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5-Hydroxytryptamine (serotonin) 2A receptor gene polymorphism is associated with schizophrenia

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***Background & objectives*: Schizophrenia, the debilitating neuropsychiatric disorder, is known to be heritable, involving complex genetic mechanisms. Several chromosomal regions associated with schizophrenia have been identified during the past; putative gene (s) in question, to be called the global signature for the pathophysiology of the disease, however, seems to evade us. The results obtained from the several population-wise association-non association studies have been diverse. We therefore, undertook the present study on Tamil speaking population in south India to examine the association between the single nucleotide polymorphisms (SNPs) at the serotonin receptor gene (*5HT2A*) and the occurrence of the disease.**

***Methods*: Blood samples collected from 266 cases and 272 controls were subjected to genotyping (PCR amplification of candidate SNPs, RFLP and sequencing). The data on the SNPs were subjected to statistical analysis for assessing the gene frequencies in both the cases and the controls.**

***Results*: The study revealed significant association between the genotypic frequencies of the serotonin receptor polymorphism and schizophrenia. SNP analysis revealed that the frequencies of GG (30%, rs6311) and CC genotypes (32%, rs6313), were higher in patients ($P<0.05$) than in controls. The study also showed presence of G and C alleles in patients. Significant levels of linkage disequilibrium (LD) were found to exist between the genotype frequencies of rs6311 and rs6313.**

***Interpretation & conclusions*: This study indicated an association between the SNPs (rs6311 and rs6313) of the serotonin receptor *5HT2A* and schizophrenia. HapMap analysis revealed that in its genotype distribution, the Tamil speaking population was different from several other populations across the world, signifying the importance of such ethnicity-based studies to improve our understanding of this complex disease.**

Key words GC/CC homozygocity - 5HT2A receptor - linkage disequilibrium - rs6313 - schizophrenia

Schizophrenia is a debilitating mental disease, the aetiology of which is still enigmatic. Investigations involving a large body of data obtained from families and twins reveal the involvement of complex genetic mechanisms leading to the high degree of heritability of the disease[1,2]. Researchers during the past two decades were able to identify several chromosomal regions associated with schizophrenia[3]. But so far, no single candidate gene locus or genetic variant that can be considered as a global signature for the pathophysiology of the disease, has been identified.

Schizophrenia has been implicated to be caused by abnormalities in neurotransmitters and its pathways. Previous studies have resulted in several lines of evidences suggesting the involvement of serotoninergic system in the onset of the disease[4,5]. Whole – genome linkage analyses have further paved the way for the identification of chromosomal regions that harbour the genes related to schizophrenia[3]. Lin and coworkers[6] in their pioneering work involving a mixed population of UK and Japanese families, have suggested a linkage between the 13q14 - q21 and schizophrenia. From then on, this part of the chromosome that houses the 5-hydroxytryptamine (5HT) receptors became the focus of attention of several investigators and this locus was suggested to enable the serotonergic system to regulate diverse neuroendocrine functions as well as depression, anxiety, and fatigue[7,8]. Subsequent attempts have provided clues on the possible involvement of two single nucleotide polymorphisms (SNPs rs6311 and rs6313) from the same gene locus (encoding 5-HT receptor, *5HT2A*), in the pathogenesis of multiple neuropsychiatric disorders[8]. The SNP at the promoter region (rs6311) has been shown to affect the promoter activity of the gene, leading to functional consequences. The inadequacy of the promoter activity (at rs6311) has been suggested to cause neuropsychiatric disorders including schizophrenia in several populations[9,10]. However, in spite of many studies published including meta-analyses, the results obtained so far have been diverse. A combined analysis involving the Russians, Tatars and Bhashkirs, revealed a significant association to exist between the genotype frequencies at rs6311 and schizophrenia in the Russian population; the Tatars and the Bashkirs, however, did not show any significant association between the SNP and schizophrenia[11]. In another association study involving rs6313, a significant allelic heterogeneity was found to exist between the East Asian and European populations[12]. In the East Asian population, including the Chinese, Japanese, Singaporean and Korean populations, a prevalence of T allele could be noticed associated with the SNP (rs6313) and schizophrenia[13,14]. Several other studies, however, found no association between the expression of either C or T allele (rs6313) and the disease[10,15]. In view of the central role played by the serotoninergic system in neural transmission and maintaining the neuropsychological balance, and the ethnicity-based discrepancies observed in various association studies, the present study was carried out to understand the association (if any) in between the serotonin receptor (*5HT2A*) gene variants and schizophrenia in Tamil speaking population in south India.

