

れる。

E. 結論

ヒトの遺伝子産物を網羅的に測定できるDNAチップを用い、統合失調症の新しい評価方法の確立を目指して研究を行った。順調に研究がすすみ、ある程度の結果が出つつある。

本研究は先端的かつ独創的なものであるが、一方で患者負担は少量通常採血のみであり、統合失調症評価への臨床応用が現実的である。プライマリーケア、健康診断、精神科診療施設などの場で早期診断、病態評価および治療評価に応用可能である。統合失調症の診断や治療に客観的な指標の導入を実現させることが期待され、社会的・医療行政的意義は大きい。

F. 健康危険情報

特になし

G. 研究発表

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H. 知的財産権の出願・登録状況（予定を含む）

1. 特許取得
該当なし。
2. 実用新案登録
該当なし。
3. その他
該当なし。

研究成果の刊行に関する一覧表

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Serotonin transporter mRNA expression in peripheral leukocytes of patients with major depression before and after treatment with paroxetine

Jun-ichi Iga^a, Shu-ichi Ueno^{a,*}, Ken Yamauchi^a, Ikuyo Motoki^a, Sumiko Tayoshi^a, Koshi Ohta^a, Hongwei Song^a, Kyoko Morita^b, Kazuhito Rokutan^b, Tetsuro Ohmori^a

^a Department of Psychiatry, Course of Integrated Brain Sciences, The University of Tokushima School of Medicine, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan

^b Department of Stress Science, Institute of Health Biosciences, The University of Tokushima School of Medicine, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan

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Abstract

Serotonin transporter (5HTT) is thought to be involved in the pathophysiology of major depression and the target of antidepressants. We hypothesized that 5HTT mRNA levels in peripheral leukocytes may be associated with depressive states and the therapeutic response to antidepressant treatments. Fifteen patients with major depression and age-, sex-matched control subjects were studied. 5HTT mRNA levels were determined with quantitative real-time PCR method. 5HTT mRNA levels in leukocytes were significantly higher in depressive patients at baseline (before medication) than in control subjects. 5HTT mRNA levels were decreased significantly after 8 weeks of paroxetine medication compared with those at baseline. Our investigation suggested that the increased expression of 5HTT mRNA in peripheral leukocytes may be related with the pathophysiology of depression and its reduction after treatment may reflect the adaptive change induced by the antidepressant. © 2005 Elsevier Ireland Ltd. All rights reserved.

The serotonin transporter (5HTT) is the initial target for many classes of antidepressants, especially selective serotonin reuptake inhibitors (SSRI). 5HTT plays a key role in the regulation of serotonergic neurotransmission [14] and is one of the potential loci for the vulnerability of depression [23]. Measurement of 5HTT located on the blood cells has been studied vigorously on the assumption that they reflect to some extent their counterparts in the CNS. Urbina et al. [24] reported that the number of binding site of [³H] paroxetine in lymphocytes was significantly reduced, while the affinity was unchanged, in patients with major depression as compared to controls. In addition, there was a partial recovery of the binding site in lymphocytes after administration of antidepressants, accompanied with clinical improvement. Decreased 5HTT binding has been also reported in platelets

of depressive patients [17,21], although some studies reported no change [3,12,16].

Recently, with the progress of experimental procedure, altered mRNA levels in leukocytes have been reported, such as decreased dopamine D4 receptor mRNA levels in major depression [20] and decreased CREB mRNA levels in treated major depression [11]. We established the procedure for a precise measurement of 5HTT mRNA levels in leukocytes and measured the levels in major depressive patients before and after the antidepressant treatment.

The subjects consisted of 15 patients with major depression (5 males, 10 females, mean age 45.9 ± 14.3) and 15 age- and sex-matched controls (5 males, 10 females, mean age 46.5 ± 13.2). Before study participation, all subjects signed an informed consent form approved by the Ethical Committee of University of Tokushima School of Medicine. All patients were diagnosed as Major Depressive Disorder according to DSM-IV (APA 1994) by at least two trained

* Corresponding author. Tel.: +81 86 633 7130; fax: +81 86 633 7131.
E-mail address: s-ueno@clin.med.tokushima-u.ac.jp (S.-i Ueno).

psychiatrists. All subjects underwent extensive medical, neurological, psychological and laboratory evaluations before participating in the study. The diagnosis and the eligibility of the patients were reconfirmed during follow-up periods.

