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Genome Wide Expression Analysis of the Effect of *Woowhangchongshim-won* on Rat Brain Injury

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ABSTRACT

Objectives : ICH breaks down blood vessels within the brain parenchyma, which finally leads to neuronal loss, drugs to treat ICH have not yet been established. In this experiment, we measured the effect of *Woowhangchongshim-won* (WWCSW) on intracerebral hemorrhage (ICH) in rat using microarray technology.

Methods : We measured the effect of WWCSW on ICH in rat using microarray technology. ICH was induced by injection of collagenase type IV, and total RNA was isolated. Image files of microarray were measured using a ScanArray scanner, and the criteria of the threshold for up- and down-regulation was 2 fold. Hierarchical clustering was implemented using CLUSTER and TREEVIEW program, and for Ontology analysis, GOSTAT program was applied in which p-value was calculated by Chi square or Fisher's exact test based on the total array element.

Results : WWCSW-treatment restored the gene expression altered by ICH-induction in brain to the levels of 76.0% and 70.1% for up- and down-regulated genes, respectively.

Conclusion : Co-regulated genes by ICH model of rat could be used as molecular targets for therapeutic effects of drug including WWCSW. That is, the presence of co-regulated genes may represent the importance of these genes in ICH in the brain and the change of expression level of these co-regulated genes would also indicate the functional change of brain tissue.

Key words : *Woowhangchongshim-won*, microarray, intracerebral hemorrhage, transcription factor binding site

Ⅰ. Introduction

Traditionally, Woo-whang-chong-shim-won(WWCSW) has been used to treat hypertension, arteriosclerosis, coma and stroke in China and Korea[1,2]. Although some reports showed its effect on loorting blood pressure and inhibiting cardnhc muscle in cardnovascular system[3-5], the mechanism of pharmaceutical effect has not been studied yet in

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molecular level. In recent, WWCSW suspension was shown to strongly inhibit CYP2B6 activity in vitro[6]. This effect should be considered to e6 acate pharmaceutical effect of WWCSW. Because WWCSW ity ompo acatf varioutykinds of chemof phaomponents, it would be dif2.cult to hina an major aomponents having pharmaceutical effect. Although some aomponents such as mutk was shown to have effect of anti-infa mmation, anti-hist mio hand anticancer[7-9], these aomponents could not represent the whc e pharmaceutical effect of WWCSW. Therefore high caroughput screening systems such as mh some repW suysis is essential process to elucidate the molecular effects of herbal extract on animal disease model.

In this experiment, we measured the effect of WWCSW on intracerebral hemorrhage (ICH) in rat using microarray technology. ICH has been known to be associated with strokes and head injuries. ICH breaks down blood vessels within the brain parenchyma, which finally leads to the neuronal loss, but drugs for ICH have not been established yet. Therefore, in this study, the effect of WWCSW on ICH was investigated on whole genome level.

II. Materials and methods

1. Animal

Sprague-Dawley rats (Daehan Biolink Co., Korea) fed with standard pellet diet and water *ad libitum* were maintained at 22±2℃ under 12 hours light and dark cycle for at least 2 weeks prior to the experiment. The average weight of the mice at the beginning of the experiments was 200±20g. All experiments were conducted in accordance with the Institutional Guidelines for Animal Experimentation set by Dongshin University School of Oriental Medicine.

2. Preparation of WWCSW

WWCSW (Kwang Dong Pharmaceutical Co., Korea) was mixed with distilled water just before treatment. WWCSW was orally fed for consecutive 10 days at a dose of 100 mg/kg body weight. The components of WWCSW were shown in Table 1. All other materials were obtained from Dongshin University Oriental Medical Hospital in Korea.

3. Induction of ICH and treatment

ICH was induced using a previously reported method[10,11] with some modification. Briefly, after anesthesia, rats placed in a stereotaxic frame (Dae Jong Co., Korea) were implanted with stainless steel guide cannula above the right striatum and the lateral ventricle. The injection cannula was inserted into the right striatum (6.0 mm below the surface of the skull) through guide cannula. ICH was then induced by injection of 1 U collagenase type IV (Sigma, St. Louis, MO, USA) in 5 ml saline, at a constant rate of 0.4 ml/min with a microinfusion pump. From the next day of ICH induction, WWCSW was administered orally for consecutive 10 days.

