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Antidepressant Pharmacogenetics: Searching for Genetics Determinants of Treatment Response

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Antidepressant Pharmacogenetics: Searching for Genetic Determinants of Treatment Response

by

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DISSERTATION

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I have always been drawn to the notion of learning something new, and I now have the opportunity to pursue knowledge as a career. I have learned that the process of acquiring knowledge is painfully slow but the prospect of improving human health or simply being the first to know something make it all worthwhile. What has also become clear is that knowledge cannot be pursued as a solo endeavor and that it takes the support and efforts of many to accomplish even a little. I was very fortunate to have the support of a tremendous group of colleagues, family, and friends. Without them, I would not have made it through graduate school.

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ABSTRACT

Antidepressant Pharmacogenetics: Searching for Genetic Determinants of Treatment Response

Jeffrey Brian Kraft Jr.

Major depressive disorder is one of the most common and debilitating psychiatric disorders. Psychopharmacological agents are the most widely used form of treatment, although they are not universally effective and can produce significant side effects in some patients. The most common psychopharmacological agents used to treat major depression are the selective serotonin reuptake inhibitors, or SSRIs. Often, these drugs take several weeks to relieve depressive symptoms. Individualized therapy would have great clinical utility by identifying patients that are likely to respond positively to SSRI therapy *a priori*. The goal of this thesis is to investigate the use of genetic markers for guiding treatment with SSRIs.

We utilized several complementary pharmacogenetic approaches and two depressed populations treated with SSRIs. The first was a small (N=96) population given fluoxetine, and the second was a large (N=1,953) population taking citalopram. We used the fluoxetine population and a variant discovery approach to uncover novel variation and previously unknown tagging SNPs in the molecular target of SSRIs, the serotonin transporter, then employed a linkage disequilibrium mapping approach to investigate variants for association to response. Several variants in the promoter region of the gene were associated with fluoxetine outcome. No markers were associated with response when investigated in our citalopram population.

We also investigated relevant candidate genes for association with citalopram response and tolerance. Variants within the FEV gene, a master transcription factor in the serotonin pathway, were associated with a number of response phenotypes and mouse work implicates this gene in citalopram response. None of our other candidate genes demonstrated association with citalopram response.

Utilizing a panel of approximately 20,000 non-synonymous cSNPs for association with citalopram response, one SNP in the gene LRP2 was significantly associated with response in the African American population. We also performed a whole genome association study using over 500,000 SNPs from across the genome. Using a two-stage study design, none of the most highly associated markers in the discovery sample were also associated in the validation sample.

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CHAPTER 1

INTRODUCTION TO MAJOR DEPRESSIVE DISORDER AND ANTIDEPRESSANT PHARMACOGENETICS

1.1 Major Depressive Disorder (MDD)

1.1.1 Scope of MDD

Major depression is one of the most common and disabling psychiatric disorders (1). Depression is a leading cause of disability worldwide and the treatment of depression costs the United States more than $40 billion annually (2-5). Depression is strongly associated with suicide, which is the eleventh leading cause of death in the US overall, and the fourth leading cause of death among 25-44 year olds (4,6). The average age of onset for major depression is 25 years, and depression is often chronic and characterized by recurrences throughout the lifespan, with some estimates of recurrence as high as 85% (7).

Major depressive disorder, as defined in the DSM-IV, is characterized by at least two weeks of pervasively depressed mood and/or diminished interest accompanied by vegetative and cognitive symptoms, including sleep and appetite disturbances, psychomotor and energy disturbances, cognitive changes and suicidal thoughts (8). Depression has high co-morbidity with other psychiatric disorders and substance abuse, and recent studies suggest that depression may be an independent risk factor for some somatic disorders such as heart disease and diabetes (9). Major depression affects 16% of the population in the United States over the course of a lifetime, with almost 6% of the population being depressed in any one year, and the societal burden due to depression is

tremendous (1,10). By the year 2020, depression is estimated to become the leading cause of years of life lost to disability in both developing and developed countries (5).

As with most psychiatric disorders, the disease we label major depressive disorder is likely to be heterogeneous with several different underlying etiologies (e.g. reaction to stressful events, induced by drugs, etc.), likely to be responsible. It appears, however, that most patients with major depression respond to a wide variety of treatments, including psychotherapies, medications, and other somatic treatments such as electroconvulsive therapy (11). Psychopharmacological treatment is currently the most common form of treatment and in particular, selective serotonin reuptake inhibitors (SSRIs) have become the most frequently prescribed antidepressant (12).

1.1.2 Antidepressant Response

While effective treatments for depression are available, it is clear that there is a large degree of clinical heterogeneity in response to antidepressants. The response rate to most antidepressants in clinical trials is on the order of 50-60%, with an even lower remission rate of 35-45% (13,14). Thus far several clinical or demographic characteristics have been associated with poor response to antidepressants including non-Caucasian ancestry, unemployment, low income or education, longer MDD index episodes, and concurrent psychiatric disorders (15). Thus far no reliable biological predictors have been found to be associated with antidepressant response (16). Because of this, patients who do not respond to their first antidepressant treatment often have to try other antidepressants in order to achieve remission. This trial and error process puts an enormous burden on the patient especially given the length of treatment required to gauge clinical effectiveness of the drugs (typically 4 weeks or longer).

Another problem is the adverse effects of antidepressants which often result in non-compliance and discontinuation of treatment. However, there is currently no way to predict whether or not a patient will experience the adverse effects. With the use of selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine and citalopram, side effects such as nausea, sexual dysfunction, headache, sleep disturbance, tremor, and weight disturbances are commonly reported. One example of the magnitude of the problem is sexual side effects, which can occur in ~50% of those taking SSRIs. A potentially devastating side effect involves increases in suicidal thinking among depressed patients, particularly adolescents, prescribed SSRIs although other studies have shown no increased risk of suicide due to SSRI treatment and this controversial issue remains highly contentious (17,18).

1.2 Antidepressant Pharmacogenetics

1.2.1 Pharmacogenetics Overview

Pharmacogenetics, defined as the study of genetic variability between individuals in response to exogenous substances, as a field dates back to the late 1950s. The earliest modern pharmacogenetic discoveries of hereditary variation in drug response involved drugs such as succinylcholine, primaquine, and isoniazid. These classic studies set the stage for subsequent pharmacogenetic investigation, which currently focus on the genes that contribute to the pharmacokinetics (the actions of the body on drugs over a period of time) and pharmacodynamics (the biochemical and physiological effects of drugs and their mechanisms of action) of a particular drug (19).

A great deal is known about the common inter-individual variation in Phase I (oxidation, reduction, or hydrolysis) and Phase II (conjugation) drug metabolizing proteins, at both the enzymatic and DNA sequence levels (20). For example, an extensive catalog of functional variants and haplotype configurations in the genes encoding cytochrome P450 enzymes has been amassed (21). A major example of the success of pharmacogenetics involves the drug metabolizing protein, thiopurine methyltransferase (TPMT). Children who inherit two defective copies of this gene can experience fatal hematological side effects when administered 6-mercaptopurine, a chemotherapeutic agent used in pediatric leukemia, while patients with two normal copies of the gene for this enzyme require higher doses of the medication (22).

Numerous examples exist for members of the cytochrome P450 family of metabolic enzymes but efforts at identifying genes involved in pharmacodynamics for particular medications have also been successful. Individual variations and haplotypes in the type 2 β-adrenergic receptor have been found to be associated with response to β-agonists in the treatment of asthma (23). In addition, the dosing of warfarin, was recently shown to be significantly influenced by the subject's genotype at a pharmacodynamic target of the drug (VKORC1) (24).

1.2.2 Pharmacogenetics of Response to Tricyclic Antidepressants

Studies performed in the 1960s and 1970s revealed that upon repeated administration of one or another class of antidepressants, both response and non-response to antidepressant class were significantly concordant between family members (25,26). This finding has been replicated more recently in relatively small samples (27,28). The important role of cytochrome P450s in tricyclic antidepressant (TCA) metabolism is

well-documented, and is reflected in the extensive work showing correlation between blood levels and response and toxicity, as well as the potential benefits of therapeutic drug monitoring for patient safety and reduced costs (29).

Pharmacogenetic analysis guided by these observations revealed in one study that patients missing CYP2D6 could not be effectively treated with tricyclics (30). This locus has been suggested to account for 34% of the variation in plasma nortriptyline levels (31). Some authors recommend that pharmacogenetic considerations be taken into account with the use of tricyclics, suggesting substantial dose reductions in persons with the "poor metabolizer" phenotype of CYP2D6 or CYP2C19 (32).

1.2.3 Pharmacogenetics of SSRI Response

The current widespread use of SSRIs in depression along with recent advances in molecular genetics have resulted in sizeable body of literature on SSRI pharmacogenetics (33). The majority of these studies focus on putative pharmacodynamic genes related to monoamine function, including the serotonin transporter (the molecular target for SSRIs), tryptophan hydroxylase 1, monoamine oxidase A, and the 1A and 2A serotonin receptors. These case-control studies as a whole examine a small number of polymorphic loci in these genes, and utilize fairly small sample sizes, often with heterogeneity in diagnosis (unipolar and bipolar depression) and medication use.

An Italian group has shown in a series of studies some evidence of an association between the long allele of a functional promoter polymorphism in the serotonin transporter and loosely defined depression, including cases of bipolar disorder in the depressed phase of the illness (34-37). Other groups have reported similar findings and these are further explored in Chapter 2 (38-40). These reports are of interest as this

polymorphism is usually defined by the long and short alleles, with the long allele leading to increased in vitro transcription of the SLC6A4 gene and serotonin uptake in cell lines (41). Additional notable findings in the pharmacogenetics of SSRI response have included associations to tryptophan hydroxylase (34), serotonin 2A receptor (42), GRIK4 (43), G protein β3 (44,45), angiotensin converting enzyme (46), and the glucocorticoid receptor FKBP5 (47).

There has been little exploration of association between genetic variants in any of these genes and adverse events related to SSRI treatment. A small study showed that the short allele of the serotonin transporter promoter polymorphism was associated with the development of insomnia and agitation in a population of 36 outpatients (48). A study by Murphy et al of 124 subjects with geriatric depression treated with the SSRI paroxetine showed that the genotype for a variant in the 2A serotonin receptor (HTR2A) predicted both greater rates of discontinuation and severity of adverse events (49).

There have also been several previous studies investigating the relationship between SSRI medications and pharmacokinetic genes. The pharmacokinetics of many SSRIs, including citalopram, are affected by CYP2D6 and CYP2C19 genotype status, although there is no evidence regarding how plasma levels of citalopram influence clinical efficacy or tolerance (50). In fact, the Evaluation of Genomic Applications in Practice and Prevention (EGAPP) initiative recently found insufficient evidence to support a recommendation for or against use of CYP450 testing in adults beginning SSRI treatment for non-psychotic depression (51). In a study of 53 Chinese patients with major depression taking citalopram, CYP2C19 genotype status was significantly associated

with clearance of citalopram and the metabolic ratio of desmethylcitalopram to citalopram, but not associated with the primary clinical outcome (52).

1.3 Genomics and Association Studies

1.3.1 Overview

The effort to sequence the human genome has dramatically altered the potential impact of pharmacogenetics on human health as the vast majority of human genes have now been localized and annotated (53,54). Perhaps one of the greatest benefits of sequencing the human genome has been the discovery of the incredible level of sequence diversity between humans. ***S***ingle ***N***ucleotide ***P***olymorphisms (single base pair substitutions) are the most abundant type of DNA variation with over 12 million individual SNPs having already been identified and referenced at dbSNP. Single nucleotide polymorphisms are effective markers for genetic studies, partly out of their abundance, but also due to the development of efficient and inexpensive methodologies for assaying SNPs (55).

In the context of the Human Genome Project, pharmacogenetics can now be broadened to "pharmacogenomics", with our new annotated knowledge of genes, proteins, and SNPs allowing a more general analysis of the many different genes that may determine drug behavior.

1.3.2 Genetic Association Studies

Pharmacogenetic phenotypes are complex traits with contributions from pharmacodynamic genetic variants (transporters, receptors), pharmacokinetic genetic variants (absorption, metabolism, elimination), and environmental factors. Given the

genetic complexity of antidepressant response, a powerful strategy for determining these genetic factors would be through use of an association study, also termed linkage disequilibrium (LD) mapping (56). In LD mapping, unobserved historical recombinations in an outbred population are used to identify genes that influence the risk for a trait by exploiting the physical proximity between a susceptibility gene and a marker locus (57).

LD mapping thus assumes that some proportion of the cases have a common ancestor who had the disease-associated variation. The individuals who share this variation are also likely to share alleles at sites neighboring the actual disease locus due to linkage disequilibrium (58). The main advantage of this approach rests on the statistical power derived from the ability to collect substantial numbers of unrelated cases and controls (59). A number of interacting factors influence the likelihood of success in LD mapping designs, including the effect size of the trait variant, frequencies of marker and trait alleles, as well as LD relationships (60).

A disadvantage of LD mapping is the reliance on the assumption that common disorders are caused by high frequency variants, which is known as the common disease-common variant hypothesis (CDCV) (61,62). This assumption is useful for LD mapping, since rare alleles (which are "newer") generally do not have significant LD with neighboring alleles, nor are common enough to be seen sufficiently often enough to allow reliable measures of association. Unless the actual causative rare allele is genotyped, it will generally not be captured by LD mapping. The CDCV hypothesis states that common alleles, each contribute by themselves very small increases in risk (e.g. odds ratios from 1.1 to 1.5 for single alleles), but when combined and interacting with each other can determine the overall genetic risk for an individual (63).

The common disease-common variant hypothesis is not universally accepted, and an alternative framework known as the common disease rare variant (CDRV) hypothesis has been proposed. This hypothesis states that for any given complex phenotype several (on the order of 100s – 1,000s) of rare variants exist in different genes and pathways that each are individually sufficient to cause the trait (64,65). This model is most similar to the molecular basis of most known Mendelian disorders. Unfortunately, using outbred populations it is difficult to collect enough samples to have adequate power to detect extremely rare variants (< 1% minor allele frequency) and family based studies are often not practical in pharmacogenetics, given the low likelihood that an extended pedigree would have sufficient numbers of members treated with the same medication.

1.3.3 Whole Genome Association Studies

A recently developed extension of LD mapping involves interrogating several thousands of markers across the entire genome and has been termed "whole genome association" (61). A major advantage of whole genome studies is that no understanding of the biological mechanism of the phenotype is required *a priori*, allowing susceptibility genes to be identified that were not considered candidate genes for the phenotype. To date a number of whole genome association studies have been published, and while there have been a few exceptional findings, results have been mixed and debate remains regarding the utility of these endeavors (66-73). For example, recent large studies of type II diabetes (T2D) found marginal genome-wide significance for a number of genes and required combining data from all these studies (FUSION, WTCCC, DBI, & DiaGen) to uncover novel susceptibility genes for T2D and also demonstrated that integrating the results from multiple genome scans can aid the prioritization of signals for replication,

and allow confirmation of genes at appropriate levels of statistical confidence not possible with individual genome-wide association studies (70-73).

1.4 Pitfalls for Linkage Disequilibrium (LD) Mapping

1.4.1 Marker Selection

There are numerous challenges to genetic association studies, whether in either a candidate gene or whole genome context. An obvious issue with these studies is which SNPs to genotype: with over 12 million known SNPs in the human genome and candidate genes often extending beyond 100kb, current genotyping technologies prohibit complete ascertainment of all the SNPs within most candidate genes or all the SNPs in the human genome in reasonably sized clinical samples. Therefore, several groups have developed methods that exploit the LD between markers in order reduce genotyping redundancy while maintaining the genetic diversity within a region. One of the simplest methods attempts to select proxies, or "tagSNPs", in order to capture allelic information at other loci based solely on the pairwise r^2 measure of LD (74). Other methods select SNPs (haplotype tagging, or htSNPs) that capture the underlying haplotype structure (75,76). There is no consensus in the field on which method has the most efficiency or power in association studies but all of this may be rendered irrelevant in the near future with whole genome resequencing. For the candidate gene studies we undertook, we selected tagSNPs based on the r^2 metric for LD alone.

For whole genome association studies the SNP marker panels are on fixed arrays in order to reduce production costs, therefore the investigator cannot change the SNPs to be genotyped. Current marker panels for whole genome studies have focused on gene-centric SNPs (ParAllele Biosciences), evenly spaced SNPs (Affymetrix), or used public

resources like HapMap to select SNPs based on patterns of LD (Illumina). In our gene-centric analysis presented in Chapter 6, we used a combination of approximately 40,000 gene-centric markers, including all known non-synonymous SNPs, by ParAllele Biosciences and in our genome-wide association discussed in Chapter 7, we used 500,000 evenly spaced SNPs utilizing the Affymetrix platform.

1.4.2 Phenotypic Heterogeneity

As with all genetic studies of complex traits, phenotypic heterogeneity is a concern. We can safely assume that the majority of clinical diagnoses in psychiatry, as has been shown with several types of cancer, are composed of different subtypes with distinct molecular mechanisms. Diagnostic techniques are limited in all fields of medicine and this is an even greater concern for psychiatric phenotypes, since these phenotypes usually require the use of structured interviews or questionnaires for diagnosis.

