## INTRODUCTION

Results from twin, family, and adoption clinical genetic studies have revealed that genetic factors play an important role in the pathogenesis of schizophrenia (Gottesman, 1994). Genetic linkage studies have demonstrated many susceptibility loci for schizophrenia (Sherrington et al., 1988; Moises et al., 1995; Vallada et al., 1995; Asherson et al., 1998; Schwab et al., 1997). However, in spite of many recent molecular genetic studies, the genes responsible for schizophrenia have not yet been identified. On the other hand, psychopharmacological studies have proposed a hypothesis for several decades that the hyperactivity of dopaminergic neurons might relate to the pathogenesis of schizophrenia, especially for the paranoid type (Snyder et al., 1970; Seeman et al., 1976). This hypothesis, which has been called 'dopamine hypothesis', based on clinical efficacy of dopamine antagonists towards schizophrenia (Carlsson and Lindqvist, 1963), and amphetamine psychosis which produce psychotic symptoms that resemble paranoid schizophrenia (Randrup and Munkvad, 1970). Association studies of dopamine receptor genes relating to the proposed dopaminergic dysfunction in schizophrenia have therefore been carried out (Arinami et al., 1994, 1997; Crocq et al., 1992; Seeman et al., 1994; Shaikh et al., 1996). However, the possibility remains that several neurotransmitters and their receptors, which enhance or interact with dopaminergic neurotransmission, are also dysfunctional in schizophrenia.

Human cholecystokinin (CCK) is a neuropeptide that is distributed mainly in gut endocrine cells, the central nervous system, and peripheral neurons. It exists in various forms (Sulfated CCK8, unsulfated CCK8, and CCK4) resulting from the posttranslational processing of prepro-CCK products (Crawley and Corwin, 1994). CCK co-exists with dopamine in dopaminergic neurons, and

mediates the release of dopamine in the nucleus accumbens (Marshall et al., 1991). Messenger RNA of the prepro-CCK gene has been found to be increased in the ventral mesenchephalon (Schalling et al., 1990), and decreased in the frontal cortex and the temporal cortex in the postmortem brain of schizophrenic subjects (Virgo et al., 1995). CCK immunoreactivity in the cerebrospinal fluid of schizophrenic patients was reduced (Verbanck et al., 1984).

There are two types of CCK receptors, CCK-A and B, classified on their binding affinities for CCK peptide forms. The CCK-A receptor (CCK-AR) shows high affinity mainly for sulfated CCK8. In contrast, the CCK-B receptor (CCK-BR) has nearly equal affinity for both sulfated and unsulfated analogs of CCK (Innis and Synder, 1980; Harty et al., 1991). CCK-ARs are found mainly in the pancreas, gall bladder, and vagus nerve. It should be noted that the receptor is also found in central brain regions, including nucleus tractus solitarius, area postrema, interpeduncular nucleus, posterior hypothalamus, and the nucleus accumbens (Hill et al., 1990; Moran et al., 1986). CCK-ARs mediate gall bladder constriction, pancreatic enzyme secretion, and may contribute to the pathogenesis of obesity, diabetes melittus, and gallstones (Ulrich et al., 1993; Miller et al., 1995). CCK-BRs exist mainly in the human brain, and mediate the neurophysiological and neuromodulatory actions of CCK in physiological and behavioral functions including anxiety and nociception (Crawley and Corwin, 1994). Pharmacological analyses have revealed that the CCK-AR mediates the actions of CCK on behavior and CCK-stimulated dopamine-release in the posterior nucleus accumbens. On the other hand, the CCK-BR mediates CCK-inhibition of dopamine release in the anterior nucleus accumbens (Crawley, 1991; Vicroy and Bianchi, 1988; Alter et al., 1989; Marshall et al., 1991). A selective antagonist of the CCK-BR decreases activity of midbrain dopamine (Rasmussen et al., 1991), and an agonist of CCK-BR

was found to decrease the affinity of dopamine D2 receptors for dopamine agonists (Dasgupta et al., 1996). The CCK-AR gene has been mapped to 4p15.2-15.1 with the dopamine D5 receptor gene (De Weerth et al., 1993; Huppi et al., 1994; Inoue et al., 1997). The chromosome 4p was recently implicated by linkage in bipolar disorder (Blackwood et al., 1996; Ginns et al., 1998), and also in schizoaffective disorder and schizophrenia (Asherson et al., 1998). In addition, the CCK-BR gene and the dopamine D4 receptor gene are located on the chromosome 11p15.4 (Song et al., 1993).

