Original Article

Investigation of the Estrogen Receptor-α Gene With Type 2 Diabetes and/or Nephropathy in African-American and European-American Populations

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The estrogen receptor-α gene (ESR1) was selected as a positional candidate under a type 2 diabetes linkage peak at 6q24-27. A total of 42 ESR1 single nucleotide polymorphisms (SNPs) were genotyped in 380 African-American type 2 diabetic case subjects with end-stage renal disease (ESRD) and 276 African-American control subjects. A total of 22 ancestry informative markers were also genotyped, and the program Admixmap was used to adjust allelic and haplotypic association tests for individual estimates of admixture. The most significant association with type 2 diabetes–ESRD was with rs1033182 in intron 2 ($P = 0.013$, admixture-adjusted $P_a = 0.021$). Genotyping 17 SNPs across a region of ESR1 intron 1–intron 2 in an expanded population of 851 case and 635 control subjects supported association with rs1033182 ($P = 0.004$, $P_a = 0.027$) and with an independent six-SNP haplotype of high linkage disequilibrium spanning 6.4 kb ($P < 0.0001$, $P_a < 0.0001$). The same 17 ESR1 SNPs were genotyped in 300 European-American type 2 diabetes–ESRD case subjects and 310 European-American control subjects. Two intron 2 SNPs, rs2431260 ($P = 0.015$) and rs1709183 ($P = 0.019$), and a four-SNP haplotype containing these SNPs ($P = 0.033$) were associated with type 2 diabetes and/or ESRD. Results suggest that intron 1 and intron 2 of the ESR1 gene may contain functionally important regions related to type 2 diabetes or ESRD risk. *Diabetes* 56:675–684, 2007

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ESRD, end-stage renal disease; LD, linkage disequilibrium; MAF, minor allele frequency; NIGMS, National Institute of General Medical Sciences; PGC1, peroxisome proliferator–activated receptor-γ coactivator 1; SNP, single nucleotide polymorphism.

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Diabetes and diabetes complications are a major cause of morbidity and mortality in the U.S., particularly in African Americans. On average, an African-American individual is twice as likely as a European-American peer to have type 2 diabetes (1). We performed a genome scan for type 2 diabetes using 638 African-American affected sibling pairs from 247 families. Nonparametric linkage analysis revealed the strongest evidence for linkage (logarithm of odds 2.26) at 163.5 cM on chromosome 6q (2). The support (LOD-1) interval for this region contains the estrogen receptor-α gene (ESR1), located at 154 cM.

Sex steroids clearly have an impact on insulin resistance (3). Male and female *Esr1* knockout mice exhibit insulin resistance, impaired glucose tolerance, and obesity (4). Similarly, the only human (male) known to be lacking a functional copy of ERα was shown to have insulin resistance, impaired glucose tolerance, obesity, and increased height (5). A restriction enzyme polymorphism in ESR1 intron 1 (rs9340799) was associated with type 2 diabetes and obesity in a Hungarian population of 49 type 2 diabetic case subjects and 138 control subjects (6).

Increased sex steroid levels in humans can lead to insulin resistance during puberty, pregnancy, and the luteal phase of the menstrual cycle, when hormone levels are elevated (3,7–9). Decreased insulin sensitivity can also result from treatment with sex steroids (oral contraceptives and anabolic steroids) (10,11). Although these observations support a role for sex steroids in insulin resistance, little information exists on the molecular basis of this effect.

ESR1 is a highly polymorphic gene containing >1,600 single nucleotide polymorphisms (SNPs) across 474 kb, including SNPs in genomic regions flanking alternatively spliced exons. SNPs were selected across the ESR1 gene, incorporating a higher density within intron 1 because it has previously been associated with type 2 diabetes (6) and related metabolic phenotypes (6,12–14). This article reports on the association between the ESR1 gene and type 2 diabetes–ESRD in African-American and European-American populations and identifies SNPs and haplotypes across the intron 1–intron 2 region of the ESR1 gene that confer protection against type 2 diabetes and/or ESRD.

TABLE 1
Demographic information for type 2 diabetic patient and control populations

	African-American case subjects, round 1	African-American control subjects, round 1	African-American case subjects, round 2	African-American control subjects, round 2	European-American case subjects	European-American control subjects
Total	380	276	851*	635†	300	310
Female	60.5 (230)	63.8 (176)	61.5 (523)	52.0 (330)	53.0 (159)	70.6 (219)
Age (years)	63.4 ± 10.8 (353)[a,b,c]	N/A	61.2 ± 10.1 (801)[a,d,e,f]	50.5 ± 10.1 (332)[b,d,g,h]	65.8 ± 10.3 (207)[e,g,i]	45.8 ± 15.2 (164)[c,f,h,i]
Type 2 diabetes diagnosis (years)	42.8 ± 11.8 (373)[j,k]	—	40.4 ± 12.0 (801)[j,e]	—	46.5 ± 12.8 (296)[k,e]	—
ESRD diagnosis (years)	60.1 ± 10.2 (375)[k,l]	—	57.7 ± 10.5 (830)[e,l]	—	63.6 ± 10.2 (298)[e,k]	—
BMI (kg/m^2)	29.3 ± 7.3 (373)[c]	N/A	29.8 ± 7.2 (819)[f,m]	29.5 ± 6.7 (336)[h]	28.5 ± 7.0 (297)[m,n]	25.7 ± 3.9 (65)[c,f,h,n]

Data are n, n (%), or the means ± SD (n). *Includes the 380 African-American case subjects from round 1 genotyping; †includes the 276 control subjects from round 1 genotyping. [a,m,n]$P <$ 0.05; [j]$P < 0.01$; [b,c,d,e,f,g,h,i,k,l]$P < 0.001$.

