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A Genome-Wide Association Study in American Indians Implicates *DNER* as a Susceptibility Locus for Type 2 Diabetes



Most genetic variants associated with type 2 diabetes mellitus (T2DM) have been identified through genome-wide association studies (GWASs) in Europeans. The current study reports a GWAS for young-onset T2DM in American Indians. Participants were selected from a longitudinal study conducted in Pima Indians and included 278 cases with diabetes with onset before 25 years of age, 295 nondiabetic controls ≥ 45 years of age, and 267 siblings of cases or controls. Individuals were genotyped on a $\sim 1\text{M}$ single nucleotide polymorphism (SNP) array, resulting in 453,654 SNPs with minor allele frequency > 0.05 . SNPs were analyzed for association in cases and controls, and a family-based association test was conducted. Tag SNPs ($n = 311$) were selected for 499 SNPs associated with diabetes ($P < 0.0005$ in case-control analyses or $P < 0.0003$ in family-based analyses), and these SNPs were genotyped in up to 6,834 additional Pima Indians to assess replication. Rs1861612 in *DNER* was associated with T2DM (odds ratio = 1.29 per copy of the T allele; $P = 6.6 \times 10^{-8}$, which represents genome-wide significance accounting for the number of effectively independent SNPs analyzed). Transfection studies in murine pancreatic

β -cells suggested that *DNER* regulates expression of notch signaling pathway genes. These studies implicate *DNER* as a susceptibility gene for T2DM in American Indians.

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A number of genetic variants associated with type 2 diabetes mellitus (T2DM) have been identified (1–6). Since most established susceptibility variants were detected by genome-wide association studies (GWASs) in Europeans, GWASs in non-European populations may identify other important variants. Except for our preliminary study with 80,044 markers in Pima Indians (7), there are few GWASs for T2DM in American Indians. In the current study, we extend this GWAS to include 453,654 single nucleotide polymorphisms (SNPs), and we genotype 6,834 additional individuals to identify reproducibly associated variants.

RESEARCH DESIGN AND METHODS

Participants

Participants were derived from a longitudinal study conducted in the Gila River Indian Community (8). Diabetes was diagnosed by a 75 g oral glucose tolerance test

according to 1997 American Diabetes Association criteria (9) or if the diagnosis was made during routine clinical care. As described previously, participants informative for analyses of young-onset diabetes or for metabolic traits related to diabetes were selected for the GWAS (10). All individuals were full-heritage American Indian, and they included 278 “cases” who developed diabetes before age 25 years (mean \pm SD age of onset = 19.4 \pm 4.4 years) and 295 “controls” who were nondiabetic and \geq 45 years old when last examined (mean age = 55.2 \pm 9.7 years). To allow for family-based analyses, discordant siblings were included. These included 129 nondiabetic siblings of cases last examined at an age older than the case’s age of onset (mean age = 28.1 \pm 8.2 years) and 138 diabetic siblings of controls who developed diabetes when they were younger than the age when the control was examined (mean age at diagnosis = 40.8 \pm 8.4 years). The diabetes GWAS involved 840 individuals in 514 sibships.

Replication studies were conducted in additional individuals from the longitudinal study. Initial studies (replication set 1) were conducted in 2,908 individuals (mean age = 40.0 \pm 16.3 years; 43.8% with diabetes) who were not part of the GWAS and who were full-heritage Pima or who had data on metabolic traits. Selected SNPs were further typed in a second group (replication set 2) of 3,926 individuals who were largely “mixed” heritage (mean age = 27.7 \pm 13.9 years; 20.2% with diabetes; mean Pima heritage = 55%). Supplementary Table 1 shows characteristics of the groups. Studies were approved by the institutional review board of the National Institute of Diabetes and Digestive and Kidney Diseases.

Association of diabetes-associated SNPs with metabolic characteristics of T2DM was assessed. Maximum BMI observed in the longitudinal study was analyzed in 6,786 individuals examined when \geq 15 years of age. Fasting serum insulin concentration was measured in 5,400 of these individuals when they were nondiabetic; measures of insulin resistance (homeostasis model assessment of insulin resistance [HOMA-IR]) and β -cell function (homeostasis model assessment of β -cell function [HOMA-B]) were calculated (11). Detailed physiologic measures were made in 400 nondiabetic full-heritage Pimas. Percentage of body fat was calculated by hydrostatic weighing or dual X-ray absorptiometry, and insulin sensitivity was determined by the hyperinsulinemic-euglycemic “clamp” (12). Acute insulin response, 3–5 min after a 25 g intravenous glucose bolus, was measured in 288 normoglycemic individuals (12).

Genotyping

Genotypes in the GWAS were generated on the Affymetrix 6.0 Human SNP Array (Affymetrix, Santa Clara, CA) using the BIRDSEED algorithm, as described previously (10). SNPs were excluded if $>$ 15% of genotype calls were missing, if genotype frequencies diverged from Hardy–Weinberg expectations ($P < 0.001$), if concordance among 100 duplicate samples was $<$ 97%, or if minor allele frequency was

$<$ 5%. Thus 453,654 SNPs were analyzed. Supplemental Fig. 1 shows the selection of SNPs in different samples.

