ApoE variant p.V236E is associated with markedly reduced risk of Alzheimer’s disease

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Abstract

Recent genome-wide association studies (GWAS) of late-onset Alzheimer’s disease (LOAD) have identified single nucleotide polymorphisms (SNPs) which show significant association at the well-known APOE locus and at nineteen additional loci. Among the functional, disease-associated variants at these loci, missense variants are particularly important because they can be readily investigated in model systems to search for novel therapeutic targets. It is now possible to perform a low-cost search for these “actionable” variants by genotyping the missense variants at known LOAD loci already cataloged on the Exome Variant Server (EVS). In this proof-of-principle study designed to explore the efficacy of this approach, we analyzed three rare EVS variants in APOE, p.L28P, p.R145C and p.V236E, in our case control series of 9114 subjects. p.R145C proved to be too rare to analyze effectively. The minor allele of p.L28P, which was in complete linkage disequilibrium (D’ = 1) with the far more common APOE ε4 allele, showed no association with LOAD (P = 0.75) independent of the APOE ε4 allele. p.V236E was significantly associated with a marked reduction in risk of LOAD (P = 7.5 × 10⁻⁵); OR = 0.10, 0.03 to 0.45). The minor allele of p.V236E, which was in complete linkage disequilibrium (D’ = 1) with the common APOE ε3 allele, identifies a novel LOAD-associated haplotype (APOE ε3b) which is associated with decreased risk of LOAD independent of the more abundant APOE ε2, ε3 and ε4 haplotypes. Follow-up studies will be important to confirm the significance of this association and to better define its odds ratio. The ApoE p.V236E substitution is the first disease-associated change located in the lipid-binding, C-terminal domain of the protein. Thus our study (i) identifies a novel APOE missense variant which may profitably be studied to better understand how ApoE function may be modified to reduce risk of LOAD and (ii) indicates that analysis of protein-altering variants cataloged on the EVS can be a cost-effective way to identify actionable functional variants at recently discovered LOAD loci.
genotype association at GWAS loci, those that alter proteins are particularly important because they can readily be investigated in model systems to search for novel therapeutic targets. The Exome Variant Server (EVS, http://evs.gs.washington.edu/EVS/) catalogs whole exome sequencing of 4300 unrelated European Americans, a series large enough to detect virtually all exonic variants with a minor allele frequency (MAF) of 0.1% (1/1000) or more. Thus expensive resequencing is no longer required to discover such variants, and it is now possible to perform a meaningful, low-cost search for “actionable” variants with MAF > 0.1% by genotyping protein-altering variants cataloged on the EVS in large European American case-control series. To evaluate the utility of this approach, we searched the EVS for protein-altering APOE variants with MAF > 0.1% and found just two, p.L28P (0.17%) and p.V236E (0.12%) in European Americans. Both were analyzed in our large European American case-control series of 4128 LOAD subjects and 4986 non-demented controls (Table 1). In addition we analyzed one extremely rare variant, p.R145C (0.026%), that did have a MAF > 1% in African Americans.

**Result**

In this proof of principle study, we used our large LOAD case-control series (Table 1) to analyze three missense variants in the APOE gene that were mined from the EVS database: rs769452 (T/c, p.L28P), rs769455 (C/t, p.R145C) and rs199768005 (T/c, p.V236E). Comparison of EVS European Americans with the control subjects in our series showed no significant difference in the MAFs for rs769452 (P = 0.27), rs769455 (P = 0.46) or rs199768005 (P = 0.075).

rs769455 (ApoE p.R145C) was successfully genotyped in 3955 AD cases and 4590 controls. With only 4 heterozygotes in the AD cases, 1 in the control group, and no homozygotes. Analysis of rs199768005 by a Fisher’s exact test showed significant (P = 1.6×10⁻³) association with increased risk of LOAD (OR = 2.39, 1.38 to 4.37). In African Americans (AA), the MAF for rs769452 on the EVS is 1.39% as compared to 0.026% in European Americans, so we evaluated this variant in our African American LOAD case control series of 168 LOAD patients and 333 non-demented control subjects. There were 9 heterozygotes in the AD cases compared to 17 in the control group and no homozygotes. A chi-square test showed no evidence of allelic association with LOAD (P = 0.91: OR = 1.05, 0.46 to 2.38), but the small series tested has relatively little statistical power as an OR of approximately 3.3 is required for 80% power to detect association at α = 0.05. Analysis in additional case-control studies is clearly needed to evaluate the association of this rare variant with LOAD.

