Replication study of candidate genes associated with type 2 diabetes based on genome-wide screening

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Submitted 18 December 2007 and accepted 17 November 2008.

Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org
ABSTRACT

Objective. The present study was conducted to confirm possible associations between candidate genes from genome-wide association studies and type 2 diabetes mellitus (T2DM) in Japanese diabetic patients and a community-based general population. A total of 11 previously reported single nucleotide polymorphisms (SNPs) from the TCF7L2, CDKAL1, HHEX, IGF2BP2, CDKN2A/B, SLC30A8 and KCNJ11 genes were analyzed.

Research Design and Methods. Candidate SNPs were genotyped in 506 T2DM patients and 402 controls, and meta-analyzed with six previous association studies in Japanese patients. Associations with fasting plasma insulin levels were investigated in a general population sample (n=1,963, 61±13 years).

Results. In our case-control subjects, susceptibility to T2DM was replicated in TCF7L2 (rs12255372), CDKAL1 (rs7756992, rs7754840), HHEX (rs7923837), IGF2BP2 (rs4402960 and rs1470579), CDKN2A/B (rs10811661) and SLC30A8 (rs13266634). In addition to these polymorphisms, meta-analysis confirmed the association of T2DM susceptibility with KCNJ11 rs5219, TCF7L2 rs7903146 and HHEX rs1111875. The TCF7L2 rs12255372 polymorphism showed the highest odds ratio for T2DM (OR; OR=1.714 (1.298 to 2.263)). Odds ratio of other polymorphisms ranged from 1.13 to 1.41. The risk allele of CDKAL1 rs7756992 was significantly associated with lower insulin levels in T2DM patients after adjustment for other confounding factors.

Conclusions. T2DM susceptibility of seven candidate genes was confirmed in Japanese. Conservation of susceptible loci for T2DM was independent of ethnic background.
A great number of studies in various populations have suggested an association between several single nucleotide polymorphisms (SNP) and type 2 diabetes mellitus (T2DM). For example, transcription factor 7-like 2 (TCF7L2) is a highly reliable predisposing gene for T2DM [1-3]. In addition, recent genome-wide association studies (GWAS) have provided new susceptible loci for T2DM [4-10]. A GWAS in French subjects, for example, identified rs13266634, a non-synonymous SNP (R325W) on the solute carrier family 30 member 8 (SLC30A8) gene, as a polymorphism involved in T2DM susceptibility [4]. The study also reported an association between T2DM and rs1111875, as well as rs7923837, located in the hematopoietically-expressed homeobox gene (HHEX). These associations were replicated in three independent GWAS in various populations [5-7].

Additional susceptible SNPs were independently identified in the insulin-like growth factor 2 mRNA-binding protein 2 gene (IGF2BP2, rs4402960 and rs1470569) [5, 6]. Involvement of SNPs rs10811661, located upstream of cyclin-dependent kinase inhibitor genes CDKN2A and CDKN2B, and “rs7754840/ rs7756992”, located in the CDK5 regulatory subunit-associated protein 1-like 1 gene (CDKAL1), has also been suggested [5, 6, 8, 9]. A recent population-based study in Danish subjects replicated the susceptible association of HHEX rs111875, CDKN2A/B rs10811661, and IGF2BP2 rs4402960 with T2DM [10].

Findings from previous GWAS, however, cannot be extrapolated to other populations with different lifestyles and environmental backgrounds. In particular, the genetic background for T2DM development in East Asians, who show lower basal insulin secretion and a marked decrease in insulin release in response to development of glucose tolerance [11], appears to be different from that in Caucasi ans or individuals of European origin. Further, SNP frequency differences are suggested to be an additional factor influencing T2DM susceptibility.

Here, based on a recent GWAS [4-10], we conducted a replication study of candidate SNPs associated with T2DM in Japanese diabetic subjects, as well as in a general Japanese population sample.

