APOE Polymorphisms and the Development of Diabetic Nephropathy in Type 1 Diabetes

Results of Case-Control and Family-Based Studies

Shin-ichi Araki, Dariusz K. Moczulski, Linda Hanna, Laura J. Scott, James H. Warram, and Andrzej S. Krolewski

The goal of this study was to examine the association between known polymorphisms in the apolipoprotein E gene (*APOE*) and diabetic nephropathy (DN) in type 1 diabetes. We used both a case-control comparison and a family-based study design known as the transmission/disequilibrium test (TDT). For the case-control comparison, we collected DNA from 223 subjects with clinically diagnosed DN and 196 control subjects with normoalbuminuria and long-duration type 1 diabetes (≥15 years). For the family-based study, we obtained DNA from both parents of 154 DN subjects and 81 control subjects. The frequency of the ε2 allele of exon 4 of *APOE* was significantly higher in DN subjects than in control subjects. The risk of DN was 3.1 times higher (95% CI 1.6–5.9) in carriers of this allele than in noncarriers. In the family study, heterozygous parents for the ε2 allele preferentially transmitted ε2 to DN offspring (64 vs. 36%, $P < 0.03$). Four additional polymorphisms (i.e., –491 A/T, –219 G/T, IE1 G/C, and *APOCI* insertion/deletion [I/D]) that flank the *APOE* locus were not associated with DN in either the case-control comparison or in the family-based study. In conclusion, the results of the case-control as well as the family-based study provide evidence that the ε2 allele of *APOE* increases the risk of DN in type 1 diabetes. The molecular mechanisms underlying this risk are unclear at present. *Diabetes* 49:2190–2195, 2000

Diabetic nephropathy (DN) is the major determinant of excess morbidity and premature mortality in type 1 diabetes (1,2). Whereas prolonged hyperglycemia is an important risk factor for the development of DN (3,4), epidemiological and family studies suggest that genetic susceptibility to this complication is also required (5,6). Despite intensive research, the genes responsible for this susceptibility are unknown (7). Recently, a polymorphism in exon 4 of the apolipoprotein E gene *(APOE)* has been investigated for association with DN (8–15).

Apolipoprotein E (apoE) is a 299–amino acid glycoprotein that plays a central role in lipid metabolism. It binds with high affinity to the LDL receptor and facilitates endocytosis of the associated lipoprotein particle (16). In addition, apoE mediates lipoprotein interactions with the LDL receptor–related protein, the VLDL receptor, other lipoprotein receptors, endothelial heparin sulfate, and plasma lipases (16–18). apoE is polymorphic, consisting of three common isoforms (E2, E3, and E4) encoded by three alleles (ε2, ε3, and ε4) in exon 4 of *APOE* (16). These isoforms differ by single amino acid substitutions that affect their structure and function (16,19,20). Whereas apoE3, the most common form, has cysteine at position 112 and arginine at position 158, apoE2 has cysteine at both positions and apoE4 has arginine at both.

Carriers of apoE4 have higher plasma levels of total and LDL cholesterol than carriers of apoE2 or apoE3 homozygotes (16). apoE4 is a significant risk factor for Alzheimer's disease and a moderate risk factor for coronary artery disease (CAD) (21,22). On the other hand, the binding of apoE2 to lipoprotein receptors is defective in comparison with that of apoE3 or apoE4 and results in delayed clearance of triglyceride-rich lipoprotein. apoE2 has been strongly associated with the development of type III hyperlipoproteinemia and subsequent CAD as well as with rare forms of lipoprotein glomerulopathy (16,23). Several recent case-control studies have examined the association of this *APOE* polymorphism with the prevalence of DN in type 1 and type 2 diabetes, but the findings have been inconclusive (8–15).

To clarify the association between *APOE* polymorphism and DN, we carried out a large case-control study, the positive results of which were examined further in a family-based association study. The latter is a bias-free study design for examining associations between DNA sequence differences and specific diseases (24). To investigate whether the *APOE* polymorphism itself or DNA sequence differences in linkage disequilibrium with *APOE* contribute to the risk of DN, we tested four additional polymorphic DNA markers in the flanking regions of *APOE* for association with DN.

