Chapter 5

Posttraumatic Stress Disorder Biomarker — p11

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Abstract

Post-traumatic stress disorder (PTSD) is a chronic and disabling anxiety disorder associated with a traumatic event [1]. It is linked to increased risk of suicide and deficits in social functioning [2, 3]. Despite extensive study in psychiatry, the underlying mechanisms of PTSD are still poorly understood [4, 5]. Currently, the diagnosis for PTSD is based on clinical observation and symptom checklist [4, 6-8] and no laboratory blood-based tests. Although biomarker discovery for PTSD is not easy [8], a reliable biomarker would significantly impact the diagnosis and therapeutic monitoring of PTSD. Developing interventions to identify and treat PTSD requires objective approaches to determining the presence of PTSD [8]. Substantial data indicate several potential biomarkers for PTSD. Of these candidate markers, p11 (S100A10) has been studied in PTSD animal models [7] and in human subjects with PTSD [6]. We found that p11 is over-expressed in both animal models and post-mortem brains of subjects with PTSD [7]. Incorporating testing of p11, a novel biomarker for PTSD, into clinical practice, along with more subjective measures, such as participants' medical history, mental status, duration of symptoms, and symptom checklist or self-report, would provide additional power to predict impending PTSD. In this chapter, we discuss the biomarker concept and the potential clinical utility of PTSD biomarkers. We further discuss the potential of p11 as a PTSD biomarker and as a tool that may enhance PTSD diagnosis and intervention in health care practice.

Keywords: PTSD, p11, biomarker

1. Introduction

Post-traumatic stress disorder (PTSD) is a chronic and disabling anxiety disorder which is a result of exposure to a traumatic event and is associated with an increased risk of suicide and marked deficits in social dysfunction [2, 3]. Currently, a PTSD diagnosis is made based on clinical observation, symptoms, and the duration of symptoms. In 2013, the American Psychiatric Association's *Fifth Edition of the Diagnostic and Statistical Manual of Mental Disorders* (DSM-5) revised PTSD diagnostic criteria to better codify it to adults, adolescents, and children over age six. The criteria currently include exposure to a traumatic event and symptoms of four symptom clusters: intrusion, avoidance, negative alterations in cognitions and mood, and alterations in arousal and reactivity. Additional criteria include the duration of symptoms [9]. Given that there are currently no laboratory biomarker tests for PTSD, this chapter will discuss the concept of using biomarkers to diagnose the potential for the existence of PTSD. Specific focus will be given to the potential of biomarker p11.

2. Biomarker concept

Biomarkers are defined as "cellular, biochemical, or molecular alterations that are measurable in biological media such as human tissues, cells, or fluids" [10-12]. Biomarkers can be measurable and quantifiable biological parameters, such as levels of protein or enzyme and hormone in the cerebrospinal fluid, saliva and blood, or mRNA specimen levels [10-12]. Those markers may be related to health and physiological conditions, metabolic processes, disease risk, psychiatric disorders, and environmental exposure, as well as disease diagnosis [13]. Biological changes, which link mechanism of drug action to clinical effectiveness, are also considered biomarkers [14]. Therefore, biomarkers may be considered as any substance, structure of molecule, or biological process that are measurable, influential and/or able to predict the incidence of outcome of disease (WHO International Program on Chemical Safety).

3. Biomarker classification and application

Biomarker research has advanced substantially as part of the Human Genome Project [15]. Initially, potential gene biomarkers were screened using high-throughput analytical instruments. Those markers were classified into four subgroups: type 0 biomarkers; type 1 biomarkers; surrogate end point-type (type 2 biomarkers); and risk markers (http://en.wikipedia.org/wiki/Human_Genome_Project). Depending on the function of those markers, they may be called imaging biomarkers (CT, PET, MRI), molecular biomarkers, or genetic markers. Molecular biomarkers include the markers in biological specimens (plasma, serum, saliva, cerebrospinal fluid, bronchoalveolar lavage, and biopsy). They can be peptides, proteins, lipids metabolites, enzymes, and other small molecules (http://en.wikipedia.org/wiki/Human_Genome_Project). Genetic markers are micromRNA, mRNA and DNA mutations, or

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polymorphisms. Finally, these markers may also be used for diagnosing the disease, determining the stage of the disease, monitoring the outcome of the treatment, and evaluating the disease prognosis.

