Genetic analysis of genes causing hypertension and stroke in spontaneously hypertensive rats: Gene expression profiles in the kidneys

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Abstract. Spontaneously hypertensive rats (SHRs) and stroke-prone SHRs (SHRSP) are frequently used as models not only of essential hypertension and stroke, but also of attention-deficit hyperactivity disorder (ADHD). Normotensive Wistar-Kyoto (WKY) rats are normally used as controls in these studies. In the present study, we aimed to identify the genes causing hypertension and stroke, as well as the genes involved in ADHD using these rats. We previously analyzed gene expression profiles in the adrenal glands and brain. Since the kidneys can directly influence the functions of the cardiovascular, endocrine and sympathetic nervous systems, gene expression profiles in the kidneys of the 3 rat strains were examined using genome-wide microarray technology when the rats were 3 and 6 weeks old, a period in which rats are considered to be in a pre-hypertensive state. Gene expression profiles were compared between the SHRs and WKY rats and also between the SHRSP and SHRs. A total of 232 unique genes showing more than a 4-fold increase or less than a 4-fold decrease in expression were isolated as SHR- and SHRSP-specific genes. Candidate genes were then selected using two different web tools: the 1st tool was the Database for Annotation, Visualization and Integrated Discovery (DAVID), which was used to search for significantly enriched genes and categorized them using Gene Ontology (GO) terms, and the 2nd was Ingenuity Pathway Analysis (IPA), which was used to search for interactions among SHR- and also SHRSP-specific genes. The analyses of SHR-specific genes using IPA revealed that B-cell CLL/lymphoma 6 (Bcl6) and SRY (sex determining region Y)-box 2 (Sox2) were possible candidate genes responsible for causing hypertension in SHRs. Similar analyses of SHRSP-specific genes revealed that angiotensinogen (Agt), angiotensin II receptor-associated protein (Agtrap) and apolipoprotein H (ApoH) were possible candidate genes responsible for triggering strokes. Since our results revealed that SHRSP-specific genes isolated from the kidneys of rats at 6 weeks of age, included 6 genes related to Huntington’s disease, we discussed the genetic association between ADHD and Huntington’s disease.

Introduction

Studies have been conducted in an attempt to identify the genes causing hypertension using 2 strains of hypertensive rats: spontaneously hypertensive rats (SHRs) and a substrain derived from the SHRs, stroke-prone SHRs (SHRSP) (1,2). Normotensive Wistar-Kyoto (WKY) rats are normally used as controls in these studies (1). Since SHRs and SHRSP are not only used as models of essential hypertension and stroke, but also as models of attention-deficit hyperactivity disorder (ADHD), it is expected that using these rats, it is possible identify the genes related not only to hypertension and stroke, but also to ADHD (3).
In our previous studies, we investigated gene expression profiles in the adrenal glands (4), and subsequently in the brain (5). Since the kidneys are logical candidate organs for studying hypertension due to their direct influence on body fluids and on the functions of the endocrine, cardiovascular and sympathetic nervous systems, in the present study, we aimed to investigate gene expression profiles in the kidneys. Since the association between kidney function and blood pressure is known to be influenced by numerous intrinsic and extrinsic factors, such as the renin-angiotensin system and catecholamine and aldosterone hormones (6), we compared gene expression profiles in the kidneys of SHRs and WKY rats and also between SHRSP and SHRs, when the rats were at 3 and 6 weeks old, a period in which rats are considered to be in a pre-hypertensive state. We isolated a total of 232 unique genes showing more than a 4-fold increase or less than a 4-fold decrease in expression.

After classifying these 232 genes into 4 groups according to their expression profiles, candidate genes were selected as significantly enriched genes, and categorized with Gene Ontology (GO) terms using the Database for Annotation, Visualization and Integrated Discovery (DAVID) web tools (7,8). Candidate genes were also selected using Ingenuity Pathway Analysis (IPA). The IPA path explorer tool revealed that B-cell CLL/lymphoma 6 (Bcl6) (9-13) and SRY (sex determining region Y)-box 2 (Sox2) (14,15) were possible candidate genes that trigger hypertension in SHRs. Moreover, our findings revealed that angiotensinogen (Agt), angiotensin II receptor-associated protein (Agtrap) (16-18) and apolipoprotein H (Apolh) (19) played pivotal roles among SHRSP-specific genes.

**Materials and methods**

**Animals, RNA extraction, microarray design, microarray analysis and microarray data analysis, reverse transcription-quantitative polymerase chain reaction (RT-qPCR), DAVID and IPA**. The details of these procedures have been described in our previous studies [Yamamoto et al (4) and Yoshida et al (5)].

**Animals.** Three strains of rat, SHR/Izm, SHRSP/Izm and WKY/Izm, were provided by the Disease Model Cooperative Research Association, Kyoto, Japan. Three-week-old rats were purchased and maintained for 2 days in our animal facility and were used as 3-week-old rats. Five-week-old rats were purchased and, after being maintained for 1 week in our animal facility, were used as 6-week-old rats.

