Association study between 5-HT_{2A} and NET gene polymorphisms and recurrent major depression disorder in Chinese han population

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Abstract: a functional NET T-182C polymorphism (rs2242446) in the promoter region, a synonymous polymorphisms G1287A in the exon 9(rs5569) and a functional serotonin 2A (5-HT2A) receptor (rs6311) genes in the promoter region were associated with MDD in different populations. However, few studies have focused on the relationship between these three polymorphisms and recurrent MDD patients in Chinese Han population. Three hundred MDD patients (112 males, 188 females) and three hundre unrelated healthy controls were enrolled in the study. POST-PCR ligase detection reaction genotype assay method was used for the genotypic analyses. There existed significant differences both in the frequencies of alleles and genotypes between patients and controls for the 5-HT2A receptor gene polymorphism ($\chi2$=9.267, p=0.01 for genotype; $\chi2$=7.615,p=0.006 for allele). No difference in genotype and allele distribution of G1287A, T182C were found in MDD patients and controls. Our results suggest that the rs6311 polymorphism seems to be the susceptibility factor in etiology of recurrent MDD. In conclusion, 5-HT2A receptor gene variants may be involved in the etiology of MDD, although the results must be verified in larger samples and different ethnicities.

Keywords: Major Depression disorder; 5-HT2A Gene; Nor epinephrine Transporter Gene; rs6311; rs2242446; rs5569

INTRODUCTION

Previous studies have demonstrated that genetic factors played an important role in the etiology of major depressive disorder (MDD). Accumulating evidences have suggested that serotonin system and nor epinephrine have been implicated in the etiology of MDD and antidepressants response (Gelder *et al*., 2000; Stein D *et al*., 2007; Warrington *et al*., 2007). Genes encoding protoins involved in the serotonin system and nor epinephrine transporter (NET) including the 5-HT2A receptors gene and NET gene are the major candidate genes of MDD (Yoshiko *et al*., 2006; Bondy *et al*., 2006; Jokela *et al*., 2007). Of all the SNPs for the 5-HT2A gene, the most extensively investigated single nucleotide polymorphism of this gene is -1438A/G (Bondy *et al*., 2006; Alessandro *et al*., 2007; Vincenzo De *et al*., 2007). Clinical evidences have suggested that the rs6311 (-1438A/G) was reported to have functional functions and not only involved in SSRI treatment response but also with other psychiatry disorder (Viikki *et al*., 2011; Andre *et al*., 2010; Kishi *et al*., 2010). The rs6311 located in the 5-HTR2A gene promoter and has been shown to modulate the expression of 5-HT2A gene and this polymorphism has been shown to be associated with increased 5-HTR2A receptor binding (Myers R *et al*., 2007), thereby making this SNP a promising candidate for an association study. Accumulating studies have also conducted the relationship between this polymorphism and MDD (Christiansen *et al*., 2007; Kishi *et al*., 2009; Illi *et al*., 2009; Tencomnao *et al*., 2010; Myong-Jin *et al*., 2004; Jansson *et al*., 2003).

The NET gene is located on chromosome 16q12.2, which spans approximately 45 kb and consists of 14 exons. Previous studies have found that compared to healthy controls, the expression of the NET gene is reduced in the locus coeruleus in MDD patients, and its role as a major target for antidepressant drugs such as desipramine, nortriptyline and venlafaxine supports the theory that the NET gene is involved in the pathophysiology of depression (Andreoli *et al*., 2002; Ferguson J *et al*., 2003; Chee Hong *et al*., 2006; Bruss *et al*., 1993; Klimek *et al*., 1997; Mizuho *et al*., 2010; Hahn M *et al*., 2008; Wenjiao *et al*., 2009; Yong *et al*., 2009). Taken together, these results suggest a decrease in the level of NE in people with depression, which is consistent with the hypothesis that central nervous system (CNS) noradrenergic dysfunction plays an important role in the pathophysiology of major depressive disorder. Recent studies found that a common T-182C (rs2242446) polymorphism located in the promoter region of this gene may regulate the degree of SLC6A2 transcription activity. Interestingly, this polymorphism has been positively

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associated with MDD (Mei *et al*., 2012). The other synonymous single nucleotide polymorphism G1287A (rs5569) located in exon 9, which has been associated with the concentration of the NE metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) in the cerebrospinal fluid (CSF) of healthy volunteers. And its polymorphism has a close relationship with MDD and antidepressant effects (Papazoglou *et al*., 2012; Ghadirivasfi *et al*., 2011).

