Distinct genetic regulation of progression of diabetes and renal disease in the Goto-Kakizaki rat

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Goto-Kakizaki (GK) rats develop early-onset type 2 diabetes (T2D) symptoms, with signs of diabetic nephropathy becoming apparent with aging. To determine whether T2D and renal disease share similar genetic architecture, we ran a quantitative trait locus (QTL) analysis in the F2 progeny of a GK × Brown Norway (BN) rat cross. Further, to determine whether genetic components change over time, we ran the QTL analysis on phenotypes collected longitudinally, at 3, 6, 9, and 12 mo, from the same animals. We confirmed three chromosomal regions that are linked to early diabetes phenotypes (chromosomes 1, 5, and 10) and a single region involved in the late progression of the disorder (chromosome 4). A single region was identified for the onset of the renal phenotype proteinuria (chromosome 5). This region overlaps the diabetic QTL, although it is not certain whether similar genes are involved in both phenotypes. A second QTL linked to the progression of the renal phenotype was found on chromosome 7. Linkage for triglyceride and cholesterol levels were also identified (chromosomes 7 and 8, respectively). These results demonstrate that, in general, different genetic components control diabetic and renal phenotypes in a diabetic nephropathy model. Furthermore, these results demonstrate that, over time, different genetic components are involved in progression of disease from those that were involved in disease onset. This observation would suggest that clinical studies collecting participants over a wide age distribution may be diluting genetic effects and reducing power to detect true effects.

T2D and DN are complex disorders that progress over time. Evidence exists to suggest that the genetic pathways involved in the onset of these disorders may differ from those involved in disease progression, as elegantly shown several years ago for rheumatoid arthritis (44). For T2D, loci have been identified specifically for age of onset (48) or early onset (45), and one study found that loci for onset of diabetes differ from those involved in progression of diabetes (7). Furthermore, when age of onset of T2D is taken into account, significance for loci associated with T2D can increase (25). Additional studies looking at the genetics of DN have found genes involved in overt proteinuria separate from those involved in decreased kidney function (review in Ref. 34). Despite these examples, however, there is a general lack of information on the genetic differences in disease onset versus disease progression. The notion of complex diseases having developmental components, potentially determined by different genetic factors, is in sharp contrast to most experimental designs in human linkage and association studies, because most studies include individuals from a wide range of ages or disease stages in a common group. Under the hypothesis of independent genetic components determining a trait at different ages or developmental stages, this pooling of individuals of different age groups may blunt the ability to detect linkage (24).

The use of an animal model can facilitate the search for genetic components involved in the onset and progression of diabetes and renal disease. The Goto-Kakizaki (GK) rat and related substrains are models of nonobese T2D and some aspects of renal disease. The strain exhibits glucose intolerance as early as 2 wk of age (high basal plasma insulin levels) and elevated plasma glucose levels after the administration of a glucose load by 4 wk of age (15, 31, 36). Aging GK rats show zones of scarring in the pancreatic islets that eventually outnumber preserved islets (35). The GK histological changes in the kidney include thickening of the glomerular basement membranes, mild mesangial matrix expansion, and glomerular hypertrophy (33, 37, 38). Some substrains exhibit increased urinary protein and albumin either naturally (37, 38) or as a result of induced hypertension (17).

Recently we developed a new rat model of DN, using GK and fawn-hooded (FHH) rats (29). T2DN rats are >97% genetically identical to GK rats and spontaneously develop diabetes with timing and intensity similar to those in GK rats. Moreover, these rats also display significant proteinuria as early as 3 mo of age and progress to ESRD by 18 mo of age. Together with overt proteinuria, T2DN rats also develop focal glomerulosclerosis, mesangial matrix expansion, and thickening of basement membranes at 3 mo of age. With time, these renal lesions progress to diffuse global glomerulosclerosis with nodular formation and arteriolar hyalinosis by 18 mo of age,
closely resembling the renal functional and structural alterations seen in DN (29). This study helped to establish the GK rat and the T2DN strain derived from it as attractive models of spontaneous diabetes-associated renal disease.

The goal of this study was to dissect the nature of the relationship between diabetes and late-onset renal disease and to determine whether genetic factors determining early stages of these disorders are independent from those controlling their progression. To this end, a linkage analysis for glucose intolerance and urinary protein excretion (proteinuria) in the segregating F2 generation of a GK × Brown Norway (BN) cross at four different time points (3, 6, 9, and 12 mo of age) was carried out. The BN rat is a well-characterized strain that has been shown to be nondiabetic (12) and not to develop any of the structural lesions or functional renal abnormalities seen in diabetic rats such as the T2DN model (29).

