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ARTICLE

Direct evidence for susceptibility genes for type 2 diabetes on mouse chromosomes 11 and 14

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Abstract
Aims/hypothesis Diabetogenic loci for type 2 diabetes have been mapped to mouse chromosome (Chr) 11 and 14 in the Nagoya–Shibata–Yasuda (NSY) mouse, an animal model of type 2 diabetes. We aimed to obtain direct evidence of these genes on each chromosome and to clarify their function and interaction in conferring susceptibility to type 2 diabetes.
Methods We established three consomic strains homozygous for diabetogenic NSY-Chr11, NSY-Chr14 or both on the control C3H background (C3H-11^{NSY}, C3H-14^{NSY} and C3H-$11^{NSY}14^{NSY}$, respectively), and monitored diabetes-related phenotypes longitudinally. The glucokinase gene was sequenced as a positional candidate gene on Chr11.
Results C3H-11^{NSY} mice showed hyperglycaemia associated with impaired insulin secretion and age-dependent insulin resistance without obesity. C3H-14^{NSY} mice exhibited hyperglycaemia mainly due to insulin resistance, with a slight increase in percentage body fat. C3H-$11^{NSY}14^{NSY}$ double consomic mice showed marked hyperglycaemia and obesity, which was not observed in single consomic strains. Sequences of the glucokinase gene were allelically variant between NSY and C3H mice.
Conclusions/interpretation These data provide direct evidence that Chr11 and Chr14 harbour major susceptibility genes for type 2 diabetes. These two chromosomes interact to cause more severe hyperglycaemia and obesity, which was not observed with the presence of either single chromosome, indicating different modes of gene–gene interaction depending on the phenotype. Marked changes in the phenotypes retained in the consomic strains will facilitate fine mapping and the identification of the responsible genes and their interaction with each other, other genes and environmental factors.

Keywords Complex trait · Interaction · Mouse chromosome 11 · Mouse chromosome 14 · *Nidd1n* · *Nidd2n* · *Nidd4n* · NSY mouse

Abbreviations

BAT	Brown adipose tissue
Chr	Chromosome
gAUC	Area under the glucose concentration curve
ipGTT	Intraperitoneal glucose tolerance test
ITT	Insulin tolerance test
NSY	Nagoya–Shibata–Yasuda
QTL	Quantitative trait locus
SNP	Single nucleotide polymorphism

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Introduction

Type 2 diabetes is a multifactorial disease caused by a complex interaction of environmental and genetic factors, with the latter consisting of multiple susceptibility genes, making it difficult to clarify their functions and interactions in conferring susceptibility to diabetes in humans. Inbred animal models of diabetes are therefore invaluable in dissecting such a complex interaction.

The Nagoya–Shibata–Yasuda (NSY) mouse was established as an inbred animal model with spontaneous development of type 2 diabetes by selective breeding for glucose intolerance from the closed colony of Jcl:ICR mice [1]. The phenotypes of the mouse resemble human type 2 diabetes in that the onset is age dependent, the animal is moderately obese and both impaired insulin response to glucose and insulin resistance contribute to the disease development [2–8]. Two quantitative trait loci (QTLs) on chromosome (Chr) 11 (*Nidd1n*) and Chr14 (*Nidd2n*), which affect glucose tolerance, have been identified in crosses of NSY mice with control C3H mice [7]. *Nidd1n* and *Nidd2n* have been suggested to affect glucose tolerance through impaired insulin secretion and insulin resistance, respectively [7]. The peaks of the linkage curve for *Nidd1n* and *Nidd2n* have been positioned in the region between *D11Mit236* (20.0 cM) and *D11Mit195* (47.0 cM) and in the region near *D14Mit59* (15.0 cM)/*D14Mit5* (22.5 cM) [7]. The regions showing significant linkage for *Nidd1n* and *Nidd2n* were broad, however, suggesting the possibility that multiple genes on the same chromosome contribute to the linkage of the regions, as was evidenced by the contribution of multiple susceptibility genes on the same chromosome to susceptibility to diabetes in the NOD mouse model of type 1 diabetes [9–11]. In fact, in addition to *Nidd1n* in the central part of Chr11, the existence of another locus near *D11Mit76* (2.0 cM), the most centromeric region on Chr11, distinct from the *Nidd1n* region, was suggested in our previous study [7].

The present study was performed to obtain direct evidence for susceptibility genes for type 2 diabetes on Chr11 and Chr14, and to clarify their function as well as interaction in conferring susceptibility to type 2 diabetes. To this end, we adopted a consomic approach [12], in which a whole chromosome of interest was introgressed onto the genetic background of the control strain. We first constructed two homozygous consomic strains, namely C3H-11^{NSY} and C3H-14^{NSY} mice, which carry an NSY-derived susceptible Chr11 or Chr14, respectively, on the control C3H background. Then, we established a double consomic strain, C3H-11^{NSY}14^{NSY}, containing both NSY-Chr11 and NSY-Chr14 in homozygous states on the C3H background. Various kinds of diabetes-related phenotypes of consomic strains have been monitored carefully and longitudinally. Finally, we performed sequence analysis of the glucokinase gene (*Gck*) on Chr11 (1.0 cM), as a functional candidate gene with peak linkage located in the centromeric region.

Methods

Animals

The NSY colony was maintained in the animal facilities of Osaka University Graduate School of Medicine. C3H/HeNcrj mice were purchased from Charles River Laboratories (Kanagawa, Japan). All mice had free access to tap water and a standard diet (CRF-1: Oriental Yeast, Tokyo, Japan) in an air-conditioned room (22–25°C) with a 12 h light–dark cycle (6:00–18:00 hours). Experimental designs were approved by the Osaka University Graduate School of Medicine Ethics Committee. Male mice were used for all experiments.

Construction of consomic strains (C3H-11^{NSY} and C3H-14^{NSY}) and double consomic strain (C3H-11^{NSY}14^{NSY})

C3H-11^{NSY} mice (Electronic supplementary material [ESM] Fig. 1) were produced by mating (NSY×C3H) F1 with C3H and selecting males that were heterozygous for the whole Chr11. These male mice were mated with C3H female mice, and their male progeny, heterozygous for the whole Chr11, were used for the next generation. In this process, we adopted a marker-assisted speed congenic method [13]. Namely, in every generation after the N3 generation, background genes were typed with polymorphic markers throughout the genome, and the best male mouse, which had the most substituted C3H genotype, was selected for breeding. This process was repeated until all the markers for background typing became homozygous for C3H genotypes (N6 or N7). Mice heterozygous for Chr11 were then intercrossed to obtain mice homozygous for Chr11. A total of four mice homozygous for NSY-derived Chr11 were obtained at the N6F1 (one out of 40 mice) and N7F1 (three out of 46 mice) generations. This line was maintained by brother–sister mating.

C3H-14^{NSY} mice (ESM Fig. 1) were constructed in the same way as for C3H-11^{NSY} mice. Five mice homozygous for NSY-derived Chr14 were obtained in the N8F1 generation (five out of 99 mice), and the line was maintained by brother–sister mating.

C3H-11^{NSY}14^{NSY} mice (ESM Fig. 1) were produced by mating (C3H-11^{NSY} × C3H-14^{NSY}) F1 with C3H-14^{NSY} and selecting mice that were homozygous for the NSY-derived allele at all loci on Chr14 and heterozygous for the NSY-derived allele at all loci on Chr11 (4/62 mice; 6.5%). These

mice were crossed with C3H-11^{NSY} to obtain mice that were homozygous for the NSY-derived allele at all loci on Chr11 and heterozygous for the NSY-derived allele at all loci on Chr14 (25/109 mice; 22.9%). Offspring that were homozygous for the NSY-derived allele at all loci on Chr14 as well as at all loci on Chr11 were selected (10/89 mice; 11.2%) and maintained by brother–sister mating.

Genotype analysis and localisation of markers

Genomic DNA was extracted from the tail. Information on microsatellite markers was obtained from the Mouse Genome Database (www.informatics.jax.org). The markers were amplified using PCR with primers with or without labels with 6FAM, NED or HEX. A total of 79 informative marker loci spanning the whole genome were analysed (ESM Table 1). In particular, we used 16 markers on Chr11 (average spanning less than 5 cM) and ten markers on Chr14 (average spanning less than 7 cM) to confirm no recombination, and to confirm none of the C3H-derived genome on Chr11 (in C3H-14^{NSY}) and Chr14 (in C3H-11^{NSY}). The non-labelled PCR products were electrophoresed on 9% polyacrylamide gels and visualised by ethidium bromide staining. The labelled PCR products were electrophoresed using an ABI 3100 sequencer (Applied Biosystems, Foster City, CA, USA) with GENESCAN 350 ROX (Applied Biosystems) as a size standard.

