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Microarray 분석법 활용을 통한 뇌출혈 흰쥐에서의 우황청심원 효능 평가

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Microarray-Based Gene Expression Profiling to Elucidate the Effectiveness of Woowhangchongshim-won on ICH Model in Rats

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ABSTRACT

Objectives : Intracerebral hemorrhage (ICH) is characterized by breakdown of blood vessels within the brain parenchyma. Fundamental therapeutic strategies for ICH, particularly those aimed at neuroprotection, have to be established. So in this experiment, the effects of Woowhangchongshim-won, a traditional prescription formula for treating Cerebral Apoplexy in Asian countries, were investigated.

Methods : After intraperitoneal injection of chloralhydrate, rats were placed in a stereotaxic frame. ICH was induced by injection of 1 U collagenase type IV and drug was administered orally for 10 days. The molecular profile of cerebral hemorrhage in rat brain tissue was measured using microarray technique to identify up- or down- regulated genes in brain tissue. These genes induced by brain damage were mainly concerned with general metabolic process such as primary metabolic process, cellular metabolic process, macromolecule metabolic process, and biosynthetic process.

Results : The number of genes increased in control and not-changed in experiment was 374, and decreased in control and not-changed in experiment was 527. We are concerned with genes that can be recovered by treatment with medicine, it is especially interesting to above types of genes.

Conclusions : Upon medicine treatment to the rat having cerebral hemorrhage, expressions of some genes were restored to normal level. Further analysis using protein interaction database identified some key molecules that can be used for elucidation of therapeutical mechanism of medicine in future.

Key words : Woowhangchongshim-won, Microarray, Gene Expression, ICH

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Introduction

Woowhangchongshim-won is a traditional presc ription formula for treating cerebral apoplexy in China, Japan and Korea, and has been widely used for treat intracerebral hemorrhage initiated symptoms[1,2].

ICH is characterized by breakdown of blood vessels within the brain parenchyma, which triggers a substantial loss of neurons that frequently leads to poor prognosis[3]. Despite accounting for about 15% of all strokes, the pathophysiology of ICH is not fully understood, and no drugs have been developed to reduce the damage[4,5]. So, fundamental therapeutic strategies for ICH, particularly those aimed at neuroprotectio n, have to be established.

Primary injury after ICH includes physical destruction of tissue, mass effects that compress surrounding structures including the ventricles, and increase intracranial pressure[4]. The secondary neurological deterioration that commonly occurs has been attributed to hematoma expansion, edema, inflammation and neuron death in the parenchyma surrounding the hematoma[6-11]. Neuron necrosis and apoptosis have been reported in animal models, and apoptotic neurons have been seen in humans, especially at the periphery of the hematoma[11-14]. Reducing secondary injury and rescuing neurons following ICH remain an attractive therapeutic goal, but a better understand ing of the pathological sequence of events is needed.

Array analysis has become a standard technique in the molecular biology laboratory for monitoring gene expression and, in recent, pharmacogenomics using microarray technology has been widely investigated to elucidate mechanism of drug and to find out novel drug candidates. For example, novel determinants of response to chemotherapy in colon cancer were identified by using pharmacogenomic approach[15-19]. This kind of study to identify drug pathway using pharmacogenomics has been increasingly reported recently. In addition to experimental method to identify drug mechanism, approach using systems biology uses all kinds of data obtained by high throughput data such as protein network, genomic expression, and single nucleotide polymorphism[20-23] . Near in future, large part of research in field of new drug identification or elucidation of drug mechanism would be necessarily carried out using bioinformatic and systemic approach. Therefore, collecting data of high throughput analysis such as genomics and proteomics for various sources will be needed greatly[24].

Here we investigated whether genetic changes are involved in experimental ICH-induced brain injury and medication involved recovery. ICH was induced by collagenase injection into the rat striatum. Test animals were treated orally with Woowhangchongshim-won.

