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Genetic and molecular markers of proteinuria and glomerulosclerosis

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The genetic predisposition for glomerulonephritis-induced glomerulosclerosis in rats is linked to chromosome 1

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Abstract

Genetic factors influence renal disease progression, and several loci have been linked to the spontaneous development of proteinuria and glomerulosclerosis in animal models. However, the role of genetic susceptibility in glomerulonephritis-induced progressive glomerulosclerosis is unknown. In a rat model of mesangial proliferative glomerulonephritis, anti-Thy-1 glomerulonephritis (antiThy1GN), Lewis/Maastricht (Lew/Maa) rats exhibit progression to glomerulosclerosis, whereas in genetically related Lewis/Møllegard (Lew/Moll) rats, glomerular lesions are repaired within 3 weeks. The genetic factors underlying this strain-related difference are not known. To identify novel quantitative trait loci (QTL) involved in progression or repair in Lewis rats, 145 female backcross rats [F1(Lew/Maa × Lew/Moll) × Lew/Maa] were studied. After induction of antiThy1GN we determined proteinuria, mesangial activation, the percentage of microaneurysms, and the glomerular damage score for each animal; a genome scan using 187 microsatellite markers was performed. QTL mapping revealed a significant QTL for glomerular damage score on chromosome 1 with a logarithm of odds (LOD) score of 3.9. Homozygosity for Lew/Maa DNA in this region was associated with a higher percentage of damaged glomeruli on day 21. Furthermore, suggestive linkage was found for the percentage of glomeruli with microaneurysms on day 3 on chromosomes 1, 6 and 11; for mesangial activation on day 7 on chromosome 18, while proteinuria was suggestively linked to chromosomes 5 (day 0), 4 (day 3) and 6 (day 7). This study identifies a QTL on rat chromosome 1 that is significantly linked to progressive glomerulosclerosis after acute glomerulonephritis.

Introduction

Progressive glomerulosclerosis is a common feature of many nephropathies that lead to end-stage renal disease¹, and the number of patients with end-stage renal disease is increasing.^{2,3}

Proteinuria is an independent risk factor for development of glomerulosclerosis and end-stage renal disease⁴; in addition, proteinuria and progressive glomerulosclerosis are risk factors for cardiovascular diseases.⁵⁻⁷ Understanding why some patients develop progressive glomerulosclerosis while others are capable of repair is important for improving treatment of both renal and cardiovascular diseases. Environmental factors and complex genetic interactions determine whether patients develop progressive glomerulosclerosis or are protected against progression^{8,9}, but it is not clear whether there are genetic factors that determine progression to glomerulosclerosis in general, or whether there are genetic progression factors that specifically affect particular renal diseases.

In humans, several lines of evidence suggest that progressive glomerulosclerosis is strongly influenced by genes. Familial focal and segmental glomerular sclerosis (FSGS) has been associated with chromosomes 19q13¹⁰⁻¹², 11q21-q22¹³, and 1q25-31¹⁴; diabetic nephropathy is associated with loci on chromosomes 10 and 18^{15,16}; and susceptibility to develop familial IgA nephropathy has been linked to chromosomes 2, 4, 6, and 17.¹⁷⁻¹⁹ Overall, the glomerulosclerosis of only a minority of all patients can be attributed to these loci.

The use of animal models, especially the comparison of inbred rodent strains that are progressors with those that are not, has shed light on the genetic factors involved in progressive glomerulosclerosis. Whole genome linkage analyses in Munich-Wistar-Frömter rats (MWF), Dahl salt-sensitive rats (Dahl S), and Fawn-hooded hypertensive (FHH) rats, which are all inbred strains that display hypertension and nephrosclerosis with aging as an inherited trait, have identified multiple chromosomal regions, termed quantitative trait loci (QTL), that are involved in the development of hypertension-mediated glomerulosclerosis.²⁰⁻²³ In addition, a QTL on rat chromosome 2 (RNO2) had been linked to progressive albuminuria and renal damage in mice.²⁴

Aitman et al. reported that copy number polymorphisms in *Fcgr3* predisposed both humans and rats to develop glomerulonephritis.²⁵ However, a genetic predisposition to progressive glomerulosclerosis after glomerulonephritis has not been demonstrated as yet in animal models. We previously described two Lewis rat substrains in which genetic differences result in considerable differences in susceptibility to progressive glomerulosclerosis following anti-Thy-1

glomerulonephritis (antiThy1GN). AntiThy1GN is an animal model of complement-mediated mesangial damage followed by activation and proliferation of residual mesangial cells and extracellular matrix deposition. The mesangial hypercellularity in anti-Thy-1GN resembles mesangial hypercellularity observed in IgA nephropathy in humans.²⁶ A single injection of anti-Thy1.1 antibody (ER4) results in full-blown mesangioproliferative glomerulonephritis in both Lewis substrains.²⁷ Lewis/Møllegard (Lew/Moll) rats are resistant to proteinuria development; as in most rat strains, glomerular lesions spontaneously resolve within 4 weeks of antiThy1GN induction. In contrast, Lewis/Maastricht (Lew/Maa) rats develop proteinuria and progressive glomerulosclerosis after induction of antiThy1GN.²⁷ This dichotomy in Lewis substrains suggests that genetic factors contribute to progression or repair after antiThy1GN.

To identify QTL that incorporate genes involved in proteinuria and remodeling or progression of damaged glomeruli, we performed a total genome scan in a backcross of Lew/Maa and Lew/Moll rats. Our results show for the first time that the genetic predisposition to develop FSGS after acute glomerulonephritis in rats is linked to a QTL on rat chromosome 1 that is homologous to loci in the human genome.

Materials and methods

Animals

All rats studied were female. Lewis/Maa rats were provided by the University of Maastricht, Maastricht, the Netherlands. Lewis/Moll rats were obtained from Taconic M&B Breeding Centre, Ry, Denmark. Animal care and experimentation were in accordance with legislation on animal experiments as determined by the Dutch Veterinary Inspection, and were approved by the Animal Experiments Committee of Leiden University Medical Center, Leiden, The Netherlands.

