Original Article

Genetic Effects of Blood Pressure Quantitative Trait Loci on Hypertension-Related Organ Damage: Evaluation Using Multiple Congenic Strains

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On rat chromosome 1, quantitative trait loci (QTLs) for susceptibility to hypertension-related renal diseases and cerebral stroke are identified in a cluster, some of which have been previously claimed to be independent of hypertension. In this study, we therefore attempted to excise genomic regions contributing to salt-induced renal damage and cerebral stroke using five congenic rats for blood pressure QTL on chromosome 1, which were constructed between SHRSP/Izm and WKY/Izm. Male rats from the five strains with different congenic segments of chromosome 1 were used in these experiments. All congenic strains harbored a fragment derived from Wistar-Kyoto (WKY) rats in the background of stroke-prone spontaneously hypertensive rat (SHRSP). Salt-loading was initiated using 1% NaCl in the drinking water when the rats were 12 week old. Histopathological evaluation of glomerulosclerosis, measurement of urinary albumin excretion, cumulative incidence of cerebral stroke and measurement of blood pressure were performed after 2 to 5 weeks of salt-loading. Substantial differences in the severity of renal damage and the incidence of cerebral stroke were observed among the five congenic strains. The cumulative incidence of cerebral stroke correlated well with the basal blood pressures of the congenic strains measured before salt-loading (Pearson's r=0.97, p=0.006), suggesting a substantial influence of blood pressure on the incidence of stroke. In contrast, the severity of glomerulosclerosis did not have a significant correlation with basal blood pressure. These results suggest that a gene (or genes) contributing to salt-induced renal damage is located in this chromosomal region. (*Hypertens Res* 2008; 31: 1773–1779)

***Key Words*: stroke-prone spontaneously hypertensive rat, cerebral stroke, glomerulosclerosis, congenic rats, quantitative trait loci**

Introduction

The stroke-prone spontaneously hypertensive rat (SHRSP) is a genetic model for hypertension and hypertension-related disorders such as cerebral stroke and renal failure (*1–3*). In previous genetic studies using SHRSP and Wistar-Kyoto (WKY) rats, we identified a potent quantitative trait locus (QTL) for blood pressure on rat chromosome (Chr) 1 (*4*, *5*), which was then confirmed in congenic strains (*6*).

Interestingly, several QTLs for renal damage as well as cerebral stroke and blood pressure were found clustering on Chr 1 of different hypertensive strains (*2*, *7–13*). Furthermore, some QTLs for renal damage and cerebral stroke were claimed to be independent of high blood pressure, implying the existence of a particular genetic susceptibility to those

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vascular disorders in this region (*11*, *14*, *15*). Accordingly, it is of interest whether the QTLs for susceptibility to such disorders can be separated from that for blood pressure.

In the present study, we employed five congenic strains harboring different regions of the Chr-1 QTL to examine whether any differences in the incidence of cerebral stroke or the severity of renal damage were observed among the strains. We found that the incidence of cerebral stroke correlated well with blood pressure among the congenic strains, while the severity of renal damage did not, suggesting an independent genetic factor for renal damage in this region.

Methods

Animals

SHRSP/Izm rats were provided by the Disease Model Cooperative Research Association (Kyoto, Japan). A congenic strain for the Chr-1 blood pressure QTL, SHRSP.WKY-(D1Wox29-D1Arb21)/Izm (abbreviated as SHRSPwch1.0), was constructed as previously described (*16*). Four additional congenic strains harboring different chromosomal regions were constructed from SHRSP.WKY-(Klk1-D1Rat116)/Izm in the same way (*6*, *16*); briefly, this congenic strain was backcrossed with SHRSP, and the resulting F_1 rats were intercrossed to obtain the F_2 generation. Recombinant individuals in the F_2 generation were selected by typing simple sequence markers located in the QTL (*17*), and homozygotes were obtained through brother-sister mating. In the present study, we employed four congenic strains, in addition to SHRSPwch1.0, as indicated in Fig. 1; they were SHRSP.WKY-(D1Rat44-D1Arb21)/Izm (SHRSPwch1.5), SHRSP.WKY-(Apbb1-D1Arb21)/Izm (SHRSPwch1.8), SHRSP.WKY-(D1Mgh5-D1Rat44)/Izm (SHRSPwch1.9), and SHRSP.WKY-(D1Mgh5-D1Wox29)/Izm (SHRSPwch1.11). Information about the strains is available at the website of the National Bio-Resource Project for the Rat (http://www.anim.med.kyoto-u.ac.jp/nbr/home.htm/).

