

Integrated microRNA and mRNA Gene Expression in Peripheral Blood Mononuclear Cells in Response to Acute Psychosocial Stress: A Repeated-Measures Within-Subject Pilot Study

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Research note

Keywords: Psychosocial stress, gene expression, microRNA, mRNA, stress, inflammation

Posted Date: April 1st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-322294/v1>

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Abstract

Objective: The impact of psychosocial stress on a variety of negative health outcomes is well documented, with current research efforts directed at possible mechanisms. Here, we focused on a potential mechanism involving differential expression of mRNA and microRNA in response to acute psychosocial stress. We utilized a validated behavioral paradigm, the Trier Social Stress Test (TSST), to induce acute psychosocial stress in a cohort of volunteers. Stress reactivity was assessed repeatedly during the TSST using saliva samples that were analyzed for levels of cortisol. Peripheral blood mononuclear cells were extracted from blood drawn at baseline and at two time points following the stress paradigm. Total RNA was extracted, and mRNA and microRNA microarrays were utilized to assess within-subject changes in gene expression between baseline and the two post-stressor time points.

Results: For microarray gene expression analysis, we focused on 12 participants who showed a robust cortisol response to the task, as an indicator of robust HPA-axis activation. We discovered a set of mRNAs and miRNAs that exhibited dynamic expression change in response to the TSST in peripheral blood mononuclear cells, further characterizing the link between psychosocial stress and cellular response mechanisms.

Introduction

Organismal stress responses are associated with widespread changes in the transcriptome of circulating peripheral blood mononuclear cells (PBMCs) of genes regulating cell signaling, adhesion, differentiation, and immune pathways [1]. Such stress-associated transcriptional changes in PBMCs are mediated, in part, through microRNAs (miRNA) [2, 3], such as miRs 7b, 16, 20b, 21, 26b, 29a, 29c, 126 and 144 [4-7].

A popular laboratory-based paradigm for the study of psychosocial stress physiology is the Trier Social Stress Test (TSST) [8]. A previous study reported TSST-associated changes in salivary levels of miR-20b, -21 and 26b [9], but their expression levels in PBMCs and their association with gene expression remain unknown. Here, we report results from a TSST pilot study examining within-subject repeated-measures of mRNA and miRNA in PBMCs.

Methods

Participants

Participants were Caucasian males recruited via flyers posted on the Stony Brook University campus and through advertisements in local newspapers in surrounding communities on Long Island, NY, USA. Study exclusion criteria included: age under 18 years, significant current or prior presence of psychiatric illness, smoking, presence of substance or alcohol abuse in the past six months, hormonal, mood-altering, heart disease and/or cardiovascular conditions related, or psychoactive drug medication, $BMI \geq 30$.

Participants were pre-screened for exclusion criteria by phone. Fifty-five participants were part of the

study, which was approved by the Institutional Review Board (IRB) and Committee on Research Involving Human Subjects (CORIHS) of Stony Brook University, Stony Brook, USA. We selected 12 participants who showed a robust cortisol response to the TSST (“Cortisol Responders”; based on a criterion suggested by Miller et al. [10]) as a discovery cohort to generate repeated-measures, within-subject genome-wide mRNA and miRNA expression data.

Psychosocial Stress Procedure: TSST

All participants were scheduled for a four-hour weekday laboratory session conducted at the General Clinical Research Center (GCRC) at the Stony Brook University Hospital from 1400h to 1800h. Upon arrival, participants were informed about the study, completed the consent procedure, and underwent insertion of an intravenous catheter into the arm for the first blood draw. Participants then rested for a period of at least 45 minutes before they were introduced to the psychosocial stress paradigm. The Trier Social Stress Test (TSST) is a standardized social stress protocol that was used to induce psychosocial stress and that has been found to elicit a strong and reliable physiological response to laboratory stress [8]. Briefly, the standardized laboratory stressor consists of a 5-min preparation period, a 5-min free speech task during which participants give a mock job interview, and a 5-min mental arithmetic task wherein participants perform serial subtraction by thirteen all in front of a non-responsive audience.

