Title: A functional variant in *HTR7,* rs7905446, is associated with good response to SSRIs in bipolar and unipolar depression

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Running title: HTR7 and antidepressant response

Abstract (249 words)

Predicting antidepressant response has been a clinical challenge for mood disorder. Although several genome-wide association studies have suggested a number of genetic variants to be associated with antidepressant response, the results are difficult to replicate. Previous animal studies have shown that knockout of the serotonin receptor 7 (*HTR7*) resulted in an antidepressant-like phenotype, suggesting it was important to antidepressant action. In this report, in the first stage, we used a cost-effective pooled-sequencing strategy to sequence the entire *HTR7* gene to investigate the association of common variants in *HTR7* and clinical response to four selective serotonin reuptake inhibitors (SSRIs) in a retrospective cohort mainly consisting of subjects with bipolar disorder. We found 80 SNPs with FDR < 0.05 associated with response to paroxetine, among which rs7905446 (T/G), located at the promoter region, also showed nominal significance in fluoxetine group. TG/GG genotypes for rs7905446 and female gender were associated with better response to two SSRIs (paroxetine and fluoxetine). In the second stage, we replicated this association in two independent prospective samples of SSRI-treated patients with major depressive disorder: the MARS (P = 0.0169) and GENDEP studies (P = 0.008). The TG/GG genotypes were consistently associated with response in all three samples. Functional study showed that the rs7905446-G allele interacted with CEBPB transcription factor, and displayed greater promoter activity in two neuronal-related cell lines while estrogen treatment decreased the activity of only the G allele. Our results provided novel pharmacogenomic evidence to support the role of *HTR7* in association with antidepressant response.

Key words: HTR7, SSRI, paroxetine, fluoxetine, antidepressant response

Background (full text: 3493)

Serotonin (5-HT) is a monoamine neurotransmitter with a broad range of physiological functions including sleep, mood, cardiovascular function, circadian rhythms, body temperature, food intake and endocrine regulation. These effects are mediated by a large number of 5-HT receptors, comprising seven families (HTR1 to HTR7) and at least 14 subtypes, among which HTR7 displays the highest affinity for 5-HT (1-3). HTR7 is a G-protein-coupled receptor that links to adenylate cyclase and transduces signals mainly through the cAMP pathway (3, 4). HTR7 has been shown to be expressed abundantly both in peripheral tissues like smooth muscle and intestine, and in brain regions including the forebrain, hippocampus, hypothalamus, brainstem and cerebellum (4-7).

A growing body of evidence has indicated that HTR7 plays a role in the pathophysiology of psychiatric disorders. Genome-wide association studies (GWAS) have suggested a relationship between *HTR7* genetic polymorphisms and schizophrenia and the development of alcohol dependence (8-10). HTR7 was also shown to influence behaviors in rodents mimic obsessive-compulsive disorder and substance abuse (11, 12). Much attention has been devoted to the possible role of HTR7 in depression. HTR7 knock-out mice or mice with pharmacological blockade of HTR7 showed antidepressant-like behavior (13-16). A recent study showed genetic polymorphisms in *HTR7* were associated with cortisol levels, suggesting HTR7 may contribute to stress and inflammation (17). Emerging preclinical evidence have suggested that HTR7 is involved in the action of antidepressants. Several antidepressants, both tricyclics and selective serotonin reuptake inhibitors (SSRIs), induce c-fos expression in a fashion that is similar to HTR7 activation, while chronic treatment by fluoxetine downregulates HTR7 expression (18, 19). In addition, blockade of HTR7 by SB-269970 was found to potentiate the effects of SSRI and norepinephrine reuptake inhibitors (NARI) (14). Indeed, several antidepressant and antipsychotic drugs with clinically established antidepressant efficacy showed high affinity for HTR7, such as amitriptyline, amoxapine, amisulpride, clozapine, aripiprazole, lurasidone, risperidone and perospirone (20-23). Thus, the above

evidence suggests HTR7 could play an important role in SSRI action and may serve as a potential target for the treatment of depression.