Phenotypic analysis

Assessment of glucose tolerance Glucose tolerance and body weight in NSY, C3H-11^{NSY}, C3H-14^{NSY}, C3H-11^{NSY}14^{NSY} and C3H mice were monitored longitudinally at 3, 6, 9 and 12 months of age. Glucose tolerance was assessed by intraperitoneal glucose tolerance test (ipGTT) (2 g glucose/kg body weight) in overnight-fasted mice, and blood glucose level was measured at 0, 30, 60, 90 and 120 min. The area under the glucose concentration curve (gAUC) was calculated according to the trapezoidal rule. Blood glucose level was measured directly by the glucose oxidase method using Glutest E (Kyoto Daiichi Kagaku, Kyoto, Japan).

Assessment of insulin secretion Insulin secretion in response to glucose was assessed by the insulinogenic index. IpGTT (2 g glucose/kg body weight) was performed as described above, and plasma insulin level was measured at 0, 15 and 30 min. Plasma insulin level was measured by ELISA (Morinaga, Yokohama, Japan). Incremental AUC ($\Sigma\Delta$gAUC) and incremental AUC ($\Sigma\Delta$iAUC) were calculated according to the trapezoidal rule from the glucose and insulin measurements at baseline (0 min), 15 and 30 min. Insulinogenic index was calculated as $\Sigma\Delta$iAUC÷$\Sigma\Delta$gAUC.

Assessment of insulin resistance HOMA-IR, as an indicator of insulin resistance, was calculated from the basal insulin and glucose concentrations (fasting glucose×fasting insulin).

Insulin tolerance test (ITT) was performed by injecting human insulin (0.25 U/kg) intraperitoneally in overnight-fasted mice at 3, 6, 9 and 12 months of age to evaluate insulin resistance longitudinally. Blood glucose level was measured at 0, 15, 30, 45 and 60 min.

Anatomical analysis Anatomical phenotypes were studied at 1 year of age. After anaesthesia by i.p. injection of pentobarbital sodium (Dainippon, Osaka, Japan), body weight and anal–nasal length were measured. BMI was calculated as body weight in grams divided by the square of anal–nasal length in centimetres Mice were killed under sevoflurane, and the epididymal, mesenteric, retroperitoneal fat pads and interscapular brown adipose tissue (BAT) were dissected and weighed.

Sequence analysis of Gck as candidate gene on chromosome 11 Genomic DNA was extracted from the livers of NSY and C3H mice. Fourteen pairs of primers (ESM Table 2) were designed so that the whole ten exons and exon–intron boundaries of *Gck* were covered. *Gck* produces two isoforms, beta cell-specific and liver-specific isoforms with alternative splicing, which are different in the promoter and first exon [14], so the primer pairs were designed separately for both. Genomic DNA was amplified by PCR with these primers, and the products were purified using a Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA). The sequencing reaction was performed using Big Dye Terminator (Applied Biosystems) according to the manufacturer's protocol, and the products were directly sequenced using an ABI 3100 sequencer (Applied Biosystems). To detect transcription binding sites, we used the TFSEARCH program (www.cbrc.jp/research/db/TFSEARCHJ.html) [15].

Statistical analysis

All results are expressed as mean ± SEM. Statistical analysis was performed by unpaired t test or one-way ANOVA. $p<0.05$ was regarded as significant.

Results

Longitudinal phenotypes of C3H-11^{NSY} mice

C3H-11^{NSY} mice had significantly higher blood glucose levels after fasting ($p<0.0001$) and at all time points after a glucose challenge ($p<0.0001$) than those in C3H mice at

12 months (Fig. 1a). No significant difference in body weight was observed between the two strains at any age, except at 3 months (Fig. 1b). In the longitudinal analysis of glucose tolerance, C3H-11^{NSY} mice showed significantly higher blood glucose levels than C3H mice at all ages studied ($p<0.0001$) (Fig. 1c). These results indicate that introduction of a single NSY-Chr11 can convert normoglycaemic C3H mice to hyperglycaemic mice without a change in body weight.

To better understand the mechanism of hyperglycaemia observed in C3H-11^{NSY} mice, we evaluated insulin secretion in response to glucose and insulin resistance longitudinally. The insulinogenic indices were significantly lower in C3H-11^{NSY} than in C3H mice at all ages (Fig. 1d). The glucose-lowering effect of insulin during ITT progressively worsened with age in C3H-11^{NSY} mice (Fig. 1e). The HOMA-IR value was significantly higher in C3H-11^{NSY} than in C3H mice at and after 6 months (Fig. 1f). These results suggest that both impaired insulin secretion in response to glucose and insulin resistance contribute to hyperglycaemia in C3H-11^{NSY} mice. Furthermore, the results suggest that impaired insulin secretion in response to glucose begins as early as at 3 months and remains constant thereafter, whereas insulin resistance begins at 6 months and gets worse with age in the C3H-11^{NSY} strain.

To further clarify the relationship of insulin resistance with body weight and abdominal fat accumulation in C3H-11^{NSY}, anatomical analysis was performed. As shown in Table 1, body weight and BMI in C3H-11^{NSY} mice were not significantly different from those in C3H mice. Fat-pad weight and the percentage of fat-pad weight/body weight were not significantly different either, suggesting that the age-dependent insulin resistance in C3H-11^{NSY} mice was independent of obesity and changes in fat accumulation.

Longitudinal phenotypes of C3H-14^{NSY} mice

C3H-14^{NSY} mice exhibited significantly higher blood glucose levels after fasting ($p<0.0001$) and at all time points after a glucose challenge ($p<0.0001$) than those in C3H mice at 12 months (Fig. 2a). Body weight was slightly heavier in C3H-14^{NSY} than in C3H mice at 3 and 6 months, but no significant difference was observed at and after 7 months (Fig. 2b). Longitudinal analysis of glucose tolerance during ipGTT showed significantly impaired glucose tolerance in C3H-14^{NSY} compared with that in C3H mice at all ages (Fig. 2c). No significant difference in the insulinogenic index was observed at any age between the two strains (Fig. 2d), suggesting that impaired insulin secretion in response to glucose did not contribute to hyperglycaemia in C3H-14^{NSY}

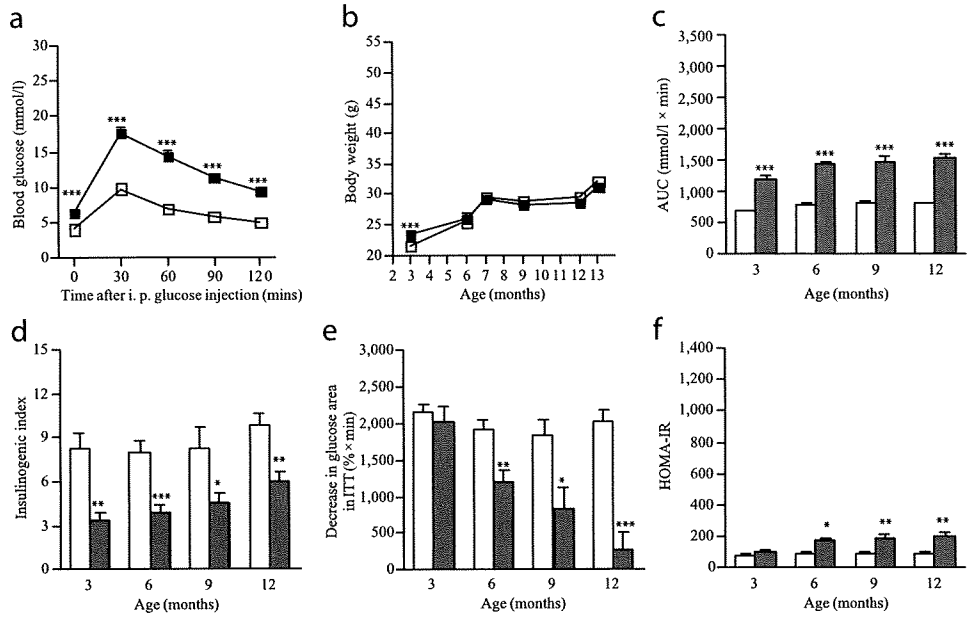


Fig. 1 Phenotypic analyses of C3H-11^{NSY}. **a** IpGTT at 12 months of age in C3H-11^{NSY} (n=28; black squares) and C3H mice (n=38; white squares). **b** Growth curve in C3H-11^{NSY} (n=17–56; black squares) and C3H mice (n=21–39; white squares). Body weight was measured after fasting (3, 6, 9 and 12 months) and under ad libitum feeding (7 and 13 months). **c–f** Longitudinal analyses of: (**c**) glucose tolerance (assessed by gAUC on ipGTT; n=28–56 C3H-11^{NSY} and n=33–39 C3H mice); (**d**) insulinogenic index (n=15–33 C3H-11^{NSY} and n=18–32 C3H mice); (**e**) insulin resistance (assessed by decrease in glucose area in insulin tolerance test; n=14–18 C3H-11^{NSY} and n=16–21 C3H mice); and (**f**) insulin resistance (assessed by HOMA-IR; n=15–33 C3H-11^{NSY} and n=18–32 C3H mice). C3H-11^{NSY} mice, grey bars; C3H mice, white bars. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.0001$ compared with C3H

