Vol. 192, No. 1, 1993
April 15, 1993
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 261-267

A NEUROPEPTIDE Y LOCUS ON CHROMOSOME 4 COSEGREGATES WITH BLOOD PRESSURE IN THE SPONTANEOUSLY HYPERTENSIVE RAT

T. Katsuya*, J. Higaki*, Y. Zhao*, T. Miki*,
H. Mikami*, T. Serikawa** and T. Ogihara*

* Department of Geriatric Medicine, Osaka University Medical School, Osaka 565, Japan

** Institute of Laboratory Animals, Faculty of Medicine, Kyoto University, Kyoto, Japan

Received March 8, 1993

SUMMARY: Recent advances in molecular biology have allowed the study of the candidate genes for essential hypertension. To identify the genes responsible for basal blood pressure in the spontaneously hypertensive rat strain, the rat model of genetic hypertension, we performed a cosegregation analysis between the genotype and blood pressure in a set of male F_2 progeny obtained from SHR and Wistar-Kyoto rats, a reference normotensive strain. Our investigation revealed that a locus on the chromosome 4 cosegregates with the blood pressure in SHR, especially at neuropeptide Y locus. The degree of cosegregation with all values of blood pressure without sodium loading was moderate but consistent. We propose that neuropeptide Y locus on chromosome 4 is a new candidate for the hypertensive effect in original SHR.

Essential hypertension is the most common clinical diagnosis. It is well known that this disease has various genetic and etiologic factors, but no definite genetic factors have been identified. Especially, since the human population has heterogeneity of genetic features(1), it is difficult to identify the role of genetic factors in the pathogenesis of essential hypertension. Consequently the inbred hypertensive rat is most suitable to study the genes responsible for hypertension. Recent advances in molecular biology have allowed the study of genetic hypertension by cosegregation analysis using F_2 progeny obtained from hypertensive rats and normotensive control rats. From the candidate gene

Abbreviations:SHR, spontaneously hypertensive rat; SHRSP, stroke-prone spontaneously hypertensive rat; WKY, Wistar-Kyoto rat; NPY, neuropeptide Y; SPR, substance P receptor; ENO2, enolase 2; ACE, angiotensin converting enzyme.

0006-291X/93 $4.00

approach, several genes, renin(2), kallikrein(3), heat shock protein 70(4) and S_A gene(5), a new candidate isolated from rat kidney cDNA library, showed positive cosegregation with blood pressure. From the genomic screening approach to identify the additional major genes, two candidate loci have been reported. One is the location of the angiotensin converting enzyme gene on rat chromosome 10, according to a study using F_2 progeny obtained from stroke-prone spontaneously hypertensive rats (SHRSP) and Wistar-Kyoto rats(6, 7). The other was reported in a study using a variable number of tandem repeat maker, 1/MCT96.1, but the chromosomal localization of this marker was not determined, except that it was not in the ACE region(8). However, the previously reported genes cannot explain all the variations in blood pressure, and it was not clear whether these genes truly associate with basal blood pressure without sodium loading in the original spontaneously hypertensive rat (SHR) strain.

In this study, we used a set of F_2 rats obtained by crossmating the original SHR with WKY, the normotensive reference strain. Using a new set of F_2 progeny, we investigated the cosegregation of directly measured basal blood pressure with microsatellites which were mapped on chromosome 4 previously(9).

MATERIALS AND METHODS

Design of Genetic Crosses

Crosses were made between 3 male SHR (191.5±1.9 mmHg) and 3 female WKY rats (128.3±0.8 mmHg) in the Laboratory Animal Science and Toxicology Laboratories, Sankyo Co., Ltd., Shizuoka, Japan. We confirmed that these substrains were genetically inbred by fingerprint analysis(10). The F_1 hybrid was intercrossed for the study of F_2 segregating generation.

Animal Maintenance

All rats were housed under identical environmental conditions at a temperature of 24±1°C, humidity of 55±5% and a 12 hour dark-light cycle, and were given a normal diet consisting of commercial pellet CE-2 (Japan Clea Co. Ltd.).

Blood Pressure Measurements

At 15 weeks of age, all rats were weighed and a catheter was implanted into the abdominal aorta via a femoral artery under light ether anesthesia. Systolic and diastolic blood pressure and heart rate were measured three times 24 hours after catheterization, while the animals were quiet at rest and in a freely mobile state. The measurements were carried out using a pressure transducer (TP-400T), monitors (AT-601G and AT-641G) and a recorder (RTA-1300), all from Nihon Koden Co., Ltd. During the measurements, animals were blinded to the observer. All measurements were taken by a single observer, who had no information on the genotyping of animals.

