Original Article

Polymorphisms of Serotonin 4 Receptor Gene Associated with Schizophrenia in Thai Ethnic

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Background: Serotonin 5-HT4 receptor (*HTR4*) is involved in learning and memory process. The location of this gene is in schizophrenia-susceptible loci.

Objective: The present study aimed to investigate the variation in *HTR4* gene in association with schizophrenia in Thai population.

Materials and Methods: A total of 75 schizophrenia patients and 170 normal controls volunteered in this study. DNAs were extracted from whole blood. The purified DNAs were amplified and genotyped by PCR using 3'-locked nucleic acid (LNA) modified SNP primers and by high resolution melting (HRM) analysis. The differences in genotype distribution between patients and controls were assessed by Chi-square test of the SPSS software version 11.5.

Results: All studied SNPs were in Hardy-Weinberg equilibrium. Two SNPs, rs2277051 and rs2277049, showed significant association with schizophrenia (p = 0.001, and p = 0.015, respectively). The genotype C of rs2277051 and G of rs2277049 existed in schizophrenia more than in control.

Conclusion: The authors found two SNPs in intronic region of serotonin 5-HT4 receptor gene, rs2277051 and rs2277049, that related to the susceptibility for schizophrenia in Thai population.

Keywords: Serotonin 5-HT4 receptor, Single nucleotide polymorphism, Locked nucleic acid, High resolution melting analysis, HRM

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Schizophrenia is a psychiatric disorder characterized by abnormalities in thought, cognition, perception, and expression of reality. The age at onset is in early adulthood or adolescence, with onset in childhood and after the fifth decade of age are rare, the prevalence for females and males is the same[1]. The incidence is approximately 1% of the population and believed to have a genetic component[2]. The dysfunctions of serotoninergic system have been suggested as the cause of several related psychiatric disorders including schizophrenia. The serotonin 5-HT4 receptor (5-hydroxytryptamine receptor 4) gene (*HTR4*) is one of the conceivable factors in schizophrenia. It was first described in culture colliculi neurons[3]. The role of serotonin 5-HT4 receptor has been well documented in the gastrointestinal tract[4]. It had a broad tissue distribution and was positively coupled to adenylate cyclase through a Gs protein[5]. The *HTR4* gene is expressed in a variety of tissue such as brain, heart, esophagus, ileum, colon, and urinary bladder. In the brain, it is expressed in amygdala, hippocampus, putamen, caudate nucleus, nucleus accumbens, globus pallidus, and substantia nigra[6]. This receptor is involved in the learning and memory process. Its gene is located on the chromosome five (5q32), the loci of which are susceptible to schizophrenia[7]. The coding sequence is highly fragmented into several exons with the possibility of variant splicing at two positions[8]. One silent nucleotide polymorphism (SNP) within the coding region and six intronic SNPs in *HTR4* gene have been studied in Japaneses schizophrenia using denaturing high performance liquid chromatography (DHPLC). It was found that there was a significant association between schizophrenia and haplotype A-T in which this haplotype may inhibit the occurrence of schizophrenia or another susceptible genetic variant may exist within linkage disequilibrium[9]. However, the relationship study between treatment-resistant schizophrenia (TRS) and SNPs in serotonin 5-HT4 receptor revealed no significant association[10].

Several techniques have been reported to determine single nucleotide polymorphisms (SNPs) in serotonin receptor gene. The locked nucleic acid (LNA) has been applied for allelic-specific analysis. It is a deoxyribonucleotide analog

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that contain a 2'-O, 4'-C methylene bridge within the ribose ring that imparts a rigid conformational structure that enhances thermal stability and improves mismatch discrimination. The LNAs substituted into DNA oligonucleotides at selective sites to enhance hybridization performance[11]. They have been used in applications in which mismatch discrimination is the criteria, such as single nucleotide polymorphism genotyping using allele-specific PCR[12] and real-time PCR[13]. LNA probes increased the specificity and sensitivity of the reaction[14]. LNA modification at 3'-end were more effective in discriminating polymorphism by real-time PCR[11]. High resolution melting (HRM) analysis can discriminate among wild-type, heterozygous and homozygous and has been used to detection SNPs in gout patients[15].

In the present study, the authors investigated the association between single nucleotide polymorphisms (SNPs) in the serotonin 5-HT4 receptor gene *(HTR4)* in Thai schizophrenia patient by PCR amplification using 3'-locked nucleic acid (LNA) primer and by high resolution melting (HRM) analysis to confirm the result.