**RNA extraction.** Briefly, total RNA was purified using a miRNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

**Microarray design.** Expression profiling was generated using a 4x44K whole rat genome oligo microarray version 3.0 G2519F (Agilent Technologies Inc., Santa Clara, CA, USA). Eighteen microarray analyses as 1 color experiment were performed using the WKY rats, SHRs, and SHRSP at 3 and 6 weeks old as biological triplicates. Each gene expression profile was compared between the SHRs and WKY rats and also between the SHRSP and SHRs.

**Microarray analysis.** Total RNA (200 ng) was reverse-transcribed into double-stranded cDNA by the AffinityScript Multiple Temperature Reverse Transcriptase (Agilent Technologies Inc.) and amplified. The resulting cDNA was used for in vitro transcription by T7-polymerase and labeled with cyanine-3-labeled cytosine triphosphate (Perkin-Elmer, Wellesley, MA, USA) using a Low Input Quick Amp Labeling kit (Agilent Technologies Inc.). After being labeled and fragmented, each cRNA sample was hybridized on Agilent 4x44K whole rat genome arrays (Agilent Design #028282). After washing, the slides were scanned using an Agilent Microarray Scanner (G2505C; Agilent Technologies Inc.). Feature Extraction software (version 10.5.1.1) was used to convert the images into gene expression data.

**RT-qPCR.** To validate the results obtained by the microarray analysis, 11 enriched genes were randomly selected from 39 enriched unique genes, and RT-qPCR was performed under 15 different experimental conditions. Statistical comparisons between the microarray and RT-qPCR data were performed using Spearman's rank correlation test.

**DAVID web tool analysis.** Annotation enrichment analysis was performed using the DAVID (http://david.abcc.ncifcrf.gov/) web tool (version 6.7, 2010) (7,8) with GenBank IDs bearing Entrez Gene ID (Table I, unique genes identified). This web-based resource provides a set of functional annotation tools for the statistical enrichment of genes categorized into GO terms. We used the GO FAT category, which filtered out very broad GO terms to identify statistically enriched functional groups. The annotated gene and protein symbols were written in italic and regular fonts, respectively.

**IPA.** IPA software (IPA®; Qiagen Redwood City, CA, USA, www.qiagen.com/ingenuity) was applied to microarray analyses that were conducted to provide functionality for the interpretation of the gene expression data. IPA was performed with Agilent probe IDs bearing Entrez Gene ID as an input for data (Table I, mapped probes). This web tool was used to overlay functions and diseases, and to categorize SHR- and SHRSP-specific genes according to disease-related or functional annotations. It identified the biological functions and/or diseases in the Ingenuity Knowledge Base (Spring 2014 version) that were the most significant to each of the category sets. The probability of the assignment was expressed by a P-value calculated using the right-tailed Fisher's exact test. The path explorer tool was also used to identify relevant interactions among SHR- and SHRSP-specific genes and to identify the shortest literature-supported paths between genes.

IPA was performed using the IPA database (Spring 2014 release of IPA) and the probe IDs of each gene. The data
obtained with DAVID were based on the database (version 6.7, 2010) and GenBank IDs of each gene. Since the renewal dates of these two databases were different, small differences were observed between these two annotation results.

Results

Isolation and classification of SHR- and SHRSP-specific genes. We compared gene expression profiles between the SHRs and WKY rats and also between the SHRSP and SHRs, at 3 and 6 weeks of age, and isolated SHR- and SHRSP-specific genes using genome-wide microarray technology. Since we expected the expression of candidate genes to be regulated before elevations in blood pressure (BP), i.e., in the pre-hypertensive period, we examined the expression profiles of each probe using RNA samples prepared from the kidneys, and isolated a total of 353 SHR- and SHRSP-specific probes showing more than a 4-fold increase or less than a 4-fold decrease in expression (Table I).

We classified the 353 probes into 4 groups, from G-1 to G-4 (Table I). G-1 probes were isolated from the rats at 3 weeks of age and contained 87 SHR-specific probes. Their expression profiles were displayed as a heatmap using the Subio Platform (Fig. 1). These 87 probes corresponded to 69 unique genes, 44 of which showed more than a 4-fold increase and 25 showed less than a 4-fold decrease in expression (Table I). G-2 contained 96 SHR-specific genes isolated from the rats at 6 weeks of age, G-3 contained 35 SHRSP-specific genes isolated from the rats at 3 weeks of age, and G-4 contained 32 SHRSP-specific genes isolated from the rats at 6 weeks of age (Table I).

Categorization and enrichment of SHR- and SHRSP-specific genes. Using the DAVID web tools, the candidate genes causing hypertension, stroke and ADHD were selected from each group as significantly enriched genes. We isolated a total of 61 enriched genes consisting of 39 unique genes (Table II).