However, few studies have focused on the relationship between this three polymorphism and recurrent MDD in Chinese Han population. Therefore, a case-control study was designed to investigate the possible role of three single nucleotide polymorphisms (SNPs) (rs2242446, rs5569, rs6311) of the NET genes among patients with MDD in Han Chinese population.

MATERIALS AND METHODS

Subjects

300 patients with MDD according to DSM-IV criteria (American Psychiatric Association 2000) (American Psychiatric Association, 1994) were recruited in the study(112males, 188famales, mean age 37±13 years, rang 18-60years),which were recruited from patients in He Nan psychiatric hospitals from October 2006 to May 2010 in this study. Severity of depression was assessed using the 17-item Hamilton Rating Scale for depression (HAMD-17). Only subjects with a minimum score of the 21 on the 17-item Hamilton Rating Scale for Depression (HAM-D) (mean score 27.6±6.5 scores, rang 21-47 scores) entered the study. To minimize the effect of ethnic differences in gene frequencies, the study participants were from the Han Chinese population in He Nan province.

Patients with substance abuse or severe organic disorder were excluded after carefully interviewed. And patients with substance abuse, severe organic disorder, organic brain disease or any concomitant major psychiatric disorder were excluded careful clinical interviews.

The normal control group included 300 healthy volunteers (127 males, 173 females, mean age 40±11 years, rang 18-60 years), recruited from the same region of the Chinese Han. We used the Chinese Version of the Modified Schedule of Affective Disorder and Schizophrenia-Lifetime to screen for psychiatric conditions in the control group. The normal control group had a physical examination in the first affiliated hospital of ZhengZhou University. Subjects were free of neurological, psychiatry, and /or past, present major, minor mental illness (affective disorder, schizophrenia, anxiety disorder, personality disorder, substance use disorders), and there was no family history of psychiatric disorder in the control subjects' first-degree relatives. The 17-item Hamilton Rating Scale for depression (HAMD-17) was used to assess the depression symptoms in control subjects, only subjects with a maximum score of the 8 on the 17-item Hamilton Rating Scale for Depression (HAM-D) were permitted into this study. All participants in this study voluntarily, and by oneself or legal guardian informed agreement signed (State Council of the People's Republic of China, 1994).

Polymorphism genotyping

The processing of the blood samples and DNA extracting were completed in the second affiliated hospital of Zhengzhou university medical center laboratory in Henan province, SNP detection completed in Shanghai Donghua University molecular biology laboratory.

Polymerase chain reaction (PCR) assay

Genomic DNA was isolated from the whole blood using a TIANGEN (genomic ext kit Beijing, China) according to the manufacture's instruction. For the SNP of rs6311, forward primer (FP) is 5'-AAATAAGGCTAGAAAACA GTATGTCC-3', and the reverse primer (RP) is 5'-CCACTCTGGACACAAACA CTG-3'. The size of PCR product is 88 bp. For rs2242446, the FP is 5'-CCCAACC TCTGTTTCCAAAT-3', and RP is 5'-CTTGCAA CTCCCAAGACCAC-3'. The size of PCR product is 122 bp. For rs5569, the PW is 5'-GCGAGAAGGAAA GTGCTGAA-3' and RF is 5'- TCCAGGGAGACC CTAATTCC-3'. The size of PCR product is 159bp.