Materials and Methods

Subjects

Seventy-five schizophrenia patients (39 male and 36 female; mean age ± standard deviation (SD), 35±10.96 years) and 170 healthy controls (115 male and 55 female; mean age ± standard deviation (SD), 42±5.19 years) participated in the present study. The patients were diagnosed according to the DSM-IV criteria. The healthy controls were also psychiatrically screened. All subjects were unrelated to each other. After diagnosis, written informed consent was obtained from each subject. This study was approved by the Ethics Committee of Faculty of Medical, Srinakharinwirot University (SWUEC/E-030/2556).

SNPs genotyping by PCR using 3'-locked nucleic acid primer

Genomic DNA was extracted from peripheral blood of all subjects. Four SNPs in intronic region of *HTR4* gene (rs2278392, rs2277051, rs3734118, and rs2277049) were selected for genotyping. The SNPs primers were modified at 3' end using locked nucleic acid (LNA) to mediate high affinities for their complementary targets and avoid mismatch hybridization. The primers sequences for the four variants are shown in Table 1. The PCR reactions were performed in a total volume of 25 µl containing 75 ng genomic DNA template, 5 µl of 10x PCR buffer, 2.5 µl of 50 mM $MgCl_2$, 1.5 µl of 10 pmol each primer [LNA forward primers (F1/F2) and reverse primer], 1.5 µl of 10 mM deoxynucleotide triphosphate (dNTP) mixture, 0.25 µl of 5 U/µl of *Taq* DNA polymerase (Invitrogen™) and de-ionized sterile-water to adjust the reaction volume. PCR amplification was performed using a thermal cycler (Touchgene Gradient, Techne™) under the following conditions; pre-denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 45 s, annealing for 45 s at optimum temperature for each primer (53°C, 60°C, 53°C and 58°C for rs2278392, rs2277051, rs3734118, and rs2277049, respectively) and extension at 72°C for 45 s. The final extension was carried out at 72°C for 4 min. The PCR products were analyzed on agarose gel (1.5%) electrophoresis. The experiments were performed three times for each SNPs.

SNPs genotyping by high resolution melting (HRM) analysis

In order to confirm the result of rs2277051 and rs2277049, the schizophrenia and control samples (>30 samples) were genotyped by PCR and HRM analysis. The DNA was primarily amplified by conventional PCR or real-time PCR and then HRM analysis were performed. The forward and reverse primers used for amplification are showed in Table 1. PCR amplification was carried out in a 20 µl total volume containing 10 ng genomic DNA template, 10 µl of QuantiMix easy probes kit (Biotools, Spain), 1 µl of each 10 pmol primers, SYTO 9 intercalating dye (Invitrogen Carlsbad, CA) and de-ionized sterile-water to adjust a total volume. Then, the DNA mixtures were amplified on a Rotor-Gene 6000 (Corbett Research, Mortlake, Australia). The amplification steps consisted of 10 min at 95°C, followed by 40 cycles of 95°C 10 s, 60°C 15 s, and 72°C 20 s. High resolution melting analysis were performed at the temperature ramping from 72 to 85°C for rs2277051 and 78 to 86°C for rs2277049 with rising by 0.1°C /2s. The melting curves were normalized using software provided with the Rotor-Gene™ 6000.

Statistical analysis

Genotype deviation from the Hardy-Weinberg equilibrium (HWE) was evaluated by Chi-square test. The association analysis of the samples and controls were performed by using Pearson Chi-square test of SPSS program Version 11.5 for Window. All SNPs within the genes were considered associated to schizophrenia if the level of significance was $p<0.05$.

Results

Genotyping of SNPs by PCR

Figure 1 showed the result from agarose gel electrophoresis of amplicons amplified by using 3'-LNA primer of rs2277051. The mismatch between the 3'-LNA primer and DNA sample resulted in no DNA band (lane 2, 5) whereas the matched nucleotides showed an enhanced binding affinity by LNA and gave a sharp band (lane 1, 3, 4, 6, 7, 8, and 9).

Genotyping of SNPs by HRM

The amplicons from PCR amplification of rs2277051 (201 bp) and rs2277049 (174 bp) were analyzed by HRM. An example of SNPs genotyping by HRM analysis is shown in Figure 2. The genotype GG, GT and TT were well distinguished from each other.

