**Table 1** Clinical characteristics of each sample set. Data are means ± SD. *BMI* Body mass index

| | Kobe | | Gunma | | Consortium | |
|---|---|---|---|---|---|---|
| | Diabetes | Control | Diabetes | Control | Diabetes | Control |
| *n* | 465 | 323 | 576 | 576 | 1,173 | 974 |
| Male participants (%) | 59.6 | 45.8 | 56.1 | 40.4 | 56.6 | 43.1 |
| Age at study (years) | 60.5 ± 10.7 | 75.6 ± 8.1 | 60.2 ± 11.5 | 67.3 ± 6.5 | 62.5 ± 8.8 | 69.2 ± 7.0 |
| BMI | 24.3 ± 3.9 | 21.4 ± 3.5 | 23.9 ± 4.2 | 23.0 ± 2.9 | 23.1 ± 2.9 | 22.6 ± 3.0 |
| $HbA_{1c}$ (%) | 8.1 ± 2.0 | 5.0 ± 0.4 | 7.8 ± 3.5 | 5.0 ± 0.4 | 7.5 ± 1.5 | 4.9 ± 0.4 |

diabetes (OR = 1.48, $P = 2.7 \times 10^{-4}$; OR = 1.39, $P = 4.6 \times 10^{-4}$; OR = 1.70, $P = 9.8 \times 10^{-5}$, respectively) in the combined sample sets. However, neither rs11196218 nor rs290487 showed a significant association. These results indicate that *TCF7L2* is an important susceptibility gene for type 2 diabetes in the Japanese population.

**Keywords** Type 2 diabetes · Polymorphism · β-cell function · Transcription factor 7-like 2 (*TCF7L2*) · Association study

## Introduction

The transcription factor 7-like 2 gene (*TCF7L2*) is one of the most convincing susceptibility genes for type 2 diabetes. Following the initial report (Grant et al. 2006), there have been a number of association studies in various ethnic groups (Florez et al. 2006; Zhang et al. 2006; Saxena et al. 2006). Regarding the Asian population, Horikoshi et al. (2007) reported that a single nucleotide polymorphism (SNP), rs7903146, in *TCF7L2* is associated with type 2 diabetes in the Japanese population but that other SNPs (rs7895340, rs11196205, rs12255372) are not. The minor allele frequencies of these SNPs in Japanese were also found to be much lower than those of Caucasians. Hayashi et al. (2007) replicated the association of *TCF7L2* with type 2 diabetes in Japanese. Contradictory results were reported for Han Chinese populations (Ng et al. 2007; Chang et al. 2007), but these two reports found that other common SNPs (rs11196218 and rs290487, respectively) were associated with type 2 diabetes. This apparent difference between Asian populations could be due to the relatively small sample sizes involved. Recently, variants in the *TCF7L2* gene also were reported to be associated with β-cell function (Schäfer et al. 2007; Lyssenko et al. 2007) and response to sulfonylureas in Caucasians (Pearson et al. 2007). To clarify the association of the *TCF7L2* gene with type 2 diabetes and β-cell function in an Asian population, we have performed association studies using a relatively large Japanese sample set: 2,214 Japanese individuals with type 2 diabetes and 1,873 normal controls.

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## Subjects and methods

### Subjects

Three sample sets were involved. The Kobe set and the Gunma set samples were recruited from hospitals in Hyogo and Gunma prefecture, respectively. The Consortium set samples were recruited from seven districts in Japan by the Study Group of the Millennium Genome Project for Diabetes Mellitus. The Kobe, Gunma, and Consortium sets were independent of one another. The inclusion criteria for normal, control subjects of the Consortium set were as follows: (1) >60 years of age; (2) $HbA_{1c}$ values <5.8%; and (3) no family history of type 2 diabetes in first- or second-degree relatives. In the Kobe and Gunma control samples, the inclusion criteria were (1) no past history of diabetes and (2) $HbA_{1c}$ values < 5.8%. The control subjects were hospital patients for annual medical checkup or unrelated disorders. Type 2 diabetes was diagnosed in accordance with WHO criteria. Other forms of diabetes were excluded based on the clinical data. The clinical and laboratory characteristics of the study subjects are shown in Table 1. Written, informed consent was obtained from all participants. The study was approved by the ethics committee of each participating institute.

### Genotyping

Five SNPs (rs7903146, rs11196205, rs12255372, rs11196218, rs290487) were genotyped using TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City,

CA) or SSP-FCS (sequence specific primer-fluorescence correlation spectroscopy) Assays (Bannai et al. 2004). Of the original five SNPs (rs7903146, rs11196205, rs12255372, rs7901695, rs7895340) in the first report (Grant et al. 2006), we selected three SNPs (rs7903146, rs11196205, rs12255372) for the following reasons: the original five SNPs are located in one linkage disequilibrium (LD) block surrounding exon 4 in the Japanese population (Supplementary Figure 1), which is similar to the case in Caucasians (Grant et al. 2006); rs7901695 and rs7895340 are in almost complete LD with rs7903146 ($r^2 = 1$) and rs11196205 ($r^2 = 0.90$), respectively, in the Japanese population (Horikoshi et al. 2007); there is no common (minor allele frequency > 10%) SNP in this LD block (HapMap JPT data). We also genotyped rs11196218 and rs290487, which were associated with type 2 diabetes in Han Chinese, to replicate this association in Japanese. To evaluate our genotyping, 180 samples in the Consortium set were genotyped by both TaqMan SNP Genotyping Assays and SSP-FCS Assays. The concordance rate between these two assays was 100%: genotypes determined by TaqMan or SSP-FCS methods were identical to those determined by direct sequencing for 48 samples.

The genotyping success rates in the three sample sets were all >93%. All five SNPs were in Hardy–Weinberg equilibrium (HWE; $P > 0.05$ in the Exact test) in both case and control groups of all sample sets.

### Clinical assessment

The clinical profile of each subject was directly determined at the time of entry. HOMA-IR and HOMA-$\beta$ were calculated as follows: HOMA-IR = (fasting insulin [pmol/l]) × glucose [mmol/l])/22.5 × 6 and HOMA-$\beta$ = (fasting insulin [pmol/l]) × 2)/(glucose [mmol/l] − 3.5) × 6. Diabetic subjects treated with insulin were excluded from analysis of HOMA-IR and HOMA-$\beta$. Assessments were performed with the combined three sample sets. Data are expressed as means ± SD.

### Statistical analysis

The differences for SNPs or estimated haplotypes between type 2 diabetic and non-diabetic subjects were compared using Chi-square test under an allelic model. We also performed multiple logistic regression analysis adjusted for age, sex, and BMI under a dominant model. Statistical analysis was performed with the Stat-View program (version 5.0-J; SAS Institute, Cary, NC). The relation of the variants in *TCF7L2* with BMI and Homeostasis model assessment (HOMA-IR and HOMA-$\beta$) by $t$ test under the dominant model for each SNP was then assessed. The HOMA-IR and HOMA-$\beta$ data were log-transformed for normality. LD and haplotype analyses were performed with SNPAlyze version 5.1 pro software (Dynacom, Mobara, Japan). We considered statistical significance at $P$ values of $< 0.01$ and $< 0.017$ in the association study for SNPs and for clinical parameters, respectively, after Bonferroni correction. The prevalence of type 2 diabetes in the Japanese population was assumed to be 0.07. Population attributable risk (PAR) was calculated as PAR = $p$(RR-1)/[$p$(RR-1) + 1], where $p$ and RR are the risk allele frequency in the general population and the relative risk, respectively, estimated by the prevalence. When the frequency of risk allele, OR, and type I error probability are assumed to be 0.03 (Horikoshi et al. 2007), 1.46 (Cauchi et al. 2007), and 0.05, respectively, based upon the previous study, the power of our combined samples (2,214 cases and 1,873 controls) to detect association between SNP rs7903146 and type 2 diabetes is 0.92. In the case of OR assumed to be 1.69 (Horikoshi et al. 2007), the power of our study is 0.99.

## Results

We performed association analyses using three independent sample sets. Regarding three SNPs (rs7903146, rs11196205, and rs12255372), which originally showed association with type 2 diabetes, the minor alleles showed a trend toward association with type 2 diabetes in the Kobe set. These SNPs also showed a marginally significant association in the Gunma set and in the Consortium set when multiple testing was considered. In the combined three sample sets (Combined set), the minor alleles of rs7903146, rs11196205, and rs12255372 showed a significant association with susceptibility to the disease (OR = 1.48, $P = 2.7 \times 10^{-4}$; OR = 1.39, $P = 4.6 \times 10^{-4}$; OR = 1.70, $P = 9.8 \times 10^{-5}$, respectively). These associations remained significant after adjustment for age, sex, and BMI (Table 2). As in a previous report (Horikoshi et al. 2007), the MAF and PAR in our study were much lower (MAF: 0.022–0.072, PAR: ~0.02 in the Combined set) than those in Caucasians. Neither rs11196218 nor rs290487 showed a significant association in any sample set (Table 2).

LD among the five SNPs in 974 control subjects in the Consortium set was then analyzed. The $D'$ and $r^2$ values are shown in Table 3. As reported previously for Japanese, three SNPs (rs7903146, rs11196205, and rs12255372) were found to be in modest to strong LD ($D' = 0.56$–$1.0$). Haplotypes then were constructed with these SNPs in the Combined set and assessed for association with type 2 diabetes. A haplotype comprising the risk allele of each

**Table 2** Association analyses for five single nucleotide polymorphisms (SNPs) in the *TCF7L2* gene. *P* values and OR were calculated with allele data by the Chi-square test. Adjusted *P* values were calculated by multiple logistic regression (dominant model) with adjustment for age, sex and BMI. *MAF* minor allele frequency, *OR* odds ratio, *CI* confidence interval

| dbSNP ID | Position on Chr10 | | Kobe | | | | | | | Gunma | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *n* | | MAF | | OR | *P* | Adjusted *P* | *n* | | MAF | | OR | *P* | Adjusted *P* |
| | | | Case | Control | Case | Control | (95% CI) | | | Case | Control | Case | Control | (95% CI) | | |
| rs7903146 | 114748339 | CC | 426 | 305 | 0.043 | 0.028 | 1.56 | 0.12 | 0.046 | 475 | 512 | 0.060 | 0.038 | 1.63 | 0.015 | 0.012 |
| | | CT | 38 | 18 | | | (0.89–2.76) | | | 63 | 42 | | | (1.09–2.42) | | |
| | | TT | 1 | 0 | | | | | | 1 | 0 | | | | | |
| rs11196205 | 114797037 | GG | 408 | 292 | 0.063 | 0.047 | 1.39 | 0.16 | 0.093 | 455 | 485 | 0.084 | 0.055 | 1.58 | 0.007 | 0.023 |
| | | GC | 55 | 30 | | | (0.83–2.18) | | | 77 | 58 | | | (1.13–2.21) | | |
| | | CC | 2 | 0 | | | | | | 7 | 1 | | | | | |
| rs12255372 | 114798892 | GG | 436 | 312 | 0.032 | 0.017 | 1.92 | 0.062 | 0.018 | 509 | 538 | 0.047 | 0.024 | 1.99 | 0.004 | 0.005 |
| | | GT | 28 | 11 | | | (0.96–3.87) | | | 48 | 27 | | | (1.24–3.20) | | |
| | | TT | 1 | 0 | | | | | | 2 | 0 | | | | | |
| rs11196218 | 114830484 | GG | 271 | 194 | 0.23 | 0.23 | 1.01 | 0.92 | 0.23 | 317 | 334 | 0.22 | 0.22 | 1.04 | 0.72 | 0.84 |
| | | GA | 170 | 106 | | | (0.80–1.29) | | | 184 | 185 | | | (0.85–1.27) | | |
| | | AA | 23 | 21 | | | | | | 25 | 25 | | | | | |
| rs290487 | 114899721 | TT | 181 | 124 | 0.37 | 0.38 | 0.94 | 0.57 | 0.90 | 209 | 236 | 0.37 | 0.34 | 1.18 | 0.072 | 0.13 |
| | | TC | 226 | 141 | | | (0.76–1.16) | | | 228 | 235 | | | (0.99–1.41) | | |
| | | CC | 57 | 49 | | | | | | 78 | 61 | | | | | |

