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Laboratory Investigation

Changes in Gene Expression in the Rat Hippocampus after Focal Cerebral Ischemia

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Objective : The rat middle cerebral artery thread-occlusion model has been widely used to investigate the pathophysiological mechanisms of stroke and to develop therapeutic treatment. This study was conducted to analyze energy metabolism, apoptotic signal pathways, and genetic changes in the hippocampus of the ischemic rat brain.

Methods : Focal transient cerebral ischemia was induced by obstructing the middle cerebral artery for two hours. After 24 hours, the induction of ischemia was confirmed by the measurement of infarct size using 2,3,5-triphenyltetrazolium chloride staining. A cDNA microarray assay was performed after isolating the hippocampus, and was used to examine changes in genetic expression patterns.

Results : According to the cDNA microarray analysis, a total of 1,882 and 2,237 genes showed more than a 2-fold increase and more than a 2-fold decrease, respectively. When the genes were classified according to signal pathways, genes related with oxidative phosphorylation were found most frequently. There are several apoptotic genes that are known to be expressed during ischemic brain damage, including Akt2 and Tnfrsf1a. In this study, the expression of these genes was observed to increase by more than 2-fold. As energy metabolism related genes grew, ischemic brain damage was affected, and the expression of important genes related to apoptosis was increased/decreased.

Conclusion : Our analysis revealed a significant change in the expression of energy metabolism related genes (Atp6v0d1, Atp5g2, etc.) in the hippocampus of the ischemic rat brain. Based on this data, we feel these genes have the potential to be target genes used for the development of therapeutic agents for ischemic stroke.

Key Words : Apoptosis · Energy metabolism · Focal cerebral ischemia · Gene expression · Hippocampus · Oxidative phosphorylation.

INTRODUCTION

To date there are only a few studies demonstrating apoptotic features in human stroke[7,14,19]. The rat middle cerebral artery thread-occlusion (MCAo) model has been widely used in stroke pathophysiology and therapeutic research. This model has many benefits, including the induction of stroke without opening the cranial vault and the option of reperfusion. In order to more accurately reflect human stroke, the use of aged animals and animals with co-morbidities has been suggested[23]. However, some previous investigators have reported that the thread-occlusion method (with a heat-treated bulb) does not reliably produce stroke in aged rats[26]. Meanwhile, others have reported successful induction of stroke using the same method, although one study failed to report success rates[1] and others experienced a relatively high rate of mortality (17% within 24 hrs)[12] and small infarcts (8% hemispheric volume).

RNA microarray technology allows for the mapping of the time course of gene responses and differential expression of thousands of genes in the brain triggered by ischemic insult[9,15,20]. Transient focal cerebral ischemia induces complex changes in the genomic profile, including the expression of new genes and upregulation and downregulation of genes, which occur distinctly in a temporal manner. Detection of gene changes after ischemia is the first step towards understanding different molecular pathways, while proteomics and peptidomics studies provide supplemental insights[3,5,21]. In this study, gene expression in the rat hippocampus after transient focal cerebral ischemia using Agilent's whole genome chip array was examined for changes in energy metabolism and apoptosis signal pathways.

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MATERIALS AND METHODS

Experimental animals

Specific pathogen-free Sprague-Dawley (SD) rats (Daehan Biolink Co. Ltd., Eumseong, Korea) were housed in an environ-

mentally controlled room at a temperature of 23±2°C, relatively humidity of 55±5%, and a 12-hr light/dark cycle. Food and water were available *ad libitum*. Experimental procedures were carried out according to the animal care guidelines of the National Institute for Health Guide and the Korean Academic of Medical Sciences.

Ischemic animal model

Male SD rats weighing 300±10 g were used. Focal cerebral ischemia was produced by a modification of the monofilament method as described by Longa et al.[13]. Briefly, rats were anesthetized with 5% isoflurane in 25% O_2/75% N_2O for induction, and then maintained throughout the operation with 1.5-2% isoflurane delivered via nasal mask. Rectal temperature was maintained at 37-38°C throughout the surgical procedure with a heating plate. A cervical parasagittal incision was made at the left common carotid artery (CCA) region. The distal left external carotid artery (ECA) was ligated, transected, and rotated 180°. The proximal CCA and distal internal carotid artery (ICA) were clamped transiently with aneurysm clips. After a longitudinal incision of the ECA, a 25-mm nylon monofilament coated with silicon (0.3 mm in diameter) was inserted into the ECA and advanced into the lumen of the ICA. The clamp on the distal ICA was released, and the filament was advanced 20 mm from the CCA bifurcation until the origin of the MCA was blocked. The filament was then tied at the ECA stump and proximal ICA, and the clamp on the CCA was released. After 3 hours of occlusion, the proximal CCA was clamped transiently with an aneurysm clip. The filament was withdrawn from the ICA, the ECA stump was tied to prevent bleeding, and the clamp on the CCA was then released.

