Journal of Psychiatric Research 47 (2013) 1572–1580

Contents lists available at ScienceDirect

Journal of Psychiatric Research

journal homepage: www.elsevier.com/locate/psychires

Psychiatric patient stratification using biosignatures based on cerebrospinal fluid protein expression clusters

Giuseppina Maccarrone [a,1], Claudia Ditzen [a,1], Alexander Yassouridis [a], Christiane Rewerts [a], Manfred Uhr [a], Mathias Uhlen [b], Florian Holsboer [a], Christoph W. Turck [a,*]

[a] Max Planck Institute of Psychiatry, Kraepelinstr. 2, D-80804 Munich, Germany
[b] Department of Biotechnology, Albanova University Center, Royal Institute of Technology, SE-10691 Stockholm, Sweden

ARTICLE INFO

Article history:
Received 13 May 2013
Received in revised form
11 July 2013
Accepted 11 July 2013

Keywords:
Psychiatric patient group stratification
Biosignatures
Multiplex analysis
Cerebrospinal fluid
Protein levels
Protein clusters

ABSTRACT

Psychiatric disorders are caused by perturbed molecular pathways that affect brain circuitries. The identification of specific biosignatures that are the result of altered pathway activities in major depression, bipolar disorder and schizophrenia can contribute to a better understanding of disease etiology and aid in the implementation of diagnostic assays.

In the present study we identified disease-specific protein biosignatures in cerebrospinal fluid of depressed (*n*: 36), bipolar (*n*: 27) and schizophrenic (*n*: 35) patients using the Reverse Phase Protein Microarray technology. These biosignatures were able to stratify patient groups in an objective manner according to cerebrospinal fluid protein expression patterns. Correct classification rates were over 90%. At the same time several protein sets that play a role in neuronal growth, proliferation and differentiation (NEGR1, NPDC1), neurotransmission (SEZ6) and protection from oxidative damage (GPX3) were able to distinguish diseased from healthy individuals (*n*: 35) indicating a molecular signature overlap for the different psychiatric phenotypes. Our study is a first step toward implementing a psychiatric patient stratification system based on molecular biosignatures. Protein signatures may eventually be of use as specific and sensitive biomarkers in clinical trials not only for patient diagnostic and subgroup stratification but also to follow treatment response.

1. Introduction

At present the diagnosis of most psychiatric disorders is based on a personal psychiatric interview according to international guidelines and classification systems (ICD-10; DSM-IV) (World Health Organization, 2010; American Psychiatric Association, 1994). Since the differentiation of psychiatric phenotypes is exclusively based on presented clinical symptoms and does not account for biological differences, the distinction of patient subgroups remains difficult. Especially in primary care settings the correct classification of a psychiatric disorder during its first onset can be very challenging in the absence of valid and objective biomarkers. For example, a first episode of major depression could either be the beginning of recurrent MDD or represent the onset of BPD. Likewise, a severe depressive episode with psychotic symptoms could be misinterpreted as a schizophrenic episode.

The classification of mental disorders would greatly benefit from objective biological markers as part of the diagnostic process (Schwarz and Bahn, 2008). This would enable improved therapeutic intervention and could result in a reduction of psychiatric patients' relapse rates. Enhanced patient stratification would also enable personalized treatment strategies in alignment with individual patient pathophysiology. In addition, and of equal importance, the knowledge of molecular signatures, including protein expression level differences, would ultimately augment our understanding of affected molecular pathways characteristic of clinical manifestation and disease course (Ditzen et al., 2012).

It is widely accepted that mood disorders and schizophrenia are of multifactorial origin (Falkai et al., 2008; aan het Rot et al., 2009). Aside from the genetic component diverse other factors can ultimately lead to a dysfunction of central nervous system pathways. Clinical symptoms of different psychiatric disorders as well as the intensity and course of the disease are likely reflected in distinct molecular patterns. Overlapping molecular signatures on the other

* Corresponding author. Tel.: +49 89 30622 317; fax: +49 89 30622 610.
E-mail address: turck@mpipsykl.mpg.de (C.W. Turck).
[1] The authors have equally contributed.

0022-3956/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jpsychires.2013.07.021

hand may illuminate common pathophysiological pathways between different psychiatric disorders (Alaerts and Del-Favero, 2009; Prasad et al., 2010; Cichon et al., 2011; Binder and Nemeroff, 2010; Blackwood et al., 2007; Moskvina et al., 2009).

Due to its close proximity cerebrospinal fluid (CSF) was chosen for the identification of brain disorder molecular biosignatures (Zougman et al., 2008). Next to blood-derived proteins CSF also contains proteins and other molecules originating from the brain which can be used to examine pathological mechanisms associated with a psychiatric disorder (Huang et al., 2006). Specifically, the analysis of CSF proteome alterations can be exploited to distinguish between disease and health as well as different types of psychiatric disorders (Yuan et al., 2002). In previous studies we have carried out proteomic analyses of CSF from psychiatric patients that have resulted in a list of biomarker candidates (Ditzen et al., 2012; Maccarrone et al., 2004). Fifty nine proteins from this list were chosen based on literature reports that have implicated them in the etiology of mood disorders, schizophrenia and neurodegenerative disease (Table S1). The goal of the present study was to interrogate these biomarker candidates with regard to their ability to distinguish controls from patients and stratify individual disease groups.

