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ONLINE METHODS

Study subjects. Stage 1 subjects were drawn from eight T2D GWAS participating in the AGEN consortium, which was organized to enable genetic studies on diverse complex traits in 2010. These eight studies included 6,952 cases with T2D and 11,865 controls from the Korea Association Resource Study (KARE), the Singapore Diabetes Cohort Study (SDCS), the Singapore Prospective Study Program (SP2), the Singapore Malay Eye Study (SiMES), the Japan Cardiometabolic Genome Epidemiology Network (CAGE), the Shanghai Diabetes Genetic Study (SDGS), the Taiwan T2D Study (TDS) and the Cebu Longitudinal Health and Nutritional Survey (CLHNS). Subjects in stage 2 included 5,843 cases with T2D and 4,574 controls from three independent GWAS, the BioBank Japan Study (BBJ), the Health2 T2D Study (H2T2DS) and the Shanghai Jiao Tong University Diabetes Study (SJTUDS), for *in silico* replication analysis. Stage 3 included up to 12,284 cases with T2D and 13,172 controls from five different studies, the Japan Cardiometabolic Genome Epidemiology Network (CAGE), the Shanghai Diabetes Study I/II (SDS I/II), the Chinese University of Hong Kong Diabetes Study (CUHKDS), the National Taiwan University Hospital Diabetes Study (NTUHDS) and the Seoul National University Hospital Diabetes Study (SNUHDS), for *de novo* replication analysis. The study design and T2D diagnosis criteria used in each study included in stages 1, 2, and 3 are described in **Supplementary Table 1** and the **Supplementary Note**. Each study obtained approval from the appropriate institutional review boards of each participating institution, and written informed consent was obtained from all participants. The three-stage design of the overall study is depicted in **Supplementary Figure 1**.

Genotyping and imputation. Subjects for the stage 1 and 2 analyses were genotyped with high-density SNP typing platforms that covered the entire human genome. In most of the studies, only unrelated samples with missing genotype call rates below 5% were included for subsequent GWAS analyses. For the genome-wide association meta-analysis, each study participating in stages 1 and 2 performed SNP imputation. IMPUTE, MACH or BEAGLE (see URLs) were used, together with haplotype reference panels from the JPT and CHB samples that are available in the HapMap database (JPT+CHB+CEU and/or YRI, in some studies) on the basis of HapMap build 36 (release 21, 22, 23a or 24). Only imputed SNPs with high genotype information content (proper info > 0.5 for IMPUTE and Rsq > 0.3 for MACH and BEAGLE) were used for the association analysis. Genotyping for the stage 3 analysis was carried out using TaqMan, Sequenom MassARRAY or the Beckman SNP Stream method. All SNPs included in stage 3 had a genotype success rate of >98% (**Supplementary Table 2**).

Statistical analyses, analysis tools and SNP prioritization for stages 2 and 3. Associations between SNPs and T2D were tested by logistic regression with an additive model (1 degree of freedom) after adjustment for sex. Other adjustments were permitted according to the situations in the individual studies. The meta-analysis was performed using an inverse-variance method assuming fixed effects, with a Cochran's *Q* test to assess between-study heterogeneity. METAL software (see URLs) was used for all meta-analyses. A plot of the negative log of the association results from the stage 1 meta-analysis, by chromosome, was generated using WGAViewer software (see URLs).The quantile-quantile plot was constructed by plotting the distribution of observed *P* values for the given SNPs against the theoretical distribution of the expected *P* values for T2D[34]. The genomic control inflation factor, λ, was calculated by dividing the median χ^2 statistics by 0.456 (ref. 35) for individual GWAS, as well as for the stage 1 GWAS meta-analysis. We did not correct for genomic control in the stage 1 analyses because the inflation was modest, suggesting that population structure is unlikely to cause substantial inflation of the stage 1 results (**Supplementary Table 2**). The selection criteria for the lead SNPs to take forward to stage 2 *in silico* replication analysis were as follows: (i) stage 1 meta-analysis $P < 5 \times 10^{-4}$ (based on the divergence between the observed and expected *P* values on the quantile-quantile plot; **Supplementary Fig. 2**); (ii) heterogeneity $P > 0.01$; and (iii) at least seven studies having been included in the stage 1 meta-analysis (**Supplementary Table 4**). After removing known variants associated with T2D, proxies for each lead SNP ($r^2 > 0.8$) were selected using the SNAP software (see URLs). The replication genotyping for stage 3 was performed for the new SNPs having a stage 2 combined $P < 10^{-5}$. Regional association results from genome-wide meta-analysis were plotted using LocusZoom software (see URLs) for SNPs reaching genome-wide significance from the combined meta-analysis of stages 1, 2 and 3.

Principal components analysis. A list of 76,534 common SNPs across the Illumina 550, 610 and 1M and Affymetrix 5.0 and 6.0 arrays were first selected. This set of SNPs in the Asian (CHB+JPT) HapMap II samples was then trained to generate a list of 44,524 SNPs having pairwise LD < 0.3 in a sliding window of 50 SNPs. Individuals from each component study and from HapMap II were plotted based on the first two eigenvectors produced by the principal components analysis.

eQTL analysis. Gene expression information from 776 adipose tissues, 667 skin tissues and 777 lymphoblastoid cell lines was obtained from the MuTHER consortium[36]. The eQTL data for eight of the ten T2D loci identified in this study were available in the MuTHER dataset. Most of those loci passed the filtering criteria, such as MAF > 5% and INFO > 0.8, except for rs16955379, which has MAF = 1.5% in the MuTHER data set. Two of the ten loci that were used in the eQTL analysis, rs6815464 (on chromosome 4) and rs17797882 (on chromosome 16), are not included in the MuTHER data set. Association between each SNP with a significant association to T2D and the normalized mRNA expression values of genes within 1 Mb of the lead SNP were performed with the GenABEL and ProbABEL package (see URLs) using the polygenic linear model incorporating a kinship matrix in GenABEL followed by the ProbABEL mmscore test with imputed genotypes. A multiple-testing correction was applied to the *cis* association results. *P* value thresholds of $P = 5.06 \times 10^{-5}$ in adipose tissue, $P = 3.81 \times 10^{-5}$ in skin and $P = 7.80 \times 10^{-5}$ in lymphoblastoid cell lines correspond to an estimated genome-wide false discovery rate of 1%.

Gene relationships among implicated loci (GRAIL) analysis. A GRAIL analysis was performed as described previously[31,33]. A total of 38 genes within T2D-associated regions were selected for the analysis. Among these genes, 28 were from the previously implicated set (**Supplementary Table 3**), and the other 10 genes were newly implicated in this study (**Table 1**). PubMed abstracts published after December 2006 were omitted from the analysis to reduce confounding by results from T2D GWAS.

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Genetics and pathogenesis of type 1 diabetes: prospects for prevention and intervention

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ABSTRACT

Type 1 diabetes is etiologically a multifactorial disease caused by a complex interaction of genetic and environmental factors, with the former consisting of multiple susceptibility genes. Identification of genes conferring susceptibility to type 1 diabetes would clarify etiological pathways in the development and progression of type 1 diabetes, leading to the establishment of effective methods for prevention and intervention of the disease. Among multiple susceptibility genes, *HLA* and *INS* are particularly important because of their contribution to tissue specificity in the autoimmune process. *DRB1*04:05-DQB1*04:01* is associated with autoimmune type 1 diabetes, idiopathic fulminant type 1 diabetes and anti-islet autoimmunity in autoimmune thyroid diseases, suggesting that this haplotype is associated with beta-cell specificity in autoimmune diseases. Genes involved in the expression of insulin in the thymus contribute to beta-cell-specific autoimmune mechanisms in type 1 diabetes. These genes and pathways are important targets for tissue-specific prevention and intervention of type 1 diabetes. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2011.00176.x, 2011)

KEY WORDS: Autoimmune disease, Genetics, Type 1 diabetes

INTRODUCTION

Type 1 diabetes is caused by destruction of insulin-producing beta-cells of the pancreas in genetically susceptible individuals. Etiologically, type 1 diabetes is classified into two major subtypes, autoimmune (type 1A) and idiopathic (type 1B). The etiologic factors and pathogenesis of idiopathic type 1 diabetes are still unknown, but recent studies suggested that fulminant type 1 diabetes may belong to this subtype[1,2]. Type 1A diabetes is an organ-specific autoimmune disease in which beta-cells of the pancreas are the target organ of the autoimmune attack.

Type 1 diabetes is a multifactorial disease caused by a complex interaction of genetic and environmental factors, with the former consisting of multiple susceptibility genes. Identification of genes conferring susceptibility to type 1 diabetes would clarify the etiological pathways in the development and progression of type 1 diabetes, leading to the establishment of effective methods for prevention and intervention of the disease. In this review, clinical problems in the treatment of type 1 diabetes are summarized in order to help understand the reason why identification of genes conferring susceptibility to type 1 diabetes is necessary, and then the current status of the molecular genetics of type 1 diabetes is reviewed with special emphasis on genes that contribute to tissue specificity of autoimmune mechanisms.

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WHY GENES?

Among patients with type 1 diabetes, heterogeneity of residual beta-cell function is observed. Some patients completely lack endogenous insulin secretion, while others have preservation of minimal insulin secretory capacity. Complete lack of endogenous insulin secretion in type 1 diabetes is associated with unstable glycemic control, so-called brittle diabetes, as evidenced by our previous studies showing an inverse correlation between unstable glycemic control and minimal residual beta-cell function in type 1 diabetes[3]. These data have recently been confirmed in fulminant diabetes[4]. This can be explained by the buffering action of endogenous insulin, whose secretion, even in a small amount, is automatically adjusted to the body's need on a minute-to-minute basis. Excess exogenous insulin can be adjusted by a decrease in endogenous insulin, whereas deficiency of insulin can be adjusted by a small increase in endogenous insulin. Type 1 diabetic patients with no residual beta-cell function lack this buffering action of endogenous insulin, and therefore have difficulty maintaining stable glycemic control, even with continuous subcutaneous insulin infusion (CSII). At the moment, pre-programmable CSII may be the only way to achieve glycemic control in such patients. Figure 1 shows the basal insulin infusion rate of pre-programmable CSII to achieve stable glycemic control in five patients with type 1 diabetes with complete lack of endogenous insulin. To achieve stable glycemic control, very dynamic adjustment of basal insulin infusion was required, with a decrease in infusion rate to avoid nocturnal hypoglycemia and an increase in infusion rate to overcome the dawn phenomenon. This in turn suggests that type 1 diabetic patients, particularly those with complete lack of endogenous

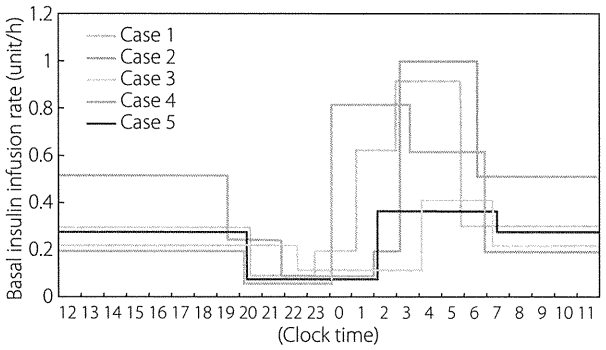

Figure 1 | Basal insulin infusion rate in type 1 diabetics with complete lack of endogenous insulin. C-peptide-negative patients with type 1 diabetes (n = 5) were treated with continuous subcutaneous insulin infusion (CSII) with a pre-programmable insulin pump. The infusion rate of basal insulin at night was adjusted to achieve target glycemic control with near-normal glycemia at bedtime and before breakfast, and no hypoglycemia (<70 mg/dL) at 3:00 AM. Very dynamic changes in basal insulin infusion rate were required. To avoid nocturnal hypoglycemia, a decrease in infusion rate to a level as low as 1/5 the daytime infusion rate was required, whereas an increase to as great as four times the daytime infusion rate was required to overcome the 'dawn phenomenon'.

