Association of single-nucleotide polymorphism of cholecystokinin receptor A gene with schizophrenia in an Eastern Indian population

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ABSTRACT

Context: Cholecystokinin A receptor (CCK-AR) gene polymorphism is being increasingly reported in schizophrenia. It varies among different population groups but is associated with several complications of schizophrenia.
Aims: The present study was undertaken to assess whether the CCK-AR polymorphism is stabilized and is more consistently associated with schizophrenia in an Eastern Indian sub-population.
Settings and Design: It was carried out as a cross-sectional, observational, hospital-based study on 95 schizophrenia patients and 138 control subjects selected by the method of convenience.
Materials and Methods: Single-nucleotide polymorphisms located in the regulatory region of the CCK-AR gene were assessed by restriction fragment length polymorphism (RFLP) in the polymerase chain reaction (PCR) amplified product of CCK-AR gene in study subjects. RFLP was done by the digestion of the PCR product by the restriction enzyme Pst-I followed by gel electrophoresis.
Statistical Analysis: Assessment of the stability of C/T polymorphism in the study population was done by applying Hardy–Weinberg equilibrium rule. The significance of difference in the allelic distribution between case and controls was analyzed by Chi-square (χ²) test and odds ratio (OR) analysis.
Result: CCK-R polymorphism was in Hardy–Weinberg equilibrium in both groups. Distribution of the C allele of this gene was significantly higher in schizophrenia patients (χ² = 4.35, OR = 1.51; confidence interval at 95% =1.04–2.20).
Conclusion: C/T polymorphism of the CCK-R gene is a stable polymorphism in our study population. Moreover, the C allele is significantly more abundant in schizophrenia patients imparting them a greater risk of development of complications like auditory hallucination.

Key words: Cholecystokinin A receptor polymorphism, Hardy–Weinberg equilibrium, restriction fragment length polymorphism, schizophrenia

INTRODUCTION

Schizophrenia is a chronic, severe, and disabling psychiatric disorder associated with a large clinical heterogeneity.[1] The global lifetime prevalence is approximately 0.3–0.7%.² The hallmark of the disease is its large clinical heterogeneity that stimulates the search for possible alternative phenotypes with high specificity. The increase in dopaminergic (DA) neurotransmission is one of the major postulated factors that are associated with many genes associated with this disorder.[2] Previous studies have reported a significant association between the short allele of serotonin transporter gene (5-HTTLPR) and the emotional response to auditory...
hallucination (AH), a cardinal feature of schizophrenia.[3] Similarly, FOXP2 polymorphism has been reported to be associated with the frequency and intensity of AH.[4] In a previous report, a significant excess of the C allele of the cholecystokinin receptor A (CCK-AR) via single-nucleotide polymorphism (SNP) +984T/C in patients with persistent AH was also found.[3] It is now well-established that CCK, a peptide neurotransmitter that was originally isolated from the gastrointestinal system, is one of the most widespread and abundant neuropeptides in the central nervous system.[5] Along with several other neuromodulators like vasoactive intestinal polypeptide (VIP), it forms a major part of the cerebral interneuronal circuits that are suggested to formulate the dis-inhibitory circuit motif which is tightly coupled to several behavioral correlates.[6,7] It mediates the release of dopamine in the nucleus accumbens and also coexists with dopamine in the DA neurons. There are two types of CCK receptor (CCKR), CCK-AR and CCK-BR of which CCK-AR acts as a mediator of DA activity and increases the release of DA while CCK-BR performs a converse action. Any alteration or hyper activity of CCK-AR leads to the increases in DA and a consequent predisposition for schizophrenia.[10]

The CCK-AR protein belongs to the seven transmembrane protein receptor superfamily linked to G-protein coupled signal transduction pathway. The human CCK-AR gene contains five exons and extends over 21.8 kb along the 4p15 chromosome region.[11] The features of the promoter region include a transcription start site (+1) located 205 bp upstream of the initiating ATG and a rich GC content.[12‑14] Studies have suggested the role of the CCK-AR gene in several mental disorders including the increased hallucination risk in patients with Parkinson disease.[15‑17] Similarly, a significant association between CCK-AR polymorphisms and alcoholic patients, with delirium tremens, was also reported.[18,19] The CCK-AR gene variant, IVS1-5T > C, has previously been found to be associated with schizophrenia in several small studies.[13‑16]

