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Original Article

Select non-coding RNA in blood components provide novel clinically accessible biological surrogates for improved identification of traumatic brain injury in OEF/OIF Veterans

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Abstract: This study was designed to identify clinically accessible molecular biomarkers of mild traumatic brain injury (mTBI) that could be used to help identify returning Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF) Veterans who are suffering from the effects of mTBI. While analyzing the expression profile of small non-coding RNAs in peripheral blood mononuclear cells (PBMCs) from an OEF/OIF veteran study cohort using a high throughput array chip platform, we identified 18 candidate small non-coding RNA biomarkers that are differentially regulated in PBMCs of mTBI compared to non-TBI control cases. Independent quantitative real-time polymerase chain reaction assays confirmed that 13 of these candidate small RNA biomarker species are, indeed, significantly down-regulated in PBMCs of mTBI compared to non-TBI control veteran cases. Based on unsupervised clustering analysis, we identified a 3-biomarker panel which was most able to distinguish mTBI from non-TBI control veteran cases with high accuracy, selectivity and specificity. The majority of mTBI cases in our biomarker study were co-morbid with Post-Traumatic Stress Disorder (PTSD), and thus our non-TBI control cases were selected to match PTSD diagnoses. Therefore, our identified panel of 3 small RNA biomarkers likely represents a biological index selective for mTBI. Outcomes from our studies suggest that additional applications of the clinically accessible small non-coding RNA biomarkers to current diagnostic criteria may lead to improved mTBI detection and more sensitive outcome measures for clinical trials. Future studies exploring the physiological relevance of mTBI biomarkers will also provide a better understanding of the biological mechanisms underlying mTBI and insights into novel therapeutic targets for mTBI.

Key words: Mild traumatic brain injury (mTBI), biomarkers, microRNA (miRNA), post-traumatic stress disorder (PTSD)

Introduction

Traumatic brain injury (TBI) is a condition often identified among Veterans deployed to the Persian Gulf region in support of Operation Enduring Freedom (OEF) or Operation Iraqi Freedom (OIF). TBI is caused by one or more concussive insults to the head or a penetrating head injury that disrupts the normal functions of the brain, leading to either transient or chronic impairments in physical, cognitive, emotional and behavioral functions [1-6]. In OEF/OIF Veterans, TBI is largely the result of concussive injuries from blast-producing weaponry [7]. Veterans exposed to blasts from prior conflicts have shown evidence of mild TBI (mTBI) and attention difficulties when compared to similar Veterans without blast exposure. Mild TBI can be difficult to diagnose and, when coupled with psychological illness, can be either misdiagnosed or missed altogether. Traditionally, physicians and scientists have viewed and interpreted diseases at the 'visual' clinical level. However, with the advent of genomics and proteomics technologies, personalized medicine offers the promise and potential of uncovering the largely 'unseen' details of disease causality, onset, and progression.

New evidence has highlighted defects in neural

circuits and synapses, and the plastic processes controlling these functions, in TBI [8-13]. While gene products relevant to these processes are expressed in the brain, some of these genes are also expressed in circulating blood cells, such as peripheral blood mononuclear cells (PBMCs) [14-17]. Consistent with this, recent studies have illustrated that PBMC-associated biomarkers may provide insights into the pathogenesis of neurological disorders such as Alzheimer's disease and may be used to monitor disease diagnosis and progression [18,19]. Thus, PBMCs may also provide an ideal clinically accessible "window" into the brain, reflecting molecular alterations following TBI which might contribute to the onset and progression of clinical TBI phenotypes.

Small non-coding RNAs, including microRNA (miRNA) and small nucleolar RNA (snoRNA), are increasingly recognized for their roles in the regulation of cellular processes in health and disease [20]. Select small non-coding RNAs, particularly miRNA, have been implicated in neurological disorders [21-23]. It is possible that miRNA and other small non-coding RNAs might contribute to the onset and/or progression of clinical complications following TBI [24]. Exploring the feasibility of identifying clinically accessible TBI biomarkers, we identified select small non-coding RNA fingerprints from clinically accessible PBMCs that may be used as independent biological indexes of mTBI in OEF/OIF Veterans. Outcomes from our studies suggest that additional applications of the clinically accessible small non-coding RNA biomarkers to current diagnostic criteria may lead to improved mTBI detection and more sensitive outcome measures for clinical trials.

Materials and methods

18 OIF and OEF Veterans (9 mTBI and 9 non-mTBI control cases) were recruited by The War Related Illness and Injury Study Center (WRIISC), Department of Veterans Affairs, New Jersey Health Care System (DVANJHCS), East Orange, NJ. Male and female participants were included if they were between 18-75 years of age and completed a clinical evaluation at the New Jersey WRIISC. Participants were included regardless of their mTBI history. Cases with inter-current infections or inflammatory-related conditions were excluded. Participants were classified as having a history of mTBI if they positively endorsed at least one of 4 items on the Veteran traumatic brain injury screening tool (VAT-BIST) [25] and had a score at least one standard deviation below the norm for age and education on the Repeatable Battery for Neuropsychological Testing (RBANS) [26] . Classification criteria for Control cases included a negative VAT-BIST score and a RBANS score less than one standard deviation below the norm.

