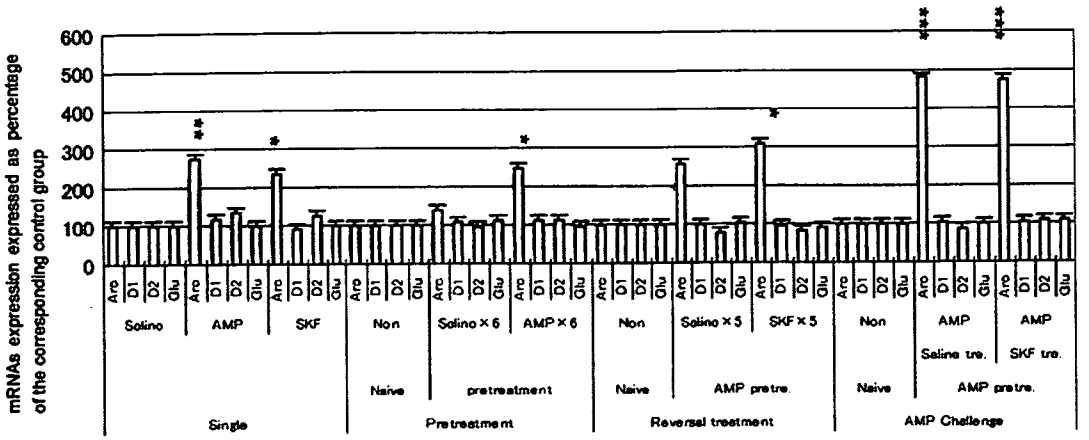

Fig. 5. Mean (±S.E.M.) mRNA expression levels after single injections in prefrontal cortex (left side). The asterisks (* and **) represent a significant difference from the mRNA expression level of the saline-treated control group ($p<0.05$ and $p<0.01$, respectively). $N=4$–6 in each group. Mean (±S.E.M.) mRNA expression levels after six intermittent administrations of AMP or saline pretreatment in prefrontal cortex (left center). The asterisk (*) represents a significant difference from the mRNA expression level of the naive control group ($p<0.05$). $N=4$–6 in each group. Mean (±S.E.M.) mRNA expression levels after reversal treatment in prefrontal cortex (right center). The asterisk (*) represents a significant difference from the mRNA expression level of the naive control group ($p<0.05$). $N=4$–6 in each group. Mean (±S.E.M.) mRNA expression levels after challenge administration of AMP in prefrontal cortex (right side). The asterisks (***) represent significant differences from the mRNA expression level of the naive control group ($p<0.001$). $N=4$–6 in each group.

0.042, NS], D_2 receptor [$F(2,9)=0.178$, NS] and mGluR1 [$F(2,9)=0.132$, NS] mRNA expression levels after the AMP challenge.

4. Discussion

This study demonstrated (i) that AMP-induced stereotypy is reversed by a D_1 agonist, and (ii) that the reversal effect of this D_1 agonist on stereotypy lasts for 4 weeks.

4.1. Reversal of behavioral sensitization by D_1 agonist

Behavioral sensitization in rodents is characterized by augmented ambulation and stereotypy, and, once established, persists for a long time. It is difficult to reverse behavioral sensitization once established; in our previous studies, chlorpromazine (Hirabayashi and Tadokoro, 1993), haloperidol and ceruletide (cholecystokinin) (Kuribara, 1993a), D_1 and D_2 receptor antagonists, namely, SCH-23390 and YM-09151-2 (Kuribara, 1993b) respectively, and MK-801, which is a noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist (Ida et al., 1995), did not reduce locomotor activity in behaviorally sensitized rats. To our knowledge, there are no other reports on reversal treatment for behavioral sensitization except for a few reports on locomotor activity (King et al., 2000) (King et al., 2000; Li et al., 2000).

In this study, we were able to reverse AMP-induced stereotypy, once established, using a D_1 receptor agonist. Stereotyped behavior is an important indicator in this animal model. We consider an increase in the rate of stereotypy as an important indicator of sensitization, first because the rate of stereotypy increased with repeated AMP administrations, and second because the increase in the rate of stereotypy prevented an increase in locomotor count in our study.

