A 3-Mbp fragment on rat chromosome 1 affects susceptibility both to stroke and kidney injury under salt loading in the stroke-prone spontaneously hypertensive rat: a genetic approach using multiple congenic strains

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Abstract: We have previously reported that a major quantitative trait locus (QTL) responsible for susceptibility to salt-induced stroke in the stroke-prone spontaneously hypertensive rat (SHRSP) is located in a 3-Mbp region on chromosome 1 covered by SHRSP.SHR-(D1Rat23-D1Rat213)/Izm (termed Pr1.31), a congenic strain with segments from SHRSP/Izm introduced into the stroke-resistant SHR/Izm. Here, we attempted to narrow down the candidate region on chromosome 1 further through analyses of subcongenic strains constructed for the target region. Simultaneously, salt-induced kidney injury was evaluated through the measurement of urinary albumin and the gene expression of renal tubular injury markers (Kim-1 and Clu) to explore a possible mechanism leading to the onset of stroke. All subcongenic strains examined in this study showed lower susceptibility to salt-induced stroke than SHRSP. Interestingly, Pr1.31 had the lowest stroke susceptibility when compared with newly constructed subcongenic strains harboring fragments of the congenic sequence in Pr1.31. Although Kim-1 and Clu expression after 1 week of salt loading in Pr1.31 did not differ significantly from those in SHRSP, the urinary albumin level of Pr1.31 was significantly lower than those of the other subcongenic strains and that of SHRSP. The present results indicated that, although the congenic fragment in Pr1.31 harbored the gene(s) related to salt-induced organ damages, further genetic dissection of the candidate region was difficult due to multiple QTLs suggested in this region. Further analysis using Pr1.31 will unveil genetic and pathophysiological mechanisms underlying salt-induced end organ damages in SHRSP.

Key words: congenic strain, quantitative trait loci (QTL), spontaneously hypertensive rat (SHR), stroke-prone SHR (SHRSP)
Introduction

The stroke-prone spontaneously hypertensive rat (SHRSP) is one of the best disease models of essential hypertension and hypertension-related end-organ damage including stroke [1, 2]. Although the known molecular mechanisms underlying the pathogenesis of stroke in SHRSP are beneficial in establishing novel therapeutic and preventive strategies for stroke in humans, the genetic determinants remain to be elucidated.

Previously, we identified major quantitative trait loci (QTL) for salt-induced stroke on chromosome (chr) 1 and 18 by linkage analysis [3]. In a recent study using reciprocal congenic strains for the stroke QTLs [4], we identified six candidate genes (Cbl protooncogene C, Cblc; C-X-C motif chemokine ligand 17, Cxcl17; zinc finger protein 45-like, LOC102548695, Zfp45L; EtHE1 persulfide dioxygenase, Ethel1; capicua transcriptional repressor, Cic; and carcinoembryonic antigen-related cell adhesion molecule 19, Ceacam 19) in a 3-Mbp fragment on chr 1 covered by a SHRSP-based congenic strain, Pr1.31. However, as the annotated molecular functions of these six genes were not directly involved in the development of stroke, it is essential to explore their functional roles in the development of stroke or to reduce the number of candidate genes further (ideally, to only one). In the present study, we applied the latter strategy and attempted to narrow down the candidate region covered by Pr1.31 by evaluating stroke susceptibility in additional subcongenic strains with smaller fragments of the original congenic region.

Hypertension is a major risk factor for nephrosclerosis that impairs glomerular filtration, leading to chronic kidney disease. Interestingly, previous studies have reported that the pathological changes in the kidney, such as increased proteinuria, preceded cerebrovascular events in SHRSP [5, 6], suggesting that kidney injury plays critical roles in the pathogenesis of stroke in this model. Based on this observation, we evaluated salt-induced kidney injury in the newly constructed subcongenic strains to explore a potential correlation between hypertension kidney injury and cerebral stroke.

