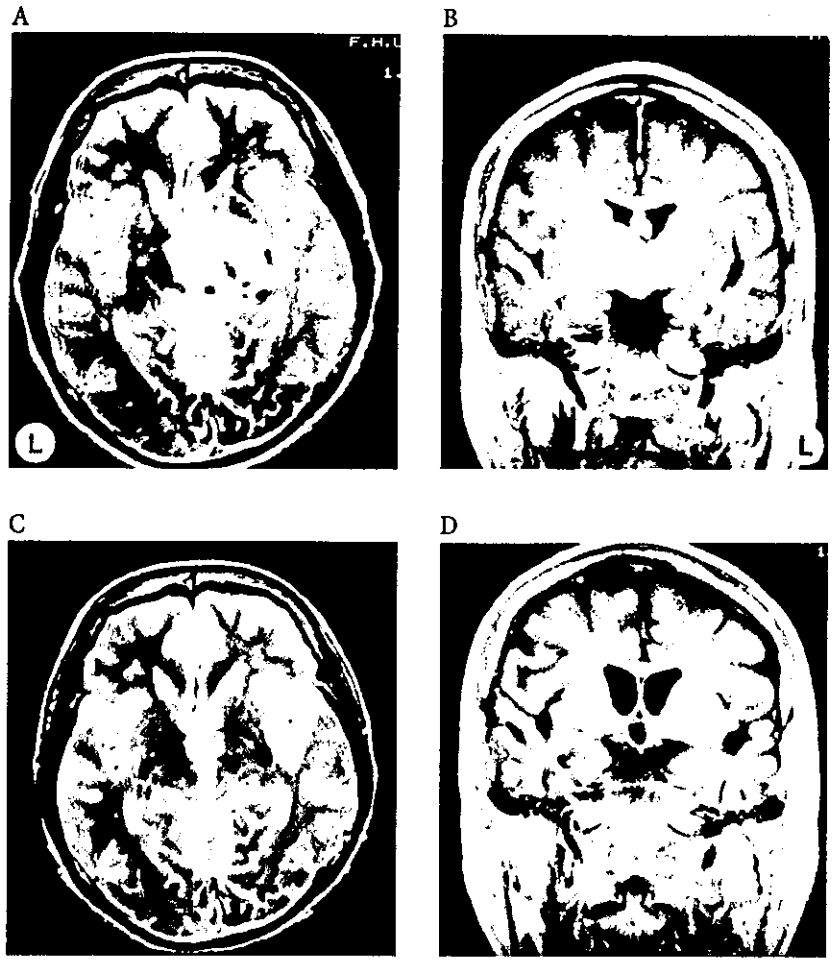


**Fig. 1** Magnetic resonance brain images. Axial T2-weighted image at admission exhibits high signal intensities in the midbrain, hypothalamus, and thalamus (**A**); coronal T1-weighted image with gadolinium exhibits enhancement in the same lesions (**B**). Axial T2-weighted image after treatment exhibits mild atrophy in the lesion (**C**); coronal T1-weighted image with gadolinium exhibits no enhanced lesion (**D**)

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# No association with the neuregulin 1 haplotype to Japanese schizophrenia

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SIR—Schizophrenia is a complex genetic disorder and has heritability of around 80%. The pathogenesis of the disease is hypothesized to be neurodevelopmental in nature based on reports of an excess of adverse events during the pre- and perinatal periods, the presence of cognitive and behavioral signs during childhood and adolescence, and the lack of evidence of a neurodegenerative process in most individuals with schizophrenia.[1]

To date, studies of the association between schizophrenia and genes coding for neurodevelopmental role has been published.[2] Recently, it has been reported that genetic variants around the gene neuregulin 1 (*NRG1*) are associated with schizophrenia in an Icelandic sample.[3] The replications of this finding have been reported independently from Scottish population[4] and Caucasians born in UK and Ireland.[5] The at-risk haplotype was found to be over-represented in schizophrenics compared to controls.

Here, we attempt to perform a replication of these results in our collection of Japanese schizophrenic patients and controls. Subjects consisted of 607 patients with schizophrenia and 515 controls. All subjects were unrelated Japanese. Most patients (93%) had more than 1-year duration of illness and 77% patients had history of hospitalization. Consensus diagnosis according to DSM-IV was made. After description of the study, written informed consent was obtained from each subject. This study was approved by the Fujita Health University and the National Center of Neurology and Psychiatry Hospital Ethics Committees.

SNP8NRG221132, SNP8NRG221533, SNP8NRG243177, and SNP8NRG433E1006 were genotyped using primer extension method by dHPLC. SNP8NRG241930 was genotyped using PCR-RFLP method. The microsatellites 478B14-848 and 420M9-1395 were genotyped following analysis on an ABI3100 capillary sequencer. Allele 0 for markers 478B14-848 and 420M9-1395 refers to PCR-product sizes of 219 and 274 bp, respectively.

Deviation from the genotype counts predicted by Hardy–Weinberg equilibrium expectations was tested using an exact test. $D$, $D'$, and $\Delta^2$ for pair-wise linkage disequilibrium (LD) were calculated. Estimation of the haplotype frequencies was performed by the expectation–maximization algorithm. Test for single-marker allelic association was performed by $\chi^2$ analysis. Test for haplotypic association was performed using the software SAS/Genetics (Release 8.2 TS2M0, SAS Institute Japan) with a permutation test to obtain the empirical significance.[6]

We genotyped the five SNPs (SNP8NRG221132, SNP8NRG221533, SNP8NRG241930, SNP8NRG243177, SNP8NRG433E1006) and two microsatellite markers (478B14-848, 420M9-1395) that constitute the core haplotype in 607 Japanese patients and 515 Japanese control individuals (Table 1). The genotypic distribution of each locus was not significantly different from the distribution expected according to Hardy–Weinberg equilibrium in this ethnic population. We found no association between each marker and schizophrenia, including the C allele of SNP8NRG221533 ($P$=0.42) that was significantly in excess in both Icelandic[3] and Scottish[4] schizophrenia ($P$=0.0028 and 0.000064, respectively). The estimated frequency of the core at-risk haplotype, which was enriched in the Scottish and Icelandic patients (10.2%, 14.4%) than in control individuals (5.9%, 7.6%) by Stefansson *et al*, had no difference between Japanese patients (4.5%) and Japanese controls (4.3%). The estimated Odds Ratio was 1.13 and the 95% confidence interval was (0.60–2.11). In order to be consistent with the literature by Williams and his colleagues, we performed three-marker 'at-risk' haplotype analysis with and without a family history of schizophrenia. However, there are no significant differences among each patent group and control.

Our results do not confirm an involvement of the NRG1 in conferring susceptibility to schizophrenia. One possible explanation for this result could stem from the clinical heterogeneity of schizophrenia. Although the disease is discussed as if it is a single disease, it probably comprises a group of disorders with heterogeneous etiologies. Indeed, the original study was based on individuals taken from large

**Table 1** Replication study showing allele frequencies for markers and haplotypes of the NRG1 gene

| *Marker* | *Allele* | *Frequency (%) Control (%)* | *(Number)* | *Schizophrenia (%)* | *(Number)* | *P (empirical)* | *OR (95% CI)* |
|---|---|---|---|---|---|---|---|
| *a. Test of the seven- and five-marker haplotypes and each SNP* | | | | | | | |
| Seven-marker haplotype | | 4.3 | 488 | 4.5 | 597 | 0.96 | 1.05 (0.59–1.89) |
| Five-marker SNP | | 45.1 | 514 | 45.2 | 603 | 0.98 | 1.01 (0.79–1.27) |
| SNP8NRG221132 | G | 100.0 | 515 | 100.0 | 607 | NA[a] | NA[a] |
| SNP8NRG221533 | C | 51.5 | 515 | 53.1 | 606 | 0.42 | 1.07 (0.85–1.35) |
| SNP8NRG241930 | G | 87.9 | 515 | 89.4 | 607 | 0.26 | 1.16 (0.80–1.68) |
| SNP8NRG243177 | T | 52.7 | 515 | 54.4 | 607 | 0.43 | 1.07 (0.55–1.36) |
| SNP8NRG433E1006 | G | 98.9 | 514 | 98.9 | 604 | 0.99 | 1.01 (0.34–3.02) |
| 478B14-848 | 0 | 42.2 | 507 | 49.1 | 603 | 0.50 | 1.32 (1.04–1.67) |
| 420M9-1395 | 0 | 39.3 | 494 | 48.3 | 605 | 0.21 | 1.44 (1.13–1.84) |

| *Test* | *Frequency (%)* | *χ* | *P-value (one tailed)* | *OR (95% CI)* |
|---|---|---|---|---|
| *b. Test of the 'at-risk' haplotype of markers SNP8NRG221533, 478B14-848, and 420M9-1395* | | | | |
| Case ($n$–600) | 5.7 | 0.51 | 0.66 | 1.13 (0.67–1.92) |
| Control ($n$=490) | 5.1 | | | |
| Familial cases ($n$=69) | 7.7 | 0.19 | 0.36 | 1.46 (0.54–3.95) |
| Nonfamilial cases ($n$=531) | 5.0 | 0.86 | 0.93 | 0.98 (0.45–2.17) |

[a]NA = not analysis.

multiply affected families from Iceland.[3] Subsequently, Williams *et al*[5] discussed sample stratification in which the genetic component might be different in their samples. Further investigations are needed by taking the effect of genetic load into consideration.

