



Proteomic-Based Approaches for the Study of Ischemic Stroke

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Ischemic stroke is the most common type of stroke. Despite the fact that there have been extensive efforts for establishing accurate diagnose and efficient therapy, ischemic stroke continues to be one of the main causes of death worldwide [1, 2]. Currently, genomics provides information about what the body “can do,” transcriptomics provides information about what the cell “will do,” while proteomics tells us “what is happening.” Proteomics complements genomics and transcriptomics and provides information about the implementation of proteins as the main functional mediators of cells, such as their post-translational modification and their interactions with other biological molecules [3]. In the last years, studies focusing on genomics and transcriptomics aimed to discover new diagnostic or prognostic evidence for ischemic stroke [4]. However, the state of brain cells after ischemic stroke is most directly related to protein function, which is also the target of most drug intervention. Therefore, investigations at the protein level are favorable for the in-depth description of neuronal injury after ischemic stroke. Innovative high-throughput proteomics techniques can help us accurately evaluate the degree of neuronal injury after ischemia, study the mechanism of drug action, and explore the role of key proteins in neuronal injury. Using these emerging proteomics techniques, diagnostic and prognostic biomarkers for ischemic stroke may be identified. For years, mass spectrometry (MS)-based proteomics has enabled us to obtain large profiling datasets with very high precision. Here, the recent advances in proteomic researches in ischemic stroke are outlined.

Studies Performed in Human Brain Tissue/Cerebrospinal Fluid/Plasma

Aiming at identifying potential biomarkers of ischemia, two-dimensional gel electrophoresis (2DE) and MS were employed to analyze the differences between post-mortem cerebrospinal fluid samples and cerebrospinal fluid from healthy subjects [5]. H-FABP was identified as a potential biomarker for ischemic stroke. Compared with plasma samples healthy controls ($n = 21$), the differential expression of apolipoprotein CI, apolipoprotein CIII, serum amyloid A, and antithrombin-III fragment were detected by one-dimensional electrophoresis coupled MS [6]. Using enzyme-linked immunosorbent assay (ELISA), apolipoprotein CI and apolipoprotein CIII were further considered to be as potential plasmatic markers to distinguish between ischemic and hemorrhagic stroke. Proteomic analysis by 2DE-MALDI-TOF/TOF MS was performed to reveal differential protein expression in endothelial progenitor cells between healthy subjects ($n = 11$) and ischemic stroke patients ($n = 11$). Two of the differentially expressed proteins, endoplasmic reticulum protein-29 and CdC-42, were only expressed in endothelial progenitor cells from healthy subjects, whereas elongation factor-2 was only identified in endothelial progenitor cells from ischemic stroke patients. Furthermore, peroxiredoxin-1 expression was identified to be 10× stronger in ischemic stroke patients than in healthy subjects [7]. Microdialysates from infarct core, penumbra, and the unaffected contralateral brain regions of patients suffering an ischemic stroke ($n = 6$) were analyzed by TMT. Compared with the contralateral samples, a total of 53 proteins showed an increasing trend in the infarct core or penumbra. Among these, the increase of glutathione S-transferase P, peroxiredoxin-1, and protein S100-B in the blood of stroke ($n = 14$) patients were further validated with ELISA [8].

Datta et al. studied the commonly regulated infarct proteome from three different brain regions (putamen, thalamus, and the parietal lobe) of female Japanese patients by an 8-plex iTRAQ-based 2D-LC-MS/MS strategy [9]. Among the identified 1520 proteins, energy metabolism-related proteins

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(SLC25A11, SLC25A12, GOT2, and MDH2) were downregulated, while proteins related to reactive gliosis (VIM, GFAP), anti-inflammatory response (ANXA1, ANXA2), and iron metabolism-related proteins (FTL, FTH1) showed an increasing trend. The deregulated proteins could be useful as potential therapeutic targets or biomarkers for ischemic stroke. Using 2DE-MALDI-TOF/TOF MS, García et al. compared the protein profiles between tissue samples from ischemic ipsilateral (infarct core and peri-infarct area) and healthy contralateral hemispheres of 3 ischemic stroke patients obtained within the first 6 h after death [10]. Among the identified 51 proteins, 8 candidates including GELS, CYTA, CNTN1, CYBP, SEPT5, PYGB, GDIR1, and DRP2 were selected from different functional categories based on their potential scientific relevance. From the 8 candidates, GDIR1, DRP2, and CNTN1 were altered in patient serum samples, which may well serve as biomarkers for long-term stroke prognosis. Utilizing MS-based proteomics, ceruloplasmin, complement component C8 gamma, and platelet basic protein have been shown to be significantly different between the ischemic patients ($n = 35$) and the controls ($n = 12$) [11]. And, platelet basic protein was further identified as a candidate serum biomarker for a transient ischemic attack. A quantitative proteomic analysis of serum from both acute ischemic stroke patients and control subjects by the iTRAQ-Based LC-MS approach revealed 389 high confidence protein identifications [12]. Among 60 proteins showing > 1.5-fold change, vWF, ADAMTS13, S100A7, and DLG4 were further investigated in serum by ELISA. Nguyen et al. examined the serum proteome of stroke patients ($n = 44$) by utilizing label-free relative quantification and correlated these with the depression rating scale scores at 3 months post-stroke [13]. The results indicated that downregulation of complement expression in the periphery in conjunction with increasing MADRS scores post-stroke may be a biomarker for incomplete recovery of brain metabolic needs, homeostasis, and inflammation following ischemic stroke damage.