Genotyping of Microsatellite Markers

The genomic DNA samples were extracted from rat livers by a standard phenol-chloroform method(11). Using the 13 microsatellites which were

Table 1. Microsatellite markers on rat chromosome 4 with polymorphism between SHR and WKY

Locus symbol	PCR primer sequence (5'-3') (upper: forward, lower: reverse)	Repeat sequences	Size (bp)	Size comparison
NPY	AGGGAGTGGCAGCATTTAG	(GA)28, (AAAT)7	135	SHR>WKY
	GAGATAGTTCAGAAGAAACCCATG			
SPR	ATCTGCAACAAGGTCCTGC	(GT)23	116	SHR>WKY
	GACGTTATCCATTTTGGGG			
ENO2	AACCTCCTTCTCCCTCACC	(GT)21	120	SHR<WKY
	CCCCTGTCATCCTGCTTTAG			

mapped on rat chromosome 4(9), we investigated the polymorphism between SHR and WKY. As a result, it was found that three markers, neuropeptide Y (NPY), substance P receptor (SPR) and enolase 2 (ENO2), were polymorphic between them (data not shown). Primer locus symbols, sequence design, repeat sequences, product size and PCR product size comparison between SHR and WKY are shown in Table 1. Sample DNA was amplified in 50 mM Tris, pH 8.5, 1 mM $MgCl_2$, 20 mM KCl, 500 μg/ml BSA, 200 μM dNTP and 10 μM of each primer. Samples were denatured for 2 min at 94°C and then cycled 30 times through the following steps: 5 sec at 94°C, 5 sec at 55°C, and then 45 sec at 72°C. All PCR were performed in a 10 μl reaction volume enclosed in glass capillary tubes using the Air Thermo-Cycler (Idaho Technology Co.). PCR products were electrophoresed for about 1 hour on 10% polyacrylamide gel. After ethidium bromide staining, F_2 progeny were divided into 3 groups: SHR/SHR, SHR/WKY and WKY/WKY according to the number of repeats in each microsatellite.

Data Analysis

One-way analysis of variance (ANOVA) was conducted utilizing the program package, Stat View (Abacus Concepts, Inc.).

RESULTS

In this study, we used the microsatellites which were previously mapped by the chromosomal assignment using a panel of rat × mouse somatic hybrid clones and the linkage analysis(9). In a preliminary study, we were impressed with the possibility that one of the candidate loci responsible for blood pressure exists on chromosome 4. Polymorphisms between SHR and WKY were found in three microsatellites as a result of screening 13 microsatellites (Figure 1). Using these three microsatellites for genetic typing, F_2 progeny were clearly divided into three groups: SHR/SHR (SS), SHR/WKY (SW) and WKY/WKY (WW) (Figure 2). On the other hand, blood pressure measurement, the most important phenotype, was performed carefully and accurately, because the variance of basal blood pressure in F_2 progeny obtained from SHR and WKY was so small in comparison with that in hybrid rats obtained from SHRSP or

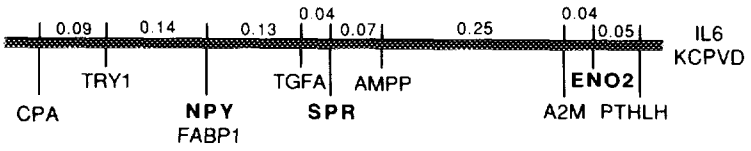

Figure 1. Genome map of rat chromosome 4 and three microsatellites with polymorphism between SHR and WKY.

The distances between markers indicated above the line are estimated from the recombination ratio. Three markers indicated in bold are polymorphic between SHR and WKY. The assignment of microsatellites, locus symbols and recombination distances was made on the basis of Serikawa's paper(9).

Dahl rats with sodium loading that it is hard to get significant difference in blood pressure. As a result of cosegregation analysis between genotype and phenotype, we concluded that the neuropeptide Y polymorphism cosegregates with systolic, diastolic and mean blood pressure in SHR (Table 2, Figure 3). This positive association with blood pressure decreases in proportion to the distance from this locus (Table 2). As the ACE locus, which was proposed to have a dominant effect in the F_2 population of SHRSP(6), the neuropeptide Y locus was also revealed to have a dominant inheritance in SHR.