SSRIs (e.g. paroxetine and fluoxetine) are the most widely used antidepressants for the treatment of major depressive disorder (MDD), however around half of the patients show poor response to SSRIs (24). Treatment resistant in MDD is common and evidence show that a substantial portion of the treatment resistant MDD patients may later be diagnosed as bipolar disorder (BD) (25). BD is a complex and chronic psychiatric condition affecting 1-2% of the population and, characterized by shifts in mood between manic and depressive states (26). Although mania is the most dramatic manifestation of BD, in reality patients spend most of their time depressed when ill (27). Though there are many effective treatments for mania, treating bipolar depression remains a considerable clinical challenge (28). The primary dilemma is the use of antidepressants; there is a risk of inducing a manic episode or rapid cycling, though the larger question is one of efficacy. Despite widespread safe and seemingly effective use in the community, many controlled trials have failed to show efficacy for antidepressants in BD (28). This suggests heterogeneity in drug response and possibly disease mechanism. Several large-scale GWAS have examined the association between genetic markers and antidepressant response, however the results are difficult to replicate and only a limited number of SNPs in *HTR7* have been covered (29-31). The overall goal of this study is to identify genes that influence SSRI response in BD. In this report, in the first stage, we utilized a cost-effective pooled-sequencing strategy to sequence the entire *HTR7* gene and its regulatory regions in a retrospectively characterized cohort mainly consisting subjects with BD, aimed to investigate the genetic association of *HTR7* and SSRIs response. In the second stage, we replicated the findings from stage one in two independent prospective cohorts consisting of patients with MDD (MARS and GENDEP).

Methods

Pooled-sequencing of HTR7 gene in a retrospective cohort

Subjects

All subjects (n=359) were ascertained as part of several cohorts collected for genetic studies of BD. All subjects were selected because they had a BD type 1 (BD-I) diagnosis or they had major depression and a first degree relative with BDI. Subjects were identified through VA and UCSD clinics, as well as, advertisement and patient support groups. All subjects provided written informed consent according to UCSD IRB approved procedures and consent form.

Assessment of SSRI response

All subjects were directly interviewed using the Diagnostic Interview for Genetic Studies (DIGS) (32) which had been modified to collect information regarding past drug trials. Interviewers underwent a training course, reliability was tested regularly and was consistently high. Information from the modified DIGS was reviewed by a panel of experienced clinicians along with medical records and information from family informants. Subjects with a BD, MDD or schizoaffective disorder bipolar type diagnosis were included in the study. Patients were queried regarding all their past medication trials including a past history of SSRI treatment. Subject's response to medications over their lifetime was assessed based on self-reporting. Blind raters considered all information about all medication trials over the patient's life in order to assess response. Good responders were those who were estimated to have 50% reduction in symptoms or episode frequency during entire illness. Subject demographic information classified by treatment groups is shown in **Table 1**.

Pooled DNA sequencing

DNA was quantified with PicoGreen and equal quantities from each subject were combined into 32 pools (ranging from 11 to 24 subjects per pool) grouped by medication (citalopram, paroxetine, fluoxetine and sertraline) and type of response (good and poor). The entire *HTR7* gene, promoter and 5' and 3' UTR regions were covered and amplified by 13 long range polymerase chain reactions, generating DNA fragments from 10 to 13 kb covering the region of Chr10: 92499978-92623668. We performed 2 x150 bp

paired-end, multiplexed sequencing on an Illumina MiSeq sequencer (Illumina, San Diego, CA). The quality of raw-reads were examined using FastQC (33) and were aligned to human reference genome (GRCh37/hg19) using BWA (34). We used CRISP (v0.7) (35) with the default setting as the variant caller and filtered the variants in the VCF files that showed EMpass, quality value >100 and minor allele frequency > 0.05. The variants were annotated by ANNOVAR (36).

Replication study I in the Munich Antidepressant Response Signature (MARS) project

The MARS project is a prospective naturalistic study of adult inpatients with depression in Germany (30, 37). Diagnoses were based on diagnostic and statistical manual of mental diseases (DSM-IV) criteria of a major depressive episode, including first-episode MDD, recurrent MDD and BD. The severity of the psychopathologic abnormality was assessed weekly based on the 21-item Hamilton Depression Rating Scale (HDRS-21) (38). In this study, we included samples with only unipolar depression diagnosis and Caucasian ancestry (n = 837) and evaluated the treatment response at week 6. We defined remission as HDRS-21 < 10, response as HDRS-21 decrease ≥ 50% and non-response as HDRS-21 decrease < 50%. For further details about the MARS project, see Hennings et al (37).