Male rats at 12 weeks of age were used in all experiments. All rats were fed with a "stroke-permissive" diet (Funabashi, Funabashi, Japan) ad libitium. The experimental protocol was approved by the local Ethics Committee for Animal Study of Shimane University School of Medicine.

Salt-Loading

At 12 weeks of age, drinking water was changed to 1% sodium chloride solution until the end of the experiments. Blood pressure was measured with the tail-cuff method (BP-98A, Softron Corp., Tokyo, Japan) before and after 2 weeks of salt-loading.

Evaluation of Phenotypes

During salt-loading, symptoms of cerebral stroke were assessed in individual rats every day. When paralysis, akinesia, spastic movements, urinary incontinence, and rapid loss of body weight and grooming were observed, rats were diagnosed with cerebral stroke. After rats were sacrificed, the brain was quickly removed and stained with 4% 2,3,5-triphenyl-tetrazolium chloride to confirm infarcted regions. The experiment was censored after 5 weeks of salt-loading. Twenty to 35 rats from each strain were used in the experiment. Evaluation of stroke latency was performed separately from the evaluation of renal injury described below.

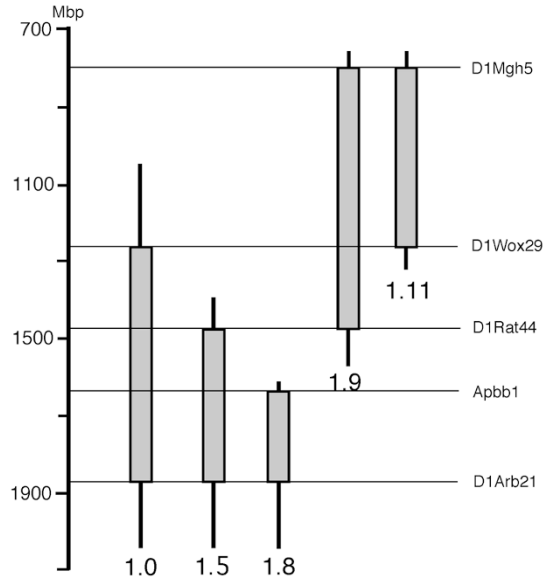

Fig. 1. *Genetic profiles of the five congenic strains. Shaded columns indicate WKY-derived chromosomal fragments introgressed into the SHRSP background. Vertical lines indicate regions having recombination.*

In the morphological study of the kidney, rats were sacrificed after 4 weeks of salt-loading. Bilateral kidneys were fixed in 10% PBS-buffered formalin, and axial slices were embedded in paraffin for light microscopy. We examined 50 to 70 glomeruli in each rat to evaluate the severity of glomerulosclerosis. Each glomerulus was classified into three categories, mild (<10%), moderate (10 to 70%) and severe (>70%), according to the size of the sclerotic area. The numbers of glomeruli with moderate to severe changes were counted by two independent pathologists. Percentages of damaged glomeruli were calculated, and averaged values for the observations by the two pathologists were used in the analysis.

Measurement of urinary albumin was performed before and after 2 weeks of salt-loading. Urine was collected in a metabolic cage for 24 h. During the collection, rats were free to access food and 1% salt solution ad libitum. Albumin was quantified with an EIA kit (NEPHRAT II; Exocell, Philadelphia, USA).

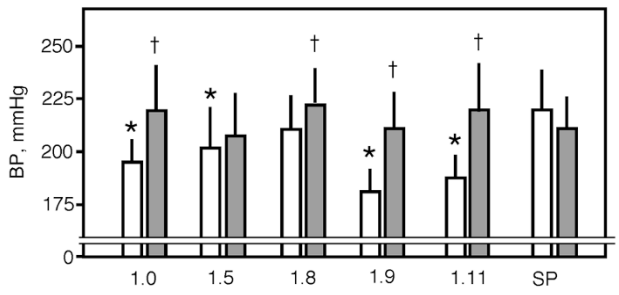

Fig. 2. *Blood pressure before and after 2 weeks of salt-loading. Open and shaded columns indicate blood pressure before and after salt-loading, respectively. Error bars represent the SD. *Significantly different from SP by the Dunnet's post-hoc test. †Significantly different from the blood pressure before salt-loading by the one-tail t-test. Twenty to 37 rats were used in each measurement, except for SHRSP where eight were used.*

