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RESEARCH ARTICLE

Quantitative trait loci that determine plasma insulin levels in F_2 intercross populations produced from crosses between DDD/Sgn and C57BL/6J inbred mice

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Abstract. When compared to C57BL/6J (B6) mice, DDD/Sgn (DDD) mice has substantially higher plasma insulin levels in both sexes. In this study, we performed quantitative trait loci (QTL) mapping of plasma insulin levels in F_2 male mice produced by crosses between DDD and B6 mice. By single-QTL scans, we identified one significant QTL on chromosome 9. When body weight was included as an additive covariate, we identified two significant QTL on chromosomes 9 and 12; the latter coincided with a QTL that was previously identified in F_2 female mice produced by the same two strains. The inheritance mode and the direction of the allelic effect of QTL on chromosome 12 were similar in both sexes, but those on chromosome 9 differed between males and females, suggesting that the QTL on chromosome 9 was sex-specific. Based on phenotypic correlations of plasma insulin levels with body weight and plasma levels of total cholesterol, triglyceride and testosterone, we subsequently assessed whether these insulin QTL explain the variation in other metabolic traits by using a point-wise significance threshold of $P = 0.05$. QTL on chromosome 12 had no significant effect on any trait. In contrast, QTL on chromosome 9 had significant effects on body weight and total cholesterol level. We postulate that *Gpr68* and *Cyp19a1* are plausible candidate genes for QTL on chromosomes 12 and 9, respectively. These findings provide insight into the genetic mechanisms underlying insulin metabolism.

Keywords. DDD/Sgn mice; plasma insulin level; quantitative trait loci mapping.

Introduction

Plasma insulin level is a representative quantitative trait controlled by multiple genes under the influence of environmental factors. Plasma insulin has clinical implications in diabetes mellitus; therefore, it is crucially important to identify genes influencing variation in its plasma level. Many quantitative trait loci (QTL) mapping studies have identified genes that influence circulating insulin levels in rodents (Schmidt *et al.* 2008). Most QTL have conserved synteny with human loci, indicating that they are a good model for biological processes in human. Nevertheless, causative genes or causal variants have not been comprehensively identified, and further QTL mapping studies using additional strain combinations are needed to identify novel genomic regions.

In the present study, we performed QTL mapping of plasma insulin levels in F_2 male mice produced by the cross between C57BL/6J and DDD/Sgn mice. We have previously performed QTL mapping studies for body weight, plasma testosterone concentration and plasma lipid concentrations in the same intercross population (Suto and Kojima 2017a, b); this provides a basis for analyses of the genetic relationship between plasma insulin level and metabolic traits. Further, we have previously performed a similar QTL mapping study for insulin levels in F_2 female mice produced by the same two parental strains (Suto 2013), enabling analyses of sex differences in the genetic mechanism underlying the control of circulating insulin levels.

Materials and methods

Mice

The inbred mouse strains DDD and B6 were maintained at the National Institute of Agrobiological Sciences (NIAS, Tsukuba, Japan). Reciprocal crosses between DDD and B6 strains produced DB (♀DDD × ♂B6) F_1 and BD (♀B6 × ♂DDD) F_1 mice, which were intercrossed to produce DB F_2 (n = 150) and BD F_2 (n = 150) male mice (Suto and Kojima 2017a, b).

All mice were weaned at four weeks of age and 4–5 mice were housed together in a cage during the experiments. All mice were maintained in a specific pathogen-free facility with a regular light cycle and controlled temperature and humidity. Food (CRF-1; Oriental Yeast, Tokyo, Japan) and water were freely available throughout the experimental period. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of NIAS.

Plasma insulin analysis

The plasma insulin level was determined at 11–14 weeks in DDD, B6 and F_1 mice, and at 11–12 weeks (71–80 days after birth) in F_2 mice.

Mice were euthanized with an overdose of ether immediately after they were weighed in the morning (from 10:00 to 12:00). Blood was collected from the heart of an individual mouse using heparin as an anticoagulant. Plasma was separated by centrifugation at 2000 $\times g$ for 15 min at 4°C and was stored at −80°C until use. The plasma insulin level was determined using the Mouse Insulin ELISA kit (Shibayagi, Gunma, Japan).

