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# Association of C/T polymorphism in intron 14 of the dopamine transporter gene (rs40184) with major depression in a northeastern Thai population

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**ABSTRACT.** Several lines of evidence suggest that the dopaminergic system is involved in the pathophysiology of major depressive disorder (MDD). Since the dopamine transporter (DAT1, also known as SLC6A3), mediates the active reuptake of dopamine from the synapses and thereby plays a vital role in the regulation of dopaminergic neurotransmission, we looked for a possible association between the C/T single nucleotide polymorphism in intron 14 of the *DAT1* gene (also referred to as rs40184) and MDD in a northeastern Thai population. One hundred and seventy-eight patients with MDD and 205 unrelated healthy controls were included in our study. Genotyping was performed using our newly established

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polymerase chain reaction-restriction fragment length polymorphism technique. We found no significant differences in genotype distributions, allele frequencies and allele carrier frequencies when comparing the two groups. Although not significant, we observed more carriers of the C allele (CC+CT genotypes) in healthy controls than in patients with MDD ($\chi^2$ = 3.20, degrees of freedom = 1, P = 0.073, odds ratio = 0.53 [95% confidence interval = 0.28-1.01]). We also detected significant differences in the allele frequencies of rs40184 between healthy subjects of Asian ancestry and those of both Caucasian and African ancestry. We concluded that there is a tendency towards an association between the homozygous TT genotype of the rs40184 single nucleotide polymorphism and an increased risk for MDD in this northeastern Thai population. Possibly, with more samples, this tendency will be confirmed.

**Key words:** Major depressive disorder; Association study; *DAT1*; rs40184; Single nucleotide polymorphism; Thai population

## INTRODUCTION

Major depressive disorder (MDD) has been a growing public health concern due to its recurrent, deliberate and lethal nature. According to projections, MDD will become the second leading cause of disability worldwide by the year 2020 (Murray and Lopez, 1997). By the year 2030, it is estimated to be the highest cause of disability in high-income countries and still the second cause of burden of disease globally (Mathers and Loncar, 2006). Having been recognized as a multifactorial disease, the total contribution of genetic factors in the origin of disease, the heritability, is estimated at nearly 40% (Sullivan et al., 2000). Notably, the mode of inheritance is complex and ambiguous. Thus, relevant DNA sequence variations in potential candidate genes contributing to the susceptibility to MDD remain to be explored.

Studies on norepinephrine and serotonin pathways have highlighted the molecular role of these neurotransmitter systems in the pathophysiology of MDD. Nevertheless, the role of dopamine (DA) neurotransmission in MDD has gained increasing attention since the earliest report in the mid-1970s (Randrup et al., 1975). Clinically, changes in both serotonergic and dopaminergic activity have been observed in patients with MDD receiving long-term treatment with antidepressants (Bonhomme and Esposito, 1998). Also, therapy using dopaminergic agents in treatment-resistant patients with MDD has been demonstrated to enhance the action of antidepressant medications (Nierenberg et al., 1998). Furthermore, antidepressants with direct dopaminergic effects have been well documented (Robinson, 2007). Research findings have consistently emphasized the molecular connection between genes associated with dopaminergic activity and the pathophysiology of MDD (Davidson et al., 2002; Dunlop and Nemeroff, 2007).

The dopaminergic system, which consists of DA-producing cells, DA receptors and DA transporters (DAT1, also referred to as SLC6A3), may play a crucial role in MDD. In particular, DAT1 proteins play a significant role in the reuptake of DA into presynaptic neurons and limit the duration of synaptic activity, thus being the key regulators of DA level in the brain. In humans, the *DAT1* gene, located at chromosome 5q35.1, contains 15 exons (Giros et al., 1992; Vandenbergh et al., 1992), and its expression occurs in all DA neurons,


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including those originating in the substantia nigra and ventral tegmentum (Ciliax et al., 1995). Although several lines of evidence suggest an association between DA and MDD, few studies have directly explored the molecular link between MDD and polymorphisms in such main dopaminergic gene as *DAT1*. For instance, no association between a 40-bp variable number of tandem repeats in the 3' untranslated region of the *DAT1* gene and MDD was found (Frisch et al., 1999). To the best of our knowledge, most subsequent studies have reported no association with regard to genetic variations of the *DAT1* gene in MDD.

