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Genotypes and Phenotypes for Apolipoprotein E and Alzheimer Disease in the Honolulu-Asia Aging Study

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Background: **The utility of apolipoprotein E (ApoE) type as an indicator of genetic susceptibility to Alzheimer disease (AD) depends on the reliability of typing. Although ApoE protein isoform phenotyping is generally assumed equivalent to genotyping from DNA, phenotype-genotype differences have been reported.**
Methods: **ApoE genotype and phenotype results were examined for 3564 older (ages 71–93 years) Japanese-American male participants of the Honolulu-Asia Aging Study, an ongoing population-based study of aging and dementia.**
Results: **Both methods demonstrated similar associations of ApoE type with AD: a direct association with ApoE4 and a less dramatic inverse association ApoE2. Advanced age did not appear to influence the ApoE4-AD association. The association with AD among ApoE4 homozygotes [odds ratio (OR) = 14.7] was higher than expected based on an observed OR of 2.0 in heterozygotes. Phenotype-genotype nonconcordance was more frequent for ApoE2 than for ApoE4. The ApoE2 phenotype occurred at a frequency of 7.9% vs a genotype frequency of 4.9%, corresponding to a probability of 56% that an individual with ApoE2 phenotype had the same genotype.**
Conclusions: **Whereas E4 and E2 phenotypes and genotypes were comparably associated with AD, neither method would be expected to substantially improve the efficiency of case finding in the context of population screening beyond prediction based on age and education. Nonconcordance of phenotype and genotype was substantial for E2 and modest for E4 in this population. The ApoE4-AD association was independent of age.**

Apolipoprotein E (ApoE)[6] plays a role in the transport and redistribution of lipids, including cholesterol. Of the three common isoforms of this glycoprotein (E2, E3, and E4), E3 is by far the most common. Isoform E4 is associated with higher plasma concentrations of cholesterol, cardiovascular disease, and Alzheimer disease (AD) *(1–4)*. The corresponding genetic alleles, which are found on chromosome 19 (ε2, ε3, ε4), account for 99% of the genetic variance *(5)*, and involve differences in codons 112 and 158.

The ApoE ε4 allele has been shown to be associated with AD in African-American, European, Japanese, and Asian-ancestry populations *(6–15)*. Increased AD risk is associated with either one or two copies of the ε4 allele and has led some to advocate ε4 genotyping for diagnostic support in early dementia or as an adjunct to the differential diagnosis of dementia *(9, 16, 17)*. Other reports have suggested that ApoE2 is protective for AD *(18, 19)*. This report addresses the reliability and comparability of ApoE phenotyping and genotyping, and the implications of using these two methods for epidemiological studies, population screening, and patient care. There have been previous reports of inconsistencies between ApoE genotypes and phenotypes, possibly related to methodological or other problems *(20–26)*. One possibility is that posttranslational protein glycation might influ-

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[6] Nonstandard abbreviations: ApoE, apolipoprotein E; AD, Alzheimer disease; HAAS, Honolulu-Asia Aging Study; and OR, odds ratio.

ence accurate characterization of the phenotype. Potential inconsistencies between ApoE genotyping and phenotyping are important considerations in assessing the utility of genetic testing for ApoE in epidemiological studies and in patient care. Although several statements about the usefulness of ApoE testing have been made, these include no mention of the possibility of laboratory errors *(27–32)*. This report aims to answer the following questions: (*a*) Are results of phenotyping and genotyping of ApoE the same? (*b*) Is the association of ApoE with AD different for phenotype and genotype? (*c*) What are the consequences of any difference in ApoE typing for specific applications (research, screening, patient care)?

Data and Methods

HONOLULU-ASIA AGING STUDY COHORT

The Honolulu-Asia Aging Study (HAAS) cohort consists of Japanese-American men born from 1900 through 1919 and living on Oahu, Hawaii when the study began. Of men followed since 1965, 3734 received evaluations for cognitive function and dementia during the 1991–1993 examination cycle *(33–35)*. Subjects were fully informed regarding participation in the study and provided informed consent. Interviews and testing were carried out by trained interviewers, either in the research center or at the subjects' homes or nursing homes, in the subjects' preferred language [Japanese (12%) or English].

