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Identification of therapeutic targets of ischemic stroke with DNA microarray

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Abstract. – OBJECTIVE: Ischemic stroke (IS) is a complex disease that resulting from the interaction of various environmental and genetic risk factors. As genetic factors exerting a direct contributory role in IS, it is one of the focus areas of identification the genetic factors of IS. This study aimed to screen bio-targets of ischemic stroke (IS), and to identify related drug molecules.

MATERIALS AND METHODS: The gene expression profile GSE22255 was downloaded from Gene Expression Omnibus (GEO) database, including 20 whole blood samples from IS patients (IS group) and 20 samples from healthy controls (control group). Differentially expressed genes (DEGs) were screened out using limma package in R. Hierarchical clustering and differences between the groups analysis were conducted for confirming these DEGs. Database for Annotation, Visualization and Integrated Discovery (DAVID) and Kyoto Enrichment of Genes and Genomes (KEGG) were used to obtain the functional genes and pathways respectively. The DEGs were then entered into the WebGestalt database and related drug molecules were retrieved.

RESULTS: Compared with the control group, 27 DEGs were identified from IS group including 25 up- and 2 down-regulated genes. Then functions and pathways enrichment analysis for DEGs were conducted and *TNF, IL1B* and *TNFAIP3* were found to be both participate in apoptosis and NOD-like receptor signaling pathway. Finally, collagenase and other most-related drug molecules were identified from the DEGs.

CONCLUSIONS: In addition to DEGs, several drug molecules were retrieved, which may be related with stroke. Our study provides some underlying bio-targets such as TNF, IL1B and TNFAIP3 for IS and potential drug molecules such as collagenase for the treatment of IS.

Key Words:
Ischemic stroke, Differentially expressed genes, Function enrichment and pathway analysis, Relevant molecule drugs

Introduction

Ischemic stroke (IS) is account for more than 80% of the stroke and is a major cause of death and disability[1,2]. Moreover, survival of IS can be also result in severe neurological impairments and physical disabilities with a high socio-economic cost[3]. IS is a complex disease that resulting from the interaction of various environmental and genetic risk factors[4]. As genetic factors exerting a direct contributory role in IS, so it is one of the focus areas of identification of the of genetic factors IS[5].

With the progress of microarray technology, a large of genetic analysis of IS has been attempted in animal models. There is considerable evidence for the inflammatory factors related to IS in acute phase in animal models. IL-1, IL-8, IL-10 and IL-17 mRNAs are elevated in peripheral blood cells in animals with ischemic stroke[6,7]. In a rat experimental studies, TNF, TNF receptor, adhesion molecules and various interleukins are elevated in peripheral blood[8,9]. There are also many other factors related to IS in acute phase in animal models. PAK1, MMP11, INI1 and E2F were provided as biomarkers in Zhang et al[10] study for cerebral artery occlusion under varied time windows. Ramos-Cejudo et al[11] study showed that the expression of many neurovascular unit development genes was altered at 24 h and 3 d including HES2, OLIG2, LINGO1 and NOGO-A; chemokines like CXCL1 and CXCL12, stress-response genes like HIF-1A, and trophic factors like BDNF or BMP4. So there are many changed expression of genes in response to IS in animals.

But there is less microarray analysis of IS has been attempted in humans. The first study of whole genome in human blood after IS was reported by Moore et al in 2005[12], who found 22

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genes derived from the Prediction Analysis for Microarrays. Tang et al[13] study provided many protein markers related to the inflammatory responses after stroke in humans, including MMP9, coagulation factor V; arginase I; carbonic anhydrase IV, S100 proteins, N-acetyl-aspartic acid and many others. However these studies address the stroke mechanisms during the acute phase or in the first months after the stroke. Krug et al[14] published their research results that TTC7B specifically increase the risk for a stroke event through investigating gene expression changes in humans at least 6 months after stroke. So there are a few changed expression of genes in IS patients in acute phase and late stage of ischemia.

