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ORIGINAL RESEARCH ARTICLE

Expression analysis of neuregulin-1 in the dorsolateral prefrontal cortex in schizophrenia

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Genetic linkage and association have implicated neuregulin-1 (NRG-1) as a schizophrenia susceptibility gene. We measured mRNA expression levels of the three major isoforms of NRG-1 (ie type I, type II, and type III) in the postmortem dorsolateral prefrontal cortex (DLPFC) from matched patients and controls using real-time quantitative RT-PCR. Expression levels of three internal controls—GAPDH, cyclophilin, and β-actin—were unchanged in schizophrenia, and there were no changes in the absolute levels of the NRG-1 isoforms. However, type I expression normalized by GAPDH levels was significantly increased in schizophrenia DLPFC (by 23%) and positively correlated with antipsychotic medication dosage. Type II/type I and type II/type III ratios were significantly decreased (18 and 23% respectively). There was no effect on the NRG-1 mRNA levels of genotype at two SNPs previously associated with schizophrenia, suggesting that these alleles are not functionally responsible for abnormal NRG-1 expression patterns in patients. Subtle abnormalities in the expression patterns of NRG-1 mRNA isoforms in DLPFC may be associated with schizophrenia.
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Introduction

Schizophrenia is a complex genetic disorder affecting 0.5–1% of the general population worldwide. Several genome-wide linkage scan studies and meta-analysis of whole-genome linkage scans show a suggestive linkage to schizophrenia on chromosome 8p.[1–10] Recently, neuregulin-1 (NRG-1), which maps to the 8p locus, has been implicated as a susceptibility gene for schizophrenia by a combination of linkage and association analyses.[11,12] NRG-1 is one of the neuregulin family of proteins, which have a broad range of bioactivities in the central nervous system and contain an epidermal growth factor (EGF)-like motif that activates membrane-associated tyrosine kinases related to ErbB receptors.[13] The EGF-like domain of NRG-1 is required for ErbB receptor binding, dimerization, tyrosine phosphorylation, and activation of downstream signaling pathways.[14] A gene-targeting approach for NRG-1-ErbB signaling revealed a behavioral phenotype in mice that overlaps with certain animal models for schizophrenia. For example, NRG-1 and ErbB4 mutant mice exhibit elevated activity levels in an open field, which was reversed by clozapine, and abnormal sensorimotor gating measured by prepulse inhibition of the startle reflex.[11,15]

The NRG-1 gene generates multiple alternative splicing variants, classified into three primary isoform groups.[16] NRG-1 type I (heregulin/ARIA: acetylcholine receptor-inducing activity/NDF: neu differentiation factor) has an immunogloblin-like domain, followed by a region of high glycosylation; type II (GGF: glial growth factor) has GGF-specific and immunogloblin-like domains; and type III (SMDF: sensory and motor neuron-derived factor) has a cysteine-rich domain. These NRG-1 isoforms play multiple and distinct functions in neuronal development, and abnormalities in brain development have been implicated in schizophrenia. Moreover, NRG-1 regulates the expression and plasticity of *N*-methyl-*d*-aspartate receptors (NMDAR), of the β2 subunit of the γ-amino butyric acid receptor, and of nicotinic acetylcholine receptor subtypes including α5, α7, and β4 subunits[17–20], some of which also may be involved in genetic risk for schizophrenia.[21,22]

Thus, while genetic evidence implicates NRG-1 as a schizophrenia susceptibility gene, and the biology of NRG-1 overlaps with diverse aspects of the putative biology of schizophrenia, there have been no published studies of NRG-1 expression in the schizophrenic brain tissue, and little is known about whether a specific NRG-1 isoform contributes to the risk for schizophrenia. Here, we employed a real-time quantitative RT-PCR technique to explore the mRNA

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expression of each type of NRG-1 in the dorsolateral prefrontal cortex (DLPFC), where prominent functional and neuroanatomical abnormalities have often been observed in schizophrenia.[23]

Materials and methods

Human postmortem tissue and RNA extraction

Postmortem DLPFC tissues from brains were collected at the Clinical Brain Disorders Branch, as previously described. [24] Diagnoses were retrospectively established by two psychiatrists using DSM-IV criteria. We endeavored within practical limits to derive a rough approximation of lifetime neuroleptic exposure, recognizing that this is an uncertain estimate. All available records, including inpatient and outpatient clinic records, were meticulously reviewed for every subject. Each reference, anywhere in the chart, to a new medication and to a change in dose of an old medication was catalogued. While it was impossible to exclude potential discontinuities in treatment (or patient noncompliance), in general, contiguous dose information was available for almost every subject. The total daily dose of neuroleptic medication given to the patients was calculated by adding the various daily medication levels and converting these levels to chlorpromazine (CPZ) equivalents, as previously formulated.[25] A median value of drug dosage was then derived from the CPZ equivalents to give the estimated average daily dose; this value was multiplied by the duration of illness (estimated from the earliest age of definable symptoms or age at first hospitalization) to give the estimated lifetime CPZ equivalents. Samples were matched for age, gender, ethnicity, brain pH, hemisphere, postmortem interval (PMI), and months in freezer (MIF). Demographic data are shown in Table 1.

The tissue blocks were dissected from the middle, superior, or inferior frontal gyrus from a 1–1.5 cm coronal slab just anterior to the corpus collosum. The blocks contained primarily gray matter and a small, but presumably random, amount of white matter. In order to test for the possibility of systematic difference in the gray matter/white matter ratio in the dissections of PFC from patients and controls, the total RNA extracted from these blocks was screened by microarray expression profiling for the content of mRNAs highly expressed in white matter such as glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP). No significant differences in GFAP mRNA levels or MBP mRNA levels in RNA from patients with schizophrenia compared to controls were found (M Vawter, personal communication). While this approach does not conclusively rule out a systematic difference in the ratio of gray to white matter compartments in the tissue sampled from the schizophrenic and control groups, it reduces the likelihood of such an artifact.

The tissues were pulverized and stored at −80°C until use. Total RNA was extracted from 300–500 mg of DLPFC using TRIZOL Reagent (Life Technologies Inc., Grand Island, NY, USA), as previously described.[26] The yield of total RNA was determined by absorbance at 260 nm and the quality of total RNA was also analyzed using agarose gel electrophoresis.

