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A novel mutation in the apolipoprotein E gene (*APOE*4 Pittsburgh*) is associated with the risk of late-onset Alzheimer's disease

M. Ilyas Kamboh[a,d,*], Christopher E. Aston[a], Jordi Perez-Tur[b], Emre Kokmen[c], Robert E. Ferrell[a], John Hardy[b], Steven T. DeKosky[a,d]

[a]*Department of Human Genetics, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA 15261, USA*
[b]*Mayo Clinic Jacksonville, Jacksonville, FL 32224, USA*
[c]*Department of Neurology, Mayo Clinic Rochester, Rochester, MN 56007, USA*
[d]*Departments of Psychiatry, Neurology and Alzheimer's Disease Research Center, Western Psychiatric Institute and Clinic, University of Pittsburgh, Pittsburgh, PA 15261, USA*

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Abstract

Using a combination of polymerase chain reaction (PCR), single-strand conformation polymorphism (SSCP) and DNA sequencing techniques, we identified a unique missence mutation (T → C) in exon 3 of the APOE gene which resulted in the substitution of pro-28 for leu-28. We screened 1118 White cases of late-onset (>60 years) Alzheimer's disease (AD) from three independent centers (Pittsburgh = 489, Indiana = 319, Mayo Clinic Rochester = 310) and 1123 controls (607 clinically assessed and 516 individuals randomly ascertained from the general population). Two of the 1123 control subjects had the pro-28 mutation (0.18%). However, this mutation was observed in heterozygous state in 2.66, 2.51 and 1.94% of the AD cases from Pittsburgh, Indiana and Mayo Clinic Rochester, respectively, with an overall frequency of 2.42%. All individuals with this mutation were carriers of the *APOE*4* allele and hence the mutation was denoted as *APOE*4 Pittsburgh* (*APOE*4P*). Compared with the non-*E*4P* carriers, the *E*4P* carriers were associated with an increased risk of AD (odds ratio (OR) 13.2) and this risk remained significant even after adjusting for the known effect of *APOE*4* (OR 5.4). The risk associated with the *E*4P/E*4* combination was about five times (OR 29.1) the risk attributed to *APOE*4* carriers alone (OR 5.7). Our data indicates that the new mutation most likely exists in cis-orientation with *APOE*4* and is associated with increased risk of developing AD.

*Keywords: APOE*4 Pittsburgh*; Late-onset Alzheimer's disease

Alzheimer's disease (AD) is a multifactorial disease with possible involvement of several genetic and environmental factors. Mutations in single genes on chromosomes 21, 14 and 1 explain the majority of early-onset, autosomal dominant familial cases of AD [3]. Genetic analysis has identified apolipoprotein E (APOE) as a major susceptibility locus for sporadic and late-onset AD [6] accounting for about 50% of the genetic risk associated with late-onset AD [2]. The *E*4* allele of APOE is a significant risk factor for AD, acting in a dose-dependent manner, and is associated with an earlier age-at-onset [1]. However, the observation that the *APOE*4* allele is neither necessary nor sufficient to cause the disease and is not a major risk factor for AD in Blacks [7], where its frequency is higher than Whites, suggest the involvement of other genetic factors which act independently or in concert with *APOE*4*. One of the hypotheses is that the *APOE*4* allele is in linkage disequilibrium with a functional mutation within the APOE gene or a gene linked to APOE on chromosome 19 which confers increased risk of AD. The aim of this study was to identify any potentially functional mutation in the APOE gene existing in cis-orientation with *APOE*4* and associated with the risk of AD.

Three case-control cohorts were examined. The first cohort was from the University of Pittsburgh Alzheimer's

* Corresponding author. Tel.: +1-412-624-3066; fax: +1-412-383-7844; e-mail: ikamboh@helix.hgen.pitt.edu

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Disease Research Center (ADRC) that included 489 sporadic late-onset (>60 years) cases (68% female; 38% autopsy confirmed) with mean age (±SD) 77.5 ± 6.3 years and mean age-at-onset 71.3 ± 6.0 years. Controls included 125 subjects (63% females, 19% autopsy confirmed) with mean age 68.1 ± 11.3 years who were found to be not demented after clinical examinations. The second cohort was recruited from the Indiana Alzheimer Disease Center (IADC) Cell Repository, that included 319 familial late-onset cases (65% female; 35% autopsy confirmed) with mean age 83.7 ± 5.7 and mean age-at-onset 72.2 ± 5.5 from 202 unrelated families. Fifty-seven clinically assessed age-matched and unrelated controls (mean age 77.8 ± 11.1, 52% female) were also available from the IADC for this study. The third cohort was from the Alzheimer's Disease Center at Mayo Clinic Rochester that included 310 sporadic late-onset AD cases (67% female; 15% autopsy confirmed) with mean age 83.2 ± 8.0 and mean age-at-onset 77.6 ± 8.0. Controls for this cohort included 425 individuals (68% female; 5% autopsy confirmed) with mean age of 83.3 ± 7.1. In addition, we analyzed 516 (age range 25–75, mean age 52.4 ± 11.5; 52% female) unrelated individuals from the general US White population, including 147 individuals > 60 years (mean age 65.9 ± 4.1). For a comparison purpose, a random African sample comprising 752 subjects (age range 19–70, mean age 40.8 ± 8.2) from Nigeria [5] was also screened for the new APOE polymorphism.