RESEARCH DESIGN AND METHODS

This study was conducted under institutional review board approval from Wake Forest University School of Medicine and adhered to the tenets of the Declaration of Helsinki. Identification, clinical characteristics, and recruitment of African-American and European-American patients and control subjects have been described previously (15–18). Briefly, 851 unrelated African-American patients with type 2 diabetes and end-stage renal disease (ESRD) were recruited. These participants were born in North Carolina, South Carolina, or Georgia; were dialysis dependent; and had at least background diabetic retinopathy or a urinalysis with 3+ or greater protein excretion in the absence of other recognized causes of nephropathy. The 471 case subjects included during the second phase of genotyping had at least one sibling with type 2 diabetes, although no relative pairs were used for these analyses. We recruited 635 unrelated African-American control subjects born in North Carolina, South Carolina, Georgia, Tennessee, or Virginia who did not have a current diagnosis of type 2 diabetes or renal disease. Finally, 300 unrelated European-American type 2 diabetic patients with ESRD (using the same criteria as above) and 310 unrelated European-American control subjects without known type 2 diabetes or renal disease were recruited. Diabetic nephropathy was diagnosed in African-American and European-American individuals with type 2 diabetes at least 5 years before the initiation of renal replacement therapy. DNA extraction was performed using the PureGene system (Gentra Systems, Minneapolis, MN).

Genotyping for admixture analyses. A total of 22 biallelic ancestry informative markers were selected from Shriver et al. (19): FY-NULL, F13B, TSC1102055, WI-11392, WI-6857, SGC30055, WI-17163, WI-9231, CYP3A4, WI-4019, LPL, CRH, D11S429, TYR-192, DRD2-*Taq*I "D," GNB3, OCA2, WI-14319, CYP19, MC1R-314, WI-14867, WI-7423, and CKM. These markers were genotyped using a MassARRAY genotyping system (Sequenom, San Diego, CA) (20) in 851 African-American case subjects and 635 African-American control subjects as well as 120 Yoruba Nigerians from the National Institute of General Medical Sciences (NIGMS) Human Variation Collection (Coriell Cell Repositories, Camden, NJ) and 282 European-American control subjects. Primer sequences are available on request.

Initial ESR1 genotyping. We chose 53 SNPs based on linkage disequilibrium (LD) information from a multiethnic panel (21) to maximally capture common haplotypes (present at a frequency >5%) in each block (P. Bretsky, J. Butler, D. Altshuler, B.E.H., J.N.H., personal communication) and 2 intron 1 SNPs (rs4870056 and rs2234693) with reported effects on transcription levels and cardiovascular disease (12). These SNPs were genotyped in 380 African-American type 2 diabetes–ESRD patients and 276 African-American control subjects (termed round 1) (Table 1). PCR and primer extension reactions were performed using a MassARRAY genotyping system (Sequenom) (20); primer sequences are available on request. A total of 11 SNPs were excluded because of low genotyping success rates (<85%), and 2 SNPs were found to be monomorphic. The final set of 42 SNPs across the ESR1 gene had an average spacing of 7.6 kb and a median spacing of 4.9 kb (range 46–31.6 kb, with highest density in intron 1). HapMap data were used to assess how well the 42 SNPs typed in round 1 tagged the ESR1 gene. Considering only SNPs with minor allele frequency (MAF) >5% in the Yoruba in Ibadan (YRI) Nigerian dataset, 19 of these SNPs had been genotyped by the HapMap project (HapMap data release no. 19). Using Tagger (22) as tags for the 299 SNPs with MAF >5% genotyped in HapMap for ESR1, these SNPs captured 66.9% of SNPs with $r^2 > 0.2$, 37.5% of SNPs with $r^2 > 0.5$, and 21.7% of SNPs with $r^2 > 0.8$. All 42 SNPs were consistent with Hardy-Weinberg equilibrium in control subjects, but rs9322331 ($P = 0.001$) and rs1033182 ($P = 0.007$) deviated from Hardy-Weinberg equilibrium in case subjects.

Genotyping additional SNPs in the intron 1–intron 2 region of ESR1. After initial association analyses, 14 additional SNPs were identified within intron 1 and intron 2 of ESR1 based on the SNP database (dbSNP) at NCBI (National Center for Biotechnology Information) Build 120, Applied Biosystem's Assays-on-Demand (Foster City, CA), HapMap data, or literature describing polymorphisms of ESR1 (12). Initial analyses of 96 African-American individuals suggested that five SNPs were monomorphic in this population, whereas two SNPs did not provide high-quality genotyping data and were removed from further analyses. The remaining 7 SNPs, together with 10 SNPs from this region from the first round of genotyping, were genotyped in the same 380 African-American case subjects and 276 African-American control subjects from round 1 and an additional 471 known familial African-American case subjects (probands) and 359 African-American control subjects (with the combined African-American population, termed round 2) (Table 1), plus 300 European-American case subjects, 310 European-American control subjects, and 120 NIGMS Yoruba Nigerians for admixture-adjusted analyses, and analyzed for association. Of the 17 SNPs, 9 had been genotyped in the HapMap YRI sample (HapMap data release no. 19). Using the locations of rs6902771 and rs11155819 to define the boundaries of the region of interest and using

Tagger (22), these 9 SNPs tagged the 27 HapMap SNPs in this region with MAF >5% at the following levels: 85.2% with $r^2 > 0.2$, 70.4% with $r^2 > 0.5$, and 55.6% with $r^2 > 0.8$. All SNPs were consistent with Hardy-Weinberg equilibrium in African-American control subjects, but rs6902771 ($P = 0.01$), rs9322331 ($P = 0.02$), and rs1033182 ($P = 0.01$) deviated from Hardy-Weinberg equilibrium in African-American case subjects. In European Americans, rs7774230 ($P = 0.02$) in control subjects and rs11155818 in case subjects ($P = 0.047$) deviated from Hardy-Weinberg equilibrium; the latter SNP is rare, with a MAF of 0.014. Across the 17 ESR1 intron 1–intron 2 SNPs, 132 quality control replicate African-American control samples (264 alleles) and 158 replicate African-American case samples (316 alleles) were 100% concordant.