**METHODS**

**Case and control subjects.** Basic clinical characteristics of subjects are summarized in Supplemental Table 1. All T2DM subjects (n=506) were in- or out-patients evaluated by diabetes specialists at Ehime University Hospital and Ehime Prefectural Hospital in Japan. Diabetes mellitus was diagnosed based on the 1998 ADA criteria [12].
Non-diabetic control subjects (n=402) were chosen based on the absence of a history of diabetes in the subject and among first-degree relatives, as well as either normal glucose tolerance, confirmed by a 75 g oral glucose tolerance test, or HbA1c levels under 5.6 with fasting plasma glucose levels under 110 mg/dl. All case and control subjects were native Japanese. Selection criteria details have been described in a previous study [13]. A total of 139 T2DM patients and 136 control subjects were overlapped with the previous meta-analysis for TCF7L2 polymorphisms [14].

**General population.** The general population subjects were selected from residents of a community of 11,000 inhabitants in Ehime Prefecture, a largely rural area located in western Japan [15]. Subjects were recruited through a community-based annual medical check-up process for self-employees, including farmers and foresters, employees of small companies, and elderly without fixed employment. The sample population consisted of 1,963 middle-aged to elderly residents (Supplemental Table 2). Overnight fasting plasma samples for the measurement of plasma insulin concentrations were available for all sample subjects. Baseline clinical characteristics were obtained from personal health records evaluated during the medical check-up. All study procedures were approved by the ethics committee of the Ehime University Graduate School of Medicine, and informed consent was obtained from each participating subject.

**Genotyping.** Genomic DNA was extracted from peripheral blood (QIAamp DNA blood kit, QIAGEN GmbH, Hilden, Germany). All SNPs were analyzed by TaqMan probe assay (Applied Biosystems Co., Ltd., Foster City, CA) using commercially available primers and probes purchased from the Assay-on-Demand system (Supplemental Table 3).

**Statistical analysis.** Values are expressed as mean±standard deviation. Linkage disequilibrium was assessed using the Haploview software (Broad Institute, Cambridge, MA) [16]. Frequency differences in each genotype were assessed by the chi-squared test. The pooled odds ratios for allele frequency with those of six other association studies in Japanese [17-22] were estimated using the fixed effects model (Mantel–Haenszel method). Differences in plasma insulin levels among genotypes (analysis of variance, and multiple regression analysis (additive model) adjusted for age, sex, body mass index were assessed using a commercially available statistical software package (SPSS Ver 14.0, SPSS Inc., Chicago, IL). Current treatment of hyperglycemia was further adjusted in T2DM patients when appropriate. Null hypotheses were rejected at a level of 0.05.
significance of p<0.05.

RESULTS

Table 1 summarizes the association between 11 candidate SNPs and T2DM in case-control subjects. The T allele of TCF7L2 (rs12255372) was significantly associated with T2DM. A tendency to association was also observed with SNP rs7903146, which was in linkage disequilibrium with rs12255372 (D'=0.854, r²=0.421). However, the risk allele frequency of these SNPs was considerably low, which is in agreement with previous reports in Japanese subjects [17, 18]. The post-hoc calculated statistical power of these SNPs (allele frequency) was 36.1% and 25.4% for rs12255372 and rs7903146, respectively, with a 5% type 1 error rate.

In addition to TCFL72 polymorphisms, a significant association was observed between T2DM and polymorphisms in CDKAL1 (rs7756992 (power: 51.0%), rs7754840 (52.9%); D'=0.920; r²=0.648), HHEX (rs7923837 (30.4%)), IGF2BP2 (rs4402960 (31.3%), rs1470579 (51.1%); D'=0.997; r²=0.918), CDKN2A/B (rs10811661 (32.6%)), and SLC30A8 (rs13266634 (10.7%)), but not HHEX (rs1111875 (8.5%). Further, a marginally significant association was observed between T2DM and the KCNJ11 polymorphism (rs5219 (21.8%)). Compared to control subjects of European descent, risk allele frequencies in Japanese control subjects were higher in the CDKAL1 gene (rs7756992 G allele, 0.470 vs. 0.258), and lower in the HHEX (rs1111875 C allele, 0.288 vs. 0.598; rs7923837 G allele, 0.177 vs. 0.597), CDKN2A/B (rs10811661 T allele, 0.555 vs. 0.850), SLC30A8 (rs13266634 C allele, 0.568 vs. 0.699), and KCNJ11 (rs5219 T allele, 0.372 vs. 0.464) genes [4, 6, 8]. In contrast, no significant frequency differences were observed in the CDKAL1 rs7754840 (C allele, 0.399 vs. 0.360) and IGF2BP2 rs4402960 (T allele, 0.287 vs. 0.304) polymorphisms.

