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Screening of differentially expressed genes related to ischemic stroke and functional analysis with DNA microarray

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Abstract. – BACKGROUND: Prognostic blood biomarkers in the setting of acute ischemic stroke have become increasingly relevant for risk stratification, monitoring disease and response to therapies, developing targets for neuroprotective treatment and as surrogate end points for treatment trials.

AIM: We aim to find the feature genes which can accurately detect acute ischemic stroke and perform function analysis of these crucial genes in peripheral blood mononuclear cells.

MATERIALS AND METHODS: The gene expression profile GSE22255 was downloaded from Gene Expression Omnibus (GEO) database which includes 20 ischemic stroke patients and 20 controls. The differentially expressed genes between patients and controls samples were identified with packages in R language. The selected differentially expressed genes were further analyzed using bioinformatics methods. Software STRING (Search Tool for the Retrieval of Interacting Genes) was used to establish co-expression network. GOTM (General Ocean Turbulence Model) software was used to obtain differentially expressed gene enriched modules. The functions of genes in modules were analyzed by using software GeneCodis.

RESULTS: A total of 37 genes were identified as differentially expressed genes by comparing peripheral blood mononuclear cells gene expression of ischemic stroke patients and 20 controls. A co-expression network was constructed within 30 differentially expressed genes, among which gene interleukin-8 (IL-8) and tumor necrosis factor (TNF) showed the highest node degree. Genes in the module containing IL-8 and TNF were significantly enriched in 6 biological functions, and the most significant function was respond to stimulation.

CONCLUSIONS: Our results highlight that genes IL-8 and TNF have close relationship with acute ischemic stroke, and the expression patterns of these genes may be valid targets for new medications able to modify the ischemic stroke process.

Key Words:

Acute ischemic stroke, Differentially expressed gene, Co-expression network, Module analysis, Enrichment analysis.

Introduction

Stroke is the third leading cause of death in the USA and the primary cause disability. The vast majority (87%) of strokes are ischemic[1]. Survival results in persistent neurological impairments and physical disabilities with a high socio-economic cost. There is a need for a credible evidence base of prognostic information for outcomes that are meaningful to patients and physicians to optimize care and allocation of healthcare resources. Prognostic blood biomarkers in the setting of acute ischemic stroke have become increasingly relevant for risk stratification, monitoring disease and response to therapies, developing targets for neuroprotective treatment and as surrogate end points for treatment trials. In this context, rapidly measurable biomarkers predicting mortality and functional outcome in stroke would be clinically helpful[2].

In the past decade, gene expression profiles based on mRNAs microarrays have been widely used to detect disease biomarkers. These profiles, measuring thousands of gene expression levels simultaneously, served as the basis for feature selection and classification methods and have been shown to provide better prognosis than prior models [3]. High-throughput technologies are being used extensively to identify physical and functional relationships between proteins and genes on a large scale.

Several mRNAs expression profiles have been reported regarding ischemic stroke. The reported

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profiling studies in humans are performed either during the acute phase or in the first months after the stroke event [4-8] and, thus, these studies address the stroke severity and/or recovery mechanisms more than the risk of the stroke event. There are no published studies investigating gene expression changes in humans that specifically increase the risk for a stroke event. In this study we collected mRNA expression microarray dataset of ischemic stroke from Gene Expression Omnibus (GEO) database and identified differentially expressed mRNAs in ischemic stroke patients compared with controls. After network analysis and functional enrichment analysis, we finally demonstrated that IL-8 and TNF might play an important role in stroke etiology.

Materials and Methods

Affymetrix genechip data

We extracted the microarray profile from the study of Krug T et al[9], which were deposited in Gene Expression Omnibus (GEO) database under the accession number GSE22255. Gene expression profiling was conducted and reported in accordance with the minimum information about a microarray experiment (MIAME) criteria. Total of 40 chips were available, including 20 chips from peripheral blood mononuclear cells (PBMC) of ischemic stroke patients and 20 chips from controls.

More stringent inclusion and exclusion criteria were applied to individuals participating in the genomic expression profiling study; ischemic stroke 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. Participants with severe anemia or active allergies were also excluded.

Data preprocessing

The probe-level data in CEL files were converted into expression measures and background correction and quartile data normalization were performed by the robust multiarray average (RMA) algorithm with defaulted parameters in R affy package[10,11].

