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GENETIC VARIANTS IN ARHGEF11 ARE ASSOCIATED WITH KIDNEY INJURY IN THE DAHL S RAT

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Abstract

A previous genetic analysis comparing the Dahl salt-sensitive (S) rat to the spontaneously hypertensive rat (SHR) identified a major locus on chromosome 2 that influences proteinuria in the S rat. In the present study, blood pressure, proteinuria, and renal hemodynamics were evaluated in congenic strains with small segments of the protective SHR genome on the S background. Proteinuria and renal function were significantly improved in the congenic strains compared to the S. The causative locus interval was narrowed to <375 kb based on congenic strains, haplotype data, comparative mapping, and concordance with human genetic studies. Sequencing of the coding region of genes in this region identified 36 SNPs (13 nonsynonymous and 23 synonymous). Gene expression profiling indicated that only few genes exhibited differential expression. *Arhgef11*, *Pear1*, and *Sh2d2* were identified as important candidate genes that may be linked to kidney injury in the S rat. In particular, *Arhgef11* plays an important role in the activation of the Rho-ROCK signaling pathway. Inhibition of this pathway using fasudil resulted in a significant reduction of proteinuria in treated S rats (compared to untreated S). However, no difference was observed between treated or untreated SHR or congenic strains. The homologous region in humans was found to be associated with estimated glomerular filtration rate (eGFR) in the Candidate Gene Association Resource (CARe) population. In summary, these findings demonstrate that allelic variants in *Arhgef11*, acting through the Rho-ROCK pathway, could influence kidney injury in the S as well as provide insight into human kidney disease.

Keywords

renal hemodynamics; fibrosis; Dahl salt-sensitive rats; genetic association studies

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CONFLICT(S) OF INTEREST/DISCLOSURE(S) None

INTRODUCTION

Chronic kidney disease (CKD) affects ~10% of adults in the United States[1]. Those with CKD initially demonstrate some sign of kidney injury (usually defined by proteinuria) and as kidney injury worsens there can be a significant decline in kidney function that results in increased risk for renal failure as well as cardiovascular disease. Extensive evidence supports the premise of genetic susceptibility for kidney disease.[2] The strong impact of genetics on kidney disease is highlighted by the significant disparity in the prevalence kidney disease in African Americans (~4 fold increase) with hypertension or diabetes compared to Caucasians[3]. A number of large-scale genome wide association analysis (GWAS) have identified single nucleotide polymorphism (SNP) associated with kidney injury, but for the most part these studies only account for a very small change in renal injury or a decline in renal function (as reviewed [4]). Therefore, there is a critical need to identify new genes and signaling pathways that might be involved in susceptibility and progression of kidney injury.

The Dahl salt-sensitive (S) rat is a commonly used genetic model to study salt-sensitive hypertension. Moreover, the S rat develops significant kidney injury, including glomerulosclerosis, tubulointerstitial fibrosis, and vascular hypertrophy[5]. Kidney injury in the S rat becomes more severe as blood pressure is increased when challenged with a high-salt diet[6, 7] similar to human patients that exhibit hypertensive nephrosclerosis [8]. In contrast, the spontaneously hypertensive rat (SHR) is resistant to hypertension associated kidney injury. Using a segregating population derived from the S and SHR, we previously demonstrated that there were 9 genomic regions associated with kidney injury in the S rat[7, 9]. Some genomic intervals were associated with both increased blood pressure as well as kidney injury, while other regions appeared to influence kidney injury independent of blood pressure changes. In particular, the locus on rat chromosome 2 demonstrated strong linkage (LOD >12) and accounted for a large amount of variation in proteinuria. Recombinant progeny testing (RPT) narrowed the region of interest on chromosome 2 to ~6Mb. The homologous region on human chromosome 1 has been also associated with various types of kidney disease[10, 11]. However, the sequence variants, genes, and/or signaling pathways linked to this locus remain to be determined in the rat or humans.

The present study utilized RPT, congenic strains, haplotype analysis, sequencing, and gene expression to establish a small number of genetic variants associated with increased susceptibility of the S rat to develop kidney injury and associated decline in kidney function. The first aim was to characterize the blood pressure and renal parameters associated with the gene or genetic variants within the quantitative trait locus (QTL) for proteinuria on chromosome 2. Thus, temporal changes in blood pressure, proteinuria, and renal hemodynamics were evaluated by comparing S rats to two congenic strains carrying small SHR chromosome 2 segments. The second aim sought to identify sequence variants and gene expression differences in genes located in the newly identified small genomic segment. Finally, to validate the importance of the genetic locus, the candidate region was evaluated using a large-scale human population of CKD patients.

MATERIAL AND METHODS

The detailed methods are described in an expanded material and methods section in online supplemental data.

Animals and Study Design

All experiments were approved by the Institutional Animal Care and Use Committee at either the Medical College of Wisconsin (MCW) or the University of Mississippi Medical

Center (UMMC). The Dahl salt-sensitive (S) and the spontaneously hypertensive rat (SHR) inbred strains are maintained at our institutional animal facility.

Study 1: Fine-Mapping using Recombinant Progeny Testing (RPT) and Congenic Strains—Recombinant Progeny Testing (RPT) was used to fine-map the proteinuria locus as done previously[12]. Proteinuria was determined at week 8 in six recombinant families (X39, n=67; X40, n=38; X41, n=37; X42, n=37; X43, n=21; X44, n=23) (Table S1). The congenic strains, S.SHR(2) X39 and X47 were developed from the recombinant families by fixing the transferred genomic interval in the homozygous SHR/SHR state (Fig. 1). These congenic strains were utilized for Studies 2-6.

Study 2: Proteinuria, Blood Pressure and Renal Hemodynamic Parameters—At 4 weeks of age, groups of age matched male S, S.SHR(2)X39, and SHR animals (n=6-8 per group) were weaned onto a low-salt diet (0.3% NaCl; TD7034; Harlan Teklad, Madison, WI). At week 12, mean arterial pressure (MAP), renal blood flow (RBF) and glomerular filtration rate (GFR) were measured.

Study 3: Time Course Measurement of Proteinuria and Telemetry Blood Pressure—At 4 weeks of age, groups of age matched male S and S.SHR(2) X47 rats (n=16 per group) were weaned to a low-salt diet (0.3% NaCl). Proteinuria and blood pressure were measured from week 8 to 20 at 4 week intervals. Blood pressure was measured using a telemetry system (Data Sciences International, St. Paul, MN) in S (n=8) and S.SHR(2)X47 (n=10) animals as done previously[13].