rs769452 (ApoE p.L28P) was successfully genotyped in 2996 late-onset AD cases and 3951 control samples. There were 36 heterozygotes in the AD cases compared to 20 in the control group and no homozygotes. Analysis of rs769452 by a Fisher’s exact test showed significant (P = 7.5×10⁻⁹) association with markedly reduced risk of LOAD (OR = 0.10, 0.03 to 0.45). rs199768005 was not genotyped in our small AA series.

rs199768005 (ApoE p.V236E) was successfully genotyped in 4128 late-onset AD cases and 4986 control samples. There were 2 heterozygotes in the AD cases compared to 23 in the control group and no homozygotes. Confirmatory genotyping using a custom TaqMan assay was 100% concordant. Analysis of rs199768005 by a Fisher’s exact test showed significant (P = 7.5×10⁻⁹) association with markedly reduced risk of LOAD (OR = 0.10, 0.03 to 0.45). rs199768005 was not genotyped in our small AA series, as its minor allele was never detected in the much larger set of 2203 EVS AA subjects.

The well-known APOE ε2, ε3, and ε4 haplotypes are formed by two APOE missense SNPs, rs429358 (T/c, p.C112R) and rs7412 (C/t, p.R158C), as shown in Table 2. The minor alleles of rs429358 and rs7412 tag the ε4 and ε2 haplotypes respectively; the ε3 haplotype has major confidence interval (95% CI) of 4.64 (0.52 to 41.56) with a p value of 0.13. In African Americans, the MAF for rs769455 on the EVS is 1.39% as compared to 0.026% in European Americans, so we evaluated this variant in our African American LOAD case control series of 168 LOAD patients and 333 non-demented control subjects.

| Table 1 Sample demographics for case-control series |
|---------------------------------------------------|
| **Series** | **n (%)** | **Mean AAD ± SD (Years)** | **Females (%)** | **ε4+ Subjects (%)** |
| AD | CON | AD | CON | AD | CON | AD | CON |
| Jacksonville | 1020 (41.2) | 1453 (58.8) | 77.7 (6.46) | 79.5 (7.86) | 623 (61.1) | 838 (57.7) | 653 (64.0) | 338 (23.3) |
| Rochester | 600 (19.9) | 2409 (80.1) | 80 (7.72) | 78.3 (5.56) | 363 (60.5) | 1294 (53.7) | 328 (54.7) | 571 (23.7) |
| Poland | 250 (100) | 0 (0) | 74.4 (5.19) | NA (NA) | 156 (62.4) | NA (NA) | 139 (55.6) | NA (NA) |
| Norway | 345 (38.5) | 552 (61.5) | 80.2 (7.25) | 75.4 (6.73) | 241 (69.9) | 330 (59.8) | 217 (62.9) | 132 (23.9) |
| NCRAD | 702 (77.1) | 209 (22.9) | 75.2 (6.76) | 78.3 (8.88) | 455 (64.8) | 129 (61.7) | 551 (78.5) | 34 (16.3) |
| Autopsy* | 1211 (76.9) | 363 (23.1) | 81.4 (8.56) | 75.9 (8.14) | 695 (57.4) | 155 (42.7) | 744 (61.4) | 98 (27.0) |
| Total | 4128 (45.3) | 4086 (45.7) | 78.7 (7.76) | 78.2 (6.91) | 2533 (61.4) | 2746 (55.1) | 2632 (63.8) | 1155 (23.2) |

*Autopsy controls unlike the clinical controls, who were neurologically normal, include some non-AD degenerative disorders.*
Table 2 APOE Haplotypes formed by three variants and their association with AD

| APOE Haplotype | rs429358 C/T | rs7412 A/T | rs199768005 G/A | rs769452 C/T |
|----------------|-------------|-------------|------------------|-------------|
| p.C121R Base   | AA          | AA          | AA               | AA          |
| p.R158C Base   | AA          | AA          | AA               | AA          |
| p.V236E Base   | AA          | AA          | AA               | AA          |
| p.L28P Base    | AA          | AA          | AA               | AA          |
| Univariate     | OR CON      | OR CON      | OR CON           | OR CON      |
| Logistic       | OR P        | OR P        | OR P             | OR P        |

| Haplotype | Minor allele frequency (%) | Univariate Logistic regression |
|-----------|---------------------------|-------------------------------|
| e2 allele | 3.15% 8.50% 0.35 (0.30-0.40) | <2x10^-16 | 0.46 (0.38-0.54) | <2x10^-16 |
| e3a allele | 58.0% 79.0% 0.35 (0.32-0.37) | <2x10^-16 | 1.00 (REF) | REF |
| e3b allele | 0.02% 0.23% 0.11 (0.02-0.36) | 2.32x10^-03 | 0.10 (0.02-0.35) | 2.16x10^-03 |
| e4a allele | 37.4% 12.3% 5.00 (4.52-5.50) | <2x10^-16 | 4.80 (4.35-5.30) | <2x10^-16 |
| e4b allele | 0.62% 0.26% 2.49 (1.45-4.41) | 1.17x10^-03 | 0.91 (0.51-1.66) | 0.75 |