From the Section on Genetics and Epidemiology, Research Division, Joslin Diabetes Center, and the Department of Medicine, Harvard Medical School, Boston, Massachusetts.

Address correspondence and reprint requests to Andrzej S. Krolewski, MD, PhD, Section on Genetics and Epidemiology, Research Division, Joslin Diabetes Center, One Joslin Place, Boston, MA 02215. E-mail: andrzej.krolewski@joslin.harvard.edu.

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S.A. is currently affiliated with the Third Department of Medicine, Shiga University of Medical Science, Otsu, Shiga, Japan. D.K.M. is currently affiliated with the Department of Internal Medicine and Diabetology, Silesian Medical School, Zabrze, Poland.

ACR, albumin/creatinine ratio; apoE, apolipoprotein E; ASO, allele-specific oligonucleotide; CAD, coronary artery disease; DN, diabetic nephropathy; ESRD, end-stage renal disease; I/D, insertion/deletion; LPG, lipoprotein glomerulopathy; OR, odds ratio; PCR, polymerase chain reaction; RFLP, restriction fragment–length polymorphism; TDT, transmission/disequilibrium test; TMAC, tetramethyl ammonium chloride.

RESEARCH AND DESIGN

Study groups. Patients attending the Joslin Clinic in the early 1990s were recruited as study subjects. During 1991–1993, a 50% sample of clinic attendees with type 1 diabetes in the age range of 15–44 years (n = 1,613) were enrolled in a follow-up study of the natural history of microalbuminuria (25). On the basis of multiple measurements of the albumin/creatinine ratio (ACR) in random urine specimens collected during a 2-year observation period, patients were classified into the following categories: normoalbuminuria (n = 1,100), persistent microalbuminuria (n = 312), and persistent proteinuria or end-stage renal disease (ESRD) (n = 201) (25).

As unrelated control subjects for the present study, we selected patients with normoalbuminuria and diabetes duration ≥15 years. Among the 1,100 normoalbuminuric patients, 420 met this additional criterion and 319 (75%) were examined. For the present study, we recruited 196 individuals who had been examined early in the study and for whom extracted DNA was already available. As unrelated cases for the present study, we selected patients with persistent proteinuria or ESRD, including the 201 such patients enrolled in the follow-up study, of whom 151 had been examined. They were supplemented with 235 patients of the Joslin Clinic with type 1 diabetes and persistent proteinuria or ESRD who had not been included in the follow-up study described above. The examination of the latter group is under way, and 72 patients have been examined so far, bringing the total number of unrelated examined subjects to 223. The two groups of patients did not differ with regard to sex, age at examination, duration of diabetes, level of HbA_{1c}, total serum cholesterol, serum triglycerides, and frequency of *APOE* alleles. Therefore, we pooled these two subgroups.

Originally we had planned to compare two subgroups of patients (i.e., those with proteinuria and those with ESRD) with a group of control subjects. However, because of the similarity in the frequency of *APOE* alleles and genotypes in both subgroups, we simplified the presentation by combining patients with proteinuria and ESRD into one group.

For the family-based association study, both parents of 154 patients and 81 control subjects had been examined as of 1998. In 41 of these families, 45 additional siblings with type 1 diabetes were identified and examined for the family-based study (we classified 18 of them as cases and 27 as control subjects).

Diabetes was considered as type 1 if it was diagnosed before age 30 years and treatment with insulin was begun within 1 year of diagnosis and continued thereafter. Only Caucasian patients were included in this study.

Examination of study participants. After consenting to participate in the study, subjects underwent a standardized physical examination and provided a diabetes history detailing their diagnosis, treatment, and occurrence of complications. Each individual provided a blood sample for biochemical measurements and DNA extraction and a random urine sample for urinalysis and determination of the ACR. For the family-based study, parents provided blood samples for DNA extraction. The Committee on Human Subjects of the Joslin Diabetes Center approved the protocol and informed consent forms.