In this study, the direct dopamine receptor agonist SKF produced effects opposite to those of the indirect dopamine receptor agonist AMP. However, from our data, it seems that the effect of reversal treatment requires a selective stimulation of the dopamine D_1 receptor, regardless of whether it is direct or indirect. For example, as mentioned in the Introduction, pergolide, a direct D_1 and D_2 dopamine receptor agonist, increases ambulation count (Li et al., 2000) and enhances cocaine craving (Haney et al., 1998). Moreover, in the cocaine relapse model, D_1 and D_2 class agonists exert opposite effects (Self et al., 1996). D_2 agonists induce cocaine-seeking behavior and enhance the priming effects of cocaine, whereas D_1 receptor agonists inhibit cocaine-seeking behavior triggered by priming injections of cocaine. De Vries et al. (1998) demonstrated that the reinstatement of cocaine-seeking behavior is associated with the expression of behavioral sensitization (De Vries et al., 1998). It is therefore important that AMP-induced stereotypy is also reversed by only D_1 receptor stimulation.

Then how would D_1 stimulation reverse AMP-induced stereotypy? It is difficult to interpret the reversal effect demonstrated here because it is associated with the AMP pharmacology. From their electrophysiological study results, Li et al. (2000) suggested that the reversal of locomotor sensitization occurs as a result of the reversal of an underlying neuroadaptation, namely, the enhanced response of neurons to D_1 receptor stimulation. Subsequently, their group reported that D_1 receptor stimulation enhances mGluR1 phosphorylation (Chao et al., 2002a,b) and mGluR1 surface expression in rat neurons (Chao et al., 2002a,b). These results suggest that reversal of stereotypy induced by D_1 stimulation also requires a reversal "neuroplastic" process as does the development and maintenance of behavioral sensitization (Wolf, 1998).

There was no significant decrease in locomotor activity after SKF treatment in this study. As shown in Fig. 2, repeated SKF treatments did not significantly reduce the sensitized locomotor response, as shown by the time course data. There are three possible reasons the SKF treatment did not reverse the sensitized locomotor response. First, as mentioned above, locomotor activity and stereotyped behavior were viewed as competing behaviors. A decrease in the time of stereotyped activity may lead to the increase in locomotor count. Second, we used a challenge dose that was the

same as the pretreatment dose so as to evaluate the effect for stereotypy. If we had used a very low challenge dose (0.1 mg/kg, for example) to minimize stereotyped behavior, we might have been able to produce a reversal effect for locomotor activity similar to that previously reported by Li et al. (2000). Finally, the rats were observed for 3 h in the test cages. Measuring locomotor activity for a longer period may yield different results.

In this study, we were also able to still reduce the reactivity of stereotypy to AMP 4 weeks after the SKF treatment, suggesting that this is not a temporary phenomenon and that the reversal effect lasts for a long time. This is very important because, if the same condition occurs in clinical situations, it would not be necessary to continue medication to remove the acquired vulnerability to AMP. Wada (2000) suggested that abusers have psychological problems after the cessation of drug abuse and most of them have no chance of receiving continuous medication in Japan. Unfortunately, it is indicated that the reversal effect of SKF becomes weaker with time, because the degree of stereotypy after 4 weeks of withdrawal is the same as that obtained after six AMP pretreatments in our study.

4.2. Reversal of behavioral sensitization by D_1 and D_2 antagonists

In this study, D_1 and D_2 antagonists did not exert a reversal effect when administered alone. The results of our study are in agreement with those of previous studies showing that sensitization, once established, is not changed by treatment with D_1 and D_2 antagonists (Kuribara, 1995b). A coadministration of the D_1 agonist SKF and the D_1 antagonist SCH cancelled the reversal effect induced by SKF, while that of SKF and the D_2 antagonist YM maintained it. Therefore, it is suggested that the reversal of AMP-induced stereotypy requires D_1 receptor stimulation.

4.3. D_1 receptor, D_2 receptor, mGluR1 and arc mRNA expression levels in PFC

The expression levels of dopamine D_1 and D_2 receptor mRNAs were not changed by our pretreatment schedule of six intermittent AMP injections (1.0 mg/kg, i.p.). As mentioned in the Introduction, Schmidt-Mutter et al. (1999) reported that repeated exposures to cocaine (20 mg/kg) for 10 days followed by a 14-h withdrawal period, induced increasing effects on D_1 and D_2 dopamine receptor mRNA expression levels in PFC. Similarly, Lu et al. (1999) reported that the mGluR1 mRNA level increased on the 3rd day of withdrawal from five daily injections of AMP (5 mg/kg/day). Nevertheless, the mGluR1 mRNA expression level showed no change in our study. Experiments using various AMP doses and time periods for withdrawal and decapitation may help explain this discrepancy.