Replication study II in the Genome-based Therapeutic Drugs for Depression (GENDEP) study

GENDEP is a multicenter part-randomized open-label pharmacogenomic study of patients with moderate to severe unipolar depression diagnosed according to DSM-IV and established in the semi-structured SCAN interview (39). Patients with personal and family history of schizophrenia or bipolar affective disorder and current dependence on alcohol or drugs were excluded from the study. Response was assessed weekly by three established measures of depression severity: the clinician-rated 10-item Montgomery–Åsberg Depression Rating Scale (MADRS) (40), the HDRS-17 (41) and the self-report 21-item Beck Depression Inventory (42). In this study, we included patients with European ancestry, and evaluated the treatment response at week 12. We defined remission as HDRS-17 ≤ 7 (43), response as

MADRS decrease ≥ 50% and non-response as MADRS decrease < 50%. For further details about the GENDEP project, see Uher et al (39).

SNP genotyping

Genotyping of rs7905446 (T/G) in UCSD samples was performed using a TaqMan SNP genotyping assay (Thermo Fisher Scientific, Waltham, MA, USA) as previously described (44). The genotyping success rate was > 95 %. Twenty percent of the samples were genotyped in duplicate, with 100% reproducibility. SNP imputation for the MARS and GENDEP cohorts see **supplementary materials/methods**.

Transfection and luciferase reporter assay

HTR7 promoter containing rs7905446 (T/G) SNP was amplified followed by ligation into pGL4.26 luciferase reporter vector (Promega, Madison, WI, USA). HT-22 and SK-N-MC cell lines were transfected with rs79054446-T or rs7905446-G vectors together with pGL4.74 Renilla Luciferase control vector (Promega) using Lipofectamine 3000 reagent (Thermo Fisher Scientific). Cells were assayed for luciferase and renilla luciferase activity using Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's instruction. Details see **supplementary materials/methods.**

Electrophoretic mobility shift assay (EMSA)

EMSA was performed using the LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific) according to the manufacturer's protocol. In brief, Hela cells nuclear extracts and biotin-labeled probes spanning rs7905446 (T/G) region were incubated at room temperature for 40 min followed by electrophoresis separation and transferring to the nylon membrane. The competition reaction was performed using 200-fold molar excess of unlabeled probe. For supershift analysis, 1 µg anti-CEBPB antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was added to the nuclear extract prior to the binding reaction. The DNA-protein complexes were detected using chemiluminescence. Details see **supplementary materials/methods.**

Statistical analysis

The association between drug response and allelic SNPs identified from pooled-sequencing were performed using logistic regression (PLINK version 1.9) (45). In this analysis, because of the pooling, Caucasians and a small portion of other ethnicities were included. However, the association between drug response and rs7905446 genotype was performed using logistic regression within the Caucasian population, adjusted for age and sex. χ^2 tests were used to compare the sex distribution between responders and non-responders. Group differences were analyzed using a student's *t*-test. A *P* value of < 0.05 was considered nominally statistically significant.

Results

Common SNPs in *HTR7* are associated with SSRI response in BD

In the UCSD sample, we performed pooled-sequencing of *HTR7* gene in total of 359 subjects (**Table 1**) and examined the association between SSRI treatment response and common SNP variations based on an allelic model. We found that 80 out of 169 common SNPs survived FDR < 0.05 in the paroxetine group and 95% (n = 76) of the significant SNPs were located in intronic regions (for the full list see **Supplementary table 1**). We are particularly interested in the SNP rs7905446 (FDR = 0.0387, **Table 2**) that was located at the promoter region, because several validated transcription factors (TFs) from ENCODE database showed binding signals around this region, implicating a functional SNP. Further, rs7905446 also showed nominal significance (*P* = 0.047) in the fluoxetine group (**Supplementary table 1**) and is in high linkage disequilibrium with the other two top SNPs in the 5' upstream, rs6583737 and rs12254390 (**Figure 1**). We validated rs7905446 in Caucasian subjects using a TaqMan SNP genotyping assay in both paroxetine and fluoxetine groups (n=266). The genotype distribution was significantly different between responders and non-responders of these two SSRIs (responders: TT vs GT vs GG = 29.7% vs 54.9% vs 15.4%; non-responders: TT vs GT vs GG = 46.0% vs 41.5% vs 12.5%; Pearson χ^2= 6.697, *P* = 0.035). Next, using logistic regression we found that TT genotype was significantly associated

with poor paroxetine response compared with TG/GG genotypes, when controlled for gender and age (TT vs TG/GG: $P = 0.005$, OR = 5.250; **Table 3**). When combining both paroxetine and fluoxetine groups, TT genotype was again shown to be associated with poor response in two SSRIs (TT vs TG/GG: $P = 0.008$, OR = 2.135; **Table 3**). Gender seemed to influence SSRI response in the BD samples, specifically, men were more likely to be poor responders ($P < 0.001$, OR = 2.623; **Table 3** and **Figure 2**). No gender × rs7905446 interaction was found in either the paroxetine group or paroxetine + fluoxetine groups. Four SNPs including rs7905446 in the fluoxetine showed nominal $P < 0.05$ (**Supplementary table 1**). No SNPs with nominal $P < 0.05$ were detected in citalopram and sertraline groups.

