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Identification of *YWHAE*, a gene encoding 14-3-3epsilon, as a possible susceptibility gene for schizophrenia

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Schizophrenia is a complex mental disorder with a fairly high degree of heritability. Although the causes of schizophrenia remain unclear, it is now widely accepted that it is a neurodevelopmental and neurodegenerative disorder involving disconnectivity and disorder of the synapses. Disrupted-in-schizophrenia 1 (*DISC1*) is a promising candidate susceptibility gene involved in neurodevelopment, including maturation of the cerebral cortex. To identify other susceptibility genes for schizophrenia, we screened for *DISC1*-interacting molecules [NudE-like (*NUDEL*), Lissencephaly-1 (*LIS1*), 14-3-3epsilon (*YWHAE*), growth factor receptor bound protein 2 (*GRB2*) and Kinesin family 5A of Kinesen1 (*KIF5A*)], assessing a total of 25 tagging single-nucleotide polymorphisms (SNPs) in a Japanese population. We identified a *YWHAE* SNP (*rs28365859*) that showed a highly significant difference between case and control samples, with higher minor allele frequencies in controls ($P_{allele} = 1.01 \times 10^{-5}$ and $P_{genotype} = 4.08 \times 10^{-5}$ in 1429 cases and 1728 controls). Both messenger RNA transcription and protein expression of 14-3-3epsilon were also increased in the lymphocytes of healthy control subjects harboring heterozygous and homozygous minor alleles compared with homozygous major allele subjects. To further investigate a potential role for *YWHAE* in schizophrenia, we studied *Ywhae*$^{+/-}$ mice in which the level of 14-3-3epsilon protein is reduced to 50% of that in wild-type littermates. These mice displayed weak defects in working memory in the eight-arm radial maze and moderately enhanced anxiety-like behavior in the elevated plus-maze. Our results suggest that *YWHAE* is a possible susceptibility gene that functions protectively in schizophrenia.

INTRODUCTION

Recent neuroimaging studies show that structural brain abnormalities are an established feature of schizophrenia and are characterized by decreased total gray matter volume (1,2). These morphological correlates of schizophrenia range from a reduction in brain size to localized alterations in the morphology and molecular composition of specific neuronal, synaptic and glial populations in specific brain areas such as the hippocampus, dorsolateral prefrontal cortex and dorsal thalamus.

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These findings have fostered the current view of schizophrenia as a disorder of connectivity (3,4) and of the synapse (5). Although the mechanism underlying the neurodevelopmental/neurodegenerative process is still unclear, a way forward is provided by the recent identification of several putative susceptibility genes, such as *Neuregulin 1* (6), *Dysbindin* (7), *G72* (8), Catechol-*O*-methyltransferase (*COMT*) (9–11) and others (12,13). For none of these genes, however, has a causative allele or the mechanism by which it predisposes to schizophrenia been identified.

Disrupted-in-schizophrenia 1 (*DISC1*) was first described as a strong candidate gene in a large Scottish family in which a balanced chromosomal translocation segregates with schizophrenia and other psychiatric disorders (12,14,15). The translocation mutation may result in loss of DISC1 function via haploinsufficiency or dominant-negative effects of a predicted mutant DISC1 truncated protein product. *DISC1* has been implicated in neurodevelopment, including maturation of the cerebral cortex (16).

DISC1 interacts with several proteins, including NudE-like (NUDEL) (17–19), lissencephaly-1 (LIS1, also called PAFAH1B1) (20), fasciculation and elongation protein zeta 1 (FEZ1) (21) and phosphodiesterase 4B (PDE4B) (16). Recently, we identified several novel DISC1-interacting molecules, including 14-3-3epsilon, Kinesin family 5A of Kinesen1 (KIF5A) and Growth factor receptor bound protein 2 (Grb2) by affinity column chromatography (22,23). Furthermore, we confirmed that DISC1 regulates the localization of the NUDEL/LIS1/14-3-3epsilon complex or Grb2 into axons as a cargo receptor (22,23) and it also regulates Neurotrophin-induced axon elongation by Grb2 (23).

In this study, we screened for the genetic association of DISC1-interacting molecules—*NUDEL* (17p13.1, OMIM: *607538), *LIS1* (17p13.3, OMIM: #607432), 14-3-3epsilon (17p13.3, OMIM: *605066), *Grb2* (17p24-q23, OMIM: *108355) and *KIF5A* (12q13, OMIM: *602821)—with schizophrenia, and identified the gene encoding 14-3-3epsilon (*YWHAE*) as a possible susceptibility gene. Our results show that a SNP of *YWHAE*, which influence the expression of 14-3-3epsilon RNA and protein, is associated with schizophrenia and seems to work protectively. We also investigated the behavioral phenotype of mice with ~50% reduction in 14-3-3epsilon protein expression and found that these mice displayed weak phenotypes consistent with some aspects of human schizophrenia.

RESULTS

Screening analysis of *DISC1*-related genes and identification of *YWHAE* as a possible susceptibility gene for schizophrenia

To investigate whether novel DISC1-interacting molecules such as NUDEL, LIS1, YWAHE, GRB2 and KIF5A are associated with schizophrenia, we performed genetic association analyses using a Japanese population.

We failed to develop the genotyping of three SNPs in *LIS1* (*rs8082331*, *rs12938775* and *rs4790348*) and one SNP in *GRB2* (*rs16967795*), therefore a total of 25 SNPs were assessed in this analysis.

Though genotype distributions of two SNPs significantly deviated from Hardy–Weinberg Equilibrium (HWE, $P_{HWF} = .0143$: *rs4789172* in case sample, and $P_{HWF} = .0171$: *rs11172247* in control sample), those of the other markers were in HWE. Six tagging SNPs in *YWHAE* were significantly associated with schizophrenia and also *YWHAE* showed gene-wide significance (permutation $P = 0.0021$), whereas we found no association of tagging SNPs in *NUDEL*, *LIS1*, *GRB2* or *KIF5A* (Table 1).

Since six tagging SNPs in *YWHAE* located in the intron region, we performed denaturing high-performance liquid chromatography (dHPLC) analysis in 5′ flanking regions and entire exon regions of *YWHAE* to identify the possible causal polymorphism, and detected two SNPs: one in the 5′ flanking region (−261 bp from the initial exon: *rs28365859*) and the other one in the 3′-UTR (*rs9393*). Since the 5′ flanking region SNP might have a functional effect due to its position, we focused on this SNP in the following analysis [linkage disequilibrium (LD) structure of first-set samples in *YWHAE* can be seen in Fig. 1).

First, to examine the association of this SNP, we expanded the sample size (1065 cases and 1386 controls in a second set of confirmation samples, for a total of 1429 cases and 1728 controls including the first set of screening samples, call rates were 100%), and significant association was obtained ($P_{allele} = 1.01 \times 10^{-5}$ and $P_{genotype} = 4.08 \times 10^{-5}$). Furthermore, the significance could be detected in either set independently (Table 2). The commonly observed feature of these analyses was that the minor allele frequencies (MAFs) of this SNP were higher in controls than in schizophrenia patients. There was no discrepancy out of 380 randomly selected samples (190 cases and 190 controls) genotyped in duplicate and by another method (TaqMan Assay: C12125119) for this marker, suggesting it is unlikely that genotyping error had occurred.

Functional analysis of the promoter SNP in *YWHAE*: *in vitro* and *in vivo* expression assays

We first investigated the influence of *rs28365859* on *YWHAE* expression by dual-luciferase assay, although there is no evidence that the region where this SNP is located on is evolutionally conserved and that any regions in *YWHAE* are match as a core promoter by in silico promoter detection software. As shown in Fig. 2, a trend for significance in a promoterless vector and significance in a promoter vector were obtained in the different cell lines. The constructs containing a minor allele (C allele) showed higher expression in the promoter vector, suggesting that the C allele plays a possible enhancer role in these cell lines.