Table 1 Anatomical analysis in three consomic and parental strains at 12 months of age

Variable	NSY	C3H-11^{NSY}14^{NSY}	C3H-11^{NSY}	C3H-14^{NSY}	C3H
Number of mice analysed	11	18	26	19	19
Blood glucose (ad lib) (mmol/l)	9.4±0.6**	9.9±0.5**,††,‡‡	8.1±0.2*	6.9±0.2	6.8±0.2
Insulin (ad lib) (pmol/l)	928.6±122.8**,§§	448.1±60.0**,††	226.0±12.8	306.1±31.5	189.7±19.1
Body weight (g)	49.5±1.5**,§§	35.1±0.6**,††,‡‡	31.0±0.5	30.9±0.7	32.1±0.5
Anal–nasal length (cm)	11.1±0.1**,§§	10.5±0.1	10.4±0.0	10.2±0.1	10.3±0.0
BMI (g/cm^2)	0.403±0.008**,§§	0.319±0.004**,††,‡‡	0.288±0.004	0.296±0.005	0.300±0.003
Total fat (g)	3.269±0.151**,§§	1.736±0.107**,†	1.366±0.083	1.480±0.092	1.258±0.071
Epididymal fat (g)	1.356±0.076**,§§	0.946±0.068**,†	0.707±0.054	0.783±0.053	0.647±0.042
Retroperitoneal fat (g)	1.044±0.049**,§§	0.207±0.020	0.156±0.013	0.187±0.018	0.134±0.014
Mesenteric fat (g)	0.869±0.055**,§§	0.584±0.026*	0.502±0.021	0.510±0.029	0.476±0.022
Total fat/body weight (%)	6.63±0.30**,§§	4.90±0.24**	4.36±0.22	4.72±0.21*	3.85±0.19
BAT (mg)	201.3±18.2**,§§	139.5±7.1**,††,‡‡	77.6±4.7	84.8±4.8	93.1±6.3

Values are total number or mean ± SEM

The strains were compared by one-way ANOVA and post hoc test (Bonferroni): $^{*}p<0.05$, $^{**}p<0.01$ vs C3H; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ C3H-11^{NSY} 14^{NSY} vs C3H-11^{NSY}; $^{\ddagger\ddagger}p<0.01$ C3H-11^{NSY} 14^{NSY} vs C3H-14^{NSY}; $^{\S\S}p<0.01$ NSY vs C3H-11^{NSY} 14^{NSY}

Ad lib, ad libitum

mice. In contrast, the glucose-lowering effect of insulin during ITT was markedly and significantly impaired in C3H-14^{NSY} compared with that in C3H mice at and after 6 months (Fig. 2e). HOMA-IR was also significantly higher in C3H-14^{NSY} than in C3H mice at and after 6 months (Fig. 2f). These observations suggest that insulin resistance, but not impaired insulin secretion, contributed to glucose intolerance in the C3H-14^{NSY} strain.

To clarify the cause of insulin resistance in C3H-14^{NSY}, anatomical analysis was performed (Table 1). No significant

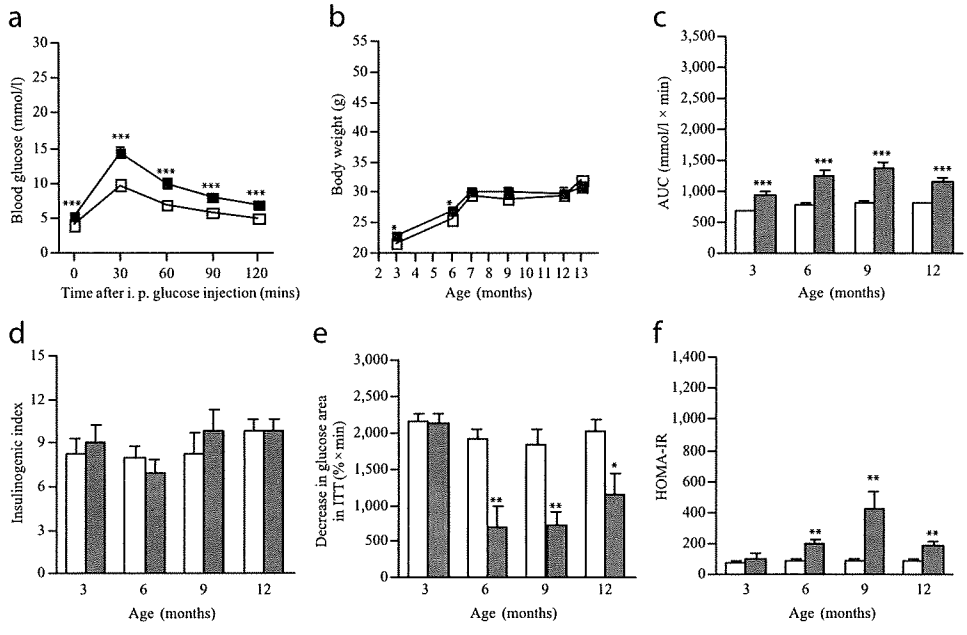


Fig. 2 Phenotypic analyses of C3H-14^{NSY}. **a** IpGTT at 12 months of age in C3H-14^{NSY} (n=32; black squares) and C3H mice (n=38; white squares). **b** Growth curve in C3H-14^{NSY} (n=15–36; black squares) and C3H mice (n=21–39; white squares). Body weight was measured after fasting (3, 6, 9 and 12 months) and under ad libitum feeding (7 and 13 months). **c–f** Longitudinal analyses of: (**c**) glucose tolerance (assessed by gAUC on ipGTT; n=26–36 C3H-14^{NSY} and n=33–39 C3H mice); (**d**) insulinogenic index (n=15–30 C3H-14^{NSY} and n=18–32 C3H mice); (**e**) insulin resistance (assessed by decrease in glucose area in insulin tolerance test; n=15–20 C3H-14^{NSY} and n=16–21 C3H mice); and (**f**) insulin resistance (assessed by HOMA-IR; n=15–30 C3H-14^{NSY} and n=18–32 C3H mice). C3H-14^{NSY} mice, grey bars; C3H mice, white bars. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.0001$ compared with C3H

difference was observed in body weight, BMI and fat-pad weight between C3H-14^{NSY} and C3H mice. The percentage of fat-pad weight/body weight, however, was slightly but significantly elevated in C3H-14^{NSY} mice compared with C3H mice (Table 1), suggesting that an increase in the percentage of body fat may play a role in insulin resistance in C3H-14^{NSY} mice.

Longitudinal phenotypes of double consomic strain (C3H-11^{NSY}14^{NSY})

C3H-11^{NSY}14^{NSY} mice showed significantly higher blood glucose levels after fasting and after a glucose challenge than C3H mice ($p<0.0001$) (Fig. 3a) as well as single consomics, C3H-11^{NSY} ($p<0.05$) and C3H-14^{NSY} mice ($p<0.01$). Hyperglycaemia in C3H-11^{NSY}14^{NSY}, however, was not as severe as in NSY mice ($p<0.0001$ at 12 months) (Fig. 3a). Longitudinal analysis of glucose tolerance revealed that gAUC in C3H-11^{NSY}14^{NSY} was significantly higher than in C3H mice at all ages ($p<0.0001$), but not as high as in NSY mice (Fig. 3c). The insulinogenic index in C3H-11^{NSY}14^{NSY} mice was significantly lower than in C3H mice, and was similar to that in NSY mice at all ages (Fig. 3d). The glucose-lowering effect of insulin in C3H-11^{NSY}14^{NSY} mice was significantly impaired as compared with C3H mice at all ages (Fig. 3e). Insulin resistance in C3H-11^{NSY}14^{NSY} mice, however, was not as severe as in NSY mice (Fig. 3e, f). These results indicate that the major components of genetic susceptibility to hyperglycaemia in NSY were located on NSY-Chr11 and NSY-Chr14, but that other component(s) are also necessary for full reconstitution of the NSY phenotypes.

