Chromosomal mapping of a quantitative trait locus for the development of albuminuria in diabetic KK/Ta mice

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Abstract

Background. The KK/Ta mouse strain serves as a suitable polygenic model for human type 2 diabetes. We previously reported a genome-wide linkage analysis of KK/Ta alleles contributing to type 2 diabetes and related phenotypes such as fasting hyperglycaemia, glucose intolerance, hyperinsulinaemia, obesity and dyslipidaemia.

Methods. Since KK/Ta mice spontaneously develop renal lesions closely resembling those in human diabetic nephropathy, we investigated the susceptibility loci using the KK/Ta × (BALB/c × KK/Ta) F1 backcross progeny in the present study.

Results. A genome-wide analysis of susceptibility loci for albuminuria with microsatellite-based chromosomal maps showed a contributing KK/Ta locus, provisionally designated UA-1, with a significant linkage with the interval on chromosome 2 at 83.0 cM close to the microsatellite marker D2Mit311 with a maximum LOD of 3.5 (χ² = 13.2, P = 0.0003). UA-1 was different from the susceptibility loci contributing to type 2 diabetes, which we earlier identified. The mode of inheritance differed from that of hypertension. The progeny homozygous for UA-1 showed significantly higher urinary albumin levels.

Conclusions. Although there were no significant correlations between urinary albumin levels and other diabetic phenotypes, the group of progeny homozygous for both UA-1 and alleles for fasting hyperglycaemia showed the highest urinary albumin levels. Thus, UA-1 appears to increase the risk of diabetic nephropathy, particularly in individuals susceptible to fasting hyperglycaemia, in a gene dosage-dependent manner. There are potentially important candidate genes that may be relevant to diabetic nephropathy.

Keywords: Diabetic Nephropathy; KK/Tamice; albuminuria; QTL

Introduction

It is widely recognized that type 2 diabetes is a complex multigenic disease. Manifestations of type 2 diabetes are not simply explained by classical Mendelian inheritance and susceptibility arises from the combined effects of multiple contributing genes. A major cause of morbidity and premature mortality is diabetic nephropathy. While it is well established that uncontrolled diabetes underlies the development of this complication, development of diabetic nephropathy is limited in up to 30% of patients who suffer from type 2 diabetes for more than 10 years [1]. Thus, it is highly feasible that genetic susceptibility to diabetic nephropathy is also involved in the glomerular injury [2]. Indeed, familial aggregation of nephropathy in type 2 diabetes has been noted in both Pima Indians [3] and Caucasoids [4]. Progress towards establishing the molecular genetic basis has been hampered, because of the complexity of susceptibility inheritance in type 2 diabetes and diabetic nephropathy in humans. In this respect, lessons from animal models are invaluable for the analysis of such complex traits, and thus far, several chromosomal intervals contributing to type 2 diabetes have been mapped in various animal models using genome-wide analysis with microsatellite-based chromosomal maps [5].

The inbred mouse strain KK/Ta established in Japan as a diabetic strain spontaneously exhibits type 2 diabetes associated with fasting hyperglycaemia, glucose intolerance, hyperinsulinaemia, mild obesity, dyslipidaemia and albuminuria [6]. Plasma insulin levels begin to increase in mice at 2–4 months of age in association with the increase of urinary glucose and microalbumin levels. Levels of albumin excretion...
markedly increase in diabetic KK/Ta mice several weeks after hyperglycaemia becomes manifest and renal lesions closely resembling those in human diabetic nephropathy [7] occur. Glomeruli of diabetic KK/Ta mice show diffuse-type and/or nodular-type hyperplasia of mesangial areas with mesangial cell proliferation. Immunohistological studies show an intense, specific fluorescence for albumin and γ-globulin along glomerular capillary walls. Therefore, KK/Ta mice are considered to serve as a suitable model for the study of type 2 diabetes and diabetic nephropathy in humans, although the nephropathy does not result in the end-stage renal failure.