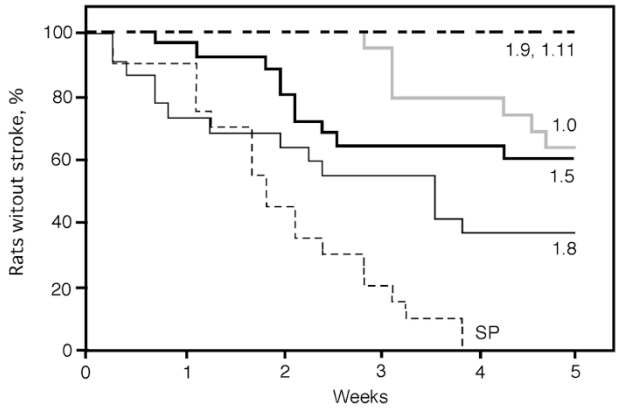

Fig. 3. *Cumulative incidence of stroke during salt-loading. Statistical significance of the inter-strain difference was discussed in Results. Twenty to 37 rats of each strain were used in the experiment.*

Statistical Analysis

Data are shown as the means±SD. The numbers of rats used in each experiment are shown in the figures. ANOVA was applied to examine the inter-strain difference of the measures. The log-rank test was used in the analysis of the cumulative stroke incidence with Bonferroni's correction. Correlations between the parameters were examined using Pearson's *r*.

Results

Figure 2 shows blood pressures before and after 2 weeks of salt-loading. "Basal" blood pressure before salt-loading was significantly lower in all congenic strains, except SHRSPwch1.8, than in SHRSP (by Dunnett's post-hoc test). This difference, however, disappeared after 2 weeks of salt-loading. The significant increase in blood pressure by salt-loading was observed in four of the congenic strains, while the increase in SHRSPwch1.5 was not significant ($p=0.16$ by the one-tail *t*-test). In fact, the difference in blood pressure was rather small in SHRSPwch1.5 (7.5 mmHg) in comparison to other congenic strains (for example, 25 mmHg difference in SHRSPwch1.0). In SHRSP, a significant increase in blood pressure was not observed after salt-loading. This was most likely because they had already suffered cerebral stroke at this time. These results indicated 1) that a chromosomal region represented by SHRSPwch1.8 did not harbor a major gene responsible for hypertension, and 2) that SHRSPwch1.5 was the only strain that did not show salt-sensitivity.

Another interesting observation was that SHRSPwch1.9 showed the lowest blood pressure among the congenic strains; the congenic fragment of this strain included the chromosomal area outside of the 100:1 confidence interval of the original QTL that was covered by SHRSPwch1.0. This implied that additional genes responsible for hypertension were located outside the Chr-1 QTL, which was supported by the observation that SHRSPwch1.11, covering the region outside the QTL (Fig. 1), had as low a blood pressure as that in SHRSPwch1.0 (Fig. 2).

Figure 3 indicates the cumulative incidence of cerebral stroke in the congenic strains. When compared with SHRSP, the four congenic strains showed significantly greater stroke latencies ($p<0.0001$ by the log rank test), except SHRSPwch1.8, which showed a marginal significance ($p=0.008$, the significance threshold became $p<0.01$ after the Bonferroni's correction).

Comparison among the congenic strains indicated no significant difference between SHRSPwch1.0 and 1.5 ($p=0.4$ by the log rank test) and SHRSPwch1.8 and 1.5 ($p=0.05$), and a marginal difference between SHRSPwch1.8 and 1.0 ($p=0.01$) after Bonferroni's correction (the significant threshold; $p<0.005$). The differences between SHRSPwch1.8 and 1.9/1.11 were highly significant ($p<0.0001$).