C3H-11^{NSY}14^{NSY} mice showed significantly higher body weight than C3H mice at all ages ($p<0.01$) (Fig. 3b), in contrast to no significant change in body weight in single consomics, C3H-11^{NSY} and C3H-14^{NSY}. These results provide direct evidence for a genetic interaction between NSY-Chr11 and NSY-Chr14, leading to obesity.

Diabetes-related phenotypes in single consomics, C3H-11^{NSY} and C3H-14^{NSY}, and double consomic, C3H-11^{NSY}14^{NSY}, in comparison with parental strains, NSY and C3H mice

As shown in Table 1, non-fasting blood glucose and insulin in C3H-11^{NSY}14^{NSY} and C3H-11^{NSY} mice were significantly higher than those in C3H mice, whereas no significant difference was observed between C3H-14^{NSY} and C3H mice. C3H-11^{NSY}14^{NSY} mice showed significantly higher body weight and BMI compared with C3H mice,

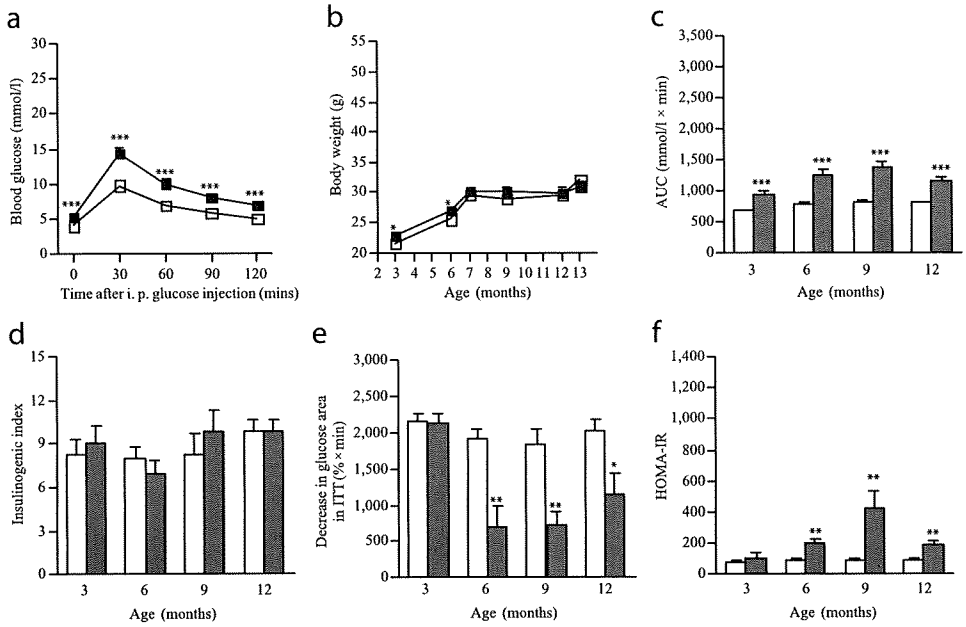


Fig. 3 Phenotypic analyses of C3H-11^{NSY}14^{NSY}. **a** IpGTT at 12 months of age in NSY (n=26; black triangles), C3H-11^{NSY}14^{NSY} (n=23; black squares) and C3H mice (n=38; white squares). **b** Growth curve in NSY (n=9–26; black triangles), C3H-11^{NSY}14^{NSY} (n=15–24; black squares) and C3H mice (n=21–39; white squares). Body weight was measured after fasting (3, 6, 9 and 12 months) and under ad libitum feeding (7 and 13 months). **c–f** Longitudinal analyses of: **c** glucose tolerance (assessed by gAUC on ipGTT; n=9–26 NSY, n=23–26 C3H-11^{NSY}14^{NSY} and n=33–39 C3H mice); **d** insulinogenic index (n=8–20 NSY, n=16–22 C3H-11^{NSY}14^{NSY} and n=18–32 C3H mice); **e** insulin resistance (assessed by decrease in glucose area in insulin tolerance test; n=9–19 NSY, n=20–23 C3H-11^{NSY}14^{NSY} and n=16–21 C3H mice); and **f** insulin resistance (assessed by HOMA-IR; n=8–20 NSY, n=16–22 C3H-11^{NSY}14^{NSY} and n=18–32 C3H mice). NSY mice, black bars; C3H-11^{NSY}14^{NSY} mice, grey bars; C3H mice, white bars. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.0001$ compared with C3H

in contrast to no change in single consomic strains, C3H-11^{NSY} and C3H-14^{NSY} mice. Fat-pad weight and BAT in C3H-11^{NSY}14^{NSY} mice were significantly greater than in C3H mice, whereas C3H-11^{NSY} and C3H-14^{NSY} mice were not significantly different from C3H mice in these respects. Histologically, the heavier BAT showed deposition of fat, resembling white adipose tissue (data not shown), as was previously reported for mice with diet-induced obesity [16]. The percentage of fat-pad weight/body weight in C3H-11^{NSY}14^{NSY} and C3H-14^{NSY} mice was significantly higher than in C3H mice, whereas no significant difference was observed between C3H-11^{NSY} and C3H mice.

As shown in Table 2, C3H-11^{NSY}14^{NSY} showed hyperglycaemia, which appeared to be simply an additive, not synergistic, effect of NSY-Chr11 and NSY-Chr14. The insulinogenic index in C3H-11^{NSY}14^{NSY} mice was similar to that in C3H-11^{NSY} and parental NSY mice at all ages (Figs. 1d, 3d and Table 2), whereas that in C3H-14^{NSY} mice was similar to that in C3H mice at all ages (Fig. 2d and Table 2), suggesting that a major component(s) for impaired insulin secretion in response to glucose in NSY mice is located on Chr11. Insulin resistance in C3H-11^{NSY}14^{NSY} mice was greater than in C3H-11^{NSY} and C3H-14^{NSY} mice, but less than in NSY mice (Table 2). This suggests that major components for insulin resistance in NSY are located on both Chr11 and Chr14, but that these two chromosomes are not sufficient to fully reconstitute the phenotypes of the parental NSY mice.

DNA sequence of *Gck* as candidate gene on chromosome 11

The nucleotide sequences of *Gck*, spanning the 5′ upstream region, 5′ untranslated region (UTR), coding region and 3′ UTR, were determined in NSY (Accession number AB255658) and C3H (Accession number AB255659) mice. As shown in Fig. 4, a total of eight variants—seven single nucleotide polymorphisms (SNPs) and one insertion/deletion—were identified between NSY and C3H mice. The SNPs in introns were not located in exon–intron boundaries. Using the TFSEARCH program, the substitution in the 5′ upstream region was shown to be not located in the known *cis* element. When the variants identified between NSY and C3H mice were compared with reference sequences, six out of eight polymorphisms found in NSY mice were identical to those in C57BL/6 mice, and five out of eight polymorphisms were identical among NSY, 129SV and C57BL/6 mice, but they were different from those in C3H mice. The insertion polymorphism in C3H mice in the 3′ UTR was not found in the other three strains.

