Development of Novel Biomental Tools for Student Mental Health

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1. Backgrounds & Aims

The mental and physical reactions to stressful life events are enormously different between individuals. Both genetic and environmental factors determine the sensitivity to it. Nurture elements interacting with nature factors profoundly affect maturing one's mental health as epigenetic factors. Mood disorders are one of the most serious problems in university students worldwide. University campus life is a particularly important stage in one's life. At the same time, it is a dangerous time for the development of mood disorders or psychiatric disorders such as major depression, since the students are apart from their parents and they meet new friends with different personalities and senses of value. We have been examining the mental state of freshmen over a period of five years and found that about 40% of freshmen have some mental problems. At the same time, around 13% of freshmen have abnormally high scores on a self-rated depression scale and the state-trait anxiety inventory (STAI) test. This is more serious in female students, because over 60 % of the female students do not satisfy their self-body image and they try to lose their body weights. As a result, only less than 30% of female students ovulate regularly, mainly due to diet and stress.

Stress plays a central role in these mental problems and possibly contributes to physical problems as well. Stressful life events trigger physiological, behavioral, and metabolic responses that are basically aimed at reinstating homeostasis. However, at present there is no integrated technology that can objectively assess the complexity and diversity of stress response. Establishment of a new biomental tool for simply, objectively assessing stress response is indispensable for improving the quality of lives of students as well as children, thus is an urgent need of society as a whole. High-throughput analysis of gene expression by microarray has a potential advantage to study the complex stress response. We have developed an original cDNA microarray specifically designed to measure the 1467 mRNA levels of stress-related genes in peripheral blood leukocytes. Using the cDNA microarray and a whole blood RNA collection system, we identified 70 genes and 24 genes useful for objective assessment of acute and chronic psychological stress responses, respectively. In addition we could detect abnormal gene expression profiles that are closely associated with major depression and chronic fatigue symdrome (CFS).

In this study, we recruited 377 students of our medical school and analyzed

1) their mental state, behaviors, and life style using 10 different questionnaires,
2) gene expression in peripheral blood cells using microarray,
3) salivary cortisol and chromogranin A responses, and
4) response of the prefrontal cortex by the light topography method.

At the same time, we pursued their mental state and stress responses observed during the regular examination period and preparation of national examination for physician's license.

To detect both environmental and biological risk factors for mental disorders, we systematically conducted these analyses and intended to establish a

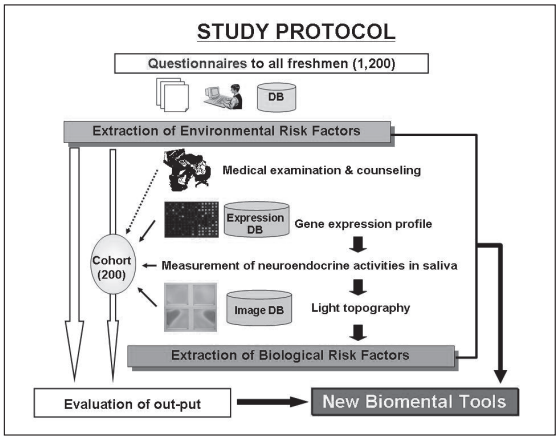

Fig. 1. Study protocol

new microarray-based, biomedical tool for student mental health. Our strategy is shown in Figure 1.

In separate experiments, using the stress DNA chip, we identified acute and chronic psychological stress-responsive genes (Biol Psychol 2007), ultraviolet B-sensitive genes (J Med Invest 2008), CFS-associated genes (Mol Med 2008), and environmental cadmium exposure-responsive genes (Toxicology 2009). All of these experimental protocols were approved by the Human Study Committee of Tokushima University Hospital. Written informed consent was obtained from each participant.

2. Results

1) Cohort study

We recruited total 377 freshmen (197 male and 179 female students) in this study. In May, we collected peripheral blood for measurement of gene expression pattern and saliva for measurement of cortisol and chromogranin A. At the same time, we asked them to fill out 10 different questionnaires; GHQ28, Zung SDS, HADS, NEO-FFI, STAI, PBI, EAT-26, PSQI, HPI, and Morningness-Eveningness Questionnaire, and we recovered them from 95% of the students. We have been following their mental state and analyzed their stress responses during regular examination period, CBT examination, and preparation of national examination for physician's license.