Genotyping in replication studies was performed by BeadXpress (Illumina, San Diego, CA) or Assays-on-Demand (Applied Biosystems, Carlsbad, CA) according to manufacturer’s protocol. To confirm accuracy of initial GWAS genotypes, all GWAS participants were retyped for each SNP in replication studies, and these genotypes were used in subsequent analyses. Forty-five SNPs with large differences in allele frequency between American Indian and European populations (13) were genotyped for estimation of the proportion of European heritage, utilizing the method of Hanis et al. (14), for use as a covariate.

Statistical Analysis

Association between young-onset T2DM and each SNP in the GWAS was analyzed by logistic regression under an “additive” model in which a numeric variable (0,1,2) is assigned based on the number of referent alleles. A class D regressive model was used to account for resemblance among siblings by including sample prevalence of young-onset diabetes among siblings as a covariate (15). Genomic control (16) was used to account for inflation of significance due to additional population stratification; the inflation parameter was calculated as the mean χ^2 statistic among all SNPs.

A family-based test of association among siblings discordant for diabetes was conducted by conditional logistic regression. To augment statistical power, the P value was calculated by combining the P value from conditional logistic regression (P_{sib}) with a truncated 1-sided P value from the case-control analysis (P_{CC1}). The family-based P value is thus taken from $\chi^2 = -2 \cdot \ln(P_{\text{sib}}) - 2 \cdot \ln(\max\{P_{\text{sib}}, [1 - (1 - P_{\text{CC1}})^2]\})$ on four df. This enhances power of the family-based test while maintaining robustness to population stratification (17).

Associations with diabetes in replication studies and in the pooled combined data were examined by logistic regression, fit by the generalized estimating equation procedure to account for dependence among siblings. Association with continuous variables was analyzed similarly with a linear “mixed” model. Values were logarithmically transformed, and the regression coefficient was exponentiated to obtain the effect estimate expressed a multiplier. The logarithms of homeostasis model assessment values were standardized within different insulin assays; effect sizes are presented in SD units. All P values presented are two-sided. To compare Pima results with those in Europeans, publicly available results were obtained from the DIAbetes Genetics Replication And Meta-analysis (DIAGRAM) consortium (1). Heterogeneity between Pimas and Europeans was analyzed by the Q -statistic.

Functional Studies

Overexpression and knockdown of *DNER* were assessed in murine pancreatic β -cells (NIT1). For overexpression,

cells were transfected with 2 μg of pCMV6 expression vector (OriGene, Rockville, MD) containing *DNER* insert or empty vector control using Lipofectamine LTX (Life Technologies, Carlsbad, CA). For knockdown studies, cells were transfected with 150 pmol small interfering RNA (siRNA) targeting *DNER* or negative control siRNA using Lipofectamine RNAiMAX. Cells were harvested after 48 h incubation, and total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA). Residual genomic DNA was eliminated by on-column DNAase digestion. First strand cDNA was synthesized using the Advantage RT-for-PCR kit (Clontech, Mountain View, CA). mRNA levels were quantified by real-time PCR using SYBR green on an ABI 7900HT-Fast RT-PCR System (Life Technologies). Relative mRNA level was taken as the ratio of experimental data to control. Data were averaged from 7–8 transfection experiments.

RESULTS

GWAS

Among the 453,654 SNPs, the genomic inflation factor was 1.13 for the case-control test and 1.02 for the family-based conditional logistic regression test. Results of the GWAS are shown in Fig. 1. There were 260 SNPs with

$P < 0.0003$ in the family-based analysis and 242 SNPs with $P < 0.0005$ in the case-control analysis (P value thresholds were taken to select ~ 250 SNPs); three SNPs met both criteria. Among these 499 SNPs, 319 tags were selected for genotyping in replication studies (with $r^2 > 0.95$ taken as indicative of redundancy).

Replication Studies

Results for replication studies are shown in Supplementary Table 2. Among 311 SNPs genotyped in the first replication sample, 41 showed directionally consistent association with $P < 0.10$. Ninety-six of the 311 SNPs, constituting 26 SNPs with $P < 0.001$ in the combined GWAS and first replication set and 70 “candidate” SNPs, were also genotyped in the second replication sample. The 20 SNPs with the strongest associations in all 7,674 individuals are shown in Table 1. Two SNPs, rs8181588 in *KCNQ1* and rs1861612 in *DNER*, showed consistent and statistically strong ($P < 5 \times 10^{-7}$) evidence for association across all samples. The *KCNQ1* SNP tags rs2283228 ($r^2 = 0.98$), a previously established marker for T2DM (4). The *DNER* SNP has not been reported in other studies of T2DM, and the effect of rs1861612 is significantly different between Europeans and Pimas ($P = 1.6 \times 10^{-6}$; $I^2 = 95.6\%$).