In order to verify the results obtained from microarray analysis, we randomly selected 11 out of the 39 genes (Table III-A), performed 15 real-time RT-qPCR experiments (Table III-B), and compared the results obtained with those of the microarray experiments by applying Spearman's rank correlation test. The results supported a correlation between the results of these two different experiments as rs=0.814 with a two-tailed P-value <0.001.

A total of 69 G-1 genes included 26 enriched genes categorized with 3 GO terms: i) GO:0005576 (extracellular region); ii) GO:0008289 (lipid binding); and iii) GO:0055114 (oxidation reduction) (Table II, G-1). A total of 96 G-2 genes included 24 enriched genes categorized with 4 GO terms: i) GO:0003013 (circulatory system process); ii) GO:0051918 (negative regulation of fibrinolysis) and ii) GO:0030097 (hemopoiesis) (Table II, G-4). Although 26 enriched G-1 genes and 5 G-4 genes did not include genes categorized with circulatory system process, 24 enriched G-2 genes included 7 genes, and 6 enriched G-3 genes included 4 genes categorized with circulatory system process, respectively (Table II).

Functions and disease-related annotations of SHR- and SHRSP-specific genes. As described above, the SHR- and SHRSP-specific genes were classified into 4 groups (Table I),

| SHRs/WKY rats          | SHRSP/SHRs          |
|------------------------|---------------------|
| G-1 3 weeks old        | G-2 6 weeks old     | G-3 3 weeks old | G-4 6 weeks old | All  |
| All probes isolated    | 87                  | 156             | 57             | 53             | 353  |
| Mapped probes          | 72                  | 102             | 35             | 32             | 241  |
| Unmapped probes        | 15                  | 54              | 22             | 21             | 112  |
| Unique genes identified| 69                  | 96              | 35             | 32             | 232  |
| Upregulated            | 44                  | 51              | 18             | 19             | 132  |
| Downregulated          | 25                  | 45              | 17             | 13             | 100  |
| Enriched GO terms      | 3                   | 4               | 2              | 2              | 11   |
| Enriched genes         | 26                  | 24              | 6              | 5*             | 61   |

Number of SHR- and SHRSP-specific probes isolated from kidneys as described in the Materials and methods section; 232 out of the 353 isolated probes corresponded to unique genes with Entrez Gene IDs. Using DAVID web tools, 232 unique genes were categorized based on GO terms and 11 significantly enriched GO terms, which included 61 enriched genes, were identified (Table II). *Three of these 5 genes were categorized into GO:0030097 (hemopoiesis) with P=0.0393 (Table II, G-4). SHRs, spontaneously hypertensive rats; SHRSP, stroke-prone SHRs; GO, Gene Ontology; WKY rats, Wistar-Kyoto rats.
Figure 1. Heatmap of SHR- and SHRSP-specific probes. A heat map of SHR- and SHRSP-specific probes isolated from the kidneys of 3- and 6-week-old rats. Data were obtained with 353 probes for 3 rat strains, WKY rats, SHRs and SHRSP, under 18 different experimental conditions (3 different rat strains, 2 different rat ages, and triplicate experiments). The data obtained with G-1 probes, i.e., 87 out of 353 probes (Table I), were clustered based on their biological function and expression profiles using a hierarchical clustering program and Spearman's rank correlation. The values used for clustering were obtained by microarray experiments as described in the Materials and methods. The color bar at the right side of the panel indicates the log2 ratio for SHRs and SHRSP at 3 or 6 weeks of age vs. WKY rats at 3 or 6 weeks of age. The bottom panel (small boxes) indicates the experimental conditions, i.e., examined at 3 or 6 weeks of age, 3 different rat strains, and triplicate experiments. SHRs, spontaneously hypertensive rats; SHRSP, stroke-prone SHRs; WKY, Wistar-Kyoto rats.
and then categorized based on disease-related or functional annotations using IPA. The results obtained are summarized in Table IV, and identified among other significantly enriched functional categories, such as ‘endocrine system disorders’, ‘cardiovascular disease’, ‘cardiovascular system development and function’ and ‘hereditary disorder’ (Table IV).

G-1 genes included 2 genes, cystic fibrosis transmembrane conductance regulator (Cftr) and serine peptidase inhibitor, Kazal type 3 (Spink3) categorized as ‘endocrine system disorders’ (idiopathic pancreatitis) (Table IV, G-1) (20,21). G-2 genes included 8 genes: angiotensin I converting enzyme (Ace), deiodinase, iodothyronine, type II (Dio2), acyl-Coenzyme A oxidase 2 (Acox2), fin bud initiation factor homolog (Fibin), flavin-containing monoxygenase 2 (Fmo2), indoleamine N-methyltransferase (Inmt), myosin XVI (Myo16) and zinc finger and BTB domain containing 16 (Zbtb16) categorized as ‘cardiovascular disease (hypertension)’ (Table IV, G-2) (22-24). G-3 genes included 6 genes: Agt, Apoh, epoxide hydrolase 2 (Ephx2), histidine-rich glycoprotein (Hrg), ryanodine receptor 1 (Ryr1) and vascular endothelial growth factor B (Vegfb) categorized as ‘cardiovascular system development and function (development of cardiovascular system)’ (Table IV, G-3) (25-30). G-4 genes included 6 genes: Btg3 associated nuclear protein (Banp), Ephx2, retinoid X receptor gamma (Rxrg), Ryr1, RNA-binding protein fox-1 homolog 1 (Rbfox1) and Zbtb16 categorized as ‘hereditary disorder (Huntington’s disease)’ (Table IV, G-4) (31-33).