Study protocol

Preliminary unpublished experiments in our laboratory indicated that both male and female Lew/Maa rats are equally susceptible to developing progressive glomerulosclerosis after induction of antiThy1GN; for this study, as in previous studies^{28;29}, we used only female rats. For the linkage analysis, we first obtained F1 hybrids by reciprocal crossing of Lew/Maa and Lew/Moll rats. All F1 animals (Lew/Maa × Lew/Moll) demonstrated proteinuria levels and renal damage closer to levels observed in the Lew/Moll strain than Lew/Maa, confirming a minor gene dose effect in susceptibility. Because of this, we choose to perform a backcross study into the background of the disease model, i.e., Lew/Maa, in order to increase the number

of animals carrying two Lew/Maa alleles at any given locus to 50% on average. We therefore generated 145 backcross rats by crossing F1 female rats with male Lew/Maa rats. AntiThy1GN was induced by intravenous injection of mAb against Thy 1.1 (ER4G) at a dose of 2 mg/kg body weight.³⁰ Monoclonal anti-Thy-1 antibody was affinity purified from hybridoma ER4 culture supernatant using protein A-Sepharose 4B (Pharmacia, Uppsala, Sweden). One group of each parental strain (n = 6 per group) and all backcross rats were phenotyped (see below), starting between 8 and 12 weeks of age. Phenotypes were subsequently studied for 3 weeks, after which the animals were killed.

Determination of phenotypes

For urine analysis, each rat was placed into a metabolic cage for 24 h before (day 0), and 3, 7, 14, and 21 days after induction of antiThy1GN. Total protein excretion was measured with the biuret method.³¹

Kidney biopsies were performed on days 3 and 7 by lateral incision. On day 21, the rats were killed and the kidney and spleen of each rat were removed. Renal tissue was fixed in 4% buffered formalin and embedded in paraffin. Splenic tissue was snap-frozen for DNA isolation.

Renal tissue sections (4 μ m) from all rats were stained with periodic acid-Schiff (PAS) followed by hematoxylin counterstaining. The renal biopsy tissue from day 7 was stained for alpha smooth muscle actin (aSMA), a marker of activated mesangial cells.³² After deparaffinization and blocking of endogenous peroxidase activity, sections were incubated with mouse anti-aSMA 1:400 (Sigma Aldrich Chemie, Zwijndrecht, the Netherlands) for 1 hour at room temperature. Primary antibodies were detected using polyclonal rabbit anti-mouse Ig/HRP, swine anti-rabbit Ig/HRP (DakoCytomation Po260 and Po217), and diaminobenzidine as substrate. Tissues were counterstained with hematoxylin.

The percentage of glomeruli with microaneurysms in Lew/Maa, Lew/Moll, F1 and all backcross rats was determined in 20 glomerular cross sections per rat on days 3 and 7 in PAS-stained sections.³³

In renal tissue obtained on days 3 and 7, the average area (calculated as a percentage) that was positive for aSMA per glomerular cross-section was scored by computer-based image analysis.³⁴ At least 20 randomly chosen glomeruli were photographed at 200x magnification with a Zeiss Axioplan microscope equipped with a Sony DXC-950P 3CCD color camera (Sony Corporation, Tokyo, Japan), and the average area percentage for aSMA per glomerulus was measured using KS-400 image analysis software version 3.0 (Zeiss-Kontron, Eching, Germany). The glomerular damage score on day 21 was used to describe the amount of glomerular damage in

both parental and backcross rats as previously reported.^{28;35} An abnormal glomerulus was defined as the appearance of microaneurysms, mesangial cell proliferation, extracellular matrix expansion, glomerular crescent formation, and collapsed capillary loops with mesangial expansion or hyaline deposits. Forty glomeruli in each section were scored in a blinded fashion by 2 independent observers. The glomerular damage score was expressed as the average percentage of abnormal glomeruli on day 21 for each individual rat. To confirm that glomerular damage mostly consists of glomerulosclerosis, we correlated glomerular damage score to collagen I deposition by image analysis (Supplemental figure S1). This shows that both measurements are significantly correlated ($R=0.72$, $P=0.006$).

Linkage analysis and QTL-mapping

Genomic DNA was extracted from splenic tissue using a DNA genomic purification kit (cat. #A1120, Promega, Leiden, the Netherlands). A complete genome scan was performed as previously described²¹; linkage with 187 polymorphic microsatellite markers was also performed as previously reported.²³ In total, > 900 microsatellite markers were tested for polymorphisms between Lew/Maa and Lew/Moll; 187 of these were found to be polymorphic (polymorphism rate 0.21). Polymorphic marker and mapping data are shown in Supplemental table S1.

Prior to linkage analysis, phenotypic distribution was tested with using the Kolmogorov-Smirnov test to assure normal distribution of the trait within the backcross population. If necessary log transformed values for parameters not normally distributed were used in linkage analysis as reported.³⁶ Thus, all parameters could be analyzed by parametric linkage analysis. QTL mapping was performed with the MapMakerQTL software.³⁷ Empirical statistical thresholds for linkage analysis were obtained by permutation testing as reported.³⁸

Statistics

Results are expressed as the mean \pm SD. The independent t-test was used to determine differences between Lew/Maa and Lew/Moll and between genotypes. Correlation coefficients were calculated by the Pearson correlation test. Statistically significant differences were defined as $P < 0.05$.

Results

Phenotypes in the Lew/Maa and Lew/Moll parental strains and F1-population

Proteinuria

Proteinuria levels associated with antiThy1GN in Lew/Maa, Lew/Moll, and backcross [F1(Lew/Maa × Lew/Moll) × Lew/Maa] animals are shown in Figure 1. In contrast to Lew/Moll rats, Lew/Maa rats showed maximum proteinuria on day 7 after induction of antiThy1GN. The mean urinary protein excretion on day 7 was six times higher in Lew/Maa rats (63 ± 16.8 mg/24 h) than in Lew/Moll rats (10.5 ± 3.6 mg/24 h, $P < 0.001$). Mean proteinuria levels in F1 (Lew/Maa × Lew/Moll) rats 7 days after antiThy1GN induction were low (21.4 ± 3.4) and were slightly but significantly higher than parental Lew/Moll rats (Table 1, $P < 0.001$).