4. Potential PTSD biomarkers

For the last 10 years, researchers have been focusing on developing a single test, such as a blood or genetic test, which may determine PTSD and simplify its treatment. In other medical fields, biomarkers have already been developed and used for diagnosis. For example, cholesterol measurements and genetic tests have been used for predicting the risks for heart attacks and Huntington's disease, respectively. In psychiatry, researchers hope to use the same strategies to identify and implement the biomarkers for predicting PTSD risk(s). However, the searching for such a test is still ongoing, though scientists are closer than ever to achieve this goal. Why is the search for biomarkers for PTSD needed when the disorder has been diagnosed by clinical-interview-based DSM diagnostic criteria for decades. Because, at least in part, biomarkers can help us better understand PTSD, its etiology and its pathologies, which in turn can lead to the development of more successful therapeutic approaches. It may help psychiatrists make faster and more effective decisions on the treatments based on the individual's pathology. In addition, biomarkers might identify high-risk populations that had not manifested any symptoms met in PTSD diagnostic criteria. To search for PTSD biomarkers, high-throughput omic approaches are required. The approaches used in biomarker studies of other diseases [16, 17] have just begun to be used in PTSD research. Although potential biomarker(s) for PTSD have been reported in the animal research [8] and human subjects research [6], the confirmation and validation of their clinical utility have not been accomplished. In PTSD biomarker studies, gene expression, metabolite levels and protein concentration in saliva [18], blood [19], cerebral spinal cord fluid [20], urine [21], and tissues [7] are compared between PTSD patients and healthy control subjects. In addition, physiological parameters (blood pressure [22], ECG [23], heartbeat [24], neurotransmitters [25-27], and brain imaging [28] are considered as biomarkers for PTSD.

PTSD biomarkers can be associated with PTSD-related behavioral characteristics (e.g., hypervigilance), the level or type of exposure to traumatic stress, genetic susceptibility, conditions of response to traumatic stress exposure, subclinical or clinical state, and conditions of response to therapy. During the course of PTSD, or after single or multiple traumatic stresses, the biomarkers can be identified. It also can be a predictor for PTSD-related risk, state, or progression. Like biomarkers identified in cancer research, PTSD biomarker research may be used for antecedent, screening, diagnostic, monitoring stage, and prognosis. One biomarker may be associated with a single or multiple phenotypes of PTSD, while a single symptom can be associated with multiple PTSD biomarkers.

Few studies have evaluated the use of multiple biomarkers for patients' risk stratification [29]. The desirable properties of biomarkers for PTSD vary with their different usages. For example, a screening test requires having high sensitivity, specificity, predictive values, large likelihood

ratios, and low cost to test a large population including normal and high-risk subjects. A diagnostic biomarker may be chosen for testing stages of the disease (acute and chronic). In those tests, sensitivity or specificity of monitoring biomarkers are less important because the marker from the same individual serves as his or her own control. The ability to monitor intra-individual variation is more important. Costs may be less important for prognostic markers since only those diagnosed with PTSD are subjects of focus.

In PTSD biomarker research, the sample size is often critical. A standard diagnostic biomarker test requires a relatively smaller sample size and a cross-sectional design. However, PTSD biomarker research requires a larger sample size and a prospective design. Although all biomarker features can be shown in one biomarker, a specific biomarker feature for use is the ultimate goal in the search for PTSD biomarkers. It would be ideal to have all the features to test PTSD and to help clinicians optimally manage PTSD patients with specific therapeutic targets. For example, the level of glucocorticoid [30] and/or concentration of epinephrine in the blood could be an indicator of responding to traumatic stress, or a plasma GABA level may evaluate whether or not the subject is recovering from trauma [31]. It is important that a biomarker test is an accurate, reproducible, standardized and acceptable procedure for PTSD diagnosis.