Study 4: Glomerular Permeability and Number—The reflection coefficient of albumin (σalb) was determined in isolated glomeruli from S and S.SHR(2)X39 raised on a low-salt diet (week 12) using modifications of the Savin technique[14]. These experiments were performed using 4-5 rats per strain with a minimum of 20 glomeruli per rat (n=80-100 total glomeruli per group). The determination of glomeruli number was performed using direct maceration/counting method[15] using kidneys isolated at two time points (week 6 and week 12).

Study 5: Renal Function and Survival Curve—At 4 weeks of age, groups of age matched male S and S.SHR(2)X39 animals (n=9 per group) were weaned to a normal rodent chow (0.7% NaCl; Purina 5010) to exacerbate hypertension and progression of injury compared to a low-salt diet. Proteinuria and creatinine clearance (CrCl) was determined at week 12. MAP, GFR, and RBF autoregulation was evaluated in a separate group of animals (n=7-8 per group). A survival study was performed on S, S.SHR(2)X39, and SHR rats (n=12 rats per group).

Study 6: Pharmacological Inhibition of Rho-ROCK Pathway—Groups of age matched male S, S.SHR(2)X39, and SHR animals (n=11 per group) were weaned onto low-salt diet (0.3% NaCl) at week 4. At week 8, animals for each strain were randomly assigned to either control (n=5 per strain) or fasudil treatment (n=6 per strain). Fasudil (LC Laboratories, MA) was provided in drinking water to achieve a low dose of ~20 mg/kg/day. Proteinuria was determined at week 8, 10, and 12 after 24-hour urine collection.

Histological Assessment

Kidney tissue was fixed in zinc formalin and embedded in paraffin, cut into 4-μm sections and stained with hematoxylin and eosin (H&E) and/or Masson's Trichrome. Glomerular injury and tubulointerstitial injury were assessed as previously described[5, 16, 17]. Immunohistochemistry was performed as previously described[5].

Molecular Methods

Genotyping and Sequencing—Genomic DNA was used to genotype congenic strains or sequencing and prepared using Wizard SV 96 Genomic DNA kit. Genotyping was done using a fluorescent-based approach on a Beckman Coulter CEQ8000 XL capillary sequencer as described previously[12]. For sequencing of coding regions of candidate genes, primers were designed from known databases (Table S2).

Real-Time PCR and Multiplex Gene Expression—RNA was extracted using Trizol® reagent (Invitrogen, Carlsbad, CA) and purified using Mini RNeasy kit (Qiagen,Valencia, CA). cDNA was obtained by reverse transcription using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). Gene expression was evaluated using SYBR-green dye chemistry on a Stratagene MX3000p Real-Time PCR machine, Bio-Rad CFX96, and Beckman Coulter GeXP platform (minimum n=6 each strain).

Western blot analysis—Tissue homogenates were prepared in RIPA lysis buffer (Santa Cruz Biotechnology) from kidney cortex isolated from S and S.SHR(2)X39 under **Study 2.** Blots were prepared using standard methods and probed with Rho-ROCK pathway proteins (Arhgef11, RhoA, Rock1, Limk1, and Cofilin) and appropriate secondary antibody and imaged using a Pierce ECL Substrate on ChemiDoc XRS+ System (Bio-Rad).

Population studies: Candidate Gene Association Resource (CARe) Consortium

The Candidate Gene Association Resource (CARe) population was used to evaluate 22 SNPs in the homologous region to the kidney injury locus identified in the present study. The CARe consortium evaluated the association of renal function measures in approximately 23,000 individuals of European descent[18] using the IBC SNP array, a 50K SNP genotyping array of candidate genes and pathways related to cardiovascular, inflammatory and metabolic phenotypes[19].

Statistical Analysis

Statistical analysis was performed using SPSS (Chicago, IL) for described animal studies. RPT data was evaluated using an independent t-test using SPSS (Chicago, IL). Congenic strain phenotype data (e.g., proteinuria, blood pressure, etc.) were evaluated by t-test and/or by one-analysis of variance followed by Bonforni correction for multiple comparisons. A $p<0.05$ was considered to be statistically significant. All data are presented as mean ± standard error (SE). Survival was evaluated by the Kaplan-Meier method (Graphpad Prism 5, La Jolla, CA). A detailed description of the statistical approach for the CARe study is provided in expanded material and methods section.

RESULTS

Fine-Mapping and Renal Hemodynamics

An overview of the approach used to fine-map and identify the genetic variants linked to the proteinuria locus (kidney injury) on chromosome 2 is shown in Figure S1. Recombinant progeny testing (RPT) was previously utilized to localize the chromosome 2 genetic locus to a ~6Mb genomic interval (D2Arb11-D2Rat230). In the present study, RPT using additional recombinant families further refined the genetic locus (in the S rat) to ~2.5 Mb (Fig. S2; Table S1). Important recombinant animals were used to generate two small congenic strains [S.SHR(2)X39 and X47] to characterize the influence of blood pressure, proteinuria, and renal hemodynamic parameters associated with the kidney injury locus (Fig. 1). At 12 weeks of age on low-salt diet (0.3% NaCl) blood pressure was similar between strains. However, the S.SHR(2)X39 congenic demonstrated a ~2-fold reduction in proteinuria and significantly

higher GFR compared to S rat (Fig. 2A). The S.SHR(2)X47 strain exhibited significantly reduced proteinuria from week 8-20 and less kidney hypertrophy (Fig. S3). At week 20, proteinuria was 109±12.2 mg/24hr compared to the 161±11.8 in the S. Similar to SHR(2)X39, no significant difference in BP was detected in the S.SHR(2)X47 compared to control S over the same period.

Proteinuria, renal hemodynamics, and survival was evaluated between the S and S.SHR(2)X39 on a normal rodent diet (0.7% NaCl) (Fig. 2B, Fig. S4). The diet exacerbated hypertension and accelerated the severity of proteinuria in both strains compared to a low-salt-diet. However, the S.SHR(2)X39 congenic still exhibited a significant reduction in proteinuria and improved kidney function (creatinine clearance) compared to S (Fig. 2B). Renal blood flow (RBF) remained relatively constant when renal perfusion pressure (RPP) varied from 100 to 140 mmHg (Fig. S4). Basal RBF was significantly higher in the congenic (4.4±0.33 ml/min/g kidney weight; $p<0.05$) compared to S (3.0±0.27); however, autoregulation of RBF was similar in the S and S.SHR(2)X39 strain (Fig. S4). Renal vascular resistance was significantly decreased in the congenic (37±3.4 mm Hg/mL/min; $p<0.05$) compared to S (53±4.2). The median survival for S.SHR(2)X39 rats was 345 days compared with median survival of S rats (176 days, $p<0.001$), whereas no SHR died during the course of the experiment (Fig. 2C).

