Fine Mapping and Functional Studies of Risk Variants for Type 1 Diabetes at Chromosome 16p13.13

Single nucleotide polymorphisms (SNPs) located in the chromosomal region 16p13.13 have been previously associated with risk for several autoimmune diseases, including type 1 diabetes. To identify and localize specific risk variants for type 1 diabetes in this region and understand the mechanism of their action, we resequenced a 455-kb region in type 1 diabetic patients and unaffected control subjects, identifying 93 novel variants. A panel of 939 SNPs that included 46 of these novel variants was genotyped in 3,070 multiplex families with type 1 diabetes. Forty-eight SNPs, all located in CLEC16A, provided a statistically significant association (\(P < 5.32 \times 10^{-5}\)) with disease, with rs34306440 being most significantly associated (\(P = 5.74 \times 10^{-6}\)).

The panel of SNPs used for fine mapping was also tested for association with transcript levels for each of the four genes in the region in B lymphoblastoid cell lines. Significant associations were observed only for transcript levels of DEXI, a gene with unknown function.

We examined the relationship between the odds ratio for type 1 diabetes and the magnitude of the effect of DEXI transcript levels for each SNP in the region. Among SNPs significantly associated with type 1 diabetes, the common allele conferred an increased risk for disease and corresponded to lower DEXI expression. Our results suggest that the primary mechanism by which genetic variation at CLEC16A contributes to the risk for type 1 diabetes is through reduced expression of DEXI.

Type 1 diabetes most frequently results from the autoimmune destruction of insulin-producing pancreatic \(\beta\)-cells, resulting in a dependence on exogenous insulin. The disease is complex and arises due to the effects of genetic and environmental factors, as evidenced by incomplete concordance between monozygotic twins (1,2). Before 2007, a small number of genetic loci that contribute to type 1 diabetes risk, including HLA, INS, IL2RA, CTLA4, and PTPN22, were identified through linkage and candidate gene studies (1). With the advent of genome-wide association studies (GWAS), more than 40 additional chromosomal regions have been identified that harbor loci that confer low to moderate risk of developing type 1 diabetes, with odds ratios (OR) of less than 1.3 (3–6). One of these regions is located at chromosome 16p13.13 and is associated with type 1 diabetes and several other autoimmune disorders, including multiple sclerosis (MS), celiac disease, primary biliary cirrhosis, systemic lupus erythematosus, Crohn disease, ulcerative colitis, and primary adrenal insufficiency (4–16).

The region on chromosome 16p13.13 implicated in autoimmunity spans \(~530\) kb and contains four known genes...
genes. The most significant evidence of an association with type 1 diabetes in this region is observed for two single nucleotide polymorphisms (SNPs), rs12708716 and rs2903692, located in different introns of the gene, CLEC16A, previously known as KIAA0350 (3,5). CLEC16A has no confirmed function but shares primary sequence similarity with C-type lectins and with a Drosophila gene whose product is reported to be involved in endosomal maturation (17,18). In contrast, neighboring genes appear to be stronger candidates for type 1 diabetic pathogenesis based on their known functions. These include CIITA, a transcription factor that is essential for the expression of HLA class II genes (19), and SOCS1, a negative feedback regulator of cytokine signaling (20). The remaining gene in the region is DEXI, a gene of unknown function whose transcript levels are reported to be upregulated in response to dexamethasone, a glucocorticoid drug commonly used as an anti-inflammatory and immunosuppressant medication (21).

Several GWAS (3–6) identified the 16p13.13 region as being associated with type 1 diabetes, and these reported associations have been independently replicated (22–26). Although the association between the 16p13.13 region and type 1 diabetes has been well established, the specific genes or SNPs responsible for the diseased risk in the region have yet to be elucidated. Most of the variants on 16p13.13 associated with type 1 diabetes occur within introns of the CLEC16A gene. It has recently been reported that an intronic region of CLEC16A can physically interact with the putative promoter region of DEXI and thereby modulate DEXI expression in monocytes and in Epstein Barr virus–transformed lymphoblastoid cell lines (27–29). It has also been separately reported that an intronic region of DEXI functions as a distal enhancer for CIITA (30,31). These functional findings suggest a potential model in which genetic variation at intronic sites in CLEC16A could modulate the expression of neighboring genes, which might in turn affect the risk of type 1 diabetes.