4.1. A strategy to identify a PTSD biomarker

The development of PTSD biomarker(s) for clinical utility is a process from preclinical development (potential biomarker identification) to clinical validation (clinical utility approval) [8]. This long process includes three major steps: screening, analytical validation, and clinical validation. In the initial stage of screening, the high-throughput approaches are used to search for the potential biomarker(s). Analytical validation is laboratory or bench work, which involves sample selection, collection, storage, and determination of the optimum analytical procedure that provide high reproducibility and accuracy. Clinical validation (clinical utility) is the final step of the development of an accurate biomarker test, including testing sensitivity and specificity of the diagnosis [8].

4.2. Screening biomarker(s) for PTSD

Biomarker screening is the first step in identifying a potential PTSD biomarker. During the screening stage, researchers can start the study from animal models or human subjects. In general, animal models needs to be validated [29]. If human subjects are used, the bias should be avoided in the sample selection process. Since current animal models of PTSD have used different stress paradigms, a wide range of behavioral responses is obtained. In many prior studies, the data are presented by the mean values (with the standard deviation or the standard error) of the entire study group. It is known that only a proportion of individuals (20%–30%) who are exposed to a traumatic event will eventually develop PTSD [29]. Therefore, it is key to identify a marker for those subjects who are in the risk population to provide a solid basis for biomarker validation research [29].

Unlike the research in other medical fields, PTSD biomarker study provides little information about the molecular mechanisms of post-traumatic psychopathology. Previously, a genome-wide screen in a validated animal model showed the possible biomarkers for PTSD [29]. A

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genomic analysis requires pure samples, which are identified by highly stringent criteria. The subjects are validated along three dimensions—analogous (similarity of phonotype), predictive (predictability of drug response or stress), and biological mechanism (regulation of gene expression and brain function). Once a potential biomarker is identified in the screening stage, an analytical validation is considered.

4.3. Analytical validation of the PTSD biomarker assay

A standardized biomarker validation process used in oncology has been considered for use in PTSD biomarker research. Adaptation of this methodological approach includes a rigorous definition and evaluation of the whole process of PTSD biomarker determination (analytical validation), and an assessment of the impact of PTSD biomarker on clinical practices (clinical validation).

Here, we briefly discuss three of the several terms that relate to analytical validation of the biomarker assay for PTSD. The first term is **precision**, which relates to reproducibility in that others should obtain similar results when following the original protocol procedures [32, 33]. Precision includes repeatability, intermediate precision, and reproducibility. Repeatability indicates the levels of the precision under the same operating conditions over a short interval of time and is referred to as intra-assay precision. Intermediate precision is within laboratory variations (different days, analysts, equipment, etc.). The second term is **accuracy**, which relates to the closeness of agreement between the value that is accepted either as a conventional true value or an accepted reference value(http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm073381.pdf). The third term is **detection limit**, which refers to the lowest quantity of a substance relative to a blank value within a stated confidence limit (generally 1%) (http://en.wikipedia.org/wiki/Detection_limit). A signal-to-noise ratio between 3:1 to 2:1 is generally acceptable for estimating the detection limit (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm073381.pdf).

There are four major phases in the analytical validation stage, including setting up and standardizing operating procedures, and obtaining an internal quality control and external quality assessment. In phase one, operating procedures are established for PTSD biomarker determination. Phase two entails the validation of the operating procedures in terms of precision and accuracy according to the standards definition of operating procedures. Phase three obtains an internal quality control by evaluation of the validated standards within the laboratory. The final phase of the analytical validation is to conduct an external quality assessment comparison and assessment of their accuracy between laboratories.

In summary, analytical validation is to examine whether the procedure is suitable for testing PTSD. The analytical procedures in the protocol should be clearly defined. Thus, analytical validation should be done in different testing sites (Lab) using the same protocol. Validation includes the biomarkers' specificity, linearity, accuracy, precision (repeatability, intermediate precision, and reproducibility), range quantitation limit, and detection limit. Analytical validations must be completed prior to their use in clinical sites. In 1999, the United States Food and Drug Administration established final guidelines for the industry validation of analytical

procedures and terminology (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm073381.pdf) [32, 33], which provides guidance on the validation of analytical procedures.

