PROCEEDING

Assessment of human stress and depression by DNA microarray analysis

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Abstract : Precise assessment of stress is an imminent issue to deal with stress-related social, medical and psychological problems. Psychological stress is known to stimulate the neuroendocrine, sympathetic nervous, and immune systems. By analyzing mRNA expression levels in leukocytes, which express receptors for hormones, neurotransmitters, growth factors, cytokines, and other stress related signals, levels of stress may be adequately measured. In a series of studies, our group has developed a cDNA microarray specifically designed to measure the mRNA levels of stress-related genes in peripheral blood leukocytes. This microarray enabled us to sensitively detect the response to psychological stress. In addition, our preliminary study suggests that the array could differentiate patients with depression from sex- and age-matched control subjects. J. Med. Invest. 52 Suppl. : 266-271, November, 2005

Keywords **:** *stress, depression, biological maker, DNA microarray*

OBJECTIVE MEASUREMENT OF STRESS

Psychological stress influences levels of quality of life. Moreover, stress has been implicated in the pathogenesis of various psychiatric and psychosomatic disorders. Precise assessment of stress is an imminent issue to deal with stress-related social, medical and psychological problems. While most studies utilize subjective questionnaires for the assessment of psychological stress, some objective methods has been introduced. Psychological stress is known to stimulate the hypothalamus-pituitary-adrenal (HPA) axis, sympathetic nervous system, and immune system. These systems interact with each other, leading to the complex stress response (1, 2). In addition to corticotrophin-releasing hormone, adrenocorticotropic hormone, and glucocorticoids, physiological stress stimulates production of cytokines and modifies inflammatory and immune responses. Measurement of one of these hormones or cytokines has been used to objectively assess the levels of stress. However, their usefulness as a biological marker is limited, because of the unsatisfactory sensitivity and/or specificity.

One of the new approaches for the assessment of stress response is to use DNA microarray. The microarray is an emerging technology that allows simultaneous measurement of thousands of mRNA transcripts in biologic samples. The microarray is now recognized as a useful clinical device to make diagnostic, therapeutic, or prognostic decisions for patients. Considerable progress has already been made in clinical cancer researches, using systematic analysis of gene expression patterns to define tumor subtypes, identify molecular markers, and investigate new therapies (3-10). The examples of special note are applications in the differential diagnosis of adult acute leukemias (4) and the identification of clinical-outcome

Received for publication September 9, 2005 ; accepted September 16, 2005.

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predictors in adult acute myeloid leukemia (5) and breast cancer (6, 7). In addition to these applications, high-throughput analysis of gene expression by microarray may have a potential advantage of being able to study complex responses, such as psychological stress response, in which the measurement of limited numbers of gene products does not always reflect the status.

Peripheral leukocytes produce various cytokines, and proinflammatory cytokines, particularly gp130 family members, directly stimulate the HPA axis (11). At the same time, leukocytes express receptors for stress mediators, such as hormones, neurotransmitters, growth factors, and cytokines. Thus, leukocytes may be a potential target for the evaluation of psychological stress response. In a series of studies, we have developed a cDNA array specifically designed to measure the mRNA levels of stress-related genes in peripheral blood leukocytes (12).

DEVELOPMENT OF STRESS-SENSITIVE MICROARRAY

Construction of stress microarray and data analysis

We started to list stress-related genes (stress hormones, neurotransmitters, cytokines, growth factors, receptors, signal transduction molecules, transcription factors, heat shock proteins, growth- or apoptosis-associated factors, and metabolic enzymes) from the UniGene database of the National Center for Biotechnology Information (Ftp://ncbi.nlm.nih.gov.repository/unigene/). Target sequences of the listed genes were designed using original software (Hitachi, Saitama, Japan), and we selected 1,467 genes that were actually amplified by reverse transcriptase-PCR using total RNA isolated from peripheral leukocytes of healthy volunteers (see http : //www.hitachi.co.jp/LS/ for the full list of genes). All PCR products were sequenced to be the corresponding cDNAs, and they were spotted on the array according to the method previously described (13). The microarray showed high reproducibility with a mean coefficient of variation of less than 20 %, and the dynamic ranges were three orders of magnitude.

Signal intensities of Cy5 and Cy3 were quantified and analyzed by subtracting the backgrounds, using QuantArray software (GSI-Lumonics). The intensity values for duplicate cDNA probes were averaged. Following global normalization, we selected several hundreds genes with a fluorescence intensity higher than the cut-off value of 300 in both conditions (labeled with Cy5 or Cy3). The relative expression values (Cy5/Cy3) for these genes were subjected to hierarchical clustering using GeneSpring 6.0 software (Silicon Genetics, Redwood City, CA, USA) and similarity analysis by standard correlation. After Cy5/Cy3 ratios of these genes were transformed to logarithms, data were statistically analyzed.

