

## Microarray Analysis in Spontaneously Hypertensive Rat Heart after Losartan Treatment

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**Objectives:** Spontaneously hypertensive rats (SHR) are frequently used as rat models of essential hypertension. The mechanism for the development of hypertension is complicated and it is unknown. The renin-angiotensin system (RAS) plays a key role in the control of blood pressure. Microarrays are a powerful tool for studying genetics. The purpose of this study was to investigate changes of gene expression in the heart tissues of SHR after losartan treatment to provide basic data that is useful in the early diagnosis of hypertension and gene treatment.

**Methods:** Rats were divided into three groups: the control (C) group; the hypertension (H) group (SHR), and the losartan (L) group; treated with losartan (10 mg/kg/day) in SHR. Rats were sacrificed at week 5 and microarray analysis was performed.

**Results:** 102 gene expressions including the genes associated with cell proliferation such as *Raf1*, *Uchl1*, *Btla*, *Spock1* were increased. The other 139 gene expressions, including the genes related to the regulation of metabolism such as *TFIID*, *Aufl*, *Bmp*, *Hub*, *Taf51* showed decreases in gene expression. A total of 31 genes were differentially expressed in the L group compared to the H group. Of these, 16 genes including the genes associated with macromolecule metabolism such as *MGC105766*, *Ppp1r1a*, *Rpl3l* showed increased expression. The other 15 genes including the genes associated with primary metabolism such as *Mcpt4*, *Ngn3*, *Tdo*, *Ak2*, *Hyal2* showed decreased expressions.

**Conclusion:** According to microarray analysis, there was significant gene expression change in SHR compared with normal rats as well as significant gene expression changes after losartan treatment in SHR. (Ewha Med J 2016;39(2):45-50)

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### Key Words

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## Introduction

Essential hypertension contributes significantly to cardiovascular morbidity and mortality. Twenty to 30% of the population worldwide suffers from hypertension [1]. Hypertension is one of the major risk factors for cardiovascular disease and can cause cerebrovascular and renal damage if not properly controlled [2].

Spontaneously hypertensive rats (SHRs) of Okamoto-Aoki strain were developed by selective breeding of Wistar-Kyoto stock for higher blood pressure. SHRs spontaneously develop

moderate-to-severe hypertension between 7 and 15 weeks of age and have served as a model of genetic hypertension in humans [3]. There are many mechanisms by which high blood pressure in SHR have been elucidated [4,5]. SHRs are commonly used in androgen-dependent genetic and experimental models. SHRs are frequently found in studies on essential hypertension [4-8].

The renin-angiotensin system (RAS) plays a central role in the control of cardiovascular and renal functions by maintaining homeostasis of blood pressure and electrolyte balance. Abnormal

activation of the RAS is associated with the pathogenesis of cardiovascular disease such as hypertension, myocardial infarction and heart failure [9–12].

Angiotensin (Ang II) is a key regulator of the RAS. Ang II is involved in many regulatory mechanisms in the cardiovascular system besides blood pressure [13]. It stimulates proliferation of smooth muscle cells in the arterial wall, increases collagen deposits and enhances matrix components. Ang II modifies the structure and the thickness of the arterial wall in both resistant and conduit arteries as well as modulates sympathetic activity. Also, it involves in endothelin-1 release from vascular endothelium and tunica adventitia and impacts the bioavailability of nitric oxide and endothelial functions. Ang II converting enzyme has been known as an important enzyme in the regulation of the RAS [14].

Losartan is a Ang II receptor antagonist, so there is a mechanism of action which involves the specific blockade of Ang II receptors by inhibiting vasoconstriction and preventing vascular and cardiac hypertrophy [6,12,15]. There were several articles about the effect of losartan in an SHR model [7,16]. The results were different according to the dose of losartan. However, reports about gene changes have been rare.

The purpose of our study was to investigate changes in gene expression of SHRs after losartan treatment using microarray analysis and to identify candidate genes responsible for causing hypertension in SHRs.

## Methods

### 1. Materials

Twelve-week-old male Wistar-Kyoto rats were used as control (C) rats for this study. The SHRs of Okamoto-Aoki strain were developed by selective breeding of Wistar-Kyoto stock for higher blood pressure. SHRs spontaneously and consistently develop moderate-to-severe hypertension between 7 and 15 weeks of age. These rats were used as hypertensive rats. All rats were housed in climate-controlled conditions with a 12 hours light: 12 hours dark cycle, and had free access to chow and water.

The rats were grouped as follows: C group (n=3), hypertension (H) group (n=3), losartan (L) group (n=3) in which losartan was administrated daily in SHR rats by gavage in a dose of 10 mg/kg/day for 5 weeks. The rats were sacrificed at week 5. The heart tissues were removed and immediately frozen

at  $-70^{\circ}\text{C}$  for microarray analysis.