We earlier reported a genome-wide linkage analysis of KK/Ta alleles contributing to type 2 diabetes and related phenotypes [8]. We observed that different diabetic phenotypes, such as impaired glucose tolerance (IGT) [Chromosome (D6Mit1, Chr 6)], and levels of fasting blood glucose [(D12Mit4, Chr 12), (D15Mit225, Chr 15)], serum triglyceride [(D4Mit336, Chr 4), (D8Mit166, Chr 8)] and total cholesterol (D3Mit12, Chr 3) are all controlled separately by different sets of susceptibility loci. In the present studies, we investigated susceptibility loci for the development of albuminuria in diabetic KK/Ta mice.

Materials and methods

Animals

Inbred mice for the present study were purchased from CLEA Japan Inc. (Tokyo, Japan). BALB/c female mice were mated with KK/Ta males to produce the F1 hybrid mice in the animal facility of Juntendo University. KK/Ta x (BALB/c x KK/Ta) F1 backcross mice were obtained by crossing female KK/Ta mice with male (BALB/c x KK/Ta) F1 mice. A total of 208 backcross mice were weaned at 4 weeks, and from 6 weeks onward mice were individually housed in plastic cages with free access to food (rodent pellet chow CE-2; 342.2 kcal/100 g, containing 4.4% crude fat) and water throughout the experimental period. Only male mice were used in this study, because KK/Ta male mice were more apt to develop type 2 diabetes than KK/Ta female. All mice were maintained in the same room under conventional conditions with a regular light cycle of 12 h light/12 h darkness, and the temperature controlled at 24 ± 1°C.

Phenotypic characterization

Body weight of each mouse was serially monitored at 8, 12, 20, 28 and 36 weeks of age. Glucose tolerance was assessed using the intraperitoneal glucose tolerance test (IPGTT) in mice at 20 weeks of age. IPGTT was performed by injecting glucose (2 g/kg in 20% solution) intraperitoneally in overnight-fasted mice. Glucose levels in blood obtained from the retro-orbital sinus were measured using Glutest E (Kyoto Daiichi Kagaku, Kyoto, Japan) at 0 (fasting blood glucose level) and 120 min after intraperitoneal glucose injection. Impaired glucose tolerance was evaluated from the sum of blood glucose at 0 and 120 min. As the urine microalbumin level is indicative of early and mild states of kidney injury (9) and as this marker is important for type 2 diabetes with nephropathy, renal dysfunction was assessed by measuring urinary albumin levels in samples obtained from mice at 20 and 28 weeks of age, using ELISA (Albuwell M Exocell, Inc. Philadelphia, PA). At 20 weeks of age, blood pressure was measured by a non-invasive tail cuff and pulse transducer system in only parental strains (Softron BP-98A, Tokyo, Japan). All mice were sacrificed at 36 weeks of age. After anesthesia with xylazine and ketamine hydrochloride (2 and 25 mg/kg body weight, respectively), the kidneys were subjected to retrograde perfusion via the abdominal aorta for 5 min at a pressure of about 150 mmHg without prior flushing of the vasculature. Some slices of kidney tissues were fixed in 10% neutral formalin. After paraffin embedding, sections were cut at 4–5 μm and stained with periodic acid–Schiff reagent. For electron microscopy a fixative containing 2% glutaraldehyde in 0.1 mol/l sodium phosphate buffer at pH 7.4 was used. After perfusion, other slices of kidneys were immersed in the same fixative overnight. Tissue was then processed by a modified postfixation and staining. All samples were finally embedded in Epon 812 by standard procedures. Semi-thin sections (1 μm) stained with toluidine blue were used for light microscopy. Ultrathin sections stained in uranyl acetate and lead citrate were observed using a HITACHI H7100 electron microscope (HITACHI, Tokyo, Japan).