We found that gene expression profiling in peripheral blood is (1) a powerful tool for detection of pathological stress response possibly linked to stress-related disorders, (2) extremely stable, but variable depending on individuals variations, and that (3) there are environment-responsive groups of genes that may determine the individual phenotype (personality?), (4) static gene expression pattern may be linked to brain activities, and (5) patients with major depression has a unique gene expression signature, which is also observed in 6-12% of healthy subjects.

In addition, we also obtained the following results:

(6) Based on the gene expression patterns of 97 students analyzed with the stress DNA chip, we identified a group of genes (396 genes) that showed marked individual variations. The group of genes classified their gene expression into five patterns. Pathway analysis with IPA clearly visualized a gene expression signature relevant to a high risk group.

(7) Adverse parenting significantly interferes with the development of brain and mind. In this study, we examined association between parent rearing attitudes, mental state, neuroendocrine functioning, and gene expression of peripheral blood cells in healthy medical students. Based on the parental bonding instrument scores of 232 medical school freshmen, we extracted 22 freshmen who perceived their parents' rearing attitudes as low care combined with high overprotection (LOW) and 30 freshmen who grew up with optimal parenting (OPT; high care and appropriate protection). LOW students had significantly higher scores of anxiety and depression scales and showed a blunted cortisol awakening response (Fig. 2). Furthermore, real-time reverse transcription-PCR showed that LOW students possessed significantly higher amounts of the glucocorticoid receptor splice variant β (*GR*β) mRNA in circulating leukocytes, whereas the classical splicing isoform *GR*α was similary expressed between LOW and OPT groups (Fig. 3). Expression of *ADRB2* encoding β 2-adrenergic receptor was also significantly down-regulated in LOW students (Fig. 4). Our results suggest that negative perception of parents'child-rearing attitudes with depressive mood may be one of the potential elements for modification of the glucocorticoid signal in healthy young adults.

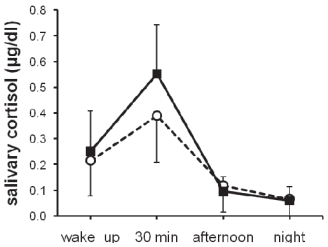

Fig. 2. Cortisol awaking response

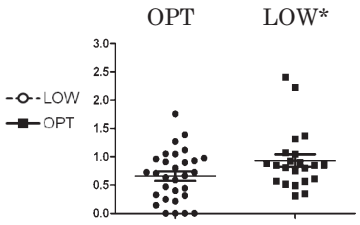

Fig. 3. Expression of GRβ mRNA.

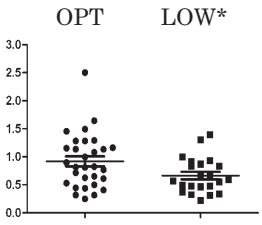

Fig. 4. Expression of ADRB2 mRNA

2) Gene expression signatures in patients with major depression

In this study, 37 patients (33 cases of the first episode and 4 cases of recurrent episode) were enrolled in this study. Out of the 37 patients, 26 patients were subjected to re-evaluation of gene expression at 8 weeks. We also recruited 91 healthy volunteers. The male-to-female ratio (63 women and 28 men) and age structure (42.9 ± 17.0, ranged 21-72 years old) were compatible with those of the enrolled patients. Differentially expressed genes between the group of 37 patients with the major depression and the group of 91 healthy individuals were extracted by *t*-test with Benjamini-Hochberg correction for multiple comparison at the 0.05 false discovery rate after gene expression analysis using a whole human genome array (Agilent). Then we selected 164 genes whose mean mRNA expression levels differed by > 1.5-fold: 61 and 103 genes were up- and down-regulated in the patients with major depression, respectively. Notably, the down-regulated genes rather than up-regulated genes appeared to mainly modify biofunctions. The most striking change was observed in cell death pathway by down-regulated genes.