Demographic information for individual mTBI and non-mTBI cases is presented in **Table 1**. The average age of mTBI and non-mTBI cases used in our interim Biomarker Discovery study was, respectively, 31.6±7.0 and 29.8+8.2 years. The interval between Veterans' last deployment and recruitment into this study was 3.9±2.7 and 2.6±2.1 years for the mTBI and non-mTBI group, respectively. The mTBI group had an average of 13.3±1.3 years of education and the non-mTBI group had an average 13.0±2.4 years of education. There was no significant difference in age, deployment interval or years of education between the mTBI and the non-mTBI groups (t-test assessments of mTBI versus non-mTBI groups: p-values of 0.59 for age, 0.30 for deployment interval, and 0.72 for duration of education). The proportion of males in the mTBI and the non-mTBI group was, respectively, 78% and 67%. Lastly, 89% of the mTBI veteran cases used in our study were comorbid with post-traumatic stress disorder (PTSD), based on a PTSD diagnosis criterion of having a score of 50 or more in the PTSD Checklist - Civilian Version [27]. Thus non-mTBI cases were selected to match for PTSD, with 78% of cases in the non-mTBI control group diagnosed with PTSD.

PBMC isolation

Blood specimens were collected by venipuncture and drawn into BD Vacutainer CPT Cell Preparation Tubes. PBMCs were isolated from freshly collected blood specimens following manufacturer's instructions (Becton, Dickinson and Company) and were stored at -80 ^{o}C until use.

RNA preparation and high throughput analysis of small non-coding RNAs

Total RNA was isolated from approximately 10-50 mg of PBMCs using RNA STAT-60 according to the manufacturer's instructions (Tel-Test,

Table 1. Demographic characteristics of mTBI and non-mTBI control cases we used in our interim Biomarker Discovery study. Mild TBI classification is based on positive endorsement of the VA traumatic brain injury screen (VAT-BIS) and a score of at least one standard deviation below the norm for age and education on the Repeatable Battery for Neuropsychological Testing (RBANS). Non-mTBI case classification is based on negative endorsement of VAT-BIS and a RBANS score of less than one standard deviation below the norm. PTSD diagnosis is based on a score of 50 or more on the PTSD Checklist – Civilian Version. Average age: mTBI group, 31.6±7.0 yrs; non-mTBI group, 29.8±8.2 yrs. Interval between their last deployment and recruitment into this study: mTBI group, 3.9±2.7 yrs; non-mTBI group 2.6±2.1 yrs. Average duration of education: mTBI group, 13.3±1.3; non-mTBI group, 13.0±2.4 yrs. Percentage of males: mTBI group, 78%; non-mTBI group, 67%. Percent of cases with co-morbid PTSD: mTBI group, 89%; non-mTBI group, 78%.

Case	mTBI/Ctl	Age	Gender	Ethnicity	Interval (yrs) since last deployment	Education (yrs)	Comorbidity PTSD
31529	mTBI	38	Male	Black, non-Hispanic	3.0	14	Yes
33297	mTBI	41	Male	Native American	4.3	12	Yes
33825	mTBI	31	Male	Black, non-Hispanic	3.4	16	Yes
33828	mTBI	42	Male	Black, non-Hispanic	0.7	14	Yes
33888	mTBI	27	Female	White Hispanic	4.5	14	Yes
33931	mTBI	23	Male	White Hispanic	1.2	12	Yes
33947	mTBI	25	Female	Black, non-Hispanic	2.8	13	No
33881	mTBI	32	Male	White Hispanic	10.2	13	Yes
33811	mTBI	27	Male	Black, non-Hispanic	4.8	12	Yes
31705	Non TBI Ctl	27	Male	Black, non-Hispanic	3.4	12	No
33565	Non TBI Ctl	25	Male	White Hispanic	1.1	12	Yes
33578	Non TBI Ctl	30	Female	White Hispanic	4.3	16	Yes
33596	Non TBI Ctl	35	Male	White Hispanic	3.8	16	Yes
33598	Non TBI Ctl	49	Male	White Hispanic	0.7	9	Yes
33821	Non TBI Ctl	26	Female	White Hispanic	0.2	16	No
33834	Non TBI Ctl	24	Male	White Hispanic	2.8	12	Yes
33913	Non TBI Ctl	22	Female	Black, non-Hispanic	6.5	12	Yes
33930	Non TBI Ctl	30	Male	Black, non-Hispanic	1.0	12	Yes

Friendswood, TX, USA). Immediately prior to RNA labeling, the purity and concentration of RNA samples were determined from $OD_{260/280}$ readings using a dual beam UV spectrophotometer and RNA integrity was determined by capillary electrophoresis using the RNA 6000 Nano Lab-on-a-Chip kit and the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) as per the manufacturer's instructions.

