Genomic biomarkers and cellular pathways of ischemic stroke by RNA gene expression profiling

T.L. Barr, PhD, RN
Y. Conley, PhD
J. Ding, PhD
A. Dillman, BS
S. Warach, MD, PhD
A. Singleton, PhD
M. Matarin, PhD

Address correspondence and reprint requests to Dr. Taura L. Barr, National Institute of Nursing Research, Tissue Injury Unit Building, 10 Hatfield Clinical Research Center, Room 2-1339, Bethesda, MD 20812
barrt@mail.nih.gov

ABSTRACT

Objective: The objective of this study was to provide insight into the molecular mechanisms of acute ischemic cerebrovascular syndrome (AICS) through gene expression profiling and pathway analysis.

Methods: Peripheral whole blood samples were collected from 39 MRI-diagnosed patients with AICS and 25 nonstroke control subjects ≥18 years of age. Total RNA was extracted from whole blood stabilized in Paxgene RNA tubes, amplified, and hybridized to Illumina HumanRef-8v2 bead chips. Gene expression was compared in a univariate manner between stroke patients and control subjects using t test in GeneSpring. The significant genes were tested in a logistic regression model controlling for age, hypertension, and dyslipidemia. Inflation of type 1 error was corrected by Bonferroni and Ingenuity Systems Pathway analysis was performed. Validation was performed by QRT-PCR using Taqman gene expression assays.

Results: A 9-gene profile was identified in the whole blood of ischemic stroke patients using gene expression profiling. Five of these 9 genes were identified in a previously published expression profiling study of stroke and are therefore likely biomarkers of stroke. Pathway analysis revealed toll-like receptor signaling as a highly significant canonical pathway present in the peripheral whole blood of patients with AICS.

Conclusions: Our study highlights the relevance of the innate immune system through toll-like receptor signaling as a mediator of response to ischemic stroke and supports the claim that gene expression profiling can be used to identify biomarkers of ischemic stroke. Further studies are needed to validate and refine these biomarkers for their diagnostic potential. *Neurology*® 2010;75:1009–1014

GLOSSARY

AICS = acute ischemic cerebrovascular syndrome; **BBB** = blood–brain barrier; **IPA** = Ingenuity Systems Pathway analysis; **PBMC** = peripheral blood mononuclear cell; **rtPA** = recombinant tissue plasminogen activator; **TLR** = toll-like receptor.

Stroke is the third leading cause of death in the United States[1] and one of the most common causes of disability in industrialized countries.[2] Despite significant advances in neuroimaging and clinical management resulting in increased survival,[3] morbidity remains high secondary to complications and a lack of alternative treatments.

The development of microarray systems for gene expression profiling permits screening of large numbers of genes for involvement in biologic processes. Gene expression profiles can be used to reflect and predict pathologic processes. Thus, gene expression profiling has been utilized for the characterization of several neurologic and immune disorders[4-6] and has facilitated the refinement of tumor subtypes,[7] distinction between good-prognosis and poor-prognosis tumors,[8] and the prediction of response to treatment.[9] The method has the potential to lead to a better understanding of the molecular mechanisms underlying disease, identify risk for secondary complications, and aid in the development of novel therapeutics.

Supplemental data at
www.neurology.org

In the context of ischemic stroke, there are 2 reports of peripheral blood mononuclear cells (PBMCs)[10,11] and 1 of peripheral whole blood[12] examining gene expression in peripheral blood. These studies provide a novel and sophisticated approach to identify biomarkers of stroke. However, the study designs are not complementary; nor are the findings. Thus, the purpose of our study was to determine the gene expression profile of peripheral whole blood following acute ischemic stroke in a larger cohort of subjects, adjusting for common stroke risk factors to provide insight into the molecular mechanisms involved in ischemic stroke and attempt to replicate previous findings.

