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Appendix 1. Supplemental Data for Table 1. Case-Control Association Analyses with Microsatellite Markers on Chromosome 20p

	Individual Allele					Global		
Marker	Allele (bp)	Case	Control	Chi-square	p	LRS	DF	p
D20S835						8.5	10	.58
	195	.169	.193	.712	.399			
	197	.003	.000	1.501	.221			
	199	.009	.013	.313	.576			
	201	.015	.013	.031	.861			
	203	.142	.138	.029	.864			
	205	.349	.388	1.197	.274			
	207	.087	.073	.505	.478			
	209	.017	.026	.633	.426			
	211	.119	.096	.987	.321			
	213	.087	.060	2.004	.157			
	215	.003	.000	1.501	.221			
D20S873						7.9	7	.34
	185	.000	.008	3.913	.048			
	187	.291	.270	.430	.512			
	189	.003	.010	1.685	.194			
	191	.100	.105	.044	.834			
	193	.026	.018	.466	.495			
	195	.537	.545	.040	.842			
	197	.034	.042	.288	.592			
	199	.009	.003	1.236	.266			
D20S882						21.3	7	.003
	72	.037	.031	.245	.620			
	74	.335	.247	7.420	.006			
	76	.126	.181	4.759	.029			
	78	.355	.316	1.316	.251			
	80	.136	.219	9.711	.002			
	82	.007	.005	.169	.681			
	84	.002	.000	1.353	.245			
	86	.002	.000	1.353	.245			
D20S95						116.6	11	.000
	95	.000	.002	1.148	.284			
	97	.009	.103	48.970	.000			
	99	.124	.111	.411	.522			
	101	.093	.022	26.940	.000			
	103	.052	.032	2.672	.102			
	105	.100	.192	17.630	.000			
	107	.339	.320	.436	.509			
	109	.098	.027	24.160	.000			
	111	.141	.148	.098	.754			
	113	.028	.020	.720	.396			
	115	.011	.020	1.480	.224			
	117	.004	.003	.065	.798			
D20S905						24.9	11	.009
	76	.003	.003	.001	.982			
	78	.038	.042	.080	.777			
	80	.008	.016	.937	.333			
	82	.014	.016	.063	.802			
	84	.264	.311	2.014	.156			
	86	.429	.376	2.187	.139			
	88	.223	.174	2.848	.092			
	90	.014	.053	9.457	.002			
	92	.000	.005	2.714	.099			
	94	.008	.000	4.268	.039			
	96	.000	.003	1.356	.244			
	98	.000	.003	1.356	.244			
D20S194						17.7	21	.67
	185	.003	.000	1.442	.230			
	187	.011	.003	2.105	.147			
	189	.011	.013	.065	.799			
	191	.003	.005	.290	.591			
	193	.025	.044	2.167	.141			
	195	.060	.065	.069	.793			
	197	.047	.047	.000	.991			
	199	.069	.063	.117	.733			
	201	.052	.076	1.707	.191			
	203	.088	.073	.569	.451			
	205	.146	.130	.373	.541			
	207	.107	.138	1.659	.198			
	209	.146	.117	1.325	.250			
	211	.060	.060	.001	.975			
	213	.096	.081	.553	.457			
	215	.025	.034	.549	.459			
	217	.016	.026	.826	.364			
	219	.019	.013	.458	.499			
	221	.008	.003	1.162	.281			
	223	.003	.003	.001	.970			
	225	.000	.005	2.672	.102			
	227	.005	.003	.397	.529			
D20S192						12.1	12	.44
	273	.000	.003	1.275	.259			
	275	.003	.000	1.507	.220			
	277	.012	.005	.939	.333			
	281	.015	.010	.261	.610			
	283	.003	.003	.007	.935			
	285	.436	.471	.929	.335			
	287	.020	.031	.838	.360			
	289	.088	.081	.115	.735			
	291	.114	.122	.121	.728			
	293	.298	.255	1.677	.195			
	295	.003	.016	3.436	.064			
	297	.006	.003	.468	.494			
	299	.003	.000	1.507	.220			
D20S156						3.7	5	.59
	181	.007	.000	1.097	.295			
	183	.149	.130	.297	.586			
	185	.518	.506	.050	.822			
	187	.298	.351	1.282	.257			
	189	.021	.013	.380	.538			
	191	.007	.000	1.097	.295			
D20S892						20.8	12	.05
	199	.006	.000	2.980	.084			
	201	.006	.000	2.980	.084			
	203	.106	.076	2.110	.146			
	205	.017	.036	2.622	.105			
	207	.011	.044	7.635	.006			
	209	.299	.284	.199	.656			
	211	.060	.047	.656	.418			
	213	.121	.130	.151	.698			
	215	.313	.307	.030	.863			
	217	.043	.057	.772	.380			
	219	.014	.013	.025	.876			
	221	.003	.003	.005	.944			
	223	.000	.003	1.292	.256			
D20S846						4.0	8	.85
	269	.349	.318	.832	.362			
	271	.009	.005	.300	.584			
	273	.261	.294	.991	.320			
	275	.071	.086	.565	.452			
	277	.017	.013	.202	.653			
	279	.153	.159	.041	.839			
	281	.102	.091	.261	.610			
	283	.034	.034	.000	.986			
	285	.003	.000	1.477	.224			

LRS, likelihood ratio statistics; DR, degrees of freedom.

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The inter-rater reliability of the Japanese version of the Montgomery–Asberg depression rating scale (MADRS) using a structured interview guide for MADRS (SIGMA)

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The inter-rater reliability of the Japanese version of MADRS (MADRSJ) was examined using the structured interview guide for MADRS (SIGMA) developed by our group. Two or more psychiatrists attended the systematic interview sessions with each subject to evaluate MADRSJ independently, using SIGMA. A total of 18 interviews was carried out for seven patients with DSM-IV major depressive disorder. The severity of each item assessed by the raters ranged from 0 to 6 for nine items and from 0 to 4 for one item. The analysis of variance intraclass correlation coefficient inter-rater reliability values for the individual items carried out by two trained personnel ranged from 0.91 to 1.00, while those achieved by three raters ranged from 0.85 to 1.00. The present results suggest that SIGMA is a potentially useful interview guide for assessing ten symptoms of MADRSJ precisely in patients with a depressive disorder. Copyright © 2004 John Wiley & Sons, Ltd.

KEY WORDS — MADRS; rating scale; SIGMA; reliability; depression; Japanese version

INTRODUCTION

The Montgomery-Asberg depression rating scale (MADRS) was originally developed as a 10-item subscale of the comprehensive psychopathological rating scale (CPRS) to assess the severity of depressive symptoms seen in patients with depressive disorders (Asberg *et al.*, 1978). It was established in 1979 to assess the severity of illness in patients with depressive disorders, designed to be particularly sensitive in gauging the effects of antidepressant therapy in patients suffering from such conditions (Montgomery and Asberg, 1979).