Total RNA was directly modified and labeled using the FlashTag™ HSR Biotin RNA Labeling Kit according to the manufacturer's instructions (Genisphere, Hatfield, PA). Verification of biotin labeling was obtained by an enzyme-linked oligoabsorbant assay (ELOSA) using Immobilizer™ Amino – 8 well strips (Nunc/Thermo Fisher Scientific, Rochester, NY, USA) according to instructions supplied by Genisphere. Labeled cRNA

(1.0 μg) was hybridized for 16hr at 48°C to Affymetrix microRNA v1.0 arrays (Affymetrix, Santa Clara, CA, USA), which contain probe sets for 1,500 small non-coding RNAs, including microRNA (miRNA), small nucleolar RNA (snoRNA), small Cajal body-specific RNA (scaRNA), and 5.8S ribosomal RNA (rRNA). Array content was derived from the Sanger miRBase miRNA database v11 (April 15, 2008, http://microrna.sanger.ac.uk), snoRNABase (www.snorna.biotoul.fr) and the Ensembl Archive (www.ensembl.org). Arrays were washed and stained on a Fluidics Station 450 (Affymetrix) according to the manufacturer's recommended procedures. The arrays were stained with phycoerythrein-conjugated streptavidin (Invitrogen/Life Technologies, Carlsbad, CA, USA) and the fluorescence intensities determined using a GCS 3000 7G high-resolution confocal laser scanner and AGCC software (Affymetrix). The scanned images were analyzed with the miRNA QC tool (Affymetrix) using RMA global background correction, quantile normalization and median polish summarization to generate quantified data (as recommended by Genisphere). Quality control metrics for arrays included normalized signal values > 1000 for five spike-in control oligo probe sets (Genisphere).

Small RNA probe sets exhibiting significant differential expression (SDE) were identified using the following steps in GeneMaths XT (Applied Maths, Austin TX): 1) Probed sets with array detection p-values ≤ 0.05 for all samples in at least one experimental group were selected for further analysis, 2) Performed Discriminant Analysis (DA) and determined the largest percentage of remaining probe sets that permitted correct group assignment of samples in unsupervised hierarchical clustering by the Unweighted Pair-Group Method using Arithmetic averages (UPGMA) based on cosine correlation of row mean centered log2 signal values; this was the top 50%-tile, 3) In the DA top 50%-tile, selected probe sets with absolute signal log2 fold changes ≥ 1.0 and independent t-test p-values ≤ 0.05 adjusted for multiple testing error by the Benjamini-Hochberg false-discovery rate (FDR) correction method [28]. Unsupervised hierarchical clustering of probes sets and heat map generation were performed in GeneMaths XT following row mean centering of log2 transformed MAS5.0 signal values; probe set clustering was performed by the UPGMA method using Cosine correlation as the similarity metric. For comparative purposes, clustered heat maps included probe sets for spike-in controls (Genisphere), or endogenous small RNAs exhibiting: 1) Array detection p-values ≤ 0.05, and 2) either a) a log2 signal value standard deviation ≤ 0.025 for all samples or b) in the DA top 50%-tile with an FC > 1.3 in the opposite direction of the selected SDE profile.

Confirmatory quantitative Real-Time Polymerase Chain Reaction (qPCR) studies

We identified specific target sequences for each of the 18 candidate small RNA biomarkers (**Table 2**). Based on the sequence information, qPCR primer sets specific for each of the biomarkers were custom-designed and synthesized commercially by Applied Biosystems (Carlsbad, CA). One microgram of total RNA was used to prepare complementary DNA (cDNA) libraries using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, California) in a total volume of 20 μL. Data were normalized relative to those for the 58S ribosomal RNA. Levels of targeted small non-coding RNA were expressed relative to those in control groups using the $2^{-\Delta\Delta Ct}$ method [29].

Results

Using the Affymetrix Human gene 1.0 ST Array chip as a high-throughput platform, we analyzed the expression profile of 1500 small non-coding RNAs in our human PBMC specimens from the mTBI and non-mTBI cases in our OEF/OIF Veteran study cohort. We detected a total of 428 small RNA species from our PBMC specimens: 190 miRNAs, 220 snoRNAs, 8 small cytoplasmic RNAs and 10 ribosomal RNAs. In an initial exploratory data analysis, we conducted a principal component analysis of all signals detected to assess the potential value of the data sets to segregate mTBI and non-mTBI cases. The analysis revealed that data sets from all cases can be clustered into an mTBI or non-mTBI group, with the exception of mTBI case #33811, which is plotted far from the mTBI and non-mTBI clusters (**Figure 1**). Results from the principal component analysis suggested that the dataset generated from case #33811 was an outlier. Based on this and the fact that the quality of RNA extracted from this case was poor (not shown), we excluded case #33811 from subsequent statistical analyses.

Table 2. Targeted nucleic acid sequences selected for qPCR analysis. Shown are names of the 18 candidate small RNA biomarkers and the corresponding targeted nucleic acid sequence used for the construction of selective probe systems for qPCR studies. Also shown is a target sequence for 58SrRNA, which we selected as an internal control for qPCR studies.