DNAse treatment and reverse transcriptase reaction

Total RNA was treated with DNase for the removal of contaminating genomic DNA using DNase Treatment & Removal Reagents (Ambion, Austin, TX, USA), according to the manufacturer's protocol. After DNase treatment, the quality of total RNA was examined using agarose gel electrophoresis. Total RNA (6.8 μg) treated with DNase was used in 50 μl of reverse transcriptase reaction to synthesize cDNA, by using a SuperScript first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Briefly, total RNA (6.8 μg) was denatured with 1 mM of dNTP and 5 ng/μl of random hexamers at 65°C for 5 min. After the addition of RT buffer, $MgCl_2$ (5 mM in final concentration), dithiothreitol (10 mM in final concentration), RNAseOUT recombinant ribonuclease inhibitor (100 U), and SuperScriptII RT (125 U), the reaction mixture was incubated at 25°C for 10 min, at 42°C for 40 min, and at 70°C for 15 min. RNAse H (5 U) was added to the reaction mixture and then incubated at 37°C for 20 min.

Real-time quantitative PCR

The structure of human NRG-1 transcripts annotated in NCBI databases and the locations of each PCR amplicon are shown in Figure 1. We designed specific primer and probe combinations to recognize each NRG-1 isoform family as follows: type I: exons 4 and 5, type II: exons 4 and 8, and type III: exons 7 and 8.

NRG-1 mRNA expression levels were measured by real-time quantitative RT-PCR, using each combination of oligonucleotides and an ABI Prism 7900 sequence detection system with 384-well format (Applied Biosystems, Foster City, CA, USA). Each 20 μl PCR reaction contained 6 μl of cDNA, 900 nM of each primer, 250 nM of probe, and 10 μl of TaqMan Universal PCR Mastermix (Applied Biosystems) containing AmpliTaq Gold DNA polymerase, AmpErase UNG, dNTPs with dUTP, passive reference, and optimized buffer components. The PCR cycling conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 59°C or 60°C for 1 min. PCR data were obtained with the Sequence Detector Software (SDS version 2.0, Applied Biosystems) and quantified by a standard curve method. This software plotted the real-time fluorescence intensity and selected the threshold within the linear phase of the amplicon profile. The software plotted a standard curve of the cycle at threshold (Ct) (where the fluorescence generated within a reaction and threshold crosses) *vs* quantity of RNA. All samples were measured in one plate for one target gene or isoform, and their Ct values were in the linear range of the standard curve. Experiments were typically performed three times with triplicate determination and each gene expression level was determined by the

Table 1 Demography in the formation of the Clinical Brain Disorder Branch cohort

Case number	*Diagnosis*	*Age*	*Sex*	*Race*	*Side*	*pH*	*PMI (h)*	*Month in freezer*	*Cause of death*	*Manner of death*	*Age of onset/ duration of illness (year)*	*Last CPZ (eq/mg)*	*Daily CPZ (eq/mg)*	*Lifetime CPZ (eq/kg)*
1	CON	68	M	AA	L	6.57	22.5	192	ASCVD	natural				
2	CON	58	F	AA	L	6.54	26.5	226	ASCVD	natural				
3	CON	39	F	AA	L	6.34	40.5	226	Cardiac arrest	natural				
4	CON	46	F	AA	L	5.93	19.5	147	Dilated cardiomyopathy	natural				
5	CON	45	M	C	L	6.61	16.0	144	Blunt force injuries	accident				
6	CON	47	M	AA	L	6.03	60.0	157	Acute bronchial asthma	N/A				
7	CON	77	M	AA	R	6.06	18.5	146	Occlusive coronary atherosclerosis	natural				
8	CON	55	M	AA	R	6.00	9.5	146	MI (ASCVD)	natural				
9	CON	60	F	C	L	6.40	8.0	145	ASCVD	natural				
10	CON	61	F	AA	R	6.15	61.0	145	Multiple blunt force injuries	accident				
11	CON	26	M	C	L	6.08	13.0	115	ASCVD	natural				
12	CON	52	F	AA	R	6.87	10.0	100	Ruptured aorta	natural				
13	CON	42	M	AA	R	6.63	40.0	97	Acute asthma attack	natural				
14	CON	24	M	AA	R	6.59	12.5	96	Fibrinous pericarditis	natural				
15	CON	38	M	AA	R	6.14	32.5	95	Pulmonary embolism	accident				
16	CON	56	M	AA	R	6.09	33.0	88	Pulmonary embolism	natural				
17	CON	57	F	AA	R	6.43	19.0	76	MI-ASCVD	natural				
18	CON	59	F	AA	R	6.57	37.0	72	Cirrhosis of the liver	natural				
19	CON	67	F	AA	L	6.69	34.0	67	Cardiomyopathy Pulmonary edema	natural				
Mean (SD)		51.4 (13.8)				6.35 (0.28)	27.0 (15.8)	130.5 (47.8)						
20	SCH/TD	71	F	C	L	6.41	47.5	181	ASCVD	natural	15/56	100	500	0.6
21	SCH	36	M	AA	R	6.56	13.0	192	Blunt force injuries	suicide	21/16	400	850	5
22	SCH	46	M	AA	R	6.35	24.5	192	ASCVD	natural	23/23	N/A	N/A	N/A
23	SCH	44	F	AA	R	6.51	32.5	191	Cardiomegaly and hypertension	natural	19/15	200	200	1.1
24	SCH	46	M	AA	R	6.73	25.0	196	Blunt force injuries	suicide	36/10	300	300	1.1
25	SCH	48	M	C	R	6.29	13.5	146	Delusional hyponatremia and hypo-osmolar coma	undetermined	33/15	300	300	1.6
26	SCH	73	M	C	R	6.00	13.5	143	ASCVD	natural	23/50	450	450	4.6
27	SCH	34	M	AA	R	6.23	34.5	141	Acute benzotropine intoxication	undetermined	26/8	N/A	N/A	N/A
28	SCH	75	M	AA	L	6.29	41.5	121	Undetermined	natural	29/46	400	400	5.3
29	SCH	64	F	AA	R	6.48	19.5	121	Asphyxia due to aspiration	accident	19/45	900	400	5.3
30	SCH	67	F	AA	R	6.63	38.5	118	Chronic obstructive pulmonary disease	natural	30/37	80	100	1.3
31	SCH	31	M	C	R	6.46	14.0	112	Cerebral edema	natural	17/14	200	N/A	N/A
32	SCH	23	M	AA	L	6.48	42.5	112	Respiratory arrest	natural	21/2	400	480	0.033
33	SCH	60	F	AA	L	6.38	19.0	110	ASCVD	natural	40/20	100	100	0.7
34	SCH	30	M	AA	L	6.32	72.5	106	Pneumonia	natural	18/12	1900	500	2.2
35	SCH	81	F	C	R	6.78	11.0	100	ASCVD	natural	27/54	100	150	2.1

