*Brief Genetics Report*

# *CHRM3* Gene Variation Is Associated With Decreased Acute Insulin Secretion and Increased Risk for Early-Onset Type 2 Diabetes in Pima Indians

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**The muscarinic acetylcholine receptor subtype M3 (*CHRM3*) gene is expressed in islet β-cells and has a role in stimulating insulin secretion; therefore, *CHRM3* was analyzed as a candidate gene for type 2 diabetes in Pima Indians. Ten variants were genotyped in a family-based sample ($n$ = 1,037), and 1 variant (rs3738435) located in the 5′ untranslated region of an alternative transcript was found to be modestly associated with both early-onset type 2 diabetes and the acute insulin response in a small subset of these subjects. To better assess whether this variant has a role in acute insulin secretion, which could affect risk for early-onset type 2 diabetes, rs3738435 was genotyped in a larger group of normal glucose-tolerant Pima Indians who had measures of acute insulin secretion ($n$ = 282) and a larger case-control group of Pima Indians selected for early-onset type 2 diabetes ($n$ = 348 case subjects with age of onset <25 years; $n$ = 392 nondiabetic control subjects aged >45 years). Genotyping in these larger sets of subjects confirmed that the C allele of rs3738435 was associated with a reduced acute insulin response (adjusted $P$ = 0.00006) and was also modestly associated with increased risk of early-onset type 2 diabetes (adjusted $P$ = 0.02).**
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Muscarinic acetylcholine (mAch) is a major neurotransmitter in the central and peripheral parasympathetic nervous systems. There are five molecularly distinct plasma membrane receptors (M1–M5) for mAch that play different roles in mediating the neurotransmitter's diverse physiological actions. mAch receptor 3 (M3) is expressed throughout the central nervous system and appears to be the predominant muscarinic receptor subtype that is functional in pancreatic β-cells (1–7). The M3 receptor is involved in several signal transduction pathways that may have important roles in obesity or type 2 diabetes. For example, M3 is involved in the central control of appetite and body weight by interfering with hypothalamic leptin signaling and the proopiomelanocortin/agouti-related protein and melanin-concentrating hormone systems. Increases in brain stem M3 receptors may activate pancreatic M3 receptors, resulting in insulin secretion and pancreatic regeneration (8). β-Cell M3 receptors are coupled to phospholipase C activation, and failure to activate this integral enzyme results in the failure to release insulin (5).

Using gene-targeting techniques, several labs have generated mutant mouse lines deficient in each of the five mAch receptor genes; among them, the M3-deficient ($M3^{-/-}$) mice have a normal life span, are hypophagic and lean, and also display a blunted insulin secretion compared with wild-type ($M3^{+/+}$) mice (6,7,9). Recently, pancreatic β-cell–specific M3 receptor knockout (β-M3-KO) mice have also been generated (10). These targeted β-cell knockout mice exhibited impaired glucose tolerance and greatly reduced insulin release. Conversely, transgenic mice overexpressing targeted β-cell M3 receptors exhibited an increase in glucose tolerance and insulin release (10). These findings suggest that β-cell M3 muscarinic receptors have an important role in maintaining appropriate insulin release and glucose homeostasis in mice (10). The human M3 receptor gene (*CHRM3*) shares high homology with the mouse M3 receptor gene; however, the relationship between *CHRM3* and glucose homeostasis has not been investigated in humans. Therefore, in this study, *CHRM3* was analyzed as a candidate gene for type 2 diabetes in the Pima Indians of Arizona, a population with the world's highest reported prevalence of type 2 diabetes (11).