Small RNA name	Target Sequence
U8	ATCGTCAGGTGGGATAATCCTTACCTGTTCCTCCTCCGGAGGGCAGATTAGAACATGATGATTGGAGATGCATGAAACGTGATTAACGTCTCTGCGTAATCAGGACTTGCAACACCCTGATTGCTCCTGTCTGATT
U58B	CTGCGATGATGGCATTTCTTAGGACACCTTTGGATTAATAATGAAAACAACTACTCTCTGAGCAGC
U27	ACTCCATGATGAACACAAAATGACAAGCATATGGCTGAACTTTCAAGTGATGTCATCTTACTACTGAGAAGT
U83A	GCTGTTCGTTGATGAGGCTCAGAGTGAGCGCTGGGTACAGCGCCCGAATCGGACAGTGTAGAACCATTCTCTACTGCCTTCCTTCTGAGAACAGC
HBII-289	ACTGAGGAATGATGACAAGAAAAGGCCGAATTGCAGTGTCTCCATCAGCAGTTTGCTCTCCATGGGCACACGATGACAAAATATCCTGAAGCGAACCACTAGTCTGACCTCAGT
U55	GTGTATGATGACAACTCGGTAATGCTGCATACTCCCGAGTGCGCGGTGGGGAAGCCAACCTTGGAGAGCTGAGC
HBII-239	TGTGTGTTGGAGGATGAAAGTACGGAGTGATCCATCGGCTAAGTGTCTTGTCACAATGCTGACACTCAAACTGCTGACAGCACACG
U38B	TCTCAGTGATGAAAACTTTGTCCAGTTCTGCTACTGACAGTAAGTGAAGATAAAGTGTGTCTGAGGAGA
U56	CCACAATGATGGCAATATTTTTCGTCAACAGCAGTTCACCTAGTGAGTGTTGAGACTCTGGGTCTGAGTGA
U15B	CTTCAGTGATGACACGATGACGAGTCAGAAAGGTCACGTCCTGCTCTTGGTCCTTGTCAGTGCCATGTTCTGTGGTGCTGTGCACGAGTTCCTTTGGCAGAAGTGTCCTATTTATTGATCGATTTAGAGGCATTTGTCTGAGAAGG
U35A	GGCAGATGATGTCCTTATCTCACGATGGTCTGCGGATGTCCCTGTGGGAATGGCGACAATGCCAATGGCTTAGCTGATGCCAGGAG
ACA48	TGTCCCTGACCTGGGTAGAGTGGCATCTGGTTGGTGATGCCCATCTCATATCAGCCAGGGACAAAGCAACTCCTTGTTCATCCCAGCTTGGCTTTTGATCCGTGCCCATGCCTGGTTCATGCCTTGGACACATAG
U91	TGGCCGATGATGACGAGACCACTGCGCAATCTGAGTTCTGGGAACCAGGTGATGGAGTATGTTCTGAGAACAGACTGAGGCCG
ENSG00000199941	TCTTAGTGACATAATTCTAATAGTTTGTTCCGACCTTCCACTGTGGACTCAATAGCAGGGAGATGAAGAGGACAGTGATTGCATGA
hsa-miR-671-5p	AGGAAGCCCUGGAGGGGCUGGAG
hcmv-miR-US4	CGACAUGGACGUGCAGGGGGAU
hsa-miR-1285	UCUGGGCAACAAAGUGAGACCU
hsa-miR-455-3p	GCAGUCCAUGGGCAUAUACAC
58SrRNA	CGACTCTTAGCGGTGGATCACTCGGCTCGTGCGTCGATGAAGAACGCAGCTAGCTGCGAGAATTAATGTGAATTGCAGGACACATTGATCATCGACACTTCGAACGCACTTGCGGCCCCGGGTTCCTCCCGGGGCTACGCCTGTCTGAGCG

Using high throughput small non-coding RNA datasets, we continued to search for candidate mTBI biomarkers that are differentially regulated in PBMCs of mTBI compared to non-mTBI cases. Two criteria were used to identify candidate small RNA biomarkers for mTBI: 1) group changes (mTBI vs. non-mTBI groups) must be associated with a magnitude of $\geq$ 1.5-fold, and 2) group changes must be statiscally significant with $p < 0.05$, based on t-test analysis followed by the application false discovery rate corrections for multi-sampling errors. We identified 18 candidate small RNA biomarkers meeting both criteria: 4 miRNAs, 13 snoRNAs and 1 small scaRNA. Interestingly, we observed that all candidate small RNA biomarkers are significantly down-regulated in mTBI versus non-mTBI cases. In an unsupervised clustering analysis using RNA expression data generated from the high-throughput gene chip platform for the 18 candidate small RNA biomarkers, we were able to correctly segregate all 17 mTBI and non-mTBI cases analyzed (**Figure 2**), implicating their potential value as surrogate biological indices for mTBI among the OEF/OIF Veteran population.