Effect of leukocyte preparation on gene expression profiles

Freshly isolated or cultured mononuclear cells are usually used to examine target genes relevant to disease pathogenesis (14). These preparations are also applied to expression analysis with microarray. It recently became possible to directly prepare RNA from whole blood using a commercially available kit, which may eliminate non-specific changes in mRNA levels during preparations (15, 16). When mRNA levels in isolated leukocytes (mixture of mononuclear cell- and neutrophil-rich fractions) were compared with those prepared from whole blood, the isolation procedures increased the expression values of 52 genes > 2-folds and decreased those for 10 genes to < 50 %. Thus, the isolation procedures, such as mechanical stimuli, significantly altered expression of stress-responsive genes. Whitney *et al.* also suggested that excessive *in vitro* handling required for isolation of monocytes from peripheral blood leads to a gene expression "signature" of cell stress, including up-regulation of v-fos (*FOS*), *CD83* and *CD69*, tumor necrosis factor (TNF)-α induced protein 3 (*TNFAIP3*), *DUSP2* (17). Based on this information, together with our findings, RNA samples directly prepared from whole blood were used to correctly assess the stress response in the following experiments.

Effects of exercise and daily activity

We examined how physical exercise affected the gene expression in peripheral leukocytes. Treadmill exercises under aerobic conditions (< 60% of VO_2 max for 1 h) did not change the expression pattern in 5 healthy volunteers, although exhaustive exercise with treadmill significantly changed it. We identified 26 genes whose expressions were significantly changed after the exhaustive exercise.

We also examined whether routine works affected the gene expression in peripheral leukocytes in daily life. It was suggested that lunch or dinner transiently changed the mRNA levels of 11 genes, such as IL-2 receptor β chain (*IL2RB*), *MYB*-related gene *BMYB* (*MYBL2*), IL-7 receptor (*IL7R*), general transcription factor IIF (*GTF2F1*), interferon inducible mRNA

fragment (*G1P3*), telomerase reverse transcriptase (*TERT*), phosphoinositide 3-kinase-associated p85 (*PIK3R1*), T-cell specific protein (*RANTES*), CDC-like kinase (*CLK1*), dihydropyrimidine dehydrogenase (*DPYD*), KIAA0822 protein (*ABCA8*). But other daily activities had no effect. In contrast, we confirmed that mRNA levels for the selected 70 genes were stable in daily life ; diet, classworks, or light exercises did not significantly alter their mRNA levels.

Assessment of stress with the stress-sensitive microarray

We asked graduate students who would take the final examination for the PhD to participate in this study. They were in good physical health, were taking no medication, and had no history of psychiatric or somatic diseases. The final examination consisted of an oral presentation of the PhD theses and a question-and-answer session. Venous blood (10 ml) was taken from each subject 2 h before and 2 or 24 h after the examination. The sample collected 4 wk before the presentation was used as a reference. All blood samples were collected under fasting conditions.

Hierarchical cluster analysis of the relative expression values identified two groups of genes, whose expressions were uniformly up-regulated or down-regulated at 2 h after the presentation. Most of the expression levels returned to the baseline within 24 h after the examination. Bayesian *t*-test (error rate = 0.05) identified 70 genes whose mRNA levels were significantly changed at 2 h after the examination.

The neuroendocrine response, activated by psychological stress, converts stress into changes in mononuclear cell functions (18), and stimulates the production of TNF-α, IL-6, IL-1 receptor antagonist, interferon (IFN)-γ, and IL-10 (19). The significantly up-regulated genes included receptors for these cytokines and their associated molecules : IL-1 receptor (*IL1R1* and *IL1R2*), TNF receptor homologue (*TNFRSF10C*), TNF-α-induced protein (*TNFAIP6*), IFN-γ receptor 2 (*IFNGR2*), interferon (*IFN*)-induced cellular resistance mediator protein (*MX2*), IFN-regulatory factor-2 (*IRF2*), and IFN-inducible proteins (*IFITM1* and *IFITM3*). This suggests that the stress-responsive cytokines may stimulate their receptor signals in peripheral leukocytes in response to the stress. In addition, the stressful event significantly up-regulated the expression values of several other cytokine/chemokine receptors and their related genes, such as colony-stimulating factor receptors (*CSF2R* and *CSF3*), *IL17R*, *IL8RA*, *IL8RB*, chemokine ligands (*SCYB5* and *GRO1*), Fc fragment of IgG (*FCGR2B*), IL-regulated nuclear factor (*NFIL3*), and selectin L (*SELL*).

The mRNAs for stress-mediating molecules including hypoxia-inducible factor 1 (*HIF1A*), *FOS*, and p38 MAP kinase (*MAPK14*), as well as those for heat shock protein 70 members (*HSPA6* and *HSPA1A*), were also significantly up-regulated. The activation of catecholamine receptors and glucocorticoid receptor indirectly or directly modifies the transcription of various genes (20, 21). Psychological stress rapidly and transiently activates nuclear factor-κB, a hallmark of inflammatory responses (22), in association with elevated levels of catecholamines and cortisol (18). Thus, psychological stress activates multiple signaling pathways ; therefore it is difficult to fully explain the biological significance of several other genes. With regard to the significantly down-regulated genes, however, the life event stress generally down-regulated mRNA expression for growth-related genes and cytochrome *c* oxidase subunits.