## RESEARCH DESIGN AND METHODS

All subjects are part of our ongoing longitudinal study of type 2 diabetes among Pima Indians living in the Gila River Indian Community. Twenty-four Pima subjects with early-onset type 2 diabetes and 23 nondiabetic Pima subjects were selected for sequence analysis of *CHRM3*. Initial genotyping of all single nucleotide polymorphisms (SNPs) was done in a family-based sample of 1,337 Pima Indians (723 subjects with diabetes at any age, which included 219 subjects with an age of onset of diabetes <25 years and 614 nondiabetic subjects) for association analysis of type 2 diabetes and BMI. DNA was isolated from blood collected at our outpatient clinic on the Gila River Indian reservation, and diabetes status was determined by a 75-g oral glucose tolerance test (OGTT) and the results interpreted according to the criteria of the World Health Organization (12). Among the nondiabetic subjects in the family-based study, 322 subjects had been additionally studied as inpatients in our clinical research center and had undergone detailed

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AIR, acute insulin response; LD, linkage disequilibrium; M3, muscarinic acetylcholine receptor 3; mAch, muscarinic acetylcholine; NGT, normal glucose tolerant; OGTT, oral glucose tolerance test; SNP, single nucleotide polymorphism; UTR, untranslated region.

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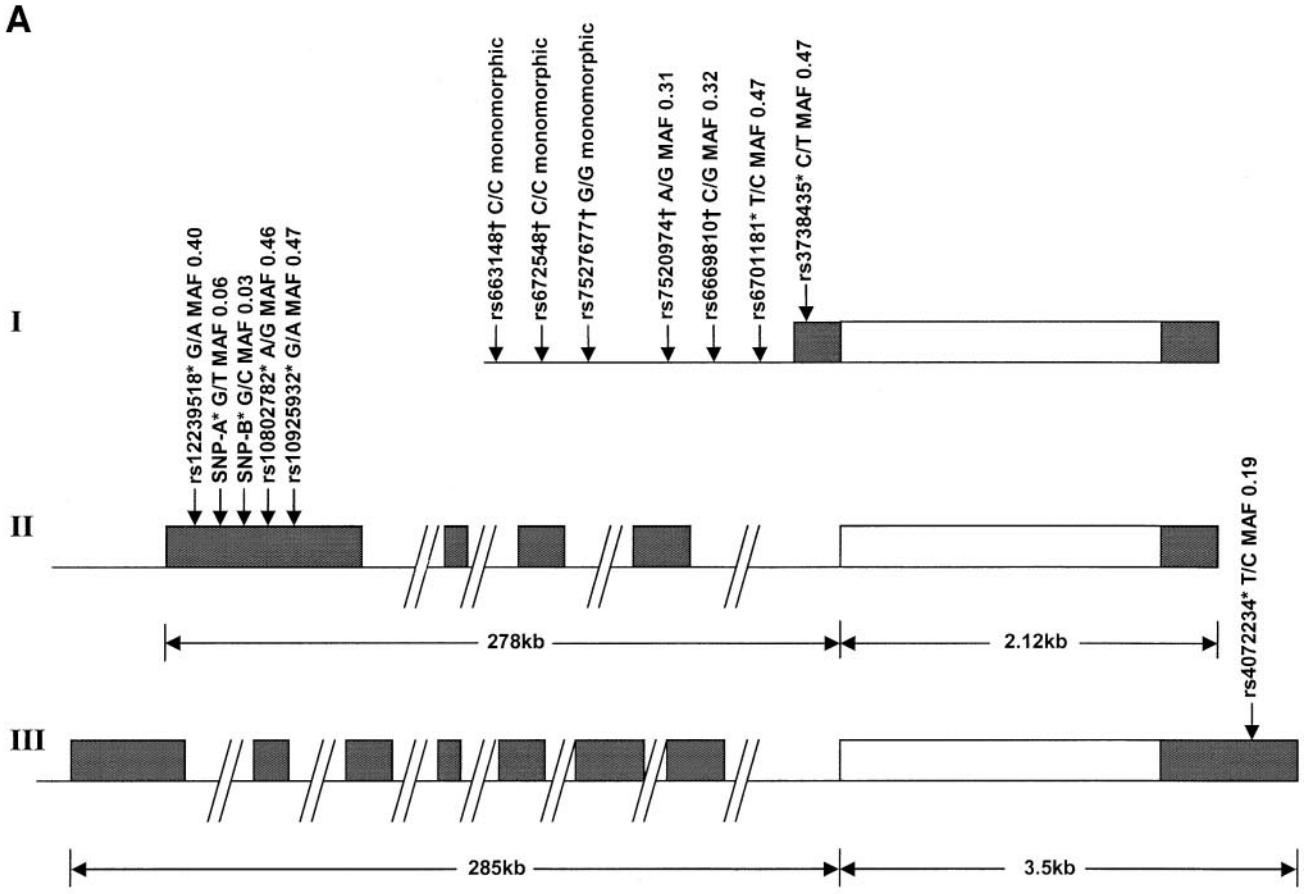