However, except for the CDKAL polymorphisms, statistical significance was not reached in the observed associations using Bonferroni’s correction, possibly due to limited statistical power. To further clarify T2DM susceptibility, seven association studies in Japanese subjects [17-22], including our present data, were meta-analyzed (Figure 1). T2DM susceptibility was confirmed in all analyzed polymorphisms, both before and after Bonferroni’s adjustment. Further, two SNPs (rs1111875 in HHEX and rs5219 in KCNJ11), which were not replicated in our data, were confirmed as susceptible polymorphisms for T2DM.

To further clarify the pathophysiological significance of the susceptibility of these seven genes for T2DM, associations with plasma insulin levels were evaluated in a community-derived population sample.
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Although differences in plasma insulin levels among the CDKAL1 rs7756992 genotype did not reach statistical significance, probably due to the limited statistical power (post-hoc calculated statistical power: 28.3% for T2DM patients, 31.8% for controls), multiple regression analysis involving the genotype as an additive model showed significantly lower insulin levels in T2DM subjects with risk genotypes after adjusting for age, sex, body mass index. The association of CDKAL1 rs7756992 remained significant after further adjustment for the current treatment of hyperglycemia (n=67, p=0.021). The risk allele of CDKAL1 rs7754840 also tended to be associated with lower insulin levels. However, no significant associations were observed in other SNPs.

DISCUSSION

In the present study, we replicated the associations of several candidate genes derived from a recent GWAS [4-10]. However, several conflicting results were observed with other replication studies in Japanese diabetic patients. Horikoshi et al. [19] and Furukawa et al. [20] observed a markedly strong association between T2DM and variants of HHEX rs1111875, whereas no association was observed in our study. However, results of our meta-analysis (Figure 1) clearly indicate the T2DM susceptibility of all candidate genes, including HHEX rs1111875. Conservation of susceptible loci for T2DM was independent of ethnic background.

However, the attributable risk of these SNPs susceptible for T2DM was different from that of European ancestries. For example, the pooled odds ratios of TCF7L2 gene polymorphisms were slightly higher than those in European ancestries [6, 23], whereas the risk allele frequencies were considerably lower. Alternatively, for HHEX gene polymorphisms, odds ratios were slightly higher in Japanese [7]. Very recently, genome-wide screening in a Japanese population identified KCNQ1 gene polymorphism as a new susceptible loci for T2DM [24]. These authors reported that the risk alleles of rs2237892 and other SNPs in linkage disequilibrium with rs2237892 were associated with an increased risk of type 2 diabetes. However, apparent associations of the KCNQ1 SNPs were not observed in previous GWAS in populations of European descent [4-10]. This discrepancy may be due mainly to the differences in allele frequencies of the susceptible SNPs.

It has been suggested that several candidate SNPs identified from genome-wide screening contribute to diabetes susceptibility primarily through effects on insulin secretion. In our quantitative trait analysis in a general Japanese population, we observed lower basal plasma insulin levels in T2DM
patients carrying the risk genotype of CDKAL1 rs7756992 SNP. Although the function of the CDKAL1 gene product is unknown, one study suggested that CDKAL1 has a role in the inhibition of cyclin-dependent kinase 5 (CDK5) activity in pancreatic β-cells [8], which prevents a decrease in insulin gene expression resulting from glucotoxicity. That study also observed reduced insulin secretion in response to glucose loading in homozygous carriers of the CDKAL1 rs7756992 polymorphism risk allele [8]. Pascoe et al [25] also reported lower insulin secretion after glucose loading in risk allele carriers of another SNP of CDKAL1 gene. Our study is the first to show a possible association between this SNP and basal insulin levels in T2DM patients. This observation provides supporting evidence for the pathophysiological role of CDKAL1 gene products in the progression of T2DM, as well as the disease susceptibility of this genetic variant.