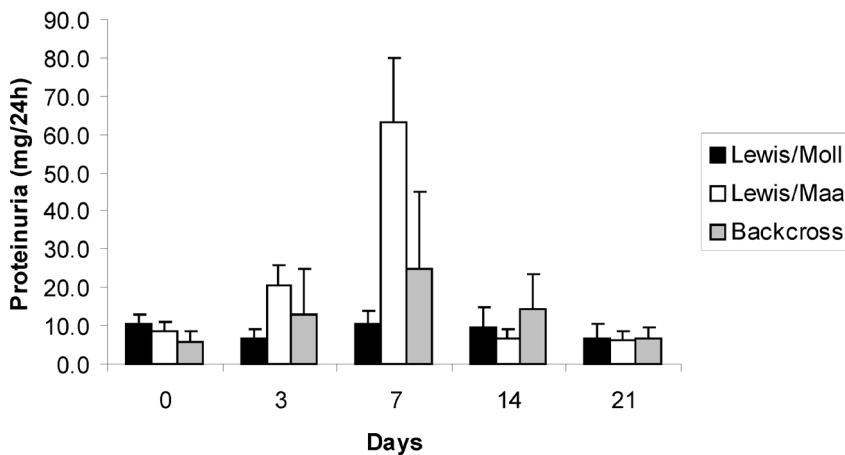


Figure 1. Proteinuria in Lew/Maa, Lew/Moll, and 145 backcross rats [F1(Lew/Maa × Lew/Moll) × Lew/Maa] at several time points after induction of anti-Thy-1 glomerulonephritis (antiThy1GN). At *day 7*, proteinuria was significantly greater in Lew/Maa vs. Lew/Moll. * $P < 0.01$ Lew/Maa vs. Lew/Moll.

Microaneurysms and mesangial cell activation

Microaneurysms were found in significantly more glomeruli in Lew/Maa than in Lew/Moll rats both on day 3 and day 7 (Table 1, $P < 0.001$). Mesangial cell activation is relatively low on day 3 in both substrains. However, on day 7 after antiThy1GN induction, there was significantly more aSMA staining (% area) in sections from Lew/Maa rats compared with Lew/Moll rats (Lew/Maa: $26.1 \pm 14.3\%$ vs. Lew/

	Lew/Maa	Lew/Moll	F-1 (Lew/Maa × Lew/moll)	Backcross (mean)	Range #
Proteinuria day 7 (mg/24 h)	63 ± 16.8*	10.5 ± 3.6	21.4 ± 3.4* ‡	25.1 ± 19.7	2.3 – 99.5
MA day 3 (%)	22.1 ± 9.3*	1.3 ± 1.9	NA	11.3 ± 9.03	0 – 48
MA day 7 (%)	6 ± 5*	0 ± 0	7.9 ± 5.4†	19.3 ± 13.5	0 – 63
aSMA day 3 (area %)	1 ± 1.7	1 ± 0.9	NA	2.42 ± 2.8	0.16 – 19.7
aSMA day 7 (area %)	26.1 ± 14.3*	9.4 ± 4.7	4.3 ± 1.2* ‡	11.1 ± 4.8	0.14 – 21
GDS day21 (%)	57 ± 3*	9 ± 1	22.9 ± 6.7§	48.9 ± 14.22	15 – 80

Proteinuria, percentage of glomeruli with microaneurysms (MA), area percentage of activated mesangial cells (aSMA), and glomerular damage score (GDS) in parental strains and backcross. Phenotypes of Lew/Maa, Lew/Moll, and backcross rats are presented as means ± SD. * $P < 0.001$ vs. Lew/Moll; † $P = 0.01$ vs. Lew/Moll; ‡ $P < 0.001$ vs. Lew/Maa; § $P < 0.05$ vs Lew/Moll and Lew/Maa; NA: not available; #: range in backcross.

Table 1. Phenotypes in Lew/Maa, Lew/Moll and backcross.

Moll: $9.4 \pm 4.7\%$, $P < 0.001$). Glomeruli of F1 (Lew/Maa × Lew/Moll) rats showed microaneurysms at day 7 comparable to parental Lew/Maa rats. On the other hand a very low area percentage of aSMA was observed ($P < 0.001$ vs. Lew/Moll).

Glomerular damage scores

The histological scores of glomerular damage 21 days after induction of antiThy1GN are shown in Table 1 The glomerular damage score was significantly higher in Lew/Maa rats than in Lew/Moll rats (Lew/Maa: $57 \pm 3\%$ vs. Lew/Moll: $9 \pm 1\%$, $P < 0.001$). Glomerular damage score in F1 animals had intermediate levels significantly lower than Lew/Maa but significantly higher than Lew/Moll (Table 1, $22.9 \pm 6.7\%$).

Cosegregation and QTL mapping analysis in backcross

Phenotypes of the backcross rats [F1(Lew/Maa × Lew/Moll) × Lew/Maa] are shown in Figures 1, 2 and in Table 1. Proteinuria ranged between 2.3 and 99.5 mg/24 h, with an average level of 25.1 mg/24h (Figure1 and Table 1). The percentage of glomeruli with microaneurysms on day 3 varied in backcross rats and ranged from 0 to 48% (average $11.3 \pm 9\%$). aSMA expression on day 7 (% area) ranged from 0.14 to 21% (average $11.1 \pm 5\%$).

