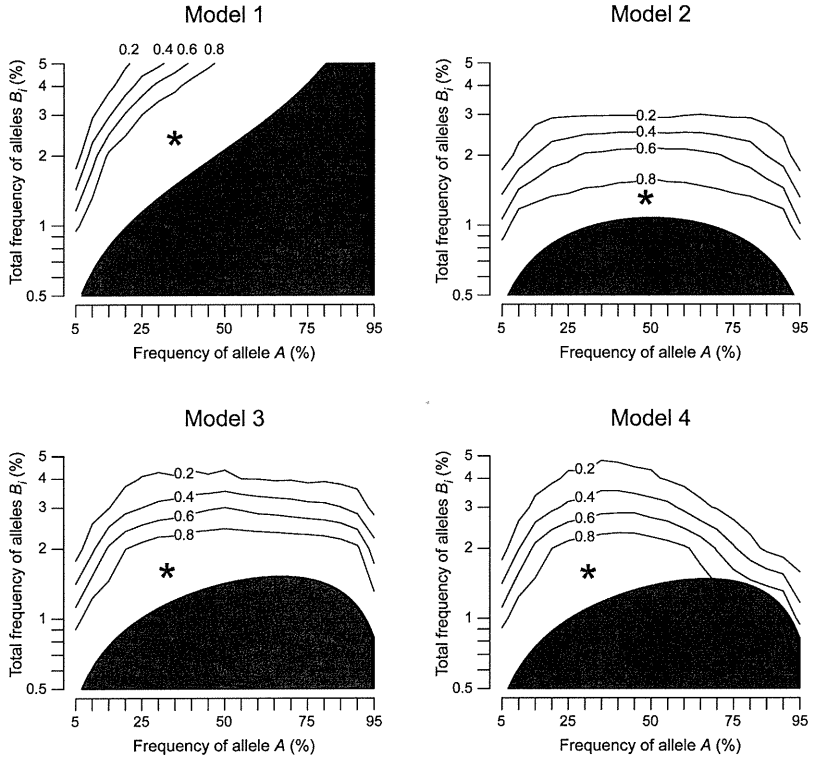


**Figure 5.** Power for detecting synthetic association by the combined test of heteroscedasticity and skewness. The power was computed from simulation under four representative genetic models, assuming the strength of marker association ($R^2_{mrk}$) of 0.00592. The format of the figure is the same as Figure 3.

the QT value is represented by a random variable $q$. The allele $B_i$ (or $C_j$) is modeled to affect the QT by $d_i$ (or $e_j$, respectively); specifically, the QT value $q$ of an individual with multivariant genotype $(y_1,\ldots,y_l,z_1,\ldots,z_m)$ has the probability density of a normal distribution with the unit variance and the mean of $\sum_{i=1}^{l} d_i y_i + \sum_{j=1}^{m} e_j z_j$. Thus, the probability density function of the genotype and QT level $(x,y_1,\ldots,y_l,z_1,\ldots,z_m,q)$ becomes

$$p(x,y_1,\ldots,y_l,z_1,\ldots,z_m,q) = p(x,y_1,\ldots,y_l,z_1,\ldots,z_m) \times \frac{1}{\sqrt{2\pi}} \exp\left(-\frac{\left(q-\sum_{i=1}^{l} d_i y_i - \sum_{j=1}^{m} e_j z_j\right)^2}{2}\right),$$

where $p(x,y_1,\ldots,y_l,z_1,\ldots,z_m)$ represents the frequency of the multivariant genotype $(x,y_1,\ldots,y_l,z_1,\ldots,z_m)$. The expectation of the QT value is

$$E[q] = \sum_{i=1}^{l} d_i E[y_i] + \sum_{j=1}^{m} e_j E[z_j],$$

where $E[\cdot]$ represents the expectation (see equation S5 in the Supplemental Notes for derivation). Similarly, when conditioned on a specific genotype $x_0$ at the marker,

$$E[q \mid x=x_0] = \sum_{i=1}^{l} d_i E[y_i \mid x=x_0] + \sum_{j=1}^{m} e_j E[z_j \mid x=x_0], \quad \text{(M1)}$$

where $E[\cdot|x=x_0]$ represents the conditional expectation.

# Methods

## Modeling the probability distribution of genotype and QT

We model a QT-associated marker SNP with alleles (referred to as the marker alleles), $A$ and $a$. We assume the low-frequency allele of each causal variant—which we call the causal allele—is linked exclusively to one of the marker alleles; $l$ causal variants each have alleles $B_1$ and $b_1$, $B_2$ and $b_2$, up to $B_l$ and $b_l$, where the causal allele $B_i$ is linked to marker allele $A$; $m$ other causal variants each have alleles $C_1$ and $c_1$, $C_2$ and $c_2$, up to $C_m$ and $c_m$, where the causal allele $c_j$ is linked to marker allele $a$. We impose one assumption for mathematical convenience. Among the haplotype classes sharing a specific marker allele ($A$ or $a$), we assume the probability distribution of causal variant alleles are independent among the variants; for example, among the haplotype classes carrying the $A$ allele, the frequency (conditional on the marker allele being $A$) of the haplotype class carrying both $B_1$ and $B_2$ should equal the product of the frequencies of classes carrying $B_1$ and $B_2$, which is very small (e.g., 0.01%, if the frequency is 1% both for $B_1$ and $B_2$). The assumed frequency would differ only marginally from the actual frequency: The haplotype class carrying both $B_1$ and $B_2$ does not exist initially if the two causal variants arose separately in the phylogeny, and increases to the assumed frequency by recombination. This assumption enables us to rewrite the test statistics into simple forms (see Supplemental Notes). As we assume Hardy-Weinberg equilibrium, the frequencies of multivariant genotypes can be calculated from those of the haplotype classes.