Rs7905446 is associated with antidepressant response in unipolar depression in MARS and GENDEP cohorts

We next investigated if rs7905446 was associated with antidepressant response in MDD in two larger-scale prospective cohorts. The treatment in MARS cohort is naturalistic, selected by clinician, which includes a variety of antidepressants such as SSRIs, SNRIs and tricyclics etc. We first examined if rs7905446 can predict antidepressant response in general, i.e. including all antidepressant drugs. We found TT genotype was significantly associated with non-remission status, while TG/GG genotypes predicted treatment remission at week 6, when controlling for gender and age (TT vs TG/GG: $P = 0.032$, OR = 1.385; **Table 3**). Next, we found similar results in patients who underwent SSRI or SNRI treatments ($P = 0.044$, **Table 3**) or were only treated with SSRI ($P = 0.017$, **Table 3**). Other top SNPs (rs6583737 and rs12254390), that are in high linkage disequilibrium with rs7905446, showed similar predictive effects. In the GENDEP cohort, two antidepressants (escitalopram and nortriptyline) that represent the two most common mechanisms of action of antidepressants, were administered in a part-randomized manner. Interestingly, we found TG/GG genotypes predicted remission only in the escitalopram-treated group, escitalopram being an SSRI ($P = 0.008$, **Table 3**) but not nortriptyline which acts like NARI ($P = 0.154$, **Table 3**). There was no significant gender effect on response to antidepressants in the MARS and GENDEP cohorts.

Functional validation of rs7905446

We used a luciferase reporter assay to test if rs7905446 was a functional SNP in two neuronal-related cell lines, SK-N-MC (neuroblastoma cell line) and HT-22 (mice hippocampal cell line). In both cell lines, we observed the rs7905446-G allele, associated with better antidepressant response, exhibited stronger luciferase signals compared with the T allele, suggesting a higher promoter activity (SK-N-MC: $P < 0.01$; HT22: $P < 0.001$; **Figure 3**). Gender seemed to play a role in modulating antidepressant response: men were more than two-fold more likely to become non-responders in the BP retrospective samples (**Table 3**), suggesting estrogen may enhance the effect of antidepressant efficacy. We treated the HT-22 cell line with different concentrations of estrogen, and found the high activity of the rs7905446-G allele was decreased after estrogen treatment at a concentration of 1μM, while the activity of the rs7905446-T allele was not influenced at any concentration tested (**Figure 3**). The ENCODE database suggests rs7905446 position overlaps with the binding sites of several potential TFs, including CEBPB, which can recruit both activators like EP300 and repressors like the estrogen receptor 1 (ESR1) (46, 47). EMSA showed the rs7905446-G allele was able to generate a shift, and when adding an anti-CEBPB antibody, a supershift was observed. In contrast, biotin-probe spanning the T allele did not show binding potentials of any TFs in the nuclear extract (**Figure 4**).

Discussion

To our knowledge, this is the first study showing a consistent association between a functional variant, rs7905446, in *HTR7* gene and SSRI response in three independent clinical cohorts. We also showed that the rs7905446-G allele which associated with better antidepressants response, displayed higher promoter activity than the T allele, and estrogen treatment decreased the promoter activity in only the G allele.

Rs7905446 is associated with response to drugs with different mechanisms of action

SSRIs are chemically diverse and therefore are different from each other in pharmacological profiles and clinical efficacy. E.g., citalopram is a racemic mixture and escitalopram is its S-enantiomer, the latter was shown to have superior efficacy (48). Paroxetine and fluoxetine have a high potential to interact with other drugs compared to citalopram and sertraline (49). In addition, paroxetine exhibits relatively high affinity to muscarinic receptors and fluoxetine shows high affinity to HTR2A/2C receptors. Whether these additional actions of SSRIs will influence HTR7 function awaits further investigation. We did not find that rs7905446 was associated with response to citalopram or sertraline, suggesting poor power, or that HTR7 is not as prominent in the mechanism of action for these two drugs. In the GENDEP cohort, we noticed that rs7905446 can predict remission only in patients treated with escitalopram but not with nortriptyline, the latter is a tricyclic antidepressant with a hundred times higher affinity to norepinephrine transporter than to the serotonin transporter (39). Consistently, in the MARS cohort, rs7905446 in predicting remission to SSRI exhibited a much lower *P*-value compared to the *P*-value predicting SSRI + SNRI together. Our result suggested *HTR7* polymorphisms were strongly associated with response to SSRIs but not inhibitors of norepinephrine reuptake.

