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ORIGINAL ARTICLE
Molecular Cardiology

Evaluation of Pharmacogenetic Algorithm for Warfarin Dose Requirements in Japanese Patients

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Background: Warfarin dosing is difficult to establish because of considerable interindividual variation. Thus, warfarin pharmacogenetics have attracted particular interest in relation to appropriate control of anticoagulation.

Methods and Results: The 200 eligible subjects were chosen from participants in a hospital cohort. Performance of a pharmacogenetic algorithm recently developed by the International Warfarin Pharmacogenetics Consortium (IWPC) was tested and compared with a clinical algorithm (without genotype data) by calculating the percentage of patients for whom the predicted dose deviated by less than 7 mg/week (1 mg/day) from the actual dose. The pharmacogenetic algorithm accurately identified a significantly ($P<0.05$) larger proportion of patients to achieve the target international normalized ratio than did the clinical algorithm (68% vs 36% for a low-dose group; and 21% vs 0% for a high-dose group). Also, an increase in warfarin dosage was found to be appropriate for the current status of alcohol drinking (4 mg/week, as against non-drinking) and smoking (3.3 mg/week, as against non-smoking).

Conclusions: The IWPC pharmacogenetic algorithm has clinical application, particularly in identifying Japanese patients who require a low dosage of warfarin and are at greater risk of excessive anticoagulation. (*Circ J* 2010; **74:** 977–982)

Key Words: Anticoagulation; *CYP2C9*; Pharmacogenetics; *VKORC1*; Warfarin

Warfarin is the most commonly prescribed oral anticoagulant drug for the prophylaxis and treatment of thromboembolic disorders, but the appropriate dose can be difficult to establish because it can vary substantially (>10 fold) among patients, in part because of differences in each patient's age, diet, race and genotype.[1–3] Incorrect doses contribute to a high incidence of adverse effects (ie, bleeding and thromboembolic events) when the effectiveness of warfarin, expressed as the international normalized ratio of prothrombin time (PT-INR), is above or below the therapeutic range.

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During the initial dosing period (ie, the first few months), patients are at the greatest risk of overanticoagulation. To reduce this risk, a number of warfarin dosing algorithms[4,5] and regimens[6,7] have been proposed, mostly incorporating clinical factors, demographic variables, and molecular variations in 2 genes: the warfarin metabolic enzyme *CYP2C9* and the warfarin target enzyme, vitamin K epoxide reductase complex subunit 1 (*VKORC1*). Regarding genetic factors, of note is the fact that, in 2007, the US Food and Drug Administration (FDA) added pharmacogenetic information to the warfarin product label.[3] Along this line, the International Warfarin Pharmacogenetics Consortium (IWPC) has recently developed a pharmacogenetic dose algorithm for warfarin using a large data set (involving a total of 5,052 patients) from diverse ethnic groups.[8] The IWPC algorithm appears to provide better predictive accuracy than the one that uses only clinical variables or a fixed-dose (5 mg/day) strategy.

In general, patients of Asian descent require a lower maintenance dose of warfarin for a similar degree of anticoagulation than patients of European descent.[5,9] Moreover, it has been reported that compared with Europeans, the incidence of thromboembolism is low in Japan, despite the less intensive regimen;[10,11] which indicates that adjusted low-dose warfarin (eg, PT-INR 1.6–2.6) is optimal for prevention of thromboembolism in Japanese patients.[12,13]

Considering these racial differences in the anticoagulation therapy, we attempted to validate the IWPC pharmacogenetic dose algorithm for warfarin in Japanese patients under low-

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Table 1. Characteristics of Study Subjects

	n=200
M/F, n	136/64
Age at entry, years	67.8±10.3
Height, cm	160.3±14.6
Body weight, kg	61.4±12.7
Daily warfarin dose, mg	3.05±1.20
Primary reason for anticoagulation, n (%)	
Atrial fibrillation	119 (59.5)
Prosthetic valve replacement	27 (13.5)
Deep vein thrombosis/pulmonary embolism	12 (6)
Other	42 (21)
Amiodarone use, n (%)	11 (5.5)
VKORC1 rs9923231 genotype, n (%)	
G/G:A/G:A/A	3 (1.5): 31 (15.5): 166 (83)
CYP2C9 genotype, n (%)	
*1/*1:*1/*3:*3/*3	195 (97.5): 5 (2.5): 0 (0)
Alcohol drinking	
Never (%)	33.5
Ex-drinker (%)	18
Current drinker (%)	48.5
Smoking	
Never (%)	38
Ex-smoker (%)	49.5
Current smoker (%)	12.5

VKORC1, vitamin K epoxide reductase complex subunit 1.

dose treatment. Also, we examined the impact of alcohol intake and smoking on warfarin dose requirements, aiming at refinements of the algorithm.

Methods

Study Population

A total of 200 eligible subjects were chosen from participants in the Hospital-Based Cohort Study in the International Medical Center of Japan (IMCJ), which was designed to investigate clinical epidemiology, pharmacogenetics and genetic susceptibility of lifestyle-related disorders such as diabetes, hypertension and cardiovascular diseases.[14] We collected information on demographic characteristics, the primary indication for warfarin treatment, the stable therapeutic dose of warfarin, the treatment INR (the INR achieved with a stable warfarin dose), the use of concomitant enzyme inducers (carbamazepine, phenytoin, rifampin, or rifampicin) and amiodarone. Anticoagulation of patients was stably controlled with a target PT-INR of 1.6–2.6 for the prevention or treatment of thromboembolic diseases. Characteristics of the patients are shown in **Table 1**. We largely divided them into 3 categories of alcohol drinking (never-drinker; ex-drinker; current drinker) and 3 categories of smoking status (never-smoker; ex-smoker; current smoker). Participants were asked to report their daily alcohol consumption, using a structured questionnaire that ascertained the consumption of typical alcoholic beverages (beer, wine, Japanese sake, shochu and spirits). Alcohol intake was denoted in terms of servings of sake (1 gou [180 ml] of Japanese rice wine is considered equal to 22 g of ethanol). As a variable of smoking conditions, the Brinkman Index was calculated from [the number of cigarettes smoked daily]×[smoking period] in addition to the categorical smoking status. All subjects were Japanese and gave written informed consent for participation in the study. The ethics committee of IMCJ approved the study protocol.

Genotyping of *CYP2C9* and *VKORC1* SNPs

Among the genetic variants of *CYP2C9* used for the IWPC algorithm (*1, *2 and *3), the *CYP2C9*2* allele (I359L) has not been reported in Asian populations.[5,15,16] Accordingly, we genotyped the *3=rs1057910 polymorphism in relation to the wild-type allele *1, thereby determining *1/*1, *1/*3 and *3/*3 genotypes at the *CYP2C9* locus. At the *VKORC1* locus, on the other hand, the −1639 G→A=rs9923231 polymorphism was genotyped, following the IWPC algorithm.[8] Both SNPs were characterized with the use of TaqMan assays (Applied Biosystems, Foster City, CA, USA).

Statistical Analysis

First, we performed multiple regression analysis to test the effects of predictor variables on interindividual variability of warfarin dose, with the square root of the warfarin dose in mg/week being used as a dependent variable, which was in accordance with the IWPC study.[8] We then evaluated the potential clinical value of 2 algorithms (the IWPC pharmacogenetic algorithm and a clinical algorithm without including genotype data) by calculating the percentage of patients whose predicted dose of warfarin was within 7 mg/week (1 mg/day) of the actual stable therapeutic dose. The IWPC pharmacogenetic algorithm for Japanese was: 5.4952–(0.2614×[age in decades])+(0.0087×[height in cm])+(0.0128×[weight in kg])−(0.8677×[*VKORC1* A/G])−(1.6974×[*VKORC1* A/A])−(0.9357×[*CYP2C9**1/*3])−(2.3312×[*CYP2C9**3/*3])+(1.1816×[enzyme inducer status])−(0.5503×[amiodarone status])=Square root of weekly warfarin dose.[8] In addition, we calculated the percentage of patients for whom the predicted dose according to each algorithm was at least 7 mg/week higher than the actual dose (overestimation) or at least 7 mg/week lower than the actual dose (underestimation). Here, we adopted 7 mg/week (1 mg/day) as a difference that clinicians would be likely to define as clinically relevant. With consideration of warfarin dose distribution in the study sample (**Figure S1**), the performance of the IWPC algorithm was assessed in 3 dose groups: low dose (≤10.5 mg/week), high dose (≥31.5 mg/week), and intermediate doses (between 10.5 and 31.5 mg/week) for stable therapeutic anticoagulation. These thresholds of 10.5 mg and 31.5 mg/week bracket the usual maintenance dose of 17.5–24.5 mg/week (2.5–3.5 mg/day) in Japanese patients.[5,15,17,18] The overall performance was measured as the coefficient of determination, R^2, which was the square of the sample correlation "R" between the predicted and therapeutic doses. Besides assessing the potential benefit of using the pharmacogenetic algorithm instead of the clinical algorithm, we computed the number needed to genotype (NNG: the number of patients who must be genotyped in order for 1 patient to have an improved dose estimate).

Furthermore, we evaluated the effects of alcohol drinking and smoking on warfarin dose requirements by multiple regression analysis in which 3 numerical models (2 categorical and 1 continuous trait models) were tested for each behavior.