Another explanation is that population stratification may exist, since the initial findings started from linkage studies that point out initial evidence for schizophrenia to 8p22-21, where *NRG1* is located.[3] In contrast, from Japanese linkage study, no loci on chromosome 8 fulfiled the criteria for significant or suggestive evidence for linkage.[7] According to the common disease/common variants (CD/CV) hypothesis, if the at-risk haplotype is linked to real susceptibility variants, haplotype association test should detect the linkage disequilibrium to the disease variants in different ethnic populations.[8] However, the sample size was not enough such that the power of analysis was reached at 0.18 (when $\alpha$=0.05). Additional studies of *NRG1* to evaluate across larger numbers of individuals and other ethnic populations should be needed to confirm the hypothesis.

However, it is unclear that single common variants will be the only relevant variants.[9] It is possible that the risk for some common diseases is due to a very large number of loci, with each having a low frequency of disease-predisposing alleles. Allelic heterogeneity may also be contributing to the association of the *NRG1* locus with schizophrenia.[10] In the study, we did not search for genetic variants at *NRG1* locus in our Japanese samples. Much greater depth of DNA resequencing should be conducted to confirm that unknown rare variants in linkage disequibrium might be a real disease gene.

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INVITED REVIEW ARTICLE

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# PHARMACOGENETICS OF ANTIPSYCHOATICS

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## ABSTRACT

Although a number of antipsychotics have been introduced for the treatment of schizophrenia, inter-individual differences of in antipsychotic response and the number of refractory schizophrenic patients have become two of the most challenging problems in clinical psychiatry. Thus, the pharmacogenetics of antipsychotics have been aimed at providing genetic components of this inter-individual variability in antipsychotic response in order to establish an individually-based pharmacotherapy for schizophrenia and to elucidate the mechanism of antipsychotic response so as to solve the refractoriness of schizophrenia. Pharmacogenetics, which is defined as the science of pharmacological response and its modification by hereditary influence can be divided into two categories: the genetic background of pharmacokinetics, i.e. the absorption, distribution, tissue localization, biotransformation and excretion of drugs, and pharmacodynamics, i.e. the biochemical and physiological consequences of a drug and its mechanism of action. Pharmacokinetics of antipsychotics has been focused mainly on the association between genetic polymorphisms in CYP genes, including CYP2D6, and the metabolism of these drugs. Polymorphism in CYP2D6 enables a division of individuals within a given population into at least two groups, i.e. poor metabolizers (PMs), extensive metabolizers (EMs), and ultrarapid metabolizers (UMs) of certain drugs. PMs have higher plasma concentrations of and more adverse effects from antipsychotics. UMs could be one of the important factors that induce treatment-refractoriness to antipsychotics. Genetic polymorphisms in serotonin and dopamine receptors that have a high affinity for antipsychotics have so far been extensively investigated in the pharmacodynamics of this type of drug. Not just one gene but multiple genes play a role in complex phenotypes, including the clinical response to medication. Thus, a multiple candidate genes approach has recently been adopted in the pharmacogenetics of antipsychotics. The new field of pharmacogenomics using DNA microarray analysis, which focuses on the genetic determinants of drug response at the level of the entire human genome, is important for development and prescription of safer and more effective individually-tailored antipsychotics.

Key Words: Antipsychotic, Genetics, Serotonin, Dopamine, Treatment response

## INTRODUCTION

Since chlorpromazine was first introduced into clinical psychiatry, various kinds of antipsychotics have been developed and used for schizophrenia. Clinicians, however, still have considerable difficulty in choosing an appropriate antipsychotic for certain patients due to the inter-individual diversities of drug response. No definitive factor that can predict drug-response has yet been identified, although many researchers have tried to discover factors that can predict drug-response, e.g. demographics, and clinical features.

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Moreover, more than 40% of schizophrenic patients demonstrate varying levels of positive symptoms resistant to medication, even though a number of additional antipsychotics have been introduced. If treatment-failure is also defined to include remission of negative, cognitive, and mood symptoms in schizophrenia, the rate of poor response beomes considerably higher. On the other hand, it is essential to identify the mechanism of action of current antipsychotics in order to discover more effective drugs for refractory patients. However, the actual pharmacological mechanism underlying the clinical effects of antipsychotics have yet to be elucidated, although many hypotheses have been proposed.

Some treatment-resistant patients some of them cannot or will not take antipsychotics because of their side effects. Even worse, antipsychotics could cause fatal adverse effects such as malignant syndrome or agranulocytosis, or irreversible side effects such as tardive dyskinesia. Susceptibility to drug-induced side effects also shows inter-individual differences. Thus, indicators that can predict not only therapeutic efficacy but also adverse effects are urgently required in clinical psychiatry.

The importance of genetic factors among inter-individual diversities of drug response has been steadily clarified since the 1950s. As a result, such clarification has given rise to the new field of "pharmacogenetics", which is defined as the science of pharmacological response and its modification by hereditary influence. The consideration of drug-effects can be divided into two categories: pharmacokinetics and pharmacodynamics, both of which are subject to genetic influence. Although pharmacokinetic studies include the consideration of the absorption, distribution, tissue localization, biotransformation and excretion of drugs, the studies have so far focused mainly on the enzymes of metabolic clearance. Pharmacodynamics is the study of the biochemical and physiological consequences of a drug and its mechanism of action. In most phamacodynamics, the structures of receptors, ion-channels or carrier proteins have been investigated.

The genetic control of a drug-metabolizing enzyme ordinarily occurs via a single locus, whereas because of the complexity of receptor structures, often involving multiple units and proteins, phamacodynamics requires multiple genes and polymorphisms. The complexity of pharmacodynamics gave rise to the new field of pharmacogenomics, which focuses on the genetic determinants of drug response at the level of the entire human genome. Pharmacogenomics can be advanced by the progress of the Human Genome Project and by high-throughput genotyping that enables the creation of high-density single-nucleotide polymorphism (SNP) map.

In this article, the author will review the pharmacokinetics, pharmacodynamics, and pharmacogenomics of antipsychotics.

## *1. Pharmacokinetics of antipsychotics*

Most antipsychotics are extensively metabolized by cytochrome (CYP) P450s that are members of a super-family of oxidative enzymes and that constitute a major system for the oxidative metabolism of therapeutic substances. Thus, the pharmacokinetics of antipsychotics has been focused mainly on the association between genetic polymorphisms in CYP genes and the metabolism of antipsychotic drugs. The CYP2D6 has been most extensively investigated in the field of psychiatry, since this enzyme is involved in the metabolism of many antipsychotics and has many genetic polymorphisms that influence the function of the enzyme. There are more than 70 variant alleles at the CYP2D6 gene locus, including the two most common variants, CYP2D6*4 and CYP2D6*45, encoding non-functional products.[1] Other variants that reduce activity, alter substrate specificity or increase activity have also been described. Compared with

efficient metabolizers (EM), poor metabolizers (PM) show no or reduced CYP2D6 activity by polymorphisms resulting in potentially increased concentrations of metabolized drugs. On the other hand, ultrarapid metabolizers (UM) that can be found in 1% of Caucasians often do not reach therapeutic concentrations and require an increased dose. Pronounced ethnic differences in the prevalence of both PM and UM have been reported; e.g., the frequency of PM is 5 to 10% among Caucasians, about 2% in Asians, and 7–8% in Africans.[2]

PMs have higher plasma concentrations of and suffer more adverse effects from antipsychotics. The incidence of the acute side effects of these drugs, including postural hypotension, excess sedation, or extrapyramidal symptoms, is disproportionately in PMs.[3] On the other hand, it is not clear whether the development of chronic side effects such as tardive dyskinesia is associated with a reduced metabolizing capacity of CYP2D6.

UMs have been reported at increased rates in select groups such as depressive inpatients, possibly due to the high percentage of insufficiently treated cases. Thus, UM can be one of the important factors that induce treatment-refractoriness in the field of psychiatry.

## *2. Pharmacodynamics of antipsychotics*

All receptor and transporter genes for neurotransmitters as well as genes located down-stream of the intracellular signaling pathways can be considered candidate genes for the pharmacodynamics of antipsychotics. It is difficult to select a good candidate gene, since the true mechanism of therapeutic action of antipsychotics has not been clarified yet. However, association studies between genetic polymorphisms in neurotransmitter system and clinical drug-response have been carried out in order to investigate the potential involvement of a specific candidate gene in clinical response. These studies have adopted a candidate gene approach that uses a priori knowledge of drug profiles to identify genes relevant to drug-response. Furthermore, appropriate polymorphisms that influence the function of the gene-product or are merely markers have to be identified for potential candidate genes. So far, genetic polymorphisms in serotonin (5-HT) and dopamine (DA) systems have been extensively investigated in the pharmacodynamics of antipsychotics.