DEMENTIA

Dementia was assessed using *Diagnostic and Statistical Manual of Mental Disorders* (DSM-III-R) diagnostic criteria *(36)* by a panel consisting of the study neurologist and at least two other physicians with expertise in geriatric medicine. Criteria of the National Institute of Neurological and Communicative Disorders-Alzheimer's Disease and Related Dementias Association were similarly applied for the diagnosis of probable or possible AD. A total of 105 cases included 61 individuals classified as having probable AD, 6 classified as having possible AD with no other cause apparent, and 38 classified as having possible AD judged the primary cause of the dementia, but with another contributing cause also recognized. Odd ratios (ORs) were calculated by comparing AD patients with the rest of the HAAS sample.

LABORATORY DETERMINATIONS OF ApoE PHENOTYPE AND GENOTYPE

ApoE phenotyping and genotyping were done on plasma and buffy coat preparations separated from anticoagulated blood within 2 h of collection and then frozen and held at −70 °C until thawed for this use. Phenotyping was done at the Northwest Lipid Research Laboratory, Seattle, under the direction of one of the authors (S.M.), using a modification of the method described by Kataoka et al. *(37)*. Briefly, 10 μL of plasma sample was incubated with dithiothreitol (0.7 g/L) and Tween 20 (2.5 mL/L) for 15 min in preparation for monodimensional isoelectric focusing. Flatbed gels of 5% polyacrylamide containing ampholytes (pH range 4.0–8.0) and 3 mol/L urea were prepared, placed in an LKB Electrophor electrofocusing unit, and prefocused by applying constant power of 20 W for 15 min, with 1 mol/L NaOH and 1 mol/L phosphoric acid as cathode and anode buffers, respectively. Plasma samples adsorbed onto filter strips were applied on the gel ~15 mm from the cathode. Known samples of the common phenotypes were included with unknowns in each gel. A constant power of 20 W for 30 min was applied to allow samples to enter the gel. Sample wicks were removed, and sample proteins were focused by further power application for 90 min. Protein fractions in the gel were eluted and transferred to a nitrocellulose filter by overnight passive capillary adsorption. The nitrocellulose filter containing the protein bands was incubated for 60 min in Tris-buffered saline (0.25 mol/L NaCl, 0.03 mol/L Tris-HCl, pH 8.0) containing 20 g/L nonfat milk. The filter was exposed for 60 min to a monospecific goat anti-human ApoE antibody (Inkstar), washed in Tris-buffered saline, and reacted with a second antibody, goat anti-rabbit conjugated with horseradish peroxidase (Kirkegaard & Perry Laboratories). After several washings, the banding patterns on the filter were visualized using an ECL chemiluminescence system (Amersham Pharmacia Biotech), and a permanent record of the results was made by exposing an autoradiographic film (Kodak).

During the early phases of the study, the procedure was validated by running 200 samples in double-blind fashion. Confirmed samples were included as quality controls in subsequent analyses. Data entry and final types were checked regularly by a second person. Phenotypes for a panel of ~20 samples identified as nonconcordant with genotype were independently confirmed at a second laboratory. Although it may be possible to identify the ApoE5 and ApoE7 phenotype patterns on gels such as those used for this study, samples of these isoforms were not ordinarily included as known positives in gels, and neither the antibody reagents nor the methods have been evaluated to determine whether these isoforms would have been identified.

ApoE genotyping was done at Duke University under the direction of one of the authors (A.S.). Genomic DNA was extracted from peripheral blood leukocytes (buffy coat samples heavily contaminated with erythrocytes) using Puregene kits (Gentra Systems) according to the manufacturer's protocol for blood. ApoE gene amplification and typing were performed as described by Saunders et al. *(7)* with the exception that reactions were nonradioactive and restriction digest fragments were visualized using a fluorimager after SYBR Green staining. Efforts to minimize human error included assigning sample-specific barcodes to all buffy coat samples, aliquots, and extracted DNA and using these to track the sample through DNA extraction, PCR set up, and reading. Risk of pipetting and transferring errors were minimized by rigorous standardization of procedures for aliquoting

DNA samples and loading gels, and by spacing of samples and controls (water, ApoE calibrators, molecular weight markers). Gels were read and genotypes transcribed by two persons independently, with correspondence checked after data entry.