Table 1 Continued

Case number	Diagnosis	Age	Sex	Race	Side	pH	PMI (h)	Month in freezer	Cause of death	Manner of death	Age of onset/ duration of illness (year)	Last CPZ (eq/mg)	Daily CPZ (eq/mg)	Lifetime CPZ (eq/kg)
36	SCH/TD	61	F	AA	R	6.74	20.0	95	Asphyxiation	accident	24/27	N/A	200	2
37	SCH/TD	38	M	AA	R	6.50	61.0	89	Acute peritonities	accident	16/27	800	60	11.8
38	SCH	41	F	AA	R	6.08	51.0	80	ASCVD	natural	16/25	2400	1135	10.4
39	SCH/TD	41	M	AA	L	6.63	32.0	76	ASCVD	natural	20/21	50	400	2.5
Mean		50.5				6.44	31.3	131.1			23.7/26.2	534	343	3.03
(SD)		(17.1)				(2.21)	(17.3)	(38.8)			(7.0/16.3)	(659)	(287)	(3.35)

Means and standard deviations are printed below the last individual in each group. CON=normal control; SCH=schizophrenia; TD=tardive dyskinesia; M=male; F=female; AA=African American; C=Caucasian; R=right; L=left; PMI=post-mortem interval; N/A=not available; CPZ=chlorpromazine; eq=equivalent.

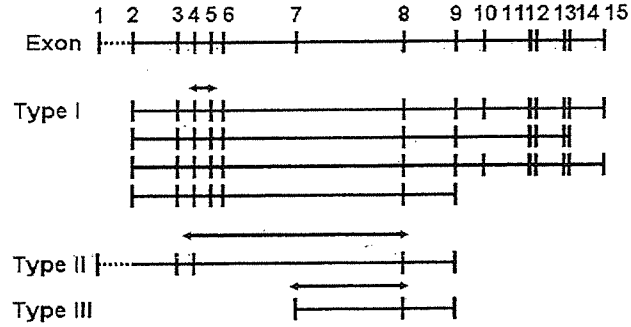

Figure 1 NRG-1 structure and probe design. Human NRG-1 mRNA has 15 exons. The exon usages of type I, type II or type III isoforms of NRG-1 are shown. Arrows indicate the location of primers and probes specific for type I, type II or type III isoforms of NRG-1.

average of three independent experiments. Predicted Ct values and sample quantities were used for statistical analysis.

Oligonucleotide primers and standard curve construction

Primer and probe sequences were designed by using PRIMER EXPRESS software (version 2.0, Applied Biosystems). Agarose gel electrophoresis was used to verify the size predictions of PCR amplicons (data not shown). The TaqMan Pre-Developed Assay Reagent kit (Applied Biosystems) was used for housekeeping genes: GAPDH, β-actin, and cyclophilin. The real-time PCR (TaqMan) detection of NRG-1 isoforms used the following oligonucleotides: type I, forward primer P3089 5′-GCCAATATCACCATCGTGGAA-3′, reverse primer P3090 5′-CCTTCAGTTGAGGCTGGCATA-3′, probe P3091 5′-FAM-CAAACGAGATCATCACTG-MGB-3′; type II, forward primer P3092 5′-GAATCA-AACGCTACATCTACATCCA-3′, reverse primer P3093 5′-CCTTCTCCGCACATTTTACAAGA-3′, probe P3094 5′-FAM-CACTGGGACAAGCC-MGB-3′; type III, forward primer P3095 5′-CAGCCACAAACAACAGAA-ACTAATC-3′, reverse primer P3096 5′-GCCAGTG-GTGGATGTAGATGTAGA-3′, probe P3097 5′-FAM-CCAAACTGCTCCTAAAC-MGB-3′(purchased from Applied Biosystems). These primers were designed to amplify specific transcripts based on the unique exon structure of each isoform. Thus, for example, because isoform II lacks exons 5–7, primers focused on exons 4 and 8, which are contiguous in the isoform II transcript, and will amplify only this isoform. Standard curves for the housekeeping genes and the three NRG-1 isoforms were prepared using serial dilutions (1:4) of pooled cDNA from total RNA derived from DLPFC of six normal control subjects (Figure 2). In each experiment, the R^2 value of the standard curve was more than 0.99 and no-template control assays resulted in no detectable signal.

SNP genotyping

DNA was extracted from brain tissue using standard methods. P3149SNP8NRG221533 and SNP8NRG24-

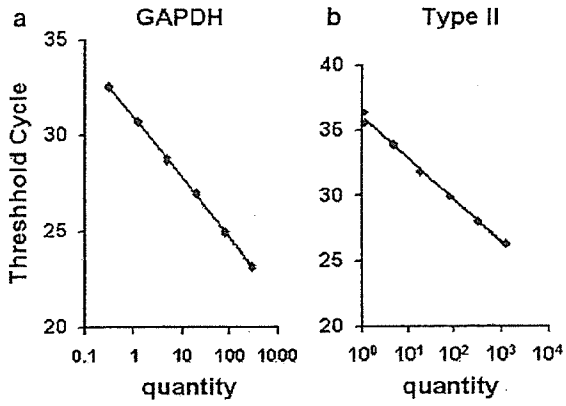

Figure 2 Standard curves for control (housekeeping genes) and NRG-1 type II. Standard curves for GAPDH (a) and type II (b). The quantity represents an amount of cDNA prepared from 1 ng of total RNA in the PCR reaction. R^2 values are 1.000 and 0.995 for GAPDH and type II.

3177P3155 genotypes were determined using the Taqman 5′-exonuclease allelic discrimination assay. These SNPs were chosen because they showed the strongest association to schizophrenia in prior studies.[11,12] Probes and primers for detection of the SNP are: SNP8NRG221533, forward primer P3151 5′-AAGGCATCAGTTTTCAATAGCTTTTT-3′, reverse primer P3152 5′-TAAGTAGAAATGGGAACTCTCCATCTC-3′, probe1 P3149 5′-FAM-TTTATTTTgCCAAATAT-MGB-3′, probe2 P3150 5′-VIC-TCTTTATTTTaCCAAATATCAT-MGB-3′; SNP8NRG243177, forward primer P3159 5′-AATTAGTAGGATTGGATGTTTGAACCA-3′, reverse primer P3160 5′-GATGGAGCGCTTCAGGAGAA-3′, probe1 P3155 5′-FAM-CCAGTATACgTTCACTTG-MGB-3′, probe2 P3156 5′-VIC-CCAGTATACaTTCAC-TTGA-MGB-3′. Each 10 μl PCR reaction contained 10 ng of DNA, 1 μM of each primer, 100 nM of each probe, and 5 μl of TaqMan Universal PCR Mastermix (Applied Biosystems) containing AmpliTaq Gold DNA polymerase, AmpErase UNG, dNTPs with dUTP, passive reference, and optimized buffer components. The PCR cycling conditions were at 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min.