All protocols were approved by the Institutional Animal Care and Use Committees of the School of Medicine of Ewha Womans University (approval No. EMS 13-0125).

### 2. Methods

#### 1) Heart microarray

##### (1) Preparation of fluorescent DNA probe and hybridization

Total RNA was extracted from the rat heart tissue using the TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. Each total RNA sample (10  $\mu\text{g}$ ) was labeled with Cyanine (Cy5) conjugated dCTP (Amersham Biosciences Corp., Piscataway, NJ, USA) by a reverse transcription reaction using reverse transcriptase, SuperScrip II (Invitrogen, Carlsbad, CA, USA). The labeled cDNA was then concentrated using ethanol precipitation method. The concentrated Cy5 labeled cDNAs were resuspended in 10  $\mu\text{L}$  of hybridization solution (GenoCheck, Ansan, Korea). After labeled cDNAs were placed on Roche NimbleGen Rat genome 12-plex array (Roche NimbleGen Inc., Madison, WI, USA) and covered by a NimbleGen H12 mixer (Roche NimbleGen Inc.). The slides were hybridized for 12 hours at  $42^{\circ}\text{C}$  MAUI system (Bio-micro Systems Inc., Salt Lake City, UT, USA). The hybridized slides were washed in  $2\times$ saline sodium citrate (SSC), 0.1% sodium dodecyl sulfate for 2 minutes,  $1\times$ SSC for 3 minutes, and then  $0.2\times$ SSC for 2 minutes at room temperature. The slides were centrifuged at 3,000 rpm for 20 seconds to dry.

##### (2) Data analysis

The Roche NimbleGen Rat genome 12-plex array was submitted to Roche NimbleGen Inc. for microarray design and manufacture using maskless, digital micromirror technology. Twelve replicates of the genome were included per chip. An average of 5 different 60-base oligonucleotides (60-mer probes) represented each gene in the genome. Sixty-mer probes were selected such that each probe had at least three mismatches compared to all other 60-mers in the target genome. A quality control check (hybridization) was performed for each array, which contained on-chip control oligonucleotides. The arrays were analyzed using an Axon GenePix 4000B scanner with associated software (Molecular Devices Corp., Sunnyvale, CA, USA). Gene expression levels were calculated with NimbleScan ver. 2.4 (Roche NimbleGen Inc.).

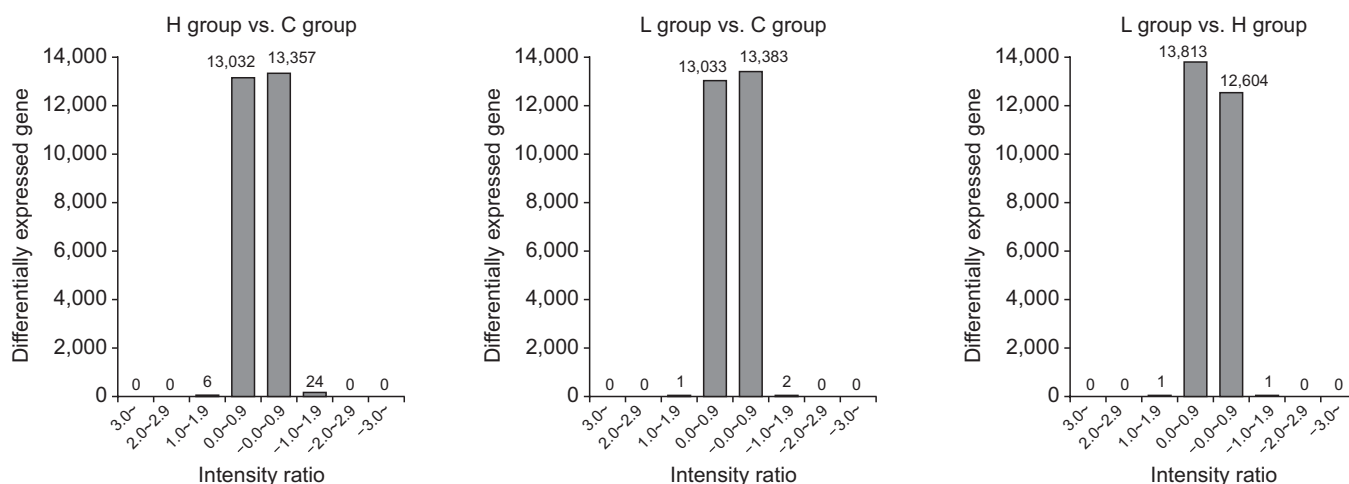


Fig. 1. Intensity ratio histogram in three groups. The graph shows a number of genes showing gene expression changes. C, control; H, hypertension; L, losartan.