In the current study, we resequenced the 16p13.13 region in a subset of type 1 diabetic individuals to identify novel variants. These variants were included in a dense genotyping panel of 939 SNPs to fine map the region associated with type 1 diabetes. We then assessed the relationship of the genotyped SNPs and the expression of the four genes in the region to discern a pattern of regulation associated with diabetes risk.

**RESEARCH DESIGN AND METHODS**

**Subjects**

This study was reviewed and approved by the University of Virginia Institutional Review Board. DNA from anonymous type 1 diabetic case subjects and control subjects was obtained from the Virginia Mason Medical Center and Puget Sound Blood Center (Seattle, WA), respectively. DNA from affected sibling-pair families was obtained from the Type 1 Diabetes Consortium (T1DGC) (32) and the Human Biological Data Interchange Repository (HBDI) (33).

**Sequencing of Region to Identify SNPs**

We targeted a 455-kb region (10,943,936–11,399,037 bp [Hg19]) on chromosome 16p13.13 for deep sequencing. This region encompassed the four genes (CIITA, CLEC16A, DEXI, and SOCS1) and all SNPs previously reported to be significantly associated with type 1 diabetes. The region was tiled with ~10-kb PCR fragments with ~0.5-kb overlaps. Amplifications were done in 48 pools that each contained DNA from 4 individuals (128 type 1 diabetic patients and 64 control subjects). Amplified PCR fragments were pooled in equimolar amounts to create DNA libraries. DNA libraries were prepared for sequencing using Illumina’s Paired-End Sample Preparation Kit (Illumina, San Diego, CA). Sequencing was performed on an Illumina Genome Analyzer IIX (Illumina) using 63 bp-end reads. Sequencing reads were assembled using the Burrows-Wheeler Aligner tool (34). Sequence Alignment/Map Tools (35) was used for conversion, indexing, and aligning of the data using the reference genome (Hg18; National Center for Biotechnology Information ver. 36) (36,37) as well as for SNP identification.

**SNP Filtering and Selection**

Putative SNPs identified from sequencing were filtered to limit false positives caused by strand bias by including only minor alleles with a frequency rate ≥0.1%, for which the proportion of forward reads was not statistically different from the proportion of reverse reads. The two proportions were only declared not statistically different if the absolute value of the t statistic for the proportions test was <1. The SNP list was then parsed into two groups: annotated SNPs and novel unreported SNPs, defined as lacking a Single Nucleotide Polymorphism Database (dbSNP) ID (http://www.ncbi.nlm.nih.gov/projects/SNP/).

SNPs annotated in dbSNP were excluded from analysis if they had a minor allele frequency (MAF) <5% (except exonic SNPs) or had <50 mapped reads. The annotated SNP list was further filtered to exclude SNPs that had previously been genotyped by the HapMap project (38), leaving 355 annotated SNPs.

Novel variants were filtered to exclude those with a MAF <5% (exonic SNPs were only excluded if MAF <1%) and those with a combination of <50 aligning reads and >20% nonaligning reads, which left 93 novel SNPs.

**Genotyping**

We designed two custom GoldenGate genotyping assays (Illumina). The first panel consisted of 123 tagging SNPs that captured information on the 505 reported SNPs in the CEU (Utah residents with ancestry from Northern and Western Europe) HapMap (r² = 0.9), as determined using Haploview 4.2 (39). The second panel consisted of 290 SNPs identified through our pooled sequencing of type 1 diabetic case subjects and control subjects. There were 16 SNPs duplicated on each panel for quality control. The compiled SNP lists were verified for designability using Illumina’s GoldenGate Panel Design software (www.illumina.com). Genotyping was performed for 413 SNPs on 1,850 HBDI
and 1,750 TIDGC samples. Data were examined in GenomeStudio (Illumina) and exported in PLINK (40) format.