Next, to examine the role of this SNP in peripheral blood of healthy control subjects, real-time RT–PCR and western blot analysis were performed. Similar to the luciferase assays, heterozygous and homozygous minor allele (G/C and C/C genotype) subjects showed higher expression levels of 14-3-3epsilon than did homozygous major allele (G/G genotype) subjects (one-way analysis of variance, ANOVA, $P = 0.0251$ and 0.0014 in real-time RT–PCR and western blot analysis, respectively). Experimental analysis were performed to examine the differences under an additive model (G/G

Table 1. Screening analysis of DISC1-related genes

Gene	SNPs		Position[a]	Missing rate (%)	MAF Cases	Controls	*P*-value Allele	Genotype
NUDEL	rs3744652	C>T	8280008	0.3	33.0	35.9	0.250	0.274
	rs8064655	C>T	8301185	0	33.2	36.3	0.228	0.246
LIS1	rs1266474	A>G	2481460	0.4	9.72	12.4	0.110	0.0876
	rs4790356	G>A	2532979	0	10.6	11.7	0.528	0.730
	rs7212450	C>G	2538690	0	42.3	41.7	0.821	0.907
YWHAE	rs34041110	C>T	1193642	0	48.9	42.5	**0.0166**	**0.00563**
	rs9393	A>G	1195142	0	27.3	27.9	0.805	0.868
	rs8064578	C>T	1201625	0	48.5	43.4	0.0562	0.117
	rs7224258	G>C	1202252	2.1	15.0	20.3	**0.0102**	**0.0342**
	rs3752826	G>T	1211814	0	48.6	42.1	**0.0139**	**0.0175**
	rs7214541	T>C	1220072	0	44.6	49.4	0.0725	0.107
	rs11655548	A>G	1230748	2.3	29.3	38.3	**0.000418**	**0.00162**
	rs2131431	A>C	1241645	0.3	13.2	18.5	**0.00598**	**0.0176**
	rs1873827	A>G	1247690	0	42.4	49.6	**0.00732**	**0.0136**
	rs12452627	C>T	1249222	0	17.7	19.6	0.367	0.662
GRB2	rs7219	T>C	70826963	0	9.07	6.85	0.125	0.239
	rs8079197	C>G	70828274	0.6	8.45	6.60	0.190	0.308
	rs4789172	C>T	70853307	0.6	24.9[b]	26.1	0.617	0.659
	rs2053156	T>G	70890035	0	6.04	4.53	0.206	0.344
	rs930296	G>A	70915763	0	5.91	4.66	0.298	0.432
KIF5A	rs11172247	C>G	56232777	0	39.4	38.3[b]	0.676	0.609
	rs11172254	G>A	56255005	0.3	19.5	21.2	0.422	0.679
	rs775250	C>A	56263307	0	20.8	21.7	0.672	0.690
	rs775251	C>T	56265007	0.4	27.7	32.2	0.0713	0.129
	rs1678536	C>G	56265457	0.1	47.9	47.4	0.833	0.644

YWHAE showed gene-wide significance (permutation $P = 0.0021$).
Bold numbers represent significant *P*-values (<0.05).
[a]Based on HapMap database release#21.
[b]deviated from Hardy–Weinberg equilibrium.
MAF, minor allele frequency.

versus G/C+C/C), again significant associations were obtained.

Furthermore, haplotype trend regression test was applied to check the effects of haplotypes of *rs28365859* and other four SNPs in intron 1 (*rs11655548*, *rs2131431*, *rs1873827* and *rs12452627*), which might also be in an enhancer region. This showed significant association in either analysis ($P = 0.0282$ and 0.0186 in real-time RT–PCR and western blot analysis, respectively), however, each SNP in intron 1 was not correlated with the expression level (data not shown).

Effect of reduction of 14-3-3epsilon protein on the cognitive functions of mice

14-3-3 proteins are highly conserved across species, from bacteria to humans, and bind to phosphoserine/phosphothreonine motifs in a sequence-specific manner (24–28). Previously we reported that 14-3-3epsilon binds to CDK5/p35-phosphorylated NUDEL and maintains NUDEL phosphorylation. To examine the protective effect of 14-3-3epsilon on schizophrenia using mice, we should investigate whether overexpression of 14-3-3epsilon results in resistance for the onset of schizophrenic symptoms. However, an assay system to evaluate the effect of a gene on the onset of schizophrenia in mice has not yet been developed. Thus, in support of a role for *YWHAE* in schizophrenia, we investigated *Ywhae* knockout mice. Null mice of *Ywhae* gene ($Ywhae^{-/-}$) show a severe cell migration defect in both the cortex and the hippocampus, whereas $Ywhae^{+/-}$ mice, in which the expression level of 14-3-3epsilon protein is reduced to ~50% compared with their wild-type littermates, show a milder migration defect (29). Because most $Ywhae^{-/-}$ mice die at birth as previously reported (29), $Ywhae^{+/-}$ mice and their wild-type littermates were analyzed by a comprehensive behavioral test battery to investigate whether the reduction in 14-3-3epsilon protein affects behavior (30,31). $Ywhae^{+/-}$ mice appeared normal, healthy and fertile (Table 3).

To examine whether reduction in 14-3-3epsilon was associated with cognitive deficits, we analyzed $Ywhae^{+/-}$ mice and their wild-type littermates in working memory and reference memory tasks (Table 3). To assess working memory of $Ywhae^{+/-}$ mice, we used a spatial working memory version of the 8-arm radial maze task (32,33). The mice were trained for 26 trials. During training, both control and mutant mice improved their performance and no significant difference was observed ($P = 0.3325$) (Fig. 3A). The number of revisiting errors of $Ywhae^{+/-}$ mice was significantly more than their wild-type littermates during trials with a delay of 300 s ($P = 0.0229$) (Fig. 3C). The number of different arms chosen during the first eight choices, which is considered a measure of working memory that is relatively independent of locomotor activity levels and the total number of choices, was not significantly affected by the deficit of 14-3-3epsilon protein during training and trials with 30, 120 and 300 s of delay ($P = 0.3325$, 0.8972, 0.6476 and 0.5077, respectively) (Fig. 3B and D). These results

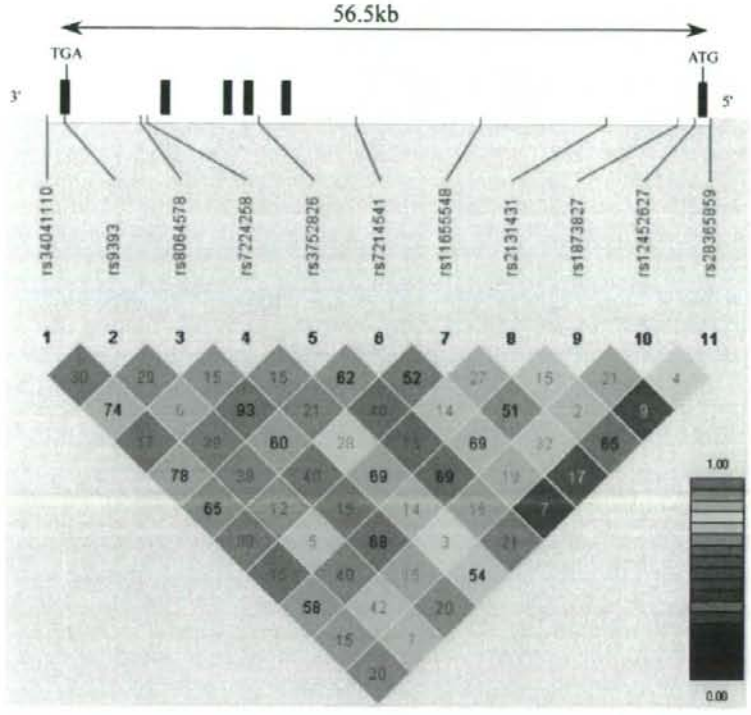

Figure 1. Tagging SNPs and LD evaluation of *YWHAE* for first-set screening samples. rs28365859 was included. Vertical bars represent exons. Numbers in boxes represent r^2 values, which should be expressed as decimals. r^2 values of 1.0 are not shown. Color scheme was based on GOLD format. Additional information is provided at the Haploview website.

Table 2. Association analysis of promoter SNP in YWHAE (rs28365859)

Samples[a]	Phenotype	*n*	Genotype G/G	G/C	C/C	MAF (%)	*P*-values HWE[b]	Allele	Genotype
Combined	Cases	1429	921	457	51	19.6	0.537	1.01×10^{-5}	4.08×10^{-5}
	Controls	1728	1000	620	108	24.2	0.366		
First-set	Cases	364	245	106	13	18.1	0.715	0.00108	0.00545
	Controls	342	192	127	23	25.3	0.748		
Second-set	Cases	1065	676	351	38	20.0	0.359	0.00123	0.00280
	Controls	1386	808	493	85	23.9	0.399		

First-set samples were identical to those used in screening analysis.
Second-set samples were independent set of samples to increase the sample size.
[a]Combined samples = first-set + second-set samples.
HWE, Hardy–Weinberg equilibrium.

suggest that *Ywhae*$^{+/-}$ mice show weak defects in working memory.

Next, we analyzed reference memory of *Ywhae*$^{+/-}$ mice, using the left–right discrimination test version of the T-maze. *Ywhae*$^{+/-}$ mice and their wild-type littermates were trained for 6 trials; then the correct side was reversed. The next 6 trials were performed under the reversal-learning condition. No significant difference was observed in the percentage of correct choices at the sixth trial (*Ywhae*$^{+/+}$, 80.647%; *Ywhae*$^{+/-}$, 77.157%; $P = 0.7516$), and no significant difference was observed under the reversal-learning condition ($P = 0.4567$) (Table 3). These results suggest that a decrease in the 14-3-3epsilon protein results in weak defects, specifically in spatial working memory.