All patients did not receive any antidepressants for the current episode before blood sampling. Twelve patients were in the first and others were in the recurrent depressive episode. Eleven out of 15 patients were treated with paroxetine for 8 weeks but other four patients left the study protocol and assessed at baseline only. The dose of paroxetine was started with 10–20 mg for the first 2 weeks and gradually increased to 40 mg based on the judgment of the trained clinician. At each visit, subjects were rated with Structured Interview Guide for the 17-item Hamilton Depression Rating Scale (SIGH-D 17, [26]; Japanese version, Nakane, 2000) before blood collection. Clinical assessment and blood collection were conducted at baseline, 4 and 8 weeks.

Peripheral blood was also collected from 15 sex- and age-matched volunteers who were in good physical health with a history of neither psychiatric nor serious somatic disease and were not taking any medication. Proband who had first-degree relatives with psychiatric disorders were excluded.

The paroxetine quantification was performed using high performance liquid chromatography with 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBDF)-derivatization, according to the method of Irie et al. [9] with slight modification in that the separation was performed on a Phenomenex C18 column (4.6 mm × 250 mm).

Total RNA was extracted from leukocytes of whole blood samples using the PAXgene Blood RNA kit (Qiagen, Tokyo, Japan) according to the manufacturer's recommendations. Residual genomic DNA was digested with RNase-free DNase I (Qiagen). Total RNA (1–5 ug) was used for cDNA synthesis by oligo (dT) primers and Powerscript Reverse Transcriptase (BD Biosciences, Japan).

Primers and hybridization probes were selected and optimized at exon–intron boundary of serotonin transporter gene by Nihon Gene Research Lab's Inc., Sendai, Japan. Primers were as followed: Forward primer; 5'-TCTATGGCATCACTCAGTT-3', Reverse primer; 5'-TGGAA-AAGTCGTAGTTGTG-3'. Hybridization probes were as followed: 5'-AACAGGAGAAACAGAGGGCTGATGGC-3'-Fluorescein, 5'-LCRed640-ACCCAGCAGATCCTCCAG-AACCACC-3'-phosphorylation. Quantitative real-time PCR was performed with LightCycler (Roche Diagnostics, Tokyo, Japan). Two housekeeping genes were used for normalization (glucose-6-phosphate dehydrogenase; G6PD and hypoxanthine guanine phosphoribosyltransferase; HPRT, Qiagen). Measurement of each gene expression was conducted in triplicate. Proper amplification of the quantitative PCR products of 5HTT and G6PD genes was confirmed by agarose gel electrophoresis in all samples.

Genomic DNA was extracted according to standard methods. Allelic variants of the 5HTTLPR (5HTT linked polymorphic region) were determined with the method described [15].

Allelic variants of the 5HTTVNTR (5HTT variable number of tandem repeats) were also genotyped [13].

Statistical calculations were carried out using the SPSS Statistical Software Package 11.5 (SPSS, Tokyo, Japan). Differences between patients and control subjects were calculated using the Mann–Whitney *U*-test. Changes during the treatment were calculated with the Friedman test followed by the Wilcoxon rank sum test.

The relative amount of 5HTT mRNA in peripheral leukocytes was standardized with G6PD mRNA as an internal standard. We also used HPRT mRNA as a standard in several samples but obtained almost the same results (data not shown). The coefficient of variance was less than 20%. 5HTT mRNA levels (5HTT mRNA/G6PD mRNA × 10⁴) was in the range of 0.07–5.34 (mean ± S.D. 1.12 ± 1.23; Fig. 1) in healthy volunteers, while 0.2–9.07 (mean ± S.D. 2.49 ± 2.21) in 15 medication-free depressed patients, showing a statistical difference (Mann–Whitney *U*-test: *P* = 0.011; Fig. 1). In patients with mean ± S.D. of 5HTT mRNA levels at baseline was 3.10 ± 3.39 in males, while 2.18 ± 1.46 in females. In controls, the level was 0.83 ± 0.46 in males, while 1.27 ± 1.49 in females. Neither patients nor controls showed significant sex difference in the 5HTT mRNA levels. 5HTT mRNA levels at baseline was 2.68 ± 2.43 in 12 first episode patients, while 1.70 ± 0.70 in three recurrent episode patients, showing no statistical difference. No significant relationship between 5HTT mRNA levels and baseline SIGH-D score was observed.