Histopathological evaluation of glomerulosclerosis is summarized in Fig. 4D. The number of sclerotic glomeruli was low in the three congenic strains, SHRSPwch1.5, 1.9 and 1.11. By contrast, SHRSPwch1.0 and 1.8 showed significantly greater numbers of sclerotic glomeruli when compared with that in SHRSPwch1.9, the strain with the least glomerulosclerosis (by Dunnett's post-hoc test). A large portion of SHRSPwch1.8 died of cerebral stroke during salt-loading. Accordingly, the value for this strain might be biased toward the lower end because only the survivors after 4 weeks of salt-loading were used in the evaluation. As no SHRSP were alive after 4 weeks of salt-loading, data after 2 weeks of salt-loading are indicated for this strain.

Figure 5A indicates urine volume and urinary albumin before and after 2 weeks of salt-loading. Although, no significant differences among the strains were observed in these parameters, SHRSPwch1.0 and 1.8 tended to have greater

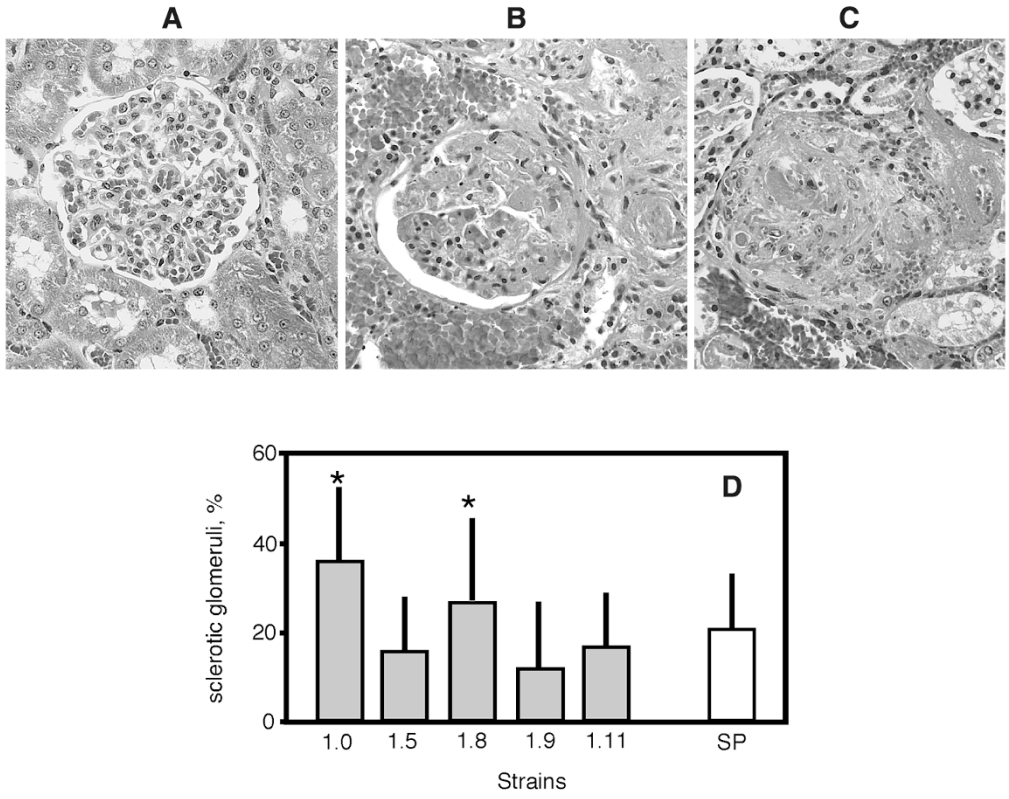

Fig. 4. *Histological evaluation of glomerulosclerosis. A–C: Typical histology of a glomerulus with mild, moderate and severe sclerotic change, respectively. D: Prevalence of glomeruli with moderate to severe sclerosis was indicated with columns. Error bars represent the SD. The evaluation was performed after 4 weeks of salt-loading in the five congenic strains and after 2 weeks of salt-loading in SHRSP. Twelve to 41 rats were used in the evaluation. Because of the difference in salt-loading duration, SP was not included in the statistical examination. *Significantly different from SHRSPwch1.9 by the Dunnett's post-hoc test.*

albumin excretion after 2 weeks of salt-loading. This was consistent with the morphological evaluation of glomerular damage as indicated in Fig. 5B. As such, the severity of glomerulosclerosis correlated well with urinary albumin excretion after salt-loading.

