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Serum and amygdala microRNA signatures of posttraumatic stress: Fear correlation and biomarker potential

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ABSTRACT

Exposure to acute traumatic stress can cause permanent changes in neurological circuitry and may lead to the development of an anxiety disorder known as posttraumatic stress disorder (PTSD). Current diagnosis of PTSD is based on clinical or behavioral symptom assessment, however, these are not definitive due to overlapping symptoms with other psychiatric disorders or mild traumatic brain injury (mTBI). No FDA approved diagnostic tests or biomarkers are currently available for diagnosis of PTSD. Recently, circulating miRNAs have emerged as novel biomarkers of many diseases. In this study, we have examined the altered expression of serum and amygdala miRNAs in an animal model of PTSD. Differentially expressed and statistically significant miRNAs in serum were validated for their presence in amygdala of corresponding animals. A panel of nine stress-responsive miRNAs viz., miR-142-5p, miR-19b, miR-1928, miR-223-3p, miR-322*, miR-324, miR-421-3p and miR-463* and miR-674* were identified, and may have potential as biomarker(s) for PTSD. Further validations by bioinformatics and system biology approaches indicate that five miRNAs such as miR-142-5p, miR-19b, miR-1928, miR-223 and miR-421-3p may play a potential role in the regulation of genes associated with delayed and exaggerated fear. To the best of our knowledge, this is the first report demonstrating the plausibility of using circulating miRNAs as biomarkers of PTSD.

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1. Introduction

Psychological distress that may follow after witnessing a traumatic event usually subsides in most cases, however, for some individuals, this distress develops into a chronic disorder which is termed as posttraumatic stress disorder (PTSD) (Sones et al., 2011). According to recent studies, it is estimated that approximately 7.3% of the global civilian population suffer from PTSD (Malan-Muller et al., 2013). PTSD is also a major psychological health issue in armed forces veterans who were previously involved in combat activities. Study shows that 10–30% of US veterans suffer from PTSD or other stress related disorders (Zhang et al., 2011). The clinical symptoms of PTSD may include feeling of helplessness, hypervigilance, irritability, exaggerated fear response or trauma-specific reenactment (Berna et al., 2012). Diagnosis of PTSD is currently based on symptoms determined from the patient's clinical history, examination of mental status, duration of symptoms and clinical symptom checklists or the patient self-report (Weathers et al., 2001). Several promising diagnosing methods and candidate biomarkers such as imaging (i.e., magnetic resonance imaging (MRI), functional MRI, single photon emission computed tomography etc.), psychological, endocrine and molecular (DNA/SNP, mRNA, protein) are currently in various phases of development (Schmidt et al., 2013). However, to date, none of these methods is in clinical use due to lack of reliability, specificity and cost efficacy (Schmidt et al., 2013).

MiRNAs are small (~22 nucleotide), endogenous, evolutionarily conserved non-coding RNAs and are posttranscriptional gene regulators of diverse biological processes. MiRNAs in circulation are considered as good biomarkers because they are highly stable in serum due to their ability to withstand repeated freeze thaw, enzymatic degradation and extreme pH conditions (Scholer et al., 2010). Circulatory miRNAs have recently shown great promise as

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non-invasive and reliable diagnostic biomarkers for different diseases and disorders such as traumatic brain injury (TBI), bipolar disorder and schizophrenia (Lai et al., 2011; Rong et al., 2011; Balakathiresan et al., 2012; Redell et al., 2010). For instance, miRNAs are implicated as plasma biomarkers in clinical samples of mild cognitive impairment (MCI) which is usually associated with the early stages of many neurodegenerative disorders such as Alzheimer's disease, vascular and frontotemporal dementia, and Parkinson's disease. It was reported that miR-132 and miR-134 families could accurately diagnose MCI in majority of patients at asymptomatic phase (Sheinerman et al., 2012). In schizophrenia, miR-34a was shown to be an accurate serum biomarker for diagnosis (Lai et al., 2011). However, currently, there are no reports on the use of circulatory miRNAs as non-invasive biomarkers for the diagnosis of PTSD.

In this study, we have used a rat model of learned helplessness stress to identify significantly modulated miRNAs in serum after traumatic stress. Our animal model mimics the pathophysiology similar to those found in PTSD subjects such as delayed and exaggerated startle response, enhanced plasma corticosterone (CORT) levels and retarded body weight gain several days after the cessation of stress (Jia et al., 2012). Among them, the delayed appearance of exaggerated acoustic startle response (ASR) corresponds to compressed time scale of the rat's life compared to a human such as 1–3 months delay in development of symptoms in PTSD patients (Jiang et al., 2011; Jia et al., 2012). The three major regions in limbic system such as amygdala, hippocampus and the pre-frontal cortex (PFC) are mainly altered in PTSD (Mahan and Ressler, 2012). Among them, hyperactive amygdala in PTSD plays a central role in fear dysregulation in response to trauma related cues (Schmidt et al., 2013). Mahan and Ressler (2012) suggested that many of the molecular tools that have been developed to study behavior in rodents could be applied to study the mechanism(s) of fear dysregulation. In this study, we have identified the significantly modulated miRNAs in serum after traumatic stress to develop a novel non-invasive PTSD diagnostic biomarker and validated their expression in amygdala since it is involved in regulating fear response under stress (Morey et al., 2012). Using *in silico* and system biology approaches, we have elucidated the regulatory role of modulated miRNAs in PTSD pathophysiology and in particular the fear response.