Discussion

This study clearly demonstrated that substitution of a single Chr11 or Chr14 from the diabetes-resistant C3H strain to the diabetes-susceptible NSY strain caused marked changes in diabetes-related phenotypes. The mechanisms of inducing hyperglycaemia, however, appeared to be different between C3H-11^{NSY} and C3H-14^{NSY} mice. NSY-Chr11 affects both insulin secretion and insulin sensitivity, whereas NSY-Chr14 affects insulin sensitivity but not insulin secretion. Interestingly, impaired insulin secretion in C3H-11^{NSY} was as severe as that in NSY mice, indicating that impaired insulin secretion in the NSY mouse could be accounted for mostly, if not totally, by Chr11. Despite the marked insulin resistance observed in C3H-11^{NSY} mice, no significant difference was observed in adiposity and obesity between

Table 2 Diabetes-related phenotypes in three consomic and parental strains at 12 months of age

Phenotypical component	NSY	C3H-11^{NSY}14^{NSY}	C3H-11^{NSY}	C3H-14^{NSY}	C3H
Glucose tolerance[a] (*n*)	2,800.9±91.5**,†† (26)	1,835.7±134.5**,‡,§§ (23)	1,535.2 ± 67.4** (28)	1,157.6±52.0** (32)	807.8±18.2 (38)
Insulin secretion[b] (*n*)	5.4±1.7** (13)	5.1±0.6**,§§ (22)	6.0±0.6* (19)	9.9±0.8 (30)	9.8±0.8 (32)
Insulin sensitivity (ITT)[c] (*n*)	−1,115.0±287.6**,†† (19)	231.9±309.2** (23)	264.2±243.2** (18)	1,146.9±299.8 (20)	2,034.0±144.0 (16)
Insulin sensitivity (HOMA-IR)[d] (*n*)	1,007.2±178.8**,†† (13)	436.3±63.9**,‡,§§ (22)	201.4±26.4 (19)	194.7±20.7 (30)	90.3±15.6 (32)

Values are total number or mean ± SEM

[a] Assessed by gAUC during ipGTT (mmol/l×min)

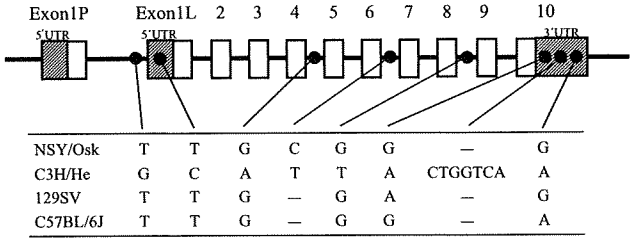
[b] Assessed by insulinogenic index (incremental AUC [ΣΔiAUC] [pmol/l]) divided by incremental gAUC ([ΣΔgAUC] [mmol/l]) during ipGTT

[c] Assessed by decrease in glucose area during ITT (%×min)

[d] Calculated from the basal insulin and glucose concentrations (fasting glucose [mmol/l]×fasting insulin [pmol/l])

The strains were compared by one-way ANOVA and post hoc test (Bonferroni): $^{*}p<0.05$, $^{**}p<0.01$ vs C3H; $^{††}p<0.01$ NSY vs C3H-11^{NSY}14^{NSY}; $^{‡}p<0.05$ C3H-11^{NSY}14^{NSY} vs C3H-11^{NSY}; $^{§§}p<0.01$ C3H-11^{NSY}14^{NSY} vs C3H-14^{NSY}

n, number of mice analysed



NSY/Osk	T	T	G	C	G	G	—	G
C3H/He	G	C	A	T	T	A	CTGGTCA	A
129SV	T	T	G	—	G	A	—	G
C57BL/6J	T	T	G	—	G	G	—	A

Fig. 4 Difference in *Gck* sequence between NSY and C3H. Exon 1P and exon 1L represent exon 1 of mRNA expressed in the pancreas and liver, respectively. Numbers represent exons. Black circles indicate different points in the sequence between NSY and C3H. Information on 129SV (Accession number L41631) and C57BL/6J strain were derived from an online database (www.ensembl.org/Mus_musculus). Dashes represent no base

C3H-11^{NSY} and C3H mice, suggesting that insulin resistance in C3H-11^{NSY} mice is independent of adiposity and obesity. In contrast, C3H-14^{NSY} mice showed a slight but significant increase in percentage fat-pad weight/body weight, suggesting that insulin resistance in C3H-14^{NSY} mice is at least in part associated with an increase in adiposity.

Type 2 diabetes develops when the pancreatic beta cell cannot compensate for insulin resistance [17]. The capacity of insulin secretion deteriorates with age, whereas insulin resistance increases with age, at least in common forms of type 2 diabetes under polygenic control. In this study, C3H-11^{NSY} mice showed that insulin secretion was impaired as early as at 3 months and continued to be impaired thereafter at any age. In contrast, insulin resistance worsened in an age-dependent manner. C3H-14^{NSY} mice did not show impaired insulin secretion at any age, but showed an age-related change in insulin resistance. These results demonstrate that each susceptibility gene(s) on Chr11 and Chr14 interacts with the ageing process in the development of insulin resistance, but not of insulin secretion.

In our previous mapping, F2 mice homozygous for NSY alleles at both *Nidd1n* and *Nidd2n* showed hyperglycaemia comparable with that in the parental NSY mouse [7]. These data, together with the marked phenotypic changes in each of the single consomic strains observed in the present study, suggest that type 2 diabetes in NSY may be oligogenic, with *Nidd1n* and *Nidd2n* accounting for most of the phenotypic changes in NSY. To ascertain whether or not *Nidd1n* and *Nidd2n* are sufficient to reconstitute most of the NSY phenotypes, a double consomic strain, C3H-11^{NSY}14^{NSY} with both *Nidd1n* and *Nidd2n*, was produced. Although the degree of hyperglycaemia in C3H-11^{NSY}14^{NSY} was greater than that observed in each single consomic, it was not as severe as that in NSY. These results suggest that additional genes on other chromosomes are necessary for the full expression of diabetes-related phenotypes of NSY mice. Moreover, in contrast to the absence of obvious obesity in each single consomic strain, C3H-11^{NSY}14^{NSY} mice showed significant obesity, demonstrating that genetic interaction between the two chromosomes plays a role in causing obesity. Thus, the present study clearly demonstrated that major components of genetic susceptibility to type 2 diabetes in NSY are located or clustered on Chr11 and Chr14, which can account for the majority of the phenotypic difference between NSY and C3H mice, but significant interaction between these two chromosomes as well as between these and other chromosomes is necessary for full reconstitution of the phenotypes.

The syntenic region on mouse Chr11 and Chr14 is human Chr17, 5q, 7p (for *Nidd1n*) and Chr3p, 10q, 8p, 13q (for *Nidd2n*). These regions are, therefore, considered to be candidate regions containing diabetogenic genes in humans. In fact, loci associated with type 2 diabetes have been mapped by whole-genome screening in humans [18–24]. More recently, many genome-wide or large-scale association studies revealed several candidate genes for type 2 diabetes and fasting glucose, such as *KCNJ11*, *KCNQ1*, *IGF2BP2*, *TCF7L2*, *MTNR1B*, *G6PC2*, and *GCKR* [25–29], although the orthologues of these genes are not located on mouse Chr11 and Chr14. In mice, linkages with type 2 diabetes were also reported on Chr11 [30–33] and Chr14 [34–37] in several independent crosses (ESM Table 3).

We previously reported sequence analyses of the genes for hepatocyte nuclear factor-1β, GLUT4 and nucleoredoxin [6, 7, 38–40]. In this study, we determined the nucleotide sequences of *Gck*, which is mapped in the centromeric region of Chr11. Although other positional candidate genes, such as genes for insulin-like growth factor binding protein (*Igfbp*) 1 and 3, are also located in the centromeric region of Chr11, *Gck*, which encodes glucokinase, a main glucose-phosphorylating enzyme acting as a glucose sensor of pancreatic beta cells, is a good functional candidate gene for QTL, which is located in the centromeric region of Chr11, because the QTL was linked to glucose/insulin ratio as well as hyperglycaemia [7]. Heterozygous mutations in the gene for human glucokinase, *GCK*, have been identified in patients with MODY [41, 42]. In the general population, a polymorphism in the beta cell-specific *GCK* promoter is associated with hyperglycaemia [43]. In mice, impaired insulin secretion and normal histology of pancreatic islets, as observed in C3H-11^{NSY} mice in the present study, were reported in mice with pancreatic beta cell-specific targeted disruption of *Gck* [44]. In a large scale mutagenesis project using *N*-ethyl-nitrosourea, it was reported that a number of mutations in *Gck* were identified in mice with the type 2 diabetes phenotype [45–47]. We found seven SNPs and one insertion/deletion polymorphism between NSY and C3H mice. The NSY allele is similar to that in C57BL/6 mice, but different from that in C3H mice. Interestingly, inbred control strains of mice have been reported to exhibit marked differences in glucose tolerance,

with C57BL/6 mice having the worst and C3H mice the best glucose tolerance [48]. It is therefore reasonable to speculate that the combination of variants with weaker effects in the non-coding SNPs in *Gck* results in susceptibility to common forms of type 2 diabetes, whereas functional mutations in exons cause a more severe form of diabetes, as in the case of MODY in humans [42]. Functional studies, including studies on insulin secretion in isolated islets, are necessary to clarify whether or not a variant of *Gck* is the cause of the insulin secretory defect in NSY, C3H-11^{NSY} and C3H-$11^{NSY}14^{NSY}$ mice.