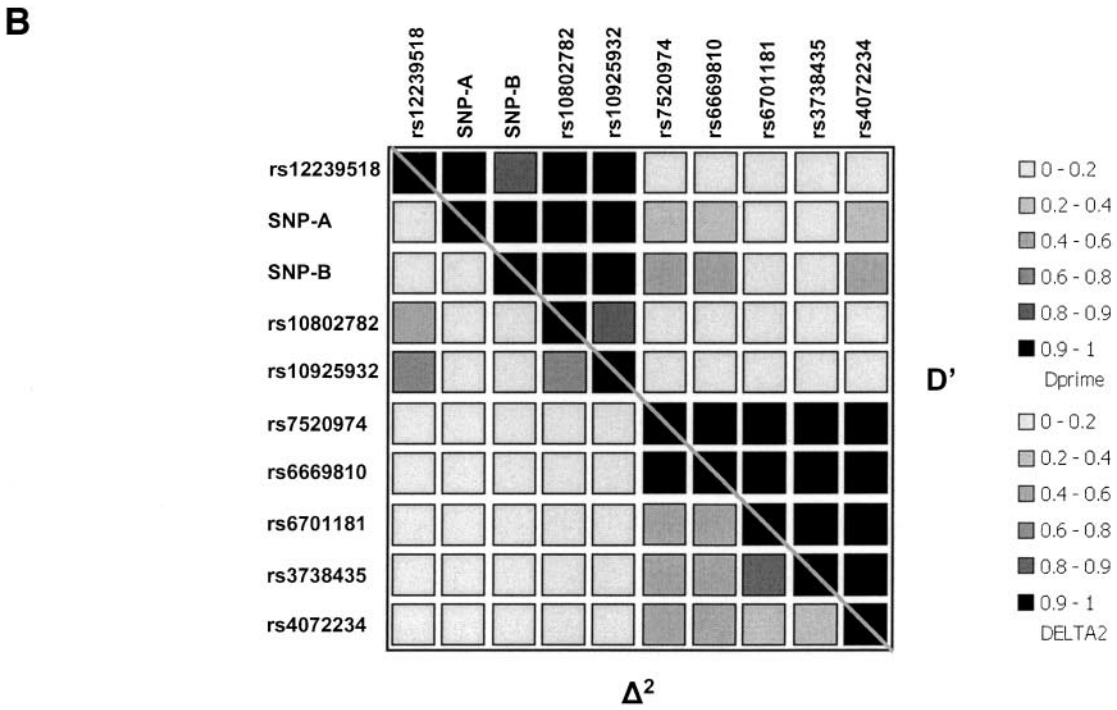


**FIG. 1.** ***A*****: Schematic showing the location of the SNPs in the three** ***CHRM3*** **transcripts. I, ENSG133019 (Ensembl); II, NM_000740 (RefSeq UCSC genome browser); III, published sequence (15). *SNPs identified by direct sequencing. †SNPs selected from the dbSNP database. ■, untranslated exons; □, translated exon.** ***B*****: Pairwise LD of SNPs in Pima Indians. D′ is shown above the diagonal and $\Delta^2$ below the diagonal.**

