

## ORIGINAL ARTICLE

# Genome-wide association study of panic disorder in the Japanese population

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Panic disorder (PD) is an anxiety disorder characterized by panic attacks and anticipatory anxiety. Although a number of association studies have been conducted, no gene has been identified as a susceptibility locus. In this study, we conducted a genome-wide association study of PD in 200 Japanese patients and the same number of controls, using the GeneChip Human Mapping 500 K Array Set. Genotypes were determined using the Bayesian Robust Linear Model with Mahalanobis (BRLMM) genotype calling algorithm. The genotype data were data-cleaned using criteria for SNP call rate ( $\geq 95\%$ ), Hardy–Weinberg equilibrium ( $P \geq 0.1\%$ ) and minor allele frequency ( $\geq 5\%$ ). The significance level of the allele  $P$ -value was set at  $1.0 \times 10^{-6}$ , to make false discovery rate (FDR)  $< 0.05$ . As a result, seven SNPs were significantly associated with PD, which were located in or adjacent to genes including *PKP1*, *PLEKHG1*, *TMEM16B*, *CALCOCO1*, *SDK2* and *CLU* (or *APO-J*). Studies with other samples are required to confirm the results.

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## INTRODUCTION

Panic disorder (PD) is an anxiety disorder characterized by panic attacks and anticipatory anxiety. A life-time prevalence of PD is 1–3% and the female/male ratio among the affected is 2:1.<sup>1</sup> Family and twin studies have consistently indicated that genes play a role in the etiology of PD. Although a number of association studies have been conducted, no gene has been identified as a susceptibility locus.

So far, in association studies, the candidate-gene approach has been employed. Particular attention has been addressed to anxiety-related genes, such as *HTR 1A*, *2A*,<sup>2–4</sup> *CCK*,<sup>5,6</sup> *ADORA2A*,<sup>7</sup> *COMT*<sup>8–10</sup> and *MAOA*.<sup>11</sup> Most studies have focused on genes related to neurotransmitter systems as mentioned above. However, these studies have produced conflicting results.

Genome-wide association studies (GWAS) have become feasible because of the recent development of a microarray system that enables the simultaneous genotyping of several hundred thousands of SNPs. GWAS has been a powerful approach to the identification of genes involved in common human diseases without selecting candidate genes. A joint GWAS has been undertaken in the British population, and it examined seven major complex diseases including bipolar disorder, coronary artery disease, Crohn's disease, rheumatoid arthritis and type 1 and 2 diabetes.<sup>12</sup> Regarding PD, Erhardt *et al.* (2007)

reported that they were conducting a GWAS in 260 patients with anxiety disorders (mostly with PD) and 260 healthy controls using the Illumina BeadArray technology, but their result has not been published. No other association study has thus far performed a genome-wide search for susceptible loci of PD. In this study, we conducted a GWAS of PD in the Japanese population using a 500 K SNP chip.

## SUBJECTS AND METHODS

### Subjects

Subjects comprised 200 patients with PD (64 male and 136 female patients; age,  $39.5 \pm 9.4$  years, mean  $\pm$  s.d.) and the same number of healthy control subjects (64 male and 136 female patients; age,  $38.2 \pm 9.3$  years). All patients and control subjects were ethnically Japanese. The patients were recruited from two clinics for anxiety disorder in Tokyo and Nagoya, Japan. Nagoya is in the central area of Japan, 300 km apart from Tokyo. The patients were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV),<sup>13</sup> and confirmed using the Mini International Neuropsychiatric Interview (M.I.N.I.) and reviewing the medical records. Healthy control subjects were mostly hospital staff who were recruited in Tokyo, Nagoya and the adjacent areas. The controls received a short interview by one of the authors and filled out a questionnaire to exclude those with a clinical history of psychiatric illness. The objective of this study was explained clearly and written informed consent was obtained from all subjects. The study was approved by the Ethical Committee of the Faculty of Medicine, the University of Tokyo.