MADRS has been used widely in clinical double-blind studies to assess the efficacy of various antidepressant therapies in western countries. In contrast to its prolific use in western countries, MADRS has not been used in either a clinical or research setting in Japan. Instead, the Hamilton depression rating scale (HAM-D) has been used exclusively, in clinical double-blind studies to assess the severity of the depressive state in patients with major depressive disorder in Japan. In order to compare data gained from clinical double-blind studies on antidepressants carried out in Japan with those carried out in other countries, it is important to assess the depressive state using the same scale and with a common method. The reliability of German (Schmidtke *et al.*, 1988), Spanish (Lobo *et al.*, 2002), French (Peyre *et al.*, 1989) and English versions (Davidson *et al.*, 1986) of MADRS has already been established. The necessity for a standardized and reliable Japanese version has become increasingly apparent. As for the Japanese translation of MADRS 10 items, Kasa and Hitomi (1987) first introduced the original Japanese version of CPRS (CPRSJ) and examined the inter-rater reliability of the CPRSJ individual items. They reported that Spearman's correlation coefficient for the depression subitems, that is, MADRS 10 items, between two doctors ranged from 0.58 to 1.00. Although they concluded that CPRSJ would become a very useful

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psychiatric rating scale in Japan, they mentioned that the raters needed to be educated and trained to obtain stable data in the use of CPRSJ (Kasa and Hitomi, 1985). Recently, Kamijima *et al.* (2003) translated ten items of the MADRS English version into Japanese and published their revised final Japanese version (MADRSJ) after having the back-translated version checked by one of the original authors. The present study was conducted to examine the inter-rater reliability of MADRSJ with a view to using it in clinical trials in Japan. Since there are fewer research psychiatrists who are interested in clinical trials in Japan, general clinical psychiatrists with little research experience are often asked to participate in clinical trials. They are often forced to evaluate the psychiatric symptoms defined in the rating scales without having been sufficiently trained, which is thought to be a factor underlying data scattering. With these limitations in mind, we have developed the structured interview guide for MADRS (SIGMA) so that clinical psychiatrists who are not familiar with the evaluation of rating scales in research can easily operate clinical trials and obtain stable and reliable data with only a small amount of training. In the present study, we examined the inter-rater reliability of MADRSJ (Kamijima *et al.*, 2003) in conjunction with SIGMA, to determine whether the SIGMA is a useful interview tool to obtain the stable data of MADRSJ in clinical trials and psychiatric research.

SUBJECTS AND METHODS

Ethical consideration

The present study was initiated after gaining the approval of the ethical committee of Nagoya University. Written informed consent to participate was obtained from all subjects, after an adequate explanation of the study was provided.

Subjects

The subjects enrolled in the present study were seven patients with major depressive disorder (four males, three females; average age 39 years), as assessed by the Diagnostic and Statistical Manual of Mental Disorders—Fourth Edition (DSM-IV), who had been followed up at the outpatients' service or hospitalized in the Department of Psychiatry, Nagoya University Hospital. They were all receiving antidepressant therapy. The patients' diagnoses were made according to the DSM-IV criteria based on information obtained from several clinical interviews. None of the subjects demonstrated any evidence of neurological disorders or had a history of substance abuse or electro-convulsive therapy.

Evaluation of reliability

The three raters, N.T., T.I. and K.T., are all psychiatrists, and the former two had completed a training course in the administration of MADRS before this reliability study was initiated. The training process consisted of reading the original MADRS itself and the instruction guide thoroughly, referring to the Japanese translation version. To assess the inter-rater reliability of MADRSJ (Kamijima *et al.*, 2003), the three raters attended each subject's MADRSJ rating interview. The interviews were carried out by one of the three raters (T.I.), but all three evaluated the ten items of the MADRSJ independently, for all subjects. A total of 18 interviews were carried for the seven subjects. One of the seven subjects was interviewed only once, three were interviewed twice, one was interviewed three times and two were interviewed four times.

Statistics

The inter-rater reliability value for each item was examined using an analysis of variance intra-class correlation (ANOVA ICC) test.

RESULTS

The inter-rater reliability results of the MADRS are shown in Table 1. The severity of the ten MADRSJ items assessed by the two raters ranged over 0–6 for nine items and 0–4 for one item. The ANOVA ICC inter-rater reliability values of two evaluators (N.T. and T.I.) for the individual scale items ranged from 0.91 to 1.00, including 1.00 for two items. Similarly, the items assessed by the three raters ranged over 0–6 for nine items and 0–4 for one item. The ANOVA ICC inter-rater reliability values for the individual scale items ranged from 0.85 to 1.00 (only one item scored 1.00).

DISCUSSION

Overall, the evaluations performed by the three raters were generally consistent and showed good agreement with each other. This is consistent with the previous inter-rater reliability study of MADRS/CPRS carried out by Montgomery *et al.* (1978). In this study, we developed a structured interview guide for MADRS

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Table 1. Results of the inter-rater reliability of MADRS using SIGMA

MADRS item	Two raters ($n = 19$)		Three raters ($n = 11$)	
	ANOVA ICC	Range evaluated	ANOVA ICC	Range evaluated
1 Apparent sadness	0.96	0–6	0.90	0–6
2 Reported sadness	0.99	0–6	0.85	0–6
3 Inner tension	0.93	0–6	0.93	0–6
4 Reduced sleep[a]	0.92	0–4	0.93	0–4
5 Reduced appetite	1.00	0–6	0.91	0–6
6 Concentration difficulties	1.00	0–6	0.96	0–6
7 Lassitude	0.96	0–6	0.98	0–6
8 Inability to feel	0.99	0–6	0.91	0–6
9 Pessimistic thoughts	0.99	0–6	1.00	0–6
10 Suicidal thoughts	0.99	0–6	0.95	0–6

[a]No patient with the severity anchor point of '6' was observed in the present study.

(SIGMA) to allow us to obtain stable and precise data. Since SIGMA consists of concrete examples of questions corresponding to each anchor point (see Appendix), it is considered that even clinical psychiatrists who are not familiar with the evaluation of research rating scales can evaluate the individual scale items precisely with only a minimal amount of training. In fact, the ANOVA ICC agreement rates for the individual items were 0.85 or higher among the three raters who participated in this study.

The advantage of using a structured interview guide for the evaluation of a rating scale is that it provides instructions to assist raters in the administration of the scoring system, thus increasing the consistency of scoring. Several studies have demonstrated that the evaluation conducted in conjunction with a structured interview guide significantly improved the scattering of the inter-rater agreement rate, when compared with that conducted without using it. For example, when the total values of the ANOVA ICC inter-rater reliability were compared with and without the use of a structured interview guide in conjunction with other rating scales, the total value of the Hamilton anxiety scale reportedly increased from 0.74 to 0.99 (Bruss *et al.*, 1994) and from 0.98 to 0.99 (Shear *et al.*, 2001), that of the Hamilton depression scale increased from 0.80 to 0.99 (Moberg *et al.*, 2001), that of the ANOVA ICC test–retest reliability of HAM-D increased from 0.77 to 0.81 (Williams, 1988), and that of the brief psychiatric rating scale increased from 0.78 to 0.91 (Crippa *et al.*, 2001). As for the individual items of HAM-D, a significant increase in the ANOVA ICC inter-rater reliability values was observed when it was used in conjunction with its structured interview guide (SIGH-D) (Moberg *et al.*, 2001): genital symptoms (0.39 to 0.94), somatic symptoms: gastrointestinal (0.34 to 0.95), obsessional and compulsive symptoms (0.02 to 1.00), worthlessness (0.25 to 1.00). The inter-rater reliability values of HAM-A also significantly increased when it was used in conjunction with its structured interview guide (HARS-IG) in the following items (Bruss *et al.*, 1994): tension (0.37 to 1.00), insomnia (0.49 to 0.99), somatic: muscular (0.43 to 0.96), respiratory (0.28 to 1.00), autonomic (0.23 to 1.00).

The results of the present study are consistent with those of the previous reliability studies carried out using structured interview guides: a high inter-rater reliability has been shown in the evaluation of ten items of MADRSJ using SIGMA, when compared with the previous reliability study of CPRSJ carried out in Japan, without using an interview guide (Kasa and Hitomi, 1985). In conclusion, the present results suggest that SIGMA is a potentially useful interview guide to obtain high inter-rater reliability of ten depressive symptoms of MADRSJ.

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APPENDIX

Structured interview guide for the Montgomery-Asberg depression scale (SIGMA): General comments

The purpose of this structured interview is to assist in the reliable assessment of MADRS by standardizing the rating method. To ensure full assessment of the domain of inquiry for each item, the interviewer should ask basically all the questions provided. In some cases, raters may also have to add their own follow-up questions to obtain the necessary information. The evaluation should be made independently, without referring to the previous results.

When two different scores are achieved, the highest (i.e. representing the more severe state) should be chosen.

If a single anchor point comprises more than one symptom and/or condition, and at least one of them is observed in a patient, the anchor point of that severity should be chosen.

If at all possible, repeated evaluations should be made at the same time of day.

The most severe symptom should be evaluated within the rating period defined by the protocol.

If it is difficult to elicit a rating score from a subject's response, an evaluation should be made based on information obtained from other involved personnel, including the patient's family and nursing staff.