The different amounts of salt intake among the strains might affect their blood pressure and the severity of renal damage. However, intake of salt water did not differ significantly among the congenic strains when estimated with the urine volume for 24 h (closed columns in the lower panel of Fig. 5A). Under the ordinary situation, urine volume was expected to be matched to intake volume (*18*).

Correlations of various parameters with basal blood pressure are summarized in Fig. 6. Stroke was indicated as the cumulative incidence of cerebral stroke after 5 weeks of salt-loading. This parameter for stroke susceptibility showed a good correlation with basal blood pressure ($r=0.97$, $p=0.006$), while no significant correlation with basal blood pressure was observed on blood pressure after salt-loading, the severity of glomerulosclerosis, or urinary albumin excretion after salt-loading. Of note, SHRSPwch1.0 showed the most severe glomerular damage as well as albuminuria when compared with other strains in spite of its modest hypertension, suggesting additional genetic factors for renal damage independent of high blood pressure.

Discussion

In this study, the effects of blood pressure on renal damage and cerebral stroke were evaluated in five congenic strains for blood pressure QTL on Chr 1. We hypothesized that, when additional genetic factors for such organ damage were located in this chromosomal region, it was possible to find dissociation between the severity of organ damage and blood pressure. If, on the other hand, blood pressure is a major determinant for organ damage, a good correlation between blood pressure and the severity of organ damage is expected.

In fact, the results indicate a good correlation between cumulative stroke incidence and basal blood pressure, suggesting a major role of blood pressure on cerebral stroke in these congenic strains. By contrast, the renal damage evaluated by the severity of glomerulosclerosis and albuminuria did not show a significant correlation with blood pressure, implying that additional genetic factors independent of blood

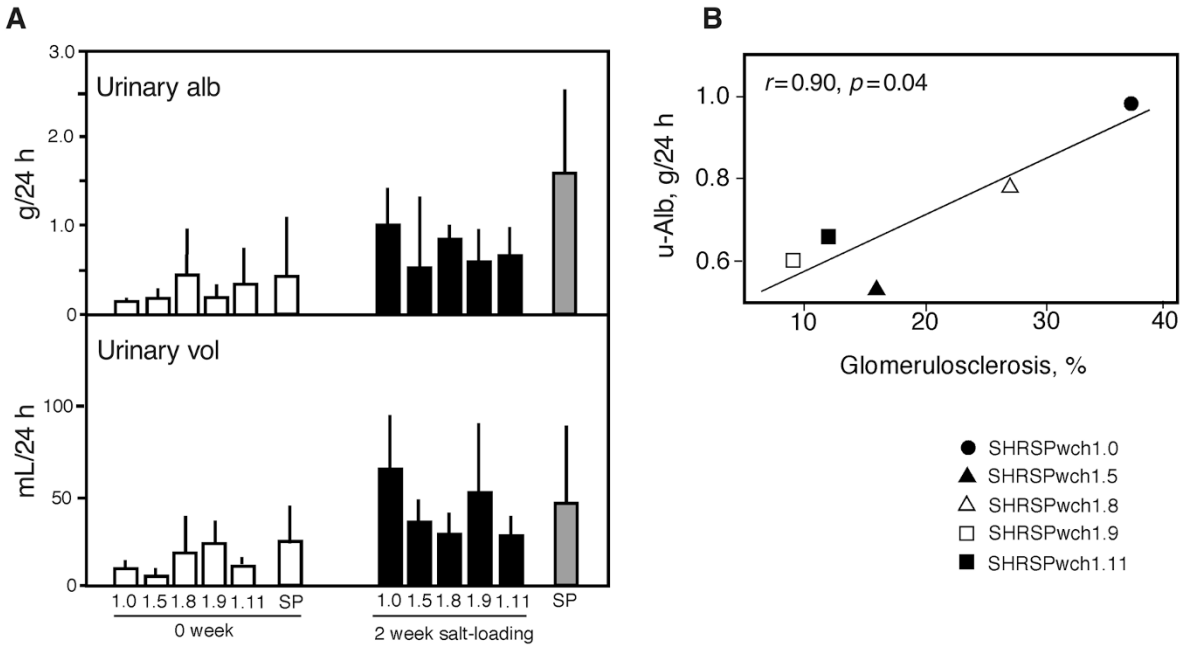

Fig. 5. *Urinary albumin excretion. A: Urine volume (lower) and urinary albumin excretion (upper) for 24 h before (open columns) and after (closed columns) 2 weeks of salt-loading are shown. Error bars indicate SD. Six to 9 rats of each strain were used, except SHRSPwch1.8 for which two were used. Shaded columns for SHRSP show the values after 1 week of salt-loading. B: Correlation between the severity of albuminuria and glomerulosclerosis. u-Alb, urinary albumin.*

pressure located in this chromosomal region.