Genotyping

Microsatellite sequence length polymorphisms were identified by electrophoresis after PCR amplification of genomic DNA. PCR amplification was performed using a TaKaRa PCR Thermal Cycler Dice (TaKaRa, Shiga, Japan) under the following conditions: 1 cycle at 94°C for 3 min; 35 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 45 s; 1 cycle at 72°C for 7 min. PCR products were separated on a 10% polyacrylamide gel (Nacalai Tesque, Kyoto, Japan) and were visualized by ethidium bromide (Nacalai Tesque) staining. In total, 117 microsatellite loci were genotyped. Their chromosomal positions were retrieved from mouse genome informatics (MGI, http://www.informatics.jax.org/). Owing to the fact that the chromosomal positions of six markers were unavailable, they were determined on the basis of our own linkage map.

QTL analysis

Normality of the trait data was assessed by the Shapiro–Wilk W test (JMP8, SAS Institute, Tokyo, Japan). If the trait data did not follow a normal distribution, Box–Cox transformation was applied to the raw trait data (JMP8).

QTL mapping was performed using R/qtl v. 1.38-4 (Broman *et al.* 2003; Broman and Sen 2009). Single-QTL scans were performed using a 1-cM interval across the entire genome with or without lineage effects (DB vs BD) as a covariate. Threshold logarithm of the odds (LOD) scores for significant ($P < 0.05$) and suggestive ($P < 0.63$) linkages were determined by performing 1000 permutations (Churchill and Doerge 1994; Lander and Kruglyak 1995). After single-QTL scans, two-QTL scans were performed. In this case, we adhered strictly to the threshold recommended by Broman and Sen (2009). Finally, the combined effects of all QTLs, including those that were significant and suggestive, were assessed using multiple QTL models (Sen and Churchill 2001).

Statistical analysis

Plasma insulin levels are represented as means ± SEM (mg/dL). Statistical differences among three groups, partitioned according to the marker locus genotype, were analysed using one-way analysis of variance. $P < 0.05$ was considered statistically significant. The strength of an association between traits was analysed using Spearman's rank correlation coefficient. Bonferroni correction was applied to account for multiple comparisons.

Results

Histograms showing the distributions of plasma insulin level in 300 F_2 male mice (data from 150 BD F_2 and 150 DB F_2 mice are combined) are presented in figure 1. The distribution of raw insulin levels was highly skewed, and was normalized by Box–Cox transformation.

Genomewide LOD score plots obtained by single-QTL scans for plasma insulin levels in F_2 male mice are shown in figure 2 (solid lines) and table 1. We identified one significant QTL on chromosome 9, and three suggestive QTL on chromosomes 4, 6 and 15. We named the significant QTL on chromosome 9 *Insdq3* based on our previous study of F_2 female mice from the cross between B6 and DDD.Cg-A^y strains in which we named two significant QTL *Insdq1* and *Insdq2* (Suto 2013). We assigned *Insdq2* and *Insdq1* to the suggestive QTL on chromosomes 6 and 12. Although there was no difference in mean insulin level between BD F_2 (0.79 ± 0.05 ng/mL) and DB F_2 (0.77 ± 0.06 ng/mL) males, we included the lineage effect (i.e., BD vs DB) as an additive covariate and repeated the analysis. As shown in figure 2 (broken lines), the results of the two analyses