Materials and Methods

Construction of subcongenic strains

SHRSP/izm rats were provided by the Disease Model Cooperative Research Association (Kyoto, Japan). Subcongenic strains harboring a SHR/izm-derived chr-1 fragment on the genetic background of SHRSP/izm were obtained as described previously [3, 4]. Briefly, Pr1.10 females (termed SHRSP.SHR-(D1Mgh5-D1Got87)/izm, NBRP Rat No.0709) were backcrossed with a SHRSP/izm male, and the resulting F1 rats were intercrossed to obtain pups of the F2 generation. Marker-assisted selection using five simple sequence repeat (SSR) markers (D1Got78, D1Rat261, D1Got82, D1Rat97, and D1Rat213; Supplementary Table 1) was performed to find F2 pups with a recombination in the target region, and ultimately, three subcongenic strains, SHRSP.SHR-(D1Got78-D1Rat97)/izm, SHRSP.SHR-(D1Got82-D1Rat97)/izm, and SHRSP.SHR-(D1Got78-D1Rat261)/izm (termed Pr1.101, Pr1.102, and Pr1.103, respectively), were established.

Rats were kept under conventional conditions at a room temperature of 23 ± 2°C, with 50 ± 10% humidity and a 12 h light-dark cycle (lights on at 7:00 and off at 19:00), in the Department of Experimental Animals, Interdisciplinary Center for Science Research, Head Office for Research and Academic Information, Shimane University. Rats were fed MF diet (Oriental Yeast Co., Ltd., Tokyo, Japan) and housed in polycarbonate resin cages (Natsume Seisakusho Co., Ltd., Tokyo, Japan) from 6 weeks of age. For experiments, the rats were fed a stroke-permissive (SP) diet (Funabashi Farm Co., Ltd., Funabashi, Japan) from 5 weeks of age and housed in stainless-steel cages (Natsume Seisakusho Co., Ltd.) from 6 weeks of age.

Evaluation of stroke latency under salt loading

Rats were given 1% salt in drinking water ad libitum from 12 weeks of age to compare stroke susceptibility under the salt loading condition [3, 4]. In this study, we used two criteria to measure stroke latency: 1) the initial sign-free period and 2) the survival period. The initial sign-free period was determined as the period in days until the first signs of stroke were observed. Signs of stroke included behavioral abnormalities (convulsive movement, slight or marked decrease in motor activity, and hyperirritability), body weight (BW) loss (>10 g for 2–3 days), and sudden death [7]. The survival period was defined as the period in days until akinesia (no walking and decreased responsiveness) was observed over several days and severe BW loss (>20% loss from maximal BW), paralysis of hind limbs, and death simultaneously or subsequently occurred. Twenty-one to twenty-three
rats were examined for each strain. Rats were carefully observed each day, and BW was measured every 2 to 3 days (Supplementary Figs. 1–6). Rats showing the severe stroke symptoms described above were sacrificed immediately by carbon dioxide inhalation. Representative macroscopic brain lesions in sacrificed rats are shown in Fig. 1C.

Blood pressure (BP), heart rate (HR), and urinary albumin measurement

Seven or eight animals were used for each strain. BP and HR were measured using the tail-cuff method (BP-98A, Softron, Tokyo, Japan) at 12 weeks of age. Two days after the tail-cuff measurement, rats were transferred to metabolic cages (TOKIWA, Tokyo Japan) to collect 24 h urine under salt-loading conditions. Urine collection was performed on days 0, 4, 7, 11, and 14 post-salt loading. The urine was centrifuged at 1,000 rpm for 10 min at 4°C, and the supernatant was stored at −80°C until use. BW changes are shown in Supplementary Fig. 7. Rats showing the severe stroke symptoms described above were immediately sacrificed by carbon dioxide inhalation.

Urinary albumin was measured using a LBIS Rat Albumin ELISA kit (FUJIFILM Wako Shibayagi Corp., Gunma, Japan) according to the manufacturer’s protocol. Each sample was analyzed in duplicate on a microplate reader (DTX 880, Beckman Coulter, Brea, CA, USA). Total excretion of albumin (mg/day) was calculated by multiplying the average concentration by the urine volume.

