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Investigation of the independent role of a rare *APOE* variant (L28P; *APOE*4Pittsburgh*) in late-onset Alzheimer disease

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Abstract

A rare missense *APOE* variant (L28P; *APOE*4Pittsburgh*), which is present only in populations with European ancestry, has been reported to be a risk factor for late-onset Alzheimer's disease (LOAD). However, due to the complete linkage disequilibrium of L28P with *APOE*4* (C112R), its independent genetic association is uncertain. The original association study implicating L28P with LOAD risk was carried out in a relatively small sample size. In the current study, we have re-evaluated this association in a large case-control sample of 15,762 White U.S. subjects and investigated its independent effect in *APOE* 3/4 subjects, as L28P has been observed only in the heterozygous state of *APOE*4* carriers and 3/4 is the most common genotype containing the *APOE*4* allele. The heterozygous carrier frequency of L28P, all with *APOE*4*, was about 3-fold higher in AD cases than in cognitively intact controls (0.845% vs 0.277%). The age- and sex-adjusted meta-analysis odds ratio (OR) was 2.87 (95% CI: 1.34 – 6.13; p= 0.0066). Among *APOE* 3/4 subjects, age- and sex-adjusted meta-analysis OR was 1.53 (95% CI: 0.70 – 3.36; p= 0.28), indicating its effect was independent of *APOE*4*. The lack of statistical significance appears mainly due to the low power of 4,138 subjects with the 3/4 genotype (12% power at α= 0.05) compared to the required sample of 139,088 subjects with the 3/4 genotype to detect an OR of 1.5 at α= 0.05 and 80% power. Our data suggesting that L28P has an independent genetic effect on AD risk is reinforced by earlier experimental findings showing that this mutation

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leads to significant structural and conformational changes in the ApoE4 molecule and can induce functional defects associated with neuronal Aβ42 accumulation and oxidative stress. Additional functional studies in cell-based systems and animal models will help to delineate its functional significance in the etiology of AD.

Keywords

Late-onset Alzheimer's Disease; Genetic Risk Factor; APOE; rare variants; rs769452

Introduction

Human apolipoprotein E (ApoE, protein; *APOE*, gene) plays a pivotal role in cholesterol/lipid transport in the peripheral and central nervous systems [1]. The most common *APOE* polymorphism due to missense mutations at codons 112 and 158 results in three allelic forms, of which *APOE*4* is associated with an increased risk and earlier age-at-onset (AAO) of Alzheimer's disease (AD), while *APOE*2* is associated with decreased risk and later AAO of AD as compared to the wild type *APOE*3* [2–6]. Since the original discovery of the association between *APOE*4* and AD, evidence that *APOE* alleles differentially influence amyloid and tau pathology, network dysfunction, and neuroinflammation has been identified [7, 8].

In 1999, two groups independently identified a novel and rare missense mutation in the *APOE* gene [9, 10] in which the leucine residue is replaced by proline at codon 28 (L28P; rs769452). This mutation occurs in complete linkage disequilibrium (LD) with the *APOE*4* allele, hence named *APOE*4Pittsburgh* and *APOE*4Freiburg*, and it was associated with an elevated risk for AD [9] and coronary artery disease [10]. Since then, this variant has been examined in additional AD case-control samples [11–13], although a consensus as to whether this mutation by itself increases the odds of developing AD is yet to be reached. The main drawbacks of aforementioned studies are the use of relatively limited samples, considering L28P is an ultra-rare variant and would require a very large sample size. The rarity of the L28P mutation, and its complete LD with the *APOE*4* allele, makes it nearly impossible to separate its unique contribution from the overwhelming effect of the *APOE*4* allele on the risk of developing AD. In the current study, we have re-evaluated this association in a large case-control sample of 15,762 U.S. White subjects and investigated its independent effect among subjects with the *APOE 3/4* genotype, as L28P has been observed in the heterozygous state only on the *APOE*4*–containing chromosome and 3/4 is the most common genotype containing the *APOE*4* allele.