METHODS

Animals

A single male GK rat obtained from the Karolinska Institutet (Stockholm, Sweden), a kind gift of Dr. Holger Lushman, was initially mated with two BN female rats from the Medical College of Wisconsin. This single GK rat is the same animal used to develop the T2DN rat described above (29).

Animals from the F1 generation were brother-sister mated to obtain 204 GK × BN F2 intercross male rats. The F2 generation animals obtained from this cross were subjected to a renal disease and diabetes characterization protocol as described below. Animals were maintained under a 12:12-h light-dark cycle and fed a standard Purina rat diet containing 1.0% NaCl by weight ad libitum. All protocols were approved by the Institutional Animal Care and Use Committee at the Medical College of Wisconsin.

Phenotypic Characterization

GK × BN F2 intercross rats were characterized for renal and diabetic phenotypes at 3, 6, 9, and 12 mo of age. At each time point animals were initially placed in metabolic cages for 24-h determination of proteinuria. Intraperitoneal glucose tolerance test (IPGTT) was also administered as described below. In addition, a plasma lipid profile consisting of plasma total cholesterol and triglycerides was obtained with kits from Sigma Diagnostics (St. Louis, MO).

Determination of lipid profiles. Rats were fasted for 12–18 h. While rats were under light anesthesia (Methoxyflurane), we collected 500–700 µl of blood from the tail. Total cholesterol and triglycerides were determined with kits from Sigma Diagnostics (St. Louis, MO).

Kidney histology. At the time of euthanasia, the right kidney was removed and placed in 10% formalin, followed by paraffin blocking. Four-micrometer sections were stained with periodic acid-Schiff and studied by routine light microscopy for analysis of patterns of injury (i.e., vascular sclerosis, interstitial fibrosis) and degree of glomerular sclerosis and mesangial expansion. Sclerosis was defined as collapse and/or obliteration of the glomerular capillary tuft, accompanied by hyaline material, increase of matrix, and/or adhesion of the tuft to Bowman’s capsule. Lesions in individual glomeruli were scored from 0 to +++, with 0 being normal, 1+ up to 25% sclerosis of the tuft, 2+ up to 50% sclerosis, 3+ up to 75% sclerosis, and 4+ more than 75% of the tuft sclerosis. A total of 30–35 glomeruli per kidney were analyzed, and an average score (sclerosis index) was calculated. A detailed description of the procedure is reported elsewhere (29).

Genetic Analysis

A genomewide scan was carried out in 204 GK × BN F2 animals. A total of 183 microsatellite markers, polymorphic between GK and BN rats, were selected from the SHR × BN V.7 rat genetic map (http://rgd.mcw.edu/GENOMESCANNER). Genotypes were assayed by PCR as previously described (27). The genotypes obtained were used to construct a genetic map with the MAPMAKER/ExP computer package.

Before analysis, phenotypes were transformed (logarithm, natural logarithm, or square root) to fit a normal distribution. Nonparametric statistics were used for phenotypes that failed to follow a normal distribution pattern after three transformation attempts. Correlation between traits was determined by simple regression analysis.

The MAPMAKER/QTL computer package was used to carry out linkage analysis in this data set. The threshold for suggestive linkage was set at α = 0.016, representing a logarithm of odds (LOD) score of 2.8, and the threshold for significant linkage was set at α = 0.0005, representing a LOD score of 4.3 (23). The particular genetic mode of inheritance fitting each quantitative trait locus (QTL) was also determined with MAPMAKER/QTL. Further analysis to detect possible allelic interactions between QTLs controlling the same trait was performed by plotting genotype effect plots that incorporated the effects of two QTLs simultaneously.

RESULTS

Longitudinal Linkage to Diabetes

Phenotypical values on the parental and F1 rats from 3 to 12 mo of age are illustrated in the supplemental material for this article and in Ref. 29.1 We identified separate loci for the early stages and late progression of diabetes (see Fig. 1). At 3 mo of age, three QTLs were identified for post-glucose injection glycemia at all time points after the glucose challenge as well as AUC. These QTLs were altered over time by becoming stronger, disappearing, or changing shape. At 12 mo of age a separate locus on chromosome 4 (LOD = 10.0 at D4Rat16), absent at the other ages (see Fig. 1), with a recessive mode of inheritance was identified for post-glucose injection glycemia.