RNA extraction and quantitative RT-PCR

A total of 10 rats at 12 weeks of age were randomly divided into the control (n=5, no salt-loading) and experimental (n=5, 1% salt-loading) groups. After a one-week experimental period with/without salt loading, the rats were deeply anesthetized by isoflurane inhalation, and their kidneys were removed. Resected kidneys were snap-frozen in liquid nitrogen and stored at −80°C until use. One part of the left or right kidney bisected by coronal section was fixed in a 10% formalin solution for histopathological evaluation [8]. Severities of glomerular sclerosis (HE staining) and tubulointerstitial fibrosis (Azan staining) were evaluated by two nephrologists independently (ME and SF), and they approved the final conclusions.

Total RNA was extracted using Sepasol RNA-I Super...
G (Nacalai Tesque, Kyoto, Japan) according to the manufacturer’s protocol. One microgram of the total RNA was reverse-transcribed to cDNA using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio, Shiga, Japan) in 20 µl reaction mixture. Quantitative RT-PCR was performed using a StepOnePlus Real Time PCR System (Applied Biosystems, Waltham, MA, USA) in a 20 µl volume of reaction mixture with TB Green Premix Ex Taq II (Takara Bio). The PCR conditions were as follows: 1 cycle at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. Each sample was analyzed in duplicate. The quantity of mRNA was normalized to that of β-actin mRNA (2-ΔCt (target -β-actin)). The primer sequences were as follows: GGAGCAGCG-GTCGATAACAACA and TCTCCACCTCAGCA-CAATACACAC for kidney injury molecule-1 (Kim-1, NM_173149), TTTACAGTTCCCGGATGTGGATTTC and ACTTCTCACACTGGCCACCA for Clusterin (Clu, NM_053021), ATATCGCTGCAGCTCGTCGTC and CCTTCTGACCCATACCCACCA for β-actin (NM_031144), and CAGGAATATGAGGCGTGTGGGA and CCCCGCACTCCTCACTGAAATA for Zfp45L (XM_039093492; formal symbol, LOC102548695).

DNA sequencing

Genomic DNA was extracted from liver using a Blood & Cell Culture DNA Mini kit (QIAGEN, Venlo, Netherlands). A genomic region including a missense variation in Ceacam 19 [4] was amplified by PCR, and the aliquots were used for subsequent sequencing reactions. The primers used were CAAAAGTCCTCCCTGAAA-CAACC and GGAGAAAGAGTGGATGGAAGACC. Sequencing reactions were performed using a SupreDye v1.1 cycle sequencing kit (Edge Biosystems, San Jose, CA, USA), and purified products were analyzed using a 3130 Genetic Analyzer (Applied Biosystems).

Statistics

Data are expressed as means ± SD. Statistical analysis was performed with Prism 8 (GraphPad, San Diego, CA, USA). Results were analyzed by log-rank test with Bonferroni correction for multiple comparisons and one-way analysis of variance (ANOVA) with Dunnett’s post hoc test, and P<0.01 and P<0.05 were considered to be significant, respectively.

Results

Evaluation of stroke susceptibility under salt loading

Figure 1A shows genomic structures of the subcongeneric strains used in this study. Each subcongeneric strain had a fragment of the SHR/izm genome, part of the chr-1 QTL region for salt-induced stroke [3, 4]. Among the five subcongeneric strains, Pr1.101, Pr1.102, and Pr1.103 were newly constructed (see Materials and Methods).

In all the subcongeneric strains, both the initial sign-free duration and the life span were significantly elongated when compared with those of SHrSP (Fig. 1B). Pr1.31 especially was more resistant to salt-induced stroke than the other four subcongeneric strains (Fig. 1B). These results suggested that multiple genes located in the 3-Mbp region between D1Rat23 and D1Rat213 covered by Pr1.31 were necessary to most effectively reduce susceptibility to salt-induced stroke in SHrSP. Although it is difficult to infer the reason for the significant difference in stroke susceptibility between Pr1.31 and Pr1.10, it might be due to deleterious genes located in congeneric regions covered by Pr1.10 but not by Pr1.31.