Each individual's dose of the capital letter alleles, $A$, $B_i$, or $C_j$, is represented by random variables, $x$, $y_i$, or $z_j$, respectively, and

## Variance

The variance of QT among individuals having a specific marker genotype $x_0$ becomes

$$\mathrm{Var}[q \mid x=x_0] = 1 + \sum_{i=1}^{l} d_i^2 \mathrm{Var}[y_i \mid x=x_0] + \sum_{j=1}^{m} e_j^2 \mathrm{Var}[z_j \mid x=x_0], \quad \text{(M2)}$$

where $\mathrm{Var}[\cdot|x=x_0]$ represents the conditional variance (see Supplemental equation S6 for derivation). Equation M2 indicates that the inflation (above one) of QT variance decomposes into a sum of terms, each corresponding to one causal variant and determined by the square of the effect-size, $d_i$ or $e_j$, and the conditional variance of genotype. Designating the frequencies of alleles $A$, $B_i$ and $c_j$ as $p_A$, $p_{B_i}$, and $p_{c_j}$, respectively, the genotype variance becomes

$$\mathrm{Var}[y_i \mid x=2] = 2\cdot\frac{p_{B_i}}{p_A}\left(1-\frac{p_{B_i}}{p_A}\right) \approx 2\cdot\frac{p_{B_i}}{p_A},$$
$$\mathrm{Var}[y_i \mid x=1] = \frac{p_{B_i}}{p_A}\left(1-\frac{p_{B_i}}{p_A}\right) \approx \frac{p_{B_i}}{p_A},$$
$$\mathrm{Var}[y_i \mid x=0] = 0,$$
$$\mathrm{Var}[z_j \mid x=2] = 0,$$
$$\mathrm{Var}[z_j \mid x=1] = \frac{p_{c_j}}{1-p_A}\left(1-\frac{p_{c_j}}{1-p_A}\right) \approx \frac{p_{c_j}}{1-p_A},$$
$$\mathrm{Var}[z_j \mid x=0] = 2\cdot\frac{p_{c_j}}{1-p_A}\left(1-\frac{p_{c_j}}{1-p_A}\right) \approx 2\cdot\frac{p_{c_j}}{1-p_A},$$

where the approximation is under $p_A, 1-p_A >> p_{B_i}, p_{c_j}$. By substituting the genotype variance into equation M2, we obtain

$$\begin{aligned}\mathrm{Var}[q \mid x=2] &= 1+2\sum_{i=1}^{l} d_i^2 \frac{p_{B_i}}{p_A},\\ \mathrm{Var}[q \mid x=1] &= 1+\sum_{i=1}^{l} d_i^2 \frac{p_{B_i}}{p_A}+\sum_{j=1}^{m} e_j^2 \frac{p_{c_j}}{1-p_A},\\ \mathrm{Var}[q \mid x=0] &= 1+2\sum_{j=1}^{m} e_j^2 \frac{p_{c_j}}{1-p_A}.\end{aligned} \tag{M3}$$

Thus, the QT variance at marker genotypes $x = 2$ ($A/A$) and $x = 0$ ($a/a$) is determined by the contribution of alleles $B_i$ and $c_j$, respectively, and the average of the two variances equals the variance at genotype $x = 1$ ($A/a$). The average of three variances weighted by marker genotype frequency becomes

$$\begin{aligned}E[\mathrm{Var}[q \mid x]] &= p_A^2\mathrm{Var}[q \mid x=2] + 2p_A(1-p_A)\mathrm{Var}[q \mid x=1]\\ &\quad + (1-p_A)^2\mathrm{Var}[q \mid x=0] = 1+2\left(\sum_{i=1}^{l} d_i^2 p_{B_i}+\sum_{j=1}^{m} e_j^2 p_{c_j}\right).\end{aligned} \tag{M4}$$

We test the heterogeneity of QT variance (i.e., heteroscedasticity) among marker genotypes using Bartlett's test (Bartlett 1937). The $\chi^2$ statistic (two degrees of freedom) is expected to become

$$\begin{aligned}&\chi^2_{\text{heteroscedasticity}}\\ &= N[\ln(E[\mathrm{Var}[q \mid x]]) - p_A^2\ln(\mathrm{Var}[q \mid x=2])\\ &\quad -2p_A(1-p_A)\ln(\mathrm{Var}[q \mid x=1]) - (1-p_A)^2\ln(\mathrm{Var}[q \mid x=0])]\\ &\approx N\left[-2\left(\sum_{i=1}^{l} d_i^2 p_{B_i}+\sum_{j=1}^{m} e_j^2 p_{c_j}\right)^2 + 2\left(\sum_{i=1}^{l} d_i^2 p_{B_i}\right)^2\right.\\ &\quad \left.+\left(\sqrt{\frac{1-p_A}{p_A}}\sum_{i=1}^{l} d_i^2 p_{B_i}+\sqrt{\frac{p_A}{1-p_A}}\sum_{j=1}^{m} e_j^2 p_{c_j}\right)^2 + 2\left(\sum_{j=1}^{m} e_j^2 p_{c_j}\right)^2\right]\\ &= N\left\{\left(\sqrt{\frac{1-p_A}{p_A}}\sum_{i=1}^{l} d_i^2 p_{B_i}\right)-\left(\sqrt{\frac{p_A}{1-p_A}}\sum_{j=1}^{m} e_j^2 p_{c_j}\right)\right\}^2,\end{aligned} \tag{M5}$$

when the sample size $N$ is large (thus, the constant for the Bartlett's test statistic equals one); for the second equality, equations M3 and M4 were substituted, and $\ln(1+x)$ was approximated as $x - x^2/2$. In the curly brackets of the final formula in equation M5, the contribution by causal alleles $B_i$ (linked to marker allele $A$) is subtracted by the contribution of alleles $c_j$ (linked to allele $a$). Thus, the statistic for heteroscedasticity is maximized when all low-frequency causal alleles are linked to the same marker allele, and diminishes when they are linked evenly to both of the marker alleles.

