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115

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Original Paper

Biomarker Discovery in Serum from Patients with Carotid Atherosclerosis

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Key Words

Asymptomatic patients · Atherosclerosis · Biomarker · Difference gel electrophoresis · Proteomics · Surface-enhanced laser desorption/ionization · Symptomatic patients

Abstract

Background: Blood-based biomarkers of atherosclerosis have been used to identify patients at high risk for developing stroke. We hypothesized that patients with carotid artery disease would have a distinctive proteomic signature in blood as compared to a healthy control population without carotid artery disease. In order to discover protein biomarkers associated with increased atherosclerotic risk, we used two different strategies to identify biomarkers from patients with clinically defined atherosclerosis who were undergoing endarterectomy for atherosclerotic carotid artery disease. These patients were compared with healthy matched controls. Methods: Serum was obtained from patients undergoing endarterectomy (EA; n = 38) and compared to a group of age-matched healthy controls (n = 40). Serum was fractionated using anion exchange chromatography and three different surface-enhanced laser desorption/ionization (SELDI) chip surfaces and then evaluated with mass spectrometry (MS) and two-dimensional difference gel

T.J.D.G., G.T.H. and P.N. contributed equally to the completion of this study.

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Cerebrovasc	Dis Extra	2011;1:115-	-129
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DeGraba et al.: Serum Biomarkers in Carotid Atherosclerosis

116

electrophoresis (2D-DIGE). Results: A random forest (RF) analysis of the SELDI-MS protein peak data distinguished these two groups with 69.2% sensitivity and 73.2% specificity. Four unique SELDI peaks (4.2, 4.4, 16.7 and 28 kDa, all p< 0.01) showed the greatest influence in the RF model. The EA patients with a history of prior clinical atherosclerotic plaque rupture manifested as either stroke or transient ischemic attack (symptomatic; n = 16) were compared to patients with carotid atherosclerosis but no clinical evidence of plaque rupture (asymptomatic; n = 22). Analysis of the SELDI spectra did not separate these two patient subgroups. A subgroup analysis using 2D-DIGE images obtained from albumin-depleted serum comparing symptomatic (n = 10) to asymptomatic EA patients (n = 10) found 4 proteins that were differentially expressed (p < 0.01) in the symptomatic patients. These proteins were identified as α_1 -antitrypsin, haptoglobin and vitamin D binding protein that were downregulated and α_2 -glycoprotein precursor that was upregulated in the symptomatic EA group. Conclusions: SELDI-MS data analysis of fractionated serum suggests that a distinct protein signature exists in patients with carotid atherosclerosis compared to age-matched healthy controls. Identification of 4 proteins in a subset of patients with symptomatic and asymptomatic carotid atherosclerosis suggests that these and other protein biomarkers may assist in identifying high-risk patients with carotid atherosclerosis. Copyright © 2011 S. Karger AG, Basel

Introduction

Atherosclerosis is an inflammatory disease that affects medium and large arteries. Immune mechanisms underlie both the formation and activation of atherosclerotic plaques that cause ischemia from vessel stenosis or plaque rupture with thrombosis [1, 2]. The discovery of biomarkers that improve current methods of diagnosis and treatment of cerebral ischemia would be a valuable addition to patient care. Several categories of protein biomarkers that reflect brain tissue damage, inflammation, vascular injury, hemostasis or neuroendocrine responses have helped to delineate mechanisms and potential pathways for new therapies [3–5]. Similarly, microarray profiles of ribonucleic acid expression in blood can distinguish ischemic stroke from controls and other types of brain injury [6]. Single nucleotide polymorphisms of genes that regulate inflammatory pathways have been associated with an increased relative risk for ischemic stroke [7-10]. However, due to the heterogeneity of causes of ischemic stroke and the lack of sufficient sensitivity and specificity, none of these markers are currently used routinely to assess risk, and diagnose, treat, or provide prognosis of cerebral ischemic events [3-5, 11].