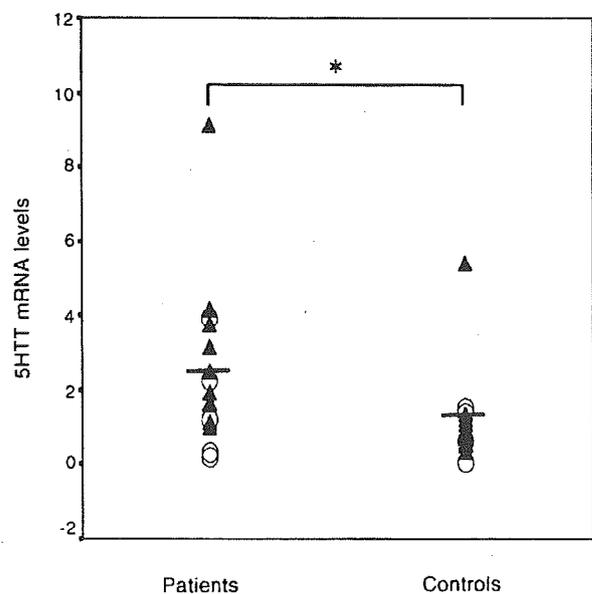


Fig. 1. The measurement of 5HTT mRNA/G6PDmRNAx4 in peripheral leukocytes in depressive patients (*n* = 15) and control subjects (*n* = 15). Mann–Whitney *U*-test: **P* < 0.05. The '▲' symbols mean the genotype of homozygous 14A type of 5-HTTLPR and homozygous 12 tandem repeats of 5-HTTVNTR. The '○' symbols mean the other genotypes. The mean are indicated by a horizontal line.

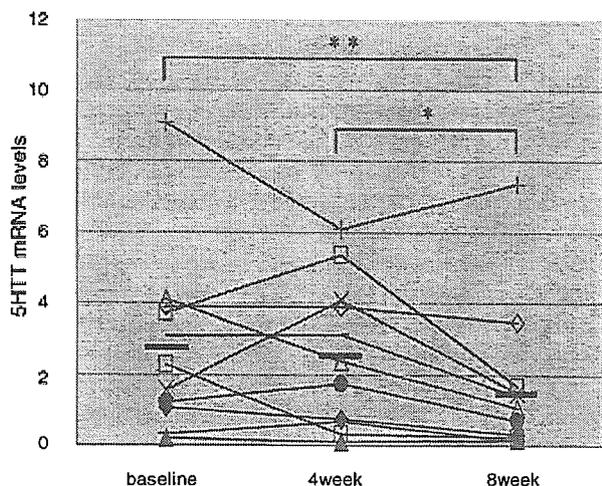


Fig. 2. The 5HTT mRNA levels in leukocytes of MDD patients were significantly decreased at 8 weeks compared with those at baseline and 4 weeks (baseline: 2.77 ± 2.52 ; 4 weeks: 2.53 ± 2.14 ; 8 weeks: 1.64 ± 2.13 ; Friedman test: $P < 0.01$ Wilcoxon rank sum test: ** $P < 0.01$ and * $P < 0.05$). The mean for baseline, 4, and 8 weeks samples are indicated by horizontal line.

Mean paroxetine doses were 18.3 ± 5.8 mg/day (10 mg/day $n=3$, 20 mg/day $n=8$, 30 mg/day $n=1$) and 32.5 ± 8.7 mg/day (20 mg/day $n=3$, 30 mg/day $n=3$, 40 mg/day $n=6$) in 4 and 8 weeks, respectively. Mean paroxetine concentration increased to 36.3 ± 23.3 and 81.4 ± 54.7 ng/ml at 4 and 8 weeks, respectively. There was a significant change in the mean SIGH-D scores during the treatment (baseline 19.6 ± 9.0 ; 4 weeks: 16.9 ± 6.8 ; 8 weeks: 11.4 ± 9.3 ; Friedman test: $P=0.018$). The score was significantly decreased in 4 and 8 weeks compared with baseline (Wilcoxon rank sum test: $P=0.028$ and 0.017 , respectively). There was a significant change in the mean 5HTT mRNA levels during the treatment (baseline: 2.77 ± 2.52 ; 4 weeks: 2.53 ± 2.14 ; 8 weeks: 1.64 ± 2.13 ; Friedman test: $P=0.006$; Fig. 2). The level was significantly decreased in 8 weeks compared with either at baseline or 4 weeks (Wilcoxon rank sum test: $P=0.003$ and 0.041 , respectively; Fig. 2). There was no significant correlation between the change of mRNA levels and the change of

SIGH-D scores from baseline to 8 weeks (Spearman's correlation coefficient by rank: $P=0.467$, $r=-0.261$).

Ten of our 15 patients had the genotype of homozygous 14A type of 5-HTTLPR and homozygous 12 tandem repeats of 5-HTTVNTR. Even when these 10 patients were compared with age-, sex- and genotype-matched control subjects, there was a significant difference in the 5HTT mRNA levels ($n=10$; Mann-Whitney U -test: $P=0.028$). Genotype information of patients and control subjects is shown in Table 1.