### Skewness

The third central moment of the QT distribution among the individuals having a specific marker genotype $x_0$ is

$$\mu_3[q \mid x=x_0] = \sum_{i=1}^{l} d_i^3\mu_3[y_i \mid x=x_0] + \sum_{j=1}^{m} e_j^3\mu_3[z_j \mid x=x_0], \tag{M6}$$

which decomposes into a sum of terms, each contributed by one causal variant (see Supplemental equation S7 for derivation); $\mu_3[\cdot \mid x=x_0]$ represents the conditional third central moment. The third central moment of the genotypes $y_i$ and $z_j$ conditional on a marker genotype becomes

$$\begin{aligned}\mu_3[y_i \mid x=2] &= 2\cdot\frac{p_{B_i}}{p_A}\left(1-\frac{p_{B_i}}{p_A}\right)\left(1-\frac{2p_{B_i}}{p_A}\right) \approx 2\cdot\frac{p_{B_i}}{p_A},\\ \mu_3[y_i \mid x=1] &= \frac{p_{B_i}}{p_A}\left(1-\frac{p_{B_i}}{p_A}\right)\left(1-\frac{2p_{B_i}}{p_A}\right) \approx \frac{p_{B_i}}{p_A},\\ \mu_3[y_i \mid x=0] &= 0,\\ \mu_3[z_j \mid x=2] &= 0,\\ \mu_3[z_j \mid x=1] &= -\frac{p_{c_j}}{1-p_A}\left(1-\frac{p_{c_j}}{1-p_A}\right)\left(1-\frac{2p_{c_j}}{1-p_A}\right) \approx -\frac{p_{c_j}}{1-p_A},\\ \mu_3[z_j \mid x=0] &= -2\cdot\frac{p_{c_j}}{1-p_A}\left(1-\frac{p_{c_j}}{1-p_A}\right)\left(1-\frac{2p_{c_j}}{1-p_A}\right) \approx -2\cdot\frac{p_{c_j}}{1-p_A},\end{aligned}$$

where the approximation is under $p_A, 1-p_A \gg p_{B_i}, p_{c_j}$. By substituting the genotype moment into equation M6, we obtain

$$\begin{aligned}\mu_3[q \mid x=2] &= \frac{2}{p_A}\sum_{i=1}^{l} d_i^3 p_{B_i},\\ \mu_3[q \mid x=1] &= \left(\frac{1}{p_A}\sum_{i=1}^{l} d_i^3 p_{B_i}\right)-\left(\frac{1}{1-p_A}\sum_{j=1}^{m} e_j^3 p_{c_j}\right),\\ \mu_3[q \mid x=0] &= -\frac{2}{1-p_A}\sum_{j=1}^{m} e_j^3 p_{c_j}.\end{aligned} \tag{M7}$$

The $z$ statistic (standard normal distribution) for testing skewness (Stuart et al. 1999) of QT among the individuals with genotype $x_0$ becomes

$$\begin{aligned}z_{x=x_0} &= \sqrt{\frac{N_{x=x_0}}{6}}\frac{\mu_3[q \mid x=x_0]}{\mathrm{Var}[q \mid x=x_0]^{3/2}}\\ &\approx \sqrt{\frac{N_{x=x_0}}{6}}\mu_3[q \mid x=x_0],\end{aligned}$$

where $N_{x=x_0}$ is the number of the individuals, and the variance in the denominator is approximated as one for this statistic. By substituting equation M7, and converting $N_{x=x_0}$ into $N$ multiplied by the marker genotype frequency,

$$\begin{aligned}z_{x=2} &= \sqrt{\frac{2N}{3}}\sum_{i=1}^{l} d_i^3 p_{B_i},\\ z_{x=1} &= \left(\sqrt{\frac{N}{3}\cdot\frac{1-p_A}{p_A}}\sum_{i=1}^{l} d_i^3 p_{B_i}\right)-\left(\sqrt{\frac{N}{3}\cdot\frac{p_A}{1-p_A}}\sum_{j=1}^{m} e_j^3 p_{c_j}\right),\\ z_{x=0} &= -\sqrt{\frac{2N}{3}}\sum_{j=1}^{m} e_j^3 p_{c_j}.\end{aligned}$$

As the QT distributions at the two homozygote marker genotypes should be skewed to opposite directions under synthetic association, $z_{x=2}$ and $z_{x=0}$ would have opposite signs. We take their difference and obtain a $\chi^2$ statistic with one degree of freedom,

$$\chi^2_{\text{skewness}} = \frac{(z_{x=2}-z_{x=0})^2}{2}, \tag{M8}$$

which we adopt as the test statistic for skewness. The test statistic is expected to become

$$\chi^2_{\text{skewness}} = \frac{N}{3}\left(\sum_{i=1}^{l} d_i^3 p_{B_i}+\sum_{j=1}^{m} e_j^3 p_{c_j}\right)^2, \tag{M9}$$

reflecting the contribution by causal alleles $B_i$ and $c_j$.

### Statistical tests of synthetic association and type I error rate

We combine two types of statistics, heteroscedasticity and skewness, to devise the combined test. Using Fisher's method, the $P$-values for testing heteroscedasticity and skewness, $p_{\text{heteroscedasticity}}$ and $p_{\text{skewness}}$, respectively, are combined as a $\chi^2$ statistic (four degrees of freedom),

$$\chi^2_{\text{combined}} = -2\ln(p_{\text{heteroscedasticity}}\cdot p_{\text{skewness}}). \tag{M10}$$

The significance level was set to 0.05 for all tests.

Before testing, we applied rank-based inverse normal transformation (Blom 1958) to the whole QT distribution. The transformation avoids detecting spurious signals when the QT distribution is skewed as a whole. The transformed QT value $q_i$ of the $i$-th individual is

$$q_i = \Phi^{-1}\left(\frac{r_i - c}{N-2c+1}\right),$$

where $r_i$ is the rank of the individual, $N$ is the total number of individuals, $c = 3/8$, and $\Phi^{-1}$ is the standard normal quantile. We strongly recommend applying the transformation, although it can

cause false positives when the marker association is extremely strong, as explained below.

Type I error rate of the tests were assessed from simulated and empirical data. Under the "null hypothesis" of indirect association, we inspected the distribution of nominal *P*-value, and assessed the test as accurate, conservative, or anticonservative, if the actual type I error rate was equal, smaller, or larger than the nominal *P*-value, respectively; an anticonservative test cannot be used. For a marker showing association at a borderline level of genome-wide significance ($R^2_{mrk} = 0.00592$), the heteroscedasticity test was accurate, but the skewness test tended to be conservative, due to the inverse normal transformation (Supplemental Fig. 4); this was not calibrated. As the tests for heteroscedasticity and skewness were not correlated, they could be combined using Fisher's method. When the marker association was as large as $R^2_{mrk} = 0.1$, which is exceptional for GWA signals, the inverse normal transformation caused spurious heteroscedasticity and skewness, thus the proposed tests were not valid. For gene expression data (Stranger et al. 2007), the heteroscedasticity test was accurate, and the skewness test was slightly conservative (Supplemental Fig. 5; Supplemental Table 2).

