Association Analysis of Variation in/Near FTO, CDKAL1, SLC30A8, HHEX, EXT2, IGF2BP2, LOC387761, and CDKN2B With Type 2 Diabetes and Related Quantitative Traits in Pima Indians

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OBJECTIVE—In recent genome-wide association studies, variants in CDKAL1, SLC30A8, HHEX, EXT2, IGF2BP2, CDKN2B, LOC387761, and FTO were associated with risk for type 2 diabetes in Caucasians. We investigated the association of these single nucleotide polymorphisms (SNPs) and some additional tag SNPs with type 2 diabetes and related quantitative traits in Pima Indians.

RESEARCH DESIGN AND METHODS—Forty-seven SNPs were genotyped in 3,501 Pima Indians informative for type 2 diabetes and BMI, among whom 370 had measures of quantitative traits.

RESULTS—FTO provided the strongest evidence for replication, where SNPs were associated with type 2 diabetes (odds ratio = 1.20 per copy of the risk allele, \( P = 0.03 \)) and BMI (\( P = 0.002 \)). None of the other previously reported SNPs were associated with type 2 diabetes; however, associations were found between CDKAL1 and HHEX variants and acute insulin response (AIR), where the Caucasian risk alleles for type 2 diabetes were associated with reduced insulin secretion in normoglycemic Pima Indians. Multiallelic analyses of carrying risk alleles for multiple genes showed correlations between number of risk alleles and type 2 diabetes and impaired insulin secretion in normoglycemic subjects (\( P = 0.006 \) and 0.0001 for type 2 diabetes and AIR, respectively), supporting the hypothesis that many of these genes influence diabetes risk by affecting insulin secretion.

CONCLUSIONS—Variation in FTO impacts BMI, but the implicated common variants in the other genes did not confer a significant risk for type 2 diabetes in Pima Indians. However, confidence intervals for their estimated effects were consistent with the small effects reported in Caucasians, and the multiallelic “genetic risk profile” identified in Caucasians is associated with diminished early insulin secretion in Pima Indians. Diabetes 58: 478–488, 2009

Although it has been known for decades that both type 2 diabetes and obesity have a genetic basis (1), remarkably few susceptibility genes with robust and reproducible effects have been identified for these diseases. The recent introduction of large-scale, high-density genome-wide association (GWA) technology has revolutionized this field. Within the past year, six high-density (>300 K) GWA studies to identify genes affecting risk for type 2 diabetes among Caucasians have been published, and replicated associations were reported with single nucleotide polymorphisms (SNPs) in/near the genes transcription factor 7-like 2 (TCF7L2), CDK5 regulatory subunit associated protein 1-like 1 (CDKAL1), zinc transporter, member 8 (SLC30A8), hematopoietically expressed homeobox (HHEX), exostosin 2 (EXT2), insulin-like growth factor 2 mRNA binding protein 2 (IGF2BP2), cyclin-dependent kinase inhibitor 2B (CDKN2B), LOC387761 (a hypothetical gene), and fat mass and obesity associated (FTO) (2–7). In a prior study, we thoroughly examined the TCF7L2 locus and determined that it was not a major gene for type 2 diabetes among Pima Indians who have a high prevalence of this disease and a high rate of obesity (8). We also recently reported results from our GWA study to identify genetic determinants for type 2 diabetes and obesity in Pima Indians (9), which did not identify SNPs in any of these genes as being among the strongest associations for type 2 diabetes. However, this prior GWA used the lower-density 100K Affymetrix array, and none of the reproducibly associated variants identified in the high-density GWA scans of Caucasians are well captured by the 100K array. Therefore, in the current study, we directly examine the specific SNPs reported in Caucasian studies and tag SNPs to examine alternative variation within CDKAL1, SLC30A8, and IGF2BP2 to evaluate their potential role in affecting diabetic status, body weight, and quantitative metabolic risk factors for diabetes in the Pima Indian population.

RESEARCH DESIGN AND METHODS

All subjects are part of an ongoing longitudinal study of the etiology of type 2 diabetes in the Gila River Indian Community in Central Arizona (10). The population-based sample included every full-heritage Pima Indian from the longitudinal study for whom DNA was available, diabetes status was known, and height and weight were measured (n = 3,501). BMI was computed for those aged ≥15 years. Among these subjects, 1,561 had type 2 diabetes (37% men, mean age of onset 37.2 ± 12.1 years, and mean maximum BMI 38.5 ± 8.4 kg/m²) and 1,940 were nondiabetic at their last exam (46% men, mean study...
age 31.1 ± 14.5 years, and mean maximum BMI 35.7 ± 8.2 kg/m²). Diabetes was
diagnosed according to World Health Organization criteria (11) when the
venous plasma glucose concentration was ≥200 mg/dl for ≥2 hours after a 75-g oral
load, the fasting plasma glucose was ≥126 mg/dl, or the diagnosis was
made by clinical means. The BMI measurement that was used for association
analyses was the BMI at the first physical examination when the subject was
not diabetic. Measurements made at or after the diagnosis of diabetes
were not included in the analysis. Subjects who were diabetic at their
first exam were excluded; therefore, the BMI analyses are restricted to 2,458
subjects.