For a sensitive and reproducible analysis of CSF proteins we have chosen the Reverse Phase Protein Microarray (RPPM) technology (Voshol et al., 2009) that allows the detection and quantification of protein analytes in a high throughput manner. Furthermore, the simultaneous measurement of a great number of patient samples enhances analytical accuracy. With the help of the RPPM technology and statistical evaluation we were able to correctly distinguish controls from diseased patients and stratify psychiatric patient groups based on CSF protein expression patterns.

2. Materials and methods

2.1. Cerebrospinal fluid specimens

Lumbar puncture was performed in sitting position at 8 am using an atraumatic needle. CSF was collected from sober patients suffering from MDD, BPD, SCZ and from healthy controls. In order to remove cellular debris, CSF samples were centrifuged, aliquoted and stored at −80 °C. Patients provided informed consent during hospital admission that their CSF can be used for scientific purposes following routine clinical analyses. The use of CSF samples for scientific studies has been approved by the *Bayerische Aerztekammer*. Samples are stored anonymously in the CSF bank of the *Max Planck Institute of Psychiatry*. For the present study CSF samples from MDD ($n = 40$), BPD ($n = 40$) and SCZ ($n = 40$) patients fulfilling ICD-10 criteria for a major depressive episode (F32/33.0–9), bipolar disorder (F31.0–9) and schizophrenia (F20.0–9), respectively, and healthy control subjects (n: 40) were selected (Supplementary Table S2). The BPD group included manic and depressed individuals. Controls represent subjects visiting the 'Neurological Outpatient Unit' at the *Max Planck Institute of Psychiatry*. Controls had unspecific and temporary complaints like vertigo or headache. For all control subjects any neurological or internal disease had been ruled out and subjects were declared "healthy". These individuals were then asked to take part in the study. After agreeing they were subjected to a psychiatric interview in order to exclude psychiatric disorders.

In addition to diagnosis, other patient information includes previous and current medication, results of physical and neurological examination and hematological, clinical chemistry as well as CSF sample laboratory data. Clinical diagnostic classification is performed by experienced psychiatrists during hospitalization of the patients. CSF samples with abnormal levels of glucose, lactate, cells, positive oligoclonal bands or disturbance of the blood–brain-barrier as well as blood-contaminated samples were not considered. Due to these strict exclusion criteria the initial number of samples for the analyses had to be reduced accordingly: MDD, $n = 36$ (age: 44 +/− 16; 56% female), BPD, $n = 27$ (age: 44 +/− 15; 56% female), SCZ, $n = 35$ (age: 37 +/− 14; 57% female), controls $n = 35$ (age: 40 +/− 16; 66% female).

2.2. Antibodies

Based on our previous studies we selected protein analytes that have been implicated in the etiology of mood disorders and SCZ. For RPPM screening 51 antibodies were provided by the *Human Protein Atlas* (HPA) program (Albanova University Center, Royal Institute of Technology, Stockholm, Sweden) (Table 1A). The polyclonal antibodies specific for the human proteins were produced by cloning and expression of 'Protein Epitope Signature Tags' (PrESTs). The experimental procedure followed for cloning, immunization and affinity purification has been described elsewhere (Agaton et al., 2003; Nilsson et al., 2005). The antibodies were provided as affinity purified reagents in Tris–HCl buffer, supplemented with 50% glycerol and 0.02% sodium azide as preservatives, and stored at −20 °C. The total protein concentration was determined by absorbance measurements at 280 nm. The binding specificity of purified antibodies was ascertained with a PrEST-array. Eight polyclonal anti-human antibodies specific for chromogranin A, cystatin C, fibronectin, glutathione peroxidase 1, glutathione peroxidase 3, presenilin-1, synapsin and synaptophysin (C-terminus) were commercially obtained. The antibodies and their suppliers are listed in Table 1B.

2.3. Immunoblot analysis

Three μg human CSF proteins were resolved by SDS-PAGE. The proteins were electroblotted onto PVDF membrane (Millipore, Schwalbach, Germany). The membranes were incubated with primary antibody (anti-fibronectin (1:1000), anti-chromogranin A (1:500) and anti-cystatin C (1:8000) in TBS-T buffer (20 mM Tris–HCl, 137 mM NaCl, 0.05% Tween 20 pH 8) for 1 h at RT, followed by a 2 h incubation at RT with a 1:2000 dilution of secondary antibody IgG-HRP (GE Healthcare, Piscataway, NJ). Proteins were visualized by enhanced chemiluminescence using the ECL reagent (GE Healthcare) and exposed to autoradiography films. For quality assessment of the commercial antibodies instructions for the immunoblots published by the manufacturers were used. Antibodies produced by the PrEST method were quality controlled by Western blot and immunohistochemistry using human tissue or cells (http://www.proteinatlas.org). Western blots for some antibodies used in the RPPM screening are depicted in Supplementary Fig. S1.

2.4. Reverse Phase Protein Microarray screening

The RPPM screening analysis was performed by NMI Technologies Transfer GmbH (NMI-TT, Reutlingen, Germany) using a protein microarray platform (Zeptosens, Witterswil, Switzerland) (Pawlak et al., 2002).

2.4.1. CSF sample preparation

Sample preparation for microarray printing was carried out by NMI-TT. CSF protein concentration was estimated using Bradford protein assay (BioRad, Hercules, CA).

CSF samples were diluted 1:2 in PBS buffer at pH 7.0 with 10% glycerol. This dilution was chosen for a final print protein concentration < 0.4 mg/ml. Two samples had to be diluted fourfold because the protein concentration was above 0.8 mg/ml. This is the upper limit of printed sample protein concentration for the RPPM method to guarantee assay signals with linear characteristics.