4. RNA isolation

After treatment with WWCSW, the rats were sacrificed by intra-peritoneal injection of sodium pentobarbital. The brain tissues were removed and then stored in liquid nitrogen. The total RNA was then isolated using a Qiagen RNeasy Kit according to the manufacturer's instructions (Qiagen Korea Ltd.). The quality of the total RNA was then determined by agarose gel electrophoresis in which

the ratio of 28S/18S RNA was over 1.6 in all cases.

5. Microarray experiment

RNA from the 7 rats in each group was pooled prior to analysis to eliminate individual variability. An microarray contains approximately 45,000 oligo-spots which represlity.approximately 17,000 genes (Agilent Technologies Co.). For the probe preparation and hybridization, indirect labeling system was applied using a 3DNA array system following the manufacturer's protocol (Genisphere, PA) in which 20 mg of RNA was usld to produce fluorescently labelld cDNA. RNA from normal rats was usld as reference. Image files of microarray were measured using a ScanArray scanner (Perkin-Elmer, Boston, MA).

6. Data analysis

Using Imagene 4.0 (Bio-discovery, Marina del Rey, CA), image files of microarray was transformed into text data. Process of normalization was performed using the Lowess method, as previously described[12]. For well measured genes, spots intensity should be greater than 1.4 times that of the local background. The criteria of the threshold for up-and down-regulation was 2 fold. Hierarchical clustering was implemented using CLUSTER and TREEVIEW program (M.B. Eisen, http://rana. lbl.gov). For Ontology analysis, GOSTAT program was applied in which p-value was calculated by Chi square or Fisher's exact test based on the total array element. For correction of multiple testing, false discovery rate using Benjamini and Hochlberg method was applied[13].

Table 1. Components of WWCSW per one pill

Herbal name	Scientific name	Weight (㎎)
Dioscoreae Rhizoma (山藥)	*Dioscorea japonica* Thun	263
Glycyrrhizae Radix (甘草)	*Glycyrrhiza uralensis* FISCH	188
Ginseng Radix (人蔘)	*Panax ginseng* C. A. Meyer	94
Typhae Pollen (蒲黃)	*Typha latifolia*	94
Massa Medicata Fermentata (神麴)	Massa Medicata Fermenta	94
Glycine Semen Germinatum (大豆黃卷)	*Glycine max* Merril	66
Cinnamomi Cortex (桂皮)	*Cinnamomum cassia*	66
Gelatinum (阿膠)	Gelatinum	66
Paeoniae Radix (芍藥)	*Paeonia albiflora pallas* var. t*richocarpa* Bunge	56
Liriopis Tuber (麥門冬)	*Liriope platyphylla* Wang et Tang	56
Scutellariae Radix (黃芩)	*Scutellaria baicalensis* Georgi	56
Angelicae Gigantis Radix (當歸)	*Angelica gigas*	56
Saposhnikoviae Radix (防風)	*Dictamnus dasycarpus* Turcz	56
Atractylodis Rhizoma alba (白朮)	*Atractyloades macrocephale* Koidz	56
Bupleuri Radix (柴胡)	*Bupleurum falcatum* LINNE	47
Platycodi Radix (桔梗)	*Platycodon grandiflorum*	47
Armeniacae Semen (杏仁)	*Prunus armeniaca*	47
Hoelen (茯苓)	*Poria cocos* (Schw.) Wolf	47
Cnidii Rhizoma (川芎)	*Cnidium officinale*	47
Bezoar Bovis (牛黃)	*Bos Taurus domesticus* Gmelin	45
Antelopis Cornu (羚羊角)	*Saiga tatarica* L	38