A subset of these full-heritage Pima Indian subjects (n = 370) was also
studied as inpatients in our clinical research center when they were nondia-
abetic. All of these subjects (aged 18–45 years) were healthy by medical
history, physical examination, and routine laboratory tests and were not
taking medications. Subjects were fed a weight-maintaining diet for 2–3 days
before they were administered an oral glucose tolerance test (OGTT). For the
OGTT, subjects underwent an overnight fast and then ingested 75 g glucose;
this glucose load, the fasting plasma glucose was 3.0–6.0 mg/dl, and 180 min
thereafter for measurement of plasma glucose and insulin concentrations. On
a different day, subjects received a 25-g intravenous glucose tolerance test
(IVGTT) to measure 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 calcu-
lated as one-half the mean increment in plasma insulin concentrations from 3
to 5 min as previously described (12). Analysis of AIR and 30-min insulin levels
during IVGTT was restricted to subjects with normal glucose tolerance
concentrations. All covariates were specified a priori based on previous studies of the
determinants of these traits. These models were also fit by generalized
estimating equations to account for correlation among siblings. Plasma insulin
concentrations, insulin-stimulated glucose disposal rates, and AIRs were log
transformed before analysis to approximate a normal distribution. Linkage
disequilibrium and haplotype block analysis were estimated by Haploview
(version 3.2). P values <0.05 were considered statistically significant.

For the analysis of heterogeneity in the odds ratios (ORs) between
Caucasians and Pima Indians, a summary OR was calculated for Pima
Indians and Europeans as a weighted sum of the logarithms of the ORs for the
individual studies with the inverse of the variance of the estimate of the
logarithm of the OR as the weight (16). Variances for the European studies were
determined from the confidence intervals for published studies; if these
variances were not available, from the genotypic counts for case and control subjects.
The Q statistic was used to test the null hypothesis that the Pima Indian and
European ORs were equal. Power to detect heterogeneity under the assump-
tion that the true OR in Europeans is the summary observed from published
studies and that the true OR in the Pima Indians is 1 was calculated by the
method of Hedges and Pigott (17).

The magnitude of the previously reported SNP associations in Caucasians
has generally been modest (ORs ~1.15). To estimate the power to detect an
association for each variant with the effect observed in Europeans, we
conducted simulations for the present set of families given the observed Pima
Indian allele frequencies under the assumption that the OR is constant
with BMI, where Pima Indian subjects carrying the risk

RESULTS
CDKAL1. Four SNPs in CDKAL1 (rs4712523, rs10946398, rs7754840, and
rs7756992) previously associated with type 2 diabetes (3–6) and 25 additional tag SNPs within
CDKAL1 were genotyped in the population-based sample of 3,501 full-heritage Pima Indians. The four
previously reported SNPs fell into one linkage disequilibrium block 1, where rs7754840 was in complete
linkage disequilibrium (D' = 1, r^2 = 0.98) with rs10946398 and rs4712523 and in high
linkage disequilibrium (D' = 0.98, r^2 = 0.68) with rs7756992 (supplementary Fig. 1, available in an online
appendix at http://dx.doi.org/10.2337/db05-0877). The allele

Statistical analysis. Statistical analyses were performed using the software
of the SAS Institute (Cary, NC). The general association of genotypes with
type 2 diabetes was assessed with logistic regression analysis and was
adjusted for age, sex, and percent body fat, when a longitudinal
model was fit with a generalized estimating equation to account for correlation among siblings.
Analyses for type 2 diabetes and BMI are given for an "additive" model in
which homozygotes for the major allele (1/1), heterozygotes (1/2), and
homozygotes for the minor allele (2/2) were coded to a numeric value for
genotype (0, 1, and 2, respectively). A within-family association analysis
among genotypically discordant siblings was also conducted by a modification of the
method of Abecasis et al. (15) to control for aggregation when the population stratafica-
tion. The association of quantitative traits with genotypes was analyzed by
linear regression using the general estimating equation method (SAS
Institute, Cary, NC). Potentially confounding covariates were included in all models.
BMI was adjusted for age, sex, and birth year. Percent body fat was
adjusted for age and sex. Insulin-stimulated glucose disposal was adjusted for
age, sex, and percent body fat. AIR was adjusted for age, sex, percent body fat,
and insulin-stimulated glucose disposal rate. The 30-min plasma insulin
concentration during an OGTT was adjusted for age, sex, percent body fat,
and infused-stimulated glucose disposal rate, and AIRs were log
transformed before analysis to approximate a normal distribution. Linkage
disequilibrium and haplotype block analysis were estimated by Haploview
(version 3.2). P values <0.05 were considered statistically significant.

For the analysis of heterogeneity in the odds ratios (ORs) between
Caucasians and Pima Indians, a summary OR was calculated for Pima
Indians and Europeans as a weighted sum of the logarithms of the ORs for the
individual studies with the inverse of the variance of the estimate of the
logarithm of the OR as the weight (16). Variances for the European studies were
determined from the confidence intervals for published studies; if these
variances were not available, from the genotypic counts for case and control subjects.
The Q statistic was used to test the null hypothesis that the Pima Indian and
European ORs were equal. Power to detect heterogeneity under the assump-
tion that the true OR in Europeans is the summary observed from published
studies and that the true OR in the Pima Indians is 1 was calculated by the
method of Hedges and Pigott (17).