Alleles in uppercase denote a major allele, alleles in lower case denote a minor allele.

| Minor allele frequency (%) | OR CON | OR P |
|---------------------------|--------|------|
| e2 allele                 | 3.15% 8.50% 0.35 | <2x10^-16 | 0.46 (0.38-0.54) |
| e3a allele                | 58.0% 79.0% 0.35 | <2x10^-16 | 1.00 (REF) |
| e3b allele                | 0.02% 0.23% 0.11 | 2.32x10^-03 | 0.10 (0.02-0.35) |
| e4a allele                | 37.4% 12.3% 5.00 | <2x10^-16 | 4.80 (4.35-5.30) |
| e4b allele                | 0.62% 0.26% 2.49 | 1.17x10^-03 | 0.91 (0.51-1.66) |

Discussion

Our results show that ApoE p.V236E occurs on the APOE e3 backbone creating a rare APOE e3b haplotype, which is significantly associated with LOAD independent of the APOE e2, e3, and e4 alleles. Comparison of the 95% CI for APOE e3b (OR = 0.10, 0.02 to 0.35) with that for APOE e2 (OR = 0.46, 0.38 to 0.54), indicates that, in our series, the e3b allele reduced risk of AD as much or more than the APOE e2 allele (Table 2, Multivariate Logistic Regression). In this regard, it is worth noting that, of the 2 LOAD patients carrying p.V236E, one developed dementia at an advanced age (98 yrs, APOE e3a/e3b genotype) and the other, who was diagnosed at 68, also carried an e4 allele (APOE e3b/e4 genotype), which likely counters the protection afforded by p. V236E. The 23 non-demented control carriers included 7 with ages of 64-88 years with e3b/e4 genotypes, 14 with ages of 68-91 with e3b/e3a genotypes, and 2 with ages of 68 and 92 with e3b/e2 genotypes. To verify the significance of the association observed in our series and to improve the OR estimate for p.V236E, replication in a similarly large series will be important, ideally a series with GWAS genotypes that can be used to adjust for the potentially confounding effect of population stratification. If APOE e2 and e3b act similarly, as seems likely, then analysis of the functional effects of e2 as compared to the novel e3b allele identified here could provide insight into the common or distinct mechanism whereby they reduce risk of LOAD.

In three previous studies [5-7], rs769452 (ApoE p.L28P) was genotyped in a total of 2630 subjects (1329 AD/1401 Control: 1118/1123 [5], 117/121 [6], 93/157 [7]. These studies also found that ApoE p.L28P occurs on the APOE e4 backbone. The risk associated with the minor allele of rs769452, which tags the rare APOE e4b allele, appeared to be greater than the risk of APOE e4 in two of these studies [5,7] but less in the other study [6]. When the results from these previous series were combined with those
presented here, the OR for APOE ε4 vs. all other alleles was 4.31 (3.96 to 4.70) as compared to 4.04 (2.74 to 6.00) when APOE ε4b was compared to the same referent group. Thus the combined results from all series, like those from our series alone (Table 2), indicate that the minor allele of p.L28P does not substantially modify the risk associated with APOE ε4 when it is present on that haplotype. Replication in additional large series will be important to confirm this finding.

ApoE is a 299 amino acid long protein with a highly hydrophobic binding domain in the C-terminal region, and a receptor binding domain in the N-terminal region. Bridged by a protease sensitive hinge region, the N- and C-terminal domains appear to interact when ApoE is delpidated, preventing lipoprotein receptor docking and internalization of unlipidated ApoE [8]. The two missense variants that create the APOE ε2 (p.C112R) and APOE ε4 (p.R158C) alleles both alter amino acids in the N-terminal region, which may interfere with receptor binding. The missense variant (p.V236E) that creates the APOE ε3b allele is the first LOAD-associated variant to alter a C-terminal amino acid [9]. The protein encoded by APOE ε3b has previously been described as APOEε2 [10] because upon isoelectric focusing it migrates similarly to the APOE2 protein encoded by the APOE ε2 allele. Studies of individuals carrying p.V236E have found no lipoprotein abnormalities [11]. Pathogenicity prediction using SIFT and PolyPhen-2 both suggest p.V236E is damaging, substituting a nonpolar, hydrophobic valine for an ionic glutamic acid is consistent with p.V236E altering the lipid binding property of ApoE, or affecting aggregation. Additionally, in light of the interaction between ApoE N- and C-terminal domains, p.V236E could alter ApoE folding and receptor binding. We are currently investigating these possibilities.

