Diabetic modifier QTL, Dbm4, affecting elevated fasting blood glucose concentrations in congenic mice

Shigeru Takeshita1,3, Susumu Kitayama1,3, Takao Suzuki2,3, Maki Moritani3, Hiroshi Inoue3 and Mitsuo Itakura3,4*

1Department of Metabolic Diseases, Pharmacology Research Labs., Astellas Pharma Inc., 21 Miyukigaoka, Tsukuba, Ibaraki 305-8585, Japan
2Department of Functional Genomics, Molecular Medicine Research Labs., Astellas Pharma Inc., 21 Miyukigaoka, Tsukuba, Ibaraki 305-8585, Japan
3Division of Genetic Information, Institute for Genome Research, The University of Tokushima, 3-18-15, Kuramoto-cho, Tokushima 770-8503, Japan
4Setagaya Memorial Hospital, 2-30-10, Noge, Setagaya-ku, Tokyo 158-0092, Japan

(Received 29 June 2012, accepted 19 October 2012)

We have previously identified four significant quantitative trait loci (QTL) affecting plasma glucose concentrations on F2 progeny of hypoinsulinemic diabetic Akita mice, heterozygous for the Ins2 gene Cys96Tyr mutation, and non-diabetic A/J mice, one of which on chromosome 15 named Dbm4 (diabetic modifier QTL 4) was shown to affect fasting plasma glucose concentrations with a maximum LOD score of 6.17. To estimate the influence of Dbm4 itself to the diabetes-related phenotypes, we constructed congenic strain with heterozygous Ins2 mutation using the Akita allele as donor of Dbm4 locus in the A/J genetic background, and measured quantitative traits including plasma glucose concentrations in glucose tolerance test (GTT). In this study, we found the Akita allele in Dbm4 was associated with higher fasting plasma glucose concentrations as in previous QTL analysis. According to gene expression assay, key enzymes of hepatic gluconeogenesis were expressed to the more increased degree in the liver of congenic mice compared to the A/J allele based control mice. Based on these results, we concluded that diabetic modifier gene(s) exist on Dbm4 locus affecting fasting plasma glucose concentrations via regulation of gluconeogenic gene expression in the hypoinsulinemic diabetic condition. Identification of the modifier gene responsible for Dbm4 would provide new drug development targets for human type 2 diabetes with hepatic insulin resistance.

Key words: congenic mouse, diabetes, gluconeogenesis, QTL

INTRODUCTION

Type 2 diabetes is a multifactorial disorder caused by both genetic (Leiter, 1989, 1993) and environmental factors. To discover causative genetic factors in complex diseases, genetic analyses of rodents and humans have been conducted. Quantitative trait loci (QTL) analysis has been widely applied to several rodent models of type 2 diabetes to identify chromosomal loci for genetic modifiers, and is rather advantageous than genetic studies in humans in that rodents offer both reduced environmental variation and genetic heterogeneity. To determine if one QTL independently contribute to phenotypic trait, it is essential to produce congenic mice that differ only in QTL. Congenic mice are useful to assess the potential effects of the locus solely on traits related to diabetes, because the difference of quantitative trait may possibly be attributed to the association with modifier genes in the multiple QTLs (Kose et al., 2002). QTL analyses of diabetes have been performed with several murine models to identify chromosomal loci of genetic modifiers (Moritani et al., 2006; Togawa et al., 2006) and the effects of a sole QTL have been verified using congenic mice (Mizutani et al., 2006; Stoehr et al., 2004). Recently novel diabetic modifier gene like Sorcs1 on chromosome 19 was identified using QTL and congenic approach (Clee et al., 2006).