The magnitude of the previously reported SNP associations in Caucasians
has generally been modest (ORs ~1.15). To estimate the power to detect an
association for each variant with the effect observed in Europeans, we
conducted simulations for the present set of families given the observed Pima
Indian allele frequencies under the assumption that the OR is constant
with BMI, where Pima Indian subjects carrying the risk
### TABLE 1
Association of SNPs in/near CDKAL1, SLC30A8, HHEX, EXT2, IGF2BP2, CDKN2B, LOC387761, and FTO with type 2 diabetes and BMI in a population-based sample of full-heritage Pima Indians

| SNP       | Gene   | Chromosome | Allele 1/2 | A. Frequency | Subjects by genotype with type 2 diabetes (top row) and nondiabetic subjects (bottom row) | BMI by genotype (top row) and number of subjects (bottom row) |
|-----------|--------|------------|------------|--------------|------------------------------------------------------------------------------------------|------------------------------------------------------------------|
| rs6935317 | CDKAL1 | 6          | C/T        | 0.25         | 763 532 83 0.70 0.97 (0.85–1.12)                                                          | 35.9 ± 8.1 36.6 ± 8.4 36.3 ± 8.8 0.13                            |
| rs1569660 | CDKAL1 | 6          | A/G        | 0.29         | 698 556 114 0.88 0.99 (0.87–1.13)                                                          | 36.0 ± 8.1 36.4 ± 8.3 36.2 ± 8.4 0.34                            |
| rs2328528 | CDKAL1 | 6          | A/T        | 0.23         | 874 474 98 0.30 0.94 (0.81–1.08)                                                          | 36.4 ± 8.4 35.9 ± 7.8 35.9 ± 7.6 0.13                            |
| rs7754840 | CDKAL1 | 6          | G/C        | 0.28         | 750 495 132 0.38 1.06 (0.93–1.22)                                                          | 36.6 ± 8.6 35.9 ± 7.9 35.1 ± 7.1 0.01                            |
| rs7756992 | CDKAL1 | 6          | A/G        | 0.34         | 610 574 178 0.15 1.10 (0.97–1.25)                                                          | 36.5 ± 8.6 36.1 ± 7.8 35.5 ± 7.8 0.09                            |
| rs9295479 | CDKAL1 | 6          | A/G        | 0.32         | 448 734 266 0.57 1.03 (0.92–1.16)                                                          | 36.0 ± 8.1 36.2 ± 8.0 36.6 ± 8.6 0.31                            |
| rs4351239 | CDKAL1 | 6          | T/C        | 0.12         | 1,068 287 17 0.11 1.16 (0.97–1.39)                                                          | 36.2 ± 8.2 36.3 ± 8.2 36.3 ± 7.8 0.98                            |
| rs1004172 | CDKAL1 | 6          | C/T        | 0.20         | 1,078 579 79 0.18 1.10 (0.96–1.27)                                                          | 36.0 ± 8.3 36.7 ± 8.1 36.0 ± 8.2 0.32                            |
| rs1498426 | CDKAL1 | 6          | T/C        | 0.24         | 840 461 101 0.16 0.91 (0.79–1.04)                                                          | 36.5 ± 8.1 35.7 ± 8.2 36.7 ± 8.7 0.05                            |
| rs9465946 | CDKAL1 | 6          | A/G        | 0.18         | 969 392 59 0.11 0.88 (0.76–1.03)                                                          | 36.4 ± 8.1 35.9 ± 8.4 37.6 ± 8.9 0.41                            |
| rs9465948 | CDKAL1 | 6          | T/C        | 0.18         | 1,233 507 46 0.11 1.12 (0.96–1.31)                                                          | 36.3 ± 8.2 35.9 ± 8.2 36.4 ± 8.3 0.49                            |
| rs10946425 | CDKAL1 | 6          | G/A        | 0.03         | 1,151 519 80 0.16 0.84 (0.68–1.00)                                                          | 36.2 ± 8.2 36.8 ± 8.8 38.2 ± 0.73                            |
| rs9295488 | CDKAL1 | 6          | G/A        | 0.03         | 1,040 118 0 0.32 0.84 (0.60–1.09)                                                          | 36.2 ± 8.2 36.3 ± 8.2 36.2 ± 8.2 0.40                            |
| rs4712580 | CDKAL1 | 6          | T/C        | 0.41         | 647 844 274 0.71 0.98 (0.86–1.10)                                                          | 36.2 ± 8.2 36.3 ± 8.2 36.2 ± 8.2 0.40                            |
| rs9295491 | CDKAL1 | 6          | G/T        | 0.15         | 1,384 453 42 0.49 0.94 (0.80–1.11)                                                          | 36.3 ± 8.1 36.2 ± 8.2 36.2 ± 8.9 0.13                            |
| rs6456396 | CDKAL1 | 6          | G/C        | 0.39         | 520 645 209 0.84 0.99 (0.88–1.11)                                                          | 36.1 ± 8.3 36.1 ± 8.0 36.7 ± 8.6 0.69                            |
| rs4076112 | CDKAL1 | 6          | G/A        | 0.04         | 1,224 115 3 0.85 1.03 (0.76–1.39)                                                          | 36.2 ± 8.2 35.1 ± 8.0 36.9 ± 6.1 0.03                            |
| rs12055480 | CDKAL1 | 6          | A/G        | 0.05         | 1,527 158 3 0.85 1.10 (0.76–1.39)                                                          | 36.2 ± 8.2 35.6 ± 8.4 NA 0.28                            |
| rs9295496 | CDKAL1 | 6          | A/G        | 0.03         | 1,178 91 0 0.61 1.10 (0.75–1.61)                                                          | 36.2 ± 8.2 35.6 ± 8.4 NA 0.28                            |
| rs9295497 | CDKAL1 | 6          | A/G        | 0.17         | 938 391 38 0.81 1.02 (0.86–1.21)                                                          | 36.2 ± 8.2 36.2 ± 8.2 37.2 ± 7.5 0.53                            |
| rs9465982 | CDKAL1 | 6          | C/T        | 0.18         | 893 401 42 0.83 1.02 (0.87–1.19)                                                          | 36.4 ± 8.1 35.9 ± 8.5 35.2 ± 6.6 0.05                            |
| SNP          | Gene   | Chromosome | Allele 1/2 | Frequency | A. 2. Frequency | Subjects by genotype with type 2 diabetes (top row) and nondiabetic subjects (bottom row) | BMI by genotype (top row) and number of subjects (bottom row) | Additive P value |
|--------------|--------|------------|------------|-----------|-----------------|------------------------------------------------------------------------------------------|------------------------------------------------------------------|------------------|
| rs0295501    | CDKAL1 | 6          | Δ/G        | 0.001     | 1,363           | 2          | 0.77 | 0.80 (0.18–3.52) | 36.2 ± 3.8      | 3.51 ± 10.6     | NA               | 0.77             |
| rs1257222    | CDKAL1 | 6          | C/T        | 0.02      | 1,330           | 50         | 0.74 | 1.08 (0.70–1.66) | 36.2 ± 3.8      | 37.0 ± 7.6      | NA               | 0.42             |
| rs89165      | CDKAL1 | 6          | C/T        | 0.16      | 1,029           | 382        | 0.63 | 0.96 (0.81–1.13) | 36.1 ± 3.8      | 36.6 ± 8.6      | 35.8 ± 8.2      | 0.62             |
| rs471965     | CDKAL1 | 6          | G/C        | 0.08      | 1,221           | 204        | 0.23 | 1.14 (0.92–1.42) | 36.3 ± 3.8      | 35.7 ± 8.4      | 34.8 ± 6.3      | 0.03             |
| rs6937610    | CDKAL1 | 6          | Δ/G        | 0.32      | 621             | 618        | 0.47 | 1.05 (0.92–1.30) | 36.5 ± 3.8      | 36.0 ± 8.2      | 35.9 ± 7.9      | 0.26             |
| rs9460612    | CDKAL1 | 6          | G/A        | 0.41      | 471             | 659        | 0.46 | 0.95 (0.84–1.08) | 36.2 ± 3.8      | 36.0 ± 8.2      | 36.9 ± 7.9      | 0.25             |
| rs7002176    | SLC30A8| 8           | T/A        | 0.25      | 756             | 500        | 0.34 | 0.93 (0.81–1.08) | 36.3 ± 3.8      | 36.1 ± 8.1      | 35.6 ± 7.8      | 0.16             |
| rs13266634*  | SLC30A8| 8           | C/T        | 0.09      | 1,156           | 209        | 0.71 | 1.04 (0.84–1.29) | 36.1 ± 3.8      | 36.5 ± 8.5      | 36.9 ± 8.0      | 0.98             |
| rs1995222    | SLC30A8| 8           | G/A        | 0.15      | 1,307           | 433        | 0.42 | 1.07 (0.90–1.27) | 36.2 ± 3.8      | 36.4 ± 8.3      | 35.4 ± 8.3      | 0.38             |
| rs1111875*   | HHEX   | 10          | T/C        | 0.40      | 499             | 661        | 0.51 | 1.04 (0.92–1.18) | 36.3 ± 3.8      | 36.1 ± 8.1      | 36.6 ± 8.8      | 0.86             |
| rs10509646   | HHEX   | 10          | T/C        | 0.46      | 414             | 680        | 0.02 | 1.15 (1.02–1.29) | 36.1 ± 3.8      | 36.3 ± 8.2      | 36.2 ± 8.4      | 0.90             |
| rs3740878‡‡  | EXT2   | 11          | G/A        | 0.13      | 1,037           | 315        | 0.57 | 1.05 (0.88–1.27) | 36.1 ± 3.8      | 36.5 ± 8.5      | 35.4 ± 8.8      | 0.53             |
| rs677038     | IGF2BP2| 3           | C/T        | 0.02      | 1,328           | 48         | 0.19 | 1.31 (0.88–1.96) | 36.2 ± 3.8      | 36.3 ± 7.9      | 46.2 ± 9.4      | 0.94             |
| rs16860234   | IGF2BP2| 3           | Δ/C        | 0.06      | 1,190           | 156        | 0.74 | 0.96 (0.74–1.23) | 36.1 ± 3.8      | 36.7 ± 7.9      | 37.4 ± 7.5      | 0.54             |
| rs4402960§   | IGF2BP2| 3           | G/T        | 0.17      | 942             | 370        | 0.32 | 1.08 (0.93–1.26) | 36.3 ± 3.8      | 35.9 ± 8.2      | 35.6 ± 7.3      | 0.14             |
| rs10811661*  | CDKN2B | 9           | T/C        | 0.17      | 1,186           | 461        | 0.08 | 1.13 (0.86–1.49) | 36.1 ± 3.8      | 37.3 ± 8.6      | 36.2 ± 6.6      | 0.08             |
| rs7480010‡   | LOC387761| 11         | A/G        | 0.16      | 976             | 368        | 0.75 | 1.03 (0.86–1.22) | 36.1 ± 3.8      | 36.6 ± 8.3      | 37.0 ± 9.4      | 0.35             |
| rs8050136†   | FTO    | 16          | C/A        | 0.15      | 1,071           | 357        | 0.03 | 1.20 (1.02–1.41) | 36.1 ± 3.8      | 36.7 ± 8.0      | 37.7 ± 8.3      | 0.002            |

