*Neuroscience* 131 (2005) 359–374

# CAROTID ATHEROSCLEROTIC PLAQUES FROM SYMPTOMATIC STROKE PATIENTS SHARE THE MOLECULAR FINGERPRINTS TO DEVELOP IN A NEOPLASTIC FASHION: A MICROARRAY ANALYSIS STUDY

**R. VEMUGANTI[a]* AND R. J. DEMPSEY[a]**

[a]*Department of Neurological Surgery, University of Wisconsin-Madison, K4/8 (Mail Stop Code CSC-8660), 600 Highland Avenue, Madison, WI 53792, USA*

**Abstract—Identification of genetic mechanisms that promote the onset of stroke and transient cerebral ischemic attack symptoms in carotid atherosclerotic patients would further our understanding of the pathophysiology of this disease and could lead to new pharmacological and molecular therapies. Using Affymetrix Human Genome 230 GeneChip set, the present study evaluated the gene expression differences in geometrically similar carotid artery plaque samples extricated from six symptomatic stroke patients and four asymptomatic patients. There was no significant difference in the degree of stenosis between the two groups. Of the 44,860 transcripts analyzed, 289 (approximately 0.6% of the total transcripts) were differentially expressed between the plaques from the symptomatic and asymptomatic groups (236 were expressed more abundantly and 53 were expressed less abundantly in the symptomatic group). Of the 236 transcripts expressed more abundantly in the symptomatic plaques, 71% (167 transcripts) indicate an active cell proliferation and neoplastic process. These include oncogenes, growth factors, tumor promoters, tumor markers, angiogenesis promoters, transcription factors, RNA splicing factors, RNA processing proteins, signal transduction mediators and those that control the metabolism. Real-time polymerase chain reaction confirmed the increased expression of 63 transcripts in the symptomatic plaques. The other groups of transcripts expressed more abundantly in the symptomatic plaques are those that control ionic homeostasis, those that participate in the progression of degenerative neurological diseases (Alzheimer's disease, amyotrophic lateral sclerosis and Huntington's disease) and epilepsy. This indicates that symptomatic plaques are molecularly and biochemically more active than the asymptomatic plaques, or active plaque growth precipitates stroke symptoms.** 

**Key words: atherosclerosis, gene expression, neoplasia, neurodegenerative disease, plaque, stroke.**

In humans, carotid artery atherosclerosis is far more common than carotid embolic disease (Rothwell et al., 2000). Proliferation of smooth muscle cells, formation of connective tissue, cholesterol deposition, calcification and extravasation of inflammatory cells were thought to promote the atherosclerotic plaque formation (Ross, 1999). At the systemic level, smoking, dietary lipid intake, diabetes, hypertension and infection might promote the plaque maturation and rupture (Burns, 2003; Linton and Fazio, 2003; Droste et al., 2003; Emsley and Tyrrell, 2002; Feigin et al., 2002; Malloy and Kane, 2001). Recent gene expression profiling studies using atherosclerotic plaques from humans and experimental animals indicated increased inflammation and second messenger signaling in the plaque tissue compared with the normal blood vessel tissue (Faber et al., 2001; Wuttge et al., 2001; Hiltunen et al., 2002; Randi et al., 2003). The Asymptomatic Carotid Atherosclerosis Study (ACAS) indicated that these factors may influence the growth of a plaque but the size of a plaque alone does not predict the development of stroke symptoms (Baker et al., 2000). The ACAS study also established that the long-term risk of ipsilateral stroke in neurologically asymptomatic patients with a >60% carotid stenosis can be reduced by carotid endarterectomy (Executive Committee of Asymptomatic Carotid Atherosclerosis Study Investigators, 1995).

