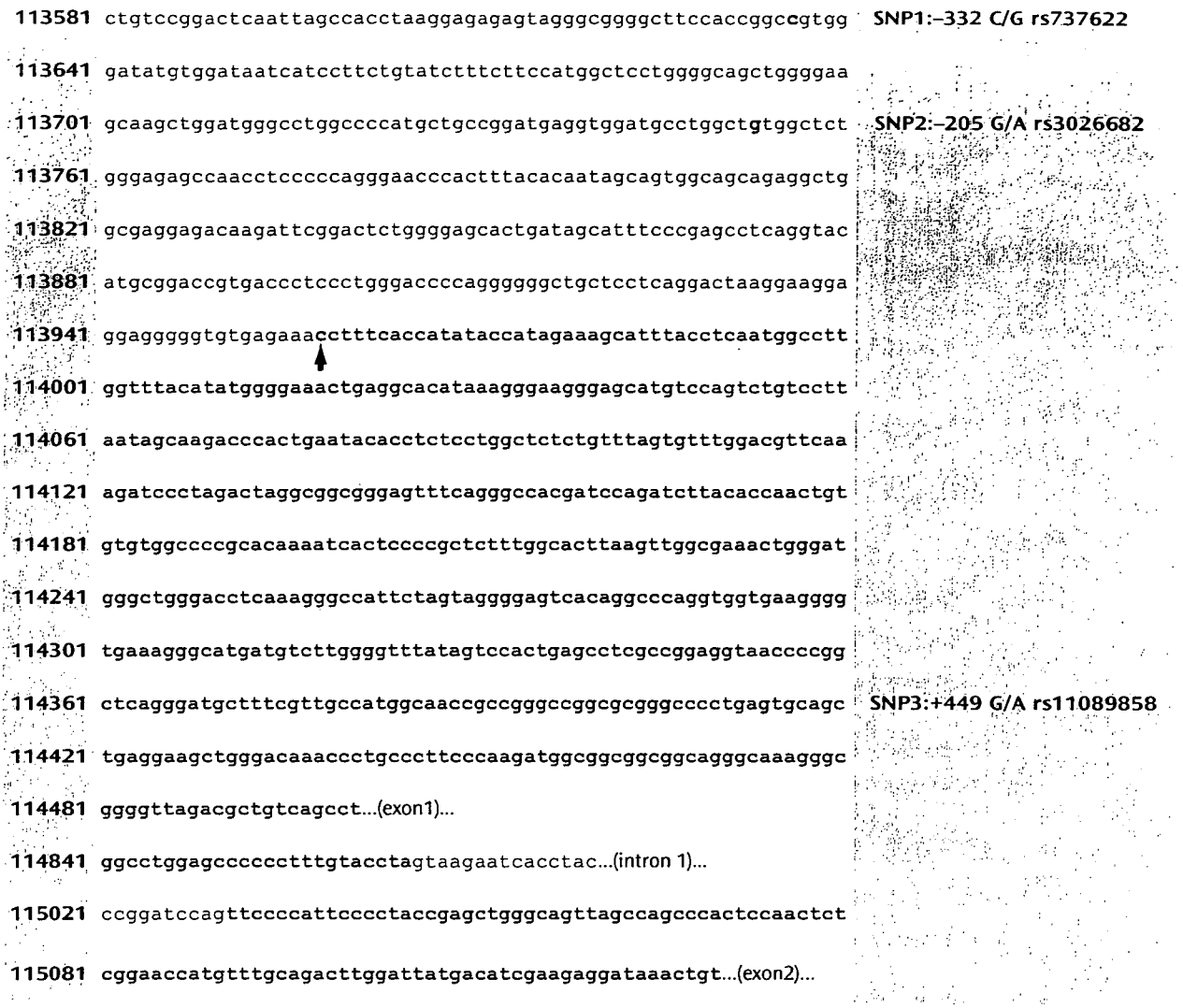
FIGURE 2. Schematic Diagram of 5'-Upstream Region of the PICK1 Gene[a]



[a] The numbers indicate the nucleotide positions cited from the NCBI database AL031587. A bold black arrow indicates the transcription start position we identified, which was 513 bp before the start position (114471) reported in the database. Blue characters indicate exons of PICK1, and the translation start codon, ATG, is orange. The positions of the three SNPs we identified are indicated in red.

distributions of SNP1, SNP2, and SNP5 were completely the same (Table 3). The allele frequencies and genotype distributions of SNP1, 3, 4, and 6 in methamphetamine abusers and comparison subjects are shown in Table 3. The genotype distributions were within the Hardy-Weinberg equilibrium.

We found significantly different frequencies between comparison subjects and methamphetamine abusers in SNP4 (Table 3). The frequency (88.7%) of carrying the T allele among the methamphetamine abusers was significantly higher (odds ratio=1.58, 95% confidence interval [CI]=1.06–2.34, p<0.03) than that of the comparison subjects (83.3%), and we also detected a different distribution of genotype (p<0.03). Positive associations were detected in the subgroup of those who experienced psychosis (alleles, p=0.007, odds ratio=1.79, 95% CI=1.17–2.74, genotype, p<0.02), transient-type psychosis (alleles, p=0.01, odds ratio=2.03, 95% CI=1.17–3.51, genotype, p<0.03), and psychosis with spontaneous relapse (alleles, p=0.003, odds ratio=2.61, 95% CI=1.35–5.07, genotype, p=0.004) and in abusers without polysubstance abuse (alleles, p<0.03, odds ratio=2.26, 95% CI=1.09–4.67, genotype, p<0.04) (Table 3). For SNP6, the frequency (48.7%) of the T allele among methamphetamine abusers who experienced psychosis with spontaneous relapse was significantly higher (odds ratio=1.62, 95% CI=1.19–2.35, p<0.02) than that of the comparison subjects (36.9%), and we also detected a different distribution of genotype (p<0.02) (Table 3). In contrast, no differences for SNP1, 2, 3, and 5 were detected between methamphetamine abusers and comparison subjects (Table 3).

TABLE 3. Genotypic and Allelic Distributions of the PICK1 Gene Polymorphisms in Comparison Subjects and Methamphetamine Abusers

