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Blood genomic profiling in extracranial- and intracranial atherosclerosis in ischemic stroke patients

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ABSTRACT

Objective: Extracranial- and intracranial atherosclerosis (ECAS and ICAS) have been suggested to have different pathogeneses. Blood genomic profiling may identify their unique molecular signatures.
Methods: Whole gene microarray of peripheral blood was performed in 24 patients with acute ischemic stroke (ECAS, n = 12; ICAS, n = 12) and 12 healthy controls. Differential gene expression and gene set enrichment analysis (GSEA) were conducted. Plasma resistin levels were compared across independent samples of stroke patients with ECAS (n = 39), ICAS (n = 20), and small vessel disease (SVD, n = 57).
Results: Microarray revealed that 144 and 24 transcripts were altered in ECAS and ICAS, respectively, compared to controls. All the transcripts that were differentially expressed in ICAS were also differentially expressed in ECAS. A total of 120 transcripts were differentially expressed only in ECAS. Gene sets related to immune response and protein metabolism were altered in both ECAS and ICAS, but the magnitude of gene alteration was higher in ECAS than in ICAS. Several genes of interest including RETN, IRF5, CD163, and CHST13 were more highly expressed in ECAS than in ICAS. Circulating resistin levels were elevated in independent samples of ECAS, but not in those of ICAS, compared to those of SVDs.
Conclusions: ECAS showed prominent genomic alteration related to immune response compared to ICAS. Although there was no ECAS-specific gene to be identified on microarray, the level of resistin expression was high on peripheral blood in ECAS, suggesting that resistin is associated with the pathogenesis of ECAS.

Introduction

Atherosclerotic stroke is a major etiologic subtype of ischemic stroke, estimated to account for up to 40% of cases of ischemic stroke [1]. White blood cell and inflammatory mediators in the peripheral blood play a major role in the development of atherosclerosis and subsequent stroke. The vascular damage caused by oxidized low-density cholesterol triggers a cascade of immune responses of white blood cells, which release various inflammatory mediators, leading to the formation of atheroma [2]. Moreover, peripheral white blood cells play a major role in the pathophysiology of stroke, including infiltration of leukocytes into the ischemic brain and secretion of circulating inflammatory mediators [3,4]. There are two subtypes of atherosclerotic stroke, based on the anatomical location where atherosclerosis develops. Extracranial atherosclerosis (ECAS) is stenosis or occlusion of arteries below the cervical portion (e.g., carotid bifurcation or orifice of vertebral artery), and intracranial atherosclerosis (ICAS) is stenosis or occlusion of major cerebral arteries above the cervical portion (e.g., major cerebral arteries or distal portion of carotid and vertebrobasilar arteries). Although both conditions had been believed to share a common pathogenesis of the atherogenic process, emerging evidence has revealed distinct pathogeneses. ICAS is common in Asians and Africans, whereas ECAS is common in Caucasians, suggesting that genetic or environmental background confers a differential risk [5,6]. Moreover, several observations have suggested that development of atherosclerosis at extracranial or intracranial arteries has different metabolic and biological mechanisms [7,8]. Extracranial arteries are more vulnerable to smoking and hyperlipidemia because their higher fluid resistance time facilitates atherogenic particle transport into vascular walls [7]. Intracranial arteries, on the other hand, have higher antioxidant capacity, but become more prone to oxidative stress as age increases [8]. Recent case-control studies have reported that inflammatory markers, such as C-reactive protein (CRP), are associated with ECAS [9,10], and metabolic syndrome is closely associated with ICAS [11,12]. These findings support the hypothesis of different pathogenic mechanisms between the two

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conditions. However, molecular pattern on peripheral blood is still obscure in these diseases.

Oligonucleotide microarray provides valuable information for understanding the molecular mechanisms of various diseases, including cerebrovascular diseases. Several microarray studies have revealed a specific molecular pattern of stroke, and microarrays can be applied as a molecular biomarker for stroke diagnosis or for identification of stroke etiology [13–15]. In the present study, we investigated the genomic profiling of peripheral blood in stroke patients with ECAS and ICAS via microarray to examine whether different molecular alterations underlie these two conditions.

Materials and Methods

Ethics Statement

All study participants gave written informed consent, and the Institutional Review Board (IRB) at CHA Bundang Medical Center approved the study protocols (IRB no. 2009-047).

Demographics of Study Participants

RNA samples were analyzed from 24 patients with acute atherosclerotic stroke at the internal carotid (ICA) or middle cerebral artery (MCA) territory (symptom onset <24 h) and 12 healthy control subjects. Cerebral infarction was confirmed by diffusion-weighted brain magnetic resonance imaging (MRI, MAGNETOM, Siemens, Germany). Determination of ECAS and ICAS was based on brain MR angiography (MRA, MAGNETOM, Siemens, Heidelberg, Germany). ECAS was defined as occlusion or stenosis of more than 50% of the extracranial portion of the ICA using the methods of the North American Symptomatic Carotid Endarterectomy Trial (NASCET) [16]. ICAS was defined as occlusion or stenosis of more than 50% of the MCA without ECAS on MRA [17]. Among the 24 patients with acute ischemic stroke, 12 patients had ECAS and 12 patients had ICAS. Patients were excluded if they met any of the following criteria: 1) age <45 years old; 2) symptom onset >24 h; 3) prior symptomatic lesion on brain MRI; 4) suspected cardioembolism, small vessel disease, or undetermined stroke subtypes as determined by Trial of Org10172 Acute Stroke Treatment (TOAST) classification [1]; 5) no brain MRA study; and 6) non-Koreans. We excluded patients with concomitant ECAS and ICAS because of the following reasons: 1) The number of cases with concomitant ECAS and ICAS is small compared to that with either ECAS or ICAS alone (only 6.4% of total large-artery atherosclerotic stroke in Koreans) [18]; and 2) the focus of the present study is to examine the difference in gene expression pattern between ECAS and ICAS.

