**Klk1** as One of the Genes Contributing to Hypertension in Dahl Salt-Sensitive Rat

Naoharu Iwai, Naomi Yasui, Hiroaki Naraba, Naomi Tago, Hideyuki Yamawaki, Hiroshi Sumiya

**Abstract**—A genome-wide quantitative trait loci analysis for blood pressure was performed using 107 male F2 rats derived from Dahl salt-sensitive and Lewis rats. Blood pressure was assessed by telemetry, and >400 microsatellite markers were used for genotyping. Two major quantitative trait loci for blood pressure were identified at chromosome 1 and chromosome 10. The expression levels of 366 transcripts around the chromosome 1 quantitative trait loci were assessed by RT-PCR, and we found that the *Klk1* (kallikrein 1) and *Ngfg* (nerve growth factor gamma) mRNA levels were significantly reduced in the kidneys of Dahl salt-sensitive rats compared with those in Lewis rats. The expression levels of kallikrein 1 protein were also suppressed in Dahl salt-sensitive rats compared with those in Lewis rats. Because the kallikrein–kinin system has been shown to be involved in renal function, including salt homeostasis, it is likely that the reduced expression of *Klk1* contributes to salt-sensitive hypertension in Dahl salt-sensitive rats. *(Hypertension. 2005; 45:947-953.)*

**Key Words:** kallikreins ■ rats, Dahl ■ genetics

Dahl salt-sensitive rats have been widely used for the investigation of salt-sensitive hypertension. Sixteen genomic regions containing quantitative trait loci (QTLs) for blood pressure (BP) regulation have been reported in this strain.1–3 However, the genes responsible for this salt-sensitive hypertension have not yet been confirmed, despite numerous genetic studies.

A possible explanation for the lack of identification of the responsible genes in rat genetic studies to date might be the incompleteness of the rat genome data. The Rat Genome Project is now almost complete,4 and thus, it is now possible to obtain information on almost all of the protein-coding genes in candidate loci for a given phenotype.

Another drawback in previous rat genetic studies was the method used to assess BP. The BP levels of F2 rats were usually measured either with a tail-cuff method or with a temporary inserted arterial catheter. The validity of these classic methods of BP measurement is not concretely established, and furthermore, largely dependent on the skill levels of the individual researchers. Moreover, with these methodologies, circadian rhythm changes in BP cannot be adequately assessed. In the present study, these drawbacks were overcome by using telemetry.

In the present study, a genome-wide QTL analysis for BP was performed using 107 male F2 rats derived from Dahl salt-sensitive (DS) and Lewis (LEW) rats. The BP was measured by a telemetry system, and the genotypes of the F2 rats were determined with >400 genetic markers throughout the genome. Major QTLs were identified for daytime and nighttime BP (systolic and diastolic) levels. Next, we assessed the expression levels of 366 transcripts in the prominent QTLs and identified 2 transcripts that were differentially expressed in the kidneys of the 2 strains.

**Materials and Methods**

**Experimental Animals**

DS and LEW rats were purchased from Sunplant (Tokyo, Japan) and Charles River Japan (Yokohama, Japan), respectively. Rats were housed in a temperature-controlled room with the light on from 7:00 AM to 7:00 PM (daytime) and fed normal rat chow (0.5% NaCl; Clea Japan) and tap water ad libitum. Male DS rats were mated with female LEW rats to produce F1 rats, and F1 rats were then intercrossed to produce an F2 population consisting of 107 male rats. F2 rats were started on an 8% NaCl diet (Oriental Yeast) at 5 weeks of age according to the protocol described by Rapp et al.1 Radiotelemetry devices (Data Sciences International) were implanted into the lower abdominal aorta of F2 rats at 9 weeks of age using sodium pentobarbital as an anesthetic agent (25 mg/kg IP). At 14 weeks of age, BP and heart rate were continuously measured for 44 hours (daytime 7:00 AM to 7:00 PM; nighttime 7:00 PM to 7:00 AM), and the data obtained during the latter 24 hours were used for analyses. The results were analyzed using Fluelet TM software (Dainippon Pharmaceutical).5 The present study was conducted in accordance with current guidelines for the care and use of experimental animals of the National Cardiovascular Center.