DNA was extracted from buffy coats or brain autopsy tissues using the QIAmp kit (QIAGEN). APOE genotyping for the known polymorphism utilized the polymerase chain reaction (PCR) as described previously [4]. Single-strand conformation polymorphism (SSCP) analysis was performed on exons 1–3 and a portion of the fourth exon of APOE in 50 White AD patients selected, based on their different APOE genotypes. With the exception of exon 3, however, no variation was observed in the other exons. APOE exon 3 was amplified using E12F 5′-TGG ACG GGG TCA GAA GGA CCC TGA CC-3′ and E12R 5′-GCC CAC CAG GAG GGT CAA GGG CCA G-3′ primers for 5 min at 95°C then submitted to 30 cycles of amplification with denaturation for 30 s at 95°C and, annealing and extension for 30 s at 70°C. SSCP analysis was performed on vertical electrophoresis at constant 100 V for 20 h at 25°C using a Brinkmann Lauda® RM 6 circulator. Gels were stained with SYBR Green II. DNA sequencing of the PCR product was done using ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Reaction kit with Amplitaq® DNA polymerase, and ABI™ 377 DNA Sequencer (Applied Biosystems). Routine DNA samples were screened by restriction analysis using *MspI* enzyme and electrophoresing on a 2% agarose gel. The wild type, leucine, was designated as 'L' and the mutant type, proline, as 'E4P', as the latter occurred on the E4 background and discovered in Pittsburgh. Allele frequencies were calculated by the allele counting method. Adherence to Hardy–Weinberg expected frequencies were tested by χ^2 goodness-of-fit tests. Comparison of genotype and allele distributions between cases and control subjects were analyzed by 2×2 χ^2 test. Logistic regression analyses were used to calculate odds ratios to determine the effects of age, gender, the known APOE polymorphism, the codon 28 polymorphism, and interaction between these variables on risk for AD.

The expected differences between cases and controls were observed in APOE allele frequencies in the three cohorts (Table 1). A relatively high frequency of *APOE*4* in the familial AD sample from Indiana than in sporadic AD samples from Pittsburgh and Rochester is consistent with prior reports [6]. PCR amplification followed by SSCP analysis of the APOE exon 3 in 50 White AD samples identified a variant pattern (Fig. 1a) which upon sequencing revealed a point mutation (T → C) at nucleotide position 2912 (Fig. 1c). This point mutation affects codon 28 (CTG → CCG) and predicts the replacement of the commonly occurring leucine by proline. Since the point mutation also creates a new restriction site for *MspI* endonuclease (Fig. 1b), the three White AD case-control cohorts and randomly selected 752 Nigerian Blacks were screened by restriction analysis. No example of the codon 28 mutation (E4P) was observed in the African sample. In the Pittsburgh cohort, the E4P mutation was observed in combination with the wild type, leucine (L), in 2.66% of the AD cases examined (Table 2), with a similar heterozygote frequency in autopsy confirmed (2.70%; $n = 185$) and clinically assessed (2.63%; $n = 304$) cases. however, no example of the E4P mutation was observed either in 125 clinically assessed controls or in 516 subjects from the general population. To confirm our initial observation that the codon 28 mutation is preferentially present among AD cases, and with a restricted distribution in combination with *APOE*4*, we examined its distribution in two additional AD samples from Indiana and Rochester (Table 2). In the familial AD sample from Indiana, the frequency of the E4P mutation was similar

Table 1

APOE allele frequencies

	Pittsburgh		Indiana		Mayo Clinic Rochester		Total		US White general population controls ($n = 516$)
	Cases ($n = 489$)	Controls ($n = 125$)	Cases ($n = 319$)	Controls ($n = 57$)	Cases ($n = 310$)	Controls ($n = 425$)	Cases ($n = 1118$)	Controls ($n = 607$)	
*APOE*2*	0.031	0.051	0.029	0.053	0.042	0.101	0.033	0.087	0.087
*APOE*3*	0.607	0.760	0.491	0.798	0.629	0.777	0.580	0.775	0.763
*APOE*4*	0.362	0.189	0.481	0.149	0.329	0.122	0.387	0.138	0.150