Relative signal intensities for each gene were generated using the robust multi-array average algorithm. The data were processed based on median polish normalization method using the NimbleScan ver. 2.4. This normalization method aims to make the distribution of intensities for each array in a set of arrays the same. The normalized, and log transformed intensity values were then analyzed using GeneSpring GX 7.3.1 (Agilent Technologies, Santa Clara, CA, USA). Fold change filters included the requirement that the genes be present in at least 150% of controls for up-regulated genes and lower than 66.67% of controls for down-regulated genes. Hierarchical clustering data were clustered groups that behave similarly across experiments using GeneSpring GX 7.3.1. Clustering algorithm was Euclidean distance, average linkage.

## 2) Statistical analysis

Results were expressed as the mean ± standard deviation. An unpaired two-tailed t-test and Mann-Whitney test were used, and a P < 0.05 was considered statistically significant. SPSS ver. 12.0 (SPSS, Chicago, IL, USA) was used for all statistical analyses.

## Results

### 1. Intensity ratio histogram in three groups

Intensity ratio histogram used log intensity and showed a continuous data of the number of differentially expressed genes according to the variation in the gene expression. X-axis

Table 1. Changes of differentially expressed gene after losartan treatment

Group	Significant increase	Significant decrease	Total
Hypertension vs. control	103	138	241
Losartan vs. control	33	23	56
Losartan vs. hypertension	16	17	33

showed a log2 ratio of comparison/control group and Y-axis showed a number of genes corresponding to the log intensity. The graph, except the two bars in the middle, showed a number of genes showing gene expression changes (Fig. 1).

This table showed the count of statistically significant differentially expressed genes. In the H group comparison with the C group, a total of 241 genes in the H group were differentially expressed with 103 genes showing significant over expression while the other 138 genes showed a significant down expression. A total of 56 genes in L group compared with C group exhibited a differential expression and of these, 33 showed over expression and 23 showed down expression. Likewise, a total of 33 genes were differentially expressed in L group compared with H group and 15 genes showed a significant over expression and the other 17 genes showed a significant down expression (Table 1).

### 2. Clustering

This is the gene expression profile expressed differentially over 1.5 fold (P < 0.05). Red color represents over expression and

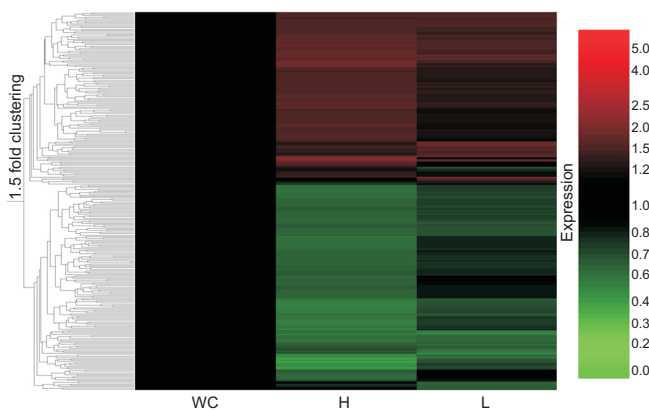
green color represents down expression (Fig. 2).

### 3. Functional analysis

This table demonstrated the functional classification of statistically significantly differentially expressed genes (Table 2). In the H group compared with the C group, a total of 241 genes showed a differential gene expression by more than 1.5 fold ( $P < 0.05$ ). Of these, 102 genes including the genes associated with cell proliferation (GO:8283,  $P = 0.0463$ ) such as *Raf1*, *Uchl1*, *Btla*, *Spock1* were over expressed by more than 1.5 fold. The other 139 genes including the genes related to regulation of metabolism (GO:19222,  $P = 0.0354$ ) such as *TFIID*, *Auf1*, *Bmp*, *Hub*, *Taf51* were down expressed by more than 1.5 fold ( $P < 0.05$ ).

In the L group sample compared with the C group sample, a total of 55 genes were differentially expressed by more than 1.5 fold ( $P < 0.05$ ). Of these, 32 genes including the genes associated with signal transduction (GO:7165,  $P = 3.66e-6$ ) such as *Olr510*, *MGC94805*, *Gadd45g*, *Prkacb* showed over expression by more than 1.5 fold. The other 23 genes including the genes for cell motility (GO:6928,  $P = 0.00083$ ) such as *Sema3b*, *Mcs* showed down expression by over 1.5 fold ( $P < 0.05$ ).