In the screening stage, high-throughput approaches, such as gene microarray and proteomics, are the important formats for simultaneous analyses of tens of thousands of molecules in a sample. These approaches require quality control and quality assurance. Many factors, including changes in the reaction conditions and calibration, influence the results in the analytical phase. Furthermore, since high-throughput approaches use many reagents or samples together in a single device, it makes the process more complex. Therefore, it is important to control analytical factors at different strategies by replicating analysis and normalization. The data with analytical validation for each operator and instrument can be provided to the next step, clinical validation.

4.4. Clinical validation of the PTSD biomarker assay

The development of a new PTSD biomarker test terminates with clinical validation that includes five phases: preclinical exploratory, clinical assay and validation, retrospective longitudinal, prospective screening, and PTSD control. Preclinical exploratory involves using a case-control approach to identify promising PTSD biomarker candidates. The second phase of clinical validation is to determine if a clinical biomarker assay can detect PTSD (population-based). The third phase uses a nested case-control approach in a population cohort; retrospective longitudinal study is conducted to verify if the biomarker is able to detect PTSD before it clinically manifests. In phase four, prospective screening is performed to determine the extent and characteristics of PTSD detected by tests in the subjects of cross-sectional cohort. Finally, if feasible, a randomized trial is conducted to examine the effect of test screening on reducing the burden of PTSD.

Clinical validation entails testing the ability of the markers to distinguish PTSD from non-PTSD cases, and to test the sensitivity and specificity. In this stage, it needs to find the true-positive rate (TPR), the proportion of PTSD cases who are biomarker-positive; the false-positive rate (FPR), and the proportion of non-PTSD subjects who are biomarker-positive. Then, a receiver operating characteristic (ROC) curve should be plotted. In this plot, the TPR versus the FPR can be used as the discrimination threshold. During clinical validation, it is important to assess factors associated with the biomarker, including demographic details such as age, gender, and race.

Clinical validation of PTSD biomarkers should include both retrospective and prospective analyses. Using stored samples, a retrospective analysis can be used to determine the detective ability of the potential biomarker(s) and the cut-point. If it involves multiple potential PTSD biomarkers, those PTSD markers can be compared and developed to algorithms together. Longitudinal observation will examine the variability within-subjects and compare the time-specific ROC. Again, the cut-off for a biomarker is determined by a ROC curve.

Longitudinal observation provides data from the sequential testing point, which may add additional power to the PTSD biomarker. The key issue is how to obtain the appropriate and

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well-characterized samples from each collecting point. Sometimes the subjects cannot be identified again or have dropped out from the study. In addition, we found that PTSD biomarkers in the brain and in the blood are not necessarily altered in the same direction. Therefore, determining the relationship between biomarkers in the peripheral nervous system and central nervous system could be more important in the clinical validation study for PTSD, though sometimes those study designs are a challenge. Finally, clinical validity of PTSD biomarker test, including its rates of false negatives and false positives, should be well established before the tests enter clinical use. Meanwhile, bridging studies are required if changes of platform device occurred after clinical validation.

5. P11 as a potential PTSD biomarker

Given that current diagnosis for PTSD rely on a clinician-administered interview, the development of a biomarker test for PTSD would be useful [7, 8]. There are no available laboratory blood biomarker tests for PTSD. We have pioneered the use of our patented blood p11 mRNA as a biomarker to differentiate PTSD from control and from other mental disorders. Our previous study demonstrates that p11 mRNA levels can be differentially detected in human blood from subjects with or without PTSD [7]. We, and others, found that p11 mRNA expression is significantly changed in post-mortem cortex of patients with PTSD [7] and depression [34]. That suggests that p11 mRNA levels in the peripheral blood cells can serve as a biomarker for PTSD.