Association analysis

Genotyping of four SNPs in serotonin 5-HT4

Table 1. The sequence of 3'-LNA primers and primers for HRM analysis

SNPs ID		Primer sequences
rs2278392 (LNA)	F1/F2	5'-CGCTATGCACATTGTTCTGTC/T-LNA-3'
	R	5'-TGTTTCCCTTTTCCCTGTTTT-LNA-3'
rs2277051 (LNA)	F1/F2	5'-AATGGTTCCCTGGTTCAATCT/C –LNA-3'
	R	5'-GACTTTGATCTTGGCTGTGGT-LNA-3'
rs3734118 (LNA)	F1/F2	5'-AAAAAGAAGGGGAAGAGAACAA/G-LNA-3'
	R	5'-GAGCTTCATTGCCCAGAGAT-LNA-3'
rs2277049 (LNA)	F1/F2	5'-CAGAGAATAAAAATAAGGAATAAAAG/T-LNA-3'
	R	5'-TCACAGGCCAGTATATCATGTTG-3'
rs2277051 (HRM)	F	5'-TCCAGCCACAGAGTCAGAGT-3'
	R	5'-AGCCTCTGAAAGAGAAATGCT-3'
rs2277049 (HRM)	F	5'-GGATGGAGCAAGGGATAGA-3'
	R	5'-ATCACTTTTCCTTTCCTCTGTT-3'

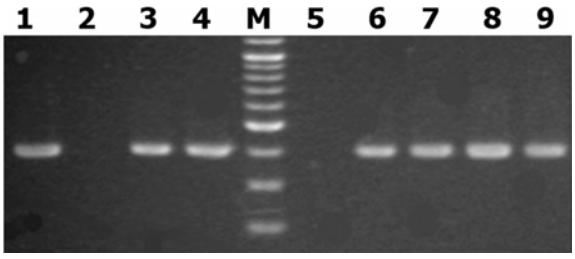

Figure 1. Agarose gel electrophoresis of PCR product amplified by using 3'-LNA primer of rs2277051. Lane M: 100 bp ladder DNA marker, lane 1 to 9: DNA from schizophrenic patients.

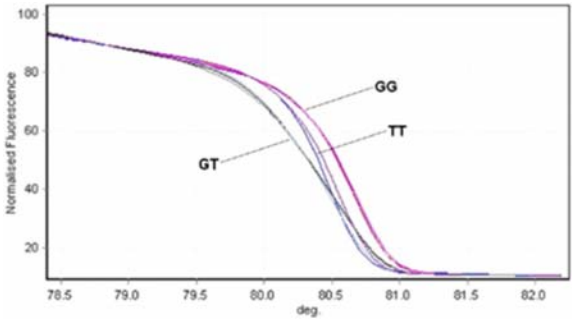

Figure 2. Normalized, high resolution melting curves of SNPs genotypes of rs2277049 from a schizophrenic patient.

receptor gene including rs2278392, rs2277051, rs3734118, and rs2277049 by PCR using 3'-LNA primer and analyzed by Chi-square test revealed that all SNPs were in Hardy-Weinberg equilibrium. The genotypic distribution, allele frequency, and *p*-value were shown in Table 2. The association between schizophrenia was statistically significant with the two SNPs, rs2277051 ($p = 0.001$) and rs2277049 ($p = 0.015$). The allelic frequencies of rs2277051 from schizophrenia were 61%T and 39%C whereas 69% and 31% were found in control. The allelic frequencies of rs2277049 in patients were 79%G and 21%T whereas 66%G and 34%T were in control. No significant association was observed between patient and control in rs2278392 ($p = 0.550$) and rs3734118 ($p = 0.256$). The SNP genotyping of rs2277051 and rs2277049 were repeated by HRM analysis to confirm the LNA result. The data in Table 3 showed that there were significant association between schizophrenia and the two SNPs, rs2277051 and rs2277049, with the *p*-value of 0.014 and 0.013, respectively. These data suggested that the polymorphism in serotonin 5-HT4 receptor may be involved in development of schizophrenia in the Thai population.

Discussion

It had been believed that the incidence of schizophrenia was unaffected by time and place, but recently this belief has been proved to be invalid. New epidemiological results showed that specific circumstances might increase the risk for schizophrenia, included obstetric complications (prenatal infections, migrant status, and seasonal effects (maternal influenza)[16]. Schizophrenia patients usually have less ability to function at social settings in school, and work. They have disorder of thought and concentration, inconsistent speech and behavior, and inability to perform a job or to care for themselves such as daily tasks and personal hygiene. Moreover, they may have diminished facial expression, and in the worst scenario being catatonic. Suicidal thoughts and substance abuse are common in schizophrenia[17].

