

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

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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 \pm SD of age, body mass index (BMI), and HbA_{1c} were 61.3 ± 9.9 years, 23.8 ± 3.4 kg/m², and $7.9 \pm 1.8\%$, respectively, for the diabetic subjects and 75.4 ± 8.1 years, 21.5 ± 3.6 kg/m², and $5.0 \pm 0.4\%$, respectively, for the control subjects. In the replication panel, those for the cases were 60.2 ± 11.5 years, 23.9 ± 4.2 kg/m², and $7.8 \pm 3.5\%$, respectively and, for the controls, 67.3 ± 6.5 years, 23.0 ± 2.9 kg/m², and $5.0 \pm 0.4\%$, 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 $\leq 5.7\%$, 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 $[\text{FIRI (pmol/l)} \times 20]/[\text{FPG (mmol/l)} - 3.5] \times 6$, and that of insulin resistance (HOMA-IR) was calculated as $[\text{FPG (mmol/l)}$

$\times \text{FIRI (pmol/l)}]/22.5 \times 6$ [7]. The serum concentration of LDL-cholesterol was calculated as $[\text{total cholesterol (mmol/l)} - \text{HDL-cholesterol (mmol/l)} - [\text{triglyceride (mmol/l)/5}]]$ [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 non-synonymous 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-

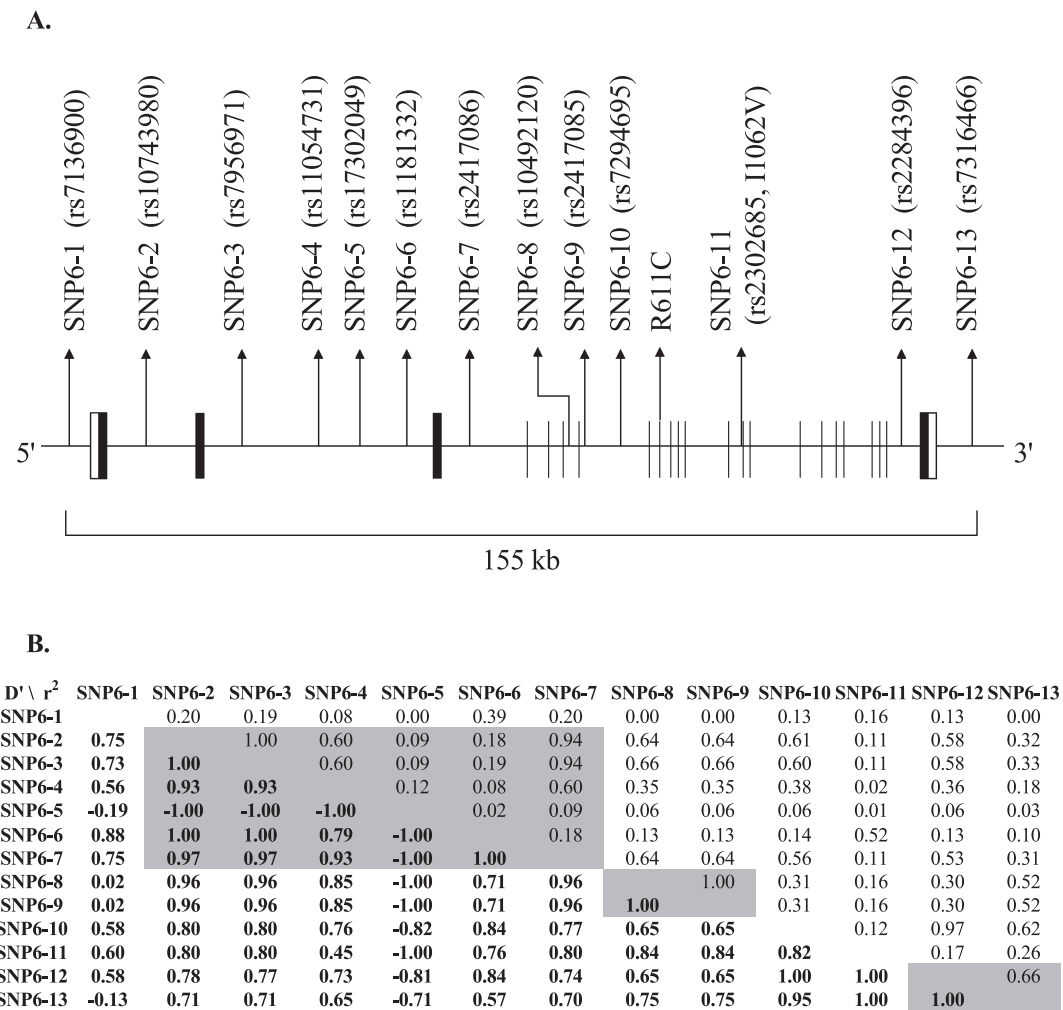


Fig. 1. Genomic organization of *LRP6* and pairwise LD analysis of SNPs. (A) Schematic representation of the *LRP6* genomic region showing the locations of SNPs. Coding and noncoding sequences of exons are shown as closed and open boxes, respectively. Details of the SNPs are provided in Table 1. (B) 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 \pm 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 non-synonymous 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

