Genetic Variants in the Gene Encoding PRKCI and Susceptibility to Diabetic Nephropathy in Pima Indians

Diabetic nephropathy occurs when the delicate filtering system in the kidney becomes impaired after many years of uncontrolled diabetes. Diabetic nephropathy has a strong genetic component and is known to be especially prevalent among certain ethnic groups, including Native Americans. We hypothesized that genetic determinants underlie susceptibility to diabetic nephropathy. The goal of this study was to identify genetic variants that predispose Native Americans to this disease. Protein kinase C (PKC) is involved in intracellular signal transduction and is upregulated by hyperglycemia. PKC mediates mesangial cell response to hyperglycemia, stimulates TGFβ production, inhibits nitric oxide synthesis, and increases permeability to albumin, all of which suggest strong etiological role in the development of diabetic nephropathy. One PKC isoform, PKC-iota (PRKCI), is highly expressed in the kidney and is located on chromosome 3q26, in a region that has been linked to diabetic nephropathy in both Pima Indians and Caucasians. In this study, we sequenced PRKCI to identify genetic variants and genotyped these variants from a population of Pima Indians with diabetes, with or without nephropathy. We compared allele frequencies between the two groups. Identification of genetic variants, which predispose individuals to diabetic nephropathy, can assist in identifying those patients who are at highest risk for developing the disease and enhances our understanding of the inheritance and pathogenesis of the disease.

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Introduction

Native Americans have a greater prevalence of type 2 diabetes (T2DM) and diabetic nephropathy. Both diseases have important genetic determinants. Diabetic nephropathy is the most common cause of end stage renal disease (ESRD) in the Western world and is associated with considerable morbidity and mortality. Furthermore, familial aggregation of diabetic nephropathy in Pima Indians suggests that the genes determining susceptibility to nephropathy are in addition to those for diabetes. A recent genome scan in Pima Indians revealed linkage of diabetic nephropathy to chromosome 3q. Protein kinase C (PKC) is involved in intracellular signal transduction and is upregulated by hyperglycemia. PKC mediates mesangial cell response to hyperglycemia, stimulates TGFβ production, inhibits nitric oxide synthesis, and increases permeability to albumin, all of which suggest a strong etiological role in the development of diabetic nephropathy. One PKC isoform, PKC-iota (PRKCI), is highly expressed in the kidney and is located on chromosome 3q26, in a region that has been linked to diabetic nephropathy in both Pima Indians and Caucasians. In this study, we sequenced PRKCI to identify genetic variants. All variants were genotyped in a population of diabetic Pima Indians with or without nephropathy, and allele frequencies were compared between the two groups.

Methods

Subjects

A total of 215 individuals of Pima/Tohono O'odham heritage were selected for genetic screening; of these, 107 individuals had ESRD (cases) and 108 were diabetic individuals without ESRD (controls). Controls were selected to match the experimental group in sex, age, and duration of diabetes. In addition, the individuals selected are not first-degree relatives of each other. All individuals are members of the Gila River Indian Community and have been participating in longitudinal studies on diabetes and its complications since 1965.

DNA Sequencing

The PRKCI genomic sequence was obtained from the genomic sequences of BAC clones 81O8 (Accession AC023891-promoter region and exons 1 and 2) and 543D10 (Accession AC073288, exons 3–18). Approximately 1 kb of the upstream regulatory region and all exons and exon-intron boundaries were sequenced using genomic DNA from 36 Pima Indian individuals (18 ESRD cases and 18 diabetic controls). DNA was amplified in a final reaction volume of 10 μL using 60ng genomic DNA, 10× standard PCR buffer, 0.8 μM dNTPs, 0.4 μM oligonucleotide primers, and 0.5 U DNA polymerase mix (AmpliTaq Gold; Applied Biosystems; Foster City, CA). PCR cycling conditions consisted of an initial denaturation at 96°C for 7 minutes, followed by 35 cycles of 96°C for 20 seconds, 57°C for 30 seconds, and 72°C for 45 seconds, ending with a final elongation step at 72°C for 5 minutes. PCR product concentration was estimated by gel electrophoresis and, depending on yield, 2.5–5 μl of PCR product was treated at 37°C for 15 min/80°C for 15 min with 1–2 μl of ExoSAP-IT® (USB; Cleveland, OH) to remove unconsumed

From the Translational Genomics Research Institute; Phoenix, Arizona.

Table 1. Estimates of LD between PRKCI SNPs

SNP	Frequency	rs1802975	133631	rs1798229	18368	rs2140825	48025	rs2454763
rs1802975	0.69 [C/T]		1.00 (0.08)	0.60 (0.08)	1.00 (0.12)	0.66 (0.10)	0.95 (0.05)	0.51 (0.04)
133631	0.84 [A/G]	13,381		1.00 (0.09)	1.00 (0.05)	1.00 (0.34)	1.00 (0.02)	1.00 (0.61)
rs1798229	0.68 [A/T]	29,355	15,974		1.00 (0.56)	0.17 (0.01)	0.07 (0.00)	0.22 (0.01)
18368	0.78 [T/C]	40,307	26,926	10,952		1.00 (0.14)	1.00 (0.03)	1.00 (0.08)
rs2140825	0.65 [C/T]	50,898	37,517	21,543	10,591		0.98 (0.21)	1.00 (0.58)
48025	0.89 [C/A]	69,964	56,583	40,609	29,657	19,066		1.00 (0.04)
rs2454763	0.76 [G/A]	74,334	60,953	44,979	34,027	23,436	4,370	

dNTPs and oligonucleotide primers. Amplicons were bidirectionally sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and 35 cycles of 96°C for 10 s, 55°C for 10 s, and 60°C for 4 m. After column purification, sequences were resolved on the AB 3730XL sequence analysis system (Applied Biosystems).