BP and HR

Systolic BP (SBP), diastolic BP (DBP), and HR at 12 weeks of age are shown in Fig. 2. The SBP of Pr1.103 was significantly lower than that of SHRSP. The DBPs of four of the subcongeneric strains, excluding Pr1.101,
were significantly lower than that of SHRSP (see Discussion). No significant differences were detected in HR between SHRSP and the subcongenic strains.

Evaluation of urinary albumin

Table 1 summarizes the time-dependent changes in urinary albumin during the 2 weeks of salt loading. Salt loading induced rapidly induced large increases in urinary albumin in all the strains examined. Among them, Pr1.31 showed a tendency to excrete less albumin both at baseline (day 0) and throughout the salt-loading period. The urinary albumin level of Pr1.31 was significantly lower at days 0 and 14 when compared with SHRSP and the other four congenic strains, while no significant differences were detected between SHRSP and the other four subcongenic strains throughout the experiment period. These findings suggested that Pr1.31 was uniquely resistant to salt-induced kidney injury among the six strains examined in this study. Although it is likely that a decrease in salt-induced BP elevation underlies this unique phenotype in Pr1.31, further analysis is necessary to clarify this.

| Strains  | Day 0     | Day 4     | Day 7     | Day 11     | Day 14     |
|----------|-----------|-----------|-----------|------------|------------|
| SHRSP    | 37.4 ± 39.4 (7) | 35.1 ± 13.3 (6) | 49.7 ± 31.6 (5) | 95.1 ± 10.1 (2) | 85.0 ± 9.4 (2) |
| Pr1.31   | 2.2 ± 1.1 (8)   | 12.2 ± 10.8 (8)  | 21.1 ± 11.2 (8)  | 33.2 ± 23.0 (7)  | 30.6 ± 13.3 (6) |
| Pr1.10   | 29.3 ± 32.0 (7) | 79.6 ± 16.6 (7)  | 76.8 ± 28.5 (4)  | 97.6 ± 22.0 (3)  | 86.0 ± 5.1 (3)  |
| Pr1.101  | 26.2 ± 27.3 (7) | 58.0 ± 20.1 (7)  | 55.8 ± 21.3 (7)  | 91.9 ± 17.9 (4)  | 85.9 ± 15.4 (2) |
| Pr1.102  | 24.0 ± 18.4 (8) | 70.1 ± 20.1 (8)  | 87.9 ± 41.9 (7)  | 78.9 ± 56.2 (4)  | 119.9 ± 42.5 (2) |
| Pr1.103  | 4.8 ± 1.8 (7)   | 27.4 ± 24.0 (7)  | 65.7 ± 52.8 (7)  | 105.0 ± 25.3 (6) | 76.7 ± 29.7 (5) |

Numbers in parentheses indicate numbers of examined rats. #P<0.05 vs. SHRSP by one-way ANOVA with Dunnett’s post hoc test. $P<0.05$ vs. Pr1.31 by one-way ANOVA with Dunnett’s post hoc test.

![Gene expression of renal tubular injury markers and histological evaluation of glomerular sclerosis and tubulointerstitial fibrosis](image)

In addition to urinary albumin, a series of renal tubular markers have been identified to predict kidney dysfunction [9]. Among them, we examined the expression of Kim-1 and Clu as proximal and distal tubular injury markers, respectively (Fig. 3A). On the other hand, Clu expression in Pr1.101 was significantly greater than that in SHRSP (Fig. 3A). These results suggested that additional unknown factors (genetic difference among the strains probably) influenced

Fig. 3. Gene expression of Kim-1 and Clu in the kidney with or without 1 week of salt loading. The expression of renal tubular injury markers was quantitatively examined in kidneys obtained from (A) non-salt-loaded control rats and (B) salt-loaded rats. The Kim-1 and Clu expression levels were standardized to β-actin expression as described in the Materials and Methods. #P<0.05 vs. SHRSP by one-way ANOVA with Dunnett’s post hoc test (n=5).
the severity of tubular damage under the baseline conditions in a manner independent of the magnitude of hypertension.