Differentially expressed microarray analysis

R limma package[12] was used to identify the differentially expressed genes among ischemic stroke patients and controls. The *p*-value was corrected for multiple hypotheses testing using Benjamini-Hochberg method[13]. The genes with a logFC| > 1 and a *p*-value < 0.01 were considered differentially expressed.

Co-expression network construction

The STRING (Search Tool for the Retrieval of Interacting Genes) database [14] provides both experimental and predicted interaction information. All associations are provided with a probabilistic confidence score. We used the STRING database to annotate functional interactions between target genes and other genes by calculated their node degree and got a network[15]. We got another network by removing the highest node degree and comparing the difference of the above two networks in order to find the most important hub [16].

Modules enrichment analysis

To functionally classify these genes in the interaction network, we performed modules enrichment analysis by mapping these genes to GOTM (Gene Ontology Tree Machine) database[17]. p <0.05 was chosen as cut-off criterion.

Significantly enriched categories were identified by comparing the distribution of genes in each Gene Ontology (GO) category between the interesting gene sets and the reference gene set which includes all genes on the Agilent 44K human whole genome oligo microarray. GOTM calculates the p value for each category and subcategories by a separate hypergeometric test.

Function enrichment analysis for the differentially expressed genes

Enrichment analysis techniques are very useful to extract biological knowledge from a set of genes or proteins. We used GeneCodis software to integrate differentially expressed genes to find groups of genes with similar biological meaning. GeneCodis [18], a web-based application, is a tool for singular and modular enrichment analysis that integrates information of diverse nature (e.g. functional, regulatory or structural) by looking for frequent patterns in the space of annotations and computing their statistical relevance. It can provide analysis of different annotations, including the three GO categories (biological process, cellular component, and molecular function), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, InterPro Motifs, and Swiss-Prot keywords. This integrative capacity sheds light on different aspects of the same information and provides a more accurate interpretation of the data.

Results

Differentially expressed genes identification

We obtained publicly available microarray dataset GSE22255 from GEO database. Using analysis of variance with multiple-testing correction on the normalized expression data (Figure 1), 37 genes were found to be differentially expressed in ischemic stroke cases compared with controls, with a threshold of $|\log FC| > 1$ and a p value < 0.05. A total of 3 genes were down-regulated and 34 genes were up-regulated.

Interaction network construction of differentially expressed genes

We mapped the differentially expressed genes to STRING database and screened significant interactions (Figure 2). By integrating these relationships above, we constructed an interaction network, which contained 123 pairs of co-expression genes formed by 30 differentially expressed genes (Figure 3A). We calculated the average degree of those 30 genes. The average degree is the average number of edges connecting all the nodes in the network. Higher values for average degree indicates a better connected network and is likely more robust. Interleukin (IL)-8 and tumor necrosis factor (TNF) had the highest average degree. After excluding the nodes with highest average degree (IL-8 and TNF), we got the second interaction network with 28 differentially expressed genes and 88 co-expressed gene pairs (Figure 3B). The average degree of each node in the second network (Figure 4) was decreased with comparison of the network including IL-18 and TNF. These results suggest that IL-8 and TNF are hub nods in the network and play critical roles in the ischemic stroke process.

