Ultra-rare mutations in \textit{SRCAP} segregate in Caribbean Hispanic families with Alzheimer disease

\textbf{ABSTRACT}

\textbf{Objective:} To identify rare coding variants segregating with late-onset Alzheimer disease (LOAD) in Caribbean Hispanic families.

\textbf{Methods:} Whole-exome sequencing (WES) was completed in 110 individuals from 31 Caribbean Hispanic families without \textit{APOE} \epsilon4 homozygous carriers. Rare coding mutations segregating in families were subsequently genotyped in additional families and in an independent cohort of Caribbean Hispanic patients and controls. SRCAP messenger RNA (mRNA) expression was assessed in whole blood from mutation carriers with LOAD, noncarriers with LOAD, and healthy elderly controls, and also from autopsied brains in 2 clinical neuropathologic cohort studies of aging and dementia.

\textbf{Results:} Ten ultra-rare missense mutations in the Snf2-related \textit{CREBBP}, activator protein (SRCAP), were found in 12 unrelated families. Compared with the frequency in Caribbean Hispanic controls and the Latino population in the Exome Aggregation Consortium, the frequency of SRCAP mutations among Caribbean Hispanic patients with LOAD was significantly enriched (\(p = 1.19e-16\)). mRNA expression of SRCAP in whole blood was significantly lower in mutation carriers with LOAD, while the expression in whole blood and in the brain was significantly higher in nonmutation carriers with LOAD. Brain expression also correlated with clinical and neuropathologic endophenotypes.

\textbf{Conclusions:} WES in Caribbean Hispanic families with LOAD revealed ultra-rare missense mutations in SRCAP, a gene expressed in the brain and mutated in Floating-Harbor syndrome. SRCAP is a potent coactivator of the CREB-binding protein and a regulator of DNA damage response involving ATP-dependent chromatin remodeling. We hypothesize that increased expression in LOAD suggests a compensatory mechanism altered in mutation carriers.

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\textbf{GLOSSARY}

\textbf{CBP} = CREB-binding protein; \textbf{CI} = confidence interval; \textbf{DLPFC} = dorsolateral prefrontal cortex; \textbf{ExAC} = Exome Aggregation Consortium; \textbf{FPKM} = fragments per kilobase per million fragments mapped; \textbf{GEE} = generalized estimating equation; \textbf{IRB} = institutional review board; \textbf{LOAD} = late-onset Alzheimer disease; \textbf{MCI} = mild cognitive impairment; \textbf{mRNA} = messenger RNA; \textbf{OR} = odds ratio; \textbf{QC} = quality control; \textbf{SNP} = single nucleotide polymorphism; \textbf{SNV} = single nucleotide variant; \textbf{WES} = whole-exome sequencing.

Progress has been made in understanding the genetics of late-onset Alzheimer disease (LOAD),\textsuperscript{1,2} but gaps in its genetic influence still need investigation. Common variants play a role in disease risk, but functionally important rare or ultra-rare variants may help to explain the remaining heritability\textsuperscript{2,3} undetected by genome-wide association studies. Sequencing of large families multiply affected by LOAD increases the ability to detect novel variants conferring risk.

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The frequency of LOAD among Caribbean Hispanic multiplex families from the Dominican Republic was 5 times higher than expected for similarly aged individuals in a non-Hispanic white population from the United States, and inbreeding was a significant predictor of LOAD in this population after adjusting for APOE ε4 genotype, an established genetic risk factor.

To identify novel variants associated with the risk of LOAD, we conducted whole-exome sequencing in 31 Caribbean Hispanic families (table e-1 at Neurology.org/ng) with 4 or more affected individuals, no mutations in known AD genes, specifically PSEN1, PSEN2, or APP, and no APOE ε4 homozygotes. For each family, we sequenced at least 2 affected and 1 unaffected member aged 65 years or older.

**METHODS Sample selection.** Families were recruited as a part of a 15-year family-based study with institutional review board (IRB) approval based in the Dominican Republic. Thirty-one families (98 affected and 12 unaffected individuals) were selected for sequencing (mean age at onset was 74.8 ± 8.3 years, and 63.1% were women) (table e-1). All family members had standard neuropsychological tests and neurologic examinations to verify their clinical status and for diagnoses based on NINCDS-ADRDA criteria.