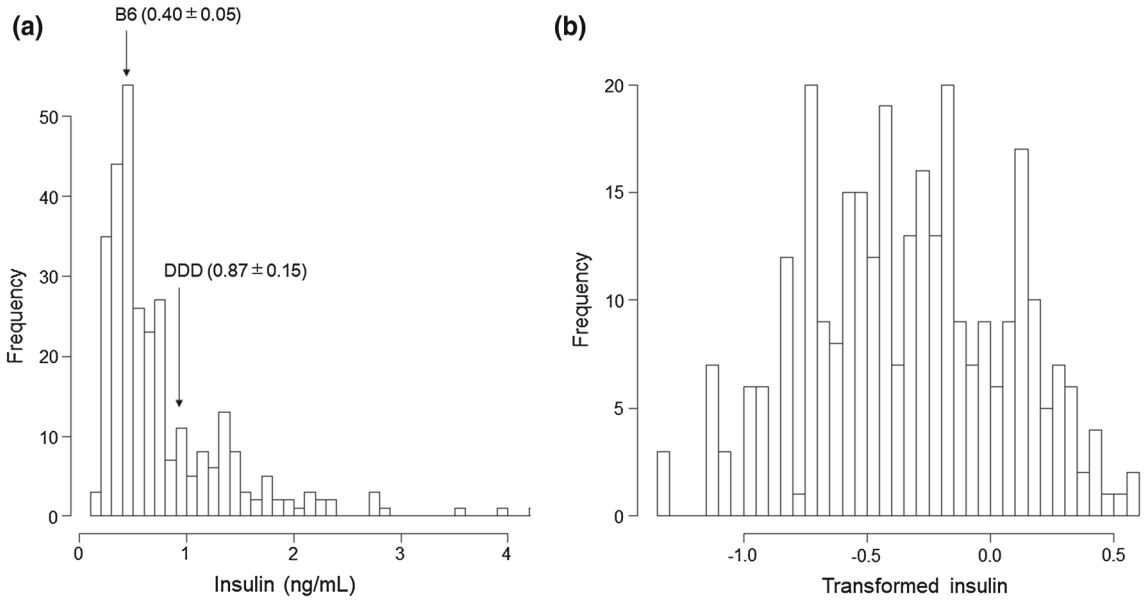

Figure 1. Histograms showing the distribution of raw plasma insulin levels (a) and transformed plasma insulin levels (b) in F_2 male mice. The distribution of raw plasma insulin levels was highly skewed, and results were normalized by Box–Cox transformation. The *x*-axis represents the plasma insulin levels, and the *y*-axis represents the number of mice. Average plasma insulin levels of B6 and DDD males are indicated by arrows.

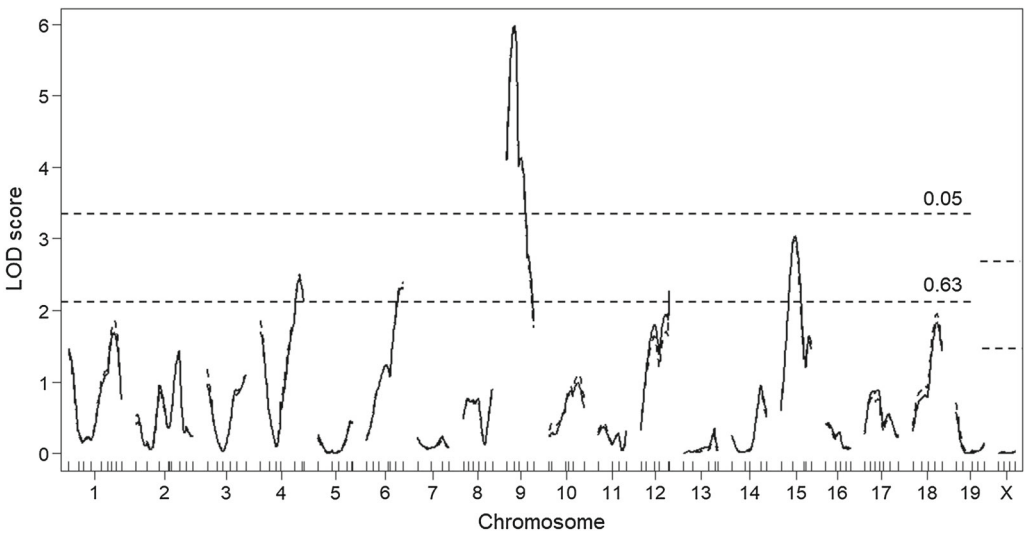

Figure 2. Genomewide LOD score plot of plasma insulin levels in F_2 male mice. Solid lines indicate LOD scores obtained without considering a lineage effect, while dashed lines indicate LOD scores obtained considering a lineage effect. The results of the two analyses were very similar. The *x*-axis represents the chromosomes and microsatellite marker positions, and the *y*-axis represents the LOD score. The horizontal broken lines indicate the genomewide threshold LOD score for significant ($P < 0.05$) and suggestive ($P < 0.63$) linkage, respectively.

were quite similar. Therefore, we did not take the effect of lineage effect into account in subsequent analyses. Further, using two-QTL scans, we did not identify significant pairwise interactions between QTL. Multiple-regression analyses indicated that the detected QTL, in combination, explain 20.4% of the variation in plasma insulin levels (table 2).