Until recently, the C/T single nucleotide polymorphism (SNP) in intron 14 of the *DAT1* gene, also referred to as rs40184, has been demonstrated to moderate the effect of perceived maternal rejection on the onset of MDD, as well as on suicidal ideation, thus signifying a gene-by-environment (G x E) interaction in the etiology of MDD (Haeffel et al., 2008). This particular SNP has also been found to play a genetic role in certain neuropsychiatric and neurological illnesses such as attention deficit hyperactivity disorder (Zhou et al., 2008), bipolar disorder (Mick et al., 2008) and migraine with aura (Todt et al., 2009). With regard to the genotyping technologies used in the research findings addressed above, the rs40184 polymorphism was genotyped on one of the high-throughput platforms, particularly Sequenom, Taqman and Illumina. At present, the rs40184 genotyping method based on polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) has not been established despite the fact that it has gained wide popularity in recent years. Since this SNP is not involved in a change of endonuclease cleavage, it is actually difficult to develop this particular PCR-RFLP genotyping method. It is worth mentioning that the functional significance of such intronic SNP as rs40184 is unclear; it is, however, hypothesized to affect gene function by certain mechanisms including alteration of the stability, splicing and localization of mRNA, as well as production of a small RNA.

Therefore, our present study aimed at performing a case-control study to investigate the association between the rs40184 polymorphism and MDD in a northeastern Thai population using our newly established, simple, economical and practical PCR-RFLP genotyping approach. In addition, the allele frequencies of this SNP in healthy subjects were compared among various populations.

## MATERIAL AND METHODS

### Subjects

We successfully genotyped genomic DNA samples obtained from 178 unrelated patients with MDD (115 women and 63 men, aged 44.79 ± 13.05 years) and 205 unrelated healthy controls (128 women and 77 men, aged 41.38 ± 9.79 years). The MDD subjects were diagnosed according to DSM-IV criteria by experienced psychiatrists of Loei Rajanagarindra Psychiatric Hospital, the organization providing mental healthcare services for Loei, Nongbualamphu and other nearby provinces of Thailand. The two groups permanently resided in the same region, and were gender and age matched. The local Ethics Committee of medical experiments on human subjects approved the study, and all participants gave their written informed consent.

### Genotyping of the rs40184 polymorphism

Genomic DNA samples were extracted from whole blood using the FlexiGene DNA kit (Qiagen, Hilden, Germany).

The utilization of NEBcutter, version 2, which is classified as internet-based bioinformatics (http://tools.neb.com/NEBcutter2/index.php), demonstrated that the *Hin*dIII restriction enzyme would be useful to distinguish the different allele of the rs40184 polymorphism. Because the natural PCR-RFLP could not be applied, we relied on mismatch PCR-RFLP for this purpose. We designed a mutagenic PCR primer complementary to the *DAT1* gene that resulted in the PCR products carrying the *Hin*dIII site (AAGCTT) corresponding to allele T, while the *Hin*dIII site would be absent in allele C because of the PCR products containing the sequence AGGCTC. Technically, the first PCR primer (5'-CTGGGGTACAATCTGGAACCAGC-3') was not mutagenic, and it was designed to locate at nucleotide position 55315, as a starting position, of the *DAT1* gene based on GenBank accession number NG_015885. The second PCR primer (5'-TGAAGTAGATCCGATGCCTCACTCAA**G**-3') corresponded to nucleotide position 55496 of the sequence NG_015885 as a starting point. In order to avoid a CG-rich string in the primer, the underlined dinucleotides, AA and TA, were changed from CC and GG of the natural *DAT1* gene sequence, respectively. The bold nucleotide (**G**) was mutated from A of the natural *DAT1* sequence, thus leading to the generation of PCR products with the first 3 nucleotides (AAG) of the *Hin*dIII site according to the 3' end of this particular primer.

Briefly, the PCR condition (35 cycles at 63°C annealing temperature based on this newly designed primer pair) in a final volume of 25 µL was carried out using *Taq* DNA polymerase with standard *Taq* buffer (New England Biolabs, Beverly, MA, USA) according to manufacturer instructions. Subsequently, RFLP was performed by digesting 10 µL of the 182-bp PCR products with 10 units of the *Hin*dIII enzyme (Fermentas, Burlington, ON, Canada) at 37°C overnight. When analyzed using 4% agarose gel electrophoresis, PCR fragments containing the C allele had one uncut band of 182 bp, whereas fragments containing the T allele were divided into two bands of 157 and 25 bp.