DISCUSSION

We used the SHR and WKY rats of Sankyo Co. colony for genetic crosses. These strains were determined to be genetically inbred(10) and SHR was one of

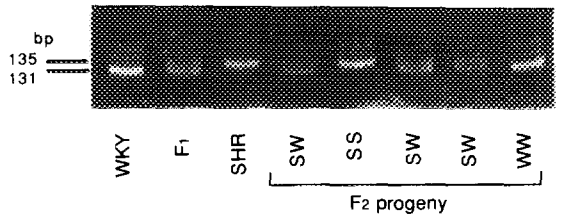

Figure 2. Genotypes of SHR, WKY and F_2 progeny determined by a microsatellite marker, neuropeptide Y.

Symbols S and W indicate the alleles specific for SHR and WKY, respectively. The PCR-based genotype specific for SHR gives rise to a fragment of 135 bp. The PCR product from WKY gives rise only to a 131 bp fragment. F_2 progeny were either homozygous for the SHR allele, SS, heterozygous for the SHR and WKY alleles, SW, or homozygous for the WKY allele, WW.

Table 2. Statistics and analysis of variance in blood pressure measurements (mean±SE mmHg) by genotype of microsatellites in the F_2 population

NPY					
Genotype	SS n=27	SW n=55	WW n=24	ANOVA	p value
Systolic BP	162±2.1	159±1.8	152±1.9	$F_{2,103}$=3.87	**0.024**
Diastolic BP	116±1.5	115±1.2	110±1.6	$F_{2,103}$=3.73	**0.027**
Mean BP	132±1.5	130±1.3	125±1.6	$F_{2,103}$=4.25	**0.017**

SPR					
Genotype	SS n=32	SW n=55	WW n=19	ANOVA	p value
Systolic BP	161±2.7	159±1.6	153±1.8	$F_{2,103}$=2.66	0.075
Diastolic BP	117±1.7	114±1.1	111±1.7	$F_{2,103}$=3.30	**0.041**
Mean BP	132±1.9	129±1.2	125±1.6	$F_{2,103}$=3.28	**0.041**

ENO2					
Genotype	SS n=30	SW n=55	WW n=20	ANOVA	p value
Systolic BP	160±2.5	159±1.6	155±1.7	$F_{2,102}$=1.36	0.263
Diastolic BP	115±1.7	115±1.1	112±1.8	$F_{2,102}$=0.92	0.403
Mean BP	130±1.9	130±1.1	126±2.0	$F_{2,102}$=1.23	0.297

P values <0.05 are indicated in bold.
ANOVA: one way analysis of variance

the colonies closely related to original stock of Okamoto(12). Thus, we consider that there was not so much genetic difference between original SHR and WKY as previously reported(13). This is supported by the fact that polymorphism between these strains is rare, contrary our expectation, regardless of the use of microsatellites that are the most powerful polymorphic DNA markers in the general population. These results suggest that the key point in the identification of the major genes is in the original process adopted in the selection of the SHR strain from WKY, where a significant elevation in the blood pressure of the strain was observed only after the passage of three generations. It is important to note, therefore, that this major change occurred during a very short period of time in contrast to the production of other hypertensive rat strains, and that SHR has been reported to be far less sensitive to salt loading than SHRSP(14). We conclude that study of the F_2 progeny obtained from SHR and WKY is necessary to demonstrate whether a gene has a major effect on blood pressure or not. For example, the ACE locus which cosegregated with blood pressure significantly after sodium loading is a

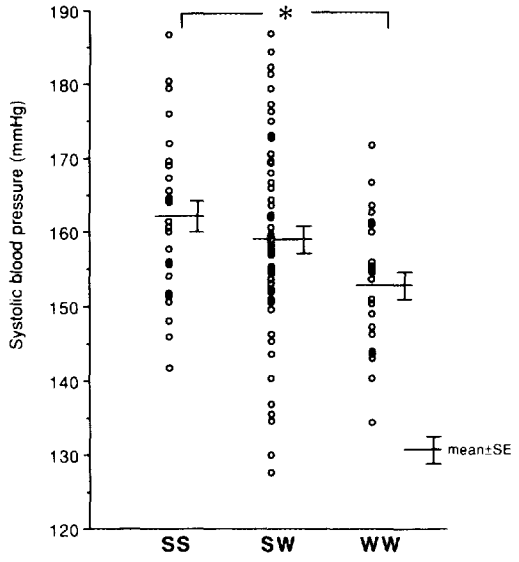

Figure 3. Distribution of systolic blood pressure in F_2 progeny classified according to the genotype of neuropeptide Y.

S and W symbols are as indicated in Figure 2. Vertical lines with small bars indicate mean ± SE. The asterisk indicates a statistical difference between SS and WW ($p<0.05$). The systolic blood pressure of F_2 progeny with homozygous SHR specific allele was 10 mmHg higher than that of F_2 population with homozygous WKY alleles.

candidate for the salt-sensitive hypertensive gene(15), but does not directly contribute to blood pressure elevation in SHR(16).