Material & Methods

This study of Tamil speaking population residing in and around Vellore (Tamil Nadu), included 266 unrelated case subjects consecutively selected, diagnosed of schizophrenia from the Psychiatry out-patient department, Government Vellore Medical College, and the Sri Narayani Hospital and Research Center (Vellore) from June 2011 to December 2012, with the following inclusion and exclusion criteria.

Inclusion criteria: those who were diagnosed as schizophrenia patients as per the Diagnostic and Statistical Manual of Mental Disorders – Fourth Edition[16], the case subjects, irrespective of drug naïve or medicated, and age ranging between 20 and 60 yr, of Tamil Nadu origin (Vellore district, India). The study population comprised ancestrally Tamil - speaking population only; information on their ethnicity was obtained through interviews and profiling (demographic data) forms.

Exclusion criteria: it was ensured that the matched control subjects 272 numbers with respect to age and ethnicity from among healthy volunteers working in VIT university chosen had neither any family history of any psychotic problems or symptoms, nor had they taken any neuroleptics in the past. Written informed consent was obtained from each subject prior to study. Necessary Ethical Clearances were obtained from the respective institutions [Institutional Review Board (IRB), Government Vellore Medical College, Vellore, Institutional Ethics Committee (IEC)/Institutional Review Board (IRB), Sri Narayani Hospital and Research Centre, Vellore, and University Human Ethical Committee, VIT University, Vellore]. The demographic details collected from patients and controls included the age, gender, marital status, mother tongue, status of current medication, age of onset of the symptoms,

Table I. Demographic details of the patients and controls

Profile	Patients (n=266)	Controls (n=272)
Age (yr)	39.66 ± 11.59	30.17 ± 10.44
Male	176	180
Female	90	92
Married	222	242
Unmarried	44	30
Mother tongue	Tamil	Tamil
Status of current medication		
Risperidone	132	----------
Clozapine	90	
Chlorpromazine (CPZ)	10	
Haloperidol	30	
Risperidone + CPZ	4	
Age of onset of symptoms (yr)		
Male	29.86 ± 9.39	----------
Female	30.68 ± 9.65	
Substance abuse	78 (males)	52 (males)

family history (if any) of schizophrenia and substance abuse (if applicable) (Table I).

Blood samples (10 ml) were collected from the subjects in EDTA-coated tubes and kept at 4° C and were transported immediately to the Molecular Endocrinology laboratory (VIT University, Vellore) and subsequently processed for DNA isolation.

Genotyping: Genomic DNA was isolated from the leucocytes using phenol-chloroform method[17]. The polymorphisms (rs6311 and rs6313) of *5HT2A* receptor gene were amplified in Applied Biosystems 9902 thermal cycler, USA in 15 µl reaction volume [0.5 µl of 100 µM primer (IDT, USA), 0.5 µl of 10 mM of dNTP mix (Merck), 1.0 µl of 10X Taq buffer A (Merck), 1.0 µl of Taq polymerase (Merck) and 50 ng of template DNA (Merck)]; primers[18,19] used for the amplification are shown in Table II. Identical amplification protocols were followed for both the polymorphisms: *i.e.* initial denaturation at 94°C for 8 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 55° C for 30 sec, chain lengthening at 72°C for 30 sec, and final extension at 72° C for 10 min.