In our study, we attempted to limit phenotypic heterogeneity through the use of response pattern analysis (77,78). SSRI and other antidepressant medications have high placebo response rates, reaching 50% in some clinical trials. It has been shown that patients who have a delayed response (>2 week) to active medication and continue to maintain their response every week until week 12 ("specific responders") are more likely to relapse if blindly switched to placebo than patients displaying an early and inconsistent response ("non-specific responders") (78). Although the delayed response may not be a critical factor in determining true drug response, a sustained response seems to strongly predict specific response status (79). Thus, a subset of patients that appear to be responding to the medication are in fact having a placebo, or non-specific response. We,

therefore, performed association tests with this phenotypic subtype in order to limit heterogeneity by accounting for non-specific response to SSRI medication.

1.4.3 Population Stratification

One of the primary concerns for population-based genetic association studies is ethnic stratification. The desire to avoid the biases due to population stratification has caused family-based association tests to become quite popular in human genetics (80). Unfortunately, as stated above, pharmacogenetic studies generally cannot efficiently collect family based samples. Population stratification occurs when cases and controls have different allele frequencies due to differences in background population that is unrelated to outcome status. In the work described in this thesis, the majority of markers investigated showed some level of differentiation in allele frequency based on self-reported race. For population stratification to have a confounding effect on genetic association studies, there also must be a difference in baseline response (or disease) rates between the ancestry subgroups (81). In the STAR*D sample set, described in Chapters 3 thru 7, using self-reported race as a proxy for ancestry, several differences in response and tolerance existed across racial groups, indicating the need to adjust for population stratification in this sample.

Uncorrected population stratification can cause false positive associations and can also mask true associations that occur within subpopulations (81). Several methods have been proposed to adjust and correct for population stratification. The simplest involves subdividing the clinical population based on self-reported race and testing for association within each substratum. It has been shown that self-reported race correlates

well with genetic ancestry based on microsatellite and large-scale SNP genotyping (82,83). We used this method in our candidate gene studies in the STAR*D sample.

Another method, known as genomic control (GC), uses unlinked markers across the genome to produce a scaling factor that is proportional to the degree of stratification (84). This scaling factor is then used to adjust the χ^2 value of individual SNP tests for differences in population background. The disadvantage of this method is that it applies the same scaling factor to all SNPs tested, when clearly some SNPs are more differentiated across populations than others. We used this GC procedure in our candidate gene studies in the fluoxetine sample, as described in Chapter 2.

An alternative to the GC procedure is structured association, which also uses unlinked markers to detect stratification, then attempts to define underlying subgroups within the stratified sample (85). After subpopulations are identified, association testing can then be performed within homogeneous subpopulations and additionally, a composite test statistic across all subpopulations can be calculated. A popular Markov chain Monte Carlo (MCMC) method for modeling population substructure is implemented in the program *structure*, which estimates the proportion of ancestry (Q) from "K" populations for each individual (86). Given that population subdivisions may be not occur as discrete clusters and the presumed levels of admixture in samples drawn from the United States, correctly choosing "K" is a difficult task. One way to select "K" is to run the model for several values of "K", and then use the estimates of the posterior probability of the model fit to select the most parsimonious value. In our whole genome association study described in Chapter 7, we used a structured association method to correct for population stratification within the STAR*D sample.

An alternative to using self-reported ancestry and structured association is a method called principal components analysis (PCA). This multivariate method utilizes genotype data to infer continuous axes of genetic variation. Intuitively, the axes of variation reduce the data to a small number of dimensions, describing as much variability as possible between the samples (87-90). A series of principal components are generated which capture variability between samples due to stratification that are then used as covariates in regression analyses. This method is not sensitive to the number of axes inferred or subject to the computational limits like structured association methods.

1.4.4 Corrections for Multiple Comparisons

Another challenge to association studies is the issue of multiple comparisons. Put another way, the likelihood of type I statistical error increases when one subjects a number of independent observations to the same significance criterion that would be used when considering a single event. In LD mapping, often several SNPs per gene are genotyped (or several thousand in whole genome studies), and some markers will reach statistical significance due to chance alone. One way to account for these multiple comparisons is to use a Bonferroni correction. For example, if we set a $p < 0.05$ Type I (α) error rate as our study-wide criteria for significance and interrogate 500 markers. A Bonferroni corrected criteria for significance would be $p < 0.0001$ (α/N) for each individual SNP comparison. Bonferroni correction assumes the individual tests are independent of each other and clearly this is not the case for closely linked SNPs due to linkage disequilibrium, therefore this correction is generally considered overly conservative by geneticists (91). Permutation based empirical significance testing can

allow for more accurate assessment of association in the presence of linkage disequilibrium (92).

An additional method for controlling type I error is to use a split sample study design (93). With this method a study sample could be split into two roughly equal halves: a discovery set, in which all markers will be genotyped, and a validation set, in which only the markers that reached the stated significance threshold in the discovery set are genotyped. Besides the cost-savings in terms of genotyping load this method also sidesteps some of the multiple testing issues since in the validation set only a subset of the total markers are tested, which requires less adjustment. However, by splitting the sample, we also greatly sacrifice power (94,95). We utilized a split sample design for our whole genome association study. There is still debate on which design is most powerful for whole genome association studies.

1.5 Summary of Chapters

In this study, we interrogated naturally occurring genetic variants for association to antidepressant response. The goal of this work is to identify genetic markers that can help guide drug choice or dosing of psychopharmacological therapy with an SSRI. This work was performed using two clinical populations of depressed subjects administered SSRIs: a small (N=96) population taking fluoxetine (Chapters 2), and a larger (N=1,953) population taking citalopram (Chapters 4 thru 7). A flow chart of the projects described in this thesis is shown in Figure 1.1.

In **Chapter 2** of this dissertation, we utilized dHPLC and direct sequencing approaches to identify new variants within the SSRI pharmacodynamic target gene, the

serotonin transporter (SLC6A4). We focused our investigation on the coding regions, intron-exon boundaries and 5' conserved non-coding sequence of this gene. To this end we screened 7.4kb in each of 96 patient samples in the fluoxetine population and discovered 27 variants of which 21 had not been previously described within the dbSNP database. Only one SNP (rs25531) showed modest association with our response phenotype ($p < 0.01$). Also of interest, the promoter length polymorphism did not show association within the fluoxetine sample (96).

Chapter 3 attempts to further explore association between the serotonin transporter and antidepressant response utilizing a much larger population taking citalopram (STAR*D). We selected tagSNPs and genotyped 10 SNPs and the promoter length polymorphism in the SLC6A4 locus. No tagSNPs or haplotypes, including the variants that were associated with fluoxetine response in Chapter 2, were significantly associated with citalopram response or response specificity (97).

In **Chapter 4**, we investigated the role of the ETS transcription factor FEV for its role in citalopram response. We utilized HAPMAP data along with the direct sequencing of coding regions, intron-exon boundaries and 5' conserved non-coding sequence to select tagSNPS and other variants of interest within FEV. We then genotyped these nine markers in the entire STAR*D clinical population searching for association with citalopram response. Several markers were significantly associated with citalopram response or response specificity ($p < 0.05$). In collaboration with Miles Berger from the laboratory of Larry Tecott, we also assessed the role FEV using knockout mice of the murine homologue to FEV, Pet-1. Utilizing the tail suspension test we observe an

approximate 50% reduction in immobility time in wild type mice however, Pet-1 knockout mice show no difference ($p < 0.01$).

Chapter 5 explores the role of the SSRI pharmacodynamic candidate genes: activator protein 2 beta (AP-2β), activator protein delta (AP-2δ), serotonin 1B receptor (HTR1B), and its adaptor protein p11 (S100A10), for association to citalopram response utilizing an LD-based candidate gene approach. We genotyped 26 tagSNPs across these four candidate genes. No tagSNPs or haplotypes were significantly associated with citalopram response or response specificity.

In **Chapter 6**, we make use of a gene-centric approach by genotyping approximately 40,000 SNP markers with about 20,000 non-synonymous, coding SNPs and 20,000 tagSNPS in the discovery set of the citalopram population. Using a combination of low p value and high odds ratio as selection criteria, we then chose SNPs that were then genotyped in the remaining half of the citalopram population, in an effort to validate the initial association. We attempted to replicate 45 SNPs that were most highly associated with our response phenotype. One of these SNPs (rs6716834) replicated their initial association in the validation set ($p = 0.005$) within the African American ethnic group. As a secondary analysis, we also attempted to utilize the power of the entire sample in a one-stage design in which 12 SNPs showed significant association with response in the entire citalopram population, 5 in the Caucasian subgroup and 7 in the African American subgroup.

In **Chapter 7**, we use a genome-wide approach by genotyping approximately 500,000 SNP markers spread across the genome in the citalopram population. We use a two-stage design in which we split the sample, and then use one group to look for

associated markers (discovery) and the other group to validate the initial associations (validation). A number of markers that were highly associated within the discovery sample were replicated in the validation sample. This work has revealed a number of interesting genes that may play an important of role in antidepressant response. It may also identify potential pathways for citalopram's molecular mechanism of action, which is not fully understood.

Chapter 8 summarizes these results and discusses the current challenges facing pharmacogenetics and complex disease association mapping in general, and offers suggestions for future directions.

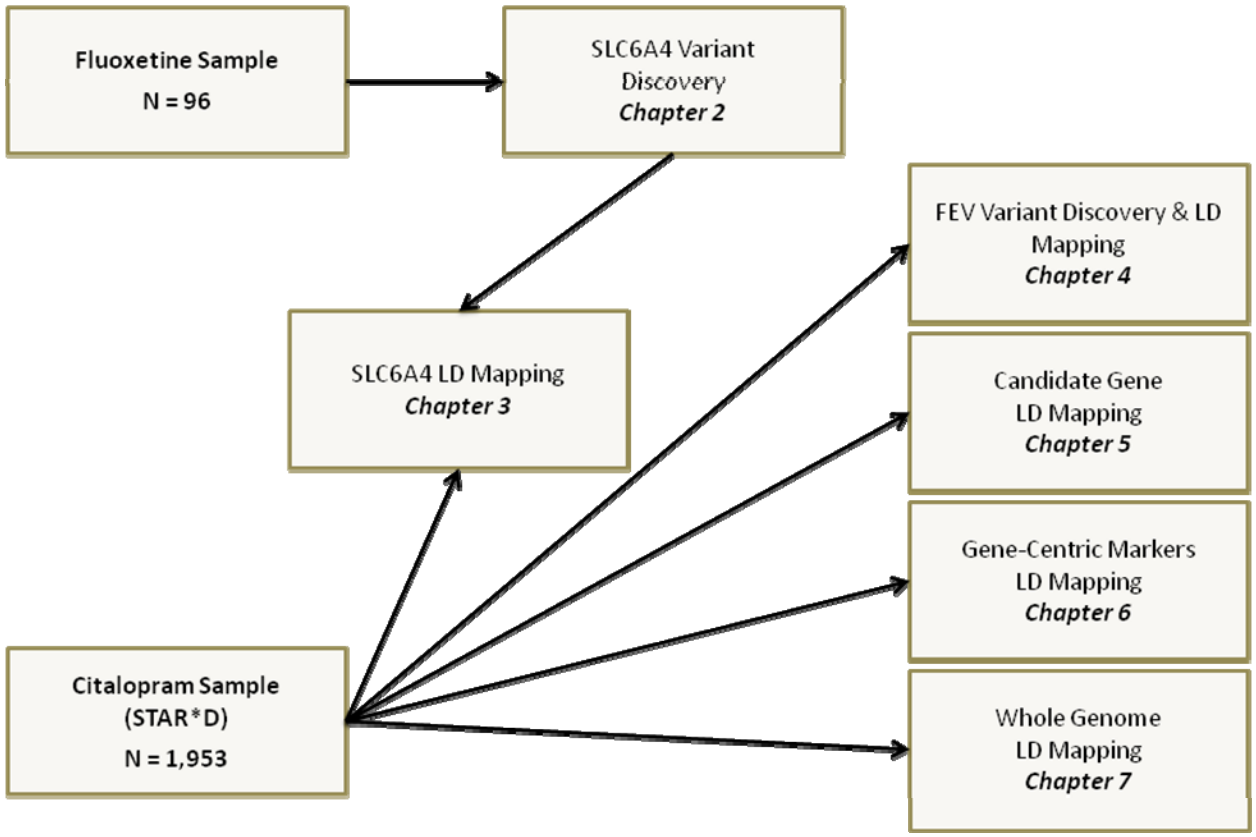

Figure 1.1: Flow chart showing the work described in this thesis dissertation.

1.6 References

1. Kessler RC, Berglund P, Demler O, Jin R and others. The Epidemiology of Major Depressive Disorder: Results From the National Comorbidity Survey Replication (NCS-R). *JAMA* 2003; 289(23): 3095-3105

2. Luppa M, Heinrich S, Angermeyer MC, Konig HH and others. Cost-of-Illness Studies of Depression: A Systematic Review. *J. Affect. Disord.* 2007; 98(1-2): 29-43

3. Berto P, D'Ilario D, Ruffo P, Di VR and others. Depression: Cost-of-Illness Studies in the International Literature, a Review. *J. Ment. Health Policy Econ.* 2000; 3(1): 3-10

4. Wong ML and Licinio J. Research and Treatment Approaches to Depression. *Nature Reviews Neuroscience* 2001; 2(5): 343-351

5. The Global Burden of Disease. 1996;

6. Hoyert DL, Heron MP, Murphy SL, and Kung HC. Deaths: Final Data for 2003. *Natl. Vital Stat. Rep.* 2006; 54(13): 1-120

7. Mueller TI, Leon AC, Keller MB, Solomon DA and others. Recurrence After Recovery From Major Depressive Disorder During 15 Years of Observational Follow-Up. *Am. J. Psychiarty* 1999; 156(7): 1000-1006

8. American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders, 4th Edition. 1994;

9. Evans DL, Charney DS, Lewis L, Golden RN and others. Mood Disorders in the Medically Ill: Scientific Review and Recommendations. *Biol. Psychiatry* 2005; 58(3): 175-189

10. Pincus HA and Pettit AR. The Societal Costs of Chronic Major Depression. *J. Clin. Psychiatry* 2001; 62 Suppl 6 5-9

11. Duval F, Lebowitz BD, and Macher JP. Treatments in Depression. *Dialogues. Clin. Neurosci.* 2006; 8(2): 191-206

12. Stafford RS, MacDonald EA, and Finkelstein SN. National Patterns of Medication Treatment for Depression, 1987 to 2001. *Prim. Care Companion. J. Clin. Psychiatry* 2001; 3(6): 232-235

13. Stahl SM, Entsuah R, and Rudolph RL. Comparative Efficacy Between Venlafaxine and SSRIs: a Pooled Analysis of Patients With Depression. *Biol. Psychiatry* 2002; 52(12): 1166-1174

14. Entsuah AR, Huang H, and Thase ME. Response and Remission Rates in Different Subpopulations With Major Depressive Disorder Administered Venlafaxine, Selective Serotonin Reuptake Inhibitors, or Placebo. *J. Clin. Psychiatry* 2001; 62(11): 869-877

15. Trivedi MH, Rush AJ, Wisniewski SR, Nierenberg AA and others. Evaluation of Outcomes With Citalopram for Depression Using Measurement-Based Care in STAR*D: Implications for Clinical Practice. *American Journal of Psychiatry* 2006; 163(1): 28-40

16. Perlis RH, Iosifescu DV, and Renshaw PF. Biological Predictors of Treatment Response in Affective Illness. *Psychiatr. Clin North Am* 2003; 26(2): 323-44, vii

17. Fergusson D, Doucette S, Glass KC, Shapiro S and others. Association Between Suicide Attempts and Selective Serotonin Reuptake Inhibitors: Systematic Review of Randomised Controlled Trials. *BMJ* 2005; 330(7488): 396

18. Simon GE, Savarino J, Operskalski B, and Wang PS. Suicide Risk During Antidepressant Treatment. *Am J Psychiatry* 2006; 163(1): 41-47

19. Weber WW. Pharmacogenetics. 1997;

20. Evans WE and Relling MV. Moving Towards Individualized Medicine With Pharmacogenomics. *Nature* 2004; 429(6990): 464-468

21. Ingelman-Sundberg M, Oscarson M, Daly AK, Garte S and others. Human Cytochrome P-450 (CYP) Genes: a Web Page for the Nomenclature of Alleles. *Cancer Epidemiol Biomarkers Prev* 2001; 10(12): 1307-1308

22. Weinshilboum R. Inheritance and Drug Response. *N. Engl. J Med.* 2003; 348(6): 529-537

23. Drysdale CM, McGraw DW, Stack CB, Stephens JC and others. Complex Promoter and Coding Region Beta 2-Adrenergic Receptor Haplotypes Alter Receptor Expression and Predict in Vivo Responsiveness. *Proc. Natl. Acad. Sci. U. S. A* 2000; 97(19): 10483-10488

24. Rieder MJ, Reiner AP, Gage BF, Nickerson DA and others. Effect of VKORC1 Haplotypes on Transcriptional Regulation and Warfarin Dose. *N Engl J Med* 2005; 352(22): 2285-2293

25. Pare CMB, Rees L, and Sainsbury MJ. Differentiation of Two Genetically Specific Types of Depression by the Response to Anti-Depressants. *Lancet* 1962; 2 1340-1343

26. Pare CMB and Mack JW. Differentiation of Two Genetically Specific Types of Depression by the Response to Antidepressant Drugs. *J. Med. Genet.* 1971; 8 306-309