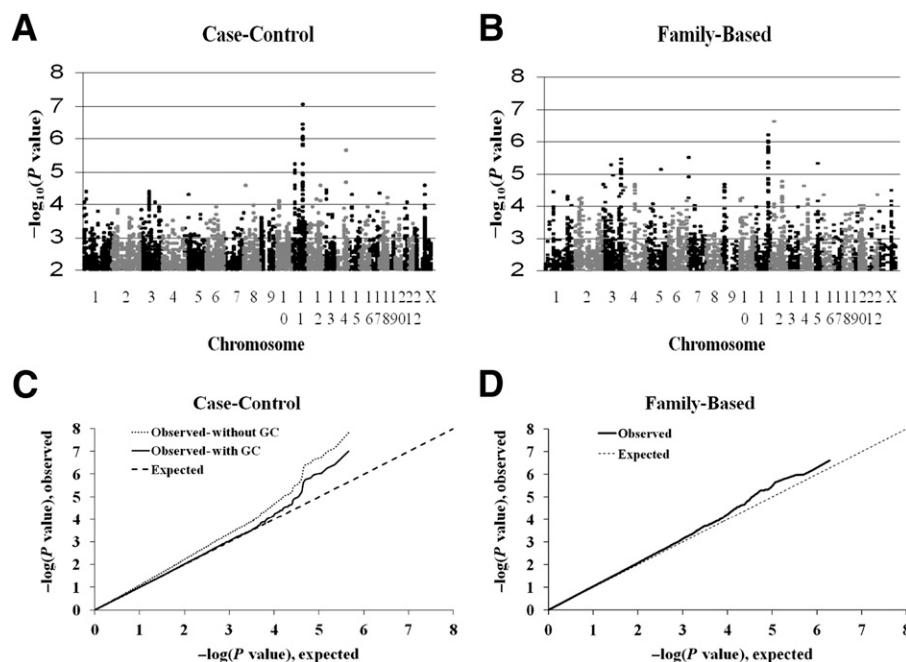


Figure 1—A: “Manhattan” plot of genome-wide association results for young-onset T2DM in American Indians in case-control study. The negative base 10 logarithm of the P value for an association with diabetes is plotted against chromosome and position (determined in Build 37). Results are shown after genomic control. B: “Manhattan” plot of genome-wide association results for young-onset T2DM in American Indians in family-based analysis. The negative base 10 logarithm of the P value for an association with diabetes is plotted against chromosome and position (determined in Build 37). C: Quantile–quantile plot of observed vs. expected (given the null distribution) P value for the case-control study. The observed distribution of P values without genomic control is shown in the dotted line, and the distribution with genomic control is shown in the solid line. D: Quantile–quantile plot of observed vs. expected (given the null distribution) P value for the family-based study. The expected distribution was estimated from simulation of 10^8 test statistics with the observed correlation ($r = 0.29$) between those for the conditional logistic regression and case-control analyses. GC, genomic control.

Table 1—Twenty SNPs with the strongest association with T2DM diabetes in all American Indian participants