Of the 37 patients, microarray analysis could be done in 26 patients two months after treatment. Their Hamilton scores were significantly improved from 21.10 ± 6.50 to 6.42 ± 5.95 (mean ± SD, n = 26). Using the same criteria, we extracted 200 treatment-responsive genes: 22 and 178 genes were up- and down-regulated after treatment, respectively.

Next, we examined whether the 164 major depression-associated genes responded to treatment and significantly changed their expression levels. As shown in Figure 5C, all down-regulated and many up-regulated genes did not change their expression even after treatment. Only 22 out of the 103 genes, which were identified as up-regulated genes in patients with major depression before treatment, significantly reduced their expression in response to treatment (Figure 5C). It was of interest that the 22 overlapped genes were all included in the depression-associated, up-regulated genes (Figs. 5A and 5B), while none of the down-regulated genes unique to major depression significantly recovered their expression levels even after treatment. These results suggest that patients with major depression may possess both trait-associated and state-related (treatment-responsive) genes in leukocytes.

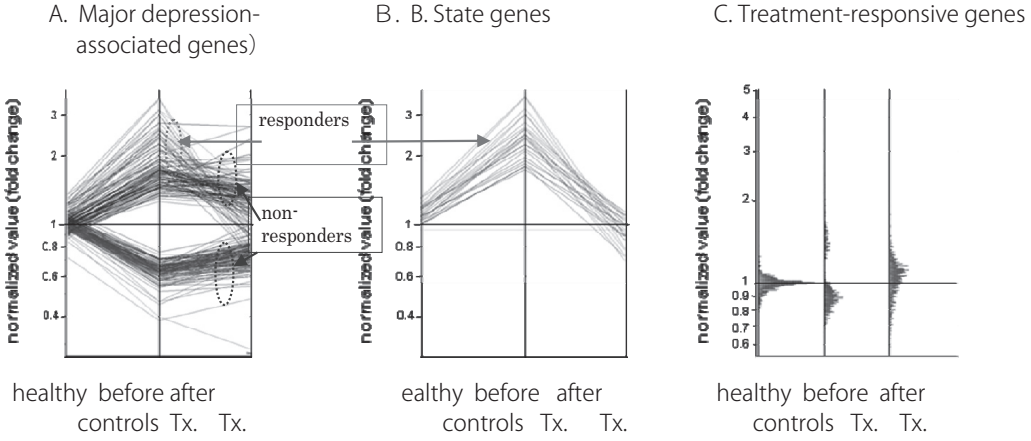

Fig. 5. Changes in gene expression two months after treatment

3) Gene expression signatures in chronic fatigue syndrome

Under the collaboration with Dr. Watanabe's group, using a whole human genome array (Agilent), we identified differentially expressed genes between 64 patients with chronic fatigue syndrome (CFS) and 70 healthy volunteers. Male-to-female ratio and age structure were compatible between the two groups. We identified differentially expressed genes by t-test with Benjamini-Hochberg correction for multiple comparison at the 0.05 false discovery rate, and then we selected 200 genes whose mean mRNA expression levels differed by > 2-fold: 68 and 132 genes were up- and down-regulated, respectively. Ingenuity Pathway Analysis revealed that top 15 canocical pathways modified were 1) Thrombopoietin Signaling, 2) IL-9 Signaling, 3) IL-15 Signaling, 4) JAK/Stat Signaling, 5) IL-3 Signaling, 6) Mitochondrial Dysfunction, 7) VEGF Signaling, 8) CNTF Signaling, 9) Oxidative Phosphorylation, 10) IGF-1 Signaling, 11) Glucocorticoid Receptor Signaling, 12) IL-2 Signaling, 13) Dendric Cell Maturation, 14) Integrin Signaling, and 15) IL-22 Signaling. Considering the pathophysiology of CFS, it was of

interest that oxidative phosphorylation was ranked as the top 5th-scored pathway. In fact, a number of components in the respiratory chain complexes were modified (Fig. 6)

