PCLO Variants Are Nominally Associated With Early-Onset Type 2 Diabetes and Insulin Resistance in Pima Indians

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OBJECTIVE—A prior genome-wide association (GWA) study in Pima Indians identified variants within *PCLO* that were associated with early-onset type 2 diabetes. *PCLO* encodes a presynaptic cytomatrix protein that functions as a Ca^{2+} sensor that may be involved in insulin secretion and/or insulin action. Therefore, *PCLO* was analyzed as a candidate gene for type 2 diabetes.

RESEARCH DESIGN AND METHODS—Sequencing of *PCLO* identified four nonsynonymous variants and a 10–amino acid insertion. These variants, together with 100 additional variants identified by sequencing or chosen from databases, were genotyped for association analysis in the same 895 subjects analyzed in the prior GWA study (300 case subjects with diabetes onset at aged <25 years, 334 nondiabetic control subjects aged >45 years, and 261 discordant siblings of the case or control subjects for within-family analyses), as well as 415 nondiabetic Pima Indians who had been metabolically phenotyped for predictors of diabetes. Selected variants were further genotyped in a population-based sample of 3,501 Pima Indians.

RESULTS—Four variants were modestly associated with early-onset type 2 diabetes in both general and within-family analyses ($P = 0.004–0.04$, recessive model), where the diabetes risk allele was also nominally associated with a lower insulin-mediated glucose disposal rate ($P = 0.009–0.14$, recessive model) in nondiabetic Pima Indians. However, their association with diabetes in the population-based sample was weaker ($P = 0.02–0.20$, recessive model).

CONCLUSIONS—Variation within *PCLO* may have a modest effect on early-onset type 2 diabetes, possibly as a result of reduced insulin action, but has minimal, if any, impact on population-based risk for type 2 diabetes. ***Diabetes* 57:3156–3160, 2008**

The Pima Indians of Arizona have an extremely high prevalence of type 2 diabetes (1). Their diabetes is characterized by obesity, dysfunction of insulin secretion, insulin resistance (decreased insulin-mediated glucose disposal), and increased rate of endogenous glucose output (2). Studies have shown that type 2 diabetes, insulin action, acute insulin response to glucose, and obesity are highly heritable in this population (3–5). To identify genes that underlie the development of type 2 diabetes in Pima Indians, we recently completed a genome-wide association (GWA) study using the Affymetrix 100K SNP genotyping array (6). Two single nucleotide polymorphisms (SNPs), rs10487656 and rs10487657, that ranked among the top 1% for a general association with early-onset type 2 diabetes (defined as age of onset <25 years) mapped within an intron of the *PCLO* gene. *PCLO* is located on chromosome 7q21 and encodes for a presynaptic cytomatrix protein that functions as a Ca^{2+} sensor that could potentially have a role in insulin secretion and/or insulin action (7–10); therefore, *PCLO* was analyzed as a positional and physiological candidate gene for type 2 diabetes.

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RESEARCH DESIGN AND METHODS

All subjects are part of our ongoing longitudinal study of the etiology of type 2 diabetes among the Gila River Indian Community in Central Arizona (1). Diabetes status is determined by a 75-g orally administered glucose tolerance test, with results interpreted according to the criteria of the World Health Organization (11). All studies were approved by the institutional review board of the National Institute of Diabetes and Digestive and Kidney Diseases. The case/control subjects analyzed for association with early-onset type 2 diabetes consisted of 300 case subjects (onset age <25 years, 38% male, mean maximum BMI 38.9 ± 8.4 kg/m^2) and 334 nondiabetic control subjects (age >45 years, 48% male, mean maximum BMI 35.4 ± 8.0 kg/m^2). To control for potential population stratification, 261 additional subjects who were discordant siblings of the case/control subjects were also genotyped to allow for within-family association analyses (total of 340 discordant sib pairs from 172 informative sibships). A larger population-based sample of 3,501 full-heritage Pima Indians consisting of 1,561 subjects with type 2 diabetes (age 37.2 ± 12.1 years, 27% male, mean maximum BMI 38.5 ± 8.4 kg/m^2) and 1,940 subjects without type 2 diabetes (age 31.1 ± 14.5 years, 46% male, mean maximum BMI 35.7 ± 8.2 kg/m^2) was also analyzed.