SNP	Gene	Ch	Mb	Als	Freq	GWAS						Replication								
						Case-control			Family-based			Set 1		Set 2		All participants		Europeans		
						OR	P value	OR	P value	OR	P value	P value	OR	P value	OR	P value	OR	P value	OR	P value
						OR	P value	OR	P value	OR	P value	P value	OR	P value	OR	P value	OR	P value	OR	P value
rs10909855	ACTRT2	1	2.91	T/C	0.38	1.76	1.4 × 10 ⁻⁴	1.56	0.0095	1.12	0.1425	1.17	0.0282	1.19	1.2 × 10 ⁻⁴	0.14	1.07	0.3013		
rs10429895	LOC440704	1	190.66	T/C	0.75	1.54	0.0056	2.44	5.2 × 10 ⁻⁵	1.18	0.0641	1.05	0.5537	1.19	4.7 × 10 ⁻⁴	0.28	1.00	0.7695		
rs10930939	STAM2	2	152.98	T/C	0.52	1.66	2.6 × 10 ⁻⁴	1.12	0.6490	1.20	0.0124	1.06	0.4829	1.16	9.4 × 10 ⁻⁴	0.25	1.00	0.7901		
rs1861612	DNER	2	230.52	T/C	0.64	1.64	5.0 × 10 ⁻⁴	1.19	0.5332	1.28	0.0016	1.28	0.0016	1.29	6.6 × 10⁻⁸	0.57	0.99	0.6358		
rs6774908	C3orf58	3	143.88	C/T	0.61	1.36	0.0290	2.13	2.1 × 10 ⁻⁴	1.19	0.0256	1.12	0.1354	1.17	6.0 × 10 ⁻⁴	0.56	1.00	0.7901		
rs1861839	MECOM	3	169.26	A/G	0.57	1.65	3.6 × 10 ⁻⁴	1.59	0.0027	1.13	0.1084	1.08	0.2635	1.16	0.0012	0.13	1.08	0.0743		
rs7649407	LPP	3	188.15	A/G	0.88	2.40	3.1 × 10 ⁻⁴	1.20	0.7060	1.31	0.0239	1.13	0.2355	1.28	4.8 × 10 ⁻⁴	0.71	1.05	0.1448		
rs33445	ITGA2	5	52.28	G/A	0.54	1.44	0.0105	1.91	1.4 × 10 ⁻⁴	1.16	0.0305	1.08	0.3341	1.15	0.0018	0.43	0.96	0.1064		
rs6901022	KIF6	6	39.31	A/C	0.61	1.47	7.2 × 10 ⁻⁴	1.94	2.1 × 10 ⁻⁴	1.11	0.1309	1.23	0.0056	1.18	2.7 × 10 ⁻⁴	0.65	1.00	0.9680		
rs4720763	ICA1	7	8.17	C/T	0.18	1.45	0.0294	3.40	1.4 × 10 ⁻⁵	1.28	0.0154	1.09	0.3536	1.21	9.0 × 10 ⁻⁴	0.55	1.03	0.4252		
rs832234*	PTPRD	9	10.06	T/A	0.37	1.70	3.5 × 10 ⁻⁴	1.19	0.4993	1.09	0.2808	1.16	0.0767	1.16	0.0020	0.15	—	—		
rs590855	MAP3K8	10	30.76	C/T	0.16	2.08	2.4 × 10 ⁻⁴	1.59	0.0487	1.07	0.4777	1.25	0.0331	1.24	1.8 × 10 ⁻⁴	0.12	1.00	0.8766		
rs11187815	PLCE1	10	95.97	G/A	0.28	1.50	0.0072	2.05	2.0 × 10 ⁻⁴	1.26	0.0048	0.96	0.6038	1.17	0.0013	0.45	1.06	0.0991		
rs8181588†	KCNQ1	11	2.83	A/G	0.48	1.36	0.0294	2.03	1.1 × 10 ⁻⁴	1.25	0.0023	1.30	0.0003	1.30	5.3 × 10⁻⁹	0.97	1.11	0.1165		
rs4310643	RAB38	11	87.68	C/T	0.80	1.89	4.1 × 10 ⁻⁴	1.45	0.0970	1.14	0.1310	1.09	0.3421	1.21	7.1 × 10 ⁻⁴	0.85	0.99	0.7941		
rs12296975	FGD4	12	32.71	G/A	0.41	1.54	0.0045	3.13	2.0 × 10 ⁻⁷	1.05	0.4984	1.13	0.1054	1.15	0.0021	0.39	1.05	0.2873		
rs659964‡	ACAD10	12	112.13	G/C	0.22	2.12	2.8 × 10 ⁻⁵	1.23	0.4575	1.12	0.2336	1.18	0.0638	1.18	0.0019	0.20	0.90	0.0457		
rs2434072	TBX3	12	114.99	T/C	0.42	1.63	4.6 × 10 ⁻⁴	1.16	0.5772	1.14	0.0849	1.04	0.6227	1.15	0.0018	0.52	1.01	0.8167		
rs523656	STARD13	13	33.72	A/G	0.45	1.78	3.9 × 10 ⁻⁵	1.32	0.1322	1.09	0.2979	1.10	0.2186	1.18	5.0 × 10 ⁻⁴	0.49	1.12	1.2 × 10 ⁻⁴		
rs17756627§	RAD51B	14	69.09	T/A	0.50	1.63	4.6 × 10 ⁻⁴	1.39	0.7431	1.10	0.2285	1.08	0.2747	1.17	0.0003	0.19	1.02	0.5437		

The nearest gene is listed, and the position (in Mb) is given from Build 37. Odds ratios are given per copy of this risk allele. Results for the GWAS analyses are adjusted for sex; results for replication sets 1 and 2 and all participants are adjusted for age, sex, birth year, and heritage. Associations with genome-wide statistical significance ($P < 5 \times 10^{-7}$ [10]) are shown in bold. Associations in Europeans, obtained from publicly available results from the DIAGRAM study (1) are shown for comparison; European allele frequencies are from the HapMap CEU population. CH, chromosome; Als, the alleles with the risk allele (in the GWAS case-control study) listed first; Freq, frequency of the risk allele; OR, odds ratio. *The PTPRD SNP rs832234 is not highly concordant with the established SNP rs17584499 ($r^2 = 0.01$ in Pimas). †Results for replication set 2 and all participants are given for rs2283228 ($r^2 = 0.98$ with rs8181588). ‡Results for ACAD10 SNPs were reported previously (25). §Results for replication set 2 and all participants are given for rs4899250 ($r^2 = 0.96$ with rs17756627).