PCR was carried out in a final volume of 20μl containing 50ng of genomic DNA, 20mM each dNTP, 2μl 10xPCR buffer, 0.6μl Mg2+, 0.2μl of Taq DNA polymerase, 4μl Q-solution, 0.4μl Primer mix, 9.8μl H2O.PCR amplification was performed with initial denaturation at 95^{L} for15 min, followed by 35 cycles of 94^{L} for 30 s, 59^{L} annealing for 1min, 72^{L} for 1min, and final elongation at 72^{L} for 7 min.2μl of PCR mixture were run on TBE gel to make sure products were amplified successfully.

Post-pcr ligase detection reaction (ldr) genotyping assay

PCR production was carried out in a final volume of 10μl containing 1μl PCR production, 6.95μl H2O, 0.05μl ligase, 1μl Probe mix, 1μl Buffer. After an initial denaturation step of 95^{L} for 2min, there were 35 cycles with two temperatures. DNA was denatured at 94^{L} for 30s and annealing and extending at 60^{L} for 20min. The LDR products would be used for allele discrimination.

Allele discrimination

Put the production of LDR 1μl ABIGS-500Rox and 1μl deion-formation together, after denaturation step of 95□ for 2min and then got them into the ice-water immediately. The above products were electrophoresed in 10% polyacrylamide and 5mol/L carbamide at the condition of 3000v for about 25 hours and the use the GENESCAN TM 672 software to collect the data, correct the line of electrophorese, measure different fragments, Adopt the Genemapper software to analyze the data genotype distributions.

STATISTICAL ANALYSIS

We used the independent samples t test to determine the difference in mean age between patients with major depression and normal control subjects and we used Pearson chi-square analysis to compare sex difference between the patient group and the control group. We assessed Hardy–Weinberg equilibrium for each group, and the frequencies of genotype and allele were also compared between patients and control subjects, using the Pearson chi-square analysis. Fisher's exact test was substituted for the Pearson's chi-square when sample sizes were smaller than expected (fewer than 5 subjects). Choose odds ratio (OR) and 95% confidence interval (CI) to assess the risk of depression. All tests were 2-tailed and alpha level was set at 0.05. Statistical analyses were performed using SPSS (version 13.0) software for Windows.

RESULTS

The genotype distributions of the rs6311, rs2242446 and rs5569 were in Hardy-Weinberg equilibrium both in the MDD patients and control subjects.

The results of the genotype distributions and allele frequencies for these three SNPs in patients and control subjects were summarized in table 1. The chi-square ($\chi 2$) test showed positive in allelic association for SNP rs6311 (OR=0.723, 95%CI=0.574~0.91, $\chi 2$=7.615, p=0.006) and in genotypic association ($\chi 2$=9.267, p=0.01), indicating that CC genotype and C allele of SNP rs6311 are higher in patients than control subjects. However, the SNP rs2242446 and rs5569 failed to detect any significant association between case- control group ($p>0.05$).

The results of the genotype distributions and allele frequencies for rs6311, rs2242446 and rs5569 in case and control groups for the female sex. (table 2). Significant difference were evident for the allele or the genotype frequencies between patients and control subjects for the same sex for rs6311 ($p<0.05$), while, no significant difference were evident for the allele or the genotype frequencies between patients and control subjects for the same sex for rs5569 and rs2242446 ($p>0.05$).

The results of the genotype distributions and allele frequencies for rs6311, rs2242446 and rs5569 in case and control groups for the male sex. (table 3).Significant difference were evident for the genotype frequencies between patients and control subjects for the same sex for rs6311 ($p<0.05$), while, no significant difference were evident for the allele or the genotype frequencies between patients and control subjects for the same sex for rs5569 and rs2242446 ($p>0.05$).

DISCUSSION

To our knowledge, this is the first study to examine the association between the 5-HT2A, NET gene polymorphism and recurrent MDD in a case-control study in the Chinese Han population.