Independent qPCR validation of candidate small non-coding RNA biomarkers

We next used an independent quantitative real-

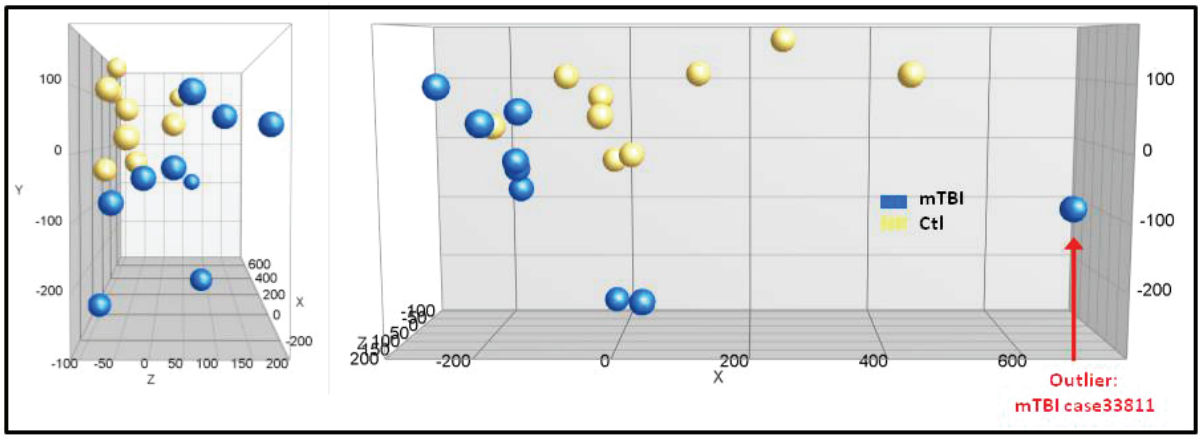

Figure 1. Principal component analysis of PBMC small RNAs from mTBI and non-mTBI veteran cases. Small RNA expression profiles for individual cases were assessed by a high-throughput Affymetrix Human gene 1.0 ST Array chip platform, which detected 428 small RNAs from PBMC specimens. Signals from all small RNA detected for each of the 9 mTBI and 9 non-mTBI cases were summarized into single points (represented by balls) plotted on a 3-dimensional plot. Blue and yellow balls represent, respectively, mTBI and non-mTBI cases. The analysis revealed that all cases can be clustered into an mTBI or a non-mTBI group, with the exception of mTBI case #33811 (indicated by a red arrow), which is plotted far away from the mTBI cluster.

time polymerase chain reaction (qPCR) procedure to assess the expression of individual candidate small RNA biomarkers in PBMC specimens from the same 9 mTBI and 9 non-mTBI cases used in our high-throughput biomarker discovery studies. Primer sets specific for each of the biomarkers were custom-designed and synthesized commercially by Applied Biosystems (Carlsbad, CA). Using these primer sets, we conducted qPCR studies and assessed the contents of individual candidate small RNA biomarkers in PBMCs of mTBI compared to non-mTBI cases. Results from our qPCR studies confirmed that 13 of the 18 candidate small RNA biomarkers are, indeed, differentially regulated in PBMCs of mTBI compared to non-mTBI veteran cases (**Figure 3**). The 13 confirmed small RNA biomarkers include 12 small nucleolar RNA (ACA48, ENSG199411, HBII-239, HBII-289, U15B, U27, U35A, U55, U56, U58B, U83A, U91) and 1 miRNA (Has-miR-671-5p) (**Figure 3**). Consistent with evidence from our RNA array platform, each of the 13 confirmed small RNA biomarkers are found in significantly lower levels in PBMC specimens from mTBI compared to non-mTBI veteran cases (**Figure 3**).

Exploring the potential value of novel small RNA biomarkers for segregating mTBI from non-mTBI veteran cases

Based on results from our qPCR biomarker confirmation studies, we next assessed the value of the 13 confirmed small RNA biomarkers as a criterion to correctly diagnose mTBI versus non-mTBI veteran cases. Using an unsupervised clustering analysis, we found the 13 confirmed mTBI biomarkers effectively segregated the cases correctly into mTBI and non-mTBI groups, with the exception of 2 of the non-mTBI cases which were incorrectly identified as mTBI (**Figure 4A**). We continued unsupervised clustering analyses to test the efficacy of individual or a combination of qPCR confirmed biomarkers to correctly segregate mTBI and non-mTBI veteran cases. Outcomes from these analyses led to the identification of a 3 small nucleolar biomarker panel, comprised of HBII-289, ENSG199411 and U35A, which is capable of distinguishing mTBI from non-mTBI veteran cases with 89% accuracy, 82% selectivity and 78% specificity (**Figure 4B**).

Discussion

Evidence has suggested that appropriate interventions can reduce functional impairment after mTBI [30,31,32,33]. In order to demonstrate the efficacy of clinical interventions, research must identify the biological, clinical, and neurological indices that are sensitive to the detection of functional impairments after mTBI. Results from our studies led to the identification of 13 novel clinically accessible small RNA mTBI