TABLE 1
Metabolic characteristics of genotypic groups for SNP rs3738435 among 282 NGT full-heritage Pima Indians studied for early insulin secretion

| | Genotype | | | *P* value | | |
|---|---|---|---|---|---|---|
| | C/C | C/T | T/T | Additive | Dominant | Recessive |
| *n* | 91 | 142 | 49 | — | — | — |
| Age (years) | 27 ± 1 | 26 ± 1 | 26 ± 1 | — | — | — |
| Percent body fat | 32 ± 1 | 31 ± 1 | 32 ± 1 | | | |
| Adjusted*†‡ | 32 ± 1 | 32 ± 1 | 31 ± 1 | 0.51 | 0.76 | 0.47 |
| Fasting plasma glucose (mg/dl) | 90 ± 1 | 87 ± 1 | 87 ± 1 | | | |
| Adjusted*†‡§ | 89 ± 1 | 87 ± 1 | 87 ± 1 | **0.02** | 0.40 | **0.008** |
| OGTT 30 min glucose (mg/dl) | 145 ± 3 | 141 ± 2 | 136 ± 3 | | | |
| Adjusted*†‡§ | 145 ± 3 | 141 ± 2 | 136 ± 3 | **0.05** | 0.08 | 0.11 |
| OGTT 60 min glucose (mg/dl) | 142 ± 4 | 140 ± 3 | 126 ± 4 | | | |
| Adjusted*†‡§ | 140 ± 4 | 141 ± 3 | 124 ± 4 | **0.03** | **0.001** | 0.45 |
| OGTT 120 min glucose (mg/dl) | 107 ± 2 | 110 ± 2 | 106 ± 3 | | | |
| Adjusted*†‡§ | 106 ± 2 | 110 ± 2 | 105 ± 3 | 0.92 | 0.20 | 0.34 |
| $Log_{10}$ fasting insulin (μU/ml) | 1.52 ± 0.02 | 1.52 ± 0.02 | 1.51 ± 0.03 | | | |
| Adjusted*†‡§ | 1.52 ± 0.01 | 1.53 ± 0.01 | 1.50 ± 0.02 | 0.78 | 0.32 | 0.62 |
| OGTT $log_{10}$ 30 min insulin (μU/ml) | 2.39 ± 0.03 | 2.31 ± 0.02 | 2.37 ± 0.04 | | | |
| Adjusted*†‡§‖¶ | 2.38 ± 0.02 | 2.30 ± 0.02 | 2.40 ± 0.03 | 0.88 | **0.03** | 0.06 |
| OGTT $log_{10}$ 120 min insulin (μU/ml) | 2.09 ± 0.03 | 2.07 ± 0.03 | 2.02 ± 0.05 | | | |
| Adjusted*†‡§ | 2.08 ± 0.03 | 2.09 ± 0.02 | 2.01 ± 0.05 | 0.28 | 0.14 | 0.75 |
| Adjusted*†‡§‖ | 2.07 ± 0.03 | 2.08 ± 0.02 | 2.05 ± 0.04 | 0.83 | 0.51 | 0.81 |
| $Log_{10}$ glucose disposal rate (mg · kg EMBS$^{-1}$ · min$^{-1}$) | 0.56 ± 0.01 | 0.57 ± 0.01 | 0.60 ± 0.02 | | | |
| Adjusted*†‡§ | 0.56 ± 0.01 | 0.57 ± 0.01 | 0.60 ± 0.02 | **0.02** | **0.05** | 0.10 |
| $Log_{10}$ AIR (μU/ml) (IVGTT) | 2.33 ± 0.03 | 2.34 ± 0.02 | 2.44 ± 0.03 | | | |
| Adjusted*†‡§‖ | 2.33 ± 0.03 | 2.33 ± 0.02 | 2.46 ± 0.03 | **0.02** | **0.00006** | 0.35 |

Data are raw means ± SE and adjusted means ± SE for each trait grouped by genotype. *P* values were calculated for the adjusted means. Significant *P* values (<0.05) are shown in bold. Covariates for adjustments are listed as: *age, †sex, ‡family membership, §percentage of body fat, ‖glucose disposal rate, and ¶30-min glucose levels. IVGTT, intravenous glucose tolerance test. EMBS, estimated metabolic body size.

metabolic phenotyping. Acute insulin secretion is measured only on normal glucose tolerant (NGT) subjects (of the 322 nondiabetic subjects, 181 were NGT). Follow-up genotyping of rs3738435 was done on every available DNA sample from the following two groups of subjects: *1*) full-blooded Pima Indians for whom there are measures of acute insulin secretion ($n$ = 282, all NGT), as assessed by an intravenous glucose tolerance tests in our clinical research center, and *2*) Pima Indians who developed type 2 diabetes before age 25 years ($n$ = 348) or were nondiabetic and at least 45 years of age ($n$ = 392), as determined by an OGTT in our outpatient clinic on the Gila River Indian Reservation. Some of these subjects are siblings.