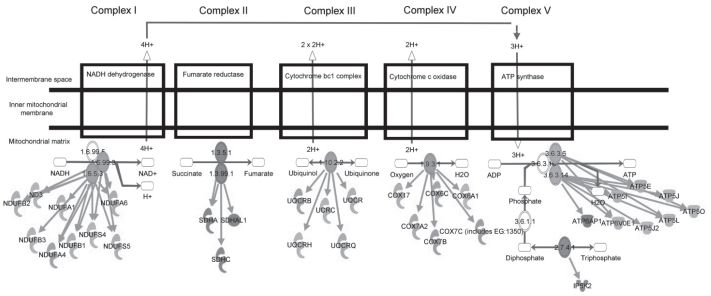

Fig. 6 Changes in expression of genes encoding components of mitochondrial respiratory chain complexes. Green and red show down- and up-regulated genes, respectively.

4) Gene expression signatures in Autism

Under the collaboration with Dr. Kamio's group, we compared gene expression between 21 patients with Autism spectrum disorders (ASDs) (ASD; 17 males and 4 females; aged 26.7 ± 5.5 years) and 21 age- and sex-matched healthy subjects (ASD control), and between 21 mothers having Autism children (Autism mothers; aged 45.0 ± 6.7 years) and 21 age-matched healthy females, using a whole human genome microarray (Agilent). We identified differentially expressed genes by t-test with Benjamini-Hochberg correction for multiple comparison at the 0.05 false discovery rate, and then we selected 25 and 101 genes whose mean mRNA expression levels differed by > 2-fold between ASD and ASD controls and between ASD mothers and control mothers, respectively. It should be noted that these differentially expressed genes were commonly observed as differentially expressed genes both in ASD and ASD mothers. We also validated the commonly changed genes both in ASD and ASD mothers with quantitative real-time PCR. These results may provide a potential approach to understand the nature and nurture elements responsible for the development of ASDs.

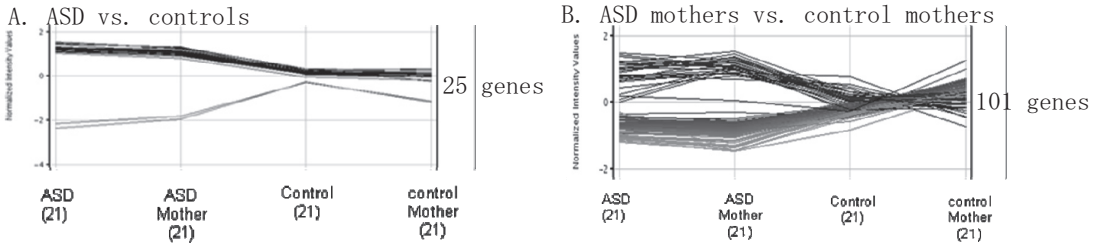

Fig. 7. Expression of 25 (A) and 101 (B) genes differentially expressed between ADS and ASD controls, and between ASD mothers and control mothers, respectively.

5) Development of a non-invasive method for stress assessment

In this cohort study, we measured 50 different cytokines in serum and saliva from 377 students and identified chronic psychological stress-responsive cytokines when 26 students were preparing for the national examination of physician's license. We established a method for assessment of chronic psychological stress by profiling 22 or 8 cytokines. At the same time, we found that cytokine profiling in saliva was also useful for assessment of chronic psychological stress. We are now trying to establish a non-invasive stress assessment method using saliva.

3. Outcomes of this cohort study

In this study, we have established a gene expression data base that includes gene expression profiles in peripheral blood cells in healthy subjects with different ages and sex. We will determine environmental and biological risk factors for development of mood disorders in university students. Based on the results of our study. We have established novel methods for assessment of chronic psychological stress, major depression, chronic fatigue syndrome, and possibly Autism. Based on these outcomes, we will develop a novel questionnaire useful for prediction of mood disorders, non-invasive stress assessment method, and diagnostic PCR array for major depression, chronic fatigue syndrome, and Autism.