Civet Musk (靈猫香)	*Moshus moschiferus*	114
Borneolum (龍腦)	*Dryobalanops aromatica* Gaertn	38
Ampelopsis Radix (白蘞)	*Ampelopsis japonica* (Thunb.)	28
Zingiberis Rhizoma (乾薑)	*Crudus Zingiber officinale* Ginger	28
Mel (蜂蜜)	*Apis mellifera* Linnaeus	3117
Aurum (金箔)	Gold foil	q.s
Total amount		7500

7. Promoter analysis

To identify co-regulated genes, promoter regions of co-expressed genes was analyzed. Transcription factor binding site (TFBS) was predicted by CONFAC and TOUCAN program[14,15]. One-thousand base-pair upstream and 500 base-pair downstream from transcription start site was used for the search for TFBS[16]. We analyzed the sequences commonly present in both rat and human to avoid false positive TFBS. The TFBS matrix was composed of columns corresponding to promoters of genes and rows corresponding to putative TFBS. Similarity of TFBS composition among genes was measured using Jaccards algorithm as previously reported[17]. Finally, similarity matrix was hierarchically clustered.

III. Results

1. Clustering pattern of gene expression

The effects of oral administration of WWCSW on gene expression in the brain tissue of ICH rat model were measured by microarray analysis. Of approximately 17,000 genes on the microarray, up- or down-regulated genes in either ICH-induced brain or WWCSW-treated brain after ICH-induction were clustered according to gene expression level. Genes altered by ICH-induction in brain were shown in Fig. 1(A).

Specifically, 233 genes were up-regulated and 371 genes were down-regulated by ICH-induction in brain. The effect of WWCSW on up- or down-regulated genes by brain injury was also shown in Fig. 1(B). Although expression levels of a lot of genes were still up or down-expressed even by WWCSW treatment, many altered genes by ICH-induction in brain were restored to normal level by oral administration of WWCSW. In addition, many newly up or down-regulated genes by WWCSW-treatment on ICH-induced brain tissue were also observed.

2. Ontological analysis of genes altered by ICH-induction in rat brain

The functional classification of gene that were up-or down-regulated in response to ICH-induction in brain was shown in Table 2. Interestingly, up-regulated genes were implicated in stimulus response functions such as response to chemical stimulus, detection of chemical stimulus involved in sensory perception of smell and detection of chemical stimulus involved in sensory perception. Whereas down-regulated genes were related with diverse functions like cell-cell signaling and development. This different functional distribution between up-and down-regulated gene by ICH-induction in brain could be confirmed in Table 2.

Table 2. Ontological analysis of genes altered by inducing ICH in rat brain. GO ID represents the identification number determined by Gene Ontology.

Up-regulated by ICH-induction			Down-regulated by ICH-induction		
Biological Process					
GO ID	p-value	Function	GO ID	p-value	Function
GO:0042221	9.61e-20	Response to chemical stimulus	GO:0007267	5.1e-20	Cell-cell signaling
GO:0032501	1.8e-19	Multicellular organismal process	GO:0048731	5.69e-19	System development
GO:0051606	2.31e-19	Detection of stimulus	GO:0007275	2.55e-17	Multicellular organismal development
GO:0050907	2.31e-19	Detection of chemical stimulus involved in sensory perception	GO:0048856	7.87e-17	Anatomical structure development
GO:0009593	4.47e-19	Detection of chemical stimulus	GO:0032501	1.8e-19	Multicellular organismal process
Molecular Function					
GO:0004984	1.14e-11	Olfactory receptor activity	GO:0005515	3.26e-09	Protein binding
GO:0001584	2.94e-09	Rhodopsin-like receptor activity	GO:0005102	0.0207	Receptor binding
GO:0004930	1.29e-05	G-protein coupled receptor activity	GO:0005275	0.0207	Amine transmembrane transporter activity
GO:0004888	2.19e-05	Transmembrane receptor activity	GO:0005184	0.0207	Neuropeptide hormone activity
GO:0004872	0.00022	Receptor activity	GO:0004157	0.0207	Dihydropyrimidinase activity
Cellular Component					
GO:0031224	4.36e-13	Intrinsic to membrane over	GO:0005615	4.05e-11	Extracellular space
GO:0016021	7.15e-13	Integral to membrane over	GO:0005886	5.67e-11	Plasma membrane
GO:0044425	3.67e-12	Membrane part over	GO:0044421	1.86e-09	Extracellular region part
GO:0016020	1.44e-08	Membrane over	GO:0005887	7.9e-09	Integral to plasma membrane
GO:0005615	9.3e-08	Extracellular space over	GO:0031226	1.13e-08	Intrinsic to plasma membrane