When the complaints of a subject are inconsistent with information gleaned from other involved personnel (e.g. the patient's family and nursing staff), the rater should evaluate the symptom as objectively as possible.

1. Apparent sadness

Observe whether the subject shows expressions or attitudes indicating sadness during the interview (i.e. sighing, calling for help, crying, talking tearfully and silence or no response).

Observe whether the subject reveals his/her sadness voluntarily or tends to respond passively to the interviewer's questions.

Observe the volume and speed of the subject's speech, and the sense of urgency in his/her tone.

Rate whether the sadness observed continues throughout the interview. Also note whether he/she sometimes smiles during the course of the interview.

2. Reported sadness

In the past week, have you been feeling down or sad?

Is the feeling of sadness with or without reason?

If something nice happened to you, would your mood brighten up without difficulty?

Were your feelings of sadness influenced by external circumstances?

Have you felt sad, miserable, and despondent throughout the day?

3. Inner tension

In the past week, have you felt on edge or restless?

Have you experienced these feelings throughout the day, just occasionally, or temporarily?

Has the anxiety ever become so intense that you felt difficulty breathing or that your heart was racing?

Were you able to control those feelings or panic state by yourself?

Have you felt unrelenting dread or anguish?

4. Reduced sleep

In the past week, have you been able to sleep as usual?

When you were well, how many hours did you sleep in a day, on average?

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What is the minimum number of hours per day that you have slept this week?

Have you experienced trouble falling asleep?

Has your sleep been broken by waking up in the middle of the night?

Have you slept poorly and feel that you have been unable to get a good night's sleep?

Have you found yourself waking up in the early hours of the morning and been unable to fall back to sleep?

5. *Reduced appetite*

In the past week, has your appetite been the same as usual?

Is your appetite reduced, as compared to before?

Do you feel that food has been as tasty as when you were well?

Have you felt that food no longer tastes as good?

Have you experienced a loss of appetite and been unable to eat?

Were you able to eat anything by persuading and forcing yourself to eat?

Have you been persuaded or forced to eat by someone?

Were you able to eat only by persuading yourself to eat, and did you need someone to persuade you to eat?

Have you experienced weight loss since your appetite was reduced?

(Evaluation of reduced appetite should be made compared with when the subject was well. When reduced appetite is observed, weight loss should be taken into consideration as collateral evidence.)

6. *Concentration difficulties*

In the past week, were you able to concentrate on things without difficulty?

Have you felt difficulty in collecting your thoughts?

Have you experienced consistent difficulty concentrating throughout the day, or only occasionally?

Have you found it difficult to read or follow TV programs because you have found it difficult to concentrate or sustain your thoughts?

Have you lost the thread of a story while reading or conversing?

Have you found that you are completely unable to read, follow programs on TV, or hold a conversations?

(Note whether the subject is unable to answer the question appropriately, i.e. whether he/she has reduced comprehension due to difficulties in concentrating.)

7. *Lassitude*

In the past week, have you found that it takes longer than usual to perform normal tasks?

Have you found it taking longer to do things you would normally be able to do quickly?

Have you had to make more effort to start daily activities, such as washing your face, getting dressed or having breakfast in the morning?

Have you found that you have relied on and got others to do things that you would normally be able to do yourself?

(When evaluating this symptom, the occurrence of a delayed response to the questions should be taken into account.)

8. *Inability to feel*

Have you been able to take as much interest as normal in things that usually give you pleasure?

Have you failed to take an interest in your surroundings?

Have you failed to feel for, or think of your close friends and relatives?

Have you felt emotionally paralyzed and experienced an inability to feel anger or pleasure?

Has it proven painful to think of your close friends and relatives?

9. *Pessimistic thoughts*

In the past week, did you ever reflect on the past and have regrets like 'I should have this done in another way' or 'It was my mistake'?

Have you felt self-reproach or self-depreciation by thinking you did something wrong or got someone into trouble?

Did such feelings of self-reproach or self-depreciation persist throughout the day or fluctuate?

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Have you been able to make plans for the future or have positive thoughts?

Do you feel that you have developed depression as a punishment for what you have done in the past?

Have you had thoughts such as 'I have no future, I'm hopeless.'

(When pessimistic thoughts are observed, confirm whether the subject has feelings (delusions) of guilt, or delusions of belittlement and, if so, evaluate the severity of these feelings/delusions.)

10. Suicidal thoughts

In the past week, have you been able to enjoy your life?

Have you been able to take your life as it comes?

Have you been weary of your life?

Have you felt this week that life isn't worth living, and you'd rather be dead?

Have you felt that you want to die, without doing anything about it?

Have you experienced such feelings only on rare occasions, or constantly?

Have you ever considered specific plans for death?

Have you ever actually attempted to commit suicide, or prepared to do so when there was an opportunity, and made active preparations for suicide?

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such a hypothesis, the sampling strategy (undiagnosed controls) could have reduced the power of our case/control analysis since major depression demonstrates a prevalence of 5–17%. Accordingly, this could partially explain the modest haplotypic association. In conclusion, owing to the potential role of TPH in affective disorders, and the restricted expression of the TPH2 isoform in brain, the results reported, together with those of Zill *et al*, clearly show the need for further investigations.

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Failure to confirm association between AKT1 haplotype and schizophrenia in a Japanese case–control population

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SIR—AKT1 is a serine/threonine kinase known as protein kinase B. Modulation of AKT1 activity appears to play a key role in the mechanism of glutamate excitotoxicity and lithium neuroprotection.[1] Glycogen synthase kinase 3β (GSK3β) is one of the substrates of AKT1 and AKT1/GSK3β signaling is known as a target of lithium. Lithium can exert its acute effect on dopamine-dependent behaviors by interfering with the action of dopamine on an Akt/GSK-3 signaling cascade in the brain of living mice.[2] Transmission of haplotypes construct from seven single-nucleotide polymorphisms (SNPs) in a highly variable region of the *AKT1* gene has been shown to have a weak evidence of association with bipolar disorder.[3]

Recently, Emamian *et al*[4] identified *AKT1* as a potential schizophrenia susceptibility gene in families of northern European origin. Levels of the AKT1 protein and phosphorylation of GSK3β at Ser9 in lymphocytes and frontal cortex were significantly lower in patients with schizophrenia in comparison with the controls. Moreover, AKT1-deficient mice treated with amphetamine show reduced prepulse inhibition (PPI). The impairment of PPI has been observed in patients with schizophrenia. Haloperidol-treated mice show increased phosphorylation of GSK3β at Ser9. In multi-SNP haplotype analysis, haplotypes containing two to five SNPs (SNP1–5) in the *AKT1* gene were associated with schizophrenia. The TCG haplotype consisting of three SNPs (SNP2/3/4) showed the highest significant association with schizophrenia (ratio of transmission to no transmission = 1.8, $P = 0.0006$). The *AKT1* core risk haplotype TC of the SNP2/3 is associated with lower AKT1 protein levels.

We attempted to confirm the association of AKT1 haplotype with schizophrenia in Japanese subjects comprising of 559 patients with schizophrenia and 567 controls. All subjects were unrelated. The TCG haplotype was estimated to confer a relative risk of 1.9 in the study by Emamian *et al*.[4] Our sample size has 0.999 power to detect the association at the haplotype frequency of 0.085, which was observed in our Japanese control subjects, at a nominal significance level of 5%.

The patients with schizophrenia analyzed were 559 unrelated Japanese (320 men, 239 women; age 18–90 years, mean 49.5 years). Diagnoses were determined after unstructured clinical interviews, and final diagnoses were carried out by consensus of at least two psychiatrists. All subjects met DSM-III-R criteria for schizophrenia. The control subjects consisted of 567 unrelated Japanese individuals (390 men, 177 women; age 29–77 years, mean 51.6 years). Control subjects were not evaluated by psychiatrists. Written informed consent was obtained from all subjects. This study was approved by the Ethics Committees of University of Tsukuba, Tokyo Medical and Dental University, the National Center of Neurology and Psychiatry, and Nagoya University. We examined the population structure with Structure software (http://pritch.bsd.uchicago.edu/)[5] to rule out the possibility that the difference in a case–control study was influenced by hidden population stratification. We genotyped 32 markers (seven microsatellite markers and 25 SNPs) and observed no subpopulation using the number of assumed subpopulation $k = 2$ and 3 (data not shown).