**Clinical tests.** Nondiabetic subjects admitted to our clinical research center underwent measures of body composition as assessed by dual-energy X-ray absorptiometry (DPX-1; Lunar Radiation, Madison, WI), as well as measures of insulin action as assessed by an OGTT, a hyperinsulinemic-euglycemic clamp (described elsewhere) (13), and measures of first-phase insulin secretory function as assessed in NGT subjects in response to a 25-g intravenous glucose bolus (14). The acute insulin response (AIR) is calculated as the average incremental plasma insulin concentration from the 3rd to the 5th min after the glucose bolus. This study was approved by the institutional review board of the National Institute of Diabetes and Digestive and Kidney Diseases and by the Tribal Council of the Gila River Indian Community. All subjects provided signed informed consent before participation.

**Expression and tissue distribution of alternative transcripts of *CHRM3*.** Primers designed for the major *CHRM3* transcript (NM_000740; RefSeq UCSC genome browser) were used to screen the following cDNA libraries: adipose, hypothalamus, pituitary (BD Marathon-Ready cDNA; BD Bioscience/Clontech, Palo Alto, CA), brain, skeletal muscle, heart, liver, kidney, spleen, pancreas (BD MTC Multiple Tissue cDNA Panels; BD Bioscience/Clontech), and pancreatic islets (kindly provided by Dr. Lorella Marcelli at Joslin Diabetes Center). Additional unique primers for the alternative *CHRM3* transcript (ENSG133019; Ensembl genome browser) were used to screen the islet cDNA library.

**Sequence variant identification and genotyping.** Screening *CHRM3* for SNPs involved sequencing the eight exons of the published sequence (15), five exons based on the RefSeq sequence (NM_000740), and an alternative *CHRM3* transcript (ENSG133019). Genomic DNA was PCR amplified and sequenced using a Big Dye Terminator version 1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). The five SNPs (rs12239518, SNP-A, SNP-B, rs10802782, and rs10925932) located in exon 1 (NM_000740) were genotyped by direct sequencing. SNPs rs3738435 and rs4072234 were genotyped by

TABLE 2
Association of rs3738435 with early-onset type 2 diabetes in Pima Indians

| | Genotype vs. diabetes status | | | | | | |
|---|---|---|---|---|---|---|---|
| | C/C | C/T | T/T | Total | Additive | Dominant | Recessive |
| Early-onset type 2 diabetes | | | | | | | |
| *n* (%) | 88 (25) | 196 (56) | 64 (18) | 348 | | | |
| *P* value | | | | | 0.48 | **0.02** | 0.2 |
| Odds ratio (95% CI) | | | | | 1.07 (0.88–1.3) | 1.44 (1.05–1.98) | 0.82 (0.61–1.1) |
| Nondiabetic subjects | | | | | | | |
| *n* (%) | 105 (27) | 188 (48) | 99 (25) | 392 | | | |

Data are *n* (%) unless otherwise indicated. *P* values were adjusted for sex, Pima heritage, and sibship. Early onset defined as age of onset <25 years. Significant *P* values (<0.05) are shown in bold.

