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Identification of potential stroke biomarkers in plasma using multiple liquid chromatography separations and nanoLC-ESI-MS detection

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Abstract

Stroke is the leading cause of disability in the USA. Rapid, reliable diagnosis *via* a point-of-care blood test may facilitate earlier treatment to reduce disability. We have recently reported detailed methods of chromatographic separations of plasma samples coupled with nanoESI-ion trap detection for identification of proteins. Herein, we report a list of proteins, which are viable candidates for further investigation as stroke biomarkers.

Keywords

Blood plasma; Biomarkers; HPLC; Stroke

Introduction

Stroke occurs in about 800,000 people per year in the United States (US) and is the leading cause of disability in the US and the world (Roger et al. 2012). Stroke is classified as ischemic or hemorrhagic. There is no proven therapy for hemorrhagic stroke, and recombinant tissue plasminogen activator (rt-PA), the only approved therapy for ischemic stroke, is given to only ~4% of ischemic stroke patients annually (Adeoye et al. 2011). The inability to rapidly and reliably distinguish ischemic from hemorrhagic stroke without advanced imaging contributes to this low rate of treatment. Development of blood biomarkers that distinguish ischemic from hemorrhagic stroke was identified as a key unresolved question by the National Institutes of Health (NIH) (2006) (Rifai et al. 2006). Whiteley et al. reviewed the diagnosis of ischemic stroke using blood biomarkers where a certain number of proteins are listed and from these, potential biomarkers were predicted based on their biological actions (Whiteley et al. 2008; Whiteley et al. 2009; Saenger and Christenson 2010). Clinical studies were also done using selected plasma biomarkers in the diagnosis of acute ischemic stroke (Kim et al. 2010; Reynolds et al. 2003). Proteomics may be helpful in the search of stroke biomarkers disease (Anderson 2005).

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Human plasma is a complex mixture of virtually all proteins expressed in humans from blood cells and/or tissue through active secretion or leakage. Our team and others have previously shown that plasma may be subjected to chromatography and mass spectrometry methods to identify potential disease biomarkers (Adkins et al. 2002; Tirumalai et al. 2003; Pieper et al. 2003; Kodali et al. 2012). In this report, we provide a brief summary of our approach and a list of novel potential biomarkers for stroke diagnosis based on our preliminary findings.

Sample Information

Stroke patients who presented to The University of Cincinnati Hospital (TUH) emergency department (ED) were enrolled in a plasma-banking project. Informed consent and Institutional Review Board (IRB) approval were obtained. Blood draws were performed in the emergency department, ED. For this project, we analyzed twenty-nine plasma samples collected within 12 hours of symptom onset. Ten samples were stroke mimics – patients admitted to the ED with complicated migraines or other symptoms initially presenting for stroke, but who did not have a stroke during that specific ED visit. Stroke mimic samples were used because these patients require distinction from true stroke patients in the clinical arena. Of the stroke cases, 9 were hemorrhagic stroke and the remaining 10 were ischemic stroke. Discussion of all the materials instrumentation and procedures used for this study can be found in our earlier report (Kodali et al. 2012).

Results and Discussion

The goal of this study was to detect and identify differentially expressed proteins in the plasma of stroke and stroke mimic patients using previously published methods (Kodali et al. 2012).

In brief, the first separation step was immunodepletion, primarily removal of HSA and IgG. This was followed by size exclusion chromatography (SEC) due to its high capacity and its wide utilization as a separation technique for biomolecules such as proteins (Lecchi et al. 2003; Alvarez-Manilla et al. 2006). SEC gave four differing molecular weight fractions, which were then analyzed by reverse phase separation. A typical set of chromatograms obtained after running SEC fraction #2 on a reverse phase C4 column, is presented in Figure 1. The protein identification analysis was carried out on all the four SEC fractions presented to nanoLC-ESIMS for MS/MS detection.

The prospect of having co-eluted proteins in a single chromatographic peak was a concern. To exclude this possibility a diode array UV-Vis detector at multiple wavelengths was used to allow evaluation of the peak purity factor. Additionally, co-eluted proteins from a reverse phase column may have similar UV-Vis absorbance spectra. To compensate for this possibility, electrospray ionization mass spectrometry (ESIMS) in the multiple reaction-monitoring mode (MRM) was used. This provides high selectivity by monitoring chromatographic co-elution of a parent ion and two product ions (called transitions). We also did method validation studies by following spike recoveries of a non-human protein (Thaumatococcus from *Thaumatococcus daniellii*) at the end of the procedure, to show that this

method is feasible. This confirmation showed that the protein was intact even after multiple chromatographic separations (see Kodali et al. 2012).