Some of the biomarkers assessing risk associated with carotid artery disease are similar to those associated with cardiovascular risk, such as C-reactive protein or fibrinogen [12]. Blood levels of cholesterol and associated lipoproteins have a well-known relationship to the risk of coronary artery disease, yet this relation is only weakly associated in ischemic stroke suggesting possible differences in mechanisms or susceptibility of the different vascular beds to atherogenesis [13-15]. A recent systematic review of biomarkers associated with stroke risk and best therapy for carotid artery disease noted the heterogeneity of results from the studies of different biomarkers [12]. Clarifying the role of inflammatory markers that are predictive of carotid artery disease within a background of systemic atherosclerosis remains challenging.

In order to discover new protein biomarkers associated with increased atherosclerotic risk, we conducted a pilot study in patients with and without carotid artery disease. We hypothesized that patients with carotid artery disease would have a distinctive proteomic signature in blood as compared to a healthy control population. Our goal was to identify serological markers that would help distinguish patients with and without carotid atherosclero-



Cerebrovasc Dis Extra 2011;1:115–129	
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sis and those who were more likely to become symptomatic. We used two different strategies to identify biomarkers from patients with clinically defined atherosclerosis who were undergoing endarterectomy (EA) for carotid artery disease. We characterized the entire proteome in these patients by fractionating serum using anion exchange and then fractionating further using three different protein chip surfaces for screening with surface-enhanced laser desorption/ionization (SELDI)-time of flight (TOF) mass spectrometry (MS). A bioinformatic approach to establish multivariate classification models based on SELDI-MS data was used to distinguish the atherosclerosis group from the no atherosclerosis group. Samples from the symptomatic and asymptomatic EA group were analyzed using two-dimensional difference gel electrophoresis (2D-DIGE), matrix-assisted laser desorption/ionization (MALDI) tandem MS (MS/MS) and liquid chromatography MS/MS. Our results demonstrate both the utility and limitations of protein profiling using these approaches.

Materials and Methods

Sample Collection

The atherosclerosis group was composed of 38 patients who had blood samples obtained prior to EA with carotid plaque removal. The control group consisted of 40 age-matched healthy subjects who were enriched for atherosclerotic risk factors but had no clinical evidence of atherosclerosis at the time of sampling. The atherosclerosis group was composed of both symptomatic (n = 16) and asymptomatic (n = 22) patients who underwent EA. The symptomatic patients underwent EA surgery after a prior stroke or transient ischemic attack (TIA) and met the North American Symptomatic Carotid Endarterectomy Trial (NASCET) criteria for symptomatic atherosclerosis [16]. The asymptomatic atherosclerotic patients had no prior clinical sequelae from their vascular disease. Table 1 lists their baseline clinical characteristics. Serum from all patients who consented to participation was collected at the National Naval Medical Center under an institutional review board-approved protocol during outpatient visits prior to EA or in outpatient visits as appropriate. The serum was stored at -80° C before use. In symptomatic patients, samples were obtained within 1–4 weeks after TIA or stroke. The strokes were mild to moderate in severity.

Sample Fractionation

Prior to generating protein expression profiles by SELDI analysis, serum samples were processed by anion exchange chromatography using a 96-well serum fractionation kit containing anion exchange resin according to the manufacturer's recommendations (Ciphergen Biosystems, Fremont, Calif., USA) and automated for high-throughput liquid handling on a Biomek 2000 automated workstation (Beckman Coulter, Fullerton, Calif., USA). Six total fractions with buffers of 50 mM Tris-HCl, 0.1% N-octyl- β -D-glucoside (OGP; pH 9; F1); 50 mM HEPES, 0.1% OGP (pH 7; F2); 100 mM Na acetate (pH 5; F3); 100 mM Na acetate, 0.1% OGP (pH 4; F4); 50 mM Na citrate, 0.1% OGP (pH 3; F5), and 33.3% isopropanol, 16.7% acetonitrile and 0.1% trifluoroacetic acid (TFA; organic, F6) were collected (fig. 1). Each of the six 200-µl fractions was then stored at -80°C until application to the ProteinChip arrays.