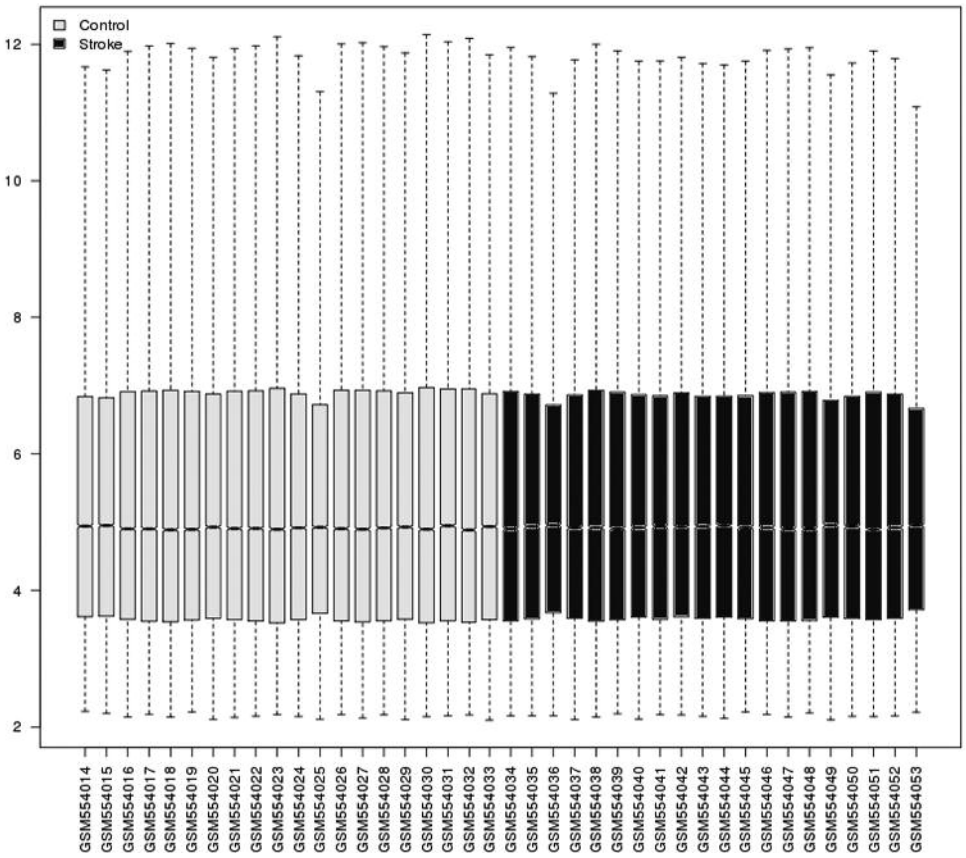

Figure 1. The box plots were generated using the normalized data of 20 ischemic stroke patients and 20 controls. Control and ischemic stroke samples are indicated with gray and black boxes, respectively. X-axis represents the data number of microarray dataset and Y-axis represents the normalized expression value. Black line in each box represents the median of each sample. All the black lines are almost in the same position, which shows minimum variability in these datasets.