Examples of normal and damaged glomeruli are shown in Figure 3. The ranges of minimal and maximal glomerular damage index levels observed in the backcross population were in agreement with the contrasting phenotypes of the parental Lew/Maa and Lew/Moll rats: Damage index levels ranged from 15 to 80% in the backcross

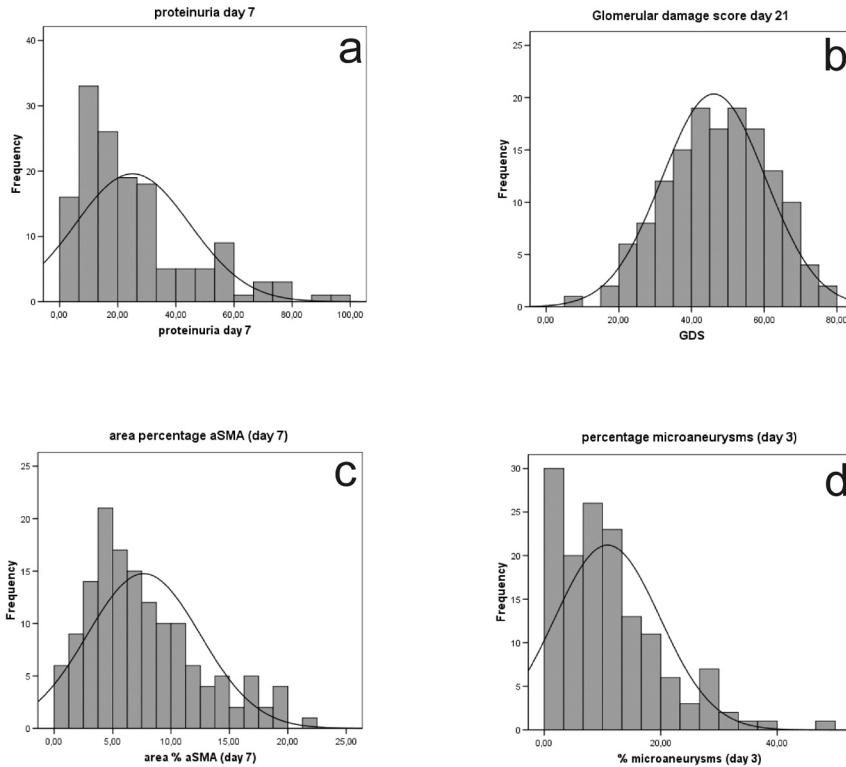


Figure 2. Frequency of traits in backcross rats [F1(Lew/Maa \times Lew/Moll) \times Lew/Maa]. a) proteinuria on *day 7*; b) glomerular damage score (GDS) *day 21*; c) area percentage of α -smooth muscle actin (aSMA) *day 7*; d) percentage microaneurysms *day 3*.

(Figure 2, Table 1). Correlation analysis revealed significant correlation between the level of proteinuria on day 7 and the magnitude of the glomerular damage score on day 21 ($R = 0.486$, $P < 0.001$); Figure 4).

QTL with suggestive and significant linkage to the glomerular damage score, the percentage of glomeruli with microaneurysms, the area percentage expressing aSMA, and proteinuria were identified on RNO1, 4, 5, 6, 11, and 18. The linkage data are shown together with the corresponding empirical statistical thresholds obtained by permutation testing in Table 2. One QTL with a significant LOD score was found. Glomerular damage score was significantly linked to a QTL on RNO1. The peak LOD score of 3.9 ($P < 0.001$) was found between markers D1rat202 and D1rat216. At this QTL, homozygosity for the Lew/Maa allele caused a 7.8% increase in the glomerular damage score compared to heterozygous backcross animals. The corresponding LOD plot for this QTL mapping result on RNO1 is shown in Figure 5. The 1-LOD support interval of the QTL for glomerular damage score represents an approximately 22

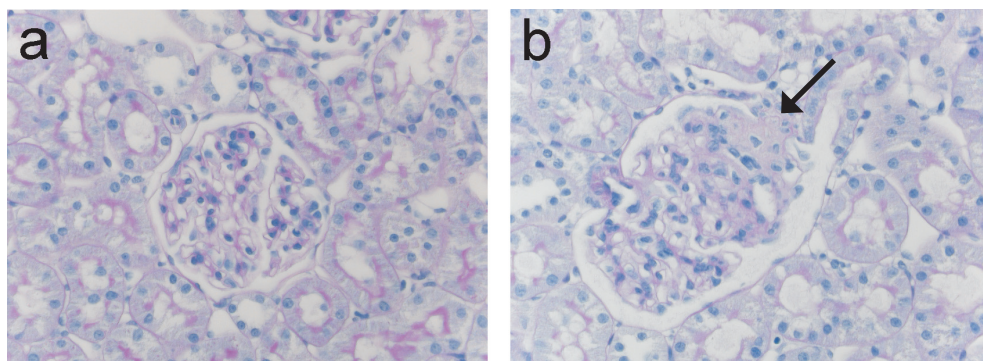


Figure 3. Histological images representing the range of glomerular damage at *day 21* in backcross rats. Periodic acid-Schiff staining. a) normal glomerulus; b) segmental sclerosis with adhesion of the tuft to Bowman's capsule (arrow).

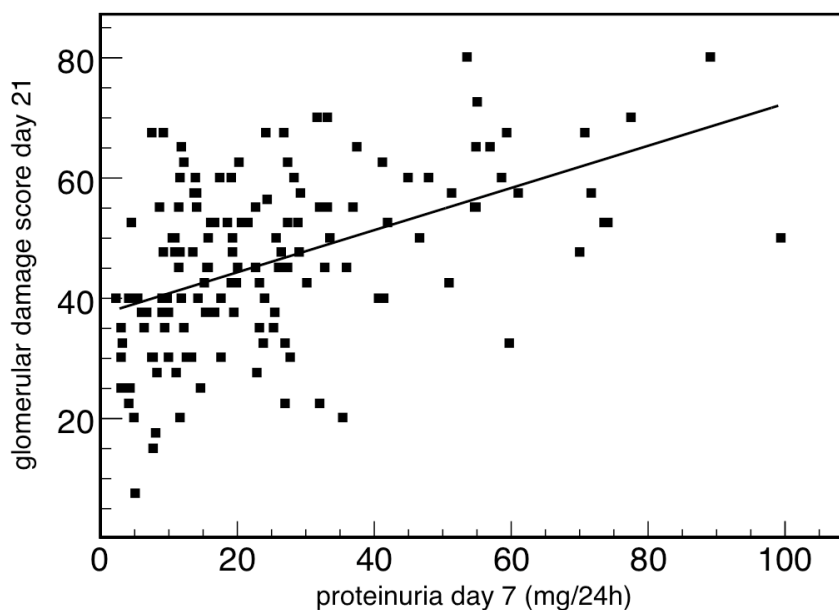


Figure 4. Correlation between proteinuria at *day 7* and glomerular damage score at *day 21*. $R = 0.486$, $P < 0.001$.