The ID numbers of these five SNPs were provided by Emamian *et al*.[4] All SNPs were genotyped with Taqman® Assays-by-Design℠ SNP Genotyping Service (Applied Biosystems)[6] and ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems).

Table 1 Pairwise linkage disequlibrium and association with schizophrenia of the five SNPs and haplotypes in the AKT1 gene

SNP name			LD[a]				Allele count (frequency)[b]			Haplotype P[c]			
dbSNP ID	Position[d]	Inter-SNP distance(bp)	D'	Delta²		n	1	2	P	2 Locus	3 Locus	4 Locus	5 Locus
SNP1	103241455	—	—	—	Schz	551	587 (0.533)	515 (0.467)	0.505				
rs3803300					Cntr	565	586 (0.519)	544 (0.481)		0.493			
		10045	0.959	0.182						0.525			
SNP2	103231410				Schz	544	931 (0.856)	157 (0.144)	0.363		0.305		
rs1130214					Cntr	566	953 (0.842)	179 (0.158)		0.601	0.654	0.479	
		13327	0.668	0.01						0.743		0.465	
SNP3	103218083				Schz	554	980 (0.884)	128 (0.116)	0.565		0.666		0.224
rs3730358					Cntr	561	1001 (0.892)	121 (0.108)		0.526	0.733	0.372	0.307
		6513	0.874	0.106						0.663		0.901	
SNP4	103211570				Schz	551	583 (0.529)	519 (0.471)	0.654		0.455		
rs2498799					Cntr	563	585 (0.520)	541 (0.480)		0.471	0.762		
		702	0.993	0.458						0.733			
SNP5	103210868				Schz	547	709 (0.648)	385 (0.352)	0.278				
rs2494732					Cntr	562	753 (0.670)	371 (0.330)					

Schz: schizophrenia, Cntr: control.

[a]LD: linkage disequilibrium of adjacent SNPs was shown by D' and delta².

[b]SNP1—allele 1: A, allele 2: G; SNP2—allele 1: G, allele 2: T; SNP3—allele 1: C, allele 2: T; SNP4—allele 1: G, allele 2: A; SNP5—allele 1: G, allele 2: A.

[c]Upper: global *P*-value (uncorrected), lower: haplotype-specific permutation *P*-value.

The haplotype-specific *P*-value of the core risk haplotype (TC for SNP2/3) reported by Emamian *et al*[4] was 0.347 and the haplotype frequencies were 0.141 in the patients and 0.154 in the controls.

The haplotype-specific *P*-value of the highest significant haplotype (TCG for SNP2/3/4) reported by Emamian *et al*[4] was 0.848 and the haplotype frequencies were 0.087 in the patients and 0.085 in the controls.

[d]Chromosome position was referred to dbSNP database.

Tests for single-marker allelic association were performed by χ^2 analysis. Haplotype was estimated with an expectation–maximization (EM) algorithm. Tests for haplotypic association were performed with COCAPHASE software (http://www.hgmp.mrc.ac.uk/~fdudbrid/software/unphased/).[7] Haplotypes with frequencies less than 3% were regarded as rare. Pairwise linkage disequilibrium (LD) was calculated with GOLD software (http://www.sph.umich.edu/csg/abecasis/GOLD/index.html).[8]

The genotypic distributions of the five SNPs did not deviate from Hardy–Weinberg equilibrium in both groups. Pairwise LD between adjacent SNPs was similar to those reported by Emamian *et al*[4] (Table 1). We found no association between each SNP and schizophrenia (Table 1). The frequency of the core risk haplotype (TC for SNP2/3) did not differ significantly between patients with schizophrenia (0.141) and controls (0.154) in our Japanese samples. Furthermore, the frequency of the highest significant haplotype (SNP2/3/4, TCG) by Emamian *et al*[4] did not differ between patients with schizophrenia (0.087) and controls (0.085) in our Japanese samples (odds ratio 1.03, 95% confidence interval 0.76–1.38) (Table 1). Significant associations were not detected between other haplotypes and patients with schizophrenia (Table 1). We further performed stratified analysis of the data by age at onset, familial loading, and sex. No significant associations were observed (data not shown).

In the present study, we could not confirm the association between *AKT1* haplotype and schizophrenia in our Japanese samples. The TCG haplotype frequency was lower in our Japanese population (0.085) than in populations of northern European origin (0.15). The power of this study to detect an association in our Japanese population is sufficient even at the haplotype frequency of 0.085; however, the different ethnical background may preclude confirmation of the genetic association. Haplotype construction is less reliable in population-based samples than in family-based samples; therefore, haplotype estimation procedure of the present study might make confirmation of the genetic association difficult. Furthermore, the samples examined by Emamian *et al*[4] included 210 proband–parent triads and 58 extended families with multiple affected individuals obtained from the NIMH Genetics Initiative. Although the χ^2 test did not show a significant difference in either allelic or haplotypic frequencies between 92 patients with first-degree relatives who had schizophrenia and controls (data not shown), it remains possible that the association is observed only in familial schizophrenia. Finally, we cannot exclude the existence of a weak association because the 95% confidence interval of the odds ratio was 0.76–1.38 in our population.

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Letter to the Editors

Failure to find association between PRODH deletion and schizophrenia ☆

Dear editors,

A deletion of the 22q11.2 region, which occurs in 1 of 4000 live births (Wilson et al., 1994), is a relatively common genetic disorder in humans. The 22q11.2 deletion has been found in 0.3–2% of adult patients with schizophrenia (Arinami et al., 2001; Karayiorgou et al., 1995) and in 6% of childhood-onset schizophrenia (Usiskin et al., 1999). It was reported that 24% of patients with VCFS met the criteria for schizophrenia (Murphy et al., 1999). Therefore, the risk of schizophrenia for patients with the 22q11.2 deletion may be 25–30 times higher than the general population risk of 1%. However, it is difficult to determine which of the specific genes in this genomic region may be associated with psychiatric problems.

The proline dehydrogenase gene (PRODH) is located in this region and has been suggested to increase susceptibility to schizophrenia because of abnormal findings in a PRODH-mutant mouse, allelic association with schizophrenia (Liu et al., 2002), and detection of a family with schizophrenia and a 350-kb deletion that includes PRODH (Jacquet et al., 2002). During our ongoing screening study for polymorphisms associated with schizophrenia in the 22q11.2 region, we identified one Japanese family with three members who carried the PRODH deletion. The father and two daughters of the family were hemizygous for at least a 100-kb region extending from single nucleotide polymorphism (SNP) rs416659 (dbSNP of National Center for Biotechnology Information (NCBI)) to D22S1638 because they each carried only one allele of the rs416659 and rs1210635 SNPs and D22S1638 and the father's allele was not transmitted to the daughters. The father's paternity was confirmed by testing of more than 400 microsatellite markers, which were used for a genome-wide linkage scan (Takahashi et al., 2003). The father's null allele should have been transmitted to the daughters.

To confirm the deletion, we devised a PCR-based homologous gene quantitative amplification screening method to detect the PRODH deletion. A primer set that amplifies an intronic region of PRODH and its counterpart, intronic region of ψPRODH was designed with the BLAST 2 (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html). PCR was done with the following primers: forward, 5′-AGCTCAGTGCCCATGTCAGT and reverse, 5′-ACTGCCCTGTCTGCCTGTAG. The reverse primer was 5′-labeled with the fluorescein dye 6-FAM. The PCR product sizes from PRODH (NCBI accession number AC008103) and ψPRODH (AC007663) were 229 and 239 bp, respectively. After denaturation at 95 °C for 10 min, amplification consisted of 26 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 30 s followed by a final extension of 72 °C for 7 min. PCR product was mixed with ROX labeled GeneScan 400 HD. Electrophoresis was carried out with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Peak height of each PCR product from PRODH and ψPRODH was measured with the GeneScan and Genotyper programs (Applied Biosystems). The peak ratio of ψPRODH/PRODH was calculated. To monitor the quality of each experiment, samples from individuals with the PRODH deletion were amplified simultaneously.