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## Genotyping

The genomic DNA was extracted from peripheral leukocytes by standard procedures. Genotyping was conducted using the GeneChip Human Mapping 500K Array Set (Affymetrix, CA, USA). A total of 500 568 SNPs on the chip were genotyped. This array set consisted of two chips (*StyI* and *NspI*) with approximately 250 000 SNPs on each chip. Genomic DNA (250 ng) was digested with restriction enzyme (*StyI* or *NspI* for each chip) and processed according to the manufacturer's protocol. The genotyping calls were analyzed using the GCOS1.4 and GTYPE4.1 software packages that employed the Bayesian Robust Linear Model with Mahalanobis (BRLMM) genotype calling algorithm.<sup>14</sup>

## Statistical analysis

Allele frequencies were compared between patients and controls, and deviations from the Hardy–Weinberg equilibrium were calculated using the  $\chi^2$  test. Statistical analyses were conducted using software developed for the analysis of large amounts of genotype data (GeneChipViewer, Dynacom, Japan).

We removed one control subject for the 'Sty' chip and one patient both for the 'Sty' and the 'Nsp' chips because the concentration of PCR products did not reach the expected level in the genotyping process. After the genotyping was completed, data cleaning was conducted as follows: (1) SNPs with a call rate of <95% in patients or controls were excluded, and (2) SNPs with a *P*-value of <0.1% for Hardy–Weinberg's equilibrium in the controls were excluded. (3) Then, the criterion regarding minor allele frequency (mAF) was determined as follows: the ratios of the observed and expected (by chance) numbers of SNPs with several levels of allele *P*-values ( $1.0 \times 10^{-2}$ ,  $10^{-3}$  and  $10^{-4}$ ) were evaluated among SNPs with mAF of  $\geq 1$ , 5, 10% in all samples according to Miyagawa *et al.*<sup>15</sup> When the observed numbers of SNPs with *P*-values were considered to be increased too much at a certain mAF compared with the expected numbers, SNPs with that (or a lower) level of mAF may be excluded, because, in that case, the 'significant SNPs' could contain too many false-positive ones. SNPs on the X chromosome were data-cleaned and analyzed statistically by sex.

The significance level of the allele *P*-value was determined by monitoring the level of the false discovery rate (FDR),<sup>16</sup> which is the proportion of false-positive SNPs among all positive (or false-positive plus true-positive) ones. Under the null hypothesis, *P*-values should be uniformly distributed between 0 and 1; FDR methods typically consider the actual distribution as a mixture of outcomes under the null (uniform distribution of *P*-values) and alternative (*P*-value distribution skewed toward zero) hypotheses. FDR was calculated using the Genstat statistical package (BioSS, Scotland, UK). To exclude as many false-positive associations as possible, we set the FDR at <0.05. Under these conditions, the significance level of allele *P*-value was estimated to be  $<1.8 \times 10^{-6}$ . Thus, we tightly set the significance level of the allele *P*-value at  $1.0 \times 10^{-6}$  in this study. For replication study, additional data at the level of the allele *P*-value  $<1.0 \times 10^{-4}$  were also shown.

The statistical power of the sample was calculated on the basis of the published method.<sup>17,18</sup>