In summary, the present study clearly provides direct evidence that Chr11 and Chr14 harbour diabetogenic genes in the NSY mouse. Introgression of each single chromosome onto control mice led to marked changes in phenotype. These two chromosomes interact to cause a more severe phenotype (hyperglycaemia) or a phenotype that was not observed in a single chromosome (obesity), suggesting a different mode of gene–gene interaction depending on the phenotype. The present study indicated the usefulness of the consomic strategy in proving the localisation as well as studying the functions and interactions of susceptibility genes for multifactorial diseases in general and diabetes-related phenotypes in particular, by dissecting disease-related phenotypes into each component. Marked changes in the phenotypes retained in the consomic strain will facilitate the fine mapping and the identification of the genes responsible and their interactions. The consomic strains established in the present study are also useful to study the interaction of genes on each chromosome with environmental factors in conferring susceptibility to diabetes. These studies are now under way.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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The Onset of Diabetes in Three out of Four Sisters: A Japanese Family with Type 1 Diabetes. A Case Report

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Abstract. Type 1A diabetes is an autoimmune disease characterized by the destruction of insulin-producing β-cells in the pancreas. The HLA-*DR* and -*DQ* genes are well established as being associated with increased risk for type 1 diabetes. Moreover, polymorphisms in *CTLA4* have been reported to be associated with susceptibility to type 1 diabetes and autoimmune thyroid disease (AITD). In both Caucasian and Japanese populations, the lifetime risk in siblings of type 1 diabetic probands is much higher than that in general populations. However, in Japan, where the prevalence of type 1 diabetes is less than one-tenth that of most Caucasian populations, it is rare for type 1 diabetes to develop in three or more siblings within a family. Here, we report a Japanese family in which type 1 diabetes occurred in three siblings amongst four sisters. Three probands of type 1 diabetes had the same combination of HLA haplotypes, *DRB1*0405-DQB1*0401/DRB1*0802-DQB1*0302*, which occurs significantly more often in type 1 diabetes patients than in control subjects in the Japanese population. With respect to the rs3087243 (+6230G>A) polymorphism of *CTLA4*, the first sister had type 1 diabetes and AITD and had the GG genotype, whereas the second and third sisters, who had type 1 diabetes without AITD, had the AG genotype. This is the first report of a family in which type 1A diabetes developed in three siblings. We performed genetic analysis of HLA-*DR*, -*DQ*, and *CTLA4* in all family members. Even in a country where the prevalence of type 1 diabetes is low, diabetic proband siblings should be monitored for the onset of type 1 diabetes.

Key words: Type 1 diabetes, Sibling, Japanese, HLA, *CTLA4*

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TYPE 1A diabetes is caused by autoimmune destruction of insulin-producing β-cells of the pancreas in genetically susceptible individuals. The major histocompatibility complex (MHC) is reported to account for 30–50% of the familial aggregation of type 1 diabetes [1]. The human leukocyte antigen (HLA)-*DR* and -*DQ* genes are well established as being associated with risk for type 1 diabetes. In the Japanese population, three haplotypes, *DRB1*0405-DQB1*0401*, *DRB1*0802-DQB1*0302*, and *DRB1*0901-DQB1*0303*, which are rare in Caucasian populations, confer susceptibility to type 1 diabetes [2, 3, 4]. In addition, several non-HLA loci have been identified as putative susceptibility genes by candidate gene approaches and/or genome-wide scanning. Among these is the gene for cytotoxic T-lymphocyte-associated-4 (*CTLA4*), which has been reported to be associated with increased susceptibility to type 1 diabetes and autoimmune thyroid disease (AITD) in Caucasian and Japanese populations [5, 6].

In Caucasian populations, the lifetime risk in siblings of type 1 diabetic probands has been reported to be much higher than that in the general population (6% versus 0.4%, λs 15), indicating that type 1 diabetes clusters in families [7]. In the Japanese population, although the prevalence of type 1 diabetes is very low and is less than one-tenth that in most Caucasian populations, studies with a large number of families with type 1 diabetic probands from different data sources have revealed much higher frequencies of type 1 dia-

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betes in siblings of diabetic probands than in the general population (1.3–3.8% versus 0.01–0.02%, λs >65) [3, 8, 9]. This indicates that type 1 diabetes clusters in families in Japan. Although the λs in the Japanese population is higher than that for Caucasian populations, the absolute number of multiplex families with type 1 diabetes is limited because of the very low incidence of type 1 diabetes in Japan. In particular, there are very few families in Japan in which type 1 diabetes has been diagnosed in three or more siblings. Here, we report a Japanese family with four sisters, in which three siblings developed type 1A diabetes and the fourth sibling was positive for islet-related autoantibodies, indicative of an ongoing type 1A process. In the present report, we determined the genotype of the HLA and *CTLA4* polymorphisms in the four siblings and in their parents to better understand the genetic basis for the familial clustering of type 1 diabetes in this family.

Case Report

A 40-year-old woman, who was the eldest of four sisters, was referred to a hospital because of general malaise, thirst, polydipsia, polyuria, and body weight loss. Although she had no prior history of diabetes, she was diagnosed as hyperglycemic and she was admitted to our hospital in April 2008. Laboratory data on admission (Table 1) revealed diabetic ketosis, elevated HbA_{1c}, and she was positive for antibodies against glutamic acid decarboxylase (GAD), insulinoma-associated protein 2 (IA-2), thyroglobulin (Tg), and thyroid peroxidase (TPO). Based on these findings, we diagnosed her with type 1A diabetes and AITD, and we started her on insulin therapy.

Figure 1 shows the pedigree of the present family. The second and third sisters developed type 1A diabetes at 6 and 12 years old, respectively. Table 2A shows the results of urinary and serum C-peptide in each of the family members, including the parents. The urinary and serum C-peptide levels were markedly decreased in the three probands of type 1 diabetes. On the other hand, the insulin secretory capacities of the fourth sibling and of the parents were not reduced, based on the their postprandial serum C-peptide levels. Table 2A also shows the characteristics of the anti-islet and anti-thyroid antibodies in each family member. In the second and third siblings, frozen sera, which were obtained 10 years ago, were used to measure the antibodies because many years had passed after their onset of type 1 diabetes. The first and third siblings had high titers of anti-GAD and anti-IA-2 antibodies. Although the fourth sibling had no prior history of diabetes, anti-GAD and anti-IA-2 antibodies were present. Because of the presence of anti-Tg and anti-TPO antibodies in the first sibling, we determined them in all of the family members. In addition to the first sibling, the mother had high titers of anti-Tg and anti-TPO antibodies, but their thyroid functions were within the normal range.

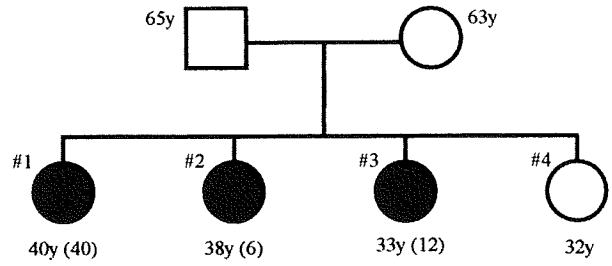

Fig. 1. Pedigree of the present family. The numbers in the brackets show the age at onset of type 1 diabetes. y = years old.

Table 1. Laboratory data for the first sibling on admission.

Postprandial plasma glucose	345 *mg/dL*	Postprandial serum C-peptide	0.4 *ng/mL*
HbA_{1c}	14.3%	Urinary C-peptide	8.0*μg/day*
3-OHBA	5819*μmol/L*	Urinary glucose	5+
ACAC	780*μmol/L*	Urinary ketone	4+
		Urinary protein	-
WBC	3600/*μL*		
Hb	14.3 *g/dL*	Anti-GAD antibody	433 *U/mL*
Plts	25*10^4/*μL*	Anti-IA-2 antibody	18 *U/mL*
Alb	4.2 *g/dL*	Anti-Tg antibody	5.2 *U/mL*
AST	58 *IU/L*	Anti-TPO antibody	50.0< *U/mL*
ALT	53 *IU/L*	TRAb	6.2%
AMY	48 *IU/L*	f-T_4	1.16 *ng/dL*
Na	134 *mEq/L*	f-T_3	1.4 *pg/mL*
K	3.9 *mEq/L*	TSH	1.65*μU/mL*
Cl	95 *mEq/L*	Arterial Blood Gas Analysis	
BUN	11 *mg/dL*	on room air	
Cre	0.36 *mg/dL*	pH	7.384
LDL-C	119 *mg/dL*	pCO_2	37.7 *mmHg*
TG	106 *mg/dL*	pO_2	102.3 *mmHg*
HDL-C	110 *mg/dL*	HCO_3^-	22 *mmol/L*
		B.E.	-2.6 *mmol/L*

TRAb = anti TSH receptor antibody. TSH = Thyrotropin. Anti-GAD, anti-IA-2, anti-Tg and anti-TPO antibodies were determined with a commercially available radioimmunoassay kit (Cosmic, Tokyo, Japan). TRAb was measured by a commercially available radioreceptor assay kit (Cosmic, Tokyo, Japan).