Table III. Validation of microarray data with RT-qPCR data.

| Group | GenBank ID | Gene symbol | FC (RT-qPCR) | FC (microarray) |
|-------|------------|-------------|--------------|----------------|
| G-1   | NM_001009626 | Apoh        | -10.236      | -61.982        |
| G-1   | NM_001044770 | Cyp4a2      | 4.808        | 12.786         |
| G-1   | NM_012564   | Gc          | 2.438        | 7.929          |
| G-1   | NM_019139   | Gdnf        | -1.036       | -5.658         |
| G-1   | NM_001009684 | Hsd17b13    | -2.432       | -9.747         |
| G-1   | NM_001108356 | LOC360919   | -1.252       | -5.247         |
| G-1   | NM_031765   | Rvrg        | -3.023       | -10.778        |
| G-1   | NM_001106121 | Ucma        | 2.370        | 7.026          |
| G-2   | NM_145770   | Acox2       | 2.908        | 7.567          |
| G-2   | NM_031506   | Cftr        | 5.125        | 6.202          |
| G-2   | NM_012564   | Gc          | 3.866        | 7.719          |
| G-2   | NM_001009684 | Hsd17b13    | -2.097       | -11.823        |
| G-2   | NM_001107295 | Oxnad1      | -1.199       | 23.616         |
| G-3   | NM_001009626 | Apoh        | 9.843        | 51.420         |
| G-4   | NM_001009626 | Apoh        | -8.046       | -69.883        |

RT-qPCR, reverse transcription-quantitative polymerase chain reaction; FC (RT-qPCR), fold change based on the results obtained with RT-qPCR; FC (microarray), fold change based on the results obtained with microarray analyses.
Interactions among SHR-specific G-1 and G-2 genes. Since our working hypothesis is that G-1 genes include genes that regulate the expression of G-2 genes, we examined the interactions between 69 G-1 and 96 G-2 genes using IPA, and found 5 direct and 3 indirect interactions (Table I and Fig. 2): Rxrg and group-specific component (Gc) interacted with cytochrome P450 subfamily 24 (Cyp24a1) (34,35); Bcl6 interacted with the following 3 genes: Zbtb16 (9,10), protocadherin 9 (Pcdh9) (11) and Spi-B transcription factor (Spib) (12); Cftr interacted with Ephx2 (36); tumor protein p73 (Tp73) interacted with tetraspanin 1 (Tspan1) (37); and Sox2 interacted with Tp73 (14).

Other than the 8 interactions between the G-1 and G-2 genes, we identified 3 interactions among the G-1 genes: Tp73 interacted with Tspan1; Sox2 interacted with Tp73; Sox2 interacted with connective tissue growth factor (Ctgf) (38); and among the G-2 genes: Gc interacted with Cyp24a1; Cftr interacted with Ephx2; Tp73 interacted with Tspan1, respec-
We also found 12 and 16 self-control genes among the SHR-specific G-1 and G-2 genes, respectively (Fig. 2). However, we did not detect any interactions between the G-1 genes and the majority of BP-controlling G-2 genes, such as Ace, Agtrap, Cft, glucagon-like peptide 1 receptor (Glp1r), kininogen 2 (Kng2), myosin light chain, phosphorylatable, fast skeletal muscle (Mylpf), Acox2, Dio2, Fibin, Fmo2, Inmt and Myo16 (Table II, G-2; GO:0003013, circulatory system process and Table IV, G-2; cardiovascular disease: hypertension).

Interactions among SHRSP-specific G-3 and G-4 genes. Since the enriched G-3 genes were expected to regulate the expression of the G-4 genes, we examined the interactions between 35 G-3 and 32 G-4 genes using IPA, and found that Agt interacted not only with Agtrap (16,17) expressed in the rats at 3 and 6 weeks of age, but also indirectly interacted with Zbtb16 (39) expressed in the rats at 6 weeks of age (Fig. 3). In addition, a total of 5 self-control genes, such as Agtrap, Ephx2, Apoh, Ryr1 and zinc finger protein 597 (Zfp597) were found to be expressed in the SHRSP at 3 and 6 weeks of age (Fig. 3).