## RESULTS

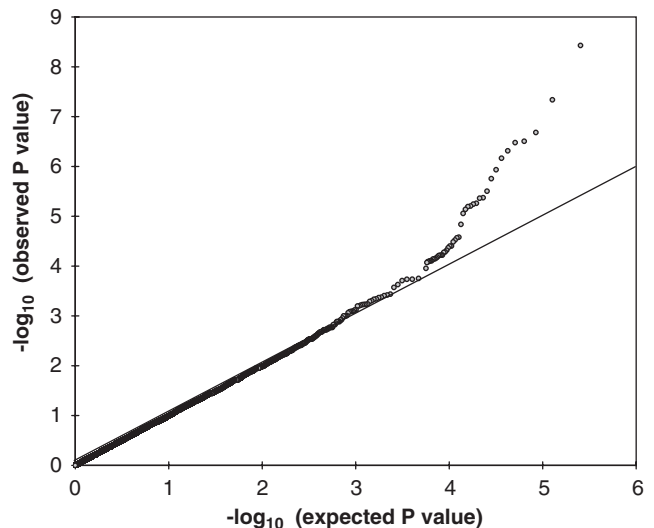
The overall call rates of the genotyping assay were adequate in both patients and controls: 97.3 and 97.3% in controls, 98.0 and 97.7% in patients for the 'Sty' and 'Nsp' chips, respectively. Data cleaning was conducted for 490 032 SNPs on autosomal chromosomes and for 10 536 SNPs on the X chromosome. Among those SNPs, 371 589 SNPs on autosomal chromosomes, 9879 SNPs on the X chromosome in male patients and 8448 on the X chromosome in female patients satisfied the data-cleaning criterion of the call rate  $\geq 95\%$  in both patients and controls. Among these SNPs, 368 721 SNPs on autosomal chromosomes and 8324 on the X chromosome in female patients satisfied the criterion of Hardy–Weinberg's equilibrium in controls ( $P \geq 0.1\%$ ).

Among them, 275 329 SNPs on autosomal chromosomes, 7102 on the X chromosome in male patients and 5921 on the X chromosome in female patients were with mAF  $\geq 1\%$ , 253 903, 6129 and 5110 were with mAF  $\geq 5\%$  and 219 349, 5265 and 4441 were with mAF  $\geq 10\%$ ,

**Table 1** Ratios of the observed and the expected number of SNP at allele *P*-values of  $<1.0 \times 10^{-2}$ – $1.0 \times 10^{-4}$  on autosomal chromosomes

<i>P</i> <	mAF $\geq 1\%$	mAF $\geq 5\%$	MAF $\geq 10\%$
$1.0 \times 10^{-2}$	4278/2753=1.53	3093/2539=1.22	2609/2193=1.19
$1.0 \times 10^{-3}$	601/275=2.19	373/254=1.47	300/219=1.37
$1.0 \times 10^{-4}$	112/27.5=4.07	48/25.4=1.89	28/22=1.27

The numbers of observed SNPs, which passed the data-cleaning criteria (HWE *P*-value  $\geq 0.1\%$  and call rate  $\geq 95\%$  in both patients and controls) were shown.



**Figure 1** Log quantile–quantile (QQ) *P*-value plot for the results after data cleaning. Cleaning criteria were single nucleotide polymorphism (SNP) call rate  $\geq 95\%$ , Hardy–Weinberg equilibrium (HWE) *P*-value  $\geq 0.1\%$  and minor allele frequency (mAF)  $\geq 5\%$ .

respectively. The ratios of the observed/expected number of SNPs on autosomal chromosomes with the allele *P*-values of  $1 \times 10^{-2}$ ,  $10^{-3}$  and  $10^{-4}$  were 1.53, 2.19 and 4.07 for the SNPs with mAF  $\geq 1\%$ , 1.22, 1.47, 1.89 for those with mAF  $\geq 5\%$ , and 1.19, 1.37 and 1.27 for those with mAF  $\geq 10\%$ , respectively (Table 1). According to these data, we employed mAF  $\geq 5\%$  in all samples, considering the balance of possible false-positive and false-negative results. As a result, 253 903 SNPs on autosomal chromosomes, 6129 on the X chromosome in male patients and 5110 on the X chromosome in female patients were included in the present analysis after data cleaning.

The log QQ *P*-value plot was shown after data cleaning using the criteria (SNP call rate  $\geq 95\%$ , HWE *P*-value  $\geq 0.1\%$  and mAF  $\geq 5\%$ ; Figure 1). Plots of *P*-values were close to the expected line ( $y=x$ ). SNPs at the level of the allele *P*-value less than 0.01 are shown in Supplementary Table 1, where 4136 SNPs on autosomal chromosomes that were at the *P* levels in all subjects and 116 SNPs on the X chromosome in female patients were included.