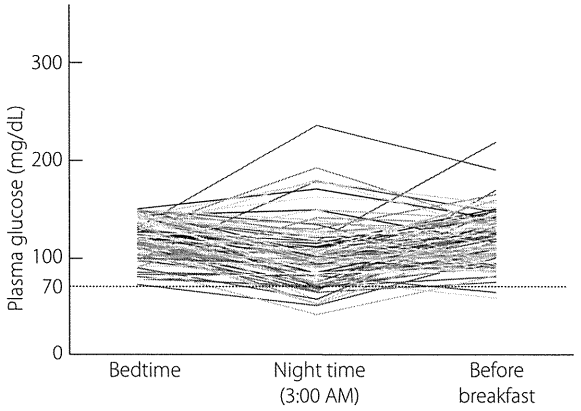

Figure 2 | Plasma glucose level at 3:00 AM compared with bedtime and before breakfast. Plasma glucose levels were monitored at bedtime (9:00 PM), during the night (3:00 AM) and before breakfast (7:00 AM) in diabetic inpatients (n = 87) treated with bedtime NPH insulin or a long-acting insulin analogue, with relatively stable glycemic control (bedtime glucose level 70–150 mg/dL). It is evident that the night time glucose level cannot be estimated from glucose levels at bedtime and/or before breakfast in some patients. Nocturnal hypoglycemia is seen in some patients, while in others an increase in glucose level with a nocturnal peak is observed.

insulin, are at high risk of nocturnal hypoglycemia when treated with bedtime NPH insulin or a long-acting insulin analogue. In fact, when plasma glucose level was measured at 3:00 AM in diabetic inpatients (n = 87) who had relatively stable glycemic control with multiple insulin injections of bedtime NPH insulin or a long-acting insulin analogue, marked variation in the glucose level was noticed (Figure 2), with nocturnal hypoglycemia occurring in 18% of patients. Nocturnal hypoglycemia was significantly more frequent in type 1 diabetic patients than in type 2 diabetic patients (31 vs 12%, P = 0.03). These data indicate the importance of preservation, or possibly regeneration, of beta-cells in type 1 diabetes. At the clinical onset of type 1 diabetes, beta-cells are not completely destroyed, and low, but significant, secretory capacity of insulin still remains in most patients. To preserve residual beta cells at an early stage of type 1 diabetes and protect regenerating beta cells from recurrent autoimmune attack, the molecular mechanisms of autoimmune beta-cell destruction must be clarified in order to establish effective methods for prevention and intervention. Identification of genes conferring susceptibility to type 1 diabetes is thus important because molecular pathways can be clarified by studying the function of genes identified.

WHICH GENES?

Initially, susceptibility genes for type 1 diabetes were studied by the candidate gene approach, and several important genes, such as *HLA* and insulin gene (*INS*), have been identified[5–9]. The random marker approach was then adopted initially in multiplex families, and more recently in a large number of cases and controls with hundreds of thousands of single nucleotide polymorphisms (SNPs), termed genome-wide association studies (GWAS) (10-12). By using these approaches, more than 40 susceptibility loci have been mapped in Caucasian populations (Figure 3). Most of them, however, were loci, and responsible genes are yet to be identified. To clarify the etiological pathway in order to develop effective methods for prevention and intervention, responsible genes must be identified.

Among multiple susceptibility genes, at least five genes, *HLA*, *INS*, *CTLA4*, *PTPN22* and *IL2RA* (*CD25*), have been shown to be responsible for type 1 diabetes susceptibility in Caucasian populations[5–12]. Although the incidence of type 1 diabetes is markedly different between Japanese and Caucasian populations, the association of candidate genes with type 1 diabetes is generally similar in both populations, and there are good reasons for the apparent differences in the genes associated with type 1 diabetes between Japanese and Caucasians[13-19].

Among these genes, *HLA* shows particularly strong susceptibility in both Japanese and Caucasian populations[20–22]. The contribution of insulin gene (*INS*) to susceptibility to type 1 diabetes is well established in Caucasian populations, but its contribution in Japanese is not as clear as that in Caucasians due to the very high frequency of risk haplotype in the Japanese general population[23]. Recent studies demonstrated that *INS* is associated with type 1 diabetes in Japanese[18]. These two genes appear to contribute to type 1 diabetes at different steps in the etiological pathway, but as discussed below, several lines of evidence suggest that not only *INS*, but also class II HLA may contribute to tissue specificity of autoimmune destruction.

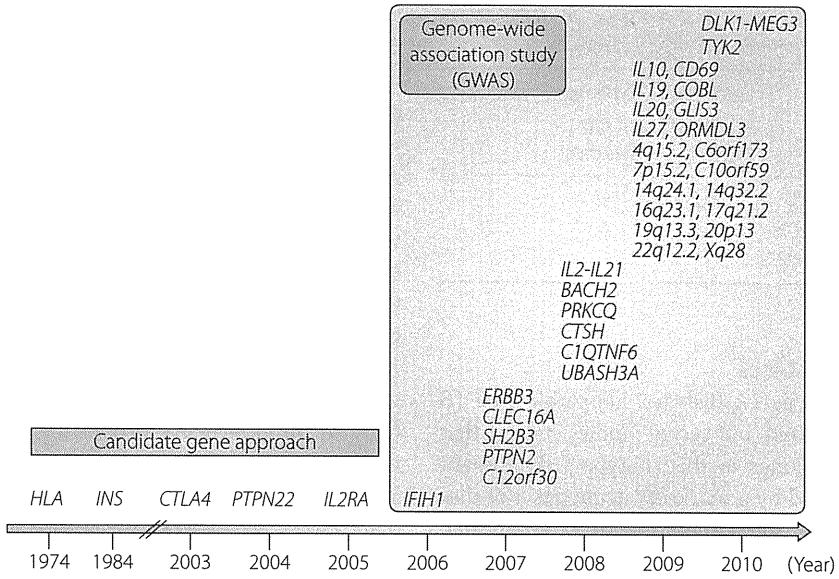

Figure 3 | Susceptibility genes or loci for type 1 diabetes identified by candidate gene approach and/or genome-wide association study (GWAS). Genes or loci for type 1 diabetes are shown relative to the year when convincing evidence was reported. *HLA*, *INS*, *CTLA4*, *PTPN22* and *IL2RA* (*CD25*) were identified by candidate gene approach. *IFIH1* was identified by genome-wide association study (GWAS) with non-synonymous SNPs, and the rest were identified by GWAS. Note that most gene symbols reported by GWAS are markers associated with the disease, but not necessarily actual genes or causal variants responsible for the disease.

MAJOR SUSCEPTIBILITY GENE: *HLA*

Class II HLA, *DRB1* and *DQB1*, have been consistently reported to be associated with type 1 diabetes in almost all ethnic groups. Differences, however, in alleles and haplotypes associated with type 1 diabetes have been reported among different ethnic groups. The DR3 (*DRB1*03:01-DQB1*02:01*) and DR4 (*DRB1*04:01-DQB1*03:02*) haplotypes are positively associated with type 1 diabetes in Caucasian populations, whereas the DR4 (*DRB1*04:05-DQB1*04:01*) and DR9 (*DRB1*09:01-DQB1*03:03*) haplotypes are associated with the disease in Japanese and most east-Asian populations[20–22]. The difference in HLA haplotypes associated with type 1 diabetes between Japanese and Caucasian populations can be explained by the presence or absence of haplotypes in each population[13,14].

HLA in Rare Multiplex Family

Type 1 diabetes clusters in families, not only in Western countries[24] but also in Japan, as evidenced by the much higher frequency in siblings of type 1 diabetic probands than in the general population[13,14,25]. Although the incidence of type 1 diabetes is much lower in Japan[26], the frequency of type 1 diabetes in siblings of type 1 diabetic probands is similar to that in white populations of European descent[13,14,25]. As a consequence, the ratio of frequencies in siblings and the general population, termed λs, which is often used to express the degree of familial clustering of a disease, is much higher in Japanese than in Caucasian populations[13,14,24,25]. A high λs value with low incidence in the general population suggests two possibilities: rare variants, that is susceptibility variants with low frequencies, but with high penetrance, cluster in families, and the shared environment within families contributes to familial clustering.

To study the former possibility, we studied a rare multiplex family in which three out of four sisters developed type 1 diabetes and the fourth sister was found to be positive for anti-GAD and anti-IA-2 antibodies[27], suggesting that the type 1 disease process existed in all four sisters, with three of them having developed the disease clinically. In this family, all four sisters shared the same HLA genotypes, *DRB1*04:05-DQB1*04:01/DRB1*08:02-DQB1*03:02*[27]. This genotype has a very low frequency in the Japanese general population, but was previously reported to show a very high odds ratio for type 1 diabetes[21,28], suggesting that this HLA genotype is a rare variant and is one of the reasons for clustering of type 1 diabetes in this family. Both the *DRB1*04:05-DQB1*04:01* and *DRB1*08:02-DQB1*03:02* haplotypes are very rare in Caucasian populations, and as a consequence, the *DRB1*04:05-DQB1*04:01/DRB1*08:02-DQB1*03:02* genotype is almost absent in Caucasian populations. However, a rare *DRB1*04:05-DQB1*03:02* haplotype does exist in Caucasian populations and was reported to be the highest risk haplotype for type 1 diabetes in Caucasians[29], although its frequency is low in the general population. These data suggest that the combination of *DRB1*04:05* and *DQB1*03:02* in either trans (Japanese) or cis (Caucasians) acts as a kind of rare variant and confers very high susceptibility to type 1 diabetes (Table 1).