Statistical analysis

An independent *t*-test was used to compare the age, brain pH, months in freezer, and postmortem interval, and a Mann–Whitney *U*-test was used to compare the gene expression levels between schizophrenic and control groups with Statistica software (release 5.5, Statsoft, Inc., Tulsa, OK, USA). The groups did not differ in gender and ethnicity. Differences in NRG-1 expression levels between groups were also analyzed by ANCOVA, with diagnosis as the independent factor and brain pH and age as covariates. Spearman rank order correlation test was used for comparison between demographic data and expression data.

Results

Control genes and NRG-1 mRNA levels

The expression levels of three standard 'housekeeping' genes—GAPDH, β-actin, and cyclophilin—were not significantly different between groups (Figure 3a). Raw (ie nonnormalized) NRG-1 isoform expression levels also did not differ between groups (Figure 3b).

Effects of demographics on NRG-1 mRNA expression levels

Expression levels of all the three NRG-1 isoforms normalized by cyclophilin were positively correlated with age in normal control subjects (Rho=0.637, P=0.006; Rho=0.573, P=0.015, and Rho=0.637, P=0.013 for type I, type II, and type III, respectively); however, there was no correlation between normalized NRG-1 isoform expression levels and age in schizophrenia patients (all $P>0.6$). Similar results were obtained with normalization to GAPDH and β-actin (data not shown). NRG-1 expression levels were not associated with sex, race or hemisphere, and did not correlate with PMI or MIF. Brain pH and type I mRNA expression normalized by cyclophilin were correlated in both normal control (Rho=−0.477, P=0.050) and schizophrenia groups (Rho=−0.500,

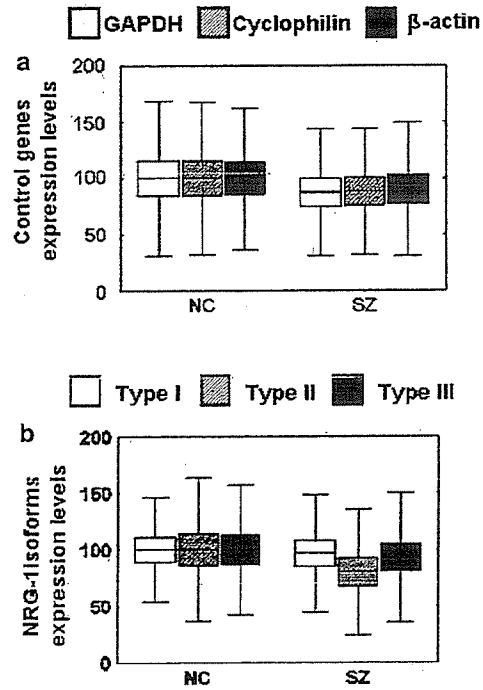

Figure 3 mRNA expression levels of NRG-1 isoforms. The expression levels of housekeeping genes (a) and NRG-1 isoforms (b) were measured in the DLPFC of normal control subjects (NC) and patients with schizophrenia (SZ). The expression levels were calculated by comparison to the percentage of average of normal control subjects. Boxes and bars outside boxes represent the standard error and standard deviation. Bars in boxes represent means.

P=0.041), with similar results normalizing by GAPDH or β-actin. Thus, brain pH (as well as age) was used as covariate for type I expression data analysis.

Normalized NRG-1 mRNA levels

NRG-1 type I expression levels normalized by GAPDH, cyclophilin or β-actin (to reduce the effects of possible mRNA degradation not detectable by electrophoresis and/or possible variations in RT efficiency) were increased by 23, 18 or 16%, respectively, in schizophrenia patients (ANCOVA: all $P<0.050$). (Figure 4a). No significant differences were observed between groups in normalized NRG-1 type II and type III expression levels (Figure 4b, c).

We further analyzed the expression ratios among the three types of NRG-1 isoforms to investigate possible isoform–isoform interactions or altered regulation of splicing (Figure 5). There was no significant difference in type I/type III expression ratio with or without covariates (age and pH) (normal control: 100.0 (mean) ±33.8 (SD) *vs* schizophrenia patients: 109.1±36.8). However, both type II/type III and type II/type I expression ratios were significantly decreased in the schizophrenic group (17 and 23%, Mann–Whitney *U*-test: P=0.010 and 0.013, respectively). ANCOVA with brain pH and age as covariates did not alter the statistical significance of this relative type II decrease (type II/type I; F=10.96, P=0.002, df=1, 32).

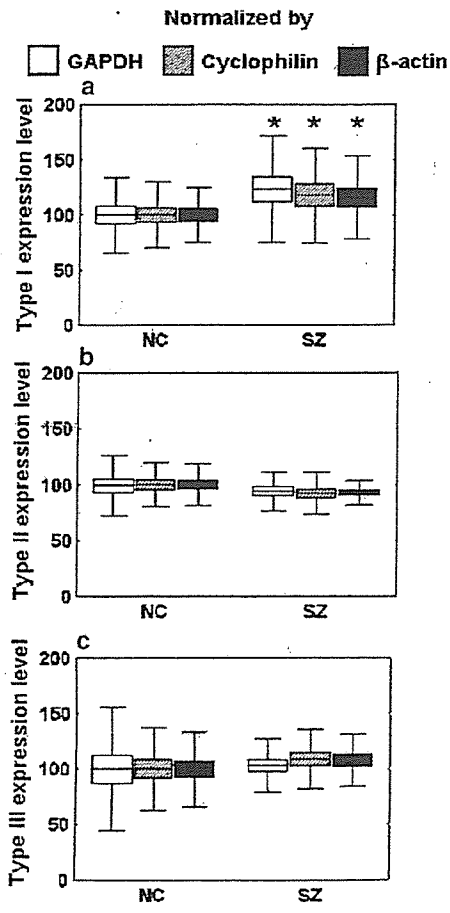

Figure 4 Relative expression levels of NRG-1 type I (a), type II (b), and type III (c) isoforms normalized by GAPDH, cyclophilin or β-actin in the DLPFC of normal control subjects (NC) and patients with schizophrenia (SZ). Significant group differences by ANCOVA are indicated by *$P<0.05$.

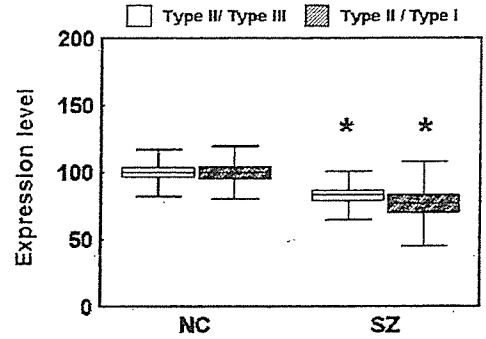

Figure 5 Relative expression ratios of Type II normalized by Type I or Type III in DLPFC of normal control subjects (NC) and patients with schizophrenia (SZ). *$P < 0.05$.