**Postmortem human brain samples.** Data were obtained from 2 clinical neuropathologic cohort studies: the ROS and the MAP. The IRB of Rush University Medical Center previously approved both studies. Clinical evaluations were used to determine NINCDS-ADRDA criteria for dementia annually. At death, a clinical diagnosis opinion was provided by a neurologist.

Neuropathologic evaluations included neuritic plaques, diffuse plaques, and neurofibrillary tangles in 5 cortical regions, scaled and averaged to obtain a composite score. Participants who met intermediate or high likelihood were rendered pathologic diagnosis of LOAD.

**WHOLE-EXOME SEQUENCING Sample preparation.** Qiagen’s Gentra Puregene and FlexiGene kits were used to extract high-molecular-weight DNA from fresh or frozen (≤ –80°C) samples. DNA from saliva was isolated using prepIT.L2P (DNA Genotypek Inc., Ottawa, ON, Canada). Cell lines from lymphocytes (in 13 probands) were used when high-quality blood DNA was not available. The concentration of DNA was determined using a NanoDrop spectrophotometer.

**Sequencing.** The Illumina TruSeq DNA preparation kit was used to prepare and index genomic DNA libraries. Custom oligonucleotide baits in the TruSeq Exome Enrichment kit were used to capture coding regions and splice sites and amplified according to the Illumina protocol. The DNA samples were multiplexed in batches of 12 samples with index “barcode” primers. These were sequenced using the Illumina Genome Analyzer IIX, HiSeq 2000, and MiSeq platforms (illumina.com) as paired-end reads over 82–307 cycles. Demultiplexing by barcode retrieved individual samples from sequencing pools. We obtained a high coverage across the samples at an average depth of >60× per sample.

**Follow-up genotyping.** Putative variants were confirmed and population frequencies estimated by genotyping the discovery samples, additional family members, and unrelated controls of the same ancestry (table e-1). Allele frequencies of novel variants were estimated from 1,949 unrelated patients and 318 healthy elderly controls similar in age and ancestry. Genotypes were generated using the KASP genotyping technology, which uses allele-specific PCR for accurate calling of single nucleotide polymorphisms (SNPs) and Indels.

**Analytical methods.** Burrows-Wheeler Aligner was used to align sequence reads to the reference genome build 37. Sequencing data quality control (QC) was performed using the Genome Analysis Toolkit (GATK), followed by variant calling using the UnifiedGenotyper and VariantRecalibrator modules. Variants that passed QC were annotated by ANNOVAR that included functional prediction by SIFT and PolyPhen.

**STATISTICAL METHODS** Association tests. Variants that were validated by follow-up genotyping were tested for association with LOAD using generalized estimating equations (GEEs), which accounts for the familial correlation. We adjusted for age and sex using data from the families and unrelated healthy controls.

We used GEE to conduct single variant and burden tests.

For joint analyses of multiple variants, we summed the number of rare variant alleles found in all individuals and tested association using GEE, adjusting for familial correlation, age, and sex, and APOE ε4. The p value threshold required to define statistical significance using a rare variant burden analysis for 20,000 genes would be 2.5 × 10⁻⁶. However, this would be difficult to achieve by assessing rare or ultra-rare variants in a data set of this size. Thus, to determine whether or not the variants discovered were enriched in families with AD in subsequent analyses, we used data from the Exome Aggregation Consortium (ExAC) combined with the Caribbean Hispanic controls.

**Exome Aggregation Consortium.** The ExAC database contains whole-exome data from 60,706 unrelated adults sequenced as part of various disease-specific (excluding AD) and population genetic studies from 6 ethnic groups. For disease association analyses, we
compared the allele frequencies of suspected variants in the healthy controls from the Caribbean Hispanic cohort to the Latino, Caucasian, and African subpopulations of the ExAC database using a Fisher exact test to avoid differences based on ancestry. Subsequent disease-associated analyses were only conducted when there were no statistically significant differences in allele frequency between the Caribbean Hispanic controls and the ExAC subpopulations. To confirm associations of genetic variants with LOAD, we compared the variant allele frequencies by selecting 1 patient from each Caribbean Hispanic family with both unaffected unrelated controls and ExAC combined using a Fisher exact test.