Table 1 | Combination of *DRB1*0405* and *DQB1*0302* as rare variant with low frequency, but high penetrance for type 1 diabetes

Population	HLA haplotype or genotype	Frequency in controls (%)	Odds ratio for type 1 diabetes	Reference
Caucasian	*DRB1*04:05-DQB1*0302*	0.2	11.4	29
Japanese	*DRB1*04:05-DQB1*04:01/ DRB1*08:02-DQB1*03:02*	0.3	42.7	28

HLA in Fulminant Type 1 Diabetes

In contrast to autoimmune type 1A diabetes, idiopathic type 1B diabetes is not well characterized, but recent studies suggest that fulminant type 1 diabetes belongs to this subtype[1,2]. Fulminant type 1 diabetes is characterized by a markedly acute onset of diabetes and an absence of islet-related autoantibodies[1], accounting for up to 20% of type 1 diabetes in Japan[2] and 7% in Korea[30]. In contrast to the relatively high frequencies in Asian populations, fulminant type 1 diabetes appears to be very rare in Caucasian and other non-Asian populations. The reason for the difference is still unknown, but one possibility may be the difference in the frequencies of risk HLAs in general populations. *DRB1*04:05-DQB1*04:01*, in particular in the homozygous form, is strongly associated with fulminant type 1 diabetes[28]. The frequency of fulminant type 1 diabetes appears to correlate with the frequency of the *DRB1*04:05-DQB1*04:01* haplotype, in that fulminant type 1 diabetes is common in Japanese and most East Asian populations, where the *DRB1*04:05-DQB1*04:01* haplotype is common in the general population, but is absent or extremely rare in Caucasian populations, where the *DRB1*04:05-DQB1*04:01* haplotype is also absent or very rare.

HLA in Autoimmune Thyroid Diseases Complicated with Islet Autoimmunity

Patients with type 1 diabetes frequently develop other organ-specific autoimmune diseases, of which autoimmune thyroid diseases (AITD) are the most frequent disorder[31,32]. In contrast to the large number of studies on autoimmunity against the thyroid gland in patients with type 1 diabetes, little is known about the anti-islet autoimmune status in patients with AITD. We recently studied the anti-islet autoimmune status in patients with AITD, and the clinical and genetic characteristics of AITD patients with anti-islet autoimmunity[33]. The prevalence of anti-islet autoimmunity as assessed by GAD Ab was significantly higher in patients with AITD than in normal control subjects. AITD patients with GAD Ab showed a significantly higher frequency of diabetes than did those without GAD Ab, and this was more pronounced in patients with a high titer of GAD Ab. Diabetes in AITD patients with GAD Ab was characterized by younger age-at-onset, lower BMI, higher HbA1c and higher frequency of insulin treatment than that in patients without GAD Ab, suggesting that diabetes in AITD patients positive for GAD Ab shows the clinical features of type 1 diabetes.

The *DRB1*04:05-DQB1*04:01* haplotype, which confers susceptibility to type 1 diabetes, was associated with AITD positive for GAD Ab, but not with AITD negative for GAD Ab[33], suggesting that the *DRB1*04:05-DQB1*04:01* haplotype is associated with anti-islet autoimmunity in subjects with as well as without AITD. In contrast, the *DRB1*08:03-DQB1*06:01* haplotype was associated with AITD without GAD Ab, but not with AITD with GAD Ab[33], suggesting that the *DRB1*08:03-DQB1*06:01* haplotype confers susceptibility to autoimmunity against the thyroid gland, but not anti-islet autoimmunity. These data suggest the contribution of HLA haplotypes not only to immune regulation, but also to organ specificity in autoimmune diseases, with *DRB1*04:05-DQB1*04:01* contributing to beta-cell specificity of the destructive process by an autoimmune mechanism in type 1A diabetes and in AITD with anti-islet autoimmunity, as well as an idiopathic mechanism in type 1B (fulminant) diabetes.

INSULIN GENE-RELATED PATHWAY

Cis Regulatory Region: *INS*-VNTR

Accumulating lines of evidence suggest that insulin is a primary autoantigen in type 1 diabetes[34-37]. Association of the insulin gene region with type 1 diabetes has been repeatedly reported in Caucasian populations[6,38-41]. Allelic variation in the variable number of tandem repeats (VNTR) located in the 5′ upstream region of *INS* has been suggested to be responsible for disease susceptibility[39,40]. In the Japanese population, the markedly high frequency (>90%) of disease-susceptible haplotype in the general population made it difficult to demonstrate the contribution of *INS* to disease susceptibility[23]. Recent large scale studies, however, demonstrated that *INS*-VNTR is associated with type 1 diabetes in Japanese as well as in Caucasian populations[18]. *INS*-VNTR is thought to contribute to type 1 diabetes susceptibility through reduced expression of insulin in the thymus, leading to impaired negative selection of insulin-specific autoreactive T-cells[41,42].

Trans-acting factor

In contrast to the association of the cis-regulatory region of *INS* with type 1 diabetes in humans, such variants have not been identified in the NOD mouse, an animal model of type 1 diabetes. Since expression of insulin is regulated not only by cis-regulatory elements, but also trans-acting factors, we studied the expression of beta-cell specific transcription factors in the thymus[43]. Among beta-cell-specific transcription factors, such as Pdx-1, Neurod 1, and MafA, only MafA was expressed in the thymus[43]. Functional polymorphisms of MafA were newly identified in the NOD mouse, which were associated with reduced expression of insulin in the thymus and susceptibility to type 1 diabetes in the NOD mouse[43]. Functional polymorphisms of human MAFA were also identified and shown to be associated with type 1 diabetes[43], suggesting that antigen-specific transcriptional factors play a critical role in induction of central tolerance to self antigens, and abnormality in such regulation may lead to organ-specific autoimmune diseases (Figure 4).

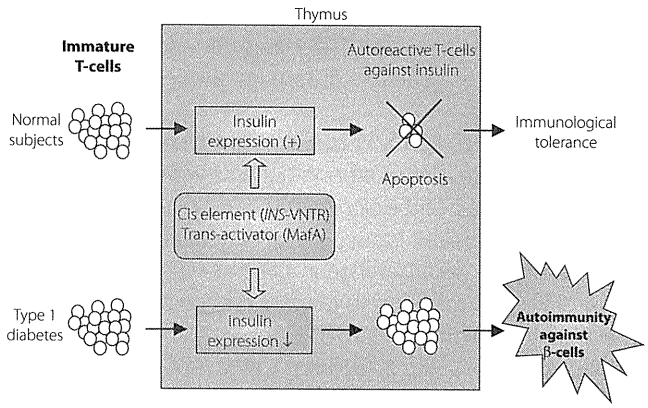

Figure 4 | Expression of insulin in thymus and negative selection of autoreactive T-cells against insulin. In subjects whose expression level of insulin is normal in the thymus, apoptosis is induced in insulin-specific T-cells upon recognition of insulin in the thymus, leading to negative selection of autoreactive T-cells and induction of central tolerance to insulin. In subjects with reduced expression of insulin in the thymus, negative selection of insulin-specific T cells is impaired, resulting in autoimmune attack against insulin-producing beta-cells of the pancreas and development of type 1 diabetes. Intra-thymic expression of insulin is regulated by cis-regulatory elements, such as *INS*-VNTR, and trans-acting factors, such as MafA. Functional variants in these elements or factors thus cause autoimmunity against pancreatic beta-cells through impaired negative selection of insulin-specific T-cells.

CONCLUSIONS

Identification of genes conferring susceptibility to type 1 diabetes is important, even if the effect of each gene is small, because each gene contributes to a step or steps in the etiological pathway, and modification of the function of the gene or gene product could contribute to prevention and intervention of the disease. In particular, genes that contribute to tissue specificity of the autoimmune process, as in the case of genes involved in the regulatory pathway of intra-thymic expression of insulin, are important because they are targets for tissue-specific prevention and intervention of autoimmunity in type 1 diabetes. Although a large number of loci have been mapped in Caucasian populations by GWAS, most of them are still loci, but not responsible genes. It will be a formidable challenge to identify genes responsible for susceptibility loci mapped by GWAS because of the relatively small effect of each locus and the multifactorial nature of the disease. To overcome this, GWAS must be performed in a population possessing haplotypes different from those in Caucasians, and genes should be identified by trans-racial studies. Such studies are now underway as a nationwide effort by the Committee on Type 1 Diabetes of the Japan Diabetes Society.

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Clinical and genetic characteristics of patients with autoimmune thyroid disease with anti-islet autoimmunity

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Abstract

In contrast to the large number of studies on autoimmunity against the thyroid gland in patients with type 1 diabetes mellitus, little is known about the anti-islet autoimmune status in patients with autoimmune thyroid diseases (AITDs). We therefore studied the anti-islet autoimmune status in patients with AITD and the clinical and genetic characteristics of AITD patients with anti-islet autoimmunity. The positivity and titer of glutamic acid decarboxylase antibody (GAD Ab) were studied in 866 Japanese patients with AITD (546 with Graves disease and 320 with Hashimoto thyroiditis), 221 patients with thyroid disease of nonautoimmune origin, and 282 control subjects. The clinical characteristics and genotypes of *HLA-DRB1*, *DQB1*, and *CTLA4* were compared between AITD patients with and without GAD Ab. The prevalence of GAD Ab was significantly higher in AITD patients than in control subjects (5.8% vs 2.1%, $P = .01$), particularly in Graves disease (7.1% vs 2.1%, $P = .0019$). The prevalence of diabetes mellitus was significantly higher in AITD patients with GAD Ab than in those without (40.0% vs 10.1%, $P < .0001$), particularly in those with a high titer of GAD Ab (high vs low titer: 64% vs 16%, $P = .001$) and also in those positive for insulinoma-associated antigen 2 (IA-2) Ab (IA-2 positive vs negative: 75.0% vs 31.3%, $P = .016$). The AITD patients with GAD Ab were characterized by younger age at onset of diabetes, lower body mass index, higher hemoglobin A_{1c} level, and higher frequency of insulin therapy than those without GAD Ab. The frequency of the *DRB1*0405-DQB1*0401* haplotype was significantly higher in AITD patients with GAD Ab than in those without GAD Ab and control subjects. A single nucleotide polymorphism (rs3087243) of *CTLA4* was significantly associated with AITD, but not with positivity of GAD Ab. These results indicate that patients with AITD, and in particular Graves disease, are prone to develop β-cell autoimmunity and insulin-requiring diabetes, particularly those with a high titer of GAD Ab and/or positive for both GAD and IA-2 Ab. Glutamic acid decarboxylase Ab positivity in AITD patients was associated with HLA, conferring susceptibility to type 1 diabetes mellitus.

1. Introduction

Both type 1 diabetes mellitus and autoimmune thyroid diseases (AITDs), including Graves disease and Hashimoto thyroiditis, are organ-specific autoimmune diseases affecting insulin-producing β-cells of the pancreas and the thyroid gland, respectively. Both are multifactorial diseases caused by a complex interaction of genetic and environmental factors, with genetic factors consisting of multiple susceptibility genes. Among susceptibility genes, HLA and CTLA4 polymorphisms have been reported to be associated with type 1 diabetes mellitus as well as AITD [1-9].