27. Franchini L, Serretti A, Gasperini M, and Smeraldi E. Familial Concordance of Fluvoxamine Response As a Tool for Differentiating Mood Disorder Pedigrees. *J Psychiatr. Res.* 1998; 32(5): 255-259

28. O'Reilly RL, Bogue L, and Singh SM. Pharmacogenetic Response to Antidepressants in a Multicase Family With Affective Disorder. *Biological Psychiatry* 1994; 36(7): 467-471

29. Burke MJ and Preskorn SH. Therapeutic Drug Monitoring of Antidepressants: Cost Implications and Relevance to Clinical Practice. *Clin. Pharmacokinet.* 1999; 37(2): 147-165

30. Chen S, Chou WH, Blouin RA, Mao Z and others. The Cytochrome P450 2D6 (CYP2D6) Enzyme Polymorphism: Screening Costs and Influence on Clinical Outcomes in Psychiatry. *Clin. Pharmacol. Ther.* 1996; 60(5): 522-534

31. Kvist EE, Al Shurbaji A, Dahl ML, Nordin C and others. Quantitative Pharmacogenetics of Nortriptyline: a Novel Approach. *Clin. Pharmacokinet.* 2001; 40(11): 869-877

32. Kirchheiner J, Brosen K, Dahl ML, Gram LF and others. CYP2D6 and CYP2C19 Genotype-Based Dose Recommendations for Antidepressants: a First Step Towards Subpopulation-Specific Dosages. *Acta Psychiatr. Scand.* 2001; 104(3): 173-192

33. Lerer B and Macciardi F. Pharmacogenetics of Antidepressant and Mood-Stabilizing Drugs: a Review of Candidate-Gene Studies and Future Research Directions. *Int. J Neuropsychopharmacol.* 2002; 5(3): 255-275

34. Serretti A, Zanardi R, Rossini D, Cusin C and others. Influence of Tryptophan Hydroxylase and Serotonin Transporter Genes on Fluvoxamine Antidepressant Activity. *Molecular Psychiatry* 2001; 6(5): 586-592

35. Smeraldi E, Zanardi R, Benedetti F, Di Bella D and others. Polymorphism Within the Promoter of the Serotonin Transporter Gene and Antidepressant Efficacy of Fluvoxamine. *Molecular Psychiatry* 1998; 3(6): 508-511

36. Zanardi R, Benedetti F, Di Bella D, Catalano M and others. Efficacy of Paroxetine in Depression Is Influenced by a Functional Polymorphism Within the Promoter of the Serotonin Transporter Gene. *J Clin. Psychopharmacol.* 2000; 20(1): 105-107

37. Zanardi R, Serretti A, Rossini D, Franchini L and others. Factors Affecting Fluvoxamine Antidepressant Activity: Influence of Pindolol and 5-HTTLPR in Delusional and Nondelusional Depression. *Biological Psychiatry* 2001; 50(5): 323-330

38. Kim DK, Lim SW, Lee S, Sohn SE and others. Serotonin Transporter Gene Polymorphism and Antidepressant Response. *NeuroReport* 2000; 11(1): 215-219

39. Pollock BG, Ferrell RE, Mulsant BH, Mazumdar S and others. Allelic Variation in the Serotonin Transporter Promoter Affects Onset of Paroxetine Treatment Response in Late-Life Depression. *Neuropsychopharmacology* 2000; 23(5): 587-590

40. Arias B, Catalan R, Gasto C, Gutierrez B and others. 5-HTTLPR Polymorphism of the Serotonin Transporter Gene Predicts Non-Remission in Major Depression Patients Treated With Citalopram in a 12-Weeks Follow Up Study. *J Clin Psychopharmacol.* 2003; 23(6): 563-567

41. Lesch K-P, Bengel D, Heils A, Sabol SZ and others. Association of Anxiety-Related Traits With a Polymorphism in the Serotonin Transporter Gene Regulatory Region. *Science* 1996; 274(5292): 1527-1531

42. McMahon FJ, Buervenich S, Charney D, Lipsky R and others. Variation in the Gene Encoding the Serotonin 2A Receptor Is Associated With Outcome of Antidepressant Treatment. *Am. J. Hum. Genet.* 2006; 78(5): 804-814

43. Paddock S, Laje G, Charney D, Rush AJ and others. Association of GRIK4 With Outcome of Antidepressant Treatment in the STAR*D Cohort. *Am. J. Psychiatry* 2007; 164(8): 1181-1188

44. Zill P, Baghai TC, Zwanzger P, Schule C and others. Evidence for an Association Between a G-Protein Beta3-Gene Variant With Depression and Response to Antidepressant Treatment. *NeuroReport* 2000; 11(9): 1893-1897

45. Lee HJ, Cha JH, Ham BJ, Han CS and others. Association Between a G-Protein Beta3 Subunit Gene Polymorphism and the Symptomatology and Treatment Responses of Major Depressive Disorders. *Pharmacogenomics. J* 2004; 4(1): 29-33

46. Baghai TC, Schule C, Zwanzger P, Minov C and others. Possible Influence of the Insertion/Deletion Polymorphism in the Angiotensin I-Converting Enzyme Gene on Therapeutic Outcome in Affective Disorders. *Molecular Psychiatry* 2001; 6(3): 258-259

47. Binder EB, Salyakina D, Lichtner P, Wochnik GM and others. Polymorphisms in FKBP5 Are Associated With Increased Recurrence of Depressive Episodes and Rapid Response to Antidepressant Treatment. *Nat Genet* 2004; advanced online publication

48. Perlis RH, Mischoulon D, Smoller JW, Wan YJY and others. Serotonin Transporter Polymorphisms and Adverse Effects With Fluoxetine Treatment. *Biological Psychiatry* 2003; 54(9): 879-883

49. Murphy GM, Jr., Kremer C, Rodrigues HE, and Schatzberg AF. Pharmacogenetics of Antidepressant Medication Intolerance. *Am J Psychiatry* 2003; 160(10): 1830-1835

50. Grasmader K, Verwohlt PL, Rietschel M, Dragicevic A and others. Impact of Polymorphisms of Cytochrome-P450 Isoenzymes 2C9, 2C19 and 2D6 on Plasma Concentrations and Clinical Effects of Antidepressants in a Naturalistic Clinical Setting. *Eur. J. Clin. Pharmacol.* 2004; 60(5): 329-336

51. Recommendations From the EGAPP Working Group: Testing for Cytochrome P450 Polymorphisms in Adults With Nonpsychotic Depression Treated With Selective Serotonin Reuptake Inhibitors. *Genet. Med.* 2007; 9(12): 819-825

52. Yin OQ, Wing YK, Cheung Y, Wang ZJ and others. Phenotype-Genotype Relationship and Clinical Effects of Citalopram in Chinese Patients. *J. Clin. Psychopharmacol.* 2006; 26(4): 367-372

53. Lander ES, Linton LM, Birren B, Nusbaum C and others. Initial Sequencing and Analysis of the Human Genome. *Nature* 2001; 409(6822): 860-921

54. Venter JC, Adams MD, Myers EW, Li PW and others. The Sequence of the Human Genome. *Science* 2001; 291(5507): 1304-1351

55. Kwok PY. Methods for Genotyping Single Nucleotide Polymorphisms. *Annu. Rev. Genomics Hum. Genet.* 2001; 2 235-258

56. Risch N and Merikangas K. The Future of Genetic Studies of Complex Human Diseases. *Science* 1996; 273(5281): 1516-1517

57. Ott J and Hoh J. Statistical Approaches to Gene Mapping. *Am J Hum. Genet.* 2000; 67(2): 289-294

58. Cardon LR and Bell JI. Association Study Designs for Complex Diseases. *Nat. Rev. Genet.* 2001; 2(2): 91-99

59. Risch N and Teng J. The Relative Power of Family-Based and Case-Control Designs for Linkage Disequilibrium Studies of Complex Human Diseases. *Genome Res.* 1998; 8(12): 1273-1288

60. Zondervan KT and Cardon LR. The Complex Interplay Among Factors That Influence Allelic Association. *Nat Rev. Genet* 2004; 5(2): 89-100

61. Collins FS, Guyer MS, and Charkravarti A. Variations on a Theme: Cataloging Human DNA Sequence Variation. *Science* 1997; 278(5343): 1580-1581

62. Reich DE and Lander ES. On the Allelic Spectrum of Human Disease. *Trends in Genetics* 2001; 17(9): 502-510

63. Newton-Cheh C and Hirschhorn JN. Genetic Association Studies of Complex Traits: Design and Analysis Issues. *Mutat. Res.* 2005; 573(1-2): 54-69

64. Weiss KM and Terwilliger JD. How Many Diseases Does It Take to Map a Gene With SNPs? *Nat. Genet.* 2000; 26(2): 151-157

65. Pritchard JK. Are Rare Variants Responsible for Susceptibility to Complex Diseases? *Am J Hum. Genet.* 2001; 69(1): 124-137

66. Maraganore DM, de Andrade M, Lesnick TG, Strain KJ and others. High-Resolution Whole-Genome Association Study of Parkinson Disease. *Am J Hum Genet* 2005; 77(5): 685-693

67. Klein RJ, Zeiss C, Chew EY, Tsai JY and others. Complement Factor H Polymorphism in Age-Related Macular Degeneration. *Science* 2005; 308(5720): 385-9

68. Lawrence RW, Evans DM, and Cardon LR. Prospects and Pitfalls in Whole Genome Association Studies. *Philos. Trans. R. Soc. Lond B Biol. Sci.* 2005; 360(1460): 1589-1595

69. Herbert A, Gerry NP, McQueen MB, Heid IM and others. A Common Genetic Variant Is Associated With Adult and Childhood Obesity. *Science* 2006; 312(5771): 279-283

70. Genome-Wide Association Study of 14,000 Cases of Seven Common Diseases and 3,000 Shared Controls. *Nature* 2007; 447(7145): 661-678

71. Salonen JT, Uimari P, Aalto JM, Pirskanen M and others. Type 2 Diabetes Whole-Genome Association Study in Four Populations: the DiaGen Consortium. *American Journal of Human Genetics* 2007; 81(2): 338-345

72. Saxena R, Voight BF, Lyssenko V, Burtt NP and others. Genome-Wide Association Analysis Identifies Loci for Type 2 Diabetes and Triglyceride Levels. *Science* 2007; 316(5829): 1331-1336

73. Scott LJ, Mohlke KL, Bonnycastle LL, Willer CJ and others. A Genome-Wide Association Study of Type 2 Diabetes in Finns Detects Multiple Susceptibility Variants. *Science* 2007; 316(5829): 1341-1345

74. Carlson CS, Eberle MA, Rieder MJ, Yi Q and others. Selecting a Maximally Informative Set of Single-Nucleotide Polymorphisms for Association Analyses Using Linkage Disequilibrium. *Am. J Hum. Genet.* 2004; 74(1): 106-120

75. Ke X and Cardon LR. Efficient Selective Screening of Haplotype Tag SNPs. *Bioinformatics.* 2003; 19(2): 287-288

76. Stram DO, Haiman CA, Hirschhorn JN, Altshuler D and others. Choosing Haplotype-Tagging SNPS Based on Unphased Genotype Data Using a Preliminary Sample of Unrelated Subjects With an Example From the Multiethnic Cohort Study. *Hum. Hered* 2003; 55(1): 27-36

77. Quitkin FM, Rabkin JD, Markowitz JM, Stewart JW and others. Use of Pattern Analysis to Identify True Drug Response. A Replication. *Archives of General Psychiatry* 1987; 44(3): 259-264

78. McGrath PJ, Stewart JW, Petkova E, Quitkin FM and others. Predictors of Relapse During Fluoxetine Continuation or Maintenance Treatment of Major Depression. *J Clin. Psychiatry* 2000; 61(7): 518-524

79. McGrath PJ, Stewart JW, Quitkin FM, Chen Y and others. Predictors of Relapse in a Prospective Study of Fluoxetine Treatment of Major Depression. *Am J Psychiatry* 2006; 163(9): 1542-1548

80. Laird NM and Lange C. Family-Based Designs in the Age of Large-Scale Gene-Association Studies. *Nat Rev Genet* 2006; 7(5): 385-394

81. Cardon LR and Palmer LJ. Population Stratification and Spurious Allelic Association. *Lancet* 2003; 361(9357): 598-604

82. Tang H, Quertermous T, Rodriguez B, Kardia SL and others. Genetic Structure, Self-Identified Race/Ethnicity, and Confounding in Case-Control Association Studies. *Am J Hum Genet* 2005; 76(2): 268-275

83. Shriver MD, Mei R, Parra EJ, Sonpar V and others. Large-Scale SNP Analysis Reveals Clustered and Continuous Patterns of Human Genetic Variation. *Hum. Genomics* 2005; 2(2): 81-89

84. Devlin B, Roeder K, and Wasserman L. Genomic Control, a New Approach to Genetic-Based Association Studies. *Theor. Popul. Biol.* 2001; 60(3): 155-166

85. Pritchard JK and Rosenberg NA. Use of Unlinked Genetic Markers to Detect Population Stratification in Association Studies. *Am J Hum. Genet.* 1999; 65(1): 220-228

86. Falush D, Stephens M, and Pritchard JK. Inference of Population Structure Using Multilocus Genotype Data: Linked Loci and Correlated Allele Frequencies. *Genetics* 2003; 164(4): 1567-1587

87. Jackson JE. A User's Guide to Principal Components. 2003;

88. Cavalli-Sforza LL, Menozzi P, and Piazza A. Demic Expansions and Human Evolution. *Science* 1993; 259(5095): 639-646

89. Price AL, Patterson NJ, Plenge RM, Weinblatt ME and others. Principal Components Analysis Corrects for Stratification in Genome-Wide Association Studies. *Nat Genet* 2006; 38(8): 904-909

90. Menozzi P, Piazza A, and Cavalli-Sforza L. Synthetic Maps of Human Gene Frequencies in Europeans. *Science* 1978; 201(4358): 786-792

91. Devlin B, Roeder K, and Wasserman L. Analysis of Multilocus Models of Association. *Genet. Epidemiol* 2003; 25(1): 36-47

92. Dudbridge F and Koeleman BP. Efficient Computation of Significance Levels for Multiple Associations in Large Studies of Correlated Data, Including Genomewide Association Studies. *Am. J. Hum. Genet* 2004; 75(3): 424-435

93. Wang H, Thomas DC, Pe'er I, and Stram DO. Optimal Two-Stage Genotyping Designs for Genome-Wide Association Scans. *Genet Epidemiol.* 2006; 30(4): 356-368

94. Skol AD, Scott LJ, Abecasis GR, and Boehnke M. Optimal Designs for Two-Stage Genome-Wide Association Studies. *Genet. Epidemiol.* 2007; 31(7): 776-788

95. Skol AD, Scott LJ, Abecasis GR, and Boehnke M. Joint Analysis Is More Efficient Than Replication-Based Analysis for Two-Stage Genome-Wide Association Studies. *Nat. Genet.* 2006; 38(2): 209-213

96. Kraft JB, Slager SL, McGrath PJ, and Hamilton SP. Sequence Analysis of the Serotonin Transporter and Associations With Antidepressant Response. *Biol Psychiatry* 2005; 58(5): 374-381

97. Kraft JB, Peters EJ, Slager SL, Jenkins GD and others. Analysis of Association Between the Serotonin Transporter and Antidepressant Response in a Large Clinical Sample. *Biol Psychiatry* 2006; advanced online publication

CHAPTER 2

SEQUENCE ANALYSIS AND VARIANT DISCOVERY IN THE SEROTONIN TRANSPORTER (*SLC6A4*) FOR ASSOCIATION WITH RESPONSE TO FLUOXETINE[†]

2.1 Introduction

Major depressive disorder (MDD) is a prevalent and disabling psychiatric disorder characterized by depressed mood, a number of neurovegetative symptoms, and functional impairment (1). Selective serotonin reuptake inhibitors (SSRIs) are effective medications for MDD and are the most widely prescribed antidepressants worldwide, and among the most commonly prescribed medications of any type. Despite recent advances in antidepressant pharmacotherapy, response rates are variable and are typically about 60% for the first drug administered (2). The underlying mechanism for this variation is complex, involving both environmental and genetic factors and their interaction (3-5).

One possible genetic mechanism involves the *SLC6A4* gene, which encodes the serotonin transporter. This protein initiates the antidepressant effect of SSRIs, which are drugs that are thought to act primarily by terminating serotonin reuptake by the presynaptic serotonergic neuron.

Many studies have tested for genetic association between deoxyribonucleic acid (DNA) variants in the serotonin transporter and SSRI response (6). Most studies have focused on a single common polymorphism located in the promoter region of *SLC6A4*. This insertion/deletion polymorphism (HTTLPR), reported to be 44 base pairs (bp) in

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length, has two common alleles, the long and the short, with the long allele having been shown to have higher in vitro transcription than the short allele (7). The results of these studies have been mixed. Two studies, including one of our own, found no association between SSRI response and the promoter variant (8,9). Multiple studies have reported a positive association with the long allele (10), and two studies have reported a negative association between the long allele and treatment response (11,12). This disparity among these studies might be partially explained by the fact that the studies finding positive association between response and the long allele were investigating Caucasian subjects, whereas those that found a negative association were studying Asian populations. No study has focused on Africans or African Americans, and the allele frequency differences between African American and other populations add complexity to association studies in diverse populations (13). Meta-analysis suggests that this polymorphism might be associated with MDD itself, although with a small effect (14). There is an equivocal imaging literature regarding the actual functional effect of HTTLPR genotype on transporter availability (15,16), although some groups have reported associations between genotype and functional imaging phenotype (17,18).