We previously performed QTL analysis using F2 intercross progeny derived from non-obese diabetic Akita and non-diabetic A/J mice (Takeshita et al., 2006). The Akita mouse is a spontaneous model of type 2 diabetes...
established from the C57BL/6 strain (Yoshioka et al., 1997), and a heterozygous Cys96Tyr mutation of the insulin 2 gene (Ins2) has been identified as the etiology for the hypoinsulinemic diabetes (Kayo and Koizumi, 1998; Wang et al., 1999), with severe and mild diabetes, respectively, in male and female mice (Yoshioka et al., 1997). The A/J mouse, used in our crosses, is genetically different from the C57BL/6 strain as exemplified by its phenotypic resistance to high-fat diet-induced diabetes (Surwit et al., 1991). In our QTL analysis, we identified the Dbm4 locus on chromosome 15 affecting fasting plasma glucose concentrations with significant logarithm of odds (LOD) score of 6.17 in the hypoinsulinemic conditions derived from heterozygous Ins2 mutation (Ins2-Hetero). The Dbm4 also weakly affects plasma glucose concentrations after glucose administration in glucose tolerance tests (GTT) with LOD score of 3.24, 2.37, and 2.91 (30, 60, and 120 min, respectively), in hypoinsulinemic Ins2-Hetero mice, all of which were detected only in male mice showing more severe diabetes than female mice (Takeshita et al., 2006). Therefore we proposed potential modifier gene(s) on the Dbm4 affecting diabetic traits in Ins2-Hetero derived hyperglycemic condition.

In this study, we report the phenotypic assessment of congenic mice with the Akita allele on Dbm4 locus in the A/J genetic background.

MATERIALS AND METHODS

Mouse strains and construction of congenic mice

Male Akita mice and female A/J mice were purchased at 7 weeks of age from SLC Japan (Shizuoka, Japan). Mice were maintained with free access to food (CRF-1, purchased from Oriental Yeast, Tokyo, Japan) and water in a temperature- and humidity-controlled environment under a 12 h light-dark cycle. Congenic mice containing the homozygote for Akita mouse genotype as a donor of Dbm4 in the A/J recipient were constructed by repeated backcrosses and intercross as follows. The male Akita mice were crossed with the female A/J mice to produce first backcross progeny (BC1) which had the Akita-derived heterozygous mutation at the Ins2 locus and heterozygous Akita allele of Dbm4 locus estimated by genotyping as described below. BC2 progeny were produced by backcrossing male BC1 with female A/J mice. Male mice containing A/J background alleles (except Ins2 and the Dbm4 locus, and Y-chromosome) were selected and used for further backcrosses five or more times to obtain mice with purified A/J genetic background, which retained more than 95% of A/J alleles. Derived backcross progeny contained A/J genetic background with Akita alleles of Dbm4 and Ins2 loci, and then male and female progenies were intercrossed to obtain congenic strains which contained the homozygous Akita alleles of Dbm4 (Ak/Ak) with heterozygous mutation of Ins2 gene, and control strains which contained the homozygous A/J alleles of Dbm4 (Aj/Aj) with Ins2 heretozygous mutation. Breeding procedures of congenic and control strains were supported by Oriental BioService, Inc (Kyoto, Japan). All animal experiments were approved by the Animal Ethical Committee of Astellas Pharma Inc.

Genetic diagnosis of the insulin 2 gene mutation and genome-wide genotyping

Genomic DNA was extracted from the tail of each mouse at four weeks of age using DNAeasy Tissue Kit (QIAGEN, Hilden, Germany). Ins2 genotype was determined to estimate the existence of Cys96Tyr missense mutation by RFLP analysis as previously described (Takeshita et al., 2006). PCR fragment length polymorphism analysis was performed for genome-wide genotyping using primers for microsatellite markers that were polymorphic between Akita and A/J mice as previously described (Takeshita et al., 2006). All primers were purchased from Applied Biosystems (Foster City, CA, USA). Thirteen well-amplified microsatellite markers were used for genotyping the Dbm4 locus and 102 markers were used for assaying genetic background (information available on request).

Phenotyping of congenic mice

Male congenic and control mice were used for phenotypic characterisation. Each congenic or control mouse was weaned at 4 weeks of age. At 10 weeks of age, individual body weight (BW) was measured after overnight fasting for 16 hours, and glucose tolerance test (GTT) was performed as follows. After fasting for 16 hours, 2 g/kg BW of glucose in distilled water was administered orally, and blood sample was collected from the retro-orbital sinus using a capillary pipette before (designated as 0 min), 30, 60, and 120 min after glucose administration. Plasma samples were obtained by centrifugation to measure glucose concentrations by the mutarotase-glucose oxidase method using the Glucose CH Test WAKO (Wako, Osaka, Japan). Mice were anesthetically sacrificed and liver and epididymal white adipose tissue (EWAT) were isolated to measure the wet tissue weight and/or to estimate liver specific gene expression at 16 weeks of age.