Influence of clinical characteristics on NRG-1 expression

None of the measurements of NRG-1 isoforms correlated significantly with the age of onset, duration of illness or last and lifetime dose of chlorpromazine equivalents (data not shown). A positive correlation between type I expression levels normalized by cyclophilin and daily dose was found (Rho=0.601, P=0.014), although daily dose was not correlated with normalized type II or type III expression levels (Rho=−0.315, P=0.218; Rho=−0.102, P=0.681, respectively). Similar results were obtained after normalization by GAPDH and β-actin (data not shown).

Allele effects on NRG-1 expression

No effect of SNP8NRG221533 genotype, which has been reported to be associated with schizophrenia in both Icelandic and Scottish populations,[11,12] was apparent in type I, type II, and type III expression levels normalized by cyclophilin, and the expression ratio of type II/type III in total subjects, normal controls or patients. For example, one allele homozygote (N=21) had mean levels of type I expression of 100.9±37.6 (SD), while two carriers (N=12) had 99.2±28.3 (SD) ($P>0.8$). Neither NRG-1 expression levels normalized by GAPDH or β-actin nor the other combinations of isoform–isoform expression ratio were affected by this genotype in any group (data not shown). Similar negative results were obtained between NRG-1 expression and SNP8NRG243177 (data not shown), which also has been associated with schizophrenia.[11,12]

Discussion

In this study, we have measured mRNA expression levels of NRG-1 isoforms in DLPFC using real-time quantitative RT-PCR in patients with schizophrenia and in normal controls. NRG-1 has been implicated as a susceptibility gene in schizophrenia. We found preliminary evidence that the pattern of expression of NRG-1 isoforms may be abnormal in schizophrenia. Specifically, there was a small increase in type I expression levels, and a small decrease of type II/type I and type II/type III ratios in the patients with schizophrenia. As consistent results were obtained from normalization of NRG-1 isoforms by all the three housekeeping genes, our findings would seem to be robust at least in comparison to results that might have been based on using only one control gene. Our data appear to add to the evidence that NRG-1 may be involved in schizophrenia, but other explanations, for example, differences in postmortem stability of the various isoforms, cannot be excluded. Moreover, as our study did not include measurement of the levels of NRG-1 proteins, of expression in other brain regions or in other psychiatric disorders, further work is necessary to clarify whether changes in NRG-1 mRNA impact on protein expression and is regionally and diagnostically specific.

NRG-1 binds to its receptor, ErbB, and NRG-1-ErbB signaling plays multiple roles in development and plasticity in the central nervous system.[13] Type I is prominently expressed early in development; type II is abundantly expressed in the adult nervous system; and type III is the major isoform produced by sensory neurons and motorneurons, and is also expressed in the rodent brain.[27] Little is known about NRG-1 expression in human brain; however, NRG-1 is present in neuronal cell bodies and synapse-rich regions in the hippocampus and type II isoform is expressed in oligodendrocytes, astrocytes, and microglia.[28,29] We detected mRNA of each of the three major classes of NRG-1 isoforms in human DLPFC, but we did not characterize the multiple spice variants within these isoforms. We also found a positive correlation between expression levels of each of the NRG-1 isoforms with age in normal subjects, suggesting that NRG-1 mRNA increases as the prefrontal cortex ages. However, the meaning of this correlation is unclear, and it was not found in the patients.

NRG-1 type I has been implicated in neuronal plasticity because it shows activity-dependent regulation, and it is involved in regulating neurotransmitter receptor expression. Multiple perturbations in neuronal activity have been shown to affect type I expression. For example, seizures, long-term potentiation, and forced locomotion induce type I expression in the hippocampus, amygdala, and motor cortex. Brain injury induces NRG-1 protein expression in astrocytes of rat cerebral cortex.[30] Curare blockade of nicotinic receptors reduces the expression of type 1 protein in chick motor neurons, an effect that can be prevented by brain-derived neurotrophic factor and neurotrophin 3.[31] In the central nervous system, NRG-1 promotes the switch from the immature form of NMDAR, which contains primarily NR2B subunits to one containing more NR2C subunits.[17] NRG-1 also potentiates $\alpha 7$ nicotinic acetylcholine receptor transmission in hippocampal neurons,[20] and expression of the $\beta 2$ subunit of the γ-amino butyric acid receptor in cerebellar granule cells.[19] Thus, the relative increase in type I expression in schizophrenia brain might alter neuronal signaling of NRG-1 *per se*, or it may be an indirect factor in putative abnormalities of NMDA, nicotinic, and/or GABA receptor-related signaling in schizophrenia brain.[21,32,33] The positive correlation between type I expression level and the daily dose of chlorpromazine equivalents suggests that this upregulation of type I could reflect a relationship between NRG-1 expression level and illness severity. Alternatively, it might be due to neuroleptic treatment. We are currently exploring in animals the potential effect of antipsychotic medication on NRG-1 expression.

NRG-1 type II (GGF) is also of central importance for neuronal and glial development. Type II is expressed in developing cortical neurons, and it promotes the transformation and differentiation of radial glial cells, which in turn support cortical neuronal cell migration and differentiation.[34] A study using NRG-1-deficient mice revealed that NRG-1-erbB2 signaling is required for the establishment of radial glia and their transformation into astrocytes in cerebral cortex.[35] In our study, decreased ratios of type II/type I and type II/type III may be due to relative underexpression of type II in DLPFC of schizophrenia patients. Neuroanatomical abnormalities have been reported in DLPFC in schizophrenia, including abnormal neuropil and cytoarchitecture.[36–40] It is unclear whether variations in NRG-1 expression could relate to these changes. In addition, a change in the balance of type I/type II to type III NRG-1 may influence cholinergic neurotransmission, as the distinct isoforms differentially induce various subunits of the nAChR.[18]

Although NRG-1 was first recognized to be critical for multiple stages of schwann cell development[41,42], its role in promoting the development of myelin-forming cells is now recognized to include oligodendrocytes. Not only is NRG-1 and various ErbB receptors expressed in the subependymal zone and the forebrain oligogenic zone, but NRG-1 can also induce the division[43] and/or promote the differentiation of oligodendrocyte precursors *in vitro*.[44–47] It is conceivable that relatively decreased type II mRNA expression may relate to putative abnormalities of oligodendroglial function implicated in schizophrenia.[48,49] Finally, we did not find evidence that NRG-1 type III mRNA expression levels are changed in schizophrenia DLPFC, although this isoform also has effects on neuronal plasticity and development.[18,50]

The multiple marker haplotype in the NRG-1 gene that has been associated with schizophrenia spans the

first exon, which is the promoter region for type II and is far upstream from the exons of all other isoforms.[11,12] The functional allele contributing to the increased risk for schizophrenia has not been identified in NRG-1, nor is there evidence that any of the associated variations would impact gene expression or function. As two single SNPs associated with schizophrenia were located around the promoter region of NRG-1 and the first exon of GGF, these SNPs might regulate the expression levels of NRG-1 isoforms and/or isoform–isoform ratios. However, no obvious allele effects of these SNPs on NRG-1 expression patterns were observed in this small sample. The estimated relative risks of each of these markers alone were less than that of the seven-marker core haplotype.[11,12] Taken together, these two SNPs do not appear to be functional alleles, at least in terms of the regulation of NRG-1 expression in human DLPFC. However, the possible relative decrease in type II expression may be regulated by an as yet unidentified allele in linkage disequilibrium with the associated haplotype.

