Review article

Large-scale analyses of schizophrenia proteome

Análise proteômica da esquizofrenia

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Abstract

Valuable knowledge about schizophrenia has been recently generated for deciphering its pathobiology and revealing biomarkers. However, efforts are still needed, especially if we take in account that this debilitating mental disorder affects approximately 30 million people worldwide. Considering that schizophrenia is a result of a complex interaction among environmental factors altered gene function and systematic differential protein expression, proteomics is likely to be a suitable tool for studying this disorder. Here we synthetize the main findings by proteomic studies and further directions to be taken in order to better comprehend the biochemistry of schizophrenia as well as reveal biomarkers. In addition, we summarize proteomic methodologies used in such studies.

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Keywords: Proteomics, schizophrenia, mass spectrometry, energy metabolism, oligodendrocytes.

Resumo

Valioso conhecimento a respeito de esquizofrenia tem sido gerado recentemente para decifrar sua patobiologia e revelar biomarcadores. Entretanto, esforços ainda são necessários, especialmente se levarmos em conta que essa debilitante desordem mental afeta aproximadamente 30 milhões de pessoas ao redor do mundo. Considerando que esquizofrenia é resultado de uma complexa interação entre fatores ambientais, função genética alterada e expressão proteica diferencial sistemática, a proteômica é provavelmente uma ferramenta adequada ao estudo dessa desordem. Aqui sintetizamos os principais achados em estudos proteômicos e posteriores direções a serem tomadas de forma a melhor compreender a bioquímica da esquizofrenia, bem como revelar biomarcadores.

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Palavras-chave: Proteômica, esquizofrenia, espectrometria de massas, metabolismo de energia, oligodendrócitos.

Introduction

Proteomics is a relatively new science which has been used for understanding and identifying affected molecular pathways providing a picture of integrated biochemical systems as well as potential biomarker candidates. Considering that the development and progression of schizophrenia (SCZ) – a chronic mental disorder which may affect up to 1% of the world population – is likely to be triggered by an intricate interaction among genetic predisposition, environmental factors and altered gene and protein expression, proteomics is consequently a suitable tool for unraveling SCZ molecular alterations and identifying biomarkers. *Post-mortem* and *in vivo* tissue such as brain, cerebrospinal fluid (CSF), liver, fibroblasts and blood serum collected from SCZ patients have been investigated extensively in the past 10 years using proteomic methodologies, which are summarized below[1].

Main outcomes

Brain, CSF and neuroendocrine system

Brain regions as frontal cortex, dorsolateral prefrontal cortex, anterior temporal lobe, anterior cingulate cortex, corpus callosum, insular cortex, posterior superior temporal gyrus, thalamus and hippocampus have been studied by proteomics. Alterations in cytoskeleton architecture through the differential expression of proteins such as GFAP, NEFL, NEFM, DNM1 and TUBB are supported by the differences observed in these brains when compared to mentally healthy controls[2]. The differential expression of these proteins may interfere in several processes, from cellular structure and communication up to synaptic transmission, which may also be disturbed due to the differential expression of calcium-related proteins such as CALN (PPP3CA), CALM and PMCA-4[3]. Calcium is an essential metabolite for the dopamine hypothesis in SCZ, especially considering its crucial role in the function of dopamine receptors D1 and D2[4].

Several proteomic differences observed in postmortem brain tissue revealed proteins related to oligodendrocytes[5]. Interestingly, these were also found in previous transcriptomic studies[6,7]. Levels of MBP and MOG, classical multiple sclerosis markers, were found altered in the CSF of SCZ patients compared to controls[8] suggesting potential degenerative processes even if not the classical neurodegeneration. In addition, the hypothesis of disturbed metabolism of cholesterol and phospholipids in SCZ is supported by proteomics data in CSF studies by the differential expression of APOE, APOA1 and PTGDS[9,10]. Furthermore, neurodegenerative processes may be triggered by dysfunction of energy metabolism in oligodendrocytes[11].

Consistent differences across the different brain regions analyzed were also observed in energy metabolism-related proteins supporting the "hypofrontality" concept, and reduced blood flow in some regions of the brain[12-14]. The consistent differential expression of ALDOC, ENO2, HK1, PGAM1, TPI1 and GAPDH place Glycolysis as one of the main biochemical pathway for further studies, basic or clinical. Interestingly, this is not the most affected pathway found in the prefrontal cortex of depression brains but oxidative phosphorylation[15].