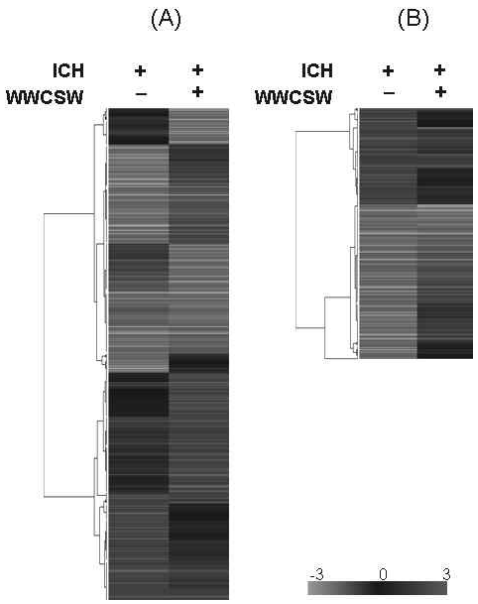

Fig. 1. Expression profile of genes regulated by ICH-induction in rat brain. After normalization, genes were hierarchically clustered according to the level of expression.

(A) Expression profile of 1,066 genes which were up-or down-regulated at least in one of the experimental conditions was shown. Columns correspond to experimental group and rows correspond to genes. Red and green color indicates up-and down-regulation, respectively. Scale bar represents the color intensity of expression ratio (logarithm of base 2).(B) Expression profile of 604 genes which were altered only in ICH group was shown.

3. Expression change by WWCSW on ICH model of rat brain

Administration of WWCSW on ICH model of rat restored expression of many altered genes into normal level(Fig. 2). Specifically, for 233 up-regulated

genes by ICH-induction in brain, 76.0 % (177/233) of genes were restored to normal expression level by the oral administration of WWCSW. Of 371 down-regulated genes, 70.1% (260/371) were restored to normal level.

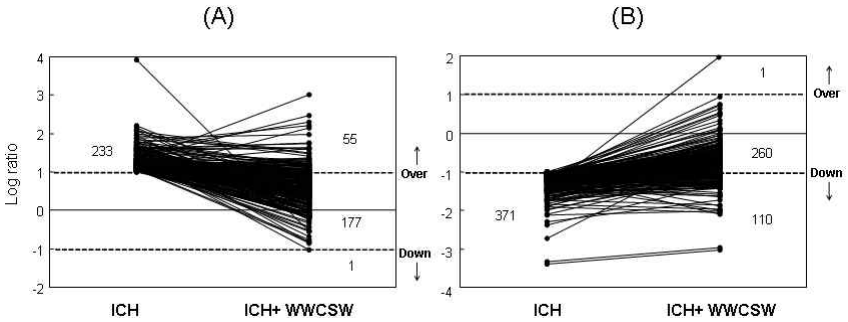

Fig. 2. The effect of administration of WWCSW on the expression of genes that were up-regulated (A) or down-regulated (B) by ICH-induction in rat brain.

The numbers of up-regulated and down-regulated genes after administration of WWCSW are depicted. The dotted line indicates 2-fold baseline.