cM region. The proximal border of the 2-LOD support interval was located within an unexplored region between marker D1rat203 and D1rat44, where no additional polymorphic markers were available. The QTL on Chromosome1 accounts for 12.8% of the variance in the glomerular damage scores in the backcross population. The

chromosomal fragment of this QTL on RNO1 is homologous to genomic regions on human chromosomes 10, 11, 15, and 16, and on mouse chromosome 7 (Figure 6).

Because levels of proteinuria at day 7 were positively correlated to the amount of glomerular damage score on day 21, we accounted for this by including proteinuria data as a covariate in the linkage analysis for glomerular damage score. When we accounted for proteinuria on day 3 and day 7, respectively, this analysis revealed maximum LOD scores of 3.6 and 2.9 by adjusting for individual proteinuria at day 3 and 7, respectively.

Three QTL with linkage to the percentage of glomeruli with microaneurysms on day 3 were found on RNO1 (LOD-score 2.4, $P = 0.003$), RNO 4 (LOD-score 1.9, $P = 0.006$) and RNO11 (LOD-score 3.6, $P < 0.001$, Table 2, Figure 5, Supplemental figure S2). The QTL for percentage of microaneurysms on Chromosome 1 did not colocalize with the significant QTL for the glomerular damage score. A QTL on RNO18 (LOD-score 2.0, $P = 0.005$) showed suggestive linkage with aSMA expression on day 7 after induction of antiThy1GN (Supplemental figure S2).

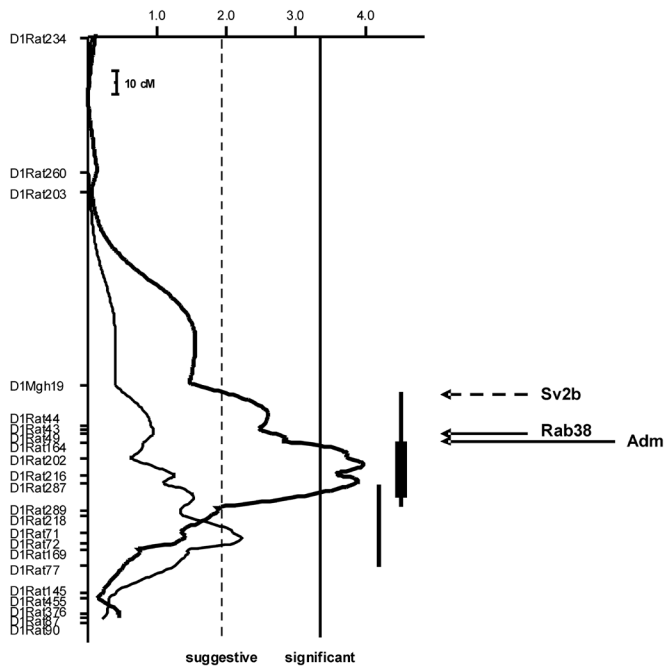


Figure 5. Genome scan logarithm of odds (LOD) plot for glomerular damage score and microaneurysms in the F1(Lew/Maa \times Lew/Moll) \times Lew/Maa population. The thick curve represents the glomerular damage score at day 21. The thin curve represents the percentage of microaneurysms at day 3. The vertical dashed line at LOD = 1.9 is the threshold for suggestive significance, and the solid line at LOD = 3.3 is the threshold for significance. The thick bar represents the 1-LOD support interval and the thin bar the 2-LOD support interval.

Phenotype	Chromosome	Locus	Maa/Maa	Maa/Moll	p-value	LOD-score	LOD-score suggestive Permutation	LOD-score significant Permutation
GDS <i>day 21</i>	RNO1	D1Rat216	50.2 ± 14	41.1 ± 12.9	<0.001	3.9	2.1	3.8
MA <i>day 3</i>	RNO1	D1Rat72	12.9 ± 9.3	8.7 ± 8.2	0.003	2.4	2.3	5.0
	RNO4	D4Rat41	12.1 ± 9.5	9.7 ± 8.5	0.006	1.9	1.5	3.5
	RNO11	D11Rat64	12.7 ± 8.9	8.5 ± 9.0	<0.001	3.6	1.5	3.6
aSMA <i>day 7</i>	RNO18	D18Rat31	8.8 ± 5.1	6.4 ± 4.2	0.005	2.0	0.9	2.4
Prot <i>day 0</i>	RNO5	D5Rat125	5.2 ± 2.4	6.7 ± 2.7	0.001	2.6	1.7	3.5
Prot <i>day 3</i>	RNO4	D4Rat33	10.2 ± 11.7	16.0 ± 12.7	<0.001	3.4	1.5	4.5
Prot <i>day 7</i>	RNO6	D6Rat1	29.7 ± 21.2	21.3 ± 17.6	0.005	2.1	1.7	4.0

Linkage results are presented for glomerular damage score at *day 21* (GDS *day 21*), percentage of glomeruli with microaneurysms (MA *day 3*), and area percentage of aSMA (aSMA *day 7*) and for proteinuria at *day 0* (Prot *day 0*), *day 3* (Prot *day 3*), and *day 7* (Prot *day 7*). Phenotypes are expressed as mean ± SD. Maa/Maa, homozygous for the Lew/Maa allele; Maa/Moll, heterozygous animals carrying 1 Lew/Maa allele and 1 Lew/Moll allele. LOD, logarithm of odds.