Table 2. Analytical parameters of the present family.

A) Urinary and serum C-peptide, plasma glucose, anti-islet antibodies, and anti-thyroid antibodies.

	Urinary C-peptide (μg/day)	Postprandial serum C-peptide (ng/mL)	Postprandial plasma glucose (mg/dL)	GAD (U/mL)	IA-2 (U/mL)	IAA (%)	TgAb (U/mL)	TPOAb (U/mL)	TRAb (%)
Father	n.d.	3.56	117	<0.3	<0.4	0.4	<0.3	<0.3	0
Mother	n.d.	2.85	127	<0.3	<0.4	<0.4	1.3	30.4	4.4
#1	8.0	0.4	345	433	18	n.d.	5.2	50<	6.2
#2	2.9	<0.03	189	<0.3	<0.4	n.d.	<0.3	<0.3	0
#3	1.1	0.05	383	3.5	0.5	n.d.	<0.3	<0.3	0
#4	n.d.	2.48	95	6.8	4.9	<0.4	<0.3	<0.3	0

B) HLA-*DRB1* and -*DQB1* haplotypes, and *CTLA4* polymorphisms.

	DRB1-DQB1	*CTLA4*	
		rs3087243 (+6230G>A)	rs231775 (+49G>A)
Father	*DRB1*0405-DQB1*0401/ DRB1*0405-DQB1*0401*	GG	GG
Mother	*DRB1*0405-DQB1*0401/ DRB1*0802-DQB1*0302*	AG	AA
#1	*DRB1*0405-DQB1*0401/ DRB1*0802-DQB1*0302*	GG	AG
#2	*DRB1*0405-DQB1*0401/ DRB1*0802-DQB1*0302*	AG	AG
#3	*DRB1*0405-DQB1*0401/ DRB1*0802-DQB1*0302*	AG	AG
#4	*DRB1*0405-DQB1*0401/ DRB1*0802-DQB1*0302*	GG	AG

Serum C-peptide and plasma glucose were measured in the postprandial state. GAD = anti glutamic acid decarboxylase antibody. IA-2 = anti insulinoma-associated protein 2 antibody. IAA = anti insulin autoantibody. TgAb = anti thyroglobulin antibody. TPOAb = anti thyroid peroxidase antibody. TRAb = anti TSH receptor antibody. n.d. = not determined.

Anti-GAD, anti-IA-2, anti-Tg and anti-TPO antibodies were determined with a commercially available radioimmunoassay kit (Cosmic, Tokyo, Japan). Anti-IAA antibody was measured by a sensitive radioimmunoassay kit (Yamasashoyu Co. Ltd., Chiba, Japan. Normal range; <0.4%). TRAb was determined with a commercially available radioreceptor assay kit (Cosmic, Tokyo, Japan).

After obtaining written informed consent, we performed genetic analysis in all of the subjects. Table 2B shows the HLA-*DRB1* and -*DQB1* haplotypes, and *CTLA4* polymorphisms in the present family. Three probands of type 1 diabetes had the same combination of HLA haplotypes, namely *DRB1*0405-DQB1*0401/ DRB1*0802-DQB1*0302*. Furthermore, the same combinations of HLA haplotypes were detected in the fourth sibling and in the mother who had no prior history of diabetes. The father had *DRB1*0405-DQB1*0401/ DRB1*0405- DQB1*0401*. Two single nucleotide polymorphisms in the *CTLA4* gene, rs3087243 (+6230G>A) and rs231775 (+49G>A), have been reported to be associated with type 1 diabetes and AITD [6, 10]. In the analysis of the rs3087243 polymorphism of *CTLA4*, the father, and the first and fourth siblings had the GG genotype. On the other hand, the mother, and the second and third siblings had the AG genotype. With respect to the *CTLA4* rs231775 polymorphism, the father and mother had the GG and AA genotype, respectively, and all four siblings had the AG genotype.

Discussion

Large families with type 1 diabetes arising from a common ancestor have been described for Bedouin Arabs [11] and in the Netherlands [12]. The diabetes susceptibility locus, *IDDM17*, was mapped by genetic linkage analysis in a Bedouin Arab family, in which 19 family members across three generations had type 1 diabetes [11]. In addition to the HLA locus, evidence for type 1 diabetes loci was observed on chromosome 8q24 and on chromosome 17q24 in an analyses of 43 subjects in the Netherlands that could be traced back

to a common ancestor within 15 generations [12]. In a study of 767 multiplex Caucasian families, which showed evidence of linkage of seven regions of the genome to type 1 diabetes, 51 families had three affected siblings, one had four affected siblings, and two with five affected siblings [13]. However, to our knowledge, no case reports have described the development of type 1 diabetes in three or more siblings or performed genetic analysis in Japanese subjects. In this report, we have described a Japanese family in which type 1A diabetes occurred in three siblings and ongoing islet autoimmunity was present in the fourth sibling of the four sisters.

Population studies have shown that HLA associations may vary depending on the geographic and ethnic origin. In Caucasian populations, predisposition to type 1 diabetes is mostly associated with the *DRB1*03-DQB1*0201* and/or *DRB1*04-DQB1*0302* haplotypes [14, 15]. Meanwhile, in Japanese populations, the *DRB1*03-DQB1*0201* haplotype is absent or very rare, and *DRB1*04-DQB1*0302* is not associated with type 1 diabetes because of differences in *DRB1*04* subtypes. Instead, three alternative haplotypes, *DRB1*0405-DQB1*0401*, *DRB1*0802-DQB1*0302*, and *DRB1*0901- DQB1*0303*, which are rare in Caucasian populations, confer susceptibility to type 1 diabetes [2, 3, 4]. Moreover, in a Japanese study, the frequencies of the *DRB1*0405-DQB1*0401/ DRB1*0802-DQB1*0302* and *DRB1* 0901-DQB1*0303/ DRB1*0901-DQB1*0303* genotypes were significantly higher in subjects with type 1 diabetes than in control subjects [2, 4]. In the present case, three siblings with type 1 diabetes had the same combination of high-risk HLA haplotypes, namely *DRB1*0405-DQB1*0401/ DRB1*0802-DQB1*0302*, suggesting an association between the onset of type 1 diabetes and HLA-linked susceptibility in the three probands.

In the present three probands, type 1 diabetes occurred in the first sibling at the age of forty, whereas the second and third siblings were diagnosed with type 1 diabetes under the age of 20 years. Several environmental agents have been reported as triggers for type 1 diabetes. However, all of the probands received their mother's breast milk and were raised in very similar situations. On the other hand, previous studies have suggested that the HLA class I region contributes to the susceptibility to and the age-at-onset of type 1 diabetes [16, 17, 18]. Indeed, the presence of HLA-*A*2402* was associated with age-at-onset of type 1 diabetes in an analysis of Caucasian sibling pairs [16]. Furthermore, the presence of HLA-A24 was shown to accelerate β-cell destruction in type 1 diabetes, and to confer susceptibility to type 1 diabetes in Japanese subjects [19, 20]. Thus, the analysis of the HLA class I gene and further studies are warranted to better understand the reasons for the difference of age-at-onset in the three probands.

The fourth sibling had a high titer of autoantibodies against GAD and IA-2, and had *DRB1*0405-DQB1*0401/ DRB1*0802-DQB1*0302*, which was the same genotypic combination as her three siblings with type 1 diabetes. In a Caucasian study, the presence of two or more anti-islet antibodies was reported to be highly predictive of the development of type 1 diabetes among first-degree relatives of type 1 diabetic patients [21]. Additionally, the risk for islet autoimmunity was found to be dramatically increased in siblings who shared both HLA haplotypes with their diabetic proband sibling compared with siblings who did not share both HLA haplotypes with their diabetic proband sibling [22]. Therefore, we speculate that this sibling is at very high risk of developing type 1 diabetes, and we need to continue to follow-up this subject with care.