## Statistical analysis

For gender data, patients with MDD and healthy control groups were compared by the chi-square ($\chi^2$) test in contingency tables. Difference in age data between the two groups was examined using the unpaired Student *t*-test. Hardy-Weinberg equilibrium (HWE) for the distributions of genotypes was estimated by the $\chi^2$ test. Genotype, allele and allele carrier frequencies were compared between groups using the $\chi^2$ test with Yates correction. Odds ratios (OR) with 95% confidence interval (CI) were calculated using the statistical Epi-Info program, version 6 (Centers for Disease Control and Prevention, 1994). When comparing allele frequencies of the rs40184 polymorphism in healthy subjects from various populations, statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Tukey honestly significant difference (HSD) test. A P value of $<0.05$ was considered to be significant for all statistical tests in this study.

# RESULTS

Regarding gender and age, characteristics of patients with MDD and healthy controls were compared. There was no statistically significant difference between these two groups with respect to gender (P = 0.660). However, there was a difference in age between the two groups (P = 0.004). In particular, proportions of 30's and 40's were increased in the control group, while proportions of 50's and 60's were elevated to some extent in the patient group.

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We successfully established the PCR-RFLP technique for genotyping of rs40184 polymorphism in a Thai population. With regard to healthy controls, their genotype distributions were in HWE ($\chi^2 = 0.091$, d.f. = 1, P = 0.763). The genotype distributions and allele frequencies for this SNP are shown in Table 1. No significant difference was detected between the two groups when examining genotype distributions, allele frequencies and allele carrier frequencies. Nevertheless, we observed more carriers of the C allele (CC + CT genotypes) in healthy controls than in patients with MDD ($\chi^2 = 3.20$, d.f. = 1, P = 0.073, OR = 0.53 [95%CI = 0.28-1.01]).

**Table 1.** Genotype distributions, allele frequencies and allele carrier frequencies of the rs40184 single nucleotide polymorphism in Thai patients with major depressive disorder (MDD) and healthy controls.

| rs40184 | MDD patients | Healthy controls | $\chi^2$ | P | OR (95%CI) |
|---|---|---|---|---|---|
| Genotype distributions | | | | | |
| CC | 88 (49.4%) | 107 (52.2%) | | | |
| CT | 64 (36.0%) | 81 (39.5%) | | | |
| TT | 26 (14.6%) | 17 (8.3%) | 3.84, d.f. = 2 | 0.150 | |
| Allele frequencies | | | | | |
| C | 240 (67.4%) | 295 (72.0%) | | | |
| T | 116 (32.6%) | 115 (28.0%) | 1.65, d.f. = 1 | 0.200 | 0.81 (0.59-1.10) |
| Allele carrier frequencies | | | | | |
| C+(CC+CT) | 152 (85.4%) | 188 (91.7%) | | | |
| C-(TT) | 26 (14.6%) | 17 (8.3%) | 3.20, d.f. = 1 | 0.073 | 0.53 (0.28-1.01) |
| T+(CT+TT) | 90 (50.6%) | 98 (47.8%) | | | |
| T-(CC) | 88 (49.4%) | 107 (52.2%) | 0.19, d.f. = 1 | 0.663 | 1.12 (0.75-1.67) |

The allele frequencies of the rs40184 SNP in healthy groups were compared among various populations, and the allele T was the minor allele for all ethnic groups (Table 2). Significant differences were detected among those of Caucasian descent, African decent and Asian descent (P = 0.009). Using one-way ANOVA followed by the Tukey HSD test for all pairwise comparisons, the allele frequencies were significantly different when compared between those of Asian ancestry and those of either Caucasian ancestry or African ancestry (P = 0.015). When comparing between those of Caucasian ancestry and African ancestry, there was no significant difference in the allele frequencies of this polymorphism (P = 0.782). Within Asian ancestry, we found that the allele frequencies of the rs40184 SNP in the Thai population were similar to those of Han Chinese, but very different from those of Japanese.

**Table 2.** Allele frequencies of the rs40184 single nucleotide polymorphism (SNP) in healthy subjects from various populations.

| Populations (reference) | Samples | rs40184 | |
|---|---|---|---|
| | | T | C |
| Caucasian ancestry | | | |
| European+ | 120 | 0.45 | 0.55 |
| North American+ | 92 | 0.39 | 0.61 |
| Russian (Haeffel et al., 2008) | 176 | 0.47 | 0.53 |
| German (Todt et al., 2009) | 272 | 0.46 | 0.54 |
| African ancestry | | | |
| Sub-Saharan African+ | 120 | 0.47 | 0.53 |
| African American+ | 90 | 0.49 | 0.51 |
| Asian ancestry | | | |
| Han Chinese+ | 90 | 0.32 | 0.68 |
| Japanese+ | 90 | 0.13 | 0.87 |
| Thai (this study) | 205 | 0.28 | 0.72 |
| P* | | 0.009 | 0.009 |

*Statistically significant difference by one-way analysis of variance (ANOVA). +Extracted data from the NCBI SNP Database (http://www.ncbi.nlm.nih.gov/projects/SNP/).