Patients with type 1 diabetes mellitus frequently develop other organ-specific autoimmune diseases, of which AITD is the most frequent disorder [10-12]. Type 1 diabetes mellitus patients complicated by AITD show some differences in clinical and genetic characteristics from those without AITD. Clinically, type 1 diabetes mellitus patients with AITD have been reported to have glutamic acid decarboxylase autoantibodies (GAD Abs) for a longer time and at higher titers

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than patients without AITD [11]. Genetically, the association with *CTLA4* was reported to be concentrated in patients with type 1 diabetes mellitus complicated by AITD [9]. In contrast to the large number of studies on autoimmunity against the thyroid gland in patients with type 1 diabetes mellitus, little is known about the anti-islet autoimmune status in patients with AITD. We therefore studied the anti-islet autoimmune status in patients with AITD, and the clinical and genetic characteristics of AITD patients with anti-islet autoimmunity were compared with those of patients without.

2. Methods

2.1. Subjects

A total of 866 Japanese patients with AITD (695 female and 171 male; age [mean ± SD], 50.2 ± 16.3 years; body mass index [BMI], 21.7 ± 3.3 kg/m^2) were studied. The AITD patients consisted of 546 patients with Graves disease (422 female and, 124 male; age, 45.8 ± 15.6 years; BMI, 21.2 ± 3.1 kg/m^2) and 320 patients with Hashimoto thyroiditis (273 female and 47 male; age, 57.8 ± 14.5 years; BMI, 22.7 ± 3.5 kg/m^2). Autoimmune thyroid diseases was diagnosed clinically by endocrinologists and confirmed by abnormal levels of thyroid hormones and autoantibodies to thyrotropin receptor, thyroid peroxidase, and/or thyroglobulin. As a control group, 221 patients (age, 57.1 ± 16.0 years; BMI, 21.8 ± 3.2 kg/m^2) with thyroid disease of nonautoimmune origin such as subacute thyroiditis or thyroid nodules and 282 healthy control subjects (age, 52.4 ± 14.4 years; BMI, 22.6 ± 3.3 kg/m^2) who underwent annual health checkup were also studied. The serum thyrotropin level and status of thyroid autoantibodies were not investigated in control subjects. Positivity of GAD Ab and its titer in patients with AITD were compared with those in patients without thyroid autoimmunity. The status of diabetes mellitus and other clinical characteristics as well as the genetic characteristics in patients with AITD positive for GAD Ab were compared with those in patients negative for GAD Ab. This study was approved by the appropriate ethical committees, and informed consent was obtained from all participants.

2.2. Methods

2.2.1. Autoantibody assay

Glutamic acid decarboxylase Ab was measured by a commercially available radioimmunoassay kit using ^{125}I-labeled recombinant human GAD65 as a tracer reagent (Cosmic, Tokyo, Japan) [13]. Samples were defined as GAD Ab positive when the level was higher than a threshold of 1.5 U/mL as suggested by the manufacturer [14,15]. This assay had a sensitivity of 82% and specificity of 92% in the first Proficiency Test of Diabetes Autoantibody Standardization Programs organized by the Immunology of Diabetes Society [16].

Autoantibody to insulinoma-associated antigen 2 (IA-2 Ab) was measured by an immunoprecipitation assay using ^{125}I-labeled IA-2 [17]. Samples were defined as IA-2 Ab positive when the level was higher than 1.0 U/mL [17].

2.2.2. Genotyping of HLA-DR, -DQ, and CTLA4

The *HLA-DRB1* and *-DQB1* alleles were genotyped in all AITD patients positive for GAD Ab (n = 42) as well as in age- and sex-matched AITD patients negative for GAD Ab (n = 158). *DRB1* and *DQB1* data from healthy subjects in our previous study (n = 230) [8] served as controls. *DRB1* and *DQB1* were genotyped by polymerase chain reaction–restriction fragment length polymorphism and polymerase chain reaction–sequence-specific oligonucleotide (SSO) and/or sequence-based typing (SBT) methods as described previously [8,18-20]. Haplotypes were determined based on the most probable haplotypes according to the linkage disequilibria in the Japanese population [21,22].

A single nucleotide polymorphism (SNP) in the CTLA-4 gene, rs3087243 (+6230G>A), which has been reported to be associated with type 1 diabetes mellitus as well as AITD [6,7,9], was genotyped in 189 patients with AITD as reported previously [9]. Genotype data of healthy subjects in our previous study served as control [9].

2.2.3. Statistical analysis

χ^2 test and Fisher exact probably test were used to determine the significance of differences in the distribution of the number of subjects and alleles. Student *t* test was used to compare the levels of clinical parameters. Statistical significance was defined as $P < .05$.

3. Results

3.1. Prevalence and clinical characteristics of AITD patients positive for GAD Ab

The prevalence of positivity for GAD Ab in AITD patients was significantly higher than that in healthy control subjects (5.8% vs 2.1%, $P = .01$) (Table 1). The prevalence in patients with Graves disease was significantly higher than that in control subjects (7.1% vs 2.1%, $P = .0019$) as well as in patients with Hashimoto thyroiditis (7.1% vs 3.4%, $P = .02$) (Table 1). The prevalence in patients with Hashimoto thyroiditis was slightly, but not significantly, higher than that in healthy control subjects (3.4% vs 2.1%, not significant [NS]). To confirm the positivity of GAD Ab, 42 AITD

Table 1
Frequency of subjects positive for GAD antibodies

			GAD Ab (+) n (%)	P^a (vs control)
AITD	Total	(n = 866)	50 (5.8%)	.01
	Graves disease	(n = 546)	39 (7.1%)	.0019
	Hashimoto thyroiditis	(n = 320)	11 (3.4%)	NS
Controls		(n = 282)	6 (2.1%)	

[a] χ^2 test.

patients positive for GAD Ab were repeatedly tested for GAD Ab. All but 2 patients positive for GAD Ab at the first test were positive for GAD Ab at the second test (Supplementary Figure 1). The 2 patients who became negative for GAD Ab at the second test had a low titer (1.6 and 1.5 U/mL) of GAD Ab at the first test (Supplementary Figure 1), suggesting the importance of the titer in studying the prevalence of GAD positivity.

To compare the titer of GAD Ab in patients with AITD with that in other conditions, patients with thyroid diseases of nonautoimmune origin (n = 221) were tested for GAD Ab. Although the prevalence of GAD Ab was not significantly different between AITD patients and patients with thyroid diseases of nonautoimmune origin (5.8% vs 4.5%), the titer of GAD Ab was markedly higher in AITD than in other conditions (Fig. 1). None of the patients with thyroid diseases of nonautoimmune origin as well as healthy control subjects showed a titer higher than 10 U/mL, whereas 23 (46%) of 50 AITD patients positive for GAD Ab showed a titer higher than 10 U/mL ($P = .017$).

The prevalence of diabetes mellitus was significantly higher in AITD patients positive for GAD Ab than in those negative for GAD Ab (40.0% vs 10.1%, $P < .0001$). When AITD patients positive for GAD Ab were divided into 2 groups according to the titer of GAD Ab, a high-titer group and a low-titer group, so that each group contained approximately the same number of subjects, the prevalence of diabetes mellitus was significantly higher in the high-titer group than in the low-titer group (64% vs 16%, $P = .001$). When the clinical characteristics of AITD patients with diabetes mellitus were compared between those with and without GAD Ab, age at onset of diabetes mellitus was significantly younger, BMI was significantly lower, and hemoglobin A_{1c} (HbA_{1c}) level and the frequency of patients treated with insulin were significantly higher in patients with GAD Ab than in those without (Table 2).

To study the positivity of other islet-related autoantibodies, we measured IA-2 Ab in patients who were

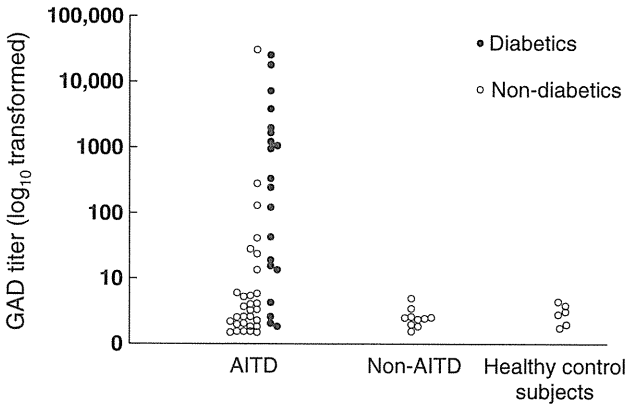

Fig. 1. Titer of GAD antibodies in antibody-positive patients with AITD, patients with thyroid diseases of nonautoimmune origin, and healthy control subjects. Closed circles, patients with diabetes; open circles, nondiabetic patients.

Table 2
Clinical characteristics of AITD patients with diabetes mellitus relative to positivity of GAD antibodies

	GAD Ab (+) (n = 20)	GAD Ab (−) (n = 83)	*P*
GAD Ab titer (mean U/mL) (range)	3176.8 (1.9-26100)		
Graves disease/Hashimoto thyroiditis	17/3	42/41	.006
Age at onset of diabetes (y)	43.2 ± 10.2	53.3 ± 14.2	.004
BMI (kg/m^2)	20.4 ± 3.8	24.1 ± 4.8	.002
HbA_{1c} (%)	8.8 ± 2.3	7.0 ± 1.3	<.0001
Treatment with insulin	85.0%	14.4%	<.0001

Fisher exact probability test for number of patients and Student *t* test for clinical parameters.

positive for GAD Ab. Among 44 patients with GAD Ab, 12 patients (27.3%) were also positive for IA-2 Ab. When positivity of IA-2 Ab was studied relative to the titer of GAD Ab, all 12 patients with IA-2 Ab had a high titer of GAD Ab (mean, 1439; range, 15.7-7310 U/mL). The prevalence of diabetes was significantly higher in patients with both GAD and IA-2 Ab than in patients with GAD Ab alone (75.0% vs 31.2%, $P = .016$).

To study the impact of age on positivity of islet-related autoantibodies, AITD patients who were younger than 30 years (n = 99) were compared with those 30 years or older (n = 767). The prevalence of GAD Ab in younger patients was not significantly different from that in older patients (4.0% vs 6.0%, NS).

3.2. Genetic background

The frequencies of the *DRB1*0405* and *DQB1*0401* alleles and *DRB1*0405-DQB1*0401* haplotype were significantly higher in AITD patients with GAD Ab than in those without GAD Ab and control subjects (Table 3, Supplemental Tables 1 and 2). When HLA in AITD patients positive for GAD Ab was compared between those with and without diabetes, the frequencies of haplotypes known to confer susceptibility to type 1 diabetes mellitus in Japanese, *DRB1*0405-DQB1*0401* and *DRB1*0901-DQB1*0303*, tended to be higher and those of haplotypes known to provide protection against type 1 diabetes mellitus in Japanese, *DRB1*1501-DQB1*0602* and *DRB1*1502-DQB1*0601*, tended to be lower in diabetic patients than in nondiabetic patients (Table 4). The frequencies of genotypes with 2 doses of susceptible haplotypes (*DRB1*0405-DQB1*0401* and *DRB1*0901- DQB1*0303*) were significantly higher in diabetic patients than in nondiabetic patients (52.6% vs 17.4%, $P < .05$). The frequencies of genotypes with at least 1 dose of resistant haplotypes (*DRB1*1501-DQB1*0602* or *DRB1*1502-DQB1*0601*) tended to be lower in diabetic patients than in nondiabetic patients (5.3% vs 30.4%, $P = .05$) (Table 4).