Rubattu *et al*. reported a QTL for the latency of salt-induced stroke on Chr 1, which overlaps with the congenic region we examined in this study (*11*). As they used SHRSP and SHR of the Heidelberg colony in the construction of F_2 and congenic strains, and as they observed no significant difference in blood pressure of these two strains, the QTL they identified on Chr 1 seemed to influence the stroke-latency independently of blood pressure (*11*). This observation was further confirmed with the study on congenic strains (*14*). On the contrary, the present observation suggested that blood pressure was a major determinant of stroke susceptibility in the congenic strains examined. This discrepancy may be due to the difference in the colonies (Izumo *vs*. Heidelberg), or in the pairs of rats (SHRSP and WKY *vs*. SHRSP and SHR) used in the two studies. The congenic strains we constructed had WKY-derived fragments on the SHRSP background, and therefore were expected to show a greater variance in blood pressure among the congenic strains. This large variance in blood pressure might eclipse additional genetic influences on stroke susceptibility.

In contrast to cerebral stroke, blood pressure did not have a clear influence on renal damage. Figure 6 indicates that the severity of glomerulosclerosis and albuminuria after salt-loading was not correlated significantly with basal blood pressure. Blood pressure after 2 weeks of salt-loading was not correlated significantly with the severity of the renal damage either (data not shown). Of note, SHRSPwch1.0 showed the most severe renal damage when evaluated both with the morphology and albuminuria, although its blood pressure was in the median of those in the five congenic strains (indicated also in Fig. 4B). SHRSPwch1.5, on the other hand, showed a lower incidence of glomerulosclerosis and a lower level of albuminuria even though its blood pressure was comparable with that of SHRSPwch1.0. SHRSPwch1.0 might have an additional susceptibility gene for salt-induced renal damage. If this is the case, the WKY-fragment between D1Wox29 and D1Rat44 includes the responsible gene(s). In this regard, it is interesting that a QTL named "Rf-2" for albuminuria was identified in Fawn-Hooded Hypertensive (FHH) rats, which was in the middle of the questionable region (*15*). Later, Rangel-Filho *et al*. identified a strong candidate for this QTL, Rab38, which had a null mutation in FHH (*19*). However, as this mutation was found only in substrains of the Long-Evans rat (*20*), albuminuria observed in SHRSPwch1.0 might not be due to this gene. Furthermore, the situation was more complicated than expected since SHRSPwch1.9 was resistant to salt-induced renal damage even though this strain had the WKY-fragment of the questionable region (Fig. 1). It may be necessary to assume that there are additional genes protective against the renal damage in the WKY-fragment of the region between D1Mgh5 and D1Wox29. Alternatively, further reduction in blood pressure in this strain might protect against the progression of renal damage in spite of sharing the sus-