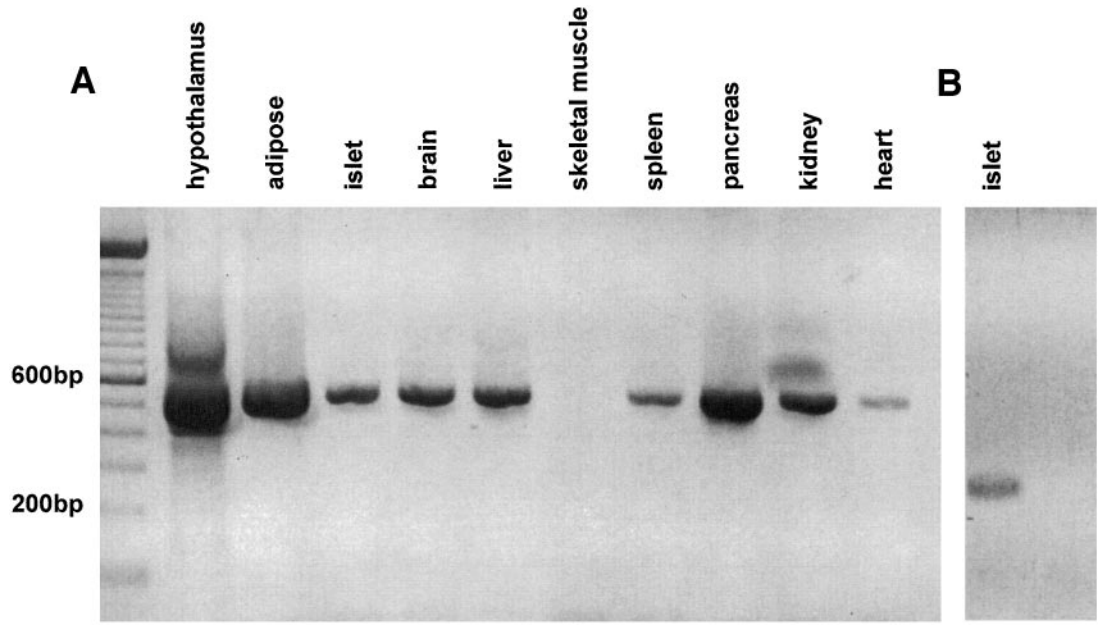


**FIG. 2. *CHRM3* gene expression profile. *A*: Expression of the major transcript (NM_000740; RefSeq UCSC genome browser) among various tissues. *B*: Expression of the alternative transcript containing rs3738435 (ENSG133019; Ensembl) in islets.**

TaqMan allelic discrimination PCR following the manufacture's protocol (Assays-on-Demand SNP Genotyping products; Applied Biosystems). Five additional database SNPs (dbSNP database) located in regions of *CHRM3*, which were not sequenced, were genotyped by SNPLex following the manufacture's protocol (SNPlex Genotyping System 48-plex; Applied Biosystems).

**Statistical analysis.** Statistical analyses were performed using the software of the SAS Institute (Cary, NC). Numeric variables are expressed as means ± SE. For continuous variables, the general estimating equation procedure was used to adjust for the covariates, including family membership, since some subjects were siblings. The association of genotypes with early onset diabetes was assessed by logistic regression model after adjusting for Pima heritage and sex. Plasma insulin concentrations and glucose disposal rates during the physiological concentration of insulin infusion were log transformed before analyses to approximate a normal distribution. For an additive model, 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, 0). The dominant model was defined as contrasting genotypic groups 1/1 + 1/2 vs. 2/2, and the recessive model was defined as contrasting genotypic groups 1/1 vs. 1/2 + 2/2. To examine pairwise linkage disequilibrium (LD), haplotype frequencies were estimated with the EH program, and these haplotype frequencies were used to calculate D′ and Δ (2). Haplotype analysis for the case-control sample was performed using both HaploView (16) and PHASE (version 2.1.1) (17). A $P$ value $<0.05$ was considered statistically significance.

## RESULTS

Sequencing of PCR amplified genomic segments and identified eight SNPs within the various *CHRM3* transcripts (Fig. 1*A*). One SNP (rs3738435) was located in the 5′ untranslated region (UTR) of an alternative transcript (ENSG133019), five SNPs (rs12239518, SNP-A, SNP-B, rs10802792, and rs10925932) were located in exon 1 of the major transcript (NM_000740), and one SNP (rs4072234) was in the 3′ UTR of an alternative published sequence (15). Of the five SNPs located in the first exon, two rare SNPs (SNP-A and SNP-B) appear to be novel, whereas the remaining three common SNPs have been reported in dbSNP (rs12239518, rs10802782, and rs10925932). To obtain better coverage of the noncoding regions of the *CHRM3* locus, five additional database SNPs were selected for analysis. Three of these SNPs were monomorphic in Pima Indians and two were polymorphic (Fig. 1*A*). Pairwise LD among all the SNPs is shown in Fig. 1*B*. The entire *CHRM3* locus was not sequenced; thus, Pima-specific tag SNPs that could capture all the genetic variation across *CHRM3* in this population were not identified.