STATISTICAL ANALYSIS METHODS

Because ApoE2, -3, and -4 alleles and the isoforms of a pair can be considered independent of each other, our analysis is based both on allele and isoform frequencies (given two observations per person), and on gene pairs and persons (where each person is one observation and can be homo- or heterozygous). ORs were calculated by logistic regression, controlling for age and education. Confidence intervals are 95% confidence intervals, and significance testing is at the 5% level, unless otherwise stated.

Multivariate logistic regression analyses to evaluate associations of E2 and E4 ApoE alleles or isoforms with AD (Table 4) were carried out as a series of four separate models. The dependent variable was the dichotomous (present or not present) diagnosis of AD, based on National Institute of Neurological and Communicative Disorders-Alzheimer's Disease and Related Dementias Association diagnostic criteria for probable or possible AD. This included the 105 cases described above and excluded other cases of possible AD in which the most important cause of dementia was not thought to be AD. All regression models included age (in single years) and education (as single years of schooling completed) as covariates. To assess possible interactions of ApoE type with age or education, all possible two-variable products of the E2 or E4 allele or isoform with age or education were registered in models in the presence of the primary variables.

Results

ApoE genotype and phenotype results were available for 3564 (95%) of the 3734 men who participated at the 1991–1993 examination. Data are presented based on individuals (Table 1) and on isoforms or alleles (two per person, n = 7128; Table 2). As expected, the most common ApoE type was 3-3. Distinguishing the probability of a phenotype-genotype nonconcordance according to isoforms, alleles, or individuals is important both for assessing the clinical utility of the two methods and for understanding the biological meaning of such nonconcordance. Of participants with genotype 3-3, 90% were also phenotyped as 3-3. Of participants with phenotype 3-3, 94% were also genotyped as 3-3. The discrepancies were largely attributable to an excess of the E2 isoform (Table 2A). When calculated as a conditional probability, 43% of ApoE phenotyped as 2 were genotyped as 3 (Table 2B). The probability of an ApoE2 phenotype among persons genotyped as 3 was 4% (Table 2C).

Frequencies of AD for each ApoE type are shown in Table 3. Of 17 men with the 4-4 phenotype and 16 men with the 4-4 genotype, 3 were found to have AD, compared with a computed expected number of 0.5 AD cases. Table 4 shows the association with AD of ApoE genotype

Table 1. Phenotype vs genotype of 2-, 3-, 4-allele/isoform of ApoE, per person.

	Genotype						
Phenotype	2-2	2-3	2-4	3-3	3-4	4-4	Total
2-2	5	7	4	2	0	0	18
2-3	2	261	2	227	5	0	497
2-4	1	2	28	0	1	0	32
3-3	0	26	3	2332	107	0	2468
3-4	0	1	0	32	497	2	532
4-4	0	0	0	0	3	14	17
Total	8	297	37	2593	613	16	3564

Table 2. Phenotype vs genotype of ApoE, per gen (twice the number of persons).

A. Cross table

	Genotype			
Phenotype	2	3	4	Total
2	317	244	4	565
3	32	5814	119	5965
4	1	38	559	598
Total	350	6096	682	7128

B. Conditional probability of genotype, given phenotype (to be read rowwise)

	Genotype			
Phenotype	2	3	4	Total
2	0.56	0.43	0.007	1.0
3	0.005	0.97	0.02	1.0
4	0.002	0.06	0.94	1.0

C. Conditional probability of phenotype, given genotype (to be read columnwise)

	Genotype		
Phenotype	2	3	4
2	0.91	0.04	0.006
3	0.09	0.95	0.17
4	0.003	0.006	0.82
Total	1.0	1.0	1.0

Table 3. AD vs genotype and phenotype of ApoE.