As mentioned in the Introduction, we analyzed the expression pattern of the neuroplasticity-related gene *arc* to gain insight into the molecular mechanism of behavioral sensitization. *Arc* expression level reportedly increases as a result of subchronic administrations of AMP (Klebaur et al., 2002; Gonzalez-Nicolini et al., 2002), MAP (Fujiyama et al., 2003; Yamagata et al., 2000; Kodama et al., 1998) and cocaine (Samaha et al., 2004; Yuferov et al., 2003; Freeman et al., 2002; Fosnaugh et al., 1995). Similarly in this study, repeated administrations of AMP enhanced *arc* expression in the cerebral cortex.

Interestingly, both single and repeated administrations of SKF significantly increased the *arc* expression level. How does D_1 stimulation enhance *arc* expression? It is suggested that D_1 receptor stimulation activates adenylyl cyclase (Cristina et al., 1998) by stimulating Gs proteins coupled to the D_1 receptor, and adenylyl cyclase activates the cAMP/protein kinase A (PKA)/cAMP-responsive element binding protein (CREB) signal transduction pathway, and CREB phosphorylation induces *arc* in dentate granule cells (Ying et al., 2002). This hypothesis is in agreement with the set of molecular mechanisms involved in learning: the stimulation of dopamine D_1 receptors, the activation of the cAMP/PKA/CREB signal transduction pathway, a transient burst of altered gene expression, and synaptic rearrangement (Berke and Hyman, 2000; Di Chiara, 2000; Dani et al., 2001). This raises the possibility that *arc* plays a role in multiple forms of synaptic plasticity, i.e., not only in AMP-induced behavioral sensitization, but also in neurobehavioral adaptations associated with the reversal effect induced by D_1 receptor stimulation.

Arc levels were elevated after AMP challenges in sensitized rats, compared with those after single AMP injection, although there was no significant difference in *arc* expression level between the saline and SKF treatment groups after the AMP challenge. Therefore *arc* expression level in the homogenate of mPFC does not correlate with the reversal effects of SKF in AMP sensitization. There is nevertheless the possibility that SKF treatment has formed novel neural circuits which are associated with the reversal effects and which also express *arc* after AMP challenge. Therefore topographical information on *arc* induction in the brain and the quantification of other cytoskeleton and synapse-associated genes will provide further insight into this finding. In addition, various time periods for decapitation will provide further information.

Noteworthily, repeated treatments with saline after AMP pretreatment showed an increase in *arc* expression level, suggesting a cross sensitization between AMP and stressful stimulants.

In summary, we have evaluated the effects of a D_1 agonist on AMP-induced behavioral sensitization (locomotor activity and stereotyped behavior) and the mRNA expression levels of the D_1 and D_2 receptors, mGluR1 and arc in the prefrontal cortex of rats. In the SKF treatment group, stereotyped behavior rate significantly decreased after both 3-day and 4-week withdrawal periods. SKF+SCH treatment inhibited the decreasing effect of SKF treatment. AMP administration significantly increased *arc* expression level. The SKF treatment group showed a marked increase in *arc* expression level after both the single SKF injection and the repeated treatments with AMP during the pretreatment period compared with the control groups. *Arc* expression level was further augmented by the treatment with saline after the AMP pretreatment.

There was no significant difference in *arc* expression level, between the saline treatment group and the SKF treatment group after the AMP challenge suggesting that *arc* was a non-specific marker in this investigation.

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Lack of association of *LRP5* and *LRP6* polymorphisms with Type 2 diabetes mellitus in the Japanese population

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Running head: Association study of *LRP5* and *LRP6* with Type 2 DM

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.*** 13 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 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

Introduction

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 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* (Figure 1A) and nine SNPs of *LRP5* (Figure 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 presented 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 Figure 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 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 (Figure 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 didn't 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, 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).

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 Figure 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 were 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 the Caucasian diabetic subjects [22]. Although we investigated 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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