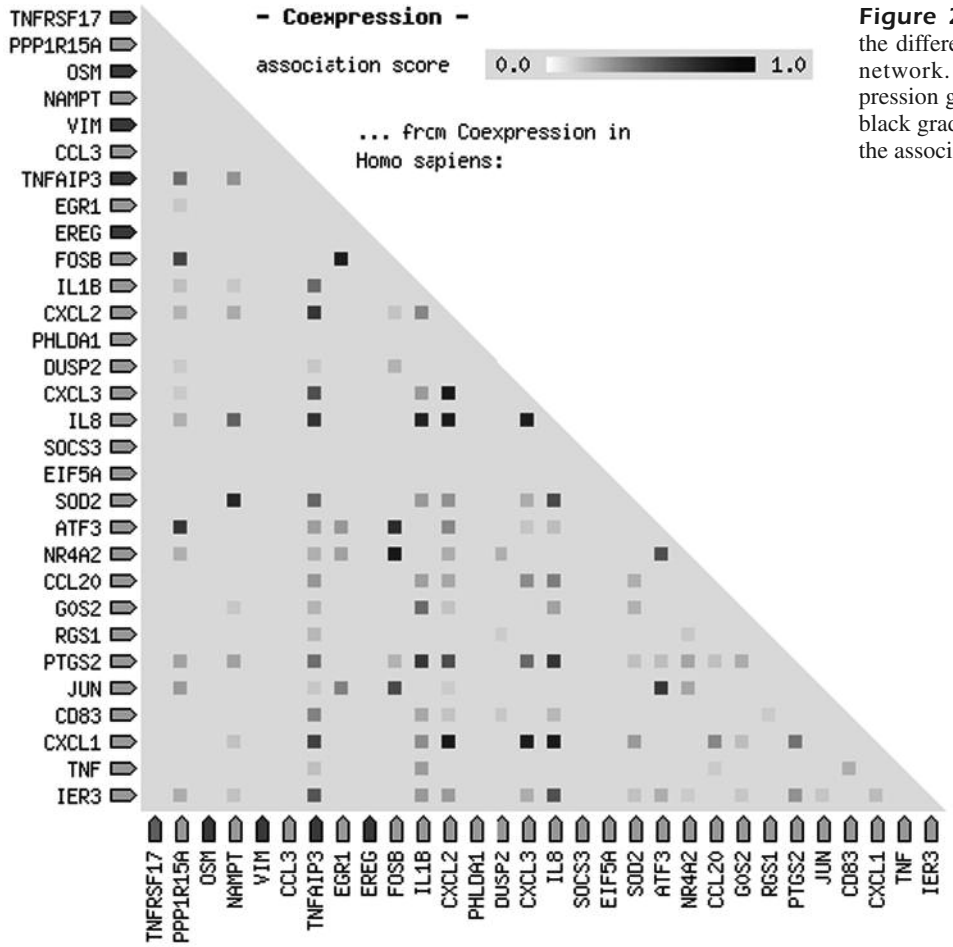

Figure 2. Co-expression levels of the differently expressed genes in the network. The boxes signify co-expression gene pair, with white-to-dark black gradient shading proportional to the association score.

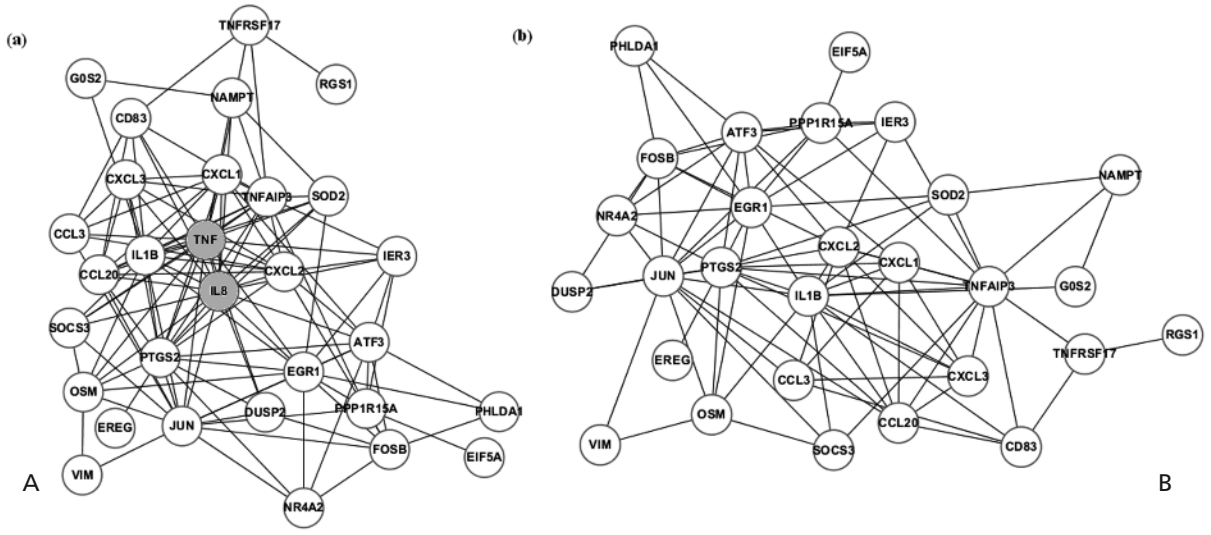

Figure 3. Co-expression network of differentially expressed genes. ***A,*** Co-expression network constructed by all the differently expressed genes. Grey color indicates the 2 genes with highest node degree (IL-8 and TNF); ***B,*** Co-expression network excluding IL-8 and TNF.