## Models for simulation

If the strength of the marker SNP association $R^2_{mrk}$, and the frequency of variants ($p_A, p_{B_i}, p_{c_j}$) are specified, we can calculate the effect-size of the causal variants ($d_i, e_j$) by solving Supplemental equation S3, and determine the genetic model. We systematically explore four representative models of synthetic association by simulation. (Plots of $d_i$ according to variant frequency are shown in Supplemental Fig. 6.)

### *Model 1*

All causal alleles linked to marker allele *A* have identical effect-size, and there are no causal alleles linked to allele *a*. By solving Supplemental equation S3 under $d_1 = \cdots = d_l$ and $m = 0$,

$$d_i = \frac{1}{\sqrt{\frac{1-R^2_{mrk}}{R^2_{mrk}} \cdot \frac{2(1-p_A)}{p_A} \left(\sum_{i=1}^{l} p_{B_i}\right)^2 - 2\sum_{i=1}^{l} p_{B_i}}}. \quad \text{(M11)}$$

By substituting this to the test statistic of heteroscedasticity (equation M5), and solving for $\sum_{i=1}^{l} p_{B_i}$,

$$\sum_{i=1}^{l} p_{B_i} = \frac{R^2_{mrk}}{1-R^2_{mrk}} \frac{p_A}{2(1-p_A)} \left( \sqrt{\frac{N}{\chi^2_{\text{heteroscedasticity}}} \frac{1-p_A}{p_A}} + 2 \right).$$

Thus, when $R^2_{mrk} = 0.00592$ and $N = 5000$, heteroscedasticity is detectable at a power >0.8 under a significance level of 0.05 (which requires a noncentrality parameter of $\chi^2 > 9.64$ for the $\chi^2$ distribution with two degrees of freedom; "+2" in the parenthesis is negligible) if

$$\sum_{i=1}^{l} p_{B_i} < 0.068 \sqrt{\frac{p_A}{1-p_A}}. \quad \text{(M12)}$$

Heteroscedasticity is detectable if the cumulative frequency of causal alleles (left term) is smaller than a certain function of the frequency of the marker allele *A* (right term); the detectable range with regard to $\sum_{i=1}^{l} p_{B_i}$ is wider when $p_A$ is larger.

### *Model 2*

Causal alleles are linked to the two marker alleles in a balanced way, such that the effect-size is uniform, as $d_1 = \cdots = d_l = e_1 = \cdots = e_m$, and the cumulative frequencies equal between causal alleles $B_i$ (linked to marker allele *A*) and causal alleles $c_j$ (linked to marker allele *a*), as $\sum_{i=1}^{l} p_{B_i} = \sum_{j=1}^{m} p_{c_j}$. By solving Supplemental equation S3 under the constraints,

$$d_i = e_j = \frac{1}{\sqrt{\frac{1-R^2_{mrk}}{R^2_{mrk}} \cdot \frac{2}{p_A(1-p_A)} \left(\sum_{i=1}^{l} p_{B_i}\right)^2 - 4\sum_{i=1}^{l} p_{B_i}}}. \quad \text{(M13)}$$

By substituting this (approximating the last term in the square-root as zero) to the test statistic of skewness (equation M9), and solving for $\sum_{i=1}^{l} p_{B_i}$,

$$\sum_{i=1}^{l} p_{B_i} = \left(\frac{R^2_{mrk}}{1-R^2_{mrk}}\right)^{\frac{3}{4}} \left(\frac{N}{6\chi^2_{\text{skewness}}}\right)^{\frac{1}{4}} (p_A(1-p_A))^{\frac{3}{4}}.$$

Thus, when $R^2_{mrk} = 0.00592$ and $N = 5000$, skewness is detectable at a power >0.8 under a significance level of 0.05 (which requires a noncentrality parameter of $\chi^2 > 7.85$ for the $\chi^2$ distribution with one degree of freedom) if

$$\sum_{i=1}^{l} p_{B_i} < 0.069 \, (p_A(1-p_A))^{\frac{3}{4}}. \quad \text{(M14)}$$

Skewness is detectable if the cumulative frequency of causal alleles $B_i$ (left term) is smaller than a certain function of the frequency of the marker allele *A* (right term); the detectable range with regard to $\sum_{i=1}^{l} p_{B_i}$ is widest when $p_A$ is around 0.5.

### *Model 3*

The effect-size of causal alleles is uniform, as $d_1 = \cdots = d_l = e_1 = \cdots = e_m$, yet the cumulative frequency of the causal alleles $B_i$ is twice the cumulative frequency of causal alleles $c_j$, as $\sum_{i=1}^{l} p_{B_i} = 2\sum_{j=1}^{m} p_{c_j}$. Then,

$$d_i = e_j = \frac{1}{\sqrt{\frac{1-R^2_{mrk}}{R^2_{mrk}} \cdot \frac{(2-p_A)^2}{2p_A(1-p_A)} \left(\sum_{i=1}^{l} p_{B_i}\right)^2 - 3\sum_{i=1}^{l} p_{B_i}}}. \quad \text{(M15)}$$

### *Model 4*

The cumulative frequencies are equal between causal alleles linked to the two marker alleles, as $\sum_{i=1}^{l} p_{B_i} = \sum_{j=1}^{m} p_{c_j}$, yet the effect-size of causal alleles $B_i$ is twice the effect-size of causal alleles $c_j$, as $d_1 = \cdots = d_l = 2e_1 = \cdots = 2e_m$. Then,

$$d_i = 2e_j = \frac{1}{\sqrt{\frac{1-R^2_{mrk}}{R^2_{mrk}} \cdot \frac{(2-p_A)^2}{2p_A(1-p_A)} \left(\sum_{i=1}^{l} p_{B_i}\right)^2 - \frac{5}{2}\sum_{i=1}^{l} p_{B_i}}}. \quad \text{(M16)}$$

## Power assessment by simulation

We assessed the power of the three tests under each of the four models by simulation. In any of the models, we assumed that effect-size equals among the causal alleles linked to the same marker allele (i.e., $d_1 = \cdots = d_l$ and $e_1 = \cdots = e_m$). In such a case, the tests remain the same if instead there was one composite allele of $B_i$'s and another composite of $c_j$'s. Using this property, we actually simulated the special case with one causal allele linked to each one of the marker alleles; the simulation results apply to the general case with multiple causal alleles.