Glomerular Morphology and Renal Pathology

Representative histology is shown for S and S.SHR(2)X39 congenic in Figure 3. Glomerular injury (mesangial expansion, glomerulosclerosis, etc) was significantly attenuated in the S.SHR(2)X39 strain compared to S as was glomerular area, whereas increased glomerular hypertrophy was observed in S rats (Fig. 3B). Increased glomerular area was significantly correlated with increased proteinuria ($r=0.72$, $p<0.001$). Nephron number was observed to be significantly increased in the congenic strain. Despite a significant difference in proteinuria between strains, glomerular permeability to FITC-albumin was similar (Fig. S4). The extent of tubular injury and degree of interstitial fibrosis (tubule atrophy, immune cell infiltration, and/or fibrosis) was significantly reduced in the S.SHR(2)X39 (Fig. 3) and S.SHR(2)X47 (Fig. S3) compared to the S**.** No difference in medial thickness of the renal arterioles between strains (Fig. 3C-D) was observed. The degree of interstitial injury was confirmed by decreased α-SMA expression and macrophage infiltration observed in the S.SHR(2)X39 (Fig. 3E-F) and S.SHR(2)X47 (Fig. S3) compared to the S.

Haplotype Analysis and Overlap with Human Kidney Disease

Haplotype analysis was performed using a panel of inbred strains known to exhibit high or low proteinuria (Table S3). There was a strong concordance between the groups (high or low) and the allelic variant (C or T) at ENSRNOSNP2786652. The S, MWF, BUF, and MNS (susceptible to proteinuria) all possess the C allele, whereas the SHR, WHY, ACI, F344, LOU, and MHS (resistant to proteinuria) possess the T allele. The location of SNP is consistent with the region identified using substitution analysis (Fig. 4). However, the SNPs above and below ENSRNOSNP2786652 did not exhibit this pattern (Table S3).

The region in humans that is homologous to the rat QTL lies on both human chromosome 1 and 4 (Fig. 4). The break point occurs around the middle of the rat QTL and provides additional confirmation and refinement of the genomic interval by comparative mapping as only the homologous region on 1q21 is linked to several forms of kidney disease (Fig. 4).

Sequencing, Expression, and Prioritization of Candidate Genes

The coding regions of genes that map into the congenic interval were sequenced from S and SHR. A complete list of genes is shown in Table S4. Only genes that exhibited sequence

differences are shown in Table 1. A total of 13 nonsynonymous (amino acid change) and 23 synonymous variants were identified in 16 genes. However, only 9 genes exhibited sequence differences in the refined genomic interval considering substitution analysis, haplotype, and comparative mapping data (summarized in Fig. 4). More than half of the sequence variants (15/26) were found within three genes, *Arhgef11*, *Pear1*, and *Iqgap3*. *Arhgef11* contained the most nonsynonymous variants (n=3), followed by *Iqgap3* (n=2) and *Pear1* (n=1).

The expression of genes in the kidney was evaluated using a multiplex approach (Fig. S5). Five genes (*Arhgef11*, *Pear1*, *Sh2d2*, *Gpatch4,* and *Lrcc*) were found to be differentially expressed (Table S5). A subset of genes located in the refined interval were confirmed using real-time PCR at an early (week 4) and late (week12) time point under low-salt diet (0.3% NaCl) (Fig. 5A). *Arhgef11*, *Pear1*, *Sh2d2*, and *Nes* were confirmed to be differentially expressed between strains, similar to the multiplex approach. Gene expression was also evaluated on isolated kidney cortex and medulla from animals exposed to a moderate increase in salt (0.7% NaCl). *Arhgef11*, *Pear1*, *Sh2d2a*, *Nes*, and *Gpatch4* were again found to be differentially expressed, but predominantly in kidney cortex (Fig. 5B). The S transcript for each gene was expressed at a higher level as compared to the congenic irrespective of diet, except for *Nes* or *Gpatch4*.

To establish gene(s) that may have the greatest likelihood for involvement in influencing kidney injury, genes were evaluated based on three criteria, including nature and type of sequence variation, differential expression, and/or possible biological role (Fig. S6). In short, genes designated as high priority are those that exhibited coding sequence variants that alter amino acids, differential expression, and evidence that the gene could have biological role in the kidney. *Arhgef11*, *Pear1*, and *Sh2d2* were identified as important candidate genes linked to kidney injury using this methodology (Fig. 4).

Western Blot Analysis of ARHGEF11, Rho-ROCK Pathway, and Inhibition by Fasudil

The potential role of *Arhgef11* in the Rho-ROCK pathway was evaluated by western blot analysis as this pathway is known to influence cardiovascular disease (Fig. 6A-B). Protein levels of ARHGEF11 were 1.6 fold higher in the S compared to the S.SHR(2)X39 congenic, consistent with detected gene expression differences (Fig. 5). RhoA, which is activated through exchange of GDP for GTP by ARHGEF11, was also increased in the S (1.5 fold) as were other downstream proteins (ROCK1, LIMK1, p-Cofilin). Protein levels of ROCK1 demonstrated the greatest change (4.7 higher in the S).

The influence of the Rho-ROCK pathway on proteinuria was examined in the S, S.SHR(2)X39, and SHR using the ROCK inhibitor fasudil (Fig. 6C). S rats treated with fasudil demonstrated a significant reduction in proteinuria compared to untreated S rats at week 10 and 12 (2 or 4 weeks treatment). Proteinuria at week 12 for S treated was similar to S.SHR(2)X39. No difference in proteinuria was detected between treated and untreated groups for either the S.SHR(2)X39 congenic or SHR.