Regional cerebral blood flow

The rat was mounted in the prone position on a dual small animal stereotaxic instrument (David Kopf Instruments, Los Angeles, CA, USA). After a cranial midline incision, a 1-mm burr hole was made with a microdrill at both sides, 2-mm-posterior and 5-mm-lateral to the bregma[24]. The rat was then placed in the supine position. A laser Doppler probe was placed on the burr hole under the rat skull, almost contacting the dura mater of the brain surface avoiding the large vessels. The parietal cortical perfusion in the territory of MCA was measured. The laser Doppler probe was connected to a BLF21D laser Doppler flowmeter (Transonic systems Inc., Ithaca, NY, USA), and the values were obtained from 10 min before the ischemic event until 15 min after reperfusion[28]. The changes in rCBF are expressed as the percentage of the baseline values. The signals were digitalized and sent to a computer for recording, storage and analysis.

Measurement of infarct size

All animals were sacrificed 24 hours after reperfusion. The brains were rapidly dissected and the forebrains were cut into six coronal sections 2 mm thick using a rat brain matrix (ASI® Instruments Inc., MI, USA). The sections were stained by incubating them in a solution of 2% 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C for 30 min[2] and then photographed. The photographic images were digitalized and used to determine the volume of each infarct and the volume of each hemisphere for each slice using a computer-assisted image analysis program (Optimas 6.1, Media Cybernetics, Silver Springs, MD, USA). The infarct volume of each slice was calculated by subtracting the normal ipsilateral volume from that of the contralateral hemisphere to reduce errors due to cerebral edema. Infarct volume is presented as the percentage of the infarct volume to the volume of the contralateral hemisphere[11].

TUNEL staining

To visualize the apoptotic cells, TUNEL staining was performed using an In Situ Cell Death Detection Kit (Roche) according to the manufacturer's protocol. Briefly, after fixation in ethanol-acetic acid (2 : 1), the sections were incubated with proteinase K (100 μg/mL) and permeabilized with 0.5% Triton X-100. The sections were then incubated in the TUNEL reaction mixture, and visualized using a Converter-POD with 3,3'-diaminobenzidine.

RNA extraction

Total RNA was extracted using the Mini RNA isolation II™ kit (Zymo Research, USA). In brief, 600 μL of ZR RNA buffer was added to isolated hippocampi. The lysate was transferred to a Zymo-Spin III Column. The column was placed into a 2 mL collection tube, and spun at full speed in a micro-centrifuge for 1 min. The collection tube was emptied and 350 μL of RNA-wash buffer was added to the Zymo-Spin III Column. It was then centrifuged again at full speed for 1 min. The column was transferred to a new 1.5 mL tube, 50μL RNase-free water was added directly to the membrane of the Zymo-Spin III column, and it was then spun briefly to elute RNA. The eluted RNA was immediately stored at -70°C until further use. Quality and quantity were measured using a ND-1000 spectrophotometer (Nanodrop Technology Inc., USA) at absorbances of 260 and 280 nm.

Agilent whole rat genome microarray analysis

The Agilent Whole Rat Genome Oligo Microarray contains 44,000 60-mer oligonucleotide probes representing 41,000 unique genes and transcripts. Amplification and labeling of 500 ng of total RNA was performed according to the manufacturer's protocol using Cy5 for MCAo RNA and Cy3 for the control RNA (Stratagene UHR). Hybridization was performed for 17 hrs at 65°C and the arrays were then scanned on an Agilent DNA microarray scanner. Images were analyzed and data was extracted using Agilent Feature Extraction Software A.7.5.1. Low normalization was performed for within array normalization between the two channels, and a linear scaling (geometric mean of each channel signal is set to a value of 1,000) was performed for between array normalization. Functional analysis of

the genes selected was carried out using KEGG PATHWAY Database (http://www.genome.jp/kegg/ pathway.html).

