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Association between *PNPO* and schizophrenia in the Japanese population

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Abstract

Accumulating evidence suggests that both homocysteine metabolism and monoaminergic neurotransmitter systems are important in schizophrenia pathology. We hypothesized that the gene *PNPO* (pyridoxine 5′-phosphatase oxidase gene) might be a candidate for susceptibility to schizophrenia because *PNPO* encodes pyridoxamine 5′-phosphate oxidase (EC 1.4.3.5), a rate-limiting enzyme in pyridoxal 5′-phosphate (PLP, vitamin B_6) synthesis. PLP is a metabolically-active form of vitamin B_6 and thus, is required as a co-factor for enzymes involved in both homocysteine metabolism and synthesis of neurotransmitters such as catecholamine. We examined 8 single nucleotide polymorphisms (SNPs) in *PNPO* and its 5′-flanking regions in 359 schizophrenia patients and 582 control subjects. Four marker regions of *PNPO* showed significant levels of allelic associations with schizophrenia (the highest was rs2325751, $P=0.004$). In addition, the haplotype case–control study revealed a significant association (permutation $P<0.00001$) between *PNPO* and schizophrenia. These findings suggest that variations in *PNPO* may contribute to overall genetic risk for schizophrenia in the Japanese population.

Keywords: Association study; Homocysteinemia; PNPO; Schizophrenia; Vitamin B_6

1. Introduction

Schizophrenia affects almost 1% of the world's population, and its etiology remains unknown yet (Bromet and Fennig, 1999). Among the multiple factors contributing to overall susceptibility are risk-conferring genes and as yet undetermined environmental variables. Despite numerous genetic studies, the detailed mechanisms that underlie

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inheritance of this disorder remain unclear. However, several putative susceptibility genes have been identified, although their pathogenic mechanisms are unresolved. Evidence indicating associations between schizophrenia and specific metabolic processes may reveal new candidate genes and provide clues to the etiology of this disorder (Thaker and Carpenter, 2001; Freeman et al., 1975; Lara et al., 2006).

High serum homocysteine levels have been reported in schizophrenic patients (Brown and Susser, 2005) and meta-analysis of eight studies indicated that elevated homocysteine serum levels were associated with a 70% increase in risk of schizophrenia (Muntjewerff et al., 2006). Homocysteine is a sulphur-containing amino acid that is derived from demethylation of the essential amino acid methionine. The metabolism of homocysteine depends on several vitamin B compounds, including folate, cobalamin, pyridoxine and riboflavin. In one subject, 5, 10-methylenetetrahydrofolate reductase (MTHFR) dysfunction was found to cause hyperhomocysteinemia with schizophrenia-like symptoms (Freeman et al., 1975). In addition, meta-analysis studies have indicated an association between a C677T substitution (Val to Ala) in MTHFR and schizophrenia (Lewis et al., 2005; Muntjewerff et al., 2006). Thus, strategies that reduce homocysteine may alleviate the symptoms experienced by chronic schizophrenic patients with hyperhomocysteinemia (Levine et al., 2006).

Pyridoxal 5′-phosphate (PLP) is the metabolically-active form of vitamin B_6 that is required as a coenzyme for many enzymes involved in homocysteine metabolism and catecholamine synthesis. High dose of vitamin B_6 decreases homocysteine serum levels in patients with schizophrenia (Miodownik et al., 2007) and we identified several patients who exhibited low peripheral blood PLP concentrations; following vitamin B_6 treatment, they experienced slightly reduced symptoms (Tada et al., *unpublished data*). Enzymes involved in vitamin B_6 metabolism include pyridoxal kinase, pyridoxine 5′-phosphatase oxidase (PNPO) and pyridoxal phosphatase. PNPO catalyzes conversion of pyridoxine 5′-phosphate and pyridoxamine 5′-phosphate to PLP and is a rate-limiting enzyme for intracellular PLP synthesis (Ngo et al., 1998). PNPO is expressed in the brain (Kang et al., 2004) and mutations in *PNPO* cause severe neonatal epilepsy, as well as low catecholamine levels in the brain (Mills et al., 2005).