SLC6A4 covers 37.8 kilobases (kb) of genomic sequence on chromosome 17q11.2, contains a 630-amino-acid open reading frame, and has 15 exons, including 2 non-coding exons, designated 1A and 1B (19). A study done by Glatt and colleagues screened the exonic regions for variants in an uncharacterized sample of 450 people and found the locus to contain no common variants with the minor allele frequency (MAF) > 10%. They did, however, find 18 rare variants (MAF < 1%) and 2 variants occurring at allele frequencies of 0.01 and 0.03 (20). A mutation screen in a much smaller disease

sample similarly yielded no coding variants (21). Given that *SLC6A4* is the therapeutic target of SSRIs, we sought to screen a well phenotyped population of persons with MDD for DNA variants in exons as well as surrounding introns to accomplish two things: 1) to identify *SLC6A4* DNA variation in a depressed population; and 2) to further investigate the role of the *SLC6A4* locus in antidepressant response. This work represents a natural extension of our previous work using the same gene and clinical sample, in which we identified association between anonymous markers and antidepressant response (9). Specifically, we found a SNP, rs25533, in the promoter region associated with antidepressant response but found no association with the HTTLPR and antidepressant response. To do this, we tested for association between variants in the sample and a response phenotype. We tested both single variants as well as haplotypes. We also describe biochemical assays suggesting that a single nucleotide polymorphism (SNP) in the promoter region of the gene might play a role in the transcription of the serotonin transporter.

2.2 Materials and Methods

2.2.1 Fluoxetine Study Sample

We genotyped 96 research subjects diagnosed with unipolar MDD enrolled in a National Institute of Mental Health–funded fluoxetine discontinuation protocol (Patrick J. McGrath, principal investigator, New York State Psychiatric Institute). Diagnoses were established with the Structured Clinical Interview for DSM-IV Axis I Disorders–Patient Edition (22). No minimum score on a depression severity rating scale was required for inclusion. Baseline medical screening included medical history, physical examination,

electrocardiogram, complete blood count, urinalysis, blood chemistry, thyroid function, and a urine drug screen.

Exclusion criteria were: significant suicide risk; pregnancy, breastfeeding, or absence of effective contraception for women; unstable physical disorders; neurological disorders significantly affecting central nervous system function, including history of seizures; lifetime history of any organic mental disorder, psychotic disorder, or mania; substance abuse or dependence active in the previous 6 months, excepting nicotine dependence; concurrent use of medications suspected to cause or exacerbate depression (e.g., β-blockers or corticosteroids) or to have significant antidepressant or anxiolytic properties; clinical or laboratory evidence of hypothyroidism without adequate and stable replacement; history of non-response to an adequate SSRI trial; or SSRI in a past or current depressive episode (defined as a 4-week trial of a minimum of 40 mg of fluoxetine or its daily equivalent). Study subjects were included if they occasionally took a non-benzodiazepine hypnotic, thyroid hormone replacement at a constant and effective dose for at least 3 months before the study, or oral contraceptives not temporally associated with onset or exacerbation of depression. Diuretics, oral hypoglycemics, and antihypertensives were permitted. Subjects in an established psychotherapy not believed to be effective for depression (i.e., other than interpersonal or cognitive behavioral therapy) were included. No subject began psychotherapy at entry or during the study.

Subjects' response patterns were categorized as responders, non-responders, or placebo-responders by pattern analysis after 12 weeks of open-label fluoxetine monotherapy (23). Subjects were subsequently followed for 12 months with double-blind fluoxetine maintenance versus placebo and again categorized by response type. Response

was judged by use of the Clinical Global Impression of Improvement score, whereby a score of "much improved" or "very much improved" was required for response. The mean (SD) baseline score on the 17-item Hamilton Rating Scale for Depression (HAMD-17) for all subjects was 23.18 (4.9) (n = 93, missing = 3). The mean end of treatment HAMD-17 score for responders was 4.42 (2.9) (n = 74, missing = 3). The mean end of treatment HAMD-17 score for non-responders was 15.6 (6.0) (n = 17, missing = 2).

An additional response phenotype was generated with response pattern analysis to attempt to decrease the apparent response to medication that is actually a placebo response. This analysis is based on the observation that a pattern of delayed but sustained response to medication characterizes "placebo" or non-specific response. Response pattern was defined in the following manner (24,25). "Specific" response was defined as response at week 12 with response beginning after the second week and sustained until week 12; "non-specific" or "placebo-pattern" response began in weeks 1 or 2 and was not sustained for all subsequent weeks until week 12. The average (±SD) age was 37.1 ± 11.6 years, and the male/female ratio was 49% to 51%. There were 77 responders (80%) and 19 non-responders (20%) to a 12-week trial of fluoxetine. Use of pattern analysis indicated that 20 of 77 responders (26%) were non-specific responders. The breakdowns of non-responder, responder, & specific responder can be seen in Figure 2.1. The subject population was 78% Caucasian, 6% African American, 8% Hispanic, 5% Asian, and 3% other. No significant differences in ethnicity (by exact test, p = 0.07) or age (by t test, p = 0.19) were found between responders and non-responders. Institutional review board approval was obtained from the New York State Psychiatric Institute and the University of California, San Francisco, and each research participant provided informed consent.

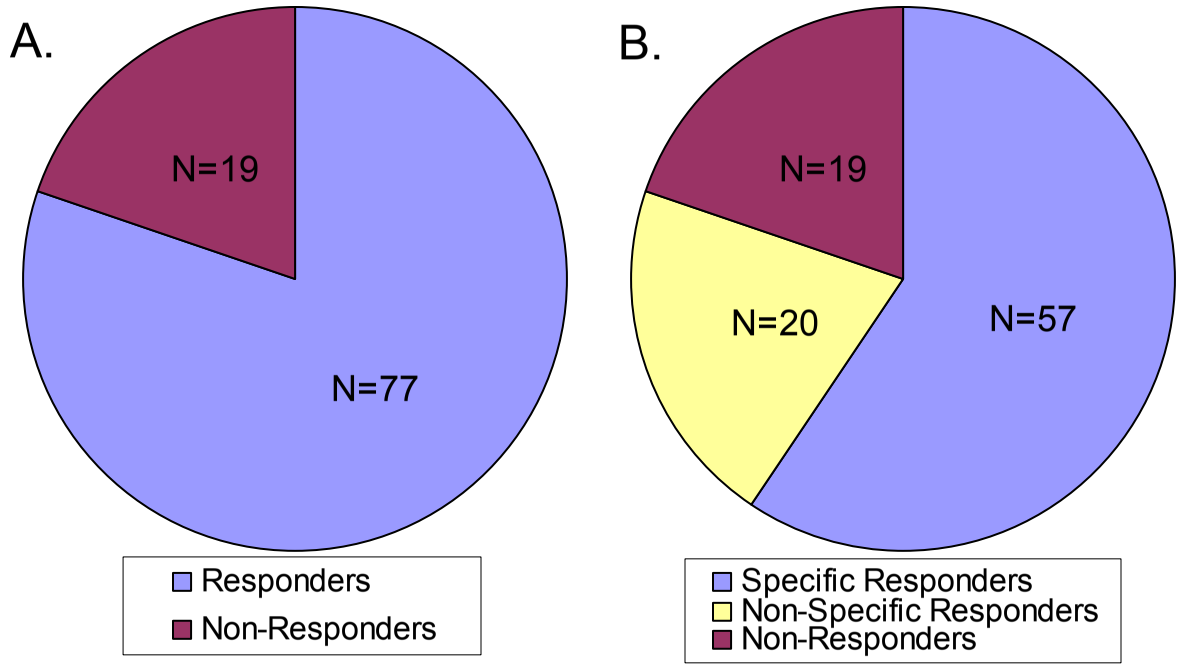

Figure 2.1: Breakdown of phenotypes in fluoxetine data set based on A.) Primary response phenotype of categorical responders versus non-responders and B.) Sub-classification of responders in to either specific or non-specific responders.

2.2.2 Gene Sequence and Polymerase Chain Reaction Primers

Genomic and complementary DNA sequences were obtained from GenBank (accession numbers AC104984 and BC069484), and primers were designed with Primer3 software (26) and manufactured by Invitrogen (Carlsbad, California). Primers were designed to give products between 350 bp and 600 bp in length. These products were designed to span exons and include flanking intronic sequence at the 5′ and 3′ ends. Eighteen primer pairs were designed to screen 7.9 kb of sequence, including all 15 exons (NT_010799) and the promoter length polymorphism (HTTLPR) (accession numbers AB061799 – AB061801) in the promoter region (Table 2.1).

2.2.3 DNA Analysis

Genomic DNA was extracted from whole blood with a Puregene genomic DNA purification kit (Gentra Systems, Minneapolis, Minnesota). Deoxyribonucleic acid was quantified with an ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, Delaware).

2.2.4 DNA Amplification

For all 96 samples, amplification was performed in a final volume of 10 μL containing 20 ng genomic DNA template, 50 μmol/L deoxyribonucleoside triphosphates (dNTPs), 1 mol/L anhydrous betaine, 50 mmol/L KCl, 20 mmol/L Tris-HCl (pH 8.4), 2.5 mmol/L $MgCl_2$, 200 nmol/L primers, and 0.25 units Platinum Taq DNA polymerase (Invitrogen, Carlsbad, California), then cycled according to a touchdown protocol at 94°C for 3 min, followed by 7 cycles at 94°C for 30 sec, 65°–59°C for 30 sec (decreased by 1°C intervals per cycle), and 72°C for 30 sec, followed by 38 cycles at 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec, with a final 10 min at 72°C. For amplicons 1

and 8, Platinum Taq High-Fidelity DNA polymerase was used along with $MgSO_4$ concentration of 2 mmol/L in place of $MgCl_2$. For amplicons 1, 8, and 16, the touchdown protocol was modified to annealing temperatures of 72°C, 60°C, and 61°C, respectively. For the HTTLPR, amplification was performed in a final volume of 6 μL containing 20 ng genomic DNA template, 200 μmol/L dNTPs, 1.5 mol/L anhydrous betaine, 15 mmol/L $(NH_4)_2SO_4$ (pH 9.3), 50 mmol/L Tris-HCl (pH 8.4), 2.5 mmoL $MgCl_2$, 0.1% Tween 20, 500 nmol/L primers, and 0.25 units JumpStart AccuTaq LA DNA polymerase (Sigma-Aldrich, St. Louis, Missouri), then cycled at 94°C for 1 min, followed by 45 cycles at 94°C for 30 sec and 68°C for 4 min, with a final 30 min at 68°C. All reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, California) in 384-well plates (MJ Research, Waltham, Massachusetts).

2.2.5 Denaturing High-Performance Liquid Chromatography Screening

All amplicons with the exception of 1, 8, 16, and the HTTLPR were screened by denaturing high-performance liquid chromatography (dHPLC) on a WAVE Nucleic Acid Fragment Analysis System (Transgenomic, Omaha, Nebraska). Denaturation temperature and column gradient conditions were determined with Wavemaker software v4.1.44 (Transgenomic, Omaha, Nebraska). All samples were amplified and screened by eye for variant waveforms. Samples with deviation from the most common waveform along with at least one sample with the common waveform were subsequently subjected to direct sequencing. Amplicons 1, 8, 16, and the HTTLPR were subjected to direct sequencing with primary screening by dHPLC.

AMPLICON	Region	PRIMERS	SIZE	Tm
LPR	*HTTLPR*	ggcgttgccgctctgaatgc gagggactgagctggacaaccac	419	*68*
1	*Exon 1A*	gagcgcaaccccatccagcgggagc cgctggggcgcatgcacctcctcg	390	*72*
2	*Exon 1B*	caccccagcatcagtaacct ccccttttgtcttggatgcta	493	*58*
3	*Exon 2*	atggactgccatgtagcaaa agctcagccactagggtggt	498	*58*
4	*Exon 2*	agagctcggaggtgatcca tcgcagcctgtgatactgac	454	*58*
5	*Exon 3*	ggatgtgatcctgcctgttc tgcctaaggcctgactgatt	519	*58*
6	*Exon 4*	gcctggagtccttggaatg actcccacccctgatagctt	501	*58*
7	*Exon 5*	tgtgctttgtggtccttcag agagagagggtgcatcatgg	454	*58*
8	*Exon 6*	tgactccaagggttgtgatctttctgc gattcaggcataaacccatccagt	515	*60*
9	*Exon 7*	ggcttcagtgtgcaagtctg ccaatcaccttcctccacac	454	*58*
10	*Exon 8*	catggcagtcagagcttcag catgcccagccttctttg	461	*58*
11	*Exon 9*	tgtcaaccacctcctcctct gccaagggacagtgcttaat	416	*58*
12	*Exon 10*	cttacccctccctcctgttc gtgggatctgcggtaaaatg	456	*58*
13	*Exon 11*	cacgcctggctaattttcac atcgggaggtcacatcttgt	487	*58*
14	*Exon 12*	gtaggaattccggcttgtca ggattacaggtgcccatcac	452	*58*
15	*Exon 13*	tcacatcttgggaatttcctg aggctttgggagatgcctta	484	*58*
16	*Exon 14*	aaggctcatcattttcttccat gggaatatgtccaggggaat	543	*58*
17	*Exon 14*	tccgcttgaatgctgtgtaa tggctagcgagatagcatcc	460	*58*

Table 2.1: PCR amplicon information including forward & reverse primer sequences, length in basepairs (bp), and primer annealing temperature.

2.2.6 DNA Sequencing

Before direct sequencing, the excess primers and deoxynucleotides in the polymerase chain reaction (PCR) products were then degraded by adding a 5-μL solution of 1 unit of shrimp alkaline phosphatase (Roche, Indianapolis, Indiana), 0.5 units of *Escherichia coli* Exonuclease I (USB, Cleveland, Ohio), 5 mmol/L $MgCl_2$, and 50 mmol/L Tris-HCl (pH 8.5). The mixture was incubated at 37°C for 90 min, followed by deactivation for 15 min at 95°C. Sequencing reactions were performed with BigDye v3.1 (Applied Biosystems, Foster City, California) chemistry at a 1/16th scale in 5 μL total volume containing 1 μL template (approximately 25 ng), 2.5 pmol primer, 0.75 μL Applied Biosystems 5× buffer, .5 μL BigDye v3.1, and 1.75 μL water. The reactions were cycled at 96°C for 3 min, followed by 25 cycles at 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min. The reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems) in 384-well plates (MJ Research). Reactions were then purified with Montage MultiScreen-SEQ (Millipore, Bedford, Massachusetts) plates and the Hamilton Microlab 4200 (Hamilton, Reno, Nevada) 96-probe liquid robotic system. Samples were analyzed on a Prism 3730xl DNA Analyzer (Applied Biosystems), and analysis of mutations was performed with Mutation Surveyor v2.30 software (SoftGenetics, State College, Pennsylvania). For SNP 1 (rs25531), samples that were homozygous for the minor G allele were amplified, run on a 2% agarose gel, then gel purified according to the manufacturer's instructions (Qiagen, Valencia, California) to separate the long and short HTTLPR alleles and then subject to direct sequencing. All variants were submitted to dbSNP. With this sample set, the probability that we would

detect variants at a minor allele frequency of 1% was 85.2%, and >99.9% for variants with minor allele frequencies of >5%.

2.2.7 Genomic Control Genotyping

To correct for population stratification within the sample collection, we previously reported the results of the genomic control method (27,28) and found minimal evidence of stratification (9). In brief, we genotyped 20 C/T SNPs distributed over the majority of chromosomes and calculated the χ^2 tests of association. The average of these statistics was used to correct for the observed stratification. In the response group versus non-response group comparison, genomic control analysis resulted in λ of 1.21, indicating a need to adjust *p* values for slight population stratification. In the other two phenotypic comparisons, specific response versus non-specific response and specific response versus all others, genomic control analysis resulted in $\lambda < 1.0$, indicating that we could not detect stratification between these two patient populations. This inflation factor is then used to adjust for potential population stratification. If there is truly no inflation due to population stratification, then the inflation factor would have a value of 1. Our estimated inflation factor indicates modest stratification, but this is not statistically significant, though our lack of significance can be due to low power (sample size = 20 markers). Regardless, we can still use our estimate of 1.21 to adjust for possible stratification.

2.2.8 Statistical Analysis

Three phenotypic comparisons were made, based on the results from the response pattern analysis described above. The comparisons made were 1) all responders (specific and non-specific) versus non-responders; 2) specific responders versus both non-specific

responders and non-responders; and 3) specific responders versus non-specific responders. Single-point association tests were performed by logistic regression with the statistical package R 1.6.1 (29). Alleles were coded as 0, 1, or 2, corresponding to the presence of 0, 1, or 2 copies of the rare allele. This coding scheme was chosen because of its robustness to departure from the true additive genetic model (30). Tests for associations using and multi-marker haplotypes were implemented in COCAPHASE v2.403 (31). This program uses an unconditional logistic regression based on a log-linear model and reports likelihood ratio tests. The expectation-maximization algorithm infers haplotypes and calculates maximum-likelihood frequency estimates. Permutation (100,000 permutations) was used to estimate the significance of the results for haplotype analyses. COCAPHASE was also used to estimate linkage disequilibrium (LD) across selected regions of *SLC6A4*.