METHODS A retrospective case-control study utilizing prospectively collected data from 2 different study sources was undertaken. Recruitment of stroke patients occurred from June 2007 through September 2008 when the following inclusion criteria were met: age ≥18 years; MRI diagnosed definite acute ischemic cerebrovascular syndrome (AICS[13]); and blood draw within 24 hours from symptom onset. Patients with probable/possible AICS and hemorrhage were excluded from this study. Time of onset was determined as the time the patient was last known to be free of the acute stroke symptoms. Recombinant tissue plasminogen activator (rtPA) was given to patients with disabling symptoms within 3 hours from onset. Premorbid deficits were determined by the modified Rankin Scale score for status prior to stroke and severity of injury was determined by the NIH Stroke Scale score at the time of blood draw after stroke. Control subjects were recruited as a consecutive convenience sample under a separate NIA/NIH protocol if they were neurologically normal per neurologist assessment at the time of enrollment. Peripheral whole blood was collected into Paxgene™ blood RNA tubes (PreAnalytiX, Qiagen) after consent. Demographic data were collected from the patient or significant other by trained neurologists.

Standard protocol approvals, registrations, and consents. This study received approval for human subject research from the institutional review boards of the National Institute of Neurological Disorders and Stroke and National Institute on Aging at NIH and Suburban Hospital, Bethesda, MD. Written informed consent was obtained from all subjects or their authorized representatives prior to performing any study procedures.

RNA extraction and amplification. Paxgene RNA tubes were inverted 8–10 times and placed in a −80°C freezer until RNA extraction. Tubes were thawed on a rotating bed at room temperature for 24 hours prior to RNA isolation. RNA was extracted per Paxgene Blood RNA Extraction Kit (PreAnalytiX, Qiagen). Globin reduction was not conducted on any sample in this study since it has been shown to have little impact on probe detection when using the Illumina platform.[14]

Biotinylated, amplified RNA was generated from the Illumina TotalPrep RNA amplification kit (Applied Biosystems). RNA quantity was determined by the Nanodrop and RNA quality was determined by $A260/A280$ ratio and the presence of 2 distinct ribosomal bands on gel electrophoresis.

Array hybridization. Samples were randomly hybridized to Illumina HumanRef-8 v2 expression bead chips, capable of analyzing >22,000 genes and alternative splice variants. Bead arrays were scanned by the Illumina BeadStation 500× and raw intensity values were saved in Illumina's Bead Studio program manager. Sample labeling, hybridization, and scanning were conducted using standard Illumina protocols.

Statistical analysis. Baseline demographic statistics were conducted in SPSS (version 15, SPSS, Inc., Chicago, IL). Comparisons were made using χ^2 analysis for gender, race, comorbidities (hypertension, diabetes, and hyperlipidemia), and medication history. Student t test was used to analyze the significance of age among the groups. The level of significance was established at 0.05 for 2-sided hypothesis testing.

Probe level analysis. Probe expression was filtered in GeneSpring GX v10 (Agilent Technologies) resulting in a 24,424 final probe set. Robust multiarray analysis normalization collated the probe data in the following order: 1) background correction—perfect match probe information; 2) quantile normalization—probe level normalization; and 3) summarization— expression measure summary in log base 2 scale with median to fit a linear model. Unsupervised clustering was performed to determine phylogenetic distances to detect outliers.

Gene expression level analysis. Gene expression analysis was conducted in Illumina BeadStudio Gene Expression (GX) Module (version 1, Illumina®, San Diego, CA) and verified in GeneSpring GX v10 (Agilent Technologies). Genes with at least a 2-fold difference in expression were compared in a univariate manner between stroke patients and control subjects through the use of Illumina's custom model (modified t test) in BeadStudio and t test comparisons in GeneSpring. The influence of multiple testing was evaluated using the Bonferroni family-wise error.

Pathway analysis. Ingenuity Systems Pathway analysis (IPA) (Ingenuity® Systems, www.ingenuity.com) was used to assess genes with a 1.5-fold difference or greater expression. Gene identifiers and signal intensities were compared to the Canonical Pathways in the Ingenuity Systems knowledge base; significant pathways ($p < 0.05$) were identified and a set of networks (35 genes/gene products) was generated. A score, derived from a p value, was generated for each network according to the fit of the set of significant genes. Scores of 2 or higher have at least a 99% confidence of not being generated by chance alone. Biologic functions were calculated and assigned to each network. The significance of the association was measured by 1) the number of molecules in a pathway that met the 1.5-fold cutoff divided by the total number of molecules of that pathway; and 2) a right-tailed Fisher exact test to calculate a p value.

