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A low-frequency *GLIS3* variant associated with resistance to Japanese type 1 diabetes

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ABSTRACT

The role of low-frequency variants in type 1 diabetes (T1D) susceptibility still remains to be clarified. In the present study, we analyzed low-frequency variants of the T1D candidate genes in Japanese. We first screened for protein-changing variants of 24 T1D candidate genes in 96 T1D patients and 96 control subjects, and then the association with T1D was tested in 706 T1D patients and 863 control subjects recruited from the collaborating institutions in Japan. In total, 56 protein-changing variants were discovered; among them, 34 were low-frequency variants (allele frequency < 5%). The association analysis of the low-frequency variants revealed that only the A908V variant of *GLIS3* was strongly associated with resistance to T1D (Haldane's odds ratio = 0.046, $p = 8.21 \times 10^{-4}$, and $p_c = 2.22 \times 10^{-2}$). GLIS3 is a zinc finger transcription factor that is highly expressed in pancreatic beta cells, and regulates beta cell development and insulin gene expression. GLIS3 mRNA is also moderately expressed in the human thymus. The precise mechanism responsible for the association is unclear at present, but the A908V variant may affect autoimmunity to the GLIS3 protein itself; the 908V containing epitope may induce central or peripheral tolerance more efficiently than that of 908A.

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1. Introduction

A number of type 1 diabetes (T1D) susceptibility loci have been revealed in Caucasians. Among them, HLA is the strongest, and contributes about one-half of the genetic component of T1D. Previous candidate gene studies have identified several non-HLA loci, which include the gene encoding insulin (*INS*), *CTLA4*, *PTPN22*, and *IL2RA* (or *CD25*) [1,2]. Furthermore, recent genome-wide association studies (GWAS) identified many weaker susceptibility loci. However, proportion of heritability explained for T1D by the confirmed T1D loci is still between two-thirds and three-quarters [2].

In Japanese subjects, it was revealed that HLA class II is also a very strong susceptibility factor with the mostly specific susceptibility haplotypes to Asian populations [3,4]. *INS* is another confirmed T1D susceptible loci in Japanese, although the susceptible class 1 allele is predominant over the protective class 3 allele; the frequency of the class 3 is less than 5% both in the T1D patients and control subjects [5]. We have also obtained evidence for an association with the *CTLA4* [6], *IL2RA* [7], *ERBB3* [8], *CLEC16A* [8] and *IL7R* [9] loci, however, it was revealed that the cumulative effect of these non-HLA susceptibility loci was much weaker than that of the HLA class II loci [9]. Thus, taking into account the much higher recurrence risk in siblings of Japanese T1D patients compared to Caucasian patients [10], non-HLA susceptibility T1D loci largely remain to be elucidated in Japanese.

It is reasonable to postulate that low-frequency variants (allele frequency < 5%) may contribute to the high risk in siblings of patients in Japanese, since they are abundant and more likely to be functional than common SNPs [11–13]. In the present study,

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we analyzed low-frequency variants of the T1D candidate genes and their potential association with T1D in Japanese.

2. Materials and methods

2.1. Subjects

A total of 1569 Japanese subjects, consisting of 706 patients with T1D and 863 control subjects recruited from the collaborative institutions were studied. The patients consisted of 418 females and 288 males with a mean (±SD) age-at-onset of 31.5 (±17.7) years. The control subjects consisted of 332 females and 531 males with a mean (±SD) age of 31.5 (±12.8) years. An ethics committee from each institute approved the study, and informed consent was obtained from all subjects.

2.2. Screening for variants by next-generation sequencing

We resequenced 24 candidate genes by a next-generation sequencer in DNA of 96 T1D patients and 96 control subjects; these subjects were among those recruited from the hospital or school of Saitama Medical University. The selected candidate genes were shown in Table 1. We selected 16 genes from the confirmed type 1 diabetes loci, four from genes responsible for monogenic diabetes, three from autoantigens, and one from genes responsible for the BB rat. Briefly, four DNA pools of 48 patients or 48 controls were prepared, and all exons and splice sites of the candidate genes were PCR-amplified, then the DNA samples were mixed and sequenced with an Illumina Genome Analyzer IIx. The obtained sequence reads (approximately 2.5 billion reads for each DNA pool) were mapped to the genes to identify variants using the software MAQ (http://maq.sourceforge.net/). Theoretically, resequencing 96 subjects provides 98% and 85% probability of detecting variants at 2% and 1% frequency, respectively.