Estrogen plays a role in antidepressant action

In accordance with our findings in the BD cohort, there are reports suggesting SSRI are more effective in women than in men (50, 51). In contrast, the effect of gender on antidepressant response was not observed in the two depression cohorts, suggesting gender may play different roles in BD and unipolar depression. While most studies showed an almost equal gender ratio in lifetime prevalence in BD, women were twice as likely than men to suffer unipolar depression (51, 52). A number of studies have suggested estrogen as antidepressant or as co-adjuvant to facilitate the effect of antidepressants like fluoxetine (53). Our work showed a novel mechanism for estrogen's antidepressant effect: via reduced *HTR7* expression. We speculate that CEBPB will predominantly recruit activators (e.g. EP300) when in conditions of absent or low levels of estrogen, thus we observed a high promoter activity in the G allele. In contrast, high levels of estrogen will trigger ESR1 (a repressor) competing with other activators to interact with CEBPB, since

we observed a significant decrease of promoter activity with 1μM β-estradiol treatment but not with 10 nM.

Multiple roles of HTR7

HTR7 has been shown to promote neurite outgrowth (54), dendritic spines and synaptogenesis (55), suggesting responders may receive more 5-HT input during neurodevelopment or in learning and memory formation. Besides, HTR7 seems to have a dual role in regulating GABAergic synaptic transmission. Activation of HTR7 in raphe nuclei reduces GABA-mediated inhibition of serotonergic neurons and consequently enhances 5-HT release. However in the hippocampus, HTR7 activation was shown to stimulate GABAergic interneuron activity (56). Thus, whether HTR7 expression level can predict SSRI response remains elusive but a decrease of HTR7 level seems to be associated with a reduction in severity of depressive symptoms.

Limitations

Treating BD with SSRIs has been controversial. Some studies show it is safe and effective in BD; however other reports express caution that SSRIs may trigger a manic switch (28, 57). While SSRIs are not used as first-line drug treating bipolar depression, they are often used as second- or third-line drugs since so many other treatments fail. The evaluation of SSRI response in the bipolar cohort was retrospective; The genetic association analyses in MARS and GENDEP studies included imputed data. We cannot provide haplotype analysis regarding the SNPs that showed significance since we used pooled-sequencing method.

Conclusion remark

Heterogeneity in drug response has been a great challenge in treating mood disorder, which may be related to different pathophysiology of the disease and metabolism of the drug, both factors thought to be influenced by an individual's genetic background (58). Understanding the relationship between genetic

factors and treatment response may allow for the clinical implementation of pharmacogenetic tests and the development of personalized treatment in patients. Our study showed a functional SNP, rs7905446 in the *HTR7* gene was associated with response to antidepressants in both bipolar and unipolar depression, which warrants further investigation as a potential novel pharmacogenetic diagnostic marker.

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Declaration of Interests

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Figure l. Rs7905446 is in high linkage disequilibrium with two top SNPs (rs6583737 and rs12254390) in the 5' upstream region of *HTR7* gene. A number of transcription factors such as CEBPB in ENCODE database showed binding signals around rs7905446, implicating a functional SNP.

Figure 2. Women gender and individual with rs7905446 GG/GT genotypes showed better response to SSRIs (paroxetine + fluoxetine).

Figure 3. The rs7905446-G allele displayed higher luciferase activity compared with the rs7905446-T allele tested in two cell lines. High concentration of β-estradiol (E2) treatment significantly reduced the activity in only the G allele. $^{**}P < 0.01$; $^{***}P < 0.001$.

Figure 4. Electrophoretic mobility shift assay showed biotin-labeled probe containing the rs7905446-G can produce a shift (arrow) when incubated with the HeLa cell nuclear extract, suggesting an interaction with transcription factors. An anti-CEBPB antibody generated a supershift (asterisk), suggesting an interaction with CEBPB transcription factor.