The 10 polymorphic SNPs in *CHRM3* were initially genotyped in a family-based study of 1,337 Pima Indians (723 with diabetes and 614 without diabetes). All the SNPs were in Hardy-Weinberg equilibrium. None of the SNPs were associated with type 2 diabetes (defined as onset at any age) or BMI (as a continuous trait) in the family-based sample; however, modest associations were observed for SNPs rs3738435 and rs6701181 (SNPs in nearly complete LD, $D' > 0.98$) when subjects with young-onset diabetes (defined as onset age <25 years; $n = 219$) were compared with the oldest subjects without diabetes (at least 45 years of age; $n = 216$). The 10 SNPs were further analyzed for associations with pre-diabetic traits among the nondiabetic subjects who had been metabolically phenotyped. Among 181 NGT subjects, SNPs rs3738435 and rs6701181 were modestly associated with acute insulin secretion as measured by an intravenous glucose tolerance test.

To better assess whether these SNPs are associated with acute insulin secretion and early-onset type 2 diabetes, rs3738435 was genotyped in a larger number of subjects who were selected for being informative for these specific phenotypes. Genotyping of rs3738435 in 282 NGT Pima Indians showed that subjects with a C allele (C/C and C/T) had a significantly lower AIR than subjects without a C allele (T/T) ($P = 0.00006$ under the dominant model adjusted for age, sex, percent body fat, family membership, and insulin action [clamp]) (Table 1). Subjects combined for C/C and C/T genotypes also had a lower mean insulin secretion at 30 min in response to an OGTT than subjects with the T/T genotype ($P = 0.03$ under a dominant model adjusted for age, sex, percent body fat, family membership, insulin action [clamp], and 30 min glucose), but the individual genotypic means underlying this association are not linear; therefore, we are cautious in interpreting this association as confirmatory (Table 1). However, the increased mean glucose level at 60 min

during an OGTT in subjects with C/C and C/T genotypes ($P = 0.001$ under a dominant model adjusted for age, sex, percent body fat, and family membership) is consistent with a reduced early insulin secretion in these subjects. Subjects with a C allele also had a slightly reduced insulin-stimulated glucose uptake rate in response to a hyperinsulinemic-euglycemic clamp, and C/C subjects also had slightly higher fasting plasma glucose levels (Table 1); however, these associations are very modest and would not remain significant after adjustment for multiple testing.

SNP rs3738435 was further genotyped in a larger early-onset diabetes case-control sample (348 diabetic subjects with onset age <25 years and 392 nondiabetic subjects aged >45 years). As shown in Table 2, rs3738435 was modestly associated with early-onset type 2 diabetes under the same model that was significant for AIR ($P = 0.02$ under the dominant model, after adjusting for sex, Pima heritage, and sibship), where the C allele that was associated with reduced insulin secretion is associated with a slightly greater risk for early-onset type 2 diabetes. Haplotype analysis did not reveal any more information than single SNP analysis for association with early-onset type 2 diabetes (data not shown). To our knowledge, this is the first report showing an association between SNPs located within the human *CHRM3* gene and type 2 diabetes–related phenotypes.

## DISCUSSION

To determine whether rs3738435 is a biologically plausible causative SNP for affecting insulin secretion, we confirmed that the *CHRM3* alternative transcript that contains this SNP within the 5′ UTR (ENSG133019) is expressed in human islets (Fig. 2). The SNP is located only 149 bp upstream of the translation start codon, and the sequence spanning this SNP may function as a potential binding site for the hepatic nuclear factor 6 (MatInspector). This transcription factor interacts with the hepatic nuclear factor 4α gene, which has been shown to be involved in the etiology of one form of an early-onset type 2 diabetes (maturity-onset diabetes of the young 1) (18,19). However, direct functional studies are needed to demonstrate that this SNP affects *CHRM3* expression levels in human β-cells.

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