Logistic regression for identification of off-target effects. Given the significant difference of age by group, a post hoc logistic regression was performed. The normalized intensities for each gene were entered separately with age and then hypertension and dyslipidemia as the covariates of interest. A Bonferroni-corrected p of ≤0.005 (0.05/9) was significant.

PCR validation. cDNA was generated per Invitrogen SuperScript III first strand synthesis kit. QRT-PCR reactions were performed using Taqman® gene expression probes (Applied Biosystems) for *ARG1, CCR7, LY96, and MMP9* by the 7900HT QRT-PCR system. β-Actin normalized the relative expression of chosen genes. Fold change differences were calculated by the δ C_T method.[15,16] Validation was confirmed if t test revealed signif-

Table 1 Univariate associations between stroke patients and control subjects

	Total sample, n (%)	Stroke, n = 39 (61.9%), n (%)	Control, n = 24 (38.1%), n (%)	Statistic/df	p Value
Gender (% female)	36 (57.1)	22 (56.4)	14 (58.3)	c^2 0.02/1	0.883
Age, y, mean ± SD	68.1 ± 14.02	73.1 ± 14	59.9 ± 9.73	t −4.4/61	0.000[a]
Hypertension	32 (50.8)	25 (64.1)	7 (29.2)	χ^2 6.6/1	0.010[a]
Diabetes	13 (20.6)	11 (28.2)	2 (8.3)	χ^2 3.3/1	0.068
Dyslipidemia	18 (28.6)	18 (46.2)	0	χ^2 14.9/1	0.000[a]
Atrial fibrillation	6 (9.5)	6 (15.4)	0	χ^2 3.9/1	0.048[a]
Myocardial infarction	6 (9.5)	6 (15.4)	0	χ^2 3.9/1	0.048[a]
Previous ischemic stroke	8 (12.7)	6 (15.4)	2 (8.3)	χ^2 0.7/1	0.414
Previous or current smoker	30 (47.6)	15 (38.5)	15 (62.5)	χ^2 7.8/2	0.020[a]
Hypertension medication	37 (47.6)	29 (74.4)	8 (33.3)	χ^2 10.3/1	0.001[a]
Diabetes medication	8 (12.7)	7 (17.9)	1 (4.2)	χ^2 2.66/1	0.103
Cholesterol medication	22 (34.9)	17 (43.6)	5 (20.8)	χ^2 3.4/1	0.066
Anticoagulant or antiplatelet	21 (33.3)	20 (51.3)	1 (4.2)	χ^2 14.8/1	0.000[a]
Family history of stroke	19 (30.2)	15 (38.5)	4 (16.7)	χ^2 3.6/1	0.169

[a] Significant.

icance ($p \geq 0.05$) and QRT-PCR results correlated with microarray signal intensity (Pearson $r \geq 0.5$ and $p \geq 0.05$).

Sample size estimation. Sample size estimation was conducted using PASS Power Analysis and Sample Size system and JMP. Twenty-two patients and 22 control subjects achieves 90.68% power for each gene to detect a difference in expression with at least a 1.5-fold change and a SD of 1.5 with a false discovery rate of 0.05 using a 2-sided 1-sample t test.

RESULTS Clinical characteristics. Ninety-two subjects (67 stroke patients and 25 control subjects) were recruited. Of the 67 stroke patients enrolled, 39 were definite AICS.[13] Based on the Trial of Org 10172 in Acute Stroke Treatment, 43.6% (n = 17) of the cases were cardioembolic stroke, 28.2% (n = 11) were undetermined, 12.8% (n = 5) were large artery embolus/thrombosis, and the remaining 15.4% (n = 6) were small vessel or other cause.

The mean time from symptom onset to blood draw was 10:06 hours ± 6:31. Nine (23.1%) patients received rtPA; only one patient had blood drawn before rtPA administration. Median prestroke modified Rankin Scale score was zero and the severity of stroke was mild with a median baseline NIH Stroke Scale score of 3 (range 0–23). There was no difference by race or gender between the groups. However, stroke patients were significantly older ($t = -4.03$; $p = 0.000$) and, as expected, vascular risk factors were more common in the stroke group (table 1).