In addition to the selection of appropriate polymorphisms, it is also important to evaluate the clinical response in psychiatric pharmacodynamics, since there is still no biological marker to reflect the degree of severity in schizophrenia. Thus, it is essential to use a reliable and validated rating scale to evaluate clinical symptoms. The Positive and Negative Syndrome Scale (PANSS) or the Brief Psychotic Rating Scale (BPRS) for antispychotics have been used for clinical ratings in pharmacogenetic studies, although some studies have lacked any exact evaluation of clinical responses or definite protocols.

Clozapine is the only antipsychotic which has proven to be effective for treatment-resistant schizophrenia, although it may also induce the fatal adverse effect of agranulocytosis. In addition, the variety of clozapine responses is considerable, ranging from near total remission to little or no response. At present, no reliable means exist to predict who will experience a favorable clozapine response, and no pharmacological mechanism is yet known to explain the efficacy of clozapine for refractory schizophrenia. Thus, pharmacogenetic studies that address antipsychotic-response have focused primarily on clozapine and its variants in candidate genes of DA and 5-HT systems.

1) DA system (Table 1)

The first candidate gene examined with regard to clozapine response was the DA4 receptor

**Table 1** Dopaminergic pharmacodynamic studies of antipsychotics

| Variant | Influence on function | Findings of pharmacogenetics | | Number of reports |
|---|---|---|---|---|
| | | Medication | Result | |
| DRD4: | | | | |
| 48 bp VNTR | Clozapine-binding | clozapine | n.s. | 5 |
| | | typical antipsychotics | n.s. | 1 |
| | | typical antipsychotics | association | 2 |
| Val194Gly | Clozapine-binding | clozapine | n.s. | 1 |
| DRD3: | | | | |
| Ser9Gly | Dopamine-binding | clozapine | n.s. | 1 |
| | | clozapine | association | 1 |
| DRD2: | | | | |
| Ser311Cys | cAMP synthesis | clozapine, typical antipsychotics | n.s. | 2 |
| -141Ins/Del | mRNA expression | clozapine | n.s. | 3 |
| | | bromperidol,nemonapride | association | 1 |
| Taq 1 A | Receptor density | Nemonapride, haloperidol | association | 2 |

gene (DRD4), because in addition to its high affinity for clozapine, the DA4 receptor is abundant in the prefrontal cortex, (a brain region thought to be related to the cognitive dysfunction of schizophrenia), and the DRD4 gene itself is highly polymorphic. Among polymorphisms in the DRD4, the 48 bp variable number of tandem repeats (VNTR) has been the most extensively investigated, since the VNTR was shown an in vitro study to influence the sodium chloride sensitivity of clozapine-binding and inhibition of c-AMP synthesis. However, this polymorphism did not seem to have any association with clozapine response.[4] On the other hand, multiple reports have shown an association between a typical antipsychotic response and the VNTR.

The DA3 receptor, which shares homologies with both the DA4 and DA2 receptors, has generated interest, since the DA3 receptor gene (DRDA3) has a known functional polymorphism, Ser9Gly, that influences dopamine binding. However, the association between the Ser9Gly and clozapine response remains controversial.[5]

The DA2 receptor is a major site of the action of conventional antipsychotics such as chlorpromazine and haloperidol, and of some atypical antipsychotics such as risperidone. One functional polymorphism (-141 Ins/Del) in the promoter region, as well as missense variants including Ser311Cer and an intronic variant (Taq 1 A), have been identified in the DA2 receptor gene (DRD2). The -141 Ins/Del polymorphism that influences the expression of the DRD2 was reported to be associated with anxiolytic and antidepressive effects during treatment with two conventional antipsychotics, bromperidol or nemonapride[6], although other studies failed to show any relation between that polymorphism and the clinical response to clozapine as well as other typical antipsychotics. Although theSer311Cer was shown to influence c-AMP synthesis, it has not been associated with clozapine or with a typical antipsychotic response. The Taq 1A that is located in the intron of DRD2 and has been reported to influence the density of the receptor was shown to have an association with the acute effects of a selective DA2 receptor antagonist, nemonapride, and haloperidol.[7]

2) 5-HT system (Table 2)

The 5-HT receptor genes have been regarded as good candidates for pharmacodynamic studies of antipsychotics, since 5-HT mediated mechanisms seem crucial to atypical antipsychotic drug action, including that of clozapine.

Among 14 human 5-HT receptors, the 5-HT2A receptor has received the most attention in

**Table 2** Serotonergic pharmacodynamic studies of antipsychotics

| Variant | Influence on function | Findings of pharmacogenetics | | Number of reports |
|---|---|---|---|---|
| | | Medication | Result | |
| HTR2A: | | | | |
| T102C | None | clozapine | n.s. | 5 |
| | | clozapine | association | 3 |
| | | risperidone | association | 1 |
| -1438A/G | mRNA expression? | clozapine | association | 2 |
| Hys452Tyr | Ca ion response | clozapine | n.s. | 2 |
| | | clozapine | association | 2 |
| HT2C: | | | | |
| Cy23Ser | m-CPP binding | clozapine | n.s. | 3 |
| | | clozapine | association | 1 |
| HTR6: | | | | |
| C267T | None | clozapine | n.s. | 1 |
| | | clozapine | association | 1 |

the field of psychophamacogenetics, since it might be involved in the pathophysiogy of hallucination, and since most atypical antipsychotics have a relatively high affinity for the 5-HT2A receptor. An association between the silent polymorphism 102T/C in the 5-HT2A receptor gene (HTR2A) and clozapine has been reported[8], although those findings have not always been replicated.[9] However, a meta-analysis showed that the 102T/C is associated with clozapine response.[10] If this association is accurate, and if we consider that the 102T/C cannot influence the function of the receptor, the other variant that is in linkage disequilibrium with the 102T/C plays a genuine role in clozapine response. The -1438A/G in the promoter region of HTR2A is in linkage disequilibrium with the 102T/C and possibly influences the expression of HTR2A. Furthermore, the -1438A/G was shown to be associated with clozapine response.[11] Another polymorphism in HTR2A, Hys452Tyr[12] was shown to influence the intracellular signal transduction of the 5-HT2A receptor, as measured by $Ca^{2+}$ mobilization induced by 5-HT stimulation. The 452Tyr was associated with both smaller peak amplitude in $Ca^{2+}$ mobilization and a different time course of response, with slower peak latency and a longer half time in the Hys452Tyr heterozygote as compared to the His452His homozygote.[13] The Hys452Tyr was shown to be associated with clozapine response although some studies could not replicate the finding[9].

In addition to the 5-HT2A receptor, other 5-HT receptors, such as 5-HT 2C and 5-HT 6, have also been investigated in psychopharmacognetic studies because atypical antipsychotics also have high affinity for these receptors. The 5-HT2C receptor has been targeted for study based on the high densities of this receptor in brain regions implicated in both the pathophysiology of schizophrenia and the mechanism of action of clozapine as well as other atypical antipsychotics. The Ser23 in the 5-HT2C receptor gene influences m-chlorophenylpiperazine (m-CPP), a nonselective5-$HT_{2C}$ agonist, binding, in comparison with Cys23. Therefore, the Ser23 may be constitutively more active and tends to be more desensitized.[14] The Cys23Ser was reported to have an association with clozapine response, although other studies could not replicate this association.[15] Although silent variant, C267T, in the 5-HT 6 receptor gene was shown to be marginally associated with clozapine response, that association also could not be replicated by any other studies.

3) Future direction: Multiple candidate genes

Not just one gene but multiple genes play a role in complex phenotypes, including the clinical response to medication. Arranz *et al.* published the most comprehensive study to date of a

pharmacogenetics screening strategy: a combination of 6 out of 19 candidate gene variants (in 5-HT2A, 2C, 5-HT transporter and Histamin 2 receptor genes) predicted response to clozapine with a prediction level of 76.9% (95.9 % sensitivity, 38.3 specificity).