The sequences of these regions are highly homologous, but the PCR product from PRODH was 10 bp shorter than that from ψPRODH (Fig. 1). In this

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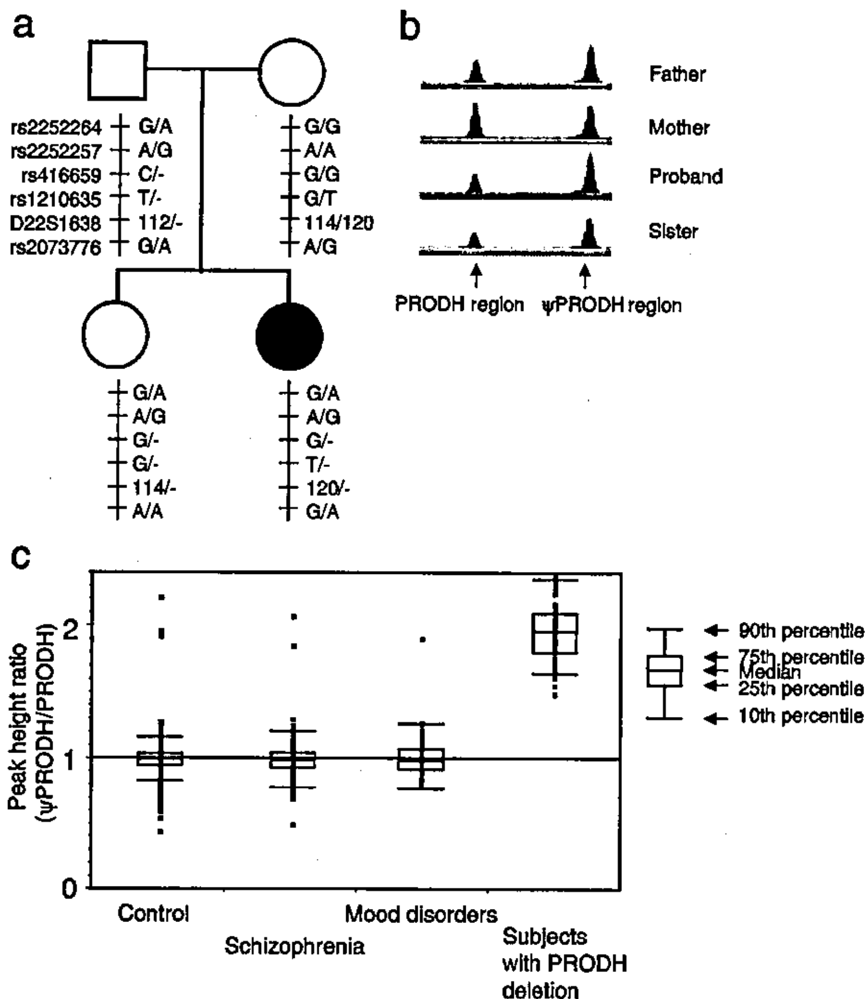

Fig. 1. A family with the PRODH deletion and detection of the deletion by a homologous gene quantitative amplification. (a) A family with the PRODH deletion and genotypes of the markers within and around the deletion. Closed circle indicates the proband with schizophrenia. Other family members are psychosis-free. Alleles of a microsatellite marker are indicated as size (bp). Minus (−), deleted (null) allele. (b) Electropherogram of deletion screening in members of the family. (c) Distribution of the peak height ratio of ψPRODH to PRODH. Six individuals among controls and patients with schizophrenia and mood disorders had a ratio of approximately 2, indicating the presence of the deletion. The distribution of subjects with PRODH deletion is that of simultaneously amplified products from three samples with the deletion to monitor the quality of each experiment.

family, the peak of the PCR product from PRODH was half as high as that from ψPRODH in the father and the two daughters, whereas the height of the two peaks was equal in the mother, supporting our finding of the deletion in the father and daughters. These data also showed that the devised homologous gene quantitative amplification method was useful for detecting the PRODH deletion.

Because the deletion did not co-segregate with schizophrenia in our family or a family reported by Jacquet et al. (2002), we tried to perform association analysis with this simple method. We screened for the PRODH deletion in patients with schizophrenia and mood disorder and controls. All subjects were unrelated Japanese. A total of 1505 unrelated Japanese subjects were screened, and six subjects carried the PRODH deletion (Fig. 1). To confirm the PRODH deletion in these six subjects, three polymorphic markers flanking PRODH, rs416659, rs1210635, and D22S1638, were analyzed. Only one allele of each marker was detected in the six subjects. The minor allele frequencies of rs416659 and rs1210635 were

0.2047 and 0.4648, respectively, in Japanese (IMS-JST Japanese SNP database, http://snp.ims.u-tokyo.ac.jp/index.html), and heterozygosity of D22S1638 was 0.8123 (our unpublished data). Although the chance that a Japanese individual is homozygous for these three markers is 6.5%, the chance that all six of the subjects were homozygous for the three markers is 7.5×10^{-8}, supporting our quantitative PCR finding that the six subjects carried the PRODH region deletion. The region includes at least PRODH and DGCR6. The deletion was found in 2 of 509 patients with schizophrenia, 1 of 107 patients with mood disorders, and 3 of 889 control subjects. Thus, 1 in approximately 250 Japanese individuals (95% confidence interval, 1 in 115 to 1 in 547) carries this deletion, indicating that the deletion is 10-fold more prevalent than the 22q11.2 deletion. However, the findings of the present study indicate that haploinsufficiency for PRODH and DGCR6 is not likely to account for the at least 10-fold increased risk of schizophrenia in individuals with a 22q11.2 deletion.

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BRIEF REPORT

No Association Between the Val66Met Polymorphism of the Brain-Derived Neurotrophic Factor Gene and Bipolar Disorder in a Japanese Population: A Multicenter Study

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Background: *Two previous studies reported a significant association between a missense polymorphism (Val66Met) in the brain-derived neurotrophic factor (BDNF) gene and bipolar disorder; however, contradictory negative results have also been reported, necessitating further investigation.*

Methods: *We organized a multicenter study of a relatively large sample of 519 patients with bipolar disorder (according to DSM-IV criteria) and 588 control subjects matched for gender, age, and ethnicity (Japanese). Genotyping was done by polymerase chain reaction–based restriction fragment length polymorphism or direct sequencing.*

Results: *The genotype distributions and allele frequencies were similar among the patients and control subjects. Even if the possible relationships of the polymorphism with several clinical variables (i.e., bipolar I or II, presence of psychotic features, family history, and age of onset) were examined, no variable was related to the polymorphism.*

Conclusions: *The Val66Met polymorphism of the BDNF gene is unrelated to the development or clinical features of bipolar disorder, at least in a Japanese population.*

Key Words: Association study, bipolar disorder, brain-derived neurotrophic factor, genetics, single nucleotide polymorphism, susceptibility

Brain-derived neurotrophic factor (BDNF) belongs to the neurotrophin family and promotes the development, regeneration, survival, and maintenance of function of neurons (Maisonpierre et al 1991). It modulates synaptic plasticity and neurotransmitter release across multiple neurotransmitter systems, as well as the intracellular signal-transduction pathway (Thoenen 1995). Growing evidence has suggested important roles of BDNF in the pathogenesis of mood disorders and in the mechanism of action of therapeutic agents, such as mood stabilizers and antidepressants (reviewed by Duman 2002). In postmortem brains of patients with bipolar disorder, BDNF protein was reduced compared with control subjects (Knable et al 2004). Chronic electroconvulsive seizure or antidepressant drug treatments increase messenger ribonucleic acid of BDNF and its receptor, tyrosine kinase receptor B (Nibuya et al 1995). Lithium might also exert its neuroprotective effect through enhancing expression of BDNF and trkB (Hashimoto et al 2002).