	Phenotype			Genotype		
Allele/ Isoform	No AD	AD	%AD	No AD	AD	%AD
2-2	18	0	0	8	0	0
2-3	490	7	1	291	6	2
2-4	30	2	6	36	1	3
3-3	2397	71	3	2526	67	3
3-4	511	21	4	586	27	4
4-4	13	4	24	12	4	25
Total	3459	105	3	3459	105	3

Table 4. Association of ApoE alleles/isoforms with AD.

		Confidence interval	
	OR[a]	CI	Years difference[b]
Phenotype			
2 homozygous	–[c]		
2 heterozygous	0.5	0.3–1.1	−2.88
4 heterozygous	1.9	1.1–3.0	2.90
4 homozygous	14.2	3.9–51.6	12.24
Genotype			
2 homozygous	–[c]		
2 heterozygous	0.6	0.3–1.4	−2.16
4 heterozygous	2.0	1.3–3.2	3.23
4 homozygous	14.7	4.0–53.6	12.36

[a] OR calculated by logistic regression, adjusting for age and education.

[b] Years difference: the risk for AD corresponds to the risk of a ApoE 3-3 person who is *x* years older.

[c] No occurrences of 2-2 allele/isoform in AD group; OR considered "low" and confidence interval "wide".

and phenotype using logistic regression adjusted for age and education. The OR for ApoE4 heterozygotes and AD was similar for genotype and phenotype and was approximately a twofold excess risk ($P < 0.01$) over non-ApoE4 individuals. There was similarity in AD risk association for genotype and phenotype comparisons of ApoE4 homozygotes with an OR of ~14 ($P < 0.001$). For ApoE2, a moderate protective effect was evident with an OR of ~0.5 ($P > 0.09$), similar for phenotype and genotype. There were insufficient 2-2 cases to determine whether a dose–response relationship existed with homozygous ApoE2 (expected numbers ≈0.5; found, none). In all models, the OR estimating the increasing occurrence of AD with a single year of age was 1.24, whereas the OR of AD for a single year of schooling completed was 0.96.

The increased frequency of AD among ApoE4 homozygotes (OR = 14.7) was higher than expected from an independent heterozygote ApoE4 effect [$(OR = 2.0)^2 = 4.0$]; the square of the upper confidence limit of the OR of ε4 heterozygotes ($3.2^2 = 10.2$) is less than the point estimate of the ε4 homozygotes (14.7), and conversely, the root of the lower confidence limit of the OR of ε4 homozygotes ($\sqrt{4.0} = 2.0$) is equal to the point estimate of the ε4 heterozygotes (2.0). This suggests that homozygosity is associated with a higher risk of AD than two independent alleles (at a 5% significance level). This "recessive" character of the ApoE4 gene risk was equally apparent when isoform typing was used.

The association of ApoE (both 2 and 4, both homo- and heterozygous) with AD appeared to be almost independent of age. The OR without age adjustment was 12.2 (3.8–39.0) for E4 homozygotes and 12.0 (3.7–38.8) for ε4 homozygotes. (For the ORs with adjustment, see Table 4.) The independence of the effects of age and ApoE4 was further supported by the introduction of an interaction term, which was nonsignificant ($P < 0.38$ for E4 homozygotes and $P < 0.36$ for ε4 homozygotes). This finding points to at least partially different mechanisms underlying the influences of age and ApoE on the development of AD, and suggests that the increased occurrence of AD with ApoE4 positivity is generally constant across the age range in this population (71–93 years).

Because of this age independence, the strength of the association of AD with ApoE4 can be expressed in a "corresponding" age difference (Table 4). Estimated from logistic regression, ApoE4 heterozygosity confers on an individual a probability of having AD approximating that of an otherwise similar 3-3 individual who is 3 years older. Similarly, an ApoE4 homozygous person has a probability of AD similar to that of a 3-3 person who is 12 years older.

Despite the considerable and significant OR (~14) for 4-homozygosity and AD, the effect of age in predicting AD is quite dominant. It is useful, therefore, to compare the number of additional cases of AD one might predict using ApoE4 status in addition to age and education, compared with age and education only. The classification table of the logistic regression, presented in the form of a so-called ROC curve *(38)* in Fig. 1, shows the trade-off of sensitivity against specificity. The two ROC curves are almost identical. In our study population, given a "fixed" sensitivity of 66.7%, the specificity in predicting AD increased from 83.0% to 83.3% when ApoE4 status was included in the logistic model in addition to age and education. Thus, in population screening, ApoE4 status contributes little to predicting dementia, if age and education are known.