There are also significant differences between the genes identified in this study and in that by Krug et al[14] in spite of using similar microarray data. In present study, gene microarray data was analyzed used GO and KEGG methods to illuminate the molecular mechanisms in late stage of ischemia and identify bio-targets of IS, then relevant molecule drugs screened in this study will provide new protective agents for IS therapeutics.

The aim of this study is to screen bio-targets of ischemic stroke (IS), and to identify related drug molecules.

Materials and Methods

Microarray Data

The microarray and other forms of high flux data produced by the scientific community can be freely downloaded from the Gene Expression Omnibus (GEO) database of NCBI, which is the biggest public database[15]. Microarray data set GSE22255 which were deposited by Krug et al[14], was downloaded from GEO database. There are a total of 40 samples available for further analysis, including 20 samples of peripheral blood mononuclear cells (PBMC) from patients with IS (IS group) and 20 samples from healthy controls (control group). All participants were adults and Spanish patients, who were classified into causative subtypes of IS. Patients were required to have suffered only one stroke episode at least 6 months before the blood collection, and controls could not have a family history of stroke. The platform was GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array. Annotation information for all probe sets is provided by Affymetrix company. We downloaded the raw CEL file and the probe annotation file.

Data Preprocessing and Differentially Expressed Genes Analysis

A total of 54675 probes were mapped into the gene names based on the annotation platform information, and expression profile data were performed log2 transformation[16]. The differential expression value between IS group and control group was calculated using limma package in R[17] and then *p*-values were adjusted for multiple comparisons using the false discovery rate (FDR) of Benjamini and Hochberg (BH) method[18]. FDR < 0.05 and $|\log FC| > 1$ were used as the threshold for identifying differentially expressed genes (DEGs).

Hierarchical Clustering Analysis

In order to collect the genes having similar expression levels together and intuitive to see the expression values of DEGs in different samples, the hierarchical clustering analysis was performed. The expression values of DEGs in each group were selected according to the probe information from the downloaded files. The R language pheatmap package (http://cran.r-project.org/web/packages/pheatmap/index.html) was used for hierarchical clustering[19] of the expression values of DEGs based on Euclidean distance[20], and the results were shown in a heat map.

The Comparison of DEGs Between Groups

Based on the DEGs found in each group, the *t*-test between groups[21] was used to compare the gene expression values of DEGs between the two groups, in order to study the expression condition of DEGs in IS group and the control group. *p*-value < 0.05 was used as the threshold value to test whether the DEGs between different groups was also significant difference.

Gene Ontology Functional and Pathway Enrichment Analysis

Gene Ontology analysis has become a commonly used approach for functional studies of large-scale genomic or transcription data[22]. The Kyoto Enrichment of Genes and Genomes (KEGG) pathway database contains information of how molecules or genes are networked, which is complementary to most of the existing molecular biology databases containing the information of individual genes[23]. We used the Database for Annotation, Visualization and Integrated Discovery (DAVID)[24] to perform functional enrichment analysis for the identified DEGs with the thresh-

old of FDR < 0.05. Pathway analysis was performed based on the KEGG database with the threshold value of p-value < 0.05.

Related Medicine Analysis

WebGestalt (WEB-based GEne SeT AnaLysis Toolkit), is an integrated data mining system for the management, information retrieval, organization, visualization and statistical analysis of large sets of genes. DEGs related medicine molecules were screened out through WebGestalt[25,26] database. In this study, the p-value < 0.05 was considered as the threshold.

Results

Identification of DEGs and Hierarchical Clustering Analysis

In order to get the DEGs with IS, we obtained publicly available microarray dataset GSE22255 from GEO. The gene profiles for IS group were compared with that of controls, and 27 DEGs met with the criteria (FDR < 0.05 and |logFC| > 1) were selected out. There were 25 up-regulated and 2 down-regulated DEGs (Table I). From the heat maps (Figure 1), the results of hierarchical clustering analysis showed us that IS group and control group can be separated by the selected DEGs in each group, which means the expression patterns of DEGs screened were very significant, and they can be used to distinguish IS and control samples.

