PROCEEDING

The quest for brain disorder biomarkers*

Christoph W Turck, Giuseppina Maccarrone, Eser Sayan-Ayata, Archana M Jacob, Claudia Ditzen, Helena Kronsbein, Isabel Birg, Can-Carlo Doertbudak, Katrin Haegler, Maria Lebar, Larysa Teplytska, Nik Kolb, Nkemdilim Uwaje, and Richard Zollinger

Max Planck Institute of Psychiatry, Molecular, Cellular, Clinical Proteomics, Munich, Germany

Abstract : The identification of disease markers in tissues and body fluids requires an extensive and thorough analysis of its protein constituents. In our efforts to identify biomarkers for affective and neurological disorders we are pursuing several different strategies. On one hand we are using animal models that represent defined phenotypes charactersistic for the respective disorder in humans. In addition, we are analyzing human specimens from carefully phenotyped patient groups. Several fractions representing different protein classes from human cerebrospinal fluid obtained by lumbar puncture are used for this purpose. Our biomarker identification efforts range from classical proteomics approaches such as two dimensional gel electrophoresis and mass spectrometry to phage display screens with cerebrospinal fluid antibodies. J. Med. Invest. 52 Suppl. :231-235, November, 2005

Keywords **:** *brain disease, biomarkers, proteomics*

INTRODUCTION

A study by the *World Health Organization* has found that diseases of the central nervous system are affecting a significant portion of the population and are amongst the leading causes of death in Western societies(2). Although great advances have been made in our understanding of the pathophysiology of psychiatric and neurological diseases, significant gaps remain in our knowledge about their ultimate causes. A major problem in the area of affective diseases is the fact that current diagnosis is mainly based on categorizing the signs and symptoms of the syndrome, which limits the ability to reliably identify biological causes and develop specific treatments. Unlike other disorders like diabetes and heart disease where biological markers are at hand that allow the physician to come up with a more reliable diagnosis there are currently no such markers available for affective diseases. Here the only means for diagnosis is the *Diagnostic and Statistical Manual of Mental Disorders* (DSM) (3). This manual systematically identifies different mental illnesses according to a list of symptoms but does in no way address the underlying cause of the disease. A major goal in the area of affective disorders is therefore the identification of markers that can categorize subsets of subjects in a consistent manner. This will allow a more precise definition and categorization of affective disorders and in turn facilitate investigations of the pathogenesis of the diseases and enhance our ability for treatment.

Proteomic technologies promise to be of great value in molecular medicine, particularly in the detection and discovery of disease markers. Whereas there are multiple lesions on the genome that differ for each individual there are common protein signatures for polygenic diseases like affective and neurological disorders. The proteome is therefore thought to be more directly related to the pheno-

*This article is a modified reprint of an article published in Laborwelt (1).

Received for publication September 9, 2005 ; accepted September 16, 2005.

Address correspondence and reprint requests to Christoph W. Turck, Ph. D., Max Planck Instiute of Psychiatry, Kraepelinstr. 2, D-80804, Munich, Germany and Fax : +49-89-30622200

type of an organism and hence protein profiling will result in the most precise understanding of disease mechanisms as well as the molecular effects of drugs.

Protein amounts and differential modifications are potential biomarkers in tissues and body fluids. The ultimate goal of proteomics in medicine is therefore to provide quantitative and qualitative data of sample proteins that reflect a certain phenotype, disease state or a response to disease treatment.

In the course of our studies we have found that animal models for disease show a high success rate for the identification of biomarkers due to the rather homogeneous genetic background that the animals have. Differences in protein expression between diseased and control animals are therefore more likely to be related to the disease process. The question remains how relevant the identified markers in the mouse model are for the disease in humans. Whereas this is less of an issue when specimens from patients are used as the source for biomarker discovery the great "background noise" due to individual variability combined with the limited sample amount makes the analysis of human samples orders of magnitude more challenging than those carried out with animal models.

BIOMARKER DISCOVERY PLATFORMS

1. Mouse Model For Trait Anxiety

The manifestation of anxiety in a number of psychiatric disorders such as anxiety disorder, depression, panic attacks, phobias, obsessive-compulsive disorders and post-traumatic stress disorders (4) highlights the importance of gaining a better understanding of associated reliable biomarkers in animal models. A relevant animal model to study behavioral, neuroendocrine and genetic concomitants of trait anxiety including psychopathology should represent a good approximation to score symptoms of anxiety disorders and also comorbid depression (4). Genetic approaches currently available in the mouse make this model organism particularly powerful for the functional analysis of candidate genes and in defining gene products underlying trait anxiety and depression (5).In order to avoid inter-strain comparisons, likely to reveal differences in more than just anxiety-related indices, my colleague Rainer Landgraf, who is heading the *Behavioral Neuroendocrinology* research group at the *Max Planck Institute of Psychiatry*, has used intra-strain breeding approaches to focus on particular traits, including anxiety-(6) and depression-like behavior (7) .The technique of selective bidirectional breeding enhances the representation of genetic material associated with a particular trait shifting the animals' phenotype bidirectionally from the strain mean. Following this approach the Landgraf group has managed to generate and validate CD1 mouse lines of hyper-anxious (HAB-M) and hypo-anxious (LAB-M) phenotypes as model of extremes in trait anxiety (8).

