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Identification and Confirmation of an Exonic Splicing Enhancer Variation in Exon 5 of the Alzheimer Disease Associated *PICALM* Gene

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SUMMARY

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by memory and cognitive impairment and is the leading cause of dementia in the elderly. A number of genome wide association studies and subsequent replication studies have been published recently on late onset AD (LOAD). These studies identified several new susceptibility genes including phosphatidylinositol-binding clathrin assembly protein (*PICALM*) on chromosome 11. The aim of our study was to examine the entire coding sequence of *PICALM* to determine if the association could be explained by any previously undetected sequence variation. Therefore, we sequenced 48 cases and 48 controls homozygous for the risk allele in the signal SNP rs3851179. We did not find any new variants; however, rs592297, a known coding synonymous SNP that is part of an exonic splice enhancer region in exon 5, is in strong linkage disequilibrium with rs3851179 and should be examined for functional significance in Alzheimer pathophysiology.

Keywords

Alzheimer; Neurodegenerative disease; PICALM; Sequencing; Exonic splicing

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Amplification protocol

Table S1: primer sequences

Table S2: Allele frequencies for rs592297

Figure S1: PICALM exon 5 sequence

Figure S2: Representation of the PICALM gene. All the coding SNPs from dbSNP (list obtained April 5th 2011) were added to the figure next to their corresponding coding region.

Figure S3: ESE results for exon 5 (http://rulai.cshl.edu/tools/ESE2/)

Figure S4: Linkage disequilibrium schematic for PICALM

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by memory and cognitive impairment, and is the leading cause of dementia in the elderly. Mutations in three genes encoding β-amyloid (APP), presenilin 1 (PS1), and presenilin 2 (PS2) cause rare Mendelian early onset AD (EOAD) (Brouwers et al. 2008). One common allele (ε4) in the apolipoprotein E gene (APOE) has been unequivocally accepted as an important risk factor for late onset Alzheimer's disease (LOAD) (Corder et al. 1993), while another allele (ε2) decreases risk (Corder et al. 1994). A number of genome wide association studies (GWAS) and subsequent replication studies have been recently published on LOAD (Carrasquillo et al. 2010; Corneveaux et al. 2010; Harold et al. 2009; Hollingworth et al. 2011; Jun et al. 2010; Lambert et al. 2009; Naj et al. 2011; Seshadri et al. 2010). These studies identified several new susceptibility genes including phosphatidylinositol-binding clathrin assembly protein (*PICALM*). Because GWAS associations rarely identify the actual susceptibility variation, additional detailed studies of the implicated genes are warranted. The aim of this study was to examine the coding sequence of *PICALM* to determine if the observed association could be explained by any previously undetected sequence variation.

Methods

Ascertainment and Samples

Our sample set is derived from the Collaborative Alzheimer Project (CAP: The Hussman Institute for Human Genomics at the Miller School of Medicine, University of Miami and The Center for Human Genetics Research at the Vanderbilt University School of Medicine). After a complete description of the study to the subjects, written informed consent was obtained from all participants in agreement with protocols approved by the institutional review board at each contributing center. For inclusion, each LOAD affected individual met the NINCDS/ADRDA criteria for definite or probable AD and had an age at onset greater than 60 years of age. Age at onset (AAO) for LOAD was determined from specific probe questions within the clinical history provided by a reliable family informant or documented significant impairment in the medical record. Cognitive controls were spouses, friends, and other biologically unrelated individuals who were frequency age and gender matched to the cases, and all were from within the same clinical catchment areas. All cognitive controls were examined and none showed signs of dementia by history and upon interview. Additionally, cognitive controls each have a documented Mini-Mental State Exam (MMSE) ≥ 27 or Modified Mini-Mental State Exam (3MS) ≥ 87 (Teng & Chui, 1987). The sample set consisted of 48 cases and 48 controls who were T/T homozygotes for the protective allele at rs3851179, the signal SNP previously associated to LOAD found at the 5' end of the *PICALM* gene (Harold et al. 2009; Jun et al. 2010; Seshadri et al. 2010).

Sequencing

Twenty one amplicons for the twenty four different exons and UTRs were optimized for amplification (see Supplementary Information for details) and the products were sequenced using ABI's 3730xl DNA analyzer (Life Technologies Corporation, Carlsbad, CA, 92008, USA) in the Vanderbilt University DNA Sequencing Core Facility. Both forward and reverse strands were sequenced to provide confirmation of any changes. Sequencher (Gene Codes Corporation, Ann Arbor, MI) was used to align the sequences; both the forward and the reverse fragment were aligned to confirm all possible variants.

ESEfinder

The program ESEfinder (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home) was used to determine the predicted effects of variation at rs592297 on the binding of exon splicing enhancers. ESEfinder 2.0 was used with a matrix library "SR proteins" and the default (thresholds) settings (Cartegni et al. 2003). We added ~20bp on each side of exon 5 as input for ESEfinder, in total 138bp was submitted to ESEfinder.