Variable		Genotype							Allele				
		C/C		C/G		G/G			C		G		
SNP1[a] (rs737622)	N	N	%	N	%	N	%	p[b]	N	%	N	%	p[b]
Comparison subjects	218	89	40.8	107	49.1	22	10.1		285	65.4	151	34.6	
Methamphetamine abusers	208	85	40.9	93	44.7	30	14.4	0.35	263	63.2	153	36.8	0.52
Psychosis	178	66	37.1	87	48.9	25	14.0	0.45	219	61.5	137	38.5	0.27
Transient	100	38	38.0	48	48.0	14	14.0	0.56	124	62.0	76	38.0	0.42
Prolonged	78	28	35.9	39	50.0	11	14.1	0.53	95	60.9	61	39.1	0.33
Spontaneous relapse													
Positive	77	32	41.6	33	42.9	12	15.6	0.37	97	63.0	57	37.0	0.62
Negative	118	48	40.7	55	46.6	15	12.7	0.73	151	64.0	85	36.0	0.74
Polysubstance abuse													
No	55	23	41.8	23	41.8	9	16.4	0.35	69	62.7	41	37.3	0.66
Yes	140	58	41.4	63	45.0	19	13.6	0.53	179	63.9	101	36.1	0.75
		G/G		G/A		A/A			G		A		
SNP3 (rs11089858)	N	N	%	N	%	N	%	p[b]	N	%	N	%	p[b]
Comparison subjects	218	180	82.5	37	17.0	1	0.5		397	91.1	39	8.9	
Methamphetamine abusers	208	167	80.3	39	18.8	2	1.0	0.71	373	89.7	43	10.3	0.56
Psychosis	178	143	80.3	34	19.1	1	0.6	0.80	320	89.9	36	10.1	0.63
Transient	100	81	81.0	19	19.0	0	0.0	0.83	181	90.5	19	9.5	0.88
Prolonged	78	62	79.5	15	19.2	1	1.3	0.47	139	89.1	17	10.9	0.52
Spontaneous relapse													
Positive	77	64	83.1	13	16.9	0	0.0	1.00	141	91.6	13	8.4	1.00
Negative	118	94	79.7	23	19.5	1	0.8	0.65	211	89.4	25	10.5	0.49
Polysubstance abuse													
No	55	44	80.0	11	20.0	0	0.0	0.75	99	90.0	11	10.0	0.71
Yes	140	112	80.0	26	18.6	2	1.4	0.58	250	89.3	30	10.7	0.44
		T/T		T/A		A/A			T		A		
SNP4 (rs713729)	N	N	%	N	%	N	%	p[b]	N	%	N	%	p[b]
Comparison subjects	218	150	68.8	63	28.9	5	2.3		363	83.3	73	16.7	
Methamphetamine abusers	208	166	79.8	37	17.8	5	2.4	<0.03	369	88.7	47	11.3	<0.03
Psychosis	178	145	81.5	30	16.9	3	1.7	<0.02	320	89.9	36	10.1	0.007
Transient	100	83	83.0	16	16.0	1	1.0	<0.03	182	91.0	18	9.0	0.01
Prolonged	78	62	79.5	14	17.9	2	2.5	0.14	138	88.5	18	11.5	0.15
Spontaneous relapse													
Positive	77	67	87.0	9	11.7	1	1.3	0.004	143	92.9	11	7.1	0.003
Negative	118	88	74.6	26	22.0	4	3.4	0.36	202	85.6	34	14.4	0.51
Polysubstance abuse													
No	55	47	85.5	7	12.7	1	1.8	<0.04	101	91.8	9	8.2	<0.03
Yes	140	109	77.9	28	20.0	3	2.1	0.16	246	87.9	34	12.1	0.11
		G/G		G/T		T/T			G		T		
SNP6 (rs2076369)	N	N	%	N	%	N	%	p[b]	N	%	N	%	p[b]
Comparison subjects	218	82	37.6	111	50.9	25	11.5		275	63.1	161	36.9	
Methamphetamine abusers	208	73	35.1	99	47.6	36	17.3	0.23	245	58.9	171	41.1	0.23
Psychosis	178	64	36.0	83	46.6	31	17.4	0.25	211	59.3	145	40.7	0.30
Transient	100	34	34.0	48	48.0	18	18.0	0.30	116	58.0	84	42.0	0.25
Prolonged	78	30	38.5	35	44.9	13	16.7	0.41	95	60.9	61	39.1	0.63
Spontaneous relapse													
Positive	77	21	27.3	37	48.1	19	24.7	<0.02	79	51.3	75	48.7	<0.02
Negative	118	46	37.9	56	47.5	16	13.6	0.77	148	62.7	88	37.3	0.93
Polysubstance abuse													
No	55	15	27.3	30	54.5	10	18.2	0.23	60	54.5	50	45.5	0.13
Yes	140	53	37.9	62	44.3	25	17.9	0.19	168	60.0	112	40.0	0.43

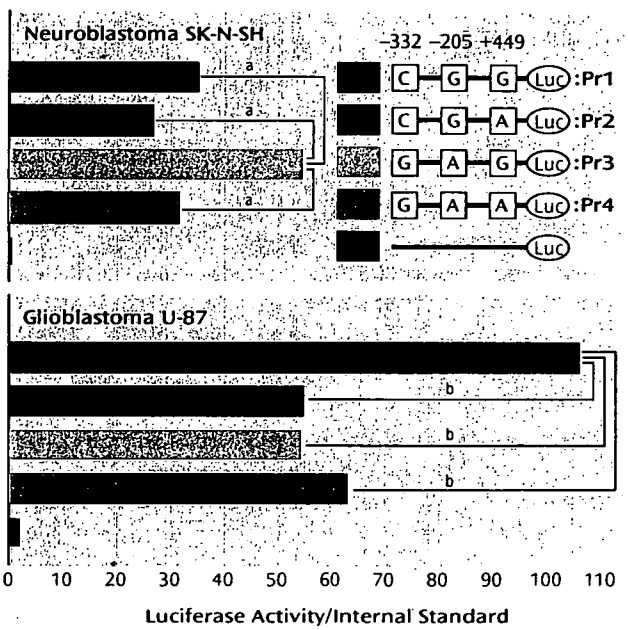
[a] The distributions of SNP2 (rs3026682) and 5 (rs3952) are the same as SNP1 (rs737622).
[b] Versus comparison subjects.

TABLE 4. Haplotype Analysis of Six Single Nucleotide Polymorphisms

Variable	Haplotype Analysis		
Overall			
Haplotype	Comparison Subjects (N=218)	Methamphetamine Abusers (N=208)	p
C-G-G-T-A-T	35.2%	33.7%	0.63
G-A-G-T-G-G	32.3%	32.3%	0.85
C-G-G-A-A-G	14.5%	9.2%	<0.02
C-G-A-T-A-G	8.3%	7.4%	0.66
C-G-G-T-A-G	5.5%	8.9%	<0.09
G-A-G-T-G-T	0.7%	3.5%	0.01
C-G-G-A-A-T	1.2%	1.7%	0.66
G-A-G-A-G-G	1.0%	0.4%	0.40
Methamphetamine abusers			
Haplotype	With Spontaneous Relapse (N=77)	Without Spontaneous Relapse (N=117)	p
C-G-G-T-A-T	42.3%	27.8%	0.001
G-A-G-T-G-G	32.1%	31.1%	0.86
C-G-G-A-A-G	4.5%	12.6%	<0.02
C-G-A-T-A-G	6.8%	6.3%	0.82
C-G-G-T-A-G	6.3%	11.8%	0.14
G-A-G-T-G-T	2.5%	4.9%	0.31
C-G-G-A-A-T	2.5%	1.3%	0.54

As shown in Figure 1, a strong linkage disequilibrium was observed in five of these six SNPs. Two haplotypes, C(SNP1)-G(SNP2)-G(SNP3)-A(SNP4)-A(SNP5)-G(SNP6) and G(SNP1)-A(SNP2)-G(SNP3)-T(SNP4)-G(SNP5)-T(SNP6), were significantly different between comparison subjects and methamphetamine abusers (Table 4). The frequency (9.2%) of the CGGAAG haplotype in the methamphetamine abusers was significantly lower (odds ratio=0.60, 95% CI=0.45–0.79, p<0.02) than that of the comparison subjects (14.5%), and the frequency (3.5%) of the GAGTGT haplotype in the methamphetamine abusers was significantly higher (odds ratio=5.2, 95% CI=2.27–11.6, p=0.01) than that (0.7%) of the comparison subjects (Table 4). Of interest, a haplotype analysis between methamphetamine abusers with and without spontaneous relapse of psychosis showed the significant difference in the most major haplotype (CGGTAT) as well as the CGGAAG type. The frequency (42.3%) of CGGTAT type in the methamphetamine abusers with spontaneous relapse was significantly higher (odds ratio=2.2, 95% CI=1.80–2.61, p=0.001) than that in those without spontaneous relapse (27.8%) (Table 4). As to the frequency of the CGGAGG type, the frequency (4.5%) in methamphetamine abusers with spontaneous relapse was significantly lower (odds ratio=0.33, 95% CI=0.23–0.47, p<0.02) than that in those without spontaneous relapse (Table 4).

FIGURE 3. Relative Luciferase Activity of the Four Haplotypes in SK-N-SH Cells (top) and U-87 Cells (bottom)[a]



[a] The phRL-TK vector used was a negative control. The pGL3 Basic vector, which does not contain any promoter sequences, was used as a negative control. Each value is shown as the mean for three independent experiments.
[b] p<0.001.

Transcriptional Effects of SNPs in the Promoter Region

The transcriptional effects of four promoter haplotypes on SK-N-SH cells and U-87 cells were also examined. As shown in Figure 3, the results for these two cell lines differed. For SK-N-SH cells, a substitution variant, Pr3 (G-332/A-205/A+449), showed significantly increased relative luciferase activity (1.54 for Pr3/Pr1, p<0.001, 2.03 for Pr3/Pr2, p<0.001, 1.74 for Pr3/Pr4, p<0.001). In contrast, for U-87 cells, every substitution showed significantly lower relative luciferase activity than that of the major type, Pr1 (C-332/G-205/G+449) (0.51 for Pr2/Pr1, p<0.001, 0.51 for Pr3/Pr1, p<0.001, 0.59 for Pr4/Pr1, p<0.001).