Statistical methods. LD was estimated between all pairs of SNPs using D′ and r^2 statistics. Haplotype block structure was determined using the Solid Spine of LD option of Haploview (23) with the block extended if pairwise D′ between SNPs was >0.80.

Each SNP was tested for allelic association by calculating a χ^2 statistic and corresponding P value using 2×2 contingency tables, except where a cell contained five or less alleles, in which case Fisher's exact test was applied (rs926848 in initial African-American genotyping and rs12664989 in European Americans). Genotypic association for dominant, additive, and recessive models was tested using logistic regression to adjust for individual estimates of admixture.

All tandem two-, three-, and four-marker haplotypes and the haplotypes within each LD block were tested for association with type 2 diabetes using a permutation test of the multinomial likelihood ratio statistic. Haplotypes with <1% overall frequency have been removed from analyses. Haplotype frequencies were estimated using the expectation-maximization (EM) algorithm in the software Dandelion (24). Statistical significance was estimated based on 10,000–10,000,000 permutations when initial estimates of the P value based on 1,000 permutations was <0.10. The haplotype-specific z score and test incorporates a continuity correction when expected cell counts are less than three.

The program Admixmap (25) was used to model the distributions of admixture and generate score tests of allelic and haplotypic association, adjusted for individual estimates of admixture. Estimates of ancestral allele frequencies were obtained from the genotyped Yoruba Nigerian and European-American control populations.

Sequencing of ESR1 exon 2. ESR1 exon 2 (191 bp) was sequenced in 48 African-American type 2 diabetic case subjects and 48 African-American control subjects. All samples were sequenced in both sense and antisense directions using BigDye Terminator version 1.1 cycle sequencing kits (Applied Biosystems, Foster City, CA) and a 3730xl DNA analyzer (Applied Biosystems). Sequences were viewed using Sequencher 4.1.4 software (Gene Codes, Ann Arbor, MI).

RESULTS

Study samples. Demographic characteristics of the populations are shown in Table 1. There were more female participants across all categories, most notably in the European-American control subjects (70.6%), which may be attributable to a combination of higher prevalence in females and participation bias. The type 2 diabetic case subjects were significantly older than control subjects in both populations: 61 ± 10.1 versus 50.5 ± 10.1 years for African-American case versus control subjects, and 65.8 ± 10.3 versus 45.8 ± 15.2 years for European-American case versus control subjects. Diagnoses of type 2 diabetes and ESRD came significantly earlier in African-American ($P <$ 0.001) than European-American case subjects. Although BMI values were close to 30 kg/m^2 in most groups, mean BMI was significantly lower in European-American control subjects ($P < 0.001$). Because age and BMI data were not collected for a substantial proportion of control subjects (Table 1), these data were not included as covariates in association analyses.

Ancestral proportions in the African-American population. Frequencies of the ancestry informative markers in the Yoruba Nigerians, African-American control and case subjects, and European-American control subjects used for admixture analyses are shown in supplementary Table 1, which can be found in an online appendix (available at http://dx.doi.org/10.2337/db06-0303). For the 380 African-American case subjects and 276 African-American control subjects used in the first round of genotyping, the mean proportions of African ancestry were estimated to be 0.80 (95% credible interval [CI] 0.78–0.83) and 0.82 (0.79–0.84), respectively. In the expanded population of 851 African-American case subjects, inclusion of known familial type 2 diabetic case subjects (0.86, 0.84–0.87) increased the overall estimated mean proportion of African ancestry to 0.83 (0.82–0.85). In the expanded control population ($n = 635$), the mean proportion of African ancestry remained similar to the control subjects from the initial screening population: 0.81 (0.79–0.83). Distributions of African ancestry in case subjects and control subjects from the expanded populations are shown in Fig. 1.