Results

The characteristics of the 200 participants in the present study are summarized in **Table 1**. Among them, the most common indications for warfarin use were atrial fibrillation

Table 2. Effect Size of Predictors on Warfarin Dose Among Japanese Subjects

Predictor	Regression of warfarin dose		Effect in the IWPC algorithm
	Effect (95%CI)	P value	
Intercept	4.940 (3.404, 6.477)	1.6E-09	3.798*
Age in decades	−0.215 (−0.334, −0.095)	5.0E-04	−0.261
Height in cm	0.001 (−0.009, 0.012)	0.82	0.009
Weight in kg	0.012 (−0.001, 0.025)	0.07	0.013
VKORC1 rs9923231			
AG vs AA	0.862 (0.545, 1.178)	2.2E-07	0.830
GG vs AA	1.677 (0.714, 2.640)	7.3E-04	1.697
CYP2C9 rs1057910			
*1/*3 vs *1/*1	−0.714 (−1.466, 0.038)	0.06	−0.936
Amiodarone status	−0.475 (−0.972, 0.022)	0.06	−0.550

Predictor variables in the multiple regression are same as those in the pharmacogenetic dosing algorithm proposed by the IWPC; enzyme inducer status is not shown because none of the subjects was taking any of the enzyme inducers listed in the IWPC algorithm (ie, carbamazepine, phenytoin, rifampin or rifampicin).
A dependent variable is the square root of the warfarin dose in mg/week.
*Intercept used for patients of Asian race with AA genotype at *VKORC1* rs9923231 and *1/*1 genotype at *CYP2C9* rs1057910.
CI, confidence interval; IWPC, International Warfarin Pharmacogenetics Consortium; *VKORC1*, vitamin K epoxide reductase complex subunit 1.

Table 3. Percentage of Patients With Ideal, Underestimated or Overestimated Dose of Warfarin, as Estimated by the Pharmacogenetic and Clinical Algorithms, in Patients Requiring Low, Intermediate or High Actual Doses of Warfarin

Actual dose required	No. of patients	Patients classified by performance of prediction			Difference between 2 algorithms, P value
		Ideal dose (error within ≤7 mg/week), %	Underestimated, %	Overestimated, %	
≤10.5 mg/week	25				0.046
Pharmacogenetic algorithm		68	0	32	
Clinical algorithm		36	0	64	
>10.5 to <31.5 mg/week	151				0.613
Pharmacogenetic algorithm		80	19	1	
Clinical algorithm		79	18	3	
≥31.5 mg/week	24				0.050
Pharmacogenetic algorithm		21	79	0	
Clinical algorithm		0	100	0	

The clinical algorithm involves clinical and demographic variables (age, height, weight, and medication), and the pharmacogenetic algorithm involves the same set of variables plus genotypes in 2 genes (*CYP2C9* and *VKORC1*).
Underestimate is the case where the predicted dose (by either pharmacogenetic or clinical algorithm) is lower than the observed dose; overestimate is the opposite.
VKORC1, vitamin K epoxide reductase complex subunit 1.

(n=119, 59.5%), prosthetic valve replacement (n=27, 13.5%), and deep vein thrombosis or pulmonary embolism (n=12, 6%). The minor allele frequencies of rs1057910 (*CYP2C9*) and rs9923231 (*VKORC1*) were 0.013 and 0.093, respectively, which were comparable to those previously reported in Japanese patients[5,15,17,18] or HapMap JPT (http://hapmap.ncbi.nlm.nih.gov/): 0.016–0.049 for rs1057910 and 0.075–0.088 for rs9923231. Each SNP was in Hardy-Weinberg equilibrium (P>0.05).

The effects of predictor variables on warfarin dose were first examined in the ordinary regression model (**Table 2**). The effect sizes of individual variables thus estimated were almost comparable to those in the IWPC algorithm.[8] When applied to the Japanese patients' data, the IWPC pharmacogenetic algorithm identified significantly (P<0.05) larger proportions of patients who required 10.5 mg or less per week (low-dose group) or those who required 31.5 mg or more per week (high-dose group) to achieve the target PT-INR than did the clinical algorithm (68% vs 36% in low-dose group; and 21% vs 0% in high-dose group; **Table 3**). We depicted the fair performance of the IWPC pharmacogenetic algorithm in the plots comparing the predicted dose and actual dose of warfarin (R^2=0.28, P=1.5×10^{-15}) (**Figure**).

A significant benefit of using genetics was further verified with the NNG analysis (**Table 4**). The NNG can be computed using the number needed to treat (NNT) method; the NNT is the inverse of the absolute risk reduction (ARR). The ARR was calculated as the absolute difference between the event rate (ER) for the pharmacogenetic algorithm and the ER for the clinical algorithm (ER = ratio of the number of patients for which an algorithm estimates a poor dose (more or less than 7 mg/week than the actual therapeutic dose) over the total number of patients). Despite different criteria for a poor dose estimate (ie, the criteria in the IWPC study[8] were >20% above or below the actual therapeutic dose), the NNG was in good agreement between the studies: 13.3 in the present study and 13.2 in the IWPC study.

Our data on Japanese patients also indicated that both

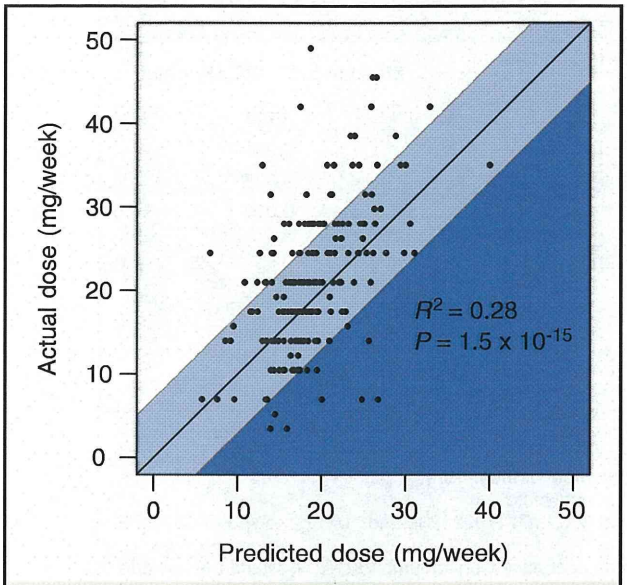

Figure. Predicted vs actual stable therapeutic warfarin dose (mg/week) for 200 Japanese patients in the present study. The diagonal solid (black) line indicates the perfect prediction, above and below which the gray lines indicate thresholds that the predicted dose according to the International Warfarin Pharmacogenetics Consortium pharmacogenetic algorithm is 7 mg/week lower than the actual dose (underestimation) or 7 mg/week higher than the actual dose (overestimation).

alcohol drinking and smoking significantly influence warfarin dose (Table 5). In the multiple regression model, the current status of drinking or non-drinking (ex-drinker+never-drinker) and that of smoking or non-smoking (ex-smoker+never-smoker) exerted approximate warfarin dose effects of 4 mg/week (P=9.5×10^{-5}, R^2=0.06) and 3.3 mg/week (P=0.03, R^2=0.02), respectively. With these predictor variables being incorporated into the IWPC algorithm, its performance was augmented (R^2=0.33, P=5.5×10^{-19}) (Figure S2).

Table 4. NNG Analysis: Clinical vs Pharmacogenetic Algorithm With ±7 mg/week Criterion

No. of events (n=200)	
Clinical >7 mg/week than actual	21
Clinical <7 mg/week than actual	51
Pharmacogenetic >7 mg/week than actual	10
Pharmacogenetic <7 mg/week than actual	47
Absolute risk reduction	0.075
NNG	13.3

NNG, number needed to Genotype.

Discussion

We have evaluated the IWPC pharmacogenetic algorithm in 200 Japanese patients under low-dose warfarin treatment. Although the target PT-INR (1.6–2.6) in the present study was slightly lower than the range (2.0–3.0) set in the IWPC study,[8] the performance of the tested algorithms in the 2 studies proved almost comparable: R^2=0.28 in the present study and R^2=0.33–0.34 for Asians in the IWPC study. Besides the reproducible performance in the whole study sample, of particular note is the fact that among patients in a low-dose group (≤10.5 mg/week), the percentage of overestimation was significantly smaller when the warfarin dose was predicted with the pharmacogenetic algorithm (32%) than with the clinical algorithm (64%) (Table 3), thus enabling us to appreciably reduce the risk of overanticoagulation. Furthermore, we demonstrated the substantial influence of alcohol drinking and smoking on warfarin dose requirements, which used to be anticipated but has not been evaluated in detail thus far.[2]

The incidence of major bleeding (eg, intracranial hemorrhage) has been reported as higher in Japanese patients (6.6% per year) than in European patients (1.6–2.5% per year) with adjusted standard-dose warfarin therapy: a target PT-INR of 2.2–3.5 in the Japanese and 2.0–4.5 in Europeans.[19–22] Because of such racial differences in bleeding tendency under warfarin, the necessity of customizing warfarin therapy has been argued.[10] Recently, a prospective study of 4,202 patients

Table 5. Effects of Alcohol Drinking and Smoking on Warfarin Doses in Different Regression Models

Model	Tested predictors	Approximate effect on warfarin dose in mg/week*		
		Effect (95%CI)	P value	R^2
Alcohol drinking				
Model 1	Stopped vs Yes	−4.1 (−6.6, −1.5)	0.003	0.06
	No vs Yes	−3.9 (−6.0, −1.7)	6.7E-04	
Model 2	Stopped/No vs Yes	−4.0 (−5.8, −2.1)	9.5E-05	0.06
Model 3	Alcohol unit (gou) per week†	0.6 (−0.05, 1.2)	0.070	0.01
Smoking				
Model 1	Stopped vs Yes	−3.5 (−6.4, −0.4)	0.03	0.02
	No vs Yes	−2.9 (−6.0, 0.4)	0.09	
Model 2	Stopped/No vs Yes	−3.3 (−6.1, −0.3)	0.03	0.02
Model 3	Brinkman index [amount per day×years]†	−0.02 (−0.1, 0.1)	0.56	0.001