Quality control measures were performed at the SNP and sample level. Signal cloud graphs were manually reviewed for each of the genotyped SNPs, and those with poor clustering were excluded. SNPs with more than five Mendelian transmission errors were identified and excluded from further analyses. SNPs genotyped in duplicate were examined for concordance using PLINK (40); those with ≥5% discordance were excluded. Also excluded were SNPs with significant deviations from Hardy-Weinberg equilibrium, >5% missingness, or <1% MAF.

For sample quality control in GenomeStudio, DNA samples with a genotype call rate of <90% were removed from further analyses. Subjects with more than five Mendelian transmission errors were identified with PLINK and excluded from the analyses. All remaining Mendelian transmission errors were identified and problematic genotypes removed with PedCheck (41).

**Imputation**

After quality control, the data set that was derived from targeted sequencing and genotyping, genotype data for 1,734 SNPs in the 16p13.13 region from the ImmunoChip data set became available and were added to the analyses (provided by Dr. Stephen Rich) (42). SNPs with significant deviations from Hardy-Weinberg equilibrium or >5% missingness were excluded from subsequent analyses. PedCheck was used to identify Mendelian errors and remove conflicting genotypes. Finally, all duplicate SNPs and SNPs with <1% MAF were removed.

**Expression Quantitative Trait Loci Analysis**

Amplifications were carried out on an ABI 7900 (Life Technologies, Carlsbad, CA) as follows: 95°C for 10 min, 35-40 cycles of 95°C for 15 s, 60° or 61°C for 30 s, 72°C for 30 s, followed by a dissociation curve analysis. The data were examined using ABI’s SDS 2.3 software, and Ct (threshold) curve were adjusted for each PCR run.

**Cell Culturing and RNA Extraction**

We procured 33 HapMap Epstein Barr virus–transformed cell lines from the Coriell Institute for Medical Research (Camden, NJ). All individuals were unrelated and healthy. Cells were grown in RPMI media supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% l-glutamine. Cells were split on a regular schedule. Harvesting of cells occurred when cells reached a density of $5 \times 10^{-7}$ and only if they were 70% viable. Cells were pelleted and stored at −80°C. RNA was isolated using TRIzol (Invitrogen), followed by a DNase treatment (Ambion, Life Technologies).

**RT-PCR**

RNA was reverse transcribed using random primers (Invitrogen) and Superscript II (Invitrogen), following the manufacturer’s protocol. The cDNA samples, RT control samples, and standards were plated in triplicate using a Biomek FX-Robotic workstation (Beckman Coulter) and air dried. PCR primers were designed based on mRNA transcripts from the National Center for Biotechnology Information database (CIITA III-BQ221200, CIITA IV-BM147267, CLEC16A Isoform I-NM_015226.2, CLEC16A Isoform II-BC112897.1, CLEC16A Isoform III-AK126771.1, DEXI-NM_014015.3, SOCS1-NM_003745.1, GAPDH: NM_002046.3) using Primer3 (48) (Supplementary Table 1). Amplifications were carried out on an ABI 7900 (Life Technologies, Carlsbad, CA) as follows: 95°C for 10 min, 35-40 cycles of 95°C for 15 s, 60° or 61°C for 30 s, 72°C for 30 s, followed by a dissociation curve analysis. The data were examined using ABI’s SDS 2.3 software, and Ct (threshold) cycle were adjusted for each PCR run.
RESULTS
Resequencing of the 16p13.13 Region Identifies Novel SNPs
A region of 455 kb from 16p13.13 was amplified in 192 individuals (128 type 1 diabetic patients and 64 control individuals) and sequenced to an average depth of coverage of 35×. The region was selected based on capturing the linkage disequilibrium block (D2 > 1) that encompassed the disease-associated SNPs for type 1 diabetes (rs12708716 and rs2903692), which contains the DEXI and CLEC16A gene. We then expanded the region to include the neighboring genes of CIITA and SOCS1 because of their important immunological function. When aligned to the human reference sequence, there were two gaps at coordinates chr16:11,028,710–11,038,336 (Hg19) and chr16:11,340,104–11,350,943 (Hg19), from which no sequence was obtained that passed quality control. When compared with the human reference genome, 962 SNPs were identified, of which 93 had no prior annotation. A genotyping panel spanning the region was designed containing tagging SNPs selected from HapMap and novel SNPs identified from our resequencing (60 of the 93 SNPs were designable and included in the panel).