One week of salt loading increased Kim-1 expression by approximately 10-fold in the rats examined in this study (Supplementary Fig. 8). In contrast, salt-induced Clu expression varied among the rats (Supplementary Fig. 8). This observation suggested that Kim-1 was a sensitive and reliable maker for salt-induced tubular injury.

Four of the subcongenic strains, excluding Pr1.103, showed lower levels of Kim-1 expression either at a significant or marginally significant level when compared with SHRSP (Fig. 3B), implying that they were more resistant to salt-induced renal tubular damage. On the other hand, no significant differences were observed in Clu expression between SHRSP and subcongenic strains.

The severities of glomerular sclerosis and tubulointerstitial fibrosis after 1 week of salt loading were compared among three representative strains: SHRSP, Pr1.31, and Pr1.102 (Fig. 1A). As shown in Supplementary Fig. 9, the sclerotic to non-sclerotic glomeruli ratio was not significantly different among the three strains, whereas two SHRSP cases showed relatively high prevalence (>10%). Additional analysis at different time points could reveal whether glomeruli of SHRSP are more fragile. No fibrotic changes were observed in the tubulointerstitial regions, even in the SHRSP cases with a high prevalence of glomerular sclerosis (Supplementary Fig. 10).

Evaluation for Zfp45L and Ceacam 19

Among the six candidate genes identified previously [4], Zfp45L and Ceacam 19 are present in the regions including recombinant breakpoints in Pr1.102 and Pr1.103 (D1Rat261-D1Got82, Fig. 1A). To check whether congenic fragments in the two subcongenic strains cover Zfp45L and/or Ceacam 19 loci, we examined gene expression levels of Zfp45L in the kidney and the nonsynonymous variation in Ceacam 19 by quantitative RT-PCR and DNA sequencing, respectively [4].

Zfp45L expression in Pr1.102 and Pr1.103 was not detectable, as in the case of SHR, indicating that both Pr1.102 and Pr1.103 harbored the SHR allele at this gene (Fig. 4A). In contrast, Pr1.102 and Pr1.103 harbored the sequence variations of SHRSP and SHR at Ceacam 19, respectively (Fig. 4B). These results indicated that Zfp45L, which is shared by both Pr1.102 and Pr1.103, is a potential candidate for susceptibility to salt-induced stroke (Fig. 4C, see Discussion).

Discussion

In the present study, we attempted 1) to narrow down the QTL for salt-induced stroke on chr 1 and 2) to examine the effects of stroke QTL on susceptibility to salt-induced kidney injury. The results indicated that 1) it was difficult to dissect the original QTL region down to a smaller fragment and that 2) the stroke QTL on chr 1 influenced the susceptibility to salt-induced kidney injury.

A previous study showed that the 2.1 Mbp region between D1Rat177 (81.581 Mbp) and D1Rat97 (Fig. 1A) putatively harbored genes responsible for stroke suscep-
tibility [4]. Thus, we focused on this region to make three subcongenic strains in the present study: Pr1.101, Pr1.102, and Pr1.103. Since six candidate genes were identified in this region (Figs. 1A and 4C), we expected to reduce this number by comparing the stroke susceptibility among these three subcongenic strains.

Interestingly, the results did not indicate any significant differences in stroke latency among the three strains (Fig. 1B). This phenomenon could be ascribed to either of two possibilities: 1) multiple genes in the region covered by Pr1.31 affected stroke susceptibility together, or 2) the gene (or genes) were located in the region between D1Rat261 and D1Got82, a part of which might be shared by both Pr1.102 and Pr1.103. Examples supporting the first hypothesis were easily found in previous studies on QTLs [15–17]. The second hypothesis was supported when the expression of Zfp45L was compared between Pr1.102 and Pr1.103 (Fig. 4A). This gene was located between D1Rat261 and D1Got82, and its expression level differed between SHR and SHRSP [4]. As its expression levels in Pr1.102 and Pr1.103 were the same as that in SHR, it could be inferred that these two subcongenic strains shared a fragment from the SHR genome including Zfp45L (Fig. 4A). In contrast, Pr1.102 and Pr1.103 harbored the SHRSP and SHR alleles at Ceacam19, respectively (Fig. 4B). Since these two subcongenic strains showed the same extent of elongation in the stroke latency (Fig. 1B), Zfp45L is a likely candidate for stroke susceptibility.