Alcohol drinking was categorized into 3 groups: current drinker (yes), abstainer (stopped), and never-drinker (no), based on the self-reported questionnaire. Likewise, smoking status was categorized into 3 groups: current smoker (yes), ex-smoker (stopped) and never-smoker (no).
*Predictors in Table 2 were included as covariates in the tested regression model.
†The square root of the value was used for the regression analysis.
CI, confidence interval.

showed an optimal PT-INR of 3.0–3.5 in the Dutch,[23] whereas the Japanese Guidelines for Pharmacotherapy of Atrial Fibrillation[24–26] (JCS 2008) have set a PT-INR of 2.0–3.0 as the therapeutic range, except for the elderly (≥70 years of age), in whom a lower dose of warfarin (PT-INR 1.6–2.6) is recommended for prevention of thromboembolism and safety from bleeding complications.[27] The intensity of warfarin control (ie, optimal PT-INR) in the Japanese remains to be further defined according to the balance between risks and benefits under individual conditions. Among the primary indications for warfarin use, the optimal therapeutic range has been debated for patients with prosthetic valve replacement,[13] corresponding to 13.5% of the current subjects (**Table 1**). Partly because of the risk of eventual valve failure of bioprosthesis, and resultant reoperation, there seems to be a tendency for increased use of prosthetic valves in Japan, with its population's long life expectancy, as compared with the USA and Europe. Including patients with prosthetic valve replacement, because the target PT-INR is often set at 1.6–2.6 in outpatient clinics in Japan,[12] our findings obtained in equivalent clinical setting should encourage clinicians to apply the IWPC algorithm to their patients. Nevertheless, in cases where the optimal therapeutic range is set differentially according to the primary disease condition, some modification of the IWPC algorithm may be required.

We have found that the average dose of warfarin in the Japanese patients is 21 mg/week (3 mg/day), which is less than the standard dose (35 mg/week) in Europeans, and in the present study one-eighth (12.5%) of the participants were categorized into a low-dose group (≤10.5 mg/week). If a fixed dose of 3 mg/day is given to these patients without conscientious monitoring of PT-INR, there is a high risk of overanticoagulation, leading to fatal bleeding events. The use of the IWPC algorithm will enable clinicians to detect approximately two-thirds (68%) of the Japanese patients in this low-dose group, which is twice as large as the proportions (36%) attainable with the clinical algorithm (**Table 3**). Although the value of adding genotype to clinical (and demographic) information seems to be less modest, benefits also accrue to Japanese patients in the high-dose group (≥31.5 mg/week); 21% of the patients were identified with the pharmacogenetic algorithm, but none (0%) with the clinical algorithm (**Table 3**).

Since the US FDA changed the labeling of warfarin to suggest that clinicians consider using genetic tests to guide dosing,[3] warfarin pharmacogenetics has drawn substantial attention towards "personalized" patient care. Although more than 30 genes may contribute to the net warfarin effect, *CYP2C9* and *VKORC1* are known to exert the most influence.[28–30] The pharmacogenetic algorithm involving these polymorphisms, developed by the IWPC, can predict approximately one-third of all dosing variations at most.[8] The question of whether the knowledge of genetic information is cost-effective in reducing bleeding and thrombotic complications is under debate.[31] Many clinical factors influence warfarin dose requirements, including diet (in particular, the vitamin K content) and concomitant drug administration, besides a list of variables that have been incorporated into the IWPC algorithm. As the clinical factors often change in individual patients, appropriate alterations in warfarin dosing must be made, regardless of genetic information. In this respect, it is important to quantitatively evaluate the individual contribution of clinical factors, as has been performed for alcohol drinking and smoking in the present study, toward refining warfarin pharmacogenetic testing for not only initial but also maintenance dose requirements.

In summary, we report the usefulness of the IWPC pharmacogenetic algorithm in its clinical application, particularly for identifying Japanese patients who require a low dose of warfarin. However, it has to be kept in mind that considering the current limitations of its application to clinical medicine, this testing alone does not make conscientious PT-INR monitoring unnecessary. When the genotype cost falls to a more reasonable level, we expect that the use of pharmacogenetic-based initial dosing will become routine clinical practice.

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Supplementary files

Figure S1. Histogram of warfarin dose.

Figure S2. Actual dose vs predicted dose with information on alcohol drinking and smoking incorporated into the International Warfarin Pharmacogenetics Consortium (IWPC) pharmacogenetic algorithm.

Please find supplementary file(s);
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REVIEW SERIES

The stroke-prone spontaneously hypertensive rat: still a useful model for post-GWAS genetic studies?

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The stroke-prone spontaneously hypertensive rat (SHRSP) is a unique genetic model of severe hypertension and cerebral stroke. SHRSP, as well as the spontaneously hypertensive rat, the parental strain of SHRSP, has made a tremendous contribution to cardiovascular research. However, the genetic mechanisms underlying hypertension and stroke in these rats have not yet been clarified. Recent studies using whole-genome sequencing and comprehensive gene expression analyses combined with classical quantitative trait loci analyses provided several candidate genes, such as *Ephx2*, *Gstm1* and *Slc34a1*, which still need further evidence to define their pathological roles. Currently, genome-wide association studies can directly identify candidate genes for hypertension in the human genome. Thus, genetic studies in SHRSP and other rat models must be focused on the pathogenetic roles of 'networks of interacting genes' in hypertension, instead of searching for individual candidate genes.
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Keywords: cerebral stroke; genetics; hypertension; QTL; SHRSP

INTRODUCTION

The stroke-prone spontaneously hypertensive rat (SHRSP) is a unique genetic model of severe hypertension and cerebral stroke. Two decades have passed since the first pioneering studies on quantitative trait loci (QTLs) of blood pressure (BP) in SHRSP.[1,2] In spite of all efforts, the genetic mechanisms underlying hypertension or cerebral stroke in this rat model remain unknown. During this period, genetic analyses in humans have progressed dramatically. Technologies have made it possible to genotype a large number of samples and analyze an enormous amount of single-nucleotide polymorphism data. Genome-wide association studies (GWAS) that rely on such advanced technologies have revealed a number of loci associated with increased BP in humans.[3–10]

Under such circumstance, what is the role of SHRSP and other models in genetic studies of cardiovascular diseases? In this review, we will address this issue and summarize the genetic studies performed thus far in SHRSP.

ESTABLISHMENT OF SHRSP

SHRSP was established from a substrain of spontaneously hypertensive rats (SHR; substrain A in Figure 1a) in 1974 by Okamoto *et al.*[11] SHRSP was created under the following circumstances: (1) the selection was started using 24th generation SHR, (2) a high stroke susceptibility was fixed only after three generations of selection and (3) severe hypertension was simultaneously fixed with the stroke susceptibility.[11] The established strain had a high incidence of stroke (80 *vs.* 10%) and severe hypertension (220–240 *vs.* 180–200 mm Hg) when compared with SHR.[11]

Although it is unknown whether strict inbreeding was applied in the initial breeding process of SHR, the genetic pool was expected to be small at the 24th generation. According to the National BioResource Project for the Rat database (http://www.anim.med.kyoto-u.ac.jp/nbr/default.aspx),[12] which collected genotypes of 357 simple sequence length polymorphism markers in 179 inbred rat strains, 7 substrains of SHR (CH, CL, B2 and Izm) and SHRSP (A1-sb, A3 and Izm), which were originally developed at Kyoto University (Figure 1a), shared 1 or 2 alleles at each of the 332 markers (93%). A total of 3 or 4 alleles were found at the other 25 simple sequence length polymorphisms among those 7 substrains. Considering the simple sequence length polymorphism markers were polymorphic enough to have 5 to 19 (or more) alleles among the 179 strains, we think it reasonable to assume the majority of the genome of SHR and SHRSP has been derived from a pair of 'ancestral' rats.

In contrast, it is important to note that the larger strain difference in the genome that does not contribute to hypertension is observed between WKY and SHR/SHRSP. This finding is principally because WKY was established independently from another pair of 'ancestral' rats in the same closed colony (Figure 1a).

SHR, SHRSP and WKY were distributed to several laboratories before they were established as fully inbred strains (Figure 1b). This process of distribution has introduced another source of variations in genetic make-up among these strains, which imposes additional difficulties when performing genetic analyses in SHR/SHRSP (see the discussion below).

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GENETIC STUDIES ON HYPERTENSION AND CEREBRAL STROKE IN SHRSP

After the seminal works published in 1991,[1,2] many QTLs for BP have been identified in SHR/SHRSP. Because of their polygenic nature, the chromosomal regions responsible for hypertension varied among pairs of hypertensive and normotensive rat strains used in QTL analyses. In fact, the Rat Genome Database (http://rgd.mcw.edu/) has compiled more than 300 QTLs influencing BP in rats,[13] and a substantial part of these QTLs were identified in experimental crosses between SHR/SHRSP and normotensive rat strains. In spite of many QTLs being identified, few causative genes have been identified thus far. In the following sections, several recent genetic studies on hypertension and stroke in SHRSP are reviewed.