Using classical proteomics approaches we have been able to identify two biomarkers that reliably distinguish the HAB-M and LAB-M phenotypes (8). One marker protein that differs in its expression level between the two mouse strains was identified as glyoxalase I. In addition, we have also been able to detect a qualitative difference of unknown nature in another protein. This difference becomes apparent by a shift in mobility in the first dimension isoelectric focusing as well as second dimension SDS gel electrophoresis.

A major goal in the area of mental disorders is the identification of biomarkers that can categorize subsets of subjects in a more reliable and consistent manner. The approach of selectively breeding mice using an intra-strain approach to focus on particular traits, in this case anxiety-related behavior, has allowed the identification of protein markers that are differentially expressed. The predictive validity of these markers to identify different levels of trait anxiety provides the basis for future testing including its impact beyond that of a biomarker, i.e. do they contribute to rather than merely parallel the manifestation of trait anxiety? To answer this question and to further pursue the functional implications of the identified markers we are planning to extend our screening studies to specimens from patients admitted to the anxiety clinic at the *Max Planck Institute of Psychiatry*. As is the case for all polygenic diseases we do not anticipate that a single marker will be able to unequivocally distinguish between clinical phenotypes. Only through a combination of markers will it be possible to gain statistical significance to differentiate complex traits like anxiety-related disorders.

2. Cerebrispinal Fluid Proteome

Short of the analysis of brain biopsies cerebrospinal fluid (CSF) is the specimen that is most relevant for human brain disorder biomarker discovery efforts. Since it constantly perfuses brain tissue CSF contains mediators that reflect metabolic

processes in the brain. Furthermore, CSF can be obtained in a controlled fashion minimizing the dangers of variability introduced at the sample collection step. It is hypothesized that the changes in CSF proteins reflect the pathological alterations in the function of the central nervous system. Therefore the comparative analysis of the proteomes of human CSF from diseased and healthy subjects is becoming increasingly important for the identification of disease-specific proteins. Proteome analysis of body fluids such as CSF is a great challenge (9). The major hurdle when it comes to patient samples is the limiting amount of starting material that is available to carry out the analysis. Another reason for the difficult analysis of body fluid proteomes is their large dynamic range reflected by the presence of very abundant proteins like albumin and, in the case of CSF, minute quantities of brain-derived proteins (9). Further complicating the analysis of CSF is the possible infiltration of serum proteins that is caused by a leaky blood-brain barrier that is especially pronounced in patients with brain disorders. As a consequence it is often impossible to know if a protein that is found in CSF is derived from the brain or serum.

Proteome analysis in general involves two stages ; protein separation followed by identification and analysis. Multidimensional separations are required in order to result in an adequate resolution of complex protein mixtures in body fluids. Classical proteomic approaches employ fractionation on the protein level with the help of two dimensional-polyacrylamide gel electrophoresis (2D-PAGE). This technique produces high resolution protein separations resulting in the display of potentially thousands of protein spots. Alternatively, tryptic peptides derived from the proteins in the mixture can be subjected to shotgun mass spectrometry analysis. In the shotgun mass spectrometry approach proteins are digested by specific enzymes into small peptides and analyzed on-line by mass spectrometry. A major advantage of the shotgun mass spectrometry approach is that low abundant proteins can be identified in the presence of high abundant proteins, a scenario that is often encountered when analyzing protein mixtures from body fluids like serum or CSF. We have been able to identify a large number (over 500) of the constituents of the human CSF proteome using a combination of isoelectric focusing and reversed phase chromatography followed by shotgun mass spectrometry (10,11). After depletion of the most abundant proteins from the CSF mixture with the help of an immunoaffinity column the peptides resulting from a proteolytic digest of the remaining proteins were first fractionated by isoelectric focusing in an IPG strip. The extracted peptides were then further fractionated on a nano reversed phase column and analyzed on-line by iontrap tandem mass spectrometry. Using this strategy we have been able to greatly increase the number of CSF proteins identified. Many of these proteins are of low abundance and represent intracellular components such as signaling proteins and transcription factors. These findings indicate that intracellular contents of cells and tissues is released into CSF presumably through apoptotic and necrotic mechanisms. Although it is difficult to know how many proteins are exclusively derived from the brain since many proteins are also expressed in other tissues and therefore could potentially be introduced into CSF through its exchange with serum, we now have evidence that many of the proteins that were identified in CSF are in fact derived from brain tissue. This is based on a comparison of the identified CSF proteins with a recently published human serum protein database(12). We found proteins with a wide range of isoelectric points using the described shotgun mass spectrometry approach. To achieve a comparable coverage of proteins with 2D-PAGE analysis one needs to run several gels with overlapping pH gradients. This requires more sample and is often prohibitive when dealing with human body fluids and tissues.