The present study is the first report to measure the 5HTT mRNA expression in leukocytes of patients with major depressive disorder (MDD) by the quantitative real-time PCR method. There are two major findings in our investigation.

First, the mean 5HTT mRNA levels at baseline was significantly elevated in patients. Two genetic polymorphisms of 5HTT gene have been reported to modulate the expression of 5HTT [4,6]. However, the 5HTT polymorphisms did not contribute to this finding, since the significant difference of gene expression was still observed between the genotype-matched depressive and control subjects. Ten of 15 patients had the genotype of homozygous 14A of 5HTTLPR and homozygous 12 tandem repeats of 5HTTVNTR. These genotype frequencies are consistent with previous reports on Japanese population [10,15]. There were two subjects with extremely high levels of 5HTT mRNA, one in the patients and one in the controls. The genotypes of these outliers are also homozygous 14A of 5HTTLPR and homozygous 12 tandem repeats of 5HTTVNTR. Removal of these two outliers from the statistical analysis did not change the results. Serotonin levels in blood are known to be different in sexes and 5HTT is a quantitative trait locus for whole blood serotonin levels in males [25]. However, in this present study, neither depressive nor control subjects showed significant difference between males and females in the 5HTT mRNA levels. Interestingly, levels of 5HTT mRNA expression in recurrent episode patients are substantially lower than those in first episode patients. Further studies need to address the potential difference between first and recurrent patients.

Our result may not be easily reconciled with previous reports showing decreased 5HTT binding sites in the

Table 1
Genotype information of patients and control subjects

5HTTLPR	5HTTVNTR	N	Sex (M/F)	Mean age	mRNA levels at baseline
Patients					
14A/14A	12/12	10	4/6	46.7 ± 13.4	2.94 ± 2.41
16D/16D	12/12	2	1/1	62.0 ± 5.6	1.74 ± 0.73
14A/16D	12/12	2	0/2	36.0 ± 7.1	2.06 ± 2.62
14A/14A	10/12	1	0/1	26	0.31
Controls					
14A/14A	12/12	10	4/6	46.1 ± 14.7	1.26 ± 1.47
14A/14A	10/12	1	0/1	29	0.68
14A/16A	10/12	1	1/0	53	0.07
14A/16D	10/12	1	0/1	53	1.52
14A/16C	12/12	1	0/1	45	1.38
14A/16A	12/12	1	0/1	57	0.63

peripheral blood cells. A possible explanation for the discrepancy may be that the increased levels of mRNA are associated with increased degradation or internalization of 5HTT protein [18]. Interestingly, however, this result could be related with the recent PET studies [8,19] reporting significantly increased 5HTT binding in the brain of depressed patients. Alternatively, this result could be secondary to increased cortisol levels, since a study showed that addition of glucocorticoid hormone caused an increase in 5HTT expression in immortalized human B-lymphoblastoid cells [5]. However, we did not measure cortisol levels in the present study.

Second, 5HTT mRNA levels were significantly decreased after 8 weeks of paroxetine treatment. This result may be consistent with the animal study showing downregulation of 5HTT sites after chronic administration with SSRIs [1,7]. One recent study [2] showed 5HTT mRNA levels in the rat dorsal raphe nucleus were increased slowly, reaching a statistically significant increase, by a maximum of 29% after 10 days of sertraline treatment, and then decreased back to baseline after 21 days of treatment. More detailed time course study may be necessary in our study. Since the decrease of 5HTT mRNA levels in the leukocytes was observed in 8 weeks but not 4 weeks, it seems to reflect the adaptive change of 5HTT after the long-term use of the SSRI.

In conclusion, our investigation revealed that the mean 5HTT mRNA levels were significantly elevated at baseline in depressive patients and were significantly decreased after paroxetine treatment. The results suggest that increased levels of 5HTT mRNA in peripheral leukocytes may be a useful biological marker that reflects the pathophysiology of depression. Admittedly, the small sample size limits the interpretation. In addition, underlying mechanisms of increased expression of 5HTT mRNA are unclear. Measurement of 5HTT binding sites or protein levels in peripheral blood cells as well as measurement of plasma cortisol levels would help to clarify the mechanisms. Further studies are necessary to confirm and extend the present results.