RT-PCR analysis

Total RNA from mouse liver was purified using RNeasy Mini Kit (QIAGEN). One μg of RNA was reverse-transcribed with Omniscript RT Kit (QIAGEN) according to manufacture’s protocol. Quantitative PCR was performed with an ABI PRISM 7900HT Real-Time PCR System (Applied Biosystems), using SYBR Premix Ex Taq Perfect Real Time (Takara Bio, Otsu, Japan) for SYBR Green intercalator assay or Premix Ex Taq Perfect Real Time (Takara Bio) for TaqMan probe assay following manufacturer’s instructions. For each sample, relative gene expression was estimated by normalizing with 18S rRNA levels to correct for differences in RNA extraction.
and reverse transcription efficiencies. The primer sequences for 18S rRNA are described as follows: sense 5'-GTGCATGCGCTCTTAGCTTG-3'; antisense 5'-CATGCAGGACTCGTTGCTT-3'. The expression level of key enzymes of hepatic gluconeogenesis, glucose-6-phosphatase (G6Pase, G6pc) and phosphoenolpyruvate carboxykinase (PEPCK, Pck1), were analyzed by TaqMan assay using probe fluorescently labeled with FAM reporter dye at the 5' end and TAMRA quencher dye at the 3' end. The primer and probe sequences used for the mouse G6Pase gene are described as follows: sense 5'-TTAAAGAGACTTG-GGCATCAAT-3', antisense 5'-ATCCACCTGAAGACGAGGTTG-3', probe 5'-TGGGTGGCCAGTGGTGGAGACT-3'. The primer and probe sequences used for the mouse PEPCK gene are described as follows: sense 5'-GTTATTGAACTGACAGACTCGC-3', antisense 5'-CACAGATATGCCCATCAGGA-3', probe 5'-CTATGTGGTGGCCAGCATGCG-3'.

Statistical analysis Data were presented as mean ± S.E.M. Statistical analysis was carried out by two-tailed unpaired Student's t test. Statistical significance was defined as p value less than 0.05.

RESULTS AND DISCUSSION

We had previously identified four major QTLs affecting diabetic traits. The most significant linkage with plasma glucose concentrations was detected on Dbm4 located on chromosome 15 with a maximum LOD score of 6.17 for fasting plasma glucose concentrations (Takeshita et al., 2006), which is the QTL overlapped with the previous report in the TSOD mouse strain (Hirayama et al., 1999). To discover diabetic modifier located on Dbm4, we constructed congenic mice, A/J.Akita, by backcrossing Akita to A/J mice, which retained the Akita mouse-derived Ins2–Hetero mutation on chromosome 7 and the homozygous Akita allele of Dbm4 locus (Ak/Ak) on chromosome 15 between the microsatellite markers D15Mit182 and D15Mit2 in the A/J allele genetic background (Fig. 1). We analyzed phenotypes of congenic mice to compare to the A/J allele control mice with Ins2–Hetero mutation and homozygote for A/J allele of the Dbm4 locus (Aj/Aj).