**Gene expression in the postmortem human brain.** RNA-Seq data came from the 541 ROS-MAP postmortem human brain samples (average age at death was 88.4 years; 63.0% were women and 97.4% self-identified Caucasian ancestry). RNA was extracted from the gray matter of frozen dorsolateral prefrontal cortex tissue using the Qiagen miRNeasy mini kit (Cat. no. 217004) and the RNase-free DNase Set (Cat. no. 79254) and quantified using Nanodrop. RNA-Seq library was prepared on the Broad Institute’s Genomics Platform using the strand-specific dUTP method with poly-A selection. Sequencing was performed on the Illumina HiSeq with 101bp paired-end reads and minimal coverage of 50M reads. Reads were aligned to the reference genome (GRCh37/hg19) using Bowtie and gene expression, measured as fragments per kilobase per million fragments mapped (FPKM), and estimated using RSEM software. Quantile normalization was applied to the FPKM calls, with batch effect removed using Combat. Logarithm base 2 transformation was applied to the gene expression level prior to the analyses.

The Student t test compared brain expression levels between LOAD and controls. Regression models were used to examine the association of gene expression with LOAD and with level of cognition proximate to death. Regression models were used to examine the relation between gene expression and a postmortem diagnosis of LOAD or with LOAD pathology. All models were adjusted for age at death, sex, education, postmortem interval RNA integrity number, and APOE e4 status. Analyses were performed using SAS software, version 9.3, of the SAS(R) system for Linux.

**RESULTS Whole-exome sequencing.** Variant calling, recalibration, and application of QC filters resulted in 290,623 single nucleotide variants (SNVs) and indels called across the 110 individuals from the 31 families (figure 1). The mean depth of sequence coverage was over 60-fold across individuals. We prioritized damaging SNVs by SIFT or by PolyPhen and indels in 78 genes segregating with LOAD in families (figure e-1, A and B), which were confirmed by genotyping in the discovery families, additional family members (265 affected and 61 unaffected individuals), and a set of 318 unrelated controls of similar age and ancestry. Using GEE adjusted for age, sex, and APOE genotype, we found 22 (28%) of the 78 selected variants (in 22 genes) significantly associated with LOAD (p = 6.4e-03 corrected for multiple testing) (table e-2). Only 4 of these 22 genes were known to be expressed in brain—ADCY6, CIT, SRCAP, and SVOPL. The variants in SRCAP,
Table 1  Missense variants in SRCAP found in WES of Caribbean Hispanic families

| Chr | Start | Gene | Ref | Obs | AA change | Discovery WES | Genotyping in discovery families | Validation genotyping cohort | ExAC frequencies |
|-----|-------|------|-----|-----|-----------|---------------|--------------------------------|----------------------------|------------------|
|     |       |      |     |     |           | AFF samples | FAM*          | AFF samples | FAM*          | Frequency in familial load cases | Frequency in elderly unaffected (>65 y) | Latino | Global frequency |
| 16 | 30731484 | SRCAP | G | A | R940Q | 1 | 1 | 1 | 1 | 4.30e-04 | 2.92e-03 | 8.64e-05  | 8.24e-06 |
| 16 | 30732143 | SRCAP | T | C | S1033P | 5 | 3 | 12 | 4 | 8.93e-03 | 5.85e-03 | 7.45e-03  | 7.41e-04 |
| 16 | 30732558 | SRCAP | C | A | T1101K | 3 | 1 | 9 | 1 | 7.63e-03 | 2.92e-03 | 2.59e-03  | 6.00e-03 |
| 16 | 30732600 | SRCAP | G | A | R1115H | 4 | 1 | 6 | 1 | 1.28e-03 | 0 | 0 | 8.24e-06 |
| 16 | 30732644 | SRCAP | C | T | P1130S | 1 | 1 | 2 | 2 | 4.71e-03 | 7.35e-03 | 2.94e-03  | 2.57e-03 |
| 16 | 30734934 | SRCAP | A | G | I1397V | 3 | 1 | 4 | 1 | 1.93e-03 | 0 | 0.00e+00  | 3.30e-04 |
| 16 | 30748932 | SRCAP | C | G | S2524C | 2 | 1 | 1 | 1 | 1.27e-02 | 2.96e-03 | 1.30e-03  | 2.04e-03 |
| 16 | 30749583 | SRCAP | C | G | P2741R | 3 | 1 | 6 | 2 | 1.28e-03 | 0 | 0 | 0 |
| 16 | 30749652 | SRCAP | T | G | V2764G | 1 | 1 | 2 | 1 | 6.66e-04 | 0 | 0 | 0 |
| 16 | 30750116 | SRCAP | C | T | L2919F | 1 | 1 | 5 | 1 | 2.58e-03 | 0 | 6.05e-04  | 1.35e-03 |