Several limitations of this study warrant mention. First, differences in linkage disequilibrium between Japanese and European subjects means that tracking of the causal variants may not be possible in SNPs based on the association study in European ancestries. Although our meta-analysis showed an association between T2DM susceptibility and the analyzed candidate SNPs, causal variants may also be strongly represented by other SNPs in Japanese subjects. Studies with multiple tag-SNPs at loci chosen based on the linkage disequilibrium pattern in Japanese may provide further clarification of this issue. Secondly, we did not investigate the class of antihyperglycemic drugs, including insulin treatment, in the general population sample. Although each drug may have affected fasting plasma insulin differently, association of the CDKAL1 genotype with plasma insulin levels was statistically significant after further adjustment for current treatment for hyperglycemia.

In the present study, we replicated several genetic variants as risk markers for T2DM susceptibility in Japanese by performing a case-control analysis and meta-analysis. These findings may be useful in advanced clinical practice and public health genomics.

ACKNOWLEDGEMENTS

We greatly appreciate the support of Dr. Masaaki Ochi, Wataru Nishida, Yasunori Takata, and Yasuhisa Fujii, and their help with sample collection. This study was supported by a Grant-in-Aids for Scientific Research from The Ministry of Education, Culture, Sports, Science and Technology of Japan; The Ministry of Health, Labour and Welfare of Japan; the Japan Arteriosclerosis Prevention Fund; and a Research Promotion Award from Ehime University.
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## Table 1 Association of candidate SNPs with type 2 diabetes in case and control subjects (n=908)

| Gene   | Frequency | Risk HWE | Allele | Dominant (95% C.I.) | Recessive (95% C.I.) | Additive (95% C.I.) |
|--------|-----------|----------|--------|---------------------|----------------------|---------------------|
|        | (rs number) | T2DM allele | p  | p  | p  | p  |
|        |           | Control (Control) |       |       |       |       |
| TCF7L2 | rs12255372 | TT/TG/GG T | 0.721 | 2.082 (1.112-3.898) | 2.059 (1.089-3.893) | 0.065 |
|        | 1/33/453  | T |       | 0.022 |       |       |       |
|        | 0/14/384  | T |       | 0.026 |       |       |       |
| TCF7L2 | rs7903146  | TT/TC/CC T | 0.501 | 1.307 (1.084-1.577) | 1.081 (0.796-1.467) | 6.8x10^-5 (1.381-2.578) |
|        | 2/45/434  | T |       | 0.005 |       |       | 2.0x10^-4 |
|        | 0/26/372  | T |       | 0.618 |       |       | 0.002 |
| CDKAL1 | rs7756992  | GG/GA/AA G | 0.053 | 1.321 (1.093-1.596) | 1.209 (0.912-1.604) | 4.6x10^-4 (1.316-2.645) |
|        | 155/217/119 | G |       | 0.004 |       |       | 0.002 |
|        | 78/217/102 | G |       | 0.187 |       |       |       |
| CDKAL1 | rs7754840  | CC/CG/GG C | 0.189 | 1.866 (1.093-1.596) | 1.209 (0.912-1.604) | 4.6x10^-4 (1.316-2.645) |
|        | 117/225/149 | C |       | 1.321 |       |       | 0.002 |
|        | 57/203/137 | C |       | 0.004 |       |       |       |
| HHEX   | rs1111875  | CC/CT/TT C | 0.593 | 1.086 (0.885-1.334) | 1.141 (0.876-1.488) | 0.602 |
|        | 44/211/235 | C |       | 0.430 |       |       |       |
|        | 35/158/203 | C |       | 0.328 |       |       |       |
| HHEX   | rs7923837  | GG/GA/AA G | 0.381 | 1.266 (1.015-1.630) | 1.432 (1.085-1.891) | 0.026 |
|        | 17/178/295 | G |       | 0.037 |       |       |       |
|        | 15/111/273 | G |       | 0.011 |       |       |       |
| IGF2BP2| rs4402960  | TT/TG/GG T | 0.972 | 1.239 (1.011-1.517) | 1.175 (0.902-1.530) | 1.714 |
|        | 66/196/231 | T |       | 0.039 |       |       | 0.016 |
|        | 33/163/203 | T |       | 0.232 |       |       | 0.050 |
| Gene      | Genotype  | Value | Frequency | Type 2 diabetes susceptibility |
|-----------|-----------|-------|-----------|---------------------------------|
| IGF2BP2   | CC/CA-AA  | C     | 0.940     |                                 |
| (rs1470579) | 77/198/216 |       |           |                                 |
|           | 35/165/198 |       |           |                                 |
| CDKN2A/B  | TT/TC/CC  | T     | 0.394     |                                 |
| (rs10811661) | 189/222/85 |       |           |                                 |
|           | 119/206/75 |       |           |                                 |
| SLC30A8   | CC/CT/TT  | C     | 0.395     |                                 |
| (rs13266634) | 162/259/72 |       |           |                                 |
|           | 133/188/79 |       |           |                                 |
| KCNJ11    | TT/TC/CC  | T     | 0.302     |                                 |
| (rs5219)  | 83/232/169 |       |           |                                 |
|           | 50/195/152 |       |           |                                 |