The frequencies of the *DRB1*0803* allele and *DRB1*0803-DQB1*0601* haplotype were significantly

Table 3
Frequency of *DRB1-DQB1* haplotype in patients with AITD with and without GAD Ab and control subjects

DRB1-DQB1	AITD		Control (n = 230)c	*P* (*Pc*[a])		
	GAD (+) (n = 42)	GAD (−) (n = 158)		GAD (+) vs GAD (−)	GAD (+) vs control	GAD (−) vs control
0101-0501	1 (2.4)	8 (5.1)	37 (16.1)	NS	.01 (NS)	.0009 (.013)
0403-0302	1 (2.4)	11 (7.0)	9 (3.9)	NS	NS	NS
0405-0401	25 (59.5)	50 (31.6)	67 (29.1)	.0009 (.013)	.0001 (.0014)	NS
0406-0302	0 (0)	11 (7.0)	14 (6.1)	NS	NS	NS
0410-0402	3 (7.1)	8 (5.1)	6 (2.6)	NS	NS	NS
0802-0302	1 (2.4)	6 (3.8)	4 (1.7)	NS	NS	NS
0802-0402	4 (9.5)	3 (1.9)	9 (3.9)	.04 (NS)	NS	NS
0803-0601	7 (16.7)	44 (27.8)	32 (13.9)	NS	NS	.0007 (.0098)
0901-0303	14 (33.3)	31 (19.6)	59 (25.7)	NS	NS	NS
1101-0301	0 (0.0)	13 (8.2)	11 (4.8)	NS	NS	NS
1201-0301	1 (2.4)	9 (5.7)	16 (7.0)	NS	NS	NS
1302-0604	1 (2.4)	13 (8.2)	27 (11.7)	NS	NS	NS
1501-0602	4 (9.5)	26 (16.5)	20 (8.7)	NS	NS	.02 (NS)
1502-0601	4 (9.5)	21 (13.3)	54 (23.5)	NS	.04 (NS)	.01 (NS)
Others[b]	9 (21.4)	46 (29.1)	75 (32.6)			

Data are number (percentage) of subjects.
[a] *P* value corrected for number of haplotypes tested (n = 14).
[b] Haplotypes with frequency <5% in each group.

higher in AITD patients negative for GAD Ab, but not in those positive for GAD Ab, than in control subjects (Table 3, Supplemental Tables 1 and 2). When GAD Ab–positive patients with and without diabetes were compared, the frequency of the *DRB1*0803-DQB1*0601* haplotype was significantly higher in nondiabetic patients (30.4%) than in diabetic patients (30.4% vs 0.0%, $P = .009$) (Table 4). The frequency of the *DRB1*0101-DQB1*0501* haplotype was significantly lower in AITD patients negative for GAD Ab than in control subjects.

The SNP rs3087243 (+6230G>A) of *CTLA4* was significantly associated with AITD. The frequency of the G allele was significantly higher in AITD patients than in control subjects (odds ratio, 1.41; 95% confidence interval, 1.07-1.87; $P = .016$) (Supplemental Table 3). No significant difference was observed in the frequency of the *CTLA4* genotype between AITD patients with and without GAD Ab (odds ratio, 1.12; 95% confidence interval, 0.58-2.19; NS).

4. Discussion

The present study demonstrated that the prevalence of anti-islet autoimmunity as assessed by GAD Ab was significantly higher in patients with AITD than in healthy control subjects. The AITD patients with GAD Ab showed a significantly higher frequency (40% vs 10%) of diabetes than those without GAD Ab; and this was more pronounced

Table 4
Frequencies of *DRB1-DQB1* haplotypes and genotypic combinations of haplotypes in AITD patients relative to GAD Ab and diabetes status

DRB1-DQB1	GAD (+)		GAD (−) (n = 158) (C)	Control (n = 230) (D)	*P* value			
	DM (+) (n = 19) (A)	DM (−) (n = 23) (B)			A vs B	A vs C	A vs D	B vs D
Haplotypes								
0405-0401	13 (68.4)	12 (52.2)	50 (31.6)	67 (29.1)	NS	.002	.0004	.023
0802-0302	0 (0.0)	1 (4.3)	6 (3.8)	4 (1.7)	NS	NS	NS	NS
0803-0601	0 (0.0)	7 (30.4)	44 (27.8)	32 (13.9)	.009	.008	NS	.036
0901-0303	9 (47.4)	5 (21.7)	31 (19.6)	59 (25.7)	NS	.01	NS	NS
1501-0602	0 (0)	4 (17.4)	26 (16.5)	20 (8.7)	NS	NS	NS	NS
1502-0601	1 (5.3)	3 (13.0)	21 (13.3)	54 (23.5)	NS	NS	NS	NS
Genotypes								
S/S	10 (52.6)	4 (17.4)	13 (8.2)	18 (7.8)	.02	<.0001	<.0001	NS
S/X	7 (36.8)	5 (21.7)	52 (32.9)	76 (33.0)	NS	NS	NS	NS
X/X	1 (5.3)	3 (13.0)	47 (29.7)	64 (27.8)	NS	.03	.03	NS
P/Y	1 (5.3)	7 (30.4)	46 (29.1)	72 (31.3)	.05	.03	.02	

Data are number (percentage) of subjects. S indicates haplotypes that confer susceptibility to type 1 diabetes mellitus, *DRB1*0405-DQB1*0401* and *DRB1*0901-DQB1*0303*; P, haplotypes that provide protection against type 1 diabetes mellitus, *DRB1*1501 -DQB1*0602* and *DRB1*1502-DQB1*0601*; X, haplotypes other than S or P; Y, any haplotype.

in patients with a high titer of GAD Ab, as shown by the prevalence of diabetes of 64% in the high-titer group as compared with 16% in the low-titer group. Diabetes in AITD patients with GAD Ab was characterized by younger age at onset, lower BMI, higher HbA_{1c}, and higher frequency of insulin treatment than that in patients without GAD Ab, suggesting that diabetes in AITD patients positive for GAD Ab shows the clinical features of type 1 diabetes mellitus.

The prevalence of GAD Ab in patients with AITD was 5.8%, which was significantly higher than that in healthy control participants in this study (2.1%) as well as the previously reported prevalence in subjects with normal glucose tolerance (0.6%) [23]. In particular, the prevalence of GAD Ab in patients with Graves disease was much higher than that in control subjects (7.1% vs 2.1%, $P < .0019$) and was similar to the prevalence reported for Graves disease in previous studies [24-26]. The prevalence of GAD Ab in Hashimoto thyroiditis, on the other hand, was slightly, but not significantly, higher than that in control subjects, indicating that the increased prevalence of GAD Ab in AITD in the present study was mostly due to its increase in Graves disease.

In addition to positivity, the importance of the titer of GAD Ab was indicated by the marked difference in titer; but the prevalence of GAD Ab positivity was similar in AITD patients and patients with thyroid diseases of nonautoimmune origin in the present study (Fig. 1). None of the patients with thyroid diseases of nonautoimmune origin positive for GAD Ab showed a titer higher than 10 U/mL, whereas 23 (46%) of 50 AITD patients positive for GAD Ab showed a titer higher than 10 U/mL. None of the GAD-positive patients with thyroid diseases of nonautoimmune origin developed diabetes, whereas 40% of GAD-positive AITD patients developed diabetes. Even within AITD patients, the frequency of diabetes correlated with the titer of GAD Ab, with a 4-fold higher prevalence in the high-titer group than in the low-titer group. The titer in healthy control subjects positive for GAD Ab was also less than 10 U/mL (Fig. 1). A high titer of GAD Ab (>10 U/mL) was previously reported to be a marker for activated T-cell response to β-cell destruction and a high risk for progression to insulin dependence in adult-onset patients with diabetes mellitus [14,27,28]. These data indicate the importance of the titer in addition to positivity of GAD Ab in evaluating anti-islet autoimmunity and β-cell destruction in patients with AITD, as in the case of patients with adult-onset diabetes mellitus.

In addition to a high titer of GAD Ab, positivity for multiple islet-related autoantibodies has been reported to more strongly predict insulin requirement in adult diabetic patients [27]. Among AITD patients positive for GAD Ab in the present study, 27.3% were also positive for IA-2 Ab. The frequency of diabetes was significantly higher in patients with both GAD and IA-2 Ab than in those with GAD Ab alone. All AITD patients positive for both GAD Ab and IA-2 Ab had a high titer of GAD Ab (>10 U/mL). These data suggest that AITD patients with a high titer of GAD Ab and/or patients positive for multiple autoantibodies are at risk for the development of diabetes. Because the present study is a cross-sectional study, prospective studies on diabetes and β-cell function in AITD patients positive for GAD Ab are necessary to further clarify whether or not nondiabetic AITD patients positive for GAD Ab develop diabetes and progress to insulin deficiency.

Genetic analysis of HLA in AITD patients with GAD Ab showed high frequencies of haplotypes known to confer susceptibility to type 1 diabetes mellitus and low frequencies of haplotypes known to provide protection against type 1 diabetes mellitus, indicating that AITD patients positive for GAD Ab differed genetically from those negative for GAD Ab, showing HLA genotypes typical of those in type 1 diabetes mellitus. These data, together with the clinical characteristics of patients with AITD positive for GAD Ab, suggest that AITD patients with GAD Ab have the genetic and clinical characteristics of type 1 diabetes mellitus.

The *DRB1*0803-DQB1*0601* haplotype was associated with AITD without GAD Ab, but not with AITD with GAD Ab, suggesting that the *DRB1*0803-DQB1*0601* haplotype confers susceptibility to autoimmunity against the thyroid gland, but not anti-islet autoimmunity. The association of the *DRB1*0803-DQB1*0601* haplotype with Graves disease has previously been reported in Japanese [4,18] and Korean populations [29]. In contrast, the *DRB1*0405-DQB1*0401* haplotype, which confers susceptibility to type 1 diabetes mellitus, was associated with AITD positive for GAD Ab, but not with AITD negative for GAD Ab, suggesting that the *DRB1*0405-DQB1*0401* haplotype is associated with anti-islet autoimmunity in subjects with as well as without AITD.

In addition to HLA, *CTLA4* has been reported to be associated with both AITD and type 1 diabetes mellitus. Previous studies showed that the association of *CTLA4* with type 1 diabetes mellitus was concentrated in patients complicated by AITD [9]. In fact, it was suggested that the weak association of *CTLA4* with type 1 diabetes mellitus may be secondary to the strong association of *CTLA4* with AITD and the high frequency of AITD complicated by type 1 diabetes mellitus. In the present study, *CTLA4* was associated with AITD as a whole; but no association with GAD Ab positivity was observed in AITD patients. These data further support the possibility that *CTLA4* is primarily associated with AITD; but the contribution of *CTLA4* to anti-islet autoimmunity and type 1 diabetes mellitus is weak, if any.