Biological Measures: Cortisol

To assess cortisol levels, ten salivary samples were obtained from each participant throughout the duration of the study. Samples were collected using Salivettes® (Sarstedt) at arrival, 2 minutes before beginning the TSST, and again at 2, 10, 20, 30, 45, 60, 90- and 105-minutes post stress task. Participants were instructed to gently chew on the cotton swab for approximately one minute during each collection. Cotton swabs were then transferred to plastic containers and immediately stored at -20°C until shipment to the analyzing laboratory (N. Rohleder, Brandeis University, Waltham, MA). Salivary cortisol concentrations were measured using a commercially available chemiluminescence immunoassay (CLIA; RE62019; IBL International, Toronto, Canada). Inter-assay variability was 3.3%, and intra-assay variability was 3.4%. Salivary cortisol concentrations have been shown to highly correlate with serum cortisol concentrations, making salivary cortisol a reliable indicator of HPA activity [11]. Participants had no oral intake except for water for 3 hours before beginning the study session. Cortisol response to the stress paradigm was assessed by subtracting the highest mean cortisol concentration following the stressor minus the baseline cortisol concentration (two minutes before the TSST). Using a criterion suggested by Miller et al. [10], those participants who showed a cortisol increase of at least 1.5 nmol/l from baseline were assigned in the “Cortisol Responder” group ($n=33$, mean cortisol increase of 8.55 nmol/l (± 5.63), while those who did not show an increase of at least 1.5 nmol/l from baseline were placed in the “Cortisol Non-Responder” group ($N=22$, mean cortisol increase of -3.889 nmol/l (± 6.45). The two groups differed in age (responders: 34.58 years \pm 17.09; non-responders: 26.18 years \pm 13.44; t-test $p<0.05$). There were no

significant differences in psychological variables of interest, as the BAI, BDI, TiCS, STAI-S and CTQ sum scores were not significantly different between the two groups (all $p>.05$). There was no significant difference in the number of stressful life events (SLEs) between the two groups ($p > .05$). Subsequent gene expression analyses with microarray profiling were conducted on the first 12 Cortisol Responders.

Biological Measures: Blood Collection

Directly upon arrival and following the informed consent procedure, an intravenous (IV) catheter was inserted into each participant's arm for the first blood draw (Time Point A). Participants then rested for a period of at least 45 minutes before they were introduced to the psychosocial stress paradigm. The second blood draw occurred 45 minutes after the stress paradigm (Time Point B) and the third blood draw occurred 105 minutes post stress (Time Point C). Ten ml of EDTA blood (Vacutette^R Greiner Bio-one) was collected at each of the three time points. Blood was immediately processed for PBMC extraction according to the Leucosep^R Instruction Manual (Greiner Bio-one), and cell pellets were frozen and stored at -80°C.

RNA Isolation, Quantification, Quality Control

Total RNA was extracted from PBMCs at each time point using the Qiagen All-Prep kit with company-recommended modification to extract small RNA (Qiagen). RNA quantity was assessed using a Nanodrop Technologies ND-1000 instrument (NanoDrop Technologies). Total RNA quality was measured using the Agilent RNA 6000 Pico kit and the Agilent Bioanalyzer 2100A (Agilent Technologies), while miRNA content was resolved using the Small RNA Kit (Agilent). RNA samples were stored at -80°C.

MiRNA Microarray Profiling

mRNA microarray profiling was performed at the ShanghaiBio Corporation using Affymetrix Human Gene ST 1.0 Arrays according to the manufacturer's protocol. Briefly, 500 ng of total RNA was used in complementary DNA synthesis and labeling, followed by hybridization, washing, scanning, and image processing of GeneChips according to standard Affymetrix protocols. Quantitative image files were loaded into the Bioconductor Affymetrix linear modeling Graphical User Interface software [12] and background adjustment, quantile normalization, probe summarization, and log2 transformation was performed using the Robust Multi-Array Average method [13].

Statistical Analysis of mRNA and miRNA Microarray Data

Time-course microarray data analysis was conducted using the Linear Models for Microarray Data (limma) Bioconductor package [14]. Linear models were fitted to distinguish differentially expressed mRNAs between the three time points (A, B, C) on the Affymetrix platform. mRNA showing limma $p<0.01$

for at least one of the time point comparisons (B to A, C to A, B to C) correcting for multiple comparisons using false discovery rate (FDR) correction, as well as with a fold change (FC) $|FC| \geq 1.3$ were highlighted as genes that show greater variation in expression in response to the TSST. The fold change threshold of $|FC| \geq 1.3$ was chosen as literature has conventionally utilized fold changes ≥ 1.3 -2 to suggest differential expression in mRNA microarray data [15]. Also, fold change of gene expression in blood has usually been less than two-fold [3, 16] and multiple studies have used a cut-off of $FC > 1.2$ for identifying changes of gene expression specifically in blood [16-18]. miRNAs generally show more subtle changes in expression than mRNAs, but even small expression changes can have significant biological effect as each miRNA has multiple gene targets and can influence multiple genes in a given pathway. Therefore, miRNAs showing a $|FC| \geq 1.2$ and limma p value < 0.01 for at least one of the time point comparisons (B to A, C to A, B to C) were highlighted as showing change in expression in response to the TSST.