Simulations were performed under the following parameter values; $R^2_{mrk} = 0.00592, 0.0118$; $p_A = 0.05, 0.10, \ldots, 0.95$; $\sum_{i=1}^{l} p_{B_i} = 0.005, 0.006, \ldots, 0.01, 0.02, \ldots, 0.05$. Other parameters—$\sum_{j=1}^{m} p_{c_j}$, $d_i$, and $e_j$—were determined according to constraints. We randomly generated 5000 (or 2500) individuals using simulation and applied the tests. The power was assessed from 1000 simulation trials. We used the R software for computation.

## Acknowledgments

We thank the participants in the lipid study and Drs. Toru Nabika (Shimane University), Tomohiro Katsuya (Osaka University), and Yukio Yamori (Mukogawa Women's University). We also thank anonymous reviewers for their constructive comments. This work was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation Organization; a Grant of the National Center for Global Health and Medicine; and the Ministry of Health, Labor and Welfare.

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*Received September 24, 2010; accepted in revised form March 9, 2011.*

Hypertension Research (2012) 35, 477–484

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# 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.**
*Hypertension Research* (2012) **35**, 477–484; doi:10.1038/hr.2012.30; published online 8 March 2012

**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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Received 24 November 2011; revised 8 January 2012; accepted 11 January 2012; published online 8 March 2012

## 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.

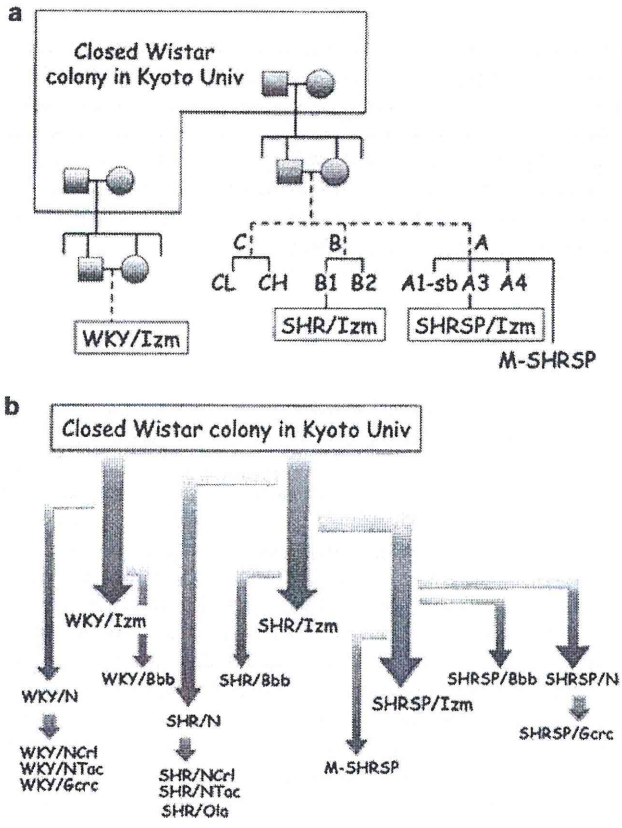


**Figure 1** Origins of the SHRSP, SHR and WKY substrains. (a) The original substrains established by Okamoto and his colleagues. (b) The substrains currently used in the world.

### 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.*

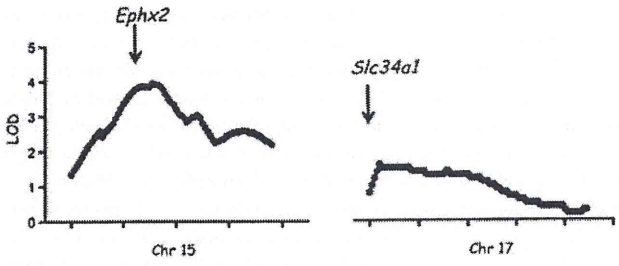


**Figure 3** QTL analysis of BP in an F2 cross between SHRSP/Izm and SHR/Izm. Lod scores for BP on Chr 15 and 17 are plotted with the location of the *Ephx2* and *Slc34a* genes. A total of 294 F2 rats were used in the analysis (male and female data were combined).

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.

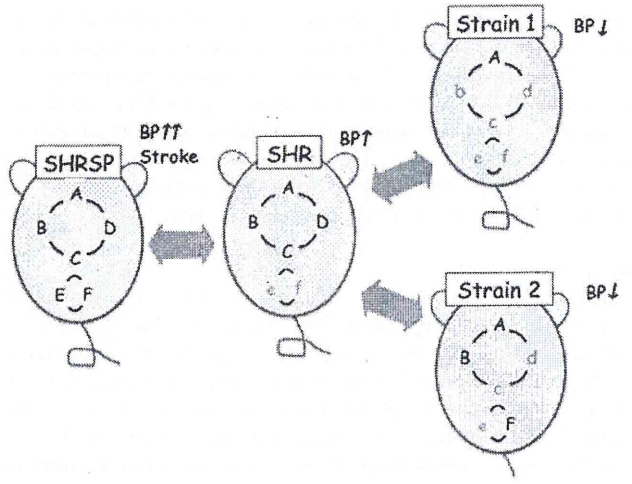


**Figure 4** Hypothetical genetic composition of hypertension in SHRSP and SHR. Networks composed of multiple interacting genes are hypothesized. They have full-blown effects on BP only when all of the included genes are the 'hypertensive' allele (shown in capital letters). Consequently, even though 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