Discussion

The major findings of the present study were the discovery of an association between PICK1 gene polymorphisms and methamphetamine abusers and the identification of functional SNPs (SNP1 and SNP2) in the promoter region of the PICK1 gene. It was of great interest to find that SNP4 and SNP6 were significantly associated with methamphet-

amine abusers who experienced spontaneous relapse of psychosis. In addition, the haplotype analysis demonstrated that specific haplotypes, C(SNP1)G(SNP2)G(SNP3)A(SNP4)A(SNP5)G(SNP6) and GAGTGT, were significantly associated with methamphetamine abusers in general. Furthermore, we also found that the frequencies of major haplotypes CGGTAT and CGGAAG were significantly different between methamphetamine abusers with and without spontaneous relapse of psychosis. Spontaneous relapse of psychosis among methamphetamine abusers is known as "flashbacks," which are known to follow nonspecific stress, even after the consumption of methamphetamine has ceased and drug treatment has begun, and it appears that a psychotic state might be induced by excess dopaminergic activity (21, 22). Given the role of dopamine systems in the pathogenesis of methamphetamine psychosis, it is possible that a functional alteration of dopamine transporter may be caused by genetic variations in PICK1 and can lead to dysfunction of the dopamine system. Taken together, these results suggest that the CGGTAT and CGGAAG haplotypes in the PICK1 gene are likely to be associated with the psychosis of methamphetamine abusers who experience spontaneous relapse. The different distributions of those two haplotypes between methamphetamine abusers with and without spontaneous relapse of psychosis also suggest the difference in genetic backgrounds between the two groups. In the present study, the group of subgroups was small. Because of the small size of subcategories, type I error cannot be ruled out. Therefore, further studies with a large group with subcategories would reveal the associations between the PICK1 gene and methamphetamine-induced psychosis.

In the 5′-upstream region of the PICK1 gene, we identified three SNPs (SNP1: −332 C/G, rs737622, SNP2: -205 G/A, rs3026682, and SNP3: 449G/A, rs11089858). A luciferase assay revealed the functional effects of these SNPs on transcriptional activities. Although the threshold scores were low, the TFSEARCH program (http://mbs.cbrc.jp/research/db/TFSEARCH.html) predicted that the major transcription factors, including GATA1 (for SNP1, score 78.3) and AML-1a (for SNP2, score 83.7), bind to either position of SNPs in the PICK1 promoter position. Of course, it is likely that unidentified transcription factors may also be involved in the transcriptional process because we found that the levels of PICK1 expression could be altered by nucleotide substitutions of these SNPs in the promoter region. After consideration of the role of PICK1 in the proper targeting and surface clustering of dopamine transporter (16), it is possible that altered PICK1 expression might lead to altered dopamine transporter function in synaptic dopamine signal transmission, which would in turn influence the pathogenesis of methamphetamine abuse and related psychotic symptoms.

In this study, we found that transcriptional effects of SNPs in the promoter region of the PICK1 gene differed in SK-N-SH and U-87 cells. The nucleotide substitutions (C→G at −332 and G→A at −205) showed significantly increased luciferase activity in SK-N-SH cells (neuronal cells), whereas the substitutions (C→G at −332 and G→A at −205) showed significantly decreased luciferase activity in U-87 cells (glial cells). Although the mechanisms underlying the discrepancy in these two cell lines are currently unknown, these findings suggest that PICK1 expression could be affected in different ways by these SNPs in neuronal and glial cells. Fujii et al. (20) reported that a haplotype, T(rs713729)-A(rs3952)-T(rs2076369), revealed a statistically significant association with disorganized schizophrenia in methamphetamine abusers in relation to comparison subjects ($p<0.02$). The TAT haplotype, discussed by Fujii and coworkers, was found to correspond to C(rs737622: SNP1)-G(rs3026682: SNP2)-G(rs11089858: SNP3)-T(rs713729: SNP4)-A(rs3952: SNP5)-T(rs2076329: SNP6) in our study, and it was the most frequent haplotype in both comparison subjects and methamphetamine abusers. As discussed, the frequency (42.3%) of the CGGTAT haplotype in methamphetamine abusers with spontaneous relapse was significantly higher ($p=0.001$) than that of those without spontaneous relapse (27.8%). These findings also suggest that methamphetamine abusers who experience a spontaneous relapse of methamphetamine psychosis might share a similar genetic susceptibility to schizophrenia.

It has been demonstrated that PICK1 interacts with other proteins, including AMPA receptors (14, 23) and metabotropic glutamate receptor 7 (mGluR7) (24, 25), which have been implicated in the pathophysiology of drug abuse as well as in schizophrenia (26–29). Thus, it seems that interactions of PICK1 with AMPA receptors and metabotropic glutamate receptors are likely to be involved in the pathogenesis of methamphetamine psychosis. Furthermore, Fujii et al. (20) identified PICK1 as a protein interactor with the D-serine synthesizing enzyme serine racemase in glial cells (30). After consideration of the role of D-serine in the pathophysiology of schizophrenia (31–35), it is likely that the interaction of PICK1 with serine racemase in glial cells may play a role in the pathophysiology of methamphetamine psychosis, although further studies will still be necessary.

In conclusion, the present findings revealed that PICK1 gene polymorphisms are associated with methamphetamine abusers, suggesting that the PICK1 gene plays a major role in a genetic susceptibility to methamphetamine psychosis.

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Letter to the Editor (Case report)

New-onset diabetic ketoacidosis in a schizophrenic patient with multiple autoimmune disease during treatment with risperidone

1. Introduction

Diabetes mellitus is due to impaired insulin secretion and variable degrees of peripheral insulin resistance leading to hyperglycemia (Stern, 1988). Available evidence suggests that clozapine and olanzapine have a high propensity to induce diabetes compared with other atypical antipsychotic drugs, risperidone and quetiapine (Newcomer and Haupt, 2006), though the conclusion is still controversial.

Autoimmune polyendocrine syndrome (APS) is characterized by the development of disorders in multiple endocrine and non-endocrine systems that are mediated by autoimmune mechanisms (Betterle et al., 2002; Betterle and Zanchetta, 2003). There are four categories of APS. Type I and II develop Addison's disease while type III does not. APS type III, which is characterized by autoimmune thyroid disease and the absence of Addison's disease, is accompanied by another autoimmune disease as type 1 DM, pernicious anemia and vitiligo with alopecia. APS III is a very rare syndrome with some cases described in the literature. APS type IV is defined as exclusion from type I, II or III.

To our knowledge, there is no information about diabetic ketoacidosis (DKA) induced by atypical antipsychotics in schizophrenic patients with autoimmune polyendocrine syndrome. Thus we describe a case of DKA in a schizophrenic patient who has coincident multiple autoimmune disease including diabetes type I that developed after treatment with risperidone.

2. Case

A 46-year-old Japanese woman with a 27-year history of schizophrenia (paranoid type) and no personal but family history of diabetes mellitus was introduced to the Department of Neuropsychiatry, Ohdate City Hospital because of persistent hallucination and delusion of injury. Her weight and height were 56 kg and 160 cm, respectively, and the BMI was 21.9. She had been treated with levomepromazine (60 mg/day) and tiapride (50 mg/day). After tiapride treatment (50 mg/day), she was switched to risperidone (3 mg/day) which was the first atypical antipsychotic agent where her mental condition ameliorated. Her routine fasting and postprandial glucose levels were between 88 and 138 mg/dL before risperidone administration, and postprandial glucose level rose to 293 mg/dL after 3 months after risperidone treatment was started. Her weight remained unchanged around 56 kg.

