BRIEF COMMUNICATION

Molecular signature of penumbra in acute ischemic stroke: a pilot transcriptomics study

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Abstract

We aimed to characterize peripheral blood gene expression profile of penumbra defined as MRI perfusion–diffusion mismatch (PD MM) in peripheral blood of patients with acute ischemic stroke. We studied 23 patients. Perfusion–diffusion mismatch volume was observed to be associated and significantly correlated with the expression of 34 genes including those related to inflammation, SUMOylation, and coagulation; while lipopolysaccharide inhibition was identified to be a candidate upstream regulator of these processes (z-score -2.38, $P = 0.04$). Penumbral volume is correlated with a specific gene expression profile in the peripheral blood characterized by overlap of inflammatory and neuroprotective pathways that are regulated by lipopolysaccharide inhibition.

Introduction

The recently completed DEFUSE-3 trial showed benefit of mechanical embolectomy in extended time windows for patients with ischemic penumbra.[1] Although collateral flow is believed to be a major factor in maintaining penumbral tissue, comprehensive understanding of neuroprotection during acute stroke remains controversial. Given the lack of clinical studies of penumbral biology, we thought to study the gene expression profiles of salvageable brain tissue in the peripheral blood of patients with acute ischemic stroke in order to identify candidate pathways that might be protective or toxic for preservation of penumbral tissue. We thought that peripheral blood may reflect molecular changes in the blood–brain interface that can be detected with highly sensitive next-generation sequencing (NGS). We hypothesize that anti-inflammatory and cytoprotective candidate pathways will be associated with penumbra that can be detected in peripheral blood of patients with acute stroke.

Methods

Patients

We enrolled patients with acute ischemic stroke between September 25, 2014 to July 21, 2015 screened by MRI ≤12 h from last seen normal (LSN) with the appropriate approval from the Ethics and Institutional Review and approved consent form for the study (NCT00009243). MRI was performed prior to thrombolysis or thrombectomy using 3 T clinical scanner.[2] Semiautomated lesion volume analysis using the planimetric method was used for diffusion weighted imaging (DWI) and mean transit time (MTT) volume measurements.[2] PD MM volume was

calculated as MTT minus DWI volume, and was treated as a continuous variable in statistical analysis.

RNA sequencing

RNA sequencing method is described in Data S1.

Statistical analysis

Statistical analysis methodology is described in Data S2.

Results

The study included 23 patients (Table 1). There were 23,444 annotated transcripts with detectable expression. One hundred and nineteen of these transcripts were deemed to have unbiased association with PD MM volume with 34 of these, each representing a unique gene, having expression significantly correlated with PD MM volume (Tables 2 and 3). Lipopolysaccharide (LPS) inhibition was identified as a candidate upstream regulator for five (CORO1A, HCAR2, HCAR3, IL1B, PF4) of these 34 genes (z-score -2.38, $P < 0.04$).

Discussion

The main finding of this pilot study is the identification of 34 genes having unbiased expression per differences in age, race, gender, DWI volume, and MTT volume that also have linearly associated expression with PD MM volume. This indicates that ischemic penumbra is associated with molecular changes that can be detected using NGS in the peripheral blood of patients with acute stroke. In

Table 1. Characteristics of patients (n = 23).

Variable	
Age, years (IQR)	73 (59.5–85.0)
Sex, male, n (%)	16 (69.6%)
African-American, n (%)	8 (34.8%)
Caucasian, n (%)	15 (65.2%)
Hypertension, n (%)	17 (73.9%)
Diabetes mellitus, n (%)	3 (13.0%)
Smoking history, n (%)	3 (13.0%)
Atrial fibrillation, n (%)	3 (13.0%)
LSN to MRI, min. (IQR)	136.0 (81.5–214.5)
NIHSS (IQR)	4 (1.5–14)
DWI volume, mL (IQR)	7.9 (4.2–22.1)
MTT volume, mL(IQR)	52.8 (6.4–288.5)
PD MM volume, mL(IQR)	41.4 (−0.7–203.7)

IQR, interquartile range; LSN, last seen normal; NIHSS, National Institutes of Health stroke scale; DWI, diffusion weighted imaging; MTT, mean transit time; PD MM, perfusion–diffusion mismatch.

Table 2. Genes negatively correlated with PD MM (downregulated).