To estimate the effect of Dbm4 locus solely on diabetic traits, we performed GTT in male congenic mice as we had done on the F2 intercross mice in the QTL analysis.
In congenic mice (Ak/Ak), fasting plasma glucose concentrations were significantly higher than in control mice (Aj/Aj) (187.51 ± 14.42 vs. 145.97 ± 8.09 mg/dL) and there were no significant differences in plasma glucose concentrations at any other time points in GTT (Fig. 2), in spite that the Akita allele-derived increases in plasma glucose concentrations had been observed at any time points during GTT in our previous QTL analysis (Takeshita et al., 2006). As for plasma insulin concentrations, the values were quite low in the hypoinsulenic Ins2–Hetero condition and no change was observed between the groups (Supplementary Table S1). Our current congenic study reproduced the result of QTL analysis only in fasting plasma glucose concentrations, however it may be considered that the phenotypes of congenic mice actually attributed to the genetic differences between Ak/Ak and Aj/Aj alleles in Dbm4, compared to the results in QTL analysis which included additional effect of whole genome association. It may also be suggested by the QTL analysis result with much weaker association of Dbm4 locus with plasma glucose concentrations in GTT at 30, 60, or 120 min rather than fasting plasma glucose concentrations (LOD scores of 3.24, 2.37, and 2.91 for 30, 60, and 120 min in GTT, rather than 6.17 for fasting plasma glucose). Therefore these data suggested that Ak/Ak alleles of diabetic modifier gene(s) located on Dbm4 is associated with severe diabetes compared to Aj/Aj allele through increased fasting plasma glucose concentrations in the hypoinsulenic condition.

From our result that the Ak/Ak alleles in Dbm4 locus was suggested to be associated with higher fasting glucose concentrations, diabetic modifier located on Dbm4 may possibly influence hepatic gluconeogenesis, because overnight fasting is well-known to lead to increased activity of gluconeogenic key enzymes (Anderson, 1974). To assess this possible hypothesis, we conducted gene expression analysis of G6Pase and PEPCK, which is two major rate-limiting enzyme for hepatic gluconeogenesis. The expression levels of both enzyme genes were significantly elevated in congenic mice compared to control mice, to the extent of 3.4 and 2.0 fold for G6Pase and PEPCK, respectively (Fig. 3). Regulation of hepatic gluconeogenesis is considered to be achieved mainly through control of the expression of these key enzymes (Radziuk and Pye, 2001; Takashima et al., 2010), although actual enzyme activities were not measured in our assay. Therefore our study suggested that the Ak/Ak allele might contribute to upregulate hepatic gluconeogenesis compared to Aj/Aj allele in Ins2–Hetero derived hypoinsulinemic diabetic condition. Diabetic modifier in Dbm4 locus is considered not to control the insulin secretion or insulin action itself due to the result of no difference in plasma insulin concentration between congenic and control groups. Therefore, it may possibly have an action on gluconeogenic pathway directly in liver or contribute to hepatic insulin sensitivity or resistance. Based on our genetic search, 115 of Ensembl genes identified on Dbm4 locus were listed in Supplementary Table S2. Some of these may have potential diabetes-related information by investigating co-citation relation between each gene and diabetes, however, there is no strong suggestion that candidate gene(s) located on Dbm4 is directly associated with gluconeogenesis or insulin sensitivity in hepatocyte from

![Figure 2](image-url)

**Fig. 2.** Higher fasting plasma glucose concentrations in congenic mice. GTT was performed in male Ins2-Hetero mice at 10 weeks of age. Open circles denote the plasma glucose concentrations at each time point in control mice whose genotypes are homozygous for Aj allele on Dbm4 (Aj/Aj, N = 34), and closed circles denote those in congenic mice whose genotypes are homozygous for Akita allele (Ak/Ak, N = 13). Data are shown as means ± S.E.M. Statistical significance was shown for p < 0.05 (*) between the congenic and control groups.

![Figure 3](image-url)

**Fig. 3.** Increased gluconeogenic gene expression in congenic mice. The gene expression of key enzymes of gluconeogenesis, G6Pase and PEPCK, was estimated in liver of male Ins2-Hetero mice. White column denotes control (Aj/Aj, N = 6) and gray column denotes congenic mice (Ak/Ak, N = 5). Data are shown as means ± S.E.M. Statistical significance was shown for p < 0.05 (*) and p < 0.01 (**) between the congenic and control mice groups.
Table 1. Body weight, wet tissue weight and plasma glucose concentrations in a fed state

| Strain                  | Body Weight (g) | Liver Weight (g) per BW ratio (mg/g BW) | EWAT Weight (g) | EWAT Weight (mg/g BW) | Plasma Glucose concentrations (mg/dL) |
|-------------------------|-----------------|----------------------------------------|-----------------|-----------------------|-------------------------------------|
| Control (Dbm4-Aj/Aj)    | 25.4 ± 0.4      | 1.27 ± 0.04 50.0 ± 0.9                 | 0.42 ± 0.04     | 16.6 ± 1.4            | 537.7 ± 48.4                         |
| Congenic (Dbm4-Ak/Ak)   | 26.4 ± 1.2      | 1.46 ± 0.11 54.9 ± 2.1                 | 0.46 ± 0.03     | 17.8 ± 1.4            | 641.1 ± 46.9                         |