**Genotyping and QTL Analysis**

Genotyping was performed by PCR using appropriate PCR primer pairs (custom-made by Amersham Pharmacia Biotech), based on information from the Rat Genome Database (http://rgd.mcw.edu/) and Ratmap (http://ratmap.gen.gu.se/). We found sequence variations between DS and LEW rats in several genes, and these
polymorphisms were also included in the genotyping data. The raw data are provided as a supplement (supplemental Table I, available online at http://hyper.ahajournals.org). We separately analyzed QTLs for daytime systolic BP (D-SBP), diastolic BP (D-DBP), nighttime systolic BP (N-SBP), and diastolic BP (N-DBP) levels using MapManager QTLb20.5 We first performed Quick Test to obtain significant thresholds for BP values. The likelihood ratio statistics (LRSs) for suggestive, significant, and highly significant QTLs were calculated to be 12.3, 17.7, and 25.9, respectively. Next, we performed marker regression for these 4 BP values. The most significant QTL was added to the background, and a second marker was added to the background to obtain the third QTL. We obtained 1 significant QTL was added to the background, and a second marker was added to the background to obtain the third QTL. We obtained 1 significant QTL (chromosome 1) and 1 or 2 suggestive QTLs (chromosome 10 and chromosome 12) for these 4 BP values. Interval mapping of chromosome 1 was performed (free model) with other some 10 and chromosome 12) for these 4 BP values. Interval mapping of chromosome 1 was performed (free model) with other QTLs included as background.

RT-PCR Analysis of Expression Levels of Transcripts

For the screening of differentially expressed transcripts, we analyzed the expression levels of transcripts in the kidneys of DS and LEW rats (5 weeks of age) on a normal diet (n = 2 in each group). The expression levels of transcripts were assessed by RT-PCR at 25 and 32 amplification stages because overamplification might obscure possible differences in expression levels. A 2.5-fold difference can be reliably detected by this method, provided the expression level of the target mRNA is between the 1/100 and 1/1000 of that level (supplemental Figure 1). After this initial screening, several possible transcripts that might be differentially expressed were subjected to precise estimation by competitive RT-PCR analyses with 18s ribosomal RNA as an internal control (QuantumRNA 18s Internal Standards Kit; Ambion). Because the 18s ribosome RNA level is extremely high, the PCR primers for 18s cDNA amplification are diluted by 18s competimers. The expression of transcripts that were analyzed and their amplification products. The expression levels were assessed in the kidneys of Transcripts have introns, and were selected to gene-specific sites to avoid cross-amplification from homologous genes. Expression was determined by RT-PCR in the kidneys. ND indicates not detected.

### Table 1. Primers for Members of the Kallikrein Gene Family

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Blast analysis in the rat genome with the Klk1 sequence identified 22 homologous sequences. Primers were set on separate exons when possible transcripts have introns, and were selected to gene-specific sites to avoid cross-amplification from homologous genes. Expression was determined by RT-PCR in the kidneys. ND indicates not detected.
Western Blotting

Rabbit polyclonal antibody against rat urinary kallikrein was obtained from Merck Biosciences (formerly Calbiochem), which recognizes the active and inactive (prepro) forms.

LEW and DS rats (5 weeks old; n=11005) were euthanized and kidneys were harvested. Kidneys were frozen in liquid N2 and were homogenized in Triton-based lysis buffer (1% Triton X-100, 20 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L Na3VO4, 1 mmol/L /H9252-glycerol phosphate, 1 mmol/L Na3VO4, 1 mmol/L leupeptin, and a 0.1% protease inhibitor mixture [Nacarai Tesque]). The protein concentration was determined with the bicinchoninic acid method (Pierce). Equal amounts of proteins (60 g) were separated by SDS-PAGE (7.5%) and transferred to a nitrocellulose membrane (BioTrace NT; Pall Corporation). After blocking with 5% BSA, membranes were incubated with primary antibody (1:1000 dilution) at 4°C overnight, and membrane-bound antibodies were visualized by horseradish peroxidase–conjugated secondary antibodies (1:10 000 dilution; 1 hour) and an ECL system (Amersham Biosciences). The expression levels were determined by densitometry.

Results

Linkage to BP

A total of 418 polymorphic markers in all of the 107 F2 rats were genotyped, and some of them gave identical genotype data. Thus, the data on 383 effective genotypes were finally obtained (supplemental Table I).