Correlation of the Region with Human GWAS

eGFR (estimated glomerular filtration rate) data was available for 23,247 individuals of European descent from the Candidate Gene Association Resource (CARe) Consortium. Among the 22 SNP examined in the region homologous to the rat locus, a significant association with eGFR was observed for rs7534418 (p= 0.001315, MAF= 0.3269, beta= 0.0069 for each copy of the T allele) after Bonferroni correction (Fig. 7). Additional nominal associations (P<0.05) for several other SNPs (rs4661229, p=0.004; rs2768744, p=0.004) were also observed. For UACR (urine albumin:creatinine ratio), (N=19,214), no associations in the region were observed (Fig. S7).

DISCUSSION

We previously reported that a region on rat chromosome 2 was associated with proteinuria, histological renal injury, but not blood pressure[9]. A congenic strain was developed [S.SHR(2)] to confirm the linkage analysis that demonstrated attenuated renal injury independent of changes in blood pressure compared to control S[12]. Subsequently, recombinant progeny testing (RPT) narrowed the genomic interval based on overlapping recombinant intervals[12]. In the present study, we utilized additional RPT to better define the genomic interval, developed two small congenic strains to characterize the cardiovascular and renal parameters associated with the genomic locus, as well as performed detailed genetic analysis (haplotype analysis, sequencing, and gene expression) to identify genetic variants that may be causative to the observed renal injury.

Both congenic strains demonstrated reduced kidney injury and improved kidney function compared to the control S. The decreased proteinuria and improved GFR was associated with reduced renal inflammation and interstitial fibrosis in the absence of measurable differences in BP between strains. Increased nephron number, along with elevated GFR in the S.SHR(2)X39 congenic, suggests that kidney development may play a role in the improved kidney function compared to the S, which exhibited significantly less nephrons. The connection between low nephron number and the risk of hypertension and kidney disease has been shown in humans and experimental animal models[20]. Glomerular permeability was similar between strains, despite the S.SHR(2)X39 congenic strain exhibiting a ~40% reduction in proteinuria compared to the S rat. This suggests that the detected difference in proteinuria is not likely due to the degree of filtered protein, but may likely reflect an increase in the reuptake of filtered protein in the proximal tubules. This finding is consistent with other genetic models of renal disease in which a large fraction of proteinuria is attributed to an impairment of protein reuptake via the proximal tubule without changes in glomerular permeability[21].

Classification of the nature and type of sequence variation, differential expression, and potential biological role identified three genes (*Arhgef11*, *Pear1*, *Sh2d2a*) as important candidates for involvement in kidney injury. In general, allelic variants (promoter, coding, or intronic) in any of these genes could play important roles in influencing the onset or progression of kidney injury, either by altering gene expression, protein function, and/or alternative splicing. For example, *Pear1* is a novel membrane protein involved in platelet contact -induced activation[22]. Platelet activation and endothelial dysfunction have been mostly linked to atherosclerotic disease[23] and results in the secretion of several types of chemokines (MCP1, CCL5, CXCL4, etc.)[24]. The release of these chemokines triggers monocyte recruitment, differentiation into macrophages[24], and perhaps immune-mediated renal injury. *Sh2d2a* is a T-cell specific adapter protein (or TSAd). Recent studies have indicated that it may function predominately in the nucleus where it acts to regulate the transcription of different T-cell target genes, including IL-2[25]. TSAd-/- deficient mice are susceptible to lupus-like autoimmune disease (when challenged with pristine) and demonstrate glomerulonephritis and proteinuria at an advanced age[26]. This type of renal injury is likely secondary to autoimmune disease by immune complexes becoming deposited in kidney glomeruli.

The most convincing evidence exists for *Arhgef11* being involved in kidney injury in the Dahl S. ARHGEF11 is Rho guanine nucleotide exchange factor that participates in the Rho-ROCK pathway. The Rho-ROCK pathway has been implicated in a variety of pathological conditions including cardiovascular disease and neurological disorders[27, 28]. The Rho family of small GTPases regulate a number of cell functions, including actin cytoskeletal organization, cell adhesion, cell motility, vascular smooth muscle contraction, and gene

expression[29]. ARHGEF11 (PDZ-RhoGEF) binds activated α12/13 (G-protein) and demonstrates specificity for activating RhoA, but not other Rho family members, Rac1 and Cdc42[30]. ARHGEF11 catalyzes the exchange of GDP for GTP thereby activating RhoA[31]. The Rho effector protein, ROCK, is a serine-threonine kinase involved in both phosphorylation of myosin light chain (MLC) which can contribute to stress fiber formation and smooth muscle contraction. Arhgef11-deficient zebrafish embryos exhibit kidney cysts (along with other phenotypes) and the distribution of F-actin in the pronephric ducts is altered[32]. This work provides some evidence that Arhgef11 can affect kidney development and play a role in stress fiber formation.

The Rho-ROCK pathway is modestly up-regulated in the S [compared to S.SHR(2)X39], suggesting that chronic stimulation of the pathway may account for progressive kidney injury in the S. At this point, it is not clear if the pathway is up-regulated due to differential expression or protein function differences based on amino acid changes between the S and SHR. However, our data is consistent with other studies that support a role for Rho-ROCK with various aspects of renal disease, including alterations in afferent arteriolar tone (via VSMC)[33], podocyte dysfunction[34], and tubular EMT leading to fibrosis (TGF-β mediated)[35, 36].

There are two ROCK inhibitors available, Y-27632 and fasudil, both of which have been extensively used to investigate the role of Rho kinase in cardiovascular disease[37]. Both compounds attenuate tubulointerstitial fibrosis in a mouse model of unilateral ureteral obstruction (UUO)[38]. Inhibition of ROCK (fasudil) has also been shown to attenuate the development of glomerulosclerosis in the Dahl salt-sensitive rat[39]. Improvement in glomerulosclerosis was not accompanied by changes in BP [39]. Inhibition of the Rho-ROCK pathway using fasudil significantly lowered proteinuria in S treated vs. non-treated and no difference was observed between treated and non-treated congenic or SHR. This suggests that low-dose treatment with fasudil does not significantly affect the Rho-ROCK pathway in the congenic or SHR because these strains are able to correctly regulate the pathway. These findings demonstrate that allelic variants in *Arhgef11*, acting through the Rho-ROCK pathway, could modulate renal protection independent of changes in blood pressure (but function on a hypertensive susceptible genetic background).