For association analysis with traits that predict type 2 diabetes, 415 nondiabetic, full-heritage Pima Indians (57.9% male, age 26.7 ± 6.2 years, BMI 34.0 ± 7.5 kg/m^2) who had been metabolically studied as inpatients in our clinical research center were genotyped. These nondiabetic inpatients were determined to be healthy by medical history, physical examination, and routine laboratory tests and were not taking medications. Oral glucose tolerance was measured after 2–3 days on a weight-maintaining diet of mixed composition. Blood for plasma glucose and insulin measurements was drawn before ingesting 75 g glucose and at 30, 60, 120, and 180 min thereafter. Subjects also received a 25-g intravenous injection of glucose over 3 min to measure the acute insulin response (AIR). Blood samples were collected before infusion and at 3, 4, 5, 6, 8, and 10 min after infusion for determination of plasma glucose and insulin concentrations. AIR was calculated as the mean increment in plasma insulin concentrations from 3 to 5 min (12). The hyperinsulinemic-euglycemic clamp technique was used to determine basal glucose appearance and insulin-stimulated glucose disappearance (uptake) rates as described elsewhere (12).

SNP identification and genotyping. To identify novel sequence variants, all 27 exons, exon-intron boundaries, 5′- and 3′-untranslated regions, and 2 kb of the putative promoter region of *PCLO* were sequenced in DNA samples from 24 non–first-degree related Pima Indians (12 developed diabetes with an onset

TABLE 1
Association of four SNPs in *PCLO* with type 2 diabetes in a case/control sample and development of type 2 diabetes in a population-based sample of Pima Indians

SNP	Risk allele and frequency	Early-onset type 2 diabetes in the case/control sample*		Development of type 2 diabetes in the population-based sample†	
		General	Within-family	General	Within-family
Ala2742Thr (rs976714)	Thr (A)	0.004	0.02	0.02	0.04
(G/A)	0.50	1.83 (1.20–2.78)	1.95 (1.09–3.52)	1.12 (1.02–1.23)	1.18 (1.00–1.38)
Val2413Ile (rs10954696)	Ile (A)	0.009	0.03	0.05	0.06
(G/A)	0.50	1.75 (1.15–2.67)	1.96 (1.07–3.58)	1.10 (1.00–1.21)	1.17 (0.99–1.39)
rs10487656	G	0.009	0.02	0.04	0.15
(A/G)	0.57	1.65 (1.13–2.42)	1.95 (1.08–3.51)	1.09 (1.00–1.19)	1.11 (0.96–1.28)
rs6950504	A	0.004	0.04	0.08	0.20
(G/A)	0.62	1.78 (1.19–2.65)	2.02 (1.02–4.00)	1.08 (0.99–1.17)	1.10 (0.95–1.28)

Data are adjusted *P* value with OR (95% CI)* and adjusted *P* value with hazard ratio (95% CI)†. The case/control sample is composed of 895 Pima subjects, 300 case subjects with diabetes onset age <25 years, 334 nondiabetic control subjects age >45 years, and 261 additional sibs of either case or control group for within-family test. The population-based sample consists of 3,501 full-heritage Pima Indians and 1,561 diabetic and 1,940 nondiabetic subjects at the last visit. Most of the case/control subjects ($n = 715$) were included in the population-based sample. *In the case/control analysis, a logistic regression was used to adjust for sex, birth year, and family membership. †For the population based-sample, a survival analysis (Poisson model) was applied to adjust for sex, birth year, age of last visit, age of onset for diabetes, and family membership. All *P* values are for a recessive genetic model.

age <25 years and 12 were nondiabetic and >45 years of age). Sequencing was performed using Big Dye Terminator technology (Applied Biosystems) on an automated DNA capillary sequencer (model 3730xl; Applied Biosystems). SNPs were genotyped by SNPlex (Applied Biosystems) following the manufacture's protocol.

Expression and tissue distribution. Primers designed for the major *PCLO* transcript (XM_929946; National Center for Biotechnology Information) were used to amplify cDNA from the following human tissues: adipose, hypothalamus, pituitary (BD Marathon-Ready cDNA; BD Bioscience/Clontech), brain, skeletal muscle, heart, liver, kidney, spleen, pancreas (BD Human MTC Multiple Tissue cDNA Panels I and II; BD Bioscience/Clontech), and pancreatic islets (kindly provided by Dr. Lorella Marcelli at Joslin Diabetes Center). PCR products were sequenced to confirm that they encoded *PCLO*.