The description and reference of each gene are summarized in Table V.

Discussion

General considerations. The first aim of the present study was to identify candidate genes that triggered hypertension in SHRs, the second was to identify genes related to stroke-prone symptoms, and the third was to identify genes related to ADHD. We compared gene expression profiles between SHRs and WKY rats and also between SHRSP and SHRs at 3 and 6 weeks of age, and isolated a total of 232 unique genes showing more than a 4-fold increase or less than a 4-fold decrease in expression as SHR- or SHRSP-specific genes (Table I). We expected a number of these genes to be related to hypertension, susceptibility to stroke and ADHD.

Interactions among SHR-specific G-1 and G-2 genes. The IPA path explorer tool suggested the presence of 5 direct interactions between 69 G-1 and 96 G-2 genes (Fig. 2): i) Rxrg interacted with Cyp24a1 (34); Bcl6 interacted with the following 3 genes: ii) Zbtb16 (9,10), iii) Pcdh9 (11) and iv) Spib (12); and v) Sox2 interacted with Tp73 (14).

i) Rxrg and Cyp24a1: Rxrg encodes a member of the retinoid X receptor (Rxr) family of nuclear receptors, which are involved in mediating the antiproliferative effects of...
retinoic acid. This receptor forms dimers with retinoic acid, thyroid hormone and vitamin D receptors, increasing both DNA binding and transcriptional function on their respective response elements. *Cyp24a1* encodes a member of the cytochrome P450 superfamily of enzymes. Cytochrome P450 proteins are monooxygenases that catalyze a number of reactions involved in drug metabolism and the synthesis of cholesterol, steroids and other lipids. By regulating vitamin D3 levels, this enzyme plays a role in calcium homeostasis and the vitamin D endocrine system.

ii) *Bcl6* and *Zbtb16*: *Bcl6* encodes a zinc finger transcription factor and contains an N-terminal POZ domain. This protein acts as a sequence-specific repressor of transcription, and has been shown to modulate the transcription of START-dependent IL-4 responses in B cells. This protein can interact with various POZ-containing proteins that function as transcription corepressors. *Zbtb16* is a member of the Kruppel C2H2-type zinc-finger protein family and encodes a zinc finger transcription factor that contains nine Kruppel-type zinc finger domains at the carboxyl terminus. This protein is located in the nucleus, is involved in cell cycle progression, and interacts with a histone deacetylase.

iii) *Bcl6* and *Pcdh9*: *Pcdh9* encodes a member of the protocadherin family, and of transmembrane proteins containing cadherin domains. These proteins mediate cell adhesion in neural tissues in the presence of calcium. The encoded protein may be involved in signaling at neuronal synaptic junctions.

iv) *Bcl6* and *Spib*: *Spib* encodes a transcriptional activator that binds to the PU-box (5'-GAGGAA-3') and acts as a lymphoid-specific enhancer.

v) *Sox2* and *Tp73*: *Sox2* encodes a member of the SRY-related HMG-box (SOX) family of transcription factors involved in the regulation of embryonic development and in the determination of cell fate. The product of this gene is required for stem-cell maintenance in the central nervous system, and also regulates gene expression in the stomach. *Tp73* encodes tumor protein p53, which responds to diverse cellular stresses to regulate the target genes that induce cell cycle arrest, apoptosis, senescence, DNA repair, and changes in metabolism. The p53 protein is expressed at low levels in normal cells and at high levels in various transformed cell lines, in which it has been suggested to contribute to transformation and malignancy. p53 is a DNA-binding protein that contains transcription activation, DNA-binding and oligomerization domains. It has been postulated to bind to a p53-binding site and activate the expression of downstream genes that inhibit growth and/or invasion, thereby functioning as a tumor suppressor.

Other than these 5 direct interactions between G-1 and G-2 genes, we identified one direct interaction between G-1 genes; *Sox2* interacted with *Ctgf* (38), which encodes a mitogen that is secreted by vascular endothelial cells. This encoded protein plays a role in chondrocyte proliferation and differentiation, cell adhesion in many cell types, and is related to platelet-derived growth factor. *Ctgf* has been linked to the
Table V. List of SHR- and SHRSP-specific genes.