Genotyping

Genome screening of KK/Ta-derived loci contributing to the development of albuminuria entailed genotyping of 208 male KK/Ta x (BALB/c x KK/Ta) F1 backcross mice with 101 microsatellite markers polymorphic between the two strains. The average marker distance was 12.2 cM and the genome coverage estimated by the percentage of each chromosome within 20 cM of marker loci was 93%. The linkage between the positive phenotype and the genotype of the microsatellite marker locus (KK/KK homozygous or KK/BALB/c heterozygous type) was examined and data from QTL analyses at a given marker locus in the backcross mice were obtained.

Genomic DNA was obtained from mouse tails by standard techniques. PCR primers flanking microsatellites were purchased from Research Genetics (Huntsville, AL). Genotyping of KK/Ta x (BALB/c x KK/Ta) F1 backcross progeny for marker loci was performed according to Dietrich et al. [10]. PCR reactions were run in 96-well plates with 8.0 ml total volume containing 10 ng of genomic DNA. A three-temperature PCR protocol (94, 55 and 72°C) was implemented for 45 cycles in a Geneamp 9600 Thermal Cycler (Perkin-Elmer-Cetus, Norwalk, CT). PCR products were diluted 2-fold with loading buffer consisting of xylene cyanol and bromophenol blue dyes in 50% glycerin and were run on 18% polyacrylamide gels. After electrophoresis, gels were visualized following ethidium bromide staining.

Linkage and statistical analyses

Linkage analysis was performed using both Pearson’s χ² test and interval mapping. Genomic interval mapping was conducted using the Map Manager QT package program originally described by Manly [11]. P-values of < 0.05 were considered statistically significant. To estimate positions of
QTL, the likelihood ratio statistics were determined using the Map Manager QT package program and LOD scores of \( \geq 1.9 \) and \( \geq 3.3 \) were used as thresholds for statistically suggestive and significant linkage, respectively, according to Lander and Kruglyak [12]. Correlations between the different parameters were analyzed by linear regression with Stat View 4.0 on the Macintosh. Analysis of variance (ANOVA) and the unpaired two-tailed \( t \)-test were used to determine differences in the extent of disease characters among each group of backcross progeny with different combinations of susceptibility alleles.

**Results**

**Morphological studies**

In KK/Ta mice, diffuse glomerulosclerosis characterized by mesangial deposition of periodic acid-Schiff-positive materials developed with aging. The glomerular lesion was generalized, involving the majority of glomeruli throughout the kidney. Increase in the number of periodic acid-Schiff-positive granules within tubular epithelial cells was remarkable (Figure 1A). Electron-microscopically, the tubular alteration was associated with increased numbers of protein-containing lysosomes (Figure 1B). In BALB/c and (BALB/c × KK/Ta) F1 hybrid mice, neither the diffuse glomerulosclerosis nor the tubular alteration was observed. There was no distinctive difference in the morphological changes between BALB/c and the F1 hybrid mice (Figure 1C and D).

**Phenotypic characterization**

Phenotypic characteristics of the diabetes and related disorders were examined in male KK/Ta, BALB/c, (BALB/c × KK/Ta) F1, KK/Ta × (BALB/c × KK/Ta) F1 backcross mice. As shown in Table 1, KK/Ta mice showed obesity, fasting hyperglycaemia and IGT compared with the findings in normal control BALB/c mice. The mean body weight of KK/Ta mice was significantly higher than that of BALB/c and F1 mice (\( P < 0.0001 \)). The values for F1 mice were intermediate between the parental strains and the differences between F1 and BALB/c mice were statistically significant (\( P < 0.0001 \)). Results of fasting blood glucose levels and sum of blood glucose levels in IPGTT of KK/Ta mice were significantly higher than those of BALB/c and F1 mice (\( P < 0.001 \)). The differences between F1 and BALB/c were not statistically significant. Thus, it

![Fig. 1.](http://ndt.oxfordjournals.org/)
appears that genes from the KK/Ta strain determine the disease phenotypes in an incomplete dominant or a recessive fashion.

