725					
	G/G	--	1.000	--	--	1.000	--	--	1.000	--
	G/T	0.884	0.978	0.722 - 1.324	0.498	0.818	0.459 - 1.461	0.792	1.064	0.672 - 1.684
	T/T	0.185	1.634	0.790 - 3.380	0.129	3.458	0.698 - 17.141	0.028	3.888	1.162 - 13.009
D‡	G/G	--	1.000	--	--	1.000	--	--	1.000	--
	G/T + T/T	0.827	1.033	0.772 - 1.383	0.888	0.961	0.554 - 1.668	0.406	1.206	0.775 - 1.879
R§	G/G + G/T	--	1.000	--	--	1.000	--	--	1.000	--
	T/T	0.174	1.647	0.803 - 3.381	0.111	3.656	0.744 - 17.978	0.029	3.806	1.150 - 12.602
					rs1042636					
D‡	A/A	--	1.000	--	--	1.000	--	--	1.000	--
	A/G	0.523	1.171	0.722 - 1.898	0.422	1.385	0.625 - 3.070	0.655	1.176	0.577 - 2.396

		rs1801726								
D‡	C/C	--	1.000	--	--	1.000	--	--	1.000	--
	C/G	0.087	0.54	0.266 - 1.093	0.048	0.310	0.097 - 0.991	0.007	0.253	0.093 - 0.687

*Univariate logistic regression.

†Logistic regression model including age, sex, age-by-sex and APOE genotypes (3/4 + 4/4 *vs* other).

‡Dominant model of inheritance.

§Recessive model of inheritance.

Table 5

PS-matched setting: estimated haplotype frequencies in AD and CH patients.

Haplotype			Protein isoform*			Frequency		χ^2	p	All
rs1801725	rs1042636	rs1801726	986	990	1011	AD	CH			
G	G	G	A	G	E	0.742	0.738	0.013	0.910	0.740
T	G	G	S	G	E	0.174	0.162	0.128	0.720	0.168
G	A	G	A	R	E	0.060	0.048	0.348	0.555	0.054
G	G	C	A	G	Q	0.017	0.050	4.139	0.042	0.034

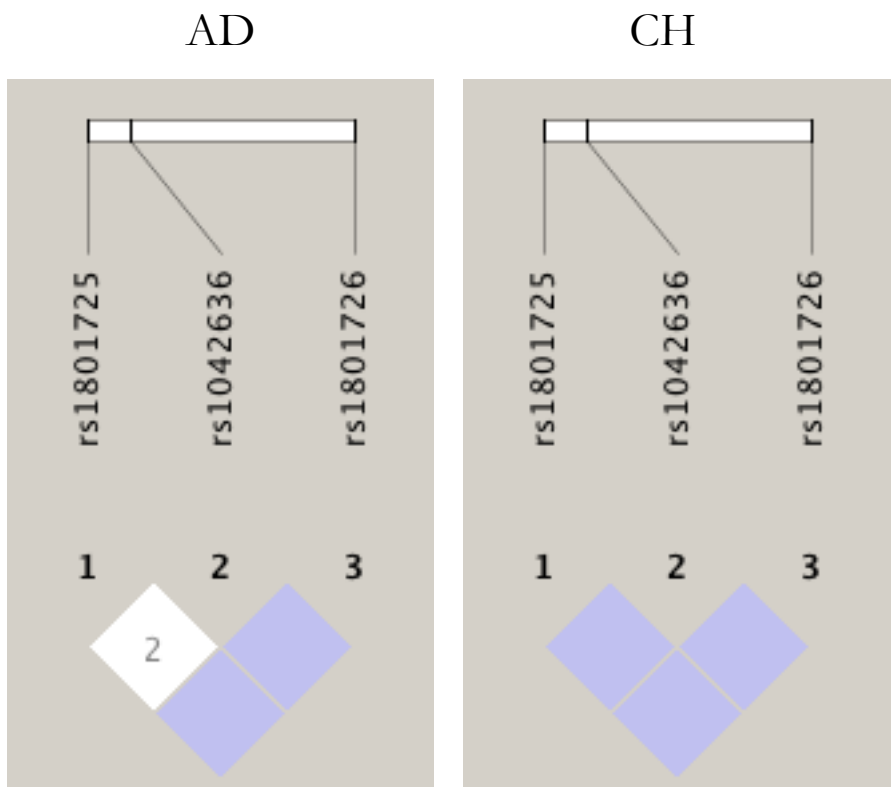
*Estimated amino acid residues (A: Ala, E: Glu, Q: Gln, R: Arg, S: Ser) in the intracellular -COOH tail of CASR protein.

Figure 1

PS-matched setting: graphical representation of the estimated linkage disequilibrium coefficient D'.

Figure(s)

Figure 1



Research in context

Systematic review: Only three reviews [1,2,3] recently suggested a role of calcium sensing receptor in AD pathogenesis and, to our knowledge, up to date no association study has been reported. Notably, using the keywords "calcium sensing receptor" and "Alzheimer's disease" to search for paper in PubMed, only one record is find [1].

Interpretation: CASR gene lies on the long arm of chromosome 3, in a putative linked region for AD (3q12.3 – q25.31). Our results contribute to the knowledgebase of AD genetics linked with this chromosomal region.

Future directions: If confirmed into wide samples of highly selected AD cases, our results are the first supporting a role of CASR gene polimorphisms in AD pathogenesis.

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