| Group | GS | Description | GenBank ID | FC     | P-value  | (Refs.) |
|-------|----|-------------|------------|--------|----------|---------|
| G-1   | Acox2 | Acyl-CoA oxidase 2, branched chain | NM_145770 | 8.194  | 1.05E-06 |         |
|       | Akr1c121l | Aldo-keto reductase family 1, member C12-like 1 | NM_001135744 | 4.772  | 1.11E-04 |         |
|       | Ankrd35 | Ankyrin repeat domain 35 | XM_001063190 | -4.364 | 9.02E-03 | (42)    |
|       | Apoh | Apolipoprotein H (β-2-glycoprotein I) | NM_001009626 | -61.982 | 2.15E-04 |         |
|       | Bcl6 | B-cell CLL/lymphoma 6 | NM_001107084 | 4.010  | 4.99E-03 | (9-13)  |
|       | Ctgf | Connective tissue growth factor conductance regulator | NM_031506 | 6.434  | 4.16E-03 | (20,36) |
|       | Cyp4a2 | Cytochrome P450, family 4, subfamily a, polypeptide 2 | NM_001044770 | 12.786 | 6.75E-04 |         |
|       | Cyp8b1 | Cytochrome P450, family 8, subfamily b, polypeptide 1 | NM_001013098 | 14.138 | 1.16E-03 |         |
|       | Dhrs7 | Dehydrogenase/reductase (SDR family) member 7 | NM_001013098 | -4.151 | 2.54E-05 |         |
|       | Endog | Endonuclease G | NM_001034938 | -4.151 | 2.54E-05 |         |
|       | Fibin | Fin bud initiation factor homolog (zebrafish) | NM_001025042 | 4.089  | 5.41E-03 |         |
|       | Galr2 | Galanin receptor 2 | NM_019172 | -5.289 | 7.51E-03 |         |
|       | Gc | Group-specific component | NM_012564 | 7.929  | 2.12E-05 | (35)    |
|       | Gdnf | Glial cell derived neurotrophic factor | NM_019139 | -5.658 | 4.27E-04 |         |
|       | Hsd17b13 | Hydroxysteroid (17-β) dehydrogenase 13 | NM_001009684 | -9.747 | 4.47E-05 |         |
|       | Il9 | Interleukin 9 | NM_01105747 | -4.291 | 7.11E-04 |         |
|       | LOC360919 | Similar to α-fetoprotein | NM_001108356 | -5.247 | 8.04E-06 |         |
|       | Neft | Neurofilament, heavy polypeptide | NM_012607 | 4.214  | 8.78E-04 |         |
|       | Nphp1 | Neurexphilin 1 | NM_012994 | 4.709  | 9.34E-03 |         |
|       | Oxnad1 | Oxidoreductase NAD-binding domain containing 1 | NM_01107295 | 27.732 | 3.78E-05 |         |
|       | Ptprj | Protein tyrosine phosphatase, receptor type, J | NM_017269 | -4.285 | 2.21E-05 |         |
|       | Rdh16 | Retinol dehydrogenase 16 (all-trans) | NM_199208 | 5.014  | 1.25E-03 |         |
|       | Rxrg | Retinoid X receptor gamma | NM_031768 | -10.778 | 1.92E-04 | (34)    |
|       | Serpina3m | Serine (or cysteine) proteinase inhibitor, clade A, member 3M | NM_01067511 | 21.327 | 1.09E-05 |         |
|       | Snap91 | Synaptosomal-associated protein 91kDa | NM_031728 | 4.424  | 2.23E-04 |         |
|       | Sox2 | SRY (sex determining region Y)-box 2 | NM_001109181 | -7.694 | 1.17E-05 | (14,15,38) |
|       | Spink3 | Serine peptidase inhibitor, Kazal type 3 | NM_012674 | 4.448  | 1.33E-03 | (21)    |
|       | Spock2 | Sparc/osteonectin, cwcv, and Kazal-like domains proteoglycan (testican) 2 | NM_001108533 | 7.515  | 5.69E-06 |         |
|       | Tp73 | Tumor protein p73 | NM_001108696 | 10.360 | 2.34E-05 | (14,37) |
|       | Tspan1 | Tetraspanin 1 | NM_001004236 | -6.728 | 4.26E-05 | (37)    |
|       | Ucma | Upper zone of growth plate and cartilage matrix associated | NM_001106121 | 7.026  | 1.06E-05 |         |
|       | Vegfb | Vascular endothelial growth factor B | NM_053549 | -340.226 | 2.71E-06 |         |
| G-2   | Ace | Angiotensin I converting enzyme | NM_012544 | -4.207 | 1.12E-05 | (22)    |
|       | Acox2 | Acyl-CoA oxidase 2, branched chain | NM_145770 | 7.567  | 1.56E-06 | (24)    |
|       | Agtrap | Angiotensin II receptor-associated protein conductance regulator | NM_001007654 | -23.157 | 2.68E-06 |         |
|       | Cftr | Cystic fibrosis transmembrane | NM_031506 | 6.202  | 1.03E-03 | (36)    |
|       | Cldn14 | Claudin 14 | NM_001013429 | -6.099 | 7.47E-03 |         |
|       | Cyp24a1 | Cytochrome P450, family 24, subfamily a, polypeptide 1 | BC100059 | 4.799  | 1.60E-04 | (34,35) |
|       | Cyp2c11 | Cytochrome P450, subfamily 2, polypeptide 11 | NM_019184 | 9.295  | 8.44E-03 |         |
|       | Cyp8b1 | Cytochrome P450, family 8, subfamily b, polypeptide 1 | NM_031241 | 38.029 | 2.17E-04 | (47)    |
|       | Dio2 | Deiodinase, iodothyronine, type II | NM_031720 | 5.290  | 1.47E-03 | (23,46) |
Table V. Continued.