We identified a single locus with a recessive mode of inheritance on chromosome 5 for fasting glycemia at 3 mo of age with a peak LOD of 4.2 at marker D5Mit10. We also identified linkage on chromosomes 1, 5, and 10 for post-glucose injection glycemia at 30, 60, 90, and 120 min after the glucose challenge, as well as AUC. Because the 60 min time point was representative of the phenomena observed at other

1 The online version of this article contains supplemental material.
post-glucose injection times, for simplicity blood glucose at 60 min after challenge will be used to discuss longitudinal dynamics observed in the QTLs across different ages (3, 6, 9, and 12 mo). Linkage at the chromosome 1 locus (D1Rat75 to D1Mgh13) decreased from 7.2 at 3 mo of age to 3.1 at 12 mo. Longitudinal linkage changes were also evident on chromosome 5, although the pattern was more complex. A peak with a LOD of 4.7 at D5Mgh11 was identified at 3 mo of age. While this peak disappeared at 6 mo, a biphasic peak mapping to a similar region emerged at 9 mo of age (LOD of 4.6 at D5Mit4 and LOD of 3.5 at D5Mit10) and remained suggestive at 12 mo of age. A third QTL mapping on chromosome 10 displayed suggestive linkage at 3 mo of age (LOD of 3.7 at D10Rat20). Interestingly, this linkage increased to significant levels at 6 mo of age (LOD of 6.1), then decreased at 9 mo of age (LOD of 4.3), and was not detected by 12 mo of age. The change in shapes and the appearance of the locus on chromosome 4 suggests that different QTLs contribute to initiation, maintenance, and progression of this trait.

Genetics of diabetes progression. To evaluate whether susceptibility to early-onset diabetes and future progression were related, the animals were separated according to their genotypes in the QTLs linked to early stages (chromosome 1, 5, and 10) and progression (chromosome 4) and their resultant phenotypes were studied. Four groups emerged: 1) G/G early-B/B progression, 2) G/G early-G/G progression, 3) B/B early-G/G progression, and 4) B/B early-B/B-progression, where G represents the GK allele, B represents the BN allele, “early” refers to genotypes at D1Rat75, D5Mgh11, or D10Rat20, and “progression” refers to genotype at D4Rat16. It was found that susceptibility to early development and progression were independent in this animal model (see Fig. 2). In other words, rats homozygous for the GK allele on chromosomes 1, 5, and 10 exhibited increased glycemia relative to rats homozygous for the BN allele on these chromosomes, and this effect was independent of the genotype on chromosome 4. The progression of glycemia from 3 to 12 mo showed that the genotype at D4rat16 is the strongest predictor of the degree of glycemia at 12 mo. Thus G/G early-B/B progression rats, which were diabetic at 3 mo, did not progress with age, indicating not only that chromosome 4 is required for progression of diabetes but also that the progression of the disease in this model is all but an inexorable event, once the disease is established. G/G early-G/G progression rats (also diabetic since 3 mo of age) become significantly more hyperglycemic at 12 mo of age, indicating that the superimposition of late effects due to the locus on chromosome 4 worsens the level of diabetes in this group. Finally, independent of the genotype on chromosomes 1, 5, and 10, at 12 mo of age BN homozygosity on chromosome 4 confers protection against hyperglycemia.

Longitudinal Linkage to Renal Disease

Phenotypical values in parental and F1 rats from 3 to 12 mo of age are illustrated in the Supplemental Material and in Ref. 29. We identified a QTL on chromosome 5 (D5Rat13) that was significantly linked to proteinuria at all time points studied (3, 6, 9, and 12 mo). The LOD score of this QTL varied from 6.2 at D5Rat13 at 3 mo to 4.7 at 12 mo (see Fig. 3). At 12 mo of age, a second QTL, on chromosome 7 (LOD of 5.2 at D7Mgh6) was linked to proteinuria (see Fig. 3). This locus appears to be critical for the progression of proteinuria, be-
cause the LOD score at this locus increases to 7.8 when linked to the change in proteinuria levels from 6 to 12 mo, an index of disease progression.

Suggestive linkage for structural damage of the kidney (glomerular and tubular sclerosis) was also identified on chromosomes 5 and 7, in similar locations as the loci for proteinuria (see Fig. 4).