Table 2—Associations of GWAS-derived SNPs with quantitative metabolic constituents of T2DM

SNP	Gene	Ch	Mb	A/G	Freq	BMI		HOMA-IR		HOMA-B		Percentage body fat		Insulin sensitivity (M)		Insulin secretion (AIR)	
						Eff	P value	Eff	P value	Eff	P value	Eff	P value	Eff	P value	Eff	P value
rs10909855	ACTR12	1	2.91	T/C	0.38	0.998	0.7719	0.002	0.8755	-0.012	0.3924	1.022	0.2226	1.006	0.6991	0.909	0.0757
rs10429885	LOC440704	1	190.66	T/C	0.75	1.002	0.6076	0.049	0.0026	0.026	0.0786	1.019	0.3662	0.963	0.0465	0.954	0.4276
rs10930939	STAM2	2	152.98	T/C	0.52	1.014	0.0008	-0.008	0.6030	-0.002	0.8876	1.040	0.0238	0.995	0.7452	0.965	0.4811
rs1861612*	DNER	2	230.52	T/C	0.64	1.001	0.8413	0.038	0.0119	0.019	0.1474	1.011	0.5420	0.991	0.5872	1.075	0.1667
rs6774908	C3orf58	3	143.88	C/T	0.61	0.997	0.4736	-0.007	0.6309	-0.006	0.6260	0.990	0.6016	0.976	0.1437	0.976	0.6460
rs1861839	MECOM	3	169.26	A/G	0.57	1.003	0.4194	0.011	0.4692	-0.005	0.7065	0.986	0.4286	1.000	0.9951	1.027	0.6050
rs7649407	LPP	3	188.15	A/G	0.88	1.007	0.2499	-0.023	0.2964	-0.042	0.0307	1.008	0.7655	0.980	0.3862	1.002	0.9839
rs33445	JTGA2	5	52.28	G/A	0.54	1.002	0.7035	0.011	0.4349	-0.002	0.8966	0.996	0.8353	0.969	0.0474	0.993	0.8926
rs6901022	KIF6	6	39.31	A/C	0.61	1.008	0.0742	-0.005	0.7242	-0.005	0.6845	1.004	0.8219	0.998	0.8983	1.087	0.0961
rs4720763	ICA1	7	8.17	C/T	0.18	1.005	0.3854	0.034	0.0685	-0.003	0.8496	1.009	0.7128	0.967	0.1156	1.005	0.9369
rs832234	PTPRD	9	10.06	T/A	0.37	1.000	0.9723	0.002	0.8984	-0.000	0.9995	0.991	0.6133	1.011	0.5010	0.964	0.5217
rs590855	MAP3K8	10	30.76	C/T	0.16	1.002	0.7147	0.026	0.1965	0.025	0.1666	1.014	0.5838	0.990	0.6490	1.102	0.1899
rs1187815	PLCE1	10	95.97	G/A	0.28	1.002	0.6734	-0.004	0.8248	-0.010	0.4834	0.998	0.9068	0.980	0.2157	0.939	0.2214
rs2283228	KCNQ1	11	2.83	A/C	0.48	0.983	4.0 × 10⁻⁵	-0.001	0.9671	-0.020	0.1406	0.977	0.1764	1.013	0.4053	0.863	0.0038
rs4310643	RAB38	11	87.68	C/T	0.80	1.008	0.1507	-0.007	0.7164	0.001	0.9483	0.996	0.8407	1.016	0.4226	0.869	0.0217
rs12296975	FGD4	12	32.71	G/A	0.41	0.996	0.3056	-0.022	0.1437	-0.011	0.4194	0.998	0.9147	0.996	0.8501	1.040	0.5052
rs659964	ACAD10	12	112.13	G/C	0.22	0.998	0.7034	-0.006	0.7457	-0.010	0.5252	0.986	0.5234	0.978	0.2463	1.134	0.0622
rs2434072	TBX3	12	114.99	T/C	0.42	1.008	0.0728	0.017	0.2519	0.003	0.8472	0.996	0.8120	0.981	0.2417	1.004	0.9422
rs523656	STARD13	13	33.72	A/G	0.45	1.006	0.1360	0.009	0.5615	0.011	0.4128	1.003	0.8698	0.982	0.3123	1.052	0.4045
rs4899250	RAD51B	14	69.09	T/C	0.49	1.006	0.1816	0.000	0.9782	-0.005	0.7090	1.005	0.7880	0.984	0.3188	1.005	0.9232

The nearest gene is listed, and the position (in Mb) is given from Build 37. The effect is given per copy of the risk allele. For BMI, percentage of body fat, insulin sensitivity, and insulin secretion the effect is expressed as a multiplier. For HOMA-IR and HOMA-B the effect is expressed as the difference in SD units per copy of the risk allele. Results for BMI are adjusted for age, sex, birth year, and heritage. Results for HOMA-IR and HOMA-B are adjusted for age, sex, birth year, heritage, and BMI. Results for percentage of body fat are adjusted for age, sex, and heritage. Results for M are adjusted for age, sex, heritage, and percentage of body fat. Results for acute insulin response are adjusted for age, sex, heritage, percentage of body fat, and M. Normally significant results ($P < 0.05$) are shown in bold. Ch, chromosome; A/G, the alleles with the diabetes risk allele listed first; Freq, frequency of the risk allele; Eff, effect; AIR, acute insulin response. *The association of rs1861612 with fasting serum insulin is effect = 0.040 SD; $P = 0.0158$.