The evaluation of the rat genomic locus using the human CKD CARe population supports our view that genetic variants/genes identified using rat models of renal disease may lead to a better understanding of genetic factors that contribute to kidney disease in humans. None of the candidate genes identified in the present study (*Arhgef11*, *Pear1*, *Sh2d2a*) have been associated with proteinuria or GFR in humans until now. Several human studies have found an association with genetic variants in these genes with other types of cardiovascular, metabolic, or inflammatory diseases. In this regard, SNPs in ARHGEF11 have been linked to diabetes[40] and susceptibility to cerebrovascular disease (intracranial aneurysm)[41]. ROCK2 (a downstream target of ARHGEF11-RHOA) is associated with lower risk of hypertension in HYPGENE study[42]. Genetic variants in PEAR1 are associated with increased platelet response[43] and genetic variants in the promoter region of SH2D2A (short allele in promoter region) are linked with susceptibility to multiple sclerosis[44] and juvenile rheumatoid arthritis[45], both chronic immune mediated diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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PRESPECTIVES

Linkage analysis usually only identifies a broad genomic region linked to a particular trait and subsequent fine-mapping is required to identify the causal genetic elements. Ultimately, it is necessary to conducted detailed physiological studies using a small congenic strain to explore the mechanism linked to the causative gene or genetic variants. Here, we provided detailed information on cardiovascular and renal hemodynamic parameters associated with genetic variants on rat chromosome 2 that strongly influences the development of kidney injury in the S rat. It is clear that functional significance of the identified candidate genes and sequence variants will require more detailed analysis, but convincing evidence exists that *Arhgef11* may be linked to kidney injury in the Dahl S via the Rho-ROCK signaling pathway. In summary, this work provides an advance in identifying genetic variants/ genes that modulate the onset and progression of kidney disease in the setting of hypertension using the Dahl S rat and establishes relevance to human CKD using the CARe population.

NOVELTY AND SIGNIFICANCE

1. What is New?

- First report on the high-resolution localization of a genomic locus linked to hypertensive related kidney disease using the Dahl salt-sensitive (S) rat, a model of human CKD.
- Few studies have performed, as we have, detailed physiological studies, histological analysis, comprehensive sequence and gene expression analysis to investigate mechanism linked to a fine-mapped genetic locus for kidney injury.
- Identified a strong candidate gene (*Arhgef11*), not previously linked to the development of kidney injury in the rat and showed the locus around this gene was associated with eGFR in humans.

2. What is Relevant?

- Our genetic studies utilize two models of hypertension (S and SHR) that differ with respect to kidney injury. The advantage of this comparison is the ability to identify loci that influence kidney injury on genetic backgrounds (either S or SHR) permissive for hypertension.
- Hypertension is the second leading cause of renal failure in humans. Thus, identification of gene/genetic variants related to kidney injury in the context of hypertension could have a significant impact on human health by serving as novel therapeutic targets.

3. Summary

Our previous studies utilized the Dahl salt-sensitive (S) rat to identify broad genomic intervals linked with cardiovascular and kidney disease. We now have successfully narrowed the genomic segment and identified specific genetic variants and genes on chromosome 2 linked with injury and reduced renal function. Thus, this work provides a significant advance in identifying genetic variants that contribute to the development of kidney disease in the setting of hypertension and establishes relevance to human CKD using the CARe population.

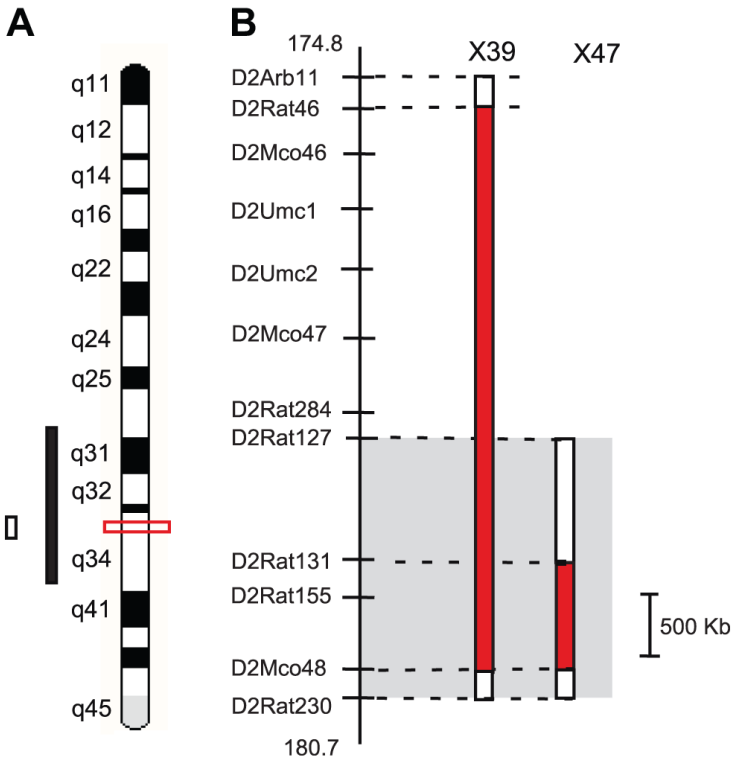

Figure 1.

High-resolution localization of genomic region linked to kidney injury on rat chromosome 2. (**A**) Ideogram showing the 95% confidence interval (CI) for the quantitative trait loci (QTL) from original linkage analysis (solid black bar)[9], 95% CI from second larger population, n=993 (open bar)[12] and current refinement of kidney injury locus to a <2 Mb based on substitution analysis (red box). (**B**) Enlargement of the genomic region and two small congenic strains important in delimiting the genomic region. The grey box denotes the location of QTL based on RPT (Fig. S2) and X47 strain. The red solid bars to the right of the physical map with relevant microsatellite markers indicate the extent of the SHR-donor regions on the S background.