Results and Discussion

PICALM is one of the four currently accepted associated genes for LOAD (along with *APOE, CLU, and CR1*). Recent studies have identified rs3851179, a SNP at the 5' end of the *PICALM* gene and thought to be part of a regulatory sequence, as being associated to LOAD with a combined odds ratio = 0.88 (95% CI, 0.86–0.91) for the T allele across all the studies (http://www.alzgene.org/meta.asp?geneid=636&polyID=2656) (Bertram et al. 2007). In the GWAS published by Harold et al. (Harold et al. 2009), several functional SNPs were identified at the PICALM locus. Two SNPs, rs561655 (meta analysis, $p=1-10^{-7}$), which is within a putative transcription factor binding site, and rs592297 (meta analysis, $p=2\times10^{-7}$), which is a synonymous SNP in exon 5 that may influence a predicted exon splicing enhancer (ESE) sequence, showed evidence for association. The association for rs561655 was confirmed in the Alzheimer Disease Genetics Consortium (ADGC) GWAS (Hollingworth et al. 2011; Jun et al. 2010; Naj et al. 2011). In addition, two longitudinal studies suggested that rs7110631 in *PICALM*, a SNP in high LD with rs3851179, is associated with age-related cognitive decline (Chibnik et al. 2011).

Further studies performed on other populations have revealed a strong architectural complexity for *PICALM* (Greene et al. 2009). A Caribbean Hispanic cohort study used to identify new LOAD associated loci and to replicate existing findings found that only rs17159904 was marginally associated in population stratification-adjusted and APOE-adjusted analyses (Lee et al. 2010). This SNP is situated between the 5' end of *PICALM* and rs3851179. A mixed Hispanic, African American, and Wadi Ari population was also examined but no association to *PICALM* was detected. It was hypothesized that the lack of association was the result of the small sample size (Jun et al. 2010). In a Han Chinese population the association between rs3851179 and LOAD could not be replicated (Yu et al. 2010). The authors concluded that rs381179 might not play a major role in the development of LOAD in the Han Chinese population. They pointed out that their study had only 266 cases and 343 controls and might be underpowered, or that population specificity might play a role, and finally that their results required further confirmation as rs3851179 was the only SNP to be genotyped (Yu et al. 2010). Another study by Li et al. confirmed the lack of association between *PICALM* and isolated Alzheimer's disease in Chinese (Li et al. 2011). No association was found between *PICALM* rs3851179 and Italian LOAD subjects, however a possible role of *PICALM* in longevity was suggested (Piaceri et al. 2011).

PICALM was first described in U937 cell lines as the clathrin assembly lymphoid myeloid leukemia (*CALM*) gene (Dreyling et al. 1996). Its N-terminus binds to phosphatidylinositol-4,5-biphosphate present in the plasma membrane and its C-terminus to clathrin and adaptor protein 2 (AP-2), recruiting both to the membrane (Baig et al. 2010). *PICALM* is important in clathrin-mediated endocytosis and intracellular trafficking of the synaptic vesicle protein VAMP2 (Harel et al. 2008). VAMP2 is a soluble N-ethylmaleimide-sensitive attachment protein receptor (SNARE) protein that is involved in neurotransmitter release at the presynaptic membrane, which is important for neuronal function and memory formation (Harold et al. 2009; Sleegers et al. 2010). Furthermore, *PICALM* may also be involved in the regulation of β-amyloid levels through the degradation of APP upon entry into endosomes and Aβ production (Kyriazis et al. 2008). *PICALM* is expressed in all tissue

types with prominent expression in neurons (Harold et al. 2009) and is present in synapses (Harel et al. 2008). *PICALM* is a large gene (112kb, http://www.ensembl.org, ENSG00000073921, GRCh37, May 2011) on 11q23, with 28 alternative transcripts, 19 of them encoding for a protein, and ranging in size from 134 residues to 660 residues. *PICALM*, like most eukaryotic genes, is comprised of multiple relatively short exons that are separated by longer introns (Fig. 1).

Point mutations in the coding regions of genes are commonly assumed to exert their effects by altering single amino acids in the encoded proteins. However, there is increasing evidence that many human disease genes harbor exonic mutations that affect pre-mRNA splicing (Cartegni et al. 2002). The mechanism of splicing is complex due to the presence of numerous intronic pseudo exons; therefore, the additional information required for "true" splicing is contained in cis-acting regulatory enhancer and silencer sequences (Cartegniet al. 2002).

