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Screening of differentially expressed genes of middle cerebral artery occlusion with DNA microarray

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Abstract. – OBJECTIVES: To screen differentially expressed genes of different days after cerebral artery occlusion and drug treatment, and identify related small drug molecules.

MATERIALS AND METHODS: The gene expression profile GSE35338 of cerebral artery occlusion was downloaded from Gene Expression Omnibus database, including a total of 14 samples. 5 samples are 1 day after cerebral artery occlusion (control), 3 samples are 7 days after cerebral artery occlusion and 3 samples are under lipopolysaccharide (LPS) treatment. Differentially expressed genes (DEGs) between different days after cerebral artery occlusion were screened ($p < 0.05$, FDR < 0.05, |logFC| > 1). The DEGs were then entered into the CMAP database and related small drug molecules were retrieved, followed by calculation of co-expression score of the genes and construction of co-expression-drug network. FuncAssociate software and DAVID were used to obtain the functional clusters of genes with p-value < 0.05 and FDR < 0.05.

RESULTS: Compared with the control group, 825, 1445, 218 DEGs and 4, 3, 2 most-related small drug molecules were respectively identified from 3, 7 days after cerebral artery occlusion and LPS treated group. Co-expression network was constructed and functional clusters were found to be 161, 146, and 6 in each group.

CONCLUSIONS: Our study provides some underlying biomarkers for cerebral artery occlusion under varied conditions and potential small drug molecules for treatment of cerebral artery occlusion.

Key Words:
Middle cerebral artery occlusion, Differentially expressed genes, Small drug molecule, Co-expression network.

Introduction

Middle cerebral artery occlusion (MCAO) is a common cause of stroke and it carries high morbidity and mortality. There are limited treatment options, so it is one of the focus areas of present biomedical research.

Many complex pathophysiological mechanisms underlie MCAO, such as loss of energy substrates (glucose and oxygen), loss of ATP, depolarization of brain cells and increase of extracellular potassium levels[1,2]. Neuronal degeneration is an important component of the sequence of events in ischemic cerebrovascular disease[3]. Many studies have indicated that excitatory transmitter glutamate and free radical[4-6] are major mediators involved in neuronal cell death. Besides, acetylcholine and calcium overload are also found to be implicated in apoptosis of neural cell[6,7]. Recent evidence suggests that progression of MCAO leads to deregulation of genes whose expression promotes ischemic neuronal death and subsequent neurological dysfunction[8-10]. However, the precise mechanisms underlying stroke-induced neuronal death and neurological dysfunction are not fully understood.

Microarray technology is powerful tool to uncover the mechanisms. In present study, gene chip data was analyzed to elucidate the molecular mechanisms of cell death and identify key genes, which will provide new insights into the development of neuro-protective agents for cerebral artery occlusion therapeutics.

Materials and Methods

Microarray Data

Microarray data set GSE35338[11] was downloaded from Gene Expression Omnibus (GEO) database. A total of 14 samples were obtained, of which 5 samples were from 1 day after cerebral artery occlusion (control), 3 samples were from 3

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days after cerebral artery occlusion, 3 samples were from 7 days after cerebral artery occlusion and 3 samples were from one day after lipopolysaccharide (LPS) treatment).

Screening of Differentially Expressed Genes (DEGs)

The original data were converted into an identifiable expression profile format using the affy package of *R*, and then the missed part in the data set was completed[12] to further standardize the data set[13]. The differential expression value between samples was calculated using limma method[14] and then p-values were adjusted for multiple comparisons using the false discovery rate (FDR) of Benjamini and Hochberg (BH) method[15]. p-value < 0.05, FDR < 0.05 and | logFC | > 1 were chosen as the threshold for identifying DEGs.

Retrieval of Related Small Molecules

The selected genes were divided into up-regulated and down-regulated sets as the entry of connectivity map (cmap). Related small medicine molecules were screened out through comparing our differentially expressed genes with expression patterns of genes collected in Comparative Mapping (CMAP) database[16]. In this study, the |score| > 0.5 was considered as the high relationship threshold.