MiRNA/mRNA Integration and Functional Profiling

Integration of mRNA and miRNA expression was performed by the computational gene network prediction tool Ingenuity Pathway Analysis (IPA, Ingenuity® Systems). Our study showed a limited number of mRNAs that were significant according to limma $p < 0.01$ and $|FC| \geq 1.3$, possibly in part due to the small study sample size and conservative statistical inclusion criteria. It is worthwhile noting that a single miRNA can subtly regulate multiple genes in a given pathway or process, having an additive biological effect. Therefore, mRNAs that showed significant changes in expression at any time contrast according to limma $p < 0.01$ were input into IPA. In parallel, human orthologues of differentially expressed miRNAs (limma $p < 0.01$ and $|FC| \geq 1.2$) were input into the IPA program as well. To integrate the two datasets, identification of negatively correlated miRNA/mRNA pairs was performed using the Target Filter Analysis in IPA. The algorithm uses experimentally validated interactions as well as predicted microRNA-mRNA interactions from TargetScan. Fold change at each miRNA and mRNA was integrated at Time B vs Time A, Time C vs Time A, and Time C vs Time B. The expression pairing analysis was used to identify negatively correlated miRNA/mRNA pairs that had experimentally demonstrated targeting or a moderate to high degree of likelihood of a targeting relationship based on prediction algorithms.

Results

Participant Characteristics and Gene Expression Analysis

Table 1 lists participant characteristics and Table 2 lists 9 mRNAs that were differentially expressed according to the criteria of limma $p < 0.01$ and $|FC| \geq 1.3$. mRNAs that showed significant changes in expression at any time contrast according to limma $p < 0.01$ were input into IPA for expression pairing and are presented in Table S1. Limma analysis revealed thirty-three miRNA probe sets on the Affymetrix array that showed significant expression changes ($p < 0.01$ and $|FC| > 1.2$) in Cortisol Responders over the course of the TSST. Only the miRNAs with identified human orthologues that readily mapped into IPA were considered in further analysis. In the instance that stem-loops were identified by the microarray, both

mature products were input into IPA when as per verbal instructions from IPA Technical Support. The list of resulting miRNAs is presented in Table 3.

MiRNA/mRNA Integration

Negatively correlated miRNA/mRNA pairs were identified at contrasts B vs A, C vs A, or C vs B using IPA's Target Filter Analysis. Since miRNAs often result in subtle changes in gene expression, mRNAs that showed significant change in expression according to limma $p<0.01$ were used in an expression-pairing analysis along with miRNAs significant according to criteria limma $p<0.01$ and $|FC|\geq 1.2$. Resultant miRNA/mRNA expression pairs at each time contrast are listed in Table S2A-C.

Table 1

TSST Participant Characteristics

Sample	Cortisol Responders profiled by microarray
N	12
Age (years)	43.17 (19.34)
Cortisol Response (in nmol/l)	13.60 (5.77)
BDI	2.36 (2.38)
BAI	3.75 (3.05)
TiCS-CSSS	10.33 (7.24)
CTQ	40.90 (14.62)
STAI-S	25.00 (5.82)
SLE	45.75 (11.08)

Table 1. TSST Participant Characteristics. BAI: Beck Anxiety Questionnaire, Sum Score; BDI: Beck Depression Questionnaire, Sum Score; TiCS-CSSS: Chronic Stress Screening Scale of the Trier Inventory for the Assessment of Chronic Stress, Sum Score; CTQ: Childhood Trauma Questionnaire, Sum Score; STAI: State-Trait Anxiety Questionnaire, SLE: Stressful Life Events (based on a structured interview). All values are mean (\pm standard deviation). Higher sum scores indicate increased symptom severity.