The BDNF gene is, therefore, an attractive candidate gene that might cause susceptibility to bipolar disorder or influence the clinical phenotype of the illness. Indeed, at least two previous studies reported a significant association between a missense polymorphism (Val66Met; National Center for Biotechnology Information Database of Single Nucleotide Polymorphisms reference number rs6265) of the BDNF gene and bipolar disorder (Neves-Pereira et al 2002; Sklar et al 2002); however, contradictory negative results have also been reported (Hong et al 2003; Nakata et al 2003). One possible reason for this inconsistency is the lack of statistical power due to small sample size. To draw any conclusion with respect to this possible association, we organized a multicenter study in which six laboratories combined their data to ensure adequate statistical power.

Methods and Materials

Subjects

Six laboratories (National Institute of Mental Health, two laboratories of the Brain Science Institute, Showa University, Tokyo Women's Medical College, and Fujita Health University) collected deoxyribonucleic acid (DNA) samples from patients with bipolar disorder and healthy control subjects. Each institute provided DNA samples of patients and control subjects matched for gender, age, and geographic area, which yielded a combined sample of 519 patients with bipolar disorder (244 male) and 588 control subjects (287 male). Mean age (± SD) for the patients was 49.3 ± 14.3 years and for the control subjects was 48.4 ± 12.7 years. All the patients and control subjects were Japanese and biologically unrelated. Consensus diagnosis of bipolar disorder was made for each patient by at least two experienced psychiatrists according to DSM-IV criteria (American Psychiatric Association 1994) on the basis of unstructured interviews and medical records. Among the patients, 347 were diagnosed as bipolar I

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Table 1. Genotype Distributions and Allele Frequencies for the Val66Met Polymorphism of the BDNF Gene Among the Patients with Bipolar Disorder and Control Subjects

	Genotype Distribution				Allele Frequency		
	n	Val/Val	Val/Met	Met/Met	*n*	Val	Met
Patients							
Total	519	188 (36.2)	239 (46.1)	92 (17.7)	1038	615 (59.2)	423 (40.8)
Bipolar I	347	123 (35.4)	166 (47.8)	58 (16.7)	694	412 (59.4)	282 (40.6)
Bipolar II	172	65 (37.8)	73 (42.4)	34 (19.8)	344	203 (59.0)	141 (41.0)
Control subjects	588	216 (36.7)	270 (45.9)	102 (17.3)	1176	702 (59.7)	474 (40.3)

Values in parentheses are percentages. Genotypewise comparisons: total patients vs. control subjects: χ^2 (2) = .0, p = .98; bipolar I vs. control subjects: χ^2 (2) = .3, p = .86; bipolar II vs. control subjects: χ^2 (2) = .8, p = .69. Allelewise comparisons: total patients vs. control subjects: χ^2 (1) = .0, p = .83; bipolar I vs. control subjects: χ^2 (1) = .0, p = .96; bipolar II vs. control subjects: χ^2 (1) = .0, p = .94.

and the remaining 172 as bipolar II. Control subjects were healthy volunteers who had no current or past contact with psychiatric services. The control subjects were recruited from the hospital staffs and their associates at each institution who showed good social functioning and reported themselves to be in good health. They were interviewed, and those individuals who had current or past contact with psychiatric services were excluded. Written informed consent for participation in the study was obtained from all subjects. The study protocol was approved by the institutional ethics committees.

Methods

Venous blood was drawn, and genomic DNA was extracted according to standard procedures. Genotyping was performed according to Neves-Pereira et al (2003). Briefly, the polymorphic site was amplified by polymerase chain reaction (PCR) and then digested with a restriction enzyme, Eco72I. The digested PCR products were visualized with gel electrophoresis and subsequent ethidium bromide staining. Genotyping for a portion of subjects was done by direct sequencing of PCR products encompassing the polymorphic site with an autosequencer (CEQ 8000; Beckman Coulter, Fullerton, California). Genotype data were read blind to the case–control status.

To examine the possible relationships of the Val66Met polymorphism with clinical variables, information on age of onset, family history, and presence of psychotic features (i.e., current or past episode with delusions or hallucinations) was obtained. We defined positive family history as having at least one first-degree relative with a history of contact with psychiatric services with a diagnosis of mood disorder or who was a suicide victim. Individuals with ambiguous clinical data were excluded from statistical analyses.

The presence of Hardy-Weinberg equilibrium for the genotype distributions in the patients and control subjects was examined with the χ^2 test for goodness of fit. The differences in the genotype and allele distributions between patients and control subjects were examined with the χ^2 test for independence. The possible relationships between the polymorphism and clinical variables were examined with the χ^2 test for independence or analysis of covariance (ANCOVA) within the patient group. All p values reported are two-tailed.

Results

Genotype and allele distributions of the Val66Met polymorphism in the patients and control subjects are shown in Table 1. The genotype distributions in the two groups were both in Hardy-Weinberg equilibrium [patients: $\chi^2(1)$ = 1.1, p = .29; control subjects: $\chi^2(1)$ = 1.2, p = .27]. The genotype and allele distributions for the patients were quite similar to those for the control group (see Table 1). The genotype and allele distributions of the patients with bipolar I and those with bipolar II were also similar.

When relationships between genotype and clinical variables were examined, genotype and allele distributions were not different according to presence of psychotic features (frequency of the Val66 allele for psychotic patients: .567; for nonpsychotic patients: .579) or family history (positive family history: .602; negative: .603). Age of onset was also similar, irrespective of the genotype (Val/Val: 35.3 ± 13.5 years; Val/Met: 37.7 ± 14.6 years; Met/Met: 36.3 ± 14.0 years). Even when ANCOVA controlling for age and gender was performed, there was no significant difference in age of onset across the three genotypic groups [$F(2)$ = .99, p = .37].

Discussion

We tried to replicate the studies of Sklar et al (2002) and Neves-Pereira et al (2002), who found a significant association between the Val66Met polymorphism of the BDNF gene with bipolar disorder. They reported excess transmission of the Val66 allele to the patients in their family-based association studies. Contrary to these findings, the genotype and allele frequencies among the patients and control subjects were similar in our sample, which is in turn consistent with more recent studies (Hong et al 2003; Nakata et al 2003), suggesting that the Val66Met polymorphism of the BDNF gene is unrelated to the development of bipolar disorder in our sample. Because our study had adequate statistical power (more than 90% to detect an odds ratio of 1.33 or more in allelic association; power analysis was performed according to Armitage and Berry 1994), the potential type II error due to lack of statistical power is unlikely. One possible explanation for this inconsistency might be a differential effect of the polymorphism depending on ethnicity, given that the majority of the subjects of Sklar et al (2002) and Neves-Pereira et al (2002) were Caucasian, whereas those of Hong et al (2003), Nakata et al (2003), and in our study were Asian. Alternatively, the positive results of Sklar et al (2002) and Neves-Pereira et al (2002) might have arisen by chance.

Concerning the possible effect of the polymorphism on clinical features, Rybakowski et al (2003) reported an earlier age of onset in Val/Val than Val/Met genotype (27 years vs. 38 years) among patients with bipolar disorder. They also found that the performance in all domains of the Wisconsin Card Sorting Test was significantly better for bipolar patients with Val/Val than for those with Val/Met genotype, suggesting a role of the Val66Met polymorphism in prefrontal cognitive function in bipolar disorder. This accords with the findings of Egan et al (2003), who

reported that the Met66 allele was associated with lower activity-dependent secretion of BDNF and poorer human memory and hippocampal function; however, we could not find any significant effect of the genotype on clinical variables of age of onset, subtype (bipolar I or II), psychotic features, or family history. Hong et al (2003) also failed to find significant difference in age of onset or suicidal history across genotypic groups in their Chinese subjects with bipolar disorder.

In conclusion, our results, together with previous two studies (Hong et al 2003; Nakata et al 2003), suggest that the Val66Met polymorphism of the BDNF gene is unrelated to the development or clinical features of bipolar disorder at least in Asian populations; however, the possibility remains that other variants of the BDNF gene might be associated with bipolar disorder in Asian populations, which requires further investigation.