In order to gauge the sensitivity of our CSF proteome mining efforts we have compared our CSF protein list against a relational database for markers for affective and neurological disorders that we have established using data from the public domain. The comparison revealed that our CSF protein list contains several candidate markers that have been previously associated with affective and neurological disorders. This finding confirms that immunodepletion of abundant proteins followed by an extensive prefactionation of peptides resulting after proteolysis of the remaining proteins leads to a rather sensitive coverage of the CSF protein constituents. This is in part due to the generation of a smaller dynamic range of the remaining protein mixture. In addition, due to the smaller amount of total protein that results from the combination of immunodepletion and prefractionation a greater equivalent of the CSF sample can be used during the shotgun mass spectrometry analysis without

the risk of overloading the reversed-phase nano column that is employed on-line with the mass spectrometer.

3. Biomarker Identification with Cerebrospinal Fluid Autoantibodies

Next to the classical proteomic efforts geared towards the analysis of the protein constituents in CSF (10,11) we have also set out to establish alternative strategies for the identification of novel disease markers for affective disorders. In an effort that has been funded as an "Exploratory Project" within the *National Genome Research Network* (NGFN-2) we want to exploit the great specificity of the body's immune system. Specifically, we are analyzing the antibody pool that is present in CSF. Autoantibodies against a variety of CNS proteins in serum and CSF of patients with affective disorders have been described. An interesting phenomenon supporting a potential involvement of an autoimmune component in affective disorders is the fact that the pattern of disease progression, the age of onset and the repeating periods of regression and recovery are very similar between autoimmune diseases and affective disorders. The identification of autoantigens could ultimately lead to novel diagnostic and therapeutic procedures for an early detection and treatment of affective disorders. Similar approaches have already provided valuable information in other disease areas. These include *Type I Autoimmune Diabetes Mellitus* and *Rheumatoid Arthritis*. Both of these disorders present examples of T cell-mediated autoimmune diseases for which autoantibodies have already found a clinical utility.

It is well known that any endogenous epitope that shares consensus sequences with exogenous antigens as well as abnormal protein expression levels, mutations or impaired chaperone activities producing misfolded proteins can raise a humoral or cellular autoimmune response. Hence, the basis of this approach stands on the hypothesis that all these factors may trigger an autoimmune response within the CNS especially in individuals with affective disorders that are propositioned to immune system dysfunction and impaired blood-brain barriers. Detection of the epitopes of CSF autoantibodies may reveal candidate mechanisms of affective disorders and help identify specific biomarkers for them. We further hypothesize that the presence of markers for affective diseases is reflected by the appearance of autoantibodies in CSF. These autoantibodies can be used for the identification of their respective autoantigen counterparts that consequently represent markers of disease.

In preliminary experiments we have used CSF antibodies in a Western blot with brain proteins as targets. For this purpose we subjected a human brain protein extract to SDS gel electrophoresis and subsequently transferred the proteins to a membrane. Each slot was then probed separately with CSF. Although the results demonstrate that specific autoantibodies are present in CSF and can be detected using biochemical techniques the identity of the autoantigens remains obscure. The realization that the identification of brain autoantigens is limited by the CSF sample that is available prompted us to explore alternative experimental strategies. The sensitivity of the phage display approach that we employ in our studies will greatly assist in the discovery of autoantigens that are expressed at low levels.

OUTLOOK

The proteomics oriented strategies that I have outlined above are only part of a multidisciplinary effort that a number of research groups are pursuing at the *Max Planck Institute of Psychiatry* in order to improve our understanding of affective and neurological disorders. Other approaches include targeted as well as global genotyping efforts and microarray studies for differential RNA expression.

An integrated evaluation of the data that are obtained by all the holistic approaches in combination with clinical and epidemiological data will eventually not only increase our understanding of disease mechanisms but subsequently also enable us to develop more specific and individualizedc medi-cines and therapies.

ACKNOWLEDGEMENTS

This work was supported by the *Max Planck Society*, the *National Genome Research Network* (NGFN-2) of the *Bundesministerium für Bildung und Forschung* and a grant from the *Bavaria California Technology Center* (BaCaTeC).

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