Among these SNPs, seven were significantly associated with PD at a *P*-value less than  $1.0 \times 10^{-6}$ . These SNPs included rs860554, rs9372078, rs17466684, rs12579350, rs941184, rs9302001 and rs3816995 (Table 2). Six of these SNPs were in genes, including *PKP1*, *PLEKHG1*, *TMEM16B*, *CALCOCO1*, *SDK2* and one unknown gene. Another SNP (rs17466684) was within 1 kb of the *CLU* (also known as *Apolipoprotein J*) gene.

**Table 2 SNPs showing significant association with panic disorder ( $P < 1.0 \times 10^{-6}$ )**

dbSNP (rs no.)	Chromosome	Nucleotide position (bp)	mAF		Allele P-value	Gene
			Patients	Controls		
rs860554	1q32	197994089	0.174	0.049	$4.60 \times 10^{-8}$	PKP1
rs9372078	6q25	151006075	0.254	0.114	$4.85 \times 10^{-7}$	PLEKHG1
rs17466684	8p21	27508764	0.214	0.086	$6.82 \times 10^{-7}$	CLU <sup>a</sup>
rs12579350	12p13	5667362	0.099	0.005	$3.73 \times 10^{-9}$	TMEM16B
rs941184	12q13	52400425	0.136	0.034	$3.33 \times 10^{-7}$	CALCOCO1
rs9302001	13q32	94261393	0.359	0.195	$3.12 \times 10^{-7}$	Unknown
rs3816995	17q25	68846855	0.126	0.026	$2.08 \times 10^{-7}$	SDK2

<sup>a</sup>SNPs were mapped in the gene except for rs17466684, which was within 1 kb of CLU.

**Table 3 SNPs showing association with panic disorder at allele P-values between  $1.0 \times 10^{-4}$  and  $1.0 \times 10^{-6}$** 