Construction of the Co-Expression Network of Small Drug Molecules

String software[17] was used to calculate the coefficient between differentially expressed genes. In this study, interactions with co-expression coefficient > 0.5 were selected to construct co-expression network under small molecular medicine regulation.

Functional Annotation

The up- and down-regulated DEGs were put into the Database for Annotation, Visualization and Integrated Discovery (DAVID)[18] and FuncAssociate[19] software for Gene Ontology (GO) term enrichment analysis. p < 0.05 and FDR < 0.05 were set as the threshold in the analysis based upon hypergeometric distribution.

Results

Differentially Expressed Genes

The gene profiles for 3 days, 7 days and LPS treatment were compared with that for 1 day, and 825, 1445 and 218 DEGs met with the criteria (p < 0.05, FDR < 0.05 and |logFC| > 1) were selected out.

Relevant Small Molecule Drugs

DEGs were divided into up-regulated and down-regulated sets as the entry of connectivity map (cmap). Based on the threshold of |score| > 0.5, 4, 3 and 2 closely related with small molecule drugs were identified in each group individually (Table I). It was obvious that MG-262, irinotecan, camptothecin and GW-8510 were the small molecule drugs closely associated with disease status 3 days after occurrence of cerebral artery occlusion; MG-262, quinostatin, and snx26 were small molecule drugs closely associated with disease status 7 days after occurrence of cerebral artery occlusion; MG-262 was present in both of the two disease statuses. These results indicate that these small molecule drugs may be effective for cerebral artery occlusion. Meanwhile, thapsigargin and mycophenolic acid were found to be closely associated with one day post-LPS treatment and the stimulation of LPS was reported to be able to give rise to cerebral inflammation in cerebral artery occlusion.

Co-expression Network

String software was used to calculate the coefficient between DEGs. Together with the 9 related small medicine molecules, we constructed small molecules regulation network. There are respectively 115, 55, and 13 co-expressed gene pairs in three groups (Figure 1).

Table I. The differentially expressed genes related small molecular medicine and their p value.

	cmap name	Score	p
1 day vs. 3 days	MG-262	–0.996	0
	Irinotecan	0.99	0.00002
	camptothecin	0.996	0
	GW-8510	0.998	0
1 day vs. 7 days	MG-262	–0.99	0
	Quinostatin	0.969	0.00167
	SNX26	0.951	0.00807
1 day vs. LPS treatment	Tthapsigargin	–0.953	0.00022
	Mycophenolic acid	0.908	0.00206