In conclusion, the present study demonstrated that the prevalence of GAD Ab was high, 5.8%, in AITD patients, and in Graves disease in particular (7.1%), and that 40% of these patients were diabetic, with clinical and genetic characteristics suggestive of type 1 diabetes mellitus, suggesting that AITD patients are prone to develop β-cell autoimmunity and insulin-requiring diabetes, in particular in those with a high titer of GAD Ab and/or positive for both GAD and IA-2 Ab. Prospective follow-up studies in nondiabetic patients with AITD positive for GAD Ab are

necessary to clarify the factors contributing to the development of diabetes, β-cell destruction, and insulin deficiency.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.metabol.2010.07.025.

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Highly Parallel and Short-Acting Amplification with Locus-Specific Primers to Detect Single Nucleotide Polymorphisms by the DigiTag2 Assay

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Abstract

The DigiTag2 assay enables analysis of a set of 96 SNPs using Kapa 2GFast HotStart DNA polymerase with a new protocol that has a total running time of about 7 hours, which is 6 hours shorter than the previous protocol. Quality parameters (conversion rate, call rate, reproducibility and concordance) were at the same levels as when genotype calls were acquired using the previous protocol. Multiplex PCR with 192 pairs of locus-specific primers was available for target preparation in the DigiTag2 assay without the optimization of reaction conditions, and quality parameters had the same levels as those acquired with 96-plex PCR. The locus-specific primers were able to achieve sufficient (concentration of target amplicon $\geq$5 nM) and specific (concentration of unexpected amplicons $<$2 nM) amplification within 2 hours, were also able to achieve detectable amplifications even when working in a 96-plex or 192-plex form. The improved DigiTag2 assay will be an efficient platform for screening an intermediate number of SNPs (tens to hundreds of sites) in the replication analysis after genome-wide association study. Moreover, highly parallel and short-acting amplification with locus-specific primers may thus facilitate widespread application to other PCR-based assays.

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Introduction

Polymerase chain reaction (PCR) is a commonly used technique in molecular biology. Several previously developed methods have employed multiplexed PCR in order to analyze genomic variations such as microsatellites or short tandem repeats (STRs), single nucleotide polymorphisms (SNPs) and insertions/deletions [1–3]. Multiplexed preparation of DNA templates in a single reaction is cost-effective, saving starting materials and run-time, while requiring careful optimization of assay conditions. The optimization process is highly empirical and time consuming, and depending on the combinations of markers, may or may not lead to successful assay development. For the conventional design of multiplex PCR, optimization of reaction conditions and careful pre-selection of targets are required in order to prevent excessive off-target priming by the numerous primers in the reaction. Moreover, the risk of generating errors in multiplex PCR, such as insufficient amplification, biased amplification and considerable primer-dimer formation within primers, tends to increase roughly as the square of the number of added primer pairs [4].

There are several approaches to resolving these drawbacks, including solid-phase assay formats (glass slide arrays, microbeads), oligonucleotides containing locked nucleic acid (LNA) residues and circularized amplification. Primers immobilized on the surface of the solid phase appear to markedly increase product yield on solid supports and may avoid the need for target pre-selection with a modification to enrich the input genomic DNA via a crude solution-phase multiplex PCR [5,6]. LNA pentamers showed high priming efficiency to achieve small biased priming in multiplex PCR [7]. Circularized amplification avoids generating artifacts associated with conventional multiplex PCR where two primers are used for each target [8]. This procedure was shown to perform a 96-plex amplification of an arbitrary set of specific DNA sequences. The arrayed primer extension-based genotyping method (APEX-2) allows efficient homogeneous 640-plex DNA amplification with locus-specific primers [9]. These approaches show effective consequences for multiplex amplification, however, a small number of approaches are practically used in the field of molecular genetics, presumably due to its cost and time consuming steps in preparation.

We developed the DigiTag2 assay for multiplex SNP typing as a simple and cost effective approach by combining multiplex PCR to enrich genetic regions including the target SNPs and an oligonucleotide ligation assay to encode all of the SNP genotypes into well-designed oligonucleotides designated DNA coded numbers (DCNs) [10]. For an effective primer design for multiplex PCR, there are several important physical properties for primer sequences, including melting temperature, Gibbs energy of duplex between primer and template, and interactions between primers and PCR amplicons. The DNA polymerase enzyme used in a multiplex PCR is one of the important factors for a successful unbiased amplification.

The DigiTag2 assay is a suitable approach to analyze an intermediate number of SNPs (tens to hundreds of locus) in the replication study after genome wide association study [11–12]. However, the most time consuming step for the DigiTag2 assay in a total running time of 13 hours is multiplex PCR for target preparation (5.5 hours). Here, we report an improved protocol for the DigiTag2 assay with a short-acting multiplex PCR through the use of Kapa 2GFast HotStart DNA polymerase, which reduces total running time and increases assay throughput. In this study, we also validate the applicability of the 192-plex PCR with locus specific primers to amplify the target regions from genomic DNA, which leads to save genomic DNA samples.

Methods

DNA samples

Genomic DNA samples from 96 unrelated healthy donors were obtained from the Japan Health Science Research Resources Bank (Osaka, Japan). All donors provided written informed consent and samples were anonymized. One microgram of purified genomic DNA was dissolved in 100 µl of TE buffer (pH 8.0) (Wako, Osaka, Japan), followed by storage at −20°C until use.

Primer design

A total of 192 pairs of primer were designed using the Visual OMP software version 7.1.0.0 (DNA software, Ann Arbor, MI, USA) with relatively long length (35–45-mer; average, 39.5-mer) to give amplicon sizes between 312 bp and 995 bp (average, 589 bp), each of which had an SNP site (Table S1). Prediction of DNA melting temperature was calculated using nearest-neighbor thermodynamic models. To avoid spurious amplification products, we employed a two-step protocol (denature and extension steps) using specifically designed primer pairs with an extension temperature at 68°C. The specificity of primer sequences was verified by Blat search in order to predict its location(s) on the human genome (GRCh37), and to confirm no unexpected SNP(s) within the primer sequence. The specificity of primer pairs was verified using MFE primer software, which can predict potential amplicon(s) generated from the human genome (GRCh37, up to 5 kb in amplicon size) [13]. All oligonucleotides (de-salted, 100 pmol/µl in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)) were purchased from Life Technologies (Carlsbad, CA, USA), and were stored at −20°C.

Multiplex PCR with Kapa 2GFast HotStart DNA polymerase

Multiplex PCR mix had a final volume of 10 µl, including 10 ng of genomic DNA, 25 nM each primer, 1.5× KAPA2G Buffer (including 2.25 mM Mg^{2+}), an additional 2.25 mM Mg^{2+} (final concentration of Mg^{2+}: 4.5 mM), 0.2 mM dNTPs and 0.4 U of Kapa 2GFast HotStart DNA polymerase (Kapa Biosystems, Woburn, MA, USA). PCR amplification was conducted using a TGradient (Biometra, Göttingen, Germany) or PTC-225 (MJ Research, Waltham, MA, USA) as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 68°C for 2 min. When necessary, the fragment length of PCR products was confirmed by capillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent, Santa Clara, CA, USA) in order to evaluate PCR efficiency. The total running times for multiplex PCR with Kapa 2GFast HotStart DNA polymerase using TGradient and PTC-225 were 1 h 48 min 55 s and 2 h 6 min 59 s, respectively.

Multiplex PCR with QIAGEN Multiplex PCR Kit

Multiplex PCR mix had a final volume of 10 µl, including 10 ng of genomic DNA, 25 nM each primer, 1× Multiplex PCR Buffer (including 3.0 mM Mg^{2+}), 0.2 mM dNTPs and HotStarTaq DNA polymerase (QIAGEN Multiplex PCR Kit; QIAGEN, Valencia, CA, USA). PCR amplification was conducted using a TGradient or PTC-225 as follows: 95°C for 15 min, followed by 40 cycles of 95°C for 30 s and 68°C for 6 min. The total running times for multiplex PCR with QIAGEN Multiplex PCR Kit using TGradient and PTC-225 were 5 h 27 min 53 s and 5 h 46 min 39 s, respectively.

96-plex genotyping by the DigiTag2 assay

The DigiTag2 assay performs multiplex SNP typing by encoding all of the SNP genotypes into well-designed oligonucleotides, designated DNA coded numbers (Figure 1, DCNs: D1_i, ED-1 and ED-2) [10]. The DCNs are assigned to the target SNPs in an unconstrained manner; therefore, the DNA chips prepared to read out the types of DCNs are universally available for any type of SNP without optimization of assay conditions. The DigiTag2 assay proceeds in four steps; target preparation, encoding, labeling and detection.

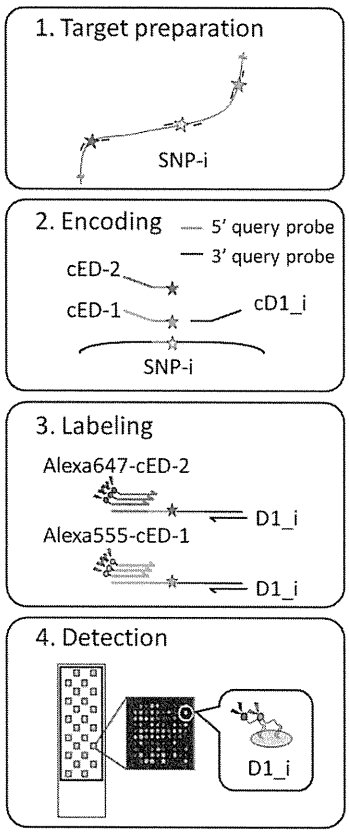

Figure 1. Schematic representation of the DigiTag2 assay. The assay has four steps: target preparation, encoding, labeling and detection. SNP genotypes are encoded into well-designed oligonucleotides, designated DNA coded numbers (DCNs: D1_i, ED-1 and ED-2). D1_i is a variable sequence assigned to each SNP. Reverse complement sequences are written by attaching the character 'c' before the sequence name.
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The encoding reactions had a final volume of 15 μl, including 0.5 μl of multiplex PCR products, 20 mM Tris-HCl, pH 7.6, 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM DTT, 1 mM NAD, 0.1% Triton X-100 (1× Taq DNA ligase buffer) with 0.33 nM of each probe and 5 U Taq DNA ligase (New England BioLabs, Ipswich, MA, USA). Encoding reactions were conducted using a TGradient or PTC-225 under the following conditions: 95°C for 5 min, followed by 58°C for 15 min. The reaction was stopped by holding the temperature at 10°C.