The molecular mechanisms that influence a carotid artery plaque to become symptomatic are not known. Microarray analysis enables the identification of molecular events underlying the disease progression in different clinical states. Recent studies used microarrays to identify the gene expression changes associated with human CNS disorders such as schizophrenia (Middleton et al., 2002), Alzheimer's disease (Blalock et al., 2004), bipolar affective disorder (Tkachev et al., 2003), alcoholism (Mayfield et al., 2002) and brain tumors (Mischel et al., 2004). Our laboratory successfully used GeneChip microarrays to decipher the neuronal mechanisms in animal models of focal ischemia (Rao et al., 2002), traumatic brain injury (Rao et al., 2003), spinal cord injury (Song et al., 2001), hepatic encephalopathy (Song et al., 2002) and cerebral ischemic tolerance (Dhodda et al., 2004).

To examine the functional consequences of plaque dynamics at the transcriptional level, the present study analyzed the gene expression patterns of surgically removed carotid artery plaques from symptomatic stroke patients and asymptomatic patients using the Affymetrix 230 Human GeneChip set representing 44,860 transcripts (Affymetrix Co., Santa Clara, CA, USA).

*Corresponding author. Tel: +1-608-263-4055; fax: +1-608-263-1728.
E-mail address: vemugant@neurosurg.wisc.edu (R. Vemuganti).

*Abbreviations:* ACAS, Asymptomatic Carotid Atherosclerosis Study; D, decrease; DAVID, Database for Annotation, Visualization and Integrated Discovery; EASE, Expression Analysis Systematic Explorer; EST, expressed sequence tag; I, increase; MD, marginal decrease; MI, marginal increase; UPS, ubiquitin–proteasome system.

0306-4522/05$30.00+0.00 
doi:10.1016/j.neuroscience.2004.08.058

**Table 1.** Patient characteristics

| Patient | Sex | Age | BMI | Stroke[a] | Heart attack | Stenosis (MRI) | Hypertension | Diabetes |
|---|---|---|---|---|---|---|---|---|
| 1 | ♂ | 85 | 28.9 | Yes | No | 70% | No | No |
| 2 | ♂ | 56 | 24.4 | Yes | No | 70% | Yes, treated | No |
| 3 | ♂ | 45 | 29.4 | Yes | No | 100% | Yes, treated | No |
| 4 | ♀ | 51 | 20.8 | Yes | No | 75% | No | No |
| 5 | ♀ | 69 | 25 | Yes | No | 80% | Yes, treated | No |
| 6 | ♀ | 48 | 27 | Yes | No | 50% | Yes, treated | Yes, controlled |
| Symptomatic Group | 3 ♂ | | | Yes | No | | 4 Yes | 1 Yes |
| | 3 ♀ | 60±15 | 25.7±3.2 | 100% | 100% | 73%±16% | 2 No | 5 No |
| 7 | ♂ | 72 | 41 | No | No | 60% | Yes, treated | No |
| 8 | ♂ | 64 | 29.7 | No | No | 70% | Yes, treated | No |
| 9 | ♀ | 71 | 20.3 | No | No | 90% | No | No |
| 10 | ♀ | 52 | 25 | No | No | 70% | Yes, treated | Yes, controlled |
| Asymptomatic Group | 2 ♂ | | | No | No | | 3 Yes | 1 Yes |
| | 2 ♀ | 63±10 | 28.0±8.9 | 100% | 100% | 72%±13% | 1 No | 3 No |

[a] Stroke is the only characteristic that is different between the two groups. Neuropathological examination indicated no significant differences in the plaque characteristics (calcification, gross lipid, hemorrhage and ulceration) between the two groups.

## EXPERIMENTAL PROCEDURES

### Plaque samples

Patients scheduled for endarterectomy were enrolled to this plaque study after describing the study and obtaining the informed consent. The patients underwent preoperative magnetic resonance imaging that identified the plaque location and the degree of stenosis. Of the 10 plaques used in this study, six are from symptomatic stroke patients and four were from asymptomatic patients. There was no significant difference in the age, body mass index, degree of stenosis, previous heart attacks, diabetes and hypertension status between the two groups. Neuropathological examination indicated no significant differences between the symptomatic and asymptomatic groups in the plaque characteristics (calcification, gross lipid, hemorrhage and ulceration). The patient characteristics are given in Table 1.