We applied this approach combined with haplotype analysis to investigate the pharmacogetics of rispreridone, one of the most widely used atypical antipychotics. In our study multiple linear regressions were used to analyze the effects of these haplotypes/genotype of six candidate polymorphisms (HTR2A -1438G>A, 102T>C, H452Y; DRD2 -141delC, *Taq* I A; COMT V158M) on PANSS scale performance of risperidone treatment. Compared with patients who had Ins-A2/Ins-A2 diplotype (n=25), PANSS total scores of patients with Ins-A2/Del-A1 diplotype (n=10) showed 40% greater improved.[16]

### *3. Pharmacogonenomics*

Since individual alleles may contribute only in a small degree to variable drug actions, phamacogenetic studies have not yet reached any definitive conclusion, as mentioned above. An alternative scenario is that it may be possible to identify collections of dozens, hundreds, or even thousands of SNPs that, taken together, might identify a patient as being at high or low risk for either beneficial or toxic drug effects. Thus, phamacogenomic studies encompass the sum of all genes, i.e., the genome, whereas the traditional pharmacogenetic approach relies on studying sequence variations in candidate genes suspected of affecting drug response.

First, progress in the human genome project has given rise to a new approach, i.e., phamacogenomic studies. Second, the high speed and specificity associated with newly emerging genomic technologies such as high-throughput DNA sequencing, gene mapping, and bioinformatics have enabled the search for relevant genes and their variants to include the entire genome.[17]

High-throughput technologies as wal are now being applied to the study of the genomic effects of antipsychotics. Profiling the expression patterns of genes in a target tissue reveals the mechanisms of drug action in a genomic context, and can serve to clarify interindividual differences in drug response that occur some time after the downstream of immediate drug effects in the body. For example, a recent DNA microarray analysis of clozapine-and haloperidol-treated rats identified a multiple differentially altered expression of the genes involved in synaptic function and in the regulation of intracellular Ca2+.[18] Transcript and protein profiling in patients could reveal an antipsychotic fingerprint for responsiveness or nonresponsiveness, as well as a signature motif that may be diagnostic of a specific phenotype. Similarly, antipsychotic-sensitive gene products could provide a new generation of pharmacological targets.

Current concepts in pharmacotherapy basically focuses large patient populations as a whole, in spite of the known inter-individual, genetically-based differences in drug response. In contrast, pharmacogenomics may help focus effective therapy on smaller patient subpopulations characterized by distinct genetic profiles although demonstrating the same disease phenotype. This strategy may also contribute to designing novel, more specific medications and to clarifying the mechanisms of the action of antipsychotics, thus furthering our knowledge of the pathophysiology of schizophrenia.

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# A Missense Variation in Human *Casein Kinase I Epsilon* Gene that Induces Functional Alteration and Shows an Inverse Association with Circadian Rhythm Sleep Disorders

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Recent studies have shown that functional variations in clock genes, which generate circadian rhythms through interactive positive/negative feedback loops, contribute to the development of circadian rhythm sleep disorders in humans. Another potential candidate for rhythm disorder susceptibility is casein kinase I epsilon (CKIε), which phosphorylates clock proteins and plays a pivotal role in the circadian clock. To determine whether variations in *CKIε* induce vulnerability to human circadian rhythm sleep disorders, such as delayed sleep phase syndrome (DSPS) and non-24-h sleep–wake syndrome (N-24), we analyzed all of the coding exons of the human *CKIε* gene. One of the variants identified encoded an amino-acid substitution S408N, eliminating one of the putative autophosphorylation sites in the carboxyl-terminal extension of CKIε. The N408 allele was less common in both DSPS ($p = 0.028$) and N-24 patients ($p = 0.035$) compared to controls. When DSPS and N-24 subjects were combined, based on an *a priori* prediction of a common mechanism underlying both DSPS and N-24, the inverse association between the N408 allele and rhythm disorders was highly significant ($p = 0.0067$, odds ratio = 0.42, 95% confidence interval: 0.22–0.79). *In vitro* kinase assay revealed that CKIε with the S408N variation was ~1.8-fold more active than wild-type CKIε. These results indicate that the N408 allele in *CKIε* plays a protective role in the development of DSPS and N-24 through alteration of the enzyme activity.
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**Keywords:** casein kinase; polymorphism, single nucleotide; phosphorylation; biological clocks; case–control studies; risk factors

## INTRODUCTION

In mammals, including humans, circadian cycles of approximately 24 h are observed in behavior and physiology, including cycles of sleep, hormone secretion, and core body temperature. The master circadian pacemaker is localized in the hypothalamic suprachiasmatic nucleus (SCN). Clock genes, *Per1/2/3*, *Cry1/2*, *Bmal1*, and *CLOCK* are expressed in the SCN and produce a nearly 24 h cycle through interacting positive/negative feedback loops (Harmer *et al*, 2001; Reppert and Weaver, 2002). BMAL1 and CLOCK proteins bind to E-box elements and activate transcription of *Per* and *Cry* genes. As the PERs and CRYs are translated, they enter the nucleus and inhibit BMAL1/CLOCK-driven transcription in the negative feedback loop. The circadian pacemaker is synchronized (entrained) to the 24 h day, primarily by the environmental light/dark cycle.

Certain human sleep disorders, designated circadian rhythm sleep disorders, are attributed to the disruption of the circadian timing system (Weitzman *et al*, 1981; Campbell *et al*, 1999; Wijnen *et al*, 2002). Patients with circadian

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rhythm sleep disorders, such as delayed sleep phase syndrome (DSPS), advanced sleep phase syndrome (ASPS), and non-24-h sleep–wake syndrome (N-24), fail to adjust their sleep/wake cycle to the daily schedule required for social life. Despite normal sleep architecture, sleep onset and offset are persistently delayed (DSPS) or advanced (ASPS) compared to the societal norm. N-24 patients suffer from daily delays of sleep onset and offset times, with the consequence of progressive cycling through the 24 h environmental day. The pathogenesis of DSPS and N-24 is not yet known, but several possible mechanisms have been proposed: reduced sensitivity of the oscillator to photic entrainment, a prolonged intrinsic period beyond the range of entrainment to 24 h day, and abnormal coupling of the sleep/wake cycle to the circadian rhythm (Weitzman *et al*, 1981; Campbell *et al*, 1999; Uchiyama *et al*, 2000). It is estimated that 0.13% (in Japan) (Yazaki *et al*, 1999), 0.17% (in Norway) (Schrader *et al*, 1993), and 0.7% (in USA) (Ando *et al*, 1995) of the general population suffer from DSPS, while the prevalence of N-24 is lower. Genetic factors reportedly confer predisposition to ASPS and DSPS (Ancoli-Israel *et al*, 2001; Jones *et al*, 1999; Reid *et al*, 2001).

Analysis of animals and humans with altered circadian rhythms demonstrated that casein kinase I epsilon (CKIε) (and presumably its most closely related homolog, CKIδ) plays a crucial role in regulating the circadian pacemaker (Eide and Virshup, 2001). CKIε (and CKI δ) phosphorylates PER proteins, leading to their destabilization and relocalization (Takano *et al*, 2000; Vielhaber *et al*, 2000; Keesler *et al*, 2000; Akashi *et al*, 2002; Camacho *et al*, 2001). CKIε/δ have long carboxyl-terminal (C-terminal) extensions, which can be autophosphorylated, with the consequence of autoinhibition of kinase activity (Graves and Roach, 1995; Cegielska *et al*, 1998). *Double-time* (*dbt*) gene, a Drosophila homolog of mammalian *CKIε*, was shown to alter or ablate circadian rhythm when functionally mutated (Price *et al*, 1998). In hamsters, a point mutation in *CKIε* that decreases kinase activity causes the semidominant short-period *tau* phenotype (Ralph and Menaker, 1988; Lowrey *et al*, 2000). A recent report showed that, in humans, familial ASPS can be induced by a *Per2* S662G mutation, which reduces CKIε-induced phosphorylation of the PER2 protein (Toh *et al*, 2001). We have reported that a *Per3* gene haplotype, in which one of the variations lies close to the CKIε target site and presumably alters PER3 protein phosphorylation, is significantly associated with DSPS (Ebisawa *et al*, 2001). These results suggest the possibility that human *CKIε* (h*CKIε*) gene may also be involved in susceptibility to circadian rhythm sleep disorders.

Accordingly, we set out to screen the complete coding region of the *CKIε* gene, as well as adjacent exon–intron boundaries for the presence of genetic variants in circadian rhythm sleep disorder patients and controls.

## MATERIALS AND METHODS

### Subjects

In all, 98 DSPS patients (60 males; 38 females; mean age: 27.1±9.1 years) and 39 N-24 patients (29 males; 10 females; mean age: 26.9±8.4 years) were recruited. Diagnosis was assigned by a trained psychiatrist according to the International Classification of Sleep Disorders (ICSD1990) criteria. All of the patients were unrelated, except for two sibling pairs, of which each consisted of a patient with DSPS and a patient with N-24. In a combined analysis of DSPS and N-24, two of the DSPS subjects with siblings of N-24 were excluded from the DSPS/N-24 group to avoid an increase in the Type I error rate. Neither of the sibling pairs carried the S408N variation. Another three patients with DSPS had relatives with probable DSPS, who were not involved in this study, and another patient with N-24 had a first-degree relative with severe insomnia. In all, 138 healthy subjects were recruited as controls (81 males; 57 females; mean age: 32.1±8.6 years). Control individuals were free from sleep disorders or psychoses. All of the study subjects were sighted. In total, 59 DSPS patients, 36 N-24 patients, and 107 control subjects of the study population were reported previously (Iwase *et al*, 2002), while the others were newly recruited for this study. In order to minimize the effect of the population stratification, which may cause false results, all of the study subjects were Japanese and recruited in mainland Japan. The controls were geographically matched to the patients. Written informed consent was obtained from the subjects. The protocol was approved by the ethics committee of Saitama Medical School and the participating institutes.