2.2.9 Electrophoretic Mobility Shift Assays

Oligonucleotides were designed for the G and A alleles of rs25531 (5′-GCATCCCCCCTGCACCCCC(G/A)GCATCCCC-3′) as well as the AP-2 consensus oligonucleotide (5′-GATCGAACTGACCGCCCGCGGCCCGT-3′) and manufactured by Invitrogen. Oligonucleotides were annealed to make double-stranded DNA (dsDNA), then labeled by 3′-end labeling with Klenow fragment (New England Biolabs, Beverly, Massachusetts) in a 50-μL reaction consisting of 5 μL of 10× Klenow buffer (100 mmol/L Tris, 500 mmol/L NaCl, 100 mmol/L $MgCl_2$, and 10 mmol/L dithiothreitol), 2 μL (2 pmoL/μL) dsDNA template, 1 μL (5 units) Klenow, 1μL alpha deoxycytidine triphosphate (αdCTP) (3000 Ci/mmol), 3 μL of dNTP mixture (100 μmol/L of deoxyadenosine triphosphate, deoxyguanosine triphosphate, and deoxythymidine

triphosphate), and 38 μL water. Labeled oligonucleotides were separated from unincorporated αdCTP by means of Sephadex G-50 spin columns (Amersham Biosciences, Piscataway, New Jersey). Binding reactions were done in 15 μL consisting of 1 μL (5 ng) HeLa cell nuclear extracts (Santa Cruz Biotechnology, Santa Cruz, California), 3 μL 5× binding buffer (50 mmol/L Tris, 750 mmol/L KCl, 2.5 mmol/L ethylenediaminetetraacetic acid, 0.5% Triton-X 100, 62.5% glycerol [vol/vol], and 1 mmol/L DTT), 1 μL (1 μg) poly dA · dT, 1 μL (approximately 300,000 counts per minute) labeled oligonucleotide, and 9 μL water. Binding reactions were loaded onto a 6% non-denaturing polyacrylamide gel and run at 180 V for 2 hours at 25°C. The gel was transferred to 3M paper and dried for 1 hour before being exposed to a PhosphorImager screen (Amersham Biosciences) overnight. The optical density of each band was determined with ImageQuant 3.3 software (Molecular Dynamics, Sunnyvale, California).

2.3 Results

2.3.1 Sequencing Results

We screened 2628 bp of exonic and 4783 bp of intronic or flanking sequence, totaling 7.4 kb in each of 96 samples. This translates to 712 kb screened in our population. Our effort screened more intronic sequence (2:1 ratio) than had been done by other studies (20,21).

2.3.2 SNP Detection

Our effort yielded 27 variants in the *SLC6A4* locus, comprising 25 SNPs and 2 insertion/deletions. Of these, 21 were newly discovered variants, including 20 nonexonic variants, 1 SNP in an untranslated exon, and 1 SNP in a coding exon (Table 2.2). Sixteen

of the 27 were singletons (3 exonic), and only 2 had MAFs greater than 10%. Of special interest was a SNP (rs25531) just upstream of the HTTLPR. This SNP, which had been previously encountered in *SLC6A4*-sequencing screens, had an MAF of 10% (21,32). It resides in the complex of repeat units that constitute the HTTLPR, and it occurs 18 bp 5′ to the site where a 43-bp insertion/deletion defines the 14-repeat (deleted, or "short") or 16-repeat (inserted, or "long") common HTTLPR alleles.

2.3.3 Single Marker Association

In our primary phenotypic comparison, response versus non-response to fluoxetine, we tested SNP rs25531, the only SNP with a sufficient minor allele frequency that had not been already analyzed in our previous work (9). We first tested for an association by using an additive model and found nominal statistical significance (p = 0.03); however, because there were only two individuals who were homozygous for the minor allele (both non-responders), we also tested for an association by using a dominant model (carrier versus noncarrier of the minor allele) and found non-significant results (p = 0.09, odds ratio .37, 95% confidence interval 0.12–1.17). The odds ratio suggested that rare allele carriers were less likely to respond to treatment. This variant showed no significant deviation from Hardy-Weinberg equilibrium.

We also tested for association between this variant and two other response phenotypes. Because we are able to determine the specificity of response pattern, we are able to separate specific ("true") responders from non-specific ("placebo") responders (see Methods and Materials for description of pattern analysis). Our two phenotypes based on this refinement consist of 1) specific responders versus all others (i.e., non-specific responders and non-responders); and 2) specific responders versus non-specific

responders. Both comparisons resulted in no significant association between the phenotype and rs25531. Four known SNPs, also found by sequencing in this population (rs6353, rs6354, rs6355, and rs140700) (Table 2.2), were previously tested for association in this same population with fluoxetine response, none of which were significantly associated with treatment response (9). The low allele frequencies seen for the remainder of the newly discovered variants provides insufficient power to detect association between the variant and phenotype in our sample, thus we did not formally test for association with these variants.

2.3.4 Haplotypic Association

To examine potential interaction between alleles from different variants within a gene, we inferred haplotypes from unphased genotypes and tested for association with our primary phenotype. For this analysis, we focused on the SNP rs25531 and two other nearby variants. One is the HTTLPR and the other is an SNP near the non-coding exon 1A (rs25533), which was nominally associated with our responder versus nonresponder phenotypic comparison in our previous work (9). Thus, we used genotypic data for the SNP rs25531 described from the previous section in conjunction with our HTTLPR and rs25533 genotypes obtained from our previously published work (9). The expectation maximization algorithm estimated six haplotypes (Table 2.3). We found a significant global test of association ($p = 0.02$); however, the 100,000 permutation global p value was nonsignificant ($p = 0.10$). Of interest, inspection of the inferred haplotypes suggests that a haplotype containing the A, 14-repeat, and T alleles of rs25531, HTTLPR, and rs25533, respectively, is more common in responders, whereas a haplotype containing complete mismatches for these variants (G, 16-repeat, C) occurs more often in non-

responders (Table 2.3). On the basis of allele frequencies of the individual markers, we would expect the A–16-repeat–T haplotype to be the most common, and indeed it was estimated to occur at nearly equivalent allele frequencies in responders (48%) and non-responders (50%).

2.3.5 Linkage Disequilibrium

We estimated LD between SNP rs25531, the HTTLPR, and the nearby SNP rs25533 by using D′ and r^2. The LD seemed to be strong among the three markers (Table 2.4) according to D′ but less so according to another metric of LD, r^2. We also estimated LD within the responders and the non-responders and observed stronger LD among the markers in the responders than in the non-responders (Table 2.4), although given the potential for wide variability due to small sample sizes, comparisons warrant caution.

2.3.6 Functional Characterization of SNP rs25531

We sought to analyze further the role that rs25531 plays in antidepressant response. To confirm that rs25531 lies upstream of the insertion/deletion constituting the HTTLPR, a matter of debate in the literature, we gel purified and sequenced separately the long and short alleles of samples homozygous for the minor G allele(32,33). The resulting sequence data show that the polymorphism is 5′ to HTTLPR and can occur in the context of a long or short HTTLPR allele. This polymorphism still lies within the greater repeat structure of the promoter region. According to the repeat architecture of a previous sequence analysis of the HTTLPR (32), this polymorphism occurs 5′ to the 43-bp (not 44-bp, as is typically reported) deletion that delineates the most common 14- and 16-repeat alleles seen in the human population. In other words, rs25531 occurs within the sixth repeat, whereas the deleted segment occurs within the seventh through ninth repeats

of the most common configuration of the HTTLPR. The A allele, also seen in chimpanzee, occurs in the 20-bp ζ allele of Nakamura et al, whereas the minor G allele corresponds to the 20-bp μ allele. Our sequence data indicate that the 43-bp deletion is defined by removing 15 bp of the o repeat, an entire 20-bp ζ repeat, and eight bases of a η repeat. This region is outlined in Figure 2.2.

The base substitution in rs25531 alters a consensus binding sequence for the activator protein 2 (AP-2) transcription factor. To test the possibility that this variation changes the binding properties of this segment of DNA for AP-2, we carried out a series of electrophoretic mobility shift assays. First, we determined that oligonucleotides containing rs25531 and flanking sequence were retarded by nuclear extracts. The banding pattern from both the G and A oligonucleotides matched that of a consensus recognition sequence for AP-2 (Figure 2.3). This protein–DNA interaction was quantitatively greater in the less common G allele oligonucleotide than that seen with major A allele oligonucleotides but less than that of consensus AP-2 oligonucleotides. This effect was inhibited by incubating nuclear extracts with excess unlabeled G or A allele oligonucleotides (data not shown), as well as excess unlabeled consensus AP-2 oligonucleotides. This effect was specific for AP-2, because unlabeled consensus SP1 oligonucleotides failed to inhibit this effect. Negative control samples lacking nuclear extract did not lead to any bands other than the free probe (data not shown).

Location	Coding	Noncoding (Exonic)	Noncoding (Intronic)	Position ‡	Variant	SNP #	Coding	No. of Chromosomes	dbSNP
Promoter	---	---	419	26247	**A** → G	1	N	19	rs25531 †
Exon 1A	---	75	315	27876	**G** → A	2	N	1	rs34845320
				28063	**C** → T	3	N	2	rs35206195
Exon 1B	---	97	396	40695	**C** → A	4	N	37	rs6354 †
				40731	**C** → A	5	N	1	rs28914831
				40784	**G** → A	6	N	1	rs28914827
				40868	**C** → T	7	N	1	rs34871173
Exon 2	343	123	174	41391	**T** → C	8	N	1	rs7212502
				41398	**G** → A	9	N	1	rs34102420
				41783	**G** → C	10	Y	2	rs6355 †
Exon 3	135	---	384						
Exon 4	220	---	281	45158-45159	ΔTC	11	N	1	rs34019821
				45549	ΔA	12	N	1	rs34459452
Exon 5	139	---	315						
Exon 6	135	---	370	47204	**G** → A	13	N	21	rs140700 †
				47244	**C** → T	14	N	1	rs35886704
				47549	**G** → A	15	N	1	rs35721756
Exon 7	104	---	267	47684	**G** → A	16	N	5	rs34956669
				47817	**G** → C	17	N	4	rs34083002
Exon 8	128	---	333	50551	**G** → A	18	N	4	rs34149483
Exon 9	113	---	303	52263	**G** → A	19	Y	1	rs6353 †
				52400	**G** → A	20	N	2	rs35842343
Exon 10	132	---	324	53113	**G** → A	21	N	1	rs34954201
Exon 11	100	---	387	54156	**G** → T	22	N	2	rs35467658
				54293	**C** → G	23	N	1	rs34332000
				54300	**G** → C	24	N	1	rs34876533
Exon 12	101	---	351	55780	**C** → T	25	Y	1	rs33919215
Exon 13	168	---	316	60197	**T** → C	26	N	1	rs34129293
Exon 14	75	440	267	65307	**C** → T	27	N	1	rs34500314

Table 2.2: Results for variant discovery sequencing in SLC6A4 within the fluoxetine sample set. Chimpanzee reference allele in boldface and number of chromosomes out of 192 indicated. † indicates a SNP was previously identified and present in dbSNP at time of study. ‡ Indicates position in reference to accession AC104984.14.

Haplotype Frequencies		
	Non-Responders	*Responders*
A - 14RPT - T	11 (29%)	67 (44%)
A - 16RPT - C	0 (0%)	2 (1%)
A - 16RPT - T	19 (50%)	74 (48%)
G - 14RPT - T	2 (5%)	0 (0%)
G - 16RPT - A	6 (16%)	6 (4%)
G - 16RPT - T	0 (0%)	5 (3%)

Table 2.3: Estimated Frequencies for haplotypes containing variants in the promoter and exon 1A region of SLC6A4. Haplotypes consist of variants: rs25531, HTTLPR, and rs25533 (in order). The number of repeats is indicated by 14RPT or 16RPT, corresponding to "short" and "long" alleles, respectively.

A.

	rs25531	***HTTLPR***	***rs25533***
rs25531		0.75	0.84
HTTLPR	0.06		1.00
rs25533	0.50	0.04	

B.

	rs25531	***HTTLPR***	***rs25533***
rs25531		1.00	0.72
HTTLPR	0.43		1.00
rs25533	1.00	1.00	

Table 2.4: Linkage Disequilibrium estimates with R^2 and D' using three markers (rs25531, HTTLPR, and rs25533). Shown in panel A.) LD estimates in the entire sample using R^2 (above diagonal) and D' (beneath diagonal) and B.) LD estimates using D' in the responders (above diagonal) and non-responders (beneath diagonal).

A

α	TGCAGCCCTCCCAGCATCCCCCC
β	TGCAACCTCCCAGCAACTCCC
γ	TGTACCCCTCCTAGGATCGCTCC
δ	TGCATCCCCCATTATCCCCCCC
ε	TTCACCCCTCGCGGCATCCCCCC
ζ	TGCACCCCC**R**GCATCCCCCC
ο	TGCAGCCC[CCCCAGCATCTCCCC
ζ	TGCACCCCCAGCATCCCCCC
η	TGCAGCCC]TTCCAGCATCCCCC
θ	TGCACCTCTCCCAGGATCTCCCC
ι	TGCAACCCCCATTATCCCCCC
κ	TGCACCCCTCGCAGTATCCCCCC
λ	TGCACCCCCCAGCATCCCCCCA
μ	TGCACCCCCGGCATCCCCCC
ν	TGCACCCCTCCAGCATTCTCCT

B

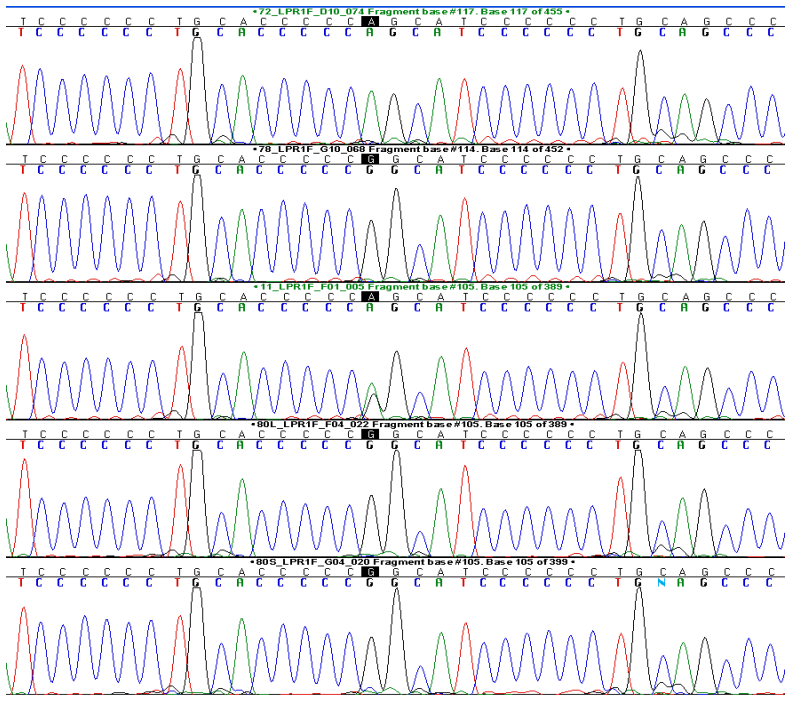

Figure 2.2: Nucleotide sequence of *SLC6A4* promoter repeat polymorphism.
A.) Repeat segments are designated as in Nakamura et al (2000) and represent a 16-repeat sequence. The location of the A/G single nucleotide polymorphism rs25531 is designated by a bolded "R" in the first repeat. The 43 base pair sequence deleted in "short" or 14-repeat alleles is marked by brackets. B.) Representative electropherograms showing rs25531 homozygote common AA (1), homozygote rare GG (2), and heterozygote AG (3). Panels 4 & 5 show the G allele on both the 14RPT (4) & 16RPT (5) backgrounds.