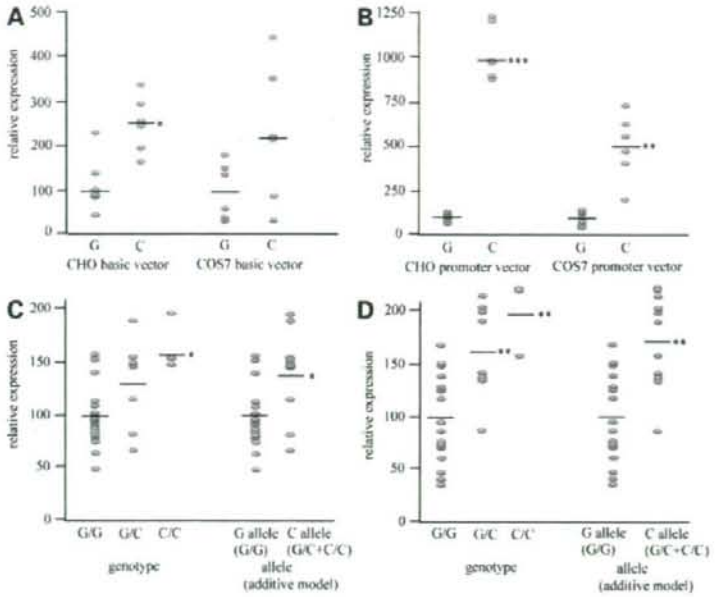

Figure 2. *In vitro* and *in vivo* expression assays. (**A**) Promoterless vector (basic vector) and (**B**) promoter vector in CHO and COS7 cells. Firefly luciferase activities were normalized with Renilla luciferase activities. Relative expression was calculated as 100 for the major allele (G allele) of *rs28365839*. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (in Student's *t*-test). Results of (**C**) real-time RT–PCR and (**D**) western blot analysis. Expression levels of 14-3-3epsilon RNA and protein were normalized with GAPDH expression. Relative expression was calculated as 100 for the major allele homozygous genotype (G/G genotype) or major allele (G allele) of *rs28365839*. Number of individuals with the distinct genotypes of *rs28365839* were 16 for G/G, 8 for G/C and 3 for C/C. $^{*}P < 0.05$, $^{**}P < 0.01$ (in *post hoc* comparison with the Dunnett test for genotype-wise analysis, and *t*-test for allele-wise analysis).

Moderately enhanced anxiety-like behavior in *Ywhae*$^{+/-}$ mice in the elevated plus-maze test

To examine the effect of 14-3-3epsilon deficit on anxiety-like behavior, *Ywhae*$^{+/-}$ mice and their wild-type littermates were analyzed in light/dark transition and elevated plus-maze tests. In light/dark transition, no significant difference was observed between *Ywhae*$^{+/-}$ mice and their wild-type littermates (Table 3). In the elevated plus-maze test, *Ywhae*$^{+/-}$ mice showed a smaller number of total entries ($P = 0.0075$) (Fig. 3E), increased time spent on closed arms ($P = 0.0195$) (Fig. 3F) and decreased time spent on center area ($P = 0.0012$) (Table 3). A significant difference was not observed in the number of entries onto open arms, total distance travelled or time spent on open arms ($P = 0.2044$, 0.1071, 0.3798, respectively) (Table 3). Thus, it is conceivable that *Ywhae*$^{+/-}$ mice have moderately enhanced anxiety-like behavior that could be detected only by the elevated plus-maze test but not by the light/dark transition or by the open-field tests.

DISCUSSION

Association between *YWHAE* and schizophrenia

In this study, we have identified *YWHAE*, the gene encoding 14-3-3epsilon, which forms a complex with DISC1 *in vivo*, as a possible susceptibility gene for schizophrenia. Genetic and expression evidence indicates that the SNP in 5′ flanking region (*rs28365859*) is associated with schizophrenia through influencing the expression level of *YWHAE*. Subjects with the C allele of *rs28365859* were thought to have a reduced risk of schizophrenia [odds ratio of combined subjects = 0.76 (95% confidence interval: 0.68–0.86)]. Our sample size was relatively large (3157samples consisting of 706 first-set and 2451 second-set samples: 1429 schizophrenics and 1728 controls), making our results reliable. In addition, another research assessing the genetic association of *YWHAE* with suicide victims [two of SNPs (*rs3752826* and *rs9393*) are identical SNPs in our study and another SNP (*rs1532976*) can be captured by *rs3752826* using HapMap information] can support our results, since it showed the same trends in the distributions of MAFs (MAFs of these SNPs were higher in controls than in cases) (34). However, a couple of limitations should be outlined. First, our results that show statistical significances may be derived from unknown population stratification, since Genomic Control was not included in this analysis. Secondly, there could be a possible effect of differential age distribution between cases and controls in the association analysis.

The *in vitro* luciferase assay suggests that the C allele might act as an enhancer, since significant luciferase induction could not be seen with the use of a promoterless vector, but luciferase activity (LA) could be assayed from the vector containing a promoter. Further, *in vivo* expression assays of RNA and

Table 3. Comprehensive behavioral test battery

Test		*Ywhae*$^{-/+}$	*Ywhae*$^{+/-}$	*P*-value	*F* value
General health					
Weight (g)		28.6	29.682	0.0262*	1.335
Rectal temperature (°C)		37.033	36.688	0.0435*	4.406
Pain test					
Hot plate (latency, s)		6.206	5.053	0.1142	2.633
Motor tests					
Grip strength (*n*)		1.044	1.085	0.2825	1.194
Wire hang (latency to fall, s)		60	50	0.0234*	5.65
Rotarod (latency to fall, s; average of six trials)		161.759	182.618	0.3391	0.941
Anxiety-like behavior					
Light dark transition					
Distance travelled (cm)	Light side	484.983	617.782	0.0728	3.434
	Dark side	1095.389	1099.288	0.97	0.001
Stay time in light side (s)		214.972	231.176	0.6043	0.274
Transitions (times)		35.111	33.588	0.6827	0.17
Latency to light side (s)		31.444	34.941	0.6683	0.187
Elevated plus-maze					
Number of entries (times)		32.556	25.118	0.0075**	8.126
Entries onto open arms (%)		31.824	26.648	0.2044	1.677
Distance travelled (cm)		1323.722	1194.329	0.1071	2.744
Time on open arms (%)		15.269	12.971	0.3798	0.793
Time on closed arms (%)		50.87	63.196	0.0195*	6.034
Time on center area (%)		35.034	23.283	0.0012*	12.495
Depression model					
Porsolt forced swim (immobility, %)	Day 1	59.369	65.648	0.0661	3.614
	Day 2	77.026	78.564	0.6256	0.243
Tail suspension (immobility, %)		26.194	22.774	0.6267	0.241
Locomotor activity					
Open field					
Total distance travelled (cm)		8745.222	9258.941	0.5822	0.309
Vertical activity (times)		208.722	393.824	0.047*	4.259
Center time (s/min)		1.432	1.107	0.6505	0.209
Stereotypic counts (times)		7260.944	6124.118	0.2251	1.528
Sensory motor gating					
Acoustic startle response		3.021	2.704	0.32	1.02
Prepulse inhibition (startle stimulus, %)					
110-dB startle		48.887	47.697	0.8496	0.037
120-dB startle		13.566	16.6	0.5617	0.344
Working memory					
8-arm radial maze					
Training					
Different arm choice in first 8 entries (times)		6.209	6.016	0.3325	0.967
Revisiting errors (times)		6.12	7.613	0.1557	2.11
Delay 30 s					
Different arm choice in first 8 entries (times)		6.5	6.471	0.8972	0.017
Revisiting errors (times)		3.417	3.676	0.7599	0.095
Delay 120 s					
Different arm choice in first 8 entries (times)		6	5.882	0.6476	0.213
Revisiting errors (times)		4.944	6.735	0.1715	1.954
Delay 300 s					
Different arm choice in first 8 entries (times)		6.167	5.971	0.5077	0.448
Revisiting errors (times)		3.778	6.294	0.0229*	5.698
Reference memory					
T-maze (correct, %)	Training	80.648	77.157	0.0696	3.519
	Reverse	61.759	59.314	0.4567	0.567
Cued and contextual fear conditioning					
Conditioning (freezing, %)		28.324	29.29	0.7581	0.096
Context testing (freezing, %)		50.998	46.611	0.5754	0.32
Cued testing with altered context freezing, %)		53.641	52.926	0.8342	0.045
Social interaction					
Total duration of contact (s)		118.386	153.383	0.1239	2.776
Number of contacts (times)		49.429	53.333	0.4968	0.494
Total duration of active contacts (s)		14.257	18.733	0.0809	3.693
Mean duration/contact		2.443	3.017	0.2889	1.241
Distance travelled (cm)		2789.357	2882.167	0.7206	0.135

Behavioral test battery was performed in the following order: general health/neurological screen, wire hang, grip strength test, light/dark transition, open field, elevated plus-maze, hot plate, social interaction (novel environment), rotarod, prepulse inhibition, Porsolt forced swim, eight arm radial maze, T-maze, cued and contextual fear condition test, latent inhibition, tail suspension test.
*$P < 0.05$, **$P < 0.01$.