DDD mice are significantly heavier than B6 mice, and have significantly higher plasma levels of total cholesterol, triglyceride and testosterone (Suto and Kojima 2017a, b). We tested whether the plasma insulin level is correlated with these metabolism-related traits in F_2 mice, and found that the plasma insulin level is significantly correlated with each of the traits (table 3). Therefore, we

Table 1. Significant and suggestive QTL identified by genome-wide scans of F_2 male mice.

QTL[a]	Chromosome	Peak cM	95% CI[b]	LOD[c]	Nearest marker	High strain[d]	Overlapping QTL	
							Name	Reference
	4	75	7–83	2.4	*D4Mit234*	DDD		
Insdq2	6	72	28–75	2.3	*D6Mit259*	DDD	*Insdq2*	Suto (2013)
Insdq3	9	26	13–32	**6.0**	*D9Mit191*	DDD	*Gluchos3*	Toye *et al.* (2005)
Insdq1	12	62	17–62	2.3	*D12Nds2*	B6	*Insdq1*, *Insq10*	Suto (2013); Kido *et al.* (2000)
	15	26	10–54	3.0	*D15Mit184*	DDD		

QTL, quantitative trait loci; CI, confidence interval; LOD, logarithm of the odds.
[a]QTL symbols, *Insdq2* was assigned to suggestive QTL on chromosome 6 because it was identified as significant QTL in our previous study of female mice (Suto 2013).
[b]95% CI was defined by a 1.5-LOD decrease.
[c]LOD scores for significant QTL are indicated in bold. Threshold LOD scores for significant and suggestive QTL are 3.3 and 2.1, respectively, for autosomes and 2.8 and 1.5, respectively, for the X chromosome.
[d]High strain-derived allele was associated with a higher plasma insulin level.

Table 2. Multiple regression analysis of the plasma insulin level.

Chromosome (cM)[a]	df[b]	Variance, %[c]	*F* value
4@75	2	2.9	5.2
6@72	2	2.1	3.8
9@26	2	6.8	12.3
12@62	2	3.4	6.2
15@26	2	2.2	4.0
Total	10	20.4	

[a]cM position on the chromosome.
[b]Degrees of freedom.
[c]Percentage of the total F_2 phenotypic variance explained by each marker.

subsequently performed single-QTL scans including each of these metabolism-related measures as an additive covariate. An additional significant QTL was identified on chromosome 12, only when body weight was included as an additive covariate (table 4; figure 3). We further performed QTL mapping for principal component 1 (PC1) extracted from these metabolism-related traits, but did not identify additional significant QTL. Finally, we assessed whether *Insdq1* and *Insdq3* influence these traits by using a point-wise significant threshold of $P = 0.05$. *Insdq1* had no significant effect on any of the traits, but *Insdq3* had significant effects on body weight and total cholesterol (table 5). Further, although it was not statistically significant, the DDD allele at *Insdq3* was associated with increased plasma levels of triglyceride and testosterone.

It would be useful to perform genomewide scans by combining the present data for males with the previously analysed data for B6 × DDD.Cg-A^y F_2 females (Suto 2013) to identify QTL × sex interactions. However, the combined trait data could not be normalized; therefore, we simply compared the allele effects of *Insdq1* and *Insdq3* between males and females. Previously analysed F_2 female mice include F_2A^y (n = 150) and F_2 nonA^y (n = 148); we analysed the data only from 148 F_2 nonA^y mice. As shown in figure 4, the DDD allele at *Insdq1* was associated with decreased insulin levels in a mostly dominant manner in both males and females. The DDD allele at *Insdq3* was significantly associated with increased insulin levels with an additive mode of inheritance in males, whereas mice with heterozygous genotype (DDD/B6) had higher insulin levels in females.

Table 3. Strength of associations between plasma insulin level and other related metabolic traits.

	Body weight	Testosterone	Total cholesterol	Triglyceride
Insulin	0.5134*	0.1813*	0.3767*	0.3513*
Body weight		0.1328	0.4855*	0.4505*
Testosterone			0.1408*	0.0971
Total cholesterol				0.4874*

Strength of associations expressed as a Spearman's rho between five metabolism-related phenotypes. According to the formula reported by Purcell-Huynh *et al.* (1995), the threshold *P*-value for significance was <0.002. Significant correlations are indicated by an asterisk.

Table 4. Significant and suggestive QTL identified when body weight was included as an additive covariate.