Fine Mapping Identifies Statistically Significant Associations With Type 1 Diabetes
Genotypes for 319 SNPs (MAF >1%) were generated from 3,370 members of 749 multiplex type 1 diabetic families. These data were merged with an overlapping data set from the same region derived from ImmunoChip, and imputation was used to fill in the missing genotypes for those subjects genotyped on only one platform.

The merged data set, after imputation, contained genotypes for 939 SNPs on 12,485 individuals (3,070 multiplex families), which included 46 of our novel variants. The family-based TDT (45) was used to test for association of alleles at these SNPs with type 1 diabetes (Fig. 1). A total of 48 SNPs were identified as having alleles significantly associated with type 1 diabetes (P < 5.32 × 10−5; Supplementary Table 2). The strongest evidence for association was obtained at rs34306440.

Figure 1—Type 1 diabetes association results on 16p13.13. A total of 939 SNPs (genotyped and imputed) were tested for association with type 1 diabetes in 3,070 multiplex families (12,485 individuals) using the TDT. The log10 of the P value for this test is plotted relative to the physical position on the chromosome for each SNP. The most significant results were obtained for rs34306440 (P = 5.74 × 10−6), marked in purple. The color of all other SNPs indicates pairwise linkage disequilibrium with rs34306440, as measured by the r2 statistic. Symbol shapes indicate those SNPs genotyped only on the custom panels (snowflake), genotyped only on the ImmunoChip (circle), and genotyped on both panels (square). cM/Mb, centimorgan/megabase.
SNPs in CLEC16A Are Associated With Variation in Expression Level of DEXI

An eQTL analysis was performed designating the expression levels of CIITA, DEXI, CLEC16A, and SOCS1 in B-cell lines as phenotypes. Primers that specifically amplified each of the common reported alternative spliced transcripts for the target genes were designed and used to assess transcript levels by quantitative RT-PCR. The full panel of 915 SNPs from the region was then tested for association with quantitative variation in the levels of each of the transcripts. The only significant associations were observed for DEXI transcript and a number of SNPs spanning the CLEC16A gene, many of which had alleles that were modestly correlated ($r^2 > 0.6$). The most statistically significant association was observed at rs7403919 ($P = 1.24 \times 10^{-6}$). A second independent biological replicate focusing on DEXI expression yielded similar results (rs7403919, $P = 5.45 \times 10^{-5}$). A full breakdown of the correlations with rs7403919 and type 1 diabetes associated SNPs based on the HapMap samples can be found in Supplementary Table 3. The rest of the genes tested did not show significant associations. The eQTL plots of all the transcripts tested can be found in Supplementary Fig. 2.