RESULTS

Changes of regional cerebral blood flow and infarct volume by MCAo

In order to confirm the ischemia model, we measured rCBF and infarct volume. MCAo immediately reduced the rCBF value to approximately 50% of the baseline level, indicating focal cerebral ischemia (data not shown). After 2 hours of occlusion, this reduction gradually recovered during reperfusion. The rCBF measurements showed similar patterns in all groups. TTC is a sensitive histochemical indicator of mitochondrial respiratory enzyme function. Brain lesions identified by TTC staining represent tissue whose mitochondrial function and oxidative respiratory enzyme systems have been irreversibly damaged[22]. Fig. 1 shows typical photographs of brain slices. The infarction volumes were assessed 24 hours post-ischemia. The MCAo group showed an average infarct volume of 45.3±0.7% (data not shown).

MCAo-induced apoptosis in the hippocampus

A TUNEL assay was performed to investigate whether cell death due to brain ischemia following MCAo was caused by apoptosis. As shown in Fig. 2, TUNEL-positive cells stained dark brown. The TUNEL assay showed that DNA strand breaks did occur, indicating the induction of apoptosis by MCAo in the hippocampal region of rats.

Analysis of microarray data

The expression of ischemia-related genes was determined by comparing MCAo-induced expression to the expression in a sham-operation group. A cDNA microarray that contained duplicate cDNA probes from 44K rat clones was used. To normalize the intensity ratio of each gene expression pattern, the global M method was used. First, the primary data were normalized by the total spots of intensity between the two groups, and then normalized by the intensity ratio of reference genes, such as house-keeping genes in both groups. Finally, the expression ratio of the MCAo group to the sham group was converted to the log2 ratio of each gene, which was represented by the scatter plot (Fig. 3).

After normalizing the data, a cut-off value of global M was set at 2 or -2. More than 44,000 genes on the arrays were analyzed after 2 hours of occlusion and 24 hours of reperfusion from three stroke and three control mice. A total of 1,882 genes were upregulated and 2337 were downregulated in the hippocampi of rats after MCAo. These genes were then divided into categories according to signaling pathways (http://www.genome.jp/kegg/pathway.html).

Of the genes whose expression changed, the majority were oxidative phosphorylation related genes. As shown in Table 1, the expression of ATPase, H^+ transporting, V0 subunit D isoform 1 (Atp6v0d1), cytochrome c oxidase subunit IV isoform 1 (Cox4i1), ATP synthase, H^+ transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 2 (Atp5g2), ATPase, H^+ transporting, lysosomal V0 subunit a isoform 1 (Atp6v0a1), cytochrome c-1 (Cyc1), NADH dehydrogenase (ubiquinone) flavoprotein 1, 51kDa (Ndufv1), ATPase, H^+ transporting, V0 subunit e (Atp6v0e1), ATP synthase, H^+ transporting, mitochondrial

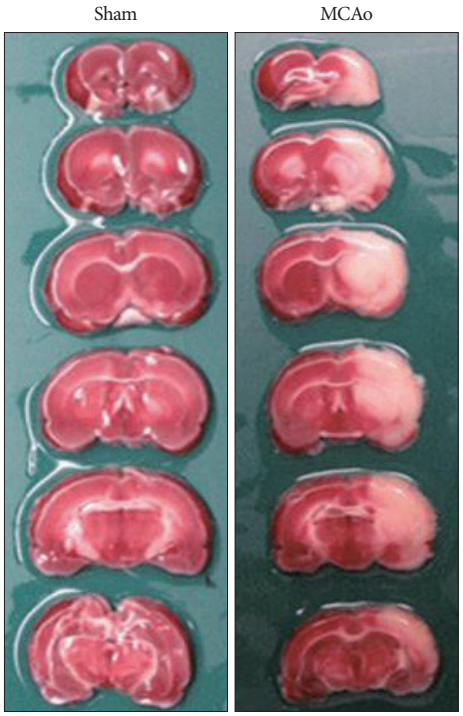

Fig. 1. Middle cerebral artery thread-occlusion (MCAo)-induced infarct formation in rats. Infarct volumes were assessed after 2 hours ischemia and 24 hours reperfusion caused by MCAo in a rat model. A representative rat brain stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) 24 h after left side middle artery/common carotid artery occlusion.