Statistical analysis. Statistical analyses were performed using version 8.0 SAS software (Cary, NC). Numeric variables are expressed as means ± SE. For continuous variables, the general estimating equation procedure was used to adjust for the appropriate covariates and to account for the correlation among siblings. For insulin-stimulated glucose disposal rates (*M*) measured during the clamp, the covariates were age, sex, and percent body fat. For AIR to an intravenous glucose tolerance test, the covariates were age, sex, percent body fat, and *M*. The glucose disposal rates during the clamp were log transformed before analyses to approximate a normal distribution.

In the case/control analysis for early-onset type 2 diabetes, a logistic regression was used to adjust for sex, birth date, and sibship. For the population-based analysis, a survival analysis (Poisson model) was used to analyze the association of genotype with development of diabetes with adjustment for sex and birth date; to account for sibship, this model was also fit with generalized estimating equations. To control for potential population stratification, the association with diabetes was also analyzed using a within-family association test (13). We estimate that the power of the case/control sample is >75% to detect a common (minor allele frequency >0.1) functional allele at $P < 0.001$ that explains 1.6% of the variance in age of onset of diabetes for the general test and 3% of the variance for the within-family test. Similarly, we estimate that the power of the population sample is 92% to detect an odds ratio (OR) of 1.2 at $P < 0.05$ for a marker with allele frequency = 0.5. However, the magnitude of the associations between several recently reported SNPs and type 2 diabetes in Caucasians has been quite modest (ORs ~1.15) (14–19). We estimate that the power of our population-based sample to detect an OR of 1.15 is ~79% for a risk allele with frequency of 0.5 and ~35% for a risk allele with frequency of 0.1. The 95% CIs for the ORs can provide a measure of the range of effect sizes consistent with the data.

Homozygotes for the risk allele (1/1), heterozygotes (1/2), and homozygotes for the protective allele (2/2) were coded to a continuous numeric variable for genotype as 2, 1, and 0, respectively. The recessive model was defined as contrasting genotypic groups 1/1 versus 1/2 + 2/2. To examine pairwise linkage disequilibrium (LD), haplotype frequencies were estimated with the Estimating Haplotype (EH) program of Xie and Ott (http://linkage.rockefeller.edu/ott/eh.htm), and these haplotype frequencies were used to calculate D′ and r^2.

RESULTS AND DISCUSSION

Sequencing of *PCLO* identified variation that predicted a 10–amino acid insertion at codon 375 and 4 amino acid substitutions at Ser4684Ala (rs2522833), Ala2742Thr (rs976714), Val2413Ile (rs10954696), and Asp287Gly (novel). These five variants, together with 100 additional SNPs identified by sequencing or selected from public databases to approximate a 5-kb dense coverage across the *PCLO* locus, were genotyped for association analysis in the same 895 early-onset type 2 diabetic case/control subjects who were analyzed in our prior GWA study (all 105 variants are listed in online appendix Table 1 and the LD pattern between these variants is shown in online appendix Fig. 1 [available at http://dx.doi.org/10.2337/db07-1800]). All of the variants were in Hardy-Weinberg equilibrium. Four common SNPs (Ala2742Thr, Val2413Ile, and intronic SNPs rs10487656 and rs6950504) were associated with early-onset type 2 diabetes under both general and within-family analyses where both recessive *P* values are <0.05 (Table 1).

The 105 variants were additionally genotyped in 415 full heritage, nondiabetic subjects who had been metabolically phenotyped for predictors of type 2 diabetes including AIR to an intravenous glucose bolus and insulin-stimulated glucose disposal rate during a hyperinsulinemic-euglycemic clamp. The four SNPs (Table 1) that were associated with early-onset type 2 diabetes in the 895 case/control subjects were also nominally associated with insulin-stimulated glucose disposal rates (Fig. 1), where the risk alleles for diabetes were associated with a lower insulin-stimulated glucose disposal rate. No haplotype that provided stronger evidence for association than these single SNPs alone was identified (data not shown).

