Pharmacogenetics of Antidepressants in Major Depressive Disorder

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Summary

In this PhD by publication thesis has been to advance the understanding of the genetic basis for antidepressant drug response. Currently, MDD is conceptualized as a gene-environment disorder of polygenic nature, its underlying biology implicates central nervous system alterations in limbic system circuits that instigate depressed mood and regulate the stress response, body weight, endocrine rhythms, and immune function. We studied 284 individuals with MDD (112 and 120 patients completed treatment with fluoxetine and desipramine, respectively) and 331 controls recruited from the same Mexican American community of Los Angeles, USA. Depressed individuals were enrolled in an outpatient randomized clinical trial (RCT), an 8-week double-blind pharmacogenetic study of treatment response to daily treatment with desipramine or fluoxetine. Six reprints were included in this thesis. In reprint 1 we reported increased response to antidepressants in highly anxious patients homozygous for the GAG haplotype of the *CRHR1* gene had a greater reduction in depressive symptoms scores when compared to heterozygous; however, in patients with low anxiety levels, these associations were not present. In reprint 2 we reported that polymorphisms in the *PDE11A* gene were significantly associated with the diagnosis of MDD and remission to antidepressants. In reprint 3 we reported the association of MDD to two genes vital to T-cell function, *PSMB4* (Proteasome ß4 subunit) and *TBX21* (T-box 21 or *TBET*). We found the nominal association between SNPs in genes that support the role of T cell [*CD3E* (CD3e molecule, epsilon), *PRKCH* (Protein kinase C substrate 80K-H), *PSMD9* (Proteasome 26S subunit, Non-ATPase 9) and *STAT3* (Signal transducer and activator of transcription 3)] and HPA axis (*UNC3,* urocortin 3) functions to antidepressant response.

In reprints 4 and 5 we re-sequenced 8 genes relevant to pharmacokinetics and pharmacodynamics of antidepressant drugs, including the *BDNF* (brain derived neurotrophic

factor gene), *ABCB1* (ATP-binding cassette, sub-family B gene, *MDR*/*TAP)*, *SLC6A2* [Solute carrier family 6 (Neurotransmitter transporter), member 2 gene/Norepinephrine transporter gene, *NAT1*/*NET1*], *SLC6A3* [Solute carrier family 6 (Neurotransmitter transporter), member 3 gene/dopamine transporter gene, *DAT1*], *SLC6A4* [Solute carrier family 6 (Neurotransmitter transporter), member 4 gene/serotonin transporter gene, *5HTT*/*SERT*/*5-HTTLPR*], *CREB1* (cAMP responsive element binding protein 1 gene), *CRHR1* and *NTRK2* (Neurotrophic tyrosine kinase, receptor, type 2) genes. We identified many novel SNPs, almost doubling the number of SNPs reported for those genes and found that in the *BNDF* gene 6 SNPs (rs12273539, rs11030103, rs6265, rs28722151, rs41282918, and rs11030101) and 2 haplotypes were significantly associated with MDD; and one SNP (rs61888800) was associated with antidepressant response. In reprint 6 we examined the role of whole-exome functional gene variants in antidepressant response and found that exm-rs1321744 achieved exome-wide significance (1.98×10^{-06}) for treatment remission; this variant is located in a brain methylation site, which suggests its involvement in epigenetic regulation of neuronal gene expression.

This body of work has increased knowledge of pharmacogenomics of antidepressant drugs in major depressive disorders, identified brain pathways involved in MDD and antidepressant response. Current work in our lab aims to expand and replicate these findings.

Declaration

I certify that: 1) This thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; 2) To the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text; 3) Published works included in this thesis have been carried out conjointly with the listed co-authors; in each of those publications I participated in initiation, conduct and direction of the conjoint research, data analyses and manuscript writing, and contributed to more than 50% of the total work presented in this thesis.

Signed: 

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Six publications are included in this thesis:

1. Licinio J, O'Kirwan F, Irizarry K, Merriman B, Thakur S, Jepson R, Lake S, Tantisira KG, Weiss ST, **Wong ML**. Association of a corticotropin-releasing hormone receptor 1 haplotype and antidepressant treatment response in Mexican-Americans. ***Molecular Psychiatry*** 2004;9:1075-1082.

2. **Wong ML**, Whelan F, Deloukas P, Whittaker P, Delgado M, Cantor RM, McCann SM, Licinio J. Phosphodiesterase genes are associated with susceptibility to major depression and antidepressant treatment response. ***Proceedings of the National Academy of Sciences USA*** 2006;103:5124-5129.

3. **Wong ML**, Dong C, Maestre-Mesa J, Licinio J. Polymorphisms in inflammation-related genes are associated with susceptibility to major depression and treatment response. ***Molecular Psychiatry*** 2008;13:800-812.

4. Licinio J, Dong C, **Wong ML**. Novel sequence variations in the Brain-Derived Neurotrophic Factor gene and association with major depression and antidepressant treatment response. ***Archives of General Psychiatry*** 2009;66:488-497.

5. Dong C, **Wong ML**, Licinio J. Sequence variations of *ABCB1*, *SLC6A2*, *SLC6A3*, *SLC6A4*, *CREB1*, *CRHR1*, and *NTRK2*: association with major depression and antidepressant response in Mexican-Americans. ***Molecular Psychiatry*** 2009;14:1105-1118.

6. **Wong M-L**, Dong C, Flores DL, Ehrhart-Bornstein M, Bornstein S, Arcos-Burgos M, and Licinio J. Clinical Outcomes and Genome-Wide Association for a Brain Methylation Site in an Antidepressant Pharmacogenetics Study in Mexican Americans. ***American Journal of Psychiatry*** 2014;171:1297-1309.

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Chapter IV
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Chapter VI
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Chapter I. Literature Review

1. Introduction

Currently major depressive disorder (MDD) is the leading cause of disability, the global burden of depression is on the rise and according to the World Health Organisation (WHO) it affects at least 350 million people worldwide (1). While the lifetime prevalence of MDD varies from 3-16.9% in different countries, it is high in Australia, where 14% of people will experience depression in their lifetime (2); 1.4 million people are affected per year and at 13.3% MDD has the third highest disease burden in Australia (3, 4). However, we know little about its underlying biology; thus, current modalities of treatment are symptomatic, aimed at reducing symptoms and do not address the basic cause of the disease.

1.1. Major depressive disorder

The following triad of clinical symptoms describes MDD: persistent depression or low mood, anhedonia and low energy or fatigue. Other symptoms including sleep, appetite, psychomotor disturbances, and guilty feelings, low self esteem, and suicide ideation, may also be present (5-7). Descriptions of depression/melancholia can be found in the ancient Greek and Roman medicine, and Hippocratic writings, as depression has been documented to plague human beings since the earliest times. As we all have at some point experienced emotions characterized as down, low, sad, depressed, dysphoric, or melancholic, the distinction between normality and disease could be challenging. Thus, these feelings part of a pathological state only in situations in which they occur almost every day, are significant, prolonged, accompanied by a cluster of other symptoms, and cause work or social dysfunction.

Our current understanding of MDD evolved from Emil Kraepelin's formulations of psychiatric disorders, his classification of depression as a disease took in consideration clinical and anatomical concepts (6). The contemporary characterisation of this disorder continues to be purely descriptive, centred on subjective symptoms. In the current classification of psychiatric disorders detailed in the Diagnostic and Statistical Manual-5 (DSM-5)(8), MDD is classified in as one of the depressive disorders. The DSM-5 changes to the classification of depressive disorders have been substantial compared to the DSM-IV (9); four conditions are now listed as depressive disorders (only 2 were described in the DSM-IV): MDD, persistent depressive disorder (which encompasses what was formerly known as dysthymia and chronic major depression), premenstrual dysphoric disorder, and disruptive mood dysregulation.

In the DSM-5 and DSM-IV the definition of MDD is unchanged [**Box. 1,** (10)]; however, the bereavement exclusion was removed from the DSM. MDD continues to be described as a cluster of five or more symptoms that need to last 2 weeks or more: at least one of two cardinal symptoms (depressed mood and loss of interest or pleasure) is present, and remaining are well-described and include vegetative symptoms (change in weight/appetite, change in sleep pattern, decreased energy level/fatigue, and decreased attention), psychomotor agitation or retardation, inappropriate guilt and recurrent thoughts of death. These symptoms need to be accompanied by a change in functioning, and should not be due to medication, substance use or medical conditions.

First, five (or more) of the following symptoms have been present during the same two-week period and represent a change from previous functioning; at least one of the symptoms is either (1) depressed mood or (2) loss of interest or pleasure (note: do not include symptoms that are clearly due to a general medical condition, or mood-incongruent delusions or hallucinations).

- Depressed mood most of the day, nearly every day, as indicated by either subjective report (for example, feels sad or empty) or observation made by others (for example, seems tearful). Note: in children and adolescents, this can be irritable mood.
- Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day (as indicated by either subjective account or observation made by others).
- Significant weight loss when not dieting or weight gain (for example, a change of more than 5% of body weight in a month), or decrease or increase in appetite nearly every day. Note: in children, consider failure to make expected weight gains.
- Insomnia or hypersomnia nearly every day.
- Psychomotor agitation or retardation nearly every day (observable by others, not merely subjective feelings of restlessness or being slowed down).
- Fatigue or loss of energy nearly every day.
- Feelings of worthlessness or excessive or inappropriate guilt (which might be delusional) nearly every day (not merely self-reproach or guilt about being sick).
- Diminished ability to think or concentrate, or indecisiveness, nearly every day (either by subjective account or as observed by others).
- Recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide.

Second, the symptoms do not meet criteria for a mixed episode.
Third, the symptoms cause clinically significant distress or impairment in social, occupational or other important areas of functioning.
Fourth, the symptoms are not due to the direct physiological effects of a substance (for example, a drug of abuse or a medication) or a general medical condition (for example, hypothyroidism).

Box 1. Criteria for Major Depressive Disorder Episode: Source: Diagnostic and Statistical Manual, American Psychiatric Association, reproduced with permission from reference 10.

Existing evidence supports the notion that MDD arises from multifactorial, complex gene-environmental interactions. It is likely that on a genetic susceptible individual, stress or adverse life event(s) will precipitate the onset of a depressive episode; thus, in a conceptual approach, depression phenotypes can the outcome of a permutation of a variety of environmental factors, encompassing loss or trauma during childhood and adulthood, to war, natural disasters and genetic vulnerability [**Fig. 1**, (6)].

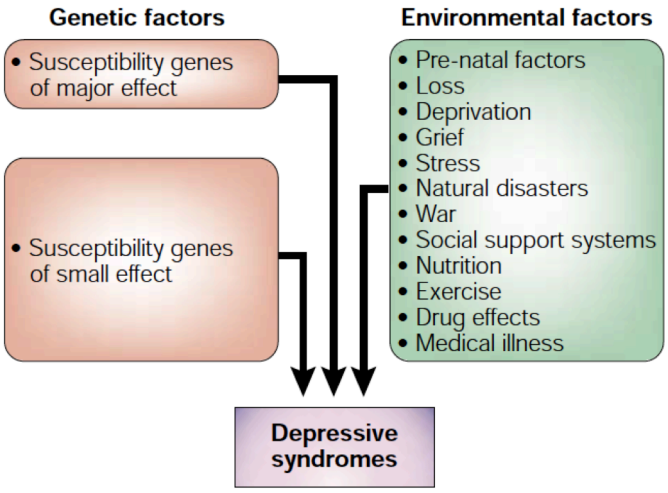

Fig. 1. A theoretical conceptualisation of MDD. A Combination of gene and environmental factors interact to influence the neurobiological substrates underlying MDD. Reproduced with permission from reference 6.

1. 2. Epidemiology of MDD

Naturalist studies revealed that the typical clinical course for MDD is a self-limiting chronic, recurrent disorder, with major depressive episodes followed by periods of recovery/remission (11, 12). Its onset is generally mid to late 20s (13, 14), and MDD is twice more prevalent in women than man (15, 16).

MDD can be extremely disabling, as about 17% of patients suffer from chronic, unremitting symptoms (17). This disorder is a major cause of morbidity, and two-thirds of suicides are associated with it (18, 19). Although suicide accounts for a small proportion (1.6%) of all deaths in Australia, it is the leading cause of death of youngster aged 15-24 years (20).

First-degree relatives of MDD individuals have a higher risk of being depressed (2-3 fold) when compared to controls (21), and disease severity and early-onset may be linked to higher risk of depression in family members (15, 22-25). Prior history of depression or dysthymia increases the risk for the occurrence of a depressive episode (26); and around 85% of people with a prior major depressive episode experience recurrent symptoms (11, 12, 27).

MDD appear to be more prevalent in primary care patients (28, 29), and there is a convincing association between depression and higher morbidity and mortality in ischemic

heart disease (30, 31). Higher incidences of MDD have been linked with acute and chronic psychosocial factors, such as an increased number of recent life events, death of a close relative, unemployment, divorce, living alone, poor social support, and alcohol and drug use (32-35).

2. Pharmacotherapy treatment

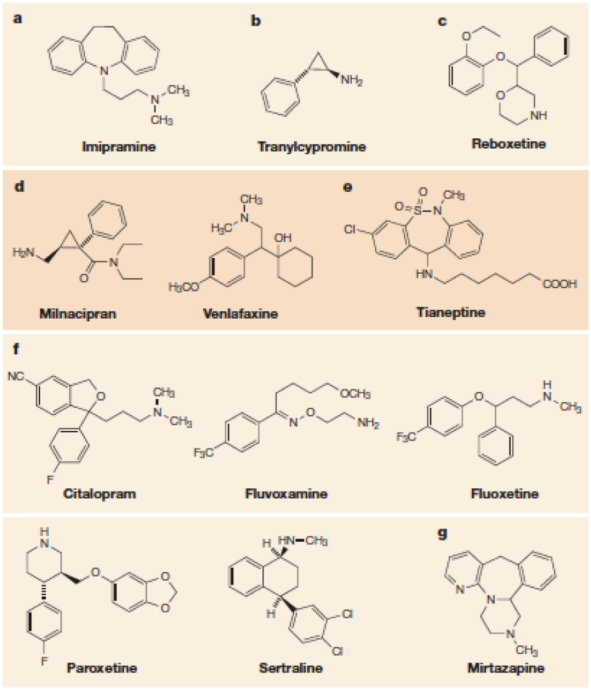

Fig. 2. Representative examples from different classes of antidepressant drugs. **a**: Prototypical TCA imipramine. **b**. Prototypical MAOI: tranylcypromine. **c**. Atypical, selective noradrenaline reuptake inhibitor: reboxetine. **d**. Atypical, selective serotonin and noradrenalin reuptake inhibitors: milnacipran and venlafaxine. **e**. Atypical, selective serotonin reuptake activator: tianeptine. **f**. SSRIs: citalopram, fluovoxamine, fluoxetine, paroxetine and sertraline. **g**. Atypical, Noradrenergic and specific serotonergic antidepressant with minimal effects on monoamine reuptake: mirtazapine (reproduced with permission from reference 10).

Available treatment modalities shown to be effective for MDD include psychotherapy (cognitive behavioural and interpersonal therapies) (36-38), electroconvulsive therapy (ECT) (39), transcranial magnetic stimulation (TMS), and complementary and alternative therapies (40). However, antidepressant drugs are the mainstay in MDD treatment. Despite the enormous burden MDD places in health care systems around the world, its treatment continues to be symptomatic, primarily with the use of antidepressant medications alone or combined with psychological therapies. This section focuses on a brief pharmacotherapy overview.

Antidepressant drugs are efficacious MDD treatment; however, FDA (USA

Food and Drug Administration) approved antidepressants have a delayed onset of action and can take several weeks to cause symptom relief. Chronic, repeated administration of antidepressant drugs is required to achieve remission from MDD, and relapse to a major depressive episode often occurs if antidepressant treatment is discontinued. Almost 60% of individuals with MDD seek treatment, 35% are treated with medication or psychotherapy (41), and of those, 30 to 40% of patients do not respond to their first antidepressant trial and several treatment trials may be needed for symptom relief (42). Antidepressants are one of the most prescribed classes of drugs in the USA, where the rate of antidepressant use increased nearly 400% in the past two decades. In Australia 23 millions scripts were written for antidepressants in 2013-14, which accounted for 67% of the prescriptions issued for mental health-related diseases (subsidised and under co-payment), making mental health medications one of the mostly costly classes of drugs subsidised by the Pharmaceutical Benefits Scheme [PBS, (43)].

Antidepressants are often classified, taking in consideration their chemical structure and mechanism of action, into 4 main classes of antidepressants: 1) monoamine oxidase inhibitor (MAOI); 2) tricyclic (TCA); 3) selective serotonin reuptake inhibitor (SSRI); 4) atypical antidepressants [**Fig. 2,** (10)].

2.1 Monoamine oxidase inhibitors (MAOIs)

The MAOI iproniazid was the first drug serendipitously described to have antidepressant effects over 50 years ago; however, it was later withdrawn from the marker due to hepatotoxicity. These first generation antidepressants increase the quantity of monoamines that can bind to postsynaptic receptors by inhibiting the monoamine oxidase enzyme, which catalyze the breakdown of monoamines (5-HT/serotonin, NE/norepinephrine and DA/dopamine) in the presynaptic neuron and in the synapse (44,

45). MAOIs, such as phenelzine, isocarboxazid, tranylcypromine and selegiline, interact with food, drinks and drugs, which limit their use because of their ability to induce severe adverse effects (very high blood pressure that can lead to stroke or heart attack). Therefore, MAOIs are usually reserved for patients with treatment resistance to first line antidepressant drugs (45).

2.2 Tricyclics (TCAs)

The cyclic imipramine was the second drug described to have antidepressant activity; cyclic antidepressants have mainly three (tricyclic), but they can have four (tetracyclic) rings in their chemical structure. They increase the monoamines level in the synapses by non-specifically inhibiting reuptake transporters activity of presynaptic neurons, which block the reuptake of monoamines. Due to their broad-spectrum action, including the blockade of histamine and muscarinic acetylcholinergic receptors, TCA antidepressants, such as amitriptyline, amoxapine, desipramine, doxepin, imipramine, notriptyline, protriptyline, trimipramine, and the tetracyclic maprotiline, tend to produce more side effects than newer classes of antidepressant drugs (46); they are currently also considered second-line agents and are recommended for patients who failed SSRI or atypical antidepressants (47).

2.3 Selective serotonin reuptake inhibitors (SSRIs)

The strategy for the second generation of antidepressant drug development (10) was focused on improving drug tolerability and safety by designing drugs with selective reuptake inhibitor properties. The prototypic SSRI drug fluoxetine was the first drug that increased the amount serotonin in the synapse by selectively blocking the serotonin reuptake transporter, without non-selectively antagonising other receptors (48); and it

replaced imipramine as the "gold-standard' for depression treatment in the 1980's. Currently, SSRIs (fluoxetine, citalopram, escitalopram, luvoxamine, paroxetine and sertraline) are the most commonly prescribed antidepressants, they have a more favorable side effect profile; however, abrupt cessation of SSRIs can result in "discontinuation syndrome" with flu-like symptoms, which can be avoided by tapering off the drugs (49).

2.4 Atypical (second generation) antidepressants

Atypical antidepressants don't fit other classes of antidepressants, and were developed after the success of second-generation SSRIs. They include serotonin-noradrenaline reuptake inhibitors (SNRIs, duloxetine, venlafaxine and desvenlafaxine), selective noradrenaline reuptake inhibitor (reboxetine), selective serotonin reuptake activator (tianeptine), noradrenaline and specific serotonergic with minimal monoamine reuptake (mirtazapine). Bupropion is a selective norepinephrine and dopamine reuptake inhibitor, which is structurally similar to atypical antidepressant trazadone, which modulate serotonin modulation by blocking type IIA serotonin receptors (5-HT2AR).

3. Pathophysiology of MDD

Our appreciation of the underlying fundamental biology of this disorder is still inadequate. Many theories about MDD's pathophysiology have been developed, the monoamine hypothesis has been the classic one, and though a number of alternative hypotheses have emerged only the main ones have been summarised below.

3.1 The Monoamine Hypothesis

The classical monoamine hypothesis of depression, developed following the discovery of the mechanism of action of efficacious antidepressant drugs (50), proposes that a deficit

of monoamines (such as serotonin, norepinephrine, and/or dopamine) exists in the brain in MDD, which is normalised with antidepressant treatment (6). A significant gap that emerged in this theory is the discrepancy between the acute onset of action of monoamines (which happen within hours) and the delayed improvement of MDD symptoms (which may take several weeks). Although contemporary antidepressants have been developed based on the monoamine hypothesis; currently, this hypothesis is considered insufficient to explain MDD. Decades of investigation into the biological basis of this disorder through understanding various neurotransmitter systems have considerably advanced our understanding of MDD; however, our understanding of MDD pathogenesis and aetiology continues to be fragmented.

3.2 *The Neuroendocrine/Neurohormone Hypothesis*

This is a prominent alternative hypothesis for MDD, as stress is considered a powerful environmental factor that predisposes/precipitates this disorder. Our body reacts to adverse situations with a distinctive stereotyped response, coined as "generalised adaptation syndrome" by Selye (51), in which activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system occurs as part of the stress response. The pathophysiological role of the stress system in MDD is corroborated by sizable body of evidence (52), including the following findings: a) increased plasma cortisol levels in a large proportion of MDD patients (50-70%); attenuated HPA axis suppression in the dexamethasone suppression test; antidepressants directly down-regulate HPA function (53-57); b) antagonism of corticotropin-releasing hormone (CRH) reduces the behavioural, autonomic and neuroendocrine aspects of the response to stress (58), and c) increased noradrenaline concentration is elevated in the cerebrospinal fluid (CSF) during 24 hours of the day, including during sleep, implying that the dysregulation of this stress-related system

is primary and not simply reactive to depressed mood (6, 59). However, the latter observation seems to directly contradict the fundamental principle of the monoamine hypothesis, i.e. the reduced monoaminergic function in MDD. Dysfunction of other systems, besides the HPA axis, established in response to acute and chronic illnesses, such as in the growth hormone (60, 61) and thyroid axes (62), have also been described in MDD. The response to persistent uncontrollable stress can be maladaptive and promote a shift to environment withdrawal precipitating the onset of MDD (63).

3.3 Neurotrophic Hypothesis

This hypothesis implicates neutrotrophic factors, specifically the brain-derived neurotrophic factor (BDNF) as a key player in the pathophysiology of MDD and antidepressant action; it proposes that in MDD there are reduced levels of BDNF, which are increased by antidepressant treatment (64, 65). Neurotrophic factors are growth factors that act directly on neurons to support cellular differentiation, growth and survival. Considerable research has focused on understanding the role of neutrophins; antidepressants increase expression of BDNF and its receptor, the neurotrophic tyrosine kinase, receptor, type 2 (NTRK2). BDNF administration has antidepressant effects (66), as antidepressants have been postulated to promote neurogenesis in the adult hippocampus (67). However, more work is required to clarify the roles of these molecules due to divergent results on BDNF's upstream transcription factor cyclic AMP response element binding protein (CREB) and BDNF mRNA (messenger ribonucleic acid) levels in the hippocampus following chronic antidepressant administration (68, 69). Moreover, animal studies have demonstrated that low BDNF activity does not produce depressed-like behaviour (70, 71).

3.4 *Neuroimmune Hypothesis*

The immune system has key roles during the stress response, and it can be suppressed or enhanced during stress. Endocrine and cytokine mediators modulate humoral and cellular aspects of the innate and adaptive immune response, and immune dysfunction can affect cognition and behaviour. The rationale for this theory has been based on the following observations: some cytokines can activate the HPA axis and brain neurotransmitter systems such as noradrenergic and serotonergic, and cytokine treatment in humans can induce depressive symptoms. Therefore, immune system dysfunction, specifically proinflammatory cytokines, could underlie the pathophysiology of MDD (72, 73). Dysregulation of the T-cell arm of the immune system has corroborated the role of immunomediators in depression (74).

3.5 *Glutamatergic Hypothesis*

The essential amino acid glutamate is an excitatory neurotransmitter and it is also the precursor of γ-aminobutyric acid (GABA) (75, 76). The glutamatergic system is largely involved in most excitatory transmission in the brain; however, excess glutamate release may lead to abnormalities in neurotransmission, cell viability, energy metabolism and excitotoxicity damage in the brain (77). Both neurons and astrocytes are indispensable to glutamate neurotransmission as astrocytes have pivotal roles in glutamate reuptake, precursors synthesis and removal, and task-dependent changes in brain energy utilisation (78-80). In this hypothesis, disturbances in astrocyte function, resulting in altered glutamatergic neurotransmission and neuroenergetic regulation, underpin the neurobiology of MDD (81).

Evidence supporting the involvement of the glutamatergic system in MDD neurobiology and treatment have been further strengthened by preliminary clinical studies

showing that the non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist ketamine, rapidly ameliorates depressive symptoms in treatment-resistant patients (82). Compounds that target glutamatergic mechanisms are currently in early clinical trials (83).

4. Pharmacogenetics Approaches to MDD

Pharmacogenetics/pharmacogenomics has been broadly described as "the study of inter-individual variations in whole-genome or candidate gene single nucleotide polymorphism (SNP) maps, haplotype markers and alterations in gene expression or inactivation that might be correlated with pharmacological function and therapeutic response" (84). This relatively recent field of investigation in psychiatry; however, the growing number of publications in this relatively new are of investigation has had variable and contradictory results.

Why is it relevant to conduct pharmacogenomics research of antidepressants? Over 20 antidepressant drugs are available in the market, in four different classes (see above). Most patients (85-90%) ultimately respond to antidepressant medication; however, the patients (30-40%) who fail their first antidepressant trial (85) have higher risk of never being effectively treated (86). Each antidepressant drug has a success rate of approximately 60%. Patients who fail one drug are generally tried with a drug from a different class, until multiple antidepressants of different classes have been tried. Currently, we do not have an objective way to predict the response to a given antidepressant drug; therefore, drug choice is regularly based on side effect profile.

Antidepressants have delayed action on ameliorating depressive symptoms; thus, each drug trial ideally would need to last 6-8 weeks, which is often followed by a wash out period and another drug trial. Therefore, highly symptomatic patients may require several antidepressant trials over many months without symptom relief until an effective drug is

found. This time consuming process may also lead to poor compliance and increased morbidity. Consequently, pharmacogenetic approaches that could lead to the *a priori* identification of an efficacious antidepressant drug for a specific patient could have significant clinical and public health value. It is also believed that pharmacogenomic approaches could identify the final genetic mediators of antidepressant action and lead to the identification of genes variants that are associated with individual susceptibility to various antidepressant drugs. Furthermore, downstream genetic targets for antidepressant action may provide novel pharmacological targets for depression treatment.

In the body of work included in this thesis, which was published from 2004 to 2014, we applied the concept of pharmacogenetics to major depressive disorders by studying the phenotype antidepressant response using two antidepressants of different classes: desipramine (TCA) and fluoxetine (SSRI). The following genetic approaches were used in the reprints included: candidate gene [Chapter II (87)], candidate pathway [Chapters III (88) and IV (89)], candidate pathway deep sequencing [Chapter V (90, 91)], and whole-exome genotyping approaches [Chapter VI (92)]. Below is a brief summary of relevant population genetic concepts and methodologies relevant to the body of work described in this thesis.

4.1 Genetic Association Studies: Case-control studies

SNPs are sequence variations in which two alleles, with distinct nucleotide residues, appear in a significant portion of the human population and are queried in association studies. In case-control studies the frequencies of SNPs are compared in two well-defined populations: cases who are affected by a certain phenotype/disorder, and controls who are unaffected. Case-control studies are commonly used in genetic association studies because they have a powerful study design. However, establishing a good match between the

genetic background for case and control populations is a crucial issue in case-control studies, because this ensures that any genetic differences between them is related to the studied phenotype/disorder and not to biased sampling or population stratification, i.e. systematic ancestry differences (93, 94).

The reprints included in Chapters II to VII are based on a prospective 8-week double-blind randomised controlled trial (RCT) in Mexican-Americans; and its pharmacogenetic data analyses used an association case (responders/remitters) and control (non-responders) design. Our primary clinical outcomes measure for antidepressant response was the Hamilton Depression Rating Scale [HAMD21, (95)]. Treatment response was classified into the following categories based on the final (week 8): remission (score of <8), non-remission (score of ≥8), responder (reduction of ≥50% in HAMD21 score), and non-

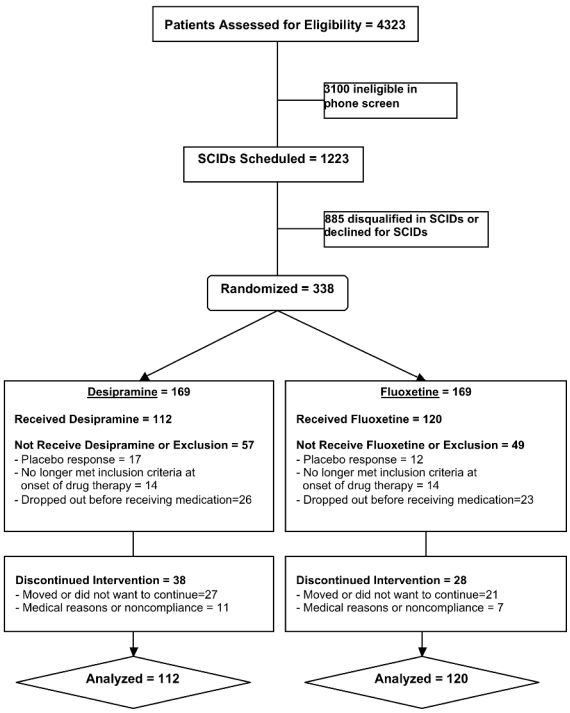

Fig. 3. Flowchart of study participants, random assignment and dropouts in a clinical trial of desipramine and fluoxetine treatment for Mexican-Americans with major depressive disorder (reproduced with permission from ref. 92).

responder (reduction of <50% in HAMD21 score). The study flow for this study is depicted in **Fig. 3**. We also recruited an age- and sex-matched control population from the Mexican-American community in Los Angeles, USA; which allowed us to also perform case (affected, n=284) and control (unaffected, n=331) genetic association analyses for MDD.

4.1.1. Mexican-Americans of Los Angeles

We studied an urban population that consisted mostly of recent immigrants. At the time that we conducted our study, Mexican-Americans were the predominant and fastest growing ethnic group in the Los Angeles area; however, virtually nothing was known about pharmacogenetics in that population. Detailed searches of the medical literature had shown that there were no publications on controlled depression treatment trials in Mexican-Americans, nor had articles on studies on the biology of pharmacological treatment responsiveness for any disease in that population found. Thus, we conducted a rigorously organised treatment trial for depression in this population as the central basis for a series of related studies conducted to elucidate at a mechanistic level the genetics and pharmacogenomics of that understudied minority population in the USA. We were aware that genetic stratification could be a potential pitfall for any type of genetic study in a heterogeneous population. We were very aware of the fact that the Mexican-American population is not homogenous; i.e., different families may have various mixtures of Caucasian and Native-American alleles. Mexico also received substantial numbers of European immigrants this century. To decrease the heterogeneity of our sample, we had an inclusion criterion that study participants needed to have at least three grandparents born in Mexico, following the guidelines of Hazuda *et al.* (96).

4.2 Hardy-Weinberg equilibrium

The Hardy-Weinberg law or principle is a fundamental theoretical principle in population genetics. It states that alleles and genotypes frequencies in a large population will remains unaltered over time in the absence of other evolutionary influences. The following conditions/hypothesis are assumed in this law: population size is infinitely large, entities are diploid and only sexual reproduction occurs, no mutations, no immigration/emigration, no natural selection, generations are non-overlapping, matting is random and allele frequencies are equal in both sexes (97, 98). Then, if p is the frequency of allele A and q is the frequency of allele a, the frequencies in the next generation will be p^2 for the AA genotype, $2pq$ for the Aa genotype and q^2 for the aa genotype. Thus, the following equation (Hardy-Weinberg equation) can be used to calculate genotypes and alleles frequencies:

$$p^2+2pq+q^2 = 1$$

The Hardy-Weinberg equilibrium is routinely used in genetic association studies. Violations of the Hardy-Weinberg equilibrium can indicate important problems, such as peculiarities or errors in the data sets that compromise crucial inferences from a genetic association study (99); thus control genotypes should be in Hardy-Weinberg equilibrium.

4.3 Genetics approaches used in the published work

4.3.1 Candidate gene and candidate pathway approaches

During the period of 1990's into the early 2000's, the candidate gene approach was the prevalent approach in genetics. In this hypothesis-driven approach, a particular variation of a nominated gene is genotyped and queried for its association with a phenotype of interest (drug response phenotype in the case of pharmacogenetics) by comparing case (affected) and control (unaffected) populations. For instance, significant difference in the frequency

of a SNP allele in cases and controls indicates that that SNP allele confers increased risk of disease/phenotype. The candidate gene was a successful approach in diseases with well-established biology. Candidate gene studies generally reported positive results for association tests that reached a nominal significant level of P=0.05.

Variations of this approach consist in testing several variants in one gene or in a candidate pathway, which were respectively used in chapters II to IV of this thesis.

4.3.2 Sequencing approach

DNA (deoxyribonucleic acid) sequencing determines the precise physical order of the four bases that form a DNA strand: Thymine (T), adenine (A), cytosine (C) and guanine (G). The work of Fred Sanger set the foundation for the current DNA sequencing technology because it was soon automated and used in the first generation of DNA sequencers (100). The capillary PCR (polymerase chain reaction) method was used in the re-sequencing/deep sequencing work presented in chapter V, which was performed by our collaborators at the Sanger Institute, UK, using the technology and protocols described in their ExoSep project (www.sanger.ac.uk/resources/downloads/human/exoseq). SNP identification was done using the ExoTrace algorythm, a base-calling for automated sequencer traces using the PHRED program (http://www/phrap.com/pred/)(101). ExoTrace uses the sense and antisense sequence reads separately and then combines the results for SNP scoring.

4.3.3 *Genome-wide association studies (GWAS) and whole exome genotyping approaches.*

The GWAS approach was developed under the assumption of the "common disease-common alleles model" (102), as multifactorial disorders are thought to fit this model, in which an altered phenotype is produced by the combination of the cumulative linear impact of gene-gene interactions (compounded common small-effect genetic variants) in the

context of genetic-environment interaction (environmental exposures that increase individual risk to exceed a biological threshold); i.e. some common [with a minor allele frequency (MAF) ≥ 1%] variants lead to susceptibility of complex polygenic disorders. The GWAS approach comprises of unbiased analyses of the entire genome, which is generally pursued using large cohorts of cases and controls.

There are about 10 million common variants in human populations (103). GWAS can test thousands to millions common SNPs to search for a common SNP that will be associated with a phenotype. Genotyping can be done at low cost (~$100/sample) using SNP arrays, as the SNPs that are close to each other in a chromosome tend to be have strong statistical relationships [i.e. they are in linkage disequilibrium (LD)], some of the SNPs can be statistically imputed/inferred (104). Using different commercial probe-based SNP array platforms one can genotype thousands to one million SNPs with >99% accuracy in a given individual in a single assay (Illumina, (105, 106). In chapter VII, we employed a variation of the GWAS approach, by using a DNA genotyping array to query the whole exome, the Illumina HumanExome-12v1_A BeadChip. This chip covers more than 250,000 markers that are putative functional exonic variants selected from more than 12,000 individuals representing diverse populations and common disorders (such as cancer, type 2 diabetes, metabolic disorders, etc).

Due to multiple testing, a typical significant threshold for a genome-wide effect for an European-descendent population is $5x10^{-8}$ (107, 108) for hypotheses that are tested on a genome scale (109). In the context of GWAS, false discovery rate (FDR)-based strategies are frequently used to correct for multiple testing (110, 111). It reduces the probability of false positive results because it uses a more conservative alpha level for each test; thus it is considered to be more powerful than the Bonferroni correction. The FDR has performed well in genome-wide analysis, with good localisation of positive results (112).

4.4 Pharmacokinectics and pharmacodynamics

Pharmacokinetics and pharmacodynamics are essential elements for the pharmacological function and therapeutic effect of any drug. Pharmacokinetics refers to processes that take place when a drug moves into, through and out of the body, such as absorption, bioavailability, distribution, metabolism, and elimination/excretion. In contrast, pharmacodynamics studies the biochemical and physiological effects of drugs. The mechanism of drug action involves the interaction of several physiological systems while the drug is in the human body (113, 114). The work included in this thesis focuses on pharmacodynamics factors; however, even though knowledge in this area has increased progressively over the past several decades, the neurobiology of major depressive disorder and the mechanisms of action of antidepressants are not completely understood.

4.5 The genetic basis of pharmacodynamics

In spite of the multitude of pharmacodynamics targets for antidepressant drugs, comprising the: i) serotonergic, noradrenergic and dopaminergic neurotransmitter systems; ii) BNDF and G-protein subunits; iii) hypothalamic-pituitary-adrenal (HPA) axis, circadian and immune systems; iv) glutamatergic and GABAergic neurotransmitter systems (115-117), pharmacogenomics of antidepressant work has been mainly focused on the monoamine neurotransmitter systems, in particular the serotonin system (118).