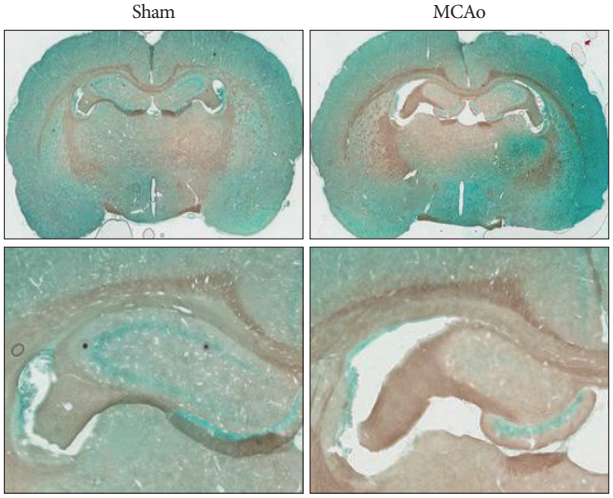

Fig. 2. Photomicrographs of terminal deoxynucleotidyl transferase-mediated dUTP nickend labeling (TUNEL) staining. TUNEL staining was performed 24 hrs after MCAo in rats. Sections are stained for TUNEL-positive cells.

F0 complex, subunit c, isoform 3 (Atp5g3), ATPase, H^+ transporting, V0 subunit C (Atp6v0c), and NADH dehydrogenase (ubiquinone) flavoprotein 2 (Ndufv2) increased more than 2-fold following brain ischemia due to MCAo. On the other hand, the expression of NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1 (Ndufa1), cytochrome c oxidase subunit VIIb (Cox7b), NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2 (Ndufb2), NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7 (Ndufa7), cytochrome C oxidase subunit Vb (Cox5b), ubiquinol-cytochrome C reductase core protein II (Uqcrc2), and Cytochrome c oxidase subunit VIII-H (heart/muscle) (Cox8h) decreased by more than 2-fold following brain ischemia due to MCAo (Table 2).

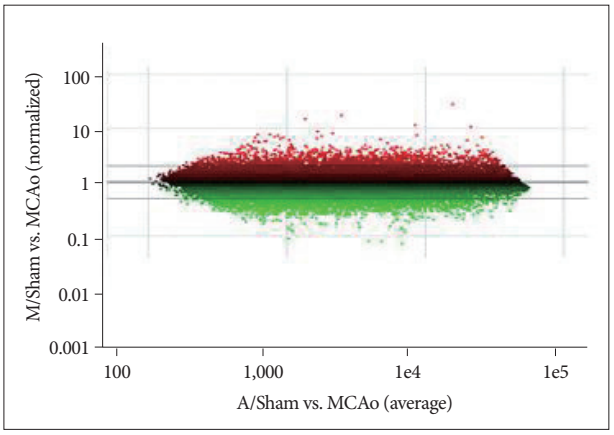

Fig. 3. Scattered plot of the normalization results by global M method in comparison of sham group versus MCAo group. The primary data were normalized by the total the intensity ratio of reference genes such as housekeeping genes in control and treatment groups. The expression ratio between two groups was converted to log2 ratio.

In addition, we looked at the changes in apoptosis-related gene expression in ischemic stroke. Table 3 shows the apoptosis-related genes that increased by more than 2-fold following brain ischemia due to MCAo. The expression of murine thymoma viral (v-akt), oncogene homolog 2 (Akt2), phosphatidylinositol 3-kinase, regulatory subunit, polypeptide (Pik3r2), tumor necrosis factor receptor superfamily, member 1a (Tnfrsf1a), protein phosphatase 3, catalytic subunit, beta isoform (Ppp3cb), apoptosis stimulating of p53 protein 1 (Ppp1r13b), inhibitor of kappa B kinase beta (Ikbkb), and protein kinase, cAMP-dependent, regulatory, type 2, alpha (Prkar2a) increased more than 2-fold. On the other hand, the expression of type II cAMP-dependent protein kinase regulatory subunit (Prkar2b), ataxia telangiectasia mutated homolog (Atm), nuclear factor kappa B (Nfkb1), and conserved helix-loop-helix ubiquitous kinase (Chuk) decreased by more than 2-fold (Table 4).