BIOLOGICAL MARKERS OF DEPRESSION

Depression affects about 10 % of the population at some point in their life and is the leading cause of disability in the nations with developed economies (23). The disease is potentially fatal because 15% of patients with severe depression eventually die by suicide (24). If treated properly, most patients would recover from the disease. However, studies have shown that depression, which lacks specific objective findings, is often missed or undiagnosed (25). Establishment of convenient and reliable biological markers would greatly improve the precise diagnosis and consequently the welfare of depressed patients.

Depression affects not only the mind but also the entire organ systems through the interaction between the brain and the body. There are convincing evidences indicating endocrinological, immunological and autonomic nervous system disturbances in depression (26). A large number of studies have been conducted to establish a diagnostic marker by detecting one of these systemic disturbances. One of the examples most extensively studied is the attempt to detect the hyperactivity of HPA axis by using DST or its modification such as Dex/CRH test (27). Measurement of neurotransmitter receptors or transporters located on the blood cells have been also studied vigorously on the assumption that they reflect to some extent their counterparts in the CNS. For example, decreased 5HTT binding have been reported in platelets of depressive patients (28, 29), although some studies reported no change

(30-32). More recently, with the progress of experimental procedure, altered mRNA levels in leukocytes have been reported, such as decreased dopamine D4 receptor mRNA levels (33) or increased serotonin transporter mRNA levels (34) in major depression and decreased CREB mRNA levels in treated major depression (35). Our group has found increased serotonin transporter mRNA levels in leukocytes. Although these and other studies with peripheral measures have contributed greatly to the understanding of the mood disorders, its usefulness as a biological marker in the clinical setting is limited, because of the unsatisfactory sensitivity and/or specificity as well as the troublesome procedure requiring the intense cooperation of patients.

ASSESSMENT OF DEPRESSION WITH THE STRESS-SENSITIVE MICROARRAY

We have been exploring to establish a new biological marker of depression by profiling mRNA expression from the leukocytes of the patients by using the stress sensitive microarray. As is the case with psychological stress, we predict that the characteristic abnormalities in the neuroendocrine, neuroimmune and autonomic nervous system in depression should have effects on the expressional pattern of mRNA in the leukocytes from depressed patients.

We have reported preliminary results with 32 patients (36). They met the criteria of Major Depressive Disorder according to Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (37), aged from 18 to 65, with no psychiatric comorbidities, no serious somatic diseases, and no use of antidepressants. Hamilton Rating Scale for Depression (HAM-D) and Clinical Global Impression Scale (CGI) were used for clinical evaluation. Peripheral blood was collected before the treatment and expression levels were compared with sex-and age-matched healthy volunteers.

The microaray analysis revealed that expression of a dozen of genes showed significant changes in the total group of the patients compared with controls. These genes may be useful as biological makers of depression. In addition to these genes, there were dozens of genes significantly altered in a half of the patients compared with controls, thus dividing the patients into two groups, although no symptomatic or demographic variables could account for the distinction. Altered expression of genes of various categories in a subgroup of depression might be relevant to the pathophysiology of this subgroup. It was also of note that some of the altered genes before treatment significantly changed toward the reverse direction after treatment.

These preliminary results revealed sets of gene expressions that could distinguish depressed patients from controls. These alterations were different from those observed in volunteers after stress or those in preliminary samples of patients with schizophrenia. Mechanisms of the alteration remain unclear. Neurotransmitter, endocrinological and immunological abnormalities are thought to have contributed to the alteration of the expression to some extent. Some of the alteration may directly reflect intracellular abnormalities of depression that might be present in the leukocytes. These altered genes provide clues for elucidating molecular mechanisms of the disease. The microarray method has a great advantage over pervious biological markers in that it can utilize hundreds of parameters from a small amount of peripheral blood cells. Thus, a complicated and probably heterogeneous disease such as depression could be adequately recognized. This approach has opened a novel and promising horizon in the search of a biological marker of depression.

CONCLUSION

Adequate biological assessment of stress would help to solve stress related social, medical and psychological problems. High-throughput analysis of gene expression by microarray may have a potential advantage of being able to study complex responses, such as psychological stress response, in which the measurement of limited numbers of gene products does not always reflect the status. We developed a cDNA microarray specifically designed to measure the mRNA levels of stress-related genes in peripheral blood leukocytes. Using this microarray, mRNA expressional change under stress was detected with good sensitivity and specificity. Moreover, preliminary results suggest that the array could differentiate patients with depression from sex-and age-matched control subjects. Thus, DNA microarray with stress-related genes may provide a new biological maker for stress response and stress-related disorders.

ACKNOWLEDGEMENT

This work was supported by a Grant-in-Aid for

Scientific Research and the Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology (KR), a Health and Labor Science Research Grant from the Ministry of Health, Labor and welfare (TO), and a Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology and a Grants-in-Aid for Scientific Research from the 21st Century COE Program, Human Nutritional Science on Stress Control Tokushima, Japan.

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