Diagnosis of diabetic nephropathy. DN status was determined on the basis of questionnaires, medical records (from the Joslin Diabetes Center and other institutions), and measurements of the ACR. Methods for measuring the ACR have been described previously (25). Patients were classified as control subjects (n = 196) if they had diabetes duration ≥15 years and their ACR (in μg/mg) was <17 (men) or <25 (women) in at least two of the last three urine specimens. Patients with microalbuminuria or intermittent proteinuria were excluded from the study. Patients were considered study subjects (n = 223) if they had persistent proteinuria or if they had ESRD caused by DN. Persistent proteinuria was defined as two of three successive urinalyses testing positive by reagent strip (≥2+ on Multistix [Bayer Corporation, Elkhart, IN]) or by an ACR (in μg/mg) ≥250 (men) or ≥355 (women). At the time of examination (1992–1997), subjects were divided into those with persistent proteinuria (n = 178) and those with ESRD (n = 45). Subjects with proteinuria were followed until the end of 1998, and 35 of them developed ESRD. Therefore, for this study we had a total of 80 patients with ESRD (45 prevalent and 35 incidence cases).

Genetic markers and genotyping. Genomic DNA was extracted from peripheral lymphocytes using a Phenol/Chloroform method. We analyzed the *APOE* ε2/ε3/ε4 polymorphism and four additional polymorphic markers flanking *APOE*: the –491 A/T and –219 G/T markers in the *APOE* promoter region (26), the IE1 G/C in the intron 1 enhancer element of *APOE* (27), and the insertion/deletion (I/D) in the 5′ end of the promoter of *APOC1* (28). All of these polymorphisms are located within a 10-kb region that includes *APOE* (Fig. 1).

Fragments containing each polymorphic site were amplified by polymerase chain reaction (PCR) methods in a 25-μl volume with 20 ng genomic DNA. The genotypes at a *Cfo*I restriction fragment–length polymorphism (RFLP) site in exon 4 of *APOE* (*APOE* ε2/ε3/ε4) and a *Hpa*I RFLP site in the 5′ end of *APOC1* (*APOC1* I/D) were determined according to previously described methods (29,28). We developed allele-specific oligonucleotide (ASO) hybridization protocols to genotype the –419A/T, –219G/T, and IE1 G/C polymorphisms as described below.

DNA fragments containing the –419A/T polymorphic site were amplified by PCR methods using the following primers: forward 5′-GAGTGCAGTGGCGA GATCTC-3′ and reverse 5′-GCTGGACAGAAGTGGGATGG-3′. The two ASO probes (5′-TCAAACTCCTGACCTTA-3′ and 5′-TCAATCTCCTGACCTTA-3′ [for each A or T allele detection]) were 5′ end–labeled with [γ-^{32}P]ATP (NEN, Boston, MA) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). PCR-amplified DNA fragments were denatured by a denaturing solution (500 mmol/l NaOH, 2 mol/l NaCl, 25 mmol/l EDTA, and 0.0001% bromophenol blue) and then dotted onto nylon membranes using a dot-blot apparatus (Bio-Rad Laboratories, Hercules, CA). After fixing the DNA on the membrane by immersion in a 2× sodium chloride–sodium citrate solution for 2 min and then heating it for 20 min in an 80°C oven, the membrane was hybridized in a tetramethyl ammonium chloride (TMAC) solution (3mol/l TMAC, 0.6% SDS, 1 mmol/l of EDTA, 10 mmol/l Na_3PO_4,5 × Denhardt solution, and 40 μg/ml yeast RNA) that contained the labeled ASO probe together with a 20-fold excess of unlabeled ASO probe corresponding to the opposite allele. After overnight hybridization at 52°C, the membrane was washed in TMAC wash buffer (3mol/l TMAC, 0.6% SDS, 1 mmol/l of EDTA, and 10 mol/l Na_3PO_4) and exposed to X-ray film. Autoradiograms were evaluated by two independent observers.

DNA fragments containing the –219 G/T and IE1 G/C polymorphic sites were amplified with previously described primers (27,30). To recognize the –219 G/T polymorphism, we used 5′-TCTGGATTACTGGGCGA-3′ and 5′-TCTGTAT TACTGGGCGA-3′ as ASO probes, and we used 5′-GGAAGCCCTGGCCTCCA-3′ and 5′-GGAACCCCTGGCCTCCA-3′ to recognize the IE1 G/C polymorphism. Allele-specific hybridizations were performed as described above.