| Consortium | | | | | | | Combined | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *n* | | MAF | | OR | *P* | Adjusted *P* | *n* | | MAF | | OR | *P* | Adjusted *P* |
| Case | Control | Case | Control | (95% CI) | | | Case | Control | Case | Control | (95% CI) | | |
| 1,020 | 879 | 0.058 | 0.041 | 1.43 | 0.014 | 0.06 | 1,921 | 1,696 | 0.055 | 0.038 | 1.48 | $2.7 \times 10^{-4}$ | 0.0011 |
| 127 | 77 | | | (1.07–1.90) | | | 228 | 137 | | | (1.20–1.84) | | |
| 3 | 1 | | | | | | 5 | 1 | | | | | |
| 1,011 | 863 | 0.071 | 0.054 | 1.32 | 0.031 | 0.12 | 1,874 | 1,640 | 0.072 | 0.053 | 1.39 | $4.6 \times 10^{-4}$ | 0.0053 |
| 153 | 99 | | | (1.02–1.70) | | | 285 | 187 | | | (1.16–1.67) | | |
| 6 | 3 | | | | | | 15 | 4 | | | | | |
| 1,068 | 906 | 0.035 | 0.023 | 1.52 | 0.026 | 0.12 | 2,013 | 1,756 | 0.037 | 0.022 | 1.70 | $9.8 \times 10^{-5}$ | $7.0 \times 10^{-4}$ |
| 76 | 42 | | | (1.05–2.21) | | | 152 | 80 | | | (1.30–2.22) | | |
| 2 | 1 | | | | | | 5 | 1 | | | | | |
| 728 | 584 | 0.20 | 0.22 | 0.87 | 0.076 | 0.11 | 1,331 | 1,115 | 0.21 | 0.22 | 0.94 | 0.26 | 0.56 |
| 370 | 321 | | | (0.75–1.01) | | | 730 | 617 | | | (0.85–1.05) | | |

**Table 2** continued

| Consortium | | | | | | | Combined | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *n* | | MAF | | OR | *P* | Adjusted *P* | *n* | | MAF | | OR | *P* | Adjusted *P* |
| Case | Control | Case | Control | (95% CI) | | | Case | Control | Case | Control | (95% CI) | | |
| 45 | 54 | | | | | | 93 | 100 | | | | | |
| 476 | 381 | 0.37 | 0.37 | 0.99 | 0.91 | 0.50 | 873 | 744 | 0.37 | 0.36 | 1.04 | 0.45 | 0.46 |
| 507 | 448 | | | (0.88–1.13) | | | 977 | 824 | | | (0.95–1.14) | | |
| 169 | 129 | | | | | | 306 | 239 | | | | | |

SNP, T-C-T, was significantly associated with type 2 diabetes ($P = 5.3 \times 10^{-5}$) (Table 4).

The relation of rs7903146, rs11196205, and rs12255372 to BMI, HOMA-IR, and HOMA-$\beta$ in the combined cases and controls were then compared. There was no association with BMI in cases or controls. The risk allele of rs7903146 was associated with lower HOMA-$\beta$ (CC ($n = 789$) versus CT/TT ($n = 83$); $52.0 \pm 87.6$ versus $35.7 \pm 35.9$, $P = 0.009$) and lower HOMA-IR (CC vs. CT/TT; $3.2 \pm 4.5$ vs. $2.2 \pm 1.6$, $P = 0.01$) in the combined diabetic subjects. However, these associations disappeared after adjustment for age, sex, and BMI. No association was found for HOMA-$\beta$ or HOMA-IR in the combined control subjects.

## Discussion

We have found that three SNPs (rs7903146, rs11196205, rs12255372) of *TCF7L2* are associated with susceptibility to type 2 diabetes in the Japanese population. Our results are consistent with previous reports for Japanese populations (Horikoshi et al. 2007; Hayashi et al. 2007), but not with other reports for Han Chinese populations (Ng et al. 2007; Chang et al. 2007). The apparent difference in the association of these SNPs in Asians could be due to the low frequencies of the SNPs and the relatively small sample sizes used in the previous studies. Since we did not detect any association of rs11196218 or rs290487 in the present study, the associations of the two SNPs in the previous reports for Chinese might be specific to that population. In this study, rs7903146, rs11196205, and rs12255372 were in modest to strong LD. Based on HapMap data (JPT), the LD block surrounding exon 4 of *TCF7L2* in Asians does not exceed the gene (Supplementary Figure 1), which is consistent with findings in Caucasians (Grant et al. 2006). Previous reports (Ng et al. 2007; Chang et al. 2007) also found that the three SNPs were in a single LD block while the other two (rs11196218 and rs290487) were not. According to meta-analysis by Cauchi et al. (2007), *TCF7L2* is the most reproducible susceptibility gene for type 2 diabetes in various ethnic groups. *TCF7L2* also was one of the most significantly associated genes in recent genome-wide association studies (Sladek et al. 2007; WTCCC 2007). While the risk alleles of this gene are not common in East Asians, including Japanese, and the population attributable risk is much lower, *TCF7L2* is nevertheless a risk gene for type 2 diabetes in East Asians as well as in other populations. On the other hand, in a very recent online report, polymorphisms in the *TCF7L2* gene were found not to be associated with type 2 diabetes in a relatively large study of Pima Indians (Guo et al. 2007). Further investigation is required to

**Table 3** Pairwise linkage disequilibrium (LD) for five SNPs in the *TCF7L2* gene. Values of $D'$ (left lower) and of $r^2$ (upper right) for pairwise LD analysis in 974 control subjects of the Consortium set

| | rs7903146 | rs11196205 | rs12255372 | rs1196218 | rs290487 |
|---|---|---|---|---|---|
| rs7903146 | | 0.24 | 0.49 | 0.002 | 0.0036 |
| rs11196205 | 0.56 | | 0.44 | 0.012 | 0.0037 |
| rs12255372 | 0.93 | 1.00 | | 0.007 | 0.0036 |
| rs1196218 | 0.45 | 0.87 | 1.00 | | 0.0002 |
| rs290487 | 0.22 | 0.19 | 0.30 | 0.02 | |

**Table 4** Association analysis for haplotypes with three SNPs (rs7903146, rs11196205, rs12255372). *P* values were calculated by the chi-square test with estimated haplotype data from the Combined set

| Haplotype | Case | Control | *P* |
|---|---|---|---|
| C-G-G | 0.91 | 0.93 | $1.5 \times 10^{-4}$ |
| C-C-G | 0.032 | 0.031 | 0.72 |
| T-C-T | 0.032 | 0.018 | $5.3 \times 10^{-5}$ |
| T-G-G | 0.020 | 0.017 | 0.30 |

elucidate the differences in the contribution of the *TCF7L2* gene to type 2 diabetes among various populations.

*TCF7L2* regulates expression of the proglucagon gene (*GCG*), which encodes the precursor of glucagon, glucagon-like peptide 1 (GLP-1) (Yi et al. 2005). Several reports have found that polymorphisms of *TCF7L2* are associated with $\beta$-cell function (Florez et al. 2006; Saxena et al. 2006; Schäfer et al. 2007; Lyssenko et al. 2007). In this study, the association between the *TCF7L2* gene and HOMA-$\beta$ was found to disappear after adjustment for the various factors. Although the relationship of this gene to $\beta$-cell function is not clear in this study, our results suggest that *TCF7L2* is an important susceptibility gene for type 2 diabetes in Japanese. The pathophysiological mechanism of this gene in susceptibility to type 2 diabetes remains to be elucidated.

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## References

Bannai M, Higuchi K, Akesaka T, Furukawa M, Yamaoka M, Sato K, Tokunaga K (2004) Single-nucleotide-polymorphism genotyping for whole-genome-amplified samples using automated fluorescence correlation spectroscopy. Anal Biochem 327:215–221

Cauchi S, El Achhab Y, Choquet H, Dina C, Krempler F, Weitgasser R, Nejjari C, Patsch W, Chikri M, Meyre D, Froguel P (2007) *TCF7L2* is reproducibly associated with type 2 diabetes in various ethnic groups: a global meta-analysis. J Mol Med 85:777–782

Chang YC, Chang TJ, Jiang YD, Kuo SS, Lee KC, Chiu KC, Chuang LM (2007) Association study of the genetic polymorphisms of the transcription factor7- like 2 (TCF7L2) gene and type 2 diabetes in the Chinese Population. Diabetes 56:2631–2637

Florez JC, Jablonski KA, Bayley N, Pollin TI, de Bakker PI, Shuldiner AR, Knowler WC, Nathan DM, Altshuler D, Diabetes Prevention Program Research Group (2006) *TCF7L2* polymorphisms and progression to diabetes in the Diabetes Prevention Program. N Engl J Med 355:241–250

Grant SF, Thorleifsson G, Reynisdottir I, Benediktsson R, Manolescu A, Sainz J, Helgason A, Stefansson H, Emilsson V, Helgadottir A, Styrkarsdottir U, Magnusson KP, Walters GB, Palsdottir E, Jonsdottir T, Gudmundsdottir T, Gylfason A, Saemundsdottir J, Wilensky RL, Reilly MP, Rader DJ, Bagger Y, Christiansen C, Gudnason V, Sigurdsson G, Thorsteinsdottir U, Gulcher JR, Kong A, Stefansson K (2006) Variant of transcription factor 7-like 2 (*TCF7L2*) gene confers risk of type 2 diabetes. Nat Genet 38:320–323

Guo T, Hanson RL, Traurig M, Li Muller Y, Ma L, Mack J, Kobes S, Knowler WC, Bogardus C, Baier LJ (2007) TCF7L2 is not a major susceptibility gene for type 2 diabetes in Pima Indians: an analysis of 3501 individuals. Diabetes. [Epub ahead of print]

Hayashi T, Iwamoto Y, Kaku K, Hirose H, Maeda S (2007) Replication study for the association of TCF7L2 with susceptibility to type 2 diabetes in a Japanese population. Diabetologia 50:980–984

Horikoshi M, Hara K, Ito C, Nagai R, Froguel P, Kadowaki T (2007) A genetic variation of the transcription factor 7-like 2 gene is associated with risk of type 2 diabetes in the Japanese population. Diabetologia 50:747–751

Lyssenko V, Lupi R, Marchetti P, Del Guerra S, Orho-Melander M, Almgren P, Sjogren M, Ling C, Eriksson KF, Lethagen AL, Mancarella R, Berglund G, Tuomi T, Nilsson P, Del Prato S, Groop L (2007) Mechanisms by which common variants in the TCF7L2 gene increase risk of type 2 diabetes. J Clin Invest 117:2155–2163

Ng MC, Tam CH, Lam VK, So WY, Ma RC, Chan JC (2007) Replication and identification of novel variants at TCF7L2 associated with type 2 diabetes in Hong Kong Chinese. J Clin Endocrinol Metab 92:3733–3737

Pearson ER, Donnelly LA, Kimber C, Whitley A, Doney AS, McCarthy MI, Hattersley AT, Morris AD, Palmer CN (2007) Variation in TCF7L2 influences therapeutic response to sulfonylureas: a GoDARTs study. Diabetes 56:2178–2182