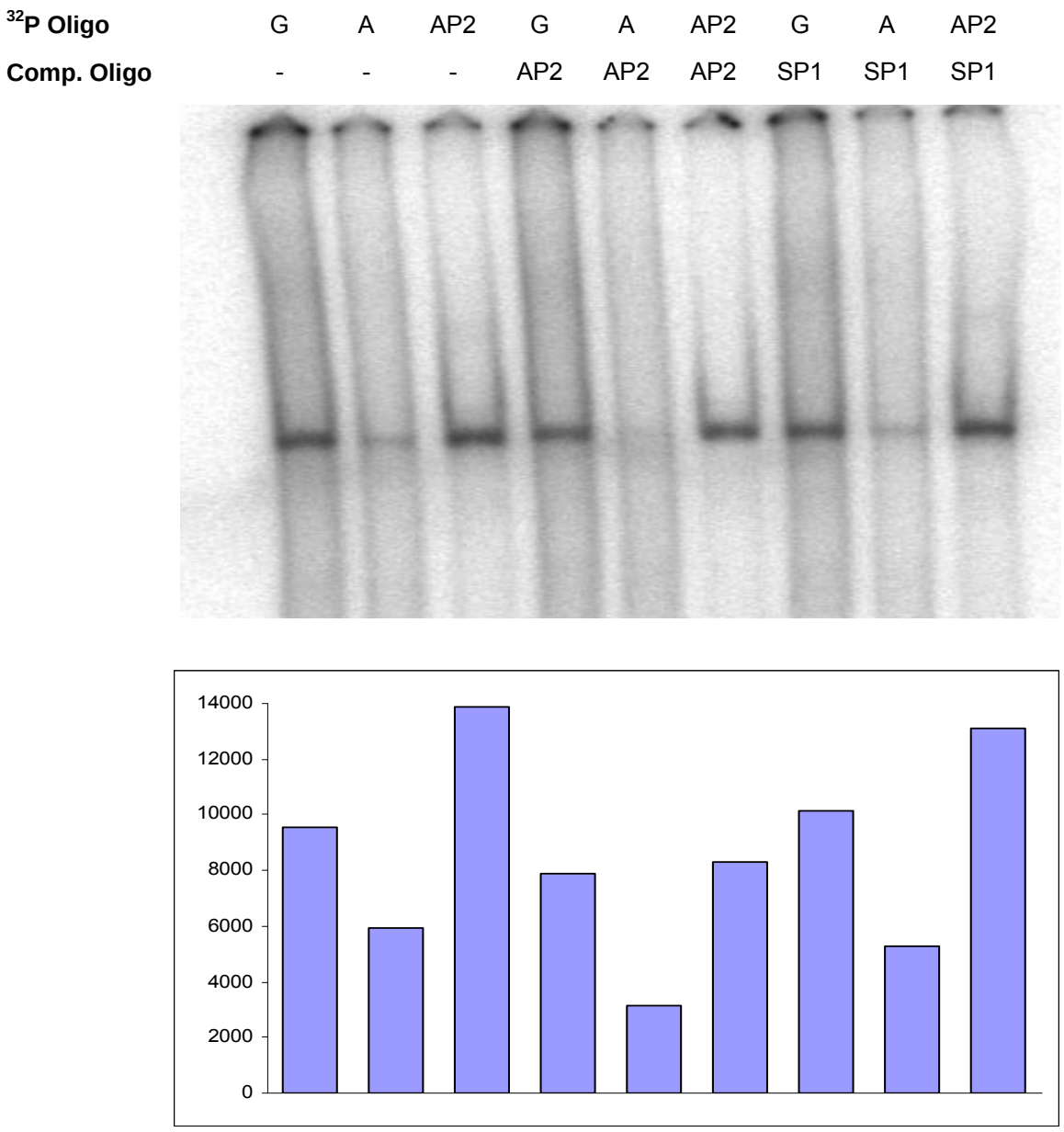

Figure 2.3: Electrophoretic mobility shift assay of the putative activator protein 2 (AP-2) consensus site surrounding the location of rs25531. **Upper panel**: Autoradiograph showing various labeled oligonucleotides (middle row) incubated with nuclear extracts (upper row) with or without various competing unlabeled oligonucleotides (bottom row). **Lower panel**: Quantitation of band patterns from the autoradiograph, expressed in units of intensity.

2.4 Discussion

Pharmacogenetic investigations are rapidly expanding, in part because of increased interest in predicting response to drug treatments based on common genetic variation. In psychopharmacology, as throughout the rest of medicine, clinicians often face variability in clinical response coupled with a lack of clinical or biological predictors of response. We have focused on a well-characterized sample with MDD being treated with a single SSRI, fluoxetine, in which we have previously identified association between anonymous markers and antidepressant response (9). We thus sought to identify variants that might explain this association, using a combination of dHPLC and direct sequencing to screen comprehensively both the exonic regions of the gene and much of the surrounding intronic regions for variation. We tested for association with a treatment response phenotype, using both single loci and multilocus haplotypes. Our SNP discovery effort yielded many new variants not previously reported, although many occurred at such low frequencies as to not be useful in association studies, unless substantially larger populations are used. Our study has confirmed what other groups have also found: that coding regions of *SLC6A4* are not particularly variable (20,21).

In our primary phenotype, categorical response versus non-response, we found suggestive evidence of an association with SNP rs25531, with the minor allele being more likely to reduce response to treatment. The HTTLPR was not significantly associated in single-locus analysis, as previously noted (9); but a test of the three marker haplotypes, including the HTTLPR and the flanking SNPs rs25531 and rs25533, was negatively associated with treatment response ($p = 0.02$), although this was not significant when the permuted global significance p value was calculated. No significant

association was found with our other two phenotypic comparisons, specific responders versus all others and specific responders versus non-specific responders. Although beyond the scope of our focused hypothesis regarding overall antidepressant response, other phenotypes, such as the genotypic effect on temporal course of response, might be of interest for subsequent investigation.

We characterized SNP rs25531 functionally to ascertain a biological basis for how this SNP could affect clinical outcome. Because rs25531 lies just upstream of the HTTLPR in the gene's promoter region, and HTTLPR genotype has been shown to affect transcriptional activity, we investigated the role of this SNP as a potential modulator of transcription factor binding. The minor G allele creates a consensus AP-2 binding sequence, and oligonucleotides containing this allele showed greater binding to nuclear extracts when compared with the major A allele. Although others have reported that the minor G allele of this SNP occurs in a region of the long (16-repeat) allele of the HTTLPR, and thus is missing from chromosomes carrying the short allele (14 repeats) we did not find this to be the case, confirmed by subsequent groups (33,34). We detected the G allele on short allele chromosomes. Given the region's repetitive nature, sequence misalignment, with the placement of the insertion/deletion in particular, might explain this discrepancy.

Many studies have been published that investigated HTTLPR in a variety of neurobehavioral phenotypes. Such studies often find association between the HTTLPR and the phenotype of interest, only to go unreplicated or to show association with the opposite allele. Because rs25531 lies just upstream of the insertion/deletion that characterizes the HTTLPR and might play a regulatory role in the gene, rs25531 could be

a confounding factor in these studies. We find evidence in our data that rs25531 is associated with both response and with HTTLPR, and as a result, the association between HTTLPR and response might depend on which allele one is considering at the rs25531 site. According to our haplotype analyses, there is support for this claim, with the long HTTLPR allele occurring more often in the non-responders, given that the minor G allele is present. When the A allele of rs25531 is present, we observe the short allele occurring more often in the responders.

Although the results of this study are promising, they are also subject to limitations. The primary disadvantage of this study is the small sample size, which contributed to a greatly reduced ability to detect associations between phenotype and genotype. This limitation is crucial because it suggests that our sample might not have been large enough to 1) detect relevant uncommon/rare variants; and 2) detect association between variants we discovered and our drug response phenotype. This type II error could presumably be addressed by a much larger clinical sample, as described in the subsequent chapter. A limitation in any case–control association study is confounding based on population stratification. By using a genomic control method in our population, we previously noted little evidence for stratification in our sample, given the constraint of our sample size and the number of markers used (9). For the amount of stratification detected in this sample, we would have to adjust the significance values of our association results minimally. In this context, we are fairly confident that we have avoided cryptic stratification or bias that can be associated with the use of racial or cultural identifiers, although a larger sample size and more markers might allow us to detect more modest stratification (35). Likewise, a larger sample will facilitate replication

of our finding of association between fluoxetine response and rs25531. The interpretation of our association data also necessitates consideration of multiple testing. The correlation between phenotypes tested, as well as the observed correlation of marker-to-marker relationships, makes traditional corrections for multiple comparisons inappropriately conservative. We have thus left our significance tests uncorrected.

Although our investigation into the biological consequences of rs25531 showed enhancement of AP-2 binding activity, the role of AP-2 in serotonin pathway biology is relatively unknown. The transcription factor AP-2 is a critical factor in mammalian neural gene expression (36). Many genes involved in brain neurotransmitter systems have AP-2 binding sites in their regulatory regions. Additionally, regional monoamine metabolite levels vary with AP-2 protein levels (37), and chronic treatment with antidepressants alters both AP-2 levels and DNA-binding activity of AP-2 (38,39); however, no clear picture immediately surfaces as to how AP-2 directly affects response to SSRIs. Thus, our observation that response to fluoxetine might be mediated by altering AP-2 function must be deemed provisional until further in vivo experiments are conducted. One approach, as described in chapter 5, is to look directly at the role of DNA variation in the gene encoding the AP-2 protein in relation to antidepressant response.

In summary, we have made an in-depth investigation into the role of DNA variants within the serotonin transporter for response to fluoxetine and found several interesting associations. We observed suggestive evidence of an association (by both single-locus and haplotypic analysis) with non-response and the minor allele of an SNP near the HTTLPR. All the associations we observed within our population were located within the promoter region of this gene. Previous studies finding association with the

HTTLPR, our own observations associating several variants within the promoter region, and functional data suggesting that at least two of these variants play a role in the regulation of serotonin transporter suggest that the 5′ region of this gene might play a role in the response of SSRIs.

2.5 Reference List

Reference List

1. Kessler RC, Berglund P, Demler O, Jin R and others. The Epidemiology of Major Depressive Disorder: Results From the National Comorbidity Survey Replication (NCS-R). *JAMA* 2003; 289(23): 3095-3105

2. Thase ME, Entsuah AR, and Rudolph RL. Remission Rates During Treatment With Venlafaxine or Selective Serotonin Reuptake Inhibitors. *Br. J. Psychiatry* 2001; 178 234-241

3. Caspi A, Sugden K, Moffitt TE, Taylor A and others. Influence of Life Stress on Depression: Moderation by a Polymorphism in the 5-HTT Gene. *Science* 2003; 301(5631): 386-389

4. Lerer B and Macciardi F. Pharmacogenetics of Antidepressant and Mood-Stabilizing Drugs: a Review of Candidate-Gene Studies and Future Research Directions. *Int. J Neuropsychopharmacol.* 2002; 5(3): 255-275

5. Malhotra AK, Murphy GM, Jr., and Kennedy JL. Pharmacogenetics of Psychotropic Drug Response. *American Journal of Psychiatry* 2004; 161(5): 780-796

6. Serretti A and Artioli P. The Pharmacogenomics of Selective Serotonin Reuptake Inhibitors. *Pharmacogenomics. J* 2004; 4(4): 233-244

7. Lesch K-P, Bengel D, Heils A, Sabol SZ and others. Association of Anxiety-Related Traits With a Polymorphism in the Serotonin Transporter Gene Regulatory Region. *Science* 1996; 274 1527-1531

8. Minov C, Baghai TC, Schule C, Zwanzger P and others. Serotonin-2A-Receptor and -Transporter Polymorphisms: Lack of Association in Patients With Major Depression. *Neuroscience Letters* 2001; 303(2): 119-122

9. Peters EJ, Slager SL, McGrath PJ, Knowles JA and others. Investigation of Serotonin-Related Genes in Antidepressant Response. *Mol Psychiatry* 2004; 9(9): 879-889

10. Serretti A and Artioli P. From Molecular Biology to Pharmacogenetics: a Review of the Literature on Antidepressant Treatment and Suggestions of Possible Candidate Genes. *Psychopharmacology (Berl)* 2004; 174 490-503

11. Kim DK, Lim SW, Lee S, Sohn SE and others. Serotonin Transporter Gene Polymorphism and Antidepressant Response. *NeuroReport* 2000; 11(1): 215-219

12. Yoshida K, Ito K, Sato K, Takahashi H and others. Influence of the Serotonin Transporter Gene-Linked Polymorphic Region on the Antidepressant Response to Fluvoxamine in Japanese Depressed Patients. *Progress in Neuro-Psychopharmacology & Biological Psychiatry* 2002; 26(2): 383-386

13. Lotrich FE, Pollock BG, and Ferrell RE. Serotonin Transporter Promoter Polymorphism in African Americans : Allele Frequencies and Implications for Treatment. *Am. J. Pharmacogenomics.* 2003; 3(2): 145-147

14. Lotrich FE and Pollock BG. Meta-Analysis of Serotonin Transporter Polymorphisms and Affective Disorders. *Psychiatr. Genet* 2004; 14(3): 121-129

15. van Dyck CH, Malison RT, Staley JK, Jacobsen LK and others. Central Serotonin Transporter Availability Measured With [123I]{Beta}-CIT SPECT in Relation to Serotonin Transporter Genotype. *American Journal of Psychiatry* 2004; 161(3): 525-531

16. Willeit M, Stastny J, Pirker W, Praschak-Rieder N and others. No Evidence for in Vivo Regulation of Midbrain Serotonin Transporter Availability by Serotonin Transporter Promoter Gene Polymorphism. *Biol Psychiatry* 2001; 50(1): 8-12

17. Hariri AR, Drabant EM, Munoz KE, Kolachana BS and others. A Susceptibility Gene for Affective Disorders and the Response of the Human Amygdala. *Archives of General Psychiatry* 2005; 62(2): 146-152

18. Heinz A, Jones DW, Mazzanti C, Goldman D and others. A Relationship Between Serotonin Transporter Genotype and in Vivo Protein Expression and Alcohol Neurotoxicity. *Biological Psychiatry* 2000; 47(7): 643-649

19. Bradley CC and Blakely RD. Alternative Splicing of the Human Serotonin Transporter Gene. *Journal of Neurochemistry* 1997; 69(4): 1356-1367

20. Glatt CE, DeYoung JA, Delgado S, Service SK and others. Screening a Large Reference Sample to Identify Very Low Frequency Sequence Variants: Comparisons Between Two Genes. *Nat. Genet* 2001; 27(4): 435-438

21. Kim SJ, Cox N, Courchesne R, Lord C and others. Transmission Disequilibrium Mapping at the Serotonin Transporter Gene (SLC6A4) Region in Autistic Disorder. *Molecular Psychiatry* 2002; 7(3): 278-288

22. Perna G, Cocchi S, Bertani A, Arancio C and others. Sensitivity to 35% CO2 in Healthy First-Degree Relatives of Patients With Panic Disorder. *Am J Psychiatry* 1995; 152(4): 623-625

23. McGrath PJ, Stewart JW, Petkova E, Quitkin FM and others. Predictors of Relapse During Fluoxetine Continuation or Maintenance Treatment of Major Depression. *J Clin. Psychiatry* 2000; 61(7): 518-524

24. Quitkin FM. Placebos, Drug Effects, and Study Design: a Clinician's Guide. *Am. J. Psychiatry* 1999; 156(6): 829-836

25. Stewart JW, Quitkin FM, McGrath PJ, Amsterdam J and others. Use of Pattern Analysis to Predict Differential Relapse of Remitted Patients With Major Depression During 1 Year of Treatment With Fluoxetine or Placebo. *Arch. Gen. Psychiatry* 1998; 55(4): 334-343

26. Rozen S and Skaletsky HJ. Primer3 on the WWW for General Users and for Biologist Programmers. 2000; 365-386

27. Bacanu SA, Devlin B, and Roeder K. The Power of Genomic Control. *Am J Hum. Genet* 2000; 66(6): 1933-1944

28. Devlin B, Roeder K, and Wasserman L. Genomic Control, a New Approach to Genetic-Based Association Studies. *Theor. Popul. Biol.* 2001; 60(3): 155-166

29. Ihaka R and Gentleman R. R: A Language for Data Analysis and Graphics. *J Comput Graph Stat* 1996; 5(3): 299-314

30. Freidlin B, Zheng G, Li Z, and Gastwirth JL. Trend Tests for Case-Control Studies of Genetic Markers: Power, Sample Size and Robustness. *Hum Hered.* 2002; 53(3): 146-152

31. Dudbridge F. Pedigree Disequilibrium Tests for Multilocus Haplotypes. *Genet Epidemiol.* 2003; 25(2): 115-121

32. Nakamura M, Ueno S, Sano A, and Tanabe H. The Human Serotonin Transporter Gene Linked Polymorphism (5-HTTLPR) Shows Ten Novel Allelic Variants. *Molecular Psychiatry* 2000; 5(1): 32-38

33. Hu XZ, Lipsky RH, Zhu G, Akhtar LA and others. Serotonin Transporter Promoter Gain-of-Function Genotypes Are Linked to Obsessive-Compulsive Disorder. *The American Journal of Human Genetics* 2006; 78(5): 815-826

34. Wendland JR, Martin BJ, Kruse MR, Lesch KP and others. Simultaneous Genotyping of Four Functional Loci of Human SLC6A4, With a Reappraisal of 5-HTTLPR and Rs25531. *Mol Psychiatry* 2006; 11(3): 224-226

35. Freedman ML, Reich D, Penney KL, McDonald GJ and others. Assessing the Impact of Population Stratification on Genetic Association Studies. *Nat Genet* 2004; 36(4): 388-393

36. Mitchell PJ, Timmons PM, Hebert JM, Rigby PW and others. Transcription Factor AP-2 Is Expressed in Neural Crest Cell Lineages During Mouse Embryogenesis. *Genes Dev.* 1991; 5(1): 105-119

37. Damberg M, Eller M, Tonissaar M, Oreland L and others. Levels of Transcription Factors AP-2[Alpha] and AP-2[Beta] in the Brainstem Are Correlated to Monoamine Turnover in the Rat Forebrain. *Neuroscience Letters* 2001; 313(1-2): 102-104

38. Berggard C, Damberg M, and Oreland L. Chronic Citalopram Treatment Induces Time-Dependent Changes in the Expression and DNA-Binding Activity of Transcription Factor AP-2 in Rat Brain. *European Neuropsychopharmacology* 2003; 13(1): 11-17

39. Damberg M, Ekblom J, and Oreland L. Chronic Pharmacological Treatment With Certain Antidepressants Alters the Expression and DNA-Binding Activity of Transcription Factor AP-2. *Life Sci.* 2000; 68(6): 669-678

CHAPTER 3

ANALYSIS OF ASSOCIATION BETWEEN THE SEROTONIN TRANSPORTER (*SLC6A4*) AND RESPONSE TO CITALOPRAM IN THE STAR*D CLINICAL SAMPLE[†]

3.1 Introduction

Pharmacologic treatment with selective serotonin reuptake inhibitors (SSRIs) is a mainstay in the treatment of major depressive disorder (MDD). There is prominent heterogeneity in response to antidepressants (1), and as of yet, there are no explanations for this observed variability. One possibility that has generated great interest, however, is that this variability results from genetic variation at the SLC6A4 locus (2). A number of studies have tested the association between DNA variations in this gene and response to various SSRIs (3). The results of these studies are inconclusive, however, particularly when different populations are investigated. In studies of the promoter variant (HTTLPR), some work finds no association with response (4,5), whereas other reports show an association with treatment response (6), although the associated allele varies with ethnicity (Table 3.1).