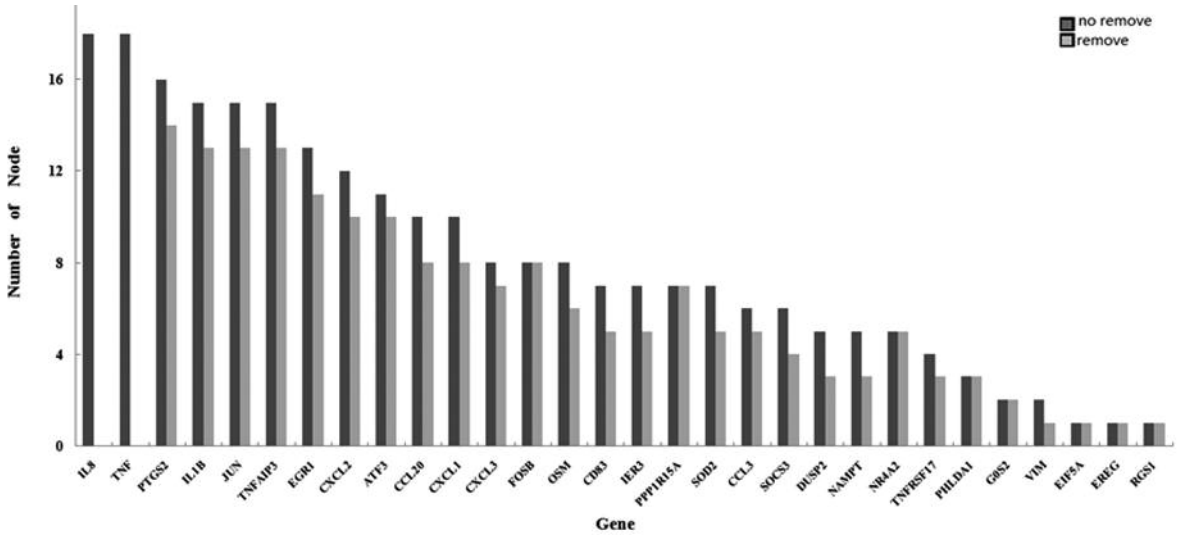

Figure 4. Node degree changes of network after excluded IL-8 and TNF. Deep grey and light grey bars represent the node degree of the network with or without IL-8 and TNF respectively.

Co-expression modules enrichment analysis

As detailed in the Materials and Methods section, we used the 37 differentially expressed genes for module enrichment detection. Modules that group together tend to contain genes with similar expression patterns. As shown in Figure 5, we got a module in which IL-8 and TNF were clustered.

Enrichment analysis for finding groups of genes with similar biological meaning in the interaction network

We performed functional enrichment analysis by mapping genes in the module we got on the last step to GeneCodis database. As shown in Table I, a total of 6 functional groups were enriched with the strict cutoff criterion ($p < 0.05$), all of which included IL-8 and TNF genes. The most significant function category was response to chemical stimulus which involved ten genes, including IL-8, TNF, chemokine (C-X-C motif) ligand 1 (CXCL1), early growth response 1 (EGR1), chemokine (C-C motif) ligand 20 (CCL20), prostaglandin-endoperoxide synthase 2 (PTGS2), CXCL3, JUN, CXCL2, and IL1B. These results suggest that those up-regulated genes may play a critical role in the pathogenic of ischemic stroke process by response to stimulus.

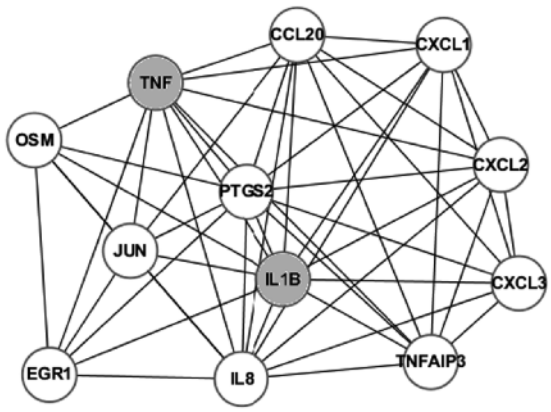

Figure 5. Result for modules enrichment analysis. IL-8 and TNF were signified with gray color.

Discussion

In this study, we compared the genetic profiles of PBMCs from 20 ischemic stroke cases and 20 controls downloaded from GEO database. A total of 37 genes displayed a significant differential expression in ischemic stroke patients compared to controls. By constructing interaction network, IL-8 and TNF were shown hub nodes of the network. This result suggests that IL-8 and TNF may play an important role in the process of ischemic stroke.

In the last decade, inflammatory mechanisms have been implicated both in the manifestation and evolution of brain ischemia[19]. Immune inflammatory processes may in time predispose to ischemic stroke through precipitation of atherosclerotic disease[20] and sustainment of atrial fibrillation[21]. Moreover, salvation of the ischemic penumbra and final size of the brain infarct, both of which strongly correlate to clinical outcome af-

Table I. Functional enrichment analysis results by mapping the module to GeneCodis database.