Gene symbol	Pearson correlation (r)*	Correlation P-value*
NUP98	−0.71	<0.001
SEMA4D	−0.64	0.001
HCAR3	−0.61	0.002
IL1B	−0.59	0.003
SENP6	−0.57	0.005
PF4	−0.56	0.005
LINC-PINT	−0.54	0.007
HCAR2	−0.52	0.011
GP9	−0.51	0.012
DYNC1LI1	−0.51	0.013
FFAR2	−0.49	0.017
ZNF117	−0.50	0.017
PUM2	−0.49	0.018
RAB27B	−0.48	0.021
KCNJ2	−0.48	0.021
TCEA1	−0.48	0.021
GNB2	−0.47	0.024

Abbreviations: NUP98, Nucleoporin 98; SEMA4D, Semaphorin 4D; HCAR-2 and 3, Hydroxycarboxylic acid receptor-2 and -3; IL1B, Interleukin-1 Beta; SENP6, SUMO1/sentrin-specific peptidase-6; PF4, Platelet factor 4; LINC-PINT, Long Intergenic Non-Protein Coding RNA; GP9, Glycoprotein IX platelet; DYNC1LI1, Dynein cytoplasmic-1 light intermediate chain-1; FFAR2, Free Fatty Acid Receptor 2; ZNF117, Zinc Finger Protein-117; PUM2, Pumilio RNA Binding-2; RAB27B, RAB27B Oncogene Family; KCNJ2, Potassium Voltage-Gated Channel Subfamily J-2; TCEA1, Transcription Elongation Factor A1; GNB2G, Protein Subunit Beta-2.

*Results observed under no leave-one-out condition.

addition, this study showed that LPS inhibition was an upstream regulator of these changes in gene expression indicating biological plausibility of detected association.

The association of LPS inhibition with PD MM may suggest neuroprotective effects of this process on preservation of penumbra. LPS has been shown to exacerbate brain damage in middle cerebral artery occlusion model of stroke.[3] LPS is the ligand of toll like receptor-4 (TLR-4) and its interaction results in activation of pro-inflammatory cytokines such as TNF, IL-1B, and IL-12 through p38 and NFkB.[4] TLR-4 activation has been shown to exacerbate brain injury in ischemia/reperfusion experimental models.[5] Barr et al. found TLR signaling to be a mediator of innate immune response in peripheral blood of patients with acute stroke.[6]

Upstream regulator analysis also showed that target genes of LPS inhibition included CORO1A, HCAR2, HCAR3, IL1B, and PF4. All these genes are involved in immune response and inflammation, except HCAR3 that represents the niacin receptor, while PF4 is a chemokine released from activated platelets promoting blood coagulation. Inhibition of these genes in the blood in association with penumbra might be another indicator of

Table 3. Genes positively correlated with PD MM (upregulated).

Symbol	Pearson correlation (*r*)*	Correlation *P*-value*
STK26	0.58	0.003
MGA	0.58	0.004
UBA2	0.57	0.004
PICALM	0.57	0.004
CDK11B	0.57	0.004
UXT	0.56	0.005
S100A6	0.55	0.006
TES	0.55	0.007
GABARAPL2	0.54	0.008
HDAC1	0.53	0.008
CORO1A	0.53	0.009
SLC38A2	0.53	0.009
POLG	0.52	0.011
CNN2	0.52	0.011
EBP	0.52	0.011
GLTP	0.49	0.018
RPS2	0.47	0.022

Abbreviations: STK26, Serine/threonine kinase 26; MGA, MAX dimerization protein; UBA2, Ubiquitin-like modifier activating enzyme 2; PICALM, Phosphatidylinositol binding clathrin assembly protein; CDK11B, Cyclin dependent kinase 11B; UXT, Ubiquitously expressed prefoldin-like chaperone; S100A6, S100 Calcium binding protein A6; TES, Testin LIM domain protein; GABARAPL2, GABA type A receptor associated protein-like 2; HDAC1, Histone deacetylase 1; CORO1A, Coronin 1A; SLC38A2, Solute carrier family-38 member-2; POLG, DNA polymerase gamma catalytic subunit; CNN2, Calponin 2; EBP, Emopamil binding protein (sterol isomerase); GLTP, Glycolipid Transfer Protein; RPS2, Ribosomal protein S2.
*Results observed under no leave-one-out condition.

biological plausibility as inflammation promotes cytotoxicity in acute cerebral ischemia.[3,5] Although all these pro-inflammatory and prothrombotic genes were inhibited except CORO1A, upregulation of this gene can be associated with both autoimmunity and immune deficiency.[7]

In addition to the LPS target genes, other differentially expressed immune response molecules associated with PD MM included inhibited SEMA4D and FFAR2, as well as upregulated CNN2. SEMA4D is a B-cell product which can compromise blood–brain barrier (BBB) integrity and the ability of neural cells to regenerate.[8] FFAR2 mediates the activation of the inflammatory and immune responses in the intestine, regulating the rapid production of chemokines and cytokines. CNN2 upregulation inhibits monocyte/macrophage proliferation, and platelet adhesion suggesting anti-inflammatory and antithrombotic effects of this molecule.[9] Inhibition of SEMA4D and upregulation of CNN2 in association with PD MM may represent another neuroprotective mechanism preserving penumbra.