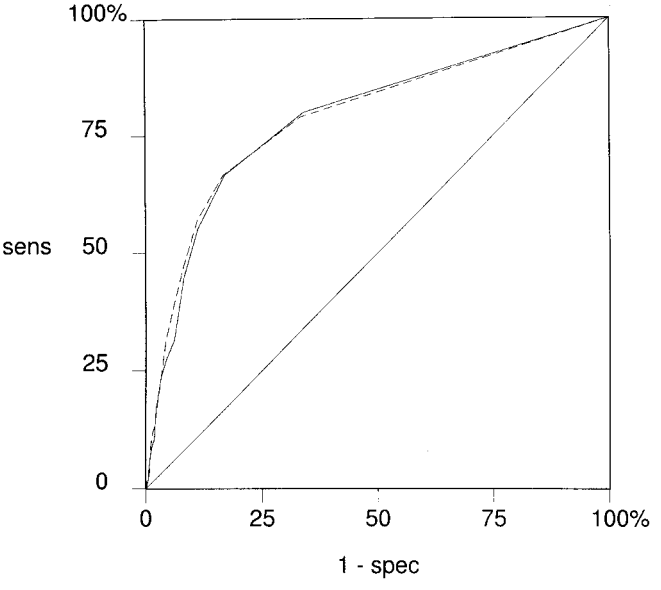

Fig. 1. ROC curve of AD prediction by a logistic model adjusted for age and education, without and with ApoE4 status.

The specificity (*spec*) and sensitivity (*sens*) of the prediction by the logistic model are shown. The predicted outcome (AD or no AD) is compared with the "true" outcome. *Solid line*, prediction by age and education only; *dashed line*, prediction by homo- and heterozygous ε4 status in addition to age and education.

Discussion

There is growing interest in identifying and using genetic polymorphisms for disease risk, including risk for AD, in the general population. This interest has been particularly strong for ApoE4 in relation to AD. Although there has been some endorsement of the use of genotyping for dementia patients, population screening of asymptomatic individuals has not been recommended *(27)*. It is, however, important to distinguish among use for epidemiological research, patient care, and population screening.

epidemiological studies

An important aspect of epidemiological research is the discovery and modeling of risk associations. An important conclusion of our study is that the phenotypes and genotypes of ApoE2 and ApoE4 are associated with similar magnitude with AD: ApoE2 appears to be moderately protective and ApoE4 is a risk factor. The robustness of these relationships supports the use of phenotyping, especially when DNA specimens are not available, as is the case in many large longitudinal population-based studies. Confirmatory observations might also support the use of specimens collected many years previously in retrospective cohort studies. Such investigations might add to existing knowledge of incidence of AD and whether differential mortality related to the presence of ApoE4 might distort subsequent relationships *(39, 40)*. However, for studies of ApoE2, considerable differences in persons identified as "at risk" by genotyping or phenotyping must be kept in mind. It has yet to be established whether the high frequency of ApoE2 phenotype-genotype nonconcordance we observed is specific to the Japanese-American subjects in the HAAS or occurs in other populations as well.

patient care

For specificity of characterization, the standard for patient care has become the genotype, and this procedure is recommended, especially because many laboratories are offering this determination. In addition, in the future it is likely that there will be other polymorphisms best studied with DNA, and these will completed in conjunction with ApoE determinations. Although the association of ApoE4 with a risk for AD is similar for genotype and phenotype, the remaining individual nonconcordance is not to be neglected, and—as is usual in clinical situations—the clinician must consider the gained information in the context of all available information bearing on the diagnosis.

population screening

Population screening for AD would involve persons without any known predisposition for this condition. The expected prevalence of AD would be mainly dependent on the age composition of the population and would be rather low. In our population sample of men 71–93 years of age, screening for AD by means of ApoE would not have been fruitful, except for identification of the limited number of ApoE4-4 persons. In the general population, use of ApoE status in addition to the freely available information on age and education only marginally improves prediction, as demonstrated by the ROC curves. Furthermore, screening is at present—without a fairly effective therapy for AD—not justified.

what might explain the high frequency of phenotype-genotype nonconcordance in this population?