SELDI ProteinChip Analysis

Mass spectra from serum protein expression profiles were obtained using immobilized metal affinity capture (IMAC30), strong anion exchange (Q10) and/or weak cation exchange (CM10) ProteinChip arrays (Ciphergen Biosystems) and run in duplicate according the manufacturer's recommended protocols. All sample preparations, including deposition of matrix, were performed on a Biomek 2000 automated work station using two 96-well Bioproces-



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Table 1. C	linical chara	acteristics	of all	patients
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	No clinical atherosclerosis (n = 40)	Atherosclerosis (n = 38)	p value
Males/females	32/8	31/7	1.0
Age, years	71.2 ± 8.3	70.0 ± 9.5	0.59
Smoking			
Never	25	11	0.004
Quit	13	20	0.45
Active	2	7	0.08
Hypertension	17	36	< 0.001
High cholesterol	12	29	0.003
Diabetes	1	9	0.023
Atrial fibrillation	0	4	0.05
CAD	0	16	< 0.001
CABG	0	8	0.002
Angina	0	8	0.01
MI	0	6	0.01
PVD	0	15	< 0.001
Claudication	0	13	< 0.001
Prior stroke or TIA	0	16	< 0.001
Glucose, mg/dl	92 ± 15	110 ± 47	0.03
Triglycerides, mg/dl	132 ± 58	151 ± 93	0.30
Cholesterol, mg/dl	203 ± 30	177 ± 34	< 0.001
HDL, mg/dl	58 ± 13	45 ± 11	< 0.001
LDL, mg/dl	123 ± 30	103 ± 26	0.003
VLDL, mg/dl	26 ± 11	28 ± 11	0.46
WBC, 10 ⁹ /l	5.92 ± 0.79	7.32 ± 2.19	< 0.001
Platelets, 10 ⁹ /l	239 ± 64	230 ± 57	0.5
Fibrinogen, mg/dl	358 ± 51	382 ± 98	0.19
ESR, mm/h	11 ± 8	18 ± 16	0.01
C-reactive protein			
<1 mg/l	36	32	0.51
1–3 mg/l	1	5	0.10
>3 mg/l	0	1	0.48
Not done	3	0	

CAD = Coronary artery disease; CABG = coronary artery bypass graft; ESR = erythrocyte sedimentation rate; MI = myocardial infarction; PVD = peripheral vascular disease. Data are means ± SD. Comparisons of the groups (no clinical atherosclerosis vs. atherosclerosis) were done using either an unpairedt test or Fisher's exact test (InStat 3, version 3.0b, GraphPad Software) with nominal p values reported.

sors from Ciphergen. For quality control purposes and to measure the chip-to-chip experimental variation, a pooled serum sample was prepared by mixing control and patient samples, and applied to 1 spot on each ProteinChip array used. The coefficients of variation (CV) of the intensity of each detectable peak were measured and the average variation across the entire range was <25%. Spectra from each sample were generated using a high and low laser intensity reading in order to optimize the detection of both low and higher molecular weight proteins. Prior to all chip reading, the instrument performance was evaluated by measuring laser energy output, resolution and sensitivity, and then externally calibrated using standard calibrants spanning the user-defined range of the mass/charge ratio. 118



Cerebrovasc Dis Extra 2011;1:115–129	
DOI: 10.1159/000334477 Published online: December 3, 2011	© 2011 S. Karger AG, Basel www.karger.com/cee



Fig. 1. Four unique SELDI-MS peaks that showed the greatest influence in distinguishing atherosclerosis samples from patient samples with no evidence of atherosclerosis.

SELDI-MS Bioinformatic Analysis

All SELDI-MS spectra were processed similarly using CiphergenExpress 2.1 data management software (Ciphergen Biosystems). Initially, both raw and processed data were analyzed. For spectrum-processing parameters, the general approach was the determination of the optimal baseline subtraction and smoothing algorithms, a total ion current normalization across all spectra, followed by a recalibration using peak alignment. A peak detection algorithm, using modifiable values for signal to noise and peak valley depth, was then employed to select peaks from a set of spectra from the same fraction and chip type. A Mann-Whitney U test was used to compare the mean intensity value of each recognized peak between two different clinical groups. The random forest (RF) program, which is a machinelearning classifier algorithm that makes group predictions based on many potentially correlated variables, was used to develop the multivariate classification models [17].