Table 2 gives the results of the analysis using the MapManager QTX program for linkage to BP in the F2 (DS×LEW) population. Separate analyses were conducted of the D-SBP, D-DBP, N-SBP, and N-DBP values. Table 2 shows only the markers that yielded an LRS of at least a suggestive level of significance (ie, >12.3). The QTLs for N-SBP were around D1Rat27 and Pex12 (Ch10). Those for N-DBP were around D1Rat27 and D10Rat98, those for D-SBP were around D1Rat27, and those for D-DBP were around D1Rat27, Pex12, and D12Arb6.

Identification of Candidate Genes in the Chromosome 1 Region

We next focused on the chromosome 1 region near D1Rat27 (at 90.3 mol/L). A total of 366 transcripts were found from this region between Cyp2a2 (82.1 mol/L) and loc292934 (99.5 mol/L), and RT-PCR expression analysis was performed (supplemental Table II). The expression of 240 genes was detected in the kidney of the 366 possible transcripts. As shown in Figure 2, the Klk1 and Ngfg mRNAs were found to be differentially expressed between the kidneys of DS and LEW rats. Klk1 and Ngfg are members of a large kallikrein...
gene family. Gene-specific primers for the kallikrein gene family were set up as described in Materials and Methods (Table 1). Direct sequencing confirmed that the PCR products originated from the Klk1 and Ngfg transcripts. Three-way ANOVA indicated that weeks of age (P=0.0023) and strain difference (P=0.0046) but not salt loading (P=0.8884) significantly affected the Klk1 mRNA levels (P=0.0006). The Klk1 mRNA level in LEW rats was 2.7-fold higher than that in DS rats. This differential expression was reconfirmed by the competitive RT-PCR method, in which a deletion mutant cRNA for Klk1 was used as an internal standard (supplemental Figure II).

Three-way ANOVA indicated that strain difference (P<0.0001) but not weeks of age (P=0.3629) or salt loading (P=0.3410) significantly affected the Ngfg mRNA levels (P=0.0005). The Ngfg mRNA level in LEW rats was 3.4-fold higher than that in DS rats at 5 weeks of age. The Ngfg mRNA level in LEW rats was 1.5-fold higher than that in DS rats at 14 weeks of age.

Sequence analysis of the entire Klk1 and Ngfg sequences including the 1-kb promoter and 1-kb 3' regions in DS and LEW rats revealed no sequence difference except in the number of TC repeats in intron 1 of Ngfg: the number was 24 and 26 in DS and LEW rats, respectively.

This differential expression was also confirmed by Western blot analysis (Figure 3). A primary antibody recognizing rat urinary kallikrein corresponding to KLK1 was used. In DS rats, KLK1 expression was significantly suppressed

**Figure 1.** Interval mapping analysis of BP QTL on chromosome 1. Interval mapping analysis of BP values was performed on chromosome 1 with other suggestive loci included as background. LRS plots for BP values are shown. The region subjected to expression analysis is indicated as a horizontal bar between D1Arb33 and D1Mgh7.

**Figure 2.** Expression levels of Klk1 and Ngfg mRNA in the kidneys of DS and LEW rats. Expression levels of the Klk1 and Ngfg mRNA were assessed by competitive RT-PCR using 18s ribosome RNA as an internal standard. The ratios of 18s primer to 18s competimer were 3:7 for the Klk1 and Ngfg mRNAs. The size of the PCR products from the Klk1, Ngfg, and 18s RNA was 721 bp, 529 bp, and 488 bp, respectively. Three-way ANOVA indicated that weeks of age (P=0.0023) and strain difference (P=0.0046) but not salt loading (P=0.8884) significantly affected the Klk1 mRNA levels (P=0.0006). Moreover, 3-way ANOVA indicated that strain difference (P<0.0001) but not weeks of age (P=0.3629) or salt loading (P=0.3410) significantly affected the Ngfg mRNA levels (P=0.0005). Each group contains 4 rats (n=4). 5W LEW indicates 5-week-old LEW rats under normal diet; 5DS, 5-week-old DS rats under normal diet; 14LEW--, 14-week-old LEW rats under normal diet; 14DS--, 14-week-old DS rats under normal diet; 14DS+, 14-week-old DS rats under a high-salt diet. The expression levels of the Klk1 and Ngfg mRNAs in 5-week-old DS rats are arbitrary defined as 1.0. Vertical bars indicate SDs.
Lower urinary excretion of kallikrein-like activity has been reported in DS rats,8,9 and adenoviral transfer of the human kallikrein 1 gene has been reported to ameliorate hypertension and hypertension-associated target organ damage in DS rats.14,15 Based on these reports, it is likely that the low expression of Klk1 in DS rats is responsible for salt-sensitive hypertension. On the other hand, mice lacking Klk1 have been reported to exhibit cardiovascular abnormalities with normal BP.16,17 The hearts of these mice exhibited septum and posterior wall thinning and a tendency for dilatation, which led to decreased cardiac function. Thus, the normal BP in these mice, despite increased vascular resistance,17 reflects a reduced cardiac function. Therefore, this knockout model is not necessarily inconsistent with our hypothesis that the reduced Klk1 expression in DS rat kidneys is responsible for salt-sensitive hypertension in DS rats.