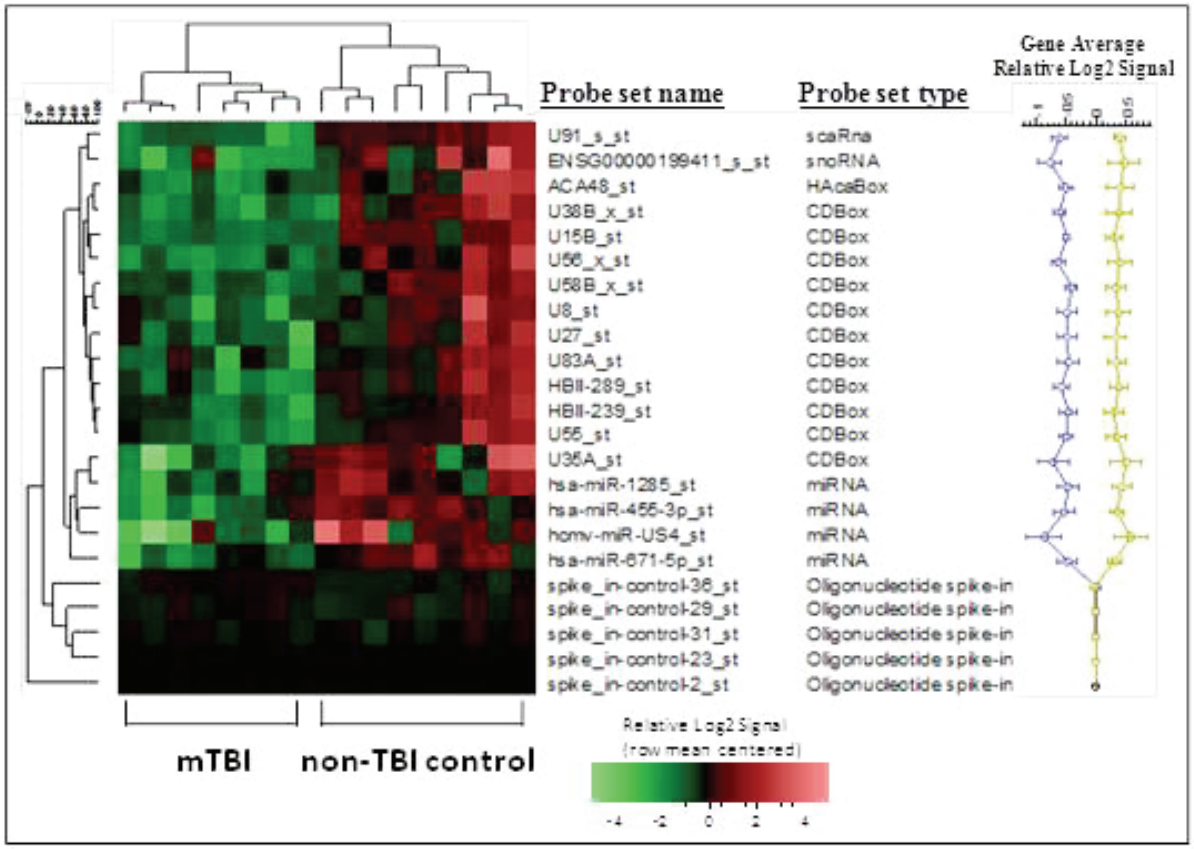

Figure 2. Unsupervised clustering analysis of 18 candidate small RNA TBI biomarker species. The 18 differentially-regulated small RNAs identified from interim high-throughput Array Chip analysis of 8 mTBI and 9 non-mTBI cases were subjected to unsupervised hierarchical clustering analysis using the UPGMA algorithm with cosine correlation as the similarity metric. Results are presented as a heat map (left panel) demonstrating that the panel of 18 small RNA biomarker species is able to correctly segregate mTBI from non-mTBI cases. Names for each of the small RNA biomarker species are identified under "Probe Set Name". Small RNA classes (and subclasses) that these 18 differentially-regulated mTBI biomarkers belong to are shown under "Probe Set Type". Vertical dendrogram (right panel) presents average (+/- SD) signal detections from mTBI versus non-mTBI groups for each of the 18 candidate small RNA biomarkers and confirmed divergent regulations of the biomarkers in PBMC specimens from mTBI vs. non-mTBI groups. Differential regulations of the 18 candidate biomarkers likely reflect true biological effects and not systematic experimental artifact(s) since there are no observable group differences for the detection of spike-in control oligonucleotides in all 17 OIF/OEF veteran cases analyzed (see heat map and vertical dendrogram). Abbreviations: miRNA, microRNA; snoRNA, small nucleolar RNA; C/D Box, the C/D box subclass of small nucleolar RNA; HAc Box, the HAc Box subclass of small nucleolar RNA; scaRNA, small Cajal body-specific RNA.

biomarkers, including 12 small snoRNA and 1 miRNA. Using qPCR, we have independently confirmed that each of these biomarkers is significantly down-regulated in PBMC specimens from mTBI compared to non-mTBI veteran cases. Among the 13 mTBI biomarkers, we demonstrated that a panel of 3 snoRNA - HBII-289, ENSG199411 and U35A – is capable of distinguishing mTBI from non-mTBI veterans with 89% accuracy, 82% selectivity and 78% specificity. Collectively, our evidence suggests that additional applications of the small RNA biomarkers we have identified, particularly the three biomarker panel, to current diagnostic criteria may improve mTBI detection and provide more sensitive outcome measures for clinical trials.