Blood samples were drawn by venipuncture and genomic DNAs were prepared from leukocytes using QIAamp DNA Blood Maxi Kit or QIAGEN Blood & Cell Culture DNA Midi Kit (QIAGEN, Hilden, Germany).

### DNA Analysis

Polymerase chain reaction/single-strand conformation polymorphism (PCR-SSCP) analysis was used to screen for variations in all coding exons of the *CKIε* gene. Fluorescein-labelled primers to amplify each of the coding exons and adjacent exon–intron junctions were derived from the genomic structure determined by alignment of the cDNA and genomic sequence of hCKIε (AB024597 and AL020993, respectively) (Table 1). PCR was performed in a total volume of 50 μl containing 100 ng DNA, 0.5 μM of each primer, 1 × PCR buffer II, 0.2 mM dNTPs, 1.5 mM $Mg^{2+}$, and 1.25 U of AmpliTaq Gold DNA Polymerase. Conditions for PCR were preincubation at 95°C for 9 min to denature the DNA and to activate the polymerase, followed by 45 cycles at 95°C for 20 s, 63–68°C for 45 s, and 72°C for 1 min, with a subsequent final extension step at 72°C for 10 min.

SSCP electrophoresis was carried out on a denaturing gel in a DSQ-500S DNA sequencer (Shimadzu, Kyoto, Japan) basically as described (Ebisawa *et al*, 2001). Briefly, 1 μl of PCR products were mixed with 19 μl of formamide buffer (90% formamide, 5 mM EDTA, 10 mg/ml Blue dextran), heated at 80°C for 7 min, and 1.5 μl of the sample mixture was electrophoresed on a 0 or 5% glycerol SSCP Gel at 20°C, according to the manufacturer's protocol. Genomic DNAs in which variants were detected by SSCP were amplified using primers that encompass the SSCP-amplified region, and purified using QIAquick PCR Purification Kit (QIAGEN). Sequence reactions were performed on both strands using internal primers and the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) according to the protocol of the

**Table 1** Primers Used for SSCP Analysis of the Human *CKIε* Gene

| Exon number | Primer name | Sequence (5′–3′) | Fragment size | Annealing temperature (°C) |
|---|---|---|---|---|
| 1 | 1F | CCA CGT CGC TGA CCC TCA TGT TCC | 234 | 68 |
| | 1R | GCC CCT GGA GCC ACA TTC TGA CTT C | | |
| 2 | 2F | CAC ACG CCA GAT CTC AGA AAT GCT TAG TGG | 266 | 63 |
| | 2R | CTG TGC TCA TGG CTG CCC ACC G | | |
| 3 | 3F | CTG CCT GCC TCT GAC CCC TGA C | 264 | 63 |
| | 3R | GGC AGG AGG CAG GGC TGG TAT C | | |
| 4 | 4F | CTG CCT GGC CCA GAG TGC TAG GCA AG | 335 | 68 |
| | 4R | AGT GGC CCC GGG TGC ACA CTG C | | |
| 5 | 5F | CCC AGA GGA TGA GTT AGG GGC CTG AGT G | 306 | 68 |
| | 5R | GCC TCA CCT TTC CCT TAG ACA GTG CCT C | | |
| 6 | 6F | GTG GCT AGG ACA GTG CTG GCT GCA G | 310 | 68 |
| | 6R | CCA GCT CAC TCT GGC CCT CTG AGT C | | |
| 7 | 7F | CTG GCC TCT GGG GCT GAC TGG TG | 271 | 68 |
| | 7R | CTG AAC CCA GCC CAC TGC CTG AGT C | | |
| 8 | 8F | GAC TCA GGC AGT GGG CTG GGT TCA G | 267 | 63 |
| | 8R | CTC AGT TCT GAG GCC CAG AGG GAC TG | | |
| 9 | 9F | ATC GCC AGC GGC TAA GGG ACT TGA C | 241 | 63 |
| | 9R | CCC ACC CCT CCA CAA CAC ATT GGT C | | |

F or R, in the primer names indicate the forward or reverse orientation of each primer.

manufacturer, and detected by an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). One of the PCR-amplified fragments in which a deletion was detected by direct sequence analysis was cloned into pGEM-T Easy vector (Promega, Madison, WI, USA), and multiple isolates were sequenced on both strands. To determine the frequency of the S408N variant, all of the samples were amplified by PCR using 9F and 9R primers in Table 1 and subjected to either SSCP and/or denaturing high-performance liquid chromatography (DHPLC) analysis, followed by sequencing reactions as described above.

For DHPLC analysis, PCR products were denatured at 98°C for 30 s and 95°C for 7 min, followed by gradual reannealing from 95 to 15°C over 40 min. The crude PCR products (5–7 μl) were then injected into a DNASep column and separated through a 13.5–15.75% acetonitrile gradient at 61°C using a WAVE DNA Fragment Analysis System (Transgenomic, Omaha, NE, USA).

## Purification of Recombinant Proteins

The partial cDNAs encoding mouse PER1 (mPER1) (amino acids 547–799), rat PER2 (rPER2) (486–793), and mouse PER3 (mPER3) (367–880) fragments, which correspond to the CKIε-binding regions (Takano, A *et al*, unpublished observation), were subcloned into pGEX4T-3 or pGEX6P-1 vector (Pharmacia, Peapack, NJ, USA) for the production of glutathione-*S*-transferase (GST)-fused recombinant proteins. The partial fragments of PERs were used for *in vitro* kinase assay, because it is practically impossible to obtain enough amount of intact full-length PER proteins due to their instability when expressed in *Escherichia coli*. The PER fragments we used correspond to the CKIε-binding domains which contain the phosphorylation sites; therefore, they can be properly used for *in vitro* kinase assay of CKIεs to compare the kinase activity against PERs.

The S408N substitution was introduced into the rat CKIε (rCKIε) cDNA by site-directed mutagenesis using PCR, generating CKIε-S408N. The amino-acid sequence of rCKIε is identical to that of hCKIε, except for two amino acids. Neither of the two amino acids is a phosphoacceptor residue. The expression constructs encoding GST-fused wild-type rCKIε (GST-CKIε-WT) and CKIε-S408N (GST-CKIε-S408N) were prepared using pGEX4T-3 (for α-casein) or pGEX6P-1 (for GST-PERs) vector. *Escherichia coli* (*E. coli*) strain BL21 (DE3) was transformed with the expression plasmids and the fusion proteins expressed were purified with glutathione sepharose 4B (Pharmacia) according to the manufacturer's protocol. GST-CKIε proteins were easily degraded, therefore, for the use in kinetic analysis

against α-casein, the fusion proteins were further purified by immunoprecipitation with the specific antibody against the C-terminal end of rCKIε to remove the contamination of partially degraded recombinant rCKIε (Takano *et al*, 2000). To perform *in vitro* kinase assay using GST-fused PER fragments as substrates, GST tag was removed from GST-CKIε using PreScission protease (Amersham) to discriminate phosphorylated GST-PERs and autophosphorylated rCKIε on electrophoretic mobility.

### *In Vitro* Kinase Assay and Kinetic Analysis

Kinase reactions were performed in buffer containing 45 mM Tris-HCl, pH 7.4, 9 mM $MgCl_2$, 0.9 mM β-mercaptoethanol, 40 μM ATP, 74 kBq of [$\gamma$-$^{32}$P], kinase and α-casein or GST-PER in a final volume of 20 μl. Approximately 40 ng of the immunoprecipitated GST-CKIε (for α-casein), 2 pmol of rCKIε (for GST-mPER1 and GST-rPER2), or 10 pmol of rCKIε (for GST-mPER3) was added to the reaction mixture. Varying concentrations of α-casein (0–100 μM), or 20 pmol of GST-mPER1, GST-rPER2, or GST-mPER3 protein, was used as a substrate. The amount of rCKIε, GST-CKIε, or GST-PER used in each reaction was confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie brilliant blue staining using bovine serum albumin as a standard, revealing that the difference in the amount of rCKIε or GST-CKIε in each experiment was smaller than 7.3% of the wild type. The kinase reactions for α-casein were allowed to proceed at 37°C for 10 min, because the enzyme activity was linear with time for up to 20 min (data not shown). Reactions were terminated by addition of 20 μl SDS-PAGE sample buffer. A part of the reaction mixture was subjected to electrophoresis on 12% (for α-casein) or 7.5% (for GST-PERs) polyacrylamide gels, and [$^{32}$P] incorporation into the substrates was determined by a BAS-2000 image analyzer. When α-casein was used as a substrate, the data were presented as a double-reciprocal plot and $V_{max}$ and $K_m$ were obtained using computer software (Kaleida Graph, Abelbeck Software).