The labeling reactions had a final volume of 12 μl, including 6 μl of ligation products, 0.5 μM each labeled primer (Alexa555-cED-1 and Alexa647-cED-2), 2.5 nM each D1 primer (D1_i), 50 mM KCl, 2 mM Mg^{2+}, 0.1 mM DTT, 0.2 mM each dNTP (N = A, G, C), 0.1 mM [^{3}H]-dTTP, 0.25 mg/ml activated salmon sperm DNA (1× *Ex Taq* Buffer) and 0.05 U of *Ex Taq*™ polymerase (TaKaRa, Shiga, Japan). Labeling reactions were conducted using a TGradient or PTC-225 under the following conditions: first held at 95°C for 1 min, followed by 30 cycles of 95°C for 30 s, 55°C for 6 min and 72°C for 30 s. The reaction was stopped by holding the temperature at 10°C. Total running times for labeling using TGradient and PTC-225 were 3 h 49 min 48 s and 4 h 8 min 48 s, respectively.

In the detection step, a hybridization mixture was prepared by mixing 6.25 μl of labeling products with 8.75 μl of hybridization buffer containing 0.5× SSC, 0.1% SDS, 15% formamide, 1 mM EDTA and 3.125 fmol of hybridization control (Alexa555-labeled D1_100 and Alexa647-labeled D1_100). The hybridization control was prepared for ensuring the hybridization step. Ten microliters of hybridization mixture was applied to each block on the universal DNA chip. Hybridization was carried out for 30 min at 37°C in a hybridization oven (ThermoStat plus; Eppendorf, Ham, Germany). After hybridization, glass slides were washed in washing buffer (0.1× SSC, 0.1% SDS) by shaking at 60 rpm for 3 min. Glass slides were consecutively washed in distilled water by shaking at 60 rpm for 1 min and then dried up by centrifugation at 500× g for 1 min. Hybridization images were scanned at photomultiplier voltages of 400 V for Alexa555 and 480 V for Alexa647 using a commercially available DNA chip scanner and fluorescence image analysis was performed using commercially available software (GenePix 4000B unit and GenePix Pro 4.1 software package; Molecular Devices, Sunnyvale, CA, USA).

Labeling with Kapa 2GFast HotStart DNA polymerase

The labeling reactions with Kapa 2GFast HotStart DNA polymerase had a final volume of 12 μl, including 6 μl of ligation products, 0.5 μM each labeled primer (Alexa555-cED-1 and Alexa647-cED-2), 2.5 nM each D1 primer (D1_i), 1.5× KAPA2G Buffer (including 2.25 mM Mg^{2+}), an additional 2.25 mM Mg^{2+} (final concentration of Mg^{2+}: 4.5 mM), 0.2 mM dNTPs and 0.4 U of Kapa 2GFast HotStart DNA polymerase. Labeling reactions were conducted using a TGradient or PTC-225 under the following conditions: first held at 95°C for 1 min, followed by 30 cycles of 95°C for 15 s, 55°C for 120 s and 72°C for 5 s. The reaction was stopped by holding the temperature at 10°C. The total running times for labeling using TGradient and PTC-225 were 1 h 29 min 48 s and 1 h 48 min 34 s, respectively.

Results

Singleplex PCR using 192 pairs of locus-specific primers

Singleplex PCR was conducted under the same reaction condition with multiplex PCR using 25 ng of genomic DNA to ensure target amplicon detection and to confirm the emergence of extra bands (unexpected amplicons). Singleplex PCR with 192 pairs of locus-specific primers revealed that most of the primer pairs are able to achieve sensitive detection (concentration of target amplicon ≥5 nM) and specific amplification without extra bands (concentration of unexpected amplicons <2 nM) except for 14 pairs of primers; low sensitivity (<5 nM) for 5 pairs of primers (61, 99, 102, 189 and 191) and low specificity with extra bands (≥2 nM) for 9 pairs of primers (40, 56, 62, 70, 91, 106, 149, 173 and 174) (Figure 2 and Table S2). Five pairs among the 9 low-specific primer pairs with extra bands (62, 70, 149, 173 and 174) resulted from heteroduplex formation of target amplicons during polyacrylamide gel electrophoresis. Despite the presence of extra bands, the remaining 4 pairs of low-specific primers had a target amplicon with a detectable concentration ≥5 nM.

Validation of efficacy of 192-plex PCR by 96-plex genotyping with the DigiTag2 assay

The DigiTag2 assay enables the simultaneous analysis of 96 target SNPs in: (1) multiplex PCR with locus-specific primers to amplify target genomic regions including target SNPs; (2) multiple oligonucleotide ligation assay with locus-specific probes to determine the genotype of each SNP; and (3) hybridization to the universal DNA chip tethered with probe sequences identical to D1_i (23-mer) (Figure 1) [10]. The validity of 192-plex PCR was assessed with 96 individual DNAs (population control samples) by comparing two sets of 96-plex genotype calls acquired from 96-plex PCR with those from 192-plex PCR (Table 1).

Conversion rate shows the proportion of successfully genotyped SNPs with fewer than 3 undetected samples after excluding low-quality genotyping data, which had more than 5 undetected SNPs in a total of 96 SNPs. However, the composition of failed SNPs in genotyping was not identical, and the conversion rate showed no differences between 192-plex PCR and 96-plex PCR. For the 1st set of 96 SNPs, 7 SNPs among 10 failed SNPs were matched between 192-plex PCR and 96-plex PCR, and for the 2nd set, 8 SNPs among the 9 failed SNPs were matched. The average call rate for successfully genotyped SNPs was over 99.79% for both sets of 96-plex genotyping, even if 192-plex PCR products were adopted for target preparation. Reproducibility was determined by independent genotyping with 96 individuals twice. As a consequence, four discordant genotype calls were observed in the duplicated genotyping data. Concordance of genotype calls between 192-plex PCR and 96-plex PCR was determined using 6,290 genotype calls for the 1st set and 7,884 genotype calls for the 2nd set. Consequently, 14,171 out of 14,174 genotype calls were matched by comparison with 83 SNPs for the 1st set and 86 SNPs for the 2nd set. In total, 3 discordant genotype calls were observed (Figure 3).

Short-acting multiplex PCR by use of Kapa 2GFast HotStart DNA polymerase

Kapa 2GFast HotStart DNA polymerase was employed to perform multiplex PCR with the locus-specific primers for target preparation in genotyping with the DigiTag2 assay. To optimize reaction conditions with Kapa 2GFast HotStart DNA polymerase, singleplex PCR was conducted using 25 ng of genomic DNA with three randomly chosen pairs of locus-specific primers. The designed amplicon sizes for the three pairs of primers were 501 bp, 671 bp and 492 bp. We performed singleplex PCR using a two-step protocol (denature and extension steps) with varied extension periods (15 s, 30 s, 60 s and 120 s) and with varied Mg^{2+} concentrations (3.0 mM and 4.5 mM) (Figure 4). The most sensitive detection and highest levels of amplification for the three

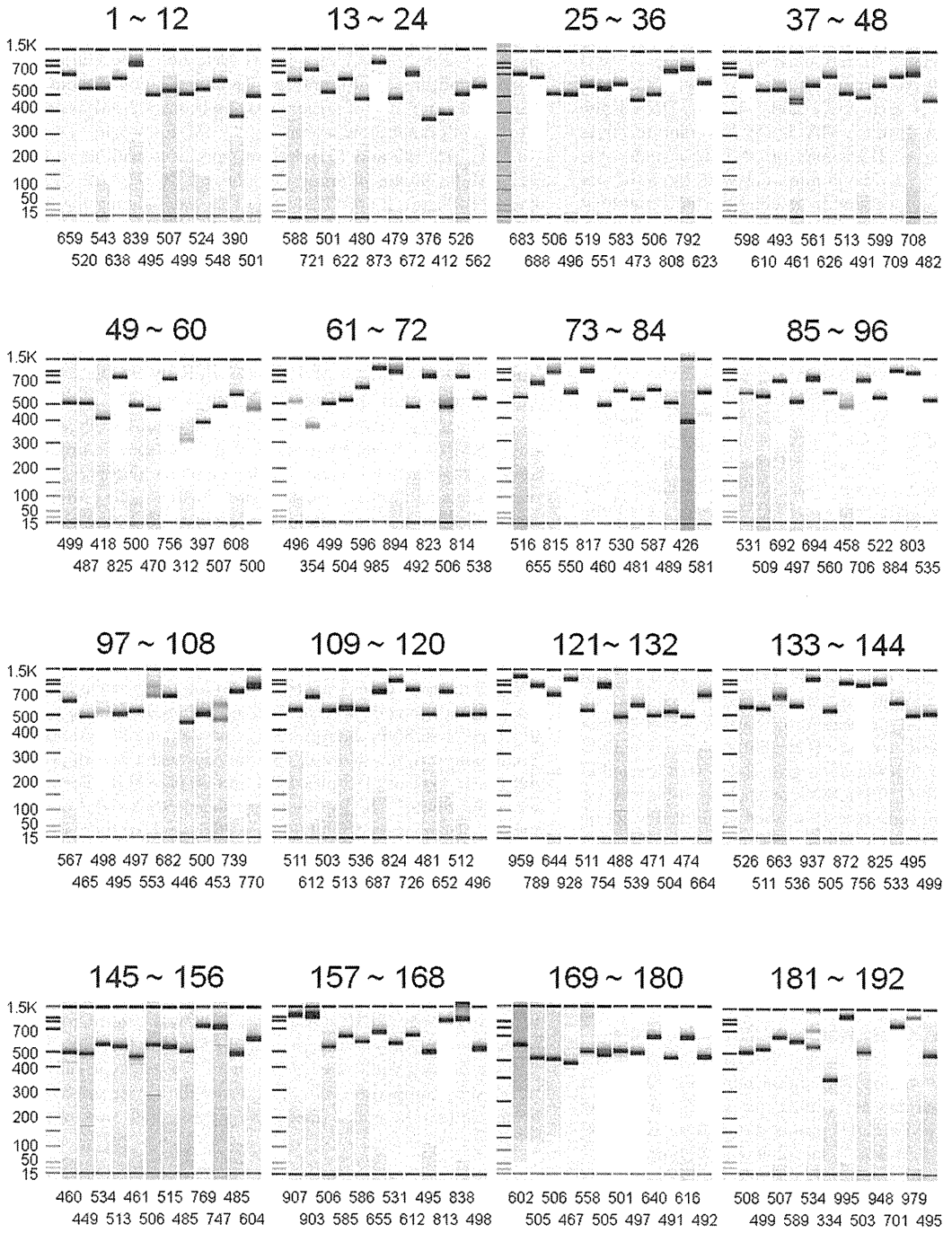

Figure 2. Electropherogram of singleplex PCR products with 192 pairs of locus-specific primers. The designed amplicon size is depicted below each lane.
doi:10.1371/journal.pone.0029967.g002

pairs of primers were observed with 120 s for the extension period and 4.5 mM for the Mg^{2+} concentration. The total running time for multiplex PCR with locus-specific primers was less than 2 hours, which is about 3 h 30 min shorter than the previous protocol (see MATERIALS AND METHODS).

The total running time of the DigiTag2 assay was markedly reduced when the labeling step was also conducted using Kapa 2GFast HotStart DNA polymerase instead of *Ex Taq* polymerase. When the DigiTag2 assay was conducted with Kapa 2GFast HotStart DNA polymerase for multiplex PCR and labeling step, the total running time of the assay was about 7 hours, which is about 6 hours shorter than the previously used protocol in combination with QIAGEN Multiplex PCR Kit for multiplex PCR and *Ex Taq* polymerase for the labeling step.