Data are shown as means ± S.E.M, n = 9 for control and n = 6 for congenic mice. Statistical significance between control and congenic mice groups is shown for $p < 0.05$ (*), according to Student’s-t test.

The change in other tissue weights (i.e., heart weight ratio), in spite of no difference in EWAT-weight, remains unclear. These genes, therefore, also seem to according to our result, even though actual relationship as a transcriptional regulator of hepatic gluconeogenesis.

Besides the diabetes-related trait, body weight and some wet tissue weights were compared between congenic and control groups, even though any wet tissue weight had not been determined in our previous QTL analysis. As for body weight, there was no statistical difference between the groups as we had no significant linkage in our previous QTL analysis. In contrast, statistically higher liver weight was observed in congenic mice compared to control mice ($p < 0.05$, for liver weight per body weight ratio), in spite of no difference in EWAT-weight (Table 1). The change in other tissue weights (i.e., heart, spleen) of congenic mice were insignificant compared to that of control mice (Supplementary Table S3). These findings suggest that phenotypic difference between congenic and control groups is mainly due to functional changes in liver including glucose metabolism (gluconeogenesis) and possibly also lipid metabolism. This suggestion is also supported by the previous report of QTL on chromosome 15 overlapped with Dbm4 that some genes including candidate responsible gene Sqle and Dgat1 mainly function in liver as a regulator of lipid metabolism (Stylianou et al., 2005).

To discover genuine modifier genes, additional genetic approach as with producing sub-congenic mice is necessary to narrow the region responsible for trait variation (i.e., gluconeogenesis). Moreover it is essential for gene identification to develop integrated approach including expression analysis, polymorphism search, functional analysis of candidate genes and also detailed etiological analysis using congenic mice. The identification of novel modifiers in the mouse will apply to mechanistic analysis of human type 2 diabetes.

Based on these, we concluded that diabetic modifier gene(s) existing on Dbm4 locus affect fasting plasma glucose concentrations possibly via regulation of gluconeogenic gene expression in the hypoinsulinemic diabetic condition. Identification of the modifier gene responsible for Dbm4 would provide new drug development targets for human type 2 diabetes with hepatic insulin resistance.

This study was supported by a grant from Cooperative Link of Unique Science and Technology for Economy Revitalization (CLUSTER). The authors thank both Dr. Youzou Takehisa and Dr. Takahiro Takehisa in Setagaya Memorial Hospital for kind consideration in preparing this article.

The current affiliation for Shigeru Takehita: Clinical Pharmacology, Development, Astellas Pharma Inc., Tokyo, Japan.

REFERENCES

Anderson, J. W. (1974) Glucose metabolism in jejunal mucosa of fed, fasted, and streptozotocin-diabetic rats. Am. J. Physiol. 226, 226–229.

Clee, S. M., Yandell, B. S., Schueler, K. M., Rabaglia, M. E., Richards, O. C., Raines, S. M., Rabura, E. A., Klass, D. M., Mui, E. T-K., Stapleton, D. S., et al. (2006) Positional cloning of Sorecs1, a type 2 diabetes quantitative trait locus. Nat. Genet. 38, 688–693.

Hirayama, I., Yi, Z., Izumi, S., Arai, I., Suzuki, W., Nagamachi, Y., Kuwano, H., Takeuchi, T., and Izumi, T. (1999) Genetic analysis of obese diabetes in the TSOD mouse. Diabetes 48, 1183–1191.