GO-ID	corr *p*-value	Description	Genes in test set
42221	4.85E-07	Response to chemical stimulus	CXCL1|EGR1|TNF|IL8|CCL20|PTGS2|CXCL3| JUN|CXCL2|IL1B
6950	2.09E-06	Response to stress	CXCL1|OSM|TNF|IL8|CCL20|PTGS2|CXCL3| JUN|CXCL2|IL1B
50896	5.07E-05	Response to stimulus	CXCL1|EGR1|OSM|TNF|IL8|CCL20|PTGS2|CXCL3| JUN|CXCL2|IL1B
50794	1.31E-02	Regulation of cellular process	CXCL1|EGR1|OSM|TNF|IL8|CCL20|PTGS2| JUN|IL1B|TNFAIP3
50789	1.84E-02	Regulation of biological process	CXCL1|EGR1|OSM|TNF|IL8|CCL20|PTGS2| JUN|IL1B|TNFAIP3
65007	2.68E-02	biological regulation	CXCL1|EGR1|OSM|TNF|IL8|CCL20|PTGS2| JUN|IL1B|TNFAIP3

ter an ischemic stroke, seem to be massively regulated by networks of post-stroke inflammatory responses[22,23]. Thus, pro-inflammatory and anti-inflammatory cytokines are important mediators of the pathophysiological events which precede an acute ischemic stroke[24] and have been further related to the clinical outcome of the disease.

Excitotoxicity and oxidative stress caused by the initial ischemic event activate microglia and astrocytes which react by secreting cytokines, chemokines and matrix metalloproteases. These inflammatory mediators lead to an up-regulation of cell adhesion molecules on endothelial cells, allowing blood-derived inflammatory cells, mainly neutrophils, to infiltrate the ischemic brain area. Neutrophils themselves also secrete cytokines which cause a further activation of glial cells. These processes all result in neuronal cell death and enhance the damage to the ischemic brain[25,26].

Activated brain endothelial cells at the site of inflammation, representing the front line of the blood-brain barrier, secrete cytokines such as TNF-α, IL-1b, IL-6, and IL-8, and express adhesion molecules such as intercellular adhesion molecule (ICAM-1), selectin E (ELAM-1), and P-selectin[27]. These stimuli may activate PBMC and result in the expression of cytokines and chemokines, as observed in this study. Our module analysis results also showed that the genes in the same module, including TNF, IL-8, CXCL1, EGR1, CCL20, PTGS2, CXCL3, JUN, CXCL2, and IL1B, participated in the response to stimulus. These results suggest that the peripheral cytokines and chemokines may be indicators of pathogenic processes of ischemic stroke[28].

Based on our network construction results, TNF and IL-8 seem to be more important than other genes. In experimental models of stroke, increased expression of the cytokines TNF-α and IL-8-like cytokines within the ischemic lesion are reported. Early increases in TNF-α are observed 1-3 h following ischemia onset[29], but it has a biphasic pattern of expression, with a second peak at 24-36 h[30]. TNF-α level may be more associated with cardioembolic strokes compared with other subtypes[31]. In a nested case-control study of 591 strokes, TNF-α also retains borderline significance after full adjustment for other inflammatory markers[32]. TNF-α238G/A polymorphisms increases the risk of ischemic stroke in Adult, Caucasian, and overall analysis[33].

IL-8 is a potent chemoattractant for polymorphonuclear leukocytes (PMNL) both *in vitro* and *in vivo*[34,35]. In a rat model, elevated concentrations of an IL-8-related neutrophil chemoattractant are detected in brain and serum after focal cerebral ischemia/reperfusion[36]. One clinical study shows that the high serum IL-8 levels are associated with prognosis[37]. In a rabbit model of cerebral reperfusion injury, systemic administration of monoclonal antibodies against IL-8 at the initiation of reperfusion can prevent polymorphonuclear infiltration and reduce the size of brain edema at 6 hours and infarction at 12 hours after reperfusion[38].

Conclusions

Increased expressions of IL-8 and TNF mRNA are observed systemically in ischemic stroke patients. Systemic up-regulation of cytokine expression may contribute to the pathogenesis of ischemic stroke through a potentiation of the secondary inflammatory process. Further studies are needed to find out whether IL-8 and TNF may represent valid targets for new medications able to modify the ischemic stroke process.

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Conflict of interest

The Authors declare that they have no conflict of interests.

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