The chromosomal locus 17p11-q25 is associated with a susceptibility to schizophrenia (William et al., 2003) and *PNPO* (OMIM 603287) is located at 17q21.32. This gene is ~7.5 kb in length and contains seven exons. The 2.4 kb mRNA transcript encodes a protein of 261 amino acids. In this study we examined whether or not genetic and haplotypic associations exist between single nucleotide polymorphisms (SNPs) in *PNPO* and susceptibility to schizophrenia.

2. Materials and methods

2.1. Subjects

All patients and controls were biologically unrelated individuals of Japanese descent. Blood samples were obtained from patients with schizophrenia (228 males and 131 females; mean age 51.1 ± 13.6 years) who attended or were hospitalized in psychiatric hospitals in the Tokushima Prefecture of Japan (population ~820,000). Patients were diagnosed by at least two experienced psychiatrists using DSM-IV criteria (American Psychiatric Association, 1994) and at the time of sampling, clinical symptoms were evaluated using Brief Psychiatric Rating Scale scores (BPRS; mean 39.1 ± 10.3; Overall and Gorham, 1962). The mean age at onset was 25.3 ± 7.5 years and the mean medication equivalent to chlorpromazine was 751.1 ± 523.4 mg/day. Extrapyramidal symptoms (EPS) were assessed using the Drug-Induced Extrapyramidal Symptoms Scale (DIEPSS; Inada and Yagi, 1996). Following psychiatric assessment, 582 individuals (332 males and 250 females; mean age 49.9 ± 12.3 years) were selected as controls. Controls were genetically unrelated residents living in Japan without either mental past histories or family histories of at least first degree relatives. Patients and controls were all of Japanese ethnicity and there is no significant population stratification in Japanese reported in several groups (Arinami et al., 2005; Kakiuchi et al., 2003; Yamada et al., 2004). There was no significant difference in age between the control and schizophrenic groups ($P = 0.172$). The genetic association studies were approved by institutional ethics committees and written informed consent to participate in those studies was obtained from all participating subjects.

2.2. Genotyping of SNPs

Genomic DNA was extracted from EDTA-containing venous blood samples according to standard procedures (Maniatis et al., 1989). We selected eight SNP markers (minor allele frequencies >0.1); rs3764396 (C/T), rs2325751 (C/A), rs16949651 (A/G), rs11079803 (A/G), rs11079804 (C/T), rs2325750 (T/C), rs2002136 (A/G) and rs7220104 (C/A) in the *PNPO* locus (~9.1 kb), which included the 5′-flanking region covered by the International HapMap Project database with population descriptors, Japanese in Tokyo, Japan (http://www.hapmap.org/cgi-perl/gbrowse/gbrowse) (Fig. 1). The SNP (rs11079804) is in exon 2 of PNPO and a synonymous polymorphism. Genotyping of polymorphisms was performed using the Assays-