| Group | GS | Description | GenBank ID | FC   | P-value | (Refs.) |
|-------|----|-------------|------------|------|---------|---------|
| Ephx2 |     | Epoxide hydrolase 2, cytoplasmic | NM_022936 | -10.816 | 1.56E-05 | (36,44) |
| Fibrin |     | Fin bud initiation factor homolog (zebrafish) | NM_001025042 | 6.062 | 7.32E-05 | (24) |
| Fmo2  |     | Flavin-containing monoxygenase 2 | NM_144737 | 5.407 | 5.45E-05 | (24) |
| Gc    |     | Group-specific component | NM_012564 | 7.719 | 6.18E-03 | (35) |
| Gdnf  |     | Glial cell derived neurotrophic factor | NM_019139 | -6.710 | 2.03E-08 | (49) |
| Glplr |     | Glucagon-like peptide 1 receptor | NM_012728 | -4.181 | 1.03E-03 | (24) |
| Fmo2  |     | Flavin-containing monoxygenase 2 | NM_144737 | 5.407 | 5.45E-05 | (24) |
| Gc    |     | Group-specific component | NM_012564 | 7.719 | 6.18E-03 | (35) |
| Gdnf  |     | Glial cell derived neurotrophic factor | NM_019139 | -6.710 | 2.03E-08 | (49) |
| Glplr |     | Glucagon-like peptide 1 receptor | NM_012728 | -4.181 | 1.03E-03 | (24) |
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| Gdnf  |     | Glial cell derived neurotrophic factor | NM_019139 | -6.710 | 2.03E-08 | (49) |
| Glplr |     | Glucagon-like peptide 1 receptor | NM_012728 | -4.181 | 1.03E-03 | (24) |
| Ephx2 |     | Epoxide hydrolase 2, cytoplasmic | NM_022936 | -12.769 | 2.99E-05 | (27) |
| Hrg   |     | Histidine-rich glycoprotein | NM_133428 | 4.578 | 2.22E-05 | (28) |
| Ephx2 |     | Epoxide hydrolase 2, cytoplasmic | NM_022936 | -12.769 | 2.99E-05 | (27) |
| Hrg   |     | Histidine-rich glycoprotein | NM_133428 | 4.578 | 2.22E-05 | (28) |
| Ephx2 |     | Epoxide hydrolase 2, cytoplasmic | NM_022936 | -12.769 | 2.99E-05 | (27) |
| Hrg   |     | Histidine-rich glycoprotein | NM_133428 | 4.578 | 2.22E-05 | (28) |
| Ephx2 |     | Epoxide hydrolase 2, cytoplasmic | NM_022936 | -12.769 | 2.99E-05 | (27) |
| Hrg   |     | Histidine-rich glycoprotein | NM_133428 | 4.578 | 2.22E-05 | (28) |
development and progression of diabetic vascular and renal disease. Low-density lipoproteins (LDL) have previously been shown to induce the expression of Ctgf in aortic endothelial cells (40) (Fig. 2).

**SHR-specific G-1 and G-2 genes related to hypertension.** Even based on the interactions, described above, we were unable to pinpoint the candidate gene(s) causing hypertension. Although these predicted interactions included the hypertension-related G-2 genes, Ephx2 and Zbtb16, they did not include other hypertension-related genes, such as Ace, Agtrap, Cfr, Glplr, Kng2, Mylpf, Accox2, Dio2, Fbin, Fmo2, Inmt and Myo16 (Table II, G-2; GO:0003013, circulatory system process and Table IV, G-2; cardiovascular disease: hypertension). In order to identify further interactions between SHR-specific G-1 and G-2 genes, we applied the IPA path explorer tool, suggested the presence of one gene that assisted in these interactions, and found such a condition when we proposed the Jun proto-oncogene (Jun) (41), which interacts directly with specific target DNA sequences to regulate gene expression. The presence of Jun has been facilitated to interact with 3 G-1 genes: Bcl6 (13), Sox2 (15) and ankyrin repeat domain 35 (Ankr35) (42), and 8 G-2 genes: Spib (43), Ephx2 (44), Tp73 (45), Dio2 (46), cytochrome P450, family 8, subfamily b, polypeptide 1 (Cyp8b1) (47), Mylpf (48), glial cell derived neurotrophic factor (Gdnf) (49) and neurofilament, heavy polypeptide (Nefh) (50) (Fig. 2).

These findings suggested that Bcl6 and Sox2 were the candidate genes responsible for causing hypertension in SHRs.