Materials and Methods

1. Medication preparation

Woowhangchongshim-won (The Original, Kwan g Dong Pharm. Co. Korea) was purchased from drug store. Just before experiment, prescription was mixed with distilled water and rats were orally fed with the dosage of 100 ㎎/㎏ body weight for 10 days.

2. Induction of intracerebral hemorrhage and prescription treatment

Experiments were conducted in accordance with the Guidelines of the United States National Institutes of Health regarding the care and use of animals for experimental procedures.

Male Sprague-Dawley rats weighing 200 to 220 g were used and maintained at constant ambient temperature (22±1℃) under a 12-h light/dark cycle. After intraperitoneal injection of chloralhydrate (400 mg/kg, Fluka, Germany), rats were placed in a stereotaxic frame (Dae Jong, Korea). Each rat was implanted with stainless steel guide cannulas (OD 0.7 ㎜) above the right

striatum (3 ㎜ lateral to midline, 0.2 ㎜ anterior to coronal suture of the bregma) and above the lateral ventricle (1.5 ㎜ lateral to midline, 0.8 ㎜ posterior to coronal suture of the bregma). The 27-gauge injection cannula, whose tip was inserted 6.0 ㎜ below the surface of the skull, was introduced through a guide cannula into the right striatum, and then ICH was induced by injection of 1 U collagenase type IV (Sigma, St. Louis, MO, USA) in 5 ㎕ saline, at a constant rate of 0.4 ㎕/min with a microinfusion pump. Drug was administered orally from the next day of ICH induction for 10 days. Vehicle were injected into the contralateral ventricle at the same time course as that for drug. After collagenase injection, the scalp incision was sutured, and rats were placed in a cage with free access to food and water.

Table 1. Components of Woowhangchongshim-won 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 (人參)	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 Blume	66
Gelatinum (阿膠)	Gelatinum	66
Paeoniae Radix (芍藥)	Paeonia albiflora pallas var. trichocarpa 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 Nakai	56
Saposhnikoviae Radix (防風)	Saposhnikovia divaricata Schischkin	56
Atractylodis Rhizoma alba (白朮)	Atractyloades macrocephale Koidz	56
Bupleuri Radix (柴胡)	Bupleurum falcatum L.	47
Platycodi Radix (桔梗)	Platycodon grandiflorum A. Jacq	47
Armeniacae Semen (杏仁)	Prunus armeniaca L.	47
Hoelen (茯苓)	Poria cocos (Schw.) Wolf	47
Cnidii Rhizoma (川芎)	Cnidium officinale Makino	47
Bezoar Bovis (牛黃)	Bos Taurus domesticus Gmelin	45
Antelopis Cornu (羚羊角)	Saiga tatarica L.	38
Civet Musk (靈猫香)	Paradoxurus hermaphroditus	114
Borneolum (龍腦)	Dryobalanops aromatica Gaertn	38
Ampelopsis Radix (白蔹)	Ampelopsis japonica Thunb.	28
Zingiberis Rhizoma (乾薑)	Zingiber officinale Roscoe	28
Mel (蜂蜜)	Apis mellifera L.	3117
Aurum (金箔)	Gold foil	q.s
Total amount		7500

3. RNA isolation

Right sides of lateral ventricle were surgically resected, immediately snap frozen, and stored in liquid nitrogen. Total RNA was isolated from frozen tissue according to the manufacturer's instructions (Qiagen), and quality of the total RNA was judged based on the ratio of 28S/18S RNA after agarose gel electrophoresis.

4. Microarray experiment

A total sequence set of ~45,000 oligo-nucleotides were generated after quality control processes. These sequence-verified clones were printed onto glass microscope slides. The probe preparation and hybridization were performed using 3DNA array detection system according to the manufacturer's protocol (Genisphere, PA) with 20 ㎍ of total RNA from tissue to derive fluorescently labeled cDNA. The arrays were washed and scanned by ScanArray scanner (Perkin-Elmer, Boston, MA). Normal RNA was used as reference which was verified to detect majority (over 85%) of cDNA spots of microarray.

