Genetic analysis of non-insulin-dependent diabetes mellitus in KK and KK-A\textsuperscript{y} mice

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Abstract
The KK mouse is considered suitable as a polygenic model for human non-insulin-dependent diabetes mellitus. To identify the quantitative trait loci (QTLs) responsible for hyperglycemia and impaired glucose tolerance in KK mice, linkage analysis using 97 microsatellite markers was carried out in a 192 F\textsubscript{2} progeny, comprising 93 mice with the a/a genotype at the agouti locus (chromosome 2) and 99 mice with the A\textsuperscript{y}/a genotype, produced by a cross between a C57BL/6J female and a KK-A\textsuperscript{y} (A\textsuperscript{y} congenic) male. In F\textsubscript{2} a/a progenies, we identified a QTL for fasting glucose levels on chromosome 6 (LOD score 6.0) and three loci with suggestive linkage on chromosomes 3, 5 and 14, but could not identify loci accounting for glucose tolerance and plasma insulin levels. In F\textsubscript{2} A\textsuperscript{y}/a progenies, there were no loci with statistically significant linkage, but three suggestive loci were identified: a locus for fasting glucose on chromosome 9, and two loci for glucose tolerance on chromosomes 1 and 8. It would thus appear that, although the fasting glucose level is controlled by QTLs in KK mice, these QTLs may be masked by the strong hyperglycemic influence of the A\textsuperscript{y} allele. Suggestive loci accounting for glucose tolerance may interact with the A\textsuperscript{y} allele, since these loci were identified only in F\textsubscript{2} A\textsuperscript{y}/a progeny. This is consistent with the finding that the impaired glucose tolerance in KK mice is moderate and becomes overt when associated with the A\textsuperscript{y} allele.

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Introduction
Non-insulin-dependent diabetes mellitus (NIDDM) is a heterogeneous metabolic disorder generally characterized by impairment of insulin secretion from pancreatic \( \beta \)-cells and insulin resistance in peripheral tissues with basal hyperinsulinemia (1, 2). Susceptibility to NIDDM appears to have a genetic basis, and, for the most part, the mode of inheritance is considered to be polygenic (1, 2). Non-genetic factors or environmental interactions may also affect the degree of NIDDM (1, 2). The genetic etiology of this disorder thus remains largely unclear in nearly all human cases. Investigation using well-defined animal models should thus be conducted (3, 4). Numerous animal models relevant to human diseases are available (5). Recent advances in mouse genetic linkage analysis using microsatellite markers and statistical strategies (6–9) has facilitated the elucidation of the genetic basis for various polygenic diseases.

The inbred mouse strain KK, established in Japan as a diabetic strain, develops NIDDM with mild obesity, mainly due to insensitivity of the peripheral tissue to insulin (10, 11). Diabetes and obesity in KK mice is relatively moderate but introduction of the A\textsuperscript{y} allele (KK-A\textsuperscript{y}) exacerbates the pathophysiological condition, with consequent overt diabetes accompanying hyperinsulinemia (12, 13). The mechanism for obesity caused by the A\textsuperscript{y} allele is thought to be as follows. Ectopically expressed agouti peptide acts as an antagonist at the melanocortin 4 receptor, to inhibit the action of \( \alpha \)-melanocyte-stimulating hormone signals (14–16).

Whether introduction of the A\textsuperscript{y} allele confers diabetic and hyperglycemic tendency in a simple additive manner or whether exacerbation caused by the A\textsuperscript{y} allele results from complex interactions between diabetic genes putatively present in the genome is not clear. A comparison of the genetic bases for NIDDM in mice populations with and without the A\textsuperscript{y} allele may thus serve to elucidate differences in the genetic etiology as well as the mechanism for morbidity due to the A\textsuperscript{y} allele.