In the L group in comparison with the H group, a total of 31 genes were differentially expressed by more than 1.5 fold ( $P < 0.05$ ). Of these, 16 genes including the genes associated with macromolecule metabolism (GO:43170,  $P = 0.0752$ ) such as *MGC105766*, *Ppp1r1a*, *Rpl31* showed more than 1.5 fold



**Fig. 2.** Changes of gene heat map after losartan treatment. This is the gene expression profile expressed differentially over 1.5 fold ( $P < 0.05$ ). Red color represents over expression and green color represents down expression. C, control; H, hypertension; L, losartan.

over expression. The other 15 genes including the genes associated with primary metabolism (GO:44238,  $P = 0.0531$ ) such as *Mcpt4*, *Ngn3*, *Tdo*, *Ak2*, *Hyal2* showed more than 1.5 fold ( $P < 0.05$ ) down expression.

## Discussion

The aim of our study was to investigate changes in gene expression of SHRs after losartan treatment using microarray analysis. Our hypothesis was that there would be changes of gene expression in the SHR group compared with the control rats, and that gene expression could be decreased after losartan treatment. The main and novel finding of this study is that in microarray analysis, there were significant gene expression changes in the hypertensive rats compared with the normal rats and there were significant gene expression changes after losartan treatment in the hypertensive rats as well.

We found that there were significant gene expression changes such as proliferation, translation, inflammatory response, immune response, cell adhesion, cell migration, cell differentiation, apoptosis, cell growth, transport, cell cycle, transcription, signal transduction and homeostasis among the C, H, and L groups.

In comparison to the C group, a total of 241 genes in the H

**Table 2.** Functional analysis after losartan treatment

Variable	H vs. C	L vs. C	L vs. H
Proliferation	10	0	0
Translation	11	2	0
Inflammatory response	4	0	0
Immune response	10	1	0
Cell adhesion	7	0	0
Cell migration	1	1	0
Cell differentiation	8	2	1
Apoptosis	3	1	0
Cell growth	3	0	0
Transport	25	4	3
Cell cycle	4	1	1
Transcription	19	2	1
Signal transduction	38	13	3
Homeostasis	1	2	0
Total	241	55	31

C, control; h, hypertension; L, losartan.

group were significantly differentially expressed, showing over or down expression. A total of 56 genes in the L group compared with the C group exhibited a differential expression. Likewise, a total of 33 genes were differentially expressed in the L group compared with the H group, and of these, 15 genes showed a significant over expression, and the other 17 genes showed a significant down expression.

In function analysis of the H group compared with the C group, a total of 241 genes showed a differential gene expression by more than 1.5 fold. Of these, 102 genes including the genes associated with cell proliferation were over expressed by more than 1.5 fold. The other 139 genes, including the genes related to regulation of metabolism, were down expressed by more than 1.5 fold.

A total of 55 genes were differentially expressed by more than 1.5 fold in analysis of the L group sample compared with the C group. In the L group, 32 genes, including the genes associated with signal transduction, showed over expression by more than 1.5 fold. The other 23 genes in the L group, including the genes for cell motility such as *Sema3b* and *Mcs*, showed down expression by over 1.5 fold.

From comparison of the L and H group samples, a total of 31 genes were differentially expressed by more than 1.5 fold in microarray. Of these, 16 genes, including the genes associated with macromolecule metabolism, showed more than 1.5 fold over expression. The other 15 genes, including the genes associated with primary metabolism, showed more than 1.5 fold down expression.

The polygenic nature of hypertension has made it difficult to isolate genes involved in the genesis of this disease [17]. Microarrays are a powerful tool for studying the genetics of hypertension as they facilitate the measurement of the expression of thousands of genes simultaneously. Since rodent models of human essential hypertension are ideal for microarray research, animal models of essential hypertension have been investigated using microarrays [18].

SHRs, the current paradigm for essential hypertension research, were developed in a breeding program based solely on selection by elevated blood pressure in Wistar rats [1]. Normotensive descendants of Wistar-Kyoto rats were used as the controls [19].

Hypertension is an important risk factor of cardiovascular diseases such as myocardial infarction, atherosclerosis, congen-

tive heart failure [20]. The RAS seems to play a key role in the development of cardiac and vascular hypertrophy [15]. Losartan is a highly specific, non-peptide antagonist of type 1 Ang II receptor. Ang II receptor antagonists are a group of drugs that are used in the treatment of hypertension. The administration of Ang II receptor blockers are widely used in the treatment of hypertension [21]. Currently, losartan is a highly popular antihypertensive agent with well-recognized clinical efficacy supported by large-scale clinical studies [22]. Blood pressure is influenced by both genomic and environmental factors, as well as their interactions [23]. However, the molecular mechanisms underlying the beneficial effects of losartan remain elusive. In the future, we want to identify the candidate genes responsible for causing hypertension in SHR and to provide basic data useful in the early diagnosis of hypertension and gene treatment.

In conclusion, we found that there were significant changes in the gene expressions of SHRs compared with normal rats as well as significant gene expression changes after losartan treatment of SHRs as shown by microarray analysis.

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