Array quality control. RNA $A260/A280$ was 1.9–2.2 and yield was >1–2 μg from 2.5 mL of whole blood. Negative control, background, and noise signals were low (<200) across all arrays and housekeeping and
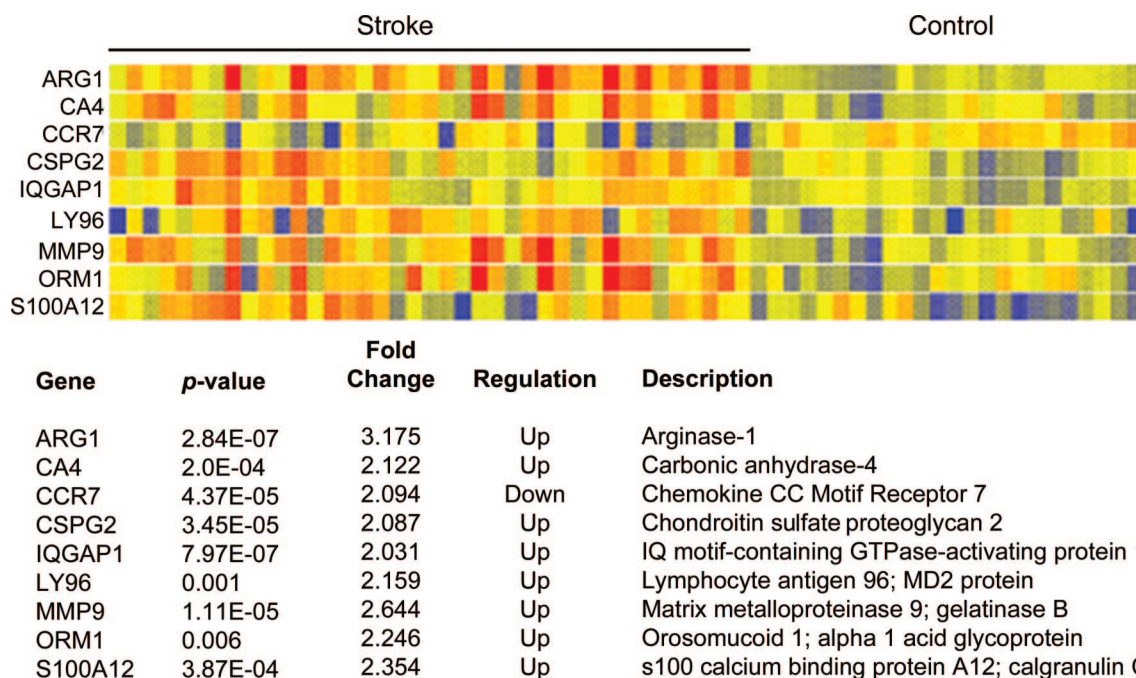
biotin signals were consistently high (>20,000). The average signal for internal controls was similar across the arrays. Control plots were consistent with high-quality data.

Nine-gene profile for stroke. BeadStudio identified 19 genes with a 2-fold difference in expression (table e-1 on the *Neurology*® Web site at www.neurology.org). GeneSpring identified 16 genes with a 2-fold difference in expression (table e-1). After comparison, there were 9 genes significantly different between stroke patients and control subjects with at least a 2-fold difference in expression and corrected $p < 0.05$ (figure 1). Five of these 9 genes were significant in the first whole blood gene expression profiling study of stroke (*ARG1, CA4, LY96, MMP9, S100A12*).[12]

Logistic regression for identification of off-target effects. *s100A12* was the only gene that fell out of the model after correction ($p = 0.014$). *ARG1* ($p = 0.002$), *CA4* ($p = 0.002$), *CCR7* ($p = 0.005$), *CSPG2* ($p = 0.003$), *IQGAP1* ($p = 0.003$), and *MMP9* ($p = 0.002$) remained significantly associated with stroke after controlling for age, history of hypertension, and dyslipidemia.