Type 2 diabetes is defined by fasting blood glucose higher or equal to 126 mg/dl, or occasional blood glucose higher or equal to 200 mg/dl and/or current use of antidiabetic agents. Differences in genotype frequency between diabetic patients and normal controls, as well as deviations from the Hardy-Weinberg equilibrium (HWE) in controls, were assessed using the chi-squared test. C.I.: confidence interval.
| Gene         | risk allele | Plasma insulin (μU/ml) | p-value | ANOVA | Multivariate |
|--------------|-------------|------------------------|---------|-------|--------------|
| TCF7L2 *     | T           | Control                 | 5.5±2.7 | (2)   | 6.2±4.7      | 6.5±4.8 | (1770) | 0.661 | 0.749 |
| (rs12255372) | T2DM        | Control                 | -       | (0)   | 4.2±1.0      | 9.0±7.3 | (136)  | 0.256 | 0.368 |
| TCF7L2 *     | T           | T2DM                   | 6.8     | (1)   | 6.5±4.3      | 6.5±4.8 | (1712) | 0.967 | 0.336 |
| (rs7903146)  | Control     | Control                 | 6.8     | (1)   | 6.5±4.3      | 6.5±4.8 | (1712) | 0.967 | 0.336 |
| CDKAL1       | G           | Control                 | 6.3±5.2 | (428) | 6.5±4.6      | 6.6±4.7 | (501)  | 0.227 | 0.457 |
| (rs7756992)  | T2DM        | Control                 | 7.9±7.0 | (38)  | 8.8±6.3      | 10.7±10.0 | (26)  | 0.269 | 0.020 |
| CDKAL1       | C           | Control                 | 6.1±4.7 | (315) | 6.4±4.8      | 6.7±4.7 | (641)  | 0.083 | 0.157 |
| (rs7754840)  | T2DM        | Control                 | 7.1±5.1 | (32)  | 9.0±6.5      | 10.3±9.4 | (40)  | 0.154 | 0.097 |
| HHEX         | C           | Control                 | 6.4±4.6 | (185) | 6.4±4.4      | 6.6±5.1 | (884)  | 0.867 | 0.883 |
| (rs1111875)  | T2DM        | Control                 | 9.8±11.6 | (15)  | 7.7±5.2      | 9.9±7.7 | (62)  | 0.323 | 0.470 |
| HHEX         | G           | Control                 | 6.1±4.1 | (112) | 6.5±4.9      | 6.5±4.7 | (1020) | 0.818 | 0.749 |
| (rs7923837)  | T2DM        | Control                 | 7.3±4.6 | (9)   | 8.8±6.9      | 9.1±7.8 | (74)  | 0.811 | 0.789 |
| IGF2BP2      | T           | Control                 | 6.2±3.9 | (215) | 6.7±5.2      | 6.3±4.5 | (842)  | 0.276 | 0.308 |
| (rs4402960)  | T2DM        | Control                 | 12.2±10.0 | (15)  | 7.8±5.4      | 9.5±8.4 | (52)  | 0.186 | 0.843 |
## Replication of type 2 diabetes susceptible genes