Cognitive impairment is a core dysfunction of schizophrenia, therefore, molecular mechanisms of cognitive function play an important role in the pathophysiology of schizophrenia[18]. Serotonin 5-HT4 receptor is considered to involve in memory process and learning. Polsinelli G, et al identified epigenetic risk and genetic variants of the serotonin 5-HT4 receptor gene (*HTR4*) for suicidal problem in schizophrenia[19]. They investigated the relationship between *HTR4* and suicidal problem in schizophrenia in 234 patients, but the result was not statistical significance. The study of

Table 2. Genotype and allele frequencies of four SNPs in *HTR4* gene analyzed by PCR using 3'-LNA modified SNPs primers

dbSNP ID	Subject/number		Genotype			*p*-value	Allele frequency (%)	
rs2278392		N	C/C	C/T	T/T		C	T
	Patient	75	20	48	7	0.550	59	41
	Control	170	51	97	22		59	41
rs2277051		N	T/T	T/C	C/C		T	C
	Patients	75	18	55	2	0.001	61	39
	Control	170	80	75	15		69	31
rs3734118		N	A/A	A/G	G/G		A	G
	Patient	75	19	53	3	0.256	61	39
	Control	170	45	108	17		58	42
rs2277049		N	G/G	G/T	T/T		G	T
	Patients	75	49	20	6	0.015	79	21
	Control	170	77	71	22		66	34

Table 3. Genotype and allele frequencies of rs2277051 and rs2277049 analyzed by using high resolution melting (HRM) analysis

dbSNP ID	Subject/number		Genotype			*p*-value	Allele frequency (%)	
rs2277051		N	T/T	T/C	C/C		T	C
	Patient	33	13	20	0	0.014	70	30
	Control	39	26	11	2		81	19
rs2277049		N	G/G	G/T	T/T		G	T
	Patients	40	25	14	1	0.013	80	20
	Control	42	16	17	9		58	42

the 5-HT4 ligand in Japanese population found an association between polymorphism and bipolar disorder such as schizophrenia in the case-control analysis ($p = 0.003$)[20]. SNPs in *HTR4* gene have been studied in Japanese schizophrenia[9]. They found significant association between schizophrenia and the haplotype A-T and suggested that the haplotype A-T—might inhibit the occurrence of schizophrenia[9]. These studies demonstrated the possible association between *HTR4* and schizophrenia which T might use as a marker for this disease. Moreover, the *HTR4* is one of serotonin receptor systems that are highlighted as suitable targets for enhancing cognition and memory for schizophrenia and other disorders[21].

The present study described the genotyping of four SNPs in an intronic region of the *HTR4* gene from DNA samples of Thai schizophrenia patients and normal controls by PCR amplification using 3'-locked nucleic acid (LNA) modified primer and high resolution melting (HRM) analysis. The LNA modified SNPs primer was used in this study, because it has been reported to enhance the binding affinity and yields consistently low amounts of mismatch products. It also increased the specificity and sensitivity of the detection by conventional PCR and real-time PCR. This method was simple, rapid, specific and cost effective for genotyping large amount of samples. Furthermore the results obtained from agarose gel electrophoresis were easily interpreted. For HRM analysis, a method that had high specificity and sensitivity for SNPs genotyping was performed using approximately half amount of both samples (33 to 42 samples) to confirm the result of rs2277051 and rs2277049. The HRM analysis gave the same results as LNA. The polymorphisms in rs2277051 and rs2277049 had a significant association with schizophrenia ($p<0.05$). The genotype C of rs2277051 and G of rs2277049 existed more in schizophrenia group compared to the control group. This finding implicated that these allele were susceptible for the risk of schizophrenia in Thai population. In addition, we used different SNPs for analysis, therefore, we could not compare the allele frequency with other population from the previous study.

Several polymorphisms have been described within each subtype of serotonin receptors such as serotonin receptor 2, 3, and 7. The association study between 5-HTR2A polymorphism and schizophrenia patients showed the C allele and the CT+CC genotype frequencies were significantly higher in patients than in control subjects[22]. The observed mutations in HTR3A were rare and played a minor role in the etiology of schizophrenia[23]. The two genetic variations of 5-HT7 also played a minor role in the development of bipolar

affective disorder and schizophrenia[24]. In addition, 5-HT7 was reported as a susceptible gene for schizophrenia in Japanese population[25].

Conclusion

Two single nucleotide polymorphisms (SNPs), rs2277051 and rs2277049, in *HTR4* (5-hydroxytryptamine receptor 4) gene were significant association with schizophrenia in Thai patient and these SNPs might be used as markers for risk of schizophrenia in Thai population.

What is already known on this topic?

The human *HTR4* gene, which encodes the 5-HT4 receptor has been reported to associate with schizophrenia and bipolar disorder.

What this study adds?

The authors found two single nucleotide polymorphisms (SNPs), rs2277051 and rs2277049, in 5-hydroxytryptamine receptor 4 (5-HT4 receptor: *HTR4*) gene were significant association with schizophrenia in Thai patients.

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Potential conflicts of interest

The authors declare no conflict of interest.

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