Differences in energy metabolism found in the brain were also observed in liver samples in a proteome comparison study analyzing 15 livers from SCZ patients and 15 controls. Oxidative stress proteins have also been found[16]. This study is particularly interesting considering that the liver releases many molecules directly into the

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bloodstream that can be indicative of altered metabolism and could be used to translate brain findings to peripheral tissues. Proteome analyses of pituitaries from patients and controls also revealed energy metabolism impairments such as differential expression of AVP and GLUT1 which are responsible to control circulating glucose levels[17]. Additionally, apolipoproteins, as in CSF, were found differentially expressed in pituitaries.

Serum and skin fibroblasts

As means to translate CNS findings to the periphery, skin fibroblast may be an interesting sample of choice since many molecular pathways are shared with neuronal cells. Skin fibroblasts can be biopsied from living patients and can also be used as a source of biomarkers. Using label-free proteomics, the proteomes of 12 SCZ patients were compared to 12 controls leading to the identification of cell cycle proteins as well as energy metabolism and oxidative[18].

Blood is the most easily accessible tissue for clinical studies. While findings in CNS and liver are useful for the comprehension of the molecular bases of SCZ, studies in blood serum can yield potential biomarkers for clinical use as for diagnosis, prognosis, patient stratification and studies of drug treatment response.

Ten proteins were found differentially expressed in a set of 55 samples from first-onset drug-naive SCZ patients in comparison to 33 controls[19]. These proteins were adapted into a DiscoveryMAP multiplex immunoassay platform (Myriad Genetics) leading to a blood test capable of distinguishing drug-naïve SCZ patients from controls. This test has been marketed under the trade name VeriPsych™ only in USA and according to the manufactures "…it is not intended to provide a definitive diagnosis of schizophrenia, or to be used as the sole means of patient treatment" but used as an aid to the diagnosis (http://www.veripsych.com/about).

In a targeted proteomic study of 66 SCZ patients compared to 68 controls, increased levels of circulating levels of proinsulin, des-31,32-proinsulin, insulin, connecting (C)-peptide and chromogranin A were observed[20]. The same research group found increased levels of insulin, chromogranin A pancreatic polypeptide, prolactin, progesterone and cortisol in the serum of 236 SCZ subjects compared to 230 controls as well as decreased levels of growth hormone. Both studies support the metabolic and HPA axis dysfunction in SCZ[21].

Conclusions

Proteomic studies in brain tissue aimed to reveal protein biomarkers which could have a diagnostic potential so as drug treatment response and prognostic biomarkers. Although several potential biomarkers candidates[22] were revealed in several brain regions, these could not be directly translated to peripheral tissue for clinical use. On the other hand, data resulting from these analyses could shed light in the comprehension of SCZ. For instance, whilst energy metabolism dysfunction was known in SCZ brain, proteomic studies could pinpointing the exact enzymes and pathways mostly involved in the disease. Similarly, protein expression differences of cytoskeleton proteins were also characterized. Impairments in oligodendrocytes metabolism could also be supported by proteomics in brain tissue, also suggesting the pivotal role of glia in SCZ, as it has been hypothesized[23]. Studies in the SCZ CSF revealed more tangible biomarkers, but the sampling is much more invasive and painful then blood serum, on which successful results could have been obtained. Serum analyses have led to the development of a product that helps diagnosis. Although this test is not a definitive product, the same strategy can be applied for studies of drug response and effectiveness. Next steps include following up the protein markers found on those proteomic studies. About SCZ pathobiology, functional studies involving knock-outs and overexpression of certain protein targets may decipher their role in preclinical models. From the biomarker point of view, follow up studies must aim turning basic findings in clinical applications. Patient stratification studies and characterization of drug response are needed.

Proteomics methodologies

Since proteomics aims for complete representation of a set of proteins expressed by a cell, tissue, or organism on a given time at a given condition, it is important that such analysis can reach the most complete visualization of the proteins in such contexts[24].

The main steps of a proteomic analysis include protein extraction, separation, identification and, in most cases, a quantitative analysis. It is important to highlight that not all extracted proteins are identified. Generally, there are groups of proteins such as extremely acidic or basic proteins as well as very large or small and hydrophobic proteins which are less represented due to their physicochemical characteristics, even if they are present at significant amounts in the analyzed cell, tissue or organism. Besides, the proteome of a given cell present oligarchic characteristic considering the wide dynamic range of proteins concentration. Therefore, in order to reach the most complete proteome visualization, the step of protein extraction becomes a critical component in any proteomic analysis, since it is necessary to extract as more proteins as possible. An interesting approach for sample preparation is to extract subproteomes by separating sets of proteins according to their characteristics or cellular localization enabling better resolution. There are several different extraction methods[25], each one presenting weaknesses and strengths. Thus, their combination may generate a wider coverage of the proteome of interest.