Zfp45L is a putative protein-coding gene with physiological functions that are fully unknown. Since zinc-finger protein families are trans-acting transcriptional regulators [18], ZFP45L may be involved in the pathogenesis of salt-induced stroke via transcriptional regulation of its target genes. Given that Zfp45L expression is high in SHRSP (Fig. 4A), the creation of Zfp45L knockout SHRSP would be one of the best ways to elucidate this hypothesis.

In the present study, we found a significant difference in stroke susceptibility between Pr1.31, the original congenic strain, and the three newly established subcongenic strains. Our previous study excluded the region upstream of D1Rat23 as the stroke QTL [4], thereby implicating another responsible gene (or genes) between D1Rat97 and D1Rat213. Furthermore, regarding the low urinary albumin excretion and low Kim-1 expression (though it was not significant) in Pr1.31, it could be hypothesized that the gene(s) between D1Rat97 and D1Rat213 influences both stroke and kidney injury (Table 1 and Fig. 3). In this region, we identified 18 protein-coding genes, 4 pseudogenes, and 1 non-coding RNA (Supplementary Table 2). Previous reports indicate that a receptor tyrosine kinase among them, Axl, is a potential candidate in terms of its pathogenic roles in (salt-dependent) hypertension [19–21]. As these previous reports suggest that Axl signaling in immune cells plays pivotal roles in the development of hypertension and salt-induced kidney dysfunction, future studies also need to focus on different types of tissues and cells.

A major limitation of this study is the fact that it remains unknown whether the stroke QTL on chr 1 affects the susceptibility to stroke and kidney injury independently; in other words, it remains unknown whether kidney injury proceeds and is causally related to the onset of stroke. Notably, Nagasawa et al. reported anatomical similarities between the pre-glomerular vessels of the juxtamedullary nephron and the cerebral vasculature [22]. This raised the possibility that a common structural basis of vasculature in the kidney and brain could explain a vulnerability to salt-induced damage in the two different tissues of the SHRSP; however further investigation is essential.

We found that the SBPs and DBPs of one and four subcongenic strains, respectively, were significantly lower than those of SHRSP (Fig. 2). This observation suggested that a decrease in BP, especially DBP, was related to a decline in stroke susceptibility in those rats. However, the correlation between stroke latency and BP in some congenic rats (for example, Pr.1.101) was inconsistent with this hypothesis. We measured the baseline BP by the tail-cuff method in this study, which might not be appropriate for evaluating the effects of BP on salt-induced stroke, thereby necessitating the measurement of BP changes under salt-loading conditions using a telemetry system [3, 4].

In conclusion, we found that the congenic fragment covered by Pr1.31 conferred resistance to both salt-induced kidney injury and stroke in SHRSP. Although the subcongenic analysis failed to narrow down the candidate region for the stroke QTL, Zfp45L may be the first candidate gene to be explored for stroke susceptibility. Given the complexity of genetic dissection of QTLs using multiple congenic strains, further analysis using Pr1.31 and SHRSP would be a reasonable strategy to identify the genetic determinants and reveal the molecular mechanisms of both salt-induced kidney injury and stroke in SHRSP.

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Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

MW and HO carried out the experiments, analyzed the data, and contributed to this work equally; ME and SF evaluated kidney sections; HM helped perform the animal experiments; HO designed the study, prepared figures and tables, and wrote and revised the manuscript; ZRG commented on drafts of the manuscript and revised the manuscript; and TN designed the study and critically revised the manuscript. All authors read and approved the final manuscript.

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