We found correlation of PD MM with genes representing different cyto- and neuroprotective pathways

including SUMOylation (inhibition of SENP6 and upregulation UBA2), anti-apoptosis (upregulation of MGA, UTX, and RPS2, downregulation of DYNCILI-1), inhibition of tumor suppression (downregulation of NUP98, LINC-PINT, RAB27B, and TCEA1), epithelial–mesenchymal transition (upregulation of S100A6), and reactive oxygen species through the voltage-gated potassium channels (KCNJ2). In a recent postmortem study of patients with ischemic stroke, we demonstrated upregulation of SUMO in neurons of the ischemic penumbra.[10] On the other hand, in the current study, we found upregulation of proapoptotic STK26 and tumor suppressor TESS genes in association with penumbra. One possible explanation of these findings is a tug of war between cytoprotection and cytotoxicity processes during acute cerebral ischemia.

We detected an upregulation of central nervous system genes in association with penumbra including EBP, POLG, SLC38A2, GABARALP2, DK11B, and PICALM. EBP is known to bind to neuroprotective agents.[11] POLG is involved in mitochondrial DNA repair in the neurons, while mutation of this gene causes neurodegeneration.[12] SLC38A2 is a fundamental part of BBB and plays an integral role in glutamine transport in neurons.[13] GABARALP2 mediates inhibitory neurotransmission.[14] PICALM plays a critical role in amyloid beta clearance from brain to circulation through endothelial cells in AD.[15] All these genes play role in preservation and function of neurovascular unit, and their upregulation might be a protective mechanism during acute cerebral ischemia. The most likely explanation for detection of these genes in circulating blood is their leakage through disrupted BBB. On the other hand, we found upregulation of DK11B which is associated with neurodegeneration in AD.[16] Although none of enrolled patients had documented diagnosis of Alzheimer's disease, we cannot completely rule out the presence of this pathology in our studied cohort.

This study has some limitations. Small sample size is one limitation of this pilot study. There is a risk of type one error and there is no replication analysis. However, this is a pilot discovery/candidate pathway analysis and, therefore, it is methodologically acceptable. Another limitation is that RNA expression was studied from whole blood and cellular sources of these molecules are unknown. Future studies are in progress to study gene expression by monocytes rather than in whole blood.

Although gene expression in blood does not necessarily reflect the gene expression in brain, detected inhibition of immune response most likely is systemic from leukocytes, lymphocytes, and monocytes. However, given the presence of cytoprotective and central neural system genes in the blood, it is likely that these genes are from glial and neuronal cells reflecting the ongoing process. As no brain

tissue is available in acute ischemic stroke, peripheral blood was used in this study to identify candidate biological processes and pathways associated with penumbra.

Since gene expression was studied in acute phase of stroke only at a single time point, the results obtained reflect temporal relationship only at this moment. Changes in penumbra with time might be associated with different gene expression profile and, therefore, repeated penumbral and gene expression measurements will be of interest. These studies are in progress. In this study, we used semiautomated lesion volume analysis using the planimetric method. It is possible that this method may overestimate perfusion deficit and might include benign oligemia. In future studies, we are planning to use automated software, which was validated in thrombectomy clinical trials (1).

This pilot study is the first step and future studies are planned with larger sample size, single-cell analysis (monocytes), in addition to use of automated software for MRI diffusion/perfusion volume analysis, and repeated measurements of penumbral volume and gene expression. Sample size calculation shows that to achieve 80% power at alpha = 0.05 when PD MM is a continuous variable similar to the current study, $n = 30$ patients will be required for future validation study. However, if PD MM represents dichotomized variable (volume ≥50 mL vs. < 50 mL), $n = 55$ patients per group (total $n = 110$) will be required to achieve 80% power at alpha = 0.05.

In conclusion, penumbral volume is correlated with a specific gene expression profile that can be detected in the peripheral blood and characterized by overlap of inflammatory and neuroprotective pathways that were regulated by LPS inhibition.

Acknowledgments

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Represents RNA sequencing method and is quoted on page 4.
Data S2. Represents statistical analysis method and is quoted on page 4.