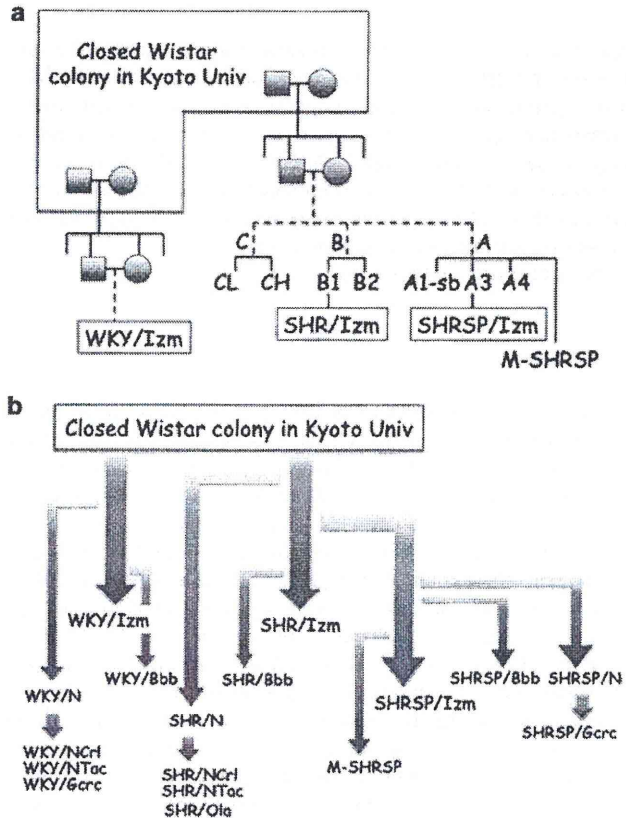

Figure 1 Origins of the SHRSP, SHR and WKY substrains. (a) The original substrains established by Okamoto and his colleagues. (b) The substrains currently used in the world.

Candidate genes detected by QTL analysis

Soluble epoxide hydrolase (Ephx2). Soluble epoxide hydrolase (sEH), encoded by the *Ephx2* gene, is an enzyme that metabolizes epoxyeicosatrienoic acids. Because epoxyeicosatrienoic acids act as vasodilators, as well as inhibitors of sodium reabsorption at the renal tubules, *sEH* is a good functional candidate gene responsible for hypertension.[14] Fornage *et al.*[14] found that mRNA expression of sEH was greatly decreased in SHRSP/Bbb (SHRSP of the Heidelberg colony) and WKY/NCrl when compared with sEH levels in WKY/Bbb and SHR/NCrl. The authors argued that the genetic variation of sEH was unlikely to contribute to the pathogenesis of hypertension in SHR, because the expression level was not in accordance with the status of hypertension among substrains of WKY and SHR. However, the fact that sEH mRNA expression was not consistently lower in all of the WKY-related strains compared with all of the SHR-related strains may not be enough to exclude a possible role for sEH in the regulation of BP.

They showed later that molecular variants in the *Ephx2* promoter were responsible for the difference in sEH expression between SHRSP/Bbb and SHR/NCrl, suggesting that low expression of sEH may be a risk factor for the stroke seen in SHRSP.[15]

An independent study by Monti *et al.*[16] showed that a genetic variation in the *Ephx2* promoter and the resulting change in sEH expression influenced the susceptibility to heart failure in SHHF, which is a model rat for heart failure derived from SHRSP.

The sEH expression was evaluated in substrains of SHR and SHRSP in several other studies.[17–19] Figure 2 summarizes the results collected from these studies. As indicated, the substrains of WKY and SHR had two haplotypes of the *Ephx2* gene, which lead to high and low sEH expression levels. We examined the *Ephx2* haplotype and sEH mRNA expression in SHR, SHRSP and WKY/Izm rats, and confirmed that SHRSP/Izm had the same haplotype as that of SHRSP/Bbb, whereas SHR/Izm and WKY/Izm shared the haplotype with SHR/NCrl. The sEH expression levels in SHRSP/Izm and WKY/Izm were low and high, respectively, which was consistent with the pattern expected from the individual haplotype (observation by Okuda *et al.*[17] and unpublished observation by Nabika *et al.*). The haplotype and expression patterns were discordant with hypertensive status among the rats examined (Figure 2). This result implied that *Ephx2* was not involved in the pathogenesis of hypertension in SHR or SHRSP. In contrast, Sellers *et al.*[20] reported that intracerebroventricular injection of an sEH inhibitor caused a significant increase in BP in SHR/NCrl, but not in WKY/NCrl. This is an interesting observation suggesting that a high sEH level in the brain of SHR opposes hypertension. In contrast, sEH expression was low in SHRSP, which may be responsible for the additional BP increase in this strain.

	-254	-112	6769	13316	13471	17284	33121	*Ephx2* exp	BP	ref.
SHR/Izm	T	G	T	A	A	T	A	High	High	u, 17
WKY/Izm	T	G	T	A	A	T	A	High	Low	u, 17
SHR/NCrl	T	G		A	A	T	A	High	High	14, 15, 17, 18
WKY/Bbb[1]				A	A	T	A	High	Low	14
SHRSP/Bbb	C	A	C	G	G	C	G	Low	High	14, 16
SHRSP/Izm	C	A	C	G	G	C	G	Low	High	u, 17
SHRSPA3	C	A						Low	High	15
WKY/Bbb[1]	C	A	C	G	G	C	G	Low[2]	Low	16
WKY/NCrl	C	A		G	G	C	G	Low	Low	14, 17, 18

Figure 2 Haplotypes and gene expression levels of *Ephx2* in the SHRSP, SHR and WKY strains. Data were compiled from the references indicated in the figure: (1) The *Ephx2* haplotype and expression pattern in WKY/Bbb appeared discrepant between Corenblum *et al.*[15] and Monti *et al.*[16] (2) Although the expression was low, it was still significantly greater than that of the SHRSP/Bbb.[16] u, unpublished observation by Nabika *et al.*

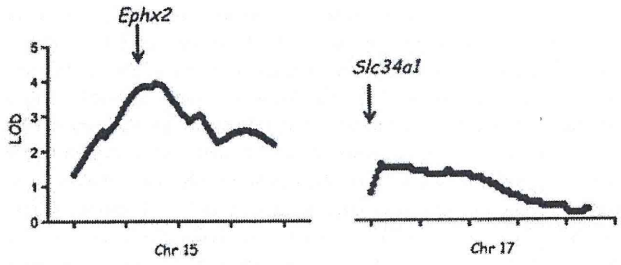

Figure 3 QTL analysis of BP in an F2 cross between SHRSP/Izm and SHR/Izm. Lod scores for BP on Chr 15 and 17 are plotted with the location of the *Ephx2* and *Slc34a* genes. A total of 294 F2 rats were used in the analysis (male and female data were combined).

We performed a QTL analysis on BP using an F2 cross between SHRSP/Izm and SHR/Izm. We found a suggestive peak on Chr 15, which included the *Ephx2* locus (Figure 3, unpublished observation). This finding may support the role of this gene on the BP difference observed between SHR and SHRSP.

Glutathione S-transferase μ-type-1 (Gstm1). McBride *et al.*[21] found that a fragment on Chr 2 was responsible for the BP difference between SHRSP/Gcrc (SHRSP of the Glasgow colony) and WKY/Gcrc through a QTL analysis and the subsequent congenic studies. On the basis of a comprehensive gene expression analysis using microarrays, they identified *Gstm1*, which had significantly lower expression in SHRSP/Gcrc, as a functional candidate gene in this chromosomal region.[21] They found that the *Gstm1* haplotype of SHRSP/Gcrc differed from that of WKY/Gcrc, which was responsible for a differential expression level of *Gstm1* between the two strains.[22]

In contrast, we found that SHRSP/Izm and WKY/Izm shared the same haplotype as SHRSP/Gcrc, and no apparent difference in *Gstm1* expression was observed between the two strains. This result was consistent with that of our QTL analysis, which showed no significant QTLs for BP on Chr 2 in the F2 cross between SHRSP/Izm and WKY/Izm (data not shown).

For both of sEH and *Gstm1*, the haplotype and the mRNA expression level were discordant with hypertensive status when substrains of WKY and SHR/SHRSP were studied. This result did not seem to support the candidacy of those genes in BP pathogenesis. However, exclusion of these genes from the list of the candidate genes should be cautiously considered; WKY may share some hypertension genes with SHR, which may raise BP only when acting in concert with other genes (see Figure 4 and the discussion below).

Sodium-dependent phosphate transport protein 2A (Slc34a1). As discussed in the first part of this review, SHR and SHRSP were derived from a small genetic pool. It is thus expected that these strains will share the same alleles in much of their genome. Doris and his colleagues[23] used such identity-by-decent areas to exclude the genomic regions that do not contribute to BP differences between SHR/B2 and SHRSP/A3. Combining the identity-by-decent information with a QTL analysis, they identified a small non-identity-by-decent area on Chr 17, which could harbor a gene (or genes) contributing to the BP difference between the two strains. On the basis of comprehensive gene expression data, they further suggested that *Slc34a1*, a sodium/phosphate co-transporter expressed in renal tubules, was a candidate gene.[23]

This is a unique strategy in that they took advantage of the common genetic backgrounds shared between SHR and SHRSP.