Table 2. Quantitative trait loci per phenotype

For proteinuria three suggestive QTL were found (Table 2, Supplemental figure S2). A QTL on RNO 5 was suggestively linked to baseline proteinuria (LOD-score 2.6, $P = 0.001$). Interestingly, heterozygosity for marker D4rat33 on RNO4 resulted in higher levels of proteinuria on day 3 after induction of antiThy1GN (LOD 3.4, $P < 0.001$) compared to animals carrying the homozygous Maa/Maa genotype. Finally, proteinuria at day 7 was suggestively linked to a QTL on RNO6 (LOD 2.1, $P = 0.005$).

Discussion

Glomerulosclerosis is a common feature of many chronic glomerular diseases.¹ It can develop without known etiology as primary glomerulosclerosis, or develop as glomerulosclerosis resulting from other disorders such as metabolic diseases, hypertension, or glomerulonephritides.³⁹ Until now, genetic predisposition has been identified for hypertension-related, diabetes-mediated, and idiopathic forms of glomerulosclerosis.^{13;15;40} However, genetic linkage analysis of inflammation-mediated glomerulosclerosis has not been thoroughly investigated. One example of inflammation-mediated glomerulosclerosis in humans is IgA nephropathy. Linkage to chromosomes 2, 4, 6, and 17 has been determined for development of familial forms of IgA nephropathy.¹⁷⁻¹⁹

The animal model for antiThy1GN in Lewis strains is suitable for analyzing the genetic basis of differing susceptibility to glomerulonephritis-induced glomerulosclerosis.²⁸ After induction of antiThy1GN, Lew/Maa rats develop glomerulosclerosis, whereas Lew/Moll rats recover within a few weeks. Adult F1 animals of the current cross between Lew/Maa and Lew/Moll rats demonstrated proteinuria and glomerular damage score values only slightly higher when compared to the Lew/Moll strain, confirming an overall recessive influence of genetic susceptibility in Lew/Maa rats. This is in agreement with data obtained for other renal disease phenotypes in both humans and animal models.⁴¹⁻⁴⁴

In this study, we identified a significant QTL on RNO1 at which homozygosity for the Lew/Maa allele was linked to more pronounced glomerulosclerosis in backcross rats. In addition, suggestive linkage was determined for the percentage of glomeruli with microaneurysms on RNO1, RNO6 and RNO11, and aSMA expression (% area) was linked to RNO18. Proteinuria was suggestively linked to RNO5 at baseline, on day 3 to RNO4, and maximal proteinuria was linked to RNO6.

The significant QTL linked to glomerulosclerosis on RNO1 was termed *GS1*. Genetic studies in rat models of renal diseases have already identified several QTL on RNO1 that are associated with renal diseases. The spontaneous development of

albuminuria and proteinuria in MWF, FHH, and DahlSS rats is linked to chromosomal regions of RNO1.^{20-22;45} Development of glomerulosclerosis was linked to RNO1 only in FHH rats, which develop severe renal damage with mild hypertension.⁴⁴ This region does not overlap with the QTL linked to glomerular damage scores that was identified in the current study. Nevertheless, the localization of multiple renal disease loci on RNO1 clearly demonstrates the importance of chromosome1 in determining renal function and in the response to renal injury that results in progression to chronic renal failure. Furthermore, this supports the previously proposed hypothesis that progression of renal disease involves a common pathway regardless of the cause of the initial disease.⁴⁶ The fact that the amount of glomerular damage score is positively linked to the percentage of glomeruli with microaneurysms at day 3 and with aSMA at day 7, might indicate that initial damage influences degree of progression. However, the percentage of microaneurysms and the area percentage of aSMA are not associated to *GS1* directly. It suggests that additional factors are required to induce progression. This is further supported by the fact that Wistar rats develop even more microaneurysms than Lew/Maa rats and also exhibit mesangial cell activation, whereas they do not develop glomerulosclerosis.³⁰ Our genome scan reveals that both the initial phase of antiThy1GN and progression or repair could be affected by the background phenotype.

Although we cannot precisely define the 2-LOD support interval of *GS1* on RNO1 based on our QTL-mapping results, this interval spans a genomic region of approximately 62 cM; it thus contains a considerable number of translated genes. To narrow down the number of candidate genes, we used two additional approaches. First, we compared the QTL on RNO1 with a previously published expression analysis of Lew/Maa and Lew/Moll rats.²⁹ Among a number of genes with differential glomerular gene expression between the two Lewis substrains, that study identified neuronal genes such as neuronal activity-regulated pentraxin (Narp) and synaptic vesicle glycoprotein 2b (sv2b). The gene coding for Sv2b is localized within the 2-LOD interval of *GS1*. Sv2b protein is present in podocytes, and it plays a role in CD2AP expression, which is a slit diaphragm molecule required for a functional glomerular filtration barrier.⁴⁷ The expression of sv2b is decreased in experimental proteinuric diseases.⁴⁸ In addition to its role in proteinuria development, sv2b may play a role in the development of antiThy1GN-induced glomerulosclerosis in Lew/Maa rats, since podocyte-associated changes are thought to be detrimental to the development of glomerulosclerosis.⁴⁹ Real-time PCR on glomerular mRNA from Lew/Maa and Lew/Moll rates during antiThy1GN-induced glomerulosclerosis revealed higher sv2b mRNA expression in the non-progressive Lew/Moll strain (Figure 6). The exact role of sv2b in progression of antiThy1GN remains to be elucidated.