Abbreviations: ExAC = Exome Aggregation Consortium; WES = whole-exome sequencing.

*AA change: amino acid change caused by the mutation.

bDiscovery WES: number of affected individuals and families identified as carrying an SRCAP variant in the WES experiment in 110 individuals (31 families).

cGenotyping in discovery families: number of affected individuals and families identified as carrying an SRCAP variant in the validation genotyping cohort in all the members of the 31 discovery families.

dAFF samples: number of familial-affected samples carrying the SRCAP variant.

eFAM: number of families carrying the SRCAP variant.

ADCY6, and SVOPL were absent in unaffected Caribbean Hispanic individuals, but only SRCAP met criteria for further analyses by having multiple, putatively damaging variants segregating in more than 1 of the 31 families.

We observed 10 ultra-rare missense mutations, defined as having an allele frequency ≤0.5%, in SRCAP in 12 families (table 1). Seven of these mutations were predicted to be damaging by either PolyPhen or SIFT and had a Combined Annotation Dependent Depletion (CADD) score > 10. Twenty-four (24.4%) of the 98 affected individuals sequenced within the 31 families were carriers of SRCAP mutations. This was significantly higher than the expected frequency based on data from ExAC database (1.79 expected mutations; p = 1.38e-05 using a binomial test).

All 10 SRCAP mutations were genotyped in additional members of the 31 families and segregated with affection status but with incomplete penetrance (figures e-3, A–D and e-4). Of the 265 patients with LOAD in the 31 families, 47 patients (17.67%) carried at least 1 of the 10 missense mutations in SRCAP compared with 2 of the 61 (3.2%) unaffected individuals from these families. Variant p.R1115H segregated with LOAD in the largest sibship in an age-dependent manner (supplementary methods, figure e-2, tables e-3, a and b) and was absent in Caribbean Hispanic controls and ExAC individuals of European, Latino, and African ancestry. p.R1115H was also found within 0.35 MB of a linkage peak (logarithm (base 10) of odds = 1.8). A single patient was homozygous for an SRCAP p.S2524C mutation and had an earlier age at onset compared with the average age in the cohort (63 vs 73.8 years). In family 1755 (figure 2), there were 2 compound heterozygous carriers (p.L2919F and p.S1033P) with earlier ages at onset (55 and 59 years) compared with LOAD patients without SRCAP mutations (p = 0.023) or patients who had a single SRCAP mutation (p = 0.018). Of interest, no unaffected family member or unrelated control was a homozygous or compound heterozygous carrier.