PTSD is commonly co-morbid with mTBI in OEF/OIF Veterans [34,35]. We note that the majority of Veterans with mTBI in our biomarker study have co-morbid PTSD and that our non-mTBI

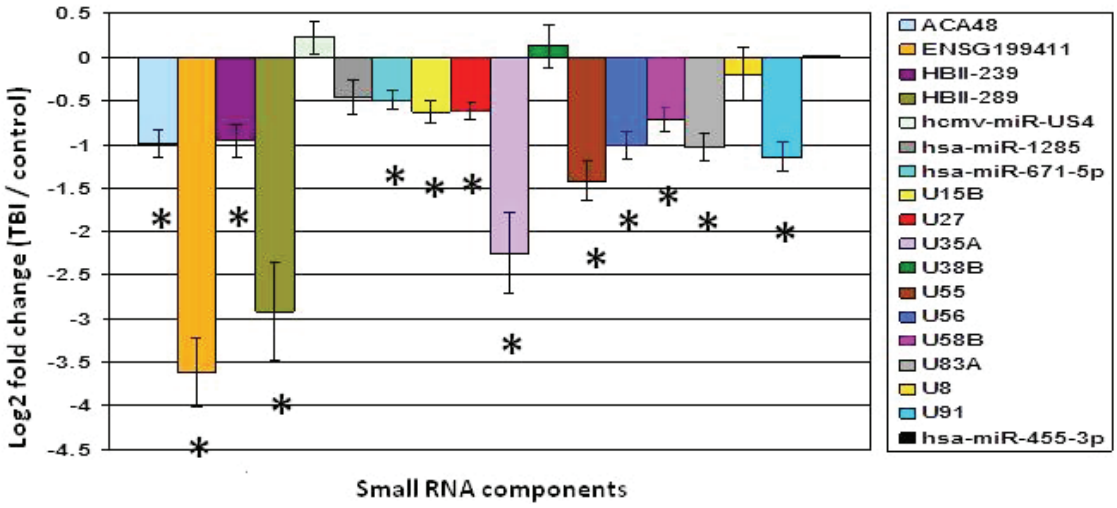

Figure 3. Independent quantitative real-time polymerase chain reaction (qPCR) assays confirmed that 13 small RNA TBI biomarkers are differentially regulated in PBMCs of mTBI relative to non-mTBI control cases. PBMC contents for each of the18 candidate small RNA biomarkers identified by the high-throughput Array Chip platform in Figure 2 were quantitatively assessed using independent qPCR assays. The same 9 mTBI and 9 non-mTBI cases (Table 1) we used in our initial high-throughput biomarker discovery assay were assessed in this qPCR biomarker confirmation study. Bar graphs represent mean small RNA biomarker contents in the mTBI group relative to the non-mTBI group; error bars represent standard errors. * False discovery rate-corrected P-value < 0.05. qPCR confirmed 13 small RNA biomarker species are significantly down-regulated in PBMC of mTBI compared to non-mTBI veteran cases. These 13 confirmed small RNA biomarkers include 12 small nucleolar RNA (ACA48, ENSG199411, HBII-239, HBII-289, U15B, U27, U35A, U55, U56, U58B, U83A, U91) and 1 miRNA (Has-miR-671-5p) species.

cases are selected to match for PTSD diagnosis. Thus, our identified 13 small RNA biomarkers likely represent biological indices selective for mTBI. Moreover, mTBI cases in our biomarker discovery studies were recruited after an average interval of 3.9 years following their last deployment (deployment-to-recruitment interval: ranging from 0.7 to 10.2 years, with a median interval of 3.4 years) (**Table 1**). Thus, changes in the regulation of these small RNA mTBI biomarkers that we observed are not acute mTBI responses, but likely represent long-term physiological consequences subsequent to mTBI experienced during deployment.

The pathological implication of our observation that select small nucleolar RNA and miRNA are differentially regulated in the PBMCs of mTBI relative to non-mTBI veteran cases is currently unknown. Small nucleolar RNA and miRNA are members of a family of non-coding RNAs that are involved in many physiological cellular processes and are also known to contribute to molecular alterations in pathological conditions [36]. SnoRNAs are short RNA sequences comprised of ~60-220 nucleotides. They are primarily known for their role as guide molecules for site-specific methylation and pseudouridylation of other RNAs, particularly rRNA, as well as tRNA and small nuclear RNAs. These chemical alterations are required for proper rRNA processing and ribosome function as well as for proper function of the spliceosome [37]. MicroRNA are short (~22 nucleotides) RNA sequences that bind to complementary sequences on target mRNA, thereby blocking translation or promoting degradation of target mRNA [37]. SnoRNA and miRNA are expressed in the brain and both classes of small RNAs have been implicated in neuroplasticity mechanisms and neurological disorders. For example, recent evidence suggests a role of the HBII52 small nucleolar RNA in the regulation of alternative splicing of the serotonin 2c receptor [38], and that patients with autism and Prader-Willi-like characteristics are found to have reduced levels of HBII52 in the brain [39]. The miRNA miR132 is induced by neuronal activity and neurotrophins in a CREB-dependent manner and plays a role in regulating neuronal morphology and cellular excitability [40]. Moreover, preclinical evidence in rodent models demonstrated that small RNA expression is affected in the brain by TBI. Redell et al. [8] reported transient elevated expression