5. Data analysis

Primary data from image files were obtained using IMAGENE 4.0 (Bio-discovery, Marina del Rey, CA) and then normalized using lowess method, as previously described. All array elements, for which the fluorescent intensity in each channel was greater than 1.4 times to the local background, were considered as well measured. Genes that were not well measured on at least 80% of the samples were excluded. The expressional ratios were hierarchically clustered by using CLUSTER and then visualized using TREEVIEW (M.B. Eisen, http://rana.lbl.gov).

Ontology analysis was performed using FatiGo algorithms (http://.babelomics.bioinfo.cipf.es).

Results

1. Quality of total RNA isolated for microarray

The quality of total RNA from rat brain was checked using gel electrophoresis method. As shown in Fig. 1, RNA from all samples including normal tissues was in good condition to perform microarray experiment. The quantity of RNA was calculated by measuring absorption of ultra-violet.

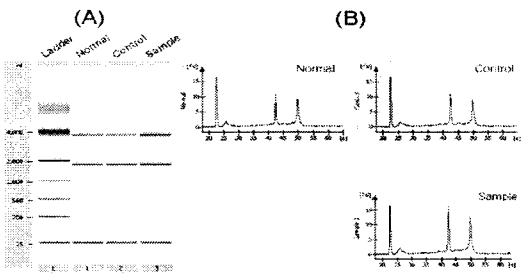

Fig. 1. Quality of total RNA isolated for microarray. The total RNA was prepared from brain tissue of rat. Image of gel electrophoresis was photographed (A) and the quality of RNA was calculated from scanned image (B).

2. Raw image of microarray

The raw images of microarray were depicted in Fig. 2. We used Agilent rat 45K oligomicroarray to analyze whole genome variation of rat. Because two colors system consisting of cy3 and cy5 fluorescent nucleotide was used, red or green spots can be seen in Fig. 2. Most spots were colored yellow because most genes were not changed significantly.

3. Normalization of microarray (MA plot)

Primary data from raw image were normalized using lowess method. Fig. 3 shows the relationship between ratio (log ratio) and intensity (log intensity) of all spots before and after normalization. By applying normalization process, skewed form of ratio pattern before normalization (Fig. 3(a)) was changed to linear pattern centered to zero of log ratio after normalization (Fig. 3(b)).

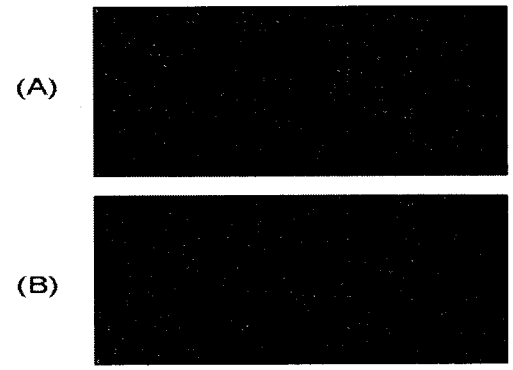

Fig. 2. Raw image of microarray. A total sequence set of ~45,000 oligo-nucleotides were printed onto glass microscope slides. The probe preparation and hybridization were performed using 3DNA array detection system with 20ug of total RNA from damaged brain tissue (A) and treated tissues (B). Normal brain tissue was used as reference RNA for all samples.

4. Between chips normalization

Besides normalization of each microarray, it is necessary to normalize between microarrays (control and experimental microarray) because the overall distribution of spots of each microarray would be different between chips. As shown in Fig. 4(a), the quantile values of microarray differed each other before between chips normalization. These different quantile values were adjusted to nearly same values after normalization between chips (Fig. 4(b)).

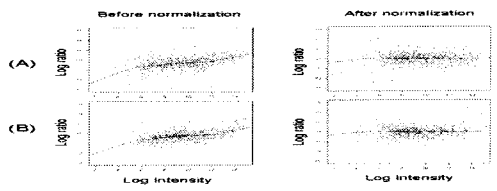

Fig. 3. Normalization of microarray (MA plot). Primary data from

raw image of control (A) and experimental sample (B) were normalized using lowess method. Vertical axis represents log ratio and horizontal axis represents log intensity of all spots before and after normalization. By applying normalization process, skewed line before normalization was changed to linear form centered to zero of log ratio after normalization.