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Expression analysis of psychological stress-associated genes in peripheral blood leukocytes

Kyoko Morita^a, Toshiro Saito^b, Masayuki Ohta^b, Tetsuro Ohmori^c, Kaori Kawai^a, Shigetada Teshima-Kondo^a, Kazuhito Rokutan^{a,*}

^a Department of Stress Science, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan

^b Life Science Group, Hitachi, Ltd., 1-3-1, Minamidai, Kawagoe, Saitama 350-1165, Japan

^c Department of Psychiatry, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan

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Abstract

In this study, we have developed a microarray including 1467 cDNAs that were selected to specifically measure stress response in peripheral blood leukocytes. Venous blood was collected from 10 graduate students 2 h before and 2 or 24 h after an open presentation for their Ph.D. The mRNA levels in leukocytes were compared with those prepared 4 weeks before the presentation. Hierarchical cluster showed that distinct groups of genes uniformly changed their expression values in response to the stress. Bayesian *t* test identified significantly up-regulated 49 genes and down-regulated 21 genes. Most of them are categorized into cytokines, cytokine receptors, growth- or apoptosis-related molecules, and heat shock proteins, suggesting that stressful life events trigger acute responses in leukocytes. Our results suggest that gene expression profile in peripheral blood leukocytes may be a potentially useful method for the assessment of complex stress responses.

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Keywords: Psychological stress; Stress response; Microarray; Bioinformatics

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 [3,6–8,11,21–23]. The examples of special note are applications in the differential diagnosis of adult acute leukemias [3] and the identification of clinical-outcome predictors in adult acute myeloid leukemia [21] and breast cancer [8,22]. 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.

Psychological stress stimulates the hypothalamus–pituitary–adrenal (HPA) axis, sympathetic nervous system, and immune system. These systems interact each other, leading to the complex stress response [5,17]. In addition to corticotrophin-releasing hormone, adrenocorticotrophic hormone, and glucocorticoids, physiological stress stimulates production of cytokines and modifies inflammatory and immune responses. Peripheral leukocytes produce various cytokines, and proinflammatory cytokines, particularly gp130 family members, directly stimulate the HPA axis [1]. 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.

* Corresponding author. Tel.: +81 88 656 9848; fax: +81 88 656 9848.
E-mail address: rokutan@basic.med.tokushima-u.ac.jp (K. Rokutan).

In this study, we developed a cDNA array specifically designed to measure the mRNA levels of stress-related genes in peripheral blood leukocytes. Here we demonstrate that gene expression profiles in leukocytes may be potentially useful for the assessment of psychological stress response.

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 1467 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 [19]. The microarray showed high reproducibility with a mean coefficient of variation of less than 20%, and the dynamic ranges were three orders of magnitude.

The protocol of this study was approved by the Human Study Committee of Tokushima University Hospital. Graduate students (2 males and 8 females aged 24.7 ± 1.1 years) participated in this study. After the experimental procedures were explained, informed consent was obtained from each subject. They were in good physical health, were taking no medication, and had no history of psychiatric or somatic diseases. After they passed the initial screening of their Ph.D. manuscripts, they took the final examination, which consisted of an oral presentation of the Ph.D. 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 weeks before the presentation was used as a reference. All blood samples were collected under fasting conditions. In a separate experiment, venous blood was collected from a male subject at 8:00, 10:00, 12:00, 14:00, 16:00, 18:00, 20:00, 22:00, and 24:00, and changes in mRNA levels were measured during regular daily activity using the 10:00 sample as a reference.

Two different methods were applied to prepare total RNA from collected blood. Venous blood anticoagulated with 3.8% sodium citrate was overlaid on the density gradient medium MonoprepTM (Nycomed, Oslo, Norway) and centrifuged at $450 \times g$ for 30 min at 4 °C. Mononuclear cell- and neutrophil-rich fractions were obtained and mixed. After being washed with phosphate-buffered saline, the cell mixture was incubated in a PAXgeneTM Blood RNA tube (Qiagen, Hilden, Germany). Apart from the isolation, the same whole blood was directly poured into the Blood RNA tube. Total RNA was extracted from isolated leukocytes (mixture of neutrophils and mononuclear cells) or from whole blood using a PAXgene Blood RNA kit (Qiagen) according to the manufacturer's protocol. Contaminated DNA was removed using a DNase kit

(Qiagen), and the resultant RNA was examined by agarose gel electrophoresis and by spectrophotometry. Five micrograms of total RNA was amplified using in vitro transcription reaction [13]. Amplified RNA (6 μ g) was reverse transcribed using random hexamer and aminoallyl-dUTP. The synthesized cDNA was labeled with dye (NHS-ester Cy5 or Cy3; Amersham Biosciences, Piscataway, NJ, USA) [10]. Cy5-cDNAs prepared from each sample were mixed with the equivalent amount of Cy3-cDNAs from the respective reference, and the mixture was applied to the cDNA microarray. Hybridization was performed at 62 °C for 12 h. After washing, fluorescence intensity at each spot was assayed using a scanner (ScanArray 5000; GSI-Lumonics, Billerica, MA, USA).