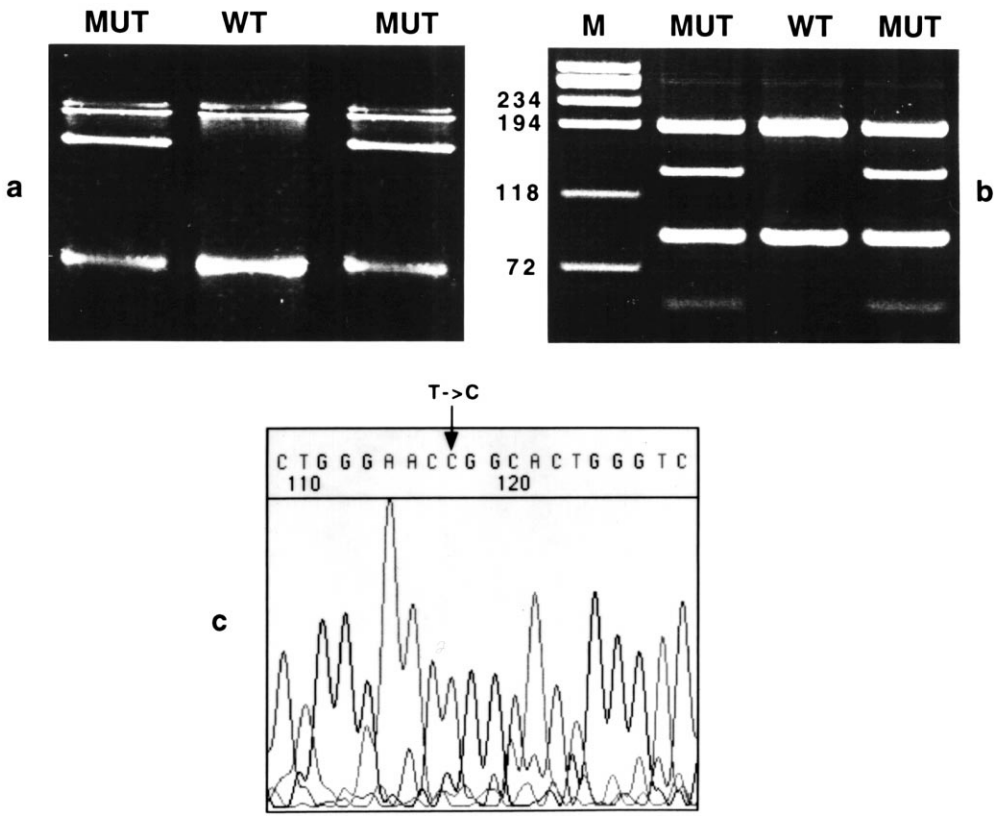

Fig. 1. Identification of the E4P mutation by SSCP (a), restriction digestion with *MspI* (b), and DNA sequencing (c). The mutant (MUT) and wild (WT) type patterns are compared directly in (a) and (b). (c) Shows nucleotide sequence of the mutant form where T is replaced by C.

whether the sample included all 319 AD cases (2.51%) or only the 202 unrelated cases (2.48%). None of the Indiana controls had this mutation. In the Mayo Clinic Rochester sample, six AD cases (1.94%) and two controls (0.47%) had the E4P mutation. We also observed two apparently normal individuals with the codon 28 mutation from two Indiana families in which this mutation was segregating with AD. Both individuals were E4/4 homozygotes with current ages of 62 and 79, respectively. These two individuals were not clinically-assessed by the IADC to determine their cognitive function, but were classified as normal based upon the information provided by members of these families. A prospective follow-up of these two individuals may reveal whether they develop AD.

In the total AD sample from three centers the frequency of the E4P mutation was 2.42% (27 of 1118) and all 27 cases with this mutation were *APOE*4* carriers, strongly indicating that the *APOE*4P* (codon 28) and *APOE*4* (codon 112) alleles occur on the same chromosome ($\chi^2 = 14.30$; $P < 0.001$). To determine the risk associated with the *APOE*4P*, *APOE*4* and combination of both, we performed logistic regression on the entire sample to estimate odds ratios (OR) (Table 3). The OR for *APOE*4P* vs. no-*APOE*4P* was 13.20 and it remained significant after accounting for the effect of *APOE*4* (OR = 5.35). The risk associated with the *APOE*4P*/*APOE*4* combination was about five times the risk explained by just *APOE*4* (OR 29.06 vs. 5.66). This difference is also reflected in a five-fold increased frequency of the E4P variant in cases compared with controls when the data is expressed as E4P/total *APOE*4* carriers (3.67 vs. 0.65%; $P < 0.01$), strongly indicating that the effect of E4P is independent of *APOE*4*. The E4P mutation was not associated with age-at-onset differences (data not shown).