Levels of the mean urinary albumin in KK/Ta mice were significantly higher than those in F1 hybrid and BALB/c mice at 20 and 28 weeks of age ($P < 0.0001$) (Table 1). Levels of urinary albumin in F1 mice were intermediate between the parental strains, and the differences between F1 and BALB/c mice were statistically significant ($P < 0.005$). Thus, it appears that genes from the KK/Ta strain determine the development of albuminuria in an incomplete dominant fashion.

For mean blood pressure, the mode of inheritance was unrelated to that of albuminuria. Although the mean blood pressure of KK/Ta mice tended to be higher than that of BALB/c mice, it was not a significant difference (89.6±1.8 mmHg vs. 82.6±3.2 mmHg, respectively). On the other hand, the mean levels in the F1 hybrid mice (101.5±0.71 mmHg) were significantly higher than those in the KK/Ta and BALB/c strains ($P < 0.0001$). Thus, the hypertension in F1 mice is suggested to occur as a result of positive epistatic interaction of the susceptibility genes derived from both parental KK/Ta and BALB/c mice.

**Mapping of susceptibility alleles**

As shown in Figure 2, the mean urinary albumin level in mice for 20 and 28 weeks of age combined showed a significant linkage with the interval on chromosome 2 at 83.0 cM close to the marker $D2Mit311$ with a maximum LOD of 3.5 ($\chi^2 = 13.2$, $P = 0.0003$), and explained 9% of the phenotypic variance (direction: KK/KK > KK/BALB). The susceptibility locus located in this interval was provisionally designated as $UA-1$ (urinary albumin level-1).

**Genotypic combination for albuminuria**

As shown in Figure 3A, when the backcross mice were separated into two groups, one homozygous and the other heterozygous for $UA-1$-linked $D2Mit311$, the former showed significantly higher mean urinary albumin levels than did the latter. However, there were no significant associations between urinary albumin levels and diabetic phenotypes such as fasting hyperglycaemia, glucose intolerance, hyperinsulinaemia, obesity and dyslipidaemia (data not shown). We then separated the backcross progeny into several groups classified according to genotypic combinations of $D2Mit311$ ($UA-1$) and several susceptibility loci for type 2 diabetes that we earlier identified. Among these, we found that, in eight groups of progeny classified according to genotypic combinations at three loci, two responsible for fasting blood glucose levels ($D12Mit4$ and $D15Mit225$) [8] and one for albuminuria ($D2Mit311$) (Figure 3B), the progeny in group A with homozygous KK/KK alleles for all three loci showed the highest urinary albumin levels, although not significantly higher than the levels seen in the groups of progeny homozygous for $D2Mit311$ (groups B–D). The progeny in group A showed significantly higher urinary albumin levels than did the groups of progeny heterozygous for $D2Mit311$ (groups E–H).