Pathway analysis. A total of 355 genes were eligible for pathway analysis ($\geq$1.5-fold difference and corrected $p < 0.05$). The 5 most significant pathways in the peripheral whole blood of stroke patients were CD28 signaling ($p = 4.03E00$), nuclear factor of activated T cells in regulation of the immune response ($p = 4.03E00$), dendritic cell maturation ($p = 3.4E00$), toll-like receptor (TLR) signaling

Gene	p-value	Fold Change	Regulation	Description
ARG1	2.84E-07	3.175	Up	Arginase-1
CA4	2.0E-04	2.122	Up	Carbonic anhydrase-4
CCR7	4.37E-05	2.094	Down	Chemokine CC Motif Receptor 7
CSPG2	3.45E-05	2.087	Up	Chondroitin sulfate proteoglycan 2
IQGAP1	7.97E-07	2.031	Up	IQ motif-containing GTPase-activating protein 1
LY96	0.001	2.159	Up	Lymphocyte antigen 96; MD2 protein
MMP9	1.11E-05	2.644	Up	Matrix metalloproteinase 9; gelatinase B
ORM1	0.006	2.246	Up	Orosomucoid 1; alpha 1 acid glycoprotein
S100A12	3.87E-04	2.354	Up	s100 calcium binding protein A12; calgranulin C

After comparison between both statistical packages, there were 9 genes differentially expressed with at least a 2-fold difference in expression and Bonferroni-corrected $p < 0.05$ between stroke patients and control subjects (*ARG1, CA4, CCR7, CSPG2, IQGAP1, LY96, MMP9, ORM1, S100A12*).

($p = 3.33E00$), and calcium-induced T-lymphocyte apoptosis ($p = 2.92E00$) (figure e-1). There were more genes expressed in the TLR signaling pathway than in any other pathway, implying it is the most significant for this dataset (figure e-2).

Taqman gene expression assay validation. QRT-PCR validated significant mRNA changes of 4 genes in all samples. Secondary to the availability of RNA, the entire gene profile could not be validated. *ARG1, LY96,* and *MMP9* were chosen because they were significant in previous work and *CCR7* was the only downregulated gene in our study (figure e-3).

DISCUSSION Peripheral whole blood gene expression profiling identified 9 genes with ≥2-fold difference in expression following ischemic stroke: Arginase 1 (*ARG1*), carbonic anhydrase 4 (*CA4*), chondroitin sulfate proteoglycan 2 (*CSPG2*), IG motif-containing GT-Pase activation protein 1 (*IQGAP1*), lymphocyte antigen 96 (*LY96*), matrix metalloproteinase 9 (*MMP9*), orosomucoid 1 (*ORM1*), and s100 calcium binding protein A12 (*s100A12*), and one downregulated gene, chemokine receptor 7 (*CCR7*).

A limitation of our study was a younger control group; however, post hoc analyses controlling for age and stroke risk factors supported the primary analysis findings. The fact that 5 of the 9 genes identified in this study were also found to be differently expressed in a previous study[12] using a different microarray platform, independent cohorts of patients and controls, and other methodologic aspects provides validation for both studies and confirms the strength of this method to unveil molecular mechanisms involved in ischemic stroke.

The majority of genes induced 2–24 hours following stroke occurs in neutrophils and monocytes.[17] Therefore, peripheral whole blood gene expression may be the most useful for identifying biomarkers of ischemic stroke in humans. The inconsistency between the first 3 peripheral blood gene expression profiling studies of stroke is most attributable to the different sources of RNA under study. Some have also interpreted this to treatment of patients with rtPA[11] and variable time between symptom onset and blood draw. In this study, there was one gene coincident with the first PBMC study (*CSPG2*)[11] and 5 genes overlapping with the previous whole blood study (*ARG1, CA4, LY96, MMP9, S100A12*).[12] Nine patients (23%) received rtPA, a similar proportion in the PBMC study, and the mean time from symptom onset to blood draw was 10:06 hours, more than the 3 hours previously suggested useful.[12] It is remarkable that besides these differences, 50% of the genes identified in the previous studies have been replicated in our study, and all of the genes shared between our study and the previous whole blood study were expressed at all time points (<3 hours, 5 hours, 24 hours). This indicates that the differences between the first expression studies were in fact due to differences in cell

populations under study and that coincident findings with the previous whole blood study are not reflecting changes secondary to administration of rtPA as previously considered, but rather changes associated with ischemic stroke. These results suggest that the relative expression of *ARG1*, *CA4*, *LY96*, *MMP9*, and *S100A12* taken together have strong evidence for diagnostic capability in acute ischemic stroke.