The LRS plots for BP values of Ch1 (Figure 1) are prominent and wide. Rapp et al reported the existence of 3 QTLs (QTL1a, QTL1b, and QTL2) on Ch1 on the basis of results in Dahl rat congenic strains with an introgressed Lew rat Ch1 segment.18 In these congenic experiments, the Klk1 locus seemed to have only a slight effect on BP (≈10mm Hg, congenic line (Ch1X3)X12; Figure 2), and an adjacent locus distal to the Klk1 locus appeared to be important (QTL1a). If 2 responsible genes reside in a very close range, the QTL plot will not give 2 separate peaks, but rather only 1 large pseudopeak. In such a case, it would not be possible to determine the precise location of the responsible genes only by linkage.

Thus, it is possible that the reduced expression of Klk1 in DS rats may have only modest effects on BP, and another gene near the Klk1 locus, which might have escaped the systematic expression analysis performed in the present study, might have profound effects on BP. Indeed, the expression analysis in the present study has several limitations; for example, subtle changes cannot be detected by the PCR-based expression analysis, the target tissue might not be the kidney, and the target gene might not be differentially expressed.

The unsolved problem of the present study is the lack of any significant sequence variations in Klk1 and Ngfg loci despite differential expression patterns. There would seem to be 2 possible explanations. One is that the regulation of Klk1 might be influenced by regulatory sequences acting over near-megabase distances. Recently, the existence of regulatory sequences acting over near-megabase distances has been suggested.19 The size of genomic regions functionally linked to a particular gene may thus need to be considerably expanded.19,20 The other explanation is that the downregulation of Klk1 in DS rats may be attributable to another gene near the Klk1 locus. The rat kallikrein gene family apparently consists of at least 22 genes encompassing >1 Mb.21–23 The physiological significance of each member in this family has not been determined. It is also possible that Klk1 expression may be modified to compensate for derangement in other members of this gene family. Such a compensatory decrease in Klk1 or Ngfg could lead to salt-sensitive hypertension in the Dahl rat.

Whatever the reason for the downregulation of Klk1 in DS rats, it has relevance to salt-sensitive hypertension because adenoviral transfer of human kallikrein 1 gene has been reported to ameliorate hypertension and hypertension-associated target organ damage in DS rats.14,15 Based on the
latter hypothesis, Klk1 might not be a causative gene but might be an effector for salt-sensitive hypertension.

Ngfg is also a member of the kallikrein gene family, and the physiological substrates for Ngfg have not been confirmed.21–23 It is currently unclear whether the differential expression of Ngfg between the 2 strains might be related to salt-sensitive hypertension. Ngfg and Klk1 were found to be downregulated in DS, although no significant sequence difference was observed between DS and LEW rats. Because the existence of large-scale copy number polymorphism has been reported in the human genome,24 we assessed the copy number of the Klk1 locus in DS and LEW rats, and found no significant difference. Coordinate suppression of Klk1 and Ngfg might support either a compensation mechanism or the existence of long-range enhancers, as mentioned above.

In the present study, an assessment was made of 4 components of BP values: N-SBP, N-DBP, D-SBP, and D-DBP. Unexpectedly, the major QTLs for these traits were almost identical. Thus, the present results exclude the possibility that nighttime and daytime BP might be influenced by completely different sets of genes.