Materials and methods
Mice and genetic crosses
The inbred mice for this study were purchased from Clea Japan Inc (Tokyo, Japan). Three C57BL/6J (coat color locus allele, aa; BB; CC) female mice were mated with a KK-A\textsuperscript{y} (A\textsuperscript{y}a; BB; Cc) male to produce F\textsubscript{1} mice. The KK-A\textsuperscript{y} mouse is a congenic strain in which the A\textsuperscript{y} allele at the agouti locus (initially from the C57BL/6J-A\textsuperscript{y} strain) had been transferred to the inbred KK strain by repetitive backcrossing. F\textsubscript{2} populations were produced...
by intercrossing F₁ mice with the Aʸ/a genotype (Aʸ/a mice could be easily recognized by their yellow coats, and the homozygote for Aʸ has been proved to be lethal at the time of implantation (5)). F₁ females assessed as being pregnant were raised separately and, on the day of birth, only dams with litters containing from five to nine pups were chosen for weaning F₂ mice. F₂ mice (more than 200) were divided into Aʸ/a and a/a genotype groups at the agouti locus according to coat color, and 93 mice (49 male and 44 female) with the a/a genotype and 99 mice (50 male and 49 female) with the Aʸ/a genotype were randomly selected so that age differences would have little effect in subsequent experimental studies. Albino F₂ mice were discarded because it was not clear whether or not they had the Aʸ allele. A total of 192 F₂ mice were weaned at 21 days after birth and each was housed in a Plexiglas cage throughout the experimental period. All the mice were maintained in a room under specific-pathogen-free conditions with a regular light cycle of 12 h light/12 h darkness and the temperature controlled at 22 ± 3°C and a relative humidity of 50%. They had free access to the diet (rodent pellet chow CE-2 (342.2 kcal/100 g, containing 4.4% crude fat); Clea Japan Inc.) with tap water available ad libitum.

**Intraperitoneal glucose tolerance test (IPGTT) and plasma insulin measurement**

IPGTT was conducted on all 133–142-day-old F₂ a/a males, 143–153-day-old F₂ a/a females, 124–130-day-old F₂ Aʸ/a males and 132–138-day-old F₂ Aʸ/a females.

A single i.p. injection of 2.8 mol/l glucose solution, which would produce 2 g/kg body weight, was given in the morning after a 16 h overnight fast. Glucose was then measured in blood from tail bleeds using the Toecho Super apparatus (Kyoto Dalichi Kagaku, Kyoto, Japan) at 0, 30, 60 and 120 min during the IPGTT. Impaired glucose tolerance was evaluated on the basis of the sum of blood glucose during IPGTT (30, 60 and 120 min). At 0 and 30 min, blood was collected into heparinized hematocrit capillary tubes for plasma insulin measurement during the early phase of IPGTT. Because of the hyperinsulinemic nature of the mice heterozygous for the Aʸ allele irrespective of genetic background, plasma insulin during IPGTT was measured only in F₂ a/a mice. Immediately after sampling, the tubes were centrifuged at 13 000 r.p.m. for 5 min and the plasma was separated. Plasma insulin was quantified using the enzyme immunoassay kit (Seikagaku Co., Tokyo, Japan). This kit requires only a 5 μl plasma sample. The intra- and interassay coefficients of variation were within 5 and 12% respectively.