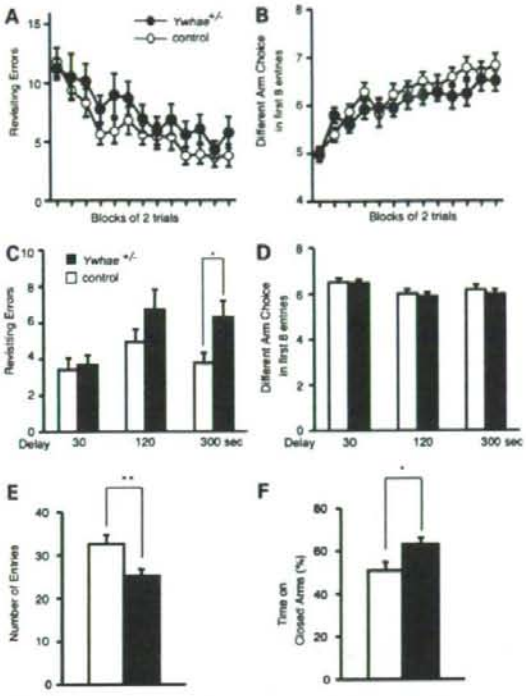

Figure 3. Behavioral abnormality of *Ywhae*$^{+/-}$ mice. (**A–D**) Working memory test of *Ywhae*$^{+/-}$ mice in the 8-arm radial maze (A and B). Total number of revisiting errors (A) and the number of different arms chosen in the first 8-arm visits (B) across training were counted. Data are presented as 2-day/trial averages. (C and D) Total number of revisiting errors (C) and the number of different arms chosen in the first 8-arm visits (D) of mice after training; exposure to delays of 30, 120 or 300 s after four pellets had been taken were counted (see *Materials and Methods*). (**E** and **F**) Anxiety-like behavior test of *Ywhae*$^{+/-}$ mice in the elevated plus-maze. (E) Number of total entries. (F) Time spent on closed arms. Number of total entries was lower and time spent on closed arms were greater in *Ywhae*$^{+/-}$ mice than in controls. Asterisks indicate a difference from the values of control mice. $^{*}P < 0.05$, $^{**}P < 0.01$.

protein in peripheral blood samples clarified the functional relevance of this SNP: Subjects who were heterozygous and homozygous with the C allele had higher expression of 14-3-3epsilon. Of note, our samples were control subjects not on medication; therefore, we could avoid the bias related to drug effects, which may be seen when studying schizophrenia subjects.

Also, haplotype trend regression analysis showed that the haplotypes consisted of five SNPs located in 5′ flanking region (*rs28365859*) and intron1 (*rs11655548*, *rs2131431*, *rs1873827* and *rs12452627*) were correlated with the expression level of *YWHAE*, whereas each SNP in intron 1 was not correlated with the expression. Therefore, we speculate that this significant result in haplotype-wise analysis may be derived mainly from the effects of *rs28365859*.

We analyzed for the homology of genome sequence between human and mice *Ywhae* gene using 500 bp upstream region from start ATG. About 200 bp upstream region from start ATG shows high identity, however, a region containing *rs2836589* SNP does not show homology. This result suggests that this SNP is not evolutionally conserved. We searched for functional motif on the sequence in the 5′ upstream region of *YWHAE* including *rs28365859* using TESS: Transcription Element Search System (http://www.cbil.upenn.edu/cgi-bin/tess/tess). In minor allele (C allele), ubiquitously expressed cellular upstream stimulatory factor (USF) -interacting motif 'CCACGT' was detected in this in silico analysis. This result may explain a possible functional effect of this SNP, an upregulation of 14-3-3epsilon in C allele-harboring people, however, further analysis would be needed to provide definitive conclusion.

Role of 14-3-3epsilon in neuronal development

Several observations of the postmortem brain suggest that alterations in neuronal cell migration, and synaptic, dendritic and axonal organizations occur in schizophrenia patients (35,36). *Ywhae*$^{+/-}$ mice show milder migration defects in both the cortex and the hippocampus, whereas *Ywhae*$^{-/-}$ mice display severe neuronal migration defects (29). Primary hippocampal neurons from *Ywhae*$^{-/-}$ mice display shorter axons and a defect in accumulation of the NUDEL/LIS1 complex in the distal part of axons (29). We confirmed that knockdown of 14-3-3epsilon by RNAi impairs not only the NUDEL/LIS1 complex transport into axons but also axon elongation (data not shown). Previously, we identified 14-3-3epsilon as an interacting molecule of *DISC1* (22). DISC1 is required to transport the NUDEL/LIS1/14-3-3epsilon complex into axons (22). Of note, depletion of endogenous DISC1 by RNAi results in a severe neuronal migration defect in the developing neocortex via regulation of the dynein complex (37). These results and reports suggest that both DISC1 and 14-3-3epsilon are required for neuronal development via transport of the NUDEL/LIS1 complex. To clarify the functional relationship between 14-3-3 epsilon and DISC1 on neuronal development via transport of the NUDEL/LIS1 complex, further genetic analysis using knockout mice will be required.

Cognitive dysfunction of *Ywhae*$^{+/-}$ mice

Ywhae$^{+/-}$ mice, in which the expression of 14-3-3epsilon protein was reduced to ~50% compared with their wild-type littermates, showed weak cognitive dysfunction specifically in working memory (Table 3). Interestingly, missense mutant mice of the *DISC1* gene show defects in working memory (38). Reduction of DISC1 or 14-3-3epsilon results in developmental defects of hippocampal neurons. These results and reports suggest that impairment of DISC1 or 14-3-3epsilon cause neuronal developmental defects, that result in cognitive dysfunction. Interestingly, impairment of working memory is one of the prominent features of schizophrenia symptomatology (39–41). Non-synonymous polymorphism of *DISC1* that consists of a serine to cysteine substitution at codon 704 (*DISC1*Ser704Cys) is reported to correlate with variations in hippocampal size and cognitive function including working memory, and is associated with

schizophrenia (42). Although relation between 14-3-3epsilon and cognitive function in human is not known, 14-3-3epsilon could be implicated in cognitive function that is associated with DISC1. Another prominent feature of schizophrenia symptomatology, prepulse inhibition (43), did not differ in *Ywhae*$^{+/-}$ mice compared with their wild-type littermates (Table 3). Schizophrenia is a complex disorder with a variety of pathology and risk factor genes. It is a reasonable assumption that modification of a single gene does not mimic all features of schizophrenia symptomatology. We think that our results using *Ywhae*$^{+/-}$ mice partly support our genetic data. However, further analysis would be required to clarify a role of 14-3-3epsilon on cognitive functions and functional relationship between *YWHAE* and *DISC1*.

YWHAE as a possible susceptibility gene for schizophrenia

In this study, we found that a SNP of *YWHAE* that correlates the expression of 14-3-3epsilon is associated with schizophrenia, and that this SNP would reduce the risk of schizophrenia. Perhaps, increased 14-3-3epsilon expression in humans affected by the identified SNP is protective, whereas decreased 14-3-3epsilon expression due to 50% reduction by heterozygous knockout in mice results in behavioral deficits. At this point, we do not know why higher expression levels of 14-3-3epsilon reduce the risk of schizophrenia, or why lower expression levels of this gene result in increase of the risk in human and behavioral changes in mice. By its susceptibility genes, schizophrenia seems to be a complex disorder with multiple symptoms and genetic risk factors. We predict that schizophrenia would be divided into several classes by its susceptibility genes. Each class would have its own molecular/signaling pathway that plays important roles in the pathogenesis. DISC1 and its interacting molecules are required in neuronal developments and adult neurongenesis (44), and would play critical roles in pathogenesis of specific classes of schizophrenia. In other classes of schizophrenia, the DISC1-pathway would not be implicated in the pathogenesis. Some genes could have redundant functions. 14-3-3epsilon is a member of adaptor proteins that interact with phosphorylated serine or threonine residue of target proteins. More than 100 of 14-3-3-binding partners involved in signal transduction, cell cycle regulation, apoptosis, stress responses and malignant transformation have been identified (45). Proteomic analysis of synapse revealed that 14-3-3epsilon forms a complex with NMDA receptor (46). Placing these results and reports in the context of the pathogenesis of schizophrenia, 14-3-3epsilon could be a susceptibility gene of not only DISC1-implicated, but also wide range of schizophrenia because of its wide variety of interacting partners. 14-3-3epsilon would be a key molecule to understand molecular mechanisms of susceptibility genes for schizophrenia.

MATERIALS AND METHODS

Subjects in genetic association analyses

In the genetic association analyses, two independent sets of subjects were examined. The first screening analysis included 364 patients with schizophrenia (188 male and 176 female; mean age ± SD 42.5 ± 14.8 years) and 342 healthy controls (191 male and 151 female; 35.0 ± 13.6 years). Patients for the second confirmation analysis included 1065 patients with schizophrenia (562 male and 503 female; 48.9 ± 14.7 years) and 1386 controls (714 male and 672 female; 42.6 ± 14.6 years). All subjects were unrelated to each other and reported to be of Japanese ethnicity. Forty patients with schizophrenia were used as subjects for a mutation search; these subjects were also included in the first-set screening scan. The schizophrenia patients were diagnosed according to criteria in the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* after at least two experienced psychiatrists reached consensus on the diagnosis on the basis of unstructured interviews and review of medical records. All healthy controls were also psychiatrically screened on the basis of unstructured interviews; to exclude subjects with any brain disorder, or psychotic disorder, or who had first-degree relatives with psychotic disorders, trained psychiatrist interviewed them to assess current and/or past mental states (psychotic, mood, anxiety, obsessive-compulsive symptoms) and family history. After description of the study, written informed consent was obtained from each subject. This study was approved by the ethics committees at Fujita Health University, Teikyo University, Okayama University, and Nagoya University Graduate School of Medicine.