Data are n or means ± SD unless otherwise indicated. *SNP previously reported to be associated with type 2 diabetes in a Caucasian GW study (2–6). For SNPs determined to be in complete linkage disequilibrium (D' > 0.99), only one representative SNP is shown. †rs7754840 (shown) is in complete linkage disequilibrium with rs10946398 and rs7112523 (not shown). ‡rs3744878 (shown) is in linkage disequilibrium with rs1113132 (not shown). €rs4402960 (shown) is in linkage disequilibrium with rs1470579 (not shown). ¶rs805136 is in linkage disequilibrium with rs939609 and rs7193144 (not shown). Data for monomorphic SNPs rs9300039 and rs17738231 are not shown. P values were adjusted for age, sex, and birth year. ORs for type 2 diabetes are expressed per copy of the underlined allele; for previously reported variants, this is the risk allele identified in Europeans (2–7), whereas for other variants it is arbitrarily set as allele 1 (major allele). BMI for these longitudinally studied subjects is defined as the maximum BMI recorded from a nondiabetic exam. In addition to the general analysis shown in the table, all variants were analyzed using a within-family analysis. With the exception of variants in FTO (detailed in Table 3), no variant showed a significant association (P < 0.05) with either type 2 diabetes or BMI using a within-family analysis. NA, analytical model not applicable due to low frequency.
allele for type 2 diabetes reported among Caucasians (C for rs7754840) were less obese (P = 0.01, adjusted for age, sex, and birth year; Table 1). These variants in CDKAL1, in contrast to variants in the genes described below, had a significant interaction with BMI for diabetes risk (e.g., P = 0.01 for interaction with rs7756992, where the interaction is in the direction that heavier Pima Indians were at greater risk if they carried the Caucasian risk allele for diabetes). The four previously reported SNPs in CDKAL1 were also modestly associated with impaired insulin secretion in Pima Indians (rs7756992 shown as representative in Table 2 and Fig. 1). Among Pima Indians with normal glucose tolerance, homozygotes for the G allele of rs7756992 (the type 2 diabetes risk allele in Caucasians) had a lower mean AIR to an IVGTT (dominant P = 0.04, adjusted for age, sex, percent body fat, and glucose disposal rate; Fig. 1A) and a lower early (30-min) mean plasma insulin level during an OGTT (dominant P = 0.0004, adjusted for age, sex, percent body fat, glucose disposal rate, and 30-min glucose levels; Fig. 1D). The reduced 30-min insulin response resulted in an elevated disposal rate; Fig. 1D) and a lower early (30-min) mean plasma insulin level during an OGTT (dominant P = 0.09, adjusted for age, sex, and percent body fat; Fig. 1C).