However, the findings regarding the relationship of schizophrenia and CCK-AR polymorphism remain inconsistent based on different geographical area. It has been reported that an association with Pst-1 polymorphic site in between intron 1 and exon 2 of CCK-AR is associated with schizophrenia in the Chinese population.[20] Wei and Hemmings (1999) also reported a significant association between the Pst-1 (+984T/C) polymorphism of the CCK-AR and different psychotic symptoms in Caucasian population of schizophrenic patients whereas in other studies undertaken in the Japanese population, no such significant association between the 779T/C polymorphism and AH was observed by Tachikawa et al.[21] Instead they found a significant association between schizophrenia and another SNP (-286A/G). Minato et al. also could not find any relationship between 779T/C polymorphism of the CCK-AR gene in schizophrenia in the Japanese population.[22] It has been observed that association with the different sites in CCK-AR gene and schizophrenia in different population is not uniform. Earlier association analysis of the CCK-AR gene in schizophrenia also yielded negative results.[23] Taken together, it is evident that there might be an inconsistent association of the CCK-AR polymorphism with schizophrenia. However, when associated, it heralds the development of several complications of the disease like AH and other complications. Keeping these factors in mind, we hypothesized that there might be an association between the CCK-AR polymorphism and schizophrenia in our region also and accordingly designed the present study to evaluate any such association between this polymorphism with its Pst-1 restriction site (rs 1800857) with an objective to find out the allelic frequency and its potential association with schizophrenia in an Eastern Indian population group.

MATERIALS AND METHODS

Study design
The present study was conducted as a cross-sectional case-control observational study in an Eastern Indian hospital during the year of 2011–2012.

Selection of cases
Inclusion criteria
Patients with schizophrenia were recruited from the outpatient department (OPD) of Psychiatry of a Tertiary Care Medical College and Hospital on a convenience basis. The diagnosis was based on the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition criteria for schizophrenia.[24] Both newly diagnosed as well as drug-treated patients were selected within the age span of 20–50 years. During selection of cases, no male-female discretion was made.

Exclusion criteria
Patients with any other inflammatory or organic or psychiatric disorder were not considered. Patients having mixed type disorders or schizoaffective disorders were also excluded. Patients with a history of addiction to any drug, smoking, or alcohol were also excluded from the study.

Selection of control subjects
Control subjects were selected from persons accompanying the patients in OPD, who were free from any metabolic, psychiatric, or any other organic disorders. Subjects with a history of addiction to any drug, alcohol or smoking were excluded. Persons having any schizophrenia patients as their first-degree relatives were also excluded. Control subjects were selected in an age- and sex-matched manner and from within the same geographical area with similar nutritional and socioeconomic status.

Ethical considerations
Written and informed consent was taken from the normal as well as individuals with schizophrenia included in the
study. The research protocol was undertaken following the guidelines of Helsinki Declaration 1975, revised in 2000[25] and was approved by the Institutional Ethical Committee.

Collection of the blood samples
Peripheral blood from both cases and healthy subjects were collected in EDTA coated vials and stored in −20°C for DNA analysis. All DNA analyses were performed within 7 days of sample collection.

DNA isolation
The genomic DNA was isolated from the blood by the standard phenol-chloroform method.[26] A polymerase chain reaction (PCR) based restriction fragment length polymorphism (RFLP) analysis was used to search for the CCK-AR gene polymorphism.[27]

Polymerase chain reaction protocol
Primer designing
The sequence of human CCK-AR gene was obtained from Ensembl database (Gene ID 886, Ensg00000163394). According to this sequence, a set of forward and reverse primers of CCK-AR gene was designed.

The forward primer was: 5'-TCAGAAGTTTGGAGAAGAGATT-3',

and

The reverse primer was: 5'-AATCTGAAACTGCGATAGAACT-3'.

The primer pair as designed was able to flank the Pst-1 restriction site at intron 1 and exon 2 (the SNP site: Rs1800857, C > T polymorphism).

The polymerase chain reaction
The amplification reaction was carried out using Veriti 96-Well Thermal Cycler. A reaction volume containing 1 µl each of primer, 0.5 µl dNTPs, 2.5 µl PCR buffer, 0.5 µl Taq Pol, and 2 µl of the isolated genomic DNA was used. The mixture was completed by adding double de-ionized and sterilized Millipore water to make the total reaction volume to 25 µl.

The initial activation at 94°C for 1 min was followed by a 38 cycle step of denaturation at 94°C for 30 s, annealing at 50°C for 50 s and then extension at 72°C for 1 min and finally a cycle of extension at 72°C for 1 min was carried out. Then the reaction mixture was put on hold at 4°C for 10 min.

Following amplification, 10 µl of the PCR product and 2 µl of the gel loading dye were electrophoresed on 1% agarose gel stained with ethidium bromide and then visualized under ultraviolet illumination in a gel documentation system.

Restriction fragment length polymorphism of the polymerase chain reaction product
Restriction digestion of the PCR product was done with the help of Pst-1 restriction enzyme (Bangalore Genei, India). The enzyme recognizes sequence CTGCA’G. On successful cleavage, the product sizes would be 1.3 kb for homozygous C/C, 760 bp and 540 bp for homozygous T/T, and 1.3 kb, 760 bp, and 540 bp for heterozygous T/C [Figure 1].

A reaction volume containing 10X assay buffer, 0.75 µl of Pst-1 enzyme, 10.5 µl PCR DNA, and 2.25 µl of Millipore to make up the total volume of 15 µl. The reaction tube was then incubated at 37°C for 2 h 30 min with intermittent light shaking. The restriction digested DNA fragments were analyzed by separation on 1% agarose gel (DNA grade, SRL India) against the DNA ladder of 100–3000 bp (Bangalore Genei, India). The gel was stained with ethidium bromide (SRL, India) and then visualized in the gel documentation system.