Rather than providing a comprehensive review on this topic, a brief review of the context in which this work began in the early 2000's is provided below.

4.6 Serotonin transporter gene (5-HTTLPR) promoter

In the 2000's SSRI drugs were already the gold standard for MDD treatment. They are the most prescribed class of antidepressants and the serotonergic transporter is their main target. Therefore, it is not surprising that this system has been the most tested in pharmacogenetic studies of antidepressant response in MDD.

The human serotonin transporter gene (*SLC6A4/5-HTTLPR*) was cloned by Ramamoorthy *et al*. and located on chromosome 17q11-1-q12 (119). Following the work of KP Lesch and colleagues describing the association of a polymorphism in the *5-HTTLPR* promoter with anxiety-related traits, that polymorphism became the focus in antidepressant response studies (120). The regulatory region of *5-HTTLPR* has an insertion/deletion region of 44 nucleotides involving repeated elements; the long variant (*l*) had more than twice the basal activity of the short form (*s*). In 1998 Smeraldi *et al.* published the first paper showing that one or more copies of long allele (*l*/*s* or *l*/*l*) for *5-HTTLPR* conferred better therapeutic response to fluvoxamine (121). They studied 102 MDD inpatients with psychotic features were randomized to 6 weeks of treatment with fluvoxamine and either placebo or pindolol, and HAMD21 was used to assess drug response. The finding that the *s* allele was associated with a worse and slower antidepressant response was independently replicate by Pollock *et al*. (122). They found that the *l*/*l* genotype had a more rapid mean reduction of depressive symptoms in elderly patients (n=95) treated with paroxetine or nortriptyline.

Similar findings were reported for citalopram (123), sertraline (124) and fluoxetine (125) in European-Ancestry populations; however, some findings in Asian populations were in the opposite direction (126, 127), or reported no association (128). Data that antidepressant-induced mania was associated with the short allele was also available (129).

4.7 Other polymorphisms associated with antidepressants

Polymorphisms in the tryptophan hydroxylase (*TPH*) gene, which encodes the rate-limiting enzyme in the synthesis of serotonin, were associated with antidepressant response. MDD or bipolar disorder patients who were homozygous for the rarer *TPH**a allele of the A218C polymorphism and who were not taking pindolol were slower respond to fluvoxamine (130). In MDD inpatients one or more copies of the *TPH**a allele was associated with worse response to paroxetine (131).

5. Hypothesis and Aims underpinning the publications

We tested the overarching hypothesis that pharmacogenetic approaches may be used to develop treatment strategies for common and complex disorders in Mexican Americans. To test this general hypothesis, we have used pharmacological treatment of depression as a proof of the concept that pharmacogenetic approaches may be used to optimise treatment strategies in this underserved and understudied minority population. Specifically, our study aimed at determining whether within the Mexican-American population, associating a particular pattern of genetic polymorphisms with responses to a drug may possibly help define subsets of the population for whom certain drugs would work best.

In 2000, when we started this study, there were over 20 antidepressants that were effective used in the treatment of depression, and could induce remission in 40-70% of patients. Clinical experience already suggested that subgroups of patients could be helpful to particular medications; however no clinically accepted method exist that can identify subgroup of patients who will respond to a specific antidepressant drug. This project studied Mexican-American subjects enrolled in a double-blind treatment trial for MDD in order to obtain a pattern of genotype profile that may serve as a pharmacogenetic predictor of treatment response.

To our knowledge, in 2000, no pharmacogenetic studies or double-blind treatment studies in MDD had been conducted in the Mexican-American population. A double-blind randomised design was used for this study: a total of 112 patients completed treatment with the SSRI fluoxetine, and another group of 120 patients completed treatment with desipramine, a drug that affects norepinephrine neurotransmission (**Figure 3**). In the course of the study we obtained detailed, standardised clinical assessments, genetic material and outcome phenotypic data on antidepressant treatment responses to be able to ascertain whether specific genetic factors contribute to drug responses by conducting specific genotype-phenotype analyses in this population. Our genotyping efforts may also have contributed to identifying pathophysiological pathways relevant to drug discovery in MDD.

5.1 Aim 1: To ascertain whether the *CRHR1* (corticotropin-releasing hormone receptor 1) gene was associated to antidepressant-mediated responses using a candidate gene approach [Chapter II (87)]

A considerable body of evidence already existed which supported a dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis function and the suppression of CRH activity in MDD, we tested the hypothesis that *CRHR1* gene variants were associated with antidepressant-mediated responses using a candidate gene approach.

5.2 Aim 2: To investigate whether genes encoding cyclic nucleotide phosphodiesterases (PDEs) were associated with antidepressant response

We studied the association of the genes enconding cyclic nucleotide phosphodiesterases (PDEs), a family of enzymes that degrade intracellular second messengers cAMP (cyclic adenosine monophosphate) and cGMP (cyclic guanosine monophosphate), using a candidate pathway approach. The second messenger cAMP is implicated in processes

relevant to learning, memory and mood, and the cGMP is a component of the nitric oxide (NO)/cGMP pathway.

5.3 Aim 3: To understand the relevance of genes related to T-cell function in MDD risk or antidepressant response [Chapter IV (89)].

We used a candidate pathway approach to query several genes that influence T-cell function, such as *PSMB4* (proteasome ß4 subunit), *TBX21* (Tbox 21, *TBET*), *CD3E* (CD3e molecule, epsilon), *PRKCH* (Protein kinase C substrate 80K-H), *PSMD9* (Proteasome 26S subunit, Non-ATPase 9), *STAT3* (Signal transducer and activator of transcription 3), and *UNC3* (Urocortin 3) genes.

5.4 Aim 4: To identify new variants in genes relevant to pharmacokinetics and pharmacodynamics of antidepressant drugs, such as the *BDNF*, *ABCB1* (ATP-Binding Cassette, Sub-Family B gene, *MDR/TAP)*, *SLC6A2* [Solute Carrier Family 6 (Neurotransmitter Transporter), Member 2 gene/Norepinephrine transporter gene, *NAT1*, *NET1*], *SLC6A3* [Solute Carrier Family 6 (Neurotransmitter Transporter), Member 3 gene/dopamine transporter gene, *DAT1*], *SLC6A4* [Solute Carrier Family 6 (Neurotransmitter Transporter), Member 4 gene/serotonin transporter gene, *5HTT*, *SERT*, *5-HTTLPR*], *CREB1* (cAMP responsive element binding protein 1 gene), *CRHR1* and *NTRK2* genes and tested their association with antidepressant response [Chapter V (90, 91)**.**

Given the importance that these genes in the pharmacokinetics (absorption, bioavailability, distribution, metabolism, and elimination/excretion of drugs) and pharmacodynamics (biochemical and physiological effects of drugs) of antidepressants. We hypothesised that new variants in these genes would be relevant to antidepressant response. We used a sequencing approach to all exons and their flanking regions of the genes

relevant to pharmacokinetics and pharmacodynamics of antidepressants, and identified variations associated with risk for MDD and antidepressant response.

5.5 Aim 5: to examine the role of functional variants in antidepressant response [Chapter VI (92)].

We applied a whole exome genotyping approach to identify functional variants associated with treatment remission, because of the likely significance of a functional SNP in the *BDNF* gene (rs6265) in the pharmacogenetics of MDD and our own previous work showing that multiple functional SNPs and environmental factors were useful in a predictive framework for MDD risk (132).

Chapter II. Reprint

Licinio J, O'Kirwan F, Irizarry K, Merriman B, Thakur S, Jepson R, Lake S, Tantisira KG, Weiss ST, **Wong ML**. Association of a corticotropin-releasing hormone receptor 1 haplotype and antidepressant treatment response in Mexican-Americans. ***Molecular Psychiatry*** 2004;9:1075-1082.

Chapter III. Reprint

Wong ML, Whelan F, Deloukas P, Whittaker P, Delgado M, Cantor RM, McCann SM, Licinio J. Phosphodiesterase genes are associated with susceptibility to major depression and antidepressant treatment response. ***Proceedings of the National Academy of Sciences USA*** 2006;103:5124-5129.

Phosphodiesterase genes are associated with susceptibility to major depression and antidepressant treatment response

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Cyclic nucleotide phosphodiesterases (PDEs) constitute a family of enzymes that degrade cAMP and cGMP. Intracellular cyclic nucleotide levels increase in response to extracellular stimulation by hormones, neurotransmitters, or growth factors and are down-regulated through hydrolysis catalyzed by PDEs, which are therefore candidate therapeutic targets. cAMP is a second messenger implicated in learning, memory, and mood, and cGMP modulates nervous system processes that are controlled by the nitric oxide (NO)/cGMP pathway. To investigate an association between genes encoding PDEs and susceptibility to major depressive disorder (MDD), we genotyped SNPs in 21 genes of this superfamily in 284 depressed Mexican Americans who participated in a prospective, double-blind, pharmacogenetic study of antidepressant response, and 331 matched controls. Polymorphisms in PDE9A and PDE11A were found to be associated with the diagnosis of MDD. Our data are also suggestive of the association between SNPs in other PDE genes and MDD. Remission on antidepressants was significantly associated with polymorphisms in PDE1A and PDE11A. Thus, we found significant associations with both the diagnosis of MDD and remission in response to antidepressants with SNPs in the PDE11A gene. We show here that PDE11A haplotype GAACC is significantly associated with MDD. We conclude that PDE11A has a role in the pathophysiology of MDD. This study identifies a potential CNS role for the PDE11 family. The hypothesis that drugs affecting PDE function, particularly cGMP-related PDEs, represent a treatment strategy for major depression should therefore be tested.

gene association | pharmacogenetics | cGMP | SNP | Mexican American

Eleven different phosphodiesterase (PDE1–11; see Table 1) families have already been identified based on their substrate specificities, kinetic properties, allosteric regulators, inhibitor sensitivities, and amino acid sequences (1–10). Within each family, several genes and splice variants have been recognized (2, 11). Each family and members within a family exhibit distinct tissue and subcellular patterns (1, 3–5, 8, 9, 12). The hydrolysis of cAMP and cGMP are controlled by multiple PDEs, and they influence numerous pharmacological processes, including mediation of inflammation, ion channel function, muscle contraction, learning, differentiation, apoptosis, lipogenesis, glycogenolysis, and gluconeogenesis (13).

As regulators of the ubiquitous second messengers cAMP and cGMP, PDEs modulate the transduction of various extracellular signals through the activation of cell-surface receptors. Intracellular concentrations of cyclic nucleotides increase and activate their target enzymes, which are PKA and PKG. These protein kinases are responsible for the phosphorylation of a number of substrates, such as ion channels, contractile proteins and transcription factors. In this manner, PDEs regulate key cellular functions and have fundamental and pharmacological interest: they have been acknowledged as important drug targets for the treatment of disparate diseases, such as congestive heart disease, depression, asthma, inflammation, and erectile dysfunction (14–17).

Table 1. Distribution of genotyped SNPs by PDE families

Family	Genes	Substrate	SNPs
PDE1	1A, 1B, 1C	cAMP/cGMP	11
PDE2	2A	cAMP/cGMP	5
PDE3	3A	cAMP/cGMP	2
PDE4	4A, 4B, 4C, 4D	cAMP	21
PDE5	5A	cGMP	1
PDE6	6A, 6C, 6D, 6G	cGMP	8
PDE7	7A, 7B	cAMP	6
PDE8	8A, 8B	cAMP	5
PDE9	9A	cGMP	4
PDE10	10A	cAMP/cGMP	10
PDE11	11A	cAMP/cGMP	5
Total	21		78

The PDE enzymes can be classified by their substrate (Table 1), whether cAMP-specific, cGMP-specific, or dual substrate (cAMP and cGMP) (14, 18, 19). The regulatory N terminus of these enzymes has considerable variation and includes regions that autoinhibit the catalytic domains and regions that control subcellular localization (20, 21). The N terminus may include a calmodulin-binding protein (PDE1), cGMP-binding sites (PDE2), phosphorylation sites for several protein kinases (PDE1–5), and a transducin-binding domain (PDE6).

The intense interest in PDE expression and activity during the last decade has advanced the understanding that in the brain, where regulation of second-messenger signaling is very complex, virtually all PDEs are expressed at high levels; their differential expression patterns and subcellular distributions are relevant to cell-to-cell communications and modulation of neuronal activity

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Conflict of interest statement: M.-L.W. and J.L. have filed a patent based on the work reported in this paper.

Freely available online through the PNAS open access option.

Abbreviations: PDE, phosphodiesterase; MDD, major depressive disorder; HWE, Hardy–Weinberg Equilibrium; LD, linkage disequilibrium; htSNP, haplotype-tagging SNP; C.I., confidence interval; GAF, cGMP-binding PDE, Anabaena adenylyl cyclase, and *E. coli* Fh1A domain.

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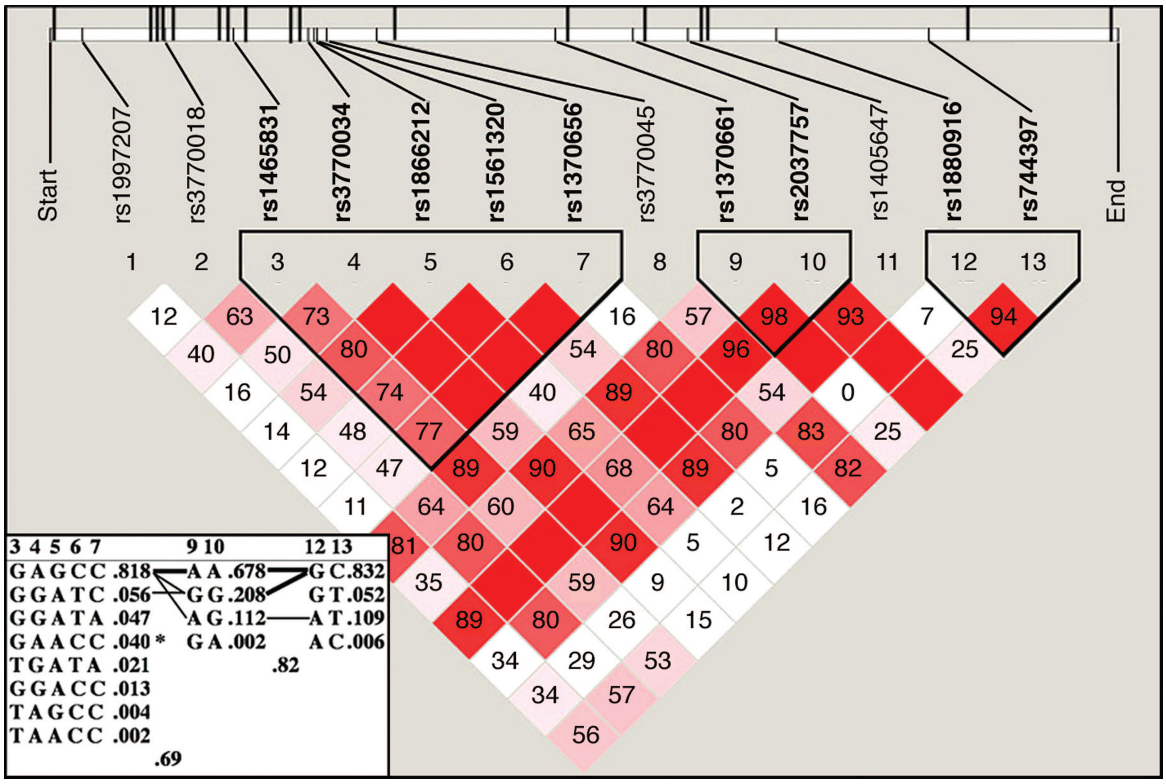

Fig. 1. LD pattern in gene PDE11A in the depressed group. Standard color scheme: white, D′ <1 and logarithm of odds (lod) <2; blue, D′ = 1 and lod <2; shades of pink/red, D′<1 and lod ≥2; bright red, D′ = 1 and lod ≥2. D′ values represent percentages and appeared inside each diamond; values of 100% are not labeled. At the top, the *PDE11A* gene structure is illustrated schematically by a long horizontal white rectangle. Short vertical lines indicate genotyped SNPs, which correspond to numbers 1–13 above the triangular image. Upward long vertical lines indicate exons (20 total some hidden because of scale). Three haplotype blocks were defined in our analyses: block 1 (38 kb, SNPs 3–7), block 2 (32 kb, SNPs 9–10), and block 3 (63 kb, SNPs 12–13). htSNPs are shown in bold. (*Inset*) Haplotypes are shown in blocks with frequency and connections from one block to the next; only htSNPs are displayed. Blocks are connected with thin lines if frequency is >5% and thick lines if >10%. Between the blocks, a value of multiallelic D′ is shown. D′ is a measure of the recombination between the two blocks. * in haplotype GAACC denotes significant association to a diagnosis of major depression ($P < 0.0001$).

(22). Several lines of investigation suggest that PDE4, a cAMP-specific enzyme, should be considered a prime target for therapeutic intervention in a range of CNS disorders, including depression and impaired cognition (23–25). The selective inhibitor of PDE4 rolipram has been shown to produce antidepressant-like and memory-enhancing effects in animals (23, 25–27). Behavioral phenotype and pharmacological data of knockout mice support the concept that PDE4D might be involved in the mediation of depressive symptoms and antidepressant response (28).

Major depressive disorder (MDD) is one of the most common psychiatric disorders; both environmental and genetic factors contribute to its etiology. Heritability of MDD is estimated at 0.36–0.7 based on twin studies (29–35). The specific genetic substrates, precipitating environmental factors, and predictors of treatment response have not yet been elucidated. The multitude of cellular responses modulated by PDEs and the strong suggestion of a potential role of PDE4 in MDD support our main hypothesis that the PDE family is a strong candidate system for susceptibility to MDD. We genotyped SNPs in 21 genes encoding PDEs and tested their association in a study of MDD cases and matched controls. We also tested the secondary hypothesis that PDE genes are associated with antidepressant treatment response.

Results

Cleaning and Filtering Steps. ***Quality control.*** The distribution of SNPs and genes across the chromosome after quality control can be seen in Table 1. In summary, 159 SNPs (80%) passed 7/7 plates; 15 (7%) passed 6/7 plates; 2 (1%) passed 5/7 plates; and 24 (12%) passed <5/7 plates. Most (92.6%) of the quality control duplicates matched. We eliminated 2.3% of observations, because they had missing data. Of our original dataset, 87.5% remained after this step.

Hardy–Weinberg Equilibrium (HWE). We examined HWE in control and depressed groups separately. We excluded 18 SNPs that were not in HWE in the control group; we also excluded 5 SNPs that were monoallelic in at least one of the groups. After quality control and HWE steps, 153 SNPs remained for linkage disequilibrium (LD) analyses.

LD. We assessed LD in each gene for control and depressed groups separately. We removed 75 of the 153 SNPs from further analysis, because they were in LD with an r^2 of ≥80% with other SNPs within a specific gene. Fig. 1 illustrates the typical LD and haplotype block structure that were obtained.

Data on 78 SNPs (of the initial 200) were used for further data analyses after quality control steps. The density of SNPs per PDE family and class was as follows: 10.7 SNPs per family of cAMP-specific PDEs, 6.6 SNPs per family of dual substrate (cAMP and cGMP) PDEs, and 4.3 SNPs per family of cGMP-specific PDE.

Haplotype Analyses. The Four Gamete Rule formed three haplotype blocks in MDD and control groups for the PDE11A gene. In the control group, the first block was defined by four haplotype-tagging SNPs (htSNPs; rs1465831, rs3770034, rs1561320, and rs1370656), whereas in the MDD group, one additional htSNP was necessary to define it (rs1866212; Fig. 1). Haplotype blocks two and three were defined each by two

Table 2. Allele frequency for SNPs significantly (*) associated and likely [(~), $P < 0.05$] to be associated with depression (MDD) when compared to control (CT)

Gene	SNP	*P* value	SNP class	Allele	Minor allele frequency	
					MDD	CT
PDE11A	rs3770018(*)	0.0005	Intron	A → C	0.058	0.11
PDE9A	rs729861(*)	0.0006	Intron	T → C	0.39	0.29
PDE5A	rs3775845(~)	0.007	Intron	A → G	0.33	0.26
PDE10A	rs717602(~)	0.009	Intron	A → G	0.46	0.38
PDE2A	rs370013(~)	0.01	Intron	A → G	0.50	0.43
PDE6C	rs650058(~)	0.01	Intron	C → T	0.41	0.48
PDE10A	rs220818(~)	0.01	Intron	T → C	0.29	0.23
PDE10A	rs676389(~)	0.03	Intron	T → C	0.24	0.23
PDE6C	rs701865(~)	0.03	Nonsynon	T → A	0.46	0.40

htSNPs (rs1370661, rs2037757 and rs1880916, rs744397, respectively).

SNP Association with MDD. Two SNPs (rs729861 in PDE9A and rs3770018 in PDE11A) were significant at the Bonferroni corrected significance level of <0.0006 for the test between control and depressed groups (Table 2).

Seven other SNPs had a *P* value ≤0.05. Those SNPs were located in four genes: PDE2A (rs376724), PDE5A (rs3775845), PDE6C (rs650058, rs701865), and PDE10A (rs220818, rs676389, and rs717602). The presence of multiple independent signals in PDE6C and PDE10A further strengthens the likelihood of an association with MDD. Table 2 shows genotype frequencies for significant SNPs in the depressed and control groups. The odds ratio for being depressed was 2.1 [95% confidence interval (C.I.) 1.3–3.3] for individuals homozygous (AA) for the major allele for rs3770018 in the PDE11A gene and 0.6 (95% C.I. 0.4–0.8) for individuals homozygous (TT) for the major allele for rs729861 in the PDE9A gene. An odds ratio of 1.4 indicates that a person with the minor allele is 40% more likely to be in the depressed group than not. Likewise, an odds ratio of 0.5 indicates that a person is half as likely to be depressed as not.

SNP Association with Antidepressant Response. Two SNPs in the PDE family had a *P* value <0.05 when tested for association with attaining remitter and nonremitter status within the entire depressed group treated with either desipramine or fluoxetine (Table 3). They were located in the PDE1A (rs1549870) and PDE11A (rs1880916) genes. The odds ratio for attaining remitter status was 4.6 (95% C.I. 1.6–13.6) for individuals homozygous (G/G) for the major allele for rs1880916 in the PDE1A gene and 3.2 (95% C.I. 1.2722–8.0092) for individuals heterozygous (A/G) for rs1880916 in the PDE11A gene.

Although each group was small, we also analyzed antidepressant response by drug and found that different SNPs and genes were associated with attaining remitter status in fluoxetine and desipramine treatment groups.

Fluoxetine Treatment. Five SNPs located in four genes were associated with remission during fluoxetine treatment (Table 3). SNPs in PDE1A (rs1549870), PDE6A (rs2544934), PDE8B (rs884162), and PDE11A (rs1880916 and rs3770018) had a difference in allele frequency with a *P* value ≤0.05 for remitters and nonremitters within the subjects treated with fluoxetine. Both SNPs associated with remission in the entire depression group were also associated with remission in the fluoxetine-treated subjects. The odds ratio for remission in the fluoxetine treatment for rs1549870 was 8.8 (1.7118–45.2382) for the major genotype; for rs1880916, 5.12 (95% C.I. 1.0602–24.738) for the heterozygous genotype; and for rs2544934, 4.4 (95% C.I. 1.1608–17.0161) for the heterozygous genotype. These confidence intervals are wide, and these results await confirmation in larger samples.

Desipramine Treatment. Two SNPs were associated with remission during desipramine treatment (Table 3). These SNPs

Table 3. Allele frequency table between remitter (R) and nonremitter (NR) groups for SNPs significantly associated to drug response at $P < 0.05$

Treatment	Gene	SNP	*P* value	Allele	Minor allele frequency	
Fluoxetine or desipramine	PDE1A	rs1549870	0.005	G → A	0.03*	0.12†
	PDE11A	rs1880916	0.04	G → A	0.16*	0.074†
Fluoxetine alone	PDE1A	rs1549870	0.007	G → A	0.022‡	0.14§
	PDE8B	rs884162	0.02	C → T	0.09‡	0.0§
	PDE6A	rs2544934	0.03	A → T	0.17‡	0.054§
	PDE11A	rs1880916	0.03	G → A	0.16‡	0.036§
	PDE11A	rs3770018	0.04	A → T	0.076‡	0.0§
Desipramine alone	PDE1C	rs992185	0.006	A → C	0.47¶	0.24‖
	PDE1C	rs30585	0.02	T → G	0.47¶	0.26‖

*R ($n = 82$).
†NR ($n = 61$).
‡R ($n = 46$).
§NR ($n = 28$).
¶R ($n = 36$).
‖NR ($n = 33$).

(rs30585 and rs992185) were located in the PDE1C gene. The odds ratio for remission with desipramine treatment for rs30585 was 5.16 (95% C.I. 1.0258–26.0228) for the minor genotype and for rs992185, 4.6 (95% C.I. 1.66–12.7) for the heterozygous genotype.

Haplotype Association with MDD. In the PDE11A gene, haplotype GAACC in block 1 (Fig. 1) was found to be significantly associated with a diagnosis of depression ($P < 0.0001$). The frequency of haplotype GAACC in the depressed group was 4.1%, and it was not present in the control group. No haplotype was found to be significantly associated with response to antidepressants.

Discussion

We found that SNPs in PDE genes are associated with MDD and antidepressant treatment response. PDEs constitute a complex family of enzymes that are essential regulators of intracellular cyclic nucleotide signaling, which have a central role in neuronal signal transduction. Through a series of rigorous processes of data cleaning, filtering steps, and analyses, we have identified two SNPs (in PDE9A and -11A genes) associated with a diagnosis of MDD and two other SNPs (in PDE1A and -11A genes) associated with treatment response. Interestingly, the PDE11A gene was associated both with drug response and depression, but different SNPs were associated with diagnosis and drug response. Almost all of the PDEs that we identified as relevant for disease or drug response catalyze cGMP; only one gene (PDE8B) identified in our study is a cAMP-specific PDE gene.

Association with MDD. Two SNPs in the PDE gene family have significantly different allele frequencies between control and depressed groups. Those SNPs were located in PDE9A and -11A genes (Table 2). PDE9A belongs to the class of cGMP-specific enzymes, and PDE11A catalyzes both cAMP and cGMP. Our data also indicate that two other members of the cGMP-specific enzymes (PDE5A and -6C) and two other members of the dual substrate (cAMP and cGMP) class of PDEs (PDE2A and -10A; refs. 14, 18, and 19) are likely to be associated with MDD. Intriguingly, five of six of these PDEs (PDE2A, -5A, -6C, -10A, and -11A) are classified as GAF-PDEs (GAF, cGMP-binding and stimulated phosphodiesterase, Anabaena adenylate cyclases, and *Escherichia coli* Fh1A; ref. 36). High amino acid sequence similarity (42–51%) is found in the catalytic region of GAF-PDEs, and catalytic domain phylogenic tree analysis of human PDEs demonstrates evolutionary relatedness among the GAF-PDE family and suggests that these genes have a common ancestor gene. Our findings are further supported by haplotype analyses of PDE11A, which showed that haplotype GAACC in block 1 is significantly associated with a diagnosis of MDD ($P < 0.0001$; Fig. 1).

Association with Drug Response. Two SNPs (rs30585 in PDE1A and rs992185 in PDE11A) have significantly different allele frequencies between remitters and nonremitters within the entire depressed group. PDE1A and -11A hydrolyze cAMP and cGMP (14, 18, 19). Individuals who have the G/G genotype for rs30585 or the A/G genotype for rs992185 are, respectively, 4.6 and 3.2 times more likely to attain remission in our sample. These two SNPs also have significantly different allele frequencies between remitters and nonremitters within the fluoxetine group but not within the desipramine group (Table 3). Different SNPs and genes were significantly associated with remitters and nonremitters in fluoxetine- and desipramine-treated patients. Three additional SNPs (rs2544934 in PDE6A, rs884162 in PDE8B, and rs3770018 in PDE11A) were also significantly associated with drug response in the fluoxetine group. Genes associated with response to fluoxetine are located in two chromosomal regions, 2q31–32 and 5q14–31. Two SNPs (rs30585 and rs992185) in the PDE1C gene were associated with treatment response in the desipramine group.

Potential CNS Role of Significant Genes. Many PDEs are expressed in high concentrations in the brain; their differential expression and subcellular compartmentalization are very suggestive that they are important in fine-tuning neuronal activity and controlling distinct physiological processes and signaling pathways.

The CNS roles for many PDEs remain elusive. Of all significant genes identified in our study, only PDE9A has a known potential role in CNS; it is relevant to cognition and neurodegeneration (22). Interestingly, all of the PDE genes that we have identified as likely to be associated with MDD have potential roles in the CNS (22): cognition and neurodegeneration (PDE2A), cognition and depression (PDE5A), retinal degeneration (PDE6C), and Huntington's disease (PDE10A; refs. 37 and 38).

Conclusions

Our data indicate that PDE genes that modulate intracellular levels of cGMP are the predominant class of PDE associated with the diagnosis and treatment outcome of major depression. All but one PDE gene (PDE8B is cAMP-specific) we identified were either cGMP-specific or dual-substrate enzymes. The cAMP-specific PDE8B, which is high-affinity and rolipram-insensitive, was associated with treatment response in our fluoxetine-treated group, but surprisingly none of the SNPs we examined in cAMP-specific PDE genes were significantly associated with diagnosis, even though in our study, SNP density was higher for that class of PDE genes.

Unexpectedly, we found that polymorphisms in the PDE11A gene are significantly associated with the diagnosis of MDD and treatment response, which strongly suggests the involvement of this enzyme in the biology of depression. PDE11, the newest member of the mammalian PDE family, was characterized 6 years ago (9). This family has a single gene (PDE11A) that has four splicing variants (PDE11A1–A4). The expression and function of this gene are not well understood, but it appears to have a role in spermatogenesis (39); however, no potential CNS role had been previously contemplated for PDE11 (22). PDE11A is phylogenetically related to GAF-containing PDEs: PDE2, -5, -6, and -10; it closely resembles PDE5 by sequence (50% identity and 70% similarity in the catalytic domain) and is located in chromosome 2q31–32 (for a recent review, refer to ref. 40). Thus, our data support the involvement of chr 2q31–32 in the susceptibility for MDD and in antidepressant response.

Pharmacological and genetic studies have indicated that cGMP could be the central mediator of the effects NO/cGMP in several brain regions (41–43). cGMP has several target proteins, including cGMP-regulated cation channels and cGMP-dependent PKs. Two cGMP-PK genes (types 1 and 2) that have been described in mammals are widely distributed in the brain (42, 44). cGMP has been implicated in neuronal maturation (45–47), directional guidance of growth cones (48–50), and learning and memory tasks (51–53). Recently, Horvath *et al.* (54) described inactivating mutations of the PDE11A gene in a condition predisposing to the development of adrenocortical hyperplasia leading to Cushing syndrome.

Further studies are necessary to establish whether polymorphisms in PDE2A, -5A, -6C, and -10A genes contribute to susceptibility to MDD. Our studies have not exhaustively examined the involvement of PDE polymorphisms in MDD or antidepressant treatment response; therefore, we cannot reject the role of any PDE gene in the genetic or pharmacogenetic of MDD. The contributions of other SNPs in the PDE family of genes should also be further scrutinized, especially in the PDE4

gene family, because PDE4D-regulated cAMP signaling may play a role in the pathophysiology and pharmacotherapy of depression (24, 25). Although we examined 17 SNPs in this gene of the PDE4 family, we have not found an association with diagnosis of MDD or drug response. Regretfully, the limited size of our sample does not permit us to comprehensively explore and detect the likely gene–gene interactions within the PDE family. Such interactions are present if an allele or SNP in one gene influences the effect of a SNP in a second gene. In a larger sample, such explorations could be conducted statistically by using stepwise logistic regression models that include effects for the SNPs within individual genes along with their interactions. In addition to detecting interactions among the SNPs already identified as significant, these analyses might also reveal SNPs within genes that play only interactive roles and thus have not yet been detected as significant.

This study identifies a potential CNS function for the PDE11 family, specifically the susceptibility for major depression and antidepressant drug response. Our results support the need for large-scale comprehensive studies focused on the role of PDE genes on the susceptibility to major depression and antidepressant treatment response. These findings suggest that drugs targeted to affect PDE function, particularly cGMP-related PDEs, could represent a new treatment strategy for major depression and should therefore be tested.

Methods

Study Population. The study population consisted of 284 depressed subjects enrolled in a pharmacogenetic study of antidepressant treatment response to desipramine or fluoxetine. We also studied 331 age- and sex-matched control subjects recruited from the same Mexican-American community in Los Angeles and studied by the same bilingual clinical research team at the Center for Pharmacogenomics and Clinical Pharmacology at the University of California, Los Angeles (55). Controls were in general good health but were not screened for medical or psychiatric illness. All patients were Mexican-American men and women aged 21–68 years, with a current episode of major depression as diagnosed by DSM-IV (56). In this study, all Mexican-American subjects had at least three grandparents born in Mexico (57). We used diagnostic and ratings instruments that have been fully validated in English and Spanish, and all assessments were conducted in the subjects' primary language.

Inclusion criteria included DSM-IV diagnosis of a current unipolar major depressive episode, with a 21-item Hamilton Depression Rating Scale (58) score of ≥18 with item number 1 (depressed mood) rated ≥2. There was no anxiety threshold for inclusion. Subjects with any primary axis I disorder other than MDD (e.g., dementia, psychotic illness, bipolar disorder, and adjustment disorder), electroconvulsive therapy in the last 6 mo, or previous lack of response to desipramine or fluoxetine were excluded. Because anxiety can be a manifestation of depression, patients who met criteria for depression and also anxiety disorders were not excluded. Exclusion criteria included active medical illnesses that could be etiologically related to the ongoing depressive episode (e.g., untreated hypothyroidism, cardiovascular incident within the past 6 mo, uncontrolled hypertension, or diabetes), current active suicidal ideation with a plan and strong intent, pregnancy, lactation, current use of medications with significant CNS activity that interferes with electroencephalogram activity (e.g., benzodiazepines) or any other antidepressant treatment within the 2 wk before enrollment, illicit drug use and/or alcohol abuse in the last 3 mo, or current enrollment in psychotherapy.

All patients had an initial comprehensive psychiatric and medical assessment and, if enrolled, had 9 wk of structured followup assessments. The study consists of two phases: a 1-wk single-blind placebo lead-in phase to minimize the impact of placebo responders followed, if subjects continue to meet the inclusion criteria after phase 1, by random assignment to one of the two treatment groups: fluoxetine 10–40 mg/day or desipramine 50–200 mg/day, administered in a double-blind manner for 8 wk, with blind dose escalation based on clinical outcomes. In the depressed group, 230 subjects received treatment in our double-blind clinical trial. Of those, 122 patients were treated with desipramine [83 female (F), 39 male (M)], and 108 were treated with fluoxetine (71F, 37M). Sixty-nine patients treated with desipramine (45F, 24M), and 72 treated with fluoxetine (52F, 20M) completed our 8-wk treatment with weekly data collection.

Genomic DNA Collection. At the initial visit, blood samples were collected into EDTA (K_2EDTA) BD Vacutainer EDTA tubes (Becton Dickinson, Franklin Lakes, NJ), and genomic DNA was isolated by using Gentra Puregene DNA purification kits (Gentra Systems, Indianapolis, IN).

Antidepressant Treatment Response. Our primary clinical outcome measure within the depressed group receiving antidepressant treatment was the Hamilton Depression Rating Scale (HAM-D21). Treatment response was classified into two categories, remission and nonremission status, based on the final (week 8) HAM-D21 score. Remission was defined as having a final HAM-D21 score of <8.

SNP Genotyping Methods. SNPs were selected from 21 of the 25 genes in the PDE family, located across 14 chromosomes. We selected an average of 10 intragenic SNPs per gene from dbSNP (build 121). SNP assays were designed and typed with the Golden Gate assay as part of a 1,536 multiplex reaction (59). DNAs with poor results (50% GC score <0.65) were removed as well as loci with a low clustering score (<0.3). The threshold for retaining individual genotype calls was set to a Genecall score of 0.25.

Cleaning and Filtering Steps. ***SNP quality control.*** Our data analysis plan included a series of data cleaning steps followed by a series of filtering steps to identify a list of significantly associated SNPs. Only data generated by SNP assays that were successfully genotyped on at least 80% of samples were included. Data quality was assessed by duplicate DNAs ($n = 26$) across all plates. Genotypes from nonmatching duplicates were dropped; they were also dropped if they had one missing data point.

HWE. We used the HWE equation ($p^2 + 2pq + q^2 = 1$; p is the frequency of the dominant allele, and q is the frequency of the recessive allele for a trait controlled by a pair of alleles) to determine the probable genotype frequencies in our study populations. Deviation from HWE was tested separately for the control and depressed groups by using the ALLELE procedure in SAS/Genetics 9.1.3 (SAS Institute, Cary, NC). PROC ALLELE uses the notation and methods described by Weir (60). SNPs that were not in HWE in the control group ($P < 0.05$) and SNPs that were monoallelic in both groups were excluded.