Methods

Study Samples

To maximize the sample size for this rare variant study, we used data from 15,762 White AD cases and controls derived from three major cohorts: Alzheimer's Disease Sequencing Project (ADSP), The Ginkgo Evaluation of Memory (GEM) study, and a cohort comprising three studies at the University of Pittsburgh: the case-control cohort at

the Alzheimer's Disease Research Center (ADRC) and two population-based cognitively normal cohorts, the Monongahela Valley Independent Elders Survey (MoVIES) and the Monongahela-Youghiogheny Healthy Aging Team (MYHAT). The demographic data on each study sample is presented in Table 1 and their detailed descriptions are given elsewhere [14–18].

Genotyping

Genotypes for the *APOE*/rs429358 (*APOE*4*) and *APOE*/rs7412 (*APOE*2*) SNPs in the Pittsburgh and GEM samples were determined using TaqMan genotyping assays followed by the determination of traditional six genotypes (2/2, 2/3, 2/4, 3/3, 3/4, 4/4) based on the three-allele *APOE* polymorphism [6]. The genotype identification of these variants in the ADSP sample were derived directly from the whole-exome sequencing (WES) data [19].

Statistical Analysis

Logistic regression was implemented for calculating odds ratios (ORs) and the 95% confidence intervals (CI) while using sex and age as covariates. The ORs were calculated individually for each of the three research studies followed by the meta-analysis. To determine the independent effect of *APOE*4Pittsburgh* L28P from *APOE*4* on AD risk, we examined the distribution of L28P among subjects with the *APOE* 3/4 genotype, since as detailed above, it has been observed in the heterozygous state only on the *APOE*4*–containing chromosome and 3/4 is the most common *APOE*4* genotype. All statistical analyses were performed in R version 3.6.1 [20] using the R package epitools [21]; meta [22]; and metafor [23]. The power analysis for the current sample size and the required sample size for 80% power were calculated in G*Power 3.1 [24] following the formula in Demidenko [25].

Results and Discussion

We examined a total of 15,762 subjects from three studies (6,390 AD cases and 9,372 controls) for the L28P variant (Table 1). As expected, the frequency of *APOE*4* carriers was higher in AD cases than in controls. Genotyping of L28P revealed two genotypes, TT and TC; no example of the rare allele homozygosity (CC) was observed. Table 2 shows the carrier frequency of the TC genotype along with the minor allele frequencies (MAF). Eighty subjects carried the L28P mutation, all with the *APOE*4* allele. The L28P carrier frequency was significantly higher in AD cases than controls (0.845% vs 0.277%; P = 1.25E-06). The meta OR was 2.87 (95% CI: 1.34 – 6.13; P = 6.60E-03).

To distinguish the independent effect of L28P from *APOE*4* on AD risk, we restricted the analysis among subjects with the *APOE* 3/4 genotype, as detailed above. Among the 4,834 AD cases and controls with *APOE*4* carriers, 85.62% had the 3/4 genotype followed by 4/4 (8.05%) and 2/4 (6.33%) genotypes. Subjects with the *APOE* 2/4 and 4/4 genotypes were excluded in order to avoid the confounding protective effect of E2 and an extra copy of E4 on L28P. The age- and sex-adjusted meta-analysis OR of L28P among *APOE* 3/4 was 1.53 (95% CI: 0.70 – 3.36; p = 0.28; Figure 1). The lack of significance is mainly due to the low power of a sample size of 4,138 in the 3/4 genotype (12% power at $\alpha = 0.05$); the calculated

required sample size was 139,088 (Figure 2). Considering that about 25% of the European Whites carry the *APOE*4* allele, the total required sample size to detect an OR of 1.5 at α = 0.05 and 80% power was 556,352 (139,088/0.25) European Whites.