The PCR amplicons were visualized by resolving in 1 per cent agarose gel (Fig. 1). The PCR products were also subjected to restriction fragment length polymorphism (RFLP) using *MSPI* restriction enzyme (NEB, England), as per manufacturer's protocol. The digested products were resolved in 2.5 per cent agarose gel.

PCR amplification resulted in the appearance of distinct bands with amplicon size of 365 bp and 446 bp for rs6311 and rs6313, respectively (Fig. 1). RFLP and subsequent electrophoresis, generated bands of 238 and 127 bp for subjects with SNP rs6311, indicating the presence of G/G genotype. For patients with SNP rs6313, bands of 255 and 191 bp were generated signifying the presence of C/C genotypes (Fig. 2). Further, for A/G genotype (rs6311), the restriction digestion generated three bands at 365, 238 and 127 bp. For C/T genotype (rs6313), the digestion generated three bands at 446, 255 and 191 bp (Fig. 2). Representative samples were sequenced using a DNA sequencer (ABI 3730XL Genetic Analyzer, USA), and the sequences were deposited in the GenBank and accession numbers were obtained (JX682563 for CC, KC603613 for AA, KC609429 for GG, KC609430 for AG, KF158420 for TC, JX656724 for TT genotype).

Statistical analysis: The allele and the genotype frequencies for the test and the controls were assessed for its possible association with schizophrenia using Chi square test (GraphPad Prism, version 5.0, USA) 95 per cent confidence interval was considered to be the requisite for rejecting null hypothesis.

Genotypic and allelic frequencies of all the HapMap populations (involving Africans, Asians and

Table II. Primer sequences and the annealing temperatures adopted for genotyping of *5HT2A* variants (rs6311 and rs6313)

rsID	Primer 5'→ 3'	Annealing temp (°C)	Restriction enzymes	Fragment size (bp)
rs6311	ACTGCGAAACCAACTTTATTTC(Forward) TTGTCAAGATTCCCATTAAGG(Reverse)[18]	55	*MspI*	365, 238 and 127
rs6313	CAAGGTGAATGGTGAGCAGAAA(Forward) TGGCAAGTGACATCAGGAAATA (Reverse)[19]	55	*MspI*	446, 255 and 191

Source: Refs 18, 19

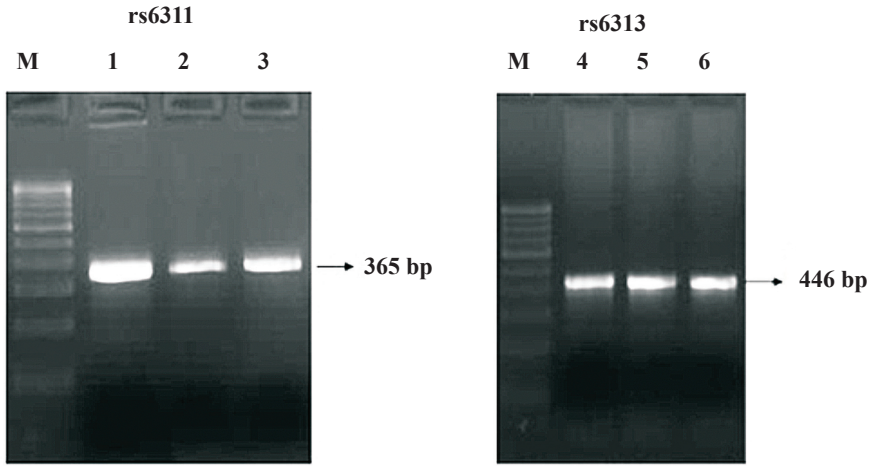


Fig. 1. PCR amplicons of *5HT2A* SNPs. M, 100 bp marker; Lanes 1 - 3, PCR amplicons of rs6311; Lanes 4-6, PCR amplicons of rs6313.