We specifically examined the expression levels of DEXI in relation to three SNPs: rs34306440 (the SNP most strongly associated with type 1 diabetes in the region), rs7403919 (the SNP most strongly associated with variation in DEXI expression in the region), and rs12708716 (most often cited in type 1 diabetes literature; Fig. 2). The risk allele for all three SNPs corresponds to the major allele. Homozygotes for the risk alleles had lower expression of DEXI, whereas the heterozygotes and homozygotes for the alternative allele displayed higher levels of expression. Figure 3 shows the OR for type 1 diabetes versus the linear regression coefficient ($\beta$) from the quantile-normalized DEXI expression level for the minor alleles of all 809 SNPs for which these data were available. Disease risk was negatively correlated with DEXI transcript levels (Pearson correlation $R = -0.66$).

In the course of designing primers for RT-PCR analysis of CIITA, several novel alternatively spliced transcripts were observed in publicly available RNA-Seq data (E-MTAB-513). The 3' untranslated region region of these CIITA transcripts overlapped with the reference transcript from DEXI in the antisense direction. This observation raised the possibility that these genes could exert some regulatory role on each other via antisense inhibition. The transcripts were validated and characterized by amplification and Sanger sequencing and were analyzed by eQTL (Supplementary Fig. 1). There was no significant evidence of association between alleles at any SNP in the 16p13.13 region and variation in the levels of these novel CIITA transcripts in the B-cell lines examined (Supplementary Fig. 2).

DISCUSSION

Chromosomal region 16p13.13 is one of a number of chromosomal regions where there is significant, replicated evidence of allelic association with type 1 diabetes (3–6) as well as with other autoimmune disorders (7–16). Repeated findings of association for multiple autoimmune disorders in a single chromosomal region such as 16p13.13 increases both confidence in the initial findings and the importance of the locus in understanding the underlying mechanism whereby autoimmunity develops. Given the evidence of genetic association at 16p13.13 with multiple diseases that are autoimmune in nature but differ in the target organ, it seems most likely that the effects of this locus would manifest in immune-related cell types, as opposed, for example, to $\beta$-cells. However, functional studies that might elucidate the mechanism depend on the ability to specify the relevant gene in the region, if not the specific causative genetic variants. In the current study, we addressed this need for the 16p13.13 region by resequencing the region in a large collection of type 1 diabetic case subjects and unaffected control subjects. The objective was to identify common variants, genotype these variants in a collection of multiplex families with type 1 diabetes, and then separately test for association of these SNPs with disease state and with transcript levels for each of the genes in the region.

There were 46 novel variants from our resequencing that were included among the 939 SNPs that we genotyped in 3,070 multiplex families with type 1 diabetes. Forty-eight of the 939 SNPs displayed a statistically significant association with type 1 diabetes. Alleles at these 48 SNPs were not all highly correlated (pairwise $r^2$ range from 0.4 to 0.9), nor were the SNPs physically clustered. All of these 48 SNPs were located in introns that spanned much of the CLEC16A gene (introns 8–22). Although the most significant disease association result was obtained at a SNP (rs34306440) initially identified from our resequencing effort, the rapid development of independent SNP resources meant that this SNP was also included in the ImmunoChip data set that subsequently became available.

The major allele at rs34306440 was associated with risk for type 1 diabetes. This is not an uncommon finding; the major allele is the risk allele for the most significantly disease-associated SNPs at approximately half of the reported risk loci for type 1 diabetes (3). Given the limited magnitude of the individual effects that each of these loci have on disease risk and the complex genetic nature of most autoimmune diseases, there might not be a significant selection pressure to limit their frequency in the population. There are also precedents, such as the DQ0602.
allele at the HLA-DQB1 locus, where the same allele confers risk for one disease (MS) (50) and protection for another (type 1 diabetes) (51).

The broad physical distribution of disease-associated SNPs and their modest correlation raise the possibility that they act through multiple mechanisms or affect multiple genes. Given their intronic locations and prior reports of cis-oriented transcriptional effects in the region, we tested for association between our panel of SNPs and transcripts levels in transformed B cell lines for each of the documented alternatively spliced forms of the four genes, CIITA, DEXI, CLEC16A, and SOCS1. Only DEXI presented statistically significant results. Similar to the disease association results, SNPs associated with DEXI transcript levels appear to be distributed across much of the CLEC16A gene. An examination of the relationship between the OR for the minor allele at each SNP in the region and the corresponding linear regression coefficient for DEXI expression for the same allele indicated that DEXI expression levels were negatively correlated with disease risk ($R = -0.66$).