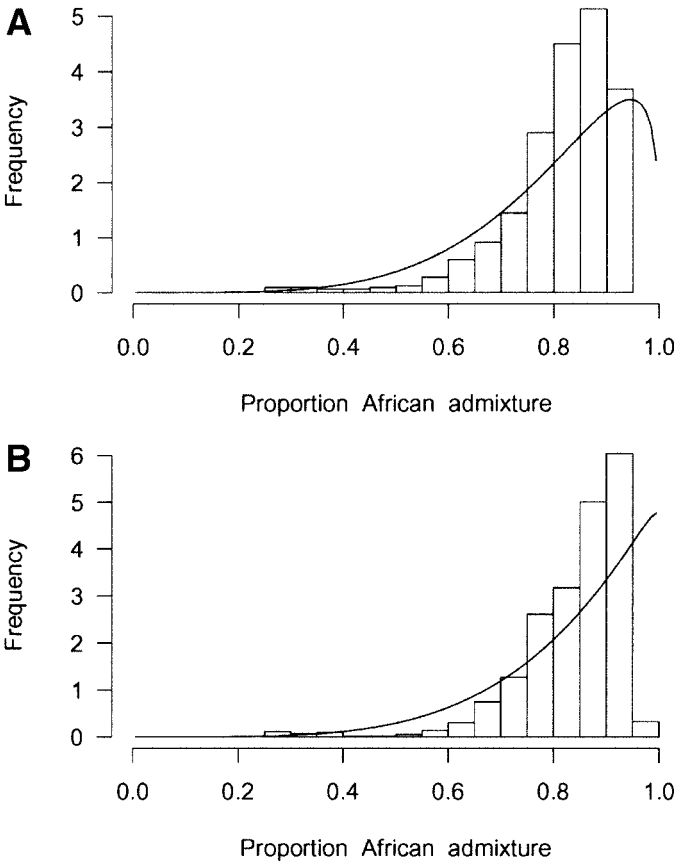

FIG. 1. Distributions of individual African ancestral proportions for 635 African-American control subjects (*A*) and 851 African-American case subjects (*B*), determined using Admixmap and ancestral prior allele frequencies from 120 Yoruba Nigerians and 282 European-American control subjects.

Initial ESR1 association analyses. Allele frequencies and single SNP association results from the initial 42 SNPs genotyped in 380 African-American type 2 diabetic case subjects and 276 African-American control subjects are summarized in supplementary Table 2. The most significant single SNP association was observed with rs1033182 ($P = 0.013$, admixture-adjusted $P_a = 0.021$). In addition, borderline allelic associations were observed with SNPs in intron 5 (rs2207232, $P = 0.049$) and intron 6 (rs2982900, $P = 0.042$).

Association analyses in the ESR1 intron 1–intron 2 region in African-American case subjects and control subjects. The strongest evidence for single SNP (supplementary Table 2) and haplotype-based association (data

TABLE 2
Single SNP type 2 diabetes association analyses of 17 SNPs spanning a contiguous region of ESR1 from intron 1 to intron 2 in African Americans

SNP	NCBI Build 35 position	Alleles (major/minor)	Control subject MAF (n = 635)	Case subject CAF (n = 851)	P value for association			
					Allelic	Genotypic recessive	Genotypic additive	Genotypic dominant
rs6902771	152159676	C/T	0.49	0.49	0.80	0.79	0.83	0.92
rs4870056	152254341	G/A	0.49	0.49	0.97	0.77	0.99	0.77
rs9322331	152254431	C/T	0.13	0.13	0.55	0.46	0.61	0.40
rs2234693	152255449	C/T	0.45	0.46	0.67	0.52	0.63	0.86
rs9340799	152255495	A/G	0.28	0.29	0.75	0.71	0.67	0.73
rs7774230	152256353	T/C	0.46	0.46	0.97	0.81	0.94	0.92
rs1709181	152267294	C/T	0.36	0.36	0.60	0.84	0.73	0.53
rs12664989	152271446	G/C	0.07	0.08	0.44	0.80	0.40	0.33
rs712221	152272355	T/A	0.37	0.35	0.28	0.88	0.33	0.21
rs1514348	152274429	G/T	0.37	0.35	0.44	0.79	0.51	0.27
rs11155818	152276244	G/A	0.09	0.11	0.16	0.66	0.17	0.16
rs827417	152281378	A/G	0.09	0.08	0.12	0.49	0.27	0.19
rs2431260	152284445	G/C	0.09	0.10	0.22	0.13	0.23	0.35
rs1709183	152286110	A/G	0.35	0.38	0.13	0.43	0.18	0.19
rs1033182	152287148	G/A	0.08	0.05	0.004*	0.82	0.009*	0.005*
rs2175898	152289066	A/G	0.06	0.07	0.45	0.59	0.36	0.39
rs11155819	152291473	T/C	0.11	0.10	0.41	0.78	0.51	0.41

*P value <0.05. CAF, corresponding allele frequency; NCBI, National Center for Biotechnology Information.

not shown) from the initial round of genotyping spanned a 41-kb region from intron 1 to intron 2 of the ESR1 gene. Because this region also contains SNPs previously reported to be associated with type 2 diabetes (6) and other metabolic phenotypes (6,12–14), we explored association in this region further by genotyping a total of 17 SNPs (10 SNPs from the first round of genotyping plus an additional 7 SNPs) in an expanded African-American sample of 851 case subjects and 635 control subjects. Genotype frequencies for these 17 SNPs in the African-American control and case subjects are shown in supplementary Table 3.

Association of rs1033182 with type 2 diabetes–ESRD remained significant in the expanded African-American population (P = 0.004, P_a = 0.027) (Table 2 and supplementary Table 4). Genotypic association analyses supported by both additive (P = 0.009, P_a = 0.016) and dominant (P = 0.005, P_a = 0.009) models. These associations were driven by the female African-American type 2 diabetes–ESRD patients, with significant associations for allelic ($P < 0.0001$, $P_a < 0.0001$) and genotypic (additive $P_a < 0.0001$, dominant $P_a = 0.0001$) tests, whereas no significant results were obtained for this SNP in male patients. The derived minor allele of this SNP (A) is relatively uncommon (0.08 in control subjects, 0.05 in case subjects), producing an odds ratio (OR) for type 2 diabetes risk of 0.65 (95% CI 0.48–0.87). Genotyping accuracy for rs1033182 was carefully reviewed. Genotyping success rates were comparable for both African-American control subjects (94.8%) and African-American case subjects (94.7%). A total of 119 individuals were genotyped twice on the Sequenom platform and showed a concordance rate of 99.2% (118 of 119 genotypes).