Our results suggest that the C/C genotype of rs6311 is a risk genotype for MDD patients in Chinese Han population. Similarly, the results of this study is consistent with those by Myong-Jin *et al* (Myong-Jin *et al*., 2004) who also found that the C/C genotype may represent risk genotypes for MDD in Korean population. On the other hand, Jansson *et al* (Jansson *et al*., 2003) and Christiansen *et al* (Christiansen *et al*., 2007) found that T/T genotype may be associated with an increased risk for MDD in Swedish and Danish population, respectively. Nevertheless, Kishi *et al* (Kishi *et al*., 2009), Illi *et al* (Illi *et al*., 2009) and Tencomnao *et al* (Tencomnao *et al*., 2010) reported no association between this polymorphism and recurrent MDD.

The rs6311 allele frequency in our samples (0.448) was not the same as Tencomnao *et al* (Tencomnao *et al*., 2010) (0.197) and Illi *et al* (Illi *et al*., 2009) (0.197) in Thai and Finnish population, respectively. The discrepancy in allele frequencies may be partly responsible for the divergent association results. The sample size in this study is different from the above mentioned previous studies, which may influence the statistic power and thus got different results.

Studies have also demonstrated that rs6311 T allele can exclusively binding site for transcription factor Th1/E47, and this allele also can specifically increased 5-HTR2A receptor binding thus differentially modulated density of the receptor critical for neurotransmitter mechanisms, thereby making this SNP a promising candidate gene for various association study (Mei *et al*., 2012; Papazoglou *et al*., 2012; Ghadirivasfi *et al*., 2011; Saiz P *et al*., 2011). Based on these evidences and our results, we speculated that rs6311 C allele decreased the 5-HTR2A receptor binding which leaded to influence the signal transduction of 5-HT thus influenced the transmission of 5-HT and might be the susceptible factors for MDD. Large replication studies with different ethnic groups are needed to determine whether there are ethnic differences in the influence of the rs6311 polymorphism on major depression.

The nor epinephrine transporter gene is a plausible candidate gene for major depression, and it provides an avenue for investigating the susceptibility to major depression and/or response to antidepressant therapy (Keizo *et al*., 2004). The results showed no association between major depression and the promoter T-182C or the exonic G1287A polymorphism of the NET gene in Han Chinese subjects.

Table 1: Genotype distributions and Allele frequencies of three SNPs between MDD patients and control subjects

SNP	Group	Genotype frequency (%)			Allele frequency (%)	
		TT	CT	CC	T	C
Rs6311	Case	93(31)	145(48.3)	62(30.7)	331(55.2)	269(44.8)
	Control	129(43)	120(40)	51(17)	378(63)	222(37)
		$\chi2$=9.267	p=0.01		$\chi2$=7.615	p=0.006
Rs2242	Case	150(50)	123(41)	27(9)	423(70.5)	177(29.5)
446	Control	158(52.7)	111(37)	31(10.3)	427(71.2)	173(28.8)
		$\chi2$=1.099	p =0.577		$\chi2$=0.065	p =0.799
Rs5569	Case	23(7.7)	126(42)	151(50.3)	172(28.7)	428(71.3)
	Control	30(10)	115(38.3)	155(51.7)	175(29.2)	425(70.8)
		$\chi2$=1.479	p =0.477		$\chi2$=0.036	p =0.849

Table 2: Genotype and allele frequencies of rs6311, rs2242446 and rs5569 gene single nucleotide polymorphism in major depression disorder and control groups in female sex.

SNP	Group	Genotype frequency (%)			Allele frequency (%)	
		TT	CT	CC	T	C
Rs6311	Case	56(52.3)	90(8.4)	42(39.3)	202(53.7)	174(46.3)
	Control	65(41.9)	88(56.8)	20(12.9)	218(63.0)	128(37.0)
		$\chi2$=8.413	p=0.015		$\chi2$=6.381	p=0.013
Rs2242	Case	70(47.6)	63(42.9)	14(9.5)	203(69.0)	91(31.0)
446	Control	88(55.0)	56(35.0)	16(10.0)	232(72.5)	88(27.5)
		$\chi2$=2.049	p =0.359		$\chi2$=0.884	p =0.347
Rs5569	Case	10(6.6)	66(43.7)	75(49.7)	86(28.5)	216(71.5)
	Control	14(9.9)	50(35.5)	77(54.6)	78(27.7)	204(72.3)
		$\chi2$=2.560	p =0.278		$\chi2$=0.048	p =0.854

Table 3: Genotype and allele frequencies of rs6311, rs2242446 and rs5569 gene single nucleotide polymorphism in major depression disorder and control groups in female sex.