To screen for new DNA sequence differences in *APOC1*, we sequenced directly the 2,000 bp of the promoter region and all four exons (together with flanking intron sequences) in 24 patients with DN. No sequence difference was

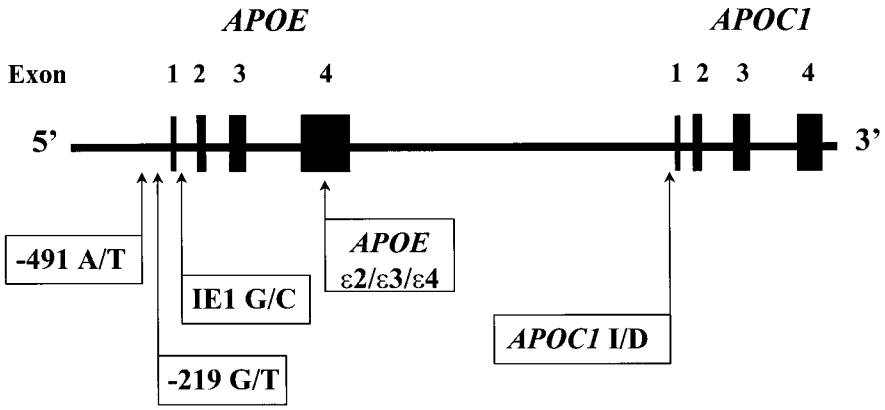

FIG. 1. Schematic diagram of the 10-kb region that includes *APOE*, showing the exons of *APOE* and *APOC1* (closed boxes) and the polymorphic sites examined in this study (as indicated by the arrows).

TABLE 1
Selected clinical characteristics of the subjects with type 1 diabetes according to nephropathy status

Clinical characteristics at the time of examination	Control subjects	Study subjects
n	196	223*
Sex (M/F)	98/98	110/113
Age at diabetes diagnosis (years)	13 ± 7	11 ± 6
Age at examination (years)	36 ± 8	35 ± 7
Duration of diabetes (years)	23 ± 7	24 ± 7
HbA_{1c} (%)	8.3 ± 1.2	9.3 ± 1.7†
Total cholesterol (mg/dl)	201 ± 48	242 ± 79†
Triglycerides (mg/dl)	117 ± 66	189 ± 139†

Data are *n* or means ± SD. *This group included 80 subjects with ESRD (45 prevalent and 35 incidence cases); †$P < 0.01$ vs. control subjects.

found except for the D/I polymorphism in the promoter previously described as the *Hpa*I RFLP site in the 5′ end of *APOCI* (28).

Analytic methods. The study groups were compared by χ^2 tests for frequencies or Fisher's exact test (SAS system for windows version 6.12; SAS Institute, Cary, NC) if the frequencies were small. Analysis of variance was used for continuous variables. Odds ratios (ORs) and their confidence intervals were used to estimate relative risk (31). Multiple logistic regression analysis was also used to assess the association of the ε2 allele of the *APOE* polymorphism with DN. The following independent variables were used: *APOE* genotype (indicator variable for ε2 allele carriers), duration of diabetes (years), and levels of HbA_{1c} (%), total serum cholesterol (mg/dl) and triglycerides (mg/dl). Pairwise linkage disequilibrium coefficients between the ε2 allele of the *APOE* polymorphism and the alleles of the flanking markers were estimated as outlined by Thompson et al. (32).

Family-based test for association. The transmission disequilibrium test (TDT) is a family-based study design that was developed to test for association between a specified allele and a disease phenotype. Unlike case-control comparisons, the TDT is valid even in the presence of population stratification (24). For the TDT, it is necessary to know the genotypes of both parents as well as their offspring with the phenotype being studied. In this study, we studied two phenotypes, DN-present and DN-absent, as manifest by normoalbuminuria. If a parent is heterozygous for any locus, transmission of a particular allele to an offspring is expected to be 50%. This is true regardless of the offspring's phenotype, as long as there is no association between the allele and the phenotype (null hypothesis). Excess transmission to the offspring is expected if the allele is associated with increased risk of the phenotype, and deficient transmission is expected if it is associated with decreased risk (McNemar's Test) (24). All diabetic offspring in a family were used in the analysis presented here. Because our case-control comparisons pointed to the ε2 allele of the *APOE* polymorphism as the risk allele, the primary hypothesis examined by the TDT was whether the transmission of that allele was significantly different from 50%. Transmission of the other two alleles was examined as secondary hypotheses to further characterize the role of the locus.