Table 2

Differentially Expressed mRNA Transcripts in Cortisol Responders

Symbol	Gene Name	Fold Change		
		B vs A	C vs A	C vs B
RNU6-82P	RNA, U6 small nuclear 82, pseudogene	1.5	1.2	-1.2
ZRANB2	zinc finger, RAN-binding domain containing 2	1.4	1.2	-1.2
SPON2	spondin 2, extracellular matrix protein	-1.3	-1.4	-1.0
AKR1C3	aldo-keto reductase family 1, member C3	-1.3	-1.4	-1.1
GOLGA8DP	golgin A8 family, member D, pseudogene	-1.1	-1.3	-1.2
TCF19	transcription factor 19	-1.1	-1.3	-1.2
RPS9	ribosomal protein S9	1.0	1.4	1.5
TGIF2LX	TGFB-induced factor homeobox 2-like, X-linked	1.1	-1.2	-1.3
RNF144B	ring finger protein 144B	1.3	-1.0	-1.3

Table 2. Differentially Expressed mRNA Transcripts in Cortisol Responders. This table presents differentially expressed mRNAs in Cortisol Responders according to the criteria limma ($p<0.01$ and $|FC|\geq 1.3$) over the course of the TSST.

Table 3
Differentially Expressed miRNAs in Cortisol Responders.

MIR	Fold-Change		
	B vs A	C vs A	C vs B
miR-92a-2	-1.2	1.0	1.2
miR-129-5p	1.2	1.1	-1.1
miR-518a-2	-1.2	1.0	1.2
miR-9-5p	-1.1	-1.3	-1.2
miR-137-3p	-1.2	-1.1	1.1
miR-624-3p	-1.1	1.2	1.3
miR-614	-1.2	-1.1	1.2
miR-3178	-1.2	-1.2	-1.0
miR-375-3p	1.1	-1.1	-1.2
miR-210-3p	1.0	-1.3	-1.3
miR-3191-3p	1.1	-1.1	-1.2
miR-1185-5p	1.1	-1.1	-1.2
miR-937-3p	1.1	1.2	1.1
miR-301b	1.1	1.1	1.2

Table 3. Differentially Expressed miRNAs in Cortisol Responders This table presents differentially expressed miRNAs in Cortisol Responders according to the criteria limma ($p<0.01$ and $|FC|\geq 1.2$) over the course of the TSST. Only miRNAs with identified human orthologues that readily mapped into Ingenuity Pathway Analysis (IPA) were considered. In the instance that stem-loops were identified by the microarray, both mature products were input into IPA.

Discussion

This pilot study examined genome-wide changes in mRNA and miRNA gene expression in PBMCs in response to TSST-induced psychosocial stress within-subjects in a repeated-measures design. By removing between-subject genomic variance that would otherwise obscure any relationship between stress and gene regulation, we could identify differentially expressed miRs and target mRNAs at FDR-corrected levels, although we replicated none of the previously reported stress-related miRs. Non-replication is common in small-scale studies, but the aggregate of our results and previous reports suggests that properly powered large-scale studies of stress-related gene regulation by miRNAs are warranted to better understand the molecular mechanisms of stress-related gene dysregulation.

Limitations

Limitations of this study include a small sample size consisting of healthy Caucasian males only. Thus, cautious interpretation is advised. Replication in a larger and more diverse samples that are more broadly representative of the variation in human stress-related phenotypes should be considered in future work.

In addition, microarray results should be confirmed by an orthologous method such as RT-qPCR in order to confirm changes in gene expression.

Declarations

Ethics approval and consent to participate

Ethical approval and a written informed consent to participate of every subject was obtained and approved by the IRB (IRB Approved by CORIHS Stony Brook University on 4/15/2011). None of the participants were under the age of 16 at the time of your study. This study complied with the most recent version of ethical principles regarding research with human participants (Declaration of Helsinki) in terms of being rigorous, using all the available good-quality data, answer the most important question(s) about the topic, present a sound statistical analysis, present the results clearly, and aim to lead to beneficial outcomes.

Consent for publication

Not applicable.

Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This research was supported by funding from NSF BCS-0843346 to T.C. The work of A. M.-A. was supported by a stipend from the German Research Foundation (MU-2847).

Authors' contributions

MJ, AMA and TC designed the study. MJ, AMA and JF collected the data.

MJ, AMA, IJ, DM, and AM analyzed the data. MJ and AMA interpreted the data.

MJ, AMA and TC were the major contributors in writing the manuscript. JF, DM and NF helped revising the manuscript. All authors read and approved the final manuscript

Acknowledgements

We thank Drs. Leighann Litcher-Kelly and Arthur Stone for their suggestions in designing the TSST, and the other undergraduate and graduate students of the Canlilab for their assistance in conducting the TSST, and Renee Wu for her statistical advice.

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Supplementary Files

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- [TableS1.mRNALimma0.01.xls](#)
- [TableS2.ExpressionPairingBMC.xlsx](#)