SNP	Group	Genotype frequency (%)			Allele frequency (%)	
		TT	CT	CC	T	C
Rs6311	Case	37(34.9)	55(51.9)	20(13.2)	129(57.6)	95(42.4)
	Control	64(50.4)	32(25.2)	31(24.4)	160(63.0)	94(37.0)
		$\chi2$=14.79	p=0.001		$\chi2$=1.453	p=0.261
Rs2242	Case	80(52.3)	60(39.3)	13(8.4)	423(70.5)	177(29.5)
446	Control	70(50.0)	55(39.3)	15(10.7)	427(71.2)	173(28.8)
		$\chi2$=0.451	p =0.798		$\chi2$=0.065	p =0.849
Rs5569	Case	13(8.7)	60(40.3)	76(51.0)	172(31.0)	428(69.0)
	Control	16(10.1)	65(38.3)	78(51.6)	175(29.2)	425(70.8)
		$\chi2$=0.212	p =0.899		$\chi2$=0.036	p =0.899

In this study, we focused on the identification of a functional polymorphism in the 5' flanking promoter region of the NET gene. Our results suggest that the C/C genotype isn't a risk genotype for MDD patients. Similarly, the results of this study aren't consistent with those by Seung-Ho *et al* (Seung-Ho *et al*., 2004), Ning *et al* (Ning *et al*., 2008) and Yong *et al* (Yong *et al*., 2009) who also found that the C/C genotype may represent risk genotypes for MDD in Korean and Chinese Han population, respectively.

Our results are inconsistent with those by Kazuyuki *et al* (Kazuyuki *et al*., 2007), though Inoue *et al*'s study was performed in Japanese population. On the other hand, Chuan-Chia *et al* (Chuan-Chia *et al.,* 2007) and Peter *et al* (Peter *et al*., 2002) reported no association between this polymorphism and MDD.

These inconsistent and contradictory results can be attributed to three factors: first, the sample size in this study is different from aforementioned previous studies, which may influence the efficiency of analysis power; second, this study focuses exclusively on recurrent MDD patients, while samples from other studies have more clinical heterogeneity. Finally, it could be expected that the different allele frequency identified among different

ethnic back-ground influenced the statistical power thus got different results.

Studies have demonstrated that the T-182C (rs2242446) polymorphism may influence NET gene expression by modifying the binding affinity of nuclear expression factors (Leszczynska R *et al*., 2002; Rippel C *et al*., 2006; Samochowiec *et al*., 2002; Tarkowski *et al*., 2000). The T→→C point mutation is located 182 bp upstream of the first codon, 20bp downstream of a CCAAT-box, 84 bp downstream of a SP1 binding site, and 98 bp downstream of a binding site for the transcription factor C/EBP (Tarkowski *et al*., 2000; Kim *et al*., 2008). Moreover, the ancestral C allele of rs2242446 may also disrupt the binding site for the cell cycle-dependent element CDF-1 (CDE/CHR-like element) a possible NET transcriptional repressor (Boung-Chul *et al*., 2008).

Furthermore, the T-182C polymorphism may be in linkage disequilibrium with other polymorphisms within the NET promoter region that are causally related to MDD. Not accounting for these additional polymorphisms may help explain previous inconsistent results. Though we did not find a significant association between T-182C and MDD, further studies on promoter activity in this gene variant are warranted to analyze potential effects on NET expression and the association with other NE-dependent behavioral traits.