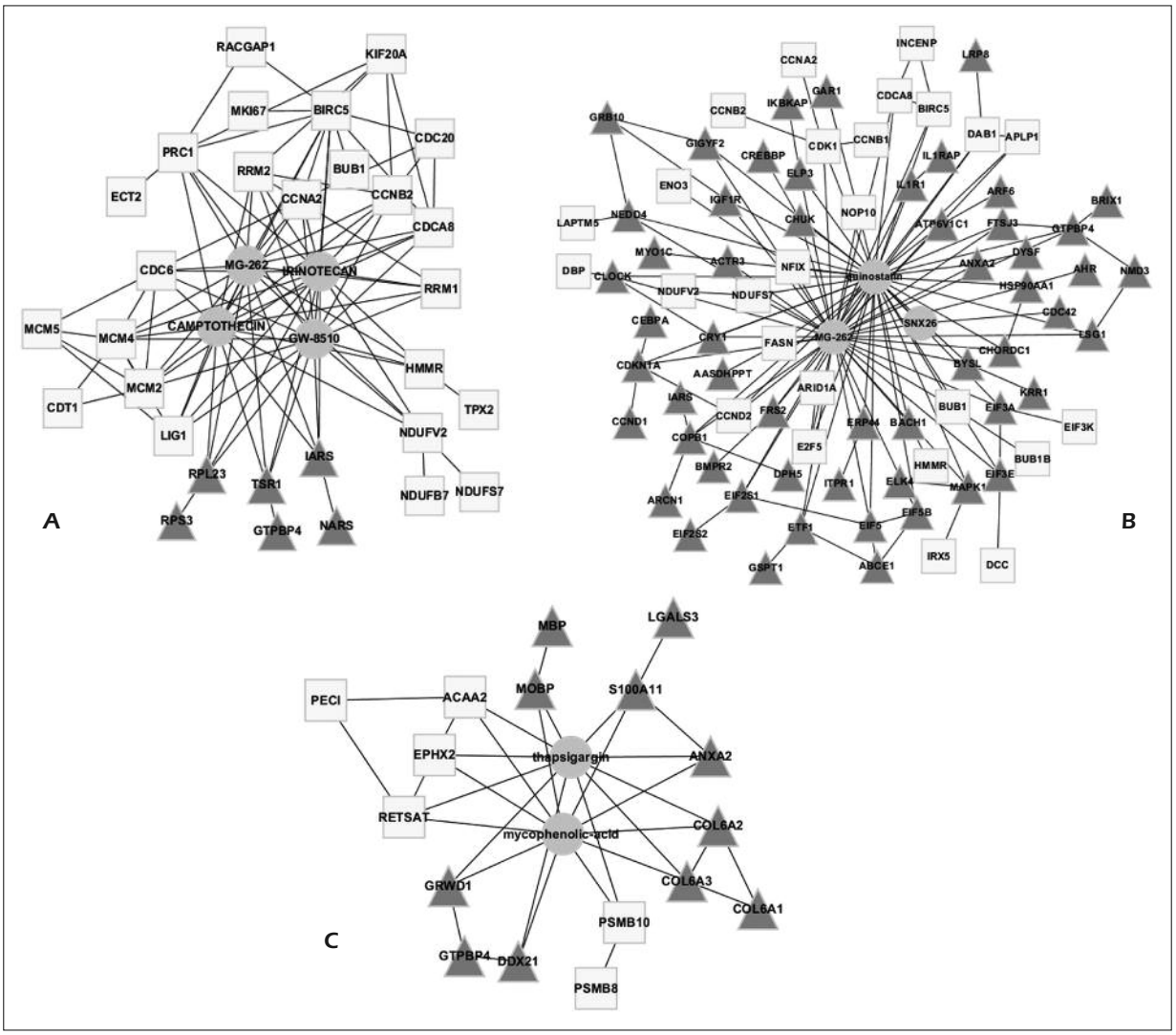

Figure 1. Small molecular medicines regulation network. The blue triangles represent the down-regulated DEGs, the yellow squares represent up-regulated DEGs, and the grey circles represent the selected small molecules.

Function Annotation Results

FuncAssociate is a web-based tool that receives a list of gene as input and returns a list of GO features that are over- or under-represented among the genes in input list. After the multiple hypothesis tests, only those representations that are significantly over- or under- represented are reported. In this study, we kept the functional annotation records of $p < 0.05$ and FDR < 0.05. The DEGs data were uploaded with co-expression relationship into the FuncAssociate software, and there were 137, 136 and 5 functional clusters from three networks. In the meantime, there were 66, 18 and 3 functional clusters from three networks using DAVID software. All in all, there were 161, 146 and 6 function clusters from 1 day vs. 3 days, 1 day vs. 7 days and 1 day vs. PLS treatment networks. The significantly enriched function in the three groups were cell cycle (GO: 0007049), condensed chromosome outer kinetochore (GO: 0000940) and structural constituent of myelin sheath (GO: 0019911).

Discussion

In present study, DEGs were screened out by comparing gene expression profiles from different time points after MCAO. There were 825, 1445 and 218 DEGs for 1 day vs. 3 days, 1 day vs. 7 days and 1 day vs. LPS treatment. It sug-

gests that active mechanisms initiate during the acute phase after experimental stroke and last for several days. Some of the DEGs have been reported to be de-regulated in stroke, such as p21 protein (Cdc42/Rac)-activated kinase 1 (PAK1) and Matrix metallopeptidase 11 (MMP11) while some are not. These genes reflect the molecular changes in stroke and may be therapeutic targets.