A joint burden analysis within the 31 discovery families using GEE adjusted for familial correlation, age, sex, and APOE genotype found a 6-fold increase in the risk of LOAD (odds ratio [OR] = 5.94; 95% confidence interval [CI] 1.59–22.27, p = 8.2e-03). Adding the additional 47 families resulted in a 2.5-fold risk of LOAD in families (OR = 2.54; 95% CI 1.03–6.21, p = 0.04). We then tested the joint burden of the 10 SRCAP variants in all the genotyped individuals in both sets of families, 1,949 unrelated LOAD cases and 318 unrelated elderly controls (table e-1b). The risk of LOAD associated with SRCAP remained increased (OR = 1.92; 95% CI 1.13–3.28, p = 0.016) after including APOE ε4 in the model (OR = 1.82; 95% CI 1.04–3.19, p = 0.036).
To reach genome-wide significance ($p = 2.5e-06$) in a gene-based test, assuming an OR of 2.0 and 5% causal SNPs and $\beta = 0.8$, a gene of average size, such as SRCAP, requires an extremely large sample size ($N = 42,000$). Therefore, we compared the combined genotyped data mentioned above with data from the ExAC database. Using a Fisher exact test, we found no statistically significant differences in the allele frequencies of the SRCAP variants in healthy Caribbean Hispanic controls compared with the Latino, African American, and white, non-Hispanics in the ExAC database. We then compared the frequencies of SRCAP variants of LOAD patients with Caribbean Hispanic controls alone or with ExAC Latino controls, ExAC African American controls, or ExAC white, non-Hispanic controls. Using one mutation carrier per family, we compared the number of variant alleles in the LOAD patients with the number of alleles in the ExAC database. Two variants, p.P2741R and p.V2764G, were not found in the ExAC database (table 1). We used the total number of alleles genotyped or sequenced for the 10 mutations as the denominator for the Fisher exact test. The enrichment of SRCAP variants in the Caribbean Hispanic patients with LOAD was highly significant at $p = 1.19e-16$. Similarly, the enrichment was statistically significant for white, non-Hispanics, and the African American ethnic groups in the ExAC database (table e-4; $p = 1.51e-21$ for white, non-Hispanics; $p = 3e-04$ for African Americans). Five of the 10 SRCAP mutations were not found in the Caribbean Hispanic controls, and 4 of these were absent in the ExAC Latino data (table 1). Restricting analyses to these 5 SNPs only, the enrichment remained significant $p = 3.55e-07$.

SRCAP is highly intolerant toward loss of function and missense mutations. SRCAP’s intolerance score (2.23) in the ExAC ranks in the 18th percentile and in the top 0.15 and 0.3 percentiles of genes ranked by the Residual Variation Intolerance Score (RVIS) using the ExAC and Exome Sequencing Project data sets, respectively.

Replication in the AD Neuroimaging Initiative data set. Using data from the Alzheimer Disease Neuroimaging Initiative (ADNI) data set, we repeated the analysis of the exonic regions of SRCAP. The analyses included 213 patients with LOAD, 304 individuals with mild cognitive impairment (MCI), and 214 healthy controls of white, non-Hispanic ancestry. We found a higher frequency of nonsynonymous mutations in LOAD and MCI cases (10.4%) vs controls (7.4%) representing a 40% enrichment. Because of the sample size, we once again compared the frequency of nonsynonymous mutations in LOAD with the ExAC database. Of interest, there was a higher frequency in LOAD vs ExAC controls (OR = 1.78, $p = 2.3e-04$).

SRCAP gene expression. Among the ROS-MAP cohorts, 218 of 541 (40.3%) individuals were diagnosed with LOAD at death, and compared with non-demented persons, SRCAP expression was significantly higher (OR = 4.9 [2.1–11.6], $p = 0.0002$) (figure 3A). Individuals with higher SRCAP expression also had lower cognitive scores prior to death ($\beta = -0.96, p < 0.0001$) (figure 3B). Similar associations were observed in episodic, semantic and working memory, and perceptual speed (table e-5). Using the clinical diagnosis, higher SRCAP expression was associated with greater likelihood of meeting the NIA-Reagan criteria for AD (OR = 4.0 [1.7–9.2], $p = 0.0015$) (figure 3C). Individuals with higher SRCAP expression had a greater burden of pathology ($\beta = 0.3, p < 0.0001$) (figure 3D). We also accessed whole-genome sequencing data from 63 participants with brain expression. Two of 69 individuals
sequenced carried 1 of the 10 SRCAP missense mutations (S1033P and L2919F), and both were diagnosed with mild cognitive impairment.

**Mutation-specific gene expression.** Relative messenger RNA (mRNA) expression of SRCAP (supplementary methods) in whole blood was measured in 5 mutation carriers with LOAD, 12 individuals without mutations with LOAD, and 12 elderly controls without dementia, all unrelated. After normalization against GAPDH, SRCAP mRNA expression was significantly lower in the mutation carriers with LOAD than in the other 2 groups (figure 4) (LOAD carriers 0.74, LOAD noncarriers 1.128, and controls 0.99, \( p = 0.004 \)). We compared the levels of exogenous mature wild-type SRCAP mRNA in HEK293 cells to the one carrying an ultra-rare mutation (p.P2741R; minor allele frequency = 0.00128 in Caribbean Hispanic cases and absent in ExAC). mRNA expression of mutant SRCAP (p.P2741R) was downregulated by 40% relative to wild-type SRCAP.