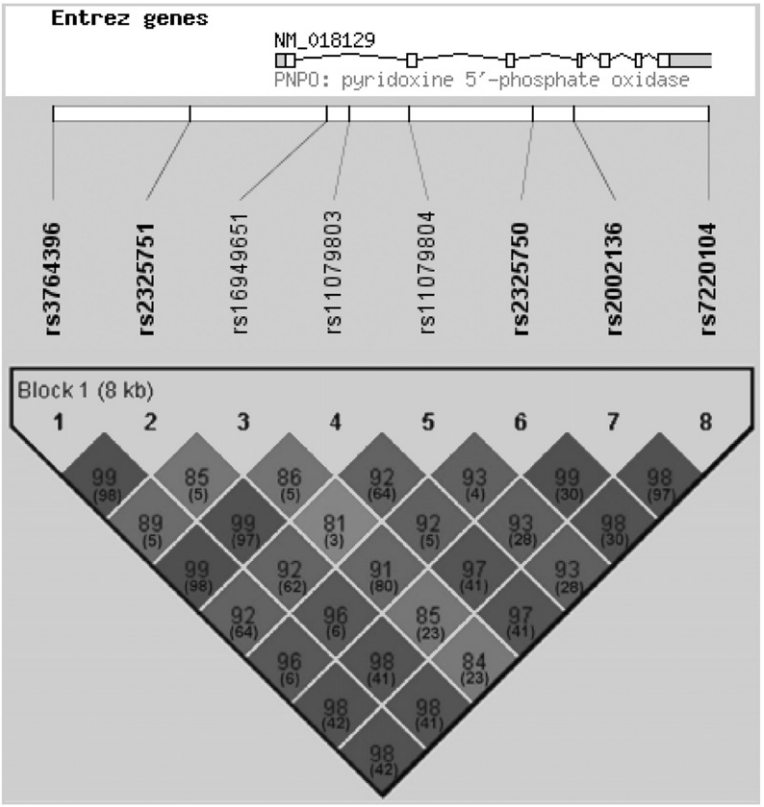

Fig. 1. Linkage disequilibrium of *PNPO*. Haplotype block structure was determined using the HAPLOVIEW program (Barrett et al., 2005). Blocks were defined according to the criteria of Gabriel et al. (Gabriel et al., 2002). Each box represents the D' value and r^2 value (in parentheses) corresponding to each pair-wise single nucleotide polymorphism.

on-Demand™ SNP kit (Applied Biosystems, Tokyo, Japan) with the Taqman® probe and an ABI 7500 Real-Time PCR system (Applied Biosystems). After genotyping with Taqman assays, SDS software (Applied Biosystems) analyzed raw data automatically and determines the contribution of each dye in the raw data using the multicomponent algorithm. The ratio of undetermined by ABI7500 was about 0.6%. We re-genotyped several samples and there was no mis-classification in genotyping.

2.3. Statistical analyses

Statistical analyses were performed using the SPSS Statistical Software Package v. 11.5 (SPSS, Tokyo, Japan). The overall distribution of alleles in schizophrenic patients was analyzed using the Fisher's exact test. Pair-wise linkage disequilibrium (LD) indices D' and r^2 were calculated for control subjects. The SNPAlyze 5.1 Pro software (DYNACOM, Shigehara, Japan) was used to estimate haplotypic frequencies and permutation P values. The criterion for significance was set at $P<0.05$ for all tests and data are presented as mean±standard deviation (SD). Haplotype block structure was determined using the HAPLOVIEW program (Barrett et al., 2005). Blocks were defined according to the criteria of Gabriel et al. (Gabriel et al., 2002). A correction for multiple testing for snps in linkage disequilibrium was performed with SNPSpD (Nyholt, 2004, http://genepi.qimr.edu.au/general/deleN/SNPSpD). Haplotype frequencies for multiple loci were estimated using the expectation-maximization method (SNPAlyze 5.1 Pro software, DYNACOM, Japan). Additionally, the permutation test was performed to test deviation of allelic frequencies of SNPs and haplotypes (Zhao et al., 2000). Distribution of the test statistic was estimated by evaluating the statistics for a random sampling of 10,000 iterated permutations by fixing the total numbers of both cases and controls to avoid false-positive results of multiple testing, which is incorporated in SNPAlyze 5.1 Pro software. In the haplotype case–control study, the threshold value for haplotype frequency was set at 2% and all frequencies below the threshold value were excluded.

3. Results

There were no significant differences in age or sex between the schizophrenia ($N=359$) and control ($N=582$)