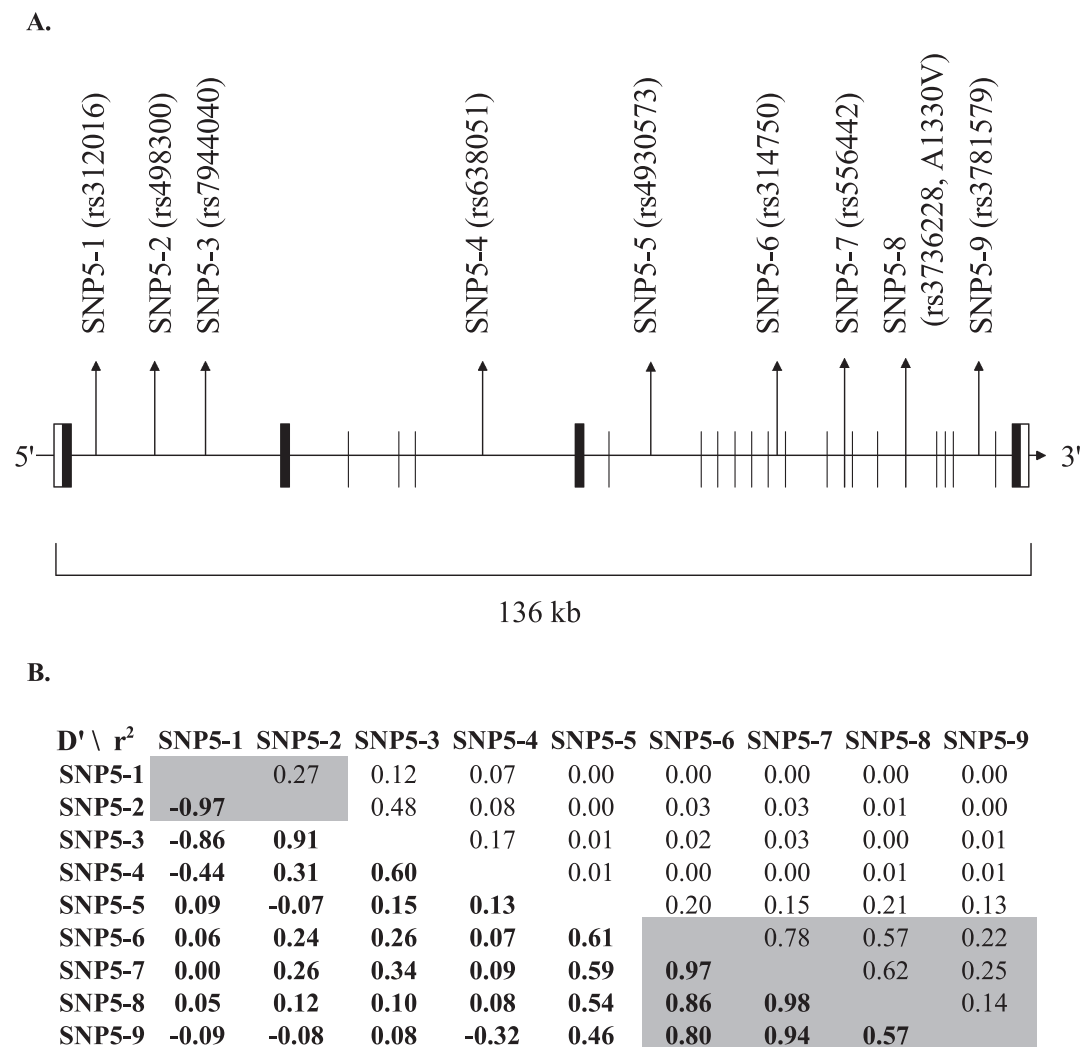


Fig. 2. Genomic organization of *LRP5* and pairwise LD analysis of SNPs. (A) Schematic representation of the *LRP5* genomic region showing the locations of SNPs. Coding and noncoding sequences of exons are shown as closed and open boxes, respectively. Details of the SNPs are provided in Table 4. (B) Values of D' (bold type) and of r² (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		<i>P</i> 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)

Initial panel							
dbSNP ID	<i>n</i>			MAF		OR (95% CI)	<i>P</i>
	Case	Control		Case	Control		
rs2417086	AA	400	215	0.19	0.24	0.74	0.008* (0.59–0.93)
	AG	188	128				
	GG	20	23				
rs17302049	AA	364	231	0.22	0.20	1.15	0.241 (0.91–1.44)
	AG	214	113				
	GG	29	16				
Combined							
dbSNP ID	<i>n</i>			MAF		OR (95% CI)	<i>P</i>
	Case	Control		Case	Control		
rs2417086	AA	705	551	0.21	0.22	0.89	0.130 (0.77–1.03)
	AG	355	303				
	GG	50	52				
rs17302049	AA	686	560	0.22	0.22	1.01	0.895 (0.87–1.17)
	AG	384	301				
	GG	57	49				

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

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.

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

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