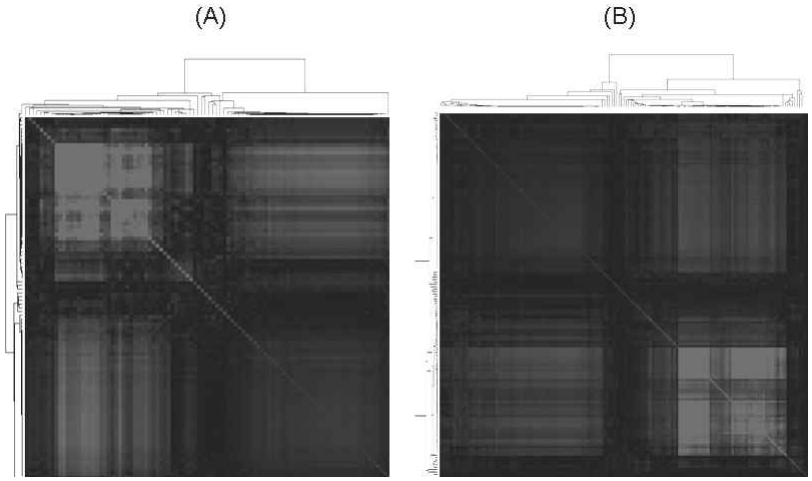

Fig. 3. Similarity matrix of co-expressed genes. The similarity in the promoter regions of altered genes by ICH-induction in rat brain was measured.

TFBS clustering was applied on up-regulated genes (A) and down-regulated genes (B). Red color represents high similarity of TFBS composition among genes while green color represents low correlation in which color intensity corresponds to correlation coefficient.

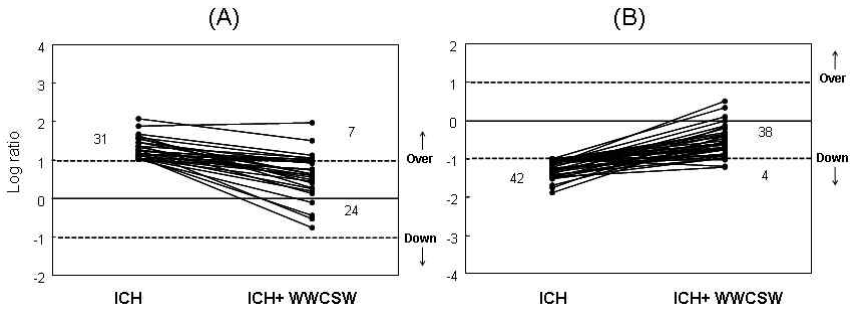

Fig. 4. Effect of administration of WWCSW on putatively co-regulated genes.

The effect of WWCSW was measured on co-up-regulated genes (A) or co-down-regulated genes (B) which were identified as having similar TFBS composition. The numbers of up-regulated and down-regulated genes after administration of WWCSW are depicted. The dotted line indicates 2-fold baseline.

4. Promoter analysis of genes altered by ICH-induction in rat brain

To identify co-regulated genes and presume the molecular target of WWCSW, promoter regions of altered genes in ICH model of rat were investigated. TFBS were predicted by CONFAC algorithm in promoter region encompassing from 1,000 bp upstream to 500 bp downstream of each gene. TFBS matrix, then, converted into similarity matrix to give gene cluster having similar TFBS composition. For up- and down-regulated genes by ICH-induction in brain, promoter regions of initial 108 and 156 genes were analyzed, respectively. The total number of tentative TFBS were 267 and 269 for up-and down-regulated genes, respectively. Fig. 3 shows the clustering pattern of up- or down-regulated genes based on similarity of TFBS composition. In both cases, clearly correlated cluster of genes can be identified in which 31 genes were co-up-regulated and 42 genes were co-down-regulated. The selected list of these correlated genes was shown in Table 3.

5. Effect of WWCSW on genes having similar TFBS

As shown in Fig. 2, WWCSW-treatment restored the gene expression altered by ICH-induction in brain to the levels of 76.0% and 70.1% for up- and down-regulated gene, respectively. This recovery rate was measured on the all genes that were altered by ICH-induction in brain. On the other hand, when small set of genes having similar TFBS composition was used, 77.4% (24/31) of recovery rate was obtained for up-regulated genes and 90.5% (38/42) for down-regulated genes. Interestingly, recovery rate was greatly increased to 90.5% from 70.1% when using correlated down-regulated genes but increase of recovery rate was not measured in the case of using correlated up-regulated genes.