P11, annexin light chain [35], is a member of the S100 protein family. It is one of the proteins within the EF-hand super-family of Ca^{2+} binding proteins [36]. The p11 gene is located in chromosome 1q21 in humans and expressed in many types of cells and tissues, such as in the lungs, intestines, kidneys, brain, and blood of many species [37]. P11 protein was originally identified in a complex with the Ca^{2+}/lipid-binding protein annexin A2 [36]. It regulated the function of exocytosis and endocytosis. Unlike other S100 proteins, it has crucial amino acid substitutions and deletions in the two EF-hand loops that render both Ca^{2+}-binding sites inactive [36, 37]. It is locked in the equivalent of a Ca^{2+}-loaded structure and in a permanently activated state. P11 binds to the target protein on hydrophobic cellular surfaces without Ca^{2+}. Thus, p11 regulates the function of several membrane proteins, such as annexin II and the 5-HT_{1B} receptor, which are associated with mental disorders [34]. In the nucleus, p11 participates in the regulation of the stress response. P11 is up-regulated by stress or stress hormones in the brain [7]. Stress-induced p11 overexpression is mediated by the glucocorticoid receptor (GR), which interacts with glucocorticoid receptor response elements (GREs) in the p11 promoter region [7]. These observations have received support from our data showing that mRNA levels of p11 increased in the post-mortem prefrontal cortex (area 46) of PTSD patients. Furthermore, in rats we showed that three days of inescapable shock induced over expression of p11 mRNA in the prefrontal cortex (PFC) and elevated corticosterone levels in the plasma [7]. This up-regulation of p11 expression can be countered by either a glucocorticoid receptor antagonist, RU486, or by mutating two of the three GREs (GRE2 and GRE3) [7]. Our preliminary data (Fig. 1, unpublished data) also demonstrated that p11 was significantly over-expressed in the blood of soldiers with PTSD who were deployed, compared to deployed soldiers who did not have

PTSD (Fig. 1a and 1b). Such overexpression is significantly associated with the severity of PTSD symptoms (Fig. 1c). Our results warrant further exploration of p11 as a potential biomarker for PTSD in a large sample size.