Albumin Depletion from Serum

Samples to be analyzed by 2D gels were albumin depleted using four Seppro Human Albumin IgY bead columns (Genway Biotech, San Diego, Calif., USA). Each serum sample was run through a column two times to obtain sufficient concentration of albumin-depleted serum proteins. Albumin-depleted and albumin-bound fractions were both recovered from the column and stored at -80°C. Symptomatic and asymptomatic samples were randomized with respect to columns. After all depletions, samples were thawed and a bicinchoninic acid assay (Pierce Biotechnology, Rockford, Ill., USA) was run to determine the protein concentration of the depleted and bound fractions for each sample. Samples were then stored at 4°C.

Cerebrovasc Dis Extra 2011;1:115-129



DOI: 10.1159/000334477 © 24 Published online: December 3, 2011 ww DeGraba et al.: Serum Biomarkers in Carotid Atherosclerosis

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2D-DIGE and Image Analysis

Detailed protocols for sample preparation, fluorescent labeling, 2D-PAGE and image acquisition are presented in the online supplementary experimental protocol (for all online suppl. material, see www.karger.com/doi/10.1159/000334477). Briefly, after sample cleaning and denaturing, 25 µg of protein were used for the Cy2 standard pool. Albumin-depleted serum samples from symptomatic and asymptomatic EA patients were labeled using either Cy3 or Cy5 dye. The remaining non-labeled samples were pooled to create a physical average for a Coomassie-stained spot picking gel. Minimal labeling uses 50 μ g of protein with 1 μ l of 400 pmol DIGE dye. Amersham IPG Immobiline Drystrips (pH 3-10 NL, 24 cm) and 18 cm 4-12% gradient Precast gels (Jule, Milford, Conn., USA) were used for the 1st and 2nd dimensions. These gels were run using the Ettan DALT 12 system (Amersham Biosciences, Piscataway, N.J., USA). All gels were imaged using a Fuji Film FLA-5000 scanner and Image-View software. The picking gel was scanned using a laser wavelength at 473 nm with a longpass blue filter. The image was saved as a 16-bit image with a resolution of 10 μ m. DIGE gels were scanned twice using appropriate laser and filter settings for Cy3 and Cy5. The 2D-DIGE images were uploaded to www.ludesi.com and analyzed for spot detection, segmentation and matching using the image analysis software, Ludesi 2D Interpreter.

Protein Identification

Tryptic in-Gel Digestion. For 2D-DIGE experiments a picking gel for protein identification, was run simultaneously and stained with Coomassie blue. Protein spots of interest were excised from the gel and the gel plugs were digested with the trypsin profile IGD kit (Sigma, St. Louis, Mo., USA) or modified trypsin (Promega, Madison, Wisc., USA) according to the manufacturers' protocols. For protein identification using high-resolution MALDI-MS/MS, the digested sample was desalted with Vivapure C18 micro spin column or C18 ZipTip (Waters, Milford, Mass., USA) and eluted with 3 μ l 60% ACN with 0.1% TFA.

High-resolution MALDI-TOF-TOF was acquired using an ABI 4700 proteomics analyzer (Applied Biosystems, Foster City, Calif., USA) operating in positive ion reflectron mode at 20 kV. The sample (0.5 μ l) was mixed with 0.5 μ l of the MALDI matrix (5 mg/ml of CHCA in 50% ACN and 0.1% TFA) and spotted onto the plate. The instrument was calibrated prior to analysis. The acquired spectra were submitted to MASCOT to search for matched proteins with human SPROT protein database.

Detailed experimental protocols are described in the online supplementary experimental protocols.

Results

Patient Characteristics

Demographics and clinical characteristics of the patients with and without atherosclerosis are summarized in table 1. Fifteen of 20 clinical variables were statistically different in the patients with atherosclerosis (e.g. coronary artery disease, peripheral vascular disease, angina, diabetes, hypertension and hypercholesterolemia). In contrast, comparisons of these clinical variables in patients with atherosclerosis who were symptomatic versus those who were asymptomatic prior to EA (table 2) showed that other than the history of a previous ischemic event, none of the clinical variables were different between these two groups.