A previous association analysis of the CCK gene (the prepro-CCK gene) in schizophrenia yielded negative results (Bowen et al., 1998). However, analyses from the clinical and symptomatic heterogeneity of the disease were not made in their study. On the other hand, a positive association for panic disorder (Wang et al., 1998) and alcoholism (Harada et al., 1998) with variants of the promoter in the CCK gene have been reported. Furthermore, familial studies suggested a relationship between schizophrenia and panic disorder (Heun and Maier, 1995). Few studies have specifically examined genetic polymorphisms in the CCK-AR (Miller et al., 1995; Inoue et al., 1997) and the CCK-BR gene (Harada et al., 1997; Kato et al., 1996; Kennedy et al., 1999). To the best of our knowledge, there have been no previous reports examining the association between polymorphism in both the CCK-AR and CCK-BR genes and schizophrenia.

These overviews of characteristics of the CCK neuronal systems, and its relationship to schizophrenia propose a hypothesis that since the dysfunction of dopaminergic neurotransmission might relate to the pathogenesis of schizophrenia, and CCK receptors may have a functional interaction with dopaminergic neurotransmission, it has been suggested that alterations in CCK peptides, CCK-AR, or CCK-BR may lead to an affect on dopamine release, which may in turn constitute a predisposition for

schizophrenia. Thus, this study aimed to prove the hypothesis to assess the possible involvement of the CCK, CCK-AR, and CCK-BR gene in the predisposition to the disease. Genetic variations in the promoter and coding regions of the CCK, CCK-AR, and CCK-BR genes were systematically analyzed, and association analyses were carried out for these variants between unrelated schizophrenic patients and healthy controls. The analysis also considered the clinical heterogeneity of schizophrenia including the subtype, course, and positive family history of the patient group.

## MATERIALS AND METHODS

### *DNA Samples*

Genomic DNA samples were prepared from whole blood collected in disodium EDTA (3 mg/liter) from 83 unrelated schizophrenic patients and from 100 healthy controls. The patient group consisted of 51 males (mean age, 46.6±12.7 years; mean age at onset, 25.7±7.6 years) and 32 females (mean age, 48.0±16.3; mean age at onset, 28.0± 10.8 years) each of whom met the DSM-IV criteria for schizophrenia (American Psychiatric Association, 1994). All the patients were interviewed by two trained psychiatrists using hospital records. Clinical details of the patients were as follows; subtypes: 18 paranoid, 25 disorganized, 4 catatonic, 28 residual, and 8 undifferentiated; longitudinal courses (re-classified simply by main characteristic features of longitudinal courses in DSM-IV criteria): 49 episodic, 20 continuous, 9 single episode, 5 other or unspecified, and 28 with prominent negative symptoms; and 38 patients who had family history (which means that the first or second degree of relatives were schizophrenics). Written, informed consent was obtained from all the patients included in the study and the

research protocol was approved by the medical ethics committee of the University of Tsukuba.

The control group consisted of 100 unrelated healthy volunteers (30 males; mean age, 32.1±11.4 years. 70 females; mean age, 43.1±12.0 years). All of the volunteers were interviewed by two psychiatrists to rule out the control with a family history of mental illness. All of the subjects participating as controls were employees of the Hospital, and resided in the same area as the patient group. Patient and control subjects were all ethnically Japanese. Genomic DNA was extracted using sodium iodide for DNA purification cell lysis (DNA Extractor WB Kit; Wako Pure Chemical Industries, Tokyo).

***PCR Conditions***

The PCR primers listed in Table 1-3 were prepared to cover the promoter region and the exons with reference to the full length sequences of the CCK-AR gene (Accession No. U23427-23430. Miller et al., 1995), the CCK-BR gene (Song et al., 1993), and the CCK gene (Takahashi et al, 1986), respectively. Nucleotide numbering for the CCK-AR, the CCK-BR, and the CCK gene followed to these reports in the present study. The 5'-terminus of each primer was labeled with indodicarbocyanine fluorescent dye (5'-cy) (Pharmacia Biotech, Uppsala) in order to permit a fluorescence-based Single-strand Conformational Polymorphism (SSCP) analysis. The amplification reaction was performed in a 25 μl volume containing 100 ng genomic DNA, 8.25 pmol of each primer, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM of each dNTP, and 1U Taq DNA polymerase (Takara Biomedicals, Tokyo, Japan). After an initial 3 min. denaturation at 94°C, 30 cycles were conducted that consisted of denaturing at 94°C for 30 sec; annealing temperatures given in Table 1-3 for 30 sec; extension at 72°C for 30 sec; followed by a final extension step at 72°C for 10 min., using a thermal cycler. (Gene Amp 9600, Perkin-Elmer, Norwalk, CT).