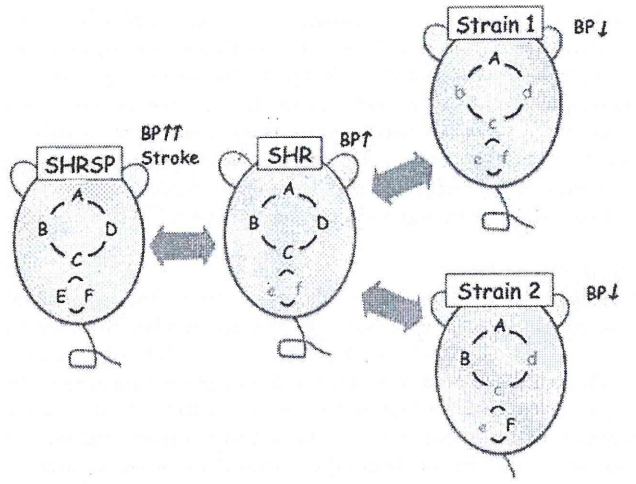

Figure 4 Hypothetical genetic composition of hypertension in SHRSP and SHR. Networks composed of multiple interacting genes are hypothesized. They have full-blown effects on BP only when all of the included genes are the 'hypertensive' allele (shown in capital letters). Consequently, even though Strain 1 harbors one hypertensive allele ('A'), it is still normotensive. If SHR and Strain 1 are used in a QTL analysis, 'gene A' would not be detected as a hypertensive gene. An SHR-based congenic strain, in which allele 'a' is substituted for 'A' would have normal BP, whereas a Strain-1-based congenic rat in which the allele 'B' is substituted for 'b' would not be hypertensive, because of the lack of alleles 'C' and 'D'. SHRSP may have another network that affects BP as well as stroke susceptibility.

Unfortunately, this QTL signal was not replicated in our classical QTL analysis using the F2 between SHRSP/Izm (=SHRSPA3) and SHR/Izm (=SHRB1; Figure 3). Thus, further studies are required to obtain a definite conclusion.

It is highly likely that substrains of SHR/SHRSP share many of the same alleles that promote hypertension. On the basis of this assumption, Doris and his colleagues[24] attempted to identify candidate genes for hypertension by exploring genes differentially expressed between the substrains of SHR/SHRSP and those of WKY. It was rather surprising that only 36 genes were selected under this criterion. They further refined the list of candidates using other criteria concerning polymorphisms in the promoter regions and gene location to select four genes as candidates. Functional studies on these genes in hypertension are expected.

Intermediate phenotype: sympathetic nerve activity

A strong QTL for BP on Chr 1 was identified in SHRSP/Izm, which was confirmed in congenic rats.[25–27] Simultaneously, our search of intermediate phenotypes indicated sympathetic hyper-responsiveness to stresses in the congenic strains constructed for this QTL.[28–30] A follow-up study using preparations of isolated neonatal brain stem confirmed that the electrophysiological nature of neurons in the rostral ventrolateral medulla, one of the most important centers for sympathetic activity regulation, were influenced by the Chr 1 QTL, suggesting that rostral ventrolateral medulla is one of the primary targets of the gene(s) in this QTL.[31] The congenic interval was further narrowed to a 1.8-Mbp region on Chr 1, in which the responsible genes are now being explored.[32]

If networks of interacting genes underlie the pathogenesis of hypertension, it is a difficult task to clarify them as a whole. Intermediate phenotypes may be regulated directly by the individual genes included in such 'causal networks', and it may be more feasible to

identify genes responsible for changes in intermediate phenotypes. Of course, many intermediate phenotypes have their own complex nature as well, and this is the key to searching for adequate target phenotypes. Information about such genes for intermediate phenotypes would be a useful resource in the investigation of gene networks underlying hypertension. The most visible example of such an intermediate phenotype is expression QTLs; this method analyzes *cis* and *trans* elements of the genes regulating mRNA expression.[33]

Stroke susceptibility

SHRSP shows a high incidence of cerebral stroke. The incidence of spontaneous stroke is approximately 80% and reaches 100% with a high-salt diet (Okamoto *et al.*[11] and unpublished observation). Cerebral stroke in SHRSP is not based on atherosclerosis, as is the major subtype of cerebral infarction in humans. It is, instead, similar to brain edema due to malignant hypertension and lacunar infarction, and cerebral hemorrhage caused by arteriosclerosis or hyalinosis of small arteries due to severe hypertension.[34] Besides hypertension, additional genetic factors were implicated in the stroke susceptibility of SHRSP, such as neuronal vulnerability to ischemic insult, dysfunctional blood–brain barrier and arterial histological abnormalities.[34]

QTL studies were performed on infarction tissue volume after artificial middle cerebral artery occlusion and stroke latency. Rubattu *et al.*[35,36] identified QTLs for stroke latency on Chr 1, 4 and 5 in an F2 intercross between SHRSP/Bbb and SHR/Bbb, of which the QTL on Chr 1 was confirmed in congenic strains. A QTL analysis in an F2 cross between SHRSP/Gcrc and WKY/Gcrc by Jeff *et al.*[37] showed a strong linkage of the markers on Chr 5 with the infarction volume after middle cerebral artery occlusion. Both studies indicated that the QTLs identified affected the stroke-related phenotypes independently of BP. Although the atrial natriuretic peptide gene was focused on in the Chr 5 QTL,[37,38] further studies on this gene and genes in other QTLs have not yet been performed.

Systematic gene expression analysis: *Cd36*

The study on *Cd36* was another seminal work that first applied comprehensive gene expression analysis in the QTL/congenic strategy, which provided a prototype for studies performed thereafter.[39] Aitman *et al.*[39] showed that an SHR/NIH derived strain had a deletion of *Cd36* located in a QTL region, suggesting that this gene was contributing to insulin resistance in this strain. Using transgenic rescue studies, Pravenec *et al.*[40,41] definitively demonstrated that genetic deficiency in the expression of *Cd36* can contribute to both insulin resistance and increased BP in rats derived from the SHR/NIH strain. In the following studies on rats and humans, substantial evidence was accumulated supporting the role of *Cd36* in hypertension and insulin resistance.[42] On the other hand, it was reported that SHR/Izm, which is a substrain of SHR used in Japan, did not have the *Cd36* deletion though it still showed insulin resistance as well as hypertension.[43] This observation indicated that *Cd36* did not have a major role in insulin resistance and hypertension in SHR/Izm.[43,44] Although the role of *Cd36* in insulin resistance was not denied, further studies on the insulin resistance in SHR are necessary to obtain a comprehensive view of this issue.

ROLE OF GENETIC ANALYSIS OF SHR AND SHRSP IN THE POST-GWAS ERA

As discussed above, it is quite common that genetic analyses gave discrepant results when different sets of SHR/SHRSP and normotensive rat strains were employed.

If the goal of QTL analyses is set to identify candidate genes in rat models, the inconsistency among the QTL studies does not matter; identified genes in rats are examined as candidates in humans anyway. Ten years ago, we did not have the tools to dissect candidate genes from the human genome, and thus, the candidate genes found in rats gave us important clues. In contrast, we are now able to extract many candidate genes (or single-nucleotide polymorphisms) directly from the human genome through large-scale GWAS.[3–10] If species differences between rodents and humans are considered, the importance of genetic model rats as a 'supplier' of candidate genes is relatively diminished.

Under such conditions, what is the role of genetic analyses in SHR/SHRSP? To answer this question, it may be useful to focus on the genetic composition of hypertension in SHR/SHRSP.

There are four possible models for this application:

(a) Single gene model: This is not likely if accumulated results of classical segregation studies as well as a number of QTL analyses are taken into consideration.
(b) Polygenic additive model: This assumes many weak causative genes distributed throughout the genome, which affect BP in an additive manner. This model is currently assumed in human GWAS. In the case of SHR/SHRSP, this model is not likely when only two to three generations were necessary to achieve substantial increases in BP during the original development process.[45]
(c) Oligogenic additive model: A limited number of causative genes with large effects additively contribute to hypertension. This may be applicable to SHR/SHRSP; however, under this model, asymmetrical effects of some QTLs in reciprocal congenic strains may be difficult to interpret (Figure 5a).
(d) Oligogenic synergistic model: Synergistic interactions among a few genes are required. This model may be the best to describe the genetic composition of hypertension in SHR/SHRSP. Many studies on congenic strains suggested that one QTL was composed of several genes interacting with one another.[46] In spite of a lack of sufficient evidence, similar interactions can be hypothesized among QTLs on separate chromosomes.

If hypertension in SHR/SHRSP is realized under the model (D), it is less useful to examine the individual candidate gene identified in SHR/SHRSP in the current genetic studies performed in humans, because in GWAS and other genetic studies in humans, gene–gene interactions are not generally considered. Instead, SHR/SHRSP needs to be analyzed as 'a total gene network' underlying hypertension and cardiovascular complications.

According to the National BioResource Project for the Rat database, SHRSP is the strain showing the highest BP among 179 strains.[47] This finding implies that SHRSP has a unique set of hypertension genes that makes it distinct from any other rat strain. Although some genes in this set may be shared with other strains, it is likely that gene–gene interactions are necessary for these genes to manifest a full-blown effect on BP (Figure 4).

It is, therefore, vital to clarify the gene network as a whole in SHRSP rather than to identify individual candidate genes. Knowledge about such a network will be useful to reveal the pathogenesis of human hypertension even if the individual genes involved in the network are not identical.