DISCUSSION

In the present study, gene expression profiles of the hippocampus after 2 hours of MCAo followed by 24 hours of reperfusion were examined using Agilent DNA microarray techniques. Ischemic damage by MCAo was confirmed using TTC

Table 1. List of oxidative phosphorylation related genes up-regulated by MCAo

Genbank	Gene symbol	Description	Normalized
NM_001011927	Atp6v0d1	ATPase, H^+ transporting, V0 subunit D isoform 1	3.0534
NM_017202	Cox4i1	Cytochrome c oxidase subunit IV isoform 1	2.8869
NM_133556	Atp5g2	ATP synthase, H^+ transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 2	2.7510
NM_031604	Atp6v0a1	ATPase, H^+ transporting, lysosomal V0 subunit a isoform 1	2.6920
XM_216944	Cyc1	Cytochrome c-1	2.5239
NM_001006972	Ndufv1	NADH dehydrogenase (ubiquinone) flavoprotein 1, 51kDa	2.4245
NM_053578	Atp6v0e1	ATPase, H^+ transporting, V0 subunit e	2.4015
NM_053756	Atp5g3	ATP synthase, H^+ transporting, mitochondrial F0 complex, subunit c, isoform 3	2.3506
NM_130823	Atp6v0c	ATPase, H^+ transporting, V0 subunit C	2.1215
NM_031064	Ndufv2	NADH dehydrogenase (ubiquinone) flavoprotein 2	2.0527

MCAo : middle cerebral artery thread-occlusion

Table 2. List of oxidative phosphorylation related genes down-regulated by MCAo

Genbank	Gene symbol	Product	Normalized
XM_343760	Ndufa1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1	0.4940
NM_182819	Cox7b	Cytochrome c oxidase subunit VIIb	0.4919
XM_342664	Ndufb2	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2	0.4199
XM_216859	Ndufa7	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7	0.4027
NM_053586	Cox5b	Cytochrome c oxidase subunit Vb	0.3818
NM_001006970	Uqcrc2	Ubiquinol-cytochrome c reductase core protein II	0.3694
NM_012786	Cox8h	Cytochrom c oxidase subunit VIII-H (heart/muscle)	0.1382

MCAo : middle cerebral artery thread-occlusion

Table 3. List of apoptosis related genes up-regulated by MCAo

Genbank	Gene symbol	Product	Normalized
NM_017093	Akt2	Murine thymoma viral (v-akt) oncogene homolog 2	3.0853
NM_022185	Pik3r2	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide	2.6432
NM_013091	Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	2.4363
NM_017042	Ppp3cb	Protein phosphatase 3, catalytic subunit, beta isoform	2.2091
XM_234555	Ppp1r13b	Apoptosis stimulating of p53 protein 1	2.2029
NM_053355	Ikbkb	Inhibitor of kappaB kinase beta	2.1485
NM_019264	Prkar2a	Protein kinase, cAMP-dependent, regulatory, type 2, alpha	2.0912

MCAo : middle cerebral artery thread-occlusion

Table 4. List of apoptosis related genes down-regulated by MCAo

Genbank	Gene symbol	Product	Normalized
M12492	Prkar2b	Type II cAMP-dependent protein kinase regulatory subunit	0.4832
XM_236275	Atm	Ataxia telangiectasia mutated homolog	0.4470
L26267	Nfkb1	Nuclear factor kappa B	0.4428
XM_219857	Chuk	Conserved helix-loop-helix ubiquitous kinase	0.3444

MCAo : middle cerebral artery thread-occlusion

staining and the TUNEL assay. Within the large-scale temporal gene expression profile of the rat hippocampus that was obtained, we found that 1,882 genes were upregulated and 2,337 genes were downregulated. Among the very large number of genes whose expression changed, we focused on the genes related to oxidative phosphorylation and apoptosis.