***Single-strand Conformational Polymorphism (SSCP) Analysis***

A DNA sequencer (ALF express; Pharmacia Biotech, Uppsala) was used to perform a fluorescence-based SSCP analysis. One microliter of the PCR product was mixed with 14 μl of the loading solution, which contained 99.5% deionized formamide and 0.5% blue dextran. The mixed solution was denatured at 97°C for 5 min., and then cooled immediately on ice. Two microlitter of single-strand PCR product was then applied to three types of polyacrylamide gel (PAG) (49:1, acrylamide: bisacrylamide ratio), which were selected for each product as follows; a 7% native PAG containing 0.5×Tris-Borate-EDTA buffer for the CCK-AR gene products (exon 3, 4, downstream of exon 5) and the CCK-BR (promoter region, exon 1, 2, 4, 5); a 7% PAG containing 10% glycerol and 0.5×Tris-Borate-EDTA buffer for the CCK-AR (exon 1, upstream of exon 5), the CCK-BR (exon 3), and the CCK (promoter region, exon 1-3); and finally an 8% PAG containing 5% glycerol and 0.5×Tris-Borate-EDTA buffer for the CCK-AR (promoter region, exon 2). Electrophoretic processing of all gels was conducted at 20 W for 4 hr at 18°C except for the promoter region, exon 1, and exon 5 of CCK-BR gene products running at room temperature. The data were analyzed using the Fragment Manager (Pharmacia Biotech, Uppsala) software package.

***Sequencing of PCR Products***

PCR products from subjects displaying altered banding patterns in the SSCP analysis were purified using electrophoresis (1% agarose gel) followed by extraction with centrifugation using Microcon tubes (Amicon, Danver, MA). DNA sequences of purified PCR products were directly determined using a Genetic Analyzer (ABI PRISM 310; Perkin-Elmer, Norwalk, CT), followed by the termination-dideoxy-cycle sequencing reaction (Sequencing Reaction Kit; Perkin-Elmer, Norwalk, CT). The same primers were used in the forward and reverse reaction, as indicated in PCR conditions.

*Restriction Enzyme Assay*

Restriction fragment-length polymorphisms (RFLPs) analysis was performed using commercially available restriction enzymes according to the recommendations of the manufacturer. Digested products were subjected to electrophoresis using 2% agarose gels, and visualized using the ethidium bromide staining method.

*Statistical Analysis*

Hardy-Weinberg disequilibrium was assessed using a $\chi^2$ test. Statistical differences in allelic frequency and genotype distributions between patients and controls were also assessed using a $\chi^2$ test, or by using the Fisher's exact probability test at a significant level of 0.05 (two-tailed). Analysis of linkage disequilibrium between two given loci was performed using the ASSOCIAT (version 2.32) software in conjunction with the LINKAGE UTILITY programs (Terwillinger and Otto, 1994). D' values for linkage disequilibrium were also calculated according to the previous report (Chen et al, 1997). Bonferroni correction for multiple comparisons was carried out for the positive result to exclude type I error.

## RESULTS

*CCK-AR gene*

Five novel polymorphisms in the CCK-AR gene were identified using SSCP and sequencing analysis. Details of the polymorphisms identified are as follows: 201A→G and 246G→A nucleotide substitutions in the promoter region, 1260T→A and 1266T→C nucleotide substitutions in intron 1, and a silent mutation Leu306Leu in exon 5. All polymorphisms found in the present study are summarized in Fig. 1. Fig. 2 shows the SSCP banding patterns and direct sequencing of the 201A→G nucleotide substitution, and Fig. 3 shows those of