Europeans) were obtained from HapMap data[20] for comparison among ethnicities (*http://hapmap.ncbi.nlm.nih.gov/ cgi-perl/gbrowse/hapmap28-B36/*), and were subjected to Chi square analysis to compare with the Tamil population (present study). Linkage disequilibrium patterns were analyzed and viewed with Haploview (Version 4.2)[21]. (*www.broadinstitute.org/scientific-community/science/programs/medical-and-populationgenetics/ haploview/haploview*).

In silico analysis: FAST SNP analysis was done to make predictions on the risk factor. The output score (obtainable from the database) is based on the risk levels in a scale of 0 - 5 (such as, no risk, very low, low, medium, high, and very high risks, respectively)[22]. Functional SNP database[23] was used to analyze both the SNPs for its deleterious effects, if any, with respect to the functional categories such as protein coding, splicing regulation, transcriptional regulation, and post-translation, assessed through the respective algorithms.

Results

Our analysis revealed that 78 of 262 (30%) patients possessed G/G genotypes, 140 patients had A/G genotypes (53%), and 44 patients (17%) had A/A genotypes (Table III). Of the 272 controls, 60 (22%) had G /G genotype, 148 and 64 individuals had A/G (54%) and A/A (24%) genotypes, respectively. Of the 266 patients, 85 had C/C genotypes (32%), while 141

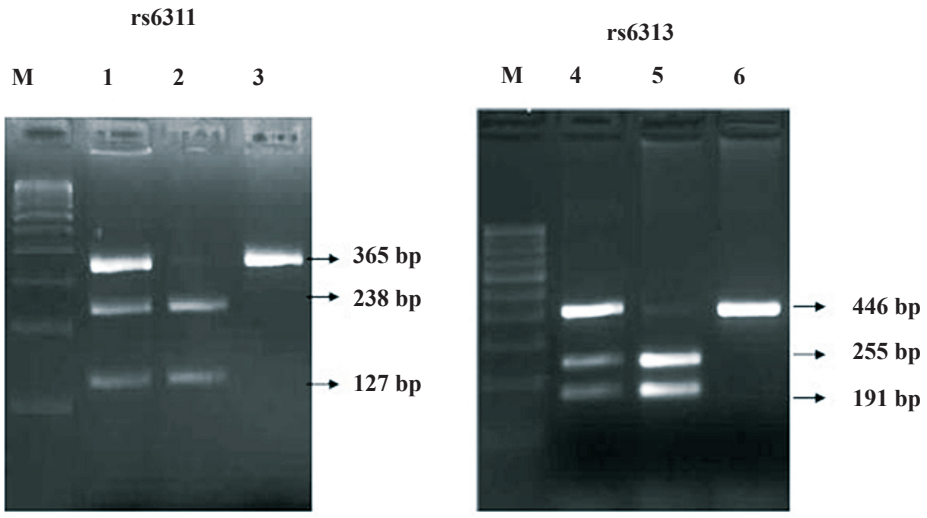


Fig. 2. RFLP products of rs6311 and rs6313. M, 100bp ladder; Lane 1, AG genotype; Lane 2, GG genotype; Lane 3, AA genotype; Lane 4, TC genotype; Lane 5, CC genotype; Lane 6, TT genotype

Table III. Genotype and allele frequencies of rs6311 and rs6313 in Tamil population

rs6311	Genotype			*P* value	Allele		*P* value
	A/A	A/G	G/G		A	G	
Cases	44 (0.17)	140 (0.53)	78 (0.30)	< 0.05	228 (0.44)	296 (0.56)	< 0.02
Controls	64 (0.24)	148 (0.54)	60 (0.22)		276 (0.51)	268 (0.49)	
rs6313	T/T	T/C	C/C		T	C	
Cases	40 (0.15)	141 (0.53)	85 (0.32)	< 0.05	221 (0.42)	311 (0.58)	< 0.02
Controls	59 (0.22)	148 (0.54)	65 (0.24)		266 (0.49)	278 (0.51)	

Figures in parentheses are percentages

patients belonged to C/T genotypes (53%); 40 patients were of T/T genotype (15%).