The brain is almost exclusively dependent on a continuous flow of glucose and oxygen for energy production through oxidative phosphorylation because it lacks stores of energy. Damage from oxygen deprivation occurs in minutes. The first consequence of CBF reduction is the depletion of substrates, particularly oxygen and glucose. This causes an accumulation of lactate via anaerobic glycolysis. Acidosis may enhance free radical formation, interfering with intracellular protein synthesis and worsening ischemic brain injury. However, the mechanisms of the deleterious effects of acidosis are still unknown[8,16,22]. Energy failure leads to the perturbation of the Na^+/K^+-ATPase and Ca^{2+}/H^+-ATPase pumps. In addition, the flow of the Na^+-Ca^{2+} transporter is reversed[18]. Subsequent ion dyshomeostasis (elevation of intracellular Na^+, Ca^{2+}, and Cl^- and elevation of extracellular K^+) causes cytotoxic edema and leads to events triggered by excess intracellular Ca^{2+}. In relation to oxidative phosphorylation, we found that 10 genes were upregulated while 7 were downregulated. Specifically, genes encoding ATPase, including Atp6v0d1, Atp6v0a1, Atp6v0e1, and Atp6v0c, were upregulated, whereas genes encoding NADH dehydrogenase, including Ndufa1, Ndufb2, and Ndufa7, were downregulated after transient focal cerebral ischemia in the rat hippocampus.

In the pathophysiology of acute ischemic stroke, cell death is in part due to the apoptotic process. Mitochondria, important source of ROS, is impaired by free radical-mediated disruption of the inner mitochondrial membrane and the oxidation of proteins that mediate electron transport, H^+ extrusion, and ATP production[4]. The mitochondrial membrane becomes leaky, in part due to the formation of a mitochondrial permeability transition pore, which promotes mitochondrial swelling, the cessation of ATP production, and an oxygen free-radical burst[10]. As a result, cytochrome C is released from the mitochondria[6] and this provides a trigger for apoptosis. Knowing this, we decided to also investigate changes in the expression of apoptosis related genes. Among the apoptosis related genes, we found that 7, including Akt2 and Tnfrsf1a, were upregulated, while 4, including Nfkb1, were downregulated after transient focal cerebral ischemia in the rat hippocampus.

Following ischemia, caspase activation occurs in response to pro-apoptotic signals such as the downregulation of Bcl-2 and the upregulation of Bax/Bid and the Death receptor family. It has been shown that caspases 1, 3, 8, and 9 are involved in cerebral ischemia[16]. However, in this study, the expression of these critical genes, including caspase, Bcl-2, and Bax, remained stable after ischemia. As the time of ischemic time was too short, these down-streaming genes of apoptosis were not demonstrated in this study.

Recently, some studies have reported gene expression profiles in the rat hippocampus after MCAo. Sun et al.[25]. found modulation of signal transducers and activators of transcription (STAT) factor pathways in the rat hippocampus after middle cerebral artery occlusion. The study suggested that the expression of STAT2, 5a, 5b, 6 and the suppressor of cytokine signaling 4 was increased by ischemia. They suggested that this may be a compensatory response by the brain and may play a protective role for damaged brain tissue. Mitsios et al.[17] demonstrated a significant difference in the number of genes affected and the time-course of expression between the human and rat brain. The total number of deregulated genes in the rat was 335 versus 126 in the human. Of the 393 overlapping genes between the two array sets, 184 changed only in the rat, 36 in the human, and 41 were deregulated in both. Interestingly, the degree of change

was much higher in the human. The expression of novel genes, including p21-activated kinase 1 (PAK1), matrix metalloproteinase 11 (MMP11), and integrase interactor 1, was further analyzed using RT-PCR, Western blotting, and immunohistochemistry. Strong neuronal staining was seen for PAK1 and MMP11. To the best of our knowledge, there has not been a previous study that analyzed the expression of genes involved with oxidative phosphorylation in the rat hippocampus after transient focal cerebral ischemia.

As the rat genome contains approximately 30,000 genes[27], the present analysis of 44,000 genes covers the entire genome. The successful identification of many novel ischemia-related genes suggests that performing a similar study using microarrays with a complete set of genes would be even more valuable. While this study focused only on those genes whose expression levels changed by more than 2.3-fold in terms of their hybridization signals, there might have been other genes whose expression was severely altered at the single cell level but were excluded by the criteria of the current study. Therefore, many other genes could be considered as candidate genes based on their physiological relevance in the post-ischemic brain.

CONCLUSION

Our analysis revealed a significant change in the expression of energy metabolism related genes (Atp6v0d1, Atp5g2, etc.) in the hippocampus of the ischemic rat brain. Based on this data, we believe that these genes have the potential to be target genes used for the development of therapeutic agents for ischemic stroke.

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