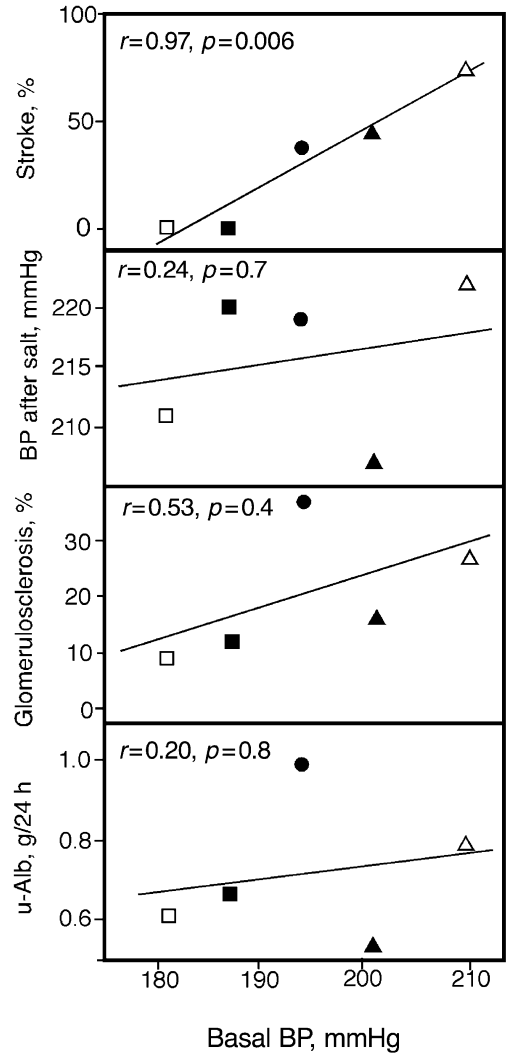

Fig. 6. *Correlation of "basal" blood pressure (BP) with cerebral stroke, BP, glomerulosclerosis, and urinary albumin (u-Alb) induced with salt-loading. Pearson's r is indicated in each panel with p values.* ●, *SHRSPwch1.0;* ▲, *SHRSPwch1.5;* r , *SHRSPwch1.8;* □, *SHRSPwch1.9;* ■, *SHRSPwch1.11.*

ceptibility gene(s) for this type of damage. Further investigation is warranted on the precise locus responsible for the renal damage observed in SHRSPwch1.0.

Radiotelemetry can monitor blood pressure continuously in rats moving freely, and thus has become a standard method to estimate blood pressure. However, it is sometimes practically difficult to obtain telemetry data from sufficient numbers of rats because of the cost. In this study, we therefore applied the conventional tail-cuff method carefully. An apparatus that gave less stress to rats was employed (*21*), a skilled investigator took measurements at least five times to obtain reliable values, and more than 20 rats of each strain were used in the experiment. A good correlation between the blood pressure measured with the indirect and the direct method was reported in a previous study (*21*). Therefore, it is an important future issue to evaluate the blood pressure of all congenic strains with radiotelemetry under unrestrained conditions.

QTL analyses and studies on congenic rats have identified the susceptibility loci for renal damage located on Chr 1 in FHH (*18*), Munich Wistar Froemter rats (*7*), Sabra Hypertensive rats (SBH) (*9*), Dahl salt-sensitive rats (*8*), SHR (*22*) and SHRSP (*2*). However, these regions did not overlap with the region examined in the present study except for the "Rf-2" locus discussed above (*15*) and a large consomic region examined in SBH (*9*). At the moment, it is quite difficult to deduce candidate genes for the renal damage in SHRSPwch1.0 because of the lack of information on the etiology. Thus, the susceptibility to renal damage may be due to exaggerated salt-appetite, rapid and large increases in blood pressure induced by salt-loading or inadequate responsiveness of the kidney to salt-loading. Further studies to identify the mechanisms of salt-induced renal damage in SHRSPwch1.0 are warranted.

Finally, SHRSPwch1.0 may be useful as an experimental model for chronic kidney disease (CKD). As SHR is known to be quite resistant to salt-induced renal damage (*3*), and as SHRSP suffers from cerebral stroke in the early stage of salt-loading, this congenic strain in combination with a "salt-resistant" strain, SHRSPwch1.5, may provide a unique experimental tool for studies on the pathophysiology and application of therapeutics for CKD.

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References

1. Okamoto K, Yamori Y, Nagaoka A: Establishment of the stroke-prone spontaneously hypertensive rat (SHR). *Circ Res* 1974; **34**/**38** (Suppl I): I-143–I-153.
2. Gigante B, Rubattu S, Stanzione R, *et al*: Contribution of genetic factors to renal lesions in the stroke-prone spontaneously hypertension rat. *Hypertension* 2003; **42**: 702–706.
3. Griffin KA, Churchil PC, Picken M, Webb RC, Kurtz TW, Bidani AK: Differential salt-sensitivity in the pathogenesis of renal damage in SHR and stroke prone SHR. *Am J Hypertens* 2001; **14**: 311–320.
4. Mashimo T, Nabika T, Matsumoto C, *et al*: Aging and salt-loading modulate blood pressure QTLs in rats. *Am J Hypertens* 1999; **12**: 1098–1104.
5. Kato N, Mashimo T, Nabika T, Cui ZH, Ikeda K, Yamori Y: Genome-wide searches for blood pressure quantitative trait loci in the stroke-prone spontaneously hypertensive rat of a Japanese colony. *J Hypertens* 2003; **21**: 295–303.
6. Kato N, Nabika T, Liang Y-Q, *et al*: Isolation of a chromosome 1 region affecting blood pressure and vascular disease traits in the stroke-prone rat model. *Hypertension* 2003; **42**: 1191–1197.