Transmission of haplotypes was also examined in our families. We examined the transmission of the six haplotypes determined by three alleles in exon 4 of *APOE* and the I/D in the 5′ end of *APOC1*. Haplotypes could be unambiguously assigned in most families. For the families with phase-unknown haplotypes, conditional probabilities of alternative configurations were calculated using all families, and the most probable haplotypes were assigned. The program Genehunter 2 (version 2.0 beta; http://waldo.wi.mit.edu/ftp/distribution/software/genhunter/gh2) was used to perform the TDT analysis for both markers separately and for haplotypes (33).

RESULTS

First, we examined the association of DN with the exon 4 *APOE* polymorphism in a case-control study. Selected clinical characteristics of the study groups are summarized in Table 1. The age at diagnosis of diabetes, age at examination, and duration of diabetes were similar in the two groups. Despite the long duration of diabetes, subjects in both groups were still young at the time of examination. Levels of HbA_{1c}, serum total cholesterol, and triglycerides were significantly higher in study subjects than in control subjects at the time of examination.

TABLE 2
Frequencies of *APOE* alleles and genotypes according to nephropathy status

	Control subjects	Study subjects
Alleles*		
ε2	3 (13)	9 (42)
ε3	82 (321)	77 (343)
ε4	15 (58)	14 (61)
Genotypes†		
ε2/ε2	0.0 (0)	1.0 (2)
ε2/ε3	6.0 (12)	14.0 (32)
ε2/ε4	0.5 (1)	3.0 (6)
ε3/ε3	65.0 (127)	61.0 (135)
ε3/ε4	28 (55)	18.0 (41)
ε4/ε4	0.5 (1)	3.0 (7)

Data are % (*n*) of chromosomes for alleles and % (*n*) of patients for genotypes. *$P = 0.002$; †$P < 0.001$ (Fisher's exact test).

Allele frequencies of the *APOE* polymorphism in exon 4 (Table 2) were significantly different between the two groups ($P = 0.002$), with the ε2 allele being more frequent in study subjects than in control subjects. The frequency of the ε3 allele had the opposite pattern, whereas the frequency of the ε4 allele was similar in the two groups. Genotype frequencies (Table 2) were also significantly different between the two groups ($P < 0.001$, Fisher's exact test). Only the genotypes that included ε3 were frequent enough to test by χ^2. Among the carriers of ε3, the heterozygotes ε2/ε3 were significantly more frequent in study subjects than control subjects, whereas the frequencies of the other two genotypes were not significantly different in the two groups ($\chi^2 = 10.9$ with 2 df, $P = 0.004$). This pattern suggested a dominant mode of inheritance of the effect of the ε2 allele, so the six genotypes were combined into two groups according to whether they were carriers or noncarriers of the ε2 allele.

Carriers of the ε2 allele were more frequent in subjects with nephropathy than control subjects (OR 3.1, 95% CI 1.6–5.9). Moreover, the strength of the association increased with the duration of diabetes (Fig. 2). The OR for carriers increased from 2.0 (95% CI 0.9–4.6) for those with shorter duration (<25 years) to 6.4 (95% CI 1.8–22.3) for those with longer duration (≥25 years). The OR for ESRD, specifically, was 3.9 (95% CI 1.8–8.4) and not significantly different from the OR for all levels of advanced nephropathy. Furthermore, the OR for prevalent cases of ESRD was the same as that for incidence cases (3.6 and 4.2, respectively). Also, in multiple logistic regression, the ORs for nephropathy were changed only slightly by adjustment for the duration of diabetes, level of HbA_{1c}, serum total cholesterol, and serum triglycerides (adjusted OR 2.8, 95% CI 1.2–6.3).