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The RNA Binding Protein TLS Is Translocated to Dendritic Spines by mGluR5 Activation and Regulates Spine Morphology

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Summary

Neuronal dendrites, together with dendritic spines, exhibit enormously diverse structure [1]. Selective targeting and local translation of mRNAs in dendritic spines have been implicated in synapse remodeling or synaptic plasticity [2, 3]. The mechanism of mRNA transport to the postsynaptic site is a fundamental question in local dendritic translation [4, 5]. TLS (translocated in liposarcoma), previously identified as a component of hnRNP complexes, unexpectedly showed somatodendritic localization in mature hippocampal pyramidal neurons. In the present study, TLS was translocated to dendrites and was recruited to dendrites not only via microtubules but also via actin filaments. In mature hippocampal pyramidal neurons, TLS accumulated in the spines at excitatory postsynapses upon mGluR5 activation, which was accompanied by an increased RNA content in dendrites. Consistent with the in vitro studies, TLS-null hippocampal pyramidal neurons exhibited abnormal spine morphology and lower spine density. Our results indicate that TLS participates in mRNA sorting to the dendritic spines induced by mGluR5 activation and regulates spine morphology to stabilize the synaptic structure.

Results

TLS, also called FUS, was first identified as a rearranged gene at a chromosomal translocation junction invariably linked to human myxoid liposarcomas [6]. Recent structural study of TLS has identified 2-folded domains: a C4 zinc finger domain and RNA recognition motif (RRM) domain [7]. Consistent with its RNA binding properties, TLS is involved in rapid nuclear-cytoplasmic shuttling by binding mRNAs in the nucleus and exporting spliced mRNA as a ribonucleoprotein complex to the cytoplasm [8] and further in the initiation of cell spreading [9]. However, no neuronal function of TLS has been reported. Our initial observation that TLS is expressed in the mouse neocortex and hippocampus led us to investigate the neuronal functions of TLS.

TLS Localization in Mouse Neuronal Dendrites

TLS is expressed in the brain, and recent proteomic analysis revealed that TLS is included in an NMDA receptor complex [10] and an RNA-transporting granule as a binding partner of conventional kinesin (KIF5) [11]. We examined the subcellular distribution of TLS in hippocampal neurons in culture. Immunostaining with anti-TLS polyclonal antibody (TLS-C) exhibited a punctate distribution of TLS within dendrites and a clustering in the nucleus (Figure 1A, upper middle panel), whereas no specific signals were observed in the preadsorbed specimen (Figure 1A, upper right panel). Consistent with this endogenous expression of TLS, when expressed in the hippocampal neurons, TLS-fused to green fluorescent protein (TLS-GFP) exhibited a similar granular distribution within dendrites in addition to staining in the nucleus (Figure 1A, upper left panel). Furthermore, double-label immunocytochemistry with anti-TLS and anti-PSD95 antibodies revealed colocalization of TLS immunoreactivity with PSD95-positive spines (Figure 1A, arrows in lower panels) (the ratio of colocalization is 65%–73%, n = 50, dendritic segments from spiny neurons). This result suggests that TLS is localized in postsynapses and is consistent with further analysis described below (see Figure S1 in the Supplemental Data available with this article online). The hippocampal neurons expressing TLS-GFP were immunostained with anti-MAP2 antibody, a somatodendritic marker, to confirm that TLS was localized in the neuronal dendrites (Figure 1B, upper panels). The result clearly showed that TLS-GFP was colocalized with MAP2-immunopositive dendrites. In contrast, TLS-GFP was absent from long thin axonal projections of hippocampal pyramidal neurons marked by antibody against phosphorylated neurofilament protein (SMI31). The SMI31-positive projections were MAP2 negative, confirming their identity as axons (Figure 1B, lower panels). These results indicate that TLS is exclusively localized in the neuronal dendrites of polarized neurons.

TLS-GFP Moves toward Dendrites

TLS localization within dendrites was examined by using an adenovirus-mediated expression system to efficiently express TLS-GFP in cultured hippocampal neurons. Movement of TLS-GFP was assayed by time-lapse confocal microscopy 48 hr after infection with adenovirus expressing TLS-GFP (Figure 2 and Movies 1

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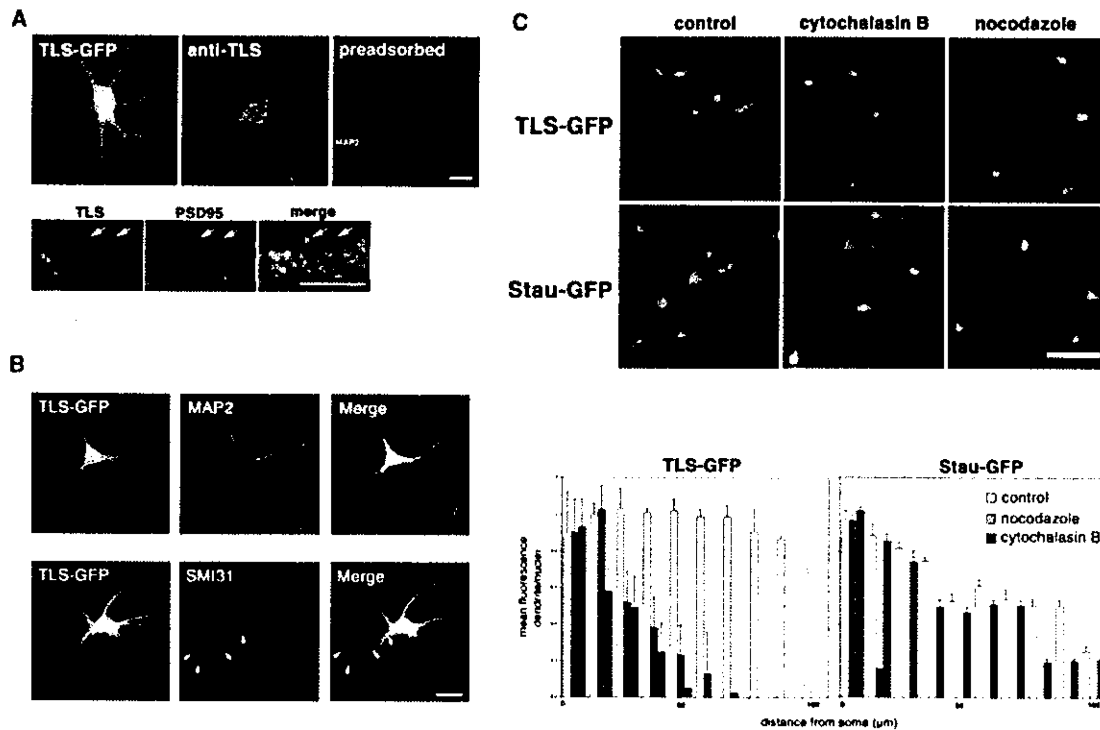

Figure 1. TLS Is Localized in Neuronal Dendrites

(A) Immunocytochemistry of mouse hippocampal-cultured neurons shows a punctate distribution of TLS within dendrites and its clustering in the nucleus (top middle). No staining with the antibody preadsorbed by excess amounts of GST-TLS fusion protein (top right, inset; costaining with anti-MAP2 antibody). Exogenously expressed TLS-GFP protein recapitulates the endogenous punctate localization of TLS within dendrites (top left). Double labeling with anti-PSD95 antibody shows that some of the TLS clusters are localized in spines (bottom arrows). Scale bars, 10 μm in upper panels and 5 μm in lower panels.

(B) TLS-GFP is localized in MAP2-positive neuronal dendrites, whereas TLS-GFP is absent in the MAP2-negative process (arrowheads). TLS-GFP is excluded from this SMI31-positive thin-axonal process (arrowheads) of a mouse hippocampal pyramidal neuron. Scale bars, 10 μm.