### Statistical Analysis

Departure from Hardy–Weinberg equilibrium was tested using a $\chi^2$ goodness-of-fit test. The allele and genotype frequencies were compared by means of Fisher's exact test. All *p*-values reported are two-tailed. Correction for multiple testing for the analyses in the previous studies was not performed since a considerable number of subjects were newly recruited for this study, which was conducted with a pre-established hypothesis (Perneger, 1998). Unpaired *t*-test was performed to compare the amounts of incorporated [$^{32}$P] into GST-PER by CKIε-WT and CKIε-S408N.

## RESULTS

Using PCR-SSCP and subsequent sequencing of the PCR-amplified fragments, all of the coding exons and flanking exon–intron boundaries of the *CKIε* gene were screened for sequence variations. In an initial screen of 35 genomic DNA samples (17 of DSPS and 18 of N-24), three sequence variants were identified (Table 2). One single-nucleotide variation (51C>T) was located in exon 1, another variation (1223G>A) in exon 9, and one intronic deletion of 4 bp (77-63_77-60delGGCG) resided upstream of exon 2. The 1223G>A exonic variation predicted an amino-acid substitution, S408N. S408 is located in the C-terminal extension of the CKIε and is conserved in CKIεs of humans, hamsters, mice, rats, and *Xenopus laevis*, as well as in CKIδs of humans and rats. Previous studies demonstrated that the C-terminal extensions of mammalian CKIε (and CKIδ) can be autophosphorylated, inhibiting the kinase activity (Graves and Roach, 1995; Cegielska *et al*, 1998), and that S408 is one of the putative phosphoacceptor residues (Gietzen and Virshup, 1999). Therefore, the S408N variation is likely to eliminate one of the autophosphorylation sites, resulting in decreased autophosphorylation and increased enzyme activity. The 51C>T exonic variation resulted in synonymous substitution. Neither the 51C>T variation nor the intronic deletion (77-63_77-60delGGCG) appeared to affect known splice sites or to create better splice donor/acceptor consensus sequences, based on visual examination of the sequence context, so functional alterations appeared unlikely (Burset *et al*, 2000). Therefore, we focused on the S408N variation for further analysis.

The frequency of the S408N variation was analyzed in a total of 137 circadian rhythm sleep disorder patients and 138 control subjects. Allele and genotype distributions are shown in Tables 3 and 4. No significant deviation from Hardy–Weinberg equilibrium was detected for the variation either in patients or in controls. The distribution analysis resulted in the detection of an additional silent sequence variation (1263A>G) in the 3′-untranslated region of the

**Table 2** Sequence Variations Identified in the Human *CKIε* Gene

| DNA polymorphism | Location | Amino-acid substitution |
|---|---|---|
| 51C>T | Exon 1 | None |
| 77-63_77-60delGGCG | Intron 1 | None |
| 1223G>A | Exon 9 | S408N |
| 1263A>G | Exon 9 (3′-untranslated region) | None |

Variations were named basically according to den Dunnen and Antonarakis (2001). Nucleotide numbers refer to the human CKIε cDNA sequence (AB024597) with the A of the ATG start codon denoted as 1. The S408N variation was submitted to DDBJ (http://www.ddbj.nig.ac.jp/, Accession no. AB080742).

**Table 3** Frequency of the S408N Variant in Patients and Controls

| | | Allele frequency | | |
|---|---|---|---|---|
| | *n* | N408 (%) | S408 (%) | *p*-value |
| Control | 276 | 34 (12.3) | 242 (87.7) | |
| DSPS | 196 | 12 (6.1) | 184 (93.9) | 0.028[a] |
| N-24 | 78 | 3 (3.8) | 75 (96.2) | 0.035[b] |
| DSPS/N-24 | 270 | 15 (5.6) | 255 (94.4) | 0.0067[c] |

[a]Odds ratio (OR) = 0.46, 95% confidence interval (CI): 0.23–0.92.
[b]OR = 0.28, 95% CI: 0.085–0.95.
[c]OR = 0.42, 95% CI: 0.22–0.79.

**Table 4** Genotype Distribution of the S408N Variant in Patients and Controls

| | | Genotype | | | | |
|---|---|---|---|---|---|---|
| | *n* | N/N (%) | N/S (%) | S/S (%) | N/N+N/S (%) | *p*-value |
| Control | 138 | 4 (2.9) | 26 (18.8) | 108 (78.3) | 30 (21.7) | |
| DSPS | 98 | 1 (1.0) | 10 (10.2) | 87 (88.8) | 11 (11.2) | 0.038[a] |
| N-24 | 39 | 0 (0) | 3 (7.7) | 36 (92.3) | 3 (7.7) | 0.061[b] |
| DSPS/N-24 | 135 | 1 (0.7) | 13 (9.6) | 121 (89.6) | 14 (10.3) | 0.013[c] |

The frequency of the N408-allele carrier is shown as (N/N+N/S). Odds ratio (OR) and 95% confidence interval (CI) are for (N/N+N/S) *vs* S/S.
[a]OR = 0.46, 95% CI: 0.22–0.96.
[b]OR = 0.3, 95% CI: 0.086–1.04.
[c]OR = 0.42, 95% CI: 0.21–0.83.
Two of the DSPS subjects, who had siblings with N-24, were excluded from the combined DSPS/N-24 group to avoid an increase in the Type I error rate. Neither of the sibling pairs carried the S408N variation.

CKIε gene, which was located 40 bp downstream of S408N polymorphic site (Table 2). One of the DSPS patients and two of the N-24 patients were heterozygous for the 1263A > G variation, while it was not detected in the control individuals. However, the frequency of the 1263A > G variation was too low to establish whether the variation affects the development of DSPS and N-24.

The N408 allele was significantly less frequent in DSPS ($p = 0.028$) and in N-24 ($p = 0.035$) than in control subjects (Table 3). The frequency of the N408-allele carrier was also significantly lower in DSPS subjects ($p = 0.038$) compared to controls, while the difference in carrier frequency between N-24 subjects and controls showed a similar tendency but did not come to statistical significance ($p = 0.061$) (Table 4). N-24 patients often suffer from DSPS during the course of the illness (Kamgar-Parsi *et al*, 1983; Oren and Wehr, 1992; McArthur *et al*, 1996), and reportedly share some of the physiological characteristics of DSPS, such as prolonged interval between natural wake time and the core body temperature trough (Uchiyama *et al*, 2000) or melatonin midpoint (Shibui *et al*, 1999; Uchiyama *et al*, 2002). These observations led to an *a priori* prediction that DSPS and N-24 are essentially the same disorder expressed with different degrees of severity (Weitzman *et al*, 1981; Campbell *et al*, 1999; Regestein and Monk, 1995). Indeed, when DSPS and N-24 subjects were combined, highly significant inverse associations were found between the N408 variant and DSPS/N-24 in both allele frequency ($p = 0.0067$, odds ratio (OR) = 0.42, 95% confidence interval (CI): 0.22–0.79) and carrier frequency ($p = 0.013$, OR = 0.42, 95% CI: 0.21–0.83), suggesting that the N408 allele protects against the development of DSPS/N-24. Our sample size had a 78% power to detect this effect of the S408N allele at a significance level of $p = 0.05$.

We next considered whether the S408N variation induces a functional alteration in CKIε, as expected from the location of the substitution. To determine whether the N408 variation in CKIε affects kinase activity *in situ*, phosphorylation of PER1 was assayed in transfected COS-7 cells by pulse-chase analysis. COS-7 cells were co-transfected with expression plasmids encoding mPer1 and either wild-type rCKIε or rCKIε with the S408N substitution. The transfected cells were pulse-labeled with [$^{35}$S]methionine for 1 h and chased for 0–6 h. After the chase period, cells were lysed and mPER1 protein expressed in COS-7 cells was immunoprecipitated using anti-mPER1 antibody. The immunoprecipitates were electrophoresed, and [$^{35}$S]-labeled mPER1 was detected. rCKIε with the S408N substitution induced a more pronounced mobility shift and reduced mPER1 protein level at 6 h post-pulse, which was indistinguishable from the effects induced by wild-type rCKIε. These results indicate that, *in situ*, wild-type rCKIε and rCKIε with the S408N substitution induce similar levels of phosphorylation and subsequent instability of the mPER1 protein (data not shown).

Previous reports suggested that subsets of autophosphorylation sites in CKIε are dephosphorylated in HEK293 and NIH3T3 cells by endogenous phosphatases, thus activating CKIε activity (Gietzen and Virshup, 1999; Rivers *et al*, 1998). Therefore, it is possible that rCKIεs transfected into COS-7 cells are dephosphorylated, consequently masking the effect of the S408N substitution on kinase activity.