To explore the possibility that the association with the ε2 allele might be caused by linkage disequilibrium with another DNA sequence difference in the same region, we examined the distribution of four additional bi-allelic markers flanking *APOE*. The *APOE* ε2 allele was in significant linkage disequilibrium (expressed as the percent of the maximum possible disequilibrium) with the –491 T allele (42%, $P < 0.001$),

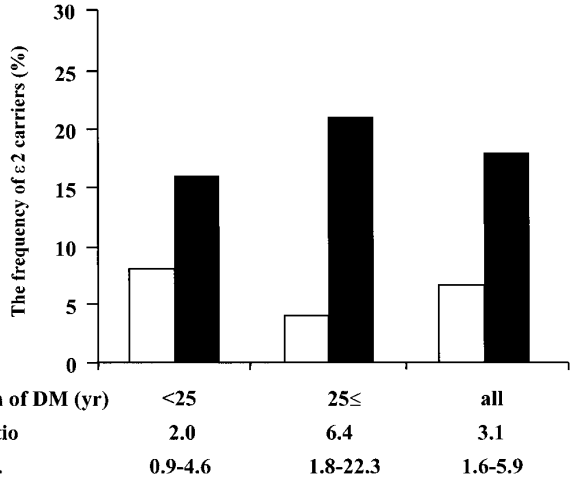

FIG. 2. The OR of DN for carriers of the ε2 allele according to the duration of diabetes. □, Control subjects; ■, study subjects.

the –219 G allele (89%, $P < 0.001$), the IE1 G allele (92%, $P < 0.001$), and the *APOCI* D allele (93%, $P < 0.001$) (Fig. 1). However, the allele frequencies of these polymorphisms were similar in both study and control subjects. The ORs for DN (shown for carriers of the minor allele of each polymorphism) were not significantly different from the value of 1.0 (Table 3).

To be sure that the association of DN with the ε2 allele of *APOE* in the case-control study was not a spurious finding due to unrecognized population stratification, we examined the association in a family-based study. Transmission frequencies from parents heterozygous for the ε2 allele of *APOE* to offspring with or without nephropathy are shown in Table 4. As expected for a risk allele, the ε2 allele was transmitted significantly more frequently than not transmitted from heterozygous parents to offspring with DN, regardless of whether ε2 was paired with ε3 or ε4 (64 vs. 36%, $P = 0.03$, McNemar's test). Conversely, ε2 was preferentially not transmitted to offspring without DN (35 vs. 65%). Whereas this departure from 50% transmission to offspring without DN was not statistically significant (McNemar's test) with the available number of control families, the pattern was consistent with ε2 being a risk allele. Moreover, the low transmission of the ε2 allele to offspring without DN was significantly different from its transmission to offspring with DN (heterogeneity test $\chi^2 = 5.3$, $P = 0.02$). This symmetrically distorted pattern of transmission strongly supports the association of the ε2 allele of *APOE* with increased risk of DN.

As a secondary question, one can consider whether the protection associated with ε3 and ε4 are equivalent. This can be tested directly by examining the transmission from parents heterozygous for ε3/ε4 to study subjects and control subjects. In the 56 families of study subjects with an ε3/ε4 heterozygous parent, ε3 was transmitted 39% of the time to the study subject ($P = 0.11$); in 47 control families with an ε3/ε4 heterozygous parent, ε3 was transmitted 55% of the time ($P = 0.47$). The heterogeneity test comparing these two transmission proportions has a χ^2 of 2.64 ($P = 0.10$). Whereas none of these tests is significant, the pattern is consistent with the hypothesis that ε3 carries a lower risk than ε4. This hypothesis remains to be tested in an independent study.

Transmission of alleles of the four polymorphisms flanking *APOE* to DN-positive or DN-negative offspring from heterozygous parents was not distorted from 50% (data not shown). Also, when each of these polymorphisms was analyzed together with *APOE* alleles, none of the complex haplotypes was preferentially transmitted from heterozygous parents to offspring with or without DN (data not shown).

DISCUSSION

The present study provides evidence that the ε2 allele of the polymorphism in exon 4 of *APOE* is a risk factor for DN in type 1 diabetes. Carriers versus noncarriers of this allele had a 3-fold risk of having advanced DN. These findings, obtained in a large case-control study, were subsequently confirmed in a family-based association study. The latter is the most reliable study design for detecting an association between DNA sequence differences and a specific disease (24).