LD among SNPs. Pairwise LD was calculated within each gene for all SNPs that passed quality control measures by using the r^2 measure. An r^2 cutoff of ≥80% was used to remove redundant SNPs from the analysis (Fig. 1). The Four Gamete Rule was used to identify haplotype blocks. This method of haplotype block definition assumes no recombination within a block but does allow for recombination between blocks (61). LD measures were assessed by using Haploview, Version 3.2 (ref. 62; Broad Institute, Cambridge, MA).

Haplotype Analyses. Haplotype block analyses and haplotype population frequency estimation were performed by using Haploview, Version 3.2 (Broad Institute) and by applying the Four Gamete Rule (61). Blocks are formed by consecutive markers where only three gametes are observed. Analyses were initially performed for depressed and control groups separately. Further haplotype anal-

yses were conducted with the depressed and control groups combined to test whether a certain haplotype was associated with a diagnosis of depression. htSNPs were defined in Haploview by using aggressive tagging (two- and three-marker haplotypes). This method selects a minimal set of markers where all other alleles to be captured are correlated ($r^2 \geq 0.8$) with a marker in that set. Then, the use of multimarker tests expands the set and includes additional markers that capture alleles not otherwise captured in the initial pairwise tagging. All haplotypes >0% were examined, and nontagging SNPs within haplotype blocks were omitted from the final analyses and figures (Fig. 1 *Inset*).

Statistical Analyses. ***SNP Analyses.*** Allele, genotype and allelic trend association tests were performed by using PROC CASE CONTROL in SAS/Genetics 9.1.3 (SAS Institute). PROC CASE CONTROL is designed to test for differences in frequency of marker data when random samples are available from populations affected and unaffected by disease and is based on case-control tests for biallelic markers described by P. D. Sasieni (63). The following criteria were used to identify a list of SNPs statistically associated with a diagnoses of depression: (*i*) SNPs were in HWE in the control group; (*ii*) the minor allele frequency in the control group was ≥5%; and (*iii*) multiple testing was corrected by using Bonferroni correction, which set the significance level at *P* value ≤0.0006 for tests between control and depressed groups. We tested our secondary hypothesis using similar criteria to identify a list of SNPs associated with treatment response: (*i*) SNPs were in HWE in the control group; (*ii*) the minor allele frequency in the control group was ≥5%; (*iii*) *P* value ≤0.05 for allele test between remitter and nonremitter groups was used. Because of the small sample size, this part of the analyses is preliminary.

Odds ratios. We compared the odds of having depression given the homozygous major, homozygous minor, or heterozygous genotype for SNPs associated with diagnoses of depression. Similarly, we compared the odds of attaining remission given the homozygous major, homozygous minor, or heterozygous genotype for SNPs associated with treatment response. Odds ratios were calculated by using PROC FREQ in SAS/Genetics 9.1.3 (SAS Institute.

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Chapter IV. Reprint

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ORIGINAL ARTICLE

Polymorphisms in inflammation-related genes are associated with susceptibility to major depression and antidepressant response

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There are clinical parallels between the nature and course of depressive symptoms in major depressive disorder (MDD) and those of inflammatory disorders. However, the characterization of a possible immune system dysregulation in MDD has been challenging. Emerging data support the role of T-cell dysfunction. Here we report the association of MDD and antidepressant response to genes important in the modulation of the hypothalamic–pituitary–adrenal axis and immune functions in Mexican Americans with major depression. Specifically, single nucleotide polymorphisms (SNPs) in two genes critical for T-cell function are associated with susceptibility to MDD: *PSMB4* (proteasome β4 subunit), important for antigen processing, and *TBX21* (T bet), critical for differentiation. Our analyses revealed a significant combined allele dose–effect: individuals who had one, two and three risk alleles were 2.3, 3.2 and 9.8 times more likely to have the diagnosis of MDD, respectively. We found associations of several SNPs and antidepressant response; those genes support the role of T cell (*CD3E*, *PRKCH, PSMD9* and *STAT3*) and hypothalamic–pituitary–adrenal axis (*UCN3)* functions in treatment response. We also describe in MDD increased levels of CXCL10/IP-10, which decreased in response to antidepressants. This further suggests predominance of type 1 T-cell activity in MDD. T-cell function variations that we describe here may account for 47.8% of the attributable risk in Mexican Americans with moderate MDD. Immune function genes are highly variable; therefore, different genes might be implicated in distinct population groups.
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Keywords: TBX21; PSMB4; major depression; genetic; SNP; cytokine

Introduction

Major depressive disorder (MDD) is a common and complex disorder of unknown etiology that affects about 15% of the population.[1] Despite recent scientific advances and its enormous social costs,[2] MDD is still currently thought to be a gene-environment disorder of polygenic nature with a descriptive diagnosis and no known biomarkers.[1] Although the contributions of immune mediators to the pathophysiology and treatment of psychiatric disorders may be traced back to over 80 years with the work of Nobel laureate Julius Wagner-Jauregg,[3] evidence from clinical and basic research have recently supported a role for dysregulation of the immune system in MDD.[4] Both acute stress and MDD are states of hyperarousal, in which a sustained focus on the threatening stimulus, fear-related behaviors and stereotyped states of cognition and affect are matched with indices of physiological hyperarousal, such as activation of the hypothalamic–pituitary–adrenal (HPA) axis and sympathetic activation, and inhibition of counterproductive neurovegetative functions during life-threatening situations.[5,6] Depression-like symptoms have been associated with activation of the HPA axis, sympathetic nervous system and inflammatory response characterized by hypercortisolaemia,[7] increased central corticotropin-releasing hormone (CRH)[8–10] and norepineprhine[11,12] functions, increased numbers of peripheral leukocytes, positive acute phase proteins and proinflammatory cytokines.[13]

We and others have proposed a role for proinflammatory cytokines in the pathophysiology of MDD, with activation of the immune system and of the cellular immune response.[14,15] Even though still underexplored in MDD, the T-cell arm of the immune system has been emerging as the centerpiece of the continued debate over the role of the immunomediators in depression.[15,16] Data supporting either

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a predominance of cytokine-producing helper T-cells, type 1 (Th1) or type 2 (Th2) have accumulated. The overactivity of the hallmarks of Th1 immunity, such as interferon-γ (IFNγ), tumor necrosis factor-α or interleukin-1 (IL-1),[17] or predominant Th2 patterns of production supported by increased levels of IL-6, IL-10 or IL-13[16] have continued to fuel this discussion. At least two fundamental processes may contribute to the role of the T-cell arm of adaptive immunity in MDD: T-cell programmed differentiation and antigen processing.

Naive $CD4^+$ T lymphocytes proliferate and differentiate into two main lineages defined by distinct cytokine profiles[18] after encountering antigen-carrying dendritic cells in secondary lymphoid organs. The balance of two main subtypes of cytokine-producing Th1/Th2 determines the immune response to pathogens. Clinically, Th1 patterns of cytokine production are associated with inflammation and autoimmune disease, whereas Th2 patterns are related to allergic responses and asthma.[19] Th1 cell-lineage commitment is controlled by the key transcriptional factor TBX21 (Tbet),[20] which is rapidly produced early in Th1 differentiation and gradually decreases at later stages.[21] TBX21 has the ability to simultaneously drive Th1 genetic programs and repress the development of the opposing Th2 subset; it may also redirect fully polarized Th2 cells into Th1 cells.

Proteasomes (prosome, macropain) are the major intracellular extralysosomal organelle for protein degradation and a central source of antigenic peptides in the endogenous pathway; they are utilized in major histocompatibility complex molecules class I (MHC1) antigen processing and protein degradation. Proteasomes are highly abundant in the cytosol and nucleus and are organized as multiunit protease complexes. Protein degradation is central to many important biological functions, including cell-cycle progression, apoptosis, synaptic reorganization, DNA repair, normal immune surveillance mechanisms and immune response networks. Disruption of the proteasomal degradation pathway has been implicated in a wide range of human disorders; immune abnormalities include the development of $CD8^+$ T lymphocytes and MHC1 molecules, and MHC1-restricted antigen presentation have been described in mice lacking proteasome subunits.[22] Independent lines of research have supported the role of protein synthesis/degradation in MDD-like neuropsychiatric symptoms in autoimmune disorders[23] and in the central actions of antidepressant drugs.[24]

We used a combination strategy consisting of genetic analyses and functional assays to assess the association of pivotal elements of acquired immunity relevant to the HPA axis modulation and T-cell function with susceptibility to MDD and antidepressant response. We genotyped a panel of single nucleotide polymorphisms (SNPs) focused on the steroid pathway[25] and on proteasome subunit genes (Table 1). We also conducted multiplex assays of 21 circulating cytokines in a subset of our patients.

Methods

Genetic study

Study population. This study was approved by the institutional review boards of the University of California Los Angeles and the University of Miami, and it has been registered in the public database ClinicalTrials.gov (NCT00265292). The study population consisted of 284 depressed Mexican Americans enrolled in a pharmacogenetic study of antidepressant treatment response as previously described.[26,27] We also studied 331 control individuals recruited from the same Mexican-American community in Los Angeles and studied by the same bilingual clinical research team. Controls for our genomic studies were in general good health but were not screened for medical or psychiatric illness. All patients were Mexican-American men and women aged 21–68 years, with a current episode of major depression as diagnosed by DSM-IV (*Diagnostic and Statistical Manual of Mental Disorders*, 4th edn).[28] In our study, all Mexican-American subjects had at least three grandparents born in Mexico.[26] All patients had comprehensive psychiatric and medical assessments. We used diagnostic and ratings instruments that have been fully validated in English and Spanish, and conducted all assessments in the subject's primary language.

Inclusion criteria included DSM-IV diagnosis of current, unipolar major depressive episode, with a 21-item Hamilton Depression Rating Scale (HAM-D21)[29] score of 18 or greater with item number 1 (depressed mood) rated 2 or greater. There was no anxiety threshold for inclusion. Subjects with any primary axis I other than MDD (for example, dementia, psychotic illness, bipolar disorder, adjustment disorder), electroconvulsive therapy in the past 6 months or previous lack of response to desipramine or fluoxetine were excluded. As anxiety can be a manifestation of depression, patients who met criteria for depression and also anxiety disorder were not excluded. Exclusion criteria included active medical illnesses that could be etiologically related to the ongoing depressive episode (for example, untreated hypothyroidism, cardiovascular accident within the past 6 months, uncontrolled hypertension or diabetes), current, active suicidal ideation with a plan and strong intent, pregnancy, lactation, current use of medications with significant central nervous system activity, which interfere with electroencephalography (EEG) activity (for example, benzodiazepines) or any other antidepressant treatment within the 2 weeks prior to enrollment, illicit drug use and/or alcohol abuse in the past 3 months or current enrollment in psychotherapy.

Depressed subjects were enrolled in an outpatient double-blind study of antidepressant treatment

Table 1 Number of SNPs investigated

Symbols	*Steroid pathway genes*	*No. of SNPs*
ABCB1	ATP-binding cassette, subfamily B (MDR/TAP), member 1	9
CD3E	CD3e molecule, epsilon (CD3–TCR complex)	2
CD4	CD4 molecule	7
CD7	CD7 molecule	2
CRH	Corticotropin-releasing hormone	8
CRHBP	Corticotropin-releasing hormone-binding protein	4
CRHR2	Corticotropin-releasing hormone receptor 2	16
CYP3A4	Cytochrome P450, family 3, subfamily A, polypeptide 4	2
GTF2F1	General transcription factor IIF, polypeptide 1	3
IL18BP	Interleukin 18-binding protein	7
IPO13	Importin 13	7
JUND	Jun D proto-oncogene	2
MFNG	MFNG *O*-fucosylpeptide 3-β-*N*-acetylglucosaminyltransferase	6
NR3C1	Nuclear receptor subfamily 3, group C, member 1	9
PFKFB4	6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	3
POMC	Proopiomelanocortin	4
PRKCSH	Protein kinase C substrate 80K-H	4
RAC2	Ras-related C3 botulinum toxin substrate 2	6
CDC42SE2	CDC42 small effector 2	3
TBX21	T-box 21	9
STAT3	Signal transducer and activator of transcription 3	4
UCN	Urocortin	1
UCN2	Urocortin 2	4
UCN3	Urocortin 3	2
Proteasome subunit genes		
α	A1, A6, A7	6
β	B2, B4, B5, B8	6
26S (non-ATPase)	D1, D2, D3, D5, D9, D13, D14	19
Inhibitor	F1	6
Total		161

Abbreviation: SNPs, single nucleotide polymorphisms.

response to desipramine or fluoxetine.[26] The treatment had two phases. Phase 1 was a 1-week, single-blind placebo lead-in phase to eliminate placebo responders. Subjects who continued to meet the inclusion criteria after phase 1 were randomly assigned to one of two treatment groups in a double-blind manner in phase 2; they received fluoxetine 10–40 mg per day or desipramine 50–200 mg per day for 8 weeks, with a dose escalation based on clinical outcomes. Depressed subjects had up to 9 weeks of structured follow-up assessments. The effect of antidepressants on HAM-D21 score was measured by the relative reduction computed as the difference in HAM-D21 score between pre- and post-treatment divided by the pretreatment HAM-D21 score. Responders were defined as the patients who had a higher than 50% reduction in HAM-D21 score on the final week (week 8).

Genotyping assays. Blood samples were collected into ethylenediaminetetraacetic acid (K_2EDTA) BD Vacutainer EDTA tubes (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) and genomic DNA was isolated from those samples using Gentra Puregene DNA purification kits (Gentra Systems Inc., Indianopolis, IN, USA). Genotyping of SNPs was performed using a SEQUENOM MassARRAY MALDI-TOF mass spectrometer (Sequenom, San Diego, CA, USA) for analysis of unlabeled single-base extension minisequencing reactions[27] or using the Golden Gate assay (Illumina, San Diego, CA, USA) as part of a multiplex reaction as previously described.[26] Our SEQUENOM protocol implemented the very short extension method proposed by Sun and colleagues[30] whereby sequencing products are extended by only one base for three of the four nucleotides (due to the presence of dideoxynucleotides for three of the four nucleotides in the minisequencing reaction) and by several additional bases for the fourth nucleotide (specified in advance so as to represent one of the two alleles at a given SNP locus). This allowed for clearly delineated mass separation of the two allelic variants at a given locus. We addressed population stratification by stratifying our analysis by self-designated ethnic group. A set of random markers across the genome was also genotyped. Cleaning and filtering steps were performed as previously described[26,27] Only data generated by SNP assays that were successfully genotyped on at least 80% of samples were included. Data quality was assessed by

duplicates DNAs across all plates. Genotypes from nonmatching or missing duplicates were dropped.

Hardy–Weinberg equilibrium. We performed both standard asymptotic test and exact test for Hardy–Weinberg equilibrium (HWE) described by Wigginton *et al*.[31] using PLINK program (http://pngu.mgh.harvard.edu/~purcell/plink/). For a locus with two alleles, the locus is in HWE in the population when the relationship between allele frequencies and genotype proportions follows the equation $p^2+2pq+q^2=1$, where p and q are the frequencies for major and minor allele, respectively. Exact test of HWE is a more appropriate approach when one allele is very rare. We detected deviation from HWE separately for control and depressed groups, and excluded those SNPs that were not in HWE in the control group.

Data analyses

SNP-based analyses of susceptibility to MDD. We performed allelic association tests using the PLINK program. Specifically, we employed χ^2-test, or Fisher's exact test when the minor allele was rare, to examine the allelic association with depression by comparing allele frequencies between cases and controls. We used the following procedures to identify a list of SNPs statistically associated with a diagnosis of depression: (1) study population and controls were randomly divided into two groups: discovery and replication samples; (2) significance level was set at $P\leqslant 0.05$ for both discovery and replication samples; (3) the minor allele frequency in controls was $\geqslant 5\%$ and (4) the Benjamini and Hochberg method was used to control for false discovery rate and the significance threshold was set at FDR_BH$\leqslant 0.05$.[32] For the SNPs associated with depression in the discovery sample, we compared the odds of having depression in individuals having a risk allele with those homozygous for a nonrisk allele.

Odds ratios and population attributable fraction. We compared the odds of having depression given the homozygous for major and minor, or heterozygous genotype for the SNPs associated with diagnoses of depression. Odds ratios (ORs) and their 95% confidence intervals (CIs) were calculated using the PLINK program. We also calculated population attributable fraction (PAF) to estimate the proportional amount by which disease risk is due to the risk genotypes in the population.[33]

SNP-based analyses of drug response. We used χ^2-test to investigate the allelic association with drug response status by comparing allele frequencies between responders and nonresponders; we used Fisher's exact tests when the minor allele was rare. We calculated the allelic OR for response status and its 95% CI using Woolf's method or fitting exact logistic regression model with SAS software when the frequency in a table cell is 0. For the SNPs with a $P<0.05$ in responder vs nonresponder analysis or nonsynonymous SNPs close to any of these SNPs, we also employed a general linear regression model to examine the additive effect of minor allele on the relative reduction of HAM-D21 score by controlling age, gender and baseline HAM-D21 score using the PLINK program.

Haplotype analyses. We used Haploview version 4.0 program (http://www.broad.mit.edu/mpg/haploview/) and applied the four-gamete rule[34] to conduct haplotype analysis with the depressed and control groups combined to test whether a certain haplotype was associated with the risk for depression. Blocks are formed by consecutive markers where only three gametes are observed, and htSNPs are defined in Haploview by using aggressive tagging (two- and three-marker haplotypes). All haplotypes $>0\%$ were examined, and nontagging SNPs within haplotype blocks were omitted from the final analyses (Figure 1).

Analyses of combined effect. We used Rothman's synergy index (S) to assess the joint effect of the two polymorphisms.[35] The S index is the ratio of the observed joint effect divided by the expected joint effect assuming additivity of the effects, defined as: $S=(OR_{11}-1)/(OR_{10}+OR_{-01}-2)$ in which subscript 0 denotes the absence of the risk genotype at the SNP and OR denotes the odds ratio. No interaction corresponds to $S=1$, whereas $S>1$ ($S<1$) can be interpreted as a measure of relative increase (decrease) in the effect among those exposed to risk genotypes at both SNPs. In addition, we conducted the Cochran–Armitage trend test to examine dose–effect relationship between the sum of risk alleles at both SNPs and the OR for depression. We used SAS Proc Genmod to calculate ORs and their 95% Wald CIs (SAS version 9.1.3;SAS Institute, NC, USA).

Immunoassay study

Study population. A subset of the genetic study population, consisting of 68 Mexican-American MDD patients (51 women (36.0 ± 8.3 years old and body mass index (BMI) 28.8 ± 5.8; mean + s.d.) and 17 male (36.1 ± 10.1 years old and BMI 28.2 ± 5.2; mean + s.d.)) and 18 Mexican-American controls (12 women (36.1 ± 9.2 years old and BMI 28.9 ± 3.6; mean + s.d.) and 6 men (31.5 ± 9.4 years old and BMI 29.1 ± 6.1; mean + s.d.)), was assessed by 21-plex cytokine assay. Controls were free of ongoing physical illness and showed no evidence of major psychiatric illness in clinical and structured interviews. Fasting blood was collected for cytokine assays one time in controls and two times in MDD patients (pretreatment at week-1 and post-treatment at week 8). Among 68 patients, 29 were assessed for the cytokines at week 8 and 1 patient was assessed only at week 8.

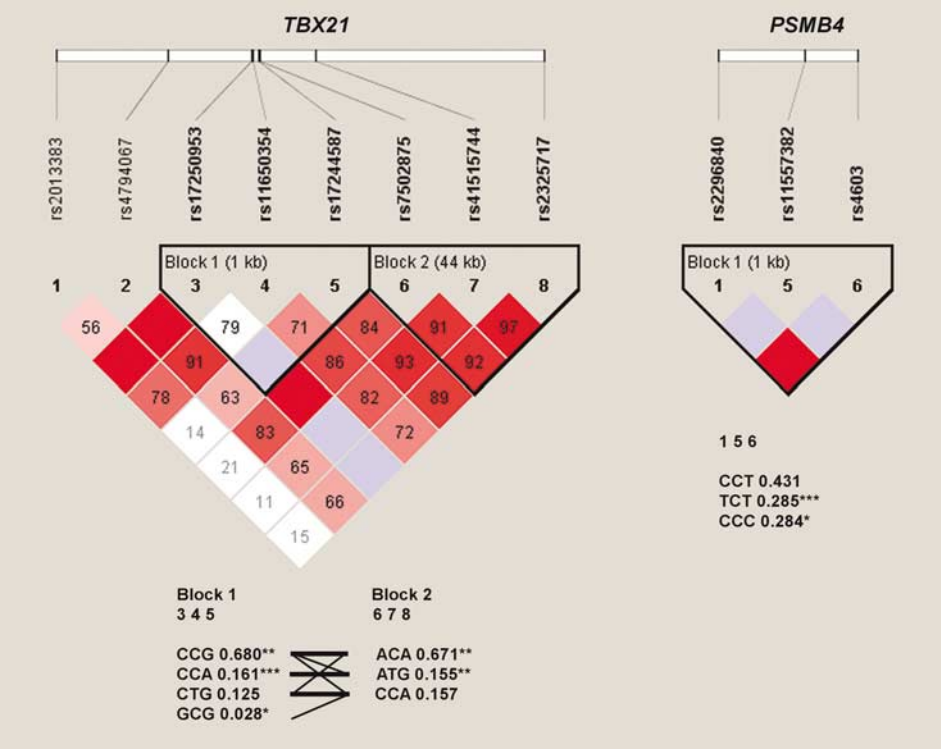

Figure 1 Linkage disequilibrium pattern in *TBX21* and *PBSM4* genes. Standard color scheme: white, $D' < 1$ and logarithm of odds (LOD) < 2; blue, $D' = 1$ and LOD < 2; shades of pink/red, $D' < 1$ and LOD $\geqslant$ 2; bright red, $D' = 1$ and LOD $\geqslant$ 2. D'-values represent percentages and appeared inside each diamond; values of 100% are not labeled. At the top of the figure, gene structures are illustrated schematically by a thick horizontal white rectangle. Short vertical lines indicate genotyped single nucleotide polymorphisms (SNPs), which correspond to the numbers above the triangular image for genes *TBX21* and *PSMB4*. Haplotype blocks were defined using the four game rule and haplotype tagging SNPs (htSNPs) are shown in bold. At the bottom of the triangular figures, haplotypes are shown in blocks with frequency and connections from one block to the next; only htSNPs are displayed. Blocks are connected with thin lines if frequency is > 5% and thick lines if > 10%. Between the blocks, a value of multiallelic D' is shown. D' is a measure of the recombination between the two blocks. *TBX21*: two haplotype blocks were defined; block 1 (1 kb; SNPs 3–5: rs17250953, rs11650354 and rs17244587) and block 2 (44 kb; SNPs 6–8: rs7502875, rs41515744 and rs2325717). Haplotype CCA in block 1 is the most significantly association with major depressive disorder (MDD) diagnosis ($P < 0.0001$). *PSMB4*: one haplotype block was defined; haplotype TCT was significantly associated with MDD diagnosis ($P = 0.0001$). $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P \leqslant 0.0001$.

Immunoassays. Plasma samples were collected before the initiation of antidepressant treatment in MDD patients. We used Human Cytokine 21 PLEX-Premixed immunoassay kits (Linco Research Inc., St Charles, MO, USA) and a multi-analyte detection system (Luminex 100 instrumentation and xMAP technology; Luminex Corp., Austin, TX, USA) to simultaneously obtain the level of several cytokines and chemokines. Assays were performed accordingly to the manufacture's instructions. Assays were run in duplicates and coefficient of variance was $\geqslant$15%.

Data analyses. Pearson's χ^2-test was performed to test for the difference of gender frequency and Student's *t*-test was conducted to compare the mean difference in age and BMI between MDD patient and control groups. No significant difference was found in age and BMI means and gender frequency between the two groups. We excluded 10 analytes from the analyses because their frequencies of undetectable level were over 30%. Of the remaining 11 analytes, we used logarithmic transformation for chemokine CXCL10/IP10 levels because of the high kurtosis (11.14) and skewness (2.73). After transformation, the kurtosis and skewness for the log10 (CXCL10/IP10) were 1.33 and 0.27, respectively. We conducted analysis of covariance analyses based on general linear model by including age, gender and BMI as covariates to compare cytokine levels between controls and pretreatment MDD patients. We performed paired *t*-test to compare pre- and post-treatment cytokine levels in MDD patients with response to antidepressant treatment. All these analyses were performed using SAS.

Results

SNP associated with MDD

In our discovery sample, the MDD diagnosis was significantly associated with SNPs in the following genes (Table 2): *PSMB4, POMC, CDC42SE2, NR3C1, ABCB1, TBX21* and *GTF2F1*. The association of four

Table 2 Polymorphisms associated with risk of depression

Polymorphism					*Allelic association*						
							Combined sample (N = 559)				
Gene	*SNP*	*Chromosome*	*Position*	*SNP type*	*P (discovery sample, N[a] = 280)*	*P (replication sample, N[b] = 279)*	*P (FDR_BH)*	*Risk/nonrisk allele*	*Case risk allele frequency*	*Control risk allele frequency*	*OR (95% CI)*
PSMB4	rs2296840[c]	1	149638671	5′ UTR	0.002	0.02	0.0001 (0.007)	T/C	0.34	0.24	1.65 (1.28, 2.12)
	rs4603		149640649	Missense	0.07	0.07	0.01 (0.17)	A/G	0.75	0.69	1.38 (1.07, 1.78)
POMC	rs2118404	2	25230833	Flank	0.02	0.30	0.02 (0.17)	T/C	0.55	0.47	1.35 (1.06, 1.73)
CDC42SE2	rs798412	5	130726373	3′ UTR	0.0009	0.53	0.005 (0.12)	A/C	0.42	0.34	1.43 (1.11, 1.83)
	rs798416	5	130720999	Intron	0.0033	0.43	0.008 (0.13)	C/T	0.41	0.33	1.40 (1.09, 1.79)
NR3C1	rs852977	5	142667687	Intron	0.02	0.12	0.007 (0.13)	A/G	0.89	0.83	1.61 (1.13, 2.27)
ABCB1	rs1002205	7	86979110	Intron	0.01	0.48	0.03 (0.25)	C/G	0.19	0.14	1.45 (1.05, 2.02)
	rs1922243	7	86981440	Intron	0.008	0.66	0.03 (0.25)	T/C	0.20	0.15	1.45 (1.04, 1.99)
TBX21	rs17244587[d]	17	43178034	3′ UTR	0.004	0.005	0.00005 (0.007)	A/G	0.21	0.12	1.97 (1.41, 2.74)
	rs41515744	17	43186946	Flank	0.04	0.004	0.0004 (0.01)	T/C	0.21	0.13	1.80 (1.30, 2.50)
	rs2325717	17	43222803	Flank	0.02	0.009	0.0004 (0.01)	C/T	0.20	0.12	1.84 (1.31, 2.56)

Abbreviations: CI, confidence interval; FDR_BH, Benjamini and Hochberg false discovery rate; OR, odds ratio; SNP, single nucleotide polymorphism; UTR, untranslated region.

[a]Includes 139 cases and 141 controls.

[b]Includes 139 cases and 140 controls.

[c]Population attributable fraction (PAF) = 23.2%.

[d]PAF = 20.1%.

Table 3 Combined effect of *TBX21* and *PSMB4* genes on the risk of depression

OR by total risk allele no.[a]				*OR by combined genotype*[b]				
No. of risk allele	*Case/control*	*OR (95% CI)*	*P*	*rs17244587*	*rs2296840*	*Case/control*	*OR (95% CI)*	*P*
0	57/93	1.00	—	GG	CC/TC	133/154	1.00	—
1	123/88	2.28 (1.49–3.50)	0.0002	GG	TT	19/8	2.75 (1.17–6.49)	0.02
2	58/30	3.15 (1.82–5.47)	0.00004	AG/AA	CC/TC	90/49	2.13 (1.40–3.23)	0.0004
3	12/2	9.79 (2.11–45.3)	0.004	AG/AA	TT	8/2	4.63 (0.97–22.2)	0.05

[a]Sum of risk alleles at rs17244587 (AA = 2, AG = 1, GG = 0) and rs2296840 (TT = 2, TC = 1, CC = 0); Cochran–Armitage trend test: $Z = -5.095$, d.f. = 1, $P = 1.74\text{-E7}$; PAF = 47.8%.
[b]Rothman synergy index = $(4.63-1)/(2.75+2.13-2) = 1.26$.

SNPs was confirmed in our replication sample; two of those untranslated regions (UTRs) SNPs: rs2296840 (T/C, 5′ UTR) in *PSMB4* (proteasome β4 subunit, β7 hs, HN3, HsN3, PROS26, O(MIM) MIM 602177) and rs17244587 (G/A, 3′ UTR) in *TBX21* (T-bet, O(MIM) MIM 604895) remained significant after Benjamini and Hochberg correction for multiple testing using the combined sample (Figure 1; Table 2). Mexican-American individuals with the minor allele T at rs2296840 in *PSMB4* were 70% more likely to be in the MDD than in the control group (OR = 1.7; 95% CI: 1.3–2.1) and 23.2% of population risk could be attributable to the genotypes (TC or TT) at 2296840 (Table 2). Individuals who had the minor allele A at rs17244587 in *TBX21* were twice more likely to be in the MDD than in the control group (OR = 2.0; 95% CI: 1.4–2.7) and 20.1% of population risk could be attributable to the genotypes (AG or AA) at rs17244587 (Table 2). Taken together, 47.8% of the population risk could be attributable to the risk genotypes at rs2296840 in *PSMB4* or rs17244587 in *TBX21* (Table 3). The joint effect of the combined genotypes of rs2296840 (recessive model) and rs17244587 (dominant model) was 26% greater than that predicted by assuming additivity of effects ($S = 1.26$) (Table 3).

Trend SNPs were located in the 3′-flanking region of *TBX21* in chromosome 17q21.3 (rs4151574 and rs2325717), and in the coding region of *PSMB4* (rs4603) in chromosome 1q21. Figure 1 depicts that we have identified three significant risk haplotypes: CCA and ATG (*TBX21*, blocks 1 and 2, respectively), and TCT (*PSMB4*), and four protective haplotypes: CCG and GCG (*TBX21*, block 1), and ACA (*TBX21*, block 2) and CCC (*PSMB4*). Notably, the 5′ UTR SNP in *PSMB4* is in linkage disequilibrium with the missense SNP rs4603 (C/T; Ile234Thr).

SNP associated with antidepressant response

Five SNPS in the steroidal pathway and proteasome genes were significantly associated with antidepressant response within the entire depressed group treated either with desipramine or fluoxetine (Table 4). They were located in the following genes: *CD3E* (rs2231449, CD3 antigen-ε subunit, OMIM 186030), *PRKCSH* (rs34095, protein kinase C substrate 80 kD, heavy chain, OMIM 177060), *PSMD9* (rs1043307, proteasome 26S non-ATPase subunit 9), *STAT3* (rs3809758, signal transducer and activator of transcription 3, OMIM 102582) and *UCN3* (rs10904481, urocortin III). The association of three genes remained significant in our general linear regression analyses after controlling for age, gender and baseline HAM-D score (Figure 2a). Among these polymorphisms, the two nonsynonymous, rs104330 (Glu197Gly) in *PSMD9* and rs10904481 (Arg91Gly) in *UCN3*, and one 3′ UTR: rs2231449 (A/C) in *CD3E* are most likely to be functionally relevant.

Fluoxetine treatment

Eight SNPs located in five genes were associated with treatment response during fluoxetine treatment (Table 4). SNPs in *CYP3A4* (rs2242480), *PSMD13* (rs1045288 and rs3817629), *CDE3* (rs2231449), *PRKCSH* (rs160841) and *PSMA7* (rs2057169, rs2057168, rs2281740, rs3746651) had a difference in allele frequency with $P \leqslant 0.05$ for responders and nonresponders within the subjects treated with fluoxetine. The association of four genes remained significant in general linear regression analyses after controlling for age, gender and baseline HAM-D score (Figure 2c). Patients who had minor allele A at missense SNP rs1045288 (Asn13Ser) in *PSMD13* had better response ($\beta = 18.13$; 95% CI: 8.29–27.96), whereas those who had minor allele C at 3′ UTR SNP rs3746651 in *PSMA7* had smaller relative reduction in HAM-D21 score ($\beta = -11.5$; 95% CI: −3.34 to −18.26). Patients who had minor allele A at 3′ UTR SNP rs2231449 in *CDE3* showed much worse response ($\beta = -37.17$; 95% CI: −55.69 to −18.65), but the number in this group was very small. Two genes (*CD3E* and *PRKCSH*) associated with response in the entire depression group were also associated with response in the fluoxetine treated subjects.

Desipramine treatment

Six SNPs located in or near five genes were associated with treatment response during desipramine treatment. SNPS in *ABCB1* (rs1202186), *CRHR2* (rs917195), *PRKCSH* (rs34095), *PSMD9* (rs1043307,

Table 4 Polymorphisms associated with response status in the treatment of depression

Medication	*Gene*	*SNP*	*Chromosome*	*Position*	*SNP type*	*Minor/major allele*	*Minor allele frequency*		*OR (95% CI)*[a]	*P*[a]
							Responder	*Nonresponder*		
Desipramine or Fluoxetine	*UCN3*	rs10904481	10	5405954	Missense	G/A	0.431	0.586	0.53 (0.30, 0.97)	0.04
	CD3E	rs2231449	11	117691515	3′ UTR	A/C	0.015	0.083	0.17 (0.04, 0.72)	0.007
	PSMD9	rs1043307	12	120838179	Missense	G/A	0.388	0.591	0.44 (0.25, 0.77)	0.004
	STAT3	rs3809758	17	37725506	Intron	A/G	0.119	0.031	4.18 (0.96, 18.2)	0.04
	PRKCSH	rs34095	19	11402685	Intron	T/C	0.365	0.625	0.35 (0.18, 0.64)	0.0005
Desipramine	*CRHR2*	rs917195	7	30694977	Flank	T/C	0.333	0.125	3.50 (1.24, 9.91)	0.01
	ABCB1	rs1202186	7	87051194	Intron	G/A	0.107	0.265	0.33 (0.12, 0.93)	0.03
	PSMD9	rs1043307	12	120838179	Missense	G/A	0.359	0.591	0.39 (0.19, 0.81)	0.01
	STAT3	rs3744483	17	37719964	3′ UTR	C/T	0.150	0.000	9.15 (1.44, ∞)	0.009
		rs3809758	17	37725506	Intron	A/G	0.171	0.000	11.28 (1.81, ∞)	0.005
	PRKCSH	rs34095	19	11402685	Intron	T/C	0.341	0.639	0.29 (0.13, 0.66)	0.002
Fluoxetine	*CYP3A4*	rs2242480	7	99199402	Intron	T/C	0.398	0.667	0.33 (0.13, 0.83)	0.02
	PSMD13	rs3817629	11	227312	Intron	T/C	0.170	0.000	6.17 (1.00, ∞)	0.04
	CD3E	rs2231449	11	117691515	3′ UTR	A/C	0.008	0.125	0.06 (0.01, 0.60)	0.002
	PRKCSH	rs160841	19	11420158	Intron	G/A	0.115	0.292	0.31 (0.11, 0.89)	0.02
	PSMA7	rs2057169	20	60145679	Intron	C/T	0.242	0.546	0.27 (0.11, 0.68)	0.004
		rs2057168	20	60145742	Intron	C/T	0.235	0.546	0.26 (0.10, 0.65)	0.003
		rs2281740	20	60145906	Intron	T/C	0.231	0.546	0.25 (0.10, 0.64)	0.002
		rs3746651	20	60151815	3′ UTR	C/T	0.230	0.500	0.30 (0.11, 0.79)	0.01

Abbreviations: SNPs, single nucleotide polymorphisms; UTR, untranslated region.
[a]*P*-values, odds ratios (OR) and 95% confidence intervals (CI) were estimated on exact logistic regression model if any cell with the frequency is 0.