7. Schulz A, Litfin A, Kossmehl P, Kreutz R: Genetic dissection of increased urinary albumin excretion in the Munich Wistar Fromter rat. *J Am Soc Nephrol* 2002; **13**: 2706–2714.
8. Siegel A-K, Kossmehl P, Planert M, *et al*: Genetic linkage of albuminuria and renal injury Dahl salt-sensitive rats on a high-salt diet: comparison with spontaneously hypertensive rats. *Physiol Genomics* 2004; **18**: 218–225.
9. Yagil C, Sapojnikov M, Katni G, *et al*: Proteinuria and glomerulosclerosis in the Sabra genetic rat model of salt susceptibility. *Physiol Genomics* 2002; **9**: 167–178.
10. Kortanje R, DiPetrillo K: Unraveling the genetics of chronic kidney disease using animal models. *Am J Physiol* 2004; **287**: F347–F352.
11. Rubattu S, Volpe M, Kreutz R, Ganten U, Ganten D, Lindpaintner K: Chromosomal mapping of quantitative trait loci contributing to stroke in a rat model of complex human disease. *Nat Genet* 1996; **13**: 429–434.
12. Garrett MR, Dene H, Walder R, *et al*: Genome scan and congenic strains for blood pressure QTL using Dahl salt-sensitive rats. *Genome Res* 1998; **8**: 711–713.
13. Saad Y, Garrett MR, Lee SJ, Dene H, Rapp JP: Localization of a blood pressure QTL on rat chromosome 1 using Dahl rat congenic strains. *Physiol Genomics* 1999; **1**: 119–125.
14. Rubattu S, Hubner N, Ganten U, *et al*: Reciprocal congenic lines for a major stroke QTL on rat chromosome 1. *Physiol Genomics* 2006; **27**: 108–113.
15. Brown DM, Provoost AP, Daly MJ, Lander ES, Jacob HJ: Renal disease susceptibility and hypertension are under independent genetic control in the fawn-hooded rat. *Nat Genet* 1996; **12**: 44–51.
16. Yao H, Cui ZH, Masuda J, Nabika T: Congenic removal of a QTL for blood pressure attenuates infarct size produced by middle cerebral artery occlusion in hypertensive rats. *Physiol Genomics* 2007; **30**: 69–73.
17. Cui ZH, Nemoto K, Kawakami K, Gonda T, Nabika T, Masuda J: Fine linkage mapping of the blood pressure quantitative trait locus region on rat chromosome 1. *Hypertens Res* 2002; **25**: 605–608.
18. Guyton AC, Hall JE: Urine formation by the kidneys: I. Glomerular filtration, renal blood flow, and their control, in Textbook of Medical Physiology. Philadelphia, Saunders Co, 1996, pp 315–330.
19. Rangel-Filho A, Sharma M, Datta YH, *et al*: Rf-2 gene modulates proteinuria and albuminuria independently of changes in glomerular permeability in the Fawn-Hooded hypertensive rat. *J Am Soc Nephrol* 2005; **16**: 852–856.
20. Ois N, Riddle SR, Serikawa T, Kuramoto T, Spritz RA: The rat Ruby (R) locus is Rab38: identical mutations in Fawn-hooded and Tester-Moriyama rats derived from an ancestral long Evans rat sub-strain. *Mamm Genome* 2004; **15**: 307–314.
21. Kuwahara M, Sugano S, Yayou K, Tsubone H, Kobayashi H: Evaluation of a new tail-cuff method for blood pressure measurement in rats with special reference ot the effects of ambient temperature. *Exp Anim* 1991; **40**: 331–336.
22. St Lezin E, Griffin KA, Picken M, *et al*: Genetic isolation of a chromosome 1 region affecting susceptibility to hypertension-induced renal damage in the spontaneously hypertensive rat. *Hypertension* 1999; **34**: 187–191.