Saxena R, Gianniny L, Burtt NP, Lyssenko V, Giuducci C, Sjogren M, Florez JC, Almgren P, Isomaa B, Orho-Melander M, Lindblad U, Daly MJ, Tuomi T, Hirschhorn JN, Ardlie KG, Groop LC, Altshuler D (2006) Common single nucleotide polymorphisms in *TCF7L2* are reproducibly associated with type 2 diabetes and reduce the insulin response to glucose in nondiabetic individuals. Diabetes 55:2890–2895

Schäfer SA, Tschritter O, Machicao F, Thamer C, Stefan N, Gallwitz B, Holst JJ, Dekker JM, T'hart LM, Nijpels G, van Haeften TW, Haring HU, Fritsche A (2007) Impaired glucagon-like peptide-1-induced insulin secretion in carriers of transcription factor 7-like 2 (TCF7L2) gene polymorphisms. Diabetologia 50:2443–2450

Sladek R, Rocheleau G, Rung J, Dina C, Shen L, Serre D, Boutin P, Vincent D, Belisle A, Hadjadj S, Balkau B, Heude B, Charpentier G, Hudson TJ, Montpetit A, Pshezhetsky AV, Prentki M, Posner BI, Balding DJ, Meyre D, Polychronakos C, Froguel P (2007) A genome-wide association study identifies novel risk loci for type 2 diabetes. Nature 445:881–885

Wellcome Trust Case Control Consortium (2007) Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature 447:661–678

Yi F, Brubaker PL, Jin T (2005) TCF-4 mediates cell type-specific regulation of proglucagon gene expression by beta-catenin and glycogen synthase kinase-3beta. J Biol Chem 280:1457–1464

Zhang C, Qi L, Hunter DJ, Meigs JB, Manson JE, van Dam RM, Hu FB (2006) Variant of transcription factor 7-like 2 (*TCF7L2*) gene and the risk of type 2 diabetes in large cohorts of U.S. women and men. Diabetes 55:2645–2648

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# The ratio of leptin to adiponectin can be used as an index of insulin resistance

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**Abstract**

The level of leptin increases with obesity, whereas that of adiponectin decreases with obesity. It is reported that the ratio of leptin to adiponectin (L/A) is associated with insulin resistance. It is difficult to evaluate insulin resistance in diabetic patients who have a dysfunction of insulin secretion. The aim of this study was to examine whether the L/A ratio is a useful marker for insulin resistance in diabetic patients. We examined L/A in the serum of a total of 139 Japanese patients with type 2 diabetes mellitus (66 women and 73 men) and 7 healthy individuals recruited in our hospital. Changes in the levels of leptin and adiponectin were observed using the oral glucose tolerance test and a hyper- and euglycemic clamp test. Twenty-one patients with type 2 diabetes mellitus were observed for more than 6 months after treatment with pioglitazone, and 31 patients with type 2 diabetes mellitus were observed for more than 6 months after the treatment with metformin. The mean value of L/A in 139 Japanese patients with type 2 diabetes mellitus was 1.22 ± 1.41 (1.68 ± 1.76 in women, 0.81 ± 0.80 in men; $P = .0002$). In the clamp tests, L/A correlated with glucose infusion rate (GIR) ($r^2 = 0.26$, $P = .0034$). The correlation of L/A and GIR indicated a stronger correlation than either leptin ($r^2 = 0.144$, $P = .03$) or adiponectin alone ($r^2 = 0.023$, $P = .41$), or the homeostasis model assessment of insulin resistance ($r^2 = 0.103$, $P = .08$). The average hemoglobin $A_{1c}$ ($HbA_{1c}$) improved from 10.2% ± 1.2% to 9.2% ± 1.6% ($P = .0037$) in 6 months after treatment with pioglitazone. Our results indicate pioglitazone to be effective for $HbA_{1c}$ improvement in subjects with high L/A and low L/A. The average $HbA_{1c}$ improved from 9.2% ± 0.9% to 8.0% ± 1.2% ($P = .0002$) in 6 months after treatment with metformin. Our results indicate metformin to be effective for $HbA_{1c}$ improvement in subjects with a low L/A. In conclusion, we demonstrate that L/A is different between male and female subjects. The correlation of L/A and GIR by the euglycemic hyperinsulinemic clamp test suggests that L/A is a useful indicator for the choice of drug to treat diabetes mellitus.


## 1. Introduction

Obesity is defined by an accumulation of adipocytes throughout the body and is also associated with a variety of metabolic diseases. Insulin resistance or metabolic syndrome is now thought to be triggered by deposition of fat in the major target organs for insulin such as liver or muscle [1]. To improve the adverse metabolic state, it is necessary to create a negative balance in energy intake and energy consumption (ie, by exercising or by enhancing the oxidation of fatty acid in the tissues using medication), thereby leading to weight loss. Leptin and adiponectin are important hormones derived from fat cells and secreted into the serum. Both hormones improve insulin resistance [2,3], although the blood concentrations are contradictory depending on adipocyte deposition. Specifically, the level of leptin increases with obesity, whereas that of adiponectin decreases [4]. Moreover, adiponectin acts against arterial sclerosis as a "good hormone" [5]. It was recently reported that the ratio of leptin to adiponectin (L/A) could act as a useful marker for metabolic disease [6,7]. Indeed, L/A was reported to display a better correlation to insulin resistance than the level of leptin or adiponectin alone [8,9]. The ratio of leptin to adiponectin is an excellent indicator of obesity and could be a useful marker for the progression of arterial sclerosis

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Table 1
Changes in the concentration of leptin and adiponectin during the OGTT

| | 0 min | 60 min | 120 min |
|---|---|---|---|
| Plasma glucose (mg/dL) | 106.1 ± 10.9 | 191.0 ± 37.4 | 131.7 ± 50.3 |
| Insulin (mol/L) | 32.5 ± 14.6 | 364.4 ± 255.0 | 209.1 ± 99.0 |
| C-peptide (mol/L) | 301.2 ± 86.0 | 1641.7 ± 711.6 | 1472.9 ± 516.3 |
| Leptin (ng/mL) | 6.34 ± 2.89 | 5.88 ± 2.69 | 5.41 ± 2.31 |
| Adiponectin (μg/mL) | 7.87 ± 3.12 | 8.04 ± 3.43 | 8.07 ± 2.87 |

The number of subjects was 7. All values are the mean ± SD. The serum level of both leptin and adiponectin showed no significant change. Comparison was made by dependent *t* test.

because the levels of the 2 hormones fluctuate in the opposite direction depending on the amount of visceral fat. Here, we evaluate L/A in patients with type 2 diabetes mellitus to assess the clinical significance.

We measured the levels of leptin and adiponectin during an oral glucose tolerance test (OGTT) and hyper- and euglycemic clamp test. In addition, we examined the relationship between insulin resistance in the muscles and L/A during the euglycemic hyperinsulinemic clamp test. We then measured the levels of leptin and adiponectin in diabetic patients and examined the possible selectivity of diabetic drugs using this index.

## 2. Subjects and methods

A total of 139 Japanese patients with type 2 diabetes mellitus (66 women and 73 men) agreed to take part in this study. The mean age of the female subjects was 62.8 ± 11.6 years, with a mean body mass index (BMI) of 24.6 ± 5.8 kg/m$^2$. The mean age of the male subjects was 59.2 ± 12.8 years, with a mean BMI of 23.8 ± 3.7 kg/m$^2$. The mean age of diabetes onset for the female and male subjects was 51.7 ± 11.7 and 50.5 ± 11.6 years, respectively. The mean hemoglobin $A_{1c}$ ($HbA_{1c}$) of the female and male subjects was 8.0 ± 1.6 and 7.8 ± 2.0, respectively. In addition, 7 healthy individuals (6 women and 1 man) who received an OGTT in our hospital also participated in this study. At the time of recruitment, informed consent was obtained from each subject. All the patients had their serum leptin and adiponectin levels measured. The OGTT was performed with 75 g glucose; and the levels of leptin and adiponectin were measured at 0, 60, and 120 minutes. Fifteen patients (7 women and 8 men) with type 2 diabetes mellitus (mean age of female and male subjects: 64.4 ± 10.2 and 44.5 ± 9.7 years, respectively; mean BMI of female and male subjects: 23.6 ± 5.1 and 25.7 ± 3.2 kg/m$^2$, respectively) received a hyperglycemic clamp test. A total of 31 patients (14 women and 17 men) with type 2 diabetes mellitus (mean age of female and male subjects: 64.8 ± 9.8 and 48.7 ± 11.7 years, respectively; mean BMI of female and male subjects: 25.1 ± 4.9 and 25.4 ± 3.8 kg/m$^2$, respectively) received a euglycemic hyperinsulinemic clamp test. The hyperglycemic-euglycemic insulin clamp study was performed according to the standard protocol [10]. We maintained the hyperglycemic clamp at the glucose level of 225 mg/dL for 60 minutes. The levels of

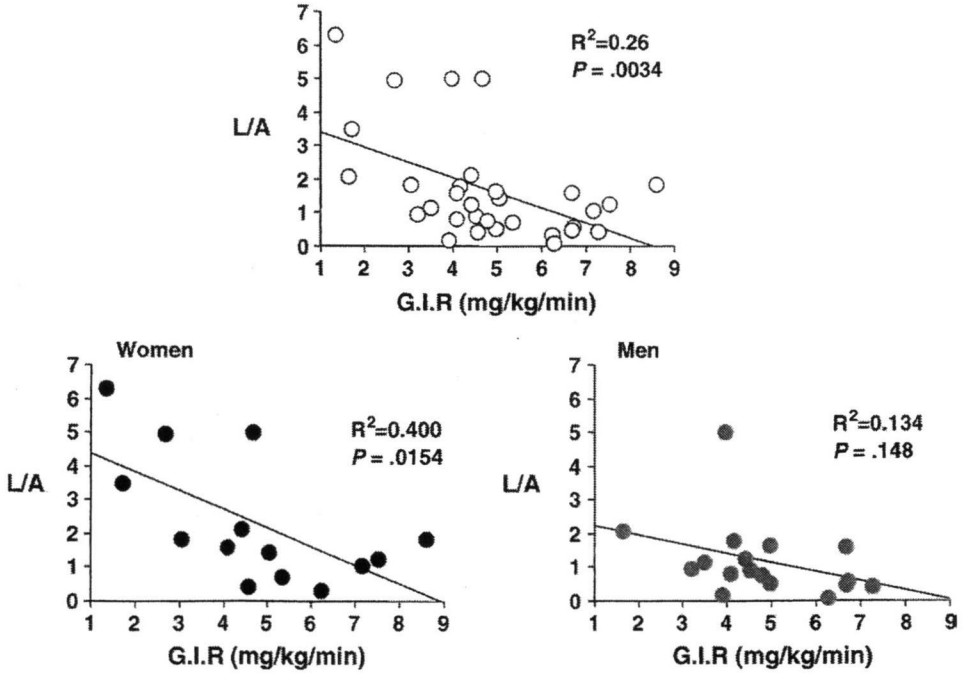


Fig. 1. Leptin to adiponectin ratio and GIR by hyperinsulinemic glucose clamp test. Comparison was made by simple linear regression analyses. Results were considered statistically significant at $P < .05$. Results examined separately for female and male subjects gave $r^2 = 0.400$ ($P = .0154$) for women and $r^2 = 0.134$ ($P = .148$) for men. Thus, a stronger correlation was found in female subjects.

Table 2
The changes of leptin and adiponectin concentrations during the hyperglycemic glucose clamp

| | 0 min | 60 min |
|---|---|---|
| Leptin (ng/mL) | 6.22 ± 4.62 | 5.51 ± 4.39 * |
| Adiponectin (μg/mL) | 5.39 ± 3.54 | 5.21 ± 3.40 |

The number of subject was 15. All values are the mean ± SD. Comparison was made by dependent *t* test.