Four months after risperidone administration, she was transferred to the emergency unit in the Ohdate City Hospital because of loss of consciousness. Her plasma glucose level was 926 mg/dL and urine ketones were positive. Her arterial blood gas analysis showed anion gap metabolic acidosis: pH was 7.14 and her base excess was −8.8 mEq/L. Her serum creatinine was 2.8 mg/dl and her blood urea nitrogen was 69 mg/dl. The CRP was 12.96 μg/ml. She was immediately hospitalized to the endocrine metabolic ward in the same hospital due to hyperglycemia, diabetic ketoacidosis (DKA) and coma.

She was treated with insulin, IV fluid replacement and antibiotics in spite of no clear finding of infection. Unconsciousness was improved with the decrease of plasma glucose concentration. After correction of her DKA, she was treated with an intensive insulin regime. The blood sugar control was improved under the insulin treatment after diabetic information was provided and she was guided about the insulin self-injection. She restarted receiving levomepromazine (70 mg/day). At present, her insulin requirements are stable and mental condition such as mild auditory hallucination and delusion of observation are maintained.

Laboratory results on the day of admission included the following: HbA_{1c} was 12.2%. Anti-GAD antibody was positive (>45,500, normal value <1.5). Urinary C-peptide was 28.8 ng/ml (normal value 20.5–198). Serum C-peptide was 0.94 ng/ml (normal value 0.94–2.80). Therefore, she was diagnosed with type 1 diabetes mellitus. A small amount of albumin in urine was positive (alb 34.4 mg/day) and she was diagnosed with diabetic nephropathy stage 2. On the other hand, her other biochemical data were as follows: TSH was 0.00 μIU/ml (normal value 0.54–4.26). T_4 was 2.80 ng/dl (normal value 0.71–1.52). T_3 was 6.82 pg/dl (normal value 2.39–4.06). Autoantibody against the thyroid TSH receptor was positive. Based on these data, she was diagnosed with Basedow's disease, and treated with antithyroid drugs and β-Blocker. The adrenal function screening was normal. Therefore, she was diagnosed with autoimmune polyendocrine syndrome (APS) type III on the basis of the presence of type I diabetes, Basedow's disease and abdominal vitiligo vulgaris.

3. Discussion

We experienced a case of DKA-onset type I diabetes in a Japanese schizophrenic patient after risperidone treatment.

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Although we have no clear explanation for the mechanism, it is likely that risperidone acts as a metabolic stressor, and leads to insulin deficiency and type I diabetes. However, the possibility that some infection promoted the onset of diabetic ketoacidosis cannot be excluded entirely.

There is no doubt that the majority of cases of diabetes seen in association with atypical antipsychotics treatment have type II diabetes (Newcomer, 2007). However, this case was diagnosed as late-onset diabetes type I because her weight did not increase after treatment with risperidone and anti-GAD antibody was positive. From patient's clinical course, she might have developed latent autoimmune diabetes in adults (LADA) (Fourlanos et al., 2005). Autoimmune diabetes in adults with slowly progressive beta-cell failure is characterized by prevalence in patients more than 30 years old and has no requirement of insulin at least during the first 6 months after diagnosis (Fourlanos et al., 2005). Thus risperidone treatment might cause metabolic stress that could shorten the period of progressive beta-cell failure.

She was also diagnosed with APS type III, which is characterized by autoimmune thyroid disease, the absence of Addison's disease and diabetes type I. To our knowledge, this is the first case of a schizophrenic patient who developed diabetic ketoacidosis with coincident multiple autoimmune disease including diabetes type I. APS might contribute to accelerating the progression of beta-cell failure because it is known that thyroid hormone increases blood sugar value.

New-onset diabetes type I and diabetic ketoacidosis in a patient taking risperidone without prior history of diabetes mellitus have been rarely reported by Mithat et al. (2005), Dibben et al. (2005) and Ananth et al. (2004). In addition, there have been several reports indicating new-onset diabetic ketoacidosis without a clear weight gain after initiation of atypical antipsychotics such as clozapine (Lafayette et al., 2003) and quetiapine (Dibben et al., 2005). Our case presents not only metabolic disturbance but also autoimmune disorder. Thus the possibility that these cases in addition to our case resulted from some autoimmune response induced by the administration of atypical antipsychotics which cannot be excluded.

In conclusion, we describe a case of DKA in a schizophrenic patient who had coincident multiple autoimmune disease including diabetes type I after treatment with risperidone.

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Influence of the tyrosine hydroxylase val81met polymorphism and catechol-O-methyltransferase val158met polymorphism on the antidepressant effect of milnacipran

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Objective Genetic polymorphisms of the noradrenergic pathway can be factors to predict the effect of antidepressants when their pharmacological mechanisms of action include the noradrenergic system. The purpose of the present study was to determine whether the tyrosine hydroxylase (TH) val81met and catechol-O-methyltransferase (COMT) val158met polymorphisms are associated with the antidepressant effect of milnacipran, a serotonin/noradrenaline reuptake inhibitor.
Method Eighty-one Japanese patients with major depressive disorder were treated with milnacipran for 6 weeks. Severity of depression was assessed with the Montgomery and Asberg Depression Rating Scale (MADRS). Assessments were carried out at baseline and at 1, 2, 4 and 6 weeks of treatment. The method of polymerase chain reaction was used to determine allelic variants.
Results The met/met genotype of the COMT val158met polymorphism was associated with a significantly faster therapeutic effect of milnacipran in the MADRS score during this study. No influence of the TH val81met polymorphism on the antidepressant effect of milnacipran was detected.
Conclusion These results suggest that the COMT val158met polymorphism in part determines the antidepressant effect of milnacipran. Copyright # 2007 John Wiley & Sons, Ltd.

key words— catechol-O-methyltransferase; major depressive disorder; milnacipran; polymorphism; tyrosine hydroxylase

INTRODUCTION

Individual genetic differences of monoaminergic pathways can have an impact on the effect of antidepressant agents, though the exact mechanism of their action is still unclear. Several lines of evidence have suggested the relationship between genetic polymorphisms of the serotonergic pathway, especially those of the 5-hydroxytriptamine transporter (5-HTT), and the antidepressant effect of selective serotonin reuptake inhibitors (SSRIs) (Binder and Holsboer, 2006).

Genetic polymorphisms of the noradrenergic pathway as well as serotonergic pathway could also affect the effect of antidepressants, especially when their pharmacological mechanisms of action include the noradrenergic system. Tyrosine hydroxylase (TH) is

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the initial and rate-limiting enzyme in the biosynthesis of catecholamine neurotransmitters including noradrenaline. The TH val81met polymorphism in exon 2 (Ludecke and Bartholome, 1995) is located in the amino-terminal regulatory domain of the tetrameric enzyme. The regulatory region is reported to have an inhibiting effect on the enzymatic function (Kumer and Vrana, 1996). Catechol-O-methyltransferase (COMT) is an important enzyme involved in degradation of catecholamine neurotransmitters including noradrenaline. The COMT val158met polymorphism located in exon 4 (Lotta et al., 1995) was reported to be associated with variation in COMT enzyme activity (Lachman et al., 1996).

No pharmacogenetic study addressed the relationship between the TH val81met polymorphism and antidepressant response. Only two studies investigated the relationship between the COMT val158met polymorphism and antidepressant response to SSRIs and mirtazapine. One reported its no overall effect on the antidepressant response to SSRIs (Arias et al., 2006), and the other reported its significant effect on the antidepressant response to mirtazapine but not paroxetine (Szegedi et al., 2005).

So far, there has been no study investigating the relationship between the TH val81met polymorphism, the COMT val158met polymorphism and the antidepressant response to serotonin noradrenaline reuptake inhibitors (SNRIs), although noradrenergic genetic factors could be one of the most plausible candidates for pharmacogenetic analysis of SNRIs. The class of SNRIs now comprises of three medications: venlafaxine, duloxetine and milnacipran. Among SNRIs, venlafaxine has a high affinity for the 5-HTT but not the noradrenaline transporter. Duloxetine has a more balanced affinity but is still more selective for the 5-HTT. Milnacipran is the most balanced and may even be slightly more noradrenergic than serotonergic (Stahl et al., 2005). Thus, the authors investigated whether the above two noradrenergic polymorphisms affect the antidepressant effect of milnacipran.