SELDI-MS Profiling

Univariate analysis of the SELDI data comparing the atherosclerosis patient group (n = 38) to non-atherosclerotic controls identified 42 peaks with p < 0.01 by Mann-Whitney t tests



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	Atherosclerosis		p value	
	symptomatic (n = 16)	asymptomatic (n = 22)		
Males/females	14/2	17/5	0.67	
Age, years	70.1 ± 12.3	70.0 ± 7.4	0.96	
Smoking				
Never	5	6	0.49	
Quit	9	11	1.0	
Active	2	5	0.67	
Hypertension	14	22	0.17	
High cholesterol	10	19	0.12	
Diabetes	5	4	0.44	
Atrial fibrillation	1	3	0.62	
CAD	6	10	0.74	
CABG	2	8	0.14	
Angina	2	6	0.42	
MI	2	4	1.0	
PVD	5	10	0.50	
Claudication	4	9	0.48	
Prior stroke or TIA	16	0	< 0.001	
Glucose, mg/dl	101 ± 16	115 ± 60	0.37	
Triglycerides, mg/dl	132 ± 54	164 ± 115	0.31	
Cholesterol, mg/dl	171 ± 35	181 ± 33	0.36	
HDL, mg/dl	42 ± 10	46 ± 12	0.20	
LDL, mg/dl	103 ± 32	103 ± 20	0.93	
VLDL, mg/dl	27 ± 11	29 ± 12	0.66	
WBC, 10 ⁹ /l	6.96 ± 1.77	7.58 ± 2.51	0.40	
Platelets, 10 ⁹ /l	220 ± 67	237 ± 48	0.36	
Fibrinogen, mg/dl	382 ± 89	381 ± 108	0.99	
ESR, mm/h	16 ± 13	20 ± 18	0.47	
C-reactive protein				
<1 mg/l	14	18	1.0	
1–3 mg/l	2	3	1.0	
>3 mg/l	0	1	1.0	
Not done	0	0		

Table 2. Clinical characteristics of the patients with atherosclerosis

CAD = Coronary artery disease; CABG = coronary artery bypass graft; ESR = erythrocyte sedimentation rate; MI = myocardial infarction; PVD = peripheral vascular disease. Data are means ± SD. Comparisons of the two groups (symptomatic vs. asymptomatic atherosclerosis) were done using either anunpaired t test or Fisher's exact test (InStat 3, version 3.0b, GraphPad Software) with nominal p valuesreported.

of mean peak intensity (listed in online suppl. table 1). We utilized 3 different ProteinChip surfaces with different binding properties. The number of unique peaks will therefore be less as some of the proteins will bind to more than one SELDI surface resulting in an overlap of the peaks between chips.

In order to better classify the groups based on SELDI protein peak data, RF analysis was applied to the data set for an unbiased estimate of correct prediction rates [18]. The RF model demonstrated 69.2% sensitivity and 73.2% specificity in atherosclerosis prediction. The metric multidimensional scaling representation of the proximity matrix is shown in figure 2. Dimension (Dim) 1 and Dim 2 are approximations to the first two principal components



Cerebrovasc Dis Extra 2011;1:115–129	
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Fig. 2. Multidimensional scaling representation of the proximity matrix for the RF analysis of atherosclerosis versus no atherosclerosis. Atherosclerosis samples are distinguished from no-atherosclerosis samples. Dim 1 and Dim 2 are approximations to the first two principal components of the similarity space defined by the model.



of the similarity space defined by the model. Based on significance weighting of specific peaks in the RF models, the signal intensity of 4 unique SELDI peaks showed the greatest influence in distinguishing atherosclerosis samples from control patient samples (online suppl. fig. 2). SELDI-derived peaks had p < 0.01 by univariate analysis. The 4 unique SELDI peaks were: 4.2 kDa (Q10, F6), 16.7 kDa (IMAC, F1), 4.4 kDa (CM10, F1) and 28 kDa (Q10, F5), with the chip type and anion-exchange fraction listed following the molecular mass of the peak. Based on our previous publication [19], the 28.1-kDa peak is apolipoprotein A₁ (ApoA₁). We did not attempt to further identify the 4.2-, 4.4- and 16.7-kDa peaks.