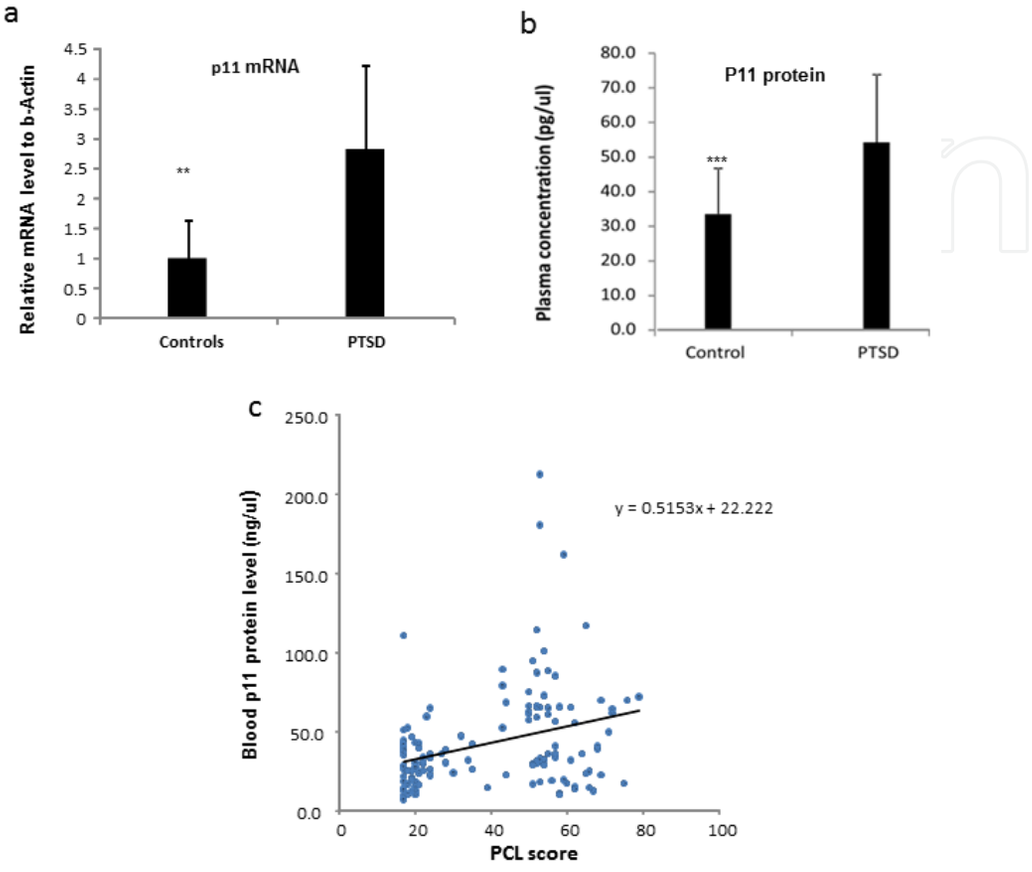

Figure 1. P11 mRNA and protein levels were significantly higher in subjects with PTSD (n=67) than that in the non-PTSD controls (n=67), and the relationship between blood p11 levels and PTSD symptom severity (unpublished data). (a) P11 mRNA level was significantly higher in subjects with PTSD than that in the non-PTSD controls (**p<0.01). (b) P11 protein level was significantly higher in subjects with PTSD than that in the non-PTSD controls (***p<0.001). (c) The relationship between blood p11 protein levels and PTSD checklist (PCL) score (p<0.0027).

6. Biorepository or biobank in biomarker study

To obtain a large quantity of good quality samples is a challenge. It requires developing a standardized protocol for sample collection, storage, and quantitative control. A biobank will provide such opportunity. A preliminary type of biobank, a biorepository can store biological samples (usually human) for use in PTSD biomarker research. The biobank market is expanding with a significant increase in using human tissues, blood, and other biomaterials to do biomarker research, and pharmaceutical and diagnostic tool development [38]. In 2008, there were 270 million specimens in biobanks. Each year, about 20 million samples have been collected for research, including biomarker studies [39], indicating worldwide changes in the nature of biomedical research. In general, more and more biobanks have been established.

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They are established by multiple research centers, leading to an infrastructure of next-generation research demand [40]. A typical example is the Psychiatric Genomics Consortium (PGC) that is comprised of more than 80 institutions. The PGC has samples from more than 150,000 people; of which, 36,989 samples are from subjects with diagnosed schizophrenia [41]. Recently, using the PGC samples, researchers have spotted 108 genetic loci in the DNA sequence of schizophrenia. The large PGC sample has also allowed for the development of an algorithm to calculate a 'risk score' for each variant's contribution to schizophrenia. PGC plans to sequence 100,000 subject samples with mental illnesses in an effort to identify the meaningful biomarker associations [41].

However, there are many issues for biobanks ahead, including ethical, legal, and social issues pertaining to their existence, the fairness of collecting donations from vulnerable populations, providing informed consent to donors, the logistics of data disclosure to participants, the right to ownership of intellectual property, and the privacy and security of donors who participate [39]. Nevertheless, biobanks will provide a great opportunity to researchers in the PTSD biomarker study.

7. Our biorepository and biomarker study, including p11 research

Since 2009, we have collected blood and saliva samples from soldiers who were deployed to Iraq and Afghanistan. To date, over 2,000 blood and 5,000 saliva samples have been collected. This study was initiated at Fort Bragg in 2009 and expanded to multiple Army National Guard units throughout California, Pennsylvania, and Guam in 2011. Biological samples and detailed mental health data, using DSM driven surveys, were voluntarily collected from soldiers, including ~500 before, during, and following a deployment to Iraq or Afghanistan, to allow for a longitudinal assessment. The mental health surveys contained established self-report inventories (e.g., PCL, GAD, and PHQ). Using our biorepository dataset to examine p11 as a potential PTSD biomarker may help scientists discover why some people, particularly those in the military, develop PTSD, depression, and suicidal behaviors while others do not.

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