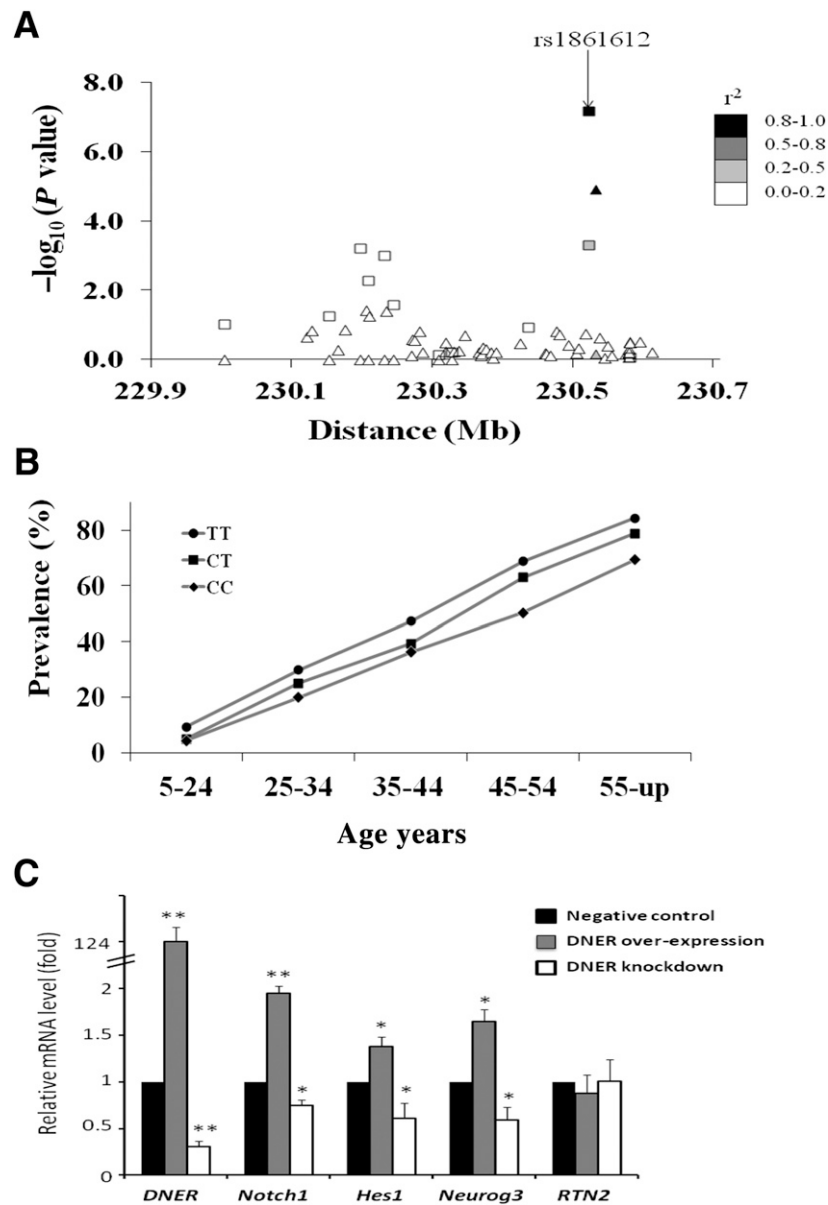


Figure 2—A: Association results for 63 variants across *DNER* with T2DM in Pima Indians. The negative base 10 logarithm of the *P* value for association is shown at the Build 37 position. Variants include 48 “tags” ($r^2 > 0.8$) for 153 SNPs from the GWAS and 14 variants identified from sequencing ~5,300 base pairs in *DNER*; one variant identified by sequencing (a short insertion/deletion at position 230.580216 Mb) is not in public databases. Results obtained in all 7,674 individuals are shown as boxes, while those obtained in the GWAS and first replication samples are shown as triangles. Symbols are shaded according to r^2 with rs1861612. B: The prevalence of T2DM by genotype at rs1861612 and age-group in 7,674 Pima Indians. *P* values for association with T2DM in each age-group are as follows: $P = 0.0005$ (5–24), $P = 0.0517$ (25–34), $P = 0.0265$ (35–44), $P = 0.0004$ (45–54), and $P = 0.0028$ (55 and up). C: Relative mRNA level in murine pancreatic β -cells after transfection experiments to overexpress *DNER* (gray bars) or to knockdown *DNER* expression (open bars) compared with negative control (black bars, which = 1 by definition). mRNA levels are shown for *DNER* itself, for notch pathway genes (*Notch1*, *Hes1*, *Neurog3*) and for *Rtn2* (which is not involved in the notch pathway). * $P < 0.001$; ** $P < 0.0001$.

Associations With Metabolic Traits

Associations with metabolic traits are shown in Table 2. The diabetes risk allele at rs2283228 in *KCNQ1* was associated with lower insulin secretion (by 13.7% per copy of the risk allele; $P = 0.0038$). The diabetes risk allele at rs1861612 in *DNER* was associated with increased HOMA-IR and increased fasting serum insulin (~0.04 SD per copy of the risk allele; $P = 0.0119$).