To test this hypothesis, we performed *in vitro* kinase assays of GST-CKIε with or without the S408N substitution, using α-casein as a substrate. Recombinant GST-CKIε proteins were expressed in *E. coli*, purified with glutathione sepharose 4B, and immunoprecipitated with the C-terminus-specific antibody for CKIε to remove partially degraded protein. The kinetic analysis was performed using varying substrate concentrations (0–100 μM). As expected, GST-CKIε-S408N exhibited higher kinase activity than GST-CKIε-WT (Figure 1a). The data were represented as a double-reciprocal plot (Figure 1b). GST-CKIε-S408N showed significantly increased $V_{max}$ (181% of GST-CKIε-WT) and a slightly decreased apparent $K_m$ (78% of GST-CKIε-WT) against casein. To investigate whether the S408N substitution in CKIε causes higher enzyme activity on endogenous clock components, *in vitro* kinase assays using GST-PERs as substrates were also performed. To distinguish the phosphorylated GST-PERs from autophosphorylated CKIεs, GST tags were removed from recombinant CKIεs. As shown in Figure 2, CKIε-S408N incorporated more [$^{32}$P] into PER1, PER2, and PER3 fragments, respectively, than CKIε-WT did.

## DISCUSSION

CKIε is one of the seven isoforms of CKI, designated α, β, γ1–3, δ, and ε (Eide and Virshup, 2001). Activity of CKIε

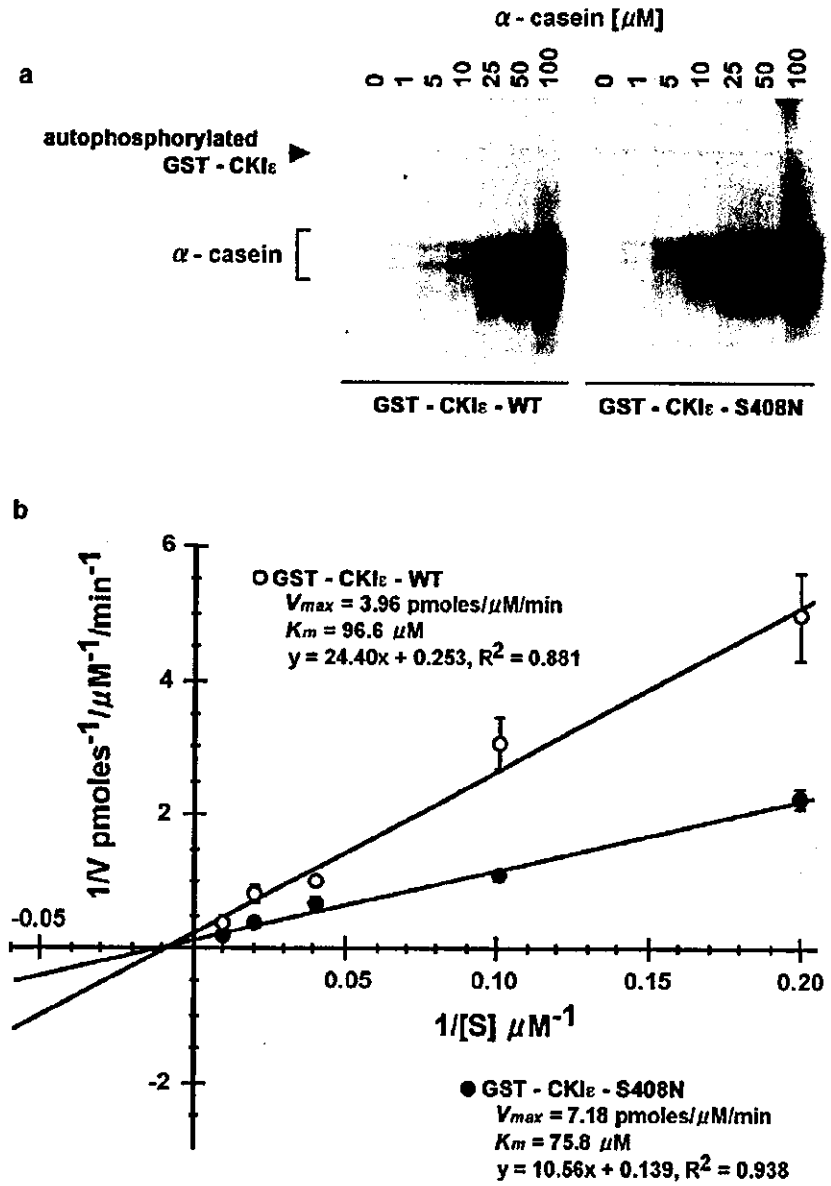


**Figure 1** Kinetic analysis of recombinant GST-CKIε for α-casein. (a) Assays were performed with various concentrations of α-casein in the presence of GST-CKIε-WT or CKIε-S408N. Autophosphorylation of GST-CKIε-WT and CKIε-S408N that were only slightly visible in this figure were readily apparent when increased amounts of recombinant enzymes were used (data not shown). (b) Double-reciprocal plot of the data derived from the kinase assay performed with various concentrations of α-casein. Open and closed circles indicate the results for GST-CKIε-WT and CKIε-S408N, respectively. Calculated $V_{max}$ and $K_m$ of GST-CKIε-WT and CKIε-S408N are shown in the upper and the lower parts of the plot, respectively (means ± standard errors (SE) from three independent experiments).

(and the closely related CKIδ) is regulated in part by autophosphorylation of the C-terminal extension (Eide and Virshup, 2001; Graves and Roach, 1995; Cegielska *et al*, 1998). *In vitro*, CKIε is highly autophosphorylated, which inhibits enzyme activity (Gietzen and Virshup, 1999; Rivers *et al*, 1998). Both dephosphorylation by phosphatase treatment and removal of the C-terminal domain reactivate the kinase (Graves and Roach, 1995; Cegielska *et al*, 1998). In a site-directed mutagenesis study, eight amino acids in the C-terminal domain were identified as probable autophosphorylation sites, including serine-408 (Gietzen and Virshup, 1999). Therefore, the amino-acid change from serine-408 to asparagine (S408N) in CKIε, which was found in this study, is likely to eliminate one of the autophosphorylation sites, and is expected to reactivate part, but not all, of the kinase activity. Indeed, in our *in vitro* kinase assay with α-casein, recombinant GST-CKIε with the S408N substitution purified from *E. coli* exhibited a moderate (1.8-fold) elevation of specific activity compared to that of wild-type GST-CKIε, while a previous study showed that a mutant CKIε, in which all of the putative autophosphorylation sites are disrupted, was eight-fold more active than wild-type CKIε (Gietzen and Virshup, 1999). The moderate elevation of CKIε activity by S408N substitution was

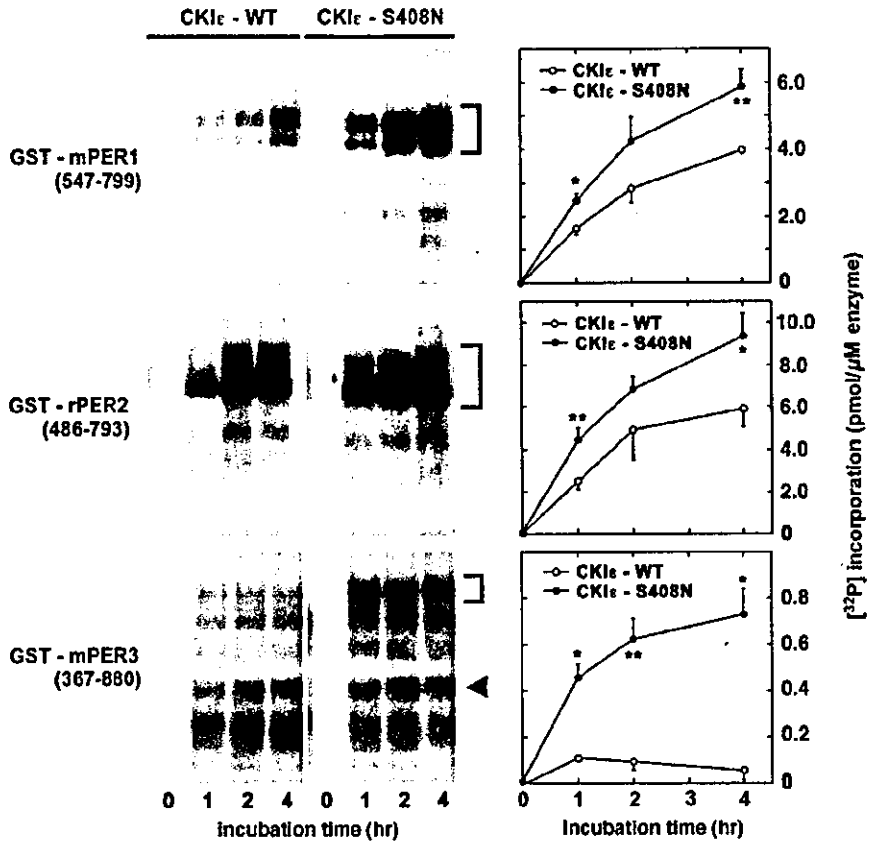


**Figure 2** *In vitro* kinase assay of recombinant CKIε using GST-fused PER fragments as substrates. GST-mPER1 (amino acids 547–799) (top panels), GST-rPER2 (486–793) (middle panels), or GST-mPER3 (367–880) (bottom panels) fragment was incubated with recombinant CKIε-WT or CKIε-S408N for the indicated duration and analyzed by 7.5% polyacrylamide gels as described in Materials and methods. Representative autoradiograms are shown. Angle brackets indicate the phosphorylated GST-PER fragments. Arrowhead indicates autophosphorylated CKIε (left panels). Incorporated [$^{32}$P] was quantified and normalized to the total amount of kinase used (means ± SE from three to five independent experiments). Statistically significant differences in [$^{32}$P] incorporation induced by CKIε-WT and CKIε-S408N are shown by asterisks (*$p<0.05$, **$p<0.01$) (right panels).