Our findings regarding the effect of the ε2 allele on the risk of DN are consistent with the results of two previous case-control studies (8,9). Chowdhury et al. (8) reported that the presence of the ε2 allele was associated with increased risk of DN in Caucasian subjects with type 1 diabetes (197 control subjects and 252 study subjects). They found an OR of 4.3 (95% CI 2.3–8.2) for DN in ε2 carriers, a value very similar to the OR of 3.1 obtained in the present study. Werle et al. (9) used multiple linear regression analysis to show that the ε2 allele was associated with elevated urinary albumin excretion in subjects with type 1 diabetes.

Two other studies in type 1 diabetes, however, did not find an association between DN and the *APOE* polymorphism in exon 4. One was a small case-control study from our group (11) that found no significant difference in the frequencies of the ε2 allele of *APOE* polymorphism among subjects having normoalbuminuria, microalbuminuria, or proteinuria. In total, only 146 subjects were examined and, at the time of examination in

TABLE 3
Comparison of allele frequencies in study subjects and control subjects for four additional polymorphic markers within the 10-kb region flanking *APOE* exon 4

Markers	Minor allele	Frequency of minor alleles		*P*	OR*	95% CI
		Control subjects	Study subjects			
–491 A/T	T	13 (51)	16 (70)	0.34	1.0	0.6–1.5
–219 G/T	T	50 (195)	47 (209)	0.40	0.9	0.6–1.5
IE1 G/C	C	40 (158)	37 (165)	0.33	0.9	0.6–1.3
APOCI I/D	D	18 (71)	22 (100)	0.12	1.2	0.8–1.7

Data are % (n) of chromosomes with minor alleles. *DN in carriers of the minor alleles are shown.

TABLE 4
Transmission frequencies of the ε2 allele of the *APOE* polymorphism from heterozygous parents to offspring according to the phenotype of the offspring

DN status in offspring	Parental genotype	Heterozygous parents	ε2 Transmitted		*P**
			Yes	No	
With DN					
	ε2/ε3	51	32	19	—
	ε2/ε4	8	6	2	—
	Total	59	38 (64)	21 (36)	0.03
Without DN					
	ε2/ε3	15	5	10	—
	ε2/ε4	5	2	3	—
	Total	20	7 (35)	13 (65)	0.18

Data are *n* or *n* (%). Heterogeneity test: $\chi^2 = 5.3$, $P = 0.02$.
*Determined by McNemar's test.

1986, they had a diabetes duration of 15–21 years, which was much shorter than the mean duration in the present study. Whereas the frequencies of the ε2 allele in subjects in the previous (8%) and the current (9%) studies are comparable, the frequency of the ε2 allele in previously studied control subjects (9%) was higher than the frequency in the present study (3%). The discrepancies between control groups could have resulted in part from the less reliable diagnoses of normoalbuminuria (only a single albumin excretion rate determination) in the former study. Only 36 patients (21 study subjects and 15 control subjects) participated in both studies. In the 21 study subjects, 12% of the chromosomes carried the ε2 allele, and in control subjects, 3% of the chromosomes carried it. For the majority of the control subjects in the earlier case-control study, the current renal status is unknown because they stopped attending the Joslin Clinic. Some may have developed DN in the intervening years. If these subjects with new cases of DN were among the carriers of the ε2 allele, the remaining normoalbuminuric control subjects would have a deficiency of this allele. Our analysis of the frequency of this allele according to duration of diabetes seems to support such a possibility.

Another study that did not find an association between DN and the ε2 allele was conducted by Hadjadj et al. (34). They examined 494 subjects with type 1 diabetes and various stages of DN and found the same frequency of the ε2 allele (5.0%) in control subjects and study subjects with established DN. Interestingly, all control subjects in that study were required to have proliferative retinopathy, even though they were normoalbuminuric. At present, it is unclear whether this eligibility criterion could have influenced the frequency of the ε2 allele in the control group.