Our findings offer preliminary evidence that abnormal expression of NRG-1 isoforms in DLPFC may be related to the pathophysiology of schizophrenia, but the evidence is weak. The biologic implications of our results are unknown, but they are at least conceptually consistent with evidence that schizophrenia involves genetic abnormalities in developmental/plasticity-related processes.[51,52] Additional studies are needed to characterize NRG-1 expression in schizophrenia, including slide-based mRNA analyses, protein analyses, neuroleptics effects, diagnostic specificity, and further exploration of genotype based variation.

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

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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.

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 [^{3}H] 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

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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. Probands 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′-TGGAAAAGTCGTAGTTGTG-3′. Hybridization probes were as followed: 5′-AACAGGAGAAACAGAGGGCTGATGGC-3′-Fluorescein, 5′-LCRed640-ACCCAGCAGATCCTCCAGAACCACC-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 × 10^4) 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.

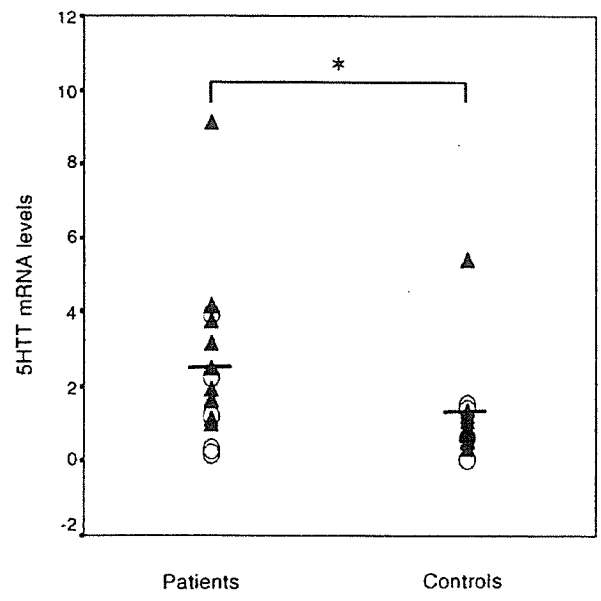

Fig. 1. The measurement of 5HTT mRNA/G6PDmRNAxE4 in peripheral leukocytes in depressive patients ($n = 15$) and control subjects ($n = 15$). Mann–Whitney *U*-test: $^*P < 0.05$. The '▲' symbols mean the genotype of homozygous 14A type of 5-HTTLPR and homozygous 12 tandem repeats of 5-HTTVNTR. The '○' symbols mean the other genotypes. The mean are indicated by a horizontal line.

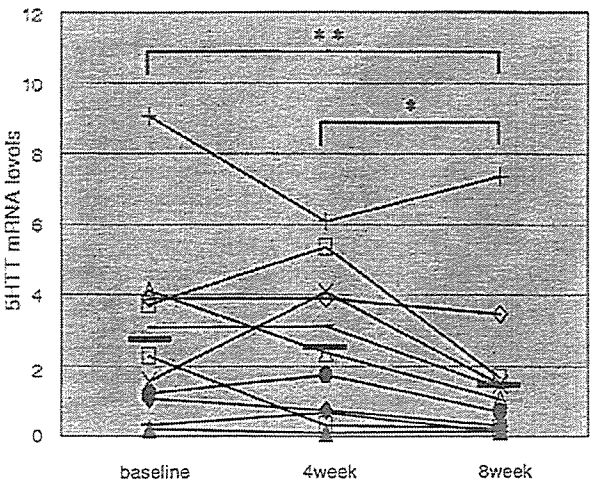

Fig. 2. The 5HTT mRNA levels in leukocytes of MDD patients were significantly decreased at 8 weeks compared with those at baseline and 4 weeks (baseline: 2.77 ± 2.52; 4 weeks: 2.53 ± 2.14; 8 weeks: 1.64 ± 2.13; Friedman test: $P < 0.01$ Wilcoxon rank sum test: $^{**}P < 0.01$ and $^{*}P < 0.05$). The 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

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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.

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, adrenocorticotropic 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.

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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 Monoprep™ (Nycomed, Oslo, Norway) and centrifuged at 450 × *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 PAXgene™ 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 μg) 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*),