QTL	Chromosome	Peak cM	95% CI[a]	LOD[b]	Nearest marker	High strain[c]
	3	89	45–77	2.2	*D3Mit110*	Heterozygote
	8	18	12–63	2.1	*D8Mit191*	B6
Insdq3	9	26	15–31	**5.3**	*D9Mit191*	DDD
Insdq1	12	62	47–62	**3.5**	*D12Nds2*	B6

QTL, quantitative trait loci; CI, confidence interval; LOD, logarithm of the odds.
[a]95% CI was defined by a 1.5-LOD decrease.
[b]LOD scores for significant QTL are indicated in bold. Threshold LOD scores for significant and suggestive QTL are 3.5 and 2.1, respectively, for autosomes and 2.8 and 1.5, respectively, for the X chromosome.
[c]High strain-derived allele was associated with a higher plasma insulin level.

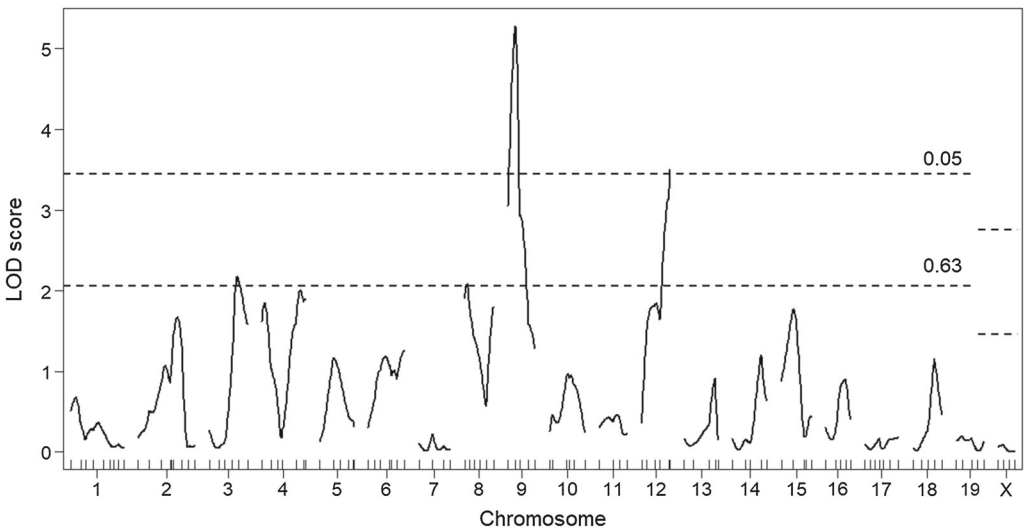

Figure 3. Genomewide LOD score plot of plasma insulin levels in F_2 male mice by including body weight as an additive covariate. A locus on chromosome 12 reached threshold LOD score for statistically significance. The *x*-axis represents the chromosomes and microsatellite marker positions, and the *y*-axis represents the LOD score. The horizontal broken lines indicate the genomewide threshold LOD score for significant ($P < 0.05$) and suggestive ($P < 0.63$) linkage, respectively.

Table 5. Effect of *Insdq1* and *Insdq3* on metabolism-related traits in F_2 male mice.

QTL (Nearest marker)	Trait	Means ± S.E. for mice with each genotype[a]			*P*-value
		DDD/DDD	DDD/B6	B6/B6	
Insdq1 (*D12Nds2*)	Body weight (g)	31.7 ± 0.4 (*n* = 76)	31.5 ± 0.2 (*n* = 155)	31.5 ± 0.4 (*n* = 69)	0.90
	Testosterone (ng/mL)	4.8 ± 0.9	4.8 ± 0.8	4.5 ± 1.0	0.60
	Total cholesterol (mg/dL)	136 ± 4	137 ± 2	143 ± 3	0.26
	Triglyceride (mg/dL)	134 ± 7	130 ± 4	149 ± 8	0.13
Insdq3 (*D9Mit191*)	Body weight (g)	32.3 ± 0.4 (*n* = 70)	31.4 ± 0.2 (*n* = 156)	31.1 ± 0.3 (*n* = 74)	0.043
	Testosterone (ng/Ml	5.2 ± 1.0	4.5 ± 0.7	4.8 ± 1.1	0.59
	Total cholesterol (mg/dL)	147 ± 4	138 ± 2	131 ± 3	0.0043
	Triglyceride (mg/dL)	145 ± 8	135 ± 5	127 ± 6	0.35

[a]Although raw data are summarized, statistical analyses were performed using normalized data, except for testosterone, which was analysed by nonparametric methods (Wilcoxon/Kruskal–Wallis tests).