In this proof of principle study, we searched the EVS for protein-altering APOE variants with MAF > 0.1% and found just two, p.L28P (0.17%) and p.V236E (0.12%). Both were tested for association with LOAD in our large case-control series, and one (p.V236E) was significantly associated with markedly decreased risk of LOAD, independent of the APOE ε2, ε3, and ε4 alleles. It will now be important to determine if this same cost-effective approach can be used to identify additional LOAD-associated, protein altering variants in genes at any of the recently discovered LOAD loci that might profitably be investigated to identify novel therapeutic targets for AD.

Materials and methods

Case-control subjects

Demographic information on the LOAD patients and non-demented control subjects that were analyzed is shown in Table 1. Approval was obtained from the ethics committee or institutional review board of each institution responsible for the ascertainment and collection of samples. Written informed consent was obtained for all individuals who participated in this study.

The Mayo case-control series consists of European Americans ascertained at the Mayo Clinic Jacksonville, Mayo Clinic Rochester, and in the Mayo Clinic autopsy-confirmed samples (Autopsy in Table 1). Additional Caucasian subjects from the United States were obtained through the National Cell Repository for Alzheimer’s Disease (NCRAD in Table 1), and European Caucasian subjects were obtained from Norway [13] and Poland [14,15]. All subjects in the Mayo clinical case-control series were diagnosed by a neurologist at the Mayo Clinic in Jacksonville, Florida, or Rochester, Minnesota. The neurologist confirmed a Clinical Dementia Rating score of 0 for all Jacksonville and Rochester subjects enrolled as controls; cases had diagnoses of possible or probable AD made according to NINCDS-ADRDA criteria [16]. Clinical LOAD cases and controls in the NCRAD, Polish, and Norwegian were ascertained similarly. In the autopsy-confirmed series, all brains were evaluated by Dr. Dennis Dickson and came from the brain bank he maintains at the Mayo Clinic in Jacksonville, FL. In the Autopsy series the diagnosis of definite AD was also made according to NINCDS-ADRDA criteria. Only samples with an age-at-diagnosis (AAD) above 60 years, with sex and APOE covariates (ε2, ε3, ε4 alleles) available, were included in this study.

Nomenclature

To conform to most of the literature on ApoE, our numbering of ApoE residues begins with the first amino acid that remains after removal of the 18 amino acid leader sequence. This is different from EVS numbering which begins with the first amino acid in the leader sequence [17]. The protein encoded by the APOE ε3b allele, which is created by the minor allele of p.V236E (see Table 2), has previously been described as APOEε2 [10,11] because upon isoelectric focusing it migrates similarly to the APOE2 protein encoded by APOE ε2 allele.

Genotyping

APOE missense variants resulting in p.L28P (rs769452), p.R145C (rs769455) and p.V236E (rs199768005) were genotyped using SEQUENOM’s MassArray iPLEX technology (SEQUENOM Inc, San Diego, CA, USA). SEQUENOM’S Typer Analyzer 4.0 was used to conduct off machine processing and genotype calling. Confirmatory genotyping of p.V236E was carried out using a custom
TaqMan assay in an ABI PRISM 7900HT Sequence Detection System with 384-Well Block Module (Applied Biosystems, California, USA). TaqMan assays were also employed to genotype the APOE missense variants resulting in p.R158C (rs7412) and p.C112R (rs429358) in order to identify the well-known APOE e2, e3, and e4 alleles. Cluster calling was carried out using SDS software v2.2.3 (Applied Biosystems, California, USA). All Sequenom and TaqMan probe sequences are available on request.

Statistical analysis

Analysis of control subjects using PLINK [18] (http://pngu.mgh.harvard.edu/~purcell/plink/), showed that all variants were in Hardy Weinberg equilibrium (P > 0.80). Allelic association was evaluated using Fisher’s exact method in PLINK. Haplotypic analysis was performed using the haplo.stats package in the R programming language (v2.14.1). Logistic regression was carried out adjusting for sex and age at diagnosis.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

Study design was developed by SGY, GB, CWM, KM, NET, and OAR. Variant discovery and preparatory bioinformatics analysis was performed by CWM and SGY. SAH, TM, LM, FZ and CWM carried out genotyping. CWM, SGY and SP carried out the statistical analysis. SGY, NET, MC, GB, LM, JS, SBS, JOA, MB, ZKW, DWD, NGR and RCP were involved in sample acquisition and/or DNA preparation. The manuscript was drafted by CWM, SGY and SAH. All authors read and approved the final manuscript.

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