Autoantibodies against GAD and IA-2 were not present in the second sibling. Type 1 diabetes occurs in genetically susceptible subjects and is probably triggered by one or more environmental agents, such as viral infections. The second sibling had rubella virus infection at the age of 6 years. Two months later, she complained of severe thirst and started insulin therapy to treat hyperglycemia. In addition, her urinary C-peptide excretion was very low. Furthermore, she had the HLA haplotypes *DRB1*0405-DQB1*0401/ DRB1*0802-DQB1*0302*. Based on these findings, we diagnosed the second sibling with type 1A diabetes, despite the absence of anti-GAD and anti-IA-2 antibodies.

CTLA4 has been reported to be associated with susceptibility to type 1 diabetes and AITD in Caucasian and Japanese populations. With respect to the *CTLA4* rs3087243 polymorphism, the frequency of the GG genotype was significantly higher in patients with type 1 diabetes and AITD than in control subjects in a Japanese population [6]. In the present family, only the first sibling of the three probands had both type 1 diabetes and AITD, and she had the GG genotype in

rs3087243 of *CTLA4*.

To our knowledge, our present report is the first to describe the development of type 1A diabetes in three siblings and to perform genetic analysis of HLA-*DR*, *-DQ*, and *CTLA4* in Japanese subjects. Even in a country where the prevalence of type 1 diabetes is very low, the onset of type 1 diabetes should be considered and anti-islet antibodies should be determined in diabetic proband siblings. Further studies on the genetic background of such families may lead to the identification of rare variants that confer susceptibility to type 1 diabetes.

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RESEARCH LETTER

Fulminant type 1 diabetes as an important exception to the new diagnostic criteria using HbA_{1c}—response to the International Expert Committee

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Keywords Criteria · Diagnosis · Fulminant · Idiopathic

Abbreviation
JDS Japan Diabetes Society

To the Editor: Taking into consideration that chronic hyperglycaemia sufficient to cause diabetes-specific complications is the defining feature of diabetes, an International Expert Committee (with members appointed by the American Diabetes Association, the European Association for the Study of Diabetes and the International Diabetes Federation) recommended a cut-off point for HbA_{1c} of 6.5% for the diagnosis of diabetes [1]. The Committee's report highlighted the limitation of HbA_{1c} in patients with rapidly evolving type 1 diabetes, where the HbA_{1c} level will not have had time to 'catch up' with the acute elevation in glucose levels. In this situation, diabetes should be diagnosed from typical symptoms and random blood glucose levels >11.1 mmol/l (200 mg/dl) despite a non-diagnostic HbA_{1c} level. In another section of the report, the Committee notes most cases of type 1 diabetes, particularly in children and adolescents, are diagnosed by the classical symptoms of polyuria, polydipsia, polyphagia, unexplained weight loss and a random blood glucose >11.1 mmol/l.

We have defined a model of rapidly evolving type 1 diabetes (fulminant type 1 diabetes) that is characterised by an absence of islet autoantibody and a markedly rapid onset [2, 3]. This is certainly an important subtype within diabetes but does not meet the new diagnostic criteria using HbA_{1c}. To confirm this, we determined the prevalence of patients with fulminant type 1 diabetes who had HbA_{1c} ≤6.1% at the onset of disease. According to the formula of the National Glycohemoglobin Standardization Program value 'HbA_{1c} ≥6.5%' in the USA is equivalent to 'HbA_{1c} ≥6.2%' in Japan (US value [%] = Japan Diabetes Society [JDS] value [%] +0.3%, determined by using Japanese standard reference material for JDS Lot 2 HbA_{1c}) [4]. Ninety-nine patients who suffered from fulminant type 1 diabetes after March 2001 were selected from a nationwide cohort of fulminant type 1 diabetes [5]. We selected the patients from March 2001 onwards because until that time the measurement of HbA_{1c} had not been standardised by using JDS Lot 2. As a result, the prevalence of patients who had HbA_{1c} ≤6.1% at disease onset was 55.6% (55/99), while all had a blood glucose level >11.2 mmol/l (200 mg/dl) and their average blood glucose level was 41.5 mmol/l (747 mg/dl). However, the average HbA_{1c} level was 12.2% at onset; this accords with the new criteria in classical type 1A diabetes in our previous report, suggesting chronic hyperglycaemia due to continuous beta cell loss [3]. Fulminant type 1 diabetes is not uncommon in the Asian population, in which it accounts for approximately 20% of ketosis-onset type 1 diabetes [3, 6]; it has also been reported in a white population [7]. In addition, it is noteworthy that almost all patients with fulminant type 1 diabetes are not children or adolescents, but adults [5]. At the onset of fulminant type 1 diabetes, patients often complain of non-specific symptoms,

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such as general fatigue and loss of consciousness, and they sometimes do not have classic symptoms of hyperglycaemia such as polyuria, polydipsia and polyphagia. This implies that sometimes the diagnosis of diabetic ketoacidosis cannot be made at onset of fulminant type 1 diabetes, which may well result in death.

From this evidence, fulminant type 1 diabetes should be highlighted as an important exception to the new diagnostic criteria using HbA_{1c}.

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SHORT COMMUNICATION

Genome-wide association database developed in the Japanese Integrated Database Project

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The establishment of high-throughput single-nucleotide polymorphism (SNP)-typing technologies has enabled astonishing progress to be made in genome-wide association studies (GWAS), and various novel genetic factors associated with complex diseases have been discovered. Our organization has created a public repository database (DB) to achieve a continuous and intensive management of GWAS data and to facilitate data sharing among researchers. In the GWAS DB, information on study design, quality control protocols, allele frequencies, genotype frequencies and statistical genetic analysis results are stored as publicly available data and can be accessed freely, whereas individual genotyping data and raw data are stored as restricted data and can only be accessed with authorization. All data are presented by a graphic viewer, which is designed to be user friendly for researchers who are not familiar with GWAS to accelerate disease-related studies. Furthermore, the DB allows users to compare various study results obtained by different institutions and on different platforms. The same data are also managed as a distributed annotation system to call up useful data from other DBs and to superimpose them on the GWAS data for help in interpretation. The DB is accessible at https://gwas.lifesciencedb.jp/.
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Keywords: database; genome-wide association; SNP

INTRODUCTION

The accomplishment of sequencing of the entire human genome[1,2] and the HapMap project,[3] coupled with the development of cost-effective high-throughput dense single-nucleotide polymorphism (SNP)-typing techniques, have enabled a genome-wide exploration of various complex disease-associated variants. Currently, the high-throughput SNP-typing methods are expected to cover about 80% of the human genome in linkage disequilibrium.[4] A number of large-scale genome-wide cohort studies and case–control studies, such as seven common disease GWAS by the Wellcome Trust Case Control Consortium (WTCCC, 2007), have been planned, and some of them are underway. So far, more than 100 loci of disease-related/causing candidates for about 40 common diseases and traits have been identified,[5] and some loci have led to new insights into pathophysiology and etiological pathways. Because GWAS yields large amounts of raw data and analysis results, the management of GWAS data has become a matter of serious concern. Furthermore, more and more grant-funding agencies, journal editors and research communities are beginning to require the disclosure of GWAS data. Disclosure and data sharing of GWAS data will primarily lead to the following three possibilities: (1) meta-analysis using data sets produced in multiple studies to find novel disease-related SNP candidates; (2) re-use of GWAS data combined with other experimental data, including pathway data and expression data, to deepen the exploration of each disease; and (3) development of methods to analyze and compute genetic statistics. In the case of meta-analysis in particular, the use of raw data is indispensable for quality control and for consideration of population structures. Some studies have successfully found additional disease-related SNP candidates on the basis of meta-analysis.[6,7]

The National Center for Biotechnology Information launched the database (DB) of Genotype and Phenotype in the fall of 2006 as a centralized GWAS system to archive and distribute GWAS data. Currently, results funded by the Genetic Association Information Network and voluntarily submitted data have been accumulated. The European Genotype Archive was created in the spring of 2008 as a repository system for phenotype–genotype relationships, and results primarily from WTCCC have been accumulated and redistributed. To achieve a continuous and intensive management of GWAS data and data sharing among researchers, we established a new DB that is publicly available. This DB is expected to have an essential role in providing easily accessible GWAS data to researchers in various biomedical fields. Some disease-related SNPs are assumed to be buried because of their insufficient *P*-values caused by an insufficient number of case–control samples. It is possible that these SNPs will be revealed by combining the GWAS analysis results with other data possessed by users.

In this paper, we introduce the GWAS DB.

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