To determine the significance of variation in *PCLO* on diabetes risk in the general Pima Indian population, the four SNPs were further genotyped in a population-based sample of 3,501 full-heritage Pima Indians, as defined as all subjects from our longitudinal study for whom there was DNA available and diabetes status was known (1,561 subjects had a diagnosis of type 2 diabetes and 1,940 were nondiabetic at their last exam). The association of these SNPs with type 2 diabetes (as defined as onset at any age)

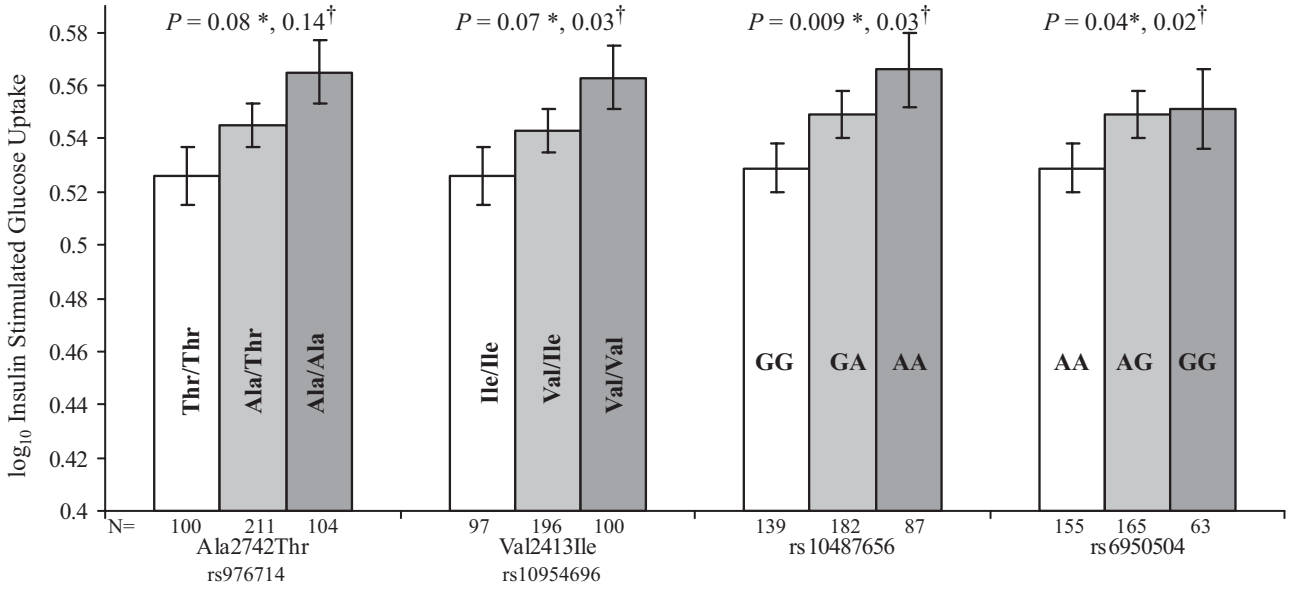

FIG. 1. Mean insulin sensitivity by genotype among nondiabetic Pima Indians. Insulin-stimulated glucose uptake (mg · kg EMBS^{-1} · min^{-1}) was measured by a hyperinsulinemic-euglycemic clamp with insulin infusion at physiologic concentrations. *P* values are given for a recessive model for both a general association* and a within family association† and are adjusted for age, sex, percent body fat, and family membership. EMBS, estimated metabolic body size.

was weaker in the larger population-based sample (Table 1). One explanation for observing a weaker association in the larger group of subjects is that *PCLO* may have a greater role in the development of early-onset type 2 diabetes compared with diabetes at older ages (mean age of diabetes onset is 19.2 ± 4.5 years in the case/control sample and 37.2 ± 12.1 years in the population sample). Alternatively, the associations among the case/control subjects for early-onset type 2 diabetes and the associations with insulin action in the nondiabetic subjects may represent false-positives. Indeed, the *P* values given in this study are unadjusted for multiple comparisons, and, if corrected for the 105 variants studied (which could be argued to be an overcorrection due to high LD among many of the variants), none of the *P* values would be significant. However, it remains controversial as to whether multiple comparison correction is appropriate for a potential physiologic candidate gene. In the present study, we instead chose to use both a case/control general and a within-family analysis to help restrict the number of false-positive associations while retaining statistical power.

It is also noteworthy that the 500 K GWA study performed at the Wellcome Trust Case Control Consortium (WTCCC) (www.wtccc.org.uk/info/summary_stats.shtml) also identified nominal associations between eight SNPs (with allele frequencies >0.02) in *PCLO* and type 2 diabetes (Table 2). Although these SNPs only showed a nonsignificant trend in another 500 K GWA study of Scandinavians (Diabetes Genetics Initiative [DGI]) (www.broad.mit.edu/diabetes), they were significantly associated with type 2 diabetes in a meta-analysis of subjects of European descent (Diabetes Genetics Replication and Meta-analysis [DIAGRAM]) (20). Five of these eight SNPs were also genotyped in the Pima Indian case/control sample, and several had nominal associations with type 2 diabetes (Table 2). The best SNPs in Pima Indians (listed in Table 1) were not represented on the 500 K chip;