SUBJECTS AND METHODS

Subjects

For the present study, one subject treated with milnacipran was added to those in our previous study (Yoshida et al., 2007). Detailed inclusion criteria have been described previously (Yoshida et al., 2007). In brief, the subjects were Japanese patients who fulfilled DSM-IV criteria for a diagnosis of major depressive disorder and whose scores on the Montgomery Åsberg Depression Rating Scale (MADRS) (Montgomery and Åsberg, 1979) were 21 or higher. Patients with other axis I and II disorders determined by clinical interview and those with severe nonpsychiatric medical disorders were excluded. The patients were 25–69 years of age (mean age (SD) = 51.1 12.3) and had been free of psychotropic drugs at least 14 days before entry into the study. After complete description of the study to the subjects, written informed consent was obtained. This study was approved by the Ethical Committee of Akita University School of Medicine and Nagoya University Graduate School of Medicine. The clinical characteristics of the patients are shown in Table 1. There was no significant difference between responders and nonresponders in regard to sex, age, number of previous episodes and presence of melancholia.

Milnacipran treatment

Milnacipran was administered twice daily (the same dose after dinner and at bedtime) for 6 weeks. The initial total daily dose was 50 mg/day, and after a week it was increased to 100 mg/day. Patients with insomnia were prescribed 0.25 or 0.5 mg of brotizolam, a benzodiazepine sedative hypnotic, at bedtime. No other psychotropic drugs were permitted during the study. Of 98 enrolled patients, 10 did not complete the study: five patients because of side effects, one patient because of severe insomnia and four patients without explanation. Of the 88 patients who completed the 6-week study, seven patients were excluded from the

Table 1. Clinical characteristics of the patients (responders and nonresponders)

	Responders (n = 51)	Nonresponders (n = 30)		p
Sex (male/female)	20/31	9/21	$x^2 = 0.70$	0.40[a]
Age (year) (SD)	50.7 12.4	51.8 12.2	$t = 0.41$	0.68[b]
No. of previous episodes (SD)	0.47 1.3	0.23 0.6	$t = 0.97$	0.34[b]
Melancholia (p/)	16/35	9/21	$x^2 = 0.017$	0.90[a]

[a]Analysis performed with the use of the x^2 test.
[b]Analysis performed with the use of the unpaired t-test.

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current analysis because plasma samples revealed very low milnacipran concentrations, indicative of poor compliance. Patients who completed the study included 52 women and 29 men, 50 outpatients and 31 inpatients.

Data collection

Depression symptom severity was assessed with the use of the MADRS. Assessments were conducted at baseline and at 1, 2, 4 and 6 weeks after initiation of antidepressant treatment. A single rater conducted each of the ratings for each patient. A clinical response was defined as a 50% or greater decrease in the baseline MADRS score. Clinical remission was defined as a final MADRS score less than 10 (Hawley et al., 2002). Collection of blood samples was performed 12 h after drug administration at bedtime, 4 weeks after initiation of antidepressant treatment.

Genotyping

The TH val81met polymorphism was determined by the method of Sharma et al. (1998). The COMT val158met polymorphism was determined by the method of Lachman et al. (1996).

Quantification of plasma milnacipran concentration

Plasma concentrations of milnacipran were measured with high performance liquid chromatography (HPLC). Details of the method have been described previously (Higuchi et al., 2003). Genotyping and measurement of plasma concentrations were performed by laboratory personnel blind to the identity and clinical antidepressant effect of the patients. Moreover, clinicians were unaware of the genotyping results and the plasma milnacipran concentrations of each patient.

Statistical analysis

Differences in patient characteristics were analysed with the use of the unpaired t-test or Chi-square test where appropriate. Differences in the MADRS scores during this study were examined with the use of two-way repeated-measures analysis of variance (ANOVA), with genotype and time as factors. Additional repeated-measures analysis of covariance (ANCOVA) was performed if necessary. When significant interaction between factors was observed, contrasts were used to enable comparisons between each two of the three genotype groups. Differences in the MADRS scores at each evaluation point were examined with the one-way factorial ANOVA followed by the Fisher's PLSD test. Genotype deviation from the Hardy–Weinberg equilibrium was evaluated by the Chi-square test. Genotype distribution and allele frequencies were analysed with the use of the Chi-square test. Plasma concentrations of milnacipran were analysed with the use of one-way factorial ANOVA in each genotype group; an unpaired t-test was then used to analyse differences between groups who were or were not responsive to milnacipran. Statistical analysis was performed using StatView version 5.0 (SAS Institute, Inc., Cary, NC) and SuperANOVA version 1.11 (Abacus Concepts, Inc., Berkeley, CA). Power analysis was performed with the use of G Power (Buchner et al., 1996). All tests were two-tailed; alpha was set at 0.05.

RESULTS

TH val81met polymorphism

The observed genotype frequencies of the TH val81met polymorphism were within the distribution expected according to the Hardy–Weinberg equilibrium. Figure 1 shows the MADRS scores over time in relation to the TH val81met polymorphism. Two-way repeated-measures ANOVA including all three genotype groups indicated no significant genotype × time interaction ($F = 0.99$, $df = 8$, $p = 0.44$). Plasma concentrations of milnacipran were not significantly different among each genotype group (val/val: 96.1 ± 32.6 (SD), val/met: 86.2 ± 30.4, met/met: 92.2 ± 47.9, $F = 0.35$, $df = 2$, $p = 0.71$). No significant differences in the genotype

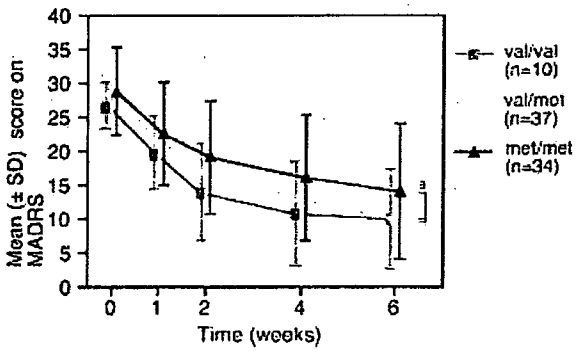

Figure 1. MADRS scores during 6 weeks of the treatment in three TH val81met genotype groups. (Each point represents the mean score ± SD. Differences in the MADRS scores during this study were examined with the use of repeated-measures ANOVA.) [a]There was no significant genotype × time interaction among all three genotype groups ($F = 0.99$, $df = 8$, $p = 0.44$)

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Table 2. Genotype distribution and allele frequencies in responders/nonresponders and remitters/nonremitters in the TH val81met polymorphism[a]

	Genotype distribution[b,c]			Allele frequency[d,e]	
	val/val	val/met	met/met	val	met
Responder	7 (13.7%)	27 (52.9%)	17 (33.3%)	41 (40.2%)	61 (59.8%)
Nonresponder	3 (10.0%)	10 (33.3%)	17 (56.7%)	16 (26.7%)	44 (73.3%)
Remitter	5 (11.4%)	23 (52.3%)	16 (36.3%)	33 (37.5%)	55 (62.5%)
Nonremitter	5 (13.5%)	14 (37.8%)	18 (48.7%)	24 (32.4%)	50 (67.6%)

[a]Analysis performed with the use of the x^2 test.
[b]No significant difference between responders and nonresponders (x^2¼ 4.25, df¼ 2, p¼ 0.12).
[c]No significant difference between remitters and nonremitters (x^2¼ 1.72, df¼ 2, p¼ 0.42).
[d]No significant difference between responders and nonresponders (x^2¼ 3.03, df¼ 1, p¼ 0.08).
[e]No significant difference between remitters and nonremitters (x^2¼ 0.45, df¼ 1, p¼ 0.50).

distribution (x^2¼ 4.25, df¼ 2, p¼ 0.12) and allele frequencies (x^2¼ 3.03, df¼ 1, p¼ 0.08) were noted between responders and nonresponders. When remitters and nonremitters were compared, there was also no significant difference in the genotype distribution (x^2¼ 1.72, df¼ 2, p¼ 0.42) and allele frequencies (x^2¼ 0.45, df¼ 1, p¼ 0.50) (Table 2).