Interestingly, MMP9 and various isoforms of S100 at the protein level have been implicated as predictors of stroke outcome. One of our recent publications suggests baseline serum MMP9 may help predict the occurrence of blood–brain barrier (BBB) disruption[18] and high level of S100 serum protein is associated with worse clinical outcome,[19] implying MMP9 and S100 may also be useful as prognostic markers in ischemic stroke.

In addition to the common genes identified by previous studies, we have also identified novel biomarkers of ischemic stroke: *CCR7*, *IQGAP1*, and *ORM1*.

Chemokine receptor 7 (*CCR7*). *CCR7* is a G-coupled chemokine receptor and the data on *CCR7* function in humans are rather sparse. However, increased *CCR7*+ T cells have been reported in peripheral blood leukocytes of patients with mild to moderate ischemic stroke 1 week following stroke.[20] We found a downregulation of *CCR7* in the peripheral blood in the acute phase of ischemic stroke. Differences in the direction of this regulation could be explained as differences in tissue/cell-specific immune response following stroke and/or blood draw time. Dramatic decreases of *CCR7* expression in the acute phase of ischemic stroke could be followed by an increase in expression with stroke progression. Further studies of *CCR7* and stroke should investigate this issue.

IQ Motif-containing GTPase activating protein 1 (*IQGAP1*). *IQGAP1* is an evolutionarily conserved scaffold protein that plays a fundamental role in cell polarity. Rho-family GTPases are small signaling G proteins that require *IQGAP1* to regulate actin cytoskeleton to produce a gradient of signaling molecules.[21] Experimental evidence suggests the expression of RhoA increases in aortic and basilar arteries with age,[22] therefore RhoA, and indirectly *IQGAP1,* may play a role in altered vascular responses associated with aging.[22] Studies in vitro have suggested that leukocyte transmigration and changes in endothelial permeability are facilitated by RhoA.[23] The upregulation of *IQGAP1* expression in the context of ischemic stroke suggests an increase in cellular signaling and transcription in the acute phase of ischemic stroke leading to increased permeability of the BBB. *IQGAP1* may mediate the disruption of the BBB as a means by which signals from the brain enter the periphery to augment cellular recruitment.

Oromucosid 1 (ORM1). Finally, *ORM1*, also known as α-1 acid glycoprotein, is an acute phase protein that suppresses lymphocyte response to lipopolysaccharides (thereby preventing ongoing tissue damage by neutrophil proteases), decreases platelet aggregation (and further platelet recruitment), and enhances cytokine secretion.[24] It exhibits anti-inflammatory effects, playing a significant role in immunomodulation of the acute phase response.[25] The upregulation of *ORM1* in the context of ischemic stroke suggests an acute phase response in ischemic stroke similar to trauma, infection, and systemic tissue injury.

IPA analysis identified TLR signaling as the most significant pathway in the peripheral blood of ischemic stroke patients. Activation of innate immunity, through TLRs, is a primary component of proinflammatory cytokine generation following ischemic brain injury.[26] Products of protein degradation damaged DNA, fibrinogen, and heat shock proteins activate the TLR pathway through a mechanism known as damage associated molecular pattern recognition.[27,28]

Activation of TLRs has been implicated as a negative moderator of the innate immune response.[29] Downregulation of TLR4 results in diminished infarct volume and better outcome in mouse middle cerebral artery occlusion. Human studies have identified that increased activation of TLR4 following ischemic stroke corresponds to worse clinical outcome.[30,31]

The predominance of the innate and inflammatory immune pathways in our study reinforces their importance in ischemic stroke. A better understanding of how these pathways react and respond to ischemic stroke may lead to the emergence of new avenues for therapeutic intervention. The TLR pathway is a promising and worthy target to be considered and studied with more detail.

The results of this study provide insight into the molecular mechanisms involved in ischemic stroke. A 9-gene profile for acute ischemic stroke has been identified and replicates previous work,[12] therefore supporting the use of gene expression profiling of peripheral whole blood to identify biomarkers of ischemic stroke. Future studies including a stroke mimic cohort and a TIA group will help determine the differential diagnostic capability and clinical utility of these results.

AUTHOR CONTRIBUTIONS
Statistical analysis was conducted by Dr. J. Ding.

DISCLOSURE

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