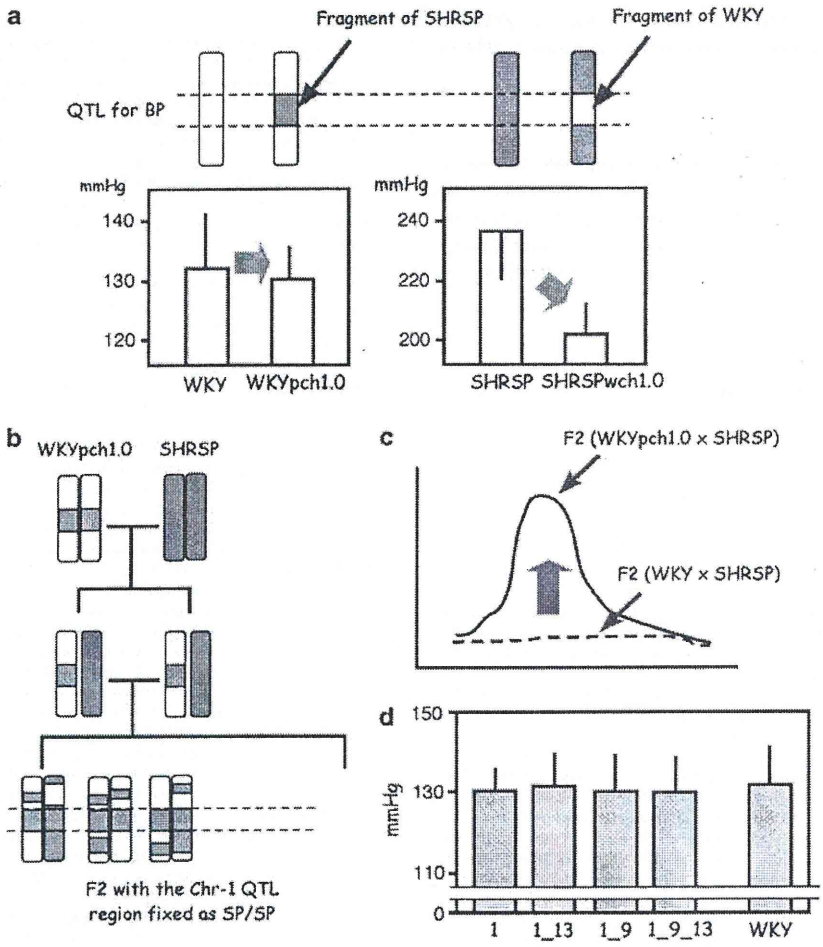


**Figure 5** A trial to show interactions among QTLs in SHRSP. (**a**) Reciprocal congenic rats for the Chr 1 QTL do not show reciprocal effects of the QTL on BP. WKYpch1.0 and SHRSPwch1.0 are reciprocal congenic strains constructed for the Chr 1 QTL.[29,31,32] (**b**) The study design of a QTL analysis 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.

## ACKNOWLEDGEMENTS

This work was partly supported by the Grant-in-Aid for Scientific Research by the Japanese Ministry of Education, Science, Sports and Culture.

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Hashimoto et al. Medical Gas Research 2011, 1:26
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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.

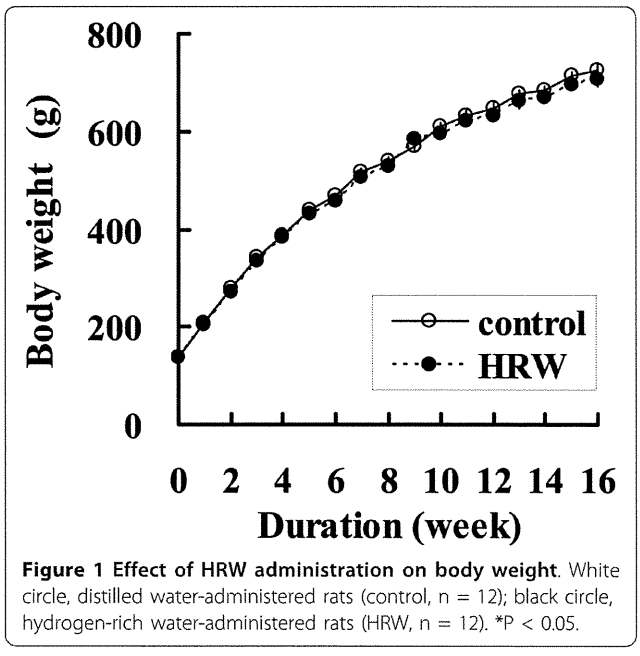


**Figure 1 Effect of HRW administration on body weight**. White circle, distilled water-administered rats (control, n = 12); black circle, hydrogen-rich water-administered rats (HRW, n = 12). *P < 0.05.

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

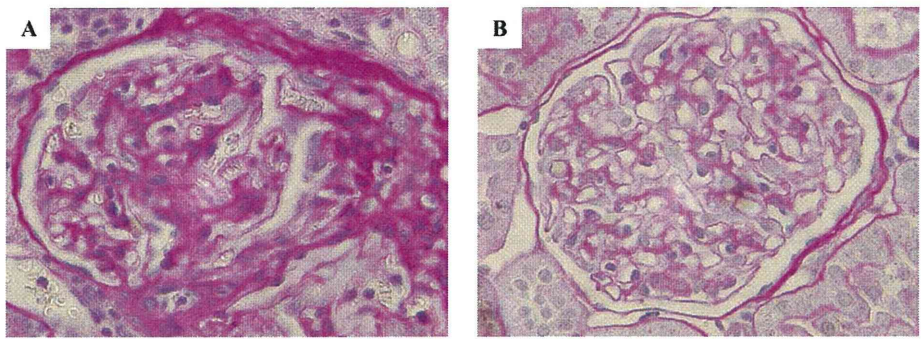


**Figure 2 Photomicrographs of coronal sections of the glomeruli from SHR-cp rats**. (A) Control group. (B) HRW group. Periodic acid-Schiff (PAS) staining of the control group revealed glomerular damage, which was characterized by segmental glomerular sclerosis and the formation of synechiae by the attachment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