TABLE 2
SNPs in *PCLO* associated with type 2 diabetes in the WTCCC 500 K GWA and their corresponding associations in the DGI 500 K GWA, the DIAGRAM meta-analysis, and Pima Indians

		WTCCC			DGI			DIAGRAM	Pima Indians			
SNP	Alleles (1/2)	Case (f1)	Control (f1)	P_{add}	Case (f1)	Control (f1)	P_{add}	P_{add}	Case (f1)	Control (f1)	P_{add}	P_{rec}
rs34608268	A/C	0.56	0.53	0.004	0.53	0.51	0.16	—	—	—	—	—
rs2715148	C/A	0.52	0.49	0.02	0.50	0.49	0.32	0.005	0.74	0.73	0.36	0.35
rs2888019	C/T	0.52	0.49	0.02	0.51	0.49	0.34	0.003	0.75	0.70	0.07	0.15
rs1986742	T/C	0.52	0.50	0.02	0.50	0.49	0.33	0.004	—	—	—	—
rs9690648	A/G	0.92	0.94	0.02	0.95	0.96	0.17	0.01	0.88	0.85	0.09	0.10
rs7781142	C/T	0.54	0.52	0.06	0.53	0.51	0.32	0.004	0.70	0.64	0.05	0.04
rs7799260	C/G	0.54	0.52	0.07	0.52	0.51	0.33	0.005	0.70	0.64	0.05	0.04
rs7778238	C/G	0.51	0.49	0.06	0.52	0.51	0.19	0.002	—	—	—	—

The frequency of allele 1 (f1) for each SNP is given for case subjects (with type 2 diabetes) and control subjects in each individual population. Frequency information was not available for the DIAGRAM meta-analysis, which combined data from the WTCCC, DGI, and FUSION studies of subjects of European descent (ref. 20). Data for WTCCC was obtained from http://www.wtccc.org.uk/info/summary_stats.shtml. Data for DGI was obtained from http://www.broad.mit.edu/diabetes. *P* values are calculated by comparison of genotypes. *P* values for WTCCC, DGI, and DIAGRAM are given for an additive model (P_{add}). *P* values for the Pima Indians are given for both an additive (P_{add}) and a recessive model (P_{rec}).