dbSNP (rs no.)	Chromosome	Nucleotide Position (bp)	mAF		Allele P-value	Gene
			Patients	Controls		
rs2806561	1p36	23250101	0.540	0.398	$6.89 \times 10^{-5}$	LUZP1
rs12061304	1p13.3	110949727	0.018	0.098	$1.17 \times 10^{-6}$	—
rs11102149	1p13.3	110953024	0.020	0.098	$3.14 \times 10^{-6}$	—
rs6587428	1q44	244675256	0.558	0.411	$4.29 \times 10^{-5}$	—
rs6733840	2q14	127376211	0.130	0.241	$6.02 \times 10^{-5}$	Unknown
rs751056	3p26	2975908	0.301	0.434	$9.92 \times 10^{-5}$	CNTN4
rs3749380	3p26	6878297	0.416	0.254	$1.76 \times 10^{-6}$	GRM7
rs3804375	4q23	102493351	0.084	0.018	$3.97 \times 10^{-5}$	PPP3CA
rs17051362	4q26	120945499	0.121	0.043	$7.14 \times 10^{-5}$	—
rs4976138	5q13	67257088	0.083	0.018	$3.27 \times 10^{-5}$	—
rs4702982	5q21	100019547	0.290	0.160	$6.23 \times 10^{-6}$	—
rs6889348	5q22	112993044	0.036	0.111	$7.27 \times 10^{-5}$	Unknown
rs2295767	6p25	2899556	0.206	0.104	$6.58 \times 10^{-5}$	SERPINB6
rs2103868	6p21	47338574	0.129	0.258	$5.48 \times 10^{-6}$	TNFRSF21
rs1853182	6q12	75186104	0.021	0.083	$9.76 \times 10^{-5}$	—
rs1190053	6q22	105851881	0.053	0.135	$8.48 \times 10^{-5}$	PREP
rs12537293	7q31	110026478	0.103	0.033	$8.58 \times 10^{-5}$	IMMP2L
rs2016795	8q12	57552940	0.182	0.086	$7.82 \times 10^{-5}$	Unknown
rs2118261	8q12	57555840	0.185	0.085	$3.95 \times 10^{-5}$	Unknown
rs10867359	9q21	79160248	0.050	0.134	$5.32 \times 10^{-5}$	—
rs7070456	10p12	25007098	0.424	0.289	$8.15 \times 10^{-5}$	ARHGAP21
rs4300338	10q24	99586654	0.141	0.058	$9.15 \times 10^{-5}$	—
rs12420498	11q23	115673824	0.472	0.330	$5.20 \times 10^{-5}$	—
rs11180828	12q12	40009387	0.248	0.138	$7.88 \times 10^{-5}$	—
rs2731006	12q12	41447092	0.448	0.289	$4.24 \times 10^{-6}$	—
rs2731000	12q12	41467541	0.433	0.293	$4.81 \times 10^{-5}$	—
rs12227600	12q12	41492743	0.424	0.280	$2.63 \times 10^{-5}$	—
rs7299940	12q24.33	129915634	0.439	0.286	$7.27 \times 10^{-6}$	Unknown
rs10872898	14q11	20890294	0.143	0.057	$6.08 \times 10^{-5}$	SUPT16H
rs4901869	14q23.1	58403881	0.092	0.018	$6.41 \times 10^{-6}$	—
rs8013992	14q31.1	80859232	0.161	0.063	$1.45 \times 10^{-5}$	RPS24P3
rs755165	15q21.1	43643884	0.087	0.020	$3.00 \times 10^{-5}$	—
rs879153	16q21	62571503	0.116	0.036	$2.71 \times 10^{-5}$	—
rs4499314	18q11	26165113	0.139	0.055	$8.11 \times 10^{-5}$	—
rs1383290	18q12	30627734	0.054	0.139	$6.17 \times 10^{-5}$	DTNA
rs11660890	18q23	71979380	0.384	0.528	$6.04 \times 10^{-5}$	—
rs3810265	19q13.33	54915078	0.173	0.065	$4.34 \times 10^{-6}$	Unknown
rs16989303	20p13	4117079	0.091	0.206	$5.70 \times 10^{-6}$	SMOX
rs4812755	20q13	41966838	0.482	0.343	$7.80 \times 10^{-5}$	—
rs2823455	21q11.2	15991037	0.119	0.034	$8.77 \times 10^{-6}$	USP25
rs132617	22q12	34858285	0.209	0.337	$7.16 \times 10^{-5}$	APOL3
rs16985077 <sup>a</sup>	Xp22.3	8409539	0.274	0.132	$4.17 \times 10^{-5}$	KAL1

<sup>a</sup>SNP rs16985077 was significant when compared between female patients and female controls.

The strongest signal was with rs12579350 (allele  $P$ -value= $3.7 \times 10^{-9}$ ), which was located in the *TMEM16B* gene at 12p13 that may function as transporters for unidentified substrates. The interpretation of this signal may, however, require caution because the mAF of this SNP in controls was quite low (0.5%), whereas it was 9.9% in patients. The second strongest association was with rs860554 at 1q32, which was in *PKP1* ( $P$ -value= $4.6 \times 10^{-8}$ ). The gene has an important role in the cytoskeleton/cell membrane interaction. The protein of *PKP1*, plakoglobin, acts as linker molecules at adherence junctions and desmosome at the plasma membrane. Among the other five SNPs, rs17466684 ( $P$ -value= $6.8 \times 10^{-7}$ ) at the chromosome 8p21 was located in the *CLU* (or *Apolipoprotein J*) gene. This gene may suppress beta-amyloid deposition in the brains of Alzheimer disease subjects. SNP rs3816995 ( $P$ -value= $2.1 \times 10^{-7}$ ) at the chromosome 17q24 was located in the *SDK2* gene, which acts as determinants of lamina-specific synaptic connectivity. SNP rs941184 was located in the *CALCOCO1* gene on 12q13. This protein binds to a highly conserved N-terminal domain and thus enhances transcriptional activation by a number of nuclear receptors.