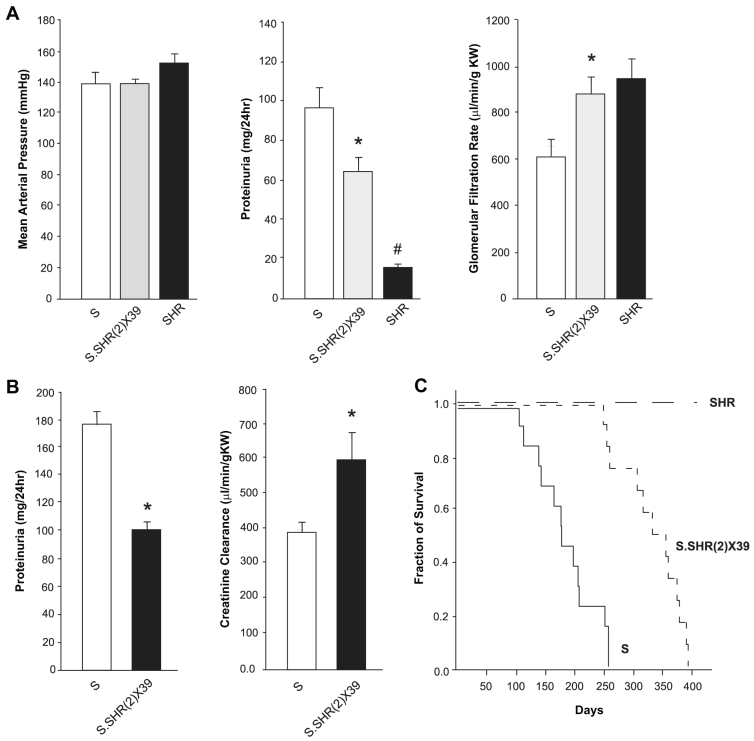

Figure 2.

Blood pressure, proteinuria, and renal hemodynamic parameters between S, S.SHR(2)X39, and SHR. (**A**) Mean arterial pressure (MAP), proteinuria and glomerular filtration rate (GFR) at week 12 for animals (n=6-8 per group) raised on low-salt. (**B**) Proteinuria and creatinine clearance (CrCl) for animals raised on Purina 5010 (0.7% NaCl) diet (n=9 per group). (**C**) Kaplan-Meier survival curve. For panels B and C**,** animals were raised on low-salt (0.3% NaCl) until 6 weeks of age and then placed on Purina 5010 (0.7% NaCl) diet for duration of experiment to accelerate injury. *, $p<0.05$ vs. S and/or SHR. #, $p<0.05$ vs. S or S.SHR(2)X39.

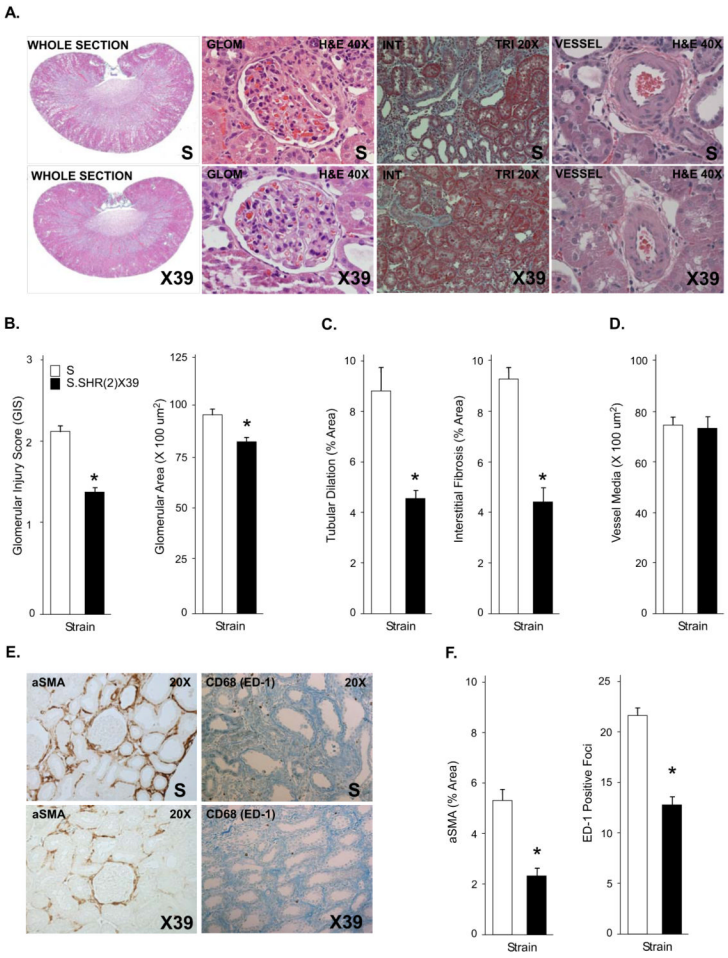

Figure 3.

Assessment of renal injury between S and S.SHR(2)X39 at week 12 raised on Purina 5010 (0.7% NaCl). (**A**) Representative light microscopy image from each strain, including whole kidney section, glomerulus, tubulointerstitial region, and renal vessel. (**B**) Semi-quantitative and morphometric analysis of individual glomeruli assessed for degree of glomerulosclerosis and mesangial expansion [scale from 0 (normal) to 4 (global sclerosis]) and glomerular area was determined. (**C**) Morphometric analysis of tubular injury and interstitial fibrosis. (**D**) Measurement of vessel wall thickening. Vessel wall thinking (vessel media, um2) was calculated by measuring the outer circumference of the vessel minus the inner circumference of the lumen (20 random images per animal, n=9 animals per group). (**E-F**) Representative immunohistochemical images of α-smooth muscle actin (SMA) and CD-68 (ED-1; macrophage). n=8 per group. *, $p<0.05$

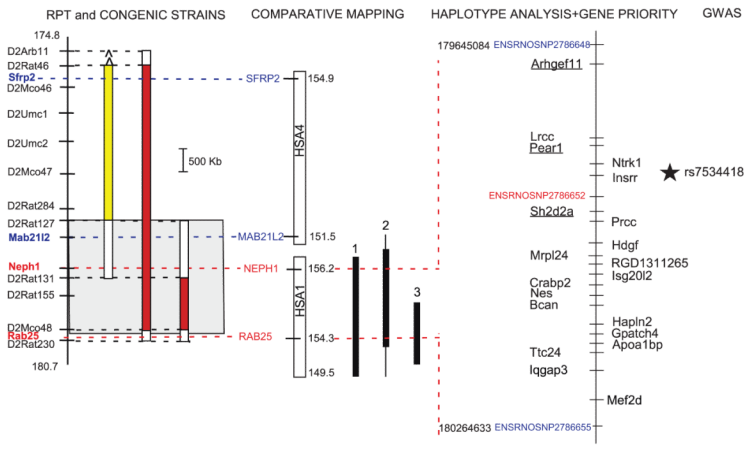

Figure 4.