PAK1 is a downstream Rac effector[20] and a major cyclin-dependent kinase 5 (Cdk5) substrate and target that co-localizes with p35/Cdk5 at neuronal peripheries[21]. P35/Cdk5 causes PAK1 hyperphosphorylation and thus affects neuronal morphology through reorganization of the actin cytoskeleton. Another study[22] also points out that FOXO proteins and Pak1 are components of a cell-intrinsic transcriptional pathway that orchestrates neuronal polarity. Rashidian et al[23] demonstrate a potential role of Cdk5/p35 in the response to ischaemic injury. Mitsios et al[24] further report that PAK1 is up-regulated in human and rat brain samples with RT-PCR, Western blotting and immunohistochemistry.

MMP11, first isolated as a breast cancer-associated protease, is not expressed in the majority of normal adult organs but is expressed during a number of pathological processes, including wound healing and atherosclerotic lesions[25]. Lijnen et al[26] report that neointima formation is accelerated in mice with MMP-11 gene inactivation. It has been report that its mRNA level and protein level are also up-regulated in human and rat brain following stroke[24], which is in accordance with our findings.

INI1 (SMARCB1) is a tumor suppressor gene and is thought to exert its function by mediating cell cycle arrest[27]. It is part of a complex that relieves repressive chromatin structures[28], allowing the transcriptional machinery to access its targets more effectively, for example HIV-1 integrase. Adler et al[29] also reported an association of the human SNF5/INI1 protein with growth arrest and DNA damage-inducible protein 34 (GADD34) that mediates growth arrest and apoptosis in response to stress signals. Accordingly, cell cycle was enriched in the functional annotation, indicating that cell cycle is an important link in the progression of MCAO.

E2F transcription factor 5 (E2F5) also plays a crucial role in the control of cell cycle[30] and action of tumor suppressor proteins. And it is slightly up-regulated in 7 days compared with 1 day, indicating the control of cell cycle starts at this time point. Di Giovanni et al[31] consider that the cycle-related genes are involved in neuronal damage and subsequent cell death and the up-regulation of E2F5 is observed in both mRNA and protein levels.

Modulations on apoptosis and cell cycle may be two strategies to treat stroke or decrease adverse consequences[32,33]. Wu et al[34] find that netrin-1 can protect infarct tissue from p53-mediated apoptosis. Adibhatla and Hatcher report the protection by D609 through cell-cycle regulation after stroke[35]. Therefore, those DEGs associated with these two functions are potential drug targets.

In addition to DEGs, several small molecules were retrieved, which may be related with stroke. Previous study[36] has indicated that proteasome inhibition could be an effective stroke therapy, while MG-262 is a reversible proteasome inhibitor. Considering ubiquitin-proteasome system plays an important role in apoptosis, the inhibitor may help to reduce the adverse effect. Conversely, thapsigargin induces apoptosis[37] through inhibits calcium-ATPase. Schreihofer and Redmond[38] find that soy phytoestrogens are neuroprotective against stroke-like injury *in vitro*, which can inhibit thapsigargin. These small molecules are valuable guides for further drug developments.

Here, we have provided substantial evidence that, although the available animal models of MCAO may well be suitable to study the pathophysiological changes following the occlusion of a cerebral vessel, they may not entirely reflect the pathophysiological process through which stroke evolves in humans. The species difference is one of the main reasons accounting for the lack of success of bench to bedside translation in the stroke area. Limit of our study includes the fact that early acute phase changes in gene expression may have been missed since genes induced and returning to normal during the first 48 hours post-ischaemia in man could not have been detected. Moreover, since we analyzed pooled RNA samples, small changes in gene expression occurring in a minority of the samples may have been missed. However, there was only a small overlap of our results with prior studies in experimental stroke involving brain tissue, and the successful identification of novel ischaemia-related genes reported here suggests that performing a further study using whole genome microarrays would be valuable.

Conclusions

The DEGs identified in present study provide good research directions, which may advance our understandings about MCAO. And the relevant small molecules are beneficial in guiding future drug discovery.