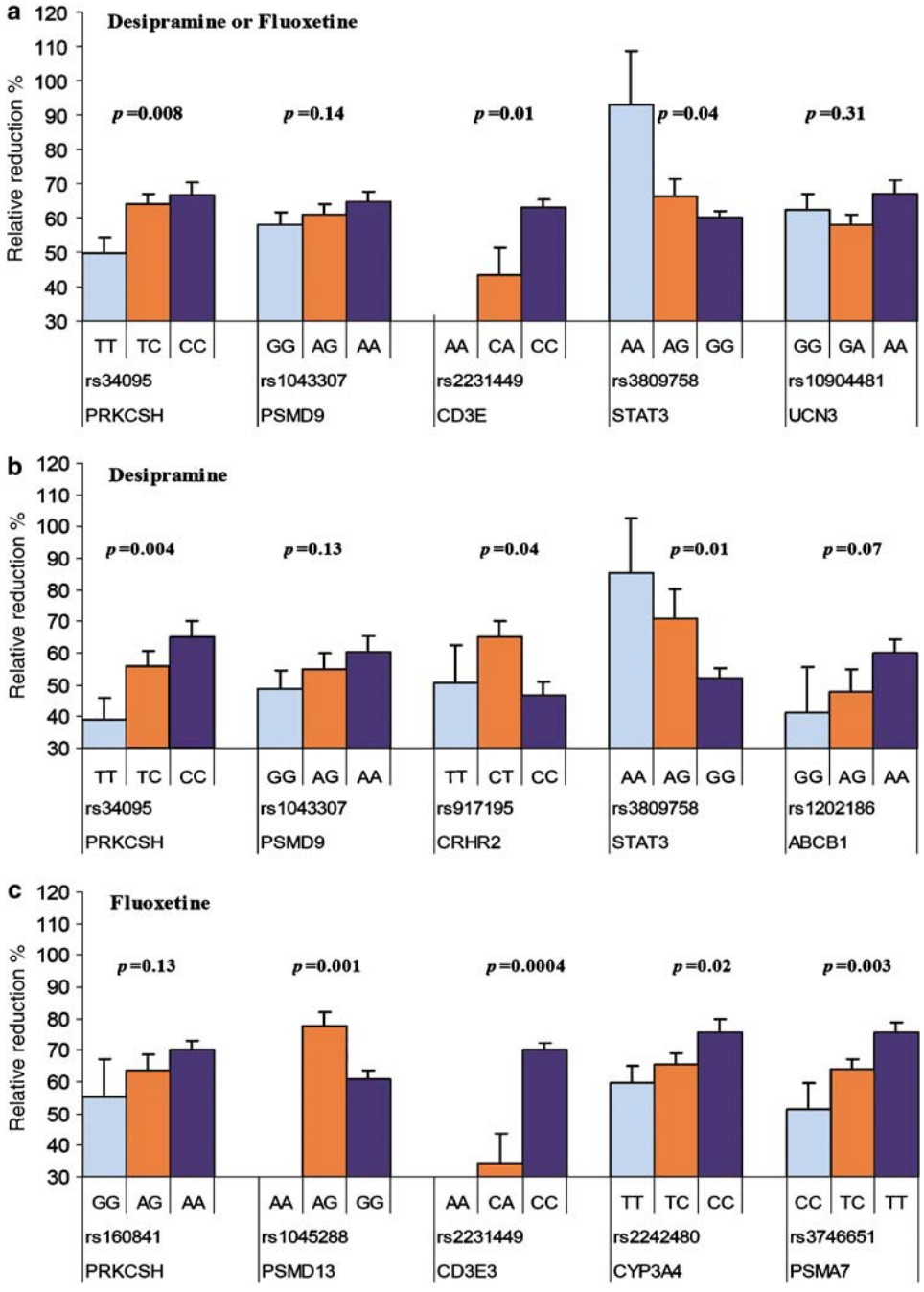

Figure 2 Genotypes and relative reduction of Hamilton Depression Rating Scale (HAM-D) score in the patients treated with desipramine and fluoxetine. Histograms represent mean and standard error of mean for relative reduction of HAM-D21 score in major depressive disorder (MDD) patients who completed 8-week antidepressant treatment with desipramine ($n = 68$) or fluoxetine ($n = 79$) by genotypes (light blue, homozygous for minor allele; orange, heterozygote; dark blue, homozygous for major allele). A general linear model was used to detect allelic additive effects on treatment response after adjustment for age, sex and baseline HAM-D21 score. The analyses were performed using all treated patients (**a**), desipramine-treated patients (**b**) and fluoxetine-treated patients (**c**).

missense) and *STAT3* (rs3744483, 3′ UTR, and rs3809758) had a difference in allele frequency with $P \leqslant 0.05$ for responders and nonresponders within the subjects treated with desipramine (Table 4; Figure 2b). Three of these SNPs (rs1043307, rs3809758, rs34095) were associated with response in the entire depression group were also associated with response to desipramine treatment.

Immunoassays

To further understand aspects of immune dysfunction relevant to MDD and/or treatment response, we

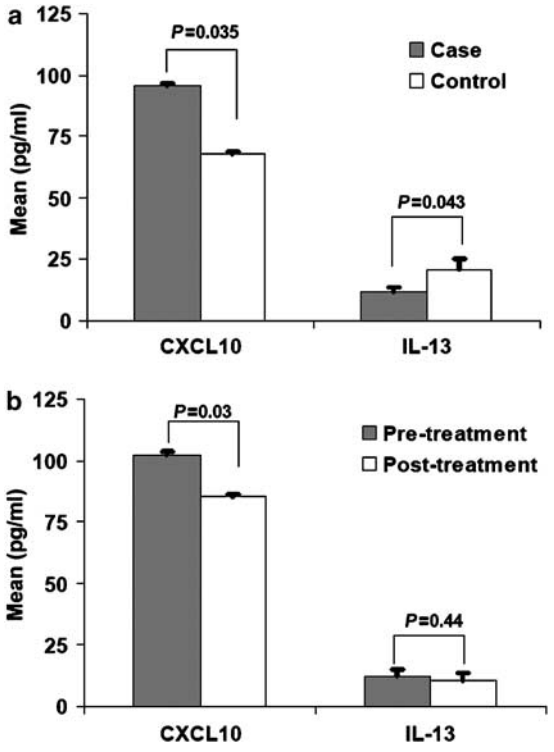

Figure 3 CXCL10 (IP10) and interleukin-13 (IL-13) levels in controls, major depressive disorder (MDD) patients and drug responders. (**a**) Histograms represent mean and standard error for CXCL10 and IL-13 levels in MDD patients before initiation of antidepressant treatment ($n=65$) and controls ($n=14$). Comparison levels between cases and controls were performed using general linear model after adjustment for age, sex and body mass index (BMI). Log arithmetic transformation was used for CXCL10 in our data analyses. (**b**) Histograms represent mean and standard error for CXCL10 and IL-13 levels before and after 8 weeks of antidepressant treatment in 19 antidepressant treatment responders (MDD patients who had higher than 50% reduction in Hamilton Depression Rating Scale (HAM-D21 score). Paired *t*-test was used to compare pre- and post-treatment levels in drug responders. Log arithmetic transformation was used for CXCL10 in our data analyses.

examined patterns of cytokines in plasma and found increased circulating plasma levels of the IFNγ-inducible chemokine CXCL10/IP10[36] in our MDD patients before initiation of antidepressant treatment when compared to controls ($P=0.035$; 95.50 ± 1.06 and $67.61\pm1.16\,\mathrm{pg\,ml^{-1}}$, respectively in MDD and control groups, mean ± s.d. calculated by using logarithmic transformation; Figure 3a) and significant decrements were found for IL-13 levels in MDD patients when compared to controls ($P=0.043$; 11.87 ± 11.77 and $20.82\pm14.77\,\mathrm{pg\,ml^{-1}}$, respectively in MDD and control groups). Patients who responded to antidepressant treatment had a significant decrement in levels of CXCL10 ($P=0.03$; $1.20\pm1.38\,\mathrm{pg\,ml^{-1}}$, paired mean difference ± s.d. calculated by using logarithmic transformation; Figure 3b).

Discussion

We found that two genes that are critical for T-cell function, *PSMB4* and *TBX21*, are associated with major depression. We found that together, 47.8% of the population risk could be attributable to the risk genotypes at rs2296840 in *PSMB4* or rs17244587 in *TBX21*. The joint effect of the combined genotypes of rs2296840 (recessive model) and rs17244587 (dominant model) was 26% greater than that predicted by assuming additivity of effects. Our analyses revealed a significant combined allele dose–effect; therefore, individuals who had one, two and three risk alleles in *PSMB4* and *TBX21* were 2.3, 3.2 and 9.8 times more likely to have the diagnosis of MDD, respectively. We also found associations of several SNPs in genes relevant to HPA axis and immune function and antidepressant response and describe in MDD increased levels of CXCL10/IP-10, which decreased in response to antidepressants. These lines of evidence are indicative of a predominance of Th1 activity in MDD.

Genetic variations in *PSMB4* and *TBX21* may also be relevant to two immune disorders, psoriasis[37] and asthma,[38] that are known to be comorbid with MDD. These two disorders are polygenic and reactive to psychosocial stressors. Susceptibility to psoriasis has been associated to the area of chromosome 1q21 (PSORS4) that encodes *PSMB4*,[30] and susceptibility to asthma and nasal polyps (O(MIM) MIM 208550)[14] has been associated with functional promoter SNPs in *TBX21* (−1993T/C), in chromosome 17q21.3.

The UTR variations in *TBX21* and *PSMB4* that we found to be significantly associated with MDD are in UTRs but they may nevertheless impact on immune response in our patients. Several roles in gene expression have been attributed to UTRs, including mRNA stability, localization and translational efficiency. The 5′ UTR, also known as the leader sequence, is a particular section of the mRNA that usually contains a ribosome-binding site; it is a major site of translational regulation and may affect the stability or translation of mRNA and gene expression. Evidence implicating the 3′ UTR of mRNA in the regulation of gene expression has accumulated recently. The 3′ UTR may influence transcript cleavage, polyadenylation and nuclear export, which determine transcript stability, level of translation and mRNA targeting.[39] It is therefore plausible that SNPs associated with treatment response may have contributed to the increased plasma levels of the IFNγ-inducible chemokine CXCL10 found in our patients.

CXCL10 is a potent angiostatic factor with antifibrotic properties[40] and its elevation is congruent with elevated leukocyte counts in peripheral blood that have been shown to be dependent on severity and treatment outcome in MDD.[41] Inflammatory immune mediators and specifically CXCL10 have also been implicated in arteriosclerosis, and they may be a link between the presence of depressive symptoms and stress, and increased risk of, morbidity and mortality

in myocardial infarction.[42] The increase of an IFNγ-inducible chemokine supports the presumption of a predominance of Th1 type activity during the symptomatic phase of MDD, as well as its role in the pathophysiology, therapeutic outcome of this disorder and immunoregulatory effects of antidepressants.[43]

We found that genetic variations affecting T-cell function and HPA axis regulation were associated with antidepressant treatment response. The following T-cell functions may be implicated in treatment response: T-cell development (*CD3E*, T-cell antigen receptor-ε subunit of T3),[44] antigen processing/degradation (*PSMD9*: proteasome 26S subunit, non-ATPase,[9,45] and intracellular signaling (*STAT3*: signal transducer and activator of transcription 3).[46] The association of a variation in the urocortin III or stresscopin gene (*UCN3*)[47] suggests a possible role for the adaptive stress response that mediates endocrine, autonomic, cardiovascular and immune systems in treatment outcome. The association of a SNP in the *CRHR2* in the treatment response to desipramine indicates that HPA axis modulation may be particularly important for tricyclic antidepressants. Notably, some of the SNPs associated with treatment response could lead to differences in immune response such as nonsynonymous variations in the *PSMD9* and *UCN3* genes, and 3′ UTR SNPs in *CD3E, STAT3* and *PSMA7* genes. Somatic variations in some of those genes have been implicated in immunodeficiencies (*CD3E*),[48,49] polycystic liver disease (*PRKCSH*, protein kinase C substrate, 80 kD, heavy chain,[50–52] type 2 diabetes[53]or autosomal dominant hyper-immunoglobulin E (IgE) syndrome, also called 'Job Syndrome'.[54–56]

We found no clear Th1 or Th2 cytokine patterns in our patients. Our results of decreased IL-13 levels in MDD contrast with a recent report of increased levels of Th2 cytokines IL-13 and IL-4 and decreased levels of Th1 cytokines.[16] Several factors could account for this discrepancy, from differences in gender and age composition to differences in environment/pathogens or differences in the phases of neuroendocrine, counterregulatory systems or severity and stage of the disorder. Moreover, cytokine profiling in Th1 and Th2 cytokine expression seem to be relative, not absolute as inconsistencies between cytokine profiles, antibody and total serum IgE have been reported.[57] Therefore, chemokines (such as CXCL10), which are low molecular weight chemotactic molecules, are emerging as a major communication system in the brain[58] as their serum and CSF levels may be correlated.[59] Chemokines are key mediators of inflammation that have major effects on migration of cells to inflammation sites as well as activation of recruited and resident central nervous system (CNS) cells, which have been implicated in a number of human pathophysiological systemic and CNS conditions[60] and their level or expression has been linked to the activity of CNS disease.

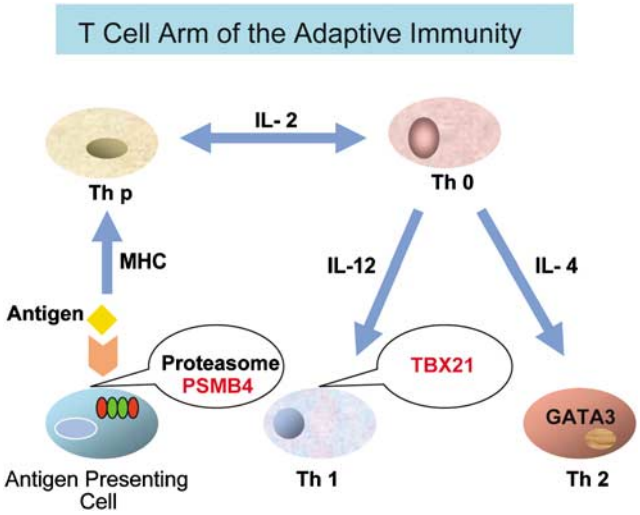

Figure 4 Schematic of sites where variations in *TBX21* or *PSMB4* could influence the T-cell arm of the adaptive immunity and contribute to susceptibility to major depressive disorder (MDD): Two crucial functions, specifically antigen processing and T cell-programmed differentiation are involved in Mexican Americans with MDD and are highlighted in red. A naive helper T-cell precursor (Th p) can become either a Th1 or Th2 cell under the instructive influence of interleukin-12 (IL-12) or IL-4, respectively; Th1 cell expresses *TBX21* and Th2 expresses *GATA3*.

Figure 4 summarizes our results of genetic variations associated with the diagnosis of MDD. These implicate that specific UTR variations in *TBX21* or *PSMB4* increase the risks for and characterize a T-cell dysfunction in MDD in Mexican Americans. These genetic variations may be involved in the immune system dysregulation described in this disorder and in known comorbidity disorders such as psoriases[38] and asthma.[37] Our patients had increased peripheral levels of the chemokine CXCL10, which decreased with response to antidepressant treatment.

These results lead to the presumption that an imbalance of Th1/Th2 activity toward a predominance of Th1 response is present in the symptomatic phase of mild to moderate forms of MDD. Replication of our findings in other ethnic groups is needed to validate the role of *TBX21* and *PSMB4* in major depression here reported in Mexican Americans. Because genes involved in immune function are highly polymorphic in human populations,[61] allele frequency may vary considerably in different ethnic populations, and variations of T-cell function may result from common variations in other genes/gene regions, which may cause a predominance of net Th1 activity. The allele frequency for rs17244587 (*TBX21*) in our subjects was similar to European populations; however, rs2296840 and rs4603 (*PSMB4*) were significantly less frequent (respectively 0 and 10%) in Europeans than in the Mexican Americans we studied (24% in Mexican-American controls and 34% in MDD). It is therefore unlikely that the *PSMB4* variations described here are significant in the

susceptibility to MDD in individuals of predominant European descendant. Consequently, characterization of neuroimmune profiles may vary depending on specific genes and SNPs involved in T-cell function variations in different populations. Moreover, given our *n* and limited numbers of patients in the desipramine and fluoxetine treatment groups, these results need to be taken with caution, pending replication by other independent studies.

Because chemokine networks already represent potentials targets for new therapies in several CNS and systemic conditions,[58] further studies are needed to fully clarify the extent of CNS immunedysregulation in the pathophysiology of MDD.

In spite of the limitations of this study, our data support the hypothesis that key T-cell functions leading to Th1 net activity are features of immune dysfunction in MDD and may also have a role in antidepressant treatment response. Different genes and polymorphisms might characterize MDD immune dysfunctions in distinct populations, as genes that influence immune functions are highly polymorphic and their allele frequency varies across human populations. We suggest that interferon-γ-inducible chemokines, such as CXCL-10, may provide viable biomarkers and might also be useful in predicting/following antidepressant response. Our findings provide a basis for conceptually innovative pharmacological approaches to MDD with a focus on T-cell function dysregulation and variations in T-cell programmed differentiation, antigen processing and cellular proteasome organelle function.

Acknowledgments

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Chapter V. Reprints

Licinio J, Dong C, **Wong ML**. Novel sequence variations in the Brain-Derived Neurotrophic Factor gene and association with major depression and antidepressant treatment response. ***Archives of General Psychiatry*** 2009;66:488-497.

Dong C, **Wong ML**, Licinio J. Sequence variations of ABCB1, SLC6A2, SLC6A3, SLC6A4, CREB1, CRHR1, and NTRK2: association with major depression and antidepressant response in Mexican-Americans. ***Molecular Psychiatry*** 2009;14:1105-1118.

ORIGINAL ARTICLE

Novel Sequence Variations in the Brain-Derived Neurotrophic Factor Gene and Association With Major Depression and Antidepressant Treatment Response

Julio Licinio, MD; Chuanhui Dong, PhD; Ma-Li Wong, MD

Context: Variations in the brain-derived neurotrophic factor gene (*BDNF*) have been associated with psychiatric disorders. Deep sequencing of the *BDNF* gene may identify new variations and bring further insight into psychiatric genetics.

Objective: To better characterize sequence variability in the *BDNF* gene by resequencing a genomic DNA region of 22 kilobases that contained all *BDNF* exons and their flanking regions.

Design: Case-control study.

Setting: University of California, Los Angeles, and University of Miami.

Participants: Two hundred sixty-four controls and 272 Mexican Americans with major depressive disorder (MDD) from Los Angeles who were assessed by the same bilingual clinical research team.

Main Outcome Measures: Identification of novel genetic polymorphisms in the *BDNF* gene and assessment of their frequencies and associations with MDD or antidepressant response.

Results: We identified 83 novel single-nucleotide polymorphisms (SNPs): 30 in untranslated regions, 4 in coding sequences, 37 in introns, and 12 in upstream regions; 3 of 4 rare novel coding SNPs were nonsynonymous. Association analyses of patients with MDD and controls showed that 6 SNPs were associated with MDD (rs12273539, rs11030103, rs6265, rs28722151, rs41282918, and rs11030101) and 2 haplotypes in different blocks (one including Val66, another near exon VIIIh) were significantly associated with MDD. One recently reported 5′ untranslated region SNP, rs61888800, was associated with antidepressant response after adjusting for age, sex, medication, and baseline score on the 21-item Hamilton Depression Rating Scale.

Conclusions: Our data support the concept that extensive resequencing of key candidate genes can lead to the discovery of substantial numbers of new variants. Further studies using larger independent samples are needed to confirm the association of the rs61888800 SNP with antidepressant response.

Trial Registration: clinicaltrials.gov Identifier: NCT00265291

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THE NEUROTROPHINS ARE SECRETED peptides that are critically involved in differentiation and survival of neuronal populations.[1-3] Brain-derived neurotrophic factor (BDNF)[4-7] is a neurotrophin that is abundantly and widely expressed in the central nervous system (CNS).[8,9] During the past decade, BDNF has emerged as a key factor implicated in complex behavioral patterns in the developing CNS and in disease. The BDNF modulates signaling pathways that rapidly affect local synaptic function but also has long-term effects on gene transcription. It promotes neuronal survival in the peripheral and CNS via the transcription factor cyclic adenosine monophosphate–response element, which influences the expression of *BCL2*, a prosurvival gene. It also has important roles in excitatory synaptic transmission and plasticity,[10-13] memory processing and storage,[13-18] and kindling and temporal lobe epilepsy.[19-22] This relevance to crucial CNS functions has raised interest in its role in neurodegenerative and psychiatric disorders.

Allelic variations of the *BDNF* gene have been implicated in several conditions. Specifically, the allelic variation Thr2Ile (substitution of isoleucine for threonine at amino acid position 2 in the coding sequence) has been implicated in congenital central hypoventilation syndrome.[23] Variations in *BDNF* have been exten-

sively studied and implicated in the susceptibility to memory and hippocampal function impairments[24] and several psychiatric disorders,[25] such as obsessive-compulsive disorder,[26] eating disorders,[27,28] bipolar disorder,[29-34] schizophrenia,[35] major depression,[36,37] and Alzheimer disease.[38-40] Despite conflicting findings in replication studies for several of these associations, it is interesting that the less frequent variation, Met66, which is associated with poorer episodic memory and abnormal hippocampal activation on functional magnetic resonance imaging, generally confers a protective effect for neuropsychiatric conditions.

The genetic factors that contribute to human disease show enormous variation in the allelic spectra in number and population frequency of disease-predisposing alleles. Common and complex disorders are multifactorial and probably composed of both common genetic variants (common disease/common allele model) with small effect and rare sequence variants (rare variant/common disease model) with larger effect.[41] Although the common allele is the prevalent view of these 2 competing models regarding the genetic basis of common and complex diseases, it has been predicted that resequencing studies may identify many rarer variants (>5%) of intermediate effect associated with common disorders; such efforts may also identify structural variations in genomic DNA, such as duplication and deletions of DNA sequences.[42,43]

Given the functional importance of BDNF in the CNS, the discovery of new *BDNF* allelic variants may be relevant to understanding the role of this gene in neurologic and psychiatric disorders. A number of studies have been conducted to examine the association of *BDNF* variants, but most of them have been focused on genotyping tag single-nucleotide polymorphisms (SNPs) or the functional coding SNP rs6265. To our knowledge, no study has comprehensively surveyed the entire *BDNF* exonic sequence variation through direct sequencing and correlated the identified genetic variants with disease susceptibility. To discover new *BDNF* genetic variants and detect rare variants, we sequenced a total 22-kilobase (kb) genomic DNA including all *BDNF* exons and their flanking regions in 536 DNA samples from 264 control subjects and 272 Mexican American individuals with major depressive disorder (MDD). We further investigated all of the identified genetic variants for association with risk for major depression and antidepressant treatment response.

METHODS

PARTICIPANTS

Participants were 264 controls and 272 patients with MDD, aged 19 to 68 years. This study was approved by the institutional review boards of the University of California, Los Angeles, and the University of Miami. Subjects gave written informed consent. All participants were Mexican Americans and had at least 3 grandparents born in Mexico. The definition of MDD was a *DSM-IV* diagnosis of current, unipolar major depressive episode and a score of 18 or greater on the 21-Item Hamilton Depression Rating Scale (HAM-D21) with item 1 (depressed mood) rated 2 or greater. All patients with MDD were enrolled in a pharmacogenetic study of antidepressant treatment response as previously described and registered at http://clinical trials.gov (No. NCT00265291).[44,45] The demographic characteristics and the numbers of subjects in each subgroup are presented in eTable 1 (http://www.archgenpsychiatry.com) and a flowchart (eFigure 1). Briefly, all patients with MDD had a comprehensive psychiatric and medical assessment in their primary language based on diagnostic and ratings instruments that had been fully validated in English and in Spanish. Exclusion criteria included active medical illnesses that could be etiologically related to the ongoing depressive episode, current or active suicidal ideation with a plan and strong intent, pregnancy, lactation, current use of medications with significant CNS activity that interfere with activity on an electroencephalogram (eg, benzodiazepines) or any other antidepressant treatment within the 2 weeks before enrollment, illicit drug use and/or alcohol abuse in the preceding 3 months, or current enrollment in psychotherapy. Control individuals for our genomic studies were in general good health but were not screened for medical or psychiatric illness; they were age- and sex-matched and recruited from the same Mexican American community in Los Angeles by the same bilingual clinical research team.

ANTIDEPRESSANT TREATMENT

All patients had an initial comprehensive psychiatric and medical assessment and, if enrolled, had weekly structured follow-up assessments for 9 weeks. The study consisted of 2 phases: a 1-week single-blind placebo lead-in phase to minimize the impact of placebo responders, followed (if subjects continued to meet the inclusion criteria after phase 1) by random assignment to 1 of the 2 treatment groups: fluoxetine hydrochloride, 10 to 40 mg/d, or desipramine hydrochloride, 50 to 200 mg/d, administered in a double-blind manner for 8 weeks. Our primary clinical outcome measure was HAM-D21 score, and clinical remission with antidepressants was defined as having a final (week 8) HAM-D21 score less than 8.[44] In addition, the relative response change was also computed as the difference in HAM-D21 score between pretreatment and posttreatment divided by the pretreatment HAM-D21 score.

GENOMIC DNA COLLECTION AND SEQUENCING

At the initial visit, blood samples were collected under informed consent from the participating individuals into EDTA (K2EDTA BD Vacutainer EDTA tubes; Becton Dickinson, Franklin Lakes, New Jersey), and genomic DNA was isolated by using DNA purification kits (Puregene; Gentra Systems, Indianapolis, Indiana). *BDNF* (OMIM 113505) DNA sequencing was completed to identify genetic polymorphisms in exonic or flanking exons by the Wellcome Trust Sanger Institute following their ExoSeq protocol (http://www.sanger.ac.uk/humgen/exoseq/). A 22-kb genomic DNA region, containing the entire *BDNF* exons and their flanking regions, was sequenced. Briefly, DNA sequences were extracted from the Vega database (http://vega.sanger.ac.uk/index.html). Primers were designed automatically by means of Primer3 (http://frodo.wi.mit.edu/) to amplify DNA, and primer pairs were checked for uniqueness before ordering and prescreened to determine the optimum conditions for amplification. After amplification, a sample of the products was visualized on an agarose gel to confirm the size of the polymerase chain reaction product. The remaining polymerase chain reaction product was then cleaned up by means of 2 enzymes, exonuclease 1 and shrimp alkaline phosphatase. Bidirectional sequencing of amplicons was carried out with a cycle sequencing kit (Big Dye Terminator, version 3.1; Applied Biosystems, Foster City, California). The SNPs were called by means of ExoTrace, a Web site algorithm (http://www.sanger.ac.uk

/humgen/exoseq/analysis.shtml) developed for the detection of heterozygotes in sequence traces, which processes the sense and antisense sequence readings separately and subsequently and combines the results to allow SNP scoring.

NUCLEOTIDE DIVERSITY AND POPULATION DIFFERENTIATION ESTIMATION

Nucleotide diversity (θ) and its standard deviation ($S[\theta]$) were calculated by SNP class under the assumption of an infinite neutral allele model as follows[46,47]:

$$\theta = K/aL,$$

$$S(\theta) = \frac{\sqrt{a\theta L + b(\theta L)^2}}{aL},$$

$$a = \sum_{i=2}^{n} \frac{1}{(i-1)},$$

$$b = \sum_{i=2}^{n} \frac{1}{(i-1)^2},$$

where K represents the number of observed SNPs among L base pairs of genomic sequence in a sample of n alleles. All calculations were based on n = 990 for all the sites given in which the average sample size was 495 individuals across all the polymorphisms. The pairwise population differentiation (F_{ST}) values were estimated for the database SNPs (dbSNPs) that were both detected in our Mexican American sample and reported in the HapMap sample and were calculated as described by Weir and coworkers.[48-50]

HARDY-WEINBERG EQUILIBRIUM TEST AND POPULATION STRATIFICATION ANALYSIS

Case-control study design is an efficient method for examining associations between candidate alleles and disease. However, to compare allele frequencies and to be able to treat chromosomes as independent observations, the genotype frequencies must be in Hardy-Weinberg equilibrium.[51] Deviation from Hardy-Weinberg equilibrium was tested separately for healthy controls and patients by using the PLINK program version 1.00 (http://pngu.mgh.harvard.edu/~purcell/plink/).[52] The SNPs that were not in Hardy-Weinberg equilibrium in the healthy control group were excluded from the allele-based association analyses of cases and controls.

Another confounding factor that may affect the internal validity of case-control studies is the presence of population stratification. We used 2 approaches to test for hidden stratification in our data. First, 54 unlinked SNPs across 22 autosomal chromosomes were used to analyze a combined sample with genotype data downloaded from 3 HapMap (http://www.hapmap.org) ethnic samples using the STRUCTURE program (http://pritch.bsd.uchicago.edu/software.html). Three distinct clusters were identified with an average proportion of at least 92% of individuals correctly assigned to the given ethnic populations (CEU [Utah residents with ancestry from northern and western Europe in the United States], CHB [Han Chinese in Beijing] + JPT [Japanese in Tokyo], and YRI [Yoruba in Ibadan, Nigeria]) (eFigure 2A). We then used this panel of SNPs to test our sample and observed an almost equal proportion assigned to each cluster given $K = 2, 3, 4$ in both cases and controls (eFigure 2B). We also ran the analysis by combining our sample with HapMap genotypes given $K = 4$ and observed very similar proportions between cases and controls of 0.467 vs 0.466, 0.056 vs 0.035, 0.018 vs 0.021, and 0.460 vs 0.479, respectively, for clusters 1, 2, 3, and 4. Second, genotype frequencies from each of the 54 unlinked SNPs were compared between cases and controls by the method described by Pritchard and Rosenberg.[53] No significant difference was found on the basis of an overall test statistic ($\chi^2_{108} = 100.50$; $P = .68$), suggesting a good match between cases and controls.

GENETIC ASSOCIATION ANALYSES OF CASES AND CONTROLS

For SNP-based association analysis, the Fisher exact test (2-tailed) was performed to compare allele frequencies and genotype distributions between depressed and healthy individuals by using the PLINK program. In the allelic association analysis, each polymorphism was tested in controls to ensure the fitting with Hardy-Weinberg equilibrium; the odds ratio (OR) on the 2×2 contingency table of allele counts and its 95% confidence interval (CI) were also estimated for the polymorphism associated with the diagnosis of depression. In the genotypic association analysis, the SNP effects were tested under a codominant model on the 2×3 contingency table of genotype counts. In addition, logistic regression analyses were performed to test whether the observed SNP-depression association remained valid after controlling for age and sex by means of the SAS package (SAS Institute Inc, Cary, North Carolina).

For haplotype-based association analysis, haplotype blocks were identified by searching for a "spine" of strong linkage disequilibrium (LD) running from one marker to another along the legs of the triangle in the LD chart, and haplotype population frequencies were estimated by using an expectation maximization algorithm performed in the computer program Haploview (Version 4; Broad Institute, http://www.broad.mit.edu/mpg/haploview/).[54] Haplotype frequencies were compared between depressed and control individuals to test whether a certain haplotype was associated with a diagnosis of depression.

To correct for multiple testing, 20 000 permutations were performed to estimate the adjusted P values for both single SNP-based analyses and haplotype-based analyses by using Haploview.

GENETIC ASSOCIATION ANALYSES OF RESPONSE TO ANTIDEPRESSANTS

Data analyses were performed using both intention-to-treat (ITT) and completed-treatment samples. The ITT sample consisted of patients who were randomized to 1 arm and received at least 1 dose of antidepressant medication, and the completed-treatment sample consisted of patients who completed 8 weeks of antidepressant treatment. The last observation carried forward approach was used to input missing outcome in the ITT analysis. For discrete outcome (remission vs nonremission), we investigated the allelic and genotypic association with the response to antidepressant treatment by using approaches similar to those in the analyses of cases and controls. For the quantitative outcome (relative reduction percentage in HAM-D21 scores between pretreatment and posttreatment), we conducted the analyses on the basis of 3 genetic models (additive, dominant, and recessive) and first performed the analyses by using the combined samples of patients treated with desipramine or fluoxetine.

Table 1. Detected *BDNF* SNPs in Mexican Americans

Location[a]	Sequence Screened, bp	No. of Novel SNPs	No. of dbSNPs	Total No. of SNPs	Nucleotide Diversity, Mean (SD)	Transition, %
Coding	792	4	2	**6**	0.0010 (0.0005)	100.0
5′ UTR	2307	16	2	**18**	0.0010 (0.0003)	72.2
Intron	10 536	37	25	**62**	0.0008 (0.0002)	71.0
3′ UTR	2928	14	8	**22**	0.0011 (0.0003)	69.6
Upstream	4989	12	10	**22**	0.0006 (0.0002)	68.2
Total	**21 552**	**83**	**47**	**130**	0.0008 (0.0002)	71.8

Abbreviations: *BDNF*, brain-derived neurotrophic factor gene; dbSNP, database single-nucleotide polymorphism (SNP); UTR, untranslated region.
[a] Intron-exon boundaries were based on multiple alternative 5′ exons in the National Center for Biotechnology Information AceView Database.

We then performed the analyses separately by antidepressant medication (desipramine only, fluoxetine only). We used a multiple linear regression model to examine the association between genotype and relative HAM-D21 score reduction by controlling for age, sex, and baseline (pretreatment) HAM-D21 score using the PLINK program.

POWER CALCULATION

Power to test the allelic association with depression was estimated with a range of effect size (OR) between 1.35 and 2.25 and minor allele frequency between 0.1 and 0.25 using the PAWE program.[55] Power analyses showed that, at a 2-sided significance level of .05, sample sizes of 265 cases and 265 controls can achieve 80% power to detect an allelic OR of 1.68, 1.57, 1.50, and 1.46 with a minor allele frequency of 0.10, 0.15, 0.20, and 0.25, respectively. Power calculations for the association of *BDNF* variants with antidepressant treatment continuous outcome were given for a range of allele frequencies and Cohen effect sizes (mean difference in unit of standard deviation) based on the dominant genetic model and using the Quanto (Version 1.2.3) program.[56,57] Sample size is assumed to be 200 for the combined sample and 100 for each antidepressant treatment group based on an ITT design. Power analyses showed that, at a 2-sided significance level of .05 and when the allele frequency is 0.15 or more, the power is greater than or equal to 89% to uncover a moderate effect size of 0.5 for a sample of 200 patients and greater than or equal to 78% to detect a medium effect size of 0.6 for a sample of 100 patients.

RESULTS

DETECTION OF SEQUENCE VARIATION

Approximately 22 kb of *BDNF* exonic sequence and its flanking regions was systematically screened for novel nucleotide sequence variations in this sample of 536 Mexican American individuals. A total of 130 nucleotide sequence variations were identified (**Table 1**). They included 83 novel SNPs and 47 dbSNPs: 40 in untranslated regions (UTRs), 6 in coding sequences, 62 in intronic sequences, and 22 in the flanking regions. Among 6 coding SNPs, 3 novel nonsynonymous SNPs (NT_009237.17_26467094 [Ala/Thr], NT_009237.17_26467235 [His/Gly], and NT_009237.17_26467246 [Gly/Asp]), and 1 synonymous SNP (NT_009237.17_26466714) were found, and their minor allele frequencies were 0.0019, 0.0019, 0.001, and 0.001, respectively, in the combined sample of cases and controls. Seventy-nine other novel polymorphisms included 30 UTR SNPs, 37 intronic SNPs, and 12 upstream SNPs (eTable 2). The minor allele frequencies for the novel polymorphisms ranged from 0.0009 to 0.2445 with an allele distribution as follows: less than or equal to 0.001, 37.6%; greater than 0.001 and less than or equal to 0.01, 50.5%; and greater than 0.01, 11.9% in the combined sample of cases and controls.

NUCLEOTIDE DIVERSITY

The nucleotide diversity was estimated in each class of sites (coding, 3′ UTR, 5′ UTR, and intronic) by correcting for both sample size and the length of the screened site (Table 1). The mean (SD) nucleotide diversities were comparable for coding (0.0010 [0.0005]), 3′UTR (0.0011 [0.0003]), and 5′ UTR (0.0010 [0.0003]) regions, but the estimate showed some lower nucleotide diversity in the intronic region (0.0008 [0.0002]) and upstream region (0.0006 [0.0002]). For the type of substitution, all of the identified coding polymorphisms were transition, whereas the transition rates were 71.0%, 69.6%, 72.2%, and 68.2% for intronic, 3′ UTR, 5′ UTR, and upstream regions, respectively.

POPULATION DIFFERENTIATION

Among the 47 dbSNPs detected, 18 were reported in 3 HapMap ethnic groups: white (CEU), black (YRI), and Asian (CHB+JPT) in the National Center for Biotechnology Information database as of June 25, 2008. Pairwise F_{ST} values between Mexican Americans and each HapMap ethnic sample were computed for the shared 18 SNPs and are shown in **Table 2**. Overall, the greatest similarity in allele frequencies was found between Mexican Americans and whites, with a lower mean F_{ST} in Mexican Americans vs whites of 0.03, compared with 0.10 in Mexican Americans vs blacks and 0.09 in Mexican Americans vs Asians. For the single-locus estimates of F_{ST} values, large F_{ST} values (>0.1) were observed at 4 SNPs (rs7124442, rs11819808, rs4923468, and rs7931755) in Mexican Americans vs blacks (22.2%) and at 5 SNPs (rs6265, rs11030102, rs11030104, rs988748, and rs10767664) in Mexican Americans vs Asians (27.8%), but less often (5.5%) in Mexican Americans vs whites (1 SNP: rs12273539).