* $P < .05$, compared with 0 min.

leptin and adiponectin were measured at 0 and 60 minutes. We set the euglycemic hyperinsulinemic clamp test (average insulin level of 98 mU/mL) at a glucose level of 100 mg/dL, which was maintained for 90 minutes; and the levels of leptin and adiponectin were measured at 0 and 90 minutes. Glucose infusion rate (GIR) at the end of the euglycemic hyperinsulinemic clamp test was also measured. Twenty-one patients (7 women and 14 men) with type 2 diabetes mellitus were observed for more than 6 months after treatment with pioglitazone (15 mg/d). Thirty-one patients (14 women and 17 men) with type 2 diabetes mellitus were observed for more than 6 months after treatment with metformin (500 mg/d). Almost all subjects in both groups of patients were taking a sulfonylurea. Serum leptin levels were measured by using the human leptin radioimmunoassay kit (LINCO Research, St Charles, MO). Serum adiponectin levels were measured by using the adiponectin enzyme-linked immunosorbent assay kit (Otsuka Pharmaceutical, Tokyo, Japan).

## 3. Statistical analyses

The clinical variables were compared by dependent and independent *t* test or by simple linear regression analysis. All statistical analyses were performed by the StatView 5.0 software (SAS Institute, Cary, NC). All values are the mean ± SD, and a value of $P < .05$ was considered statistically significant. Statistical methods are included in the tables and figures.

## 4. Results

### *4.1. Changes of serum leptin and adiponectin during OGTT and hyper- and euglycemic hyperinsulinemic clamp tests*

Changes in the mean values of leptin and adiponectin during OGTT are shown in Table 1. During the test, the mean plasma glucose values at 0, 60, and 120 minutes were 106, 191, and 131 mg/dL, respectively. The serum level of both leptin and adiponectin showed no significant change.

Changes in the mean values of leptin and adiponectin during the hyper- and euglycemic hyperinsulinemic clamp tests are shown in Table 2. The level of leptin decreased significantly 60 minutes ($P = .0017$) after the hyperglycemic clamp test, but no significant change in the level of adiponectin was observed. Indeed, our results concur with a previous study that concluded that the serum level of adiponectin is unaffected by hyperinsulinemia and hyperglycemia [11].

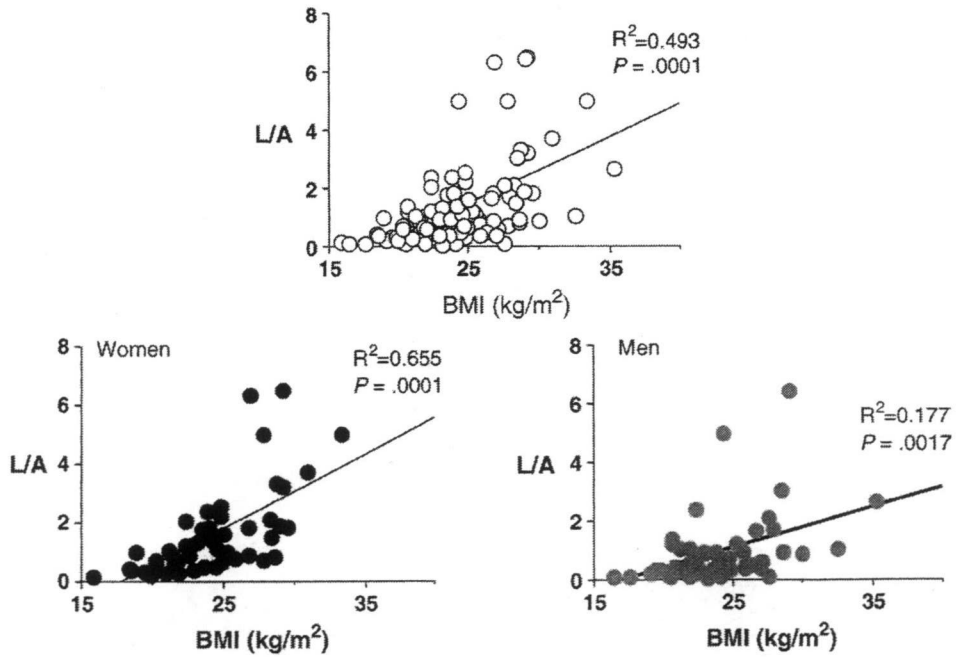


Fig. 2. Leptin to adiponectin ratio and BMI. Comparison was made by simple linear regression analyses. Results were considered statistically significant at $P < .05$. Results examined separately for female and male subjects gave $r^2 = 0.655$ ($P = .0001$) for women and $r^2 = 0.177$ ($P = .0017$) for men. Thus, a stronger correlation was found in female subjects. The correlation of L/A and BMI indicated a stronger correlation than leptin ($r^2 = 0289$, $P = .001$) or adiponectin alone ($r^2 = 0.052$, $P = .020$).

Table 3
Clinical characteristics of the study population before treatment

| | Treatment by pioglitazone | Treatment by metformin |
|---|---|---|
| Sex (F/M) | 7/14 | 14/17 |
| Age (y; F/M) | 60.0 ± 13.1/59.2 ± 14.3 | 58.9 ± 9.2/58.4 ± 8.8 |
| BMI (kg/m$^2$; F/M) | 28.0 ± 3.1/26.9 ± 4.4 | 24.3 ± 3.5/24.1 ± 4.6 |
| Onset of diabetes (y; F/M) | 51.2 ± 9.2/49.2 ± 8.3 | 49.1 ± 8.3/48.5 ± 8.8 |
| $HbA_{1c}$ (%; F/M) | 10.1 ± 0.7/10.7 ± 1.1 | 9.2 ± 1.0/9.3 ± 0.9 |
| Leptin (ng/mL; F/M) | 11.4 ± 2.6/3.9 ± 1.8 | 11.9 ± 5.5/4.0 ± 2.5 |
| Adiponectin (μg/mL; F/M) | 6.2 ± 2.4/5.0 ± 1.6 | 8.1 ± 3.0/7.4 ± 2.9 |
| L/A ratio (F/M) | 2.06 ± 0.79/0.92 ± 0.77 | 1.87 ± 1.54/0.79 ± 0.84 |

All values are the mean ± SD. F/M indicates female/male.

### 4.2. L/A correlated with GIR by euglycemic hyperinsulinemic clamp test

The mean values of L/A, BMI, and GIR in this clamp test were 1.62 ± 1.57 (2.18 ± 1.88 in women, 1.14 ± 1.12 in men), 25.4 ± 4.44 kg/m$^2$ (25.4 ± 4.69 in women, 25.4 ± 4.38 in men) and 4.76 ± 1.75 mg/(kg min) (4.82 ± 2.08 in women, 4.72 ± 1.48 in men), respectively. In the euglycemic hyperinsulinemic clamp tests, L/A correlated with GIR ($r^2$ = 0.26, $P$ = .0034) (Fig. 1). The correlation of leptin and GIR was weak ($r^2$ = 0.144, $P$ = .035), and adiponectin ($r^2$ = 0.023, $P$ = .41) or the homeostasis model assessment of insulin resistance (HOMA-IR) ($r^2$ = 0.103, $P$ = .08) was not correlated with GIR. The L/A correlation in women ($r^2$ = 0.400, $P$ = .0154) was stronger than that in men ($r^2$ = 0.134, $P$ = .148) (Fig. 1). The L/A was also correlated with BMI ($r^2$ = 0.240, $P$ = .003). The relationship is particularly good for female patients. It was also reported that L/A in women with polycystic ovary syndrome is related with insulin resistance [12]. However, these sex-based differences might be related to age variation within the subject group.

### 4.3. L/A in different BMI populations with type 2 diabetes mellitus

The L/A correlated with BMI ($r^2$ = 0.279, $P$ < .001), as shown in Fig. 2. The mean L/A was 1.22 ± 1.41. The mean L/A was significantly higher in women (1.68 ± 1.76) than in men (0.81 ± 0.80). However, this sex difference was not apparent when BMI ranged from 20 to 24. We then divided the subjects into 3 groups: BMI less than 20 (9 women and 8 men), BMI of 20 to 24 (24 women and 31 men), and BMI more than 24 (31 women and 31 men). The L/As of women and men were 0.48 ± 0.37/0.27 ± 0.11 for BMI less than 20 ($P$ = .078), 0.91 ± 0.60/0.75 ± 0.92 for BMI 20 to 24 ($P$ = .480), and 2.64 ± 2.13/0.95 ± 0.69 for BMI more than 24 ($P$ = .0001), respectively. No differences were observed in terms of the BMI between men and women in the 3 groups. Comparison was made by independent $t$ test.

### 4.4. L/A ratio and the effects of pioglitazone and metformin

The clinical parameters of the patients before treatment are shown in Table 3. The average $HbA_{1c}$ improved from 10.25% ± 1.2% to 9.2% ± 1.6% ($P$ = .0037) 6 months after treatment with pioglitazone. The average weight significantly changed from 66.4 ± 11.7 to 69.7 ± 12.9 kg ($P$ = .0025). Treatment with pioglitazone was considered effective because an $HbA_{1c}$ decline was observed in subjects with high L/A and low L/A (Fig. 3A). The average $HbA_{1c}$ improved from 9.2% ± 0.9% to 8.0% ± 1.2% ($P$ = .0002) 6 months after treatment with metformin. No change in average weight was observed (from 60.2 ± 11.9 to 60.5 ± 12.4 kg, $P$ = .66). Treatment with metformin was considered effective because an $HbA_{1c}$ decline was observed in subjects with a low L/A (Fig. 3B). Comparison was made by dependent $t$ test.

## 5. Discussion

Methods to alleviate or prevent insulin resistance and obesity have been intensively studied. The target molecules of these therapies are leptin, adiponectin, tumor necrosis factor α, and plasminogen activator inhibitor 1 derived from

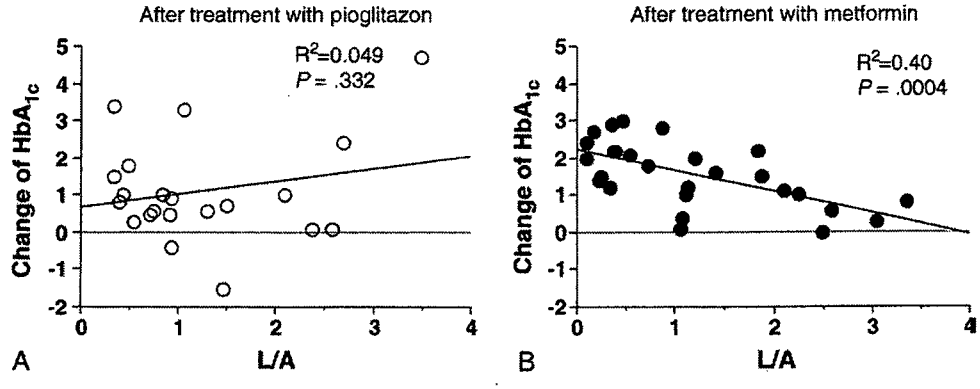


Fig. 3. Correlation of L/A and change of $HbA_{1c}$ after treatments with pioglitazone and metformin. In Fig. 2, the L/A ranges in 50% middle persons are from 0.580 to 2.100 for women and 0.335 to 1.041 for men. In this study, *low L/A subjects* are defined as those with less than 0.580 for women and 0.335 for men; *high L/A subjects* are those with more than 2.100 in women and 1.041 in men. Number of subjects undergoing treatment with pioglitazone having low L/A or high L/A was 3 and 7, respectively. Number of subjects undergoing treatment with metformin having low L/A or high L/A was 8 and 11, respectively. Comparison was made by simple linear regression analyses. Results were considered statistically significant at $P$ < .05.

fat cells [13-16]. In the present study, we measured leptin and adiponectin, which regulate insulin sensitivity, during the OGTT and hyper- and euglycemic clamp test. Our results show that the level of leptin decreases significantly during the hyperglycemic clamp test, but the change is not significant during the OGTT. These observations indicate that the duration of hyperglycemia is the determining factor of leptin concentration in the serum. Thus, long-term hyperglycemic conditions may obstruct appetite suppression as the level of leptin decreases. However, the precise mechanism remains unclear.