Statistical analysis
Significance of statistical differences in allelic frequency and genotype distributions between patients and controls was also assessed using a Chi-square (χ²) test, odds ratio (OR), and their value of significance at 95% confidence interval (95% CI) [Tables 1-3].

RESULTS
Restriction fragment length polymorphism pattern
The RFLP pattern is shown in Figure 1. Homozygotes for CC were not cut by the restriction enzyme and showed a single band at 1300 bp region, homozygotes for TT were cut into two fragments and showed two bands at 760 and 540 bp regions, whereas the heterozygotes containing both alleles for CC and TT on two chromosomes showed three

Figure 1: The restriction fragment length polymorphism pattern of the Pst-1 restriction enzyme digestion. Lane A: DNA ladder for marking the DNA fragments. Lane B and E: homozygous for TT, Lane C: homozygous for CC, Lane D and F: heterozygote for both TT and CC
bands at the region of 1300 bp for the CC allele containing chromosome, and at the 760 bp and 540 bp regions for the CC allele for the TT allele containing chromosome.

**Restriction fragment length polymorphism profile**

Among the 105 samples for the case study, 22 individuals were C/C homozygous, 32 were T/T homozygous, and 51 were C/T heterozygous [Table 1]. χ² value for the allelic frequency and genotype frequency was calculated to be 0.034 that was much less than the minimum value of 3.68 for the difference to be significant at 95% confidence limit. Thus, lack of any significant difference from the χ² test showed that the genotype frequencies in the case subjects followed the Hardy–Weinberg equilibrium rule and hence the distribution of the C > T polymorphism to be established in the schizophrenia population in our study group.

Of 129 normal control samples collected, we found 19 homozygous C/C, 55 homozygous T/T, and 55 heterozygous C/T [Table 2]. The allele frequency of C and T was 0.360 and 0.640, respectively. The genotype frequency of C and T as calculated by Hardy–Weinberg law is given. The χ² value calculated was 1.04, less than the minimum value of 3.68 to be significant at 95% confidence limit that signified the distribution of genotype frequency to be in equilibrium in the normal population also according to the Hardy–Weinberg rule.

After analyzing the genotype frequency, any difference in the distribution of the C and T alleles was assessed in both case and control groups by χ² test and OR calculation for the range at 95% CI. Results [Table 3] showed a χ² value of 4.35 suggesting a significant predominance of C alleles among the case group. Furthermore, since the OR range of 1.04–2.20 at 95% CI does not include 1, we concluded that the cases are more likely to have the C allele than the control subjects.

**DISCUSSION**

Results of the Hardy–Weinberg equilibrium study showed that the C > T polymorphism in the CCK-AR gene was well-established in both the case and control groups of our study population. It signified that this polymorphism is internal to our population group and is not introduced recently.

The χ² and odds test analysis regarding the distribution of these alleles between the schizophrenia cases and healthy subjects, however, showed a significantly higher distribution of the C alleles in schizophrenia patients than found in normal control population (χ² = 4.35, OR = 1.51, range of a lower limit of 1.04 and 2.20 at 95% CI). The present observations suggest that CCK-AR gene polymorphism for the C/T allele is closely related to the disease process of schizophrenia. As this polymorphism is located at the intron 1/exon 2 of CCK-AR gene, it may result in an alternation in splicing mechanism resulting in formation of defective protein transcript that may play a contributory role in the disease development. Reduced cholecystokinin CCK levels in the limbic lobe and reduced high-affinity CCK binding in the hippocampus and frontal cortex have been observed in schizophrenia. Reduced cholecystokinin CCK levels in the limbic lobe and reduced high-affinity CCK binding in the hippocampus and frontal cortex have been observed in schizophrenia. The genes coding for CCK-R could be considered as candidate genes for studying the mechanism of pathogenesis of schizophrenia. Alternately, it could be in linkage disequilibrium with some other causal gene variant. However, the exact mechanism remains still unclear, and observations are not consistent and differ significantly region wise.

Our observations are in close agreement with some recent studies made by Koefoed et al. in Denmark but do not conform with the observations found by Minato et al. in the Japanese population. These variations reflect that the polymorphism of this gene is not distributed uniformly throughout the world and hence necessitates the need of region specific observations. The results of the present study are important in this context by providing a significant association of C alleles of the CCK-AR gene with the disease process of schizophrenia. Association of this
allelic variation has been reported with several symptoms of the disease, e.g., AHs in several population groups\(^5,21,23\) that suggest a link of this polymorphism with the symptomatic complications of schizophrenia also. However, the exact role of this association with the overall pathogenesis is still not clear and merits further research.

**CONCLUSION**

C > T polymorphism was found to be well-established in our study population of Eastern India with C alleles to be more associated with schizophrenia. Further studies involving the association of this polymorphism with different symptoms of schizophrenia are necessary to elucidate the causal relationship of the disease with this genetic polymorphism.

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