Still, many single-nucleotide polymorphisms influencing BP have been identified in human GWAS, and there are strong arguments against the clinical significance of these single-nucleotide polymorphisms due to

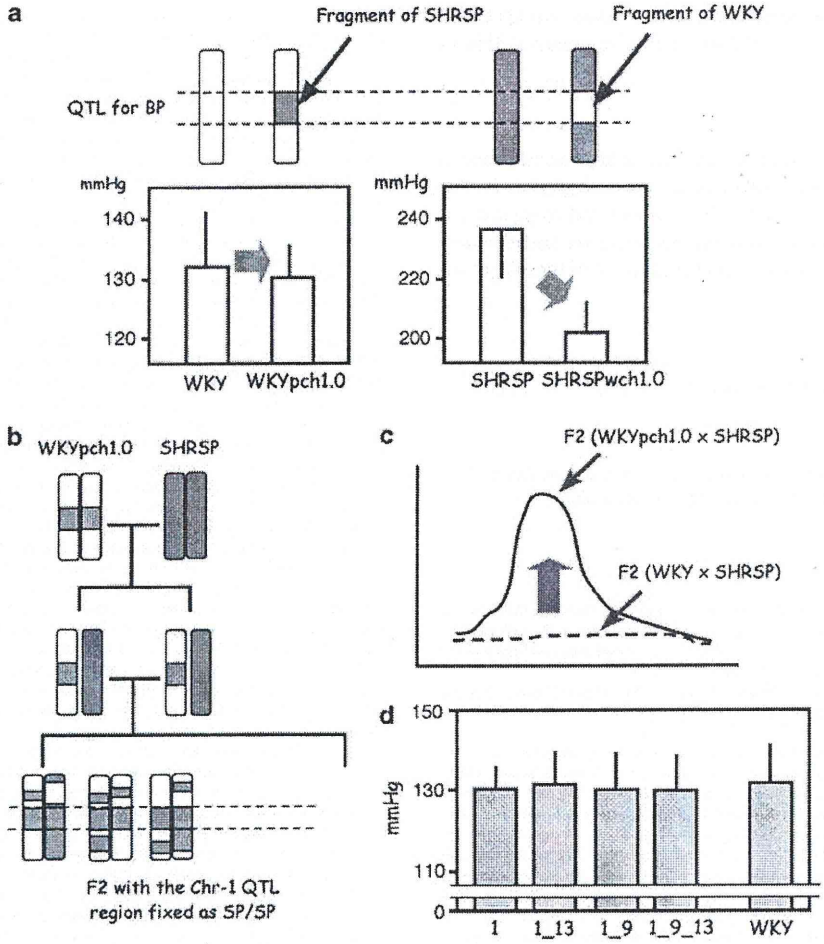

Figure 5 A trial to show interactions among QTLs in SHRSP. (**a**) Reciprocal congenic rats for the Chr 1 QTL do not show reciprocal effects of the QTL on BP. WKYpch1.0 and SHRSPwch1.0 are reciprocal congenic strains constructed for the Chr 1 QTL.[29,31,32] (**b**) The study design of a QTL analysis using a congenic rat (WKYpch1.0) and SHRSP. In all the F2 rats, the Chr 1 QTL is fixed as homozygous for the SHRSP allele. (**c**) An expected, result of the QTL analysis illustrated in (**b**). A new QTL may appear only in the F2 between the congenic rat (WKYpch1.0) and SHRSP, because of the requirement of interactions with the Chr 1 QTL. (**d**) BP of double and triple congenic rats. On the basis of the QTL analysis shown in (**b**), double congenic rats for Chr 1 and 9 (shown as 1_9) and for Chr 1 and 13 (1_13) QTLs and a triple congenic rat for the three QTLs (1_9_13) were constructed. Evaluation of BP indicated that no significant rise in BP was observed in these congenic strains.

their weak effects.[48,49] If a network of interacting genes is shown to be essential in the pathogenesis of hypertension in rats, it may provide new insights into the pathogenic mechanisms of human hypertension.

In this regard, whole-genome sequencing of multiple rat strains, including several SHR and SHRSP substrains, which is ongoing in the EuraTrans project (http://www.euratrans.eu/), will provide useful information. A comprehensive analysis of the genome sequence combined with the analysis of a gene expression network recently succeeded in identifying a new gene responsible for type I diabetes mellitus.[50] A similar bioinformatics strategy may be able to dissect the network underlying hypertension in SHR/SHRSP.[19,51]

In addition to such bioinformatics studies, another tool may be useful to promote physiological, cell biological and biochemical studies: a 'reconstructed' SHR with a few genomic fragments of SHR/SHRSP on the WKY background.

The initial process of development of SHR as well as a classical segregation study by Tanase *et al.*[52] suggested that only a limited number of genetic loci (or QTLs) were involved in hypertension in SHR.[45] This observation implies that the appropriate combination of several genomic regions of SHR/SHRSP can 'reconstruct' hypertension on the WKY background to some extent. Such a 'reconstructed' SHR/SHRSP can then be used to evaluate the effects of interacting QTLs on various biochemical and physiological processes in combination with WKY.

To test this possibility, we performed a QTL analysis on an F2 cohort constructed by crossing WKYpch1.0 (a WKY-based congenic strain for the Chr 1 QTL) and SHRSP. Under this study design, the Chr 1 QTL was fixed as homozygous for the SHRSP allele in all the F2 progenies; and thus, additional detected QTLs would be those interacting with the Chr 1 QTL (Figure 5b and c). The results indicated that two regions on Chr 9 and 13 showed a weakly suggestive linkage with BP (unpublished observation). However, as these linkage signals were detected in an F2 cross between WKY and SHRSP, it did not seem that these QTLs interacted with the Chr 1 QTL originally identified. In fact, a confirmation study using double and triple congenic strains for these QTLs showed no apparent increase in BP, translating to a failure of 'reconstructing' SHR on the WKY background (Figure 5d, unpublished observation). This result may

indicate that complex interactions between more than two QTLs are necessary to raise BP. We continue the attempt to 'reconstruct' SHR in our laboratory.

CONCLUSIONS

SHRSP will continue to have an important role in the genetic research of hypertension, if the putative networks of interacting genes in this model become better understood. To obtain direct and more convincing evidence, additional information and resources for better understanding of the genetic and genomic architecture of SHR/SHRSP are required.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Effects of hydrogen-rich water on abnormalities in a SHR.Cg-*Lepr*^{cp}/NDmcr rat - a metabolic syndrome rat model

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Abstract

Background: Hydrogen (H_2), a potent free radical scavenger, selectively reduces the hydroxyl radical, which is the most cytotoxic of the reactive oxygen species (ROS). An increase in oxygen free radicals induces oxidative stress, which is known to be involved in the development of metabolic syndrome. Therefore, we investigated whether hydrogen-rich water (HRW) affects metabolic abnormalities in the metabolic syndrome rat model, SHR.Cg-*Lepr*^{cp}/NDmcr (SHR-cp).

Methods: Male SHR-cp rats (5 weeks old) were divided into 2 groups: an HRW group was given oral HRW for 16 weeks, and a control group was given distilled water. At the end of the experiment, each rat was placed in a metabolic cage for 24 h, fasted for 12 h, and anesthetized; the blood and kidneys were then collected.

Results: Sixteen weeks after HRW administration, the water intake and urine flow measured in the metabolic cages were significantly higher in the HRW group than in the control group. The urinary ratio of albumin to creatinine was significantly lower and creatinine clearance was higher in the HRW group than in the control group. After the 12-h fast, plasma urea nitrogen and creatinine in the HRW group were significantly lower than in the control group. The plasma total antioxidant capacity was significantly higher in the HRW group than in the control group. The glomerulosclerosis score for the HRW group was significantly lower than in the control group, and a significantly positive correlation was observed between this score and plasma urea nitrogen levels.

Conclusion: The present findings suggest that HRW conferred significant benefits against abnormalities in the metabolic syndrome model rats, at least by preventing and ameliorating glomerulosclerosis and creatinine clearance.

Keywords: hydrogen-rich water, renal glomerulosclerosis, metabolic syndrome model rats, oxidative stress

Background

Hydrogen (H_2), a potent free radical scavenger, selectively reduces the hydroxyl radical, which is the most cytotoxic of the reactive oxygen species (ROS). In addition, water saturated with H_2 (H_2-rich water) (HRW) orally administered to rats reduces oxidative stress in the animals, suggesting the molecule's anti-oxidative potency. Molecular H_2 reportedly acts as a therapeutic antioxidant by reducing cytotoxic oxygen radicals [1]; however, its beneficial effects on pathophysiological functions remain unknown.

Oxidative stress represents an imbalance between the production of ROS and the activity of the antioxidant defense system. An increase in oxygen free radicals induces oxidative stress, which is known to be involved in the development of metabolic syndrome. Metabolic syndrome is characterized by a cluster of metabolic risk factors for atherosclerosis, including obesity, insulin resistance, hyperglycemia, hyperlipidemia, and hypertension [2-4]. Metabolic syndrome also increases susceptibility to chronic renal disease [5]. Drinking HRW is a potentially novel therapeutic and preventive strategy

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against metabolic syndrome [6]. Thus, the antioxidative potency of HRW may affect the development of metabolic syndrome.

Here, with the use of the SHR.Cg-*Lepr*^{cp}/NDmcr (SHR-cp) rat, a metabolic syndrome rat model, we investigated whether HRW affects the rats' metabolic abnormalities. SHR-cp rats spontaneously develop obesity, hypertension, hyperlipidemia, hyperglycemia, and hyperinsulinemia, i.e., metabolic syndrome [7]. The syndrome is comprised of several risk factors for organ damage that operate at high levels of intensity [8]. Thus, this rat model appears well suited for assessing the renal changes induced by broad metabolic abnormalities and the development of glomerular damage such as focal and segmental glomerulosclerosis.