This investigation focused on the most prominent QTLs for BP levels (ie, those around the D1Rat27 locus). Although no assessment was made of the expression levels of transcripts around the second (Pex12 [peroxisomal biogenesis factor 12] at Ch10) or third (D12Arb6) QTL, we noted the existence of differentially expressed transcripts in the kidneys of DS and LEW rats, namely Pnpo (pyridoxine 5′-phosphate oxidase) at Ch10 and P2rx4 (purinergic receptor P2X, ligand-gated ion channel 4) at Ch12, the differences for which had been revealed by chance in our previous microarray analyses.25 However, a more thorough assessment will be necessary to identify candidate genes in these QTLs that could contribute to hypertension.

Garrett et al reported the existence of 9 BP QTLs (Ch1, 2, 3, 5, 8, 10, 16, 17, and 18) using F2 derived from DS X LEW, the same strains used in the present study. The most prominent BP QTLs were reported on Ch5 and 10. Of these 9 QTLs, the QTLs on Ch1, 5, 8, 10, and 17 were confirmed by the establishment of congenic strains.1,26–27 In the present study, BP QTLs were found on Ch1, 10, and 12. The most prominent BP QTL was on Ch1. Garrett et al measured BP by the tail-cuff method, and BP measured by this method is likely to be influenced by stress (eg, by heating of the tails and constrains). On the other hand, the BP QTLs detected by the telemetry system are free from such stress. The discrepancy observed between the 2 studies may be mainly attributable to the difference in the methods of BP measurement.

**Perspectives**

The Rat Genome Project is almost complete,4 and precise maps of protein-coding genes are now available. Furthermore, the rat is an appropriate animal for the physiological assessment of cardiovascular functions. Therefore, the rat is a suitable model animal for identifying genes that are related to cardiovascular diseases by means of a positional cloning strategy. On the other hand, the existence of various noncoding RNAs has been confirmed, including microRNAs, and it is highly probable that these noncoding RNAs are involved in gene regulation and gene–gene interactive network regulation.28–29 Moreover, the existence of large-scale copy number polymorphism in the human genome has also been reported.24 An expression analysis of just the protein-coding genes might not be sufficient for the identification of genes that contribute to salt-sensitive hypertension.

**Acknowledgments**

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**References**


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Table I Genotypes of F₂ rats

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Table II: RT-PCR analysis of transcripts in chromosome 1 QTL.
Supplementary Figure I. Validity of RT-PCR assessment of mRNA expression levels.

The sensitivity of simple RT-PCR quantification was tested. We prepared RNA samples in which the expression levels of Ngfg mRNA were artificially altered by mixing kidney and brain RNAs. The expression level of Ngfg mRNA in brain was too low to be detected with 35 cycles of PCR amplification. Sample 1 is kidney RNA without brain RNA. Sample 2 RNA was prepared by mixing 1.0 kidney RNA with 1.5 brain RNA. Sample 3 RNA was prepared by mixing 1.0 kidney RNA with 9.0 brain RNA. Sample 4 was prepared by mixing 1.0 sample 3 RNA with 1.5 brain RNA, and so on. As shown in the figure, 2.5 fold differences were consistently made discernable by 25 and 32 cycles of amplification.
Supplementary Figure II.
Assessment of KLK1 expression levels by the competitive RT-PCR method.

Rat KLK1 cDNA was subcloned into pCR4·TOPO (Invitrogen). The resultant plasmid was digested with HindIII and BglII, blunt-ended with Klenow enzyme, and self-ligated. The deletion mutated cRNA was synthesized by T7 RNA polymerase from the resultant plasmid. Two micrograms of the total kidney RNA (5 week-old, n=5) mixed with 2X10^6 molecules of the deletion mutant rat KLK1 cRNA were reverse-transcribed. The resultant cDNAs were amplified by PCR with the KLK1-specific primers. The KLK1 mRNA and the deletion-mutated KLK1 cRNA yielded 720bp and 427bp PCR products, respectively. The validity of this competitive RT-PCR method has been repeatedly confirmed previously (1-3). As shown in the figure, the KLK1 mRNA expression levels were 3.65 times higher in LEW than in DS. KLK1 and ΔKLK1 indicate the PCR product from the KLK1 mRNA and the deletion-mutated KLK1 cRNA, respectively. L and D indicate LEW and DS, respectively. The expression level of the KLK1 mRNA in DS was arbitrarily defined as 1.0.

Reference
1. Iwai N, Inagami T. Quantitative analysis of rennin gene expression in extrarenal