References

1) Lo EH, Dalkara T, Moskowitz MA. Mechanisms, challenges and opportunities in stroke. Nat Rev Neurosci 2003; 4: 399-415.
2) Mehta SL, Manhas N, Raghubir R. Molecular targets in cerebral ischemia for developing novel therapeutics. Brain Res Rev 2007; 54: 34-66.
3) Mattson MP, Duan W, Pedersen WA, Culmsee C. Neurodegenerative disorders and ischemic brain diseases. Apoptosis 2001; 6: 69-81.
4) Schulz JB, Henshaw DR, Siwek D, Jenkins BG, Ferrante RJ, Cipolloni PB, Kowall NW, Rosen BR, Beal MF. Involvement of free radicals in excitotoxicity in vivo. J Neurochem 1995; 64: 2239-2247.
5) Facchinetti F, Dawson VL, Dawson TM. Free radicals as mediators of neuronal injury. Cell Mol Neurobiol 1998; 18: 667-682.
6) Lipton P. Ischemic cell death in brain neurons. Physiol Rev 1999; 79: 1431-1568.
7) Solberg Y, Belkin M. The role of excitotoxicity in organophosphorous nerve agents central poisoning. Trends Pharmacol Sci 1997; 18: 183-185.
8) Mitsios N, Gaffney J, Kumar P, Krupinski J, Kumar S, Slevin M. Pathophysiology of acute ischaemic stroke: an analysis of common signalling mechanisms and identification of new molecular targets. Pathobiology 2006; 73: 159-175.
9) Zhai F, Zhang X, Guan Y, Yang X, Li Y, Song G, Guan L. Expression profiles of microRNAs after focal cerebral ischemia/reperfusion injury in rats. NRR 2012; 7.
10) Slevin M, Krupinski J, Kumar P, Gaffney J, Kumar S. Gene activation and protein expression following ischaemic stroke: strategies towards neuroprotection. J Cell Mol Med 2007; 9: 85-102.
11) Zamanian JL, Xu L, Foo LC, Nouri N, Zhou L, Giffard RG, Barres BA. Genomic analysis of reactive astrogliosis. J Neurosci 2012; 32: 6391-6410.
12) Troyanskaya O, Cantor M, Sherlock G, Brown P, Hastie T, Tibshirani R, Botstein D, Altman RB. Missing value estimation methods for DNA microarrays. Bioinformatics 2001; 17: 520-525.
13) Fujita A, Sato JR, Rodrigues Lde O, Ferreira CE, Sogayar MC. Evaluating different methods of microarray data normalization. BMC Bioinformatics 2006; 7: 469.
14) Yoshida R, Imoto S, Higuchi T. Estimating time-dependent gene networks from time series microarray data by dynamic linear models with Markov switching. Proc IEEE Comput Syst Bioinform Conf 2005; 289-298.
15) Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I. Controlling the false discovery rate in behavior genetics research. Behav Brain Res 2001; 125: 279-284.
16) Lamb J, Crawford ED, Peck D, Modell JW, Blat IC, Wrobel MJ, Lerner J, Brunet JP, Subramanian A, Ross KN. The Connectivity Map: using gene-expression signatures to connect small molecules, genes, and disease. Sci Signal 2006; 313: 1929-1935.
17) Szklarczyk D, Franceschini A, Kuhn M, Simonovic M, Roth A, Minguez P, Doerks T, Stark M, Muller J, Bork P, Jensen LJ, von Mering C. The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored. Nucleic Acids Res 2011; 39: 561-568.
18) Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 2009; 4: 44-57.
19) Berriz GF, Beaver JE, Cenik C, Tasan M, Roth FP. Next generation software for functional trend analysis. Bioinformatics 2009; 25: 3043-3044.
20) Edwards DC, Sanders LC, Bokoch GM, Gill GN. Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. Nat Cell Biol 1999; 1: 253-259.
21) Rashid T, Banerjee M, Nikolic M. Phosphorylation of Pak1 by the p35/Cdk5 kinase affects neuronal morphology. J Biol Chem 2001; 276: 49043-49052.
22) de la Torre-Ubieta L, Gaudilliere B, Yang Y, Ikeuchi Y, Yamada T, DiBacco S, Stegmuller J, Schuller U, Salih DA, Rowitch D, Brunet A, Bonni A. A FOXO-Pak1 transcriptional pathway controls neuronal polarity. Genes Dev 2010; 24: 799-813.
23) Rashidian J, Rousseaux MW, Venderova K, Qu D, Callaghan SM, Phillips M, Bland RJ, During MJ, Mao Z, Slack RS, Park DS. Essential role of cytoplasmic cdk5 and Prx2 in multiple ischemic injury models, in vivo. J Neurosci 2009; 29: 12497-12505.
24) Mitsios N, Saka M, Krupinski J, Pennucci R, Sanfeliu C, Wang Q, Rubio F, Gaffney J, Kumar P, Kumar S, Sullivan M, Slevin M. A microarray study of gene and protein regulation in human and rat brain following middle cerebral artery occlusion. BMC Neurosci 2007; 8: 93.
25) Ketelhuth DF, Back M. The role of matrix metalloproteinases in atherothrombosis. Curr Atheroscler Rep 2011; 13: 162-169.
26) Lijnen HR, Van Hoef B, Vanlinthout I, Verstreken M, Rio MC, Collen D. Accelerated neointima formation after vascular injury in mice with stromelysin-3 (MMP-11) gene inactivation. Arterioscler Thromb Vasc Biol 1999; 19: 2863- 2870.