The effect of the ε2 allele on the risk for DN has also been examined in case-control studies in type 2 diabetes. Eto et al. (10) reported that in Japanese patients with type 2 diabetes, carriers (compared with noncarriers) of the ε2 allele had an OR of 3.0 (95% CI 1.2–7.7) for DN. On the other hand, Kimura et al. (12) did not find an association between ε2 allele carriers and ESRD in Japanese patients with type 2 diabetes. Furthermore, several studies in Caucasians with type 2 diabetes reported a decreased risk of DN in carriers of the ε2 allele (13,14).

Reasons for the discrepant results of the case-control studies are unknown; however, one may consider the following possibilities: different diagnostic criteria for DN, variable degrees of linkage disequilibrium if ε2 allele is a marker only of susceptibility, interactions with other genetic or environmental factors among the different populations, and population stratification. Of these possibilities, the effect of population stratification can be excluded by the TDT family study (24). In our family study, transmission of the ε2 allele from heterozygous parents to subjects with DN was significantly more frequent than the expected 50% transmission and significantly more frequent than transmission of that allele from heterozygous parents to diabetic control subjects without DN. This dependence of the transmission frequency on the phenotype of the offspring provides the strongest evidence that the ε2 allele is involved directly or is associated (as a marker) with the development of DN.

The previous positive studies did not investigate whether *APOE* ε2 may be a marker only of susceptibility as a result of linkage disequilibrium with polymorphisms at other loci flanking *APOE*. In this study, we examined the association of DN with four known polymorphisms in the 10-kb region flanking exon 4 of *APOE*. None of these polymorphisms could account for the association of the ε2 allele with DN. Therefore, these findings may be seen as further evidence that the apoE protein itself influences susceptibility to DN, with the apoE2 isoform increasing the risk. However, this interpretation must be qualified because an unknown polymorphism flanking exon 4 of *APOE* may influence susceptibility to DN and may be in linkage disequilibrium with *APOE* alleles.

The stages of DN that are influenced by apoE isoforms remains undetermined. Because the ORs for proteinuria and ESRD were similar in carriers of apoE2, we hypothesize that this isoform influences the risk of the earliest stages of DN (e.g., the onset of microalbuminuria or its progression to proteinuria). Recently, Werle et al. (9) reported that the ε2 allele was a positive predictor of the level of urinary albumin excretion in type 1 diabetic patients, suggesting that the ε2 allele may have an impact on the earliest stage of DN. Further studies, however, are required to examine this hypothesis.

The mechanism(s) by which apoE isoforms may influence the development of DN are also unclear. At least two explanations can be considered, the first of which is related to the lipid abnormalities associated with apoE isoforms. Compared with apoE3, individuals with apoE2 have lower cholesterol and higher triglyceride levels (22); therefore, dyslipidemia caused by the apoE2 isoform may promote the development of DN. The second explanation is related to apoE functions other than its effect on lipid metabolism. Interestingly, a high level of apoE localizes to the extracellular matrix after nerve injury, suggesting that apoE has some role on the tissue repair system (35). In the kidney, immunofluorescence studies have shown massive staining for apoE in the mesangial area of patients with kidney diseases, including DN (36,37). Furthermore, apoE2 is associated with the development of lipoprotein glomerulopathy (LPG), which is a newly recognized, rare renal disease (23). Although the exact pathogenesis of LPG remains to be elucidated, the recurrence of LPG in transplanted kidneys has been reported (38,39). This suggests that the primary abnormality of LPG is not related to the kidney itself but to systemic factors, including the apoE2 phenotype. Thus, one may hypothesize that apoE2 protein accumulates in the mesangial area under diabetic conditions and changes the properties of mesangial matrix or cell functions.

Finally, because the study included prevalence cases of DN, one must consider the possibility of spurious findings resulting

from differential mortality related to the *APOE* genotype. This hypothesis is suggested by the observation that nondiabetic people with the ε2 allele live longer than those without the allele (40). Although only a large follow-up study will be able to resolve this hypothesis unequivocally, the data from our study do not support that interpretation. First, our study and control subjects were enrolled in the study at an age when mortality is relatively low (1). Second, when the analysis was conducted in those aged <36 (below median) and ≥36 years, the effect of the ε2 allele of *APOE* on DN risk was not changed. Third, the effect of the ε2 allele on ESRD risk was similar, regardless of whether we used prevalent or incidence cases of ESRD.

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