**Genotype analysis**

A total of 97 microsatellite marker loci (except for sex chromosomes) were genotyped in all F₂ mice. Genomic DNA was prepared from the tail following standard procedures. Microsatellite sequence length polymorphism was detected by electrophoresis subsequent to PCR. Most microsatellite primers were purchased as MapPairs (Research Genetics, Huntsville, AL, USA) while some were synthesized. Amplification was carried out in a Takara PCR thermal cycler MP (Takara Biomedicals, Tokyo, Japan) under the following conditions: one cycle at 94°C for 5 min; 35 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 45 s; one cycle at 72°C for 7 min. PCR products were electrophoresed on 10% polyacrylamide gels (PAGEL; Atto Co., Tokyo, Japan) for 70 min and visualized by ethidium bromide staining.
presence of the A\textsuperscript{y} allele at the agouti locus. Before statistical analysis, all phenotypic data were processed, standardized by subtracting the mean and divided by standard deviation within each sex to minimize gender differences. Linkage analysis was conducted using interval mapping using the Mapmaker/QTL 1.1b computer program (7). Quantitative trait locus (QTL) analysis was carried out on a/a mice and A\textsuperscript{y}/a mice independently. After QTL identification, the mice were divided into KK/KK, KK/B6 or B6/B6 genotype groups on the basis of the genotype at the closest marker to the putative QTL region having a maximum LOD score in both F\textsubscript{2} a/a and F\textsubscript{2} A\textsuperscript{y}/a mice. Two-way ANOVA was then carried out on raw data for both F\textsubscript{2} a/a and F\textsubscript{2} A\textsuperscript{y}/a mice for assessment of the statistical significance of genotypes at marker loci, gender, and their interaction for all traits. A LOD score exceeding 4.3 (threshold of statistical significance at the $\alpha = 0.05$ level) was considered an indication of significant linkage, and one of more than 2.8 but less than 4.3 as suggestive of linkage. The $\alpha$ level for suggestive linkage implies the expectation that there will be one false positive in a genome-wide search (9). The dominance of each QTL was determined by comparison of dominant, recessive and additive models by the Mapmaker/QTL program, with a LOD score of 1.0 or higher scoring as the criterion for determination.

**Results**

For all traits examined, interactions between gender and genotype at marker loci were not statistically significant (ANOVA tables not shown). Males and females were
thus examined independently using one-way ANOVA for each genotype group at the agouti locus.

Plots of fasting glucose levels and glucose levels during IPGTT in each parental strain and F1 and F2 individuals are presented in Fig. 1, and the results of IPGTT for parents and F2 progeny are shown in Fig. 2. Various diabetes-related phenotypic values for the F2 mice are indicated in Table 1. Blood glucose during fasting in KK-Ay males was higher than in KK males, and that in KK males was essentially the same as in C57BL/6J females (Fig. 1). Although fasting glucose levels were comparable for KK males and C57BL/6J females, hyperglycemia during IPGTT clearly differed (Fig. 2).

In F2 a/a mice, four loci were found for fasting glucose on chromosomes 3, 5, 6 and 14. A locus on chromosome 6 was mapped near D6Mit268 with statistical significance (Fig. 3 and Table 2; LOD score 6.0 and by one-way ANOVA, \( P = 0.0029 \) in males and \( P = 0.0016 \) in females). The constrained genetic model of the

<table>
<thead>
<tr>
<th>Table 1 Phenotypic values for F2 progeny. Data expressed as means ± s.e.m. (range: minimum to maximum value).</th>
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<tr>
<td></td>
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<td>-----------------</td>
</tr>
<tr>
<td>Body weight (g)</td>
</tr>
<tr>
<td>(30.0–55.9)</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
</tr>
<tr>
<td>(4.3–12.3)</td>
</tr>
<tr>
<td>Glucose during IPGTT (mmol/l)</td>
</tr>
<tr>
<td>(22.8–78.1)</td>
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<tr>
<td>Insulin at 0 min during IPGTT (pmol/l)</td>
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<tr>
<td>(56.9–1499.2)</td>
</tr>
<tr>
<td>Insulin at 30 min during IPGTT (pmol/l)</td>
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<td>(65.5–1102.9)</td>
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ND, not done since insulin level was beyond the detection of the assay system in most cases.
The Mapmaker/QTL program indicated possible recessive action of the KK allele at this locus in that there were more than 1.0 LOD score differences in other genetic models (Fig. 3). The results of post-hoc testing in the Scheffe’s F test are shown in Fig. 4. In males and females, mice homozygous for the KK allele had significantly higher blood glucose than the heterozygous mice, but not significantly different from mice homozygous for the C57BL/6J allele. Plasma insulin after fasting and during IPGTT did not differ significantly for mice grouped according to genotype at D6Mit268 (data not shown). The loci responsible for impaired glucose tolerance and insulin concentration at fasting and during IPGTT could not be found.