Table 2. Allele Frequencies and F_{ST} Values for *BDNF* dbSNPs Shared by Mexican Americans and HapMap Samples

		Minor Allele Frequency						F_{ST}		
		Mexican American			HapMap Sample					
SNP	Major/Minor Allele	Cases	Controls	All	CEU	YRI	HCB + JPT	MA vs CEU	MA vs YRI	MA vs HCB + JPT
rs7124442	T/C	0.23	0.26	0.25	0.37	0.54	0.07	0.03	0.18	0.09
rs6265	G/A	0.10	0.15	0.12	0.18	0.00	0.48	0.01	0.08	0.31
rs11030101	A/T	0.26	0.33	0.29	0.40	0.12	0.31	0.02	0.07	0.00
rs11819808	C/T	0.01	0.00	0.01	0.00	0.28	0.00	0.00	0.45	0.00
rs11030102	C/G	0.17	0.18	0.18	0.29	0.07	0.01	0.04	0.04	0.11
rs12273539	C/T	0.35	0.23	0.29	0.00	0.34	0.13	0.20	0.00	0.06
rs11030104	A/G	0.12	0.15	0.14	0.20	0.00	0.49	0.01	0.08	0.29
rs11030109	G/A	0.02	0.03	0.03	0.04	0.00	0.00	0.00	0.01	0.01
rs988748	C/G	0.15	0.16	0.15	0.22	0.05	0.49	0.01	0.04	0.25
rs4923468	C/A	0.01	0.02	0.02	0.02	0.19	0.02	0.00	0.26	0.00
rs10767664	A/T	0.14	0.16	0.15	0.20	0.04	0.49	0.00	0.04	0.26
rs7931755	A/G	0.01	0.01	0.01	0.00	0.25	0.00	0.00	0.41	0.00
rs2030324	G/A	0.37	0.41	0.39	0.57	0.55	0.56	0.06	0.05	0.05
rs12273363	T/C	0.15	0.16	0.16	0.19	0.07	0.01	0.00	0.03	0.09
rs908867	C/T	0.04	0.05	0.04	0.12	0.10	0.04	0.04	0.03	0.00
rs7931247	C/T	0.37	0.42	0.39	0.57	0.55	0.56	0.06	0.05	0.05
rs12288512	G/A	0.14	0.15	0.15	0.19	0.07	0.01	0.00	0.02	0.09
rs11030123	G/A	0.04	0.05	0.04	0.12	0.10	0.04	0.04	0.02	0.00

Abbreviations: *BDNF*, brain-derived neurotrophic factor gene; CEU, Utah residents with ancestry from northern and western Europe in the United States; CHB, Han Chinese in Beijing; dbSNP, database single-nucleotide polymorphism (SNP); F_{ST}, population differentiation; JPT, Japanese in Tokyo; MA, Mexican American; YRI, Yoruba in Ibadan, Nigeria.

Table 3. *BDNF* Polymorphisms Associated With Depression

SNP	Chr Position	SNP Type	Risk/Nonrisk Allele	Control Risk Allele Frequency	OR (95% CI)	*P* Value[a]
rs41282918	27635356	3′ UTR	A/C	0.84	2.13 (1.18-3.86)	.01 (.02)
rs6265	27636492	Nonsynonymous	G/A	0.85	1.66 (1.14-2.41)	.009 (.008)
rs11030101	27637320	Intronic	A/T	0.67	1.37 (1.05-1.78)	.02 (.04)
rs28722151	27637752	Intronic	C/G	0.68	1.48 (1.10-1.99)	.01 (.009)
rs11030103	27638909	Intronic	G/A	0.19	1.80 (1.18-2.74)	.008 (.03)
rs12273539	27639887	Intronic	T/C	0.23	1.75 (1.32-2.31)	<.001 (<.001)

Abbreviations: *BDNF*, brain-derived neurotrophic factor gene; Chr, chromosome; CI, confidence interval; OR, odds ratio; SNP, single-nucleotide polymorphism; UTR, untranslated region.

[a] Results are based on the Fisher exact test for comparisons of allele and genotype (in parentheses) frequencies between depressed patients and controls.

SINGLE SNP-BASED ASSOCIATION ANALYSES OF CASES AND CONTROLS

Analyses of SNP-based allelic associations showed that 6 polymorphisms were associated with MDD (rs12273539, $P<.001$; rs11030103, $P=.008$; rs6265, $P=.009$; rs28722151, $P=.01$; rs41282918, $P=.01$; and rs11030101, $P=.02$) (**Table 3**). All of these 6 SNPs had a minor allele frequency of 0.14 or greater, and their genotypes were in Hardy-Weinberg equilibrium in controls. Genotyped-based analyses also showed that the 6 polymorphisms were associated with depression status with $P\leq.04$ (Table 3). Among the 6 associated SNPs, 4 were intronic variants with ORs ranging from 1.37 to 1.80; 1 SNP was a 3′ UTR variant (rs41282918) with an effect of OR=2.13 (95% CI, 1.18-3.86); and 1 SNP was a nonsynonymous variant (rs6265) with an effect of OR=1.66 (95% CI, 1.14-2.41). Logistic regression analyses did not show a significant difference in age or sex between cases and controls, and the associations of the 6 SNPs with depression remained similar after adjusting for age and sex. Permutation analysis showed that only SNP rs12273539 remained significant after adjusting for multiple tests with a corrected *P* value of .002.

HAPLOTYPE-BASED ASSOCIATION ANALYSES OF CASES AND CONTROLS

The **Figure** shows that 7 haplotype blocks were identified by searching for the solid spine of strong LD. Among the 130 detected polymorphisms, 33 SNPs with a minor allele frequency of 1.5% or greater were included in the haplotype analyses. Several haplotypes were found to be associated with the diagnosis of depression in block 3 (5 SNPs: rs56820186, rs6265, rs11030101, rs28722151, and rs11030102) and block 4 (4 SNPs: rs57083135, NT_009237.17_26469156, rs110303103, and rs12273539). Block 3 included 3 SNPs associated with depression (Table 3). The most significant association in block 3 was found for a common haplotype TGACC, and

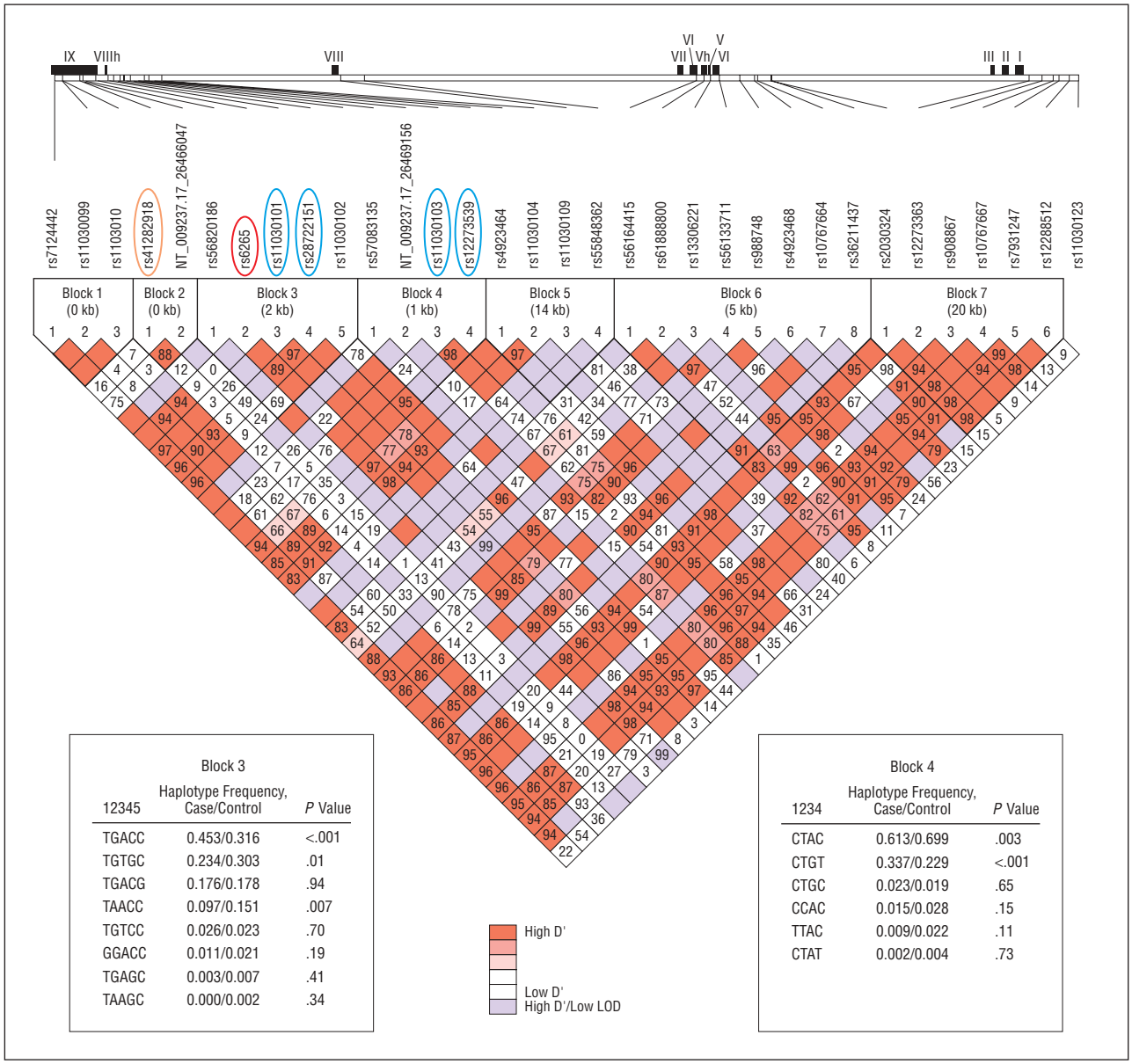

Figure. Linkage disequilibrium (LD) pattern in brain-derived neurotrophic factor gene (*BDNF*). Standard color scheme in Haploview program is used to display the level of logarithm of odds (LOD) and the D′ (right key). Estimated statistics of the D′ are shown in each box. They indicate the LD relationship between each pair of single-nucleotide polymorphisms (SNPs) and are not labeled if D′ = 1.00. The *BDNF* gene structure is illustrated by the long horizontal white bar at the top, with vertical lines indicating the relative positions of SNPs and black boxes representing alternative exons named by Pruunsild et al.[7] The SNPs associated with depression are marked in orange (untranslated region), red (coding), and blue (intronic) circles. The left inset shows the haplotype frequencies in the cases and controls and the *P* values for the association analysis between haplotype and diagnosis of depression in blocks 3 and 4. kb indicates kilobase.

the haplotype frequency was 0.453 in cases and 0.316 in controls ($\chi^2 = 20.80$, $P < .001$; permutation adjusted $P < .001$). In block 4, the most significant association was found for haplotype CTGT, and the haplotype frequency was 0.337 in cases and 0.229 in controls ($\chi^2 = 15.06$, $P < .001$; permutation adjusted $P = .002$). No other haplotypes were associated with depression after adjusting for multiple testing in the permutation tests.

GENETIC ASSOCIATION ANALYSES OF RESPONSE TO ANTIDEPRESSANTS

In the present study, there were 200 patients with MDD who received at least 1 dose of antidepressant treatment (ITT sample of 103 received desipramine and 97 received fluoxetine) and 142 patients with MDD who completed 8-week antidepressant treatment (completed-treatment sample of 68 with desipramine and 74 with fluoxetine). For the discrete outcome (remission vs nonremission), no detected polymorphisms were found to be significantly associated with the remission status in allelic and genotype-based analyses with the use of ITT or completed-treatment samples. For the quantitative outcome (relative reduction in HAM-D21 score), 1 newly reported 5′ UTR SNP, rs61888800, was found to be associated with the better response to antidepressant treatment ($P = .02$) after adjusting for age, sex, medication, and baseline HAM-D21 score in the combined sample of pa-

Table 4. *BDNF* Polymorphisms Associated With Response to Antidepressant Treatment With Desipramine

SNP (Type[a])	Chr Position	Genotype	Intent-to-Treat Analysis[b] No.	Mean (SD)	β (95% CI)	P Value	Complete-Case Analysis[b] No.	Mean (SD)	β (95% CI)	P Value
rs7124442 (3′ UTR)	27633617	CC/CT	43	37.97 (30.35)	14.88 (2.99 to 26.77)	.02	28	50.17 (25.58)	14.60 (3.09 to 26.11)	.02
		TT	58	48.17 (30.77)			40	60.88 (22.58)		
rs11030102 (intronic)	27638172	GG/GC	33	35.64 (30.60)	15.72 (3.14 to 28.31)	.02	21	46.64 (27.50)	15.09 (3.08 to 27.11)	.02
		CC	65	49.45 (30.04)			47	60.86 (21.56)		
rs12273539 (intronic)	27639887	TT/TC	53	47.30 (30.89)	−13.70 (−25.95 to −1.45)	.03	35	61.13 (21.34)	−14.16 (−26.10 to −2.23)	.02
		CC	44	36.35 (31.42)			28	49.66 (27.49)		
rs61888800 (5′ UTR to intronic)	27678854	TT/TG	33	36.05 (30.83)	17.12 (4.19 to 30.05)	.01	21	47.28 (27.64)	18.41 (6.24 to 30.57)	.004
		GG	60	49.84 (31.14)			41	63.84 (20.96)		
rs56133711 (intronic)	27679910	AA/AG	28	36.54 (29.80)	15.52 (2.64 to 28.40)	.02	18	48.57 (28.44)	14.12 (1.20 to 27.04)	.04
		GG	63	50.16 (29.58)			45	61.64 (21.44)		
rs2030324 (intronic)	27683491	AA/AG	61	40.35 (30.63)	12.81 (0.34 to 25.29)	.05	37	52.44 (24.80)	14.20 (2.78 to 25.62)	.02
		GG	39	48.79 (32.27)			29	63.70 (21.84)		
rs12273363 (upstream)	27701435	CC/CT	29	34.59 (28.61)	15.81 (2.54 to 29.09)	.02	19	48.29 (24.16)	14.28 (1.47 to 27.09)	.03
		TT	71	46.39 (31.99)			48	59.44 (24.00)		
rs7931247 (upstream)	27703567	TT/TC	63	39.90 (30.23)	13.20 (0.92 to 25.49)	.04	38	51.72 (24.86)	14.94 (3.53 to 26.35)	.01
		CC	39	48.79 (32.27)			29	63.70 (21.84)		

Abbreviations: *BDNF*, brain-derived neurotrophic factor gene; Chr, chromosome; CI, confidence interval; dbSNP, database single-nucleotide polymorphism (SNP); UTR, untranslated region.

[a] Intron-exon boundaries were based on multiple alternative 5′ exons in the National Center for Biotechnology Information AceView Database.

[b] Means are average relative reductions in 21-Item Hamilton Depression Rating Scale (HAM-D21) score. β values are regression coefficients for allele effect based on the dominant model after adjusting for sex to age and baseline HAM-D21 score.

tients treated with desipramine or fluoxetine in completed-treatment sample analysis. Patients who had GG genotype showed a larger average reduction of HAM-D21 score of 66.3% (95% CI, 62.0%-70.7%) compared with those who had non-GG genotype and had an average relative reduction of HAM-D21 score of 56.5% (95% CI, 48.6%-64.57%). For the medication-specific analyses, 8 *BDNF* polymorphisms were found to be associated with the HAM-D21 score reduction among the patients treated with desipramine in both ITT and completed-treatment analyses with $P \leq .05$ after controlling for age, sex, and baseline HAM-D21 score (**Table 4**). Among the 8 SNPs associated with response to desipramine treatment, all showed a 14% larger reduction in HAM-D21 score in the patients homozygous for a major allele except rs12273539, which showed 14% smaller reduction in patients homozygous for a major allele in completed-treatment analysis and showed a similar pattern but with a smaller reduction in ITT analysis. No polymorphism associated with desipramine treatment response remained significant after adjusting for multiple testing through permutation, and no detected SNPs were found significantly associated with the reduction of HAM-D scores in the fluoxetine-treated group.

COMMENT

Our results provide a detailed description of *BDNF* sequence variations in Mexican Americans. Among the 130 SNPs that we detected in this study, 83 are novel and only 47 have been reported in the National Center for Biotechnology Information dbSNP database, which has collected 254 *BDNF* SNPs to date (http://www.ncbi.nlm.nih.gov/projects/SNP). Most of these new polymorphisms (89%) are rare variants with a minor allele below 1% (eTable 2). This is not surprising because our study was conducted in a large sample of 537 subjects of a specific ethnic group that has not been investigated extensively. The overall nucleotide diversity in that genomic region is 0.0008. Pairwise F_{ST} values showed a substantial population differentiation in 18 dbSNPs using frequency data available from the National Center for Biotechnology Information database of 3 ethnic groups (CEU, YRI, and CHB + JPT). For example, a high divergence of allele frequency was noted for nonsynonymous SNP rs6265 across ethnic populations; minor allele (A allele) frequencies of 0.12, 0.18, 0.00, and 0.48 were found in Mexican Americans, whites, blacks, and Asians, respectively. Our findings suggest that the genetic variation in the *BDNF* gene across different populations may be large, and this heterogeneity may contribute to explain controversial findings in associations of *BNDF* with depressed patients from different populations.

It is noteworthy that rare variants in relevant genes in neurodevelopmental pathways have been associated with schizophrenia,[58] further supporting the rare variant/common disease model. The discovery of 83 mostly rare variants in *BDNF*, a gene that is found to be relevant to several psychiatric disorders, may therefore be of widespread interest.

We report herein that 5 SNPs in the *BDNF* gene were significantly associated with depression, in addition to the nonsynonymous SNP rs6265 that we reported previously.[36] Among the 6 SNPs, rs12273539, an intronic variant located 3.4 kb away from rs6265 and near alternative 5′ exon VIIIh (Figure), showed the most significant association with depression and remained significant after adjustment for multiple testing. Unlike rs6265, rs12273539 showed much less similarity in allele frequency between Mexican Americans and whites, with a large F_{ST} value of 0.20. Haplotype analyses showed a strong LD ($D' = 1.00$) between rs6265 and rs12273539, but they mapped to 2 LD blocks (blocks 3 and 4 in the Figure). Two common haplotypes, TGACC that includes *BDNF*

Val66 allele (G) in exon IX in block 3 and CTGT in block 4 near exon VIIIh, were found to be significantly associated with increased risk of depression after correcting for multiple testing.

We also found that 8 SNPs were associated with response to desipramine treatment in both ITT and completed-treatment samples, although the association did not remain significant after adjustment for multiple testing. Among the 8 SNPs, there were one 3′ UTR SNP (rs7124442) in block 1, 2 newly reported SNPs (5′ UTR SNP rs61888800 in exon Vh and intronic SNP56133711) in block 6, 3 SNPs (rs2030324 in the intron; and rs12273363 and rs7931247 in the upstream region) in block 7, and 1 in each of block 3 (rs11030102) and block 4 (rs12273539) (Figure). Interestingly, SNP rs12273539, which showed the most significant association with depression status, was also associated with the drug response to desipramine treatment ($\beta = -14.16\%$; $P = .02$) in 8-week completers.

There are several implications to our findings. First, they support the concept that *BNDF* genetic variants may differ in frequency and/or effect among different ethnic groups. For example, our data support that, in the variant rs6265 (Val66Met), the Val (G allele) carriers are at increased risk for depression, which is consistent with the data of several studies in whites.[59-61] However, several studies in Asians have reported no association between depression and Val66Met[62-64] or the association of the Met (A allele) variant with susceptibility to depression.[65,66] Our population differentiation analysis also showed that Mexican Americans and whites have a comparable Val66Met allele frequency ($F_{ST} = 0.01$), but they have substantial allele difference when compared with Asians ($F_{ST} = 0.31$). The observed results across ethnic groups may suggest heterogeneity in *BDNF* allele frequencies and genetic polymorphisms among populations. Second, they suggest that other *BDNF* genetic variants besides Val66Met may contribute to susceptibility to depression. In this survey, we found 6 *BDNF* polymorphisms that were associated with depression risk. The strongest association was found to an intronic variant: rs12273539. We also identified 2 haplotypes in different haplotype blocks, one containing rs6265 and the other containing rs12273539, that are significantly associated with depression after multiple testing adjustment ($P \leq .002$). Third, they suggest that the association of *BDNF* genetic variants with drug response to antidepressant treatment may be medication-specific and do not support a major role of Val66Met variant in antidepressant action in this population. Among the 6 polymorphisms associated with depression in this study, only SNP rs12273539 was found to be associated with HAM-D21 score reduction in desipramine treatment in our sample. However, 7 other SNPs were found to be associated with desipramine treatment by showing greater than or equal to 14% more average reduction in patients who are homozygous for a major allele.

Three studies[62,64,67] have recently assessed the association between Val66Met polymorphism and antidepressant response in patients with MDD, but only 1 reported that Met carriers had a better response to 8-week citalopram hydrobromide treatment.[62] Gratacòs et al[67] reported an SNP rs908867 and a haplotype (TAT at rs12273363, rs908867, and rs1491850) in 5′ upstream region associated with antidepressant response. Interestingly, in this region, we found 3 SNPs (rs2030324, rs12273363, and rs7931247 in block 7) associated with desipramine treatment, although the association of rs908867 with response to antidepressant treatment was not significant in our study. The differential findings could be due to a number of factors such as medication type, outcome assessment, sample size, population substructure, and, very importantly, the complexity and rich diversity in the regulation of *BDNF* multiple transcripts, in the coding and noncoding sequences, and in the pro*BDNF* and mature *BDNF* translation product sequences.[7,68]

Limitations of this study are related to the sample size, which is relatively small, particularly for analyses of antidepressant treatment response. Power analyses showed that, at a single 2-sided significance of .05 and allele frequency of 0.15 or more, a sample size of 200 patients can achieve 89% power to detect a moderate effect size of 0.5, which is close to what we observed in the desipramine group; however, the power should be much lower if the genetic effect is medication-specific, as our results suggest. Another limitation is that the population stratification analysis was not based on ancestral informative markers, and the potential risk of hidden population substructures in this admixed sample could not completely be eliminated. Given the relatively small sample size, the lack of a replication sample, and the potential risk of a population substructure, the observed association should be interpreted with much caution and considered exploratory.

In conclusion, we have identified 83 novel *BDNF* genetic variants. Our data support the concept that extensive resequencing of key candidate genes can lead to the discovery of substantial numbers of new variants. Our results further implicate *BDNF* in the susceptibility to MDD and in the therapeutic response to antidepressants. To our knowledge, this work is the most comprehensive genetic association study to date to have examined the association between *BDNF* sequence variation with both depression and antidepressant response. Given that a number of alternative *BDNF* transcripts have been found to display complex splicing and expression patterns and that the findings in different studies remain inconsistent, further comprehensive studies in larger independent samples are clearly warranted for conclusive results. Moreover, we suggest that deep sequencing of relevant genes in large numbers of patients can disclose substantial numbers of novel variants that may be useful targets for future association studies.

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ORIGINAL ARTICLE

Sequence variations of *ABCB1*, *SLC6A2*, *SLC6A3*, *SLC6A4*, *CREB1*, *CRHR1* and *NTRK2*: association with major depression and antidepressant response in Mexican-Americans

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We studied seven genes that reflect events relevant to antidepressant action at four sequential levels: (1) entry into the brain, (2) binding to monoaminergic transporters, and (3) distal effects at the transcription level, resulting in (4) changes in neurotrophin and neuropeptide receptors. Those genes are ATP-binding cassette subfamily B member 1 (*ABCB1*), the noradrenaline, dopamine, and serotonin transporters (*SLC6A2*, *SLC6A3* and *SLC6A4*), cyclic AMP-responsive element binding protein 1 (*CREB1*), corticotropin-releasing hormone receptor 1 (*CRHR1*) and neurotrophic tyrosine kinase type 2 receptor (*NTRK2*). Sequence variability for those genes was obtained in exonic and flanking regions. A total of 56 280 000 bp across were sequenced in 536 unrelated Mexican Americans from Los Angeles (264 controls and 272 major depressive disorder (MDD)). We detected in those individuals 419 single nucleotide polymorphisms (SNPs); the nucleotide diversity was 0.00054 ± 0.0001. Of those, a total of 204 novel SNPs were identified, corresponding to 49% of all previously reported SNPs in those genes: 72 were in untranslated regions, 19 were in coding sequences of which 7 were non-synonymous, 86 were intronic and 27 were in upstream/downstream regions. Several SNPs or haplotypes in *ABCB1, SLC6A2, SLC6A3, SLC6A4, CREB1* and *NTRK2* were associated with MDD, and in *ABCB1, SLC6A2* and *NTRK2* with antidepressant response. After controlling for age, gender and baseline 21-item Hamilton Depression Rating Scale (HAM-D21) score, as well as correcting for multiple testing, the relative reduction of HAM-D21 score remained significantly associated with two *NTRK2*-coding SNPs (rs2289657 and rs56142442) and the haplotype CAG at rs2289658 (splice site), rs2289657 and rs2289656. Further studies in larger independent samples will be needed to confirm these associations. Our data indicate that extensive assessment of sequence variability may contribute to increase understanding of disease susceptibility and drug response. Moreover, these results highlight the importance of direct re-sequencing of key candidate genes in ethnic minority groups in order to discover novel genetic variants that cannot be simply inferred from existing databases.
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Introduction

Major depressive disorder (MDD) is a common, complex and recurrent disorder of gene–environment interactions. The estimated heritability may range from 0.36 to 0.66.[1,2] Following up on previous study on the pathophysiology of MDD and on the prevailing hypotheses for treatment response, we sought to identify genes that influence susceptibility for MDD or treatment response in the central nervous system pathways relevant to stress reactivity and to the pathways of action of antidepressant drugs. Current data point out to roles for genes involved in drug transport, serotonin neurotransmission, neurotrophin signaling and response to stress. Promising linkage results are located in several chromosomes,[3] which highlight the multilocus nature of the genetic vulnerability to MDD.

Recently, rapid technological advances have started unraveling the contributions of common (frequency >1%) and rare genetic variants in complex disorders. In a topical review, Bodmer and Bonilla[4] have synthesized current views, implications and

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integration of the competing hypotheses of common disease–common variant and common disease–rare variant. For most common variants, the disease-associated variant is unlikely to be functionally relevant; it may be closely linked to the functional variant, and it will cause a small increase in disease risk (odds ratio smaller than 2, generally between 1.1 and 1.4). In contrast, rare variants generally have functional and large phenotypic effects; in many cases they are missense variants that reflect amino-acid changes relevant to protein–protein interactions. Diverse scenarios may occur in the pathophysiology of common complex disorders: Common variants may be modifiers of genes with rare variant effects, such as recently described for the *MC4R* gene.[5] Moreover, areas near common variants may contain candidate genes in which there are rare variants. The identification of rare variants may significantly affect our understanding of complex disease etiology.

We re-sequenced seven candidate genes of importance in the pathophysiology of MDD.[6] Conceptually, we sought a group of genes that reflects a sequence of events relevant to drug action at four levels: (1) entry into the brain, (2) binding to monoaminergic transporters, and (3) distal effects at the transcription level, resulting in (4) changes in neurotrophin and neuropeptide receptors. Specifically, we studied a blood–brain barrier drug transporter pump (*ACCB1*, also called *MDR1*), which regulates drug entry into the brain (level 1), the norepinephrine, dopamine, and serotonin transporters (*SCL6A2*, *SLC6A3* and *SL6A4*) (level 2), an antidepressant-regulated transcription factor (cyclic AMP-responsive element binding protein 1 (*CREB1*)) (level 3) and two receptors (level 4): neurotrophic tyrosine kinase type 2 receptor (*NTRK2*), important in synaptic function and neural plasticity, and corticotropin-releasing hormone receptor 1 (*CRHR1*), which regulates the response to stress at the behavioral, neuroimmune and neuroendocrine—hypothalamic–pituitary–adrenalaxis levels.

Materials and methods

Patients and controls

The study consisted of 272 patients (66% female, 34% male; mean age: 38 ± 10) with MDD and 264 healthy control individuals (60% female, 40% male; average age: 36 ± 11). MDD was defined as a DSM-IV (Diagnostic and Statistical Manual of Mental Disorders, 4th Edition) diagnosis of current, unipolar major depressive episode and a 21-item Hamilton Depression Rating Scale (HAM-D21) score of $\geqslant 18$ with item number 1 (depressed mood) rated $\geqslant 2$. All MDD patients were screened for the pharmacogenetic study of antidepressant treatment response as previously described.[7] All MDD patients had comprehensive psychiatric and medical assessments in their primary language, on the basis of diagnostic and ratings instruments that had been fully validated in English and in Spanish. Exclusion criteria included active medical illnesses that could be etiologically related to the ongoing depressive episode, current or active suicidal ideation with a plan and strong intent, pregnancy, lactation, current use of medications with significant central nervous system activity, which interfere with electroencephalogram (EEG) activity (for example, benzodiazepines) or any other antidepressant treatment within the 2 weeks before enrollment, illicit drug use and/or alcohol abuse in the last 3 months or current enrollment in psychotherapy. All MDD patients were Mexican-Americans and had at least three grandparents born in Mexico.

All patients had an initial comprehensive psychiatric and medical assessment and, if enrolled in the pharmacogenetic study of antidepressant treatment response, had weekly structured follow-up assessments for 9 weeks. The study consisted of two phases: a 1-week single-blind placebo lead-in phase to minimize the impact of placebo responders followed, if subjects continued to meet the inclusion criteria after phase 1, by random assignment to one of the two treatment groups: fluoxetine 10–40 mg per day or desipramine 50–200 mg per day, administered in a double-blind manner for 8 weeks. Our primary clinical outcome measure was HAM-D21 score and clinical remission on antidepressants was defined as having a final (week 8) HAM-D21 score < 8. In addition, the relative response change was also computed as the difference in HAM-D21 score between pre- and post-treatment divided by the pretreatment HAM-D21 score.

Age-, gender- and ethnicity-matched healthy control individuals were recruited from the same Mexican-American community in Los Angeles by the same bilingual clinical research team. Controls for our genomic studies were in general good health but were not screened for medical or psychiatric illness.

Genomic DNA collection, amplification and sequencing

At the initial visit, after informed consent was obtained from the participating individuals, blood samples were collected into EDTA (K2EDTA) BD Vacutainer EDTA tubes (Becton Dickinson, Franklin Lakes, NJ, USA), and genomic DNA was isolated by using Gentra Puregene DNA purification kits (Gentra Systems, Indianapolis, IN, USA). DNA sequencing for seven genes was carried out in collaboration with the Sanger Institute by following ExoSeq protocol (http://www.sanger.ac.uk/humgen/exoseq/). Briefly, the known protein-coding regions, novel coding sequences and transcripts, exons and their flanking sequence were extracted from the Vega database (http://vega.sanger.ac.uk/index.html). Primers were designed automatically using Primer3 (http://frodo.wi.mit.edu/) to amplify DNA and primer pairs were checked for uniqueness before ordering and pre-screened to determine the optimum conditions for amplification. After amplification, a sample of the products were visualized on an agarose gel to confirm the size of the PCR product. The remaining PCR product was then cleaned up using two enzymes,

Exonuclease 1 and Shrimp Alkaline Phosphatase. Bidirectional sequencing of amplicons was carried out using Big DyeTM chemistry (Big Dye Terminator, Version 3.1; Applied Biosystems, Foster City, CA, USA). Single nucleotide polymorphisms (SNPs) were called using ExoTrace http://www.sanger.ac.uk/humgen/exoseq/analysis.shtml, a novel algorithm developed in-house for the detection of heterozygotes in sequence traces , which processes the sense and antisense sequence reads separately and subsequently, and combines the results to allow SNP scoring. All polymorphisms reported here had a genotyping rate of $\geqslant$80% and an average nucleotide call rate of 93%.

Genomic control genotyping

To detect potential bias due to population stratification, two approaches were used to test for hidden stratification in our data. First, 54 independent SNPs across 22 autosomal chromosomes were selected to analyze a combined sample using the genotype data download from three HapMap ethnic samples using STRUCTURE program (http://pritch.bsd.uchicago.edu/software.html)[8,9] and showed that three distinct clusters were well identified with an average proportion of at least 92% of individuals correctly assigned to the given ethnic populations (CEU, CHB + JPT, YRI). This panel of SNPs were then used as genomic control to test our sample and showed an almost equal proportion assigned to each clusters, given $K = 2$, 3, 4 in both cases and controls. Second, genotype frequencies from each of the 54 unlinked SNPs were also compared between cases and controls using the method described by Pritchard and Rosenberg [10] and no significant difference was found based on an overall test statistic ($\chi^2 = 100.50$, d.f. = 108, $P = 0.68$). Therefore, no population stratification adjustment was necessary for our association analyses.

Nucleotide diversity, population differentiation and Hardy–Weinberg equilibrium

Nucleotide diversity (θ) and its standard deviation ($S(\theta)$) were calculated under the assumption of an infinite neutral allele model,[11,12] and all calculations were based on $n = 946$ for all the sites given that the average sample size was 473 individuals across all the polymorphisms. Population differentiation estimation was based on the pairwise F_{ST} values for the dbSNPs (single nucleotide polymorphism database hosted at the National Center for Biotechnology Information), which were both detected in our Mexican-American sample and reported in HapMap sample. F_{ST} values were calculated as described byWeir,[13] Weir and Cockerham,[14] and Weir and Hill.[15] In order to compare allele frequencies and to be able to treat chromosomes as independent observations, the genotype frequencies must be in Hardy–Weinberg equilibrium (HWE).[16] Exact testing of HWE was performed separately for healthy controls and MDD patients using the PLINK program Version1.00 (http://pngu.mgh.harvard.edu/ ~purcell/plink/).[17] SNPs that were not in HWE in the healthy control group were excluded from the allele-based association analyses of cases and controls.

Statistical analysis

Data preparation and descriptive statistics were carried out with SAS software (SAS Version 9.1.3, SAS Institute, Cary, NC, USA). For SNP-based association analyses of case vs control or remitter vs non-remitter, Fisher's exact test (two-tailed) was performed to compare allele and genotype distributions between depressed and healthy individuals using PLINK. In the allelic association analysis, each polymorphism was tested in controls to ensure the fitting with HWE; the odds ratio on the 2×2 contingency table of allele counts and its 95% confidence interval were estimated using Woolf's method or fitting exact logistic regression model with SAS software when the frequency in a table cell is zero.[18] In genotypic association analysis, SNP effects were tested under a codominant model on the 2×3 contingency table of genotype counts.

For the quantitative outcome (relative reduction % in HAM-D21 scores between pre- and post-treatment), the analyses based on dominant model were performed, separately, for the joint sample of patients treated with desipramine or fluoxetine and for medication-specific sample. General linear regression models were used to examine the association between genotype and relative HAM-D21 score reduction by controlling for age, gender and baseline (pretreatment) HAM-D21 score using the PLINK program. The Benjamini and Hochberg method was used to control for false discovery rate and the significance threshold was set at FDR_BH $\leqslant 0.05$[19].

For haplotype-based association analysis, Haploview (Version 4.1, Broad Institute of MIT and Harvard, http://www.broad.mit.edu/mpg/haploview/), was first used to identify the haplotype blocks by applying the Four Gamete Rule[20] based on the SNPs with a minor allele frequency (MAF) $\geqslant 0.01$ in the combined sample of cases and controls and HWE exact test $P > 0.01$ in controls. The PLINK program was then used to examine the association of specific haplotype with depression diagnosis, clinical remission, as well as quantitative outcome of antidepressant treatment.

Results

Identification of sequence variations

A total of 419 single nucleotide sequence variants (Table 1) were identified by re-sequencing of $\sim$105 kb of exonic sequence and their flanking regions in the selected seven genes in an ethnically homogeneous sample of 264 healthy controls and 272 MDD patients. Among the 419 SNPs, 204 (49%) are novel polymorphisms, not previously described, including 86 in introns, 72 in untranslated regions (UTRs), 19 (12 synonymous) in coding regions, 18 in upstream and 9 in downstream regions. Overall, 95% of the novel polymorphisms had a MAF lower than 5%,

Table 1 Single nucleotide polymorphisms (SNPs) detected in seven candidate genes for depression in Mexican-Americans

		SNP type									
Gene (Location)	*SNP*[a]	*Downstream*	*3′ UTR*	*Intronic*	*SYN*	*NS*	*5′ UTR*	*Upstream*	*All*	*Sequence screened (kb)*	*Nucleotide diversity (s.d.) × 10⁻⁴*
CREB1 (2q34)	New	2	5	5	0	0	0	0	12	7.5	3.2 (0.9)
	dbSNP	1	2	2	1	0	0	0	6		
	Total	3	7	7	1	0	0	0	18		
SLC6A3 (5p15.3)	New	0	2	6	2	1	0	7	18	12.3	5.3 (1.2)
	dbSNP	0	7	10	5	1	0	7	30		
	Total	0	9	16	7	2	0	14	48		
ABCB1 (7q21.1)	New	0	0	20	1	3	4	0	28	28.5	3.8 (0.8)
	dbSNP	0	4	37	2	5	4	1	53		
	Total	0	4	57	3	8	8	1	81		
NTRK2 (9q22.1)	New	0	43	10	2	2	0	0	57	23.6	5.1 (1.0)
	dbSNP	0	24	6	2	0	0	0	32		
	Total	0	67	16	4	2	0	0	89		
SLC6A2 (16q12.2)	New	0	4	10	2	1	1	11	29	13.3	5.6 (1.2)
	dbSNP	0	4	9	2	2	0	9	26		
	Total	0	8	19	4	3	1	20	55		
SLC6A4 (17q11.1-q12)	New	5	4	23	0	4	1	0	37	12.5	7.8 (1.6)
	dbSNP	4	1	23	4	1	2	0	35		
	Total	9	5	46	4	5	3	0	72		
CRHR1 (17q12-q22)	New	2	8	12	0	1	0	0	23	6.9	10.9 (2.4)
	dbSNP	1	12	16	3	1	0	0	33		
	Total	3	20	28	3	2	0	0	56		
All seven genes	New	9	66	86	7	12	6	18	204	104.5	5.4 (1.0)
	dbSNP	6	54	103	19	10	6	17	215		
	Total	15	120	189	26	22	12	35	419		

Abbreviations: *ABCB1*, ATP-binding cassette subfamily B member 1; *CREB1*, cyclic AMP-responsive element binding protein 1; *CRHR1*, corticotropin-releasing hormone receptor 1; NS, non-synonymous; SYN, synonymous; *NTRK2*, neurotrophic tyrosine kinase type 2 receptor; UTR, untranslated region.
[a]New: not reported in NCBI dbSNP database as of 30 June 2008.