Four haplotype blocks were identified in this region in African-American control subjects (Fig. 2*A*). Haplotype analyses of the 17 SNPs spanning a contiguous region of intron 1–intron 2 were conducted by investigation of two-, three-, and four-marker moving windows and each of the four haplotype blocks from Fig. 2*A*. Of the four haplotype blocks, only block 1 was significantly associated with type 2 diabetes (overall $P < 0.0001$, $P_a < 0.0001$). Table 3 summarizes the results for this haplotype block, shown as the specific haplotype, the estimated frequency of the haplotype in the case and control subjects, and a z score for each haplotype, in which a negative sign shows a higher proportion of this haplotype in control subjects and a positive sign a higher proportion of the haplotype in type 2 diabetic case subjects. Also reported are the ORs of a haplotype versus all other haplotypes pooled, haplotype-specific P values for significance of the z score, an overall empirical P value for the difference between type 2 diabetic case and control subjects, and admixture-adjusted P values. Haplotype block 1 contained three haplotypes (CGCTAT, TACCAC, and TATCGC) that were not found in African-American case subjects but were present in African-American control subjects with frequencies of 3, 2, and 1%, respectively ($P \leq 0.022$). The uncommon haplotypes were observed only in female patients; therefore, in sex-stratified analyses, only the female analyses were significant (global $P_a < 0.0001$). Given the importance of rs7774230, which distinguishes protective haplotypes from common haplotypes, genotyping accuracy was evaluated. The genotyping success rate was 94.5 and 93.4% for case and control subjects, respectively. A total of 56 individuals (27 case and 29 control subjects) with both sequence and Sequenom data for rs7774230 displayed 100% concordance. For 371 individuals, this SNP was genotyped twice on the Sequenom platform, with 100% concordance. Although a continuity correction of 0.5 was added to each of the four haplotype–by–type 2 diabetes cells to minimize the influence of rare haplotypes on the test statistic when the expected count was <5, comparisons of uncommon haplotypes (frequency <5%) should be interpreted with caution. Moving-windows analyses showed that all smaller subhaplotypes of this block that contained the distal two SNPs of this block (rs9340799 and rs7774230) were also significantly associated with type 2 diabetes ($P \leq 0.012$). Haplotype block 2 (P = 0.06, P_a = 0.09), block 3 (P = 0.29, P_a = 0.54), and block 4 (P = 0.035, P_a = 0.21) did not show significant evidence of association with type 2 diabetes after adjustment for admixture.