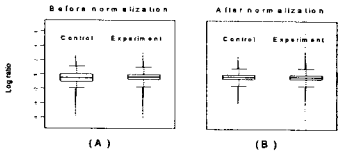

Fig. 4. Between chips normalization. After normalization of each microarray, it is necessary to normalize between microarrays (control and experiment). The log ratio of the quantile values of microarray was represented before (A) and after (B) between chips normalization. These different quantile values were adjusted to nearly same values after normalization between chips.

5. Expressional profile of microarray and expression of genes in 8 subclasses

Fig. 5 shows the gene expressional profile of samples. This pattern of gene expression can be classified into 8 classes; (A) genes increased in control and not-changed in experiment, (B) decreased in control and not-changed in experiment, (C) increased in control and increased in experiment, (D) decreased in control and decreased in experiment, (E) increased in control and decreased in experiment, (F) decreased in control and increased in experiment, (G) not-changed in control and increased in experiment, and (H) not-changed in control and decreased in experiment. The position of each pattern in overall gene expression profile is depicted in Fig.5.

Expressional change of genes in each class was shown in Fig. 6. In accordance with Fig. 5, most of genes showing different expression were gathered in class A and class B. Because we were looking for genes that were restored to normal level by treatment of medicine, class A and class B could be the most important genes.

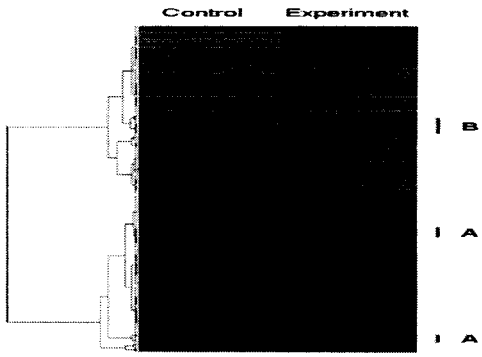

Fig. 5. Expressional profile of microarray. The normalized ratios were hierarchically clustered by using CLUSTER and then visualized using TREEVIEW program. Red, green, and black color represents up-regulation, down-regulation, and no change of gene expression, respectively. The color intensity correlates with expression level of the gene. Genes increased in control and not-changed in experiment were clustered in A. Genes decreased in control and not-changed in experiment were clustered in B.

Discussion

Generally, Woowhangchongshim-won is administered once or twice a day by chewing or dissolving in warm water. For adults, 1 pill per time; for children, 2/3 pill1 (5~8 years old), : 1/2 pill (7~5 years old), 1/3 pill (4~2 years old), and 1/4 pill (1 year and under). 27 medicinal materials composes this prescription, for example, Dioscoreae Rhizoma, Glycyrrhizae Radix, Ginseng Radix, Bezoar Bovis, Aurum, etc. In Korea, this formula can be bought at any drug store without doctor's prescription for being classified over-the-counter drug[1,2]. This formula has been used for treating various types of heart or brain damaged diseases but it's pathway of relieving those symptoms were still not clearly investigated.

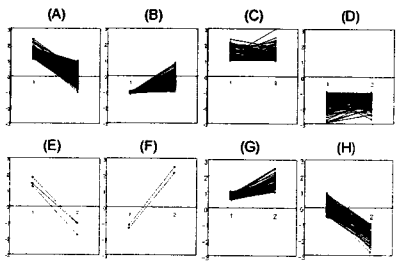

Fig. 6. Expression of genes in 8 subclasses defined in Fig. 5. (A) genes increased in control and not-changed in experiment, (B) decreased in control and not-changed in experiment, (C) increased in control and increased in experiment, (D) decreased in control and decreased in experiment, (E) increased in control and decreased in experiment, (F) decreased in control and increased in experiment, (G) not-changed in control and increased in experiment, and (H) not-changed in control and decreased in experiment.