Leptin has been reported to be a good hormone because it improves insulin resistance [2]. Adiponectin is also a good hormone because it improves insulin resistance and has action on anti–arterial sclerosis [3]. However, the level of leptin increases with obesity, whereas that of adiponectin decreases [4]. Thus, we reasoned that the L/A ratio may be an excellent predictor for insulin resistance in diabetic patients. When diabetic patients are evaluated for insulin resistance using HOMA-R, it is essential that the fasting plasma glucose is greater than 140 mg/dL to avoid erroneous results [17]. This study clearly demonstrates that L/A correlates with GIR more closely than leptin and adiponectin alone or HOMA-R. We therefore conclude that L/A could be a useful index for insulin resistance in clinical practice.

It has been reported that both leptin and adiponectin in the peripheral tissues indicate oxidation enhancement of fatty acid through adenosine monophosphate–activated protein kinases [18,19], resulting in an improvement of insulin resistance and obesity. Metformin has been reported to decrease gluconeogenesis in the liver and increase uptake of glucose in the peripheral tissues through adenosine monophosphate–activated protein kinases [20]. Moreover, it has also been confirmed that pioglitazone, an insulin-sensitizing drug, is a powerful tool to increase plasma adiponectin. We reasoned that the balance of leptin and adiponectin in the body could influence the effect of antidiabetic drugs. Therefore, we examined the effect of these drugs using L/A as an index. Our results show that pioglitazone was particularly potent in subjects with a high L/A and in those with a low L/A compared with the midrange L/A population. This may be because many subjects with high L/A are low in adiponectin, making them particularly receptive to increases in the level of adiponectin. However, because the sample number is too small, we cannot explain why low L/A subjects are more amenable to $HbA_{1c}$ improvement. Further studies involving greater subject numbers will be required to investigate this mechanism in more detail.

On the other hand, metformin was potent in subjects with low L/A. Low L/A subjects in this study had a high serum adiponectin and low BMI. This may be because the condition of high adiponectin is working as a good balance for the communication of the signal. These results appear to contradict previous studies using metformin, which concluded that the drug is particularly effective for obese individuals [21]. However, more recent studies reported that there was no difference in the change of $HbA_{1c}$ between nonobese and obese subjects with type 2 diabetes mellitus [22]. Finally, we anticipate reinforcement of the effect of using metformin after an increase in adiponectin after treatment with pioglitazone. Further studies are required to confirm this hypothesis. In conclusion, we found that L/A is a good predictor for insulin resistance in diabetic patients. In addition, L/A may be a good indicator for assessing the effectiveness of antidiabetic drugs.

## Acknowledgments

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## References

[1] Matsuzawa Y, Funahashi T, Nakamura T. Molecular mechanism of metabolic syndrome X: contribution of adipocyte-derived bioactive substance. Ann NY Acad Sci 1999;892:146-54.

[2] Kamohara S, Burcelin R, Halaas JL, Friedman JM, Charron MJ. Acute stimulation of glucose metabolism in mice by leptin treatment. Nature 1997;389:374-7.

[3] Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, et al. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. Nat Med 2001;7: 941-6.

[4] Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, et al. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. Biochem Biophys Res Commun 1999;257:79-83.

[5] Kumada M, Kihara S, Sumitsuji S, Kawamoto T, Matsumoto S, Ouchi N, et al. Association of hypoadiponectinemia with coronary artery disease in men. Coronary artery disease. Arterioscler Thromb Vasc Biol 2003;23:85-9.

[6] Satoh N, Naruse M, Usui T, Tagami T, Suganami T, Yamada K, et al. Leptin-to-adiponectin ratio as a potential atherogenic index in obese type 2 diabetic patients. Diabetes Care 2004;27:2488-90.

[7] Kotani K, Sakane N, Saiga K, Kurozawa Y. Leptin:adiponectin ratio as an atherosclerotic index in patients with type 2 diabetes: relationship of the index to carotid intima-media thickness. Diabetologia 2005;48: 2684-6.

[8] Inoue M, Maehata E, Yano M, Taniyama M, Suzuki S. Correlation between the adiponectin-leptin ratio and parameters of insulin resistance in patients with type 2 diabetes. Metabolism 2005;54:281-6.

[9] Inoue M, Yano M, Yamakado M, Maehata E, Suzuki S. Relationship between the adiponectin-leptin ratio and parameters of insulin resistance in subjects without hyperglycemia. Metabolism 2006;55: 1248-54.

[10] DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. Am J Physiol 1979;237:E214-23.

[11] Heliovaara MK, Strandberg TE, Karonen SL, Ebeling P. Association of serum adiponectin concentration to lipid and glucose metabolism in healthy humans. Horm Metab Res 2006;38:336-40.

[12] Xita N, Papassotiriou I, Georgiou I, Vounatsou M, Margeli A, Tsatsoulis A. The adiponectin-to-leptin ratio in women with polycystic ovary syndrome: relation to insulin resistance and proinflammatory markers. Metabolism 2007;56:766-71.

[13] Ebihara K, Masuzaki H, Nakao K. Long-term leptin-replacement therapy for lipoatrophic diabetes. N Engl J Med 2004;351:615-6.

[14] Kumada M, Kihara S, Ouchi N, Kobayashi H, Okamoto Y, Ohashi K, et al. Adiponectin specifically increased tissue inhibitor of metalloproteinase-1 through interleukin-10 expression in human macrophages. Circulation 2004;109:2046-9.

[15] Samad F, Uysal KT, Wiesbrock SM, Pandey M, Hotamisligil GS, Loskutoff DJ. Tumor necrosis factor alpha is a key component in the obesity-linked elevation of plasminogen activator inhibitor 1. Proc Natl Acad Sci U S A 1999;96:6902-7.

[16] Ma LJ, Mao SL, Taylor KL, Kanjanabuch T, Guan Y, Zhang Y, et al. Prevention of obesity and insulin resistance in mice lacking plasminogen activator inhibitor 1. Diabetes 2004;53: 336-46.

[17] DeFronze RA, Ferrannini E, Simonson DC. Fasting hyperglycemia in non–insulin-dependent diabetes mellitus contributions of excessive hepatic glucose production and impaired glucose uptake. Metabolism 1989;38:387-95.

[18] Minokoshi Y, Kim YB, Peroni OD, Fryer LG, Muller C, Carling D, et al. Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. Nature 2002;415:339-43.

[19] Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, et al. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat Med 2002;8:1288-95.

[20] Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest 2001;108:1167-74.

[21] Shikata E, Yamamoto R, Takane H, Shigemasa C, Ikeda T, Otsubo K, et al. Human organic cation transporter (OCT1 and OCT2) gene polymorphisms and therapeutic effects of metformin. J Hum Genet 2007;52:117-22.

[22] Ong CR, Molyneaux LM, Constantino MI, Twigg SM, Yue DK. Long-term efficacy of metformin therapy in nonobese individuals with type 2 diabetes. Diabetes Care 2006;29:2361-4.

*Endocrine Journal* 2008, **55** (4), 699–707

# Lack of Association of *LRP5* and *LRP6* Polymorphisms with Type 2 Diabetes Mellitus in the Japanese Population

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**Abstract.** *Aims.* A missense mutation in the low density lipoprotein receptor-related protein 6 gene (*LRP6*) was recently shown to be responsible for a disorder characterized by early-onset coronary artery disease as well as diabetes mellitus (DM), hyperlipidemia, hypertension, and osteoporosis. Mice deficient in LRP5, a closely related paralog of LRP6, manifest a marked impairment in glucose tolerance. The aim of the present study was to examine whether common variants of *LRP5* and *LRP6* are associated with Type 2 DM or dyslipidemia in Japanese individuals. *Methods.* Thirteen single nucleotide polymorphisms (SNPs) of *LRP6* and nine SNPs of *LRP5* were genotyped in a total of 608 Type 2 DM patients and 366 nondiabetic control subjects (initial study). An association analysis was then performed for each SNP and for haplotypes. For some of the SNPs, we provided another sample panel of 576 cases and 576 controls for the replication study. The relation to clinical characteristics was also examined in diabetic subjects. *Results.* In the initial study, three SNPs of *LRP6* were found to be associated with susceptibility to Type 2 DM. However, this association was not detected in the replication panel. None of SNPs in *LRP5* were associated with Type 2 DM in the initial panel. Neither *LRP6* nor *LRP5* was associated with body mass index, HOMA-β, HOMA-IR or serum lipid concentrations. *Conclusions.* We found no evidence for a substantial effect of *LRP5* or *LRP6* SNPs on susceptibility to type 2 diabetes or clinical characteristics of diabetic subjects in Japanese population.

*Key words*: *LRP5*, *LRP6*, Single nucleotide polymorphism, Association study, Type 2 diabetes mellitus

(*Endocrine Journal* **55**: 699–707, 2008)

**THE** common form of Type 2 diabetes mellitus (DM) results from a complex interaction between genetic background and the environment. Identification of susceptibility genes for Type 2 DM has proven difficult because of the multifactorial nature of the disease. Genes responsible for monogenic disorders are potential contributors to similar conditions with a multifactorial etiology. A missense mutation (R611C) in the low density lipoprotein (LDL) receptor-related protein 6 gene (*LRP6*) was recently shown to be causally linked to a dominant form of early-onset coronary artery disease in an Iranian family. This mutation was also linked to DM, hyperlipidemia, hypertension, and osteoporosis in the same family [1]. Mice deficient in LRP5, a closely related paralog of LRP6, manifest a marked impairment in glucose tolerance [2]. LRP5 and LRP6 are members of the LDL receptor family [3] and function as co-receptors for Wnt ligands, playing an important role in Wnt signaling [4]. The transcription factor 7-like 2 gene (*TCF7L2*) shows a reproducible association with Type 2 DM [5] in multiple populations, and the encoded protein also plays a role in Wnt signaling [6].

These various observations suggest that *LRP5* and *LRP6* are potential susceptibility genes for Type 2

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DM. We therefore examined whether common variants of *LRP5* and *LRP6* might be associated with Type 2 DM or dyslipidemia in Japanese individuals.

## Subjects and Methods

*Subjects*

A total of 608 unrelated individuals with Type 2 DM and 366 unrelated nondiabetic control subjects were enrolled for the initial study. We provided another sample panel of 576 cases and 576 controls for the replication study (replication panel). In the initial panel, the mean ± SD of age, body mass index (BMI), and $HbA_{1c}$ were 61.3 ± 9.9 years, 23.8 ± 3.4 kg/m$^2$, and 7.9 ± 1.8%, respectively, for the diabetic subjects and 75.4 ± 8.1 years, 21.5 ± 3.6 kg/m$^2$, and 5.0 ± 0.4%, respectively, for the control subjects. In the replication panel, those for the cases were 60.2 ± 11.5 years, 23.9 ± 4.2 kg/m$^2$, and 7.8 ± 3.5%, respectively and, for the controls, 67.3 ± 6.5 years, 23.0 ± 2.9 kg/m$^2$, and 5.0 ± 0.4%, respectively. The diagnosis of Type 2 DM was based on the criteria of the American Diabetes Association (1997). The nondiabetic subjects were selected according to the following criteria: age of >60 years (only for the initial panel), no past history of glucose intolerance, $HbA_{1c}$ content of ≤5.7%, and no family history of DM. The study was performed with written informed consent from all subjects and was approved by the Ethics Committee of Kobe University Graduate School of Medicine or of Gifu University School of Medicine.