SELDI-MS Profiling of Symptomatic versus Asymptomatic EA Groups

We analyzed SELDI peak data to compare asymptomatic EA patients to symptomatic EA patients. RF analysis failed to develop models with high sensitivity and specificity. The balanced RF (average over 10 RF runs) yielded a sensitivity of 28.8% and a specificity of 52.7%.

2D-DIGE with Protein Identification of Differentially Expressed Proteins in the Symptomatic and Asymptomatic EA Groups

2D-DIGE analysis of albumin-depleted serum on the 20 randomly selected samples revealed 11 spots with p < 0.01 (fig. 3). Absolute spot intensity and volumes, p values and protein identification of these differentially expressed spots are shown in table 3. Using MALDI-MS/MS and liquid chromatography MS/MS, 8 of the 11 proteins that were differentially expressed were identified (table 3). Four of these spots were identified as belonging to the same parental protein molecule haptoglobin (HPT) and 2 were related to α_1 -antitrypsin (A₁AT), both of which were downregulated in patients in the symptomatic EA group. It is likely that these spots represent proteins that are alternative splice variants or posttranslationally modified versions of the parental protein. Vitamin D binding protein (VTDB) was downregulated in the symptomatic EA, and leucine-rich α_2 -glycoprotein precursor (LRG) was upregulated in the symptomatic EA group (fig. 4).

Discussion

Carotid atherosclerosis is one of the primary causes of ischemic stroke [20]. Endothelial activation due to inflammation at the site of the carotid plaque as well as systemic inflammation may contribute to thrombus formation [20–22]. Examination of carotid plaques re-



Cerebrovasc Dis Extra 2011;1:115–1	129
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DeGraba et al.: Serum Biomarkers in Carotid Atherosclerosis



image consisting of a single Cy5labeled symptomatic EA patient serum, a Cy3-labeled asymptomatic EA patient serum and a Cy2-labeled internal standard control sample. The spots indicated with circles locate the position of the 11 differentially expressed spots with p < 0.01.

Fig. 3. A representative 2D-DIGE

Table 3. Protein identification by MALDI-MS/MS and liquid chromatography-MS/MS for spots differentially expressed between symptomatic and asymptomatic EA patients using 2D-DIGE

Spot No. (identifier)	Mean average spo asymptomatic	$\frac{\text{ot volume} \pm \text{SD}}{\text{symptomatic}}$	p value	Protein ID	MASCOT score	Peptides
1 (16,674)	1,092±313	767 ± 165	0.0039	НРТ	141	GSFPWQAR VGYVSGWGR VTSIQDWVQK DIAPTLTLYVGK SCAVAEYGVYVK (C) YVMLPVADQDQCIR (C) YVMLPVADQDQCIR (C) (M) SPVGVQPILNEHTFCAG (C) (M)
2 (16,681)	780±219	535±121	0.0029	HPT	82	YEELQITITAGR LALDLEIATYR WELLQQVDTSTR THNLEPYFESFINNLR
				HPT	45	VGYVSGWGR YVMLPVADQDQCIR (C) (M)
3 (16,682)	563±68	426±82	0.0007	VTDB	84	YTFELSR THLPEVFLSK HLSLLTTLSNR EDFTSLSVLYSR
4 (16,723)	535±87	375 ± 66	0.0001	KAC	58	SGTASVVCLLNNFYPR (C) TVAAPSVFIFPPSDEQLK
				ADPGK	22	LAAAWDALIVRPVRR
				PF21B	21	LASNYLNNPLFLTAR GSELQNEHQQLEEERDR
5 (16,770)	169±58	317±106	0.0005	НРТ	66	VGYVSGWGR VTSIQDWVQK YVMLPVADQDQCIR (C) YVMLPVADQDQCIR (C) (M)