For our sequencing of the exonic regions of *PICALM*, we picked 48 cases and 48 controls that were homozygous for the protective allele for rs3851179 and that were of mixed APOE status (see Supplementary Information for details on primer sequences and PCR protocol). Our study was designed to detect rare variants with a minor allele frequency of 0.01, and even though we selected for homozygotes at rs3851179 to provide a more consistent background for the search of new variants, we did not find any new exonic polymorphisms or rare variants; only heterozygotes for the previously known SNPs rs76719109 and rs592297 were detected (see supplementary material, Fig. 1 for details). Thus the strong association in this region is unlikely to be explained by a novel coding variant in *PICALM*.

We saw altered, but non-significant (when corrected for multiple comparison), allele frequencies for rs592297 in exon 5 (see Table S2). Even though this SNP is approximately 143 kb from rs3851179, they are in strong linkage disequilibrium (LD). A slight increase in LD is observed between the ADGC GWAS data (Jun et al. 2010) and the HapMap data (GWAS D'=0.937, r^2=0.40 and HapMap D'=0.923, r^2=0.28). The bias in sampling, only T/T homozygotes, and the high correlation between the two SNPs likely explains the altered genotype frequencies that we see in our sample set.

Alteration of the allele for rs592297 from a G to an A changes the predicted exon enhancer binding at this site from an SF2/ASF protein to SRp40 (see Fig. S2). This alteration of the ESE could change the splicing pattern at exon 5. However, there are limitations in using ESEfinder; the presence of a high-score motif in a sequence, as seen in Figure S2, does not necessarily identify that sequence as a functional ESE, and in general, there is not a very strict quantitative correlation between numerical scores and ESE activity (Cartegni et al. 2003). Direct experimental evidence is necessary before we can reach a final conclusion. Reverse transcriptase PCR experiments could be designed for the different cell lines, with the different genotypes, to detect possible splicing differences. The lack of additional variation within the coding sequence of the gene suggests that novel variation in *PICALM* does not explain the association results and that the known variation should be explored further. Expression studies could be designed using the known coding variant in order to see if the variation could indeed change the expression of *PICALM*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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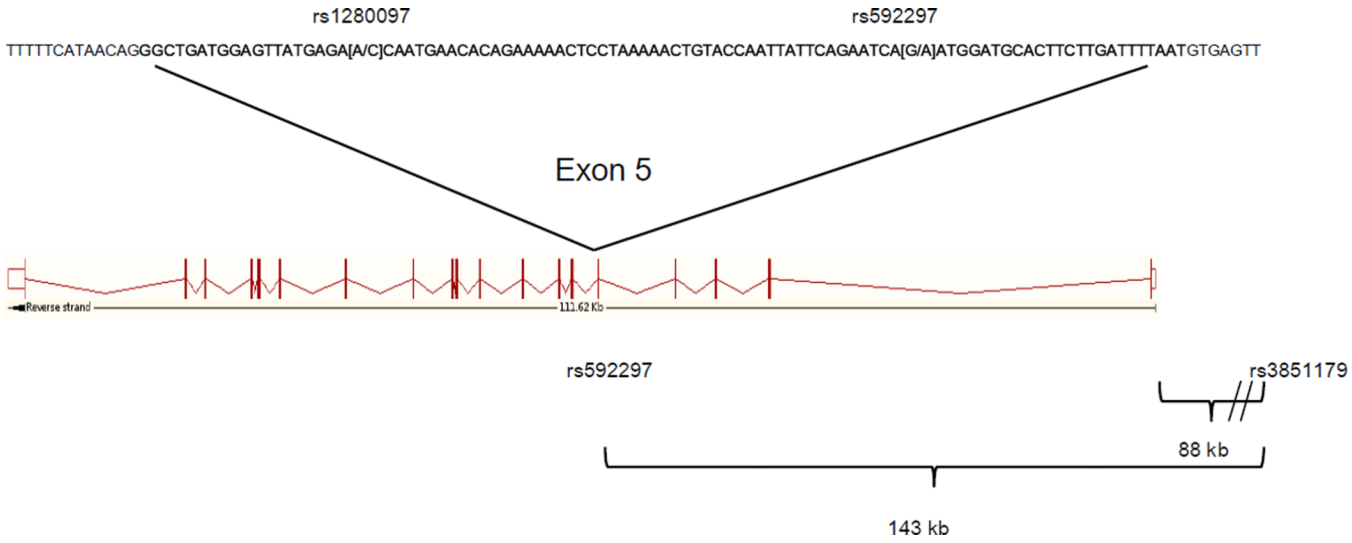

Figure 1.

A *PICALM* representation from http://www.ensembl.org. The full sequence of exon 5 is represented in bold and the location of the SNPs and base pair change can be seen on the sequence. Rs3851179, has a combined OR= 0.88 for all studies (http://www.AlzGene.org, April 2011), and is 88kb from the 5' end of the gene, and 143kb from rs592297 that has a p value of 2×10^{-7} in theHarold et al. (2009) meta analysis.