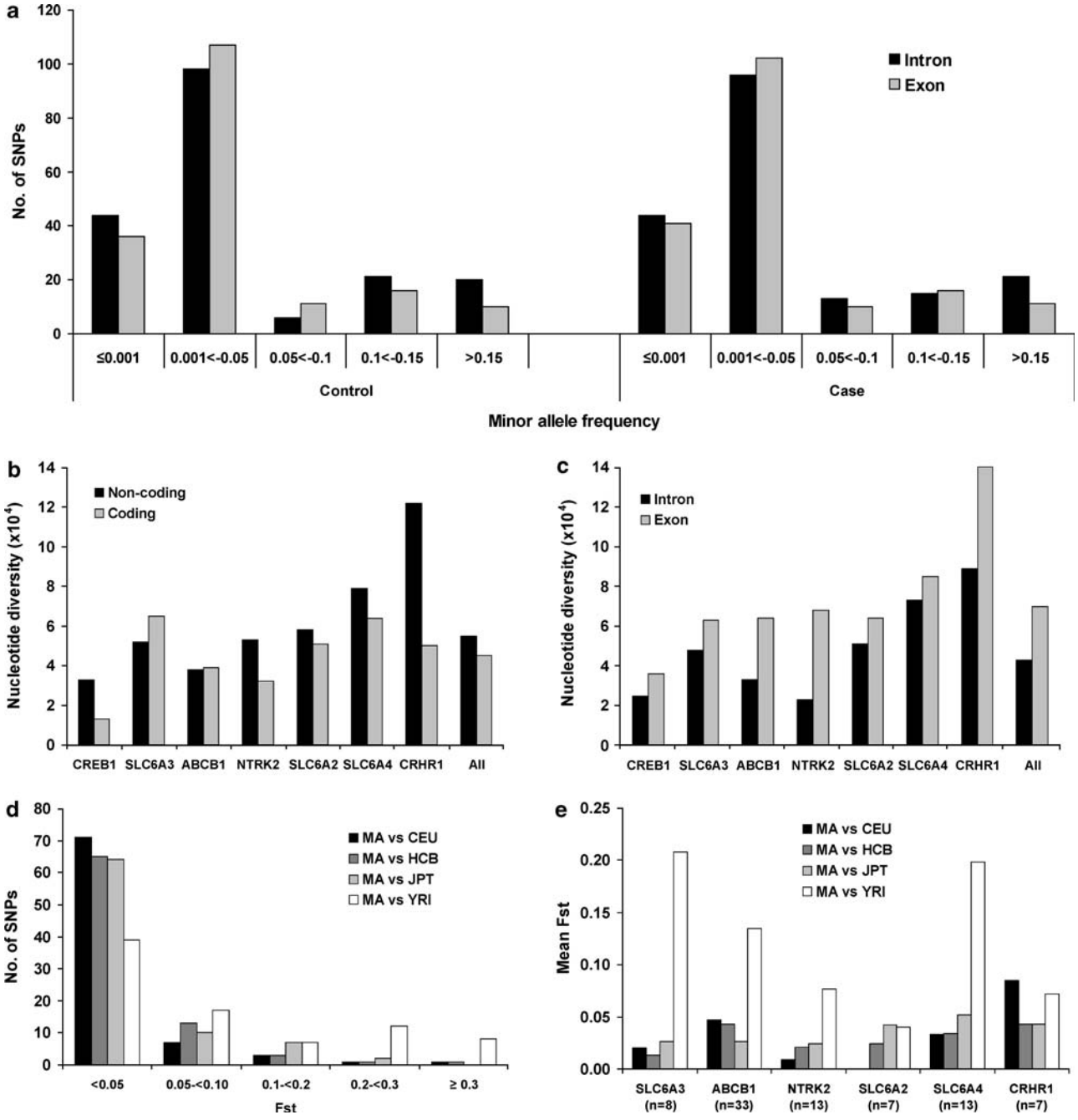

Figure 1 Minor allele frequency (MAF), nucleotide diversity and F_{ST} measure in seven candidate genes in Mexican-American major depressive disorder (MDD) patients and controls. Histograms show the total number of single nucleotide polymorphisms (SNPs) detected in intronic (black bar) and exonic (gray bar) regions in the seven genes by MAF in 272 MDD patients and 264 healthy controls (**a**); the nucleotide diversity in noncoding (black bar) and coding (gray bar) (**b**) or intronic (black bar) and exonic (gray bar) regions (**c**) by gene in the combined sample of 272 MDD patients and 264 healthy controls; the total number of SNPs shared by Mexican-American (MA) sample and HapMap samples by pairwise F_{ST} value (**d**) or represent the average F_{ST} by gene (**e**) in MA vs CEU (black bar), MA vs HCB (dark gray bar), MA vs JPT (gray bar) and MA vs YRI (White bar).

whereas the corresponding proportion was 57% for dbSNPs (Supplementary Table 1). Similar distribution of MAFs was seen between cases and controls for both SNPs in intronic and in exonic regions (Figure 1a). Among the 419 SNPs, the proportion of SNPs with HWE exact test *P*-value $\geqslant 0.05$ was 92% for controls and 91% for MDD cases (Supplementary Table 1).

Nucleotide diversity was estimated for each gene by correcting for both sample size and length of the screened site (Table 1). Nucleotide diversities were comparable in *SLC6A3* (0.00053 ± 0.00012), *NTRK2* (0.00051 ± 0.0001) and *SLC6A2* (0.00056 ± 0.00012), but were lower in *CREB1* (0.00032 ± 0.00009) and ATP-binding cassette subfamily B member 1 (*ABCB1*)

(0.00038 ± 0.00008) and appeared higher in *SLC6A4* (0.00078 ± 0.00016) and *CRHR1* (0.00109 ± 0.00024). This led to an overall nucleotide diversity of 0.00054 for all the seven genes investigated. When the nucleotide diversity was estimated separately for coding and noncoding sequence, five out of seven genes (except for *SLC6A3* and *ABCB1*) showed higher nucleotide diversity in noncoding regions when compared with coding segments (Figure 1b). However, when the nucleotide diversity was estimated separately for exonic and intronic sequence, all the seven genes showed higher nucleotide diversity in exonic regions than in intronic segments (Figure 1c). This is because of the high nucleotide diversity in untranslated regions (0.00088 ± 0.00017).

Among the 215 dbSNPs detected, 83 were reported in all four HapMap ethnic groups: CEU (Caucasian), YRI (African), CHB (Han Chinese) and JPT (Japanese) in the NCBI database as of 25 June 2008. Pairwise F_{ST} values between Mexican Americans (MA) and each HapMap ethnic sample were computed for the shared 83 dbSNPs. Overall, the greatest difference in allele frequencies was found between Mexican Americans and Africans with a highest mean F_{ST} of 0.126, compared with mean F_{ST} of 0.035 in MA vs CEU, 0.033 in MA vs CHB and 0.032 in MA vs JPT (Figure 1d). For the gene-specific mean F_{ST} in MA vs YRI, larger mean F_{ST} values were observed for *SLC6A3* (0.208) and *SLC6A4* (0.198), but much lower for *SLC6A2* (0.04) (Figure 1e).

SNP-based genetic association analyses of cases and controls

Single nucleotide polymorphism-based allelic and genotypic association analyses revealed that 16 polymorphisms were associated with MDD with a nominal $P<0.05$ in five genes (Table 2), including two common 3′ UTR polymorphisms in *NTRK2* (rs7020204 and rs2013566) and one rare 5′ UTR polymorphism in *SLC6A4* (rs28914831). Among the nine SNPs with a nominal $P<0.05$ in both allelic and genotypic tests, seven were uncommon polymorphisms with a MAF <0.03 in controls, including one in *CREB1* (rs3732076), two in *ABCB1* (rs4728697, rs58898486) and four in *SLC6A4* (rs7212502, rs28914831, NT_010799.14_3288789 and rs56355214) (Table2 and Supplementary Table 1). Three *SLC6A4* common polymorphisms (rs7224199 and rs3813034 in upstream and rs140701) showed genotypic association, but with a small allelic odds ratio <1.3 and allelic test nominal $P>0.05$. No associated SNPs remained significant after adjusting for multiple tests with an FDR_BH $\leqslant 0.05$.

SNP-based genetic association analysis of antidepressant response

In this study, there were 142 MDD patients who enrolled in the pharmacogenetic trial and completed 8-week antidepressant treatment (68 treated with desipramine and 74 treated with fluoxetine). For the discrete outcome (remission vs non-remission), SNP-based allelic or genotypic association analyses revealed that clinical remission status was associated with several polymorphisms in or near three genes, *ABCB1*, *NTRK2* and *SLC6A2* (Table 3). All of the nine associated *NTRK2* SNPs were in 3′ UTR or coding regions except for rs2289658 at a splice site, whereas the two associated *SLC6A2* SNPs were in intron or upstream region. For the *ABCB1* gene, the associated SNPs included two in UTR, two in introns and one in coding sequence. No associated SNPs remained significant after adjusting for multiple tests with an FDR_BH $\leqslant 0.05$ in the discrete outcome analysis.

For the quantitative outcome (relative reduction in HAM-D21 score) after controlling for age, gender and baseline HAM-D21 score, general linear regression analyses revealed that relative reduction of HAM-D21 scores was associated with six *NTRK2* SNPs (three in 3′ UTR, two synonymous and one intronic at splice site) and one *SLC6A3* intronic SNP rs8179029 in desipramine-treated patients, two *SLC6A2* upstream SNPs in fluoxetine-treated patients and one *SLC6A3* intronic SNP rs8179029 for combined sample, with a nominal $P<0.01$ (Table 4). Among the associated SNPs, only two *NTRK2* synonymous SNPs, rs2289657 and rs56142442, remained statistically significant after correcting for multiple testing with an FDR_BH = 0.05 in the sample of patients treated with desipramine. Desipramine-treated patients who are homozygous for C allele at synonymous SNP rs2289657 or at rs56142442 had higher levels of improvement with 27% larger reduction in HAM-D21 scores, compared with those who are not homozygous for C allele at rs2289657 or rs56142442.

Haplotype-based analyses

Haplotype analysis identified a total of 17 haplotype blocks in the seven genes using the Four Gametes Rules with the Haploview program, including one block in *CREB1*, two blocks in each of *SLC6A3*, *SLC6A4* and *CRHR1*, three blocks in each of *ABCB1* and *SLC6A2*, and four blocks in *NTRK2* (Figure 2). For the association analysis of case and control, the diagnosis of depression was found to be associated with five haplotypes with a nominal *P*-value between 0.01 and 0.05 in *CREB1*, *SLC6A3*, *ABCB1*, *NTRK2* and *SLC6A2*. Among the five depression-associated haplotypes, four included at least one SNP showing an association with depression in the single SNP-based analysis (Table 5). For the association of remitter and non-remitter, eight haplotypes were found to be associated with remission status, including two in *ABCB1* (ACA in block 1 for desipramine-treated patients and GCGCACACGAGAC in block 2 for fluoxetine-treated patients), two in *NTRK2* (TCG and CAG in block 3 for desipramine-treated patients), one in *SLC6A2* (GCCAGT in block 4 for desipramine-treated patients) and three in *SLC6A4* (TAGC and TAGA in block 1 and ATTGTAACCC in block 2 for the combined sample of desipramine- or fluoxetine-treated patients). Among the eight remission-associated haplotypes, three showed an association with a

Table 2 Polymorphisms associated with depression in Mexican-Americans

Gene	SNP	Chromosome	Position	SNP type	Risk/non-risk allele	Risk allele frequency		OR[a] (95% CI)	P	
						Case	Control		Allelic	Genotypic
CREB1	rs3730276	2	208140591	INT	A/G	0.998	0.977	11.45 (1.46–89.77)	0.004	0.003
SLC6A3	rs8179029	5	1462985	INT	C/T	0.910	0.856	1.71 (1.12–2.58)	0.02	0.02
	rs2550936	5	1464256	INT	A/C	0.839	0.799	1.31 (0.94–1.83)	0.13	0.004
ABCB1	rs4728697	7	86986874	INT	A/G	0.060	0.028	2.24 (1.18–4.28)	0.01	0.02
	rs2032583	7	86998497	INT	A/G	0.936	0.899	1.64 (1.04–2.59)	0.04	0.10
	rs58898486	7	87063142	INT	G/T	0.998	0.984	8.40 (1.05–67.39)	0.02	0.02
NTRK2	rs7020204	9	86616007	INT, 3′ UTR	C/T	0.899	0.850	1.57 (1.07–2.32)	0.02	0.05
	rs2013566	9	86616118	INT, 3′ UTR	A/G	0.876	0.828	1.47 (1.02–2.10)	0.04	0.13
SLC6A4	rs7224199	17	25547852	Downstream	G/T	0.411	0.369	1.19 (0.92–1.55)	0.19	0.004
	rs3813034	17	25548930	Downstream	A/C	0.454	0.399	1.25 (0.98–1.60)	0.08	0.040
	rs140701	17	25562658	INT	C/T	0.454	0.400	1.25 (0.97–1.61)	0.09	0.01
	rs7212502	17	25573328	INT	A/G	1.000	0.987	8.56 (1.23–∞)	0.01	0.01
	rs28914831	17	25573988	5′ UTR	G/T	1.000	0.988	8.37 (1.20–∞)	0.02	0.01
	rs2066713	17	25575791	INT	A/G	0.329	0.263	1.37 (1.04–1.80)	0.03	0.004
	NT_010799.14_3288789	17	25575922	INT	C/G	1.000	0.988	8.63 (1.24–∞)	0.01	0.01
	rs56355214	17	25576325	INT	G/A	1.000	0.988	8.47 (1.22–∞)	0.01	0.01

Abbreviations: *ABCB1*, ATP-binding cassette subfamily B member 1; CI, confidence interval; *CREB1*, cyclic AMP-responsive element binding protein 1; INT, Intronic; *NTRK2*, neurotrophic tyrosine kinase type 2 receptor; OR, odds ratio; SNP, single nucleotide polymorphism; UTR, untranslated region.
[a]OR: calculation based on allele count and 95% CI based on exact logistic model when one cell count is 0.

Table 3 Polymorphisms associated with remission after 8-week antidepressant treatment with desipramine or fluoxetine

Medication	*Gene*	*SNP*	*Chromosome*	*Position*	*SNP type*	*Better/poor response allele*	*Better allele frequency*			*Fisher's exact* P	
							Remitter	*Non-remitter*	*OR*[a] *(95% CI)*	*Allelic*	*Genotypic*
Desipramine or fluoxetine ($N=142$)	*ABCB1*	rs3842	7	86971302	3′ UTR	C/T	0.203	0.094	2.44 (1.14–5.24)	0.02	0.11
		rs17064	7	86971406	3′ UTR	A/T	0.100	0.028	3.81 (1.08–13.5)	0.03	0.11
	NTRK2	rs45596934	9	86618439	INT, 3′ UTR	A/G	0.171	0.123	1.47 (0.74–2.94)	0.31	0.002
		NT_023935.17_16593339	9	86618627	INT, 3′ UTR	T/C	0.177	0.123	1.53 (0.77–3.05)	0.24	0.002
		NT_023935.17_16593340	9	86618628	INT, 3′ UTR	G/A	0.181	0.123	1.58 (0.79–3.15)	0.24	0.001
		rs1624327	9	86619110	INT, 3′ UTR	A/G	0.321	0.224	1.64 (0.95–2.83)	0.08	0.021
		NT_023935.17_16651920	9	86677208	INT, 3′ UTR	G/C	0.961	0.880	3.35 (1.14–9.86)	0.02	0.05
		rs2289658	9	86753190	INT, splice site	T/C	0.885	0.778	2.19 (1.12–4.28)	0.03	0.05
Desipramine ($N=68$)	*ABCB1*	rs17064	7	86971406	3′ UTR	A/T	0.132	0.018	8.39 (1.03–68.4)	0.02	0.14
	NTRK2	NT_023935.17_16593339	9	86618627	INT, 3′ UTR	T/C	0.157	0.113	1.47 (0.53–4.05)	0.61	0.03
		NT_023935.17_16593340	9	86618628	INT, 3′ UTR	G/A	0.167	0.113	1.57 (0.57–4.35)	0.45	0.02
		rs11140793	9	86681300	INT, 3′ UTR	A/C	0.882	0.726	2.83 (1.12–7.14)	0.03	0.05
		rs2289658	9	86753190	INT, splice site	T/C	0.909	0.742	3.48 (1.26–9.59)	0.02	0.04
		rs2289657	9	86753280	SYN	C/A	0.955	0.823	4.53 (1.20–17.1)	0.02	0.04
		rs56142442	9	86826085	SYN	C/T	0.957	0.906	2.31 (0.55–9.65)	0.31	0.05
	SLC6A2	rs5564	16	54283476	INT	G/A	0.141	0.000	11.92 (1.81–∞)	0.004	0.04
Fluoxetine ($N=74$)	*ABCB1*	rs1128503	7	87017537	SYN	G/A	0.522	0.304	2.49 (1.18–5.30)	0.02	0.05
		rs10276036	7	87018134	INT	T/C	0.550	0.320	2.59 (1.24–5.44)	0.01	0.04
		rs2235020	7	87037201	INT	T/A	0.539	0.318	2.50 (1.15–5.43)	0.02	0.09
	NTRK2	rs1624327	9	86619110	INT, 3′ UTR	A/G	0.348	0.212	1.99 (0.90–4.39)	0.09	0.04
		NT_023935.17_16651920	9	86677208	INT, 3′ UTR	G/C	0.972	0.864	5.52 (1.06–28.7)	0.05	0.04
	SLC6A2	rs1362621	16	54245985	Upstream	A/G	0.872	0.731	2.52 (1.06–5.96)	0.04	0.03

Abbreviations: *ABCB1*, ATP-binding cassette subfamily B member 1; CI, confidence interval; INT, intronic; *NTRK2*, neurotrophic tyrosine kinase type 2 receptor; OR, odds ratio; SNP, single nucleotide polymorphism; UTR, untranslated region; SYN, synonymous.
[a]OR: calculation based on allele count and 95% CI based on exact logistic model when one cell count is 0.

Table 4 Polymorphisms associated with relative reduction of HAM-D21 score after 8-week antidepressant treatment with desipramine or fluoxetine

Medication	*Gene*	*SNP*	*Chromosome*	*Position*	*SNP type*	*Genotype*	*N*	*Mean (s.d.)*	*Delta[a] (95% CI)*	*P[b]*	*FDR_BH[c]*
Desipramine or fluoxetine	*ABCB1*	rs2214103	7	87062884	INT	CC	132	64.26 (21.35)	21.45 (5.29–37.61)	0.01	0.72
						CG	8	46.44 (32.92)			
	NTRK2	rs9969765	9	86679605	INT, 3′ UTR	CC	46	69.48 (19.13)	10.96 (3.11–18.81)	0.007	0.72
						GG/CG	87	58.79 (23.81)			
		rs2289657	9	86753280	SYN	CC	106	65.38 (20.35)	12.74 (3.32–22.15)	0.009	0.72
						AA/AC	26	52.48 (29.11)			
	SLC6A2	NT_010498.15_9300464	16	54243766	Upstream	TG	15	77.25 (21.72)	18.49 (6.60–30.39)	0.003	0.72
						TT	121	60.69 (22.06)			
Desipramine	*SLC6A3*	rs8179029	5	1462985	INT	CC	54	61.32 (20.05)	29.79 (9.04–50.54)	0.007	0.56
						TT/TC	6	27.05 (39.26)			
	NTRK2	rs7038236	9	86678222	INT, 3′ UTR	CC	37	63.52 (18.98)	16.97 (4.73–29.22)	0.009	0.56
						AA/AC	18	48.02 (24.77)			
		rs11140793	9	86681300	INT, 3′ UTR	AA	43	62.71 (23.47)	18.30 (5.74–30.86)	0.006	0.56
						CC/AC	22	46.39 (23.30)			
		rs2289658	9	86753190	INT, splice site	TT	44	62.94 (20.19)	18.00 (5.32–30.69)	0.007	0.56
						CC/TC	20	43.57 (27.86)			
		rs2289657	9	86753280	SYN	CC	51	62.11 (19.93)	26.83 (13.39–40.27)	0.0002	0.05
						AA/AC	13	36.40 (29.89)			
		rs56142442	9	86826085	SYN	CC	59	60.70 (19.89)	33.17 (17.15–49.19)	0.0001	0.05
						TT/TC	8	28.92 (34.90)			
Fluoxetine	*SLC6A2*	NT_010498.15_9300464	16	54243766	Upstream	TG	10	85.09 (13.13)	20.55 (8.23–32.87)	0.002	0.46
						TT	60	65.69 (18.37)			
		rs4783899	16	54244713	Upstream	TT	24	76.99 (15.15)	15.02 (5.37–24.68)	0.003	0.47
						GG/TG	48	64.22 (20.09)			

Abbreviations: *ABCB1*, ATP-binding cassette subfamily B member 1; CI, confidence interval; HAM-D21, 21-item Hamilton Depression Rating Scale; INT, intronic; *NTRK2*, neurotrophic tyrosine kinase type 2 receptor; SNP, single nucleotide polymorphism; UTR, untranslated region; SYN, synonymous.
[a]Delta: adjusted mean difference in relative reduction of HAM-D21 score.
[b]*P*: based on general linear model after controlling for age, gender and baseline HAM-D21 score.
[c]Benjamini-Hochberg false discovery rate after correcting for multiple testing.

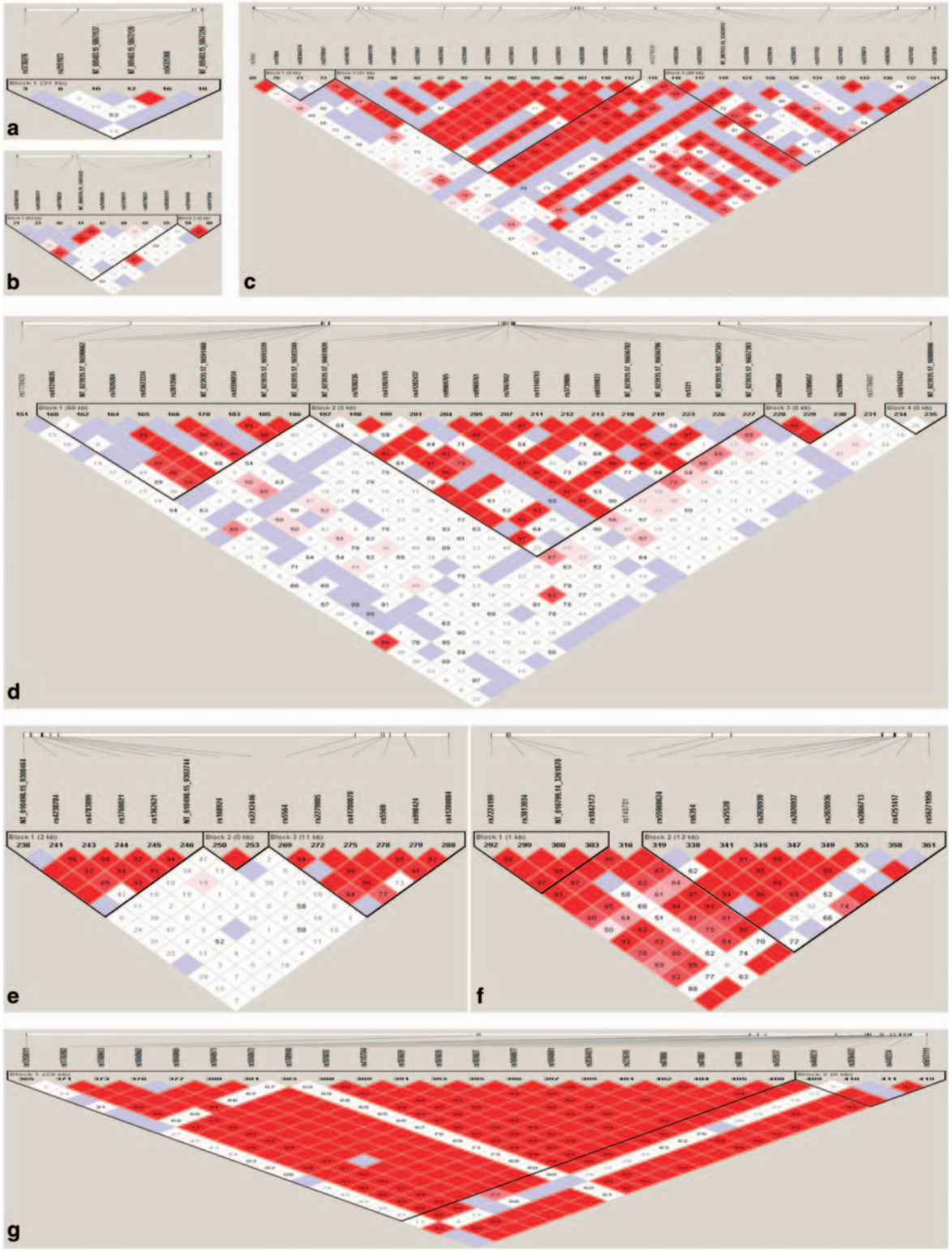

Figure 2 Linkage disequilibrium (LD) pattern in seven genes: cyclic AMP-responsive element binding protein 1 (*CREB1*) (**a**), *SLC6A3* (**b**), ATP-binding cassette subfamily B member 1 (*ABCB1*) (**c**), neurotrophic tyrosine kinase type 2 receptor (*NTRK2*) (**d**), *SLC6A2* (**e**), *SLC6A4* (**f**) and corticotropin-releasing hormone receptor 1 (*CRHR1*) (**g**). Standard color scheme in Haploview program is used to display the level of logarithm of odds (LODs) and the D′. Shown in each box are estimated statistics of the D′, which indicates the LD relationship between each pair of single nucleotide polymorphisms (SNPs) and are not labeled if D′ = 1.00. Regions are shown in bright red, light blue, shades of pink/red and white for $D' = 1 + \mathrm{LOD} \geqslant 2$, $D' = 1 + \mathrm{LOD} < 2$, $D' < 1 + \mathrm{LOD} \geqslant 2$ and $D' < 1 + \mathrm{LOD} < 2$, respectively. Vertical lines on the long horizontal white indicate the relative positions of SNPs in the gene.

Table 5 Haplotypes associated with depression or clinical remission after 8-week antidepressant treatment

Sample	*Gene*	*Block no.*	*Haplotype*[a]	*Frequency*		χ^2	*d.f.*	P
				Case	Control			
MDD patients and healthy controls	*CREB1*	Block 1	GCACGG	0.003	0.018	5.71	1	0.02
	SLC6A3	Block 1	CGTGCGGT	0.089	0.134	4.83	1	0.03
	ABCB1	Block 2	GCACACACGAGAC	0.060	0.028	6.12	1	0.01
	NTRK2	Block 1	GTTAGGGCA	0.094	0.133	3.96	1	0.05
	SLC6A2	Block 3	ACCAGA	0.004	0.017	3.98	1	0.05
				Remitter	Non-remitter			
MDD patients treated with desipramine or fluoxetine	*ABCB1*	Block 1	ACA	0.101	0.029	4.72	1	0.03
	NTRK2	Block 3	TCG	0.839	0.717	5.63	1	0.02
	SLC6A4	Block 1	TAGC	0.006	0.047	5.11	1	0.02
		Block 1	TAGA	0.048	0.003	4.92	1	0.03
		Block 2	ATTGTAACC	0.028	0.081	4.00	1	0.05
MDD patients treated with desipramine	*ABCB1*	Block 1	ACA	0.132	0.018	5.31	1	0.02
	NTRK2	Block 3	TCG	0.865	0.679	6.35	1	0.01
		Block 3	CAG	0.045	0.177	5.74	1	0.02
	SLC6A2	Block 3	GCCAGT	0.134	0.012	6.79	1	0.009
MDD patients treated with fluoxetine	*ABCB1*	Block 2	GCGCACACGAGAC	0.503	0.682	4.08	1	0.04
	SLC6A4	Block 1	TAGC	0.000	0.085	8.37	1	0.004

Abbreviations: *ABCB1*, ATP-binding cassette subfamily B member 1; *CREB1*, cyclic AMP-responsive element binding protein 1; MDD, major depressive disorder; *NTRK2*, neurotrophic tyrosine kinase type 2 receptor; SNP, single nucleotide polymorphism.
[a]Letters with underline indicate the SNPs also showing an association with a nominal $P<0.05$ in the corresponding SNP-based analysis.

nominal $P\leqslant 0.01$: TCG in block 3 of *NTRK2* and GCCAGT in block 4 of *SLC6A2* ($P=0.009$) for desipramine-treated patients, and TAGC in block 1 of *SLC6A4* for fluoxetine-treated patients ($P=0.004$) (Table 5).

For quantitative outcome analysis of antidepressant treatment, 15 haplotypes were found to be associated with the relative reduction in HAM-D21 score after controlling for age, gender and baseline HAM-D21 score (Table 6). Among the 15 associated haplotypes, 2 in *SLC6A3* and 3 in *NTRK2* showed a correlation with a nominal $P<0.004$ in desipramine-treated patients, and 2 in *SLC6A2* showed an association with a nominal $P<0.008$ in fluoxetine-treated patients. The most significant association was found between *NTRK2* haplotype CAG (rs2289658, rs2289657 and rs2289656) and relative reduction of HAM-D21 score with a nominal $P=0.0002$ and an effect size of squared $R=0.20$ (Table 6).

Discussion

In this study, we analyzed the fine structure of seven genes that are relevant to the pathophysiology of MDD or to antidepressant response at four sequential levels: (1) entry into the brain, (2) binding to monoaminergic transporters, and (3) distal effects at the transcription level, resulting in (4) changes in neurotrophin and neuropeptide receptors. We observed new alleles in all seven genes in Mexican-Americans. We described a total of 204 novel SNPs (Table 1), which almost doubled the number of reported SNPs in these genes that was detected in these individuals (total of dbSNPs was 215). The number of novel SNPs identified in these Mexican-American subjects ranged from 12 to 57, and in the case of *CREB1*, the total number of SNPs tripled from 6 to 18. Most of the novel SNPs reported here had MAF lower than 5% (Supplementary Table 1). Higher nucleotide diversity was found in the exonic regions of these genes, particularly in UTRs (Figure 2b). Only a small number of the novel SNPs were in coding regions[19] and of those <40% (7) were non-synonymous. Analyses of HapMap data on four ethnic groups found different allele frequencies, with the greatest differences between Mexican Americans and Africans (Figure 1d).

Our analyses revealed nominal associations of eight SNPs and four haplotypes with susceptibility for MDD; those SNPs and haplotypes were located in four genes, *ABCB1*, *CREB1*, *NTRK2* and *SLC6A3*. In addition, eight SNPs in *SLC6A4* and one haplotype

Table 6 Haplotypes associated with relative reduction of HAM-D21 score after 8-week antidepressant treatment

Medication	*Gene*	*Block no.*	*Haplotype*[a]	N	β[b]	R^2 [c]	t	P
Desipramine or fluoxetine	*SLC6A3*	Block 1	CGCGAAGT	133	40.95	0.07	3.14	0.002
		Block 1	CGTGCGGT	133	17.01	0.05	2.69	0.008
	ABCB1	Block 3	TCTTACCGATCG	141	27.17	0.05	2.58	0.01
	NTRK2	Block 2	GCACGCCATTGGCAC	140	−5.85	0.03	−2.20	0.03
		Block 3	CAG	132	14.58	0.07	3.16	0.002
		Block 3	TCG	132	−7.49	0.04	−2.35	0.02
		Block 4	CA	134	−11.22	0.04	−2.36	0.02
	SLC6A2	Block 1	GATGAT	140	−15.88	0.04	−2.46	0.01
		Block 1	TAGGGT	140	10.00	0.03	2.10	0.04
Desipramine	*SLC6A3*	Block 1	CGTGCGGT	65	36.51	0.15	3.29	0.002
		Block 1	CGCGAGGT	65	−15.77	0.12	−2.99	0.004
	NTRK2	Block 2	GAACCCGCTCGGTAC	66	13.69	0.09	2.47	0.02
		Block 2	GCACGCCATTGGCAC	66	−9.68	0.08	−2.30	0.02
		Block 3	CAG	64	24.02	0.20	3.90	0.0002
		Block 3	TCG	64	−11.55	0.09	−2.49	0.02
		Block 4	CA	65	−20.49	0.15	−3.29	0.002
		Block 4	TA	65	22.05	0.13	3.02	0.004
	SLC6A4	Block 1	GAGA	67	10.65	0.08	2.37	0.02
Fluoxetine	*NTRK2*	Block 2	CCACCCCACCATCTC	72	14.21	0.06	2.16	0.03
	SLC6A2	Block 1	GATGAT	73	−18.92	0.10	−2.88	0.005
		Block 1	TAGGGT	73	14.54	0.10	2.73	0.008
		Block 2	GT	67	−13.66	0.06	−2.03	0.05
	SLC6A4	Block 1	GAAA	74	28.05	0.06	2.08	0.04

Abbreviation: *ABCB1*, ATP-binding cassette subfamily B member 1; HAM-D21, 21-item Hamilton Depression Rating Scale; *NTRK2*, neurotrophic tyrosine kinase type 2 receptor; SNP, single nucleotide polymorphism.

[a]Letters with underline indicate the SNPs also showing an association with a nominal $P<0.05$ in the corresponding SNP-based analysis.

[b]β: regression coefficient after controlling for age, gender and baseline HAM-D21 score.

[c]R^2: proportion variance explained.

in *SLC6A2* were also associated with MDD (Tables 2 and 5). However, some of these SNPs were not very common (MAF <0.03 in controls).

Nominal associations with several polymorphisms were also found for treatment response of 142 MDD patients who completed 8-week antidepressant treatment with desipramine or fluoxetine. Discrete outcome analyses (remitters vs non-remitters) showed that SNPs and haplotypes in *ABCB1* and *NTRK2* were associated with response. Variation in *SLC6A2* and one haplotype in *SLC6A4* were also associated with remission status. Quantitative outcome analyses showed that SNPs and haplotypes in *ABCB1*, *NTRK2* and *SLC6A2* were associated with relative HAM-D21 score reduction, but only two SNPs and one haplotype in *NTRK2* remained significant for desipramine treatment after correcting for multiple testing.

Our data show that variations in six out of seven genes were associated with MDD or antidepressant response. Briefly, (i) SNPs in *ABCB1* (located at 7q21.1), which is also called multidrug resistance 1, were associated with MDD and antidepressant response. *ABCB1* encodes a large transmembrane transporter protein that acts as an active efflux pump transporting a wide range of drugs from the brain to the blood. Polymorphisms in this gene have been reported to predict the response to antidepressant treatment to drugs that are substrates for this transporter.[21] (ii) SNPs in the *CREB1* gene were associated with MDD. *CREB* (cyclic AMP response element-binding protein, located at 2q32.2-q34) encodes a transcription factor that modulates key growth factors important for synaptogenesis and neurogenesis. Sequence variations in the promoter and intronic regions of the *CREB1* gene have previously been described to be cosegregated with mood disorders in women.[22] 3) SNPs in *NTRK2* (located at 9q22.1) were associated with susceptibility to MDD and antidepressant response. Furthermore, two SNPs and one haplotype in *NTRK2* continued to be significantly associated with relative reduction of HAM-D21 scores in the desipramine-treated group, after controlling for age, gender and baseline HAM-D21 scores. *NTRK2*, also known as tyrosine kinase receptor B, and its ligand, brain-derived neurotropic factor, regulate short- and long-term synaptic functions and neural plasticity. *NTRK2* variants have been recently associated with obsessive-compulsive

disorder in female patients.[23] (iv) SNPs in *SLC6A2* (noradrenaline transporter, located at 16q12.2) were associated with remission status and relative reduction of HAM-D21 scores, and one haplotype in this gene (ACCAGA) was associated with MDD. *SLC6A2* gene encodes a transporter, which regulates norepinephrine (noradrenaline) homeostasis and the reuptake of norepinephrine into presynaptic nerve terminals.[24] *SLC6A2* polymorphisms have been reported to be associated with depression[25,26] and response to antidepressants.[27,28] (v) SNPs or haplotypes in *SLC6A3* (dopamine transporter or DAT1, located at 5p15.33), which encodes a transporter that is important in dopaminergic neurotransmission, were associated with risk for MDD or relative reduction of HAM-D21. This transporter mediates the active re-uptake of synaptic dopamine.[29] Variations in this gene have already been implicated in susceptibility for mood disorders[30,31] and antidepressant action.[32] Other neuropsychiatric conditions have also been associated with SLC6A3, such as parkinsonism,[33] attention-deficit hyperactivity disorder,[34,35,36] Tourette's syndrome and addictive behavior.[37,38] 6) *SLC6A4* (serotonin transporter, located at 17q11.1-q12) encodes a transporter, which mediates antidepressant action, and behavioral effects of cocaine and amphetamines. Sequence variations in *SLC6A4* have been extensively queried and they may be associated with several neuropsychiatric conditions, including MDD,[39,40,41] anxiety-related personality traits[42] and antidepressant response.[43,44] Our findings support that variations in *SLC6A4* are associated with MDD risk. Haplotypes in *SCL6A4* have also been associated with remission status and reduction of HAM-D21 scores.