The comparison of DEGs Between Groups

The gene expression values of DEGs were extracted from expression profile. Then t-test was used as statistical test analysis between groups. The results were shown in Figure 2, the DEGs in the two groups also have significant differences between groups (p = 0.01703).

Function Annotation Results

In this study, we kept the functional annotation records of FDR < 0.05. The DEGs data were up loaded into the DAVID software, and GO enrichment analysis was performed for DEGs. There were 14 functional clusters and the significantly enriched functions by the DEGs were apoptosis

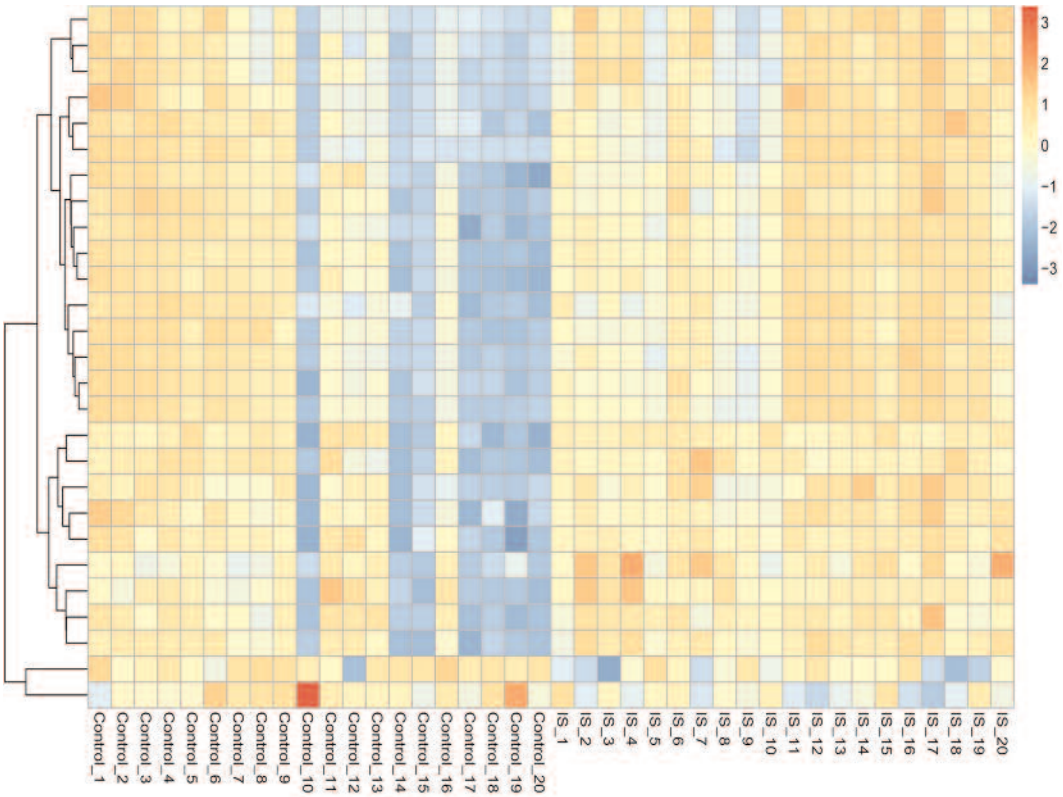

Figure 1. Hierarchical clustering heat maps of the differentially expressed genes. The gradient color from blue to orange represent expression values (IS /normal controls) from the lower to raise.

Table I. List of differentially expressed genes.