2.3. Estimated frequencies of the variant and wild-type alleles based on sequence reads from DNA pools

In the present study, we focused on the variants that change coding proteins. For the identified variants, we first estimated the allele frequencies by the sequence reads (variant and wild-type alleles) generated from the DNA pools containing 96 chromosomes of T1D patients or control subjects each. Before the large-scale genotyping, we validated the accuracy of the frequency estimates in the DNA pools by next-generation sequencing in 14 variants by comparing allele frequencies in individually genotyped DNA samples.

2.4. Association analysis of low-frequency variants

Next, we conducted association analysis of low-frequency variants in 706 T1D patients and 863 control subjects enrolled from collaborating institutions. Individual genotypes of variants were determined by DigiTag2 assay [14] and/or TaqMan SNP Genotyping Assay (Applied Biosystems; Foster City, CA). Theoretically, association test based on 706 cases (1412 alleles) and 863 controls (1726 alleles) has 86% power to detect association at false-positive rate $\alpha = 0.05$ for allele frequency 2% and OR = 2 or 61% power for allele frequency 1% and OR = 2 (GraphPad StatMate 2.00, GraphPad Software, Inc., USA).

2.5. Statistical analysis

The correlation of the frequency estimates in the DNA pools and allele frequencies in individually genotyped DNA samples were assessed by Pearson's correlation coefficient (r). The differences in the allele frequencies determined by individually genotyping were assessed by two-sided Fisher's exact test, and each p-value was corrected by multiplying the number of analyzed variants (p_c-value; Bonferroni correction). The odds ratios (OR) for the variant allele were calculated using Haldane's method. StatsDirect Ver. 2.6.5 (StatsDirect, Cheshire, UK) were used for these tests. Statistical significance was defined as $p_c < 0.05$.

3. Results

3.1. Screening for variants by next-generation sequencing

As shown in Table 2, 56 variants that change coding proteins (55 non-synonymous and one splice site) were discovered in 17 among the 24 genes studied by next-generation sequencing: the average coverage of depth for the positions of these variants was 10536x. Among them, 34 (61%) were low-frequency variants both in T1D patients and control subjects.

3.2. Validation for the frequency estimates in the sequenced DNA pools

We analyzed the correlation of the frequency estimates in the DNA pools and allele frequencies in individually genotyped DNA samples in 14 variants. A good correlation was found between these, in the total variants (Fig. 1A) and in the low-frequency variants only (Fig. 1B) ($r = 0.98$ in both).

3.3. Association analysis low-frequency variants

Thirty-four low-frequency variants were genotyped by the DigiTag2 assay and/or the TaqMan SNP assay; the DigiTag2 assay was reported to have high accuracy and reproducibility [14]. Actually, we genotyped five variants of this study by both DigiTag2 assay and TaqMan SNP assay, and found that 99.9% of the typing results were identical. Seven variants (*CCR5* M49V, *CLEC16A* T773P, *CLEC16A* D1053A, *ERBB3* Y603S, *SLC30A8* S230AR, *WFS1* I421M and *WFS1* H763P) were excluded from association analysis, since these variants were not detected by the DigiTag2 assay (possibly due to typing failure), assuming that the variant allele frequencies were at

Table 1
Studied genes.

Category	Gene
Type 1 diabetes loci	*CCR5, CD226, CD28, CLEC16A, CTLA4, ERBB3, GLIS3, IFIH1, IL2, IL21, IL2RA, IL7R, INS, PTPN2, PTPN22, SH2B3*
Autoimmune polyglandular syndrome (APS) type 1	*AIRE*
Wolfram syndrome	*WFS1*
Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome	*FOXP3*
Neonatal diabetes	*KCNJ11*
Type 1 diabetes autoantigens	*GAD2, PTPRN, SLC30A8*
BB rat	*IAN4L1*

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