The analyses presented here have not shown that variations in *CRHR1* (located at 17q12-q22) gene are associated with susceptibility to MDD or antidepressant response. It can be noted that the current analyses have not taken anxiety levels into consideration. *CRHR1* encodes the receptor of CRH, a key stress hormone that regulates the response to stress at the behavioral, immune, autonomic and neuroendocrine levels, through the activation of the hypothalamic–pituitary–adrenalaxis. Polymorphisms in *CRHR1* were reported to be associated with antidepressant response, but only when anxiety scores are taken in consideration,[45,46] and with seasonal pattern and early onset of first depressive episode.[47]

In summary, we show that substantial levels of sequence variation, especially those that are not very common (MAF $>5\%$), are likely to be found in candidate genes in an ethnically defined and understudied group. In this population group, for example, half of the SNPs detected were novel. Therefore, deep sequencing data may be relevant to our understanding of common and complex disorders, such as major depression, particularly in minority populations. Our analyses showed that several sequence variations and haplotypes in six out of seven selected genes were nominally associated with MDD risk and/or antidepressant treatment response and that after controlling for age, gender and baseline HAM-D21 score, as well as correcting for multiple testing, there was a significant association of antidepressant response with two *NTRK2*-coding SNPs and one haplotype. Our findings suggest that these variants may be implicated in the pathophysiology of MDD. The Mexican Americans are the most rapidly growing population group in the United States, but remain under represented in research studies. These results highlight the importance of direct re-sequencing of key candidate genes in ethnic minority groups in order to discover novel genetic variants that cannot be simply inferred from existing databases.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Chapter VI. Reprint

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Article

Clinical Outcomes and Genome-Wide Association for a Brain Methylation Site in an Antidepressant Pharmacogenetics Study in Mexican Americans

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Objective: The authors compared the effectiveness of fluoxetine and desipramine treatment in a prospective double-blind pharmacogenetics study in first-generation Mexican Americans and examined the role of whole-exome functional gene variations in the patients' antidepressant response.

Method: A total of 232 Mexican Americans who met DSM-IV criteria for major depressive disorder were randomly assigned to receive 8 weeks of double-blind treatment with desipramine (50–200 mg/day) or fluoxetine (10–40 mg/day) after a 1-week placebo lead-in period. Outcome measures included the Hamilton Depression Rating Scale (HAM-D), the Hamilton Anxiety Rating Scale, and the Beck Depression Inventory. At week 8, whole-exome genotyping data were obtained for 36 participants who remitted and 29 who did not respond to treatment.

Results: Compared with desipramine treatment, fluoxetine treatment was associated with a greater reduction in HAM-D score, higher response and remission rates, shorter time to response and remission, and lower incidences of anticholinergic and cardiovascular side effects. Pharmacogenetics analysis showed that exm-rs1321744 achieved exome-wide significance for treatment remission. This variant is located in a brain methylated DNA immunoprecipitation sequencing site, which suggests that it may be involved in epigenetic regulation of neuronal gene expression. This and two other common gene variants provided a highly accurate cross-validated predictive model for treatment remission of major depression (receiver operating characteristic integral=0.95).

Conclusions: Compared with desipramine, fluoxetine treatment showed a more rapid reduction of HAM-D score and a lower incidence of side effects in a population comprising primarily first-generation Mexican Americans with major depression. This study's pharmacogenetics approach strongly implicates the role of functional variants in antidepressant treatment response.

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Efficacy and side effect data for psychotropic medications in the United States have been investigated primarily in non-Hispanic Caucasian populations. Currently, the Hispanic population is the largest ethnic minority group in the country, representing over 37 million people (1). Within this group, almost 70% are Mexican Americans. Although this population is growing dramatically, there is insufficient research regarding psychiatric diagnosis and treatment in this group (2).

Major depressive disorder is a serious public health problem worldwide, with a lifetime prevalence of 10%–20% in the general population (3). Vega et al. (4) reported that the lifetime prevalence of major depression in U.S.-born Mexican Americans is 14.8%. Although Hispanics have participated in antidepressant treatment studies, it has been difficult to ascertain whether there are any major differences in antidepressant efficacy in that population, for several reasons, including methodological differences among studies, small sample sizes, and the inclusion of several Hispanic subgroups in an attempt to illustrate a "Hispanic response" (5).

A retrospective review (6) suggested that pharmacokinetic factors play a role in the differential sensitivity to tricyclic antidepressants in depressed Puerto Rican American females as compared with Anglo females, resulting in greater efficacy, higher rates of adverse drug reactions, and higher dropout rates in the former group. In an open-label study, nefazodone (7) was found to have similar efficacy but a higher dropout rate in a predominantly Hispanic Caribbean female sample as compared with non-Hispanics from previous nefazodone trials. An open-label study of two selective serotonin reuptake inhibitors (SSRIs) (8) found that although the drugs were comparable in efficacy, Mexican American females had a higher dropout rate even though there were more severe adverse drug reactions reported in the non-Hispanic group. A more recent study of an SSRI reported no differences in efficacy, rates of adverse drug reactions, or dropout rates between Hispanic and non-Hispanic patients with HIV (9).

Three recent meta-analyses on the pharmacogenetics of antidepressants in major depression were not able to show genome-wide significant variations (10–12). The nonsynonymous single-nucleotide polymorphism (SNP) rs6265 in the *BDNF* (brain-derived neurotrophic factor) gene may have a minor impact on susceptibility to major depression

(13) and antidepressant drug response (11); however, the overall conclusion of these meta-analyses was that the results do not support any major effect of any single gene variation in the pharmacogenetics of antidepressants in major depression.

In the present study, we focused on functional SNPs, based on 1) the likely significance of a functional SNP in the *BDNF* gene (rs6265) in the genetics and pharmacogenetics of major depression; 2) our own recent work proposing a predictive framework for the diagnosis of major depressive disorder using interactions of multiple functional SNPs and environmental factors (14); and 3) a growing body of evidence supporting the involvement of epigenetic mechanisms in major depression and antidepressant action (15–19). Here we present data on the efficacy and adverse drug reaction profiles of desipramine and fluoxetine, two extensively used off-patent antidepressants, and new pharmacogenetic leads that could advance our understanding of genetic variants implicated in antidepressant treatment response.

Method

The study protocol was approved by the University of California Los Angeles and University of Miami institutional review boards and the Australian National University Human Ethics Committee. This was a single-site prospective double-blind 8-week trial with fluoxetine and desipramine conducted in the greater Los Angeles area. All study participants had an initial medical evaluation consisting of a detailed history, physical examination, and blood collection for routine testing and genotyping, followed by two consecutive study phases: a 1-week single-blind placebo lead-in phase to minimize the effect of placebo response, and subsequent random assignment to receive either 10–40 mg/day of fluoxetine or 50–200 mg/day of desipramine, with weekly follow-up visits to assess clinical status. Participants provided written informed consent after receiving a complete description of the study.

Given the proven efficacy of these antidepressant medications, we used a placebo lead-in period followed by active treatment for all patients in order to minimize risk to participants (20, 21).

Participants

All participants met the following inclusion criteria (22): at least three of their four grandparents born in Mexico (23); a DSM-IV diagnosis of current unipolar major depressive episode; a score ≥18 on the 21-item Hamilton Depression Rating Scale (HAM-D) (24), with item 1 (depressed mood) rated ≥2; and age between 18 and 70 years. Exclusion criteria were any axis I disorder other than major depressive disorder or primary anxiety disorders; an active medical illness that could be related to the ongoing depression (e.g., untreated hypothyroidism, myocardial infarction or cerebrovascular incident within the previous 6 months, uncontrolled hypertension or diabetes); current suicidal ideation with a plan and strong intent, or recent serious suicide attempt; history of ECT in the previous 6 months; current use of medications with CNS activity that interferes with EEG activity or any other antidepressant treatment within 2 weeks before enrollment; history of poor response to treatment with desipramine or fluoxetine; illicit drug use or alcohol abuse in the previous 3 months; and current enrollment in counseling or psychotherapy treatment. In addition, women who were pregnant or lactating or were of childbearing age and not using contraception were excluded.

Recruitment and Outcome Measures

Participants were recruited by advertisements in bilingual newspapers, radio, and television. Informed consent forms, questionnaires, and assessment scales were given in their preferred language (English or Spanish). In addition, clinical staff also participated in health fairs, conferences, and cable network programs through which they recruited participants, and some participants were referred by regional outpatient community clinics.

The presence of a current major depressive episode was determined by the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID) (25) (mean kappa score for sensitivity and specificity among raters, 0.84–0.85), and diagnoses were confirmed by a research psychiatrist. Symptom severity was rated by experienced bilingual clinical personnel using Spanish or English versions of the HAM-D (24), the Hamilton Anxiety Rating Scale (HAM-A) (26), the Global Assessment Scale (GAS) (27), the Beck Depression Inventory (BDI) (28), and the Center for Epidemiologic Studies Depression (CES-D) Scale (29). Participants also completed an acculturation questionnaire to determine their language preference, education level, and generation status.

Interventions and Treatment

All participants received 1 week of single-blind placebo to minimize the effect of placebo response, which was defined as a decrease of 25% or more in HAM-D score compared with the screening visit or a HAM-D score <18. Those who did not show a placebo response were randomly assigned in a 1:1 ratio to receive either fluoxetine or desipramine for an 8-week double-blind phase. Participants initially received 10 mg/day of fluoxetine or 50 mg/day of desipramine, which increased at week 2 to 20 mg/day of fluoxetine or 100 mg/day of desipramine. At week 4, for participants who had less than a 25% decrease in their HAM-D score, dosages were increased to 30 mg/day of fluoxetine or 150 mg/day of desipramine. At week 6, for participants whose HAM-D score was >12, dosages were increased to 40 mg/day of fluoxetine or 200 mg/day of desipramine. The staff and participants were aware of dosage escalation, which occurred only if the previous dosage was well tolerated, but they were blind to the drug. At the end of the study, participants were referred to a psychiatric clinic of their choice for follow-up treatment. Random antidepressant blood levels were collected to ascertain medication adherence but not to obtain therapeutic levels.

Statistical Analysis

Analyses were performed using SAS, version 9.1.3 (SAS Institute, Cary, N.C.), and included all participants who received at least 1 week of study drug. The primary outcome measure was change in HAM-D score from week 0 to week 8. The secondary outcomes of interest were change in HAM-A, BDI, CES-D Scale, and GAS scores. Remission was defined as a HAM-D score <8, response was defined as a reduction of ≥50% in HAM-D score, and nonresponse was defined as a reduction of <50% in HAM-D score. Remission and response were compared between treatment groups. Student's t test was used to compare the mean values for age, acculturation score, and baseline clinical measurements, and chi-square or Fisher's exact test was used to compare the percentages of demographic characteristics and side effect events.

For repeated continuous outcome measure analyses, the likelihood-based mixed-effects model as the primary analysis of efficacy was used to assess differences between treatment groups in changes from baseline across 8 weeks. The model included the categorical effects of treatment, treatment week, treatment-by-week interaction, and gender, as well as continuous covariates of

TABLE 1. Least Squares Mean of Changes From Baseline in Mexican American Patients With Major Depression Who Received Desipramine or Fluoxetine[a]

	HAM-D			HAM-A			BDI			CES-D Scale			GAS		
Week and Treatment	N	LSM	SE	N	LSM	SE	N	LSM	SE	N	LSM	SE	N	LSM	SE
Week 1															
Desipramine	111	–1.5	0.5	107	–0.8	0.6	100	–1.9	0.6	109	–1.0	0.8			
Fluoxetine	120	–2.0	0.5	116	–1.3	0.6	111	–1.6	0.6	115	–1.8	0.8			
Week 2															
Desipramine	105	–4.2	0.5	102	0.2	1.5	96	–4.9	0.7	100	–3.1	0.8			
Fluoxetine	113	–4.3	0.5	111	–3.9	1.5	104	–3.4	0.7	106	–4.1	0.8			
Week 3															
Desipramine	99	–6.2	0.5	98	–2.8	0.6	92	–6.8	0.8	95	–5.7	0.8			
Fluoxetine	110	–6.0	0.5	105	–4.4	0.6	97	–6.5	0.8	104	–6.1	0.8			
Week 4															
Desipramine	97	–7.3	0.5	95	–3.8	0.6	88	–8.5	0.9	92	–7.0	0.8	96	7.7	0.9
Fluoxetine	106	–7.6	0.5	101	–5.1	0.6	96	–7.8	0.9	103	–7.1	0.8	103	8.7	0.9
Week 5															
Desipramine	86	–9.3	0.6	88	–5.7	0.7	84	–10.1	0.9	82	–8.5[b]	0.9			
Fluoxetine	100	–9.8	0.5	97	–7.5	0.7	95	–10.3	0.9	91	–11.1[b]	0.8			
Week 6															
Desipramine	82	–10.0	0.6	81	–5.8[b]	0.7	77	–9.7[b]	0.9	75	–8.6[c]	0.9			
Fluoxetine	95	–11.1	0.5	89	–8.2[b]	0.7	88	–12.1[b]	0.8	86	–11.9[c]	0.8			
Week 7															
Desipramine	75	–10.5[d]	0.6	74	–6.1[d]	0.7	75	–11.8[b]	0.8	71	–10.2[c]	0.9			
Fluoxetine	93	–13.2[d]	0.5	88	–9.7[d]	0.7	83	–14.2[b]	0.8	86	–13.7[c]	0.9			
Week 8															
Desipramine	74	–12.0[d]	0.6	72	–7.4[d]	0.7	71	–12.2[b]	0.9	72	–11.1	0.9	64	18.5[c]	1.0
Fluoxetine	92	–14.6[d]	0.5	90	–10.6[d]	0.6	85	–15.3[b]	0.9	81	–13.4	0.9	83	22.9[c]	0.9

[a] Mixed-model repeated-measures analyses included all available observations for all participants at all time points. Least squares mean change from baseline was calculated after adjustment for gender, age, and baseline score. HAM-D=Hamilton Depression Rating Scale; HAM-A=Hamilton Anxiety Rating Scale; BDI=Beck Depression Inventory; CES-D Scale=Center for Epidemiologic Studies Depression Scale; GAS=Global Assessment Scale; LSM=least squares mean.
[b] Significant difference between treatment groups, $p \leq 0.05$.
[c] Significant difference between treatment groups, $p \leq 0.01$.
[d] Significant difference between treatment groups, $p \leq 0.001$.

baseline score and age. Mixed-model repeated-measures analyses of changes from baseline were conducted using PROC MIXED in SAS. A macro was written in SAS for covariance structure selection by comparing Akaike's information criterion and Schwarz's Bayesian criterion using compound symmetry, unstructured, first-order autoregressive (AR[1]), and Huynh-Feldt covariance structures based on the "smaller is better" criterion. For each outcome comparison, the covariance structure was used if the sum of Akaike's information criterion and Schwarz's Bayesian criterion was smallest.

For repeated analyses of dichotomous outcomes (remission and response), the modified Poisson regression model with robust error variance estimated by the generalized estimating equation approach (30) was performed with PROC GENMOD in SAS to estimate the adjusted relative risk. To compare the time to response or remission between the two treatment groups, Cox regression analysis was performed with PROC TPHREG in SAS using the DISCRETE option to handle ties in event time. Gender, age, and baseline scores were included as covariates in all models. For all analyses, the threshold for significance was a p value ≤ 0.05 (two-sided).

Pharmacogenetics Procedures

Whole-exome genotyping. Genomic DNA of 65 participants who completed the 8-week treatment course (36 of whom had remitted at week 8 and 29 of whom had not responded) was subjected to whole-exome genotyping, performed by the Australian Genome Research Facility (Melbourne), an Illumina Certified Service Provider for the Infinium Genotyping Service. We used the Illumina HumanExome-12v1_A BeadChip, which covers putative functional exonic variants selected from over 12,000 individuals. The exonic content consists of >250,000 markers representing diverse populations (including European, African, Chinese, and Hispanic individuals) and a range of common conditions, such as type 2 diabetes, cancer, metabolic disorders, and psychiatric disorders. Samples with calls below the Illumina-expected 99% SNP call rate were excluded. To test genotyping reliability and quality, an individual was duplicated. The identity by descent between all pairs of individuals was estimated and used for quality control.

Quality control and filtering. GenomeStudio data were imported to SVS, version 7.6.7 (Golden Helix, Bozeman, Mont.; http://www.goldenhelix.com), an integrated collection of analytic tools for managing, analyzing, and visualizing multifaceted genomic and phenotypic data.

Parameters for excluding markers from analyses included 1) deviations from the Hardy-Weinberg equilibrium with $p<2\times10^{-7}$ (0.05/250.000 markers) in both case and control subjects (this avoids the exclusion of major effect causal variants); 2) a genotype call rate <90%; 3) more than two alleles; and 4) monoallelism. Genotype and allelic frequencies were estimated by maximum likelihood.

Genetic stratification analysis. We estimated the inbreeding coefficient in order to detect the presence of hidden biological relatives in the sample, which might reduce the independence of the data. The fixation index between pairs of subpopulations,

TABLE 2. Remission and Response Rates in Mexican American Patients With Major Depression Who Received Desipramine or Fluoxetine[a]

		Remission				Response			
Week and Treatment	N	Rate (%)	Relative Risk	95% CI	p	Rate %	Relative Risk	95% CI	p
Week 1									
Desipramine	111	0.9		Reference		1.7		Reference	
Fluoxetine	120	1.7	1.90	0.17–21.08	0.600	3.3	1.87	0.35–10.10	0.466
Week 2									
Desipramine	105	3.7		Reference		11.9		Reference	
Fluoxetine	113	3.5	0.95	0.24–3.72	0.936	9.4	0.79	0.37–1.68	0.534
Week 3									
Desipramine	99	3.9		Reference		15.2		Reference	
Fluoxetine	110	5.5	1.40	0.40–4.84	0.598	14.9	0.98	0.52–1.83	0.945
Week 4									
Desipramine	97	4.4		Reference		21.6		Reference	
Fluoxetine	106	10.2	2.33	0.81–6.69	0.117	30.7	1.42	0.90–2.26	0.135
Week 5									
Desipramine	86	11.9		Reference		36.0		Reference	
Fluoxetine	100	19.9	1.67	0.84–3.33	0.143	50.2	1.39	1.00–1.94	0.049
Week 6									
Desipramine	82	21.4		Reference		44.0		Reference	
Fluoxetine	95	25.0	1.17	0.68–2.00	0.575	59.4	1.35	1.02–1.79	0.036
Week 7									
Desipramine	75	31.3		Reference		59.0		Reference	
Fluoxetine	93	51.0	1.63	1.11–2.39	0.012	75.1	1.27	1.04–1.56	0.020
Week 8									
Desipramine	74	43.1		Reference		60.7		Reference	
Fluoxetine	92	59.1	1.37	1.01–1.87	0.046	79.7	1.31	1.09–1.59	0.005

[a] Generalized estimating equation model for repeated-measures analysis included all available observations. Rates were estimated after adjustment for gender, age, and baseline score.

case subjects, and control subjects was estimated to evaluate the potential presence of genotype stratification (microdifferentiation), a common cause of spurious associations. Independently, an additional correction of putative population stratification was applied with 10 principal-components analyses to normalize genotypic data by its actual standard deviation.

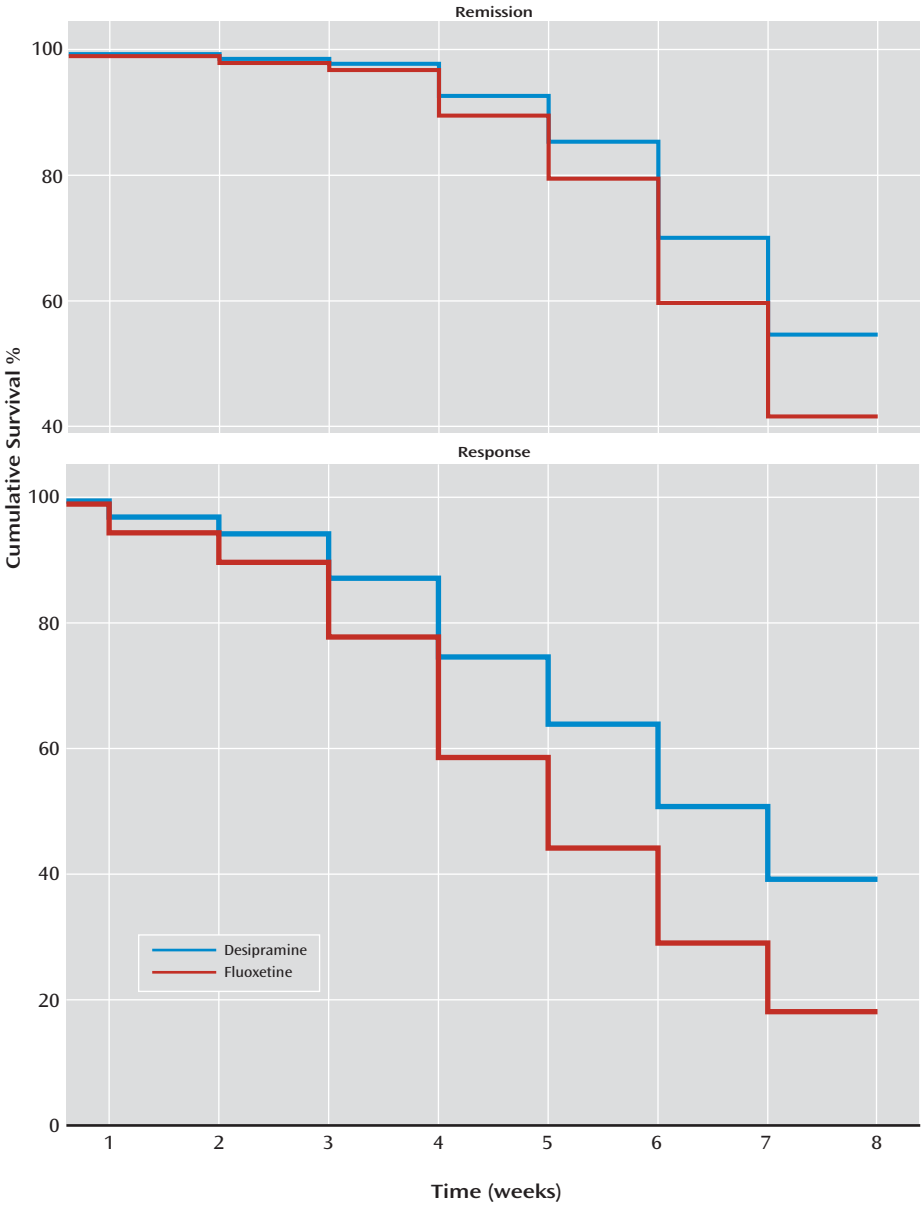
Exome-wide association analysis. The genotypic (additive model) and allelic tests of association were applied. Multiple test correction to determine exome-wide significance was performed using the false-discovery-rate approach. Mixed linear models were applied as a tool to include in the analyses fixed factors (sex, age, treatment) and random effects (family or population structure) and to contrast with other analytical tools, different from principal-components analysis, the effects of potential inbreeding (by inclusion of the kinship matrix as defined by identity by descent). We applied the single-locus mixed model (which assumes that all loci have a small effect on the trait) and the multilocus mixed model (which assumes that several loci have a large effect on the trait) as implemented in SVS. Linkage disequilibrium analysis was also implemented using SVS.

Advance recursive partitioning (tree-based) approach (ARPA). Rao has suggested that recursive partitioning techniques should be highly recommended for genetic dissection of complex traits (31). ARPA is widely used in predictive analyses, as it accounts for nonlinear and interaction effects, offers fast solutions to reveal hidden complex substructures, and provides truly nonbiased statistically significant analyses of seemingly unrelated high-dimension data (14).

ARPA accounts for the effect of hidden interactions better than alternative methods and is independent of the type of data (categorical, continuous, ordinal, etc.) and data distribution type (normal or nonnormal) (31). Furthermore, results supplied by tree-based analytics are easy to interpret visually and logically (14). Therefore, to generate the most comprehensive and parsimonious classificatory model to predict remission of major depression at end of treatment, we applied ARPA using a set of different modules implemented in the Salford Predictive Modeler (SPM) software package, namely, CART, random forest, and Tree-Net (http://www.salford-systems.com). SPM is a highly accurate and ultra-fast analytics and data-mining platform for creating predictive, descriptive, and analytical models from databases of any size, complexity, or organization. One important advantage of SPM compared with other available software is that it can use raw data with sparse or empty cells (a common problem when dealing with genetic data).

CART is a nonparametric approach in which a series of recursive subdivisions separates the data by dichotomization (32). The aim is to identify, at each partition step, the best predictive variable and its best corresponding splitting value while optimizing a splitting statistical criterion so that the data set is successfully divided into increasingly homogeneous subgroups (32). We used a battery of different statistical criteria as splitting rules (e.g., Gini index, entropy, and twoing) to determine the splitting rule that decreased the relative cost of the tree the most while increasing the prediction accuracy of target variable categories (32). The best split at each dichotomous node was chosen by either a measure of between-node dissimilarity or iterative hypothesis testing of all possible splits to find the most homogeneous split (lowest impurity) (32). Similarly, we used a wide range of empirical probabilities (priors) to model numerous scenarios recreating the distribution of the targeted variable categories in the population (32). Following this iterative process, each terminal node was assigned to a class outcome (remitter or nonresponder).

FIGURE 1. Survival Curves Showing Time to Remission or Response for Mexican American Patients With Major Depression Who Received Desipramine (N=112) or Fluoxetine (N=120)



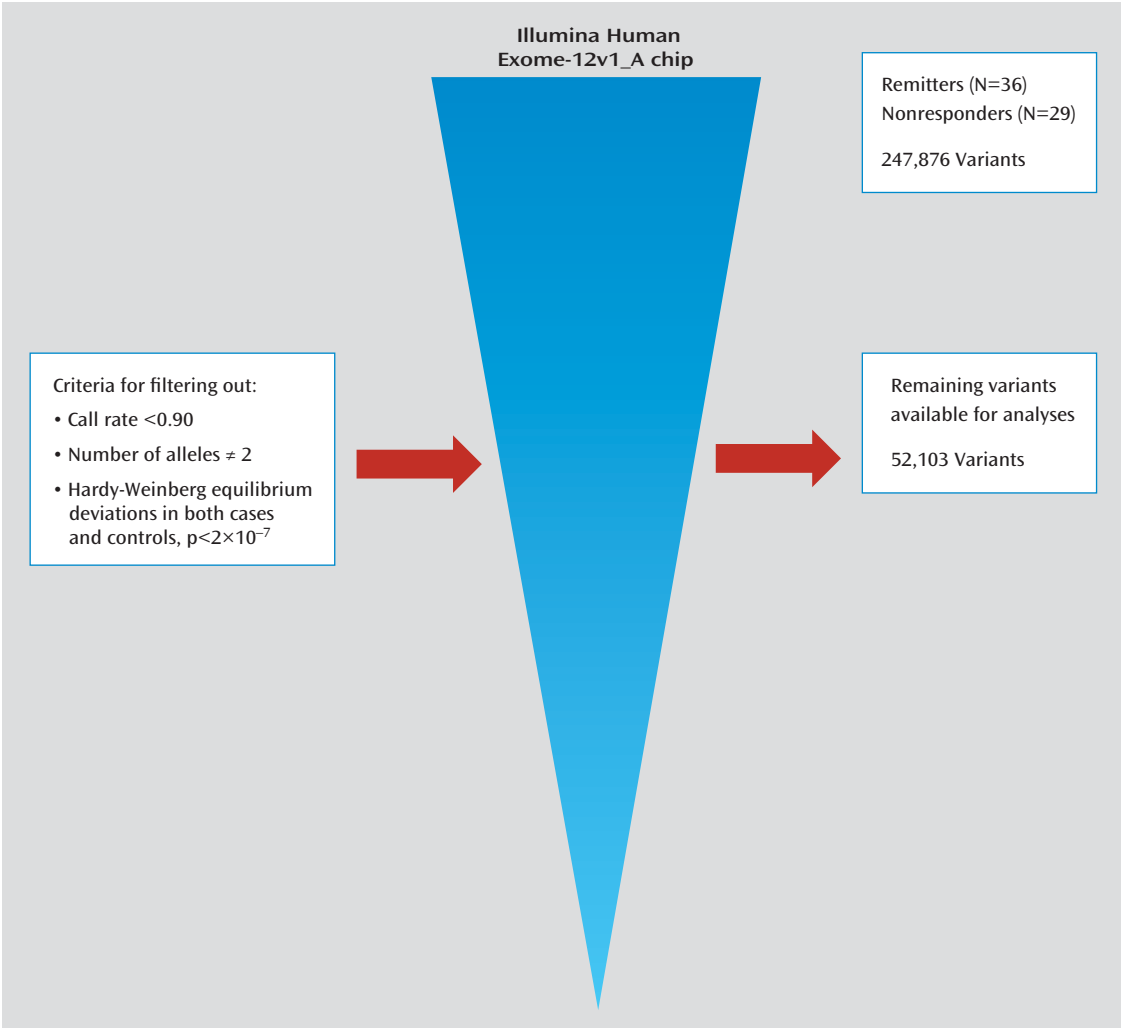
To avoid finishing with an overfitted CART predictive model (a common problem in CART analysis) and to ensure that the final splits were well substantiated, we applied tree pruning (32). During this procedure, predictor variables that were close competitors (surrogate predictors with comparable overall classification error to the optimal predictors) were pruned to eliminate redundant commonalities among variables, so the most parsimonious tree would have the lowest misclassification rate for an individual not included in the original data (32).

Furthermore, to exactly identify the most important set of variables predicting remission of major depression, we applied the random forest method using a bagging strategy (33). The random forest strategy differs from CART in the use of a limited number of variables to derive each node while creating 100 to 1000 trees (33). This strategy has proved to be immune to the overfitting generated by CART (33). In the random forest strategy, variables that appeared repeatedly as predictors in the trees were identified. The misclassification rate was recorded for each approach.

The TreeNet strategy was used to complement the CART and random forest analyses because it reaches a level of accuracy that is usually not attainable by single models (CART) or by ensembles such as bagging (random forest) (34). The TreeNet algorithm generates thousands of small decision trees built in a sequential error-correcting process converging on an accurate model (34).

To obtain honest assessments of the derived models and have a better view of their performance on future unseen data, we applied a cross-validation strategy in which both training with all the data and then indirect testing with all the data were performed. To do so, we randomly divided the data into 10 separate partitions (folds). This strategy allowed us to conduct a cross-validation and review the stability of results across multiple replications (32). Cross-validation is a model validation technique for assessing how the results of a statistical analysis will generalize to an independent

FIGURE 2. Process Used to Filter Out Single-Nucleotide Polymorphism (SNP) Exomic Variants in Mexican American Patients With Major Depression Who Received Desipramine or Fluoxetine, to Evaluate Pharmacogenetic Response as Remitters (N=36) and Nonresponders (N=29)[a]



[a] From 247,876 SNPs, 195,773 were discarded because they were monoallelic, because they had more than two alleles, because they had a call rate <90%, or because their genotype proportions deviated from the expected ones as defined by the Hardy-Weinberg equilibrium theorem in both case subjects (remitters) and control subjects (nonresponders) at a p value $<2\times10^{-7}$. The remaining 52,103 SNPs were used for exome-wide association analysis.

data set; the n-fold cross-validation technique is designed to get the most out of data sets that are too small to accommodate a holdout or test sample. For our specific problem, we used a maximization algorithm that allowed us to validate the entire original results of our genome analysis by considering n-fold subsamples of the original set. So we used cross-validation to be able to both train with all the data and then indirectly test with all the data as well.

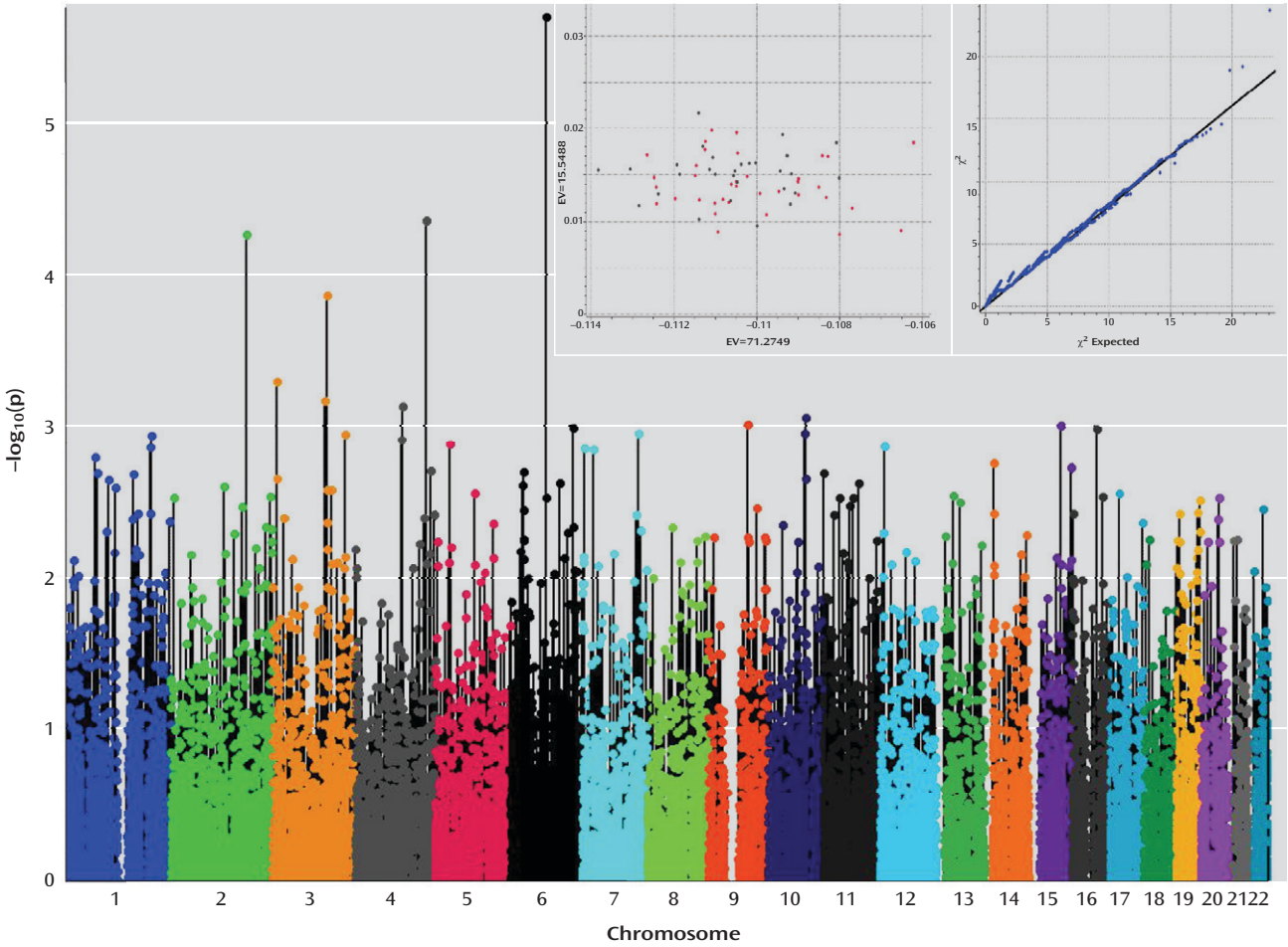
We also applied a categorical approach to link the set of genotypes of the associated marker (rs1321744) by using latent class analysis to identify unobservable subgroups within the subset of completers who were genotyped (remitters and nonresponders).

Results

Disposition and Baseline Characteristics of Patients

After assessing 4,323 people for eligibility by telephone, we scheduled 1,223 structured clinical interviews (a diagram of participant flow in the study is presented in Figure S1 in the data supplement that accompanies the online edition of this article). Of these, 885 individuals either were disqualified for the study by the SCID results or declined to participate, and a total of 338 participants were enrolled. Of those enrolled, 232 received at least one dose of study drug (112 in the desipramine group and 120 in the fluoxetine group) and were included in the intent-to-treat analysis; 166 participants completed 8 weeks of treatment and 66 did not complete the study because they dropped out, were nonadherent, or were terminated (34% and 23% in the desipramine and fluoxetine groups, respectively, not a statistically significant difference). No significant differences were found in mean retention time in noncompleters or in baseline demographic and clinical characteristics between treatment groups (see Table S1

FIGURE 3. Manhattan Plot to Evaluate Pharmacogenetic Response in Mexican American Patients With Major Depression Who Received Desipramine or Fluoxetine, as Remitters (N=36) and Nonresponders (N=29)[a]



[a] Genotype model (additive) after correcting stratification by principal-components analysis was used. A unique significant peak at chromosome 6 remained significant after correction for multiple comparisons by false discovery rate. In the inset, the principal-components analysis shows absence of stratification between remitters and nonresponders (left box), and a Q-Q plot depicts the fitting of chi-square against the chi-square expected distribution (right box). EV=eigenvalue.

in the online data supplement). Our participants were primarily (60%) first-generation Spanish-speaking-only Mexican Americans with 6–12 years of education (see Table S1).