Promising biomarker candidates have been investigated in several studies involving animal models, followed by validation in human body fluids. By DiART-Label proteomic analysis of ischemic mouse brain, Song et al. identified clusterin as a potential biomarker for severity of an acute ischemic stroke, which was validated in human serum [14]. As a result of LC-MS/MS analysis, a total of 1600 proteins were identified in the thrombus derived from four acute ischemic stroke patients. Among the identified proteins, 341 proteins are commonly detected in all the four patients included in this study [15]. To verify proteomic biomarkers for minor stroke and transient ischemic attack from a list of 141 candidate proteins, Penn et al. applied the multiple reaction monitoring-mass spectrometry (MRM-MS) in plasma samples from 545 participants [16]. Among the 141 candidate proteins, 9 proteins including L-selectin, insulin-like growth factor-binding protein 3,

hyaluronan-binding protein 2, coagulation factor X, serum paraoxonase/lactonase 3, thrombospondin-1, heparin cofactor 2, apolipoprotein B-100, and von Willebrand factor were significant univariate predictors of a transient ischemic attack. Based on label-free spectral counting, 30 differentially expressed proteins between infarcted and contralateral areas were identified. Among them, SAHH2 was subsequently verified in the plasma of ischemic stroke patients and considered meaningful as a prognostic biomarker of neurological improvement [17].

Tissue Proteomics Studies in Animal Ischemia Model Without Intervention

Using 2DE analysis and MALDI-TOF MS, there were four studies performed in rats to test the different protein expression in ischemic cortex between middle cerebral artery occlusion (MCAO) group and sham group at 24 h after surgery [18, 19], a 30-min ischemia group and 2-h ischemia group [20], and a nondiabetic group and diabetic group [21]. Compared with sham group, upregulation of 60 kDa heat shock protein, dehydropyrimidinase-related protein 2, t-complex protein 1, Rho GDP dissociation inhibitor, spectrin alpha II chain, heat shock cognate protein 70 pseudogene 1, and tropomodulin 2 after focal cerebral ischemia in rats was proved [18, 19]. The 2-h ischemia group indicated near complete loss of ATP production, synaptic dysfunction with degraded cytoarchitecture, more conservative chaperone activity, and additional cell survival factors than those seen in the 30-min ischemia group [20]. Compared with nondiabetic MCAO rats, expression of metabolism-related enzymes, including isocitrate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, adenosylhomocysteinase, pyruvate kinase, and glucose-6-phosphate isomerase was decreased in diabetic MCAO rats, suggesting that the diabetic condition may enhance brain ischemic damage via downregulating metabolism-related protein expression [21]. In addition, the 2DE-coupled MALDI-TOF MS was used to test ischemia-induced changes in protein level in the pituitary, adrenal gland, splenic lymphocytes, hippocampus, plasma, spinal cord, and sciatic nerve [22–25]. Based on label-free quantitation, TDP43, FUS, hnRNPA1, PSF/SFPQ, and p54/NONO were found to be aggregated in ipsilateral neocortical tissue following ischemia/reperfusion [26]. Furthermore, iTRAQ-MALDI-MS/MS was employed for the discovery of changes in protein associated with ischemia in the penumbra, ventricular zone, and serum [27–29]. Law et al. found that inflammation and neurogenesis-relevant proteins were dysregulated in the penumbra of the *Macaca fascicularis* stroke model [28]. Similarly, Gunton et al. found that inflammatory mediators, such as antiproteases and cytokine/growth factor-binding proteins, were upregulated in the ventricular zone after cortical stroke in mice [27].

And, upregulation of RHOA and CDC42 in the penumbra was proved in a rat ischemic model, which may serve as a potential serum biomarker [29].

Tissue Proteomics Studies in Animal Ischemia Model with Intervention

First, 2-DE combined with MALDI-TOF analysis was performed to identify proteomic changes in the penumbra and cortex induced by preconditioning and hypothermia in the murine MCAO model [30–33]. The expression of HSP70, HSP27, HSP90, guanylyl cyclase, muskellin, platelet-activating factor receptor, and beta-actin was increased in the ipsilateral cortex at 24 h after preconditioning induced by a 10-min transient MCAO [30]. And, both cPKC β II-interacting proteins and nPKC ϵ -interacting proteins were found to be involved in hypoxic preconditioning-induced neuroprotection against cerebral ischemia in mice [31, 32]. Pharmacological preconditioning using the immune activator D192935 induced a proteomic signature following cerebral ischemia that is associated with neuroprotection in the rhesus macaque [34]. Hypothermia was typically associated with opposite protein expression changes in the penumbra (cortex) to those induced by stroke under normothermic conditions but not in the core (striatum) in a rat ischemic model induced by endothelin-1 [33].