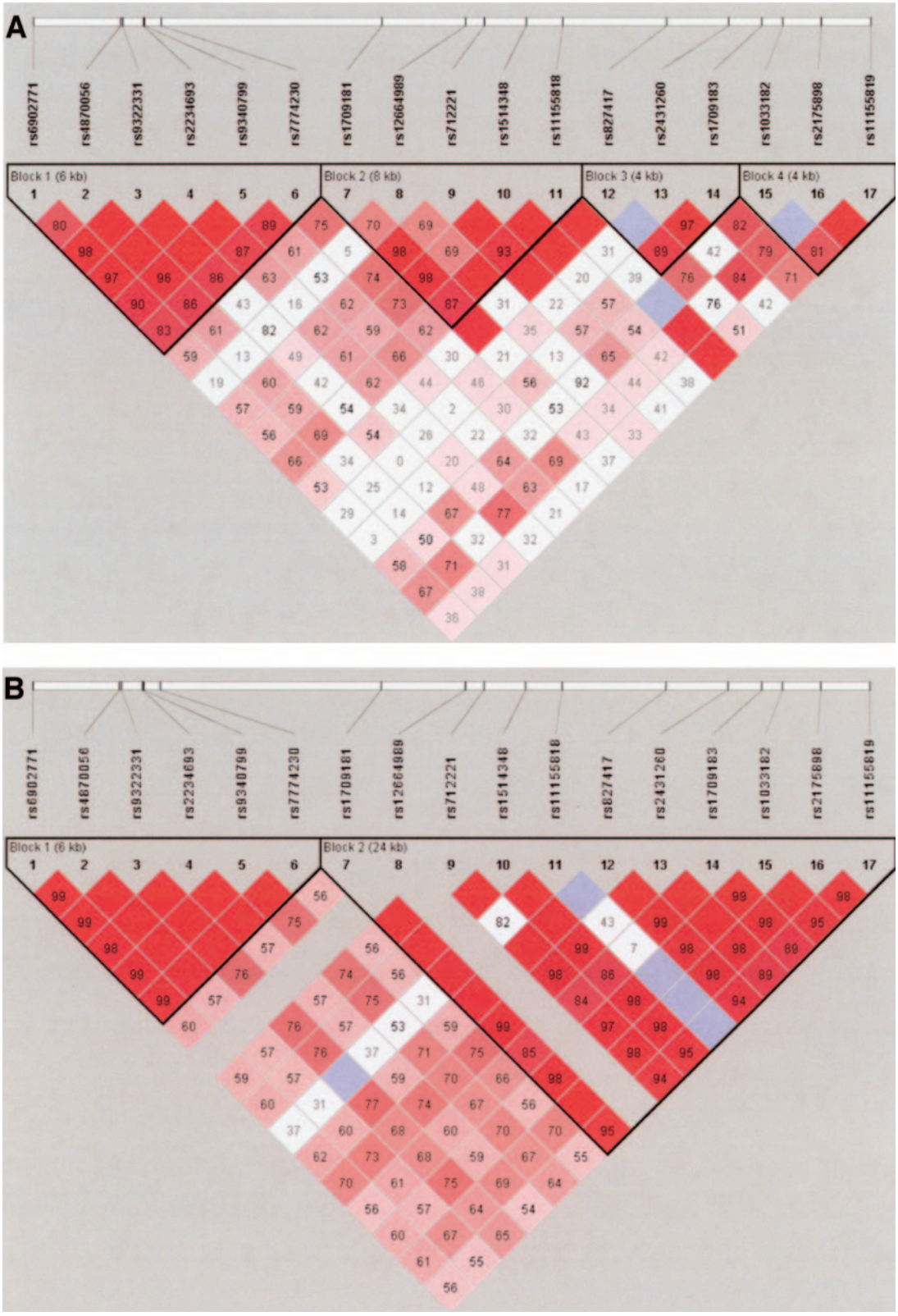

FIG. 2. LD structure of a 41-kb region spanning ESR1 intron 1–intron 2 in African-American control subjects (n = 635) (A) and European-American control subjects (n = 310) (B), determined using the solid spine of LD option of Haploview, with blocks extended if pairwise D′ >0.80. D′ values are displayed in the squares. Empty red squares have a pairwise D′ of 1.00. Red squares indicate high pairwise LD, gradually coloring down to white squares of low pairwise LD. Blue squares indicate high LD but low significance. Empty gray squares present along axes with rs12664989 in European Americans are attributable to its low allele frequency (0.003).

Sequencing of ESR1 exon 2 in African Americans. Exon 2 falls within the most significantly associated African-American haplotype block (block 1) and was sequenced to assess whether coding mutations contribute to the observed associations. DNA from 48 African-American case subjects and 48 African-American control sub-

TABLE 3
Haplotype type 2 diabetes association analysis in African Americans for haplotype block 1 spanning a contiguous region of ESR1 intron 1 to intron 2

Haplotype	African-American control subject frequency	African-American case subject frequency	*Z* statistic	Haplotype-specific *P* value	OR	Overall *P* value	Admixture-adjusted haplotype *P* value	Admixture-adjusted overall *P* value
CGCTAT	0.03	0	−5.04	<0.0001*	—	<0.0001	<0.0001	<0.0001
TACCAC	0.02	0	−4.37	<0.0001*	—	—	<0.0001	—
TATCGC	0.01	0	−2.08	0.038*	—	—	0.0001†	—
TATCGT	0.13	0.13	−0.06	0.96	0.99	—	0.72	—
TGCCAT	0.04	0.04	0.58	0.56	1.17	—	0.39	—
TACCAT	0.16	0.17	0.51	0.61	1.07	—	0.61	—
CACCAT	0.05	0.04	−0.32	0.75	0.92	—	0.68	—
TACCGT	0.13	0.15	1.25	0.21	1.21	—	0.11	—
CGCTAC	0.42	0.45	1.43	0.15	1.16	—	0.11	—

Block 1: rs6902771, rs4870056, rs9322331, rs2234693, rs9340799, and rs7774230. Haplotypes with overall frequency >0.01 are shown. ORs were not calculated when there were less than three occurrences of the haplotype in either case or control subjects. *P values <0.05; †includes TATCGC and remaining rare haplotypes combined.

jects was sequenced, but no coding variations were detected (data not shown).

Association analyses of the ESR1 intron 1–intron 2 region in European-American case subjects and control subjects. Figure 2*B* shows the Haploview assignment of haplotype blocks for the 17 intron 1–intron 2 SNPs in the European-American population. Haplotype block 1 is the same in both populations (Fig. 2). Although the region from rs1709181 to rs11155819 forms blocks 2–4 in African Americans, in the European-American population, this region forms a single block in high LD.

Of the 17 ESR1 SNPs, 2 showed significant single SNP association in the European-American population (rs2431260, $P = 0.015$; and rs1709183, $P = 0.019$) (Table 4). These SNPs are in high LD ($r^2 = 0.93$). The derived allele of rs2431260 (African-American control subject MAF 0.09) and ancestral allele of rs1709183 (0.35) appear to be protective (rs2431260: OR 0.70, 95% CI 0.53–0.93; rs1709183: 0.74, 0.57–0.95) (Table 4).

There was no evidence in the European-American sample of haplotypic association with either of the two haplotype blocks from Fig. 2*B* (data not shown). The four-SNP moving-window haplotype analysis provided modest evidence of association with a single four-SNP haplotype associated with type 2 diabetes composed of the two significantly associated single SNPs and the two flanking SNPs (overall $P = 0.033$) (Table 5). This result is primarily caused by an increased frequency of one common haplotype in the case subjects (AGAG: 0.38 in case subjects, 0.33 in control subjects) and one common haplotype in control subjects (ACGG: 0.24, 0.30), although neither haplotype was significant at the 0.05 level. The two SNPs that differ between these haplotypes are those individually associated with type 2 diabetes (rs2431260 and rs1709183), suggesting that these two haplotypes and the two associated SNPs in high LD may potentially be tagging the same functional variant. Overall association analyses of two-

TABLE 4
SNP type 2 diabetes association analyses of 17 SNPs spanning a contiguous region of ESR1 from intron 1 to intron 2 in European Americans