**Discussion**

We identified a susceptibility locus, provisionally designated $UA-1$, contributing to the development of albuminuria in diabetic KK/Ta mice. $UA-1$ differs from previously identified susceptibility alleles for fasting hyperglycaemia, glucose intolerance, hyperinsulinaemia, obesity and dyslipidaemia in these mice [8]. This
gene acts to control the level of urinary albumin in an incomplete dominant fashion. It is well known that uncontrolled diabetes increases the incidence of diabetic nephropathy. However, recent evidence showed that elevated urinary albumin excretion in type 2 diabetes is familial [13], and that elevated urinary albumin excretion also occurred in non-diabetic siblings and offspring of probands with diabetic nephropathy. Fogarty et al. [14] assessed familial aggregation of urinary albumin excretion in 96 Caucasian families with type 2 diabetes, demonstrating the significant impact of genetic factors on the levels of urinary albumin excretion in family members with and without type 2 diabetes. These findings are consistent with our present data. Compared to findings in KK/Ta × (BALB/c × KK/Ta) F1 backcross mice heterozygous for UA-1, the mice homozygous for UA-1 showed significantly higher urinary albumin levels than the heterozygous progeny did. Thus, UA-1 appears to increase the risk of diabetic nephropathy in a gene dosage-dependent manner. Furthermore, since the group of progeny homozygous for both UA-1 and alleles for fasting hyperglycaemia showed the highest urinary albumin levels, UA-1 may be more effective in individuals genetically susceptible to fasting hyperglycaemia.
In this study, the mean blood pressure in KK/Ta mice was controlled separately from other diabetic phenotypes including diabetic nephropathy. Although hypertension may affect the levels of urinary albumin excretion by increasing vascular permeability that permits glomerular leakage of albumin, it seems unlikely to be the basic mechanism for diabetic nephropathy. The interval closely linked to UA-1 contains several interesting candidate genes, including the Hnf4 gene for hepatocyte nuclear factor - 4 α (HNF4α), Ghrh gene for growth hormone releasing hormone (GHRH), Smstr4 gene for somatostatin receptor gene 4, Agpt4 gene for angiopoietin 4 and Thbd gene for thromboxanomodulin (TM) (Figure 3).

One of the MODY genes, HNF4α, is a transcription factor and is known to activate numerous genes involved in cholesterol, fatty acid, and glucose metabolism. Recently, Klupa et al. [15] identified the susceptibility locus on Chr 20 for type 2 diabetes. In this study, they concluded that Hnf4 is not a candidate gene in their familial cases, but they identified the susceptibility locus for type 2 diabetes on Chr 20q13.1–13.2 that located near Hnf4. This locus is also located within UA-1 locus. Further study is needed to determine whether Hnf4 is a candidate gene or not.

Doi et al. [16] obtained evidence that the significant glomerular enlargement and progressive severe glomerular lesions characterized by initial mesangial hypercellularity followed by the appearance of severe sclerosis were observed in GH or GHRH transgenic mice. GHRH and somatostatin are thought to play a major competitive role in growth control. It has been shown that therapy with octreotide, a somatostatin analog, decreases the glomerular filtration rate (GFR) and the effective renal plasma flow in patients with type 1 diabetes and renal hyperfiltration [17]. It was speculated that octreotide reduces GFR by suppression of GHRH [18]. Thus, both GHRH and somatostatin receptor gene 4 are potential candidate genes for diabetic nephropathy.

The angiopoietins have recently joined the vascular endothelial growth factor family as the only known growth factors largely specific for vascular endothelium. There is little information on angiopoietins although vascular endothelial growth factor (VEGF) exerts its effect during the early stages of vessel development (44–46), whereas angiopoietin-1 and angiopoietin-4 act later to promote angiogenic remodelling as well as vessel maturation and stabilization [19]. Although the function of angiopoietin-4 is not fully understood, it may be that angiopoietin-4 promotes angiogenic remodelling after damage related to metabolic abnormalities in diabetes. TM is a membrane glycoprotein on the vascular endothelial cell surface. There are several reports suggesting a significant positive correlation between the serum concentration of TM and severity of diabetic microangiopathy including nephropathy in humans [20]. The serum elevation of TM is considered to reflect progressive endothelial damage, which is due to the release of TM from the surface of endothelial cells after damage mediated by metabolic abnormalities in diabetes.

Final identification of UA-1 will require further investigation. To limit the numbers of potent candidate genes in the chromosome interval, the generation of interval-congenic KK/Ta mouse strains is underway in our laboratories. A more thorough understanding of genetic factors involved in type 2 diabetes and nephropathy may provide clues on the pathogenesis, and then prophylactic and therapeutic clinical approaches can be better designed.

Acknowledgements. We thank Mr M. Otsuji, Miss T. Shibata and Dr H. Okura for their skillful technical support.

Conflict of interest statement. None declared.

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*Received for publication: 24.4.04*

*Accepted in revised form: 1.12.04*