COMT val158met polymorphism

The observed genotype frequencies of the COMT val158met polymorphism were within the distribution expected according to the Hardy–Weinberg equilibrium. Figure 2 shows the MADRS scores over time in relation to the COMT val158met polymorphism. Two-way repeated-measures ANOVA including all three genotype groups indicated a significant genotype × time interaction (F¼ 2.00, df¼ 8, p¼ 0.046). Contrast analysis indicated a significant genotype × time interaction between the val/met and met/met genotype groups (F¼ 3.31, df¼ 4, p¼ 0.011). The MADRS score of the val/met genotype group was significantly lower than that of the met/met genotype group at the 0 week (p¼ 0.0098). Contrast analysis indicated a significant genotype × time interaction between the val/val and met/met groups (F¼ 3.19, df¼ 4, p¼ 0.011). The MADRS score of the val/val genotype group was significantly lower than that of the met/met group at the 0 week (p¼ 0.013). Contrast analysis indicated no significant genotype × time interaction between the val/val and val/met genotype groups (F¼ 0.49, df¼ 4, p¼ 0.74). There was no significant difference in the MADRS score at any evaluation point between the val/val and val/met genotype groups. To determine whether the initial difference of the MADRS scores affect the subsequent scores, a repeated measures ANCOVA was performed with the initial MADRS score as a covariate. This analysis revealed no significant time × the initial MADRS score interaction (F¼ 0.46, df¼ 3, p¼ 0.71), indicating that the initial MADRS score was not a significant covariate.

To determine which aspects of depressive symptoms contributed to overall differences over time of the MADRS scores, the results of factor analyses of depression symptomatology using MADRS (Parker et al., 2003; Suzuki et al., 2005) were applied to the present results. Suzuki et al. (2005) identified three factors labelled dysphoria, retardation and vegetative symptoms. Figure 3 shows the dysphoria scores over time in relation to the COMT val158met polymorph-

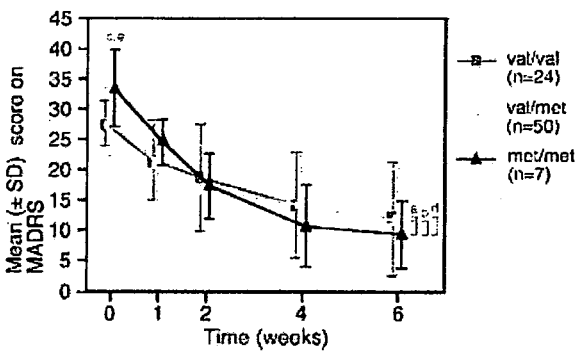

Figure 2. MADRS scores during 6 weeks of the treatment in three COMT val158met genotype groups. (Each point represents the mean score ± SD. Differences in the MADRS scores during this study were examined with the use of repeated-measures ANOVA. Differences in the MADRS scores at each evaluation point were examined with the use of one-way factorial ANOVA followed by Fisher's PLSD test.) [a]Significant genotype × time interaction among all three genotype groups (F¼ 2.00, df¼ 8, p¼ 0.046). [b]Significant genotype × time interaction between the val/met and met/met groups (F¼ 3.31, df¼ 4, p¼ 0.011). [c]Significant difference at the 0 week between the val/met and met/met groups (p¼ 0.0098). [d]Significant genotype × time interaction between the val/val and met/met groups (F¼ 3.19, df¼ 4, p¼ 0.011). [e]Significant difference at the 0 week between the val/val and met/met groups (p¼ 0.013)

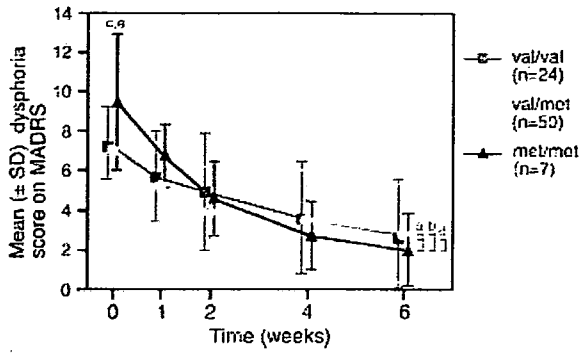

Figure 3. MADRS dysphoria scores during 6 weeks of the treatment in three COMT val158met genotype groups. (Each point represents the mean score SD. Differences in the MADRS dysphoria scores during this study were examined with the use of repeated-measures ANOVA. Differences in the MADRS dysphoria scores at each evaluation point were examined with the use of one-way factorial ANOVA followed by Fisher's PLSD test.) [a]Significant genotype time interaction among all three genotype groups ($F = 2.68$, $df = 8$, $p = 0.0074$). [b]Significant genotype time interaction between the val/met and met/met groups ($F = 4.43$, $df = 4$, $p = 0.0017$). [c]Significant difference at the 0 week between the val/met and met/met groups ($p = 0.016$). [d]Significant genotype time interaction between the val/val and met/met groups ($F = 3.23$, $df = 4$, $p = 0.013$). [e]Significant difference at the 0 week between the val/val and met/met groups ($p = 0.049$)

ism. Two-way repeated-measures ANOVA for the dysphoria scores including all three genotype groups indicated a significant genotype time interaction ($F = 2.68$, $df = 8$, $p = 0.0074$). As in the case of overall results, contrast analysis indicated a significant genotype time interaction between the val/met and met/met genotype groups ($F = 4.43$, $df = 4$, $p = 0.0017$), and between the val/val and met/met genotype groups ($F = 3.23$, $df = 4$, $p = 0.013$). Contrast analysis indicated no significant genotype time interaction between the val/val and val/met genotype groups ($F = 1.10$, $df = 4$, $p = 0.36$). Two-way repeated-measures ANOVA for the scores of retardation and vegetative symptoms did not indicate significant genotype time interactions (data not shown). Parker et al. (2003) identified three factors labelled dysphoric apathy/retardation, psychic anxiety and vegetative symptoms. Two-way repeated-measures ANOVA for the scores of psychic anxiety including all three genotype groups indicated a significant genotype time interaction ($F = 3.24$, $df = 8$, $p = 0.0015$). As in the case of overall results and those based on the factor analyses by Suzuki et al. (2005), contrast analysis indicated a significant genotype time interaction between the val/met and met/met genotype groups ($F = 5.97$, $df = 4$, $p = 0.0001$), and between the val/val and met/met genotype groups ($F = 4.47$, $df = 4$, $p = 0.016$). Contrast analysis indicated no significant genotype time interaction between the val/val and val/met genotype groups ($F = 0.63$, $df = 4$, $p = 0.64$). Two-way repeated-measures ANOVA for the scores of dysphoric apathy/retardation and vegetative symptoms did not indicate significant genotype time interactions (data not shown).

Plasma concentrations of milnacipran were not significantly different among each genotype group (val/val: 82.7 21.6 (SD), val/met: 94.7 44.9, met/met: 81.1 34.7, $F = 0.97$, $df = 2$, $p = 0.38$). No significant differences in the genotype distribution ($\chi^2 = 1.79$, $df = 2$, $p = 0.41$) and allele frequencies ($\chi^2 = 0.81$, $df = 1$, $p = 0.37$) were noted between responders and nonresponders. When remitters and nonremitters were compared, there was also no significant difference in the genotype distribution ($\chi^2 = 0.93$, $df = 2$, $p = 0.63$) and allele frequencies ($\chi^2 = 0.16$, $df = 1$, $p = 0.69$) (Table 3).

Power

This study had a power of 0.12 to detect a small effect, 0.67 to detect a medium effect and 0.99 to detect a

Table 3. Genotype distribution and allele frequencies in responders/nonresponders and remitters/nonremitters in the COMT val158met polymorphism[a]

	Genotype distribution[b,c]			Allele frequency[d,e]	
	val/val	val/met	met/met	val	met
Responder	14 (27.5%)	31 (60.8%)	6 (11.8%)	59 (57.8%)	43 (42.2%)
Nonresponder	10 (33.3%)	19 (63.3%)	1 (3.3%)	39 (65.0%)	21 (35.0%)
Remitter	13 (29.5%)	26 (59.1%)	5 (11.4%)	52 (59.1%)	36 (40.9%)
Nonremitter	11 (29.7%)	24 (64.9%)	2 (5.4%)	46 (62.2%)	28 (37.8%)

[a]Analysis performed with the use of the χ^2 test.
[b]No significant difference between responders and nonresponders ($\chi^2 = 1.79$, $df = 2$, $p = 0.41$).
[c]No significant difference between remitters and nonremitters ($\chi^2 = 0.93$, $df = 2$, $p = 0.63$).
[d]No significant difference between responders and nonresponders ($\chi^2 = 0.81$, $df = 1$, $p = 0.37$).
[e]No significant difference between remitters and nonremitters ($\chi^2 = 0.16$, $df = 1$, $p = 0.69$).