Putative stroke biomarkers are listed in Table 1. The protein scores in blue highlight indicate that the protein was found by two database search engines (Mascot and Spectrum mill), via accessing the Uniprot database. The other proteins were found by only one search engine and are reported based on having good protein scores. Four of the proteins were found earlier (Kodali et al. 2012). These are kinesin heavy chain isoform 5A (SEC fraction 1), Low-density lipoprotein receptor-related protein 2 precursor (SEC fraction 2), Endothelin-converting enzyme-like 1 (SEC fraction 3) and Low-density lipoprotein receptor-related protein 1B precursor (SEC fraction 4). Certain proteins from Table 1 merit brief discussion vis-à-vis biomarker potential. Protein kinase C η type (PRKCH), which was found in the SEC fraction #1 and mainly in 80% of hemorrhagic stroke patients. PRKCH is a phospholipid, diacylglycerol (DAG)-dependent serine/threonine-protein kinase and calcium-independent, which is involved in the regulation of cell differentiation in pre-B cell receptor and keratinocytes and mediates regulation of epithelial tight junction integrity (Lee et al. 2010). Non-synonymous single nucleotide polymorphism (SNP) in PRKCH has been shown to increase the risk of cerebral infarction (Kubo et al. 2007). Further, the 1425G/A SNP in PRKCH is associated with ischemic stroke and cerebral hemorrhage as reported for a Chinese population (Wu et al. 2009).

Fibrinogen gamma chain was also found in SEC fraction #1 (mainly found in 70% of ischemic stroke patients) and fibrinogen beta and alpha chain were found in SEC fraction #2 (mostly found in 80% of stroke mimic and 70% of hemorrhagic stroke patients). Plasma fibrinogen level and fibrin clot structure may be of importance in the pathogenesis of both ischemic and hemorrhagic stroke. Fibrin clot structure has also been reported to predict recurrent stroke and acute coronary heart events in stroke survivors (Beamer et al. 1998; Tanne et al. 2001; Di Napoli and Papa 2002; Rothwell et al. 2004; Woodward et al. 2005). An association between ischemic stroke and genetic variation at the fibrinogen gamma and fibrinogen alpha genes, independent of plasma fibrinogen levels, has been previously reported (Jood et al. 2008).

Complement C3 was found in SEC fraction #3 and in 80% of ischemic stroke patients. C3 plays a central role in the activation of the complement system. Complement factor C3 and C4 have been associated with atherosclerosis and cardiovascular risk factors. Plasma levels of C3 and C4 have been found to be risk factors for the incidence of cardiovascular disease (Engstrom et al. 2007). Methylenetetrahydrofolate reductase (MTHFR) was found in SEC fraction #4 and in 90% of hemorrhagic stroke samples. It catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate for homocysteine remethylation to methionine (Goyette et al. 1998). Methylenetetrahydrofolate reductase gene polymorphisms are associated with ischemic and hemorrhagic stroke (Sazci et al. 2003). Two polymorphisms in the MTHFR gene, C677T, A1298C result in reduced enzyme activity (Kara et al. 2003). Antithrombin-III was found in SEC fraction #3 and in 80% of stroke mimic patients. It is the most important serine protease inhibitor in plasma that regulates the blood coagulation cascade. Antithrombin-III associated with fibrinogen predicts the risk of cerebral ischemic stroke (Meng et al. 2011). Collagen alpha-1(IV) chain

was found in SEC fraction #4 and in 90% of hemorrhagic stroke patients. Type IV collagen is the major structural component of glomerular basement membranes. Mutations of the COL4A1 gene may predispose to hemorrhagic stroke (Gould et al. 2006).

Using the intensities of the precursor ions from the MRM experiments on the proteins mentioned above, an ANOVA one-way analysis with Bonferroni correction was performed. As a summary of the statistical differences between the three groups, the p levels are shown in the Table 2. From the set of possible biomarkers it is interesting that Fibrinogen, on its three isoforms is the only protein found with statistical differences across all the groups; while all the proteins except Antithrombin-III, shows statistical differences between the AIS and ICH groups.

The fact that the sample numbers is limited makes the use of a more powerful statistical tools, such as multivariate predictive methods, not viable choices for this study, however the fact that it is possible to show statistically significant differences in the levels of stroke related proteins by an extensive separation/detection, double-blind study, opens the way for a larger scale study with more specific detection of the target proteins proposed as biomarker candidates in this study.

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Figure 1.

Reversed phase separation of *SEC fraction 2*. The chromatogram is the overlay of stroke mimic (blue), hemorrhagic (red) and ischemic (green) patient. Six regions were collected from the reversed phase separation for SEC fraction 2, which showed clear differences in intensities between different sets of samples (stroke mimic, hemorrhagic and ischemic stroke). The chromatogram for hemorrhagic stroke samples was different when compared to stroke mimic and ischemic stroke samples. The observed differences were consistent for all stroke mimic, hemorrhagic and ischemic patients. The black dot boxes represent the fractions that were further analyzed with nanoLC-ESIMS for protein identifications.

Table 1

Table shows potential protein biomarkers obtained from different size exclusion fractions (Fraction 1,2,3 and 4). The first column is protein I.D's followed by protein biomarkers, SEC fraction, proteins found in different types of samples, protein score and number of matched peptides. Proteins of matched peptides of two or less were confirmed by Blast. The proteins in red bold were also found in our earlier study. The protein scores in blue highlight indicate that particular protein was found in two search engines (Mascot and Spectrum mill). SM= Stroke mimic; AIS= acute ischemic stroke; ICH= intracerebral hemorrhage. For MASCOT, the score of 51 ($p < 0.05$) and for Spectrum Mill, the score of 25 are considered validated.