**DISCUSSION** A statistically significant enrichment of SRCAP ultra-rare mutations was found among patients within Caribbean Hispanic families multiply affected by LOAD. These mutations were observed to segregate imperfectly with disease consistent with the presence of phenocopies in large LOAD families.

Reduced mRNA expression of SRCAP was found in whole blood of mutation carriers compared with noncarriers and controls. Higher SRCAP expression was expressed in noncarriers in both blood of Caribbean Hispanic patients with LOAD and brains of Caucasian patients with LOAD, irrespective of differences in ancestry. Pathologic evidence indicates that SRCAP expression is altered in LOAD with or without mutations, correlating with characteristic clinical manifestations. Taken together, the combined data implicate a putative role for SRCAP in LOAD.

Snf2-related cyclic AMP-responsive element-binding protein (SRCAP) binds to the CREB-binding protein (CBP), which influences the transcription of CREB. CREB and the related transcription factors are involved in memory retention and consolidation by hippocampal neurogenesis. In AD-transgenic mice and in humans with LOAD, CREB and CBP are decreased or disrupted by the accumulation of amyloid-β. Reduced levels have also been observed for pCREB, CBP, and related CREB co-activators p300 and cAMP-dependent protein kinase in the human AD brain and blood compared with elderly controls. We also observed reduced mRNA expression of SRCAP in mutation carriers compared...
with healthy elderly controls and unrelated, noncarriers with LOAD, and a reduction in SRCAP expression in cell lines transfected with SRCAP mutations. We reported an association between episodic memory and SNP rs2526690 in CBP as well as nominal significance for common variants in CREB1 and RBAI48 in the CREB pathway.38

SRCAP regulates the CREB pathway by catalyzing H2A.Z into chromatin, which is required for gene expression and interactions with co-activators of CREB such as CBP.39,40 In the absence of mutations, the increased mRNA expression of SRCAP in postmortem LOAD brain suggests a compensatory response to promote CREB activation in the presence of Aβ accumulation. Paradoxically, in persons with SRCAP mutations, disruption of the binding of SRCAP to CBP would decrease CREB-mediated transcription in brain over the lifetime. CREB may have neuroprotective qualities against Aβ toxicity, and persistent downregulation of CREB resulting from decreased SRCAP could promote neurodegeneration.41 This suggests that SRCAP has a regulatory role in the CREB pathway in LOAD, regardless of the presence or absence of SRCAP mutations. Ample evidence indicates disruption of CREB transcription by Aβ, but the effects of these ultra-rare mutations on protein function and the LOAD phenotype will need to be fully elucidated.

Truncating mutations in SRCAP cause Floating-Harbor syndrome,42 a childhood disorder characterized by short stature, delayed speech, and facial abnormalities. The majority of SRCAP mutations reduced expression and create a nonfunctioning protein. Mutations in CBP, the substrate of SRCAP activation, cause a developmental disorder similar to Floating-Harbor syndrome and Rubinstein-Taybi syndrome.

This study does have limitations. We found a modest increase in the risk of LOAD among carriers of these ultra-rare SRCAP mutations in Caribbean Hispanic families, which makes replication difficult. Validation and replication efforts in other ethnic backgrounds would be essential to generalize the findings.

AUTHOR CONTRIBUTIONS

Conception and design of the study: B.N.V., G.T., S.B., R. Lefort, D.A.B., P.L.D.J., E.R., P.S.G.-H., and R.M. Acquisition and analysis of data: B.N.V., R. Lefort, P.L.N., D.R.-D., J.H.L., R.C., L.Y., D.A.B., P.L.D.J., M.M., R. Lantigue, and R.M. Drafting the manuscript or figures: B.N.V., G.T., E.R., P.S.G.-H., and R.M.

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