Application of the Hardy-Weinberg equilibrium and subsequent Chi-square analysis revealed the existence of perceptible differences between the genotype distributions and allelic frequencies of the patients and the controls. Frequencies of the G/G (30%) and the C/C genotypes (32%) (rs6311 and rs6313, respectively), were found to be significantly higher in patients than in the controls; the G allele (56%) and the C allele (58%) frequencies were also found to be higher in patients (Table III). Chi-square analysis on the rs6311 and rs6313 revealed significantly higher frequencies of A/A (rs6311) (24%) and T/T (rs6313) (22%) genotypes in controls compared with cases (Table III). The power analysis revealed a power of 91 per cent for both the SNPs rs6311 and rs6313, with a relative risk of 1.5 ($\alpha = 0.05$), under a multiplicative model.

In silico analysis: The linkage disequilibrium analyses with Haploview (Version 4.2) generated an LOD value (Logarithm of Odds) as high as 82.37, evincing the occurrence of LD in our study population.

Comparison of genotype frequencies of both SNPs of the Tamil speaking population with those of other populations, obtained from HapMap Database revealed the existence of considerable discrepancy in terms of the relative distribution of the genotypes. For example, GG/AA ratios of rs6311 and CC/TT ratios of rs6313 appeared to differ between the Tamil population (present study) and other populations such as LWK, ASW, GIH, MEX, YRI and MKK. Comparison with some other populations such as CHB, CHD, JPT and TSI however, did not show any significant difference in the genotype distribution (Fig. 3A, B).

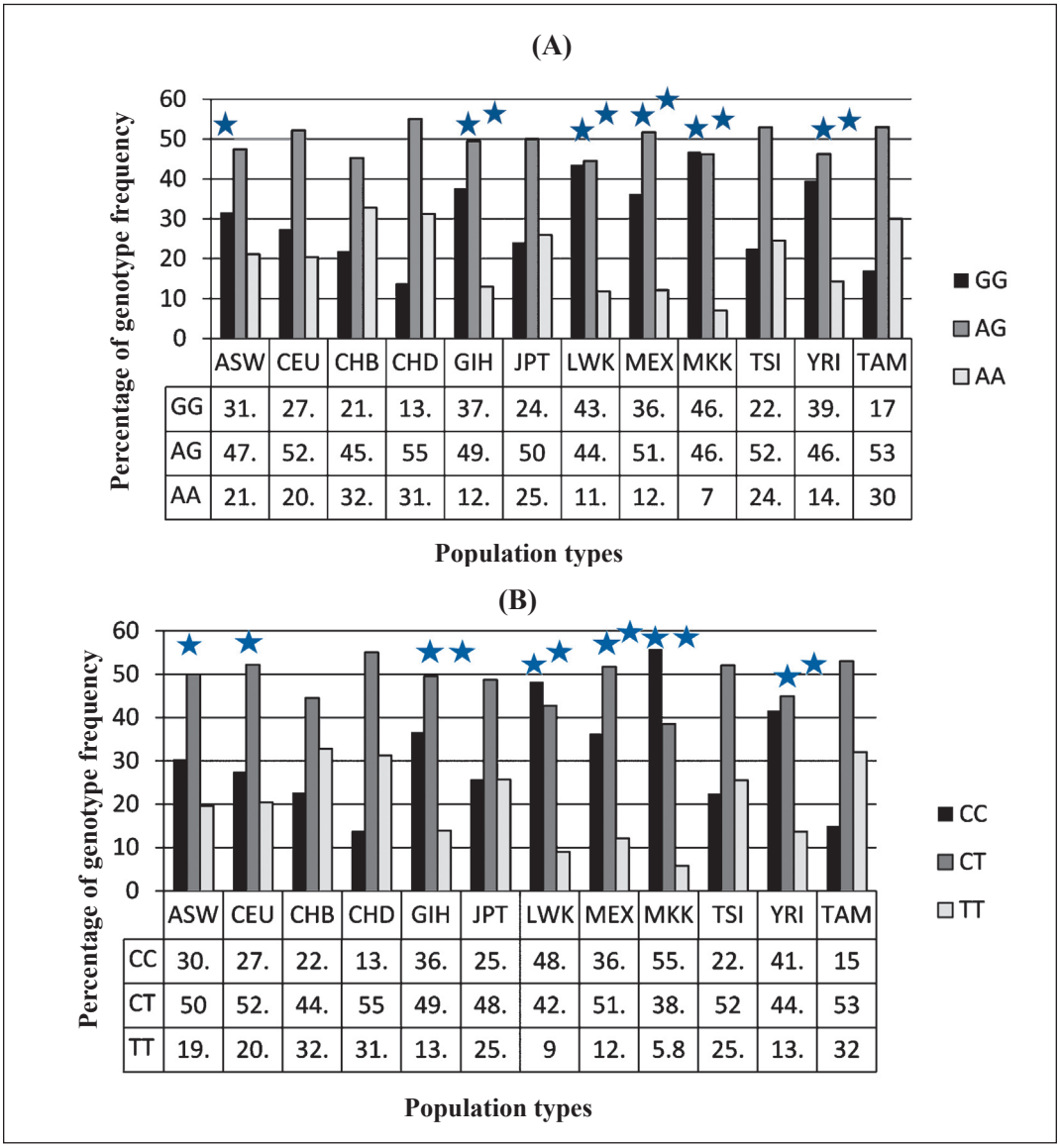
F-SNP provided the functional information on the SNPs in coding region with respect to splicing and transcriptional regulations[23]. F-SNP analysis of our data revealed that rs6311 had a significant role in the transcriptional regulation with the FS score of 0.5 and rs6313 has an evident role in the splicing regulation with the FS score of 0.33.