Materials and methods

Animals

Male SHR-cp rats (5 weeks old) supplied by the Disease Model Cooperative Research Association (Kyoto, Japan) were randomly divided into 2 groups: an HRW group (n = 12) was given oral HRW for 16 weeks, and a control group (n = 12) was given distilled water. Nakao *et al.* have described the production and characterization of HRW [6]. HRW was prepared by dipping a plastic-shelled product (stick) consisting of metallic magnesium (99.9% pure) and natural stones (Doctor SUISOSUI®; Friendear Inc., Tokyo, Japan) into distilled water. HRW was freshly prepared every other day in a 200-mL bottle containing the stick, and the H_2 concentration was maintained between 0.3 and 0.4 ppm during the experiment. The HRW contained 23 mg/L of calcium, 5 mg/L of magnesium, 19 mg/L of sodium, less than 1 mg/L of potassium and a pH of 7.2. SHR-cp rats were housed in an air-conditioned animal room with a 12:12-h dark: light cycle under controlled temperature (23 ± 2°C) and humidity (50 ± 10% relative humidity). They were given free access to a Quick Fat diet (CLEA Japan Inc., Tokyo, Japan) and a bottle containing either HRW or distilled water. The water intake of the rats was measured every 2 days. All animal experiments were carried out in accordance with the procedures outlined in the Guidelines for Animal Experimentation of Shimane University compiled from the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science.

Urine and blood collection

After 16 weeks of HRW ingestion, each rat was weighed and placed in a metabolic cage for 24-h urine collection. Following this, the rat was fasted for 12 h and anesthetized with intraperitoneal sodium pentobarbital (65 mg/kg); its blood was then collected and its kidneys excised.

Biochemical measurements in blood and urine

Plasma total cholesterol, triglycerides, glucose, creatinine and blood urea nitrogen (BUN) concentrations were determined with an automatic analyzer (BiOLiS 24i; Tokyo Boeki Medical System Ltd., Tokyo, Japan). The concentration of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in plasma was determined by enzyme immunoassay (Highly Sensitive 8-OHdG Check; Japan Institute for the Control of Aging, Shizuoka, Japan). The plasma total antioxidant capacity levels were determined by the biological antioxidant potential (BAP) test (Free Radical Analytical System 4; H&D srl, Parma, Italy). The BAP measurement is based on the ability of a colored solution containing a source of ferric (Fe^{3+}) ions adequately bound to a special chromogenic substrate (thiocyanate derivative) to discolor when Fe^{3+} ions are reduced to ferrous ions (Fe^{2+}) in response to the reducing activity of blood samples [9]. Urine albumin and creatinine levels were measured using the Nephrat kit for the quantitation of rat urinary albumin and the Creatinine Companion kit (Exocell, Philadelphia, PA) according to the manufacture's instructions. The ratio of the concentrations of albumin to creatinine (AC ratio) in urine was used as an index of urinary albumin excretion. Endogenous creatinine clearance (CrCl) was determined as $CrCl = Ucr \times V \times Pcr^{-1}$, where Ucr and Pcr are urinary and plasma creatinine concentrations, respectively, and V is urine flow. The Ucr and V values were calculated from the data of SHR-cp rats in metabolic cages, and Pcr values were cited from Table 1. The CrCl was used as an index of glomerular filtration rate (GFR).

Morphological analysis

Coronal sections of renal tissue (3-4 μm thick) were stained with periodic acid-Schiff (PAS) and examined by light microscopy in a blinded fashion.

Table 1 Biochemical parameters of plasma

	Control group (n = 12)	HRW group (n = 12)
Triglyceride (mg/dL)	443.9 ± 34.5	548.8 ± 50.4
Total cholesterol (mg/dL)	151.3 ± 4.8	153.3 ± 7.6
Glucose (mg/dL)	217.0 ± 35.8	229.2 ± 45.6
BUN (mg/dL)	24.0 ± 0.7	20.9 ± 0.7*
Creatinine (mg/dL)	0.25 ± 0.02	0.20 ± 0.01*
BAP (μmol/L)	2148 ± 91.6	2620 ± 159*
8-OHdG (μg/mL)	0.266 ± 0.02	0.250 ± 0.01

BAP, biological antioxidant potential; BUN, blood urea nitrogen; HRW group; rats orally administered with hydrogen-rich water; 8-OHdG, 8-hydroxy-deoxyguanosine.

At the end of this study, each rat was weighed and placed in a metabolic cage for 24-h urine collection. After urine collection, the rat was fasted for 12 h and anesthetized, and its blood was collected. Values represent mean ± SE. *$P < 0.05$.

Glomerulosclerosis was semi-quantitatively evaluated according to criteria developed by Uehara *et al* [10]. Briefly, 50 glomeruli were randomly selected from each animal for morphometric analysis. Glomerulosclerosis, defined as synechiae formation by PAS staining with focal or global obliteration of capillary loops, was graded as follows: 1+, < 30% of glomerular area affected; 2+, 30% to 70% affected and 3+, > 70% affected. The overall glomerulosclerosis score per animal was the average grade of all the glomeruli evaluated.

Statistical analysis

All data are expressed as the means ± SE. Significant differences between HRW and control groups were determined by the unpaired Student's *t*-test. Correlation was determined by Pearson's correlation analysis. Differences of $P < 0.05$ were considered significant. PASW Statistics 18 was used for the statistical analysis (SPSS Inc., Chicago, IL, USA).

Results

Body weight and HRW intake

HRW administration did not affect the body weight of SHR-cp rats throughout the experimental period (Figure 1). The volume of water intake per 24 h measured in the metabolic cages was larger in the HRW group than in the control group (Table 2).

Plasma biochemical data, water intake, and parameters of renal functions

The plasma biochemical data in the control and HRW rats fasted for 12 h after 16 weeks are listed in Table 1.

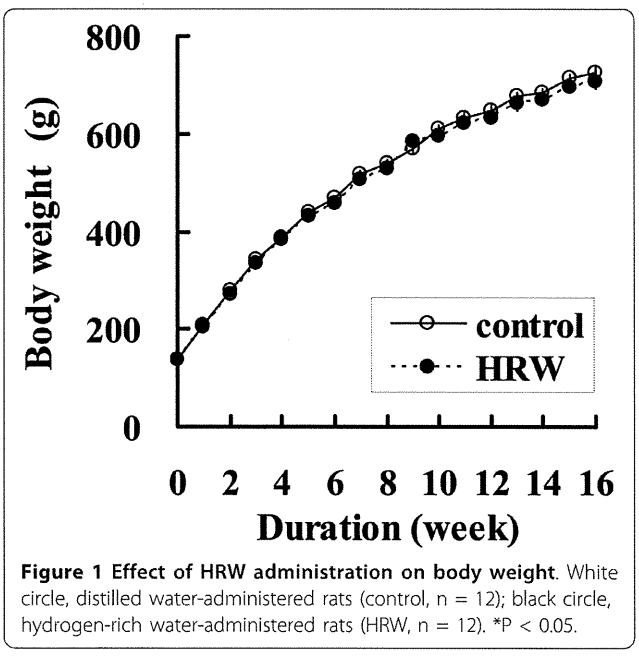

Figure 1 Effect of HRW administration on body weight. White circle, distilled water-administered rats (control, n = 12); black circle, hydrogen-rich water-administered rats (HRW, n = 12). *P < 0.05.

Plasma BUN and creatinine concentrations were significantly lower in the HRW group than in the control group. There was no significant difference in the concentrations of plasma triglyceride, total cholesterol, or the level of plasma 8-OHdG between the 2 groups; however, the plasma BAP level in the HRW group was significantly higher.

The water intake and renal function parameters measured in the metabolic cages at 16 weeks of HRW administration are listed in Table 2. Water intake and urine flow measured for 24 h in the metabolic cages were significantly higher in the HRW group than in the control group. Urine albumin was lower, but not significantly so, in the HRW group than in the controls ($0.05 < P < 0.1$), leading to an albumin to creatinine ratio of 24.1% in the HRW group that was significantly lower than that in the control group. CrCl increased with HRW administration in SHR-cp rats, with a 21.7% potentiation compared with the control group.

Effect of HRW administration on glomerular sclerosis

HRW administration inhibited histological damage to the kidneys of SHR-cp rats (Figure 2). The glomerular sclerosis score was significantly lower in the HRW group (1.46 ± 0.06) than in the control group (1.75 ± 0.11) (Figure 3A). Simple regression analyses were performed to determine whether an alteration in the glomerulosclerosis score was associated with plasma BUN and other parameters used as indices of kidney damage. A significantly positive correlation was observed only between the glomerulosclerosis score and plasma BUN levels (Figure 3B), while correlation of the former with other variables such as water intake, urine flow, and CrCl was not statistically significant.

Discussion

The hydroxyl radical and peroxynitrite are the strongest oxidants that react indiscriminately with nucleic acids, lipids, and proteins, resulting in DNA fragmentation, lipid peroxidation, and protein inactivation. Molecular H_2 reduces the hydroxyl radical and peroxynitrite *in vitro* and induces therapeutic antioxidant activity in the rat middle cerebral artery occlusion model [1]. HRW ingestion reduces oxidative stress in human subjects with potential metabolic syndrome, suggesting that HRW represents a potentially novel therapeutic and preventive strategy for metabolic syndrome [6]. Oxidative stress represents an imbalance between the production of ROS and the activity of the antioxidant defense system. Cardinal *et al.* reported that both local and systemic concentrations of H_2 measured in the kidneys and serum following oral administration of HRW peaked within 15 min after ingestion, proving that HRW is an effective mode of delivery for H_2 [11]. The continuous

Table 2 Effects of hydrogen-rich water (HRW) on water intake and renal functions in SHR-cp rats

	Water intake	Urine flow	Urine			Creatinine clearance
			Albumin (A)	Creatinine (C)	AC ratio	
	(mL/kg BW. day)	(mL/kg BW. day)	(mg/kg BW. day)	(mg/kg BW. day)		(mL/min)
Control group (n = 10)	71.1 ± 1.8	47.5 ± 2.4	161.7 ± 14.0	24.4 ± 1.3	6.81 ± 0.64	5.16 ± 0.4
HRW group (n = 11)	113.7 ± 3.5	62.0 ± 6.3	129.9 ± 10.9	25.1 ± 0.6	5.17 ± 0.42	6.28 ± 0.36

Values are mean ± SE. *P < 0.05, BW, body weight; AC ratio, albumin to creatinine ratio.
These data were obtained from SHR-cp rats housed for 24 h in metabolic cages, except that of plasma creatinine concentrations, which were cited in Table 1.

incorporation of H_2 from the stomach into the blood may alter the state of blood components to a reductive one. Indeed, the plasma BAP levels of SHR-cp rats in the HRW group were significantly higher than that in the control group (Table 1) in this study. Therefore, continuous exposure to H_2 may influence the oxidative state in organ tissues.