*Clinical assessment*

The BMI of each individual was directly measured at the time of collection of blood samples. The fasting plasma glucose concentration (FPG), fasting plasma immunoreactive insulin concentration (FIRI), serum concentrations of total cholesterol and high density lipoprotein (HDL)-cholesterol, and $HbA_{1c}$ level were determined by standard laboratory techniques calibrated with uniform standards. Indices of basal insulin secretion and resistance were derived by homeostasis model assessment (HOMA). The HOMA of β cell function (HOMA-β) was calculated as [FIRI (pmol/l) × 20]/[FPG (mmol/l) − 3.5] × 6, and that of insulin resistance (HOMA-IR) was calculated as [FPG (mmol/l) × FIRI (pmol/l)]/22.5 × 6 [7]. The serum concentration of LDL-cholesterol was calculated as [total cholesterol (mmol/l) − HDL-cholesterol (mmol/l) − [triglyceride (mmol/l)/5]] [8]. Among the 608 diabetic subjects of the initial panel, the 467 individuals who had not been treated with insulin were evaluated for HOMA-IR, HOMA-β, and FPG, whereas the 422 individuals who had not taken lipid-lowering drugs were evaluated for lipid parameters.

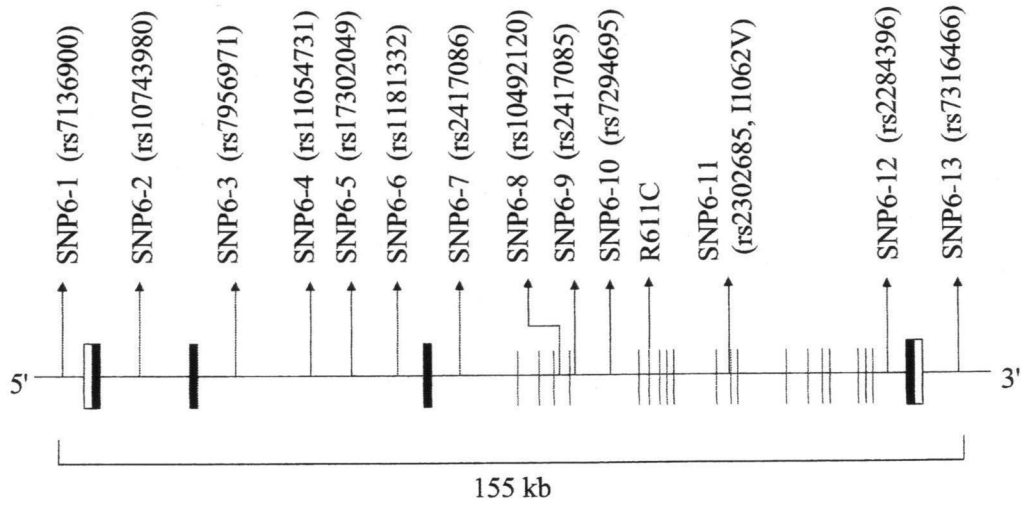
*DNA analysis*

We selected 13 single nucleotide polymorphisms (SNPs) of *LRP6* (Fig. 1A) and nine SNPs of *LRP5* (Fig. 2A) from the HapMap database (http://www.hapmap.org) according to the inclusion criteria as follows: minor allele frequencies >0.10 (except a nonsynonymous polymorphism, rs2302685 in *LRP6*) and linkage disequilibrium (LD) by $r^2$<0.8 in the Japanese data (JPT). Genomic DNA was extracted from blood with the use of a QIAamp DNA Blood Maxi Kit (Qiagen, Valencia, CA), and genotypes for the SNPs were determined with the TaqMan procedure (Applied Biosystems, Foster City, CA). The polymerase chain reaction was performed with ABsolute QPCR ROX Mixes (ABgene, Epsom, UK) and an ABI PRISM 7700 Sequence Detector System (Applied Biosystems); the amplification protocol included incubation at 95°C for 15 min followed by 40 cycles of 92°C for 15 s and 60°C for 1 min. Sequencing of exon 9 of *LRP6* was performed with the use of a Big Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems) and an automated DNA capillary sequencer (model 3100, Applied Biosystems).

*Statistical analysis*

We assessed association and Hardy-Weinberg equilibrium with the chi-square test. Linkage disequilibrium and haplotype analyses including permutation tests were performed with SNPAlyze version 5.1 pro software (Dynacom, Mobara, Japan). Haplotype estimation was performed by the expectation-maximization algorithm [9]. If we assume a minor allele frequency of 0.24, odds ratio of 1.3, and type I error probability (α) of 0.05, the power of our initial sample (608 cases and 366 controls) computed by the PS program [10] is 0.82. In case of combined sample (1184 cases and 942 controls), the power is 0.98. Averaged data are pre-

A.




B.

| D' \ r² | SNP6-1 | SNP6-2 | SNP6-3 | SNP6-4 | SNP6-5 | SNP6-6 | SNP6-7 | SNP6-8 | SNP6-9 | SNP6-10 | SNP6-11 | SNP6-12 | SNP6-13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SNP6-1 | | 0.20 | 0.19 | 0.08 | 0.00 | 0.39 | 0.20 | 0.00 | 0.00 | 0.13 | 0.16 | 0.13 | 0.00 |
| SNP6-2 | 0.75 | | 1.00 | 0.60 | 0.09 | 0.18 | 0.94 | 0.64 | 0.64 | 0.61 | 0.11 | 0.58 | 0.32 |
| SNP6-3 | 0.73 | 1.00 | | 0.60 | 0.09 | 0.19 | 0.94 | 0.66 | 0.66 | 0.60 | 0.11 | 0.58 | 0.33 |
| SNP6-4 | 0.56 | 0.93 | 0.93 | | 0.12 | 0.08 | 0.60 | 0.35 | 0.35 | 0.38 | 0.02 | 0.36 | 0.18 |
| SNP6-5 | -0.19 | -1.00 | -1.00 | -1.00 | | 0.02 | 0.09 | 0.06 | 0.06 | 0.06 | 0.01 | 0.06 | 0.03 |
| SNP6-6 | 0.88 | 1.00 | 1.00 | 0.79 | -1.00 | | 0.18 | 0.13 | 0.13 | 0.14 | 0.52 | 0.13 | 0.10 |
| SNP6-7 | 0.75 | 0.97 | 0.97 | 0.93 | -1.00 | 1.00 | | 0.64 | 0.64 | 0.56 | 0.11 | 0.53 | 0.31 |
| SNP6-8 | 0.02 | 0.96 | 0.96 | 0.85 | -1.00 | 0.71 | 0.96 | | 1.00 | 0.31 | 0.16 | 0.30 | 0.52 |
| SNP6-9 | 0.02 | 0.96 | 0.96 | 0.85 | -1.00 | 0.71 | 0.96 | 1.00 | | 0.31 | 0.16 | 0.30 | 0.52 |
| SNP6-10 | 0.58 | 0.80 | 0.80 | 0.76 | -0.82 | 0.84 | 0.77 | 0.65 | 0.65 | | 0.12 | 0.97 | 0.62 |
| SNP6-11 | 0.60 | 0.80 | 0.80 | 0.45 | -1.00 | 0.76 | 0.80 | 0.84 | 0.84 | 0.82 | | 0.17 | 0.26 |
| SNP6-12 | 0.58 | 0.78 | 0.77 | 0.73 | -0.81 | 0.84 | 0.74 | 0.65 | 0.65 | 1.00 | 1.00 | | 0.66 |
| SNP6-13 | -0.13 | 0.71 | 0.71 | 0.65 | -0.71 | 0.57 | 0.70 | 0.75 | 0.75 | 0.95 | 1.00 | 1.00 | |

**Fig. 1.** Genomic organization of *LRP6* and pairwise LD analysis of SNPs. (**A**) Schematic representation of the *LRP6* genomic region showing the locations of SNPs. Coding and noncoding sequences of exons are shown as closed and open boxes, respectively. Details of the SNPs are provided in Table 1. (**B**) Values of $D'$ (bold type) and of $r^2$ (nonbold type) for pairwise LD analysis in 92 control subjects. Three estimated LD blocks are highlighted in gray.

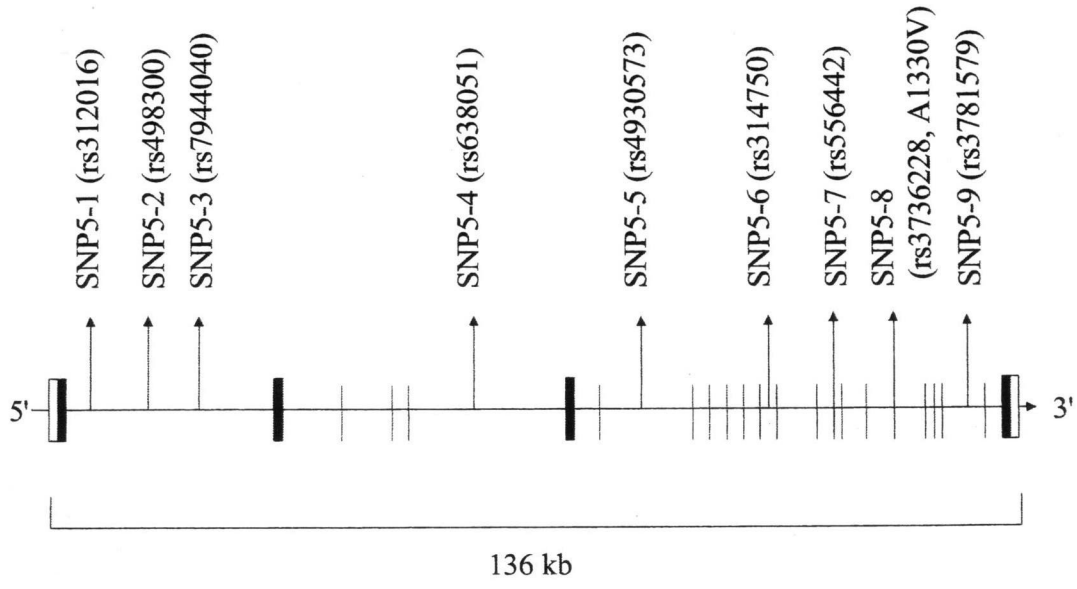
sented as means ± SD, and differences between groups were analyzed by ANOVA; if necessary, data were log transformed. Statistical analysis was performed with StatView software version 5.0-J (SAS Institute, Cary, NC). A $P$ value of <0.05 was considered statistically significant.

## Results

### *LRP6*

For analysis of LD in the *LRP6* genomic region, we genotyped 13 SNPs in 92 nondiabetic control subjects. The $D'$ and $r^2$ values for the 92 control subjects are shown in Fig. 1B. Two SNPs (SNP6-3, SNP6-8) were excluded from further genotyping because of their absolute LD. The remaining 11 SNPs, including a nonsynonymous polymorphism (I1062V, SNP6-11), were genotyped in all 608 Type 2 DM subjects and 366 control subjects. All SNPs with the exception of SNP6-13 were in Hardy-Weinberg equilibrium ($P$>0.01). Association results for the 11 genotyped SNPs are shown in Table 1. We found associations between three SNPs (SNP6-1, SNP6-2, SNP6-7) and susceptibility to Type 2 DM. SNP6-7 showed the strongest association (odds ratio = 0.74, 95% confidence interval = 0.59 to 0.93, $P$ = 0.008). SNP6-2 and SNP6-7 were in strong LD

A.