Even with non-statistically significant p-value, the OR of 1.53 among 3/4 subjects suggests that the effect of L28P on AD risk is independent of *APOE*4*. This genetic observation is further supported by a comprehensive experimental study in which the L28P mutation was associated with significant structural and conformational changes in the wild type (WT) ApoE4 that resulted in intraneuronal Aβ42 accumulation and oxidative stress [26]. As compared to lipid-free WT ApoE4, lipid-free L28P induced the intracellular accumulation of Aβ42 in SK-N-SH human neuroblastoma cells and mouse primary neurons. Furthermore, lipidated L28P significantly reduced the viability of SK-N-SH cells when compared to lipidated WT ApoE4, which was due to greater cellular oxidative stress induced by L28P than WT ApoE4 [26]. Regardless of its lipidation state, if L28P promotes the *in vivo* neuronal accumulation of Aβ42 followed by induction of increased oxidative stress and ensuing AD pathogenesis, this would represent a gain of function over the WT ApoE4, that itself does not induce the intracellular accumulation of Aβ42. WT ApoE4 is more susceptible to proteolysis than the other ApoE isoforms (E2 and E3) and ApoE4 fragments have been found in brains of AD patients [27]. In this regard, a specific ApoE4 fragment, ApoE4[Δ(166-299)], has previously been found to promote the cellular uptake of extracellular Aβ42 and resulted in increased oxidative stress [28], similar to the effect of L28P. Since the intraneuronal accumulation of Aβ42 and the resulting persistent oxidative stress are considered early events in the pathogenesis of AD and the naturally occurring L28P mutation is associated with both these events as well as with AD risk, it will be important in future studies to examine the role of L28P in cell-based systems, such as induced pluripotent stem cells (iPSCs), which can successfully recapitulate the pathology of AD [29, 30] and/or animal models.

In summary, our genetic data among *APOE* 3/4 subjects suggest that L28P has an effect independent of *APOE*4* on AD risk, which is reinforced by earlier experimental findings. Further confirmation of our genetic data in much larger APOE 3/4 subjects would help validate this independent association.

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University of Pittsburgh

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ADSP

The study was supported in part by NIH grants AG041718, AG030653, AG064877, AG005133, AG023651, and AG07562. The Alzheimer's Disease Sequencing Project (ADSP) is comprised of two Alzheimer's Disease (AD) genetics consortia and three National Human Genome Research Institute (NHGRI) funded Large Scale Sequencing and Analysis Centers (LSAC). The two AD genetics consortia are the Alzheimer's Disease Genetics

Consortium (ADGC) funded by NIA (U01 AG032984), and the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) funded by NIA (R01 AG033193), the National Heart, Lung, and Blood Institute (NHLBI), other National Institute of Health (NIH) institutes and other foreign governmental and nongovernmental organizations. The Discovery Phase analysis of sequence data is supported through UF1AG047133 (to Drs. Schellenberg, Farrer, Pericak-Vance, Mayeux, and Haines); U01AG049505 to Dr. Seshadri; U01AG049506 to Dr. Boerwinkle; U01AG049507 to Dr. Wijsman; and U01AG049508 to Dr. Goate and the Discovery Extension Phase analysis is supported through U01AG052411 to Dr. Goate, U01AG052410 to Dr. Pericak-Vance and U01 AG052409 to Drs. Seshadri and Fornage. Data generation and harmonization in the Follow-up Phases is supported by U54AG052427 (to Drs. Schellenberg and Wang).

The ADGC cohorts include: Adult Changes in Thought (ACT), the Alzheimer's Disease Centers (ADC), the Chicago Health and Aging Project (CHAP), the Memory and Aging Project (MAP), Mayo Clinic (MAYO), Mayo Parkinson's Disease controls, University of Miami, the Multi-Institutional Research in Alzheimer's Genetic Epidemiology Study (MIRAGE), the National Cell Repository for Alzheimer's Disease (NCRAD), the National Institute on Aging Late Onset Alzheimer's Disease Family Study (NIA-LOAD), the Religious Orders Study (ROS), the Texas Alzheimer's Research and Care Consortium (TARC), Vanderbilt University/Case Western Reserve University (VAN/CWRU), the Washington Heights-Inwood Columbia Aging Project (WHICAP) and the Washington University Sequencing Project (WUSP), the Columbia University Hispanic- Estudio Familiar de Influencia Genetica de Alzheimer (EFIGA), the University of Toronto (UT), and Genetic Differences (GD).