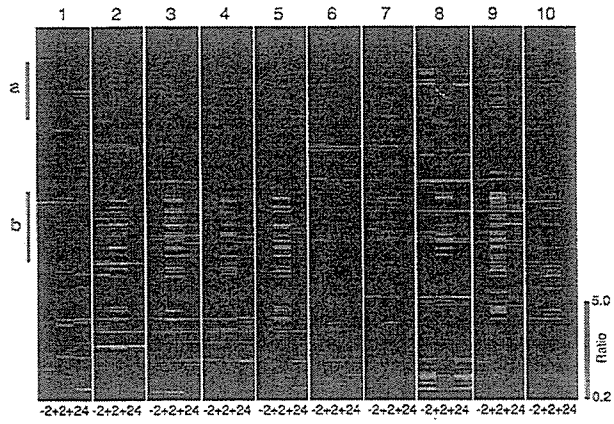
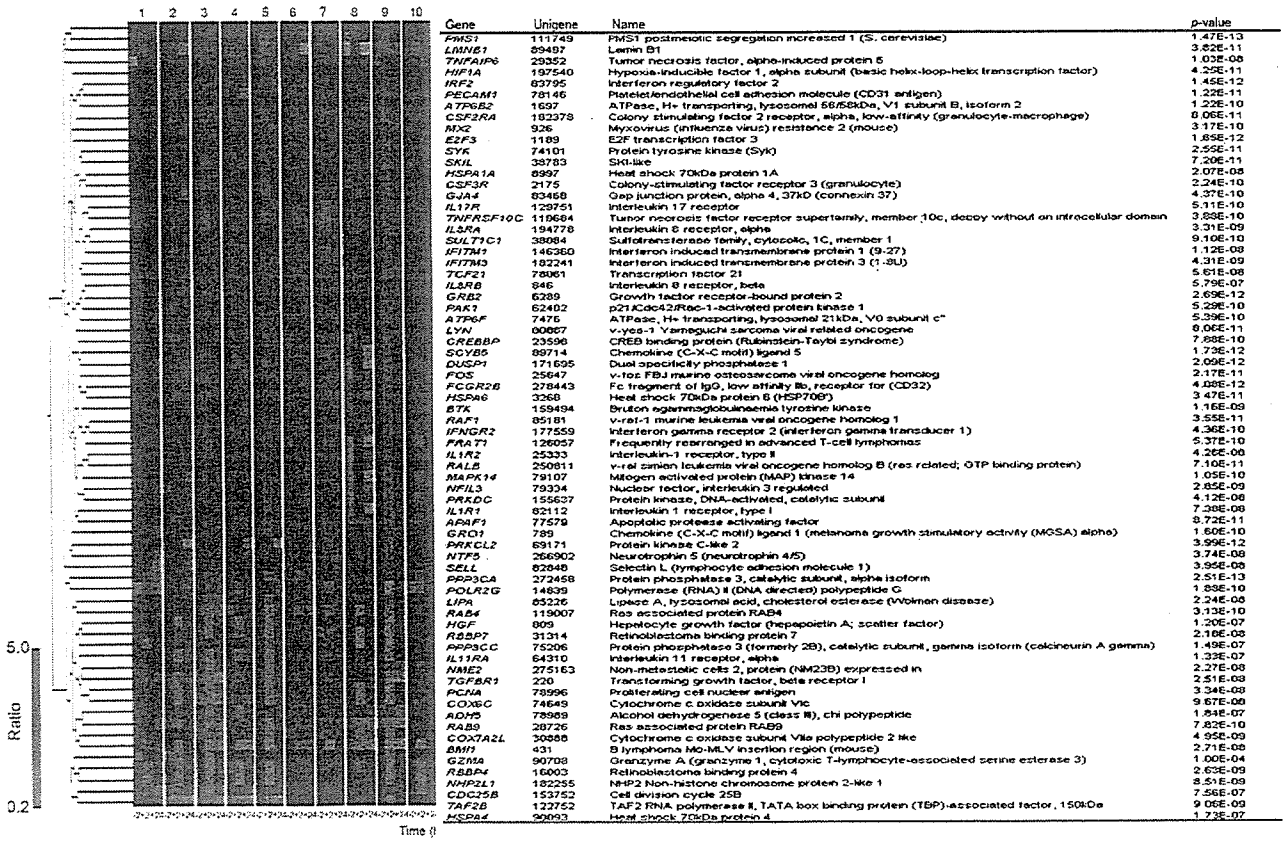


Fig. 1. Hierarchical clustering of psychological stress-associated changes in gene-expression pattern in peripheral leukocytes. Venous blood was collected 2 h before and 2 or 24 h after the examination, and levels of the detectable 519 mRNAs in leukocytes were measured using the sample collected 4 weeks 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 (*MX1*), signal transducer and activator of transcription 2 (*STAT2*), GTPase-activating protein 6 isoform 4 (*ARHGAP6*), prostaglandin endoperoxide synthase 1 (*PTGS1*). 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-



Gene	Unigene	Name	p-value
PMS1	111749	PMS1 postmeiotic segregation increased 1 (S. cerevisiae)	1.47E-13
LMNB1	89497	Lamin B1	3.82E-11
TNFAIP6	29352	Tumor necrosis factor, alpha-induced protein 6	1.03E-08
HIF1A	197540	Hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	4.25E-11
IRF2	83795	Interferon regulatory factor 2	1.45E-12
PECAM1	78146	Proteleridendothelial cell adhesion molecule (CD31 antigen)	1.22E-11
ATP6B2	1697	ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B, isoform 2	1.22E-10
CSF2RA	182378	Colony stimulating factor 2 receptor, alpha, low-affinity (granulocyte-macrophage)	8.06E-11
MX2	926	Myxovirus (influenza virus) resistance 2 (mouse)	3.17E-10
E2F3	1189	E2F transcription factor 3	1.85E-12
SYK	74101	Protein tyrosine kinase (Syk)	2.55E-11
SKIL	38783	SKI-like	7.20E-11
HSPA1A	8997	Heat shock 70kDa protein 1A	2.07E-08
CSF3R	2175	Colony-stimulating factor receptor 3 (granulocyte)	2.24E-10
GJA4	83468	Gap junction protein, alpha 4, 37kD (connexin 37)	4.37E-10
IL17R	129751	Interleukin 17 receptor	5.11E-10
TNFRSF10C	119684	Tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain	3.88E-10
IL3RA	194778	Interleukin 3 receptor, alpha	3.31E-09
SULT1C1	38084	Sulfotransferase family, cytosolic, 1C, member 1	9.10E-10
IFITM1	146360	Interferon induced transmembrane protein 1 (9-27)	1.12E-08
IFITM3	182241	Interferon induced transmembrane protein 3 (1-8U)	4.31E-09
TCF21	78061	Transcription factor 21	5.61E-08
IL8RB	846	Interleukin 8 receptor, beta	5.79E-07
GRB2	6289	Growth factor receptor-bound protein 2	2.69E-12
PAK1	62402	p21/Cdc42/Rac-1-activated protein kinase 1	5.29E-10
ATP6F	7475	ATPase, H+ transporting, lysosomal 21kDa, V0 subunit c"	5.39E-10
LYN	90867	v-yes-1 Yamaguchi sarcoma viral related oncogene	9.06E-11
CREBBP	23596	CREB binding protein (Rubinstein-Taybi syndrome)	7.88E-10
SCYB5	89714	Chemokine (C-X-C motif) ligand 5	1.73E-12
DUSP1	171695	Dual specificity phosphatase 1	2.09E-12
FOS	25647	v-fos FBJ murine osteosarcoma viral oncogene homolog	2.17E-11
FCGR2B	278443	Fc fragment of IgG, low affinity IIb, receptor for (CD32)	4.08E-12
HSPA6	3268	Heat shock 70kDa protein 6 (HSP70B')	3.47E-11
BTK	159494	Bruton agammaglobulinaemia tyrosine kinase	1.16E-09
RAF1	85181	v-raf-1 murine leukemia viral oncogene homolog 1	3.55E-11
IFNGR2	177559	Interferon gamma receptor 2 (interferon gamma transducer 1)	4.36E-10
FRA7I	126057	Frequently rearranged in advanced T-cell lymphomas	5.37E-10
IL1R2	25333	Interleukin-1 receptor, type II	4.26E-08
RALB	250811	v-ral simian leukemia viral oncogene homolog B (ras related; GTP binding protein)	7.10E-11
MAPK14	79107	Mitogen activated protein (MAP) kinase 14	1.05E-10
NFIL3	79334	Nuclear factor, interleukin 3 regulated	2.85E-09
PRKDC	155627	Protein kinase, DNA-activated, catalytic subunit	4.12E-08
IL1R1	82112	Interleukin 1 receptor, type I	7.38E-08
APAF1	77579	Apoptotic proteese activating factor	8.72E-11
GRO1	789	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulatory activity (MGSA) alpha)	1.60E-10
PRKCL2	69171	Protein kinase C-like 2	3.99E-12
NTF5	266902	Neurotrophin 5 (neurotrophin 4/5)	3.74E-08
SELL	82848	Selectin L (lymphocyte adhesion molecule 1)	3.95E-08
PPP3CA	272458	Protein phosphatase 3, catalytic subunit, alpha isoform	2.51E-13
POLR2G	14839	Polymerase (RNA) II (DNA directed) polypeptide G	1.83E-10
LIPA	85226	Lipase A, lysosomal acid, cholesterol esterase (Wolman disease)	2.24E-08
RAB4	119007	Ras associated protein RAB4	3.13E-10
HGF	809	Hepatocyte growth factor (hepapoietin A; scatter factor)	1.20E-07
RBBP7	31314	Retinoblastoma binding protein 7	2.18E-08
PPP3CC	75206	Protein phosphatase 3 (formerly 2B), catalytic subunit, gamma isoform (calcineurin A gamma)	1.49E-07
IL11RA	64310	Interleukin 11 receptor, alpha	1.33E-07
NME2	275163	Non-metastatic cells 2, protein (NM23B) expressed in	2.27E-08
TGFBR1	220	Transforming growth factor, beta receptor I	2.51E-08
PCNA	78996	Proliferating cell nuclear antigen	3.34E-08
COX6C	74649	Cytochrome c oxidase subunit VIc	9.67E-08
ADH5	78969	Alcohol dehydrogenase 5 (class III), chi polypeptide	1.84E-07
RAB9	28726	Ras associated protein RAB9	7.82E-10
COX7A2L	30888	Cytochrome c oxidase subunit VIIa polypeptide 2 like	4.95E-09
BMI1	431	B lymphoma Mo-MLV insertion region (mouse)	2.71E-08
GZMA	90708	Granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	1.00E-04
RBBP4	16003	Retinoblastoma binding protein 4	2.63E-09
NHP2L1	182255	NHP2 Non-histone chromosome protein 2-like 1	8.51E-09
CDC25B	153752	Cell division cycle 25B	1.56E-07
TAF2B	122752	TAF2 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 150kDa	9.06E-09
HSPA4	90093	Heat shock 70kDa protein 4	1.73E-07