The next step is the fractionation of the extract proteins. These can be classified in general as "in gel" or "off gel" fractionation. The main in gel approach for proteomics is two-dimensional electrophoresis (2-DE). In principle, proteins are separated firstly according to their isoelectric point (pI) followed by an SDS-PAGE step on which are separated according to their molecular mass (MW). The result is a Cartesian plot on which the "x" axis represents the pI and the "y" axis represents the molecular masses of the proteins. Each one of the dots of this plot – the so called spots – represents, in theory, different proteins. Resultant gels can be stained post-electrophoresis by classical protocols such as coomassie blue or silver staining. An alternative technique is the two-dimensional differential gel electrophoresis (2D-DIGE) on which protein samples are labeled with fluorescent dyes and mixed prior 2-DE. Only one gel is needed and proteins can be observed and quantified using a fluorescence scanner. Specific computational programs for image analysis perform densitometry measurement, allowing relative quantification of protein spots and comparison among different gels. Spots found significantly different among compared samples can be excised from the gel and processed for further analyses. The protein identification is performed by mass spectrometry (MS) using a methodology named "peptide mass fingerprinting" (PMF). Here, after spot digestion with a defined protease, molecular masses from the resulting peptides are obtained by MS. These masses are compared to theoretical masses obtained by *in silico* digestion of the whole translated genome of interest, leading to the protein identification. Drawbacks of the combination 2-DE/MS are the existing variation among different gels – which are reduced using 2D-DIGE, since different samples can be compared on a single gel – the representation of extremely acidic and basic proteins as well as hydrophobic proteins.

Off-gel approaches are a milestone on proteomics since data acquisition throughput, sensitivity and accuracy have improved significantly and several drawbacks associated to 2-DE were overcome. The main principle of "shotgun proteomics"[26] is to digest the whole proteome of interest before separation. Peptides are then separated by liquid chromatography and usually eluted online into a mass hybrid spectrometer (LC-MS/MS). Depending on the sample complexity steps of pre-fractionation before LC-MS may be needed. Pre-fractionation methods can be other types of chromatography, isoelectric focusing or in-gel electrophoresis. Protein identification relies strongly on computational resources that combine and interpret data generated by MS/MS.

Quantification can be performed by different methods. Stable isotopes labeling *in vitro* such as ICAT[27], and ICPL[28] allow the

comparison from two to four samples. Prior to LC-MS/MS, protein or peptide of each sample are labeled with two to four stable isotopes – so called tags. The tags are chemically identical, but may carry different combination of heavy isotopes, usually ^{2}H, ^{13}C or ^{15}N. Since the mass of the tags are known, it is possible to identify by MS labeled peptides, allowing the identification of peptides. The relative quantification is performed by comparing the peak intensities of each peptide labeled with different tags. Another quantitative methodology which has been used on psychiatric proteomic research is the isobaric tags for relative and absolute quantification – iTRAQ[29] which allows the quantification of up to eight samples at once. iTRAQ tags are composed by three parts: a reactive site, a balance region (BR) and the reporter ion (RI). BR and RI of each tag have different masses which are complementary in order to make all tags isobaric. Thus, the same peptides species from different samples present the same molecular mass even when labeled with different tags. But once submitted to MS/MS, the BR is lost and each RI will present its different mass, providing data for relative quantitation through the analysis of their intensities. The disadvantage of methods utilizing such labels is that the labeling procedure occurs after sample extraction and preparation, what can lead to the insertion of technical errors and loss of accuracy on quantification procedure. *In vivo* labeling is also an alternative as discuss in the article of Dr. M. Filiou, in this same edition. Another well characterized *in vivo* labeling approach is SILAC (stable isotope labeling by amino acids in cell culture)[30]. Here, cell culture is grown on a media with heavy aminoacids – preferably lysine and arginine to do the large use of trypsin as enzyme in proteomics. This allows the comparison to cells grown in non-labeled media. SILAC can also be applied in animal models. Alternatively, shotgun proteomic data can be analyzed using label-free quantification. This technique is based on the fact that the concentration of a certain peptide of a given sample is directly proportional to its peak chromatographic area[31]. Based on this principle, it is possible to analyze an unlimited number of samples what is suitable for clinical and biomarker studies.

Not all proteomic methods are explored by psychiatric research. For instance, not so much has been done using large-scale studies of protein post translational modification as well as targeted analyses as selected/multiple reaction monitoring (SRM/MRM). The proteomic toolbox may still provide more solutions and answers to psychiatric studies[25].

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