B.

| D' \ $r^2$ | SNP5-1 | SNP5-2 | SNP5-3 | SNP5-4 | SNP5-5 | SNP5-6 | SNP5-7 | SNP5-8 | SNP5-9 |
|---|---|---|---|---|---|---|---|---|---|
| **SNP5-1** | | 0.27 | 0.12 | 0.07 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **SNP5-2** | **-0.97** | | 0.48 | 0.08 | 0.00 | 0.03 | 0.03 | 0.01 | 0.00 |
| **SNP5-3** | **-0.86** | **0.91** | | 0.17 | 0.01 | 0.02 | 0.03 | 0.00 | 0.01 |
| **SNP5-4** | **-0.44** | **0.31** | **0.60** | | 0.01 | 0.00 | 0.00 | 0.01 | 0.01 |
| **SNP5-5** | **0.09** | **-0.07** | **0.15** | **0.13** | | 0.20 | 0.15 | 0.21 | 0.13 |
| **SNP5-6** | **0.06** | **0.24** | **0.26** | **0.07** | **0.61** | | 0.78 | 0.57 | 0.22 |
| **SNP5-7** | **0.00** | **0.26** | **0.34** | **0.09** | **0.59** | **0.97** | | 0.62 | 0.25 |
| **SNP5-8** | **0.05** | **0.12** | **0.10** | **0.08** | **0.54** | **0.86** | **0.98** | | 0.14 |
| **SNP5-9** | **-0.09** | **-0.08** | **0.08** | **-0.32** | **0.46** | **0.80** | **0.94** | **0.57** | |

**Fig. 2.** Genomic organization of LRP5 and pairwise LD analysis of SNPs. (A) Schematic representation of the LRP5 genomic region showing the locations of SNPs. Coding and noncoding sequences of exons are shown as closed and open boxes, respectively. Details of the SNPs are provided in Table 4. (B) Values of D' (bold type) and of $r^2$ (nonbold type) for pairwise LD analysis in 92 control subjects. Two estimated LD blocks are highlighted in gray.

with each other ($r^2 = 0.94$) in the 92 control subjects tested for LD. We also sequenced exon 9 of *LRP6*, which contains the previously identified missense mutation R611C [1]. No polymorphism was detected in the 24 diabetic and 24 control subjects subjected to such direct sequencing.

An LD block spanning SNP6-2 to SNP6-7 (Fig. 1B) encompassed a region containing exons 2 and 3 of *LRP6* but did not include exon 9. Although we performed haplotype analysis with SNP6-7 and the other SNPs, we did not detect an association with Type 2 DM more significant than that of SNP6-7. A haplotype comprising SNP6-5 = A and SNP6-7 = G showed an association with Type 2 DM similar to that of SNP6-7 alone (estimated haplotype frequencies of 0.19 and 0.24 in diabetic and control subjects, respectively; permutation *P* value computed by 10,000 permutations = 0.006).

When we consider multiple testing for the number of SNPs ($P<0.05/9$ SNPs; where four of 13 SNPs are not counted because of strong LD of $r^2>0.8$), the LD block including SNP6-7 is the most likely to be associated with the susceptibility to Type 2 DM. Therefore, we did not include SNP6-1 for further analysis (*P* value of SNP6-1 = 0.042). In order to examine a replication for the association of the SNPs or the LD block,

**Table 1.** Association analysis for SNPs of *LRP6* and Type 2 DM in the initial panel

| Position on chromosome 12 | SNP name | rs number | Major allele | Minor allele | MAF | | *P* value | Odds ratio (95% CI) |
|---|---|---|---|---|---|---|---|---|
| | | | | | Case | Control | | |
| 12314360 | SNP6–1 | rs7136900 | G | A | 0.08 | 0.10 | 0.042* | 0.72 (0.52–0.99) |
| 12304062 | SNP6–2 | rs10743980 | C | T | 0.19 | 0.23 | 0.019* | 0.77 (0.61–0.96) |
| 12279248 | SNP6–3 | rs7956971 | T | C | | | | |
| 12267732 | SNP6–4 | rs11054731 | G | A | 0.28 | 0.31 | 0.106 | |
| 12256592 | SNP6–5 | rs17302049 | A | G | 0.22 | 0.20 | 0.241 | |
| 12253186 | SNP6–6 | rs1181332 | A | G | 0.05 | 0.05 | 0.837 | |
| 12241380 | SNP6–7 | rs2417086 | A | G | 0.19 | 0.24 | 0.008* | 0.74 (0.59–0.93) |
| 12224619 | SNP6–8 | rs10492120 | C | T | | | | |
| 12222642 | SNP6–9 | rs2417085 | T | C | 0.16 | 0.18 | 0.186 | |
| 12214885 | SNP6–10 | rs7294695 | G | C | 0.19 | 0.21 | 0.293 | |
| 12193165 | SNP6–11 | rs2302685 | T | C | 0.06 | 0.05 | 0.333 | |
| 12166202 | SNP6–12 | rs2284396 | C | T | 0.18 | 0.22 | 0.094 | |
| 12159793 | SNP6–13 | rs7316466 | T | C | 0.16 | 0.16 | 0.984 | |

SNP position is indicated as base-pair number in NCBI build 127. MAF, minor allele frequency. *P* values for the difference in the minor allele frequency between cases and controls were calculated by the chi-square test; the odds ratio and 95% confidence interval (CI) were also calculated for the minor allele. Asterisks indicate *P* values of <0.05.

**Table 2.** Association analysis for rs2417086 (SNP6–7) and rs17302049 (SNP6–5)

| dbSNP ID | | Initial panel | | | | | |
|---|---|---|---|---|---|---|---|
| | | *n* | | MAF | | OR (95% CI) | *P* |
| | | Case | Control | Case | Control | | |
| rs2417086 | AA | 400 | 215 | 0.19 | 0.24 | 0.74 (0.59–0.93) | 0.008* |
| | AG | 188 | 128 | | | | |
| | GG | 20 | 23 | | | | |
| rs17302049 | AA | 364 | 231 | 0.22 | 0.20 | 1.15 (0.91–1.44) | 0.241 |
| | AG | 214 | 113 | | | | |
| | GG | 29 | 16 | | | | |

| dbSNP ID | | Replication panel | | | | | |
|---|---|---|---|---|---|---|---|
| | | *n* | | MAF | | OR (95% CI) | *P* |
| | | Case | Control | Case | Control | | |
| rs2417086 | AA | 305 | 336 | 0.23 | 0.22 | 1.06 (0.86–1.31) | 0.569 |
| | AG | 167 | 175 | | | | |
| | GG | 30 | 29 | | | | |
| rs17302049 | AA | 322 | 329 | 0.22 | 0.23 | 0.92 (0.75–1.13) | 0.451 |
| | AG | 170 | 188 | | | | |
| | GG | 28 | 33 | | | | |

| dbSNP ID | | Combined | | | | | |
|---|---|---|---|---|---|---|---|
| | | *n* | | MAF | | OR (95% CI) | *P* |
| | | Case | Control | Case | Control | | |
| rs2417086 | AA | 705 | 551 | 0.21 | 0.22 | 0.89 (0.77–1.03) | 0.130 |
| | AG | 355 | 303 | | | | |
| | GG | 50 | 52 | | | | |
| rs17302049 | AA | 686 | 560 | 0.22 | 0.22 | 1.01 (0.87–1.17) | 0.895 |
| | AG | 384 | 301 | | | | |
| | GG | 57 | 49 | | | | |

MAF, minor allele frequency. *P* values for the difference in the minor allele frequency between cases and controls were calculated by the chi-square test; the odds ratio and 95% confidence interval (CI) were also calculated for the minor allele. Asterisks indicate *P* values of <0.05.

SNP6-5 and SNP6-7 were genotyped in an independent sample panel (replication panel). However, none of these two SNPs or haplotypes were associated with Type 2 DM in the replication panel (Table 2 for SNPs, data not shown for haplotypes). No association was apparent when we combined the initial panel and the replication panel (Table 2).

Finally, we examined the relation of SNP6-7 to clinical characteristics in the diabetic subjects of the initial panel. However, no apparent association was found with BMI, HOMA-IR, HOMA-β, or serum lipid parameters (Table 3).

**Table 3.** Clinical characteristics of Type 2 DM subjects in the initial panel according to genotype for rs2417086 (SNP6–7) of *LRP6*.

| Parameter | AA | AG | GG | *P* value |
|---|---|---|---|---|
| Sex (male/female) | 234/166 | 107/81 | 8/12 | |
| Age (years) | 61 ± 10 ($n$ = 400) | 61 ± 10 ($n$ = 188) | 59 ± 12 ($n$ = 20) | 0.710 |
| BMI (kg/m$^2$) | 23.8 ± 3.5 ($n$ = 399) | 23.9 ± 3.3 ($n$ = 188) | 25.0 ± 4.5 ($n$ = 20) | 0.245 |
| FPG (mmol/l) | 7.7 ± 2.5 ($n$ = 307) | 7.5 ± 2.0 ($n$ = 139) | 8.2 ± 2.8 ($n$ = 19) | 0.285 |
| HOMA-IR* | 2.90 ± 2.39 ($n$ = 299) | 3.14 ± 3.32 ($n$ = 136) | 2.79 ± 2.06 ($n$ = 19) | 0.920 |
| HOMA-β* | 54.0 ± 95.4 ($n$ = 298) | 60.5 ± 80.2 ($n$ = 133) | 44.3 ± 50.3 ($n$ = 19) | 0.342 |
| Total cholesterol (mmol/l)* | 5.3 ± 1.0 ($n$ = 271) | 5.2 ± 1.0 ($n$ = 119) | 5.4 ± 0.6 ($n$ = 15) | 0.614 |
| HDL-cholesterol (mmol/l)* | 1.4 ± 0.4 ($n$ = 269) | 1.4 ± 0.4 ($n$ = 118) | 1.3 ± 0.4 ($n$ = 15) | 0.832 |
| LDL-cholesterol (mmol/l)* | 3.3 ± 0.9 ($n$ = 267) | 3.2 ± 0.9 ($n$ = 116) | 3.3 ± 0.5 ($n$ = 14) | 0.689 |
| Triglyceride (mmol/l)* | 1.4 ± 0.8 ($n$ = 271) | 1.7 ± 2.6 ($n$ = 119) | 1.8 ± 1.3 ($n$ = 15) | 0.302 |
| $HbA_{1c}$ (%)* | 8.0 ± 1.9 ($n$ = 400) | 7.7 ± 1.7 ($n$ = 187) | 7.7 ± 1.7 ($n$ = 20) | 0.312 |

Data are means ± SD. *P* values were calculated by ANOVA. *These parameters were log transformed before analysis.

**Table 4.** Association analysis for SNPs of *LRP5* and Type 2 DM in the initial panel.

| Position on Chromosome 11 | SNP name | rs number | Major allele | Minor allele | MAF | | *P* value |
|---|---|---|---|---|---|---|---|
| | | | | | Case | Control | |
| 67838979 | SNP5–1 | rs312016 | C | T | 0.48 | 0.48 | 0.920 |
| 67845407 | SNP5–2 | rs4988300 | G | T | 0.25 | 0.24 | 0.626 |
| 67857932 | SNP5–3 | rs7944040 | C | T | 0.14 | 0.15 | 0.584 |
| 67897990 | SNP5–4 | rs638051 | A | G | 0.26 | 0.28 | 0.442 |
| 67920032 | SNP5–5 | rs4930573 | C | G | 0.22 | 0.23 | 0.591 |
| 67938604 | SNP5–6 | rs314750 | A | G | 0.33 | 0.36 | 0.244 |
| 67949266 | SNP5–7 | rs556442 | A | G | 0.37 | 0.40 | 0.171 |
| 67957871 | SNP5–8 | rs3736228 | C | T | 0.29 | 0.30 | 0.587 |
| 67966294 | SNP5–9 | rs3781579 | T | C | 0.16 | 0.16 | 0.820 |

SNP position is indicated as base-pair number in NCBI build 127. MAF, minor allele frequency. *P* values for the difference in the minor allele frequency between cases and controls were calculated by the chi-square test.