2. Methods and materials

2.1. Animals and stress protocol

Twenty four male albino Sprague Dawley rats (Taconic Farms, Germantown, NY, USA) weighing 76–100 g and aged between 4 and 6 weeks old were used for this study. These animals were kept for acclimation for a week and then the rats were grouped into two groups of 12 animals each for stress and control. Young animals were used for this study to give sufficient time for simulating PTSD progression as seen in the battlefield scenario. Development of PTSD like symptoms may take at least two weeks after the cessation of stressors in the animal model and hormonal changes occur immediately after stress exposure as compared to the molecular level changes (Servatius et al. 1995). Hence, young animals were used to give sufficient time for studying the molecular level changes like protein or gene expression during PTSD development. Housing conditions, acclimation of rats and the stress protocol were followed as previously described (Jia et al., 2012). The stress protocol consisted of a 2 h per day session of immobilization along with tail shocks for three consecutive days. These animals were restrained and exposed to 40 electric shocks (2 mA, 3 s duration) at varying intervals of 150–210 s. Control groups were handled similar to stress group such as acclimation and housing except for the stress protocol. The Institutional Animal Care and Use Committee of the USUHS approved all the experimental procedures.

2.2. Samples collection

Animals from both the groups of control and post stress were sacrificed immediately (day 0; $N = 6$ each) and day 14 ($N = 6$ each) after the last stress exposure and the samples were collected between 1100 and 1200 h. Trunk blood was collected in 15 ml centrifuge tubes (VWR International, Radnor, PA, USA) and was left to clot at room temperature for 30 min for serum extraction. Blood was centrifuged (Allegra 6R centrifuge, Beckman Coulter) at 3500 rpm for 30 min at 4 °C and supernatants were harvested in clean tubes. The supernatant was again centrifuged at 3500 rpm for 10 min at 4 °C to pellet down remaining cellular fraction. The serum obtained was aliquoted into 1.5 ml microfuge tubes and stored at −80 °C until further use. Brain dissection and subsequent collection of amygdala was carried out as described previously (Jia et al., 2012). Amygdala tissues were immediately submerged into RNAlater-RNA stabilization reagent (Qiagen, Valencia, USA) in microfuge tubes and then stored at −80 °C until further use.

2.3. RNA isolation, quantity and quality check

Total RNA including miRNA was isolated from the serum samples using the miRNeasy Serum/Plasma Kit (Qiagen, Valencia, USA) according to the manufacturer's protocol. QIAzol lysis reagent (1 ml) was added to the serum sample (200 μL) and vortexed. After incubating at room temperature for 5 min, 200 μL of chloroform was added and the samples were incubated at room temperature for 2–3 min and centrifuged for 15 min at 12,000×*g* at 4 °C. The aqueous phase obtained after centrifugation was mixed with 1.5 volume of 100% ethanol and loaded into an RNeasy MiniElute spin column in a 2 ml collection tube. The flow through after centrifugation was discarded and the column was washed with 700 μL of Buffer RWT, 500 μL of Buffer RPE, 500 μL of 80% ethanol and then finally eluted with 14 μL of RNAse-free water.

Total RNA was isolated from the amygdala tissue by combining a protocol of TRIzol reagent (Ambion/Life Technologies, Carlsbad, CA, USA) and the mirVana miRNA isolation kit (Ambion/Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, 2 volumes of TRIzol were added to the samples along with 1 volume of chloroform. After centrifugation, the aqueous layer was collected and mixed with 1.25 volume of absolute ethanol and passed through the RNAqueous micro kit cartridge and RNA eluted in TE buffer. Quality and quantity of small RNA for both serum and amygdala samples were analyzed using Agilent Small RNA kit (Agilent Technologies, Santa Clara, CA, USA) in Agilent 2100 Bioanalyzer. Bioanalyzer data indicated the presence of good quality miRNA in total serum RNA extractions. However, the miRNA quantity in serum was an average of 15 ng/μl (Fig. S1). This is expected since miRNAs are reported to be present in serum at low concentration and most of them are secreted out of the cells (Sayed et al., 2013). miRNA concentrations of 30 ng of serum and 5 ng of amygdala miRNAs were used for the PCR reactions.

2.4. Reverse transcription, pre-amplification and real-time quantitative PCR

Reverse transcription (RT) was performed with TaqMan miRNA RT Kit (Life Technologies, Carlsbad, CA, USA) as described with slight modifications (Balakathiresan et al., 2012). miRNA quantity was measured from the total RNA of bioanalyzer data and used as template RNA (5 ng-brain miRNA; 30 ng-serum miRNA) for RT