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Cerebrovasc Dis Extra 2011;1:115–129		
DOI: 10.1159/000334477 Published online: December 3, 2011	© 2011 S. Karger AG, Basel www.karger.com/cee	124
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Spot No. (identifier)	Mean average spot volume \pm SD		p value	Protein	MASCOT	Peptides
	asymptomatic	symptomatic		ID	score	
6 (17,006)	283±74	153±42	0.0001	HPT	19	VGYVSGWGR YVMLPVADQDQCIR (C) (M)
				FHL1	11	VSHPVSKAR
7	128±51	293 ± 238	0.0028	MELT2	13	LSRLLKPGGMMLLR (M)
(16,816)				SUCB1 PCDG9	12 11	ALIADSGLKILACDDLDEA (C) GRLVLLCSLLGMLWEAR (C)
8 (16,843)	103 ± 46	240 ± 83	0.0015	LRG	35	DLLLPQPDLR TLDLGENQLETLPPDLLR
10 (16,862)	106 ± 27	63±38	0.0115	A ₁ AT	116	FLENEDR WERPFEVK DTVFALVNYIFFK LQHLENELTHDIITHDIITK VFSNGADLSGVTEEAPLK DTEEEDFHVDQVTTVK TLNQPDSQLQLTTGNGLFLSEGLK
11 (16,868)	142 ± 48	74±36	0.0015	A ₁ AT	134	FLENEDR WERPFEVK DTVFALVNYIFFK TDTSHHDQDHPTFNK LQHLENELTHDIITH VFSNGADLSGVTEEAPLK DTEEEDFHVDQVTTVK LYHSEAFTVNFGDTEEAK ELDRDTVFALVNYIFFK LYHSEAFTVNFGDTEEAKK GTEAAGAMFLEAIPMSIPPEVK (M)

Table 3 (continued)

Spot 9 was identified as keratin. (C) = Carbamidomethyl; (M) = oxidation.

moved from patients with ischemic strokes found that ulceration and thrombosis are more common in symptomatic patients [21]. Identifying proteins from the blood proteome that influence inflammation in patients with carotid atherosclerosis may provide some insight into the cause of thrombus formation and serve as potential biomarkers to identify patients at risk for developing symptomatic carotid atherosclerosis.

We hypothesized that differences in serum proteome expression between the atherosclerosis and control groups would be due to the presence of mature atherosclerotic plaques and not simply induced by the presence of atherosclerosis risk factors. We attempted to identify serological markers that would help us distinguish not only those patients with and without carotid atherosclerosis but also those who were more likely to become symptomatic. Using RF analysis of the entire protein spectra of fractionated serum from patients with and without carotid atherosclerosis revealed a 69.2% sensitivity and a 73.2% specificity in predicting the presence of atherosclerosis (table 1; fig. 1). These results suggest that a distinctive pattern is present in patients with carotid atherosclerosis. The 4 peptide peaks showing the greatest effect in the RF analysis that discriminated between these groups had apparent molecular masses of 4.2, 4.4, 16.7 and 28 kDa. However, this approach did not discriminate between the pa-



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DeGraba et al.: Serum Biomarkers in Carotid Atherosclerosis



Fig. 4. Normalized fluorescent intensity values from each albumin-depleted sample of the three most statistically significant, differentially expressed spots observed by 2D-DIGE. The gray lines represent the mean from each group, while the red lines delineate standard deviations. Sample numbers are shown on the x-axis of each graph.

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DeGraba et al.: Serum Biomarkers in Carotid Atherosclerosis

tients with symptomatic versus asymptomatic carotid artery disease. This may reflect a limitation of the SELDI-TOF methodology as well as the smaller sample size of this subgroup analysis. The 28.1-kDa peak was reduced in patients with atherosclerosis and identified as ApoA₁ based on a comparison of the signal characteristics to our SELDI database [19]. ApoA₁ is a hepatic immediate-early response protein and a major component of high-density lipoprotein (HDL). It has antioxidant and anti-inflammatory actions and low HDL cholesterol and low ApoA₁ levels have been found to be significant risk factors for all types of stroke [23, 24].