SNP Genotyping

SNPs were genotyped by a method known as allelic discrimination. Sixty nanograms of each sample of genomic DNA were combined with the Taqman Universal PCR Master Mix (No AmpErase UNG at 1× concentration), SNP assay mix at 1× concentration, 0.25 U AmpliTaq Gold, and 0.3 ug RNase A. PCR cycling conditions consisted of an initial denaturation at 95°C for 10 min, followed by two-step thermocycling for 40–55 cycles of 95°C/15 s and 60°C/60 s. After amplification, fluorescence was measured on the ABI 7000 Sequence Detector using the allelic discrimination software Sequence Detection System Software Version 2.0 (Applied Biosystems). Genotypes were derived from FAM:VIC ratios, following normalization to a ROX reference signal.

Data Analysis

Sequencing chromatograms were analyzed using Sequencher software (Gene Codes Corporation; Ann Arbor, MI) and polymorphisms (single nucleotide polymorphisms [SNPs] and insertion/deletions) were identified by visual inspection. Genotypic concordance was determined by assessing the pairwise correlation of alleles at one locus with alleles at a second locus.

The statistical evidence for association in cases and controls was assessed by analysis of contingency tables. The strength of the association between genotypes and affection status was assessed by the odds ratio, calculated by logistic regression. Logistic regression analysis was used to control for potentially confounding variables. The degree of pairwise linkage disequilibrium between alleles was quantified by the disequilibrium coefficient D′, and standardized by the square root of the product of the two locus-specific variances r^2 using the emLD program (http://linkage.rockefeller.edu/soft/). The observed genotype frequency for each SNP was assessed for deviation from that expected under Hardy-Weinberg equilibrium using Chi-square analysis. Control measures (ie, encrypted samples and determination of Mendelian incompatibility) were employed to assess data quality.

RESULTS

We sequenced approximately 12 kb of the gene encoding PRKCI and identified a total of 13 SNPs, including 8 SNPs that were found in the public SNP database (dbSNP) and 5 novel variants. Six SNPs were in 100% genotypic concordance, based on sequencing in 36 individuals, thus 7 SNPs were genotyped in total.

We first quantified the degree of pairwise LD between PRKCI SNPs. LD was quantified using the disequilibrium coefficient D′, which represents the proportion of the maximum possible allelic association given allele frequencies and the direction of the association, and r^2, which is a measure of concordance such that absolute values of 1 only occur when there is complete linkage disequilibrium and when the associated alleles have identical frequencies. As shown in Table 1 the seven PRKCI SNPs varied substantially in LD, as estimated by both D′ (0.07–1.00) and r^2 (0.00–0.61).

The frequency of the more common allele is presented in Table 1. Linkage disequilibrium between SNPs, expressed as D′ or r^2 (in parenthesis), is shown above the diagonal. Distance in base pairs between SNPs is shown below the

Table 2. Association of PRKCI SNPs with ESRD in Pima Indians

SNP	GT	N (%ESRD)	OR (95% CI)	*P*-value
48025	CC	175 (0.50)		
	CA	34 (0.50)		
	AA	0	1.02 (0.48, 2.17)	.95
rs1082975	CC	87 (0.52)		
	CT	85 (0.52)		
	TT	19 (0.37)	1.23 (0.79, 1.92)	.36
rs1798229	AA	95 (0.56)		
	AT	91 (0.44)		
	TT	18 (0.50)	1.31 (0.84, 2.04)	.23
rs2140825	CC	91 (0.48)		
	CT	87 (0.47)		
	TT	25 (0.68)	0.70 (0.46, 1.07)	.09
rs2454763	GG	119 (0.48)		
	GA	67 (0.49)		
	AA	17 (0.71)	0.65 (0.41, 1.02)	.06
18368	TT	129 (0.53)		
	TC	64 (0.44)		
	CC	11 (0.55)	1.27 (0.79, 2.06)	.32
133631	AA	143 (0.46)		
	AG	54 (0.57)		
	GG	10 (0.70)	0.57 (0.34, 0.95)	.03

diagonal. For all measures of pairwise LD, $P<.001$.

We next examined association between PRKCI SNPs and ESRD in Pima Indians. Genotypic comparisons were assessed in 107 diabetic Pima individuals with ESRD and 108 diabetic control individuals with diabetes duration $>$ 10 years. As shown in Table 2, the more common genotype (GT) is listed first and the number of individuals with each genotype is given as *N* with the percentage of those with ESRD shown parenthetically. Odds ratios (OR) were calculated under an analytical model assuming an additive allele effect (eg, expressed per difference in number of alleles) with adjustment for age, sex, and duration of diabetes. We found evidence for association with the novel SNP, 133631, ($P=.03$) and a trend toward association with rs2140825 ($P=.09$).

CONCLUSION

We have sequenced 12 kb of PRKCI sequence and identified 13 SNPs. We genotyped a total of 7 informative SNPs and tested them for association with ESRD in American Indians. We found evidence that one SNP, located in intron 2 (rs13631) is associated with ESRD in our population.

It is not yet clear what the functional effects of SNP 133631 are. Future studies should include sequencing of the PRKCI gene to identify additional SNPs. If association with ESRD is confirmed, a functional assessment of the relevant SNPs should be conducted. Results may aid in the development of improved therapeutic interventions for diabetic nephropathy and/or provide a means to identify those individuals who are at greatest risk for developing the disease.

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