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.

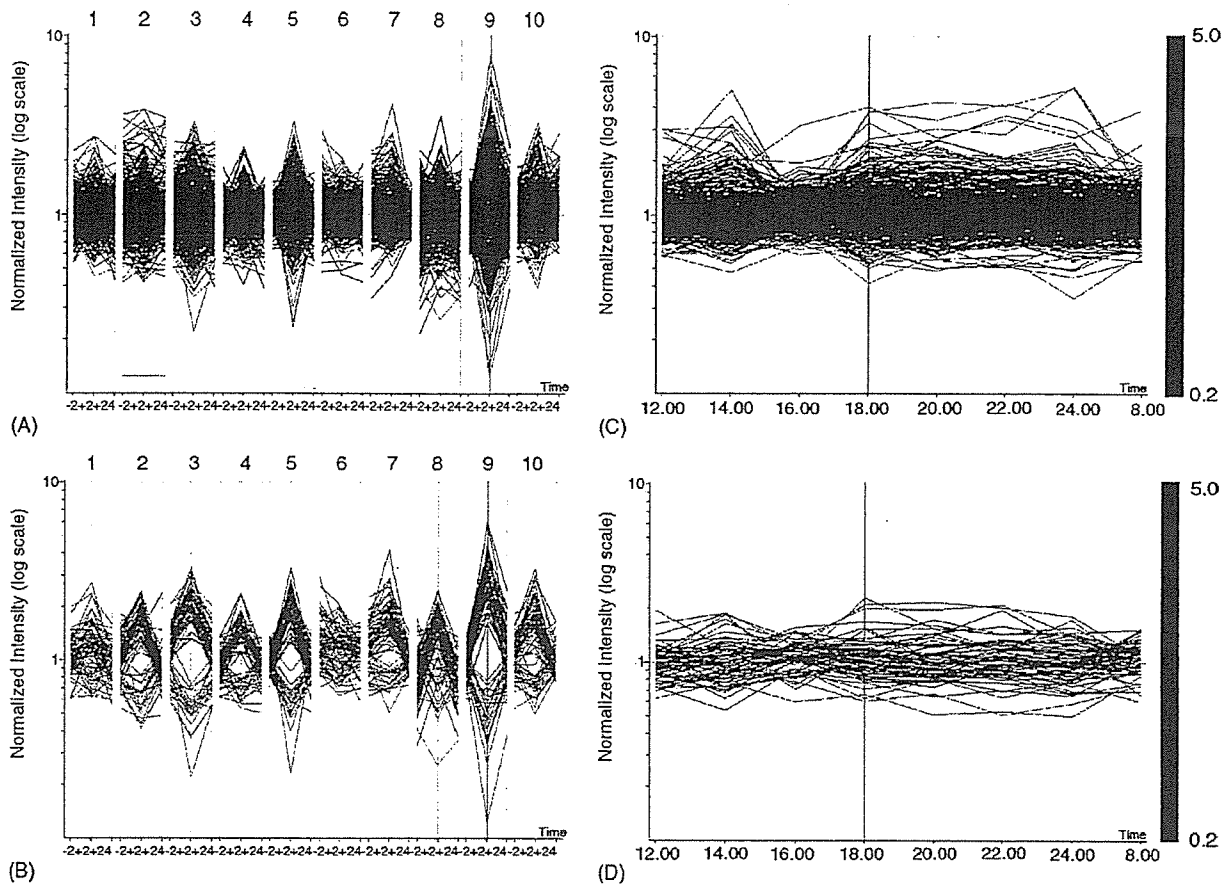


Fig. 3. Time courses of expression values of 519 genes or selected 70 genes during stress exposure or daily activity. Time-dependent changes in 519 mRNA levels 2 h before and 2 or 24 h after the examination (A), and those at the indicated times during routine daily activity (C) are shown. Time-dependent 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-κB, 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 591 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 (*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 (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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