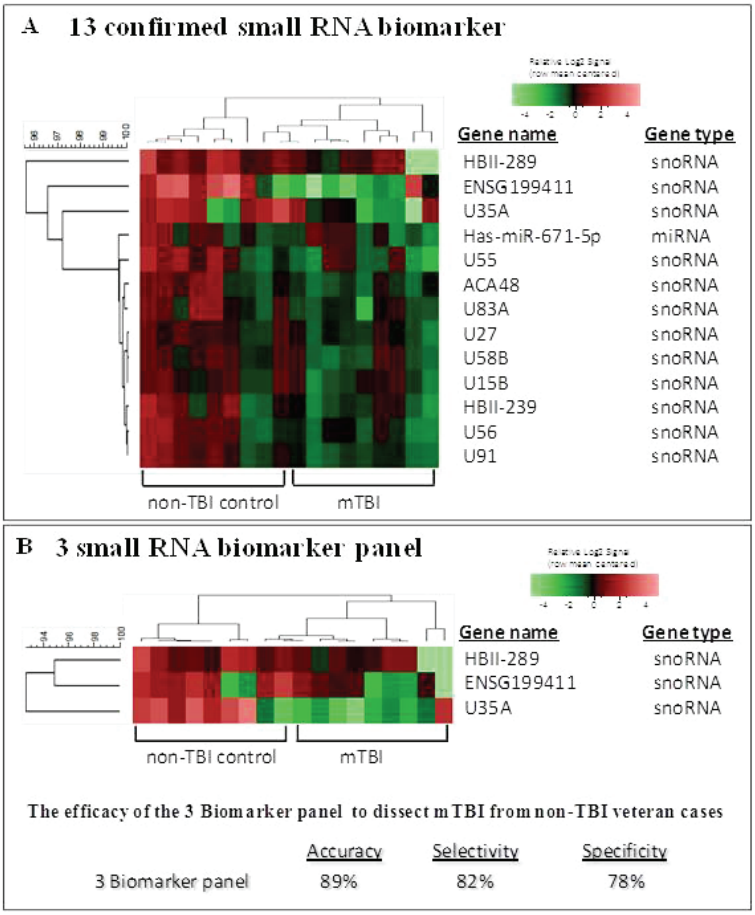

Figure 4. The content of small RNA biomarkers in clinically accessible PBMCs provides a sensitive and specific criterion for dissecting mTBI from non-mTBI veteran cases. We tested the role of the 13 qPCR confirmed small RNA mTBI biomarkers as a criterion for distinguishing mTBI from non-mTBI veteran cases. Biomarker contents in banked PBMC specimens from the same 9 mTBI and 9 non-mTBI cases (Table 1) were used in our biomarker discovery studies and quantified by qPCR. (A,B) The efficacy of using biomarker contents from clinically accessible PBMCs as a criterion to correctly segregate mTBI and non-mTBI cases was tested by unsupervised clustering analysis using the UPGMA algorithm with cosine correlation as the similarity metric. Results are presented as heat maps demonstrating the efficacy of using all 13 small RNA biomarkers (A) or using a panel of three small nucleolar RNA biomarkers (B) to correctly segregate mTBI from non-mTBI cases. (B) A three small nucleolar RNA biomarker panel (HBII-289, ENSG199411 and U35A) is capable of distinguishing mTBI from non-mTBI cases with 89% accuracy, 82% selectivity and 78% specificity (accuracy is the percentage of all mTBI and non-mTBI cases that are correctly identified; sensitivity is the probability that a case identified as mTBI actually is a mTBI case; specificity is the probability that a case identified as a non-mTBI case is actually a non-mTBI case). Abbreviations: snoRNA, small nucleolar RNA; miRNA, microRNA.

of the miRNA miRNA-21 in rats following an impact injury to the brain. Using a high-throughput Array Chip platform, Lei et al. [41] reported potential aberrant up- or down-expression of 203 miRNA species in the rat cerebral cortex up to 72 hrs following fluid percussion injury to the brain. Redell et al [42] also identified potential up-regulation of 35 and down-regulation of 50 miRNA species in the hippocampus of rats within 72 hrs following an impact TBI to the brain; altered regulations for a smaller subset of 8 (4 up-regulated and 4 down-regulated) miRNA species in the hippocampus were subsequently confirmed by independent qPCR.

It is possible that the altered regulation of select small nucleolar RNA and miRNA that we observed in PBMCs of veteran mTBI cases might have implications in the central nervous system. Genes relevant to neural circuits, synapses and neural plasticity processes are also expressed in circulating blood cells, such as PBMCs. Thus, the fact that we observed significant down-regulation of select small RNA biomarkers in PBMC specimens from our veteran mTBI cases long after their deployment might reflect long-term molecular alterations in the central nervous system contributing to the onset and progression of clinical TBI phenotypes. Future studies exploring the physiological relevance of mTBI biomarkers will provide a better understanding of the biological mechanisms underlying mTBI and insights into novel therapeutic targets for mTBI.

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