However, insulin action as assessed by insulin-stimulated glucose uptake during a hyperinsulinemic-euglycemic clamp did not differ among the genotypic groups (dominant P = 0.97, adjusted for age, sex, and percent body fat; Fig. 1B). Two database tag SNPs (rs9295479 and rs2328528) in CDKAL1, each from different linkage disequilibrium blocks (supplementary Fig. 1), were also associated with both AIR and early (30-min) plasma insulin levels, the most notable being rs9295479 because of its common frequency (allele frequency 0.96 – 0.97). Three additional tag SNPs within/near CDKAL1 were also genotyped in Pima Indians. Neither rs7002176 nor rs1995222 was associated with type 2 diabetes or BMI (Table 1), and rs17738231 was monomorphic.

**SLC30A8.** One SNP in SLC30A8 (rs13266634) was associated with type 2 diabetes in multiple GWA studies (2–6). In Pima Indians, rs13266634 was not associated with type 2 diabetes or BMI (Table 1) and was not associated with insulin or glucose responses to an OGTT, insulin sensitivity, or insulin secretion among the 370 metabolically phenotyped nondiabetic Pima Indian subjects (data not shown; specific traits analyzed are listed in Table 2). The frequency of the C allele (the risk allele in the other studies) was higher among the Pima Indians (0.91) compared with Caucasians (0.61–0.70) and Chinese (0.52–0.56), but this allele was also very frequent among Africans (0.96–0.97). Three additional tag SNPs within/near SLC30A8 (rs7002176, rs1995222, and rs17738231) were also genotyped in Pima Indians. Neither rs7002176 nor rs1995222 was associated with type 2 diabetes or BMI (Table 1), and rs17738231 was monomorphic.

**HHEX.** A SNP near HHEX (rs1111875) was previously associated with type 2 diabetes in multiple studies of Caucasians (2,4–6,18) and Asians (19–22) but not African Americans (23). Based on these findings, rs1111875 and another SNP within this region (rs10509646) were genotyped in the Pima Indian subjects. Rs1111875 was not associated with type 2 diabetes or BMI (Table 1); however, among Pima Indians who were normal glucose tolerant, those homozygous for the previously reported risk allele (C/C) at rs1111875 had a decreased AIR and a reduced early (30-min) insulin response to an OGTT (adjusted P dominant = 0.02 and 0.01, respectively; Table 2). A nominal association with type 2 diabetes was observed with...
rs10509646 (OR 1.15 [95% CI 1.02–1.29], \( P = 0.02 \), adjusted for age, sex, and birth year; Table 1).

**EXT2.** Two SNPs in **EXT2** (rs3740878 and rs1113132) that are in complete linkage disequilibrium in Caucasians and Chinese were associated with type 2 diabetes in a GWA study of French Caucasians (2) but were not replicated in other GWA studies of Caucasians (4–6) or Japanese populations (19,22). These SNPs were also in complete linkage disequilibrium in Pima Indians (supplementary Fig. 1) and were not associated with type 2 diabetes (rs3740878 shown in Table 1). However, among nondiabetic Pima Indians, these SNPs were associated with several measures of insulin resistance, including a lower insulin-stimulated glucose disposal rate in response to a hyperinsulinemic-euglycemic clamp (\( P = 0.04 \), adjusted for age, sex, percent body fat; Fig. 2A), and elevated glucose and insulin levels during an OGTT (\( P = 0.04, 0.03, \) and 0.008 for 1-h glucose, 1-h insulin, and 2-h insulin levels, respectively, adjusted for age, sex, and percent body fat; Fig. 2B). Pima Indians carrying the type 2 diabetes risk allele reported in the French Caucasians (A allele for rs3740878) were more insulin resistant. However, this risk allele is much less common among Pima Indians compared with Caucasians (frequency of A allele for rs3740878 0.13 in Pima Indians and 0.29 in Caucasians). These two SNPs were in high linkage disequilibrium in Pima Indians (\( D^2 = 0.98 \), the risk alleles were less common among Pima Indians than among Caucasians (frequencies of T allele of rs4402960 and C allele of rs1470579 = 0.17 in Pima Indians and 0.29 and 0.30 in Caucasians). These SNPs and two additional tag SNPs within **IGF2BP2** (rs6777038 and rs16860234; supplementary Fig. 1) were genotyped in the Pima Indians, but none was associated with type 2 diabetes or BMI (Table 1) or any of the diabetes-related quantitative traits (data not shown).