Summary of refinement of genomic locus using substitution analysis, comparative mapping, haplotype analysis, and identification of key genes. The gray box (left panel) denotes the location of the QTL based on RPT (Fig. S2) and smallest congenic strain, S.SHR(2)X47 (Fig. 1). The region in humans that is homologous to the rat QTL lies on both human chromosome 1 and 4 (middle panel). The break point (between 178.523 and 179.114 Mb) occurs around the middle of the rat QTL and provides a means to further refine the genomic interval. Human chromosome 1 has been linked to kidney disease, (1) Adult onset nephropathy and hypertension [10]; (2) MCDK1, medullary cystic kidney disease [46-48]; and (3) creatinine clearance (HyperGen study) [11], whereas no human linkage or GWAS studies have been associated with kidney disease on chromosome 4. The panel on the right shows important SNP used for haplotype analysis (Table S3). The SNP shown in red demonstrated an association between high or low proteinuria strains, whereas those SNP in blue did not. The underlined genes (*Arhgef11*, *Pear1*, *and Sh2d2*) were identified as high priority using gene priority decision-tree (Fig. S6) and exhibit sequence variants that altered amino acid(s), exhibited differential expression, and linked to a possible biological role in the kidney. The remaining genes on the left of the physical map either exhibit sequence variants, or differential expression. The genes on the right of the physical map exhibited no coding sequence variants or differential expression. The star denotes the SNP (rs7534418) associated with eGFR in the CARe study described in this study (Fig. 7).

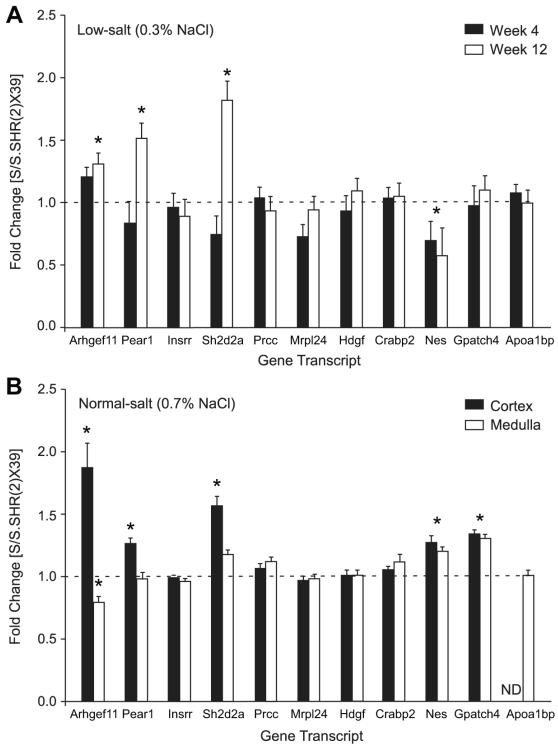

Figure 5.

Quantitative real-time PCR of candidate genes. (**A**) Gene expression at week 4 and week12 under low-salt (0.3% NaCl). (**B**) Gene expression evaluated on isolated kidney cortex and medulla from animals exposed to a moderate increase in salt (0.7% NaCl). *, $p<0.01$. Ratio >1, S transcript expressed at higher level than congenic; Ratio <1, congenic expressed at higher level than S transcript. ND=not detected, n=6 per group.

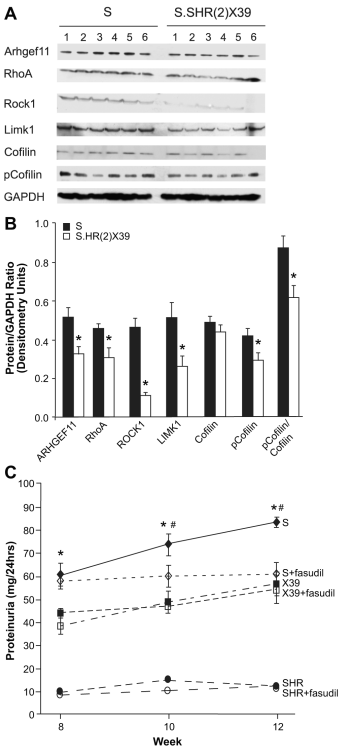

Figure 6.

Western blot of Rho-ROCK pathway and pharmacological inhibition of the using fasudil. (**A**) Western blot analysis of the Rho-ROCK pathway between the S and S.SHR(2)X39 on Purina (0.7% NaCl) at week 12 (same samples as Fig. 3). Protein levels of ARHGEF11 and key proteins in the Rho-ROCK signaling pathway (n=6 per group). A representative image of GAPDH is shown to demonstrate similar protein loading. (**B**) Densitometry measurement of each protein normalized to GAPDH. GAPDH was probed along with each protein (i.e. each protein was normalized to GAPDH probed on same blot as protein). (**C**) Treatment of S, S.SHR(2)X39, and SHR with Rock inhibitor fasudil. At week 8, animals for each strain were randomly assigned to either control (n=5 per strain) or fasudil treatment (n=6 per strain). Fasudil was provided in drinking water to achieve a low dose of ~20 mg/kg/day. * $p<0.05$ S verse other groups; #, $p<0.05$ S versus S treated with fasudil.

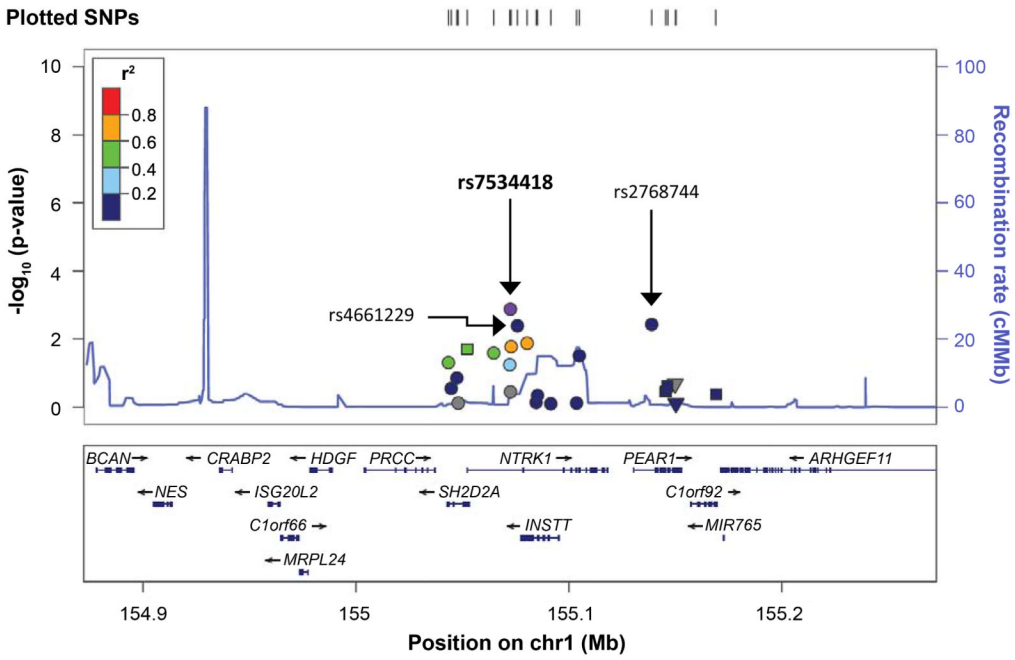

Figure 7.