Our results suggest that genetic variation over a broad region spanning much of the CLEC16A gene influences the expression of a neighboring gene, DEXI, and that reduced DEXI expression is correlated with an increased risk for type 1 diabetes. This model is consistent with a prior report that sequences in intron 19 of CLEC16A physically interact with the promoter region of DEXI (27), providing a plausible mechanism for how genetic variation in CLEC16A can act at a distance to regulate DEXI expression. Our results suggest that the region of interaction may be large or that distal sequences may act indirectly to affect

**Figure 2**—Expression levels of DEXI grouped by genotypes of SNPs rs7403919 (A), rs12708716 (B), and rs34306440 (C). Alleles associated with type 1 diabetes are in boldface. The centerlines represent the median value, box area represents upper and lower quartiles, the whiskers represent 1 SD from the mean.
the stability or structure of the DNA loops formed. Consistent with this, examination of ENCODE data (52) reveals a number of peaks of H3K27 histone acetylation in the same region as the SNPs associated with disease risk and variation in DEXI expression.

Our study only examined expression in a single cell type, B lymphoblastoid cell lines, and only in cell lines derived from unaffected individuals. The possibility cannot be excluded that there may be different, or additional, effects on gene expression associated with the SNP alleles studied here in distinct tissues or specific to affected individuals. However, lower DEXI and/or SOCS1 expression has also been reported in thymic samples for risk alleles associated with MS for SNPs rs12708716, rs7206912, and rs6498169 located in CLEC16A (53). Two of these three SNPs (rs12708716 and rs6498169) were also included in our panel and showed a similar relationship to DEXI expression. Finally, the Genotype-Tissue Expression Portal (GTEx) reports a significant eQTL for rs12708716 in a third tissue type, thyroid; again, with the same allelic association with expression.

Our results and those from prior studies focus attention on DEXI as a candidate gene affecting the risk for type 1 diabetes in the 16p13.13 region. Little is known about the function of DEXI and its predicted 95 amino acid protein product. The protein is well conserved, with orthologous genes readily detectable in multiple species. There is 95% identity between the human and mouse orthologs and 84.5% between human and zebrafish.

There are no identifiable domains in DEXI that might suggest a possible function for the protein. However, Miyaki et al. (54) showed that small interfering RNA-mediated knockdown of DEXI reduced the incidence of apoptosis in cells treated with the chemotherapy agent camptothecin. A role in a basic cellular function, such as apoptosis, would be consistent with the involvement of DEXI in multiple autoimmune diseases with different target tissues.

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**Author Contributions.** M.J.T. designed and performed the experiments, analyzed data, produced Tables and Figures and wrote the manuscript. A.P. analyzed sequencing data, analyzed expression data (eQTL), produced genotype expression plots, and reviewed and edited the manuscript. R.P. assisted genotyping the custom panel based on sequencing data and reviewed and edited the manuscript. M.M. helped design and perform long-range PCR and RT-PCR experiments and reviewed and edited the manuscript. K.L.K. helped design and perform initial genotyping on the 123 custom tagging panel and reviewed and edited the manuscript. X.H. coanalyzed sequencing results and reviewed the manuscript. J.M. coanalyzed sequencing results, coanalyzed the raw ImmunoChip data, and reviewed and critiqued the manuscript. W.-M.C. coanalyzed the raw ImmunoChip data and reviewed and critiqued the manuscript. P.C. helped design and supervise the project and reviewed and edited the manuscript: S.O.-G. helped design and supervise the project, performed experiments, and reviewed and edited the manuscript. M.J.T. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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