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Biological samples and associated phenotypic data used in primary data analyses were stored at Study Investigators institutions, and at the National Cell Repository for Alzheimer's Disease (NCRAD, U24AG021886) at Indiana University funded by NIA. Associated Phenotypic Data used in primary and secondary data analyses were provided by Study Investigators, the NIA funded Alzheimer's Disease Centers (ADCs), and the National Alzheimer's Coordinating Center (NACC, U01AG016976) and the National Institute on Aging Genetics of Alzheimer's Disease Data Storage Site (NIAGADS, U24AG041689) at the University of Pennsylvania, funded by NIA, and at the Database for Genotypes and Phenotypes (dbGaP) funded by NIH. This research was supported in part by the Intramural Research Program of the National Institutes of health, National Library of Medicine. Contributors to the Genetic Analysis Data included Study Investigators on projects that were individually funded by NIA, and other NIH institutes, and by private U.S. organizations, or foreign governmental or nongovernmental organizations.

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Highlights

- A rare missense *APOE* (L28P) has been reported to be a risk factor for late-onset Alzheimer's disease (LOAD).
- However, due to the complete linkage disequilibrium of L28P with *APOE*4*, its independent genetic association is uncertain.
- we have re-evaluated the L28P association in 15,762 White U.S. case-control subjects and in 4,139 *APOE* 3/4 subjects, since *APOE 3/4* is the most common genotype containing the *APOE*4* allele
- Our data suggesting that L28P has an independent genetic effect on LOAD.

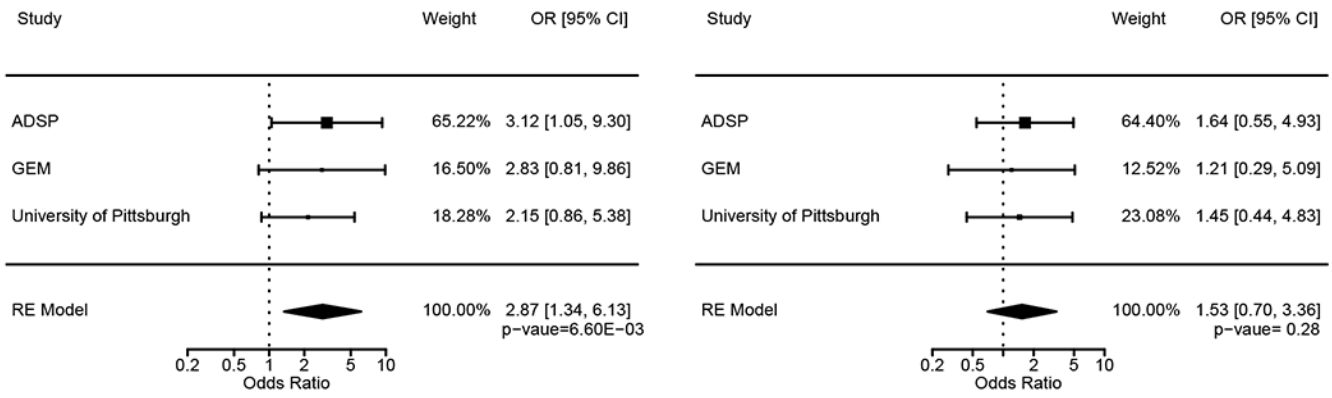

Figure 1.

Meta-analysis of L28P in the total sample (left) and among subjects with the *APOE* 3/4 genotype (right).