Although major discrepancies between ApoE phenotypes and genotypes have been reported previously, the extent of nonconcordance has varied dramatically. The initial descriptions of phenotype-genotype nonconcordance were focused on their associations with diabetes and hyperglycemia, leading to speculation that a glucose-driven posttranslational modification of the protein might lead to alteration in the band pattern on isoelectric focusing and ultimately to errors in typing *(23–26)*. For the most part, these observations have not been confirmed, and the glycation of the molecule appears an unlikely cause for substantial nonconcordance. A second possibility is clerical or laboratory error, occurring by chance *(22)*. A third possibility, as yet not demonstrated to be an important cause of phenotype-genotype nonconcordance, is that there are rare genetic polymorphisms associated with ApoE2 that affect protein expression and/or alter the primary structure of the gene product. Some reports of "rare" polymorphisms have appeared, notably in the Japanese population (ApoE-ϵ7, ApoE-E1, ApoE-E5, ApoE-E7) *(41–43)*, but unusual mutations have also been reported that cause an ApoE-ϵ4/E4 discrepancy with usual assessment methods *(44)*. Finally, "normal" variations in test reproducibility may explain part of the nonconcordance; these may become visible especially in studies with large numbers of subjects. In fact, there is a point to be made for routinely genotyping DNA in duplicate, as soon as "cheaper" techniques allow the extra effort.

etiology

Our finding of the independence of risk for AD associated with age and ApoE (in a group of men over age 70) may reflect the presence of AD and not its progress. Independence was also found by others *(19, 45)*. However, some dependencies have been found in clinical case-control studies with a wider age range *(11, 46)*. It has also been reported that the influence of ApoE4 may diminish rather dramatically after age 80 *(47)*. ApoE4 does not seem to be related to the progress of AD *(48–50)*, which is in contrast to the association of ApoE with the progress of cognitive impairment in similar age groups *(51–53)*.

The choice of genotyping or phenotyping should also be informed by the other correlates of ApoE type, especially as related to lipid metabolism. Before identification of the relationship between ApoE4 and AD, most work

with ApoE type related to cardiovascular disease and lipid concentrations. Because this research goes back several years and because of limitations in technology, ApoE serum proteins were measured either directly or in the VLDL subfraction *(22)*. As genotyping became available, there were some attempts to compare results. In one such comparison, serum triglyceride concentrations were higher in the phenotyped ApoE2 subgroup compared with the genotyped designation *(22)*. The reasons for this discrepancy were unclear. The possibility that unrecognized genotypes or different intermediary processes might affect physiologic and metabolic measurements has been suggested *(22)*. Such phenomena could vary with ethnicity, comorbidity, or other factors.

The observation of Lahoz et al. *(22)* that many ApoE phenogenotype-genotype discrepancies could be attributed to errors in labeling or handling must not be minimized. When ApoE typing is important for the care or diagnosis of an individual, only very low laboratory error is tolerable. When typing is done as part of epidemiologic research, the identification of true phenotype-genotype differences may well lead to a better understanding of several illnesses, including AD, atherosclerosis, and diabetes. The importance of reliable typing for research or clinical purposes is obvious. Although duplicate testing has rarely been done for genetic assays, this or some other method for detecting test inconsistencies could ultimately be an important quality-control strategy for laboratories conducting such tests.

In conclusion, the HAAS provided a valuable opportunity to evaluate various aspects of the use of ApoE phenotyping and genotyping in a population-based epidemiological study. In this specific population, either genotyping or phenotyping was adequate for determining associations of ApoE4 with AD. Genotype and phenotype showed similar associations, although mild discrepancies for ApoE4 and substantial discrepancies for ApoE2 produced some differences in which individuals were identified. The effect of age and ApoE status on AD presence appeared to rather independent. Our findings indicate that the use of ApoE status for screening of the general population would provide only minimal improvement in prediction of cases over the use of education and age only.

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