Light microscopy has shown that SHR-cp rats develop glomerular damage, mesangial expansion, and focal and segmental glomerular sclerosis; thus, the glomerulosclerosis score in SHR-cp rats is higher than that in Wistar Kyoto (WKY) rats [8]. In the present study, the glomerulosclerosis score in the HRW group was lower than that in the control group (Figure 3A), suggesting a preventive effect of HRW administration on the development of histologically evident glomerular injury observed in the SHR-cp rats. Increases in plasma creatinine and/or BUN levels were considered indices of damage to renal function. Indeed, the BUN level in SHR-cp rats is 1.65 times greater than that in WKY rats [8]. In this study, HRW administration decreased the plasma BUN and creatinine levels of the SHR-cp rats compared with those of the control rats (Table 1). The HRW administration-induced decreases in plasma BUN and creatinine levels were consistent with the results recently reported by Nakashima-Kaminura *et al* [12]. They reported that HRW prevented metamorphosis-associated decreased apoptosis in the kidney and nephrotoxicity as assessed by serum creatinine and BUN levels. Moreover, HRW ingestion significantly decreases plasma creatinine levels in human subjects with potential metabolic syndrome [6]. These results suggest that continuous HRW administration appears to prevent and ameliorate histological damage to the kidneys.

Recent studies have indicated that metabolic syndrome increases susceptibility to chronic kidney disease [5]. Glomerular and tubulointerstitial damage characteristic of human type II diabetic nephropathy (e.g., focal and segmental glomerular sclerosis) develops in SHR-cp rats together with evidence of increased oxidative stress [13]. In this study, continuous administration of HRW did not affect the body weight or plasma levels of triglycerides, total cholesterol, or glucose in SHR-cp rats, but significantly inhibited the deterioration of glomerulosclerosis. Continuous HRW administration also decreased the urinary AC ratio, which can be used to diagnose the early stages of diabetic nephropathy in patients with diabetes [14]. In clinical practice, the measurement of CrCl remains the most widely used method for obtaining a GFR index. SHR-cp rats develop

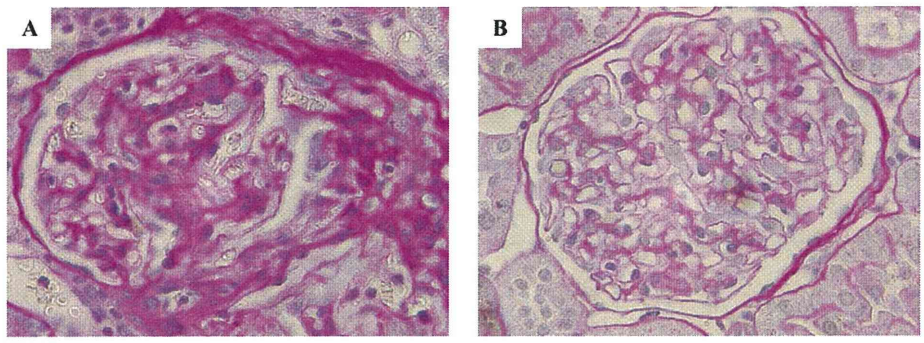

Figure 2 Photomicrographs of coronal sections of the glomeruli from SHR-cp rats. (A) Control group. (B) HRW group. Periodic acid-Schiff (PAS) staining of the control group revealed glomerular damage, which was characterized by segmental glomerular sclerosis and the formation of synechiae by the attachment of parietal epithelial cells to the denuded glomerular basement membrane (PAS stain, original magnification ×400).

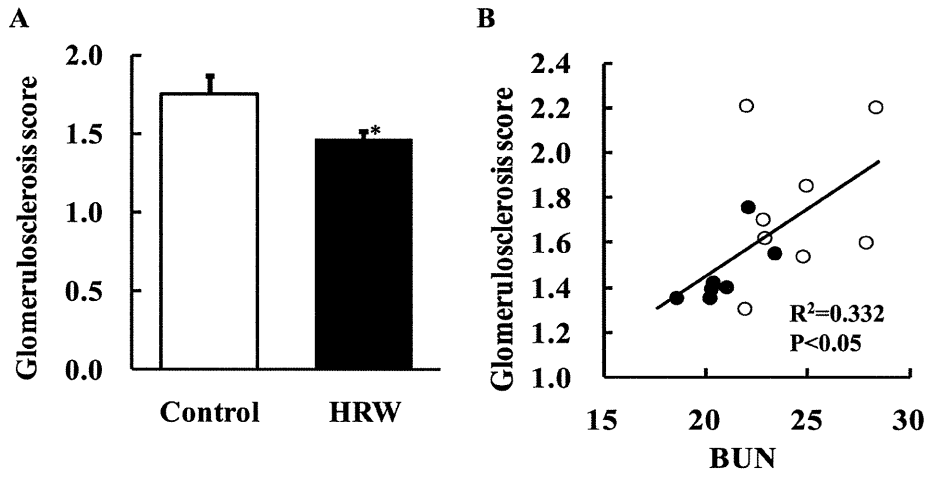

Figure 3 Effect of HRW administration on glomerular sclerosis. After 16 weeks of HRW administration, coronal sections of the renal tissue from distilled water-administered rats (control, n = 8) and HRW-administered rats (HRW, n = 7) were stained with PAS and examined by light microscopy in a blinded fashion (Figure 3A). Glomerulosclerosis was semi-quantitatively evaluated according to the criteria developed by Uehara et al. [10]. *P < 0.05. Data represent mean ± SE. Figure 3B illustrates the relationship between the glomerulosclerosis score and concentrations of blood urea nitrogen. White and black circles indicate control and HRW rats, respectively.

progressive diabetic nephropathy with severe proteinurial and histological abnormalities, which are associated with a decrease in CrCl as compared with WKY rats [15]. In this experiment, HRW administration significantly increased the levels of CrCl in SHR-cp rats (Table 2). These results suggest that the intake of HRW inhibited renal dysfunction in metabolic syndrome model rats. The mechanisms of the increased water intake and urine flow in HRW-administered SHR-cp rats remain to be elucidated. From the present data, it is difficult to clarify the possible causes and consequences of these increments. Typically, urine flow, urinary flow of creatinine, and/or CrCl as GFR indices are multifactorial phenomena. The increase in GFR and the decrease in the AC ratio observed in HRW-administered SHR-cp rats suggest that continuous HRW administration inhibits the development of renal dysfunction, leading to the increased urine flow and, presumably, the increased water intake. Further experiments are required to confirm this.

From the data obtained in this study, it is difficult to clarify the mechanisms underlying the beneficial effects of HRW on renal diseases. The HRW administration-induced increase in water intake, urine flow, and CrCl, and/or the decrease in oxidative stress observed in this study may play a role in this ameliorating effect in SHR-cp rats. Cardinal *et al.* recently reported that oral HRW administration prevents chronic allograft nephropathy after renal transplantation via the ability of molecular H_2 to reduce oxidative stress-induced damage [11]. Antioxidant enzymes do not detoxify the hydroxyl radical and peroxynitrite, which are target oxidants of molecular H_2, because no enzyme detoxifies these radicals. H_2 therapy reduces apoptosis by suppressing caspase activity in the neonatal hypoxia-ischemia rat model [16]. It is also reported that a sufficient supply of H_2-rich pure water may prevent or delay the development and progression of type II diabetes mellitus by providing protection against oxidative stress [17]. Therefore, our studies suggest that HRW may have direct effects on kidney function and that its administration appeared to ameliorate glomerular damage in a rat model of metabolic syndrome, possibly by limiting oxidative stress. Further studies are needed to confirm these mechanisms.

Conclusions

The present study was designed to evaluate whether HRW ingestion would have an ameliorative effect on a host of metabolic abnormalities, including glomerulosclerotic damage, blood creatinine and BUN levels, oxidative potentials, urinary flow, and GFR in metabolic syndrome model rats. Based on the biochemical and renal parameter results and morphological changes in the kidneys, the present study clearly indicates that HRW conferred significant benefits against these abnormalities in metabolic syndrome model rats.

List of abbreviations

The abbreviations used are: AC ratio: ratio of the concentrations of albumin to creatinine; BAP: biological antioxidant potential; BUN: blood urea nitrogen; CrCl: creatinine clearance; GFR: glomerular filtration rate; HRW: hydrogen-rich water; 8-OHdG: 8-hydroxy-2'-deoxyguanosine; PAS: periodic acid-Schiff; ROS: reactive oxygen species; SHR-cp: SHR.Cg-*Lepr*^{cp}/NDmcr; WKY: Wistar Kyoto.