A limitation of the previous studies is that most did not systematically study variation across this gene, instead focusing almost exclusively on the promoter variant or, to a lesser extent, on an intronic tandem repeat polymorphism (7). Although our group failed to find association between the HTTLPR and SSRI response, we did detect nominal association between two single nucleotide polymorphisms (SNPs), rs25531 and

[†] This chapter has been published previously: Kraft JB, Peters EJ, Slager SL, Jenkins GD, Reinalda MS, McGrath PJ., & Hamilton SP. "Analysis of association between the serotonin transporter and antidepressant response in a large clinical sample." *Biological Psychiatry* 2007; 61(6): 734-742. Reprinted with permission.

rs25533, near the HTTLPR in the 5′ region of this gene in studies of 96 subjects treated with fluoxetine (5,8). This work was described in detail in the previous chapter. Table 3.1 summarizes the findings of these previous studies, which were chosen for inclusion if the study analyzed variants in the serotonin transporter in depressed samples being treated with an SSRI. In several of these studies, analyses were carried out by modeling recessive or dominant transmission of the short allele, but this was not done in others.

In this study, we sought to conduct a more definitive study by genotyping variants in the SLC6A4 gene in a large sample of subjects with major depression whose response to treatment had been carefully characterized. All of the subjects were treated with the SSRI citalopram. This sample was collected in the course of Sequenced Treatment Alternatives to Relieve Depression (STAR*D), a prospective, multicenter, randomized clinical trial involving 4,041 outpatients in both primary and specialty care settings (9,10).

AUTHORS	*YEAR*	*POPULATION†*	*UPD*	*DRUG*	*VARIANT*	*RESULT*
Smeraldi et al.	1998	Itailian	69	Fluvoxamine	5-HTTLPR	S = Poor Response
Pollock et al.	2000	???	51	Paroxetine	5-HTTLPR	S = Slow Response
Zanardi et al.	2000	Italian	58	Paroxetine	5-HTTLPR	S = Poor Response
Kim et al.	2000	Korean	120	Fluoxetine Paroxetine	5-HTTLPR, Intron 2 VNTR	L = Poor Response; Int2 VNTR '12' Allele = Good Response
Zanardi et al.	2001	Italian	108	Fluvoxamine	5-HTTLPR	S = Poor Response
Rausch et al.	2002	???	51	Fluoxetine	5-HTTLPR	S = Poor Response
Yu et al.	2002	Chinese	121	Fluoxetine	5-HTTLPR	S = Poor Response
Yoshida et al. Ito et al.	2002 2002	Japanese	66	Fluvoxamine	5-HTTLPR, Intron 2 VNTR	L = Poor Response
Arias et al.	2003	Spanish	131	Citalopram	5-HTTLPR	S = Non Remission
Yoshida et al.	2004	Japanese	96	Milnacipran	5-HTTLPR, Intron 2 VNTR	No Association
Durham et al.	2004	Caucasian‡	106	Sertraline	5-HTTLPR	L/L = Early Response
Murphy et al.	2004	Caucasian‡	121	Paroxetine	5-HTTLPR	No Association
Peters et al. Kraft et al.	2004 2005	Caucasian‡	96	Fluoxetine	5-HTTLPR, Intron 2 VNTR, Intron 7 STR, 18 SNPs	rs25531 & rs25533 Minor Allleles = Poor Response

Table 3.1: Summary of studies examining SLC6A4 genotypes for association with antidepressant response in unipolar major depression. UPD represents the number of subjects with unipolar depression within each study. UPD, number of subjects with unipolar major depression. Result, s = short allele of 5-HTTLPR; l = long allele of 5-HTTLPR; Int2 VNTR, intron 2 variable number tandem repeat; Int7 STR, intron 7 short tandem repeat. †, ethnicity populations in publications that are without "???" are specifically described in the publications. ‡, populations for Durham et al, 95% Caucasian, 3% African American, <1% Asian, 1% other; Murphy et al, 89% Caucasian, 5% African American, 2% Asian , 4% other; and Peters et al and Kraft et al, 78% Caucasian, 6% African American, 7% Hispanic, 5% Asian, 3% other.

3.2 Materials and Methods

3.2.1 Sample

Of the 4,041 subjects, DNA was obtained from 1,953 subjects as part of the National Institute of Mental Health (NIMH) Human Genetics Initiative. The design of STAR*D was to enroll adults experiencing a major depressive episode who exhibited neither an inadequate response nor intolerance to an adequate trial of any of the STAR*D protocol treatments during the current episode. The overall aim of STAR*D (principal investigator, A. John Rush, NIMH Contract N01-MH-90003) was to determine prospectively which of a number of treatments are beneficial for subjects experiencing an unsatisfactory clinical outcome following treatment with citalopram. Because the STAR*D trial design has been described extensively (9-11), it is summarized only briefly here.

To make the findings as generalizable as possible, STAR*D used broad inclusion criteria (10,11) and enrolled a diverse population, including good minority representation. Diagnoses were made using the Psychiatric Diagnostic Screening Questionnaire (12), and depressive symptoms were assessed with the 16-item Quick Inventory of Depressive Symptomatology Self-Report version (QIDS-SR) collected at clinic visits. The QIDS-SR is highly correlated with the 17-item Hamilton Rating Scale for Depression ($HRSD_{17}$), and scores can be converted readily between the two instruments (13). Subjects meeting criteria and providing consent were administered citalopram as the initial treatment. The protocol encouraged 12 weeks of treatment with vigorous dosing of open-label citalopram (20–60 mg/day).

The subsample of 1,953 participants who consented to provide DNA samples was 61.8% female and 38.2% male, with ethnic proportions of 78.1% Caucasian, 16.1% African American, 3.5% multiracial, 1.1% Asian, 1.2% Pacific Islander/Native American, and 0.1% unspecified; 14.0% of the sample reported being Hispanic, and 43.5% of the sample came from primary care clinics, with the remaining 56.5% coming from specialty clinics. For this analysis, we report on analyses on DNA from 1,914 participants (98%). Baseline demographic and clinical data on these 1,914 subjects are presented in Table 3.2.

Access to the DNA samples and clinical data was approved by the STAR*D Ancillary Studies Committee, and clinical data were obtained from the Data Coordinating Center of STAR*D. Approval to carry out the work described here was obtained by the Committee on Human Research at the University of California, San Francisco.

3.2.2 Marker Selection

To provide adequate coverage of the entire SLC6A4 locus, tagging SNPs were chosen based on our previous genotyping and variant discovery efforts (5,8). Tagging SNPs were selected from a data set of SNP variants that were common (> 5% minor allele frequency) in a subset of Caucasian patients (n = 75) from our previous studies. Thirteen SNPs met this criteria, and seven were selected as tagging SNPs using a linkage disequilibrium (LD) threshold of $r^2 > 0.8$, as implemented in the program "ldselect.pl" (14). Because our initial variant characterization efforts were performed using a largely Caucasian population, we compared our tagging SNP set to HapMap data for this genomic region, which contained 10 common SNPs in a sample of 30 Yoruban trios (HapMap build 16c.1, June 2005). Two additional tagging SNPs from HapMap data were

selected for this study (rs16965628 and rs2020933) because they had large (> .25) minor allele frequency differences between Yoruban samples and Caucasian samples. In addition, two markers that were previously reported to be associated with antidepressant response (5-HTTLPR and rs25531); (3,5,8) were also included in this study, for a total of 11 markers (Table 3.3). The distribution of markers at the SLC6A4 is schematized in Figure 3.1.

3.2.3 Sample DNA Analysis

DNA was quantified using the Quant-IT DNA Assay Kit, Broad Range (Molecular Probes, Eugene, OR, USA) and fluorescence read on the VICTOR2 1420 Multilabel Counter (PerkinElmer Life Sciences, Boston, MA, USA). Gender was verified by the use of a PCR based assay of the sexually dimorphic amelogenin locus (15).

3.2.4 DNA Analysis and Genotyping

DNA from subjects was quantified and then used as a template to amplify specific regions of the gene via polymerase chain reaction (PCR). Variants were assayed by either fluorescence polarization detection of template-directed dye-terminator incorporation (FP-TDI) or by the use of restriction fragment length polymorphism analysis (RFLP).

3.2.5 DNA Amplification

All samples were amplified using polymerase chain reaction (PCR) in 5 microliters (μL) reactions containing 200 nM of the forward and reverse primers (Table 3.3), 5 ng genomic DNA template, 50 μM dNTPs (Roche, Indianapolis, IN, USA), 1 M anhydrous betaine (Acros Organics, Geel, Belgium), 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2 mM $MgCl_2$, and 0.2 units (U) Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). All primers and TDI probes were designed using Primer3 software

(16) and manufactured by Invitrogen. Samples were cycled using a touchdown protocol at 94°C for 3 min, followed by seven cycles of 94°C for 30 s, 65-59°C for 30 s (decreased by 1°C intervals per cycle), and 72°C for 30 s, followed by 38 cycles of 90°C for 30 s, 58°C for 30 s, and 72°C for 30 s, with a final 10 min at 72°C. The reactions were performed on either an Applied Biosystems GeneAmp PCR System 9700 (Foster City, CA, USA) or a DNA Engine Tetrad PTC-225 thermal cycler (MJ Research/Bio Rad, Hercules, CA, USA). For SNP rs25533, reactions were changed to include 10 ng genomic DNA template, 500 μM dNTPs, 300 nM forward and reverse primers, and 0.3 units (U) Platinum Taq DNA polymerase and the following protocol was used: 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 68°C for 30 sec, 72°C for 30 sec with a final 10 min at 72°C.

For SNP rs25531 and the 5-HTTLPR, conditions were changed so that amplification was performed in a final volume of 10 μL containing 25 ng of genomic DNA template, 300 μM dNTPs, 1.5 M anhydrous betaine, 15 mM ammonium sulfate (pH 9.3), 50mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 0.1% Tween 20, 500 nM primers, and 0.5 units Platinum Taq DNA polymerase then amplified using the following protocol: 94°C for 3 min, followed by 35 cycles of 94°C for 45 sec, 68°C for 30 sec, 72°C for 60 sec with a final 10 min at 72°C.

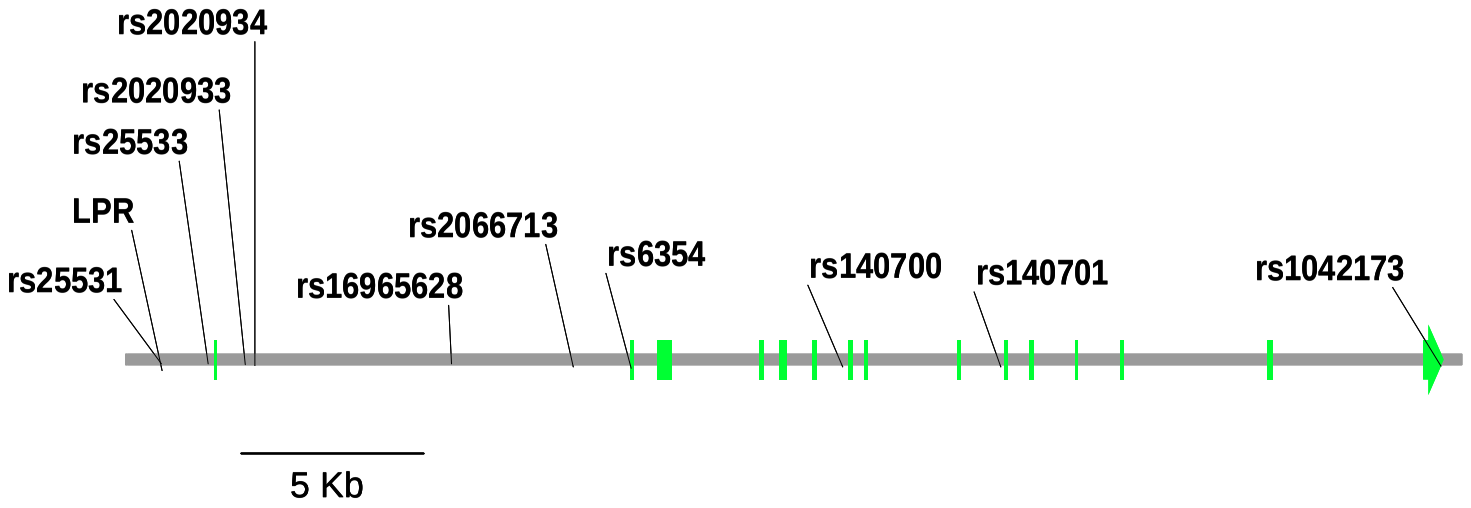

Figure 3.1: Schematic of the SLC6A4 gene region. The relative location of each of the 11 genotyped variants is shown with respect to the first 2 non-coding and subsequent 13 coding exons of SLC6A4 gene. The direction of transcription is indicated by the arrow.

Baseline Variable	Total subjects in Analysis (N=1,914)	Subjects classified as Responders (N=991)	Subjects classified as Non-Responders (N=669)	p value	Subjects classified as Specific Responders (N=679)	Subjects classified as Non-Specific Responders (N=187)	Subjects Classified as Remitters (N=826)
Number in Race / Ethnicity (%)							
Caucasian	1,501 (78.4)	799 (80.6)	509 (76.1)	0.06	559 (82.3)	146 (78.1)	679 (82.2)
African American	299 (15.6)	130 (13.1)	121 (18.1)		77 (11.3)	28 (15.0)	100 (12.1)
Other (Multi-racial)	68 (3.6)	36 (3.6)	23 (3.4)		23 (3.4)	9 (4.8)	25 (3.0)
Asian	21 (1.1)	15 (1.5)	5 (0.7)		12 (1.8)	2 (1.1)	13 (1.6)
Pacific Islander/Native American	24 (1.2)	11 (1.1)	11 (1.6)		8 (1.2)	2 (1.1)	9 (1.1)
Unspecified	1 (0.1)	0	0				
Number of Hispanic (%)	269 (14.1)	122 (12.3)	102 (15.2)	0.09	82 (12.1)	25 (13.4)	96 (11.6)
Number of Females (%)	1,179 (61.6)	621 (62.7)	404 (60.4)	0.35	424 (62.4)	126 (67.4)	518 (62.7)
Mean Age in Years (SD)	42.6 (13.4)	42.2 (13.4)	43.0 (13.2)	0.11	41.7 (13.3)	43.4 (14.0)	42.0 (13.7)
Mean Years of Schooling (SD)	13.6 (3.3)	14.1 (3.3)	13.2 (3.1)	<0.001	14.2 (3.3)	13.9 (3.3)	14.2 (3.3)
Marital Status							
Married	819 (42.8)	435 (43.9)	290 (43.3)	0.27	301 (44.3)	76 (40.6)	377 (45.6)
Never Married	536 (28.0)	278 (28.1)	185 (27.7)		190 (28.0)	53 (28.3)	239 (28.9)
Divorced	483 (25.2)	249 (25.1)	162 (24.2)		169 (24.9)	52 (27.8)	189 (22.9)
Widowed	76 (4.0)	29 (2.9)	32 (4.8)		19 (2.8)	6 (3.2)	21 (2.5)
Clinical Characteristics							
Age at first MDE (SD)	26.1 (14.9)	26.2 (14.4)	25.6 (14.9)	0.19	26.4 (14.6)	24.8 (13.7)	26.4 (14.5)
Months in current MDE (SD)	25.0 (53.9)	21.6 (45.8)	31.7 (65.8)	<0.001	20.4 (45.5)	24.6 (51.5)	21.3 (45.9)
Index Length 24+ Months (%)	487 (25.4)	230 (23.2)	203 (30.3)	0.001	142 (20.9)	53 (28.3)	187 (22.6)
Presence of Recurrent Depression (%)	1,347 (70.4)	701 (70.7)	469 (70.1)	0.71	481 (70.8)	130 (70.0)	574 (69.5)
Presence of Family History of Depression (%)	1,037 (54.2)	557 (56.2)	346 (51.7)	0.09	382 (56.3)	103 (55.1)	466 (56.4)
Baseline QIDS (SD)	16.4 (3.4)	16.2 (3.3)	16.6 (3.4)	0.008	16.2 (3.3)	16.1 (3.1)	15.8 (3.3)
Years Since 1st MDE (SD)	16.6 (13.9)	16.0 (13.8)	17.5 (14.0)	0.018	15.4 (13.8)	18.6 (14.6)	15.7 (13.8)

Table 3.2: Summary of baseline clinical and demographic data from STAR*D participants giving DNA. Significant differences found between responders versus non-responders included race, having Hispanic ancestry, mean years of schooling, months in current major depressive episode (MDE), length of MDE 24+ months, family history of MDD, baseline MDD score, & years since first MDE.