Gerald *et al* (Gerald *et al*., 1996) reported that the G1287A polymorphism is a silent mutation with no functional consequences. Our results are consistent with the findings by Kazuyuki *et al* (Kazuyuki *et al*., 2007) Chuan-Chia *et al* (Chuan-Chia *et al*., 2007) and Peter *et al* (Peter *et al*., 2002), which also demonstrated no association between this polymorphism and MDD in Caucasian, Chinese and Japanese population respectively. However, our findings are inconsistent with those of Yong *et al* (Yong *et al.*, 2009) in Chinese population. Yong *et al* (Yong *et al.*, 2009) study found that only rural women carrying the G/G genotype of the G1287A polymorphism were susceptible to MDD.

However, the affinity of the binding or the transport of neurotransmitters may be affected by this exon or another neighboring exon, which may influence the NET expression (Higuchi *et al*., 2009). Furthermore, Kim *et al* (Kim *et al*., 2008) confirmed that disturbance in NET expression signaling may be the risk factors of the psychiatric disorder by modifying nor epinephrine transmission. Therefore, the future studies should focus on the exon activity in this gene variant to analyze its potential effects on NET expression.

Furthermore, Jonsson *et al* (Jonsson *et al*., 1998) reported that CSF MHPG concentrations were higher in the G/G genotype than in either the G/A or A/A genotypes of healthy volunteers. Hahn *et al* (Hahn *et al*., 2008) reported that the urinary concentration of D-MHPG in depressive parents was 1.3 times higher than in healthy volunteers. Yoshida (Yoshida *et al*., 2002) reported that the A/A genotype is associated with a lower response to the SNRI milnacipran than the G/A genotype in Japanese major depressed patients, while Kim *et al* (Kim *et al*., 2008) reported that the G/G genotype was associated with a better response to nor epinephrine reuptake inhibitors (NRIs) than selective serotonin reuptake inhibitors (SSRIs) in elderly Korean depression patents. These observations suggest that the NET G1287A (rs5569) polymorphism may be involved in the development of MDD and may also useful for predicting the response to NET-targeted antidepressants. Moreover, the G1287A polymorphism is located in a region encoding an uncharacterized domain of the protein between two trans membrane domains (Ebmeier *et al*., 20062).

It has also been reported that in the United States, the lifetime prevalence of MDD in women is approximately twice that in men (Suiho *et al*., 2011; David *et al*., 2005), we hypothesized that the 5-HTR2A, NET polymorphism is associated with gender differences in MDD. Therefore, we compared genotype and allele frequencies of the rs6311, rs5569 and rs2242446 single nucleotide polymorphism in MDD and control groups within the same gender, and again detected a signaficant relationship between rs6311 and MDD. However, no significant association was found between rs5569, rs2242446 and MDD.

Our conclusion is still needed to explain with great caution to interpret the 5-HT2A gene polymorphisms to MDD patients. Because MDD is a complex disorder caused by multiple genes and parameters such as family history, sex, self-directedness, some personality traits, negative events (Wai *et al*., 2011). Furthermore, studies have indicated that the genetic factor of MDD is approximately 60% (Levinson, 2006; Nobile *et al*., 2004), thus future studies should take genetic, environment and other factors into account to fully investigate the etiology of MDD.

The main limitation of our study is that the small sample size. It is generally accepted that case-control association studies are influenced by sample size and that small sample size can lead to false results. Another limitation of this study is that we only focused on one SNP located on the 5-HT2A gene. It is possible that other SNP of 5-HT2A gene may be important in conferring susceptibility to MDD patients. Studies have also demonstrated that rs6313 which had a strong linkage disequally with 6311 also associated with some psychiatry disorder. Thus, future studies should also take rs6313 into consideration to fully explore its role in the etiology of MDD.

In conclusion, we report a significant association of a 5-HT2A polymorphism with recurrent MDD. The results

presented here suggest that the investigated genetic variants of the NET gene do not play a major role in increasing susceptibility to recurrent major depression. Prospective studies with a much larger group, preferably in family-based samples, are necessary to confirm the results of our study.

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