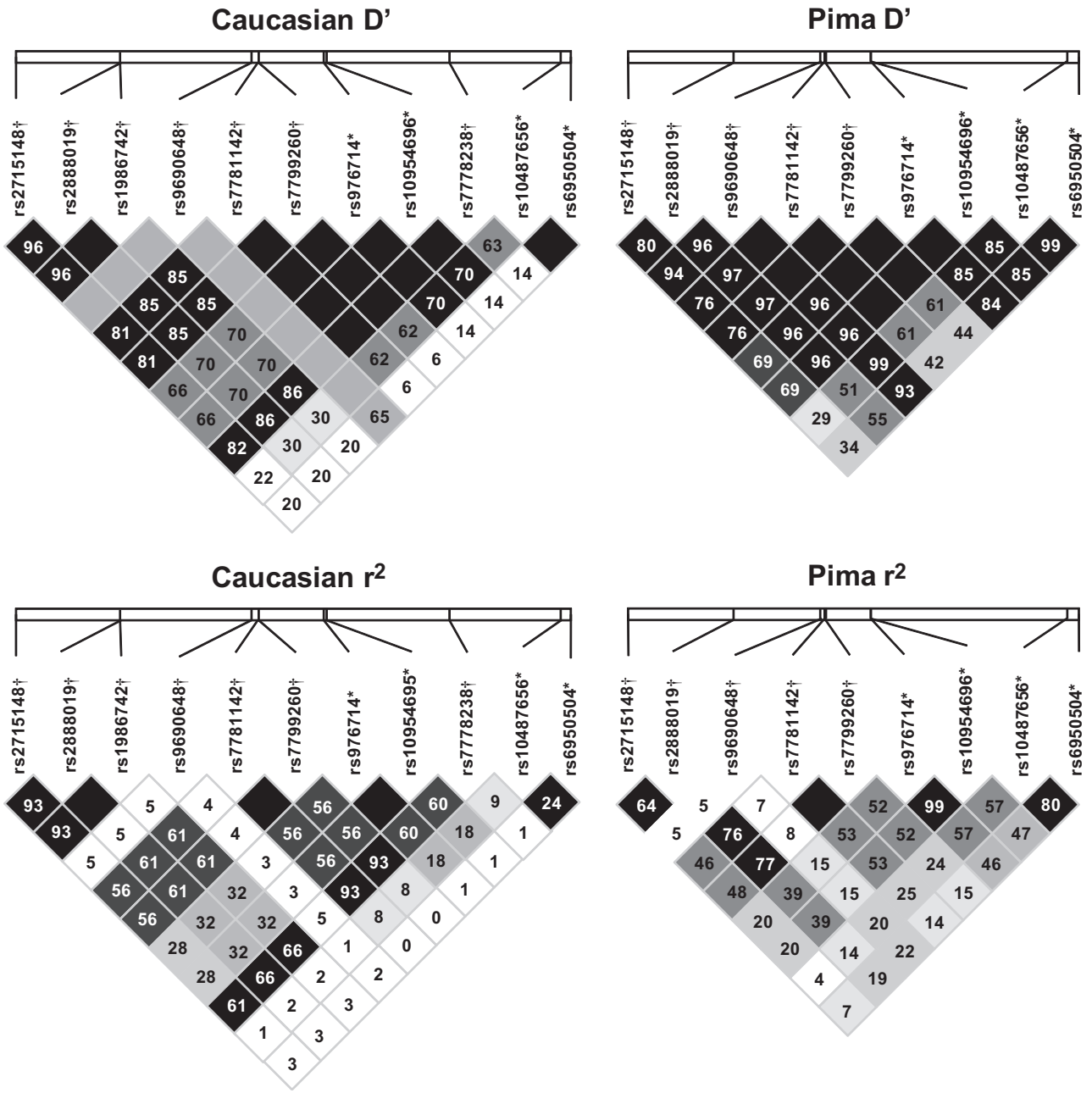

FIG. 2. LD pattern between SNPs in Table 1* and Table 2† in Caucasians and Pima Indians. LD in Caucasians was determined by CEU data (www.HapMap.org) using HaploView (www.broad.mit.edu/mpg/haploview). rs34608268 from Table 2 could not be included in this figure because there was no publicly available HapMap data for this SNP, and two SNPs (rs1986742 and rs7778238) were omitted from the LD pattern in Pima Indians because they were not genotyped in Pima Indians.

however, several of the WTCCC/DIAGRAM SNPs nominally associated with type 2 diabetes were in high LD with these SNPs in both Caucasians and Pima Indians (Fig. 2). For example, rs7781142 and rs7799260 (Table 2), which are in complete LD ($D' = 1$; $r^2 = 1$) in both Caucasians (of European descent) and Pima Indians, were in high LD with rs976714 (Ala2742Thr) and rs10954696 (Val2413Ille) in both Caucasians ($D' = 1$; $r^2 = 0.56$) and Pima Indians ($D' = 1$; $r^2 = 0.53$) (Fig. 2).

PCLO is known to be a Ca^{2+} sensor in pancreatic β-cells (7), and the formation of a cAMP-GEFII·Rim2·Piccolo complex in β-cells is important in insulin secretion (8). However, variants in this gene were not associated with the AIR to a 25-g intravenous glucose bolus infusion or the 30-min insulin response to a 75-g oral glucose tolerance test among normal glucose-tolerant Pima Indians (data not shown). Based on our associations, we propose that *PCLO* may also have a subtle role in the regulation of insulin sensitivity in insulin-responsive tissues. As a first step to test this hypothesis, we have shown that *PCLO* is ubiquitously expressed, with notable levels of expression in insulin-responsive tissues such as adipose and liver (online appendix Fig. 2). Another example of a Ca^{2+} sensor having a role in modulating insulin sensitivity is the α1-subunit of the voltage-dependent calcium channel, which has been previously associated with insulin resistance and type 2 diabetes in animal models (21) and humans (22). The Ca^{2+} sensor *PCLO* could potentially interact with the voltage-dependent calcium channel to influence insulin sensitivity.

In summary, we found a modest association of *PCLO* variants with early-onset type 2 diabetes, where the association was further supported by quantitative trait analysis in nondiabetic Pima Indians. However, this association was not well substantiated in a population-based sample of Pima Indians. We conclude that *PCLO* variants may modestly increase risk for early-onset type 2 diabetes, possibly as a result of altered insulin action, but variation in this gene does not appear to influence the population-based risk for developing diabetes among full-heritage Pima Indians.

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