SNP	Alleles (major/minor)	European-American control subject MAF ($n = 310$)	European-American case subject CAF ($n = 300$)	*P* value for association			
				Allelic	Genotypic recessive	Genotypic additive	Genotypic dominant
rs6902771	C/T	0.45	0.47	0.69	0.72	0.63	0.29
rs4870056	G/A	0.45	0.46	0.84	0.84	0.63	0.35
rs9322331	C/T	0.33	0.34	0.83	0.65	0.71	0.83
rs2234693	T/C*	0.45	0.47	0.59	0.60	0.50	0.13
rs9340799	A/G	0.34	0.36	0.44	0.41	0.34	0.46
rs7774230	C/T*	0.42	0.44	0.69	0.77	0.48	0.21
rs1709181	T/C*	0.38	0.41	0.32	0.41	0.25	0.30
rs12664989	G/C	0.003	0.002	1.00	1.00	0.64	0.64
rs712221	A/T*	0.37	0.39	0.64	0.57	0.57	0.70
rs1514348	T/G*	0.37	0.41	0.22	0.41	0.18	0.19
rs11155818	G/A	0.01	0.01	0.95	0.34	0.96	0.84
rs827417	A/G	0.35	0.36	0.71	0.37	0.58	0.88
rs2431260	G/C	0.30	0.23	0.015†	0.42	0.014†	0.008†
rs1709183	A/G	0.32	0.26	0.019†	0.42	0.016†	0.008†
rs1033182	G/A	0.34	0.37	0.39	0.24	0.38	0.69
rs2175898	A/G	0.26	0.21	0.09	0.55	0.09	0.08
rs11155819	T/C	0.30	0.33	0.36	0.21	0.34	0.63

CAF, corresponding allele frequency. *Minor allele in European Americans differs from minor allele in African Americans; †$P < 0.05$.

TABLE 5
The most significantly associated haplotype from the 17 SNP analysis spanning ESR1 intron 1 to intron 2 in European Americans

Haplotype*	European-American control frequency	European-American case frequency	*Z* statistic	Empirical haplotype-specific *P* value	OR	Overall *P* value
ACGG	0.30	0.24	−1.62	0.11	0.74	0.033†
GGAA	0.34	0.36	0.41	0.68	1.07	—
AGGG	0.02	0.02	0.42	0.67	1.28	—
AGAG	0.33	0.38	1.28	0.20	1.24	—

Haplotypes with overall frequency >0.01 are shown. *rs827417, rs2431260, rs1709183, and rs1033182; †$P < 0.05$.

and three-marker moving windows in this region approached significance ($P = 0.059$ to $P = 0.081$).

DISCUSSION

We investigated ESR1 as a positional candidate gene for type 2 diabetes because of evidence for linkage to 6q24-27 in an African-American type 2 diabetic population. After an initial survey of variation across the ESR1 gene, we focused on a contiguous region across intron 1, exon 2, and intron 2. Genotyping of additional SNPs in this region, together with haplotype analyses, suggest that protection from type 2 diabetes and/or ESRD is associated with two distinct regions of the ESR1 gene: a six-SNP haplotype encompassing intron 1, exon 2, and part of intron 2 in African Americans and a second more distal region of intron 2 in African Americans and European Americans.

Initial observation of significant association between rs1033182 and type 2 diabetes–ESRD was supported by results from an expanded African-American case-control sample ($P = 0.004$, admixture-adjusted $P_a = 0.027$) (Table 2 and supplementary Table 4), with the level of significance of the admixture-adjusted results similar for both analyses. SNP rs1033182 was in Hardy-Weinberg equilibrium in African-American control subjects, European-American case subjects, and European-American control subjects, but it deviated from Hardy-Weinberg equilibrium in the African-American type 2 diabetic case subjects. In both the initial genotyping phase and the expanded African-American population, this deviation was in the direction of reduced heterozygosity, which may indicate a functional role for this SNP in diabetes susceptibility. SNP rs1033182 minor allele A was not detected in the 120 African samples genotyped but was present at 34% in European-American control subjects, suggesting it entered the African-American population through admixture with Europeans. The four-SNP intron 2 haplotype significantly associated with type 2 diabetes in European Americans encompasses the two associated European-American SNPs, rs2431260 and rs1709183, as well as rs1033182, which is individually associated in African Americans (Tables 2, 4, and 5).

The strongest evidence for association in African Americans was observed with haplotype block 1 (Fig. 2 and Table 3), with an empirical overall *P* value <0.0001, although the differences between case and control subjects are attributable to three uncommon (1–3%) protective haplotypes. The African-American block 1 protective haplotypes are not present in the European-American population, which probably explains why association with this block was not observed in European Americans. Haplotype block 1 encompasses 6.5 kb of the ESR1 gene, commencing in intron 1 and with only rs7774230 located in intron 2. SNPs within this region of association, primarily located in intron 1, including rs2234693 (also known at the *Pvu*II polymorphism in the literature) and rs9340799 (known as the *Xba*I polymorphism), have been associated with various metabolic traits, including type 2 diabetes and obesity (6), BMI and/or waist circumference (26–28), and lipid profile (12,14,29–31). Although we genotyped rs2234693 and rs9340799, we did not detect associations between these individual SNPs and type 2 diabetes–ESRD. It is possible that the rare protective haplotypes detected in the African-American population in this study and the SNPs reported to be associated with metabolic traits are in fact tagging the same (unknown) functional variant(s) in ESR1 intron 1.

The final two SNPs of ESR1 block 1 flank exon 2. We found no evidence for a coding polymorphism in the associated region in an African-American population. Noncoding intronic variants could be responsible for the observed associations by causing changes in expression levels or alternative splicing. Associated SNPs are not located within putative consensus splice donor/acceptor or branch sites and are located some distance from exon 2 (rs9340799 is 350 bp proximal to exon 2 and rs7774230 317 bp distal) but may be in LD with untyped SNPs with these functions. Poola et al. (32) reported the presence of 20 splice variants of ESR1 and that variants with deletions encompassing exon 2 were the most prevalent in normal and tumor tissues (33). In vitro studies indicate that variant proteins with exon 2 deletions remain in the cytoplasm and are functionally inactive, while deletions of other exons (such as exons 3 and 5) still produce functionally active protein (34). If polymorphisms within the associated region affect exon 2 splicing, this could influence transcription of downstream genes involved in the pathogenesis of diabetes. Some genes, such as p21-*ras* and c-*jun*, shown to be altered in insulin resistance (3,35), contain estrogen receptor response elements (36,37).