Proteomics analysis was performed to understand the mechanisms underlying the repair function of adipose-derived mesenchymal stem cell administration and human cerebral endothelial cell (hCMEC/D3) transplantation in experimental ischemia models. A total of 2416 proteins in adipose-derived mesenchymal stem cell secretome were identified [35], while mitochondrial dysfunction (paraplegin matrix AAA peptidase subunit, SPG7), neuroinflammation (peroxiredoxin 6, PRDX6), and neuronal death (zinc finger protein 90, ZFP90) were markedly reduced in the hCMEC/D3 treatment group compared with the control group [36].

Ferulic acid exerts a neuroprotective effect in cerebral ischemia through its anti-oxidant and anti-inflammation activity. Koh et al. conducted a series of proteomics experiments by 2DE combined with MALDI-TOF analysis to analyze the changes in proteomics caused by ferulic acid [37–40]. The results showed that ferulic acid attenuated ischemia-induced decrease of adenosylhomocysteinase, isocitrate dehydrogenase [NAD(+)], mitogen-activated protein kinase kinase 1, glyceraldehyde-3-phosphate dehydrogenase, protein phosphatase 2A subunit B, hippocalcin, peroxiredoxin-2 and thioredoxin, and increase of pyridoxal phosphate phosphatase and heat shock protein 60 in rat MCAO model.

In addition, proteomics analysis has been used to light on the mechanisms underlying pleiotropic actions of rt-PA [41] and the neuroprotection of traditional Chinese medicines

(baicalin [42], *Ginkgo biloba* extract (EGb761) [43], curcumin [44], Danhong injection [45], quercetin [46], rhubarb [47], hydroxysafflor yellow A [48], Xiao-Xu-Ming decoction [49]), asialoEPO [50], melatonin [51], estradiol [52], PACAP38 [53], tetrandrine [54], simvastatin [55], resveratrol [56], PPAR α agonist [57], tetramethylpyrazine nitron [58], and grape seed extract [59] against ischemic injury).

Studies in Cell Lines Further Verified in In vivo Ischemia Model

Using 2-DE analysis and MALDI-TOF MS, nucleoside diphosphate kinase A has been identified to be upregulated in primary cortical neuronal cultures exposed to oxygen-glucose deprivation (OGD) [60]. And then, the authors verified a neuroprotective role of nucleoside diphosphate kinase A against ischemia-related injury in cultured neurons by using RNAi and an adenoviral vector-induced overexpress. Based on stable isotope labeling with amino acids (SILAC) followed by LC-MS/MS analysis, 19 proteins were found differentially secreted between OGD and normoxia/normoglycemia conditions in cultured hCMEC/D3 [61]. Western blot was used and further verified a set of 9 differentially secreted proteins. Among them, COL1A2, CLU, IGFBP2, ANXA1, and PRDX3 were analyzed in serum samples of ischemic stroke patients ($n = 38$), stroke-mimicking conditions ($n = 18$), and healthy controls ($n = 18$), which suggested a potential of IGFBP2 for the diagnosis of cerebral ischemia. Based on existing reports and author's findings that hypoxic-conditioned medium derived from bone marrow mesenchymal stromal cells protects against ischemic stroke in rats, cytokine antibody arrays and label-free quantitative proteomics analysis were used to analyze the differences between hypoxic-conditioned medium and normoxic-conditioned medium from bone marrow mesenchymal stromal cells. A total of 163 proteins were differentially expressed between the two groups, including 107 upregulated proteins and 56 downregulated proteins [62].

Challenges and Shortcomings

Although proteomics studies have contributed with a long list of potential biomarkers for diagnosis, prognosis, and monitoring of ischemic stroke, most of them have not been implemented in clinical application successfully. When reviewing the existing reports, shortcomings, including small sample size; ignoring cell types [63]; ignoring the age needs of laboratory animals [64]; and using single-sex experimental animals [65], it seems to be that they are responsible for blocking these results to achieve clinical implementation. Moreover, study designs were frequently not close to the clinical

situation, such as reperfusion [66]. Additionally, proteomics analysis of clinical or preclinical intervention strategies, such as Glasgow oxygen level-dependent technology [67] and low oxygen postconditioning [68], may yield more meaningful findings. In addition, a combination of proteomics studies with a real-world study with a follow-up is required [69].

There is no doubt that there is an urgent demand for biomarkers of brain injury after ischemic stroke to maximize benefit from the existing treatment schemes. Therefore, using proteomics as a tool to integrate clinical needs with drug development may be conducive to the discovery of effective biomarkers and their clinical application.

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Compliance with Ethical Standards

The study was approved by the Ethics Committee of the First Affiliated Hospital of Soochow University. And there is no human or animal subject in this article.

Conflict of Interest The authors declare that they have no conflict of interest.

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