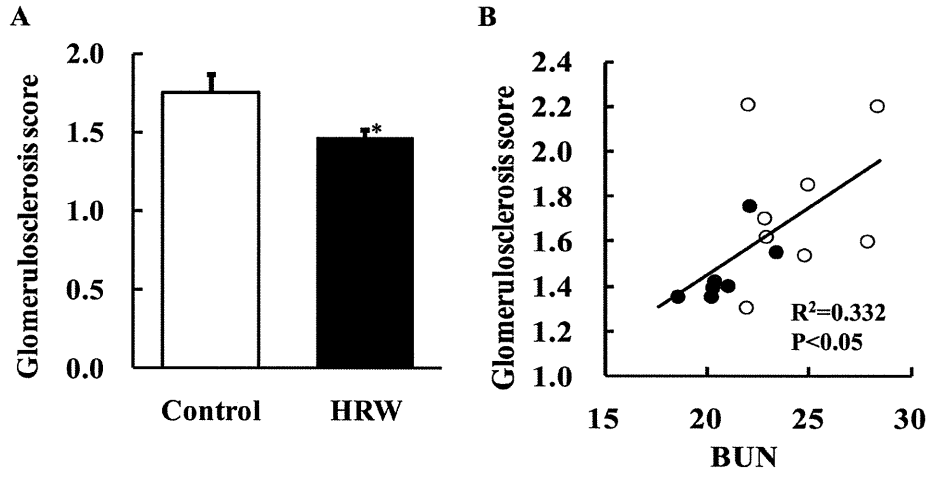


**Figure 3 Effect of HRW administration on glomerular sclerosis**. After 16 weeks of HRW administration, coronal sections of the renal tissue from distilled water-administered rats (control, n = 8) and HRW-administered rats (HRW, n = 7) were stained with PAS and examined by light microscopy in a blinded 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.

**Acknowledgements**
The authors thank Professor Hideki Okunishi and technician Keiko Shimoura of the Department of Pharmacology at Shimane University Faculty of Medicine, for preparing coronal sections of renal tissues. We also thank Friendear Inc. (Tokyo, Japan) for its generous gift of metallic magnesium sticks (Doctor SUISOSUI®, Friendear Inc.). This study was supported in part by a grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (19500324 to MH).

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**Authors' contributions**
MH, MK, and YT carried out experiments. MH, TN, ST, and OS participated in the design of the study. MK and YT performed the statistical analysis. MH and SH wrote the manuscript. All authors read and approved the final manuscript.

**Competing interests**
The authors declare that they have no competing interests.

Received: 7 June 2011 Accepted: 3 November 2011
Published: 3 November 2011

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doi:10.1186/2045-9912-1-26
**Cite this article as:** Hashimoto *et al.*: Effects of hydrogen-rich water on abnormalities in a SHR.Cg-*Lepr*$^{cp}$/NDmcr rat - a metabolic syndrome rat model. *Medical Gas Research* 2011 1:26.

ORIGINAL CONTRIBUTIONS

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# Role of Complement 3a in the Synthetic Phenotype and Angiotensin II-Production in Vascular Smooth Muscle Cells From Spontaneously Hypertensive Rats

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**BACKGROUND**

Spontaneously hypertensive rats (SHR)-derived vascular smooth muscle cells (VSMCs) show exaggerated growth with a synthetic phenotype and angiotensin II (Ang II)-production. To evaluate the contribution of complement 3 (C3) or C3a toward these abnormalities in SHR, we examined effects of a C3a receptor inhibitor on proliferation, phenotype, and Ang II-production in VSMCs from SHR and Wistar–Kyoto (WKY) rats.

**METHODS**

Expression of pre-pro-C3 messenger RNA (mRNA) and C3 protein was evaluated by reverse transcription-PCR and western blot analyses, and C3a receptor mRNA was evaluated by reverse transcription-PCR analysis in quiescent VSMCs from SHR and WKY rats. We examined the effects of the C3a inhibitor, SB290157, on proliferation and the expression of phenotype-marker and Krueppel-like factor 5 (KLF-5) mRNAs in VSMCs from SHR and WKY rats. We examined effects of C3a receptor inhibitor, SB290157, on Ang II-production in conditioned medium of VSMCs from SHR and WKY rats by a radioimmunoassay.

**RESULTS**

Expression of pre-pro-C3 mRNA and C3 protein was significantly higher in SHR VSMCs than WKY VSMCs. SB290157 significantly inhibited proliferation of VSMCs from SHR, but not in cells from WKY rats. Relative to WKY VSMCs, SB290157 significantly increased the low expression of SM22α mRNA and decreased the high expression of osteopontin mRNA in SHR VSMCs. SB290157 significantly decreased the high expression of KLF-5 and Ang II-production in VSMCs from SHR, but not in cells from WKY rats.

**CONCLUSIONS**

C3a induces exaggerated growth, a synthetic phenotype and Ang II-production in SHR-derived VSMCs. C3a may be primarily involved in cardiovascular remodeling in hypertension.

***Keywords:*** *angiotensin II; blood pressure; complement 3; hypertension; Kruppel-like factor 5; phenotype; proliferation; spontaneously hypertensive rat; vascular smooth muscle cell*

*American Journal of Hypertension*, advance online publication 17 November 2011; doi:10.1038/ajh.2011.214

Patients with essential hypertension, a hereditary polygenic disease, are eventually complicated with stroke, cardiovascular remodeling, and nephrosclerosis. These complications are practical targets for therapy of essential hypertension with antihypertensive medicines. Spontaneously hypertensive rats (SHR), a genetic animal model for essential hypertension, show exaggerated growth of cardiovascular organs in comparison with normotensive Wistar–Kyoto (WKY) rats.[1,2] Enhanced DNA synthesis and organ hypertrophy have been described in SHR even as early as the day of birth.[3–5] SHR-derived vascular smooth muscle cells (VSMCs) in culture show a higher specific growth rate, abnormal contact inhibition, accelerated entry into S phase of the cell cycle, and nonspecific hyperproliferation in response to various growth factors in comparison to cells from WKY rats.[6,7] These behaviors may reflect intrinsic abnormalities in SHR that are not caused by pressure overload because there is no blood pressure in culture. Therefore, these characteristics of VSMCs from SHR appear to be associated with genetic abnormalities. We found that SHR-derived VSMCs generate angiotensin II (Ang II) in homogenous cultures.[8] We have reported that the mechanism underlying this enhanced generation of Ang II in SHR-derived VSMCs appears to be a change from the contractile to the synthetic phenotype in comparison to cells from WKY rats.[9,10]

It is possible that genetic abnormalities are involved in the exaggerated growth and synthetic phenotype of VSMCs from SHR. We investigated the genes that are responsible and found by microarray analysis that the messenger RNA (mRNA) encoding complement 3 (C3) is expressed only in VSMCs from SHR and is associated with both the synthetic phenotype and exaggerated growth.[11] At the same time, we demonstrated