Plotting the proteinuria levels according to the genotypes at D5Mgh11 and D7Mgh6 revealed strong epistasis between the two loci, as shown in Fig. 5A. Rats harboring GK alleles at D5Mgh11 had moderately or considerably more proteinuria than those that had BN alleles at the same locus, depending on the genotype at D7Mgh6. Thus progression of proteinuria only occurs if the genotype at D7Mgh6 is GK/GK, but for progression to occur at least one GK allele is also required at D5Mgh11. In addition, the effect plot between these two loci demonstrates an interaction similar to that found for proteinuria (Fig. 5B), providing corroborative evidence that these loci are involved in kidney disease in this rat model. This pattern contrasts with that seen in diabetes, where the progression locus on chromosome 4 influenced glycemia independently from the early-stage diabetes QTLs.

**Correlational Analysis of Chromosome 5 Locus**

The QTL on chromosome 5 for proteinuria, which displays an additive mode of inheritance, overlaps with the QTLs for both fasting and post-glucose injection glycemia described above, immediately raising the possibility that this might reflect an interaction between renal disease and T2D in the GK rat model. Nevertheless, no significant correlation was found between glycemia and proteinuria in the F2 rats (data not shown). The analysis of correlation was carried out in subgroups of animals, breaking down the 204 animals into groups according to genotype at D5Mgh11 (maximum likelihood of linkage for glycemia) and D5Rat13 (maximum likelihood of linkage for proteinuria). No evidence of a possible interaction between these two traits at these loci was observed (data not shown).

**Linkage to dislipidemia.** Plasma cholesterol and triglyceride levels were assayed at 6 and 12 mo of age. Figure 6 shows the linkage for these blood parameters. The only QTL identified for triglycerides mapped, at both ages, to chromosome 7 at D7Mgh6, overlapping with the QTL for proteinuria. At 6 mo of age, the maximum LOD score at this locus was 7.1, and at 12 mo of age it was 6.4.

Plasma cholesterol levels mapped to chromosomes 1 and 8. At 6 mo, the QTL on chromosome 1 had a suggestive LOD score of 2.9 at D1Pas1. On chromosome 8, the LOD score was 2.4 at D8Rat47. At 12 mo of age the QTL on chromosome 1 disappeared, while the LOD score on chromosome 8 increased to 4.1.

**DISCUSSION**

Through genomewide analysis of a GK × BN F2 intercross, we have identified several loci involved in glucose tolerance and urinary proteinuria. Over time, loci for both phenotypes change, demonstrating that separate genetic mechanisms are involved in disease onset versus disease progression. These results highlight the importance of taking into account the stage of disease in genetic studies of complex traits. To our knowledge, this is the first study to look at the influence of genetics on the progression of diabetes and renal disease in a rat model of DN.

We confirmed three loci involved in the presence of glucose tolerance (chromosomes 1, 5, and 10) and one locus for the onset of proteinuria (chromosome 5). In addition, we identified one locus involved in the progression of T2D (chromosome 4) and a locus involved in the progression of urinary proteinuria (chromosome 7). While loci for both glycemia and proteinuria were found on chromosome 5, no correlation was found between these traits, suggesting that separate genetic mechanisms are involved in these phenotypes in this rat model.

The early-stage diabetes QTLs (chromosomes 1, 5, and 10) decreased in significance over time, such that they were no longer present when the animals were 12 mo old. Each of these three QTLs has previously been identified in other F2 intercrosses. The chromosome 1 locus was the most significant and has been identified previously by multiple investigators using the GK rat (10, 12) and other rat models of T2D (19, 47). Recently, congenic and expression studies have shown that multiple loci within this region play a role in glycemic control (14, 46). Another study in GK substitution congenic rats found separate loci within the chromosome 1 region at 3 versus 6 mo (20). This QTL also coincides with age-of-onset QTL identified in the human population (7), as well as several other loci identified in human linkage and association studies for T2D.
In addition, several genes identified in recent genomewide association studies reside in this region (see Ref. 9). Specifically, TCF7L2, whose introns 3 and 4 have repeatedly shown the strongest association with the risk of developing T2D in humans (9, 41), maps within this QTL. Nevertheless, a recent study in congenic strains from GK rats excluded Tcf7l2 from the minimal critical region linked to diabetes (14). However, SORCS1, a gene recently identified for T2D in the mouse (5), resides in one of the minimal congenics (14) and therefore may be a candidate gene in the GK rat. Both the chromosome 5 and 10 loci have also previously been identified for glucose tolerance in the GK rat (10, 12), while the chromosome 5 locus has also been found in a cross using the diabetic Otsuka Long-Evans Tokushima fatty (OLETF) rat (47). Interestingly, CDKN2A/B, recently identified by human genomewide association studies (41), resides within this chromosome 5 locus.