SNP selection and genotyping

For LD-based association analysis using the first set of screening samples, we first consulted the HapMap and dbSNP databases to pick-up 'tagging SNPs'. From the HapMap database (Data Release #21: population JPT: MAF of >0.05: regions 8275000.8320000 for NUDEL, 2440000.2537000 for *LIS1*, 1193000.1256000 for *YWHAE*, 70823000.70917000 for *GRB2*, 56227000.56266000 for *KIF5A*), we selected a total of 27 tagging SNPs (one SNP for *NUDEL*, six SNPs for *LIS1*, nine SNPs for *YWHAE*, six SNPs for *GRB2* and five SNPs for *KIF5A*) with a threshold criterion of $r^2 > 0.8$ in pairwise tagging mode using Tagger software (47). Two SNPs (one for *NUDEL*, rs3744652 and one for *YWHAE*, rs34041110) were added for denser mapping.

All SNPs were genotyped by TaqMan assays, primer extension using dHPLC and polymerase chain reaction–restriction fragment length polymorphism assays as described previously (48). More detailed assay information can be found in Supplementary Material, Table S1.

Mutation search

After we detected significant association of *YWHAE* in screening samples, we used dHPLC analysis for a mutation search, the details of which are described in a previous paper (48). Primer pairs (Supplementary Material, Table S2) were designed with the use of information from the GenBank sequence (accession number: NT 010718.15) into 10 amplified regions, which covered all the coding regions, the branch sites and the 5′ flanking region 1026 bp upstream from the initial exon of *YWHAE*.

In vivo and *in vitro* expression assays

We used a dual-luciferase assay, real-time RT–PCR and western blot analysis to examine the influence of SNP *rs28365859* in the 5′ flanking region on expression levels of *YWHAE*. For the dual-luciferase assay, 497-bp fragments that included *rs28365859* were PCR amplified (Supplementary Material, Table S1). Genomic DNAs with identified genotypes were used as templates, and PCR products of either genotypes were cloned into a pGL3-basic vector and a pGL3-promoter vector (Promega, WI). These vectors with both alleles, the Renilla luciferase vector and the phRL-TK vector, were transiently transfected into Chinese hamster ovary (CHO) cells and COS-7 cells with the use of Lipofectamine 2000™ (Invitrogen, CA). All inserts were sequenced to confirm the containing alleles. After 48 h, cell extracts were prepared and assayed for firefly LA (LA_F) and Renilla LA (LA_R) as described by the manufacturer (Pikka-Gene Dual SeaPansy™ Luminescence Kit, Tokyo Ink, Japan) on a Fluoroskan Ascent FL (Thermo Labsystems, Finland).

For *in vitro* assays (real-time RT–PCR and western blot analysis), we processed and analyzed a total of 27 peripheral blood samples from normal control subjects to determine the amount of *YWHAE* transcript or protein: 16 subjects with homozygous major alleles (G/G genotype: 7 male and 9 female; 32.6 ± 6.4 years) in *rs28365859*; 8 subjects with heterozygous major alleles (G/C genotype: 4 male and 4 female; 33.5 ± 7.7 years) and 3 subjects with homozygous minor alleles (C/C genotype: 1 male and 2 female; 51.3 ± 17.0 years). These subjects were healthy controls who had not received any medication within at least 1 month before the collection of RNA and protein.

In the real-time RT–PCR assay, total RNA was isolated with the use of a QIAamp RNA Blood Mini Kit (QIAGEN, Inc., CA). Complementary DNA was generated with the use of a High-Capacity cDNA Archive Kit (Applied Biosystems). Real-time PCR constituents were 50 ng DNA, 2× TaqMan Universal Master Mix and 20× primer/probe mix (Hs00356749_g1, Applied Biosystems) in a 50-μl final volume. The amplification was done according to the manufacturer's instructions, and signals were recorded during PCR with the use of an ABI PRISM 7900 instrument. All gene expression results were normalized to the expression of glyceryl aldehyde-3-phosphate dehydrogenase (*GAPDH*).

In the western blot analysis, lymphocytes were purified (Axis-Shield, Oslo, Norway) and protein concentrations were determined with bovine serum albumin as the reference protein. The antibody against 14-3-3epsilon and alpha-tubulin were purchased (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Proteins were subjected to SDS–PAGE, followed by immunoblot analyses with anti-14-3-3epsilon or anti-alpha-tubulin antibody. The amount of 14-3-3epsilon was detected by chemiluminescence in a linear range using serial dilutions of standards and was estimated with Densitograph (ATTO, Tokyo, Japan). Alpha-tubulin was used as the standard for quantification. The results of these *in vivo* and *in vitro* expression assays were representative of three independent experiments.

Animals and experimental design

Ywhae$^{+/-}$ mice and their wild-type littermates were obtained as previously reported (29). Genetic background of mice is mixed 129/S6 × NIH Black Swiss. All behavioral tests (8-arm radial maze test, elevated plus-maze test, T-maze test, light/dark transition test and startle response/prepulse inhibition tests) were carried out with male mice that were 9–10 weeks old at the start of the testing. Heterozygous knockout mice and wild-type littermates were compared in experiments. Mice were housed in a room with a 12-h light/dark cycle (lights on at 7:00 a.m.) with access to food and water ad libitum. Behavioral testing was performed between 9:00 a.m. and 6:00 p.m. After the tests, all apparatus was cleaned with super hypochlorous water to prevent a bias on the basis of olfactory cues with the apparatus. Detailed description of each behavioral test (neurological screen, neuromascular strength, rotarod test, open-field test, light/dark transition test, elevated plus-maze test, hot plate test, startle response/prepulse inhibition tests, social interaction test in a novel environment, sociability and social novelty preference test, social interaction test in home cage, T-maze test and contextual and cued fear conditioning) can be seen in Supplementary methods.

Statistical analysis

Tests for HWE and marker-trait association were evaluated by χ^2 test (SAS/Genetics, release 8.2, SAS Institute Japan Inc., Tokyo, Japan). Gene-wide significance of single-SNP test was estimated by permuting phenotype status to generate 10 000 data set of SNPs in each gene under null hypothesis of no association (49). Differences in relative expression between alleles (for luciferase assay) and genotypes (for real-time PCR and western blot) were evaluated by a two-tailed Student's *t*-test and one-way ANOVA, respectively (JMP5.1J, SAS Institute Japan Inc.). When a significant difference was obtained in ANOVA, *post hoc* comparison with the Dunnett test [with homozygous major alleles (G/G genotype) set as controls] was used to identify specific group differences. Also to check the effects of haplotypes on gene expression, haplotype trend regression test with permutation (10 000 times) was applied (Power Marker V3.25 by Jack Liu, www://power-marker.net/). In behavior analysis, statistical analysis was conducted by using STATVIEW (SAS Institute, Cary, NC). Data were analyzed by ANOVA or repeated-measures ANOVA. Values in graphs were expressed as mean ± SEM. The level of significance was set at 0.05.

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SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* Online.

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Variants of dopamine and serotonin candidate genes as predictors of response to risperidone treatment in first-episode schizophrenia

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Aims: Abnormalities in dopaminergic and serotonergic transmission systems are thought to be involved in the pathophysiology of schizophrenia and the mechanisms underlying the therapeutic effects of antipsychotics. We conducted a pharmacogenetic study to evaluate whether variants in dopamine-related genes (*DRD1–DRD5*, *AKT1* and *GSK3β*) and serotonin receptor genes (*HTR1A*, *HTR1B*, *HTR1D*, *HTR2A*, *HTR2C*, *HTR6* and *HTR7*) can be used to predict the efficacy of risperidone treatment for schizophrenia.
Materials & methods: A total of 120 first-episode neuroleptic-naive schizophrenia patients were treated with risperidone monotherapy for 8 weeks and clinical symptoms were evaluated by the Positive and Negative Syndrome Scale. **Results:** Among the 30 variants that we examined, two SNPs in *DRD2* (-241A>G [rs1799978] and TaqIA [rs1800497]) and two SNPs in *AKT1* (*AKT1*-SNP1 [rs3803300] and *AKT1*-SNP5 [rs2494732]) were significant predictors of treatment response to risperidone. **Conclusion:** These data suggest that the SNPs in *DRD2* and *AKT1* may influence the treatment response to risperidone in schizophrenia patients.

Keywords: dopamine, pharmacogenetics, risperidone, schizophrenia, serotonin

Schizophrenia is a severe psychiatric disorder with a lifetime risk of 1%. Its pathophysiology is unknown, as are the mechanisms underling therapeutic response to treatment. Similarly, the reasons for variable individual responses to treatment are not known and, at present, the choice of particular antipsychotic treatments for individual patients is effectively a trial and error process. However, since genetic factors contribute to treatment response [1], pharmacogenetic approaches offer at least the potential for predicting treatment response at an individual level

With respect to the use of antipsychotic medication in schizophrenia, pharmacogenetic research has focused on dopamine- and serotonin-related genes. In particular, several groups [1–13] have targeted genes encoding the dopamine D2 receptor (*DRD2*: -141Ins/del [rs1799732] and TaqIA polymorphisms [rs1800497; although TaqIA is now known to be located in another proximal gene: X-kinase or ANKK1]), the D3 receptor (*DRD3*: Ser9Gly [rs6280]), the D4 receptor (*DRD4*: 48-bp repeat in exon III), and serotonin 5-HT2A (*HTR2A*: T102C [rs6313]) and 5-HT1A receptors (*HTR1A*: -1019C/G [rs6295]). However, the results of these studies have been discrepant [1,2]. This may be because variation in each individual gene may have weak effects [14]. It has been postulated that interaction between variants in dopaminergic and serotonergic systems may be of greater magnitude in predicting responses to treatment of schizophrenia [15]. The candidate polymorphisms in such genes should be integrated for precise analysis; therefore, we can evaluate the individual gene effects and gene–gene interaction to antipsychotic treatment.