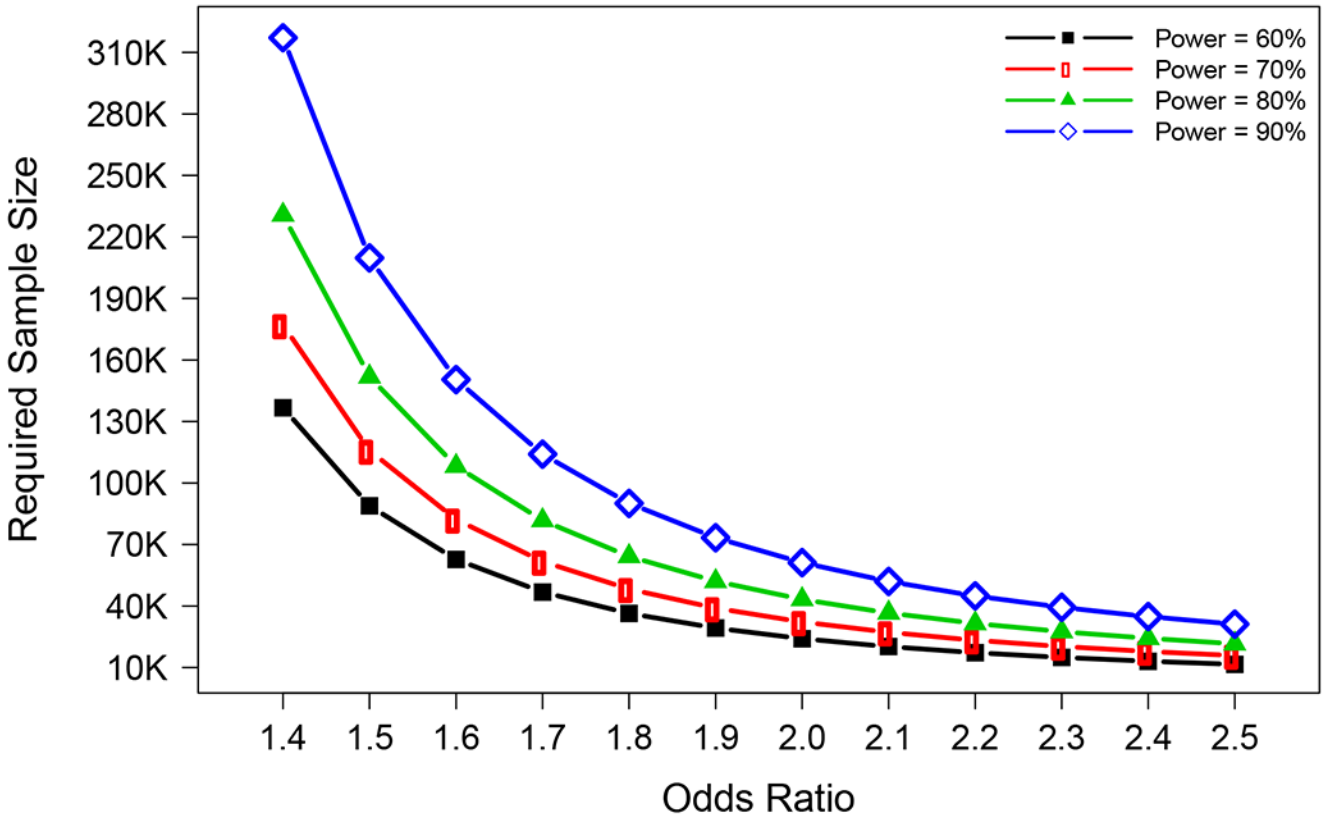

Figure 2.
Required sample size for *APOE* 3/4 genotype individuals with different combinations of the odds ratio and the power (α=0.05).

Table 1.

Demographic information on the study samples

Study	ADSP		GEM		Univ Pittsburgh		Total	
	AD Case	Control	AD Case	Control	AD Case	Control	AD Case	Control
N	4316	5964	384	2217	1690	1191	6390	9372
Mean Age ± SD	73.7 ± 7.95	85.6 ± 4.10	79.9 ± 3.64	78.3 ± 3.11	72.0 ± 8.02	77.2 ± 7.79	73.6 ± 7.97	82.8 ± 5.88
Sex Female (%)	1973 (45.7%)	2355 (39.5%)	181 (47.1%)	980 (44.2%)	1077 (63.7%)	742 (62.3%)	3231 (50.6%)	4077 (43.5%)
*APOE*4* Carrier N (%)	2201 (51%)	814 (14%)	150 (39%)	461 (21%)	974 (58%)	234 (20%)	3325 (52%)	1509 (16%)

Table 2.

Distribution of L28P (**rs769452**) Carriers in AD cases and Controls

Study	ADSP		GEM		Univ Pittsburgh		Total	
	AD Case	Control	AD Case	Control	AD Case	Control	AD Case	Control
N	4316	5964	384	2217	1690	1191	6390	9372
L28P Carrier N (%)	29 (0.672%)	10 (0.168%)	4 (1.04%)	9 (0.406%)	21 (1.243%)	7 (0.588%)	54 (0.845%)	26 (0.277%)
MAF %	0.335%	0.084%	0.521%	0.203%	0.621%	0.294%	0.423%	0.139%