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large effect in the genotype distribution (n¼ 81). For the allele frequency analysis (n¼ 162), this study had a power of 0.25 to detect a small effect, 0.97 to detect a medium effect and 0.99 to detect a large effect. In the power analysis, effect size conventions were determined according to the method of Buchner et al. (1996) as follows: small effect size¼ 0.10, medium effect size¼ 0.30 and large effect size¼ 0.50 (alpha¼ 0.05).

DISCUSSION

The present study revealed that the COMT val158met polymorphism affected the antidepressant effect of milnacipran. The met/met genotype of this polymorphism was associated with a significantly faster therapeutic effect in the MADRS scores during this study, although the difference in final therapeutic response was not significant between the met/met and other genotype groups.

Lachman et al. (1996) reported that individuals with the met/met genotype of the COMT val158met polymorphism had a threefold to fourfold reduction in enzymatic activity compared with those with the val/val genotype, and heterozygous individuals had intermediate enzymatic activity between that of homozygous individuals. However, the impact of the COMT val158met polymorphism on the metabolism of catecholamines appears to be minimal in usual physiological condition, even though it is a functional polymorphism. The high-affinity neuronal reuptake is an efficient elimination system for the released catecholamines, being responsible for most of their elimination both in the peripheral tissues and the brain (Mannisto and Kaakkola, 1999).

When exogenous levodopa, a dopamine precursor, is administered, the situation is dramatically altered for dopamine. During the combination therapy of levodopa and dopa decarboxylase inhibitor, the majority of surplus levodopa is preferably metabolised by COMT (Mannisto and Kaakkola, 1999). Individual differences of COMT activity become important for the pharmacological effect of levodopa in this situation.

The similar situation can occur to noradrenaline when its synaptic concentration is pharmacologically increased by the reuptake inhibition induced by milnacipran, though it has not been investigated yet. As the individuals with the met/met genotype of the COMT val158met polymorphism have a lower enzymatic activity, the synaptic concentration of norepinephrine may remain higher in patients with the met/met genotype than those with other genotypes. One possibility to explain the present result is that prolonged higher synaptic concentration of norepinephrine potentiates its neurotransmission particularly in patients with the met/met genotype, resulting in a faster antidepressant effect.

The present result about the COMT val158met polymorphism is not consistent with that of a previous study using an antidepressant mirtazapine (Szegedi et al., 2005). Szegedi et al. (2005) reported that carriers of the val/val and val/met genotype had significantly greater antidepressant effect than those of the met/met genotype. The initial pharmacological action of milnacipran and mirtazapine is not identical: that of the former is blockade of noradrenaline transporters, and that of the latter is blockade of a_2-adrenergic autoreceptors. However, the discrepancy of the present results and those of Szegedi et al. (2005) cannot be explained by the difference of the initial pharmacological action of milnacipran and mirtazapine, because both drugs commonly result in enhanced noradrenergic transmission. Detailed mechanisms underlying the discrepancy of the present results and those of Szegedi et al. (2005) remain unclear.

Additional analyses based on the results of factor analyses of depression symptomatology revealed that the factor of dysphoria (Suzuki et al., 2005) and psychic anxiety (Parker et al., 2003) contributed to overall differences over time of the MADRS scores among each COMT val158met genotype group. The factor of dysphoria identified by Suzuki et al. (2005) and that of psychic anxiety identified by Parker et al. (2003) shares the symptoms of pessimistic and suicidal thoughts. Although serotonergic dysfunction in brain has been reported to be responsible for these symptoms (Carroll, 1994), this conclusion is not adequately justified by current evidence. For example, Poelinger and Haber (1989) found anxiety ratings decreased more with maprotiline (noradrenaline selective agent) than with fluoxetine (serotonin selective agent). Akkaya et al. (2006) reported that response rate for anxiety of reboxetine (noradrenaline selective agent) group was significantly higher than venlafaxine groups in the middle of treatment in patients with anxious depression, though the final response rate for anxiety was not significantly different. These findings and the present results suggest that the noradrenergic system in brain play a role in improvement of anxious symptoms of depression, and its genetic polymorphisms might affect the onset of therapeutic efficacy of milnacipran for anxiety in depression.

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The present study also revealed that the TH val81met polymorphism did not affect the antidepressant effect of milnacipran. The TH val81met polymorphism is reported to be associated with early-onset alcoholism (Dahmen et al., 2005) and the left ventricular structure (Linhart et al., 2002). However, Ishiguro et al. (1998) reported that TH val81met polymorphism was not likely to play a major role in the genetic predisposition to schizophrenia, mood disorders or alcohol dependence. Kunugi et al. (1998) also reported no evidence for involvement of the TH val81met polymorphism in schizophrenia or Parkinson's disease. The functional effect of the TH val81met polymorphism is still unknown, and the present results indicate no important role of the TH val81met polymorphism on the antidepressant effect of milnacipran.

One major limitation of this study is the relatively small number of subjects. A second limitation is the relatively small endpoint treatment differences in the analysis for the COMT val158met polymorphism. These limitations make it difficult to definitely conclude that the COMT val158met polymorphism is the genetic factor to predict the antidepressant effect of milnacipran. Difference in allele frequencies of the TH val81met polymorphism between responders and nonresponders seems marginal ($p \approx 0.08$), and increased number of subjects may reveal significant difference. Serotonergic effects of milnacipran cannot be neglected, and are probably independent of genetic differences in enzyme activities affecting catecholamine biosynthesis and elimination. Therefore, genetic polymorphisms of TH and COMT only have limited predictive value, and if any, can be at most partial predictors for the overall response to milnacipran. The authors performed collection of blood samples 4 weeks after initiation of antidepressant treatment. This schedule makes it impossible to perform an intent-to-treat analysis in relation to genetic polymorphisms, because the authors have no information of genotypes of dropout subjects. Further studies with a larger number of subjects are needed not only to confirm the results of this study but also to investigate the interaction of many genes, including the COMT gene, on the mechanisms of antidepressant action.

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Relationship between three serotonin receptor subtypes (*HTR3A, HTR2A* and *HTR4*) and treatment-resistant schizophrenia in the Japanese population

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Abstract
The proportion of treatment resistant schizophrenia (TRS) has been estimated as 20 to 40% in the schizophrenic patients. Genetic factors are considered to be involved in the development of this condition. Serotonin subtypes are hypothesized to be the candidate genes. In the present study, single marker and haplotype analyses between several mutations of serotonin receptor subtypes (*HTR2A, HTR3A* and *HTR4*) and TRS (TRS=101, Non-TRS=239) were performed to determine a possible relationship with the development o f TRS. Additionally, we also compared the daily neuroleptic dosage among each genotype. No significant association was observed between TRS and each allele, genotype, and haplotype. However, the daily neuroleptic dosage that patients had been receiving during their maintenance therapy was significantly higher in patients with the T/T genotype of *HTR3A* polymorphism (rs1062613, p=0.041). The present results support further research to examine the relationship between *HTR3A* polymorphism and the development of TRS in the Japanese population.
Keyword: *HTR*, serotonin, antipsychotic drug, refractory, gene, haplotype