Many candidate genes have been proposed to be of pharmacogenetic relevance to antipsychotic treatment; compared with typical antipsychotics, second-generation antipsychotics, including risperidone, have lower (to similar) affinity for the D2 receptor and a higher degree of occupancy at four other dopamine receptors (D1, D3, D4 and D5) and at some serotonin receptors (5-HT1A, 1B, 1D, 2A, 6 and 7, and other receptors) [16]. In addition, variation in signaling cascades downstream of D2 receptor blockage may be associated with treatment of schizophrenia [17]. Among these, the AKT/glycogen synthase kinase 3 (GSK3) signaling cascade is a particularly attractive candidate. First, antipsychotics, including risperidone, alter the expression level of GSK3 protein in the rat medial prefrontal cortex and striatum [18]. Second, increased phosphorylation of AKT1 and GSK3β have been reported in mice exposed to haloperidol [19]. Third, in a study of dopamine transporter knockout and wild-type mice, AKT1–GSK3β signaling cascades partially mediated DA-dependent behaviors in response to manipulation by exposure to lithium and amphetamine [20]. Lastly, genetic aspects of susceptibility to schizophrenia and antipsychotic response may be closely associated [21]. This is of

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relevance to AKT1–GSK3β signaling, since several case–control and family-based association studies have provided some evidence for association between *AKT1* and schizophrenia [19,22–26], although the findings are not universal [27–30].

The aim of this study was to conduct a pharmacogenetic study of risperidone response and variants in genes encoding dopamine and serotonin receptors (*D1–D5, HTR1A, HTR1B, HTR1D, HTR2A, HTR2C, HTR6* and *HTR7*), *AKT1* and *GSK3β*. In a small sample, we previously reported that diplotypes at *DRD2* were associated with clinical performance after risperidone treatment, although no association was found between risperidone response and gene variants in *5-HT2A* and *COMT* [12]. In this study, we expanded the sample size of first-episode neuroleptic-naive samples (from 31 to 120 patients) and controlled for nongenetic factors such as clinical charactistics and environmental variables (gender, age, duration of untreated psychosis [DUP] and baseline Positive and Negative Syndrome Scale [PANSS] total score) by multiple linear regression analysis.

Materials & methods

Subjects & collection of clinical data

In total, 131 first-episode, neuroleptic-naive schizophrenic patients were included in this open-label pharmacogenetic study. For *DRD2* and *HTR2A*, 31 patients were the same as those included in our previous report [12]. We excluded ten patients whose DUP was longer than 5 years in accordance with another study [31]. Genotypes could not be determined in one subject, and this patient was also excluded. Consequently, 120 patients were analyzed.

Patients were entered into the study if they met diagnostic and statistical manual of mental disorders (DSM)-IV-TR criteria for schizophrenia, were physically healthy and had all laboratory parameters within normal limits, or if they had neither current nor past DSM-IV-TR diagnosis of mood disorders or substance abuse. Consensus diagnoses were made by at least two experienced psychiatrists on the basis of unstructured interviews with patients and families and review of medical records. DUP was defined as the period from the onset of psychotic symptoms to that of first antipsychotic exposure.

Subjects received risperidone monotherapy (starting dosage: 0.5–4 mg/day; mean starting dosage: 2.5 mg) and dosage was adjusted in accordance with patients' symptoms by trained psychiatrists (1–8 mg/day; mean dosage: 3.4 mg at 8 weeks) for 8 weeks. Patients with insomnia were prescribed brotizolam 0.25 or 0.5 mg at bedtime. No other psychotropic drugs were permitted.

Clinical symptoms were evaluated at the first visit and after 8 weeks of treatment by the use of the PANSS. Evaluations were carried out by trained psychiatrists and a psychologist (interrater reliability: intraclass correlation coefficient [ICC's] = 0.90 [Yamanouchi & Iwata, Unpublished Data]).

The clinical characteristics of subjects that were used as potential covariates in the stepwise linear regression analysis were selected from a previous paper [32]: gender (58 male, 62 female), age (31.2 ± 8.7 years), DUP (13.7 ± 11.4 months) and baseline PANSS total score (79.1 ± 20.5).

All patients were unrelated and were ethnically Japanese. After explanation of the study, written informed consent was obtained from each subject. This study was approved by the Ethics Committee at Fujita Health University, University of Occupational and Environmental Health and Nagoya University Graduate School of Medicine.

Variant selection & genotyping

In total, 30 variants were selected from previous studies (**Table 1**). We specifically targeted potential functional polymorphisms and those which were previously associated with treatment response or with schizophrenia itself. These include: four SNPs for *DRD1* [33]; three variants for *DRD2* [12,34]; two SNPs for *DRD3* [35]; four variants for *DRD4* [36,37]; one SNP for *DRD5* [38]; five SNPs for *AKT1* [22]; two SNPs for *GSK3B* [39]; one SNP for *HTR1A* [40]; one SNP for *HTR1B* [41]; one SNP for *HTR1D* [42]; one SNP for *HTR2A* [12]; two SNPs for *HTR2C* [43,44]; one SNP for *HTR6* [10]; and two SNPs for *HTR7* [45]. Genotyping methods can be seen in **Table 1** and primer sequences are available on request.

In *DRD1*, -1251*HaeIII* was not polymorphic while the three other SNPs (-800HaeIII, -48DdeI and +1403Bsp1286I) were in absolute linkage disequilibrium (LD; $r^2 = 1$) in our sample. Thus, we included only -800HaeIII in *DRD1* to the following regression analysis. At *DRD3*, -205G>A and Ser9Gly were similarly in LD ($r^2 = 1$), so we analyzed only Ser9Gly (**Table 1**). For the 48-bp repeat in exon III of *DRD4*, the allele frequencies of the variant differ considerably between populations, and the seven-repeat allele is quite rare in the Japanese population. Therefore, alleles with five or more repeats were grouped with the long L allele in accordance with another study [36]. For

Table 1. Distribution of genotypes and direct association between PANSS improvement and genotypes.

Gene symbol	SNP ID	Methods	n	Genotype			p-value*
				M/M	*M/m*	*m/m*	
DRD1‡	-1251HaeIII (G>C)	PCR-RFLP	120	120	0	0	NA
	-800HaeIII (C>T)	PCR-RFLP	120	99	21	0	0.686
DRD2	-241A>G	PCR-RFLP	120	96	24	0	0.447
	-141 Ins/Del (Ins>Del)	PCR-RFLP	120	90	30	0	0.435
	TaqIA (A2>A1)	PCR-RFLP	120	54	54	12	0.0239§
DRD3¶	Ser9Gly (Ser>Gly)	PCR-RFLP	120	60	55	5	0.989
DRD4	120 bp duplication (L>S)	PCR-RFLP	120	73	44	3	0.403
	-616G>C	Direct sequencing	120	56	57	7	0.193
	-521T>C	Direct sequencing	120	38	66	16	0.925
	48bp repeat in exon III (S>L)#	PCR	120	114	6	0	0.969
DRD5	1481C>T	PCR-RFLP	120	62	46	12	0.456
AKT1	SNP1 rs3803300 (G>A)	PCR-RFLP	120	34	56	30	0.102
	SNP2 rs1130214 (G>T)	PCR-RFLP	120	71	45	4	0.949
	SNP3 rs3730358 (C>T)	PCR-RFLP	120	89	31	0	0.676
	SNP4 rs2498799 (A>G)	PCR-RFLP	120	32	60	28	0.210
	SNP5 rs2494732 (C>T)	PCR-RFLP	120	65	44	11	0.0286§
GSK3B	SNP6 rs1574154 (C>T)	PCR-RFLP	120	37	56	27	0.525
	SNP8 rs2037547 (C>T)	PCR-RFLP	120	106	14	0	0.844
HTR1A	-1019C>G	TaqMan®	120	73	45	2	0.799
HTR1B	861G>C	PCR-RFLP	120	25	73	22	0.151
HTR1D	rs674386	TaqMan®	120	64	50	6	0.597
HTR2A	102T>C	PCR-RFLP	120	31	58	31	0.948
HTR2C	-759C>T	TaqMan®	120	105	7	8**	0.315
	-697C>G	TaqMan®	120	104	8	8**	0.222
HTR6	267C>T	PCR-RFLP	120	60	56	4	0.580
HTR7	SNP2 rs3808932	Primer extension	120	74	33	13	0.535
	SNP5 rs12412496	PCR-RFLP	120	60	40	20	0.0437§

p-value for direct association (analysis of variance).