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 519 genes with fluorescence intensity higher than the cut-off value of 300 in both conditions (labeled with Cy5 or Cy3) among all 30 samples. The relative expression values (Cy5/Cy3) for 519 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 519 genes were transformed to logarithms, statistical significance between 4 weeks before the examination and 2 h before, 2 h after, or 24 h after the oral presentation was examined by the Bayesian *t* test using the modified R software package (available at www.r-project.org) [12]. Statistical significance was defined as *P* value of <0.05 .

Freshly isolated or cultured mononuclear cells are usually used to examine target genes relevant to disease pathogenesis [9]. 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 [16,20]. 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 less than twofolds and decreased those for 10 genes to $<50\%$. The up-regulated genes included interleukin (IL)-8 (*IL8*), Bcl-2-related protein A1 (*BCL2A1*), Bcl-2-interacting killer (*BIK*), histidine triad nucleotide-binding protein 1 (*HINT*), guanine nucleotide-binding protein 10 (*GNG10*), dual specificity phosphatase 1 (*DUSP1*), Fc fragment of IgE (*FCER1A*), purinergic receptor (*P2Y5*), 5-hydroxytryptamine (serotonin) receptor 2C (*HTR2C*), granzyme A (*GZMA*), cytochrome *c* oxidases (*COX6C*, *COX7B*, *COX7C*, *COX7A2*), heat shock proteins (*HSBP1*, *HSPE1*, *HSJ2*, *HSPCA*, *HSF4*), ATPases (*PSMC6*, *ATP5J*, *ATP6J*, *ATPase inhibitor precursor*). The down-regulated genes included myeloproliferative leukemia virus oncogene (*MPL*), Bcl-2 like 1 (*BCL2L1*), platelet-derived growth factor α polypeptide (*PDGFA*), guanine nucleotide-binding protein α z polypeptide (*GNAZ*), caspase 2 (*CASP2*),

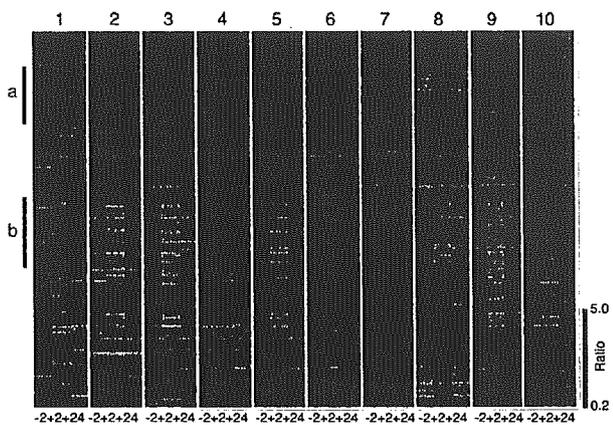


Fig. 1. Hierarchical clustering of psychological stress-associated changes in gene-expression pattern in peripheral leukocytes. Venous blood was collected 2 h before and 2 or 24 h after the examination, and levels of the detectable 519 mRNAs in leukocytes were measured using the sample collected 4 weeks before the examination as a reference. The relative expression values were subjected to a hierarchical clustering algorithm. The relative expression levels are visualized in red and green colors. The up-regulated and down-regulated cluster groups are indicated as “a” and “b”, respectively. -2; 2 h before, +2; 2 h after, +24; 24 h after oral presentation.

myxovirus resistance 1 (*MXI*), signal transducer and activator of transcription 2 (*STAT2*), GTPase-activating protein 6 isoform 4 (*ARHGAP6*), prostaglandin endoperoxide synthase 1 (*PTGSI*). 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* [24]. 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.

As shown in Fig. 1, hierarchical cluster analysis of the relative expression values of 519 genes identified two groups of genes (indicated as groups “a” and “b”), 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 (Fig. 2).

The neuroendocrine response, activated by psychological stress, converts stress into changes in mononuclear cell func-