**CDKN2B.** One SNP in **CDKN2B** (rs10811661) was associated with type 2 diabetes in several studies of Caucasians (4–6). This association was strongly replicated in a Danish population (18) and modestly replicated in Asians (19,21,22) but not in African Americans (23). These two SNPs were in high linkage disequilibrium in Pima Indians (\( D^2 = 0.99 \), \( r^2 = 0.98 \)), and the risk alleles were less common among Pima Indians than among Caucasians (frequencies of T allele of rs4402960 and C allele of rs1470579 = 0.17 in Pima Indians and 0.29 and 0.30 in Caucasians). These SNPs and two additional tag SNPs within **IGF2BP2** were associated with type 2 diabetes or BMI (Table 1) or any of the diabetes-related quantitative traits (data not shown).
LOC387761 and intergenic chromosome 11p. One SNP in the predicted gene LOC387761 (rs7480010) and a second SNP in an intergenic region on chromosome 11p at \(42\) Mb (rs9300039) were highly associated with type 2 diabetes in two GWA studies of Caucasians (2,5). Among the Pima Indians, there was no evidence of association between rs7480010 and type 2 diabetes or BMI (Table 1) or a diabetes-related quantitative trait (data not shown; specific traits analyzed are listed in Table 2). SNP rs9300039 was monomorphic for the C allele.

**FTO.** SNPs within a region of high linkage disequilibrium in intron 1 of FTO (rs8050136 and rs9939609) were initially reported to be associated with BMI and obesity in two studies of Caucasians (25,26). Although these SNPs were also associated with type 2 diabetes, their association with type 2 diabetes was due to the higher BMI of the diabetic subjects (25). The association of the SNPs with BMI has been widely replicated in additional studies of Caucasians and non-Caucasian populations (27–31), although there have been a few reports of a lack of association (32,33). The association of rs8050136, rs9939609, and rs7193144, which were in complete linkage disequilibrium among the Pima Indians (supplementary Fig. 1), with BMI was replicated in Pima Indians (\(P = 0.002\) and 0.002 for the general and within-family analyses, respectively, where \(P\) is adjusted for age, sex, and birth year; Table 3). Subjects

| Trait                  | CC          | CA          | AA          | Additive | Recessive |
|------------------------|-------------|-------------|-------------|----------|-----------|
| BMI (kg/m\(^2\))       | 36.1 ± 8.2  | 37.7 ± 8.3  | 36.7 ± 8.0  | 0.002    | 0.005     |
| Fat cell size (ng lipid/cell) | 0.76 ± 0.21 | 0.84 ± 0.20 | 0.86 ± 0.25 | 0.006    | 0.002     |

\(P\) values are given for both additive and recessive models (CC vs. CA + AA) due to the small number of AA (\(n = 6\)) with measures of fat cell size. All \(P\) values were calculated after adjusting for age, sex, and birth year. \(P\) values for fat cell size were additionally adjusted for percent body fat. SNP rs8050136 is in linkage disequilibrium with rs9939609 and rs7193144 (not shown).
TABLE 4
Analysis of heterogeneity between Pima Indians and Caucasians in the association of sentinel SNPs in CDKAL1, SLC30A8, HHEX, IGFBP2, CDKN2B, and FTO with type 2 diabetes

| SNP         | Gene   | Pima Indian risk allele | Caucasian risk allele | Heterogeneity |
|-------------|--------|-------------------------|-----------------------|---------------|
| rs7756992   | CDKAL1 | 0.59                    | 1.10 (0.97–1.25)      | 0.49          |
| rs13266634  | SLC30A8| 0.29                    | 1.04 (0.84–1.29)      | 0.22          |
| rs1111875   | HHEX   | 0.58                    | 1.04 (0.92–1.18)      | 0.49          |
| rs44029660  | IGFBP2 | 0.43                    | 1.08 (0.93–1.26)      | 0.49          |
| rs10811661  | CDKN2B | 0.38                    | 1.13 (0.86–1.49)      | 0.27          |
| rs8050136   | FTO    | 0.55                    | 1.20 (1.02–1.41)      | 0.45          |

ORs and 95% CIs are given per copy of the risk allele, as determined in studies of Caucasians. Results for Pima Indians are derived from the present study, whereas those from Caucasians represent a combined estimate from other published studies (2–6,18) (see RESEARCH DESIGN AND METHODS). The heterogeneity power is defined as the power needed to detect significant heterogeneity at \( P < 0.05 \) given the Caucasian OR, the SE of its logarithm, and the SE of the logarithm of the Pima OR, under the assumption that the “true” OR in the Pima Indians is 1. The heterogeneity \( P \) value is given for the null hypothesis that the ORs in Pima Indians and Caucasians are the same.