In F2 Ay/a mice, there were no loci with statistically significant linkage, but three loci with suggestive linkage were identified: one for fasting glucose on chromosome 9 (LOD score 2.8), and two for glucose tolerance on chromosomes 1 (2.8) and 8 (3.0) (Table 2). The results of IPGTT appear in Fig. 5, in which the F2 Ay/a mice were grouped according to genotype at D8Mit191 on chromosome 8. Differences in glucose tolerance were more apparent in females.

### Table 2: Identification of QTLs for hyperglycemia at fasting and during IPGTT.

<table>
<thead>
<tr>
<th>F2 mice genotype at agouti locus</th>
<th>Chromosome (closest marker)</th>
<th>Trait</th>
<th>LOD score</th>
<th>Variance explained (%)</th>
<th>Gene symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>a/a</td>
<td>3 (D3Mit102)</td>
<td>Fasting glucose</td>
<td>2.8</td>
<td>21.9</td>
<td></td>
</tr>
<tr>
<td>a/a</td>
<td>5 (D5Mit113)</td>
<td>Fasting glucose</td>
<td>2.8</td>
<td>15.1</td>
<td></td>
</tr>
<tr>
<td>a/a</td>
<td>6 (D6Mit268)</td>
<td>Fasting glucose</td>
<td>6.0</td>
<td>30.9</td>
<td>Fgq1</td>
</tr>
<tr>
<td>a.a</td>
<td>14 (D14Mit165)</td>
<td>Fasting glucose</td>
<td>3.0</td>
<td>14.4</td>
<td></td>
</tr>
<tr>
<td>A/y/a</td>
<td>9 (D9Mit229)</td>
<td>Fasting glucose</td>
<td>2.8</td>
<td>12.8</td>
<td></td>
</tr>
<tr>
<td>A/y/a</td>
<td>1 (D1Mit303)</td>
<td>Glucose tolerance</td>
<td>2.8</td>
<td>12.2</td>
<td></td>
</tr>
<tr>
<td>A/y/a</td>
<td>8 (D8Mit191)</td>
<td>Glucose tolerance</td>
<td>3.0</td>
<td>12.8</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4** Fasting blood glucose in males and females of F2 a/a mice. At the bottom of each column, numbers of mice grouped according to genotypes at D6Mit268 are indicated. Data are expressed as means ± S.E.M. KK/KK, homozygote for KK allele; KK/B6, heterozygote for KK and C57BL/6J allele; B6/B6, homozygote for C57BL/6J allele.
Discussion

Various non-genetic factors genetically predispose to polygenic diseases and modulate traits (1, 2). To eliminate or minimize such factors as much as possible, the mice used in the present study were maintained under constant room temperature, moisture and diet. To avoid competitive male aggression in the KK strain, each mouse was individually housed throughout the experimental period. Body weight at weaning appeared to be a significant factor for hyperglycemia in KK mice (17). Mice less than 8.5 g at weaning did not develop hyperglycemia at later stages even when fed a high caloric diet. Litters of less than five pups and more than nine pups were thus discarded. In F2 mice, the weight of an individual in a five to nine pup litter always exceeded 8.5 g.