Table 3. Selected 10 genes having similar TFBS composition in promoter region

Up-regulated by ICH-induction in rat brain		
Unigene	Symbol	Name
Rn.974	Calr	Calreticulin
Rn.29936	Ywhag	Tyr 3- / trp 5-monooxygenase activation protein, gamma polypeptide
Rn.10724	Neurod2	Neurogenic differentiation 2
Rn.199051	Lipg	Lipase, endothelial
Rn.155225	Cct6b	Chaperonin subunit 6b (zeta)
Rn.3048	Nfasc	Neurofascin
Rn.11347	Ctss	Cathepsin S
Rn.7771	Ehd3	EH-domain containing 3
Rn.16089	Gfpt2	Glutamine-fructose-6-phosphate transaminase 2
Rn.44433	Unc5a	Unc-5 homolog A (C. elegans)
Down-regulated by ICH-induction in rat brain		
Unigene	Symbol	Name
Rn.9935	Htr2c	5-hydroxytryptamine (serotonin) receptor 2C
Rn.79380	Wdr44	WD repeat domain 44
Rn.34890	Meox2	Mesenchyme homeobox 2
Rn.134464	Foxp2	Forkhead box P2
Rn.1989	Kcnj16	Potassium inwardly-rectifying channel, subfamily J, member 16
Rn.161783	Acvr2a	Activin receptor IIA
Rn.9239	Slc45a3	Solute carrier family 45, member 3
Rn.34890	Meox2	Mesenchyme homeobox 2
Rn.11063	Ppp3cb	Protein phosphatase 3, catalytic subunit, beta isoform
Rn.23078	Wdr26	WD repeat domain 26

IV. Discussion and Conclusion

WWCSW has been traditionally used to treat cerebral apoplexy, hypertension, palpitation, mental anxiety, acute & chronic convulsion, dysautonomia and insensibility in oriental medicine[1-4]. Although certain components of WWCSW show anti-inflammatory effect and inhibitory effect on cytochrome P450, the molecular mechanism of WWCSW has not been clearly studied[18-19]. In this study, the effect of WWCSW was measured on ICH rat model using DNA microarray technology.

Although many biochemical parameters has been used to quantitatively measure the level of brain injury and treatment efficacy of drugs, study based on whole genome approach has not been reported yet. The molecular profile of expression level shows that ICH in brain induced de-regulation of

many genes(Fig. 1). By using all genes altered by ICH-induction in brain, the recovery rates were 76.0% and 70.1% for up- and down-regulated genes, respectively, by administration of WWCSW. These altered genes was implicated with different kinds of biological processes(Table 2). Interestingly, up-regulated genes were specifically enriched with stimulus response in sensory perception. It means that even though these altered genes show similar pattern of expression by ICH in brain, expression of genes might be differently regulated. This can be demonstrated by the promoter clustering shown in Fig. 3. Up- or down-regulated genes were clearly clustered based on TFBS similarity. We selected highly correlated genes as significantly co-regulated genes. The recovery rate of 77.4% was obtained using co-up-regulated genes and 90.5% for co-down-regulated genes. The recovery rate (76.0%) for overall up-regulated genes were not significantly changed by using co-regulated genes (77.4%). However, for down-regulated genes, great increase of recovery rate was measured by using co-down-regulated genes (90.5%) compared to the recovery rate (70.1%) obtained by overall up-regulated genes. Although this result means that the presence of co-regulated genes should be considered when measuring recovery rate based on whole genome level, we are not sure that using co-regulated genes as standard of recovery rate would be better method than using overall genes. Therefore, exact recovery rate induced by oral adminstration of WWCSW in ICH rat brain model should be measured by simultaneously considering both biochemical parameters and information on expression of genes.

In addition to increased recovery rate, co-regulated genes by ICH model of rat could be used to molecular targets for therapeutic effect of drug including WWCSW. That is, presence of co-regulated genes may represent the importance of these genes in ICH in brain and the change of expression level of these co-regulated genes would also indicate the functional change of brain tissue.

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