Table 1
Genotypic and allelic distributions of *PNPO* polymorphisms

ID	N	Genotype			HWE	Genotypic *P*			Allele		Allelic *P*	Odd ratio (95% C.I.)
						Dominant	Recessive	Codominant				
snp1: rs3764396		C/C	C/T	T/T					T	C		
Schizophrenia	356	192	138	26	0.96	0.054	**0.024**	**0.030**	522	190	**0.010**	1.33(1.07–1.66)
Control	581	357	199	25	0.77				913	249		
snp2: rs2325751		C/C	C/A	A/A					C	A		
Schizophrenia	357	190	140	27	0.97	**0.040**	**0.012**	**0.013**	520	194	**0.004**	1.38(1.11–1.72)
Control	581	359	197	25	0.85				915	247		
snp3:rs16949651		A/A	A/G	G/G					G	A		
Schizophrenia	356	243	101	12	0.85	0.548	0.228	0.271	587	125	0.426	n.d.
Control	577	371	191	15	0.72				933	221		
snp4: rs11079803		A/A	A/G	G/G					A	G		
Schizophrenia	344	183	135	26	0.97	0.052	**0.019**	**0.020**	501	187	**0.007**	1.36(1.09–1.69)
Control	580	355	200	25	0.84				910	250		
snp5: rs11079804		C/C	C/T	T/T					C	T		
Schizophrenia	357	221	120	16	0.97	0.133	**0.019**	**0.037**	561	153	**0.012**	1.36(1.07–1.72)
Control	581	402	164	15	0.38				968	194		
snp6: rs2325750		T/T	T/C	C/C					T	C		
Schizophrenia	357	241	106	10	0.82	1	0.669	0.860	588	126	0.711	n.d.
Control	581	383	182	16	0.38				948	214		
snp7: rs2002136		A/A	A/G	G/G					A	G		
Schizophrenia	356	106	182	68	0.59	0.286	**0.020**	0.059	394	318	**0.030**	1.23(1.02–1.49)
Control	580	216	270	94	0.59				702	458		
snp8: rs7220104		C/C	A/C	A/A					C	A		
Schizophrenia	356	108	182	66	0.56	0.372	**0.047**	0.117	398	314	0.060	n.d.
Control	580	214	272	94	0.69				700	460		

HWE means *P* values for Hardy–Weinberg equilibrium. Statistical differences in genotypic and allelic distributions were evaluated using the Fisher's exact test. genotypic *P* values of three types of models are shown. n.d means not determined. Values of $P \leq 0.05$ are shown in bold.

subjects. Single marker analyses are presented in Table 1. SNP frequencies for both the schizophrenia and control groups were within the Hardy–Weinberg equilibrium. Significant differences in both genotypic and allelic distributions were observed between schizophrenia and controls in rs3764396, rs2325751, rs11079803 and rs11079804 (rs2325751 exhibited the highest, genotypic *P*, dominant, recessive and codominant model = 0.040, 0.012 and 0.013, respectively; allelic, $P = 0.004$). No significant differences were observed between the SNP frequencies of males and females for either the schizophrenia or control groups.

LD analyses were performed in the blocks defined by the criteria of Gabriel et al. (Gabriel et al., 2002). There was a significant haplotypic association of the SNPs in *PNPO* between schizophrenic patients and control subjects (Table 2, $P < 0.00001$, permutation *P* value). For example, the frequencies of CCAACTAC and CCGACCGA were significantly lower in schizophrenia patients than in control subjects (0.518 and 0.585, permutation $P = 0.004$; 0.133

Table 2
Estimated haplotype frequencies and association significance for *PNPO*

Haplotype	% of individuals			Chi-Square	*P*-value	Permutation *P*-value
	Overall	Control	Schizophrenia			
CCAACTAC	0.560	0.585	0.518	8.76	**0.003**	**0.004**
TAAGTTGA	0.168	0.154	0.193	5.01	**0.025**	**0.026**
CCGACCGA	0.160	0.177	0.133	6.36	**0.012**	**0.012**
TAAGCTGA	0.058	0.056	0.062	0.24	0.624	0.612
Selected loci	Chi-square	*d.f.*	Overall *P*-value	Overall permutation	*P*-value	Replications
SNPs1-8	90.14	27	<**0.00001**	<**0.00001**		10000

8 SNP haplotype block defined according to the criteria of Gabriel et al. (Gabriel et al., 2002) was analyzed. Values of $P \leq 0.05$ are shown in bold. *d.f.* means degree of freedom. Replications of permutation were 10,000.

and 0.177, permutation $P=0.012$, respectively), whereas that of TAAGTTGA was vise versa (0.193 and 0.154, respectively; permutation $P=0.026$). Although we sequenced the region between rs2325751 and rs11079803 (from the 5′-flanking region to intron 1 of *PNPO*) and all exons in samples obtained from eight schizophrenic patients, only single nucleotide variants reported were identified. No gender differences were detected in the *PNPO* polymorphisms of schizophrenic patients or control subjects. Furthermore, no correlations were detected between *PNPO* genotypes and BPRS scores, chlorpromazine equivalent amounts of antipsychotics, or extrapyramidal signs (DIEPSS scores).