Analysis of the sequence data generated from the amplicons of rs6311 and rs6313 revealed the synonymous nature of these SNPs. Fast-SNP database was used as a tool to foresee the role, if any, of these synonymous SNPs in the regulatory and/or the coding regions of the candidate gene. Fast-SNP predicted a risk ranking of low to medium *i.e.* 1-3 (in 0-5 scale) for rs6311. Presumably, this situation might lead to alteration in the promoter/regulatory region; those for rs6313, however, was found to have a very low – very low risk ranking *i.e.* 1-1 (in 0-5 scale).

Discussion

Our study showed a significant association of the homozygocities of G/G (rs6311) and C/C (rs6313) genotypes with schizophrenia patients and significantly higher frequencies of A/A (rs6311) and T/T (rs6313) in the control population compared with cases.

Although the Haploview analysis revealed the existence of LD between rs6311 and rs6313 in the present study population, data on other populations showed varying results. While the Jewish, Asturian (Northern Spain) and Caucasian populations, for instance, demonstrate the existence of LD between these polymorphisms[24], LD does not seem to occur in the ASW population (African ancestry in South West USA), wherein these candidate SNPs are known to be placed in different blocks (Block 9 and 10 respectively, Haploview Version 4.2)[21].



	ASW	CEU	CHB	CHD	GIH	JPT	LWK	MEX	MKK	TSI	YRI	TAM
GG	31.	27.	21.	13.	37.	24.	43.	36.	46.	22.	39.	17
AG	47.	52.	45.	55	49.	50	44.	51.	46.	52.	46.	53
AA	21.	20.	32.	31.	12.	25.	11.	12.	7	24.	14.	30

	ASW	CEU	CHB	CHD	GIH	JPT	LWK	MEX	MKK	TSI	YRI	TAM
CC	30.	27.	22.	13.	36.	25.	48.	36.	55.	22.	41.	15
CT	50	52.	44.	55	49.	48.	42.	51.	38.	52	44.	53
TT	19.	20.	32.	31.	13.	25.	9	12.	5.8	25.	13.	32