| Gene          | Genotype | Control       | T2DM         | p-value | p-value* |
|---------------|----------|---------------|--------------|---------|----------|
| IGF2BP2       | C        | 6.1±3.8       | 11.6±9.6     | 0.118   | 0.314    |
| (rs1470579)   | CC       | 6.8±5.2       | 7.8±5.4      | 0.276   | 0.869    |
|               | CA       | 6.3±4.5       | 9.5±8.5      |         |          |
|               | AA       | 6.3±4.5       | 9.5±8.5      |         |          |
| CDKN2A/B      | T        | 6.4±4.7       | 8.1±8.0      | 0.719   | 0.858    |
| (rs10811661)  | TT       | 6.5±4.6       | 8.9±6.5      | 0.346   | 0.169    |
|               | TC       | 6.6±5.2       | 10.2±8.0     |         |          |
|               | CC       | 6.6±5.2       | 10.2±8.0     |         |          |
| SLC30A8       | C        | 6.5±4.9       | 9.8±7.1      | 0.151   | 0.332    |
| (rs13266634)  | CC       | 6.4±4.4       | 7.9±5.6      | 0.939   | 0.910    |
|               | CT       | 6.6±5.2       | 9.8±11.5     |         |          |
|               | TT       | 6.6±5.2       | 9.8±11.5     |         |          |
| KCNJ11        | T        | 6.5±4.8       | 6.8±3.8      | 0.088   | 0.156    |
| (rs5219)      | TT       | 6.3±4.6       | 8.8±7.2      |         |          |
|               | TC       | 6.7±4.9       | 10.3±8.7     |         |          |
|               | CC       | 6.7±4.9       | 10.3±8.7     |         |          |

Values are mean±standard deviation. Number of subjects in each genotype is provided in the parentheses. Statistical significance was assessed using log-transformed insulin value. Multiple regression analysis involving each genotype as additive model adjusted for age, sex, and body mass index. * T allele dominant model.
Replication of type 2 diabetes susceptible genes

**Figure 1 Meta-analysis of type 2 diabetes genetic association studies in Japanese**

Estimation of odds ratios and 95% confidence intervals in each study are displayed as a closed square and horizontal line, respectively. Square size represents the study weighting. Combined odds ratio is represented as the diamond. Study A, present study; Study B, Hayashi T et al., Diabetologia 2007 (reference 18); Study C, Horikoshi M et al., Diabetologia 2007 (reference 17); Study D, Horikawa Y et al., J Clin Endocrinol Metab 2008 (reference 19); Study E, Furukawa Y et al., J Clin Endocrinol Metab 2008 (reference 20); Study F, Horikoshi M et al., Diabetologia 2007 (reference 21); Study G, Omori S et al., Diabetes 2008 (reference 22).

| Gene     | SNP          | Study       | Odds ratio (95% confidence interval)               |
|----------|--------------|-------------|---------------------------------------------------|
| TCF7L2   | rs12255372   | ABC         | 1.714 (1.298-2.263), p=2.0*10^-4                 |
| TCF7L2   | rs7903146    | CBA         | 1.412 (1.139-1.750), p=0.002                      |
| CDKAL1   | rs7754840    | ABD         | 1.290 (1.185-1.405), p=5.3*10^-9                  |
| HHEX     | rs1111875    | EBDGA       | 1.258 (1.181-1.400), p=1.2*10^-12                 |
| HHEX     | rs7923837    | EADG        | 1.258 (1.171-1.351), p=3.0*10^-10                  |
| CDKN2AB  | rs10811661   | DAFG        | 1.234 (1.161-1.311), p=1.3*10^-11                  |
| CDKAL1   | rs7756992    | ADFG        | 1.223 (1.152-1.300), p=5.2*10^-11                  |
| IGF2BP2  | rs4402960    | ADFGF       | 1.196 (1.121-1.276), p=5.9*10^-8                   |
| IGF2BP2  | rs1470579    | ADGF        | 1.185 (1.099-1.277), p=9.7*10^-6                   |
| SLC30A8  | rs13266634   | DEFAE       | 1.144 (1.078-1.213), p=7.8*10^-6                   |
| KCNJ11   | rs5215       | AGF         | 1.126 (1.039-1.219), p=0.004                      |