ID	FDR	logFC	Gene symbol
201123_s_at	0.00183776	-1.215456	EIF5A
206641_at	0.00243299	-1.0267375	TNFRSF17
202643_s_at	0.0189855	1.0193335	TNFAIP3
238893_at	0.00458577	1.043154	
LINC00936			
227697_at	0.02907835	1.04877	SOCS3
202672_s_at	0.03064402	1.0737905	ATF3
1555827_at	0.00178348	1.075209	CCNL1
37028_at	0.01187828	1.1483435	PPP1R15A
230170_at	0.00679872	1.166787	OSM
201631_s_at	0.0045455	1.18707	IER3
204794_at	0.01145231	1.213562	DUSP2
1569599_at	0.00482087	1.2228775	SAMSN1
1555938_x_at	0.00079963	1.3024035	VIM
243296_at	0.00762286	1.302593	NAMPT
216834_at	0.02587638	1.3467345	RGS1
215078_at	0.00975942	1.402818	SOD2
1568768_s_at	0.03867819	1.408874	BRE-AS1
235490_at	0.00007097	1.5177265	TMEM107
207113_s_at	0.00590229	1.5194	TNF
204621_s_at	0.045301	1.5297155	NR4A2
205067_at	0.04309577	1.605887	IL1B
204748_at	0.02015769	1.793512	PTGS2
201466_s_at	0.00088106	1.8006055	JUN
227404_s_at	0.00476479	1.809806	EGR1
209774_x_at	0.02071469	2.270752	CXCL2
213524_s_at	0.00431627	2.288196	G0S2

(GO: 0006915, FDR = 0.049), response to extracellular stimulus (GO: 0009991, FDR = 0.035) and negative regulation of cell proliferation (GO: 0008285, FDR = 0.028) (Table II). A total of 8 DEGs were enriched in cell apoptosis, including *OSM*, *IER3*, *TNF*, *JUN*, *IL1B*, *TNFAIP3*, *PPP1R15A* and *SOD2*.

Pathway Enrichment Analysis of DEGs

KEGG analysis was performed in order to gain further insights into the pathways of DEGs screened in our study. Only 4 pathways (Table III) were enriched among the DEGs and NOD-like receptor signaling pathway (p = 1.32E-05) was the most enriched pathway. Genes involved in the NOD-like receptor signaling pathway were *TNF*, *IL8*, *CXCL2*, *IL1B*, and *TNFAIP3*, among these genes, *TNF*, *IL1B* and *TNFAIP3* also enriched in the most significant function of GO.

Relevant Molecule Drugs

Based on the DEGs we screened, molecule drugs were identified in WebGestalt database (Table IV). It was obvious that collagenase (p = 4.67e-08), anakinra (p = 5.75e-06) and nitric oxide (p = 5.75e-06) were the small molecule drugs closely associated with IS. There were 5 DEGs enriched in collagenase, including *JUN*, *TNF*, *PTGS2*, *IL1B* and *IL8*. *TNF* and *IL1B* were both enriched in the most significant function and pathway.

Discussion

IS occurs in approximately 6 million persons per year worldwide and treatment options for IS are highly limited[27]. Tissue plasminogen activator