homozygous for the risk allele (A for rs8050136) had a mean BMI that was 1.6 kg/m\(^2\) greater than that of individuals homozygous for the nonrisk allele (C). The risk allele for high BMI is less common among Pima Indians than Caucasians (frequency of A allele of rs8050136 is 0.45 vs. 0.15, respectively). Among the metabolically phenotyped nondiabetic Pima Indians who had undergone abdominal subcutaneous adipose tissue biopsies, subjects carrying the risk allele for increased BMI (A allele for rs8050136) also had larger individual fat cells, even after adjustment for their higher percentage of body fat (\( P = 0.02 \) and 0.002 for the general and within-family analyses, respectively; Table 3). SNPs in FTO were also modestly associated with type 2 diabetes in Pima Indians (Table 1; OR 1.20 [95% CI 1.02–1.41], \( P = 0.03 \), adjusted for age, sex, and birth year); however, the association was weakened and no longer met statistical significance after adjusting for BMI (1.16 [0.98–1.38], \( P = 0.08 \)), suggesting that the type 2 diabetes association was largely due to the effect on BMI.

Analysis of heterogeneity and multiallelic association. Associations of many of these SNPs in CDKAL1, SLC30A8, HHEX, IGFBP2, CDKN2B, and FTO with type 2 diabetes have been well-replicated in populations of European origin; however, the magnitude of these associations has generally been modest (ORs ~1.15). To assess whether the effects seen in the present study of Pima Indians were consistent with those observed in previous studies of European ancestry, we conducted a test for heterogeneity of the ORs (Table 4). Although, with the exception of rs8050136 in FTO, none of the SNPs were associated with diabetes at \( P < 0.05 \) in Pima Indians, the confidence intervals for the OR in Pima Indians invariably included the point estimate for Caucasians. Furthermore, none of the ORs were significantly different between Pima Indians and Caucasians in a formal heterogeneity test. In contrast, omitted from Table 4 but shown previously (8), there was significant heterogeneity between Pima Indians and Caucasians in the ORs for diabetes and SNPs in TCF7L2, which were not associated with this disease in Pima Indians.

Results of the multiallelic analysis of all SNPs shown in Table 4 (plus rs7903146 in TCF7L2 (8)) are shown in Fig. 3A. There was a modest but statistically significant increase in prevalence of type 2 diabetes among Pima Indians carrying increasing numbers of risk alleles, where risk is defined in Caucasian studies (2–7) (OR 1.10 per copy of a risk allele [95% CI 1.03–1.19], \( P = 0.006 \)). If rs8050136 in FTO was excluded from the analysis, this effect was reduced (1.07 [95% CI 1.00–1.16], \( P = 0.06 \); supplementary Fig. 2A, available in the online appendix), and if rs7903146 in TCF7L2, which has been previously shown to have heterogeneous effects between Pima Indians and Caucasians, was further excluded, the OR was 1.09 (95% CI 1.01–1.17) (\( P = 0.03 \); supplementary Fig. 2B). These results were largely unmodified by further adjustment for BMI (e.g., 1.13 [1.05–1.22], \( P = 0.001 \) for multiallelic effect including all variants).

Results for the multiallelic analyses for percent body fat, insulin sensitivity, and AIR among individuals who had undergone extensive metabolic phenotyping are shown in Fig. 3B–D. There was a modest inverse relationship with body fat, such that an increased number of risk alleles was associated with lower percent body fat, but this was not statistically significant (\( P = 0.07 \)). Similarly, an increased number of risk alleles had a nonsignificant trend toward increased insulin sensitivity as assessed by the hyperinsulinemic-euglycemic clamp (\( P = 0.06 \)). In contrast, there was a marked decrease in the AIR associated with each copy of a risk allele (\( P = 0.0001 \)). These results were largely unchanged when the SNPs in FTO and TCF7L2 were excluded (supplementary Fig. 2C and D).

DISCUSSION
The common forms of type 2 diabetes and obesity are thought to be complex polygenic diseases. However, it remains unknown how many genes contribute to these diseases and whether any single susceptibility gene will be shared among all ethnic groups or whether all will show some degree of population specificity. Recent high-density GWA studies in humans have identified specific variants in several genes that are associated with type 2 diabetes or obesity in more than one population and some variants that are highly associated with type 2 diabetes in one group of subjects but not others. In our study, we sought to determine whether these variants and/or genes also contribute to type 2 diabetes or obesity in the Pima Indian population in Arizona. In addition, because type 2 diabetes is a complex heterogeneous disease, a single risk factor or diabetes-related quantitative trait may be influenced by fewer physiological pathways and thus be determined by fewer genetic loci than the development of type 2 diabetes itself. Therefore, we also sought to determine whether any of the variants associated with type 2 diabetes in another population were associated with a diabetes-related quantitative trait in Pima Indians. We have presented the \( P \)
values in the present study without any adjustment for multiple comparisons. For SNPs that are well replicated in other populations (e.g., rs8050136 in FTO), the prior probability of a “true positive” is high and, thus, $P < 0.05$ is probably sufficient to conclude that these variants are also associated in this population. For the additional tag SNPs, however, this may not be the case, and none of the nominally significant results reported here would remain significant if a Bonferroni correction for the 31 tag SNPs were applied.

Genotyping of SNPs previously associated with type 2 diabetes in CDKAL1, SLC30A8, HHEX, EXT2, IGF2BP2, CDKN2B, LOC387761, and an intergenic region on chromosome 11p provided no evidence that any of these are significantly associated with type 2 diabetes or obesity among full-heritage Pima Indians living in the Gila River Indian Community in Arizona. However, it remains possible that other genes, with much larger effects on type 2 diabetes, exist in the Pima population and that the effect of these genes masks any minor role of CDKAL1, HHEX, and EXT2 in increasing type 2 diabetes risk.