Fine Mapping and Functional Studies

Sixty-three variants in *DNER* were selected from the GWAS and from direct sequencing of exons and ~2 kb of the promoter region in 12 diabetic and 12 nondiabetic Pimas. Results for association with diabetes are shown in Fig. 2A. The initial GWAS SNP, rs1861612, was the most strongly associated variant. The association was consistent across age-groups (Fig. 2B).

To investigate the role of *DNER* in regulating the notch signaling pathway, *DNER* mRNA was overexpressed (124-fold enrichment) and knocked down via siRNA (69% reduction) in murine β -cells, and expression levels of four genes were measured. The mRNA of notch pathway-specific genes *Notch1*, *Hes1*, and *Neurog3* were increased by 2.0-, 1.4-, and 1.6-fold, respectively ($P < 0.001$), in response to *DNER* overexpression and decreased by 25, 40, and 41% ($P < 0.001$) in response to *DNER* knockdown (Fig. 2C). In contrast, *Rtn2*, which is not involved in the notch pathway, was largely unaffected.

DISCUSSION

A number of genetic variants have recently been identified as associated with T2DM (1–6). Most of these variants were identified in GWASs in Europeans, but associations for many are consistent in other ethnic groups, including American Indians (18,19). However, some associations are heterogeneous across ethnic groups (5,6,20). In Pima Indians, for example, *TCF7L2* variants, which are strongly associated in most ethnic groups, show little association with diabetes (20). In addition because of ethnic differences in allele frequencies, relative importance of different diabetes-susceptibility alleles varies. For these reasons, GWASs in non-European populations might yield additional T2DM susceptibility variants. Indeed, studies in East Asians and South Asians have identified additional diabetes associations (4–6).

There have been few GWASs in American Indians despite their high risk for T2DM. In the current study, we extend our initial GWAS in Pima Indians (7) to include 453,654 SNPs. We also conduct additional replication studies in the population, so associations were ultimately assessed in 7,674 individuals. These studies implicate *DNER* as a novel locus conferring susceptibility to T2DM. The association with rs1861612 in *DNER* is reproducible across different groups of Pimas but is not observed in Europeans. Other ethnic-specific genetic associations for T2DM have been described (5,6). Such associations may occur due to difference in frequency of functional alleles, differences in linkage disequilibrium, or interaction with other genetic or environmental factors. Studies of *DNER* polymorphisms in other populations are required to determine generalizability.

Stringent criteria for statistical significance are generally applied in GWASs on account of multiple statistical testing. In non-African populations, $P < 5 \times 10^{-8}$ is conventionally considered genome-wide significance. In the current study, only a variant in the established diabetes gene *KCNQ1* achieved this threshold. Associations with *KCNQ1* variants, which are subject to parent-of-origin effects, are particularly strong in Pimas (21). Conventional criteria for genome-wide significance were derived from estimates of the number of effectively independent common variants in European and East Asian

populations (22,23), and this number is likely smaller in Pimas. In fact, we estimate that $P < 5 \times 10^{-7}$ is an appropriate level for genome-wide statistical significance in Pimas (10), and the *DNER* association achieves this criterion. Although many of the other established T2DM-susceptibility loci appear to influence diabetes risk in Pimas (19), their effects are modest and difficult to detect at genome-wide significance with the current sample. The present GWAS was small and had little power in itself to detect associations at genome-wide significance.

The mechanism by which variation in *DNER* might cause T2DM is not clear. *DNER* encodes a Δ /notch-like epidermal-growth-factor-related receptor, and it mediates notch signaling. We show that *DNER* expression affects expression of several notch pathway genes in pancreatic β -cells. The diabetes risk allele at rs1861612 was associated with fasting hyperinsulinemia and elevated HOMA-IR, but there was no association with directly measured insulin resistance. A speculative mechanism is that *DNER*-mediated alterations in notch signaling may produce fasting hyperinsulinemia, which increases risk of diabetes independently of insulin resistance (24), but further mechanistic studies are required. Regardless of the mechanism, the current study implicates *DNER* as a T2DM-susceptibility gene in American Indians.

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References

- Zeggini E, Scott LJ, Saxena R, et al.; Wellcome Trust Case Control Consortium. Meta-analysis of genome-wide association data and large-scale replication identifies additional susceptibility loci for type 2 diabetes. *Nat Genet* 2008;40:638–645
- Voight BF, Scott LJ, Steinthorsdottir V, et al.; MAGIC investigators; GIANT Consortium. Twelve type 2 diabetes susceptibility loci identified through large-scale association analysis. *Nat Genet* 2010;42:579–589
- Morris AP, Voight BF, Teslovich TM, et al.; Wellcome Trust Case Control Consortium; Meta-Analyses of Glucose and Insulin-related traits