## DISCUSSION

Several lines of evidence in experimental, pharmacological and physiological research suggest that the *DAT1* gene is involved in MDD. The impact of the rs40184 polymorphism on the susceptibility of MDD is of immense interest since it has been recently shown to be associated with specific neuropsychiatric and neurological disorders (Haeffel et al., 2008; Zhou et al., 2008; Mick et al., 2008; Todt et al., 2009). Although the molecular connection between this SNP and cellular DAT1 level has not been demonstrated, many reports in the literature link polymorphisms of unknown function to certain psychiatric diseases (Cook Jr. et al., 1995). Thus, we carried out a study on the genotype distributions, allele frequencies and allele carrier frequencies of the rs40184 SNP in northeastern Thai using a case-control design. To the best of our knowledge, the PCR-RFLP genotyping approach has never been utilized for this particular SNP. The natural PCR-RFLP method could not be utilized here since this SNP was not directly involved in the endonuclease recognition site. The NEBcutter program was used for designing and evaluating the mutagenic primer. In fact, another web-based program called SNP Cutter has been reported to be more user-friendly with regard to an SNP PCR-RFLP assay design (Zhang et al., 2005). Eventually, we successfully developed this approach based on the mismatch PCR-RFLP technique that has been previously proven to be practical, reliable and inexpensive (Haliassos et al., 1989a,b; Xiao et al., 2006). We are the first to establish and employ this method for the rs40184 SNP in the present study.

No significant difference was found between the two groups when considering genotype distributions, allele frequencies and allele carrier frequencies. Although not significant in every analysis, we observed more carriers of the C allele (CC + CT genotypes) in healthy controls than in patients with MDD, thus suggesting a tendency towards an association between the homozygous TT genotype and an increased risk for MDD. According to the previous investigation in a Russian population, subjects who reported maternal rejection and also had the TT genotype of the rs40184 SNP were the most likely to have a current episode of MDD (Haeffel et al., 2008), thus being in agreement with our study. Nevertheless, our present study design was different from that of Haeffel and colleagues, mainly the aspect of G x E interaction. In other words, we focused on the main effect of genotype, while they examined the interaction of genotype and environment. It should be additionally noted that we are the first to report the significant difference in the healthy subjects' allele frequencies of the rs40184 between those of Asian ancestry and those of either Caucasian ancestry or African ancestry. This finding truly supports the idea that association studies should be performed in each population.

With respect to the subjects recruited in this study, both patient and control groups resided in the same locality, and their psychosocial backgrounds were similar, supporting the strength given to this study. Regarding a point of concern, the noted difference in age between the two groups in the present study may have influenced the outcome in the association test. For instance, certain healthy controls may become ill due to MDD in the future. Although proportions of 50's and 60's were increased in the patient group, we had no data concerning the number of patients with MDD with younger age of onset. It is worth stating that we successfully carried out the study by taking the utmost precaution in genotyping accuracy. This concern is of enormous significance to prevent misgenotyping and misinterpretation. In particular, we have recently discovered specific misgenotyping results of SNP in one of the major genes of dopaminergic system, dopamine receptor D1 gene -48A/G polymorphism (Tencomnao and Boonmalert, 2009).

The possible association between *DAT1* gene polymorphisms and susceptibility to MDD should be investigated in a larger group in order to provide higher statistical power. We highly

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suggest that the potential association between the rs40184 SNP and MDD should be studied in other populations. Given the role of DA in the etiology of MDD, we also recommend that future research explore the contribution of other candidate genes in the dopaminergic system.

In conclusion, the present study offers a newly established PCR-RFLP method to genotype the rs40184 SNP. We found no significant difference between case and control groups with respect to genotype distributions, allele frequencies and allele carrier frequencies of this particular SNP. Nevertheless, the present investigation provides evidence that there is a tendency towards an association between the homozygous TT genotype and an increased risk for MDD in the northeastern Thai population. The result of this study also indicates that the allele frequencies of this SNP are different among ethnic groups.

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