On the other hand, NPY is a 36 amino acid peptide isolated from the brain in 1982(17). It is a vasoconstrictor peptide widely distributed in the central and peripheral nervous system(18). NPY is proposed to act as a regulator of appetite(19), non-adrenergic sympathetic activity(20), pituitary hormones(21) and renin secretion(22). Although no difference was observed among SHR, SHRSP and WKY with regard to the plasma concentration of NPY, the platelet content of NPY in SHR was higher than that in WKY(23). All reports suggest the importance of NPY as a potent vasoconstrictor. However, there has been only one association study, which reported no significant association between *TaqI* restriction fragment length polymorphisms of human NPY gene and the presence of hypertension in the human population(24).

In this study, NPY polymorphism was shown to cosegregate with basal blood pressure moderately ($p=0.02$). It was not as significant as the ACE locus, but we inferred that the NPY gene itself or a nearby linked gene on chromosome 4 acts to elevate basal blood pressure in SHR. Hilbert *et al.* have also performed genomic screening of chromosome 4, but they did not examine the NPY locus(6). We conclude that the NPY locus is a new candidate for the

hypertensive effect in original SHR, and that further study is necessary to explain clearly the difference in basal blood pressure between SHR and WKY using the genomic screening approach.

ACKNOWLEDGMENTS

We thank H. Tanase, H. Yagisawa and K. Kohara for rat breeding and blood pressure measurements and Y. Yoshikawa and K. Hayashi for technical support. This work was supported by grants from the Special Coordination Funds for Promoting Science and Technology of Japan.

REFERENCES

1. Oshima, T. *et al.* (1992) *J. Hypertens.* **10(9),** 1089-1094.
2. Rapp, J. P., Wang, S.-M. & Dene, H. (1989) *Science* **243,** 542-544.
3. Pravenec, M. *et al.* (1991) *Hypertension* **17(2),** 242-246.
4. Hamet, P. *et al.* (1992) *Hypertension* **19,** 611-614.
5. Lindpaintner, K. *et al.* (1993) *J. Hypertens.* **11,** 19-23.
6. Hilbert, P. *et al.* (1991) *Nature* **353,** 521-529.
7. Jacob, H. J. *et al.* (1991) *Cell* **67,** 213-224.
8. Nabika, T., Nara, Y., Ikeda, K., Endo, J. & Yamori, Y. (1993) *J. Hypertens.* **11,** 13-18.
9. Serikawa, T. *et al.* (1992) *Genetics* **131,** 701-721.
10. Katsuya, T. *et al.* (1991) *Tohoku J. Exp. Med.* **165,** 253-260.
11. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning, A Laboratory Manual* , 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
12. Okamoto, K. & Aoki, K. (1963) *Jpn. Circ. J.* **27,** 282-293.
13. Johnson, M. L., Ely, D. L. & Turner, M. E. (1992) *Hypertension* **19(5),** 425-427.
14. Luft, F. C. *et al.* (1988) *Cli. Sci.* **74(6),** 577-585.
15. Deng, Y. & Rapp, J. P. (1992) *Nature Genet.* **1,** 267-272.
16. Katsuya, T. *et al.* (1992) *Biochem. Biophys. Res. Commun.* **187(3),** 1359-1366.
17. Tatemoto, K., Carlquist, M. & Mutt, V. (1982) *Nature* **256,** 659-660.
18. Walker, P. H., Grouzmann, E., Burnier, M. & Waeber, B. (1991) *Trend. Pharmacol. Sci.* **12,** 111-115.
19. Stanley, B. G. & Leibowitz, S. F. (1984) *Life Sci.* **35,** 2635-2642.
20. Kahan, T., Daddei, S., Pedrinelli, R., Hjemdahl, P. & Salvetti, A. (1992) *J. Card. Pharmacol.* **19,** 587-592.
21. McDonald, J. K., Lumpkin, M. D., Samson, W. K. & McCann, S. M. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 561-564.
22. Aubert, J.-F. *et al.* (1992) *Clin. Exp. Pharmacol. Phys.* **19,** 223-228.
23. Ogawa, T., Kitamura, K., Kanagawa, K., Matsuo, H. & Eto, T. (1992) *J. Hypertens.* **10(8),** 765-771.
24. West, M. J., Summers, K. M. & Huggard, P. R. (1992) *Clin. Exp. Pharmacol. Phys.* **19,** 315-318.