(Uniprot) I.D	List of proteins to study further as stroke biomarkers	SEC fraction	Protein found in samples	Protein Score (Mascot)	Matched Peptides	Protein Score (Spectrum Mill)	Matched Peptides
P01023	Alpha-2-macroglobulin	1	AIS $\gg$ ICH > SM	142	6	260	11
P04114	Apolipoprotein B-100	1	ICH > AIS	110	4	180	7
P02751	Fibronectin	1	SM $\gg$ ICH > AIS	125	5	188	9
P24723	Protein kinase C ϵ type	1	ICH > AIS	90	4	72	3
O75204	Transmembrane protein 127	1	ICH > AIS	78	3	----	----
P02679	Fibrinogen gamma chain	1	AIS $\gg$ ICH > SM	84	3	126	7
P10909	Clusterin	1	AIS > ICH	117	3	74	3
Q12840	Kinesin heavy chain isoform 5A	1	ICH $\gg$ AIS > SM	----	----	68	2
P27169	Serum paraoxonase/arylesterase 1	1	ICH > SM	96	3	72	3
P03952	Plasma kallikrein	1	SM > ICH	76	1	52	2
Q9NS23	Ras association domain-containing protein 1	1	ICH > SM	54	2	----	----
P02675	Fibrinogen beta chain	2	SM $\gg$ ICH > AIS	105	4	124	7
P00738	Haptoglobin	2	AIS $\gg$ ICH > SM	123	4	65	2
P05155	Plasma protease C1 inhibitor	2	ICH	128	5	74	3
P98164	Low-density lipoprotein receptor-related protein 2 precursor	2	ICH > AIS	52	2	----	----
P02649	Apolipoprotein E	2	ICH	110	4	56	3
P00450	Ceruloplasmin	2	ICH	116	5	55	3
P02671	Fibrinogen alpha chain	2	ICH $\gg$ AIS > SM	82	4	104	6
O43526	Potassium voltage-gated channel subfamily KQT member 2	2	ICH > AIS	94	3	----	----
P01024	Complement C3	3	AIS > ICH	129	3	114	7
P01008	Antithrombin-III	3	SM > ICH	76	2	64	2

(Uniprot) ID	List of proteins to study further as stroke biomarkers	SEC fraction	Protein found in samples	Protein Score (Mascot)	Matched Peptides	Protein Score (Spectrum Mill)	Matched Peptides
P02647	Apolipoprotein A-I	3	AIS>>>ICH>SM	140	3	112	7
P02774	Vitamin D-binding protein	3	AIS>>>SM>ICH	104	2	77	3
P00734	Prothrombin	3	AIS > ICH	62	2	54	2
O95672	Endothelin-converting enzyme-like 1	3	AIS>>>SM>ICH	74	3	64	3
P01019	Angiotensinogen	3	SM>>>ICH>AIS	63	1	72	2
Q96RK4	Bardet-Biedl syndrome 4 protein	3	SM>>>AIS>ICH	94	4	----	----
O15078	Centrosomal protein of 290 kDa	3	AIS>>>ICH>SM	92	4	72	3
P02766	Transthyretin	3	AIS>>>ICH>SM	51	1	62	2
P31327	Carbamoyl-phosphate synthase [ammonia], mitochondrial	4	ICH>>>AIS>SM	84	3	----	----
P42898	Methylenetetrahydrofolate reductase	4	ICH	96	4	84	4
P02462	Collagen alpha-1(IV) chain	4	ICH	98	4	62	3
Q9NZR2	Low-density lipoprotein receptor-related protein 1B precursor	4	AIS>>>ICH>SM	----	----	62	3
P51787	Potassium voltage-gated channel subfamily KQT member 1	4	ICH > AIS	98	4	----	----
Q8WW38	Zinc finger protein ZFPM2	4	ICH > SM	84	3	----	----
P35579	Myosin-9	4	ICH > AIS	92	4	----	----

Table 2

Statistical significance for the differences (p values via ANOVA one-way with Bonferroni correction), of the signal intensities for the proposed proteins by sample groupings. For example, with fibrinogen gamma chain protein, the stroke mimic (SM) shows significant differences to the hemorrhagic stroke sample (ICH). Between the SM and acute ischemic stroke (AIS) samples there is no significant difference (NS) between the protein kinases C-eta type.

Protein	SM vs ICH	SM vs AIS	ICH vs AIS
Fibrinogen gamma chain	< 0.01	< 0.01	< 0.01
Protein kinase C eta type	< 0.05	NS	< 0.01
Fibrinogen gamma chain	< 0.05	< 0.01	< 0.01
Fibrinogen beta chain	< 0.01	< 0.01	< 0.01
Fibrinogen alpha chain	< 0.01	NS	< 0.01
Complement C3	NS	< 0.05	< 0.01
Methylenetetrahydrofolate reductase	< 0.01	NS	< 0.01
Antithrombin-III	< 0.01	< 0.01	NS
Collagen alpha-1(IV) chain	< 0.01	NS	< 0.01