Introduction
The proportion of treatment resistant schizophrenia (TRS) has been estimated as 20 to 40 % in the schizophrenic patients, and this unfortunate situation in the clinical psychiatric field still remain unchanged even after the introduction of several atypical antipsychotic agents [1]. Among the atypical antipsychotics, only clozapine has been reported to be effective for 30-60 % of schizophrenic patients refractory to typical and atypical antipsychotics [2, 3]. Clozapine is known to provide antipsychotic effects through binding to the several serotonin receptor subtypes (5-HT) [4]
although the actual mechanism of clozapine for TRS has not been elucidated yet. In order to clarify this mechanism several researches investigated the predictable genetic factors for the clinical response to clozapine, as a result a significant association with the 5-HT receptor subtypes has been reported in a number of studies as follows.
Clozapine has a high affinity for 5-HT2A receptor [5] and produces a significant downregulation of cortical 5-HT2A receptor in the radioligand binding studies [6]. In addition, two PET studies have shown that the systemic administration of clozapine to schizophrenic patients produces an 84-90% occupation of cortical 5-HT2A receptor [7, 8]. A couple of researches have reported the association between 5-HT2A receptor gene (*HTR2A)* polymorphism and TRS [9, 10] or response to clozapine [11], although no association study has been reported in the Japanese subjects with TRS.
Since the 5-HT3A receptor has been reported to have potential anxiolytic and anti-psychotic properties from animal studies, 5-HT3A receptor antagonists are being explored as therapeutic agents for a variety of behavioral disorders [12]. Additionally, 5-HT3A receptor gene (*HTR3)* is located on 11q23.1, where linkage with schizophrenia has been suggested in several studies [13, 14]. These results suggest that *HTR3* may be related to the treatment response in the schizophrenic patients. Gutierrez have reported no association between *HTR3A* polymorphism and clozapine response [15], however, this study did not take haplotype block structure into consideration and did not cover whole genomic region of *HTR3A*.
5-HT4 receptor gene (*HTR4)* also has been reported to be associated with schizophrenia in the Japanese population [16]. Therefore, this gene could also be a candidate gene for TRS.
Thus, the *HTR2A, 3A, 4* could be considered as plausible genes related to the development of the TRS. Therefore, in the present study, we performed Linkage disequilibrium (LD) analysis of *HTR3A*, followed by the case-control association studies between *HTR3A* polymorphisms and TRS using

single-marker association analyses and haplotype analyses. In addition, the association was also examined between *HTR2A* polymorphism, *HTR4* polymorphism and TRS.

Materials and Methods

Subjects

This study was initiated after the approval by the Ethics Committee of the Nagoya University School of Medicine. Written informed consent was obtained from all subjects at study entry.

A total of 340 patients with schizophrenia (male = 200, female = 140, age: 54 ± 12.8, duration of illness: 33.6 ± 12.4 years, daily neuroleptic dosage: 1021 ±1857 mg/day) who had been diagnosed using the criteria of DSM-IIIR (American psychiatric association, 1987) were selected in this study. All patients were Japanese descent and had been hospitalized and receiving antipsychotic drugs for more than one year.

Definition of TRS

The definition of TRS is described elsewhere in the previous study [17]. Briefly, information about the neuroleptic therapy that the schizophrenic patients had been receiving was obtained from their clinical records. The daily neuroleptic dosage was calculated from the recent one year neuroleptic prescription history. Schizophrenic patients were diagnosed as having TRS when they had been hospitalized for more than 1 year and had been receiving antipsychotic therapy at dosages of at least 1,000 mg/day chlorpromazine equivalents for more than one year.

SNP selection and Genotyping

Using the information obtained from the HapMap Database and the dbSNP Database, two single nucleotide polymorphisms— rs1062613 and rs1176713—were selected as haplotype tag SNPs (htSNPs) that covered the whole coding region, 5' flanking region upstream 500bp, and 3' UTR region downstream 500bp of *HTR3A*. The LD block was defined using HAPLOVIEW version 3.0 (http://www.broad.mit.edu/mpg/haploview/) as a region of D'>0.8. In each LD block, haplotype frequency was estimated by the expectation-maximization (EM) algorithm and htSNPs were selected using the same program. Additionally, a SNP (rs6313) of *HTR2A* and two SNPs (rs2278392, rs3734119) of *HTR4* which have been reported to be associated with schizophrenia in the previous study [16] were selected. Genotyping was carried out using polymerase chain reaction-restriction fragment length polymorphism assays or direct sequence assays for each SNP. Sequences of each primer pairs are available on request.

Statistics

Genotype deviation from the Hardy-Weinberg equilibrium (HWE) was evaluated by Chi square test. Single-marker and haplotype analyses were performed using SPSS version 11.0J (Tokyo, Japan) and Cocaphase 2.403 (http://www.rfcgr.mrc.ac.uk/~fdudbrid/software/unphased/), respectively.

Comparison of the daily neuroleptic dosage among each genotype was performed using Mann-Whitney *U* test. Power calculation was performed by Power Caluculator (http://calculators.stat.ucla.edu/powercalc/). The level of significance for all statistical tests was set at 0.05.

Results

A total of 101 schizophrenic patients were identified as the TRS (TRS: male=67, female=34, age=50±10.5, onset age=20±5.3; NON-TRS: male=133, female=106, age=56±13.1, onset age=23.5±8.2). The male ratio tended to be higher in the TRS patients ($p<0.1$), and the age at onset was significantly younger in this group ($p=0.009$). However, no significant difference was observed in the incidence of any psychiatric symptom between the two groups, such as delusion and hallucination, bizarre behavior, disorganization, and negative symptoms at their first episode, as reported in our previous report [18]. The genotype distributions of the polymorphisms did not deviate significantly from the HWE in each study group for any polymorphism. The genotype and allele frequencies of 3 kinds of serotonin receptor genes in TRS and NON-TRS groups are shown in Table1.

No significant association was observed in the single marker analysis of *HTR2A*, *HTR3A*, and *HTR4*, and in haplotype analysis of *HTR3A* and *HTR4* (Table1).

The characteristics of neuroleptic treatment among the three subgroups showing each SNP polymorphism are shown in Table2. In rs1062613 of *HTR3A*, the daily neuroleptic dosage during maintenance therapy was significantly higher in patients with the T/T genotype than the others ($p = 0.041$).

When the proportion of TRS was set to be 30% [19], we obtained more than 80% power to detect an association with the SNPs of which the minor frequency is more than 10%.

Discussion

The results presented here suggest that *HTR3A* may be involved in the development of TRS in the Japanese population. In this study, significant difference in the daily neuroleptic dosage received during maintenance therapy was observed in schizophrenic patients with the T/T genotype of *HTR3A* polymorphism (rs1062613).

The SNP rs1062613 is located on the promoter

region of *HTR3A* and has been reported to regulate the expression of this gene [20]. Since presynaptic 5-HT3A receptors modulate the release of several neurotransmitters in various brain regions [21, 22], the abnormal expression of *HTR3A* might increase the dopamine concentration in the synaptic cleft. This may lead to increase the therapeutic antipsychotic doses in the schizophrenic patients with this mutation.

Additionally, several antipsychotic drugs reduce the dopaminergic neurotransmission by antagonizing the 5-HT3A receptor[23]. Therefore, reduction in the expression of 5-HT3A receptor may weaken the therapeutic effect of antipsychotics through this pathway; even higher dose of most antipsychotic drugs may not reduce the dopaminergic neurotransmission.

Furthermore, this SNP has been reported to have a critical role in the amygdala activity leading to the facial expression recognition [24], and the defect of facial expression recognition has been reported to be a specific symptom to the schizophrenia including TRS [25, 26]. Therefore, this SNP may have a role in the development of TRS based on the effect of the SNP on the impairment of facial expression recognition.

The definition of TRS in the present study is different form that proposed by Kane et al (27).

Since the polypharmacy is widely prevalent in the antipsychotic treatment of schizophrenia in Japan. In the present study, the psychopathology of TRS was defined by the total antipsychotic doses that the schizophrenic patients had been receiving during the recent 1 year, that is, the severity of illness was extrapolated by the total antipsychotic doses. In addition, they had been hospitalized for more than 1 year, indicating that they had been no good level of functioning over this period. In fact, age at disease onset had been observed to be significantly younger in the TRS subjects, suggesting that the younger onset patients tend to less response to the antipsychotic therapy. Therefore, we consider that virtually no essential difference exists between the present definition of TRS enrolled in Japan and that proposed by Kane et al (27).

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