In this study, we investigated the function of Woowhangchongshim-won on brain injury induced by cerebral hemorrhage in rat using microarray technique to identify genes related with treatment. ICH is characterized by breakdown of blood vessels within the brain parenchyma, which triggers a substantial loss of neuron, and despite accounting for about 15% of all strokes, the pathophysiology and drugs still not have been well developed[3-5].

In recent, microarray technology has been widely investigated to elucidate mechanism of drug, and novel determinants of response to chemotherapy in colon cancer were identified by using pharmacogenomic approach[15-19]. This kind of study to identify drug pathway using pharmacogenomics has been increasingly reported recently[20-23].

We investigated whether genetic changes are involved in experimental ICH-induced brain injury and Woowhangchongshim-won medication involved recovery. And we could identify that expression levels of most genes were not changed significantly by brain damage. Only 1153 genes (up-regulation of 393 genes and down-regulation of 760 genes) showed expressional variation, which correspond to only about 2.5% of total genes. Besides, the number of down-regulated genes is more predominant than up-regulated genes, which implicate that brain damage induced by cerebral hemorrhage impaired normal function of many genes in brain. Since many genes showing differential expression were selected, it is not possible to view these genes at individual level.

Gene expressional profile of samples were shown in Fig. 5. Genes showing expressional variations were selected for comparison. Interestingly, experimental sample shows similar gene expression pattern with brain injury sample. Most genes that were up regulated or down regulated in brain injury sample were also up or down regulated in experimental sample. However, some genes show different expressional pattern between control and experiment.

Although the effect of medicine in brain damage can be studied with biochemical method, it is more fundamental approach to measure the genetic variation in brain tissue. As shown in Fig. 5, the overall pattern of gene expression upon treatment with medicine was similar with that of control gene expression. This result can be supported by annotational analysis of genes showing expressional variation.

As control of brain damage tissue, genes related with general metabolic process were mainly selected as significantly up- or down-regulated genes. However, more careful observation makes it possible to isolate genes differentially expressed between control and treated sample.

Briefly we could define 8 subclasses; (A) genes increased in control and not-changed in experiment, (B) decreased in control and not-changed in experiment, (C) increased in control and increased in experiment, (D) decreased in control and decreased in experiment, (E) increased in control and decreased in experiment, (F) decreased in control and increased in

experiment, (G) not-changed in control and increased in experiment, and (H) not-changed in control and decreased in experiment. The positions of these classes in overall gene expression profile and change of expressional ratio were depicted in Fig. 5 and Fig. 6, respectively. Because we are concerned with genes that can be recovered by treatment with medicine, it is especially interesting to investigate genes involved in class A and class B. The number of genes in class A was 374 and in class B was 527. As mentioned above, the functional roles of these genes were mainly involved in general metabolic processes. However, it should be studied further in detail whether effect of treatment with medicine comes from simple recovery of metabolic genes or from other key genes in minor category of annotation.

Now we are planning to find out the possible key components inducing treatment effect, protein interaction analysis will be done, and intensive studies on these gene products should be conducted in future.

Conclusion

We investigated whether genetic changes are involved in experimental ICH-induced brain injury and Woowhangchongshim-won medication involved recovery using microarray based gene expression profiling. The results of our study are summarized as follows.

1. 1153 genes (up-regulation of 393 genes and down-regulation of 760 genes) showed expressional variation.

2. Experimental sample shows similar gene expression pattern with brain injury sample. However, some genes show different expressional pattern between control and experiment.

3. Genes related with general metabolic process were mainly selected as significantly up- or down-regulated genes.

4. The number of genes increased in control and not-changed in experiment is 374, the number of genes decreased in control and not-changed in experiment is 527

We defined the molecular profile of rat brain upon damage and Woowhangchongshim -won administered recovery. By treatment with medicine, expressions of some genes were restored to normal level although these genes were mainly involved in general metabolic process. Further analysis using protein interaction database should be identified in future.

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