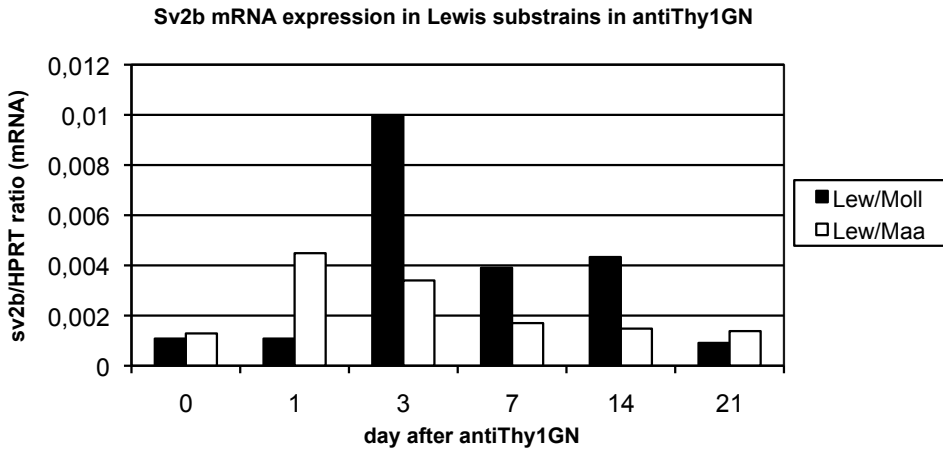


Figure 6. Real-time PCR for sv2b corrected for hypoxanthine phosphoribosyl transferase (HPRT) in glomeruli of Lew/Maa and Lew/Moll at several time points after induction of antiThy1GN. Higher expression of Sv2b was found in Lew/Moll three days after induction of antiThy1GN.

Our second approach to identifying genes in *GS1* was to search for candidates within the 2-LOD support interval of *GS1* by comparative mapping analysis of the human, rat, and mouse genomes.⁵⁰ In addition to sv2b, interesting candidate genes are Rab38 and adrenomedullin. Rab38 encodes a small GTP-binding protein that is thought to play a role in the vesicular transport of proteins.^{51;52} Rab38 is linked to increased urinary protein excretion in FHH rats (locus *RF2*) and appears to influence tubular reabsorption and degradation of filtered proteins.⁵³ However, Rab38 was not linked to development of glomerulosclerosis in an (FHHxACI) F2 intercross.⁴⁴ Adrenomedullin is a potent vasodilative peptide⁵⁴. In the kidney, adrenomedullin is expressed in glomeruli, tubuli, and collecting ducts. It is thought to be anti-proliferative, pro-apoptotic, and anti-fibrotic.⁵⁴ In previous studies, adrenomedullin mRNA and protein expression were upregulated during the resolution phase of antiThy1GN.⁵⁵ Glomerular levels of adrenomedullin mRNA were not different between Lew/Maa and Lew/Moll in our study (data not shown).

There is a large difference in the degree of proteinuria between Lew/Maa and Lew/Moll rats. However, we did only find one suggestive QTL linked to the amount of proteinuria at day 7. One possible explanation is that proteinuria is influenced by multiple QTL with small effects that are below the detection level using our QTL-mapping strategy that involves 145 backcross animals. Furthermore, proteinuria-promoting alleles and alleles protecting against proteinuria may complicate linkage analysis. Interestingly, with regard to the proteinuria phenotype on day 0 and day

3, respectively, suggestive QTL were identified at which the animals homozygous for the *Lew/Maa* allele showed significantly lower proteinuria levels compared to the heterozygous animals (Table 2). This highlights the complex interaction between genetic factors accounting for proteinuria.

As evident from the data in Figure 4 there is a strong and significant correlation between proteinuria at day 7 and subsequent glomerular damage determined at day 21 ($r=0.486$, $p<0.001$). Consequently, we accounted for this by including proteinuria data as a covariate in the linkage analysis for glomerular damage score. This analysis indicated that the variance of glomerular damage score attributable to the genetic effect on chromosome 1 after adjusting for proteinuria at day 3 and day 7 lies between 9.6 and 11.7% as compared to 12.8% in the unadjusted analysis. Thus, the QTL-mapping result for glomerular damage score on *RNO1* remained essentially the same after adjusting for proteinuria. It indicates that *GS1* influences progressive glomerular damage independent of proteinuria. However, in clinical practice progressive glomerulosclerosis without proteinuria is rarely seen. The results of our current study suggest that proteinuria coincides with progressive glomerular damage, but its severity is not correlated to progression of renal disease.

A major limitation of our study results from the fact that two genetically very closely related Lewis strains have been tested. This makes the identification of polymorphic markers a difficult task, as previously reported in other strains.⁵⁶ Consequently, the genome screen analysis is still incomplete (supplemental table S1). In our mapping analysis we covered about a total genetic distance of approximately 1043 cM, while the total genetic size of the rat genome as obtained in two different mapping crosses varies between 1562-1631 cM (including all chromosomes except the Y-chromosome) (data available at <http://rgd.mcw.edu/maps/>, assessed July 9, 2008). Consequently, we covered only about 64-67% of the genetic size of the rat genome in our linkage analysis. Although significant progress has been made in the development of genomic tools for the rat⁵⁷ that are deposited in rat genome databases and can be easily retrieved, this does not necessarily apply to polymorphic markers for specific strains and crosses such in the case of the two closely related Lewis strains analyzed in this study. Very recently, a screening tool with a limited panel of SNPs based on 34 inbred rat strains has been established in which, however, the Lewis strain was not included.⁵⁸

It is important to consider the relevance of the linkage analysis in rat models to human kidney disease. Concordance between rodent and human QTL underlying chronic kidney disease have been described.⁴⁵ In our study, *GS1* is concordant to human chromosomes 10q26, 11p15.2-p15.5, 11q13.4-q14.3, 15q26.1-26.3, 16p12.3-p11.2, and to mouse chromosome 7. Furthermore, the region on human chromosome 10 that