- Consortium (MAGIC) Investigators; Genetic Investigation of ANthropometric Traits (GIANT) Consortium; Asian Genetic Epidemiology Network–Type 2 Diabetes (AGEN-T2D) Consortium; South Asian Type 2 Diabetes (SAT2D) Consortium; DIAbetes Genetics Replication And Meta-analysis (DIAGRAM) Consortium. Large-scale association analysis provides insights into the genetic architecture and pathophysiology of type 2 diabetes. *Nat Genet* 2012; 44:981–990
4. Unoki H, Takahashi A, Kawaguchi T, et al. SNPs in *KCNQ1* are associated with susceptibility to type 2 diabetes in East Asian and European populations. *Nat Genet* 2008;40:1098–1102
 5. Cho YS, Chen CH, Hu C, et al.; DIAGRAM Consortium; MuTHER Consortium. Meta-analysis of genome-wide association studies identifies eight new loci for type 2 diabetes in east Asians. *Nat Genet* 2012;44:67–72
 6. Saxena R, Saleheen D, Been LF, et al.; DIAGRAM; MuTHER; AGEN. Genomewide association study identifies a novel locus contributing to type 2 diabetes susceptibility in Sikhs of Punjabi origin from India. *Diabetes* 2013; 62:1746–1755
 7. Hanson RL, Bogardus C, Duggan D, et al. A search for variants associated with young-onset type 2 diabetes in American Indians in a 100K genotyping array. *Diabetes* 2007;56:3045–3052
 8. Knowler WC, Pettitt DJ, Saad MF, Bennett PH. Diabetes mellitus in the Pima Indians: incidence, risk factors and pathogenesis. *Diabetes Metab Rev* 1990;6:1–27
 9. American Diabetes Association Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 1997;20: 1183–1197
 10. Malhotra A, Kobes S, Knowler WC, Baier LJ, Bogardus C, Hanson RL. A genome-wide association study of BMI in American Indians. *Obesity (Silver Spring)* 2011;19:2102–2106
 11. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985;28:412–419
 12. Bunt JC, Krakoff J, Ortega E, Knowler WC, Bogardus C. Acute insulin response is an independent predictor of type 2 diabetes mellitus in individuals with both normal fasting and 2-h plasma glucose concentrations. *Diabetes Metab Res Rev* 2007;23:304–310
 13. Tian C, Hinds DA, Shigeta R, et al. A genomewide single-nucleotide-polymorphism panel for Mexican American admixture mapping. *Am J Hum Genet* 2007;80:1014–1023
 14. Hanis CL, Chakraborty R, Ferrell RE, Schull WJ. Individual admixture estimates: disease associations and individual risk of diabetes and gall-bladder disease among Mexican-Americans in Starr County, Texas. *Am J Phys Anthropol* 1986;70:433–441
 15. Schnell AH, Karunaratne PM, Witte JS, Dawson DV, Elston RC. Modeling age of onset and residual familial correlations for the linkage analysis of bipolar disorder. *Genet Epidemiol* 1997;14:675–680
 16. Devlin B, Bacanu SA, Roeder K. Genomic control to the extreme. *Nat Genet* 2004;36:1129–1130; author reply 1131
 17. Hanson RL, Knowler WC. Design and analysis of genetic association studies to finely map a locus identified by linkage analysis: assessment of the extent to which an association can account for the linkage. *Ann Hum Genet* 2008;72:126–139
 18. Haiman CA, Fesinmeyer MD, Spencer KL, et al. Consistent directions of effect for established type 2 diabetes risk variants across populations: the Population Architecture Using Genomics and Epidemiology (PAGE) Consortium. *Diabetes* 2012;61:1642–1647
 19. Rong R, Hanson RL, Ortiz D, et al. 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. *Diabetes* 2009;58:478–488
 20. Guo T, Hanson RL, Traurig M, et al. *TCF7L2* is not a major susceptibility gene for type 2 diabetes in Pima Indians: analysis of 3,501 individuals. *Diabetes* 2007;56:3082–3088
 21. Hanson RL, Guo T, Muller YL, et al. Strong parent-of-origin effects in the association of *KCNQ1* variants with type 2 diabetes in American Indians. *Diabetes* 2013;62:2984–2991
 22. Dudbridge F, Gusnanto A. Estimation of significance thresholds for genomewide association scans. *Genet Epidemiol* 2008;32:227–234
 23. Hoggart CJ, Clark TG, De Iorio M, Whittaker JC, Balding DJ. Genome-wide significance for dense SNP and resequencing data. *Genet Epidemiol* 2008; 32:179–185
 24. Weyer C, Hanson RL, Tataranni PA, Bogardus C, Pratley RE. A high fasting plasma insulin concentration predicts type 2 diabetes independent of insulin resistance: evidence for a pathogenic role of relative hyperinsulinemia. *Diabetes* 2000;49:2094–2101
 25. Bian L, Hanson RL, Muller YL, et al.; MAGIC Investigators. Variants in *ACAD10* are associated with type 2 diabetes, insulin resistance and lipid oxidation in Pima Indians. *Diabetologia* 2010;53:1349–1353