As additional data, we show SNPs with the allele  $P$ -values between  $1.0 \times 10^{-6}$  and  $1.0 \times 10^{-4}$  in Table 3. Eleven SNPs were at the significance level of  $1.0 \times 10^{-6} \leq P < 1.0 \times 10^{-5}$ . Among the eleven, approximately a half (or six SNPs) was in or adjacent to genes, and the others were not. Thirty-one SNPs were at the significance level of  $1.0 \times 10^{-5} \leq P < 1.0 \times 10^{-4}$ , and a half of them (or 16 SNPs) were in or adjacent to genes, and the rest were not.

## DISCUSSION

We conducted a GWAS of panic disorder (PD) in Japanese, using a 500 K SNP chip. This may be the first report on GWAS of PD, to the best of our knowledge. We employed a set of data-cleaning criteria (SNP call rate  $\geq 95\%$ , HWE  $P$ -value  $\geq 0.1\%$  and mAF  $\geq 5\%$ ) for the data cleaning. The criterion of the mAF was determined considering the ratio of the observed and expected numbers of SNPs at the allele  $P$ -values of  $1 \times 10^{-2}$ – $10^{-4}$  in the comparison between patients and controls. The significance level for the allele  $P$ -value was set at  $P < 1.0 \times 10^{-6}$  by setting the false discovery rate (FDR)  $< 0.05$ .

At the significance level of  $P < 1.0 \times 10^{-6}$ , seven SNPs were associated with PD. Seven SNPs were within ( $n=6$ ) or adjacent ( $n=1$ , within 1 kb) to genes, one of which is unknown. Among the seven SNPs, rs9302001, located on 13q32, is within the area of *PAND1*, which was associated with panic disorder/bladder syndrome.<sup>19</sup> When mAFs in the controls are compared with those in the Japanese subjects in the HapMap database (<http://www.ncbi.nlm.nih.gov/SNP/>), the mAFs are similar in a portion of the SNPs including rs12579350 (mAF=0.5% in controls in this study and 0.0% in Japanese in the HapMap database, respectively) and rs860554 (mAF=4.9 and 6.4% respectively), whereas the mAFs are rather different in the rest of the SNPs. The results in them might be interpreted with caution. The SNP rs12579350 showed the strongest association (allele  $P$ -value= $3.7 \times 10^{-9}$ ) that should however also be interpreted with caution, because the mAF of this SNP was very low ( $< 1\%$ ) in controls in contrast to close to 10% in patients.

As additional data, we showed SNPs at a significance level between  $1.0 \times 10^{-6}$  and  $1.0 \times 10^{-4}$  (Table 3). Forty-two SNPs were found at this level. It is interesting that fewer portions of the SNPs (22 of the 42) were located within the genes when this level of significance was employed, than at the level of  $P < 1.0 \times 10^{-6}$ . The mAFs in the controls are quite similar to those in Japanese in the HapMap database in a portion of the SNPs including rs132617 in *APOL3*

and rs1383290 in *DTNA*, whereas the mAFs are rather different in another portion of the SNPs.

Several limitations may be acknowledged in this study. First, the sample size is limited (200 patients and 200 controls). The statistical power is estimated to be more than 0.9 for  $\alpha=1.0 \times 10^{-6}$ , assuming that the prevalence of PD is 1.4% in the Japanese population, genotypic relative risk is 2.5 and minor allele frequency of the polymorphism is 0.3. For SNPs with less effects (or relative risks) or less allele frequencies, the sample size may be too small to detect the association with PD. Owing to the lack of power, several SNPs that are truly associated with PD might not be detected in this study. Candidate genes that were investigated in earlier association studies were not detected, including *HTR 1A*, *2A*, *ADORA2A*, *COMT* and *MAOA*. This could be due to the lack of power in this study. Second, the number of SNPs in the statistical examination for the association with PD was decreased approximately to a half (or 254 000 SNPs) by the data cleaning. This might also affect the result of the genetic association to a degree, although the estimation is that 300 000 SNPs might capture most of the common genetic variations in a population.<sup>20</sup> Finally, this study is a one-stage investigation, and therefore the SNPs with a significant association may be considered as candidates for future studies with other samples. The GWAS data will be available publicly in the Integrated Database (<http://gwas.lifesciencedb.jp/>).

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