Using 2D-DIGE, we examined a subset of 20 patients with symptomatic or asymptomatic carotid atherosclerosis. Four proteins were differentially expressed and significantly different between these two subgroups and were identified as HPT, A₁AT, VTDB and LRG. Two of these proteins, HPT and A₁AT are part of a panel of acute-phase reactants termed inflammatory-sensitive plasma proteins (e.g. fibrinogen, A₁AT, HPT, ceruloplasmin and orsomucoid) that have been shown in large cohort studies to be major risk factors for stroke [13]. Hypercholesterolemia in association with elevations in inflammatory-sensitive plasma proteins increases the risk of cardiovascular disease, ischemic stroke and myocardial infarction whereas in the absence of the proteins, no association was found between hypercholesterolemia and ischemic stroke [13]. These plasma biomarkers modify the risk of stroke in patients with hypercholesterolemia, hypertensive patients, and patients with obesity and diabetes [13, 18, 25]. A recent study showed similar findings using an inflammatory score based on white blood cell count, HPT and C-reactive protein that added predictive information above that of total cholesterol and triglycerides to predict major cardiovascular events (stroke, myocardial infarction or heart failure) in a healthy cohort over 1 decade [26].

HPT has several functions that may serve as a cofactor in symptomatic atherosclerotic disease. It is an acute-phase reactant that binds free hemoglobin, serves as an antioxidant and induces anti-inflammatory cytokines [27]. HPT also binds to ApoA₁ on HDL, a site that is critical for lecithin cholesterolacyl transferase activity and may help bind hemoglobin to HDL making it a potentially proatherogenic molecule [27, 28]. A strong association of increased risk of micro- and macrovascular cardiovascular disease in patients with the Hp2:2 genotype and diabetes has been described [27]. Its role in non-diabetic cardiovascular disease remains to be defined [29].

VTDB is a major plasma carrier of vitamin D and its metabolites [30]. In addition, VTDB has other important functions, including scavenging actin, fatty acid transport, macrophage activation and enhancement of neutrophil and macrophage chemotaxis to C5 des Arg [30, 31]. Stressed endothelial cells release VTDB that has both a chemotactic function and serves as a growth factor for vascular smooth muscle cells [32]. Actin filaments released from damaged cells have the potential to damage the vasculature and enhance thrombosis [33]. Recently, VTDB has been found in both the serum and thrombotic plaques of patients with acute myocardial infarction [34]. VTDB administration in vitro decreased platelet aggregation and prolonged coagulation [34]. Further, patients with acute coronary syndromes have persistent elevation of plasma VTDB over 6 months compared to healthy volunteers [35]. We show that VTDB is present in patients with carotid artery atherosclerosis and that patients who were symptomatic prior to EA have lower levels of VTDB. Lower levels of VTDB in our symptomatic EA patients may have enhanced atherogenesis or contributed to an unstable atheromatous plaque.

LRG was elevated in symptomatic EA patients. It is an acute-phase reactant induced by proinflammatory cytokines, and increased expression has been found in several inflammatory conditions, including the sera of patients with graft-versus-host disease, lung and pancreatic cancer and in the cerebrospinal fluid of patients with normal pressure hydrocephalus [36]. LRG is upregulated during neutrophilic differentiation and is localized in azurophilic granules [37]. It binds to cytochrome c, a proinflammatory mediator, and is toxic to lympho-

	Cerebrovasc Dis Extra 2011;1:115–129		
Cerebrovascular Diseases	DOI: 10.1159/000334477 Published online: December 3, 2011	© 2011 S. Karger AG, Basel www.karger.com/cee	127

cytes [36, 38]. The inflammation-modifying effects of LRG in the pathogenesis of atherosclerosis remain to be defined.

Multiple strategies are necessary to find new biomarkers associated with carotid atherosclerosis [39, 40]. Our study describes preliminary observations regarding biomarkers associated with symptomatic and asymptomatic carotid artery disease. Limitations of our study are based in part on its size and methodology. The SELDI platform is a semiquantitative tool for proteomic discovery and hypothesis generation. It provides spectral patterns that are rich with information, but limitations of this approach have been noted [39, 41]. In our patients, the timing of blood sampling after the acute event may have influenced our analysis. Some proteins may be upregulated during recovery from the acute event rather than representing a specific marker of carotid artery disease. Biomarkers measured in serum may not necessarily reflect biologic activity at the level of the plaque and may also be influenced by other factors, such as degradation in the serum, tissue metabolism and excretion. Lastly, our study did not control for the effects of different therapies (i.e. statins, cyclooxygenase inhibitors or antihypertensives) that may have unrecognized effects on the differentially expressed proteins. Our observations will require validation in other well-defined cohorts at risk for cerebral ischemia.

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