‡*-48Ddel and +1403Bspl1286I were in absolute LD.*

§*Significant p-values.*

¶*-205G>C was in absolute LD.*

#*Five patients had 2/2 repeat, 25 patients had 4/2 repeat, 84 patients had 4/4 repeat, five patients had 4/5 repeat, one patient had 4/7 repeat.*

**Hemizygotes.*

LD: Linkage disequilibrium; M: Major allele; m: Minor allele; NA: Not analyzed; PANSS: Positive and Negative Syndrome Scale.

the two SNPs (-759C>T and -697C>G) in *HTR2C* that are located on the X chromosome, we applied a dominant genetic model to the following regression analysis: wild-type homozygote and the combined group of heterozygotes and mutant homozygotes (however, there is no sample with mutant homozygotes in our sample: eight samples in men have hemizygotes both of -759C>T and -697C>G. Seven and eight samples in women have heterozygotes of -759C>T and -697C>G, respectively).

Statistical analysis

All SNPs were tested for deviation from the Hardy–Weinberg equilibrium using an exact test (SAS/Genetics, release 8.2, SAS Institute Inc., Tokyo, Japan).

To check first if there was evidence for association between PANSS improvement and genotype, one-way analysis of variance (ANOVA) was applied (JMP, 6.J, SAS Institute Inc.). We next performed a stepwise backward selection procedure with a p-value threshold of 0.10 for excluding

covariates. In those analyses, the dependent variable was improvement rate in total scores of PANSS (calculated as shown below) and the independent variables included genotype, gender, age, DUP and baseline PANSS total score (JMP, 6.J).

$$\text{Improvement rate} = \frac{\left(\text{PANSS at week 0}\right) - \left(\text{PANSS at week 8}\right)}{\text{PANSS at week 0}}$$

For the baseline PANSS score, we expected that total symptoms, positive symptoms, negative symptoms and general psychopathology would be correlated. Therefore, we initially calculated the correlation by Spearman's rank correlation test. We also selected total PANSS score as the covariate due to its generality (all correlations between the total PANSS score and the other subscores showed significance, but other combinations were not always significant [data not shown]).

When the significant variables were obtained, the adjusted means of improvement rate in PANSS score for each genotype were estimated by the method of least squares, and the protected least square difference test was used to compare individual groups. The significance level for all statistical tests was set at a p-value of less than 0.05.

Results

The distributions of all SNPs were within the values expected from Hardy–Weinberg equilibrium.

The improvement in total PANSS scores followed a normal distribution (Shapillo–Wilk test: $W = 0.991$; $p = 0.629$). Using one-way ANOVA, we found that three polymorphisms (TaqIA, *AKT1*-SNP5 and *HTR7*-SNP5) were nominally associated with PANSS improvement (Table 1). By contrast, the stepwise regression analysis suggested that two SNPs in *DRD2* (-241A>G polymorphism: $p = 0.031$; TaqIA polymorphism: $p = 0.0075$) and two SNPs in *AKT1* (*AKT1*-SNP1: $p = 0.018$; *AKT1*-SNP5: $p = 0.02$) were significant contributors (Table 2). Considering clinical background, only the baseline PANSS total score was a significant contributor ($p = 0.0058$) to predicting response (worse scores at 0 week showed better response). We found no difference in diagnosis subtypes between genotype groups for the four SNPs that were found to be predictors for treatment response (data not shown).

To evaluate the quantitative risk of predictor genotypes, the adjusted improvement rate among each genotype was compared by *t*-statistic. The patients with the A/A genotype in -241A>G, the A1/A1 genotype in TaqIA or the T/T genotype in AKT1-SNP5 showed significantly better improvement in total PANSS score than those without the above genotypes in each SNP. For AKT1-SNP1, individuals with the G/A genotype showed a significantly better improvement than patients with the A/A genotype, and a nearly significant improvement compared with those with the G/G genotype ($p = 0.0697$) (Figure 1).

Discussion

In this study, we found that SNPs in *DRD2* and *AKT1* (-241A>G [rs1799978] and TaqIA [rs1800497] in *DRD2*, and *AKT1*-SNP1 [rs3803300] and *AKT1*-SNP5 [rs2494732] in *AKT1*) were significant predictors of the improvement in total PANSS score after risperidone treatment.

However, we found a number of disagreements between the ANOVA and the regression analyses (-241A>G, *AKT1*-SNP1 and *HTR7*-SNP5 in *HTR7* [rs12412496]), although two findings, TaqIA and *AKT1*-SNP5 were consistent between the two analyses. It is not surprising, and we hypothesize that this is derived from the effects of gene–gene or gene–environmental interaction, since multiple linear regression analysis can reflect or adjust the above effects.

Our data support a recent report regarding an association between -241A>G in *DRD2* and treatment response for schizophrenia [11], but do not support previous significant associations between other polymorphisms in dopamine- and serotonin-related genes [1]; in the Chinese population, the 'A' allele of the -241A>G polymorphism is the predictor allele of better treatment response to risperidone [11]. Our data replicated this finding, suggesting that it might be a true predictor for risperidone treatment in Asian populations in general. However, we need further replications for clinical use. In another study, the authors found that the 'G' allele (and -141Ins/Ins homozygote) is associated with a faster response time to antipsychotic treatment [9], but we cannot directly compare our present results with this finding, as our study did not contain multiple longitudinal assessment points.

Our previous pharmacogenetic study suggested that diplotypes in *DRD2* were associated with clinical response: compared with patients with the Ins-A2/Ins-A2 diplotype, PANSS total scores of patients with Ins-A2/Del-A1 showed 40% greater improvement [12]. In the present study, we decided not to use this type of analysis

Table 2. Genotypes and other clinical backgrounds associated with improvement rate in PANSS total score by a stepwise backward selection procedure.

Variable	p-value
Baseline PANSS total score (one-point increments)	0.0058
DRD2 -241A>G	0.0311
DRD2 TaqIA	0.0075
AKT1-SNP1 rs3803300	0.0183
AKT1-SNP5 rs2494732	0.0201

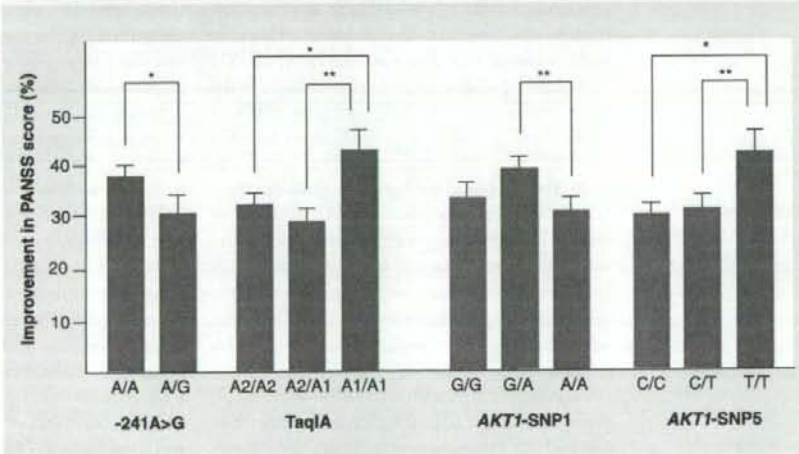
r^2: 0.25.
PANSS: Positive and Negative Syndrome Scale.

as a primary method because of weak LD between these variants (D': 0.38; r^2: 0.045), but here we note that we undertook such an analysis but we could not confirm our previous findings (data not shown). The reasons for the discrepancies may derive from the background of the target samples; in our previous study, the subjects in the 'New' and 'Switched' groups were combined in order to increase the sample size, which might have caused a heterogeneous sample. By contrast, the present study included only first-episode, neuroleptic-naive subjects, resulting in pure phenotypes. In addition, the larger sample size in this study provided more statistical power than that in our previous study. In the previous report, TaqIA did not reach significance as a predictor (p = 0.08 [Yamanouchi & Iwata, Unpublished Data]), which might have been due to a type II error. However, a very likely explanation is that our previous positive report represented a type I error.

Concerning *AKT1*, it is an interesting finding that the identical variant, SNP5, may be a schizophrenia-susceptibility SNP [22] and a predictor SNP for risperidone treatment in schizophrenic patients. A recent pharmacogenetic study [25] showed no relationship between any of the tested SNPs in *AKT1* and therapeutic response to first- or second-generation antipsychotics. However, differences in LD among populations may also be responsible for such inconsistent results.

Emamian and colleagues showed that certain haplotypes (combination of SNP2 and 3) influenced the expression level of *AKT1* [19], but whether SNP1 or SNP5 are associated with function is unclear. To examine this, we developed a real-time reverse transcriptase PCR assay using lymphocyte-derived cell lines (25 subjects with schizophrenia and 25 age-matched healthy controls) to measure the expression of *AKT1* and *DRD2*. However, we found no evidence for association between any genotype and expression phenotype (data not shown). Since *AKT1* is an important intermediary between the D2 receptor and GSK3 proteins [19,20], *AKT1*-SNP5 and *AKT1*-SNP1 and/or SNPs in *DRD2* (-241A>G and TaqIA) may influence several signaling cascades in a gene–gene interaction manner.

Figure 1. Adjusted mean improvement rate in total PANSS score stratified by genotype in possible predictor SNPs.



Data shown are the mean ± SE. *p < 0.05; **p < 0.01.
PANSS: Positive and Negative Syndrome Scale.