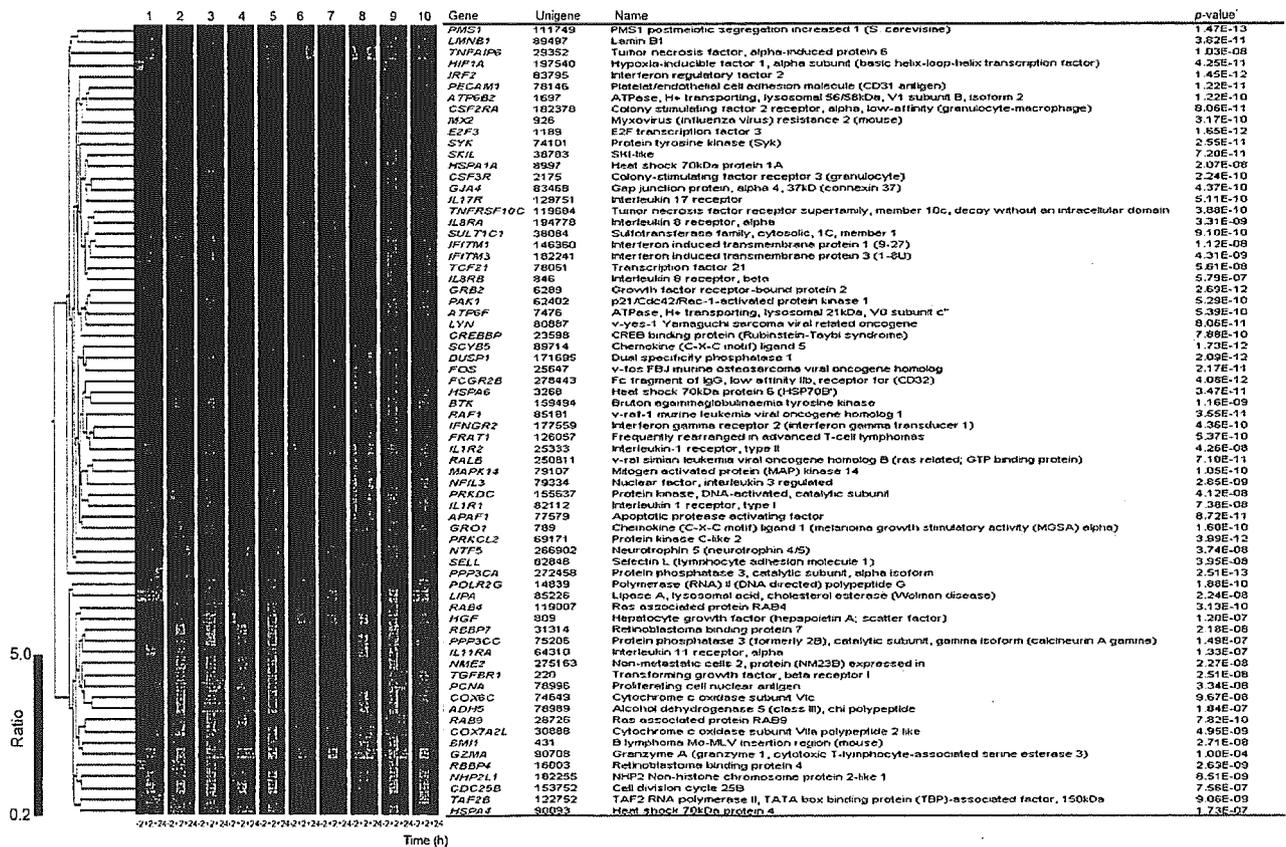


Fig. 2. Hierarchical clustering of significantly responsive genes to psychological stress. Significantly responsive genes were analyzed by the Bayesian *t* test at $P < 0.05$. The results of hierarchical clustering for the responsive 70 genes among 10 healthy subjects are displayed with a gene tree, and individual genes are listed in a clustered order (right panel). -2; 2 h before, +2; 2 h after, +24; 24 h after oral presentation.