4. Discussion

The purpose of this study was to investigate the relationship between *PNPO* and schizophrenia using case–control genetic and haplotype-based analyses. In samples from our Japanese subjects, highest significant association was detected between schizophrenia and alleles of the marker, rs2325751, with permutation *P* value, ($P=0.004$ for allele, 10,000 replication). Multiple testing of snps in *PNPO* would increase the type I error rate under nominal thresholds, although the strong LD exists between snps which are assumed to be completely independent. We used SNPSpD (Nyholt, 2004) for correction for multiple testing. With SNPSpD, the thresholds of *P* to keep the type I error rate at 5% were less than 0.0092. The *P* values of rs2325751 and rs11079803 for allele were still significant. Furthermore, a significant haplotypic association was found between *PNPO* and schizophrenia (8 SNPs, permutation $P<0.00001$, Table 2).

High serum homocysteine levels have been reported in schizophrenic patients (Brown and Susser, 2005). Homocysteine metabolism depends upon several vitamin B compounds and strategies that reduce homocysteine appear to improve the symptoms experienced by chronic schizophrenic patients with hyperhomocysteinemia (Levine et al., 2006). High-dose vitamin B_6 supplements can also decrease homocysteine levels in the serum of schizophrenic patients (Miodownik et al., 2007). Since PNPO is a rate-limiting enzyme responsible for decreasing homocysteine levels in the blood, mutations or variants of the gene encoding this enzyme (*PNPO*) may play a role in susceptibility to schizophrenia. In addition, the human *PNPO* gene is located on chromosome 17q21.32, a locus known to be associated with susceptibility to schizophrenia (William et al., 2003).

PNPO (7743 bp) contains seven exons that encode a 30 kDa protein of 261 amino acids (Kang et al., 2004). In all human tissues, two forms of transcript (2.4 and 3.4 kb) are generated via selection of polyA signal sites in exon 7 and not by alternative splicing. PNPO mRNA is not only expressed in the liver but also in the brain and that expression is developmentally regulated in both regions (Ngo et al., 1998). Homologous mutations in *PNPO* exon 2 can cause severe neonatal epilepsy, as well as low catecholamine levels in the brain (Mills et al., 2005). These findings support the suggestion that mutations in *PNPO* can affect function of the central nervous system like schizophrenia. Although the Stanley Institutes' DNA microarray analyses database (http://www.stanleygenomics.org/) indicated that *PNPO* mRNA expression levels were similar in the brains of both schizophrenic patients and control subjects, lower and higher expression levels have been linked to sudden death and lifetime use of antipsychotics (>45,000 mg equivalent to chlorpromazine), respectively. In the 8 SNPs of *PNPO*, we observed that the haplotype frequency of CCAACTAC and CCGACCGA is significantly lower in schizophrenia patients than in controls ($P=0.004$ and 0.012, respectively), whereas that of TAAGTTGA is the reverse ($P=0.026$). We hypothesized that *PNPO* expression differed between schizophrenic and control subjects developmentally and that these differences could relate to schizophrenic susceptibility. However, at present the relationship between expression of *PNPO* and these haplotypes remains unknown. The snp (rs2325751) yield the best *P*-value (0.004) and is located on the 5′-flanking region of PNPO. The snp (rs2325751) seems to have susceptibility of schizophrenia. It is intriguing to perform a reporter gene assay and examine whether this SNP alters transcriptional activity.

Our study suggests that certain *PNPO* polymorphisms contribute to the risk of schizophrenia. Our findings should be replicated in additional samples because it is essential for all the findings from genetic association studies (Ioannidis et al., 2001). Further genetic and functional investigations will be required to determine how differences in PNPO relate to schizophrenia symptoms and homocysteine concentration. Linking different homocysteine concentrations to specific *PNPO* haplotypes may be an important step in the etiological subgrouping of schizophrenia.

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Contributors

Song H and Ueno S designed the study. Nuamta S and Iga J wrote the protocol. Ohmori T and Ueno S managed the literature search. Shibuya-Tayoshi S, Nakataki M, Tayoshi S, Yamauchi K, Sumitani S, Tomotake M and Tada T collected some of samples and advised this search. Tomotake T, Tanahashi T and Itakura M undertook the statistical analysis. Song H wrote first draft of the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest

There are none.

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