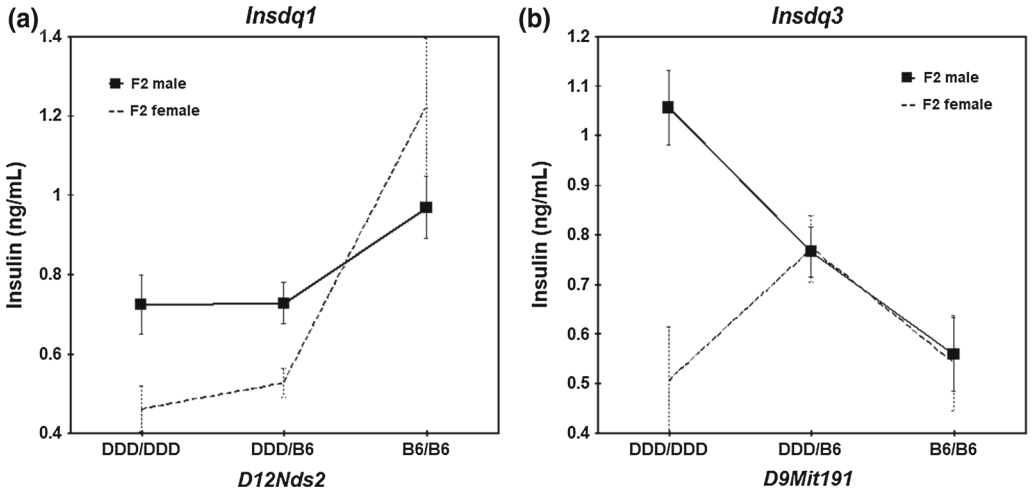

Figure 4. Allelic contributions of *Insdq1* (a) and *Insdq3* (b). The *x*-axis shows the genotypes of F_2 mice partitioned according to the nearest marker locus genotypes: homozygous DDD alleles are represented by DDD/DDD, homozygous B6 alleles are represented by B6/B6, and heterozygous alleles are represented by DDD/B6. The *y*-axis shows the average raw plasma insulin level, and the error bars are SEM. Solid lines: male, and broken lines: female.

Table 6. List of candidate genes for *Insdq1* and *Insdq3*.

QTL (Chromosome)	Gene	Position, cM	Phenotypic effect on various traits[a]				
			Insulin	Body weight	Cholesterol	Triglyceride	Testosterone
Insdq1 (12)	*Gpr68*	51.1	▼	–	–	–	–
	Mark3	61.05	▼	▼	–	–	–
Insdq3 (9)	*Panx3*	20.79	▼	–	–	–	–
	Bsx	21.65	▲	▼	–	–	–
	C1gtnf5	24.62	▼	–	–	–	–
	Cbl	24.72	▼	▲	–	▼	–
	Pdzd3	24.84	▼	–	–	–	–
	Sidt2	25.36	▼	▼	–	–	–
	Sik3	25.36	▼	▼	▼	▼	–
	Dixdc1	27.75	▼	–	–	–	–
	Sik2	27.75	▲	–	–	▲	–
	Cyp19a1	29.49	▲	▲	▲	▲	▲
	Neil1	30.89	▲	▲	–	▲	–
	Bbs4	32.01	▲	▼	▲	–	–

[a]Data for phenotypic effects were retrieved from MGI. '▲' denotes either an increased body weight and/or increased circulating level of each plasma component, while '▼' denotes a decreased body weight and/or decreased circulating level of each plasma component. '–' denotes that phenotypic changes are currently not known.

Finally, we searched for candidate genes associated with QTL using the term 'abnormal circulating insulin level' as a query using the MGI database (mammalian phenotype browser). We retrieved 959 genotypes with 978 annotations (MGI search was performed on 28 November 2017). Based on the chromosomal localization, we identified two and 12 candidate genes for *Insdq1* and *Insdq3*, respectively (table 6). Based on the kind of traits affected by the QTL and the direction of the effect of the DDD allele, we postulated that *Gpr68* and *Cyp19a1* were the most plausible candidate genes of *Insdq1* and *Insdq3*, respectively.