The following conclusions were drawn from the present results. (a) Polygenic control of hyperglycemia in fasting KK mice was confirmed. In F2 Ay/a progeny, only a locus in fasting glucose control was identified, possibly because of the strong diabetic influence of the Ay allele. With regard to QTL for fasting glucose on chromosome 6 identified in F2 a/a mice, no suggestive evidence for linkage was detected in F2 Ay/a mice (LOD score in F2 Ay/a at D6Mit268, 1.4). The Ay allele would thus appear to annul the effect of QTL on chromosome 6. (b) Loci for impaired glucose tolerance were identified only in F2 Ay/a progeny, although only suggestive. This is consistent with the finding that impaired glucose
tolerance in KK mice is moderate and becomes overt in response to the A\(^Y\) allele. Suggestive loci possibly responsible for glucose tolerance may interact with the A\(^Y\) allele, since these loci were identified only in F\(_2\) and A\(^Y/a\) progeny. Obesity caused by this allele may affect the glucose tolerance (Fig. 2a) and is a possible risk factor for insulin resistance, as is evident from the finding that the lighter F\(_2\) a/a females showed less severe glucose intolerance (Table 1 and Fig. 2b). Loci for hyperglycemia at fasting and during IPGTT were not identical with those for body weight and obesity identified previously (18). (c) Glucose levels during fasting and feeding were regulated by distinct genetic components in general, but a locus on chromosome 8 for glucose tolerance in F\(_2\) A\(^Y/a\) mice also had an effect on fasting glucose particularly in females (Fig. 5). (d) Loci for plasma insulin during fasting and IPGTT could not be identified; thus glucose may not depend so much on insulin concentration as on peripheral sensitivity to insulin. Hyperinsulinemic KK-A\(^Y\) mice exhibited more severely impaired glucose tolerance than KK mice (Fig. 2a). Thus peripheral insulin sensitivity is crucial to glucose concentration in KK and KK-A\(^Y\) mice.

Two previous studies postulate modes of inheritance of diabetes in KK mice using genetic crosses. According to Butler (19, 20), in crosses between KK and C57 mice, the inheritance of glucosuria in KK mice may be explained by assuming that there is a dominant gene which may possibly enhance penetrance to 75%. Nakamura & Yamada (21) conclude the mode of inheritance to be polygenic, in consideration of glucose tolerance. The present fasting glucose concentration showed a major contributing locus, which but acts recessively. This may be at variance with Butler’s results.

With regard to QTL for fasting glucose on chromosome 6 in the F\(_2\) a/a cohort, ANOVA indicated significant differences between KK/KK and KK/B6 genotypes only, with no significant differences between KK/KK and B6/B6. The fact that fasting blood glucose in C57BL/6j mice was nearly the same as that in KK mice, and that the KK allele at this locus in the heterozygous form could not sustain glucose but the B6 allele in the KK allele at this locus in the heterozygous form could, may explain this.

In the neighboring region of QTL on chromosome 6 for fasting glucose, two candidate genes are located. One is leptin (Lep) and the other neuropeptide Y (Npy). These genes may function as important regulators of body weight by controlling feeding behavior (22). Further investigation of the interactions between them is required. We would like to designate QTL on chromosome 6 as Fgq1 (fasting glucose QTL no. 1). Warden et al. (23) have reported an obesity-related locus on chromosome 6 using BSB mice, and designated the locus as Mob-2 (23). Mob-2 is located near D6Mit1, the proximal edge of chromosome 6, and has been found to affect femoral fat pad weight and plasma total cholesterol, and has suggestive linkage for insulin. This suggests an influence on diabetes, but the relative chromosomal location of the present Fgq1 appears not to be identical with that of Mob-2. According to QTL analysis of the GK rat, Gauguier et al. (4) identified loci for fasting glucose on chromosomes 1 and 17 and loci for glucose tolerance on chromosomes 1 and 5, and Galli et al. (3) identified loci for fasting glucose on chromosomes 2 and 10 and loci for glucose tolerance on chromosomes 1, 2 and 10. In most cases, these rat chromosomes do not appear to correspond to the mouse chromosomes on which the loci identified in the present investigation are situated (according to the homology rat vs mouse, Mouse Genome Database (MGD)).

The distal portion of chromosome 2 (surrounding the agouti locus) and the mid part of chromosome 7 (surrounding the tyrosinase locus) are to be examined in the near future since parental KK-A\(^Y\) mice were heterogeneous with regard to both loci. The X chromosome could not be studied here because of the paucity of polymorphic markers. Mapping of human loci corresponding to the mouse QTL may facilitate identification of causal genes for diseases.

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References


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