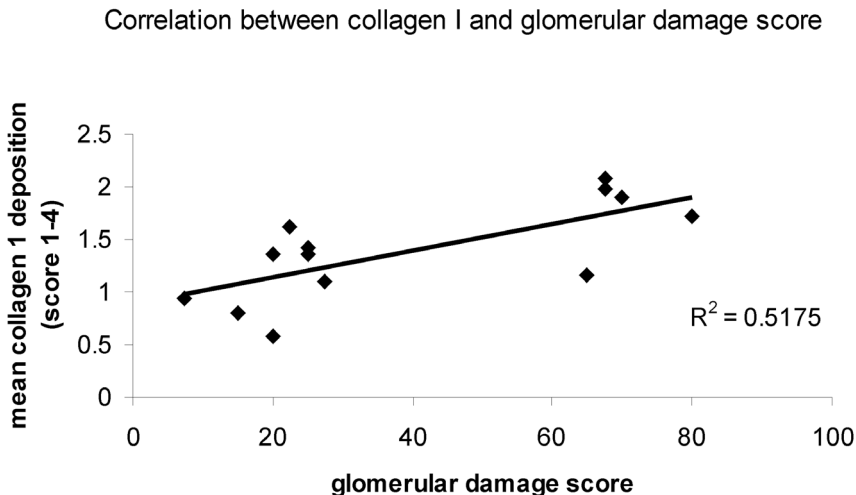
is homologous to *RF1* in FHH rats contributes to ESRD susceptibility.^{59;60} Familial juvenile hyperuricemic nephropathy (FJHN) maps to a region on chromosome 16 that is homologous to our QTL.⁶¹ FJHN is an autosomal dominant renal disease characterized by juvenile onset of hyperuricemia, gouty arthritis, and progressive renal failure at an early age. Mutations in uromodulin, also known as Tamm-Horsfall protein, are responsible for the development of FJHN in some families.^{62;63}

In conclusion, this is the first time a QTL on *RNO1* has been linked to progressive glomerulosclerosis after acute glomerulonephritis. This locus cosegregated with glomerulosclerosis independently of acute phase proteinuria development. In the future, fine mapping and comparison with expression analysis of genes within this locus should identify the gene(s) responsible for glomerulosclerosis secondary to glomerular inflammation.

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Supplemental figures and table

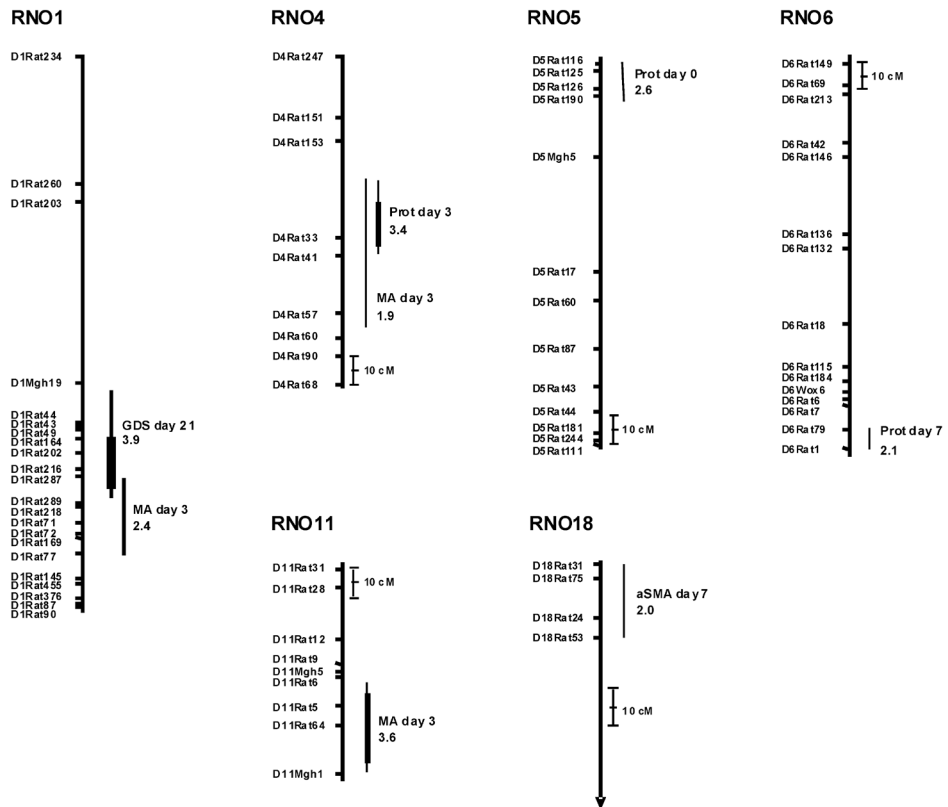


Supplemental figure S1. Correlation between mean glomerular collagen I deposition and glomerular damage score at day 21. Collagen I was depicted as mean collagen I deposition (score 1-4).

RNO	average spacing (cM)	chromosomal coverage (cM)*	marker number	gap 20 - 30 cM	gap > 30 cM
1	4.9	93.3*	22		1
2	10.6	108.5	15	1	
3	10.1	70.6	12		1
4	13.8	80.5	9		
5	12.3	87.6	13		1
6	9.0	72.8	15		
7	6.5	13.0	4		1
8	9.4	81.1	15	1	
9		0.0	2		
10	6.2	49.8	11		1
11	9.8	27.6	9		
12	5.7	56.8	11		
13	12.8	12.8	3		1
14	14.8	59.2	5	1	
15	10.8	57.9	8		
16	6.8	44.6	10		
17	15.2	15.2	3		1
18	6.4	19.1	4	1	
19	9.8	37.8	7		
20	9.9	19.7	4		1
21	11.5	34.6	6	1	

*large gaps >30cM in size were not included for the estimation of chromosomal coverage by linkage analysis.

Supplemental table S1. Polymorphic marker and mapping data per rat chromosome (RNO) in the Lew/Maa x Lew/Moll backcross.



Supplemental figure S2. Rat chromosome (RNO) linkage maps and QTL localization for glomerular damage score at day 21 (GDS day 21), percentage of glomeruli with microaneurysms (MA day 3), and area percentage of aSMA (aSMA day 7) and for proteinuria at day 0 (Prot day 0), day 3 (Prot day 3), and day 7 (Prot day 7). Chromosomal markers are given on the left of each chromosome and ordered according to the linkage map at distances of centiMorgan (cM). A genetic distance of 10 cM is indicated. Thick solid bars at the right of each chromosome represent the 1-LOD interval, thin solid bars the 2-LOD interval for placement of the QTL. The chromosomal size of RNO18 was only given in part.

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