(C) TLS translocation to dendrites requires intact cytoskeletal polymers and is actin dependent. In control cells, TLS-GFP is distributed both in the cell bodies and dendrites ($n = 27$) as well as staufen-GFP. Treatment with either cytochalasin B (0.2 μM) ($n = 48$, over 50 μm distant from the soma, $p < 0.01$) or nocodazole (2 μM) ($n = 51$, over 70 μm distant from the soma, $p < 0.01$) for 12 hr reduces the amount of TLS-GFP within dendrites, although the neuronal extensions are not retracted. Nocodazole treatment blocks dendritic localization of staufen-GFP, however, cytochalasin B has no effect on its somatodendritic localization. Scale bar, 50 μm. Graphs showing quantitative data for pharamacological experiments with inhibitors described above are presented. Error bars indicate SEM (standard error of mean) for each experiment.

and 2). When TLS-GFP was expressed in immature dendrites with few spines (culture day 13, Figure 2A), rapid fusion (Figure 2A, 0–5 min, left arrows) and dissociation (Figure 2A, 0–5 min, right arrows) of a fraction of the TLS-GFP particles took place. Active movement of TLS-GFP particles over a short distance within dendrites was also observed (Figure 2A, 15–25 min, arrowhead). To measure the exchange rate of TLS-GFP molecules in the particles, we performed fluorescence recovery after photobleaching (FRAP) of TLS-GFP clusters present in dendrites. After photobleaching, fluorescent signals of TLS-GFP were recovered rapidly, and half recovery of fluorescence was observed within 20 s (data not shown). This rapid time course of FRAP recovery indicates a dynamic exchange of TLS molecules between particulate and soluble fractions. The movement of TLS-GFP was also revealed to be bidirectional, and some populations of TLS-GFP particles formed stationary clusters within the dendritic shaft (Figures S1Ba–S1Bc). These clusters also repeatedly gathered and dispersed within the dendrites. By double labeling with anti-cortactin binding protein (CortBP), as an independent marker that identifies the morphology of the spines, and TLS-GFP, we further analyzed whether TLS-GFP clusters are localized in spines (Figure S2). The distribution of spines/filopodia labeled with anti-CortBP was different from that of TLS-GFP clusters, indicating that TLS is not selectively translocated into spines/filopodia at the early developmental stage. In mature dendrites at culture day 23, there was a significant shift of the localization of TLS-GFP particles from the dendritic shafts to spines (Figure 2B, arrow; see also Figure S1A and S1Ca–S1Cc), and these particles within the spines did not show the rapid movement that was evident in the immature dendrites. TLS-GFP clusters within spines were of a stationary nature. These data suggest that TLS may move dynamically within dendrites before spine maturation. However, once im-

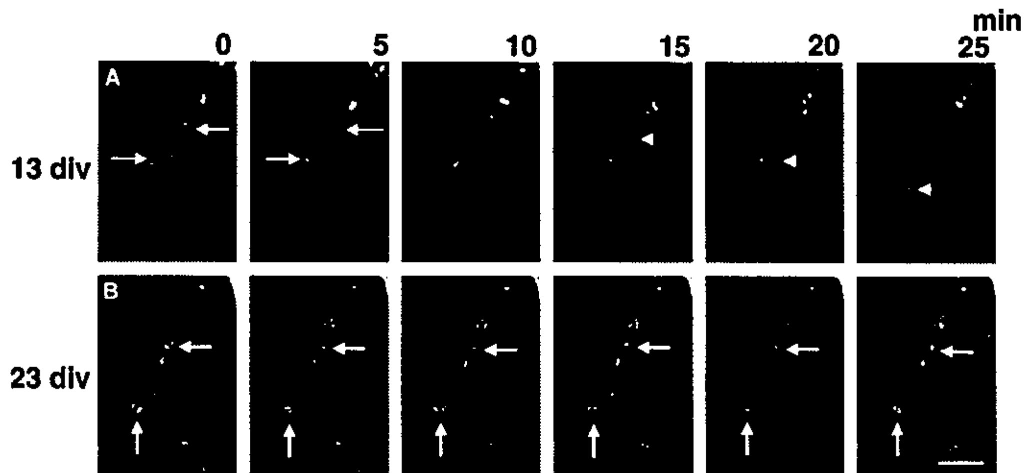

Figure 2. TLS-GFP Clusters Show Distinct Dynamics at Different Stages of Dendritic Maturation

TLS-GFP was expressed in mouse hippocampal neurons by use of the adenoviral expression system. TLS-GFP was assayed by time-lapse confocal imaging of TLS-GFP-expressing adenovirus 24–48 hr after the infection. (A) When TLS-GFP was expressed in immature dendrites with few spines (13 div: 13 days of in vitro culture), some TLS-GFP clusters fused together or dissociated during the short observation period of the time-lapse sequences (arrows in [A], 0–5 min). A fraction of TLS-GFP clusters actively moved a short distance in dendrites (arrowheads in [A], 15–25 min). (B) In mature dendrites (23 div: 23 days of in vitro culture), TLS-GFP clusters were stationary within spines and did not move as fast as they did in the immature dendrites. However, TLS-GFP clusters changed their shape actively, possibly because of the overall change of spine shape affected by actin-dependent motility (B, arrows). Scale bar, 5 μm.

mature spines are committed to form stable synapses, TLS may become preferentially recruited to and accumulated within spines.

Requirement of Intact Actin Polymers for TLS Translocation

To examine how the cytoskeletal organization of the dendrites is involved in the movement or transport of TLS, we treated primary cultured hippocampal neurons either with cytochalasin B or nocodazole, potent inhibitors of the assembly of actin filaments or microtubules, respectively. Both reagents affected the distribution of TLS within dendrites, and the TLS-GFP disappeared from the dendrites (Figure 1C). Quantitative analysis revealed a significant decrease in the TLS-GFP signal intensity in dendrites after treatment with either cytochalasin B or nocodazole (Figure 1C). On the other hand, the distribution of Staufen, whose dendritic localization is known to be microtubule dependent [12, 13], was affected by the nocodazole treatment, but not by cytochalasin B (Figure 1C). These data indicate that the dendritic localization of TLS-GFP required both actin filaments and microtubules.

Synaptic Activity-Dependent TLS Translocation to the Dendritic Spines

Mature dendrites expressing TLS-GFP were immunostained with anti-synapsin I antibody, anti-vesicular glutamate transporter-1 (VGLUT1) antibody, and anti-CortBP antibody to reveal the precise localization of TLS in dendrites. CortBP completely overlapped TLS-GFP at the synaptic sites (Figure S1F), whereas punctate structures immunopositive for either synapsin I, a marker of presynaptic vesicles, or VGLUT1, a marker of excitatory presynaptic structures, were closely apposed to the fluorescent clusters of TLS-GFP (Figure S1D and S1E, respectively), suggesting that TLS was specifically localized in the spines of excitatory postsynaptic sites, as described above.

Local protein synthesis subsequent to translocation of mRNA to dendrites is known to be stimulated by DHPG, a group 1 mGluR agonist [3], as well as neurotrophin, BDNF [14, 15]. Using DHPG to transiently activate mGluRs in dendrites, we tested to see if TLS-GFP accumulation in dendrites and dendritic spines depends on the state of synapse activation. When cultured hippocampal neurons expressing TLS-GFP were stimulated with DHPG (100 μM) over a 60-min period, the amount of TLS-GFP clusters in dendrites increased (Figures 3A–3D) and the movement of the particles accelerated (data not shown). Furthermore, the TLS-GFP clusters gradually accumulated in the dendritic spines (Figures 3A–3D, inset) where retrospective immunocytochemistry with synapsin I antibody revealed the presence of a presynaptic component at the sites of TLS accumulation (Figures 3D–3F). To the contrary, other synaptic proteins such as PSD95, Homer-1c (PSD-Zip45), Shank, and GKAP were not translocated into spines by DHPG treatment (see Figure S3). These results indicate that TLS is likely to be involved in the translocation of mRNA to the dendritic spines for local translation in dendrites. In DHPG-treated mature hippocampal neurons, the relative fluorescence intensity in spines was increased significantly (n = 26, cluster index 35% in average) by 5-fold compared with that of control neurons (n = 17, cluster index 7% in average) (Figure