identically observed in the *in vitro* kinase assay with each of the three subtypes of PER proteins, which are endogenous substrates for CKIε. It is intriguing that CKIε-S408N induced more phosphorylation of PER3 than CKIε-WT did, because we have previously reported that a *Per3* gene haplotype, which presumably alters PER3 protein phosphorylation, is significantly associated with DSPS (Ebisawa *et al*, 2001). However, it should be noted that we observed much less phosphorylation of PER3 compared with that of PER1 or PER2, which is consistent with the previous reports showing CKIε-induced phosphorylation of PER3 (Takano *et al*, 2000; Akashi *et al*, 2002) and unstable interaction of PER3 with CKIε in the absence of PER1 (Akashi *et al*, 2002; Lee *et al*, 2004). We could not find any elevation of enzyme activity in pulse-chase analysis *in situ*, presumably because of dephosphorylation by endogenous phosphatases as described in 'Results', or because the analysis was insufficiently sensitive to detect a moderate difference of activity.

The *tau* mutation in hamster *CKIε* decreases kinase activity by as much as eight-fold (Lowrey *et al*, 2000), whereas the S408N variant in h*CKIε* results in only 1.8-fold change (an increase) in the activity. This difference might explain the reason why the N408 allele of h*CKIε* induces a significant but modest effect (~2-fold reduction in the risk to develop DSPS/N-24), compared with the *tau* mutation in hamster *CKIε*, which causes a semidominant short-period phenotype (Ralph and Menaker, 1988).

Studies in flies and mammals suggest that CKIε binds to and phosphorylates PER proteins, leading to instability and intracellular relocalization of the PERs (Takano *et al*, 2000; Vielhaber *et al*, 2000; Keesler *et al*, 2000; Akashi *et al*, 2002). Mutant *CKIε* in the Syrian Golden hamster is deficient in PER phosphorylation (Lowrey *et al*, 2000). *Per2* S662G mutation in a reported familial ASPS cause hypophosphorylation by CKIε (Toh *et al*, 2001). In both cases, the PER protein(s) seems to undergo delayed degradation and accelerated accumulation, leading to hastened nuclear entry and shortened circadian period. In contrast, in flies with $dbt^{L}$ or $dbt^{ar}$ (long-period alleles of *dbt*, the Drosophila homolog of *CKIε*), it is likely that delayed phosphorylation and increased nuclear stability of PER protein slow the rate of PER elimination from the nucleus and lengthen circadian rhythm (Price *et al*, 1998; Rothenfluh *et al*, 2000). Therefore, hypophosphorylation of PER protein appears to cause different phenotypes depending on the subcellular localization of the stabilized PERs. hCKIε with an S408N substitution appears more active than wild type only when

the protein is autophosphorylated. A recent study suggests that the autophosphorylation level of CKIε, in neuroblastoma N2a cells, is dynamically regulated through transient dephosphorylation and subsequent phosphorylation, thus regulating the kinase activity (Liu *et al*, 2002). Additionally, in clock-relevant cells, CKIε intracellular localization is under circadian control (Lee *et al*, 2001); therefore, it is possible that a dynamic autophosphorylation/dephosphorylation cycle could differentially regulate CKIε activity at different subcellular locations in pacemaker cells. The S408N variation of h*CKIε* might alter circadian rhythmicity through increased phosphorylation and decreased stability of PER protein; the expected phenotypic consequences, however, would differ depending on the levels of CKIε autophosphorylation in each subcellular location. It will be of interest to investigate the autophosphorylation status of CKIε-S408N and to clarify its functional role in circadian clock machinery.

Although a significant inverse association was observed between the N408 variant and DSPS/N-24, 10.3% of the patients carried the N408 allele, indicating that DSPS/N-24 is genetically heterogeneous and multiple genes affect susceptibility to the development of DSPS/N-24.

The 1263A>G variation in the 3′-untranslated region of h*CKIε* was detected only in three of the rhythm disorder subjects, but not in controls. A larger sample size will be necessary to ascertain its relevance to DSPS/N-24.

Owing to the potential role of CKIε in the circadian rhythm, all of the coding exons in h*CKIε* gene were screened for variations in circadian rhythm sleep disorder patients and controls. We found a missense variation S408N, for the first time, which eliminates one of the putative autophosphorylation sites in hCKIε and confers 1.8-fold higher enzyme activity *in vitro*. There was a significant difference in the frequency of N408 allele between controls and DSPS or N-24, respectively, with an excess of N408 allele in controls. When considering the whole sample of circadian rhythm sleep disorders (DSPS/N-24), we found a highly significant inverse association between N408 allele and DSPS/N-24 ($p=0.0067$, OR=0.42, 95% CI: 0.22–0.79). These results indicate that the N408 allele of the h*CKIε* gene is a marker for decreased risk of DSPS/N-24. S408N variation would also be useful to investigate other disorders related to disturbed circadian rhythm or interindividual differences of circadian rhythmicity in apparently normal subjects (Johansson *et al*, 2003). Our results will yield a new insight into the mechanism of DSPS/N-24 and raise a question in the role of CKIε autophosphorylation on mammalian clock functioning.

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# No association was found between a functional SNP in ZDHHC8 and schizophrenia in a Japanese case–control population

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**Abstract**

ZDHHC8 is a new and attractive candidate for a schizophrenia-susceptibility factor. First, several lines of linkage studies showed that 22q11, on which ZDHHC8 is located, is a "hot" region. Second, fine linkage disequilibrium mapping revealed a significant association around ZDHHC8. Moreover, a very recent study reported that one single nucleotide polymorphism (SNP: rs175174) in ZDHHC8 might affect the splicing process, the ZDHHC8 knock-out mice showed the gender-specific phenotype, and the transmission disequilibrium test (TDT) using this SNP also showed significant association with human female schizophrenia. Thus, we attempted a replication study of this SNP using relatively large Japanese case–control samples (561 schizophrenics and 529 controls). No association was found between schizophrenia and controls even after dividing samples by gender. Because our sample size provided quite high power, ZDHHC8 may not play a major role in Japanese schizophrenia. And our results did not support the gender-specific effect of this SNP.


*Keywords:* Chromosome 22q11; Gender difference; Candidate gene

The 22q11 region (OMIM: #600850 SCZD4) is associated with increased risk for schizophrenia [2]. Two independent meta-analyses of linkage studies showed the linkage around 22q11 [1,5], although one negative result was also reported [8]. This chromosome region contains at least three genes, COMT [12], PRODH2 and DGCR6 [7], implicated as susceptibility genes for schizophrenia.

Recently, ZDHHC8 was reported as a new and attractive candidate gene on 22q11 from the evidence of a genetic association study and animal study [6,9]. In the initial genetic association study, Liu et al. showed that three single nucleotide polymorphisms (SNPs) in ZDHHC8 were associated with schizophrenia. One of these SNPs (rs175174), which was located in intron 4 of ZDHHC8, showed the most highly significant *P* value [6]. This intronic SNP seemed to modify ZDHHC8 expression by causing imperfect splicing, intron retention and reduced enzyme activity. In addition, *Zdhhc8* knockout mice had a gender-dependent dimorphic deficit in prepulse inhibition similar to schizophrenia and reactivity to the psychomimetic *N*-methyl-D-aspartate (NMDA) receptor blocker dizocilpine. In the light of these findings, the transmission disequilibrium test (TDT) divided samples according to gender differences, revealing that human female schizophrenia was significantly associated with this SNP [9]. Thus, we here provide a replication study of rs175174 in ZDHHC8 using Japanese case–control samples.

A total of 561 patients with schizophrenia (259 female; mean age ± standard deviation (S.D.) 49.6 ± 16.4 years: 302

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