Regional plot for the homologous candidate region associated with eGFR in humans (23,247 individuals). X-axis shows the genomic region and genes, and the y-axis shows the -log10 p values for associations. The panel also shows the recombination rate in the region estimated from HapMap CEU data and pairwise linkage disequilibrium (r^2) between SNPs in the region and the lowest p-value SNP (labeled in purple) estimated from the meta-analyses. The r^2 values are color coded according to the scale on each panel.

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Table 1

Summary of Coding Sequence Variants in Refined Genomic Region Associated withKidney Injury

Ensembl Transcript ID	Gene ID	Start(bp)	End(bp)	Position	BN(Ref)	S	SHR	Type	AminoAcid
ENSRNOSNP2786648		179645084							
ENSRNOT00000049112	Arhgef11	179676236	179796785	179744835	*G*CC	*G*CC	*A*CC	nonsynonymous	ala/thr
				179774065	*G*AC	*G*AC	*A*AC	nonsynonymous	asp/asn
				179788780	AC*C*	AC*T*	AC*C*	synonymous	thr
				179791299	A*A*A	A*A*A	A*G*A	nonsynonymous	lys/arg
				179792553	*G*CA	*G*CA	*A*CA	nonsynonymous	ala/thr
ENSRNOT00000020147	Lrcc	179798050	179808855	179806328	GCT	GCT	GC*C*	synonymous	ala
				179800777	GCG	GCG	GC*A*	synonymous	ala
				179802405	A*C*G	A*C*G	A*T*G	synonymous	thr
ENSRNOT00000019502	Pear1	179810529	179820653	179819959	GC*C*	GC*T*	GC*C*	synonymous	ala
				179817163	GG*C*	GG*C*	GG*T*	synonymous	gly
				179816423	AGT	AGT	AG*C*	synonymous	ser
				179812075	CTT	CTT	C*C*T	nonsynonymous	leu/pro
				179810631	CC*C*	*CCG*	CCC	synonymous	pro
ENSRNOSNP2786652		179892073							
ENSRNOT00000017798	Sh2d2a	179916108	179924625	179923584	CA*G*	CA*G*	CA*T*	nonsynonymous	gln/his
ENSRNOT00000035271	Mrpl24	179995713	180001750	180000830	AC*T*	AC*T*	AC*G*	synonymous	thr
ENSRNOT00000025314	Nes	180052034	180060546	180057831	CA*G*	CA*A*	CA*G*	synonymous	gln
				180058176	GA*A*	GA*A*	GA*G*	synonymous	glu
ENSRNOT00000025496	Bcan	180067864	180080942	180073710	*G*AT	*G*AT	*A*AT	nonsynonymous	asp/asn
ENSRNOT00000050476	Ttc24	180138942	180144393	180143444	*C*GT	*C*GT	*T*GT	nonsynonymous	arg/cys
				180141671	*G*CG	*G*CG	*T*CG	nonsynonymous	ala/ser
				180138974	*T*CC	*T*CC	*C*CC	nonsynonymous	ser/pro
ENSRNOT00000040822	Iqgap3	180156261	180198814	180166501	TG*C*	TG*T*	TG*C*	synonymous	cys
				180178988	C*G*T	C*A*T	C*G*T	nonsynonymous	arg/his
				180179073	AA*T*	AA*T*	AA*C*	synonymous	asn
				180187672	CC*C*	CC*C*	CC*T*	synonymous	pro
				180189860	AT*T*	AT*T*	AT*G*	nonsynonymous	Ile/met
ENSRNOSNP2786655		180264633							

Ensembl Transcript ID	Gene ID	Start(bp)	End(bp)	Position	BN(Ref)	S	SHR	Type	AminoAcid
ENSRNOT00000026396	Rhbg	180320785	180333050	180323710	AG*C*	AG*C*	AG*T*	synonymous	ser
				180321328	CC*C*	CC*T*	CC*C*	synonymous	pro
				180325286	AT*C*	AT*A*	AT*C*	synonymous	ile
ENSRNOT00000026153	Kpp	180438070	180441660	180439470	GC*C*	GC*T*	GC*C*	synonymous	ala
				180441222	CT*C*	CT*C*	CT*T*	synonymous	leu
ENSRNOT00000026514	Smg5	180448929	180474674	180464043	GC*C*	GC*T*	GC*C*	synonymous	ala
ENSRNOT00000036143	Paq6	180478768	180481707	180481614	GC*C*	GC*C*	GC*A*	synonymous	ala
ENSRNOT00000026530	Bglap2	180482313	180483290	180482514	GA*C*	GA*C*	GA*T*	synonymous	asp
ENSRNOT00000034816	Slc25a44	180514428	180526461	180517963	CT*C*	CT*T*	CT*C*	synonymous	leu
				180522533	*C*GG	C*A*G	C*G*G	nonsynonymous	arg/gln
ENSRNOT00000026733	Sema4a	180552405	180573625	180553168	C*C*T	C*T*T	C*C*T	nonsynonymous	pro/leu

The italicized Ensembl transcript ID designates those genes residing in the refined genomic region based on congenic strain, haplotype analysis, and comparative mapping.SNP (ENSRNOSNP) important for haplotype analysis (Table S3) are also shown. Brown Norway (BN) is the rat genome reference sequence. S is SS/jr and SHR is SHR/Hsd. Sequence variants different from the reference are underlined and nucleotide in italics is the SNP. Each SNP was confirmed on at least 2 independent samples from each strain. Table S4 provides a summary of all genes in the genomic interval. The genes in bold in Table S4 were sequenced between S and SHR and only those exhibiting a sequence difference are shown inthis table.