Kayo, T., and Koizumi, A. (1998) Mapping of murine diabetogenic gene Mody on chromosome 7 at D7Mit258 and its involvement in pancreatic islet and beta cell development during the perinatal period. J. Clin. Invest. 101, 2112–2118.

Kose, H., Moralejo, D. H., Ogino, T., Mizuho, A., Yamada, T., and Matsumoto, K. (2002) Examination of OLETF-derived non-insulin-dependent diabetes mellitus QTL by construction of a series of congenic rat. Mamm. Genome 13, 558–562.

Leiter, E. H. (1989) The genetics of diabetes susceptibility in mice. FASEB J. 3, 2231–2241.

Leiter, E. H. (1993) Obesity genes and diabetes induction in the mouse. Crit. Rev. Food Sci. Nutr. 33, 333–338.
Mizutani, S., Gomi, H., Hirayama, I., and Izumi, T. (2006) Chromosome 2 locus Nidd5 has a potent effect on adiposity in the TSOD mouse. Mamm. Genome 17, 375–384.

Moritani, M., Togawa, K., Yaguchi, H., Fujita, Y., Yamaguchi, Y., Inoue, H., Kamatani, N., and Itakura, M. (2006) Identification of diabetes susceptibility loci in db mice by combined quantitative trait loci analysis and haplotype mapping. Genomics 88, 719–730.

Radziuk, J., and Pye, S. (2001) Hepatic glucose uptake, gluconeogenesis and the regulation of glycogen synthesis. Diabetes Metab. Res. Rev. 17, 250–272.

Riu, E., Ferre, T., Mas, A., Hidalgo, A., Franckhauser, S., and Bosch, F. (2002) Overexpression of c-myc in diabetic mice restores altered expression of the transcription factor genes that regulate liver metabolism. Biochem. J. 368, 931–937.

Stoehr, J. P., Byers, J. E., Clee, S. M., Lan, H., Boronenkov, I. V., Schueler, K. L., Yandell, B. S., and Attie, A. D. (2004) Identification of major quantitative trait loci controlling body weight variation in ob/ob mice. Diabetes 53, 245–249.

Stylianou, I. M., Clinton, M., Keightley, P. D., Pritchard, C., Tymowska-Lalanne, Z., Bürger, L., and Horvát, S. (2005) Microarray gene expression analysis of the Fob3b obesity QTL identifies positional candidate gene Sqle and perturbed cholesterol and glycolysis pathways. Physiol. Genomics 20, 224–232.

Surwit, R. S., Seldin, M. F., Kuhn, C. M., Cochrane, C., and Feinglos, M. N. (1991) Control of expression of insulin resistance and hyperglycemia by different genetic factors in diabetic C57BL/6J mice. Diabetes 40, 82–87.

Takashima, M., Ogawa, W., Hayashi, K., Inoue, H., Kinoshita, S., Okamoto, Y., Sakae, H., Watsoka, Y., Emi, A., Senga, Y., et al. (2010) Role of KLF15 in regulation of hepatic gluconeogenesis and metformin action. Diabetes 59, 1608–1615.

Takeshita, S., Moritani, M., Kunika, K., Inoue, H., and Itakura, M. (2006) Diabetic modifier QTLs identified in F2 intercrosses between Akita and A/J mice. Mamm. Genome 17, 927–940.

Togawa, K., Moritani, M., Yaguchi, H., and Itakura, M. (2006) Multidimensional genome scans identify the combinations of genetic loci linked to diabetes-related phenotypes in mice. Hum. Mol. Genet. 15, 113–128.

Wang, J., Takeuchi, T., Tanaka, S., Kubo, S. K., Kayo, T., Lu, D., Takata, K., Koizumi, A., and Izumi, T. (1999) A mutation in the insulin 2 gene induces diabetes with severe pancreatic β-cell dysfunction in the Mody mouse. J. Clin. Invest. 103, 27–37.

Yoshioka, M., Kayo, T., Ikeda, T., and Koizumi, A. (1997) A novel locus, Mody4, distal to D7Mit189 on chromosome 7 determines early-onset NIDDM in nonobese C57BL/6 (Akita) mutant mice. Diabetes 46, 887–894.