the 246G→A nucleotide substitution and silent mutation Leu306Leu. The 246G→A nucleotide substitution was apparently quite a rare variant, and was found in only one sample from the control group and from two samples from the schizophrenic group. The silent mutation Leu306Leu was found in only one sample in the control group, and in one sample in the schizophrenic group. The remaining two polymorphisms detected in the present study have been described previously (Inoue et al., 1997). They were a 608G→A substitution in intron 1 and a 3849C→T (Ile296Ile) substitution in exon 5. The 1266T→C nucleotide substitution in intron 1 and the 3849C→T (Ile296Ile) in exon 5 were confirmed with *Msp* I and *Rsa* I, respectively. There were no commercially available restriction enzymes for the remaining polymorphic sites. Note that other mutations have been reported for the CCK-AR gene, as well as an aberrant splicing pattern (Inoue et al., 1997; Miller et al., 1995). However, we did not detect these in the present study. Six banding patterns arising from the SSCP analysis corresponding to the nucleotide sequencing of the 1260T→A and 1266 T→C substitution are shown in Fig. 4. Polymorphic status of both loci suggested a strong linkage disequilibrium between the 1260T allele and the 1266T allele (delta value = 0.1611, D' value = 0.667, $P < 0.00001$). Another possible linkage disequilibrium was found between the 608G allele and the 3849C allele (delta value = 0.0465, D' value = 0.945, $P < 0.00001$).

Table 4 shows the polymorphisms of the CCK-AR gene and the genotype distributions between patients and control groups. There was a significant difference in the allelic frequencies of the 201A→G substitution in the promoter region between the patient and the control groups ($P = 0.0181$, Odds ratio = 1.972). However, the difference was not significant after Bonferroni correction ($P = 0.0905$). The genotype distributions and allelic frequencies for the other polymorphisms were not significantly different between the two groups. A significant difference in the allelic frequencies between patients with paranoid

type and controls ($P$ = 0.0274, Odds ratio = 3.667) was found. Again, the difference was not significant after Bonferroni correction ($P$ = 0.137). No significant differences between the patient with other subtypes, longitudinal courses, or positive family history and the controls were found. Our analyses suggested that the 201A allele frequency was higher in the schizophrenic group, especially in paranoid type, than in the control group at a rate that was not quite significant after Bonferroni correction.

***CCK-BR gene***

Three novel variants of the CCK-BR gene were identified by SSCP analysis and sequencing as shown in Fig. 5. The first was the -215C→A nucleotide substitution in the promoter region. The second was characterized by a single base pair substitution at the nucleotide position 109C→T in exon 1, and resulted in amino acid change Leu37Phe. The third mutation, identified in only one control, was a rare variant with amino acid change Arg319Glu at the nucleotide position 2811G→A in exon 5. Additionally, three mutations in the coding region and one variant in the non-coding region reported previously (Harada et al., 1997; Kato et al., 1996) were also identified: namely, the missense mutation at the nucleotide position 1550 (G→A) in exon 2 (Val125Iso), the silent mutation at the 1962 (T→C) in exon 3 (His207His), the missense mutation at 1985 (G→A) in exon 3 (Arg215His), and the 2491C→A nucleotide substitution in intron 4. All variants found in the present study are summarized in Fig. 6. All of these variants were identified as heterozygotes, and no evidence of a linkage disequilibrium was found among them.

Genotype distributions of the CCK-BR gene for the schizophrenic patients and controls are given in Table 5. The genotype distribution of the -215C→A substitution in the promoter was 6.25% for schizophrenic patients (5/80), and was significantly higher than that of controls ($P$ = 0.0359, Odds ratio = 5.600). However, the observed $P$ value was 0.25 after the Bonferroni correction. The

distribution of genotypes regarding Leu37Phe, Val125Iso, His207His, Arg215His, 2491C→A and 2811G→A showed no significant differences between patients and controls. No positive correlation was found between genotype frequencies of variants of the CCK-BR gene and subtypes, longitudinal courses, and positive family history. Variants of 2491C→A (4 cases) and Arg215His (3 cases) were found only in the patients whose longitudinal courses were episodic. Furthermore, variants of Arg215His (3 cases) were present only in those patients with a positive family history. However, statistical analysis revealed no significant difference between genotypes and clinical features.

***CCK gene***

Polymophisms of -45C→T, 1270C→G, and 6662C→T reported in the previous studies (Bowen et al., 1998; Wang et al., 1998; Harada et al., 1998) in addition to a novel polymorphism (-196A→G) shown in Fig. 7 were identified in the present study. They are summarized in Fig. 8. A complete linkage disequilibrium between -45C→T and 1270C→G loci reported by Bowen et al. (1998) was found in the present study. Moreover, a possible linkage between the -196A allele and the -45C allele (delta value = 0.1356, D' value = 0.556, $P < 0.00001$) was also found.

Distribution of genotypes and allele frequencies of these polymorphisms showed no significant difference between patients and controls as shown in Table 6. Moreover, no positive correlation was found amongst genotype and allele frequencies of variants of the CCK gene and subtypes, longitudinal courses, and positive family history of the patient group.