Only one locus (chromosome 4) was identified for glucose intolerance when the animals were 12 mo old, suggesting that this locus is involved in the progression of a decline in glycemic control. Another diabetes-related QTL was identified previously in a GK × BN cross, linked to plasma insulin levels, but not glucose levels, in 4-mo old rats (12). This QTL does not overlap with the one that we found linked to glycemic levels at 12 mo. This diabetes progression QTL that we uncovered has not previously been identified, likely because a longitudinal study design was not employed, and appears to act independently from the three early-stage diabetes QTLs. A GK allele at this locus results in a worsening of glycemic control even if the alleles at the early-stage QTLs (on chromosome 1, 5, or 10) are from the BN strain. In fact, animals with a GK

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We identified a QTL for proteinuria on chromosome 5 at a location similar to the locus identified on this chromosome for glucose tolerance. However, no correlation was found between glycemia and proteinuria in the F2 generation, suggesting that similar genetic mechanisms are not involved in these traits in the GK rat. Interestingly, genes identified in recent genome-wide association studies for T2D (e.g., TCF7L2, HHEX) differ from those recently identified for DN (e.g., ELMO1 and CNDP1) (16, 50), supporting a separate genetic basis for these two traits.

In addition to the chromosome 5 locus for proteinuria, a second locus was identified for this trait on chromosome 7. The chromosome 7 locus was only found when the animals were 12 mo old, suggesting that it is involved in the

allele at chromosome 4 and a BN allele at one of the early-stage loci achieve the same degree of hyperglycemia at 12 mo of age as those animals that carry a GK allele at both chromosome 4 and any one of the early-stage QTLs. Interestingly, a decline in glycemic control does not progress over time in animals that have a BN allele at the chromosome 4 locus, even in animals that carry a GK allele at one of the early-stage QTLs and therefore initially exhibit hyperglycemia. This important locus, which is involved in determining disease prognosis, would not have been identified if we had not studied the animals at 12 mo of age, emphasizing the importance of timing in genetic studies of complex traits.
progression of proteinuria. In contrast to the progression QTL for glucose tolerance, the proteinuria QTL on chromosome 7 acts in conjunction with the onset QTL on chromosome 5 such that high levels of proteinuria are only found if both the chromosome 5 and 7 alleles are from the GK strain. While the chromosome 5 locus has not previously been identified in other F2 intercrosses, the chromosome 7 locus has previously been identified for progression of albuminuria in the Munich Wistar Frontler (MWF) rat (39), while it has not been identified in the Dahl salt-sensitive (SS) rat strain (11). Suggestive QTLs on both chromosomes 5 and 7 were also identified for glomerular and tubular sclerosis, corroborating the linkage for proteinuria at these loci. Interestingly, of the genes that have recently been identified in human genomewide association studies for DN (see Refs. 1, 16, 32) or chronic kidney disease (22), none falls in the homologous region of the rat for the proteinuria QTLs identified in the present study. As many more genes are likely to be identified for both DN and kidney disease in the coming years, this finding is not surprising and suggests that the GK rat may prove useful in identifying novel proteinuria genes.

A QTL on chromosome 7 for high triglyceride levels overlaps with the progression QTL for proteinuria, suggesting a link between dislipidemia and renal disease in these rats. Because high cholesterol and triglyceride plasma levels have been shown to be independent risk factors for progression of renal disease in humans (42), this finding suggests that the GK rat could be used to dissect the genetic mechanisms linking these traits. QTLs for increased triglycerides have also been found at this locus in the Wistar Ottawa Carlsburg W (WOKW) rat (21) and in the OLETF rat (30). In addition, TRIB1, a gene in this region, was recently identified in human genomewide association studies for triglyceride levels (20).

We have identified independent loci involved in onset and progression of glycemia and proteinuria in the GK rat. While the early-stage loci for glucose intolerance were identified previously, this is the first time a progression locus for glucose tolerance has been found on rat chromosome 4. We also identified a novel locus for proteinuria on rat chromosome 5 as well as a progression locus for proteinuria on rat chromosome 7. Corroborating recent findings in human studies, no overlap was found for loci involved in T2D versus renal disease. In addition to providing evidence for novel loci involved in glucose tolerance and proteinuria, these results highlight the importance of taking into account time of disease onset as well as disease severity when conducting genetic studies in humans and animals. Clustering of participants across a wide range of ages in studies using humans is likely to reduce power to detect true effects. Longitudinal studies offer the opportunity to investigate other loci that may be driving the chronic phase of disease.

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tion of “OLETF”, a rat model for non-insulin-dependent diabetes mellitus. 