The control group comprised 12 non-stroke subjects who had visited the outpatient clinic for a routine health examination. Data from 8 stroke patients and 12 controls were previously described elsewhere [19]. All stroke patients were screened using a neurological examination, history taking of vascular risk factors, a routine blood test, brain imaging, and cardiac examinations [electrocardiography (ECG), transthoracic and trans-esophageal echocardiography, and/or 24-hour ambulatory ECG monitoring] during hospitalization. Assessment of vascular risk factors included: hypertension (baseline systolic blood pressure ≥ 140 mmHg, diastolic blood pressure ≥ 90 mmHg, or history of anti-hypertensive medication), diabetes mellitus (fasting blood glucose ≥ 7.0 mmol/L, postprandial blood glucose ≥ 11.1 mmol/L, or history of hypoglycemic medication) and hyperlipidemia (fasting blood total cholesterol ≥ 6.2 mmol/L or history of anti-lipidemic medication).

Plasma resistin level was measured in independent samples from 116 patients with acute atherosclerotic or lacunar stroke (symptom onset <24 h). According to the TOAST classification [1], 59 patients were classified as large artery atherosclerotic stroke, and 57 patients were classified as small vessel disease (SVD). Patients with large artery atherosclerotic stroke were further divided into ECAS (n = 39) and ICAS (n = 20) based on MRA finding. For these cohorts, ECAS was defined as occlusion or ≥50% stenosis of all extracranial arteries relevant to infarction (proximal portion of ICA or vertebral arteries), and ICAS was defined as occlusion or ≥50% stenosis of all major intracranial arteries relevant to stroke (anterior, middle, posterior cerebral artery, distal portion of ICA, and vertebrobasilar arteries) on MRA as previously described [17]. Methods for demographic and risk factor assessment in these cohorts were identical to those in the microarray study.

mRNA Microarray Test

Blood Sampling and RNA Preparation

Whole blood (10 ml) was collected by antecubital venipuncture within 24 h (median sampling time: 8.0 h, interquartile range [IQR]: 7.3 to 12.5 h) after symptom onset in stroke patients. Total RNA was extracted as quickly as possible (within 20 min of sampling) using Trizol (Invitrogen Life Technologies, Carlsbad, USA) and purified using RNeasy columns (Qiagen, Valencia, USA). After processing with DNase digestion and clean-up procedures, RNA samples were quantified, aliquoted, and stored at -80 °C until use. For quality control, RNA purity and integrity were evaluated by denaturing gel electrophoresis. The optical density (OD) 260 nm/280 nm ratio was measured, and samples were analyzed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). The OD 260/280 ratio was above 1.7, and negative control, background and noise signals were low (<200) across all arrays, while housekeeping (>15,000) and biotin (>30,000) signals were high.

Labeling and Purification

RNA was amplified and purified using the Ambion Illumina RNA amplification kit (Ambion, Austin, USA) to yield biotinylated cRNA. Briefly, 550 ng of total RNA was reverse-transcribed to cDNA using a T7 oligo (dT) primer. Second-strand cDNA was synthesized, *in vitro* transcribed, and labeled with biotin-NTP. After purification, the cRNA was quantified using the ND-1000 Spectrophotometer (NanoDrop, Wilmington, USA).

Hybridization and Data Export

Samples were hybridized to Illumina HumanHT-12 v.3 expression bead chips (Ambion), which are capable of analyzing >27,000 protein-coding transcripts and alternative splice variants. Labeled cRNA samples (750 ng) were hybridized to each mouse-8 expression bead array for 16-18 h at 58 °C, according to manufacturer's instructions (Illumina, Inc., San Diego, USA). Detection of the array signal was carried out using Amersham fluorolink streptavidin-Cy3 (GE Healthcare, Little Chalfont, UK) according to the bead-array manual. Arrays were scanned with an Illumina bead-array reader confocal scanner. Array data-export processing and analysis were performed using Illumina GenomeStudio v2009.2 (Gene Expression Module v1.5.4).

Statistical Analysis of Microarray Data

The quality of hybridization and overall chip performance was monitored by visual inspection of both internal quality control checks and the raw scanned data. Raw data were extracted using the manufacturer's software (Illumina GenomeStudio v2009.2: Gene Expression Module v1.5.4). The selected gene signal value was log-transformed and normalized by the quartile method. Statistical analysis was conducted using independent t-test to examine the differentially expressed genes in ECAS- and ICAS groups compared to controls. Statistical significance of the expression data was determined using fold-differences (FD). Corrected p values (bh-p) were determined after correction of Benjamini-Hochberg's method for multiple comparisons of microarray data. Go-ontology analysis for a significant probe list was performed via the online PANTHER database (http://www.pantherdb.org/panther/ontologies.jsp), using text files containing the Gene ID list and the accession numbers of the Illumina probe IDs. Gene set enrichment analysis (GSEA) of a total of 99 and 169 genes with FD ≥ 2 and uncorrected $p < 0.05$ from microarray data was performed in ECAS and