### GeneChip analysis

The methods used for sample preparation, hybridization, data analysis, sensitivity and quantification have been described in our recent papers (Rao et al., 2002, 2003; Song et al., 2001, 2002; Dhodda et al., 2004) and are based on the Affymetrix GeneChip Expression Analysis Manual (Affymetrix, Santa Clara, CA, USA).

### GeneChip set

We used Affymetrix Human Genome U133 set consisting two GeneChip arrays (U133A and U133B) containing 44,860 probe sets representing more than 39,000 transcripts derived from approximately 33,000 well-substantiated human genes and approximately 6000 expressed sequence tags (ESTs; unknown genes). The Human U133 GeneChip set design used the sequences selected from GenBank, dbEST, and RefSeq. The sequence clusters were created from the UniGene database (Build 133, April 20, 2001) and refined by analysis and comparison with other publicly available databases including the Washington University EST trace repository and the University of California, Santa Cruz, Golden Path human genome database (April 2001 release). The HG-U133A Array includes representation of the RefSeq database sequences and probe sets related to sequences previously represented on the Human Genome U95Av2 Array. The HG-U133B Array contains primarily probe sets representing EST clusters. Further details about this GeneChip set can be obtained from the Affymetrix website (http://www.affymetrix.com).

### Sample preparation

Total RNA was extracted from each plaque sample with the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The RNA was cleaned (RNeasy Mini Kit; Qiagen Inc., Valencia, CA, USA) and converted to double-stranded cDNA (Gibco BRL Superscript Choice System; Life Technologies, Rockville, MD, USA), and then to biotinylated cRNA (Bioarray High Yield RNA Transcription Labeling Kit; Enzo Diagnostics, Farmingdale, NY, USA) according to the manufacturer's protocols. Following fragmentation and quality confirmation with the Affymetrix Test3 Array, the biotinylated cRNA was hybridized to the Affymetrix Human Genome U133A and U133B chips. Following hybridization, each chip was washed, stained with streptavidin–phycoerythrin and scanned with a probe array scanner.

### Data analysis

GeneChip data were analyzed by Affymetrix Microarray Suite MAS 5.0 software which used one-sided Wilcoxon's signed rank test to generate a 'detection *P*-value' (set at $P<0.05$) to statistically decide whether a transcript is expressed on a chip. Based on the *P*-value, the software generated a present ($P<0.04$), marginal ($P<0.04$ to $P<0.05$) or absent ($P>0.05$) call for each transcript. On each chip, human β-actin, GAPDH, hexokinase, 5S rRNA, and B1/B2 repeats of γ-crystalline served as housekeepers, several rat, murine and yeast probe sets served as negative controls and externally spiked bacterial bioB, bioC, bioD and cre served as positive hybridization controls. While comparing two chips, MAS 5.0 software normalized and scaled the data for each chip and then generated a 'change *P*-value', a 'difference call' and a 'signal log ratio' using the Wilcoxon's signed rank test to derive statistically significant results from the raw probe cell intensities of expression arrays. During the comparison analysis, each probe set on the experimental array was compared with its counterpart on the control array to calculate the change *P*-value which was used to generate the difference call of increase (I; $P<0.04$), marginal increase (MI; $P<0.04$ to $P<0.06$), decrease (D; $P>0.996$), marginal decrease (MD; $P>0.994$ to $P>0.996$) and no change (NC; $P>0.06$ to $P<0.0.996$). Fold changes were computed using the formulae: $Fold\ Change = 2^{signal\ log\ ratio}$ (if signal log ratio is $>0$; upregulated transcripts) and $Fold\ Change = (-1)\times 2^{-(signal\ log\ ratio)}$ (if signal log ratio is $<0$; decreased transcripts). In this study, we used 10 plaque samples (six from