The nominal associations of SNPs in CDKAL1 and HHEX with two independent measures of insulin secretion (namely, AIR assessed by an IVGTT and 30-min insulin assessed by an OGTT), where both of these measures were adjusted for insulin sensitivity, is consistent with prior association studies. Previous reports that homozygous carriers of the type 2 diabetes risk allele (G/G) for rs7756992 in CDKAL1 had a lower corrected insulin response to an oral glucose load than carriers of the A allele (3) and that carriers of the type 2 diabetes risk allele (C) for rs10946398 in CDKAL1 had a reduced 30-min insulin response (24) suggest a mechanism of impaired insulin secretion as the basis of the association with type 2 diabetes. Direct evidence that CDKAL1 affects insulin secretion has recently come from a large study of European Caucasians who had undergone both measures of insulin secretion using an IVGTT and insulin sensitivity using a hyperinsulinemic-euglycemic clamp (35). Similarly, previous studies of HHEX in Caucasians have shown that type 2 diabetes risk allele (C) carriers of rs1111875 had a lower mean insulin response in Caucasians (18,24), which is also consistent with the known physiological role of HHEX in pancreatic development. HHEX is highly expressed in pancreatic islet tissue, and HHEX knockout mice have a complete loss of ventral pancreas (36).

The strongest replication in this study was the association of variation (rs8050136, rs9939609, and rs193144, all in complete linkage disequilibrium) in FTO with BMI, where subjects homozygous for the previously reported risk allele were on average 1.6 kg/m² heavier than homozygotes for the nonrisk allele (heterozygotes were interme-
diate) among the population-based sample of Pima Indians. In addition, among the subset of subjects who had undergone subcutaneous adipose biopsies, subjects homozygous for the risk allele had larger individual adipocytes, even after adjusting for their higher percentage of body fat. FTO encodes a 2-oxoglutarate–dependent nucleic acid demethylase, which is highly expressed in hypothalamic nuclei and is thought to be regulated by fasting and feeding conditions (37). FTO is also expressed in adipose tissue, but FTO mRNA is not correlated with rs9939609 or rs8050136 (38,39). Healthy women homozygous for the obesity-protective FTO allele have an ~30% increased in vivo lipolytic activity (assessed as circulating glycerol corrected for total body fat), which is likely due to their increased spontaneous (basal) fat cell lipolysis (39). This finding is consistent with our observation of a difference in fat cell size by FTO genotype, where homozygotes for the protective genotype had significantly less lipid per cell (Table 3).

Most of the variants identified in previous GWA studies have fairly modest effects in Caucasian populations with ORs in the range of 1.1–1.2. Even with the present sample size of ~3,500 Pima subjects, there is only moderate power to detect (or exclude) associations of this magnitude. If most of these variants do have modest effects in Pima Indians, one would expect power to be increased in a multiallelic analysis that considers the total number of risk alleles across all variants. The present analyses did show a nominally significant, albeit modest, association of the number of risk alleles with type 2 diabetes. Furthermore, there was a marked association between number of risk alleles at these loci and a diminished AIR but not with other quantitative metabolic risk factors for type 2 diabetes, such as percent body fat or insulin sensitivity. These data are consistent with the hypothesis that many of the diabetes risk variants in Caucasians also have subtle effects in Pima Indians, although the statistical power of the present study is not sufficient to identify individual risk variants with confidence. In addition, the multiallelic analysis is consistent with the hypothesis that most of these variants influence the risk of type 2 diabetes through their effect on insulin secretion.

We have previously reported that variation in TCF7L2, which has shown the strongest association with type 2 diabetes across many populations, has minimal, if any, impact on this disease in Pima Indians (8). The variants presented in this paper, which also have well-replicated associations with both type 2 diabetes and measures of insulin secretion among Caucasians, do not have a major effect on type 2 diabetes in Pima Indians, although an association with AIR was observed. It is possible that these findings may be explained by a relatively smaller contribution of insulin secretory dysfunction to the occurrence of diabetes in Pima Indians compared with Caucasians, but insufficient data are available at present to evaluate this hypothesis. To determine whether additional variants exist with stronger effects in Pima Indians or other populations at high risk for diabetes among whom the relative contributions of obesity, insulin resistance, and insulin secretory dysfunction may differ will require mapping studies specific for these populations. In the present study, genotyping of additional tag SNPs in CDKAL1, SLC30A8, and IGF2BP2 did not reveal alternative variants associated with type 2 diabetes in Pima Indians; however, important variation could have been missed because these genes were interrogated by tag SNPs with a criteria of $r^2 \geq 0.5$ rather than the more customary $r^2 \geq 0.8$.

In summary, when examined individually, variants in CDKAL1, SLC30A8, HHEX, EXT2, IGF2BP2, CDKN2B, and LOC387761 associated with type 2 diabetes in other populations were not significantly associated with type 2 diabetes in Pima Indians. However, taken together, they had a modest additive association with type 2 diabetes and a strong association with decreased insulin secretion. In addition, previously reported variation in FTO does have a role in determining BMI and type 2 diabetes in the Pima Indian population. A recent report by the Diabetes Genetics Replication and Meta-Analysis consortium has described six additional type 2 diabetes susceptibility loci identified via GWA methods (40). Studies of these loci in Pima Indians are ongoing.

ACKNOWLEDGMENTS

R.R. has received a mentor grant from the American Diabetes Association. This study was supported by the intramural research program of the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health.

No potential conflicts of interest relevant to this article were reported.

We gratefully acknowledge the volunteers from the Gila River Indian Community, whose cooperation made these studies possible.

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