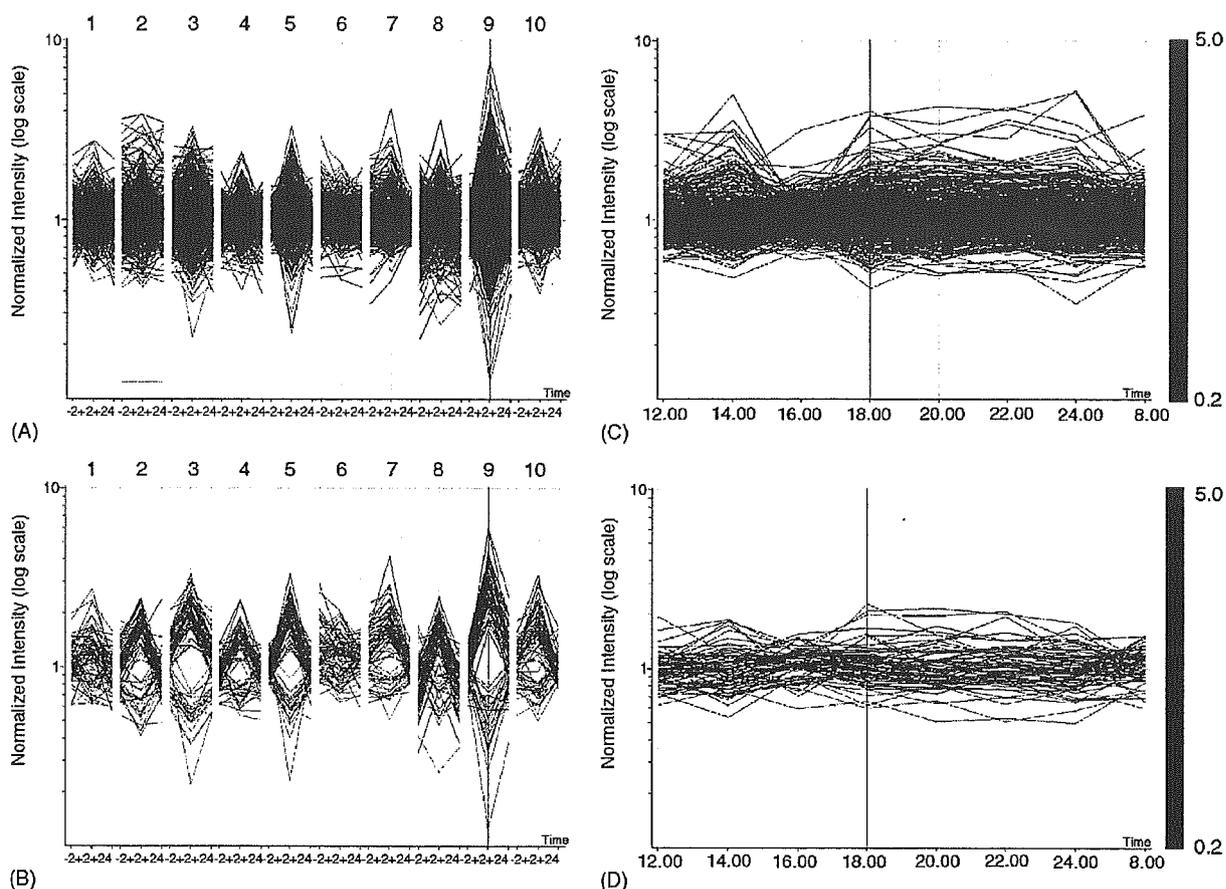


Fig. 3. Time courses of expression values of 519 genes or selected 70 genes during stress exposure or daily activity. Time-dependent changes in 519 mRNA levels 2 h before and 2 or 24 h after the examination (A), and those at the indicated times during routine daily activity (C) are shown. Time-dependent changes in the expression levels of significantly up-regulated 49 genes and down-regulated 21 genes were compared among 10 individuals (B). The mRNA levels of the responsive 70 genes were also measured at the indicated times (D).

tions [2], and stimulates the production of TNF- α , IL-6, IL-1 receptor antagonist, interferon (IFN)- γ , and IL-10 [14]. As shown in Fig. 2, 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*), 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 [15,18]. Psychological stress rapidly and transiently activates nuclear factor- κ B, a hallmark of inflammatory responses [4], in association with elevated levels of catecholamines and cortisol [2]. Thus, psychological stress activates multiple signaling pathways; therefore it is difficult to fully explain the biological significance of several other genes listed in Fig. 2. 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.

Although the official examination is one of the most stressful events for graduate students, most mRNA levels returned to the baseline levels within 24 h, and none of the subjects complained of any mental or physical distress.

We assessed the specificity of the genes identified here as psychological stress-responsive genes. Overall changes in 519 mRNA levels and the levels of 70 mRNAs (listed in Fig. 2) before and after the presentation are shown in Fig. 3A and B, respectively. The overall response of 519 genes to the stress varied among the subjects (Fig. 3A), and the expres-

sion profiles of the 70 genes also showed individual variations both in magnitude and in time-course among the 10 subjects (Fig. 3B). We examined whether routine works affected the gene expression in peripheral leukocytes in daily life. Figs. 3C and D respectively show changes in mRNA levels for 519 genes and for the significantly affected 70 genes in daily life of one male subject. As shown in Fig. 3C, 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*), IFN inducible mRNA fragment (*GIP3*), 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 (Fig. 3D). In separate experiments, 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 five healthy volunteers, while exhaustive exercise with treadmill significantly changed it. We identified 26 genes whose expressions were significantly changed after the exhaustive exercise (unpublished observations). Of these 26 genes, 7 genes were included in the 70 genes identified as psychological stress-responsive genes. Thus, a majority of the selected 70 genes may be potential makers of acute psychological stress.

We report here that the stressful life event uniformly changes the expression of a distinct group of genes in peripheral leukocytes. The specific behavior of individual genes was informative; therefore, the defined cluster genes may be useful to objectively assess psychological stress response. We are now using this microarray analysis to detect pathological responses in stress-related disorders.

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