27) Kuwahara Y, Charboneau A, Knudsen ES, Weissman BE. Reexpression of hSNF5 in malignant rhabdoid tumor cell lines causes cell cycle arrest through a p21(CIP1/WAF1)-dependent mechanism. Cancer Res 2010; 70: 1854-1865.

28) Ranneberg-Nilsen T, Rollag H, Slettebakk R, Backe PH, Olsen O, Luna L, Bjoras M. The chromatin remodeling factor SMARCB1 forms a complex with human cytomegalovirus proteins UL114 and UL44. PLoS One 2012; 7: e34119.

29) Adler HT, Chinery R, Wu DY, Kussick SJ, Payne JM, Fornace AJ, Jr., Tkachuk DC. Leukemic HRX fusion proteins inhibit GADD34-induced apoptosis and associate with the GADD34 and hSNF5/INI1 proteins. Mol Cell Biol 1999; 19: 7050-7060.

30) Gaubatz S, Lindeman GJ, Ishida S, Jakoi L, Nevins JR, Livingston DM, Rempel RE. E2F4 and E2F5 play an essential role in pocket protein-mediated G1 control. Mol Cell 2000; 6: 729-735.

31) Di Giovanni S, Knoblach SM, Brandoli C, Aden SA, Hoffman EP, Faden AI. Gene profiling in spinal cord injury shows role of cell cycle in neuronal death. Ann Neurol 2003; 53: 454-468.

32) Rashidian J, Iyirhiaro GO, Park DS. Cell cycle machinery and stroke. Biochim Biophys Acta 2007; 1772: 484-493.

33) Rosenblum WI. Apoptosis and stroke pathogenesis. Stroke 1999; 30: 1154-1155; author reply 1156.

34) Wu TW, Li WW, Li H. Netrin-1 attenuates ischemic stroke-induced apoptosis. Neurosci 2008; 156: 475-482.

35) Adibhatla RM, Hatcher JF. Protection by D609 through cell-cycle regulation after stroke. Mol Neurobiol 2010; 41: 206-217.

36) Cvek B, Dvorak Z. The ubiquitin-proteasome system (UPS) and the mechanism of action of bortezomib. Curr Pharm Des 2011; 17: 1483-1499.

37) Min KJ, Kim HS, Park EJ, Kwon TK. Melatonin enhances thapsigargin-induced apoptosis through reactive oxygen species-mediated upregulation of CCAAT-enhancer-binding protein homologous protein in human renal cancer cells. J Pineal Res 2012; 53: 99-106.

38) Schreihofer DA, Redmond L. Soy phytoestrogens are neuroprotective against stroke-like injury *in vitro*. Neurosci 2009; 158: 602-609.