Our study has a number of limitations. First, since we did not correct the p-values for multiple testing and sample size is relatively small, the results must be considered hypothesis generating and preliminary, and our nominally significant data need to be confirmed by independent samples. Second, we selected the patients who had completed 8 weeks of treatment, which might yield a false positive due to sampling bias as an open-label study. Third, we did not perform a comprehensive evaluation of any of the genes, for example, through a systematic LD-based study (e.g., tagging SNPs). Last, we did not detect the functional effect of these significant polymorphisms or did not include the fine mutation scan to search the actual causal variants. Therefore, the relationship between *DRD2* and *AKT1* polymorphisms and their functional relevance needs further study.

Conclusion & future perspective

We found predictor SNPs in *DRD2* and *AKT1* for risperidone treatment in schizophrenia patients. Further studies will be required for conslusive results, however, this evidence may lead to an improvement of response prediction and/or treatment selection for antipsychotics.

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved

Executive summary

Risperidone response & dopamine- & serotonin-related genes

- In total, 120 first-episode neuroleptic-naive schizophrenic patients were examined to see if variants in dopamine-related genes (*DRD1–DRD5*, *AKT1* and *GSK3β*) and serotonin receptor genes (*HTR1A*, *HTR1B*, *HTR1D*, *HTR2A*, *HTR2C*, *HTR6* and *HTR7*) predicted the efficacy of risperidone treatment for schizophrenia.
- To reflect the gene–gene and gene–environmental interactions, multiple linear regression analysis was applied.

SNPs in* DRD2 & AKT1 *were associated with risperidone response

- Two SNPs in *DRD2* (-241A>G and TaqIA) and two SNPs in *AKT1* (*AKT1*-SNP1 and *AKT1*-SNP5) were significant predictors of treatment response to risperidone.
- These data suggest that SNPs in *DRD2* and *AKT1* may influence treatment response to risperidone in schizophrenia patients.

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PHARMACOGENETICS

Genetic and pharmacokinetic factors affecting the initial pharmacotherapeutic effect of paroxetine in Japanese patients with panic disorder

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Abstract
Objective The objective of this study was to evaluate genetic and pharmacokinetic factors affecting the initial pharmacotherapeutic effect of paroxetine (PAX) in Japanese patients with panic disorder (PD).
Method Plasma concentration of PAX was determined by high performance liquid chromatography. Serotonin transporter gene-linked polymorphic region (5-HTTLPR) variants were determined by polymerase chain reaction techniques. PD severity was assessed using the Panic and Agoraphobia Scale (PAS).
Results Multiple regression analysis revealed that the plasma concentration of PAX, 5-HTTLPR genotype, and comorbid physical illness were significant factors affecting the initial pharmacotherapeutic effect of PAX in PD and indicated that these factors accounted for 52.4% (R^2=0.524) of the variability in the percent reduction in PAS score. The final model was described by the following equation (P=0.001): percent reduction in PAS score (%) = 68.5 − 1.2 × [plasma concentration of PAX (ng/ml)] − 33.0 × (L/S=1, S/S=0) − 21.8 × (with comorbid physical illness=1, without comorbid physical illness=0).
Conclusion The high plasma concentration of PAX, the L/S genotype of 5-HTTLPR, and comorbid physical illness might be associated with a poor response to the initial phase of pharmacotherapy of PD with PAX.

Keywords Pharmacotherapy · Panic disorder · Paroxetine · 5-HTTLPR · SSRIs

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Introduction

Panic disorder (PD) is characterized by sudden onset and repeated episodes of intense apprehension, fearfulness, or terror accompanied by physical symptoms that may include chest pain or discomfort, heart palpitations, shortness of breath, choking or smothering sensations, dizziness, or abdominal distress. The prevalence of PD has been estimated at 1–3% [1]. A gender difference has also been observed, with greater prevalence in females than in males [2]. Moreover, family and twin studies have suggested genetic liability for PD [3–5]. In a meta-analysis study, PD heritability was estimated to be about 48% [6].

Selective serotonin reuptake inhibitors (SSRIs) are thought to interact with the serotonergic nervous system and are believed to be effective for the treatment of PD. The efficacy of SSRIs including paroxetine (PAX) in the treatment of PD has been established in several placebo-

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controlled trials [7–11], indicating that SSRIs have been recognized as the first-line agents for the treatment for PD.

Sandmann et al. investigated the relationship between the plasma level of fluvoxamine, one of the representative SSRIs, and the clinical response to fluvoxamine (50–300 mg/day, 1–5 weeks) in 16 PD patients in accordance with the DSM-III-R criteria [12]. In their study, six out of eight patients treated with fluvoxamine who showed full remission of panic attacks had a plasma level below 100 ng/ml [12], suggesting that increasing the plasma level of SSRIs might be associated with poor clinical effect in PD patients.

In accordance with the study of Sandmann et al. [12], Watanabe et al. reported that increasing the plasma concentration of PAX might be associated with poor clinical effect in PD patients [13]. Specifically, they investigated the clinical improvement of 21 unrelated Japanese patients who fulfilled the DSM-IV-TR criteria for a diagnosis of PD and who were treated with PAX (10 mg/day) for 2 weeks as initial treatment. Improvement of PD symptoms was assessed using the Panic and Agoraphobia Scale (PAS). In the range of plasma concentrations of PAX > 20 ng/ml, none of the subjects showed a percent reduction in PAS score > 20%. The subjects whose plasma concentration of PAX was < 20 ng/ml had a significantly higher mean percent reduction in PAS score than those whose plasma concentration of PAX was > 20 ng/ml. Multiple regression analysis showed that the plasma concentration of PAX was the only significant factor affecting the percent reduction in PAS score and accounted for 28.0% of the variability in the percent reduction in PAS score of the subjects. The final model of correlation was as follows: percent reduction of PAS score= 42.3−0.9 × [plasma concentration of PAX (ng/ml)] [R=0.529, P=0.014, coefficient of determination (R^2) = 0.280]. Assuming that the percent reduction in PAS score was 20% in the equation above, the plasma concentration of PAX is calculated to be about 25 ng/ml, which is suggested to be the upper end of the therapeutic window for the initial phase of PD treatment with PAX.

Meyer et al. reported a relationship between the serum concentration of PAX and the proportion of serotonin (5-HT) transporter (5-HTT) sites blocked. Striatal 5-HTT binding potential was measured with [^{11}C](N,N-dimethyl-2-(2-amino-4-cyanophenylthio) benzylamine ([^{11}C]DASB) and by positron emission tomography (PET) before and after 4 weeks of treatment with PAX. 5-HTT occupancy increased in a nonlinear fashion with the serum concentration of PAX such that its plateau of occupancy occurred at around 85% for a serum concentration of PAX > 28 ng/ml [14], suggesting no accumulation of clinical effect after reaching 28 ng/ml.

5-HTT removes serotonin from the synaptic cleft, and this protein is the primary target of action of SSRIs. The 5-HTT gene-linked polymorphic region (5-HTTLPR), which is located in the promoter region, has been identified as a functional polymorphism. The polymorphism consists of a 44-base-pair insertion or deletion involving repeat elements 6 to 8 [15]. In vitro, the basal activity of the long variant (L) was found to be more than twice that of the short variant (S) in 5-HTT mRNA synthesis and 5-HTT expression [15, 16]. These two different transcriptional efficiencies suggest that 5-HTT gene transcription is modulated by 5HTTLPR genetic variants [15, 16]. However, association studies have reported the absence of a significant difference in the 5-HTTLPR allele frequencies between PD subjects and normal controls [17–20].

Recent investigations have also been focused on the impact of genetic polymorphisms of 5-HTT on the clinical effect of SSRIs in PD because 5-HTT is the primary target of action of SSRIs. Pena et al. investigated the relationship between the allelic variation of 5-HTT and the clinical response to PAX in 92 PD patients who completed treatment with variable doses of PAX for 12 weeks. Both homozygotes for the long variant (L/L) of the 5-HTT promoter and heterozygotes (L/S) showed a better response to PAX than homozygotes for the short variant (S/S) (P< 0.03). This result was observed in the whole sample but was related to only female patients (P<0.02) [21].

In the present study, we investigated the association between therapeutic response to PAX 2 weeks after treatment initiation and the plasma concentration of PAX, 5-HTTLPR, and other clinical factors in Japanese PD patients.

Patients and methods

Patients

Thirty-eight unrelated Japanese patients who met the DSM-IV-TR criteria for a diagnosis of PD and who were receiving PAX (10 mg/day) participated in the present study. They were all drug-naïve outpatients at Dokkyo Medical University Hospital. The age of the patients (male=11, female=27) ranged from 21 to 72 years (mean±SD=34.3±9.8 years). Body weight ranged from 40 to 95 kg (57.3±11.2 kg). Six patients had comorbid major depressive disorder. The exclusion criteria of the present study were as follows: (1) axis I diagnosis other than PD and major depressive disorder, (2) presence of axis II diagnosis, (3) major laboratory abnormalities, (4) suicidal risk, (5) history of substance abuse, (6) use of antidepressants, antipsychotics, and benzodiazepines before the study, or (7) pregnancy. Written informed consent was obtained from each patient after the procedure was fully explained. The study protocol was approved by the Ethics Committee of Dokkyo Medical University Hospital.