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Received 29 June 2011; first decision 18 September 2011; accepted 29 September 2011.

that C3 also changes renal mesangial cells to the synthetic phenotype.[12] We investigated mechanisms underlying the C3-induced phenotypic changes and found that C3 stimulates Kruppel-like factor 5 (KLF-5) promoter activity through extracellular signal-regulated kinase (ERK) signaling.[13]

C3 is a 190 kDa glycoprotein that consists of two polypeptide chains, which are produced from liver, monocytes, and macrophages, and it is essential for eliciting the complement response.[14] C3 from pre-pro-C3 mRNA is secreted after cleavage of the heterodimer, and then C3 is proteolytically cleaved into C3a (molecular weight 9,000) and C3b (molecular weight 185,000). C3a is an anaphylotoxin, and C3b serves as an opsonizing agent.[15] We have demonstrated that the increases in Ang II-production in VSMCs in homogeneous culture are associated with changes to the synthetic phenotype in VSMCs.[8,16]

In the current study, in order to evaluate whether C3 or C3a is associated with the exaggerated growth of the synthetic phenotype and increased Ang II-production in VSMCs from SHR, we examined effects of the C3a receptor inhibitor on proliferation, phenotype, and Ang II-production in VSMCs from SHR and WKY rats.

## METHODS

*Ethics and animals.* Our investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, 1996). The ethics committee of the Nihon University School of Medicine examined every research protocol involving the use of living animals. SHR/Izm and WKY/Izm rats were obtained from Japan SLC (Hamamatsu, Japan).

*Cell culture and establishment of quiescence.* VSMCs were obtained from aortic explants from 3-week-old prehypertensive male SHR/Izm and WKY/Izm rats. VSMCs were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum (Gibco Life Technologies, Gaithersburg, MD), 100 U/ml penicillin, and 100 mg/ml streptomycin. Experiments were performed on cells between the 5th and 10th passages. Trypsinized cells were plated into 24-well culture dishes at a density of $10^5$ cells/cm$^2$. They were allowed to grow in DMEM containing 10% calf serum for 24 h, and the culture medium was then changed to DMEM with 0.2% calf serum. The cells were incubated in this medium for 48–72 h to establish quiescence.

*Proliferation of VSMCs.* VSMCs from SHR and WKY rats were inoculated and grown into DMEM containing 5% calf serum in the absence or presence of 0.1 μmol/l SB290157 in 24-well culture dishes at a density of $10^5$ cells/cm$^2$. Cells were trypsinized with 0.05% trypsin at 24, 48, and 72 h after inoculation, and cell numbers were counted in a Coulter counter (Coulter Electronics, Luton, UK).

*Semiquantitative reverse transcription-PCR analysis.* Total RNAs from samples were extracted from quiescent VSMCs from SHR and WKY rats with ISOGEN (Nippon Gene, Tokyo, Japan). Primer sequences are listed in **Table 1**. 18S ribosomal RNA was used as an internal control. To confirm that no genomic DNA was co-amplified by PCR, control reverse transcription-PCR experiments were performed with each set of primers but without reverse transcriptase; no product was amplified. For semiquantative analysis of mRNA, the kinetics of the PCR reaction were monitored; the number of cycles at which the PCR products were detectable on the gel was compared between samples.[17] Serial tenfold dilutions of complementary DNA (100, 10, and 1 ng) were amplified; the PCR products were detectable at earlier cycles with increasing amounts of complementary DNA. PCR was performed for 30 cycles in a DNA thermal cycler (Perkin-Elmer Cetus, Waltham, MA), and products were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and visualized by ultraviolet illumination.

*Western blot analysis for C3 protein in VSMCs.* VSMCs ($5 \times 10^4$ cells/cm$^2$) were disrupted with lysis buffer (50 mmol/l Tris-HCl (pH 8.0), 150 mmol/l NaCl, 0.02% sodium azide, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1% Triton X-100). Total proteins were extracted and purified with 100 μl of chloroform and 400 μl of methanol. Protein samples were boiled for 3 min and subjected to electrophoresis on 8% polyacrylamide gels and then transblotted to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Blots were incubated with rabbit polyclonal antibodies specific for C3 (Santa Cruz Biotechnology, Santa Cruz, CA) or a mouse monoclonal antibody specific for α-tubulin (Sigma, St Louis, MO) as an internal control, and were then incubated with goat anti-rabbit immunoglobulin G or goat anti-mouse immunoglobulin G (Bio-Rad Laboratories). Immunocomplexes were detected by enhanced chemiluminescence (ECL, Amersham, UK).

*Measurement of Ang II in conditioned medium.* VSMCs ($10^6$) from SHR and WKY rats were inoculated in 10 cm$^2$ wells with

**Table 1 | PCR primers and product sizes**

| Target mRNA | | Primer Sequence | PCR product (bp) |
|---|---|---|---|
| Pre-pro-C3 | 5′ | 5′-CAGCAGACCTCAGTGACCAA-3′ | 351 |
| | 3′ | 5′-ATAGCTGTCAGCCAGGTGCT-3′ | |
| C3a receptor | 5′ | 5′-GACCTACACTCAGGGC-3′ | 376 |
| | 3′ | 5′-ATGACGGACGGGATAAG-3′ | |
| SM22α | 5′ | 5′-TTGAAGGCCAATCACGTGCTT-3′ | 312 |
| | 3′ | 5′-AAGCCAGTGAAGGTGCCTGAG -3′ | |
| Osteopontin | 5′ | 5′-TGGCTTACGGACTGAGGTCA-3′ | 486 |
| | 3′ | 5′-GACCTCAGAAGATGAACTCT-3′ | |
| KLF-5 | 5′ | 5′-ACCTACTTTCCCCCATCACC-3′ | 205 |
| | 3′ | 5′-CCGGGTTACTCCTTCTGTTG-3′ | |
| 18S rRNA | 5′ | 5′-CGACGACCCATTCGAACGTCT-3′ | 312 |
| | 3′ | 5′-GCTATTGGAGCTGGAATTACCG-3′ | |

KLF-5, Kruppel-like factor 5; mRNA, messenger RNA; pre-pro-C3, pre-pro-complement 3; rRNA, ribosomal RNA.