### *LRP5*

Nine SNPs including a non-synonymous SNP (A1330V, SNP5-8) were genotyped in 92 control subjects. The $D'$ and $r^2$ values for these subjects are shown in Fig. 2B. Then all polymorphisms were genotyped in the initial panel of 608 Type 2 DM subjects and 366 control subjects. They were in Hardy-Weinberg equilibrium (P>0.01).

The results of association tests for susceptibility to Type 2 DM are shown in Table 4. No association between SNPs of *LRP5* and Type 2 DM was apparent in this panel. Next, we assessed the relations between all SNPs and clinical characteristics, BMI, HOMA-IR, HOMA-β, or serum lipid parameters in the diabetic subjects. However, no association was detected (data not shown).

## Discussion

We found no evidence for a substantial effect of *LRP5* or *LRP6* SNPs on susceptibility to type 2 DM in Japanese population. The association of rs2417086 (SNP6-7) or haplotype analysis in *LRP6* observed in the initial panel could be false positive due to the small sample number. A previous study showed that a mutation in *LRP6* was genetically linked with a familial disorder characterized by early-onset coronary artery disease as well as hyperlipidemia, hypertension, DM, and osteoporosis [1]. Genes that cause rare monogenic disorders might also confer susceptibility to similar conditions with a multifactorial etiology, although we failed to detect such a case. For example, genes responsible for maturity-onset diabetes of the young, an autosomal dominant monogenic form of DM, have also been associated with Type 2 DM [11–14].

LRP5 and LRP6 are co-receptors for Wnt ligands [4, 15]. Wnt signaling is necessary for embryogenesis but also plays important roles in postnatal development and tissue homeostasis. Mouse embryos homozygous for an insertion mutation in *Lrp6* exhibit a variety of severe developmental abnormalities, including midbrain defects, truncation of the skeleton, and limb anomalies [4]. *Lrp6* mutations cause early-onset osteoporosis in mice [16]. *Lrp5*$^{-/-}$ mice exhibit low bone density and frequent bone fractures. In human, mutations in *LRP5* cause the autosomal recessive disorder osteoporosis-pseudoglioma syndrome (OPPG) [17, 18]. Recently, some reports showed that polymorphisms of LRP5 were associated with bone mineral density [19–21]. Meanwhile, LRP5 plays an important role in glucose and lipid metabolism, with *Lrp5* knockout mice showing a marked impairment in glucose tolerance as a result of a reduced level of glucose-induced insulin secretion. Maintenance of these knockout mice on a high-fat diet also increases the plasma concentration of cholesterol to levels greater than those apparent in similarly fed normal mice [2]. We assessed whether polymorphisms of *LRP5 or LRP6* were associated with HOMA-IR, HOMA-β, or lipid parameters in patients with Type 2 DM. However, no such association was detected. We did not evaluate whether the polymorphisms were associated with osteoporosis or cardiovascular disease because information was not available for these disorders. Recently, Guo *et al.* showed that a haplotype including rs4988300 (SNP5-2) in *LRP5* was associated with BMI in Caucasian diabetic subjects [22]. Although we investigated the association between BMI and this polymorphism or haplotypes comprising SNP5-1 to SNP5-3, there was no association (data not shown).

To date, *TCF7L2* (also known as *TCF4*) has been the gene most reproducibly associated with Type 2 DM [5]. TCF7L2 is a transcription factor that partners with β-catenin in the canonical Wnt signaling pathway [6]. Wnt signaling and β-catenin are necessary for the proliferation of pancreas including β cells in mice [23–25]. Elucidation of the mechanisms by which this signaling pathway contributes to regulation of glucose metabolism may provide insight into the pathogenesis of Type 2 DM.

In conclusion, our results failed to reveal an association between Type 2 DM and SNPs or haplotypes of *LRP5* and *LRP6*. Furthermore, we found no association between these genes and any clinical characteristics such as serum LDL-cholesterol in the subjects with Type 2 DM. Similar studies are needed to clarify whether variants of *LRP5* and *LRP6* may be associated with coronary artery disease, hyperlipidemia, hypertension as well as Type 2 DM.

## Acknowledgments

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## References

1. Mani A, Radhakrishnan J, Wang H, Mani A, Mani MA, Nelson-Williams C, Carew KS, Mane S, Najmabadi H, Wu D, Lifton RP (2007) LRP6 mutation in a family with early coronary disease and metabolic risk factors. *Science* 315: 1278–1282.
2. Fujino T, Asaba H, Kang MJ, Ikeda Y, Sone H, Takada S, Kim DH, Ioka RX, Ono M, Tomoyori H, Okubo M, Murase T, Kamataki A, Yamamoto J, Magoori K, Takahashi S, Miyamoto Y, Oishi H, Nose M, Okazaki M, Usui S, Imaizumi K, Yanagisawa M, Sakai J, Yamamoto TT (2003) Low-density lipoprotein receptor-related protein 5 (LRP5) is essential for normal cholesterol metabolism and glucose-induced insulin secretion. *Proc Natl Acad Sci USA* 100: 229–234.
3. Caskey CT, Todd JA, Hess JF (1998) Isolation and characterization of LRP6, a novel member of the low density lipoprotein receptor gene family. *Biochem Biophys Res Commun* 248: 879–888.
4. Pinson KI, Brennan J, Monkley S, Avery BJ, Skarnes WC (2000) An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature* 407: 535–538.
5. Grant SF, Thorleifsson G, Reynisdottir I, Benediktsson R, Manolescu A, Sainz J, Helgason A, Stefansson H, Emilsson V, Helgadottir A, Styrkarsdottir U,

Magnusson KP, Walters GB, Palsdottir E, Jonsdottir T, Gudmundsdottir T, Gylfason A, Saemundsdottir J, Wilensky RL, Reilly MP, Rader DJ, Bagger Y, Christiansen C, Gudnason V, Sigurdsson G, Thorsteinsdottir U, Gulcher JR, Kong A, and Stefansson K (2006) Variant of transcription factor 7-like 2 (TCF7L2) gene confers risk of type 2 diabetes. *Nat Genet* 38: 320–323.

6. Yi F, Brubaker PL, Jin T (2005) TCF-4 mediates cell type-specific regulation of proglucagon gene expression by β-catenin and glycogen synthase kinase-3β. *J Biol Chem* 280: 1457–1464.
7. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC (1985) Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 28: 412–419.
8. Friedewald WT, Levy RI, Fredrickson DS (1972) Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 18: 499–502.
9. Excoffier L, Slatkin M (1995) Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population. *Mol Biol Evol* 12: 921–927.
10. Dupont WD, Plummer Jr WD. (1998) Power and sample size calculations for studies involving linear regression. *Control Clin Trials* 19: 589–601.
11. Bonnycastle LL, Willer CJ, Conneely KN, Jackson AU, Burrill CP, Watanabe RM, Chines PS, Narisu N, Scott LJ, Enloe ST, Swift AJ, Duren WL, Stringham HM, Erdos MR, Riebow NR, Buchanan TA, Valle TT, Tuomilehto J, Bergman RN, Mohlke KL, Boehnke M, Collins FS (2006) Common variants in maturity-onset diabetes of the young genes contribute to risk of type 2 diabetes in Finns. *Diabetes* 55: 2534–2540.
12. Gudmundsson J, Sulem P, Steinthorsdottir V, Bergthorsson JT, Thorleifsson G, Manolescu A, Rafnar T, Gudbjartsson D, Agnarsson BA, Baker A, Sigurdsson A, Benediktsdottir KR, Jakobsdottir M, Blondal T, Stacey SN, Helgason A, Gunnarsdottir S, Olafsdottir A, Kristinsson KT, Birgisdottir B, Ghosh S, Thorlacius S, Magnusdottir D, Stefansdottir G, Kristjansson K, Bagger Y, Wilensky RL, Reilly MP, Morris AD, Kimber CH, Adeyemo A, Chen Y, Zhou J, So WY, Tong PC, Ng MC, Hansen T, Andersen G, Borch-Johnsen K, Jorgensen T, Tres A, Fuertes F, Ruiz-Echarri M, Asin L, Saez B, van Boven E, Klaver S, Swinkels DW, Aben KK, Graif T, Cashy J, Suarez BK, van Vierssen Trip O, Frigge ML, Ober C, Hofker MH, Wijmenga C, Christiansen C, Rader DJ, Palmer CN, Rotimi C, Chan JC, Pedersen O, Sigurdsson G, Benediktsson R, Jonsson E, Einarsson GV, Mayordomo JI, Catalona WJ, Kiemeney LA, Barkardottir RB, Gulcher JR, Thorsteinsdottir U, Kong A, Stefansson K (2007) Two variants on chromosome 17 confer prostate cancer risk, and the one in TCF2 protects against type 2 diabetes. *Nat Genet* 39: 977–983.
13. Love-Gregory LD, Wasson J, Ma J, Jin CH, Glaser B, Suarez BK, Permutt MA (2004) A common polymorphism in the upstream promoter region of the hepatocyte nuclear factor-4α gene on chromosome 20q is associated with type 2 diabetes and appears to contribute to the evidence for linkage in an Ashkenazi Jewish population. *Diabetes* 53: 1134–1140.
14. Silander K, Mohlke KL, Scott LJ, Peck EC, Hollstein P, Skol AD, Jackson AU, Deloukas P, Hunt S, Stavrides G, Chines PS, Erdos MR, Narisu N, Conneely KN, Li C, Fingerlin TE, Dhanjal SK, Valle TT, Bergman RN, Tuomilehto J, Watanabe RM, Boehnke M, Collins FS (2004) Genetic variation near the hepatocyte nuclear factor-4α gene predicts susceptibility to type 2 diabetes. *Diabetes* 53: 1141–1149.
15. He X, Semenov M, Tamai K, Zeng X (2004) LDL receptor-related proteins 5 and 6 in Wnt/β-catenin signaling: Arrows point the way. *Development* 131: 1663–1677.
16. Kokubu C, Heinzmann U, Kokubu T, Sakai N, Kubota T, Kawai M, Wahl MB , Galceran J, Grosschedl R, Ozono K, Imai K (2004) Skeletal defects in ringelschwanz mutant mice reveal that Lrp6 is required for proper somitogenesis and osteogenesis. *Development* 131: 5469–5480.
17. Gong Y, Slee RB, Fukai N, Rawadi G, Roman-Roman S, Reginato AM, Wang H, Cundy T, Glorieux FH, Lev D, Zacharin M, Oexle K, Marcelino J, Suwairi W, Heeger S, Sabatakos G, Apte S, Adkins WN, Allgrove J, Arslan-Kirchner M, Batch JA, Beighton P, Black GC, Boles RG, Boon LM, Borrone C, Brunner HG, Carle GF, Dallapiccola B, De Paepe A, Floege B, Halfhide ML, Hall B, Hennekam RC, Hirose T, Jans A, Jüppner H, Kim CA, Keppler-Noreuil K, Kohlschuetter A, LaCombe D, Lambert M, Lemyre E, Letteboer T, Peltonen L, Ramesar RS, Romanengo M, Somer H, Steichen-Gersdorf E, Steinmann B, Sullivan B, Superti-Furga A, Swoboda W, van den Boogaard MJ, Van Hul W, Vikkula M, Votruba M, Zabel B, Garcia T, Baron R, Olsen BR, Warman ML, Osteoporosis-Pseudoglioma Syndrome Collaborative Group (2001) LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. *Cell* 107: 513–523.
18. Kato M, Patel MS, Levasseur R, Lobov I, Chang BH, Glass DA 2nd, Hartmann C, Li L, Hwang TH, Brayton CF, Lang RA, Karsenty G, Chan L (2002) Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. *J Cell Biol* 157: 303–314.
19. Ezura Y, Nakajima T, Urano T, Sudo Y, Kajita M, Yoshida H, Suzuki T, Hosoi T, Inoue S, Shiraki M, Emi M (2007) Association of a single-nucleotide varia-