Table 2

APOE codon 28 genotype distributions in AD cases and control subjects

	N	Codon 28 genotype	
		L/L (%)	L/E4P (%)
AD cases			
Pittsburgh	489	476 (97.34)	13[a] (2.66)
Indiana	319	311 (97.49)	8[b] (2.51)
Mayo Clinic Rochester	310	304 (98.06)	6[c] (1.94)
Total Cases	1118	1091 (97.67)	27 (2.42)
Controls			
Pittsburgh – clinical	125	125 (100.0)	0 (0.0)
Indiana - clinical	57	57 (100.0)	0 (0.0)
Mayo Clinic Rochester - clinical	425	423 (99.53)	2[d] (0.47)
US Whites general population	516	516 (100.0)	0 (0.0)
Total controls	1123	1121 (99.82)	2 (0.18)

[a]Four of 13 were APOE 4/4, seven were 3/4 and two were 2/4.
[b]Five of eight were APOE 4/4 and three were 3/4.
[c]Five of six were APOE 3/4 and one was 4/4.
[d]One was APOE 3/4 and the other 2/4.

Table 3
Age- and sex-adjusted odds ratios

*APOE*4* vs. no *APOE*4*	5.66 (4.68–6.84; $P = 0.0001$)[a]
*APOE*4P* vs. no *APOE*4P*	13.20 (3.12–55.93; $P = 0.0005$)
*APOE*4* vs. no *APOE*4* (adjusted for the codon 28 polymorphism)	5.49 (4.53–6.65; $P = 0.0001$)
*APOE*4P* vs. no *APOE*4P* (adjusted for the APOE polymorphism)	5.35 (1.26–22.78; $P = 0.023$)
*APOE*4P*/*APOE*4* vs. no *APOE*4P*/*APOE*4*	29.06 (6.83–123.53; $P = 0.0001$)

[a]Confidence interval (95%) with *P*-values in parentheses.

Since this association was confirmed in three independent AD samples, we believe this finding does not represent a chance observation. Three possibilities may explain an increased frequency of a mutation in late-onset AD cases: (a) the mutation is over-represented in older people in general due to differential survival; (b) the mutation is etiologically related to a late-onset phenotype; or (c) the association of the mutation with *APOE*4* and AD is an historical coincidence. Since we observed only two examples of this mutation in 607 clinically assessed older controls and 516 random, healthy individuals from the general population, we may discard the first possibility. However, since this mutation is strongly associated with the *APOE*4* allele, our inability to detect this mutation in controls may relate to a lower observed frequency of *APOE*4* carriers in controls (27.25%) than in AD cases (65.83%). The number of *APOE*4* carriers observed among controls ($n = 306$), however, was reasonably matched with the number of *APOE*4* carriers in each AD sample from Pittsburgh ($n = 304$), Indiana ($n = 260$), and Mayo Clinic Rochester ($n = 172$). Furthermore, when we expressed our data as E4P/total *APOE*4* carriers in cases (27/736) and controls (2/306) the frequency of the E4P mutation was significantly elevated in cases, indicating its independent effect of *APOE*4*. There is the alternative possibility that the association between this mutation, *APOE*4* and AD is an historical coincidence. At some point in evolutionary history, a yet unknown functional mutation leading to AD occurred on an *APOE*4* background. Through decay of linkage disequilibrium with time we now perhaps see non-*APOE*4* AD carriers who carry this unknown functional mutation. More recently, the E4P mutation occurred on an AD/*APOE*4* background among Whites, as no example of this mutation was observed among African Blacks where the *APOE*4* allele frequency is about three times the frequency in Whites [5]. However, there has not been sufficient time for this linkage disequilibrium to decay, resulting in the E4P mutation only occurring on this background and therefore only occurring among AD cases despite the possibility that this mutation is otherwise unrelated to the etiology of AD. The observation of two controls with the E4P mutation (Table 2), and two apparently normal individuals from two IADC families in which this mutation was segregating with AD, support the latter assumption. However, this observation may also be explained if we assume that this is a functional mutation with incomplete penetrance. A prospective follow-up of these and other normal individuals with this mutation may help to clarify the functional nature of this mutation. The substitution of proline for leucine by itself may have direct impact on the function of APOE, as the proline residue is associated with less conformational freedom of the protein at its location because its side chain is fixed by a covalent bond to the main chain. Functional studies aiming to evaluate these and related aspects may provide answers to these questions.

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