Primary Outcome Measure

Table 1 summarizes the mean HAM-D score changes (least squares means after adjustment for age, gender, and baseline score). Mixed-model repeated-measures analysis revealed a between-subject effect of treatment ($F=4.45$, df=1, 227, $p=0.036$; least squares mean change difference=1.0, 95% CI=0.1–1.9), within-subject effect of treatment time ($F=76.38$, df=1, 1312, $p<0.001$), and no interaction between treatment and time. At endpoint (week 8), fluoxetine showed an advantage over desipramine of 2.6 points on the HAM-D score (−14.6 compared with −12.0) from baseline after adjustment for age, gender, and baseline score (95% CI=1.1–4.2; $F=10.71$, df=1, 131, $p=0.001$). No gender-specific effect was found on HAM-D score reductions, but significant effects were observed in baseline HAM-D scores (F values ≥23.27, df=1, 227, p values <0.001). Mixed-model repeated-measures analysis revealed an age effect on reduction in HAM-D score (coefficient=−0.1; $F=5.89$, df=1, 227, $p=0.016$).

Table 2 presents the response and remission rates after adjusting for age, sex, and baseline HAM-D score. At endpoint, response and remission rates were significantly 1.3–1.4 times higher in the fluoxetine group than in the desipramine group. Survival analysis (using a Cox regression model including all participants with response or remission or right-censoring time) revealed that fluoxetine was associated with a shorter time to response (hazard ratio=2.01, 95% CI=1.40–3.13; $\chi^2=13.08$, df=1, $p<0.001$) and a shorter time to remission (hazard ratio=1.57, 95% CI=0.98–2.51; $\chi^2=3.54$, df=1, $p=0.060$) than desipramine (Figure 1).

Secondary Outcome Measures

The results of mixed-model repeated-measures analysis revealed a significant between-subject effect of treatment

TABLE 3. Exome-Wide Association Analysis Results[a]

Marker	Chr	Position	Reference/Alternate Allele	Major Allele	p (Fisher's Exact Test)	FDR-Corrected p (Fisher's Exact Test)	Allele Frequency (Cases)
exm-rs1321744[b,c]	6	85,891,744	T/C	C	1.98E–06	0.05	0.19
exm-rs16867321[c]	2	181,362,379	C/T	C	5.37E–05	0.42	0.58
exm433050	4	169,083,694	A/C	A	4.41E–05	0.51	0.50
exm-rs6583826[c]	10	94,347,830	G/A	A	8.85E–04	1.00	0.57
exm2259477[c]	9	93,072,009	C/T	C	9.88E–04	1.00	0.57
exm2265531[c]	3	174,131,106	A/G	A	1.13E–03	1.00	0.39
exm660951[c]	7	138,455,988	A/G	G	1.13E–03	1.00	0.39
exm345346[c]	3	124,731,689	T/A	T	6.76E–04	1.00	0.31
exm2270591	7	27,223,563	G/T	T	1.43E–03	1.00	0.49
exm-rs3729931	3	12,626,516	G/A	A	5.04E–04	1.00	0.54

[a] FDR=false discovery rate; HGVS=Human Genome Variation Society.
[b] Exome-wide significance.
[c] MeDIP-seq raw signal indicates that this is a DNA methylation site (UCSC Genome Browser, http://genome.ucsc.edu/). Data from postmortem human frontal cortex gray matter of a 57-year-old male. This was done to investigate the role of intragenic, tissue-specific CpG island methylation plays in controlling gene expression. Hardy-Weinberg equilibrium exact p values were greater than 0.05.

on the difference in mean change in HAM-A, BDI, and GAS scores (Table 1). The fluoxetine group showed greater improvement on HAM-A, BDI, CES-D Scale, and GAS scores than the desipramine group. Significant interactive effects of treatment and time were observed in BDI scores ($F=2.09$, df=7, 199, $p=0.005$) and GAS scores ($F=4.92$, df=7, 135, $p=0.028$). The fluoxetine group had a smaller reduction in BDI score in the first 4 weeks and a greater reduction in the last 3 weeks. CES-D Scale scores showed a treatment effect ($F=5.30$, df=1, 211, $p=0.022$) at weeks 5–7, but not at week 8. Baseline scores showed significant effects on specific score changes ($p<0.05$). No gender effect was observed, and age showed a significant effect only on increase in GAS score (mixed-model repeated-measures analysis: $F=4.59$, df=1, 190, $p=0.033$; last observation carried forward model: $F=10.39$, df=1, 198, $p=0.002$).

Adverse Drug Reactions

The data on adverse drug reactions indicated that 78% (182/232) of participants had one or more side effects (see Table S2 in the online data supplement). There was no significant difference in the overall occurrence of adverse drug reactions between treatment groups (90/112 in the desipramine group and 92/120 in the fluoxetine group). However, compared by category, patients treated with desipramine had significantly higher rates of anticholinergic adverse drug reactions ($\chi^2=4.96$, df=1, $p=0.026$), cardiovascular adverse drug reactions ($\chi^2=7.22$, df=1, $p=0.007$), perspiration, tingling/paresthesias, blurred vision, orthostatic hypotension (by pulse), and palpitations (p values <0.05).

Exome-Wide Association Analysis

After we filtered out markers that did not meet either quality control criteria or variability requirements, 52,103 variants remained for further analysis (Figure 2). The estimation of the fixation index coefficient reported a value of 0.001 that according to Price et al. (35) may be corrected by principal-components analysis using 50,000 SNPs. After principal-components analysis correction, we found that a single SNP marker located on chromosome 6 (exm-rs1321744) achieved exome-wide significance ($p=1.98\times10^{-6}$; false-discovery-rate-corrected $p=0.05$) (see Figure 3 and Table 3). This marker is located in a methylated DNA immunoprecipitation sequencing site (MeDIP-seq raw data) obtained using postmortem human frontal cortex gray matter (UCSC Genome Browser; http://genome.ucsc.edu), which suggests that it is involved in epigenetic regulation of neuronal gene expression. Interestingly, other top markers, which did not attain exome-wide significance, are also located in methylated DNA sites (Table 3). Linkage disequilibrium block harboring exm-rs1321744 does not harbor any gene; however, there are three genes surrounding exm-rs1321744: *TBX18*, *NT5E*, and *SNX14*.

Advance Recursive Partitioning (Tree-Based) Approach (ARPA)

The ARPA analysis generated a classification tree in which the three main splitter variants were exm-rs1321744, exm-rs350035, and exm-rs7679 (Figure 4A). Both left and right terminal nodes have a conspicuous power to discriminate between remitters (category 1) and nonresponders (category 0). The receiver operating characteristic (ROC) integral to evaluate the predictive accuracy of this derived model is 0.9454, which describes the high sensitivity and specificity of the tree in discriminating between remitters and nonresponders (Figure 4B). The TreeNet and random forest analyses (see Figure S2 in the online data supplement) show that after 150 simulated trees, the classification error is lower than 10% for both the training and the testing sample, and the ROC curve converges to values higher than 90% for both the training and the testing samples.

Allele Frequency (Controls)	Classification	Gene	HGVS Coding	HGVS Protein
0.45	Intergenic			
0.28	Intergenic			
0.38	Coding nonsynonymous	ANXA10	c.211A>C	p. Met71Leu
0.24	Intergenic			
0.28	Intergenic			
0.43	Intergenic			
0.43	Coding nonsynonymous	ATP6V0A4	c.5G>A	p.Ala2Val
0.33	Coding nonsynonymous	HEG1	c.2734A>T	p. Thr912Ser
0.48	Intronic	HOXA11		
0.26	Intronic	RAF1		

Furthermore, the random forest and TreeNet analyses disclosed the same set of variables as the main predictors of remission and also discarded the possibility of model overfitting described for the CART analyses.

Finally, we applied a categorical approach to link the set of genotypes of the associated marker (exm-rs1321744) using latent class analysis, which shows how the patterns of remission, as depicted by HAM-D scores, split the set of patients into two significantly different clusters associated with the three genotypes CC, CT, and TT (Figure 5). Cluster 1, constituted by remitters, is associated with the CC genotype, exm-rs1321744. Cluster 2, constituted by nonresponders, is associated with the CT genotype, exm-rs1321744. Treatment with either desipramine or fluoxetine did not produce any significant additional splitting of these two clusters.

Discussion

In this single-site double-blind antidepressant trial of predominantly first-generation Mexican American patients, we found that both fluoxetine and desipramine were effective; however, patients treated with fluoxetine had significantly better scores at endpoint on the HAM-D, HAM-A, BDI, and GAS than desipramine-treated patients across all analytical approaches. The advantage of fluoxetine over desipramine was also evidenced by a shorter time to response in the survival analysis, a lower occurrence of anticholinergic and cardiovascular adverse drug reactions, and a lower dropout rate. These clinical outcomes contrast with previous studies showing similarities in efficacy between SSRIs and tricyclic antidepressants (36) and no difference in the response rate between SSRIs and tricyclics in an intent-to-treat analysis (37).

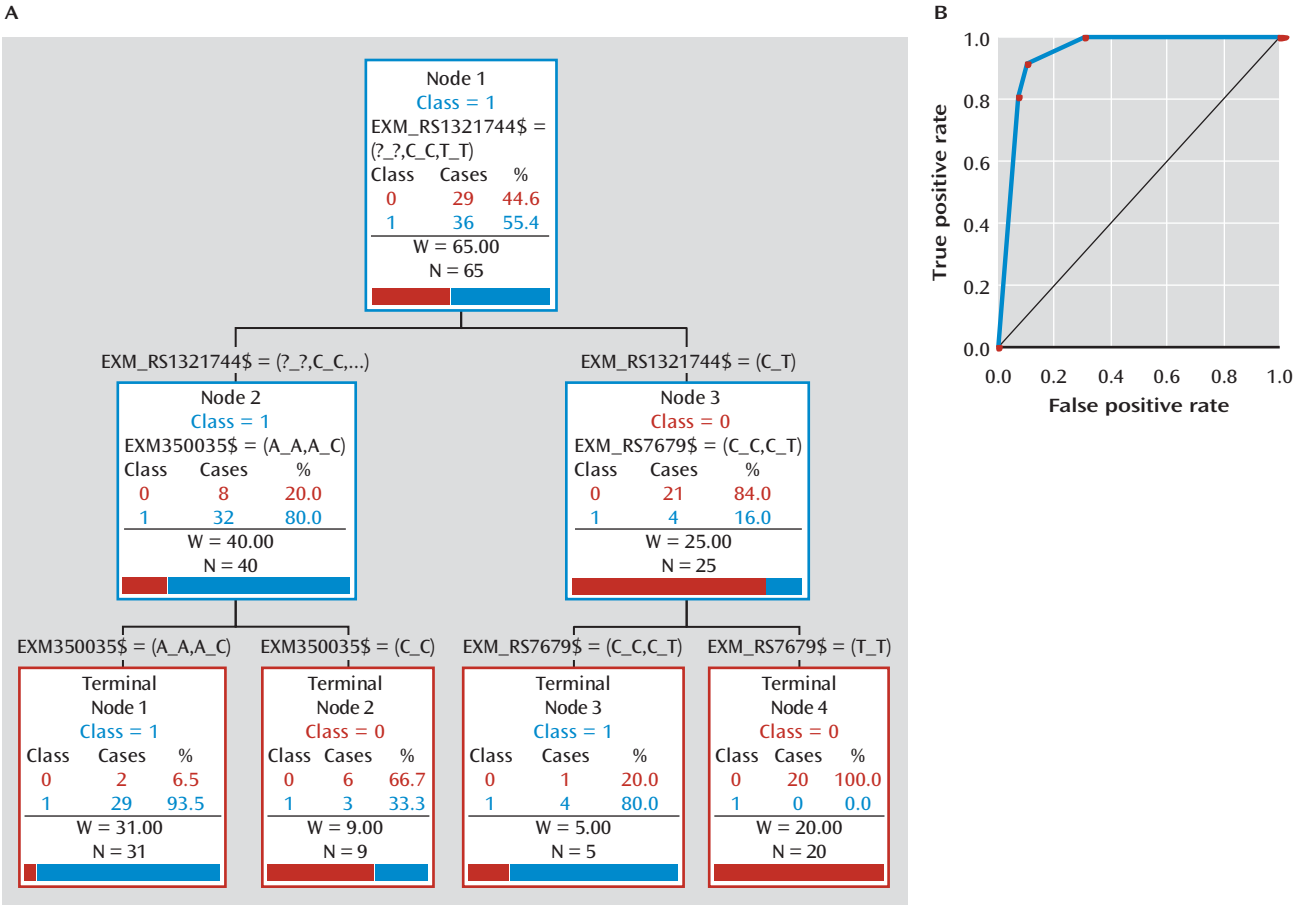
Our results are in concordance with data showing higher dropout rates among patients treated with tricyclic antidepressants because of lack of efficacy or adverse reactions (30.0% and 24.7%, respectively) (37). Tricyclic antidepressants have historically been found to be associated with moderate to severe adverse drug reactions (38); SSRIs are associated with milder adverse drug reactions that may diminish as treatment continues, and they also have lower toxicity and lower lethality when taken in an overdose situation (39). The overall comparability we observed in tolerability between desipramine and fluoxetine could be related to the close monitoring of our patients, a practice that improves outcomes of treatment with tricyclic antidepressants in depression (40). Our data supported a higher dropout rate and occurrence of anticholinergic and cardiovascular side effects in desipramine-treated patients. Previous studies have indicated that Africans are more sensitive to tricyclic antidepressants (41, 42), and populations with higher African genetic admixture rates, such as Puerto Rican Americans, have elevated adverse drug reaction rates and dropout rates with tricyclic antidepressants (43). This could also have contributed to a greater sensitivity to tricyclic antidepressants in our Hispanic subgroup with an African genetic admixture.

To our knowledge, this is the first randomized double-blind placebo lead-in trial conducted in the United States comparing the clinical efficacy and tolerability of antidepressants in depressed individuals of Mexican descent. The reasons for the lack of prospective randomized clinical trials in ethnic minorities are multiple and include difficulties in recruitment and retention of appropriate subjects and significantly less adherence to antidepressant therapy during the initial 100-day period (44). Considerable resources were needed for our study. Recruitment was challenging despite the fact that over 8 million persons of Mexican descent live in California (1), including 3 million in Los Angeles County. We conducted more than 4,300 telephone interviews and scheduled 1,223 screening visits in order to obtain 166 study completers.

A World Health Organization study (45) found that treatment with tricyclic antidepressants was more cost-effective than treatment with SSRIs in 14 different populations (including Mexico and the United States), particularly in lower-income subregions. However, in our study, fluoxetine treatment produced a better and faster response than desipramine in first-generation Mexican Americans with mild to moderate major depressive disorder, which suggests that fluoxetine may constitute a better drug of first choice for patients of Mexican descent.

Our whole-exome genotyping approach identified one functional intergenic SNP in chromosome 6 with exome-wide association with remission ($p=1.98\times10^{-6}$; false-discovery-rate-corrected p=0.05), which is remarkable given the small number of remitters (N=36) and nonresponders (N=29). Mexicans are an admixture of Europeans, Native Americans, and Africans, but a considerable proportion of their ancestry is Caucasian (46). For European-descendent populations, a p value $\leq7.2\times10^{-8}$ is regarded as compellingly significant for a genome-wide effect (47), but this assumption is appropriate for hypotheses that are tested on a genome scale (48). Our results strongly support the involvement of common functional variants in

FIGURE 4. Results of Advance Recursive Partitioning (Tree-Based) Approach (ARPA) Analysis[a]



[a] In panel A, a reconstructed classificatory tree shows those variants involved in the process of branching. Nonresponders (class 0) are indicated in red, and remitters (class 1) are indicated in blue. In panel B, the receiver operating characteristic (ROC) integral=0.9454.

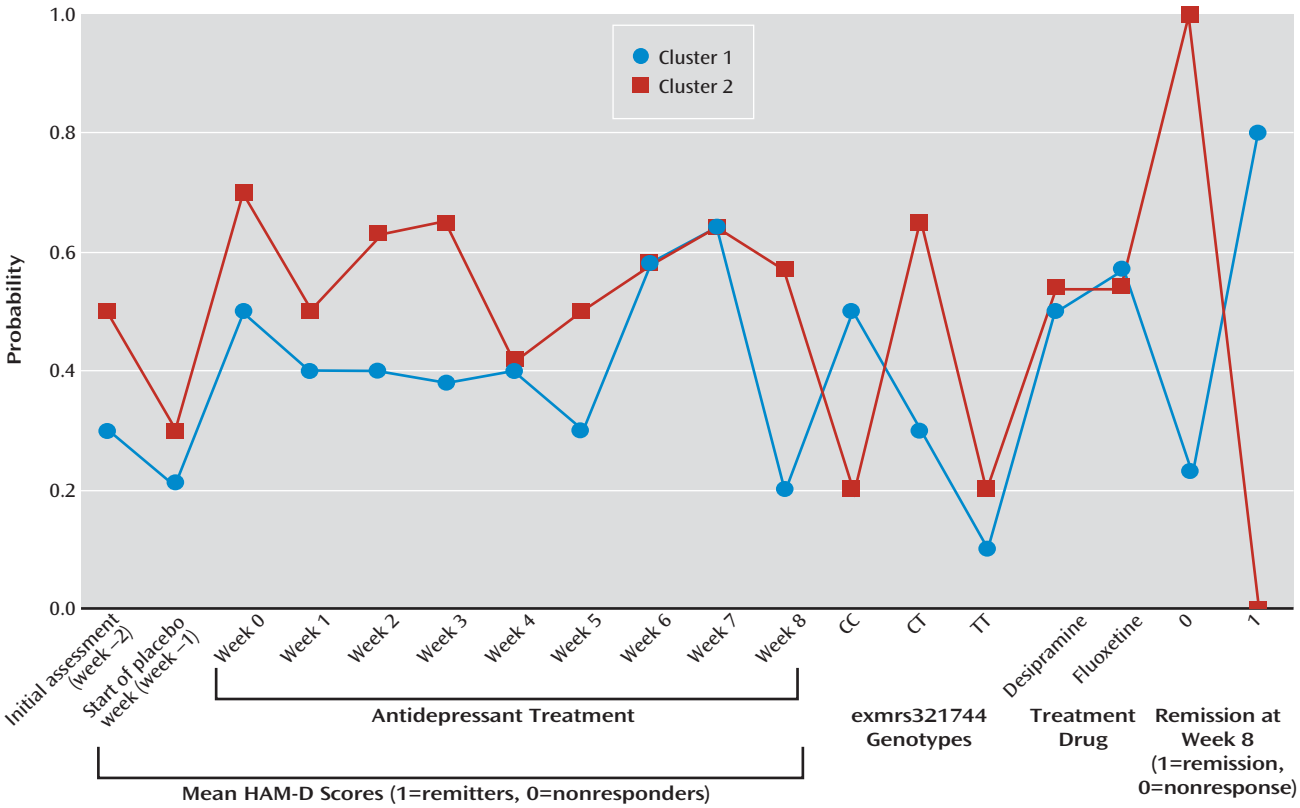
antidepressant drug response, specifically of brain methylation sites. Not much is known about the function of the intergenic exm-rs1321744, but growing clinical and preclinical evidence has implicated brain epigenetic changes in stress, depression, and antidepressant action (15–19); however, that body of work has focused mainly on regulation of the hypothalamic-pituitary-adrenal axis.

Even though linkage disequilibrium block harboring exm-rs1321744 does not harbor any gene, there are three genes surrounding the significant associated peak: *TBX18*, *NT5E*, and *SNX14*. *TBX18* encodes a member of an evolutionary conserved family of transcription factors that plays a crucial role in embryonic development. *NT5E* encodes a plasma protein membrane that catalyzes the conversion of extracellular nucleotides to membrane-permeable nucleosides. *SNX14* encodes a member of the sorting nexin family that is involved in intracellular trafficking. The encoded protein also contains a regulator of G protein signaling domains that act as GTPase activating proteins for G alpha subunits of heterotrimeric G proteins.

Our ARPA results suggest that the phenotype for antidepressant response is polygenic, as tree analysis showed that three common functional SNPs could predict remitter versus nonresponder status with 94% accuracy in our population. The top main splitter, exm-rs1321744, was discussed above, but, intriguingly, the other main splitter variants are located in regions relevant to lipoprotein function; rs7679 (in the *PCIF1* gene, chromosome 20) has genome-wide association with blood lipoprotein concentrations (49), and intergenic exm-rs350035 on chromosome 5 is 10 nucleotides away from rs351629, a genome-wide SNP associated with triglycerides phenotype (50) (source, dbGaP; http://www.ncbi.nlm.nih.gov/gap).

Our study had several limitations. One is that the genotyping was done in only 65 of 166 participants who completed 8 weeks of treatment and 232 intent-to-treat subjects. We found no differences in week 8 HAM-D scores between completers who were genotyped and those who were not (see Table S3 in the online data supplement), which supports the absence of any ascertainment bias. A second limitation is that antidepressant blood levels were not included as a covariate, as they were randomly collected at different times of the day; some patients could come for follow-ups only in the evening. The absence of

FIGURE 5. Patterns of Hamilton Depression Rating Scale (HAM-D) Scores Indicating Two Significantly Different Clusters Associated With the exm-rs1321744 Genotypes[a]



[a] Cluster 1 is constituted by remitters and is associated with the CC genotype. Cluster 2 is constituted by nonresponders and is associated with the CT genotype. Treatment with either desipramine or fluoxetine did not produce any significant additional splitting of these two clusters.

placebo is another important limitation. However, as the efficacies of desipramine or fluoxetine were well known when we designed our study, it would be ethically problematic to justify a placebo arm. To minimize placebo response in our study, patients received placebo during the first week of their treatment. Placebo was given single-blinded; patients knew that they were going to receive 1 week of placebo at some point in their treatment course, but staff knew that this would occur in the first week of treatment. In our study, we determined a priori that placebo response was a reason to remove participants from the study, and therefore we believe that the SNPs we identified are likely to predict response to antidepressant treatment and not merely improvement.

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The authors report no financial relationships with commercial interests. An intellectual property application has been prepared to include the pharmacogenetics findings of this work.

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Chapter VII. Conclusion

In summary, our findings support that: 1) Highly anxious patients homozygous for the GAG haplotype (for rs1876828, rs242939 and rs242941) of the *CRHR1* gene had 70% and 31% greater reduction in anxiety symptoms and depressive symptoms scores when compared to heterozygotes; 2) Polymorphisms in the *PDE11A* gene were significantly associated with the diagnosis of MDD and remission to antidepressants; one SNP in the *PDE9A* gene was significantly associated with MDD diagnosis and one SNP in the *PDE1A* gene was also associated with antidepressant response. 3) One SNP in the *TBX21* gene and one in the *PSMB4* gene were associated with MDD diagnosis. SNPs in genes that support the role of T cell functions were associated with antidepressant treatment response (*CD3E*, *PRKCH*, *PSMD9*, *STAT3,* and *UNC3*). 4) Re-sequencing of pharmacokinetics and pharmacodynamics genes showed several new variants. *BDNF* gene variants were associated with MDD diagnosis and one SNP was associated with antidepressant treatment response. Variants in several other genes were nominally associated with the diagnosis of MDD and antidepressant response. We found significant associations between two coding SNPs in the *NTRK2* gene and one haplotype with antidepressant response. 5) A variation located in a brain methylation site was associated with remission at the whole exome level, suggesting the involvement of epigenetic regulation in antidepressant response.

The body of work presented in this thesis has increased knowledge of pharmacogenetics of antidepressant in MDD and identified potential brain pathways involved in MDD and antidepressant response. The relevance of our results has been replicated/supported by clinical and pre-clinical studies.

1. *CRHR1* gene

Liu *et al.* replicated our findings on the haplotype GAG (for rs1876828, rs242939 and

rs242941) of the *CRHR1* gene. They studied the response of 127 Chinese MDD patients to fluoxetine treatment and found that that haplotype was significantly associated with antidepressant response in MDD patients with high anxiety (133). The SNP rs472888 in the *CRHR1* gene has been modestly associated with duloxetine response in patients with generalised anxiety disorder (134). Recently, a functional polymorphism at the 3' UTR (rs28364032) of the *CRHR1* gene and three haplotypes containing that SNP were reported to be significantly associated with remission to antidepressant drugs (135).

In contrast to our findings, where we did not found a significant association with *CRHR1* variations to MDD susceptibility, Liu *et al*. studied 206 Chinese hospitalised with MDD (83 males/123 females) and 195 health controls and reported that the SNP rs242939 in the *CRHR1* gene was significantly associated with MDD in allele (P=0.0008) and genotype (P=0.0002) analyses, and the haplotype G-G-T for rs1876828, rs242939 and rs242941 was significantly over-represented in MDD patients (136). Papiol *et al*. reported the association of *CRHR1* gene polymorphism with increased risk for seasonal pattern and early onset MDD in a Spanish cohort (137). *CRHR1* gene polymorphisms have also been found to moderate the impact of stress on physical health (138).

Variations in other genes important for the stress hormone-regulating HPA axis, notably the *FKBP5* (FK506 Binding protein 5) gene SNPs rs1360780, rs1334894 and rs755658, which is a glucocorticoid receptor-regulating cochaperone of hsp-90 (heat shock protein-90), were associated with recurrence of MDD and antidepressant response (139) in a cohort of 294 patients (86.6% with MDD, 12% with bipolar disorder, and 1.2% with dysthymia) hospitalised for the treatment of depression with SSRIs (n=70), TCA (n=48) and mirtazapine (n=55). Individuals homozygous for the TT genotype in rs1360780 had more depressive episodes and a better response to antidepressant. These findings were replicated in an independent sample of 85 inpatients; 339 controls matched for ethnicity were also

recruited. The rs1360780 findings were replicated in a sample of white non-Hispanic of the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) cohort (140).

2. *PDE* genes

Our findings in the variations of the *PDE11A* and *PDE1A* genes have not been replicated in non-Hispanic MDD patients treated with citalopram (141) and in the STAR*D cohort (142). Treatment response to duloxetine was not associated with variations in the *PDE1A*, *PDE1C*, *PDE6A* or *PDE11A* genes in MDD (143) but rs1549870 in the *PDE1A* gene was associated with duloxetine response in generalised anxiety disorder (134). However, PDE signaling system has been reported to be disrupted in post-mortem cerebella tissue of individuals with schizophrenia, bipolar disorder and MDD (144). Furthermore, rs11628551 in the *SSTR1* (Somatostatin receptor 1) gene, which is involved with brain signal transduction and glutamate receptor signaling, and has been found to be associated with monoamine metabolite levels in human CSF at a genome-wide level, controls the expression of *PDE9A* (145).

3. Genes relevant to T-cell function (*TBX21, PSMB4, CD3E, PSMD9, STAT3, etc.)*

Thus far, no other publication has focused on the variations in the *TBX21* or *PSMB4* genes that were significantly associated to the diagnosis of MDD in our study. We also found that polymorphisms in several genes relevant to T-cell function that were nominally associated with antidepressant response. Interestingly, Tbx21/Tbet deficient mice were shown to be resilient to stress-induced depressive-like behaviors (146), and CD4+CD25+ regulatory T (Treg) cells have also been shown to have role in the modulation of anxiety- and depression-like behaviors (147); CD4+CD25+ Treg cell level was decreased in MDD patients (148).

Variations in the *PSMD9* gene has been linked with generalised anxiety disorder,

depression and insomnia (149-151). The *PSMD9* gene is known to be relevant to type 2 diabetes, as it is within the NIDDM2 (non-insulin-dependent-diabetes 2) locus, a locus linked to type 2 diabetes in genome wide association studies (152, 153), SNPs in this gene have been significantly associated with type 2 diabetes onset in Italian families and its neuropathy (154-156).

STAT3 has been found to control interleukin-6-dependent regulation of serotonin transporter function and depressive-like behavior (157, 158). Fragments of urocortin 3 have been found to have anxiolytic action (159, 160).

4. Genes relevant to the pharmacokinetics and pharmacodynamics of antidepressant drugs (*BDNF*, *ABCB1*, *SLC6A2*, *SLC6A3*, *SLC6A4*, *CREB1*, *CRHR1* and *NTRK2)*

We reported that in the *BNDF* gene, rs12273539, rs11030103, rs6265, rs28722151, rs41282918 and rs11030101, and 2 haplotypes were significantly associated with MDD. The rs61888800 SNP in the *BDNF* gene was associated with antidepressant response in our Mexican-American cohort. We also found that two SNPs (rs2289657 and rs56142442) in the *NTRK2* gene and one haplotype (CAG at rs2289658, rs2289657 and rs228956) were significantly associated with antidepressant response. No other publication to date has focused on rs61888800 (*BNDF*) or rs2289657/rs56142442 (*NTRK2*); therefore, our findings are yet to be confirmed.

The rs6265 variation in the *BDNF* gene is a non-synonymous SNP that may have a minor influence on MDD diagnosis (161). This SNP has also been the most investigated in pharmacogenetics, and contrasting to our findings heterozygous individuals in the rs6265 have been reported to have better antidepressant response (162-167). Results from the Munich Antidepressant Response Signature (MARS) project have supported the possible (nominal) association of seven *BDNF* SNPs and nine *NTRK2* SNPs in antidepressant

treatment response (168).

5. Whole-exome genotyping of functional variants

We have recently reported that exm-rs1321744 in chromosome 6 achieved exome-wide significance (1.98×10^{-06}) for treatment remission; this variant is located in a brain methylation site, which suggests its involvement in epigenetic regulation of neuronal gene expression.

Epigenetic modifications, such as DNA methylation and histone changes, are candidate mechanisms for environmentally induced long-lasting CNS functional changes. Epigenetic studies to understand MDD susceptibility or antidepressant response have focused on the following genes: *NRC3C1* (Nuclear receptor subfamily 3, group C, member 1, GRL/GCR/CR) that encodes for the glucorticoid receptor variant 1, the glucorticoid response element of the *FKBP5* gene, the promoter region of the *SLC6A4* gene, and the CpG sites in the promoter region of the *BDNF* gene (169). A lower methylation in the *BDNF* gene and a higher methylation of the *NRC3C1* exon IF promoter were observed respectively in responders to antidepressant treatment (170) and in successful psychotherapy treatment outcome in post-traumatic stress disorder (171). Methylation levels of the CpG sites 21 to 22 in the *IL-11* (interleukin 11) gene predicted antidepressant response in the Genome-Based Therapeutic Drugs for Depression (GENDEP) project, in which MDD patients were treated with escitalopram or nortriptyline (172).

6. Overall significance of the work

Pharmacogenetic approaches that could identify an efficacious antidepressant drug for a given patient based on their genetic profile would be of enormous clinical and public health value. Furthermore, because antidepressants of different classes have common

antidepressant effects after chronic use, it is believed that downstream regulation in gene transcription may mediate their therapeutic effects. Pharmacogenetic approaches to identify the final genetic mediators of antidepressant action may lead to the identification of gene variants that are associated with individual responsiveness to various antidepressant drugs. Moreover, identification of such downstream genetic targets for antidepressant action may provide clues for novel pharmacological targets in the treatment of depression.

The development of an effective *a priory* predictor that could be used to identify the most effective medication or class of medications for a particular patient prior would increase the rate of response and shorten the course of a depressive episode. Decrease health care costs would result from a combination of factors: decreased expenditures on ineffective medication trials, decreased utilisation of inpatient and outpatient psychiatric services, and decreased in the high utilisation rate of general medical services by depressed patients.

7. Pitfalls

Recent meta-analyses showed no variations that achieved genome-wide significant for antidepressant response in MDD (92). Only a minor impact on the susceptibility to MDD and antidepressant drug response was found in relation to the *BDNF* rs6265 SNP. Currently, we understand that for phenotypes for which the underlying biology is not well understood, candidate gene studies/candidate pathway studies have a very small probability of detecting a true association. Due to insufficient understanding of the effects of multiple hypotheses testing in the earlier days of disease/phenotype-associated genes studies, many positives results were found by chance (false positives) due to the number of hypothesis tested for each phenotype (173). Prospective pharmacogenetic double-blind RCT are very expensive to conduct; therefore, large cohorts that are needed to perform whole genome

approaches have not been available. Though our sample size seems small for a genetic study, it is not small in comparison to other prospective pharmacogenetics RCT cohorts.

8. Future directions of the work

Our current efforts have been focused on following up our most recent findings (92). Thus, we have been examining the involvement of DNA methylation in antidepressant response. We have also obtained whole genome sequencing from a small sub-cohort of remitters and non-responders and we are currently analysing those data. Our long-term goal is to be able to conduct a large prospective pharmacogenetics of antidepressant study in Australians with MDD.

Abbreviations

A: Adenine
ABCB1: ATP-Binding Cassette, Sub-Family B gene, MDR/TAP
BDNF: Brain-derived neurotrophic factor
BDNF: Brain-derived neurotrophic factor gene
C: Cytosine
cAMP: Cyclic adenosine monophosphate
CD3E: CD3e molecule, epsilon gene
cGMP: Cyclic guanosine monophosphate
CNS: Central nervous system
CSF: Cerebrospinal fluid
CREB: cyclic AMP response element binding protein gene
CRH: Corticotropin-releasing hormone
CRHR1: Corticotropin-releasing hormone receptor 1 gene
DA: Dopamine
DNA: Deoxyribonucleic acid
DSM-5: Diagnostic and Statistical Manual-5
DSM-IV: Diagnostic and Statistical Manual-IV
ECT: Electroconvulsive therapy
FDA: USA Food and Drug Administration
FDR: False discovery rate
FKBP5: FK506 Binding protein 5 gene
G: Guanine
GABA: γ-aminobutyric acid
GENDEP: Genome-Based Therapeutic Drugs for Depression
GWAS: Genome-wide association studies
HAMD21: Hamilton Depression Rating Scale
HPA: Hypothalamic-pituitary-adrenal
Hsp-90: Heat shock protein-90
5-HT: Serotonin
5-HTTLPR/*SLC6A4*: Serotonin transporter gene
5-HT2AR: Type IIA Serotonin receptors
IL-11: Interleukin 11 gene
l: *5-HTTLPR* promoter long form/variant
LD: Linkage disequilibrium
MAOI: Monoamine oxidase inhibitor
MARS: Munich Antidepressant Response Signature
MDD: Major depressive disorder
mRNA: Messenger ribonucleic acid
NRC3C1: Nuclear receptor subfamily 3, group C, member 1 gene, GRL/GCR/CR
NE: Norepinephrine
NIDDM2: Non-insulin-dependent-diabetes 2
NMDA: N-methyl-D-aspartate
NO: Nitric oxide
NTRK2: Neurotrophic tyrosine kinase, receptor, type 2
NTRK2: Neurotrophic tyrosine kinase, receptor, type 2 gene
PBS: Pharmaceutical Benefits Scheme
PCR: Polymerase chain reaction
PDE: Cyclic nucleotide phosphodiesterase

PDE1A: Cyclic nucleotide phosphodiesterase 1A gene
PDE1C: Cyclic nucleotide phosphodiesterase 1C gene
PDE9A: Cyclic nucleotide phosphodiesterase 9A gene
PDE11A: Cyclic nucleotide phosphodiesterase 11A gene
PRKCH: Protein kinase C substrate 80K-H gene
PSMB4: Proteasome ß4 subunit gene
PSMD9: Proteasome 26S subunit, Non-ATPase 9 gene
RCT: randomized controlled trial
s: *5-HTTLPR* promoter short form/variant
SLC6A2: Solute Carrier Family 6 (Neurotransmitter Transporter), Member 2 gene/norepinephrine transporter gene, *NAT1*/*NET1*
SLC6A3: Solute Carrier Family 6 (Neurotransmitter Transporter), Member 3 gene/dopamine transporter gene, *DAT1*
SLC6A4/5-HTTLPR: Solute Carrier Family 6 (Neurotransmitter Transporter), Member 4 gene/serotonin transporter gene, *5HTT*/*SERT*
SNP: Single nucleotide polymorphism
SNRI: Serotonin-noradrenaline reuptake inhibitor
SSRI: Selective serotonin reuptake inhibitor
SSTR1: Somatostatin receptor 1 gene
STAT3: Signal transducer and activator of transcription 3 gene
STAR*D: Sequenced Treatment Alternatives to Relieve Depression
T: thymine
TBX21: T-box 21 gene, *TBET*
TCA: Tricyclic
TMS: Transcranial magnetic stimulation
TPH: Tryptophan hydroxylase gene
Treg: Regulatory T
UK: United Kingdom
UNC3: Urocortin 3 gene
USA: United States of America
WHO: World Health Organisation

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