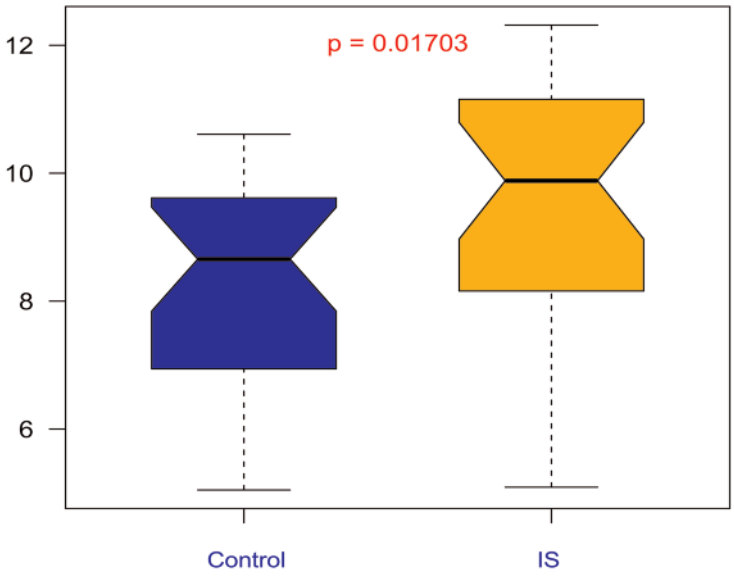

Figure 2. Comparison chart of the different expressed genes between the groups. Blue and orange respectively represent blood samples of normal control group and ischemic stroke group.

Table II. Function enrichment results of differentially expressed genes.

Term	Count	FDR
GO:0006915~apoptosis	8	0.049044
GO:0009991~response to extracellular stimulus	6	0.035333
GO:0008285~negative regulation of cell proliferation	7	0.027839
GO:0060548~negative regulation of cell death	7	0.027405
GO:0043069~negative regulation of programmed cell death	7	0.026976
GO:0006916~anti-apoptosis	6	0.025732
GO:0043066~negative regulation of apoptosis	7	0.024912
GO:0016265~death	9	0.016383
GO:0008284~positive regulation of cell proliferation	8	0.004235
GO:0010941~regulation of cell death	10	0.003777
GO:0043067~regulation of programmed cell death	10	0.003663
GO:0042981~regulation of apoptosis	10	0.003374
GO:0042127~regulation of cell proliferation	10	0.002824
GO:0007610~behavior	9	0.000634

Table III. Pathways enrichment results of differentially expressed genes.

ID	KEGG pathway	*p*-value
hsa04621	NOD-like receptor signaling pathway	1.32E-05
hsa04060	Cytokine-cytokine receptor interaction	3.20E-04
hsa04620	Toll-like receptor signaling pathway	0.001882
hsa04010	MAPK signaling pathway	0.027661

(tPA) is the first and only available effective therapy for acute IS[28]. However, tPA must be administered within 3 hours of symptom onset and there is also an associated risk of hemorrhagic transformation after tPA therapy[29,30]. So we began to actively looking for other biological treatments of IS which safely and effectively. With the ultimate goal of uncovering novel bio-targets for IS, DEGs were screened out by comparing gene expression profiles in whole blood from IS patients sampled at least 6 months after their first and only stroke episode, relative to controls. Through GO and KEGG analysis, we found that *TNF*, *IL1B* and *TNFAIP3* were both participate in apoptosis and NOD-like receptor signaling pathway. Based on the DEGs we screened, molecule drugs were identified and collagenase was the most markedly molecule drugs closely associated with IS.

TNF is a tumor necrosis factor family gene and tumor necrosis factor-α (TNF-α) is the main member that has effects in inflammation, sepsis, lipid and protein metabolism, haematopoiesis, angiogenesis and host resistance to parasites and malignancy[31,32]. TNF-± is secreted by M1 macrophages in the brain in response to the pathological processes of IS[33,34]. TNF-α mRNA is rapidly produced in response to IS within 1 h, reaches a peak at 6-12 h post ischemia, and subsides 1-2 days later. TNF-α has been demonstrated to activate the expres-