Table 1. Validation of efficacy of 192-plex PCR by 96-plex genotyping.

		192-plex PCR	96-plex PCR
1st set	Conversion rate	86/96 SNP	86/96 SNP
	Call rate	99.84% (7,728/7,740 genotype)	99.81% (6,695/6,708 genotype)
	reproducibility	99.99% (7,288/7,289 genotype)	100% (6,121/6,121 genotype)
	concordance	99.98% (6,289/6,290 genotype)	
2nd set	Conversion rate	87/96 SNP	87/96 SNP
	Call rate	99.79% (8,074/8,091 genotype)	99.79% (8,161/8,178 genotype)
	reproducibility	99.97% (7,792/7,794 genotype)	99.99% (7,712/7,713 genotype)
	concordance	99.97% (7,882/7,884 genotype)	

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Table 2 summarizes the quality parameters (conversion rate, call rate, reproducibility and concordance) when genotyping was conducted with 192-plex PCR or 96-plex PCR by use of Kapa 2GFast HotStart DNA polymerase. The conversion rate was slightly decreased when multiplex PCR was conducted in 192-plex form. However, the conversion rates were better than those observed when multiplex PCR was conducted with the QIAGEN Multiplex PCR Kit. The composition of failed SNPs in genotyping was not consistent for the 1st set of 96 SNPs, in which 4 SNPs were matched between 192-plex PCR and 96-plex PCR. For the 2nd set, a total of 8 failed SNPs in the 96-plex PCR were completely matched to those in the 192-plex PCR. When the composition of failed SNPs were compared between Kapa 2GFast HotStart DNA polymerase and QIAGEN Multiplex PCR Kit, the 1st set had 5 matched SNPs in a total of 8 failed SNPs for 192-plex PCR, and 4 matched SNPs in 5 failed SNPs for 96-plex PCR. From the 2nd set, 5 SNPs in a total of 9 failed SNPs were matched when 192-plex PCR was conducted and 4 SNPs in a total of 8 failed SNPs were matched when 96-plex PCR was conducted. The average call rate for successfully genotyped SNPs was over 99.76% for both sets of 96-plex genotyping, even if 192-plex PCR products were adopted for target preparation. The reproducibility was 100% for the 2nd set; however, three discordant genotype calls were observed for the 1st set. With regard to the concordance of genotype calls between 96-plex PCR and 192-plex PCR, only one discordant genotype call was observed in the comparison for the 1st set, and no discordant genotype calls were observed in the 2nd set.

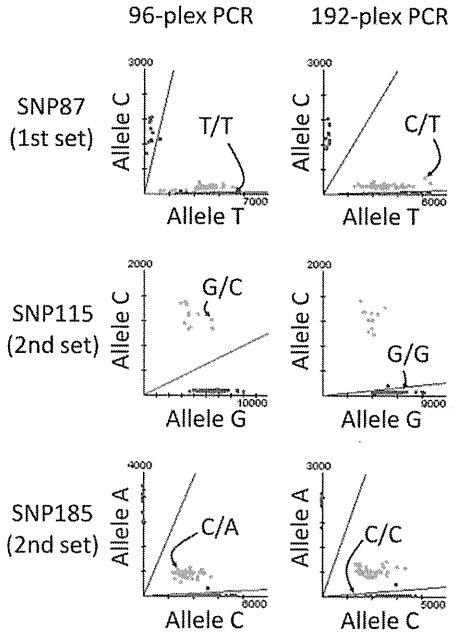

Figure 3. Scatter plots for three SNPs with 3 discordant genotypes. Scatter plots in genotyping with 192-plex PCR and 96-plex PCR are depicted side-by-side. The genotypes of discordant samples are indicated in the scatter plots by arrows.
doi:10.1371/journal.pone.0029967.g003

Table 3 shows the concordance rate in comparison with the genotype calls by the use of Kapa 2GFast HotStart DNA polymerase or QIAGEN Multiplex PCR Kit for multiplex PCR. For the 1st set, there were 4 discordant genotype calls with 96-plex PCR and 8 discordant genotype calls with 192-plex PCR. For the 2nd set of 96 SNPs, there was one discordant genotype call in genotyping with 96-plex PCR and 192-plex PCR.

Discussion

The locus specific primers sufficiently worked in a multiplex form under the same reaction conditions without any optimization processes, either 96-plex PCR or 192-plex PCR. We also found that either 96-plex PCR or 192-plex PCR could be accomplished within two hours through the use of Kapa 2GFast HotStart DNA polymerase. The total running time of the DigiTag2 assay was shortened by 6 hours over the original 13-hour long protocol using Kapa 2GFast HotStart DNA polymerase for both multiplex PCR and the labeling step. The quality parameters (conversion rate, call rate, reproducibility and concordance) observed in genotyping with the new protocol were the same as those observed in the original protocol using QIAGEN Multiplex PCR Kit for multiplex PCR and *Ex Taq* polymerase for the labeling step. The DigiTag2 assay worked with a conversion rate of over 93.2% (179 / 192 SNPs), average call rate of over 99.80% (16,789/16,823 genotypes) and reproducibility of over 99.99% (16,135/16,136 genotypes) using 96-plex PCR under the new protocol. The composition of successfully genotyped SNPs was different when the genotype calls were acquired using the different polymerases (Kapa 2GFast HotStart DNA polymerase and QIAGEN Multiplex PCR Kit), which would result from a varying amplification bias in multiplex PCR. We also found that 192-plex PCR with locus-specific primers worked in 96-plex genotyping with the DigiTag2 assay, giving the same quality parameter data as those observed in genotyping with 96-plex PCR. However, the

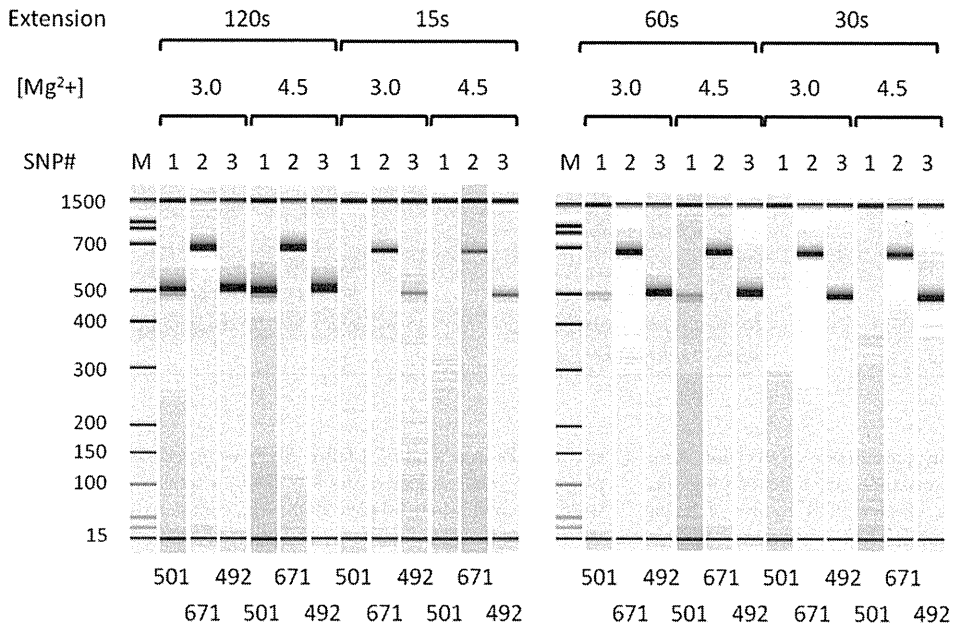

Figure 4. Electropherogram of singleplex PCR products using Kapa 2GFast HotStart DNA polymerase. Singleplex PCR was performed with varied extension periods (15 s, 30 s, 60 s and 120 s) and with varied Mg^{2+} concentrations (3.0 mM and 4.5 mM) using three pairs of locus-specific primers. The designed amplicon size is depicted below each lane.
doi:10.1371/journal.pone.0029967.g004

composition of successfully genotyped SNPs was not consistent between 192-plex PCR and 96-plex PCR, which may be explained by changing the interactions between primer pairs in 192-plex PCR and in 96-plex PCR. The composition of successful SNPs was not consistent when using different polymerases or multiplex systems in the multiplex PCR, which casts some shadows on the reliability of the assay. Regardless of the existing shadows, indeed, 96-plex and 192-plex PCR work with a high conversion rate in genotyping over 93.2%. To clear the existing shadows, it is necessary to continuously accumulate genotyping data.

In this study, fifteen discordant genotype calls were in total observed in the comparison of genotype calls with: i) duplicated genotyping data; ii) genotyping data by use of 192-plex PCR and 96-plex PCR; and iii) genotyping data with different types of polymerases (Table S3). Table S3 shows the genotype calls acquired 8 times under different conditions. All fifteen discordant genotype calls were analyzed with direct sequencing, of which 13 genotype calls were determined. In 8 of 15 discordant genotype calls, the genotype calls were completely different depending on the type of polymerase. The genotype calls acquired using Kapa 2GFast HotStart DNA polymerase were 100% concordant (6 of 6) with those acquired by direct sequencing. This suggests that SNP allelic bias in PCR amplification readily occurred with the QIAGEN Multiplex PCR Kit; however, the error rate in genotyping was only 0.04% (6 out of 14,886 genotypes). The remaining 7 discordant genotype calls were randomly observed in 1 out of 8 different conditions. This shows that the random error rates were almost equal in the genotype data acquired with both types of polymerases (4 out of 62,227 genotypes for QIAGEN Multiplex PCR Kit and 3 out of 66,008 genotypes for Kapa 2GFast HotStart DNA polymerase).

Among the five low-sensitivity primer pairs found on singleplex PCR (61, 99, 102, 189 and 191), no amplicons were detected by primer pair 189 and low concentrations (<5 nM) of amplicon were detected by the 4 other primer pairs (Table S2). Therefore, the SNP189 failed in genotyping, independently of the type of

Table 2. Validation of efficacy of 192-plex and 96-plex PCR with Kapa 2GFast HotStart DNA polymerase.

		192-plex PCR	96-plex PCR
1st set	Conversion rate	88/96 SNP	91/96 SNP
	Call rate	99.84% (8,259/8,272 genotype)	99.76% (8,443/8,463 genotype)
	reproducibility	99.97% (8,069/8,071 genotype)	99.99% (8,339/8,340 genotype)
	concordance	99.99% (7,982/7,983 genotype)	
2nd set	Conversion rate	87/96 SNP	88/96 SNP
	Call rate	99.91% (8,171/8,178 genotype)	99.83% (8,346/8,360 genotype)
	reproducibility	100% (7,705/7,705 genotype)	100% (7,796/7,796 genotype)
	concordance	100% (8,161/8,161 genotype)	

doi:10.1371/journal.pone.0029967.t002