**Interactions among SHRSP-specific G-3 and G-4 genes.** Since the candidate gene(s) found to cause stroke in SHRSP were expected to be included in the G-3 genes, we focused on the interaction between G-3 and G-4 genes (Fig. 3). Our results revealed that G-3 genes included 3 typical blood pressure-related genes, Ephx2, Kng2 and Agrp (Table II, G-3; GO:0003013, circulatory system process). These 3 genes isolated from the SHRSP at 3 weeks of age were not isolated from the SHRs at 3 weeks of age, but were isolated from the SHRs at 6 weeks of age (Table II). These results indicated that the expression of genes related to BP control proceeds more rapidly in SHRSP than in SHRs during their development.

The IPA path explorer tool revealed one interaction among G-3 genes and 8 self-controlling genes (Fig. 3). One of the G-3 genes, Agrp, interacted with another G-3 gene, Agtrap, and Agrp also interacted with 2 G-4 genes, Agtrap and Zbtb16 (Fig. 3). These results suggest that Agrp, Agtrap and Zbtb16 play pivotal roles in causing stroke-prone symptoms. Moreover, G-4 genes including 9 self-controlling genes (Fig. 3), and self-control genes, such as Agtrap, Ephx2, Apoh, Ryr1 and Zfp597, were expressed in the 3- and 6-week-old SHRSP.

In order to detect further interactions between SHRSP-specific G-3 and G-4 genes, we applied the IPA path explorer tool, suggested the presence of one gene that assisted these interactions, and found such a condition when we proposed the Hrg gene in host protection from atherosclerosis and susceptibility to strokes. Moreover, 2 enriched G-3 genes, Agrp (18) and Apoh (19), and 3 G-4 genes, Apoh, Vegtub (52) and Banp (51) (Fig. 3).

**SHRSP-specific G-3 and G-4 genes related to stroke.** Four enriched G-3 genes were categorized as GO:0003013 (circulatory system process). These genes were expected to participate in blood pressure control and the pathogenesis of stroke. Moreover, 2 enriched G-3 genes, Apoh and Hrg; were categorized as GO:0051918 (negative regulation of fibrinolysis) (Table II, G-3). Since Apoh has been implicated in various physiological pathways, including lipoprotein metabolism, coagulation and the production of antiphospholipid autoantibodies, we hypothesized that it may participate in the genesis of atherosclerosis and stroke. Hrg possesses antimicrobial activity, and the incorporation of Hrg into fibrin clots facilitates bacterial entrapment and killing and promotes inflammation. Since vascular inflammation is known to trigger atherosclerosis, Hrg influences atherosclerosis and susceptibility to strokes.

Two out of the 5 enriched G-4 genes, Apoh and Hrg were categorized as GO:0051918 (negative regulation of fibrinolysis), while the remaining 3 genes, chemokine (C-C motif) receptor 1 (Ccr1), leukocyte immunoglobulin-like receptor B-3-like (Lilrb3l) and Zbtb16 were categorized as GO:0030097 (hemopoiesis) (Table II, G-4): Ccr1 encodes a member of the β-chemokine receptor family. Knockout studies on the mouse homolog suggested roles for these genes in host protection from...
inflammatory responses, and susceptibility to viruses and parasites. Lirb3l is a receptor for the major histocompatibility complex class I antigen (MHC-I), and may play a physiological role in the brain for neuronal circuitry stability by inhibiting synaptic plasticity. Zbhlb6 encodes a protein which is located in the nucleus. It is involved in cell cycle progression and interacts with a histone deacetylase.

Genes related to ADHD and Huntington's disease. We previously examined gene expression profiles in the brain, and found that 6 SHRSP-specific genes isolated from the rats at 6 weeks of age (Agtr1b, Arc, Egr2, Fos, Hspa1b and Snca) were annotated to ‘behavior’ and were suggested to participate in the genesis of ADHD (5). In the present study, we investigated gene expression profiles in the kidneys, and unexpectedly found that 6 SHRSP-specific genes isolated from the rats at 6 weeks of age (Banp, Ephx2, Rfxb1, Rxrg, Ryr1 and Zbhlb6) were annotated to ‘Huntington's disease’ (Table IV, G-4). Tp53 was also found to be involved in ‘Huntington's disease’ (33,52). These findings suggested the participation of common genes in the genesis of symptoms related to ADHD and Huntington's disease (Table IV, G-4).

Conclusion. SHR-specific genes isolated from the kidneys of 3-week-old rats included possible candidate genes that trigger hypertension (Bcl6 and Sox2), and SHRSP-specific genes isolated from the kidneys of 3-week-old rats included possible candidate genes that trigger stroke, such as Agt, Agtrap and Apoh. The results obtained from SHRSP-specific genes isolated from the kidneys of 6-week-old rats included 6 genes that have been functionally annotated to Huntington's disease (Banp, Ephx2, Rfxb1, Rxrg, Ryr1 and Zbhlb6). These results implicate these genes in the involuntary movement associated with Huntington's disease as well as 'attention-deficit hyperactivity' observed in ADHD.

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