Peroxisome proliferator–activated receptor-γ coactivator 1 (PGC1) (38) coactivates estrogen receptor-α by interacting at the hinge domain (encoded by exon 4) and the AF2 domain (encoded by exon 8) of estrogen receptor-α (39). SNPs in PGC1 are associated with type 2 diabetes in Danish, British, and Japanese patients (40–42), but not in French Caucasians (43), and PGC1 haplotypes are associated with carbohydrate metabolism and type 2 diabetes in white Europeans (44). In addition, PGC1 and its target genes have been shown to be downregulated in type 2 diabetes (45,46). Because PGC1 is a coactivator of peroxisome proliferator–activated receptor-γ, estrogen receptor-α, and HNF-4α, this may indicate the convergence of key signaling pathways in diabetes.

We determined that the African-American case and control populations show similar proportions of African ancestry (mean 0.83 and 0.81 in African-American case and

control subjects, respectively), comparable to reports from several other U.S. African-American populations (47). Familial type 2 diabetes–ESRD case subjects (probands) had higher African ancestry (0.86, 95% CI 0.84–0.87) than unrelated case subjects (0.80, 0.78–0.83) or control subjects (0.81, 0.79–0.83). However, only 22 AIMs were genotyped, and it is unclear how accurately estimates of ancestry were captured and subsequently accounted for in adjusted analyses. There is little empirical data on this issue, and studies of larger numbers of AIMs are underway in this population.

A limitation of this study is that the ascertainment scheme means that we cannot distinguish whether associations are with type 2 diabetes and/or nephropathy. However, given the growing prevalence and public health importance of both diseases, any insights into susceptibility to either disease is of value. African-American siblings concordant for type 2 diabetes produced greater evidence for linkage at 6q24-q27 than the subset of siblings from these families concordant for renal impairment (2). Animal studies of ESR1 suggest that it is more likely that this gene contributes to type 2 diabetes susceptibility/protection because knockout mice had increased adiposity and insulin resistance (4,48). However, upregulation of ESR1 expression in the diabetic kidney (49) and a role for estrogen receptor-α in compensatory kidney growth has also been observed (50). Additional studies will be needed to determine the trait responsible for these associations.

The inferences of association reported above are based on nominal significance levels without correction for multiple testing. Because the 17 SNPs typed in the expanded panel fall into four haplotype blocks of sizes 6, 5, 3, and 3 SNPs, there is a question of how to correct the correlated tests. Salyakina et al. (51) have computed a simple metric of effective number of tests (M_{eff}) based on the mean LD (E [r^2]) between SNPs in a block. Using Fig. 1 from their article and the estimated LD in each block, we estimate the total correction as:

$$\sum_{block\,=\,1}^{4} M_{eff}(block) = 15$$

rather than the Bonferroni value of 17. This modest reduction indicates that the apparent Bonferroni correction is not unduly conservative, a result that has been noted in other studies, in both genetics and other biostatistical applications. Using $M_{eff}(total) = 15$, the unadjusted and admixture-adjusted P values for rs1033182 in African-American type 2 diabetes–ESRD and associations with rs2431260 and rs1709183 in European-American type 2 diabetes–ESRD would not survive correction; however, female-stratified analyses for rs1033182 and haplotype block 1 in African-Americans remain significant. Although the SNPs used in the initial round of genotyping successfully detected a region of association, given the large size of this gene and its highly polymorphic nature, it may be informative to screen the ESR1 gene at a higher SNP density.

Using the Genotype-IBD Sharing Test (GIST) (52), none of the 17 SNPs in the intron 1–intron 2 region individually account for the 6q linkage signal; however, it is possible that combinations of SNPs may be required to have an impact on linkage. This is especially relevant because the strongest association was seen with a six-SNP haplotype block, and the association was driven by uncommon haplotypes, but there is no generally accepted method to account for the effect of multiple SNPs on a linkage peak.

It is not surprising that associated SNPs differ between African-American and European-American subjects because SNPs and haplotypes have marked frequency differences across these populations, especially for the majority of intron 2 SNPs in the distal end of the genotyped region (rs11155818–rs11155819l) (Tables 2 and 4). The results are consistent with protective alleles having arisen independently in each population. It is possible that existing protective alleles may not have been represented in African subpopulations that migrated elsewhere. Given the highly polymorphic nature of the ESR1 gene, it is possible that there may be multiple uncommon ESR1 variants contributing to disease risk/protection (possibly present only in the African diaspora) and that we have been fortunate to detect evidence of two such examples. However, this hypothesis can only be tested by a more comprehensive evaluation of ESR1 gene variation. These studies are currently underway. These apparent racial differences can only be clarified when the causative variants have been identified. Given the relatively low frequencies of the associated protective alleles and haplotypes, the contribution of this gene to diabetes risk is likely to be relatively modest; however, this may be the model for most diabetes loci, and the influence of this locus may be greater in the context of epistatic or environmental interactions. Estrogen receptor-α has pleiotropic influences, including effects on reproductive fitness (53), so these uncommon alleles may have been retained in the population because of positive selective pressure unrelated to diabetes or ESRD.

Although different SNPs and haplotypes are associated with type 2 diabetes and/or ESRD in African Americans and European Americans, associations in both populations are in ESR1 intron 1 or intron 2, suggesting these may be important functional regions of this gene in relation to type 2 diabetes or ESRD. It is critical that future studies attempt to replicate these results, although functional studies will be necessary to explain the influence of these intronic regions on diabetes susceptibility.

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