Discussion

In this study, we identified a multifactorial basis for plasma insulin levels in F_2 male mice produced by crosses between B6 and DDD inbred mice. We identified two significant QTLs, *Insdq1* and *Insdq3*, on chromosomes 12 and 9, respectively. It is important to note that *Insdq1* was identified as significant QTL only when body weight was included as an additive covariate. This suggests that the effect of *Insdq1* may be indirect, acting via body weight. An effect of *Insdq1* was clearly identified in both male and female mice, and the mode of inheritance and the direction

of the allelic effects were similar in both sexes. A significant effect of *Insdq3* was identified only in males, suggesting that this QTL was sex-specific. Comparison with analyses of females are needed because most QTL studies of insulin levels have focussed on males (Schmidt *et al.* 2008). It is notable that the 95% confidence interval (CI) of *Insdq3* overlaps with a consensus region from a meta-analysis with significant linkage to circulating insulin levels (Schmidt *et al.* 2008).

Insdq1 and *Insdq3* correspond with coincidental QTL reported by others. *Insq10* was identified in male F_2 intercross population between B6 and 129SvIR inbred mice (Kido *et al.* 2000). *Insq10* was located on chromosome 12 at a peak position of 50.87 cM. However, in contrast with *Insdq1*, the B6 allele was associated with decreased insulin levels, suggesting that *Insdq1* and *Insq10* differ. *Gluchos3* was identified in a male intercross population between B6 and C3H/HeH inbred mice (Toye *et al.* 2005). *Gluchos3* was located on chromosome 9 near *D9Mit1001*, which is located at 20.98 cM. Similar to *Insdq3*, the B6 allele was associated with decreased insulin levels, and both loci may be allelic. Toye *et al.* (2005) suggested that *Atp5l* and *Isl2* are candidate genes for *Gluchos3*. We did not detect any mutations of these genes in the DDD sequence in our whole exome sequencing analysis (data not shown).

In addition to significant QTL, in an overlapping region of suggestive QTL on chromosome 4, we have previously identified significant QTL associated with diabetes mellitus in DDD.Cg-A^y male mice (Suto and Satou 2013, 2015). In this diabetic mouse model, the DDD allele was associated with a decreased body weight and decreased plasma insulin level due to extensive loss of pancreatic beta cells. The action of the locus on chromosome 4 may be inverted by the introgression of the A^y allele not in female mice, but in male mice, if the two loci were allelic. Tentatively, we did not assign a gene symbol to the suggestive QTL on chromosome 4.

We previously performed QTL mapping for body weight and plasma lipids in the same F_2 intercross population. By single-QTL scans, we identified four significant QTL for body weight on chromosomes 1, 2, 5 and 17 (Suto and Kojima 2017a), and three significant QTL for plasma lipids on chromosomes 1, 17 and 19 (Suto and Kojima 2017b). Despite significant correlations between the traits (table 3), there were no overlapping QTL. However, this does not necessarily mean that a genetic link among these traits was absent. *Insdq3*, not *Insdq1*, showed significant effects on body weight and plasma total cholesterol level. Based on these results, we searched for candidate genes associated with significant QTL. By searching against the MGI database, we identified two and 12 candidate genes for *Insdq1* and *Insdq3*, respectively (table 6). Based on the traits affected by the QTL and the direction of the action of the DDD allele, we postulated that *Gpr68* and *Cyp19a1* were plausible candidate genes for *Insdq1* and *Insdq3*, respectively. On the basis of comparative maps between humans and mice (https://www.ncbi.nlm.nih.gov/projects/homology/maps/index.shtml), the 95% CI of *Insdq1* is homologous to a portion of chromosome 14 in humans, and *GPR68* is located at 14q32.11 with genomic coordinates (GRCh38) of 14 : 91,232,531–91,264,580. On the other hand, the 95% CI of *Insdq2* is homologous to portions of chromosomes 11 and 15 in humans, and *CYP19A1* is located at 15q21.2 with genomic coordinates of 15 : 51,208,056–51,338,597. However, when compared to the reference sequence, we did not find any nucleotide changes in the DDD sequence. Therefore, the suitability of these candidate genes needs to be validated by additional studies. Nevertheless, our results provide insight into the genetic mechanisms underlying insulin metabolism.

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