Fig. 3. Genotype frequencies of **(A)** rs6311 and **(B)** rs6313 in Tamil speaking population and the HapMap populations. $P^{*}<0.05$, $^{**}<0.001$; ASW, African ancestry in Southwest USA; CEU, Utah residents with Northern and Western European ancestry from the CEPH collection; CHB, Han Chinese in Beijing, China; CHD, Chinese in Metropolitan Denver, Colorado; GIH, Gujarati Indians in Houston, Texas; JPT, Japanese in Tokyo, Japan; LWK, Luhya in Webuye, Kenya; MEX, Mexican ancestry in Los Angeles, California; MKK, Maasai in Kinyawa, Kenya; TSI, Tuscan in Italy; YRI, Yoruban in Ibadan, Nigeria; TAM, Tamil speaking population, India.

The present findings on the schizophrenia patients with G/G and C/C genotypes at rs6311 and rs6313, respectively, are in consonance with those of several other populations. In a meta-analysis performed on the Chinese population, an association was found to exist between rs6311 and schizophrenia in most of the sub-populations assessed[25]. In another study involving the European population, a significant association between rs6313 and schizophrenia was reported[26]; the C allele was associated with diagnosis of schizophrenia in this population. Another meta-analysis revealed the occurrence of strong correlation between rs6313 and schizophrenia in Caucasians[12]. The allele frequency analyses in the Han population in China, have revealed the predominance of T allele over C allele (rs6313) in schizophrenia patients[27]. In another study, involving 93 patients from Korea, no significant difference was found between the genotype frequencies (rs6313) and schizophrenia[28]. In a study on the Turkish population involving 102 patients and 107 controls, no correlation

was found between schizophrenia and the genotype frequencies of either of the SNPs (rs6311 and rs6313)[15]. A positive correlation existed between the genotype frequencies at rs6313 and schizophrenia in a cohort of Japanese population[29]. However, no association (between rs6313 and schizophrenia) was found in a similar study, but using a larger population from Japan (Kyushu Islands)[30].

The SNPs rs6311, rs6313 are the results of synonymous mutations that may lead to functional inadequacies at mRNA levels and disorder in transcriptional activities. In humans, synonymous mutations are suggested to result in deficient protein synthesis caused by functionally defective RNA[9]. The allelic variant rs6311 is suggested to result in inadequate promoter activity, which in turn may affect the transcription of the gene[9]. The two variants (rs6311 and rs6313) are in complete LD, the C allele of rs6313 is shown to be associated with lower mRNA and lower protein expression than the T variant[13], which may possibly explain the prevailing occurrence of C allele (and CC genotype) in schizophrenia patients in the present study.

Fast SNP analysis suggested that rs6311 was functionally significant with the risk ranking from low - medium (1-3), leading to altering the promoter/ regulatory region. Both the SNPs with FS score of 0.5 and 0.33, respectively for rs6311 and rs6313 have been suggested to play a significant role in the transcriptional regulation[22,23].

To conclude, the present study showed an association between the SNPs occurring in *5HT2A* and schizophrenia in Tamil speaking population in India. That these two SNPs exhibited considerable degrees of LD and had their origin in the same receptor (*5HT2A*). This association (between the SNPs and the disease) poses a question if these point mutations (at *5HT2A*) could be considered as genetic marker for the disease. Due to the proposed polygenic mode of inheritance of the disease and the variable distribution of these genotypes in several populations distributed across the different continents, these SNPs cannot be considered as exclusive genetic markers. Our study had two major limitations: *(i)* The study relied on the data obtained exclusively from the Tamil speaking population residing in and around Vellore at Tamil Nadu. Samples from other parts of Tamil Nadu State were not collected. *(ii)* The population sub-types (based on caste) could not be considered.

However, that these two SNPs could potentially contribute to the genesis of the disease in Tamil speaking population should be addressed through future research involving expression studies.

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