Table IV. Screened results of significant related drugs

Drug	ID	Parameters	Gene
collagenase	DB_ID:PA449107	O = 5; raw p = 4.67e-09; adj p = 4.67e-08	JUN, TNF, PTGS2, IL1B, IL8
anakinra	DB_ID:PA10799	O = 3; raw p = 1.05e-06; adj p = 5.75e-06	TNF, IL1B, IL8
nitric oxide	DB_ID:PA450635	O = 4; raw p = 1.15e-06; adj p = 5.75e-06	TNF, PTGS2, IL1B, SOD2
pentoxifylline	DB_ID:PA450864	O = 2; raw p = 4.73e-05; adj p = 0.0002	TNF, IL8
interferon alpha-2b	DB_ID:PA165958353	O = 2; raw p = 8.77e-05; adj p = 0.0002	TNF, SOCS3
erythromycin	DB_ID:PA449493	O = 2; raw p = 7.29e-05; adj p = 0.0002	IL1B, IL8
sulfasalazine	DB_ID:PA451547	O = 2; raw p = 4.73e-05; adj p = 0.0002	TNF, IL8
sulindac	DB_ID:PA451565	O = 2; raw p = 8.77e-05; adj p = 0.0002	PTGS2, ATF3
rofecoxib	DB_ID:PA451268	O = 2; raw p = 5.94e-05; adj p = 0.0002	NR4A2, PTGS2

sion of pro-adhesive molecules on the endothelium, which results in leukocyte accumulation, adherence, and migration from capillaries into the brain. Furthermore, TNF-α also induces the expression of other cytokines such as interleukin-6 (IL-6) and interleukin-1 (IL-1) and they play a key role in pro-inflammatory together[35]. In the acute phase of ischemia, TNF-α and IL-1β (also called *IL1B*) as inflammation factors, play a role of acceleration of inflammatory lesions, and induce cell necrosis or apoptosis[36]. Moreover, as the ischemic cascade progresses, cell death leads to a new phase of the inflammatory response[37]. In our study, after 6 months of the ischemic damage, the expression level of *TNF* and *IL1B* were still higher than the controls. This showed that damage had continued to exist and not fully recovered at the molecular and cellular level, although histologic damage might be not shown on the imaging and manifestations in patients. So it suggest that TNF-α and IL-1β are still risk factors of IS, and can be used as biological targets for treatment through agents that suppress TNF-α and IL-1β production.

We found that *TNF*, *IL1B* and *TNFAIP3* were enriched in NOD-like receptor signaling pathway. The innate immune system comprises several classes of pattern recognition receptors, including Toll-like receptors (TLRs), NOD-like receptors (NLRs), and RIG-1-like receptors (RLRs). TLRs recognize microbes on the cell surface and in endosomes, whereas NLRs and RLRs detect microbial components in the cytosol[38]. Two NLRs, NOD1 and NOD2, sense the cytosolic presence of the peptidoglycan fragments meso-DAP and muramyl dipeptide, respectively, and drive the activation of mitogen-activated protein kinase (MAPK) and the transcription factor NF-κB[39]. A different set of NLRs induces caspase-1 activation through the assembly of large protein complexes named inflammasomes[40]. TNF-α promotes inflammation by stimulation of capillary endothelial cell pro-inflammatory responses and thereby provides leukocyte adhesion and infiltration into the ischemic brain. On the other hand, NF-κB induce the activation of IL-1 and TNF and activated caspase 1resulting in post-translational processing of IL-1β and IL-18[41]. TNF and IL-1β play a role of acceleration of inflammatory lesions of IS may be through the NOD-like receptor signaling pathway.

In addition to DEGs, several drug molecules were retrieved, which may be related with stroke. Previous study has indicated that collagenase could be an effective stroke therapy drug, while combination with thrombolytic drug such as t-PA. In our study, *TNF* and *IL1B* were enriched in collagenase, so it revealed that collagenase may play a role in IS through *TNF* and *IL1B*. Moreover, anakinra and nitric oxide were also screened as the small molecule drugs closely associated with IS which might beneficial in guiding future treatments.

Conclusions

We reveal that TNF and IL-1β play a role of acceleration of inflammatory lesions of IS may be through the NOD-like receptor signaling pathway. So the DEGs such as *TNF*, *IL1B* and *TNFAIP3* identified in present study provide good bio-targets, which may advance our understandings about mechanisms of IS. Then the relevant drug molecule such as collagenase is beneficial in guiding future drug discovery. Moreover, anakinra and nitric oxide also screened out may beneficial in guiding future treatments.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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