3.2.6 Polymorphism Genotyping

All markers except rs25531, rs25533, and the 5-HTTLPR were genotyped using fluorescence polarization detection of template-directed dye-terminator incorporation (FP-TDI). Following PCR, the excess primers, deoxynucleotides, and pyrophosphate in the PCR reaction were then degraded by adding a 0.1 μl of 10X PCR Clean-Up Reagent, containing a mixture of shrimp alkaline phosphatase and exonuclease I (PerkinElmer, Wellesley, MA, USA), 0.1μl of inorganic pyrophosphatase (Roche Applied Science, Indianapolis, IN, USA), and 0.8 μl of PCR Clean-Up Dilution Buffer to each 5 μl PCR reaction (PerkinElmer, Wellesley, MA, USA). The mixture was then incubated at 37°C for 60 min, followed by inactivation for 15 min at 80°C. The final step was the addition of a 4 μl solution containing a final concentration of 0.5 μM TDI probe, 1 μl of 10X TDI Reaction Buffer, 0.5 μl of AcycloTerminator Mix (containing R110 and TAMRA-labeled AcycloTerminators, corresponding to the polymorphic base), and 0.025 μl of AcycloPol DNA polymerase (PerkinElmer). This mixture was cycled at 95°C for 2 min, followed by 25 cycles of 94°C for 15 s and 55°C for 30 s. Following template-directed incorporation, fluorescence polarization was read using a VICTOR2 1420 Multilabel Counter (PerkinElmer), and genotypes were read using custom software.

For rs25531 and rs25533, genotyping was carried out using restriction fragment length polymorphism (RFLP) analysis. The reason for this is that traditional hybridization-based or single-base extension techniques will not work for the highly repetitive region surrounding rs25531. The minor allele in both these SNPs introduces an *Hpa* II digestion site which was exploited for genotyping. Following PCR amplification, samples were then digested in a 10 μl reaction containing 1 μl 10X Buffer 1 (New

England Biolabs, Ipswich, MA, USA), 5 μl PCR product, and 1 unit of *Hpa* II enzyme (New England Biolabs). The reaction was incubated at 37°C for 90 minutes followed by heat inactivation at 80°C for 15 min. Samples were then separated on a 3% NuSieve (Cambrex Corporation, East Rutherford, NJ, USA) agarose gel at 150V for 1 hour and genotyped based on the presence of cut DNA of predicted sizes. The 5-HTTLPR marker was genotyped by separating the PCR product on a 2.5% agarose gel at 150V for 1 hour and determining the size of the bands. To verify genotypes for the HTTLPR and rs25531, a subset of 384 samples from the total number of 1,914 samples (~20%) was subjected to direct sequencing. All genotypes were concordant.

3.2.7 Phenotypic Definitions

We define five interrelated response phenotype definitions of response to citalopram, building upon those described in the previous chapter. The first two are responders and non-responders: responders are subjects who had at least 42 days of treatment and whose QIDS-SR on their final clinical visit shows ≥ 50% reduction in score; the remaining subjects, who had at least 42 days of treatment, were then considered non-responders. The ≥ 50% reduction in symptom severity on the $HRSD_{17}$ is the conventional definition of response in clinical trials. We used the QIDS-SR score to estimate severity because all subjects had this rating, and it correlates highly with the $HRSD_{17}$ scores (13). We required this 42-day (or 6-week) threshold to ensure an adequate exposure to citalopram and to enhance the power to find associations between genotype and response by reducing potential heterogeneity. Using this threshold, we found no statistical difference in the average total dosage of citalopram between those who were on the trial for at least 42 days (average total dosage = 29.88 mg) and those who were not

(average total dosage = 30.43 mg). The 254 subjects with < 42 days of treatment were excluded from analysis.

The third phenotype definition is remission. Remission was defined as a QIDS-SR score ≤ 5, which closely corresponds to the conventional definition of an HRSD score of ≤ 7 (13). The final two phenotypes are based on our attempt to reduce heterogeneity further by attempting to separate placebo response from true drug response in antidepressant trials (17). Some response to antidepressant medication is a placebo response, which we posit may have either no genetic determinant or a different genetic substrate than "true" drug response. Thus, it is of interest to limit our definition of response to true pharmacologic response rather than placebo response. For these phenotypes, a "specific" pattern of response was defined by persistence, or the maintenance of response for the remainder of the study once it was attained. Previous studies considered "specific" patterns to be further characterized by delayed response, that is, after the first 2 weeks (18,19). We were unable to employ this criterion because the STAR*D study design did not include ratings before week 2. We defined persistent responders as those subjects who had a sustained response at all consecutive visits following the first visit with a response, as measured by ≥ 50% reduction in QIDS-SR scores. Those whose response occurred only at the last visit were removed from the analysis. In contrast, "non-specific" responders were those subjects who responded using QIDS-SR reduction criteria, but did not maintain their response following the first visit with a response.

Note that "specific" and "non-specific" responders are a subset of responders (as defined by the response phenotype described earlier). Moreover, because visits were at

least 2 weeks apart, we assumed that intervening weeks were characterized by the response defined by the previous visit. We compared "specific" responders to non-responders, allowing us to test the hypothesis that the "specific" response to citalopram represented a more genetically homogenous group of persons taking citalopram. We also compared "specific" responders to "non-specific" responders to test whether there are genetic difference between "true" drug responders and "placebo" responders, as suggested in our previous work (5).

3.2.8 Statistical Analysis

The frequency distributions of demographic and clinical variables were examined in the combined sample and by the five phenotypes. To control for any potential population stratification, all analyses were stratified by race categories: Caucasian and African American. Other racial categories were not considered because of the small numbers of those samples. We tested for Hardy–Weinberg equilibrium within each of the Caucasian and African American groups, and all subjects from a stratum were used in the analysis because all subjects had depression, and the evaluated polymorphisms were not suspected to influence risk of depression. Linkage disequilibrium was estimated using r^2.

We used unconditional logistic regression analysis to examine associations of the 11 genetic polymorphisms and each of the four phenotypic comparisons. These comparisons are 1) Responder versus Non-responder, 2) Remitter versus Non-remitters, 3) "Specific" Responders versus Non-responders, and 4) "Specific" Responders versus "Non-specific" Responders. Each polymorphism was modeled individually as gene–dosage effects in the regression models. This coding scheme was chosen because of its robustness to departure from the true additive genetic model (20). Regression analyses

were either unadjusted or adjusted for potential confounding effects, including gender, age, education (years of school), months in current major depressive episode (MDE), and years since first MDE. We estimated odds ratios (OR) and 95% confidence intervals (CIs) for the carriers of the minor allele versus non-carriers of the minor allele. Because of the large number of statistical tests, significance threshold was set at 0.01, and permutation tests were performed on any test that resulted in an asymptotic *p* value of 0.01 or less.

Association between haplotypes and the four phenotypic comparisons were calculated using a score test implemented in the computer program HAPLO.SCORE (21). This test uses the expectation–maximization algorithm to estimate the posterior probability of each person's haplotype. These posterior probabilities are then used to calculate a person's expected haplotype score in the logistic regression analyses. All haplotypes with frequencies > 0.01 were simultaneously tested in the analysis. Global *p* values and individual haplotype *p* values were obtained. Statistical tests were performed in SAS version 8.2 or Splus version 6.2.1 statistical packages. All statistical analyses were carried out in collaboration with Dr. Susan L. Slager (Mayo Clinic).

3.2.9 Post-Hoc Analysis Methods

For the intent to treat analysis and the longitudinal analyses, we stratified the analyses by race (Caucasian or African American). For the intent to treat analysis, all subjects with at least 1 follow-up were analyzed. We determined response as any subject whose QIDS-SR on his/her final clinical visit shows at least a 50% reduction in score. Tests for association were then analyzed as discussed in Statistical Analysis. For the longitudinal analysis, we used generalized estimating equations (GEE), which is an

extension of generalized linear models that accounts for correlated repeated measurements within individuals. We used the exchangeable correlation structure and all subjects with at least one follow up were analyzed for the association between the raw QIDS-SR score and each DNA variant.

Polymorphism	Major / Minor Allele	Product Size	Forward Primer	Reverse Primer	Probe Sequence
5-HTTLPR	16 RPT / 14 RPT	Variable	atgccagcacctaacccctaatgt	ggaccgcaaggtgggcggga	N/A
rs25531	A / G	Variable	atgccagcacctaacccctaatgt	ggaccgcaaggtgggcggga	N/A
rs25533	A / G	249 bp	aagttaaagagcaggaaagtcag	agaatttttgcgtcactttg	N/A
rs2020933	T / A	145 bp	tccttttccatttgggactct	gctagcaggctcataaataatcca	cagttttgtccagaaaagtgaacc
rs2020934	T / C	125 bp	tctgtgtgaagccactgagg	ttgctcaatttgcacaaacc	ggtggcagtgaccgttccaa
rs16965628	G / C	121 bp	gtcttgtgggcctcagtttc	tctagcacagggaagcatca	gctagggtatgaagtagaaaggca
rs2066713	C / T	104 bp	ctctctacccaggcccaga	actgctcactgctgctgcta	gatggaccgcatttcccttc
rs6354	T / G	256 bp	ccagagctgagctgacttcc	cactgctgctcaccatttgt	gctaagccccttgttattctgcaa
rs140700	C / T	110 bp	gaggtgggtgaatggatgtc	atccgatccctgtgtgactc	tgaagaccttgagaaaggaggg
rs140701	C / T	139 bp	agtgtgaggacgcacttggt	agaggaggaggtggttgaca	aaaactcagccacaacaacagtta
rs1042173	A / C	130 bp	aaactgcgtaggagagaacagg	cttcctttcctgatgccaca	aggttctagtagattccagcaataaaatt

Table 3.3: Summary for oligonucleotide sequences of PCR primers and FP-TDI probes. dbSNP accession numbers for the SNPs and PCR fragment sizes are provided. Genotyping methods are described in detail in section 3.2.6 - Polymorphism Genotyping.

3.3 Results

Of the 1,953 subjects who consented to give DNA, data for 1,914 subjects are described in this report. The 39 samples that were unavailable for genotyping did not differ from the remaining samples in demographic or clinical variables. Using our responder versus non-responder phenotype (R/NR), 1,660 of the 1,914 samples could be categorized, with 991 responders and 669 non-responders, for a response rate of 59.7%. We excluded 254 because they did not reach the 6-week treatment threshold. The clinical and demographic characteristics of the 1,914 samples are shown in Table 3.2. Significant demographic and clinical differences between responders and non-responders within the current study included years of schooling ($p < 0.001$), months in current MDE ($p < 0.001$), length of current MDE >24 months ($p = 0.001$), baseline QIDS-SR ($p = 0.008$), and years since first MDE ($p = 0.02$). The presence of recurrent depression or a family history of depression did not differ between responders and non-responders. Of the responders, 826 (83.3% of responders) were considered to be remitters. For the analysis of specificity of the 991 responders, 679 (68.5%) were categorized as "specific" responders, and 187 (18.9%) as "non-specific" responders, with the remaining 125 (12.6%) responders unclassifiable for the specificity response phenotype. The ratio of "specific" to "non-specific" responders is similar to that seen in our previous work (5).

We used the phenotypes described to test two general hypotheses. First, we sought to determine whether SLC6A4 variants are associated with general indicators of response (responders vs. non-responders, remitters vs. non-responders) based on changes in the QIDS-SR. Second, we sought to examine whether SLC6A4 variants influences response in a subgroup of responders likely to exhibit a "true" drug response ("specific"

responders versus non-responders, “specific” responders versus “non-specific” responders). All analyses were stratified by ethnicity, which for this analysis are Caucasian and African American. None of the variants showed significant deviation from Hardy–Weinberg equilibrium within any of the ethnic groups (results not shown). Linkage disequilibrium (LD) was present among the polymorphisms. Figure 3.2 shows the extent of LD for each ethnic group, using the r^2 metric. Given our SNP ascertainment strategy, we did not expect prominent association between markers.

The association results for the 11 polymorphisms for the response versus non-response comparison for each of the two ethnic groups are shown in Table 3.4. The minor allele frequencies among the non-responders within the Caucasians ranged from 5% (rs2020933) to 46% (rs2020934). None of the variants were found to be associated with response at a relaxed significance threshold of $p < 0.01$. We found similar results for our other three phenotypic comparisons: “specific” responders versus non-responders; “specific” responders versus “non-specific” responders; and remitters versus non-responders (Table 3.4). We also found no evidence of confounding adjusting for gender, age, education, months in current MDE, and years since first MDE in the regression analyses.

We then constructed haplotypes and tested for association with the four phenotypic comparisons to account for possible interactions among the 11 variants within the serotonin transporter. Our global test of association with the responder versus non-responder phenotypic comparison was found to be non-significant in either Caucasian or African-American groups ($p = 0.55$ & $p = 0.28$), respectively. Similar results were observed for the other phenotypic comparisons (results not shown).

A.

	R vs NR		*SR vs NS*		*SR vs NR*		*Rt vs NRt*	
Polymorphism	**P-value**	**MAF (R / NR)**	**P-value**	**MAF (S / NS)**	**P-value**	**MAF (SR / NR)**	**P-value**	**MAF (Rt / NRt)**
HTTLPR	0.2062	0.07 / 0.08	0.8146	0.08 / 0.07	0.6823	0.08 / 0.07	0.2069	0.08 / 0.07
rs25531	0.3686	0.44 / 0.42	0.6853	0.43 / 0.43	0.0626	0.43 / 0.44	0.3111	0.42 / 0.44
rs25533	0.4699	0.06 / 0.06	0.9761	0.07 / 0.07	0.5251	0.07 / 0.06	0.3719	0.07 / 0.06
rs2020933	0.6206	0.05 / 0.05	0.742	0.04 / 0.04	0.5288	0.04 / 0.05	0.3929	0.04 / 0.05
rs2020934	0.0868	0.46 / 0.49	0.6848	0.50 / 0.47	0.3926	0.50 / 0.46	0.026	0.50 / 0.45
rs16965628	0.8468	0.06 / 0.07	0.468	0.06 / 0.05	0.0324	0.06 / 0.06	0.5169	0.06 / 0.07
rs2066713	0.1156	0.38 / 0.42	0.0055	0.40 / 0.50	0.4662	0.40 / 0.38	0.0547	0.42 / 0.38
rs6354	0.8943	0.20 / 0.20	0.7324	0.21 / 0.20	0.1412	0.21 / 0.20	0.9462	0.20 / 0.20
rs140700	0.0604	0.07 / 0.10	0.7662	0.10 / 0.09	0.5913	0.10 / 0.07	0.0321	0.10 / 0.07
rs140701	0.6545	0.44 / 0.42	0.2405	0.42 / 0.38	0.0389	0.42 / 0.44	0.1691	0.41 / 0.44
rs1042173	0.4831	0.43 / 0.45	0.4372	0.43 / 0.41	0.0726	0.43 / 0.45	0.1413	0.42 / 0.46

B.

	R vs NR		*SR vs NS*		*SR vs NS/NR*		*Rt vs NRt*	
Polymorphism	**P-value**	**MAF (R / NR)**	**P-value**	**MAF (S / NS)**	**P-value**	**MAF (SR / NS+NR)**	**P-value**	**MAF (Rt / NRt)**
HTTLPR	0.5152	0.27 / 0.25	0.409	0.29 / 0.32	0.1848	0.29 / 0.25	0.2387	0.28 / 0.24
rs25531	0.6077	0.22 / 0.20	0.4775	0.19 / 0.21	0.7838	0.19 / 0.20	0.9213	0.21 / 0.21
rs25533	0.0425	0.13 / 0.07	0.5552	0.14 / 0.12	0.0109	0.14 / 0.07	0.009	0.14 / 0.08
rs2020933	0.3732	0.36 / 0.32	0.02	0.31 / 0.48	0.975	0.31 / 0.32	0.5923	0.32 / 0.35
rs2020934	0.3966	0.18 / 0.23	0.4946	0.14 / 0.20	0.0416	0.14 / 0.23	0.3258	0.18 / 0.23
rs16965628	0.8403	0.35 / 0.33	0.0047	0.29 / 0.48	0.41	0.29 / 0.33	0.3454	0.32 / 0.35
rs2066713	0.2894	0.29 / 0.26	0.7834	0.32 / 0.29	0.2004	0.32 / 0.26	0.7359	0.28 / 0.28
rs6354	0.8494	0.34 / 0.34	0.7268	0.33 / 0.34	0.697	0.33 / 0.34	0.6274	0.34 / 0.34
rs140700	0.026	0.09 / 0.04	0.9791	0.08 / 0.08	0.1075	0.08 / 0.04	0.4044	0.08 / 0.05
rs140701	0.9653	0.28 / 0.27	0.1768	0.23 / 0.35	0.2965	0.23 / 0.27	0.9114	0.28 / 0.27
rs1042173	0.9948	0.22 / 0.23	0.6859	0.22 / 0.26	0.9908	0.22 / 0.23	0.2629	0.24 / 0.21

Table 3.4: Association results for SLC6A4 and citalopram response stratified by ethnicity. Panel (A) is Caucasians and (B) is African Americans. Minor allele frequencies (MAF) are shown for each variant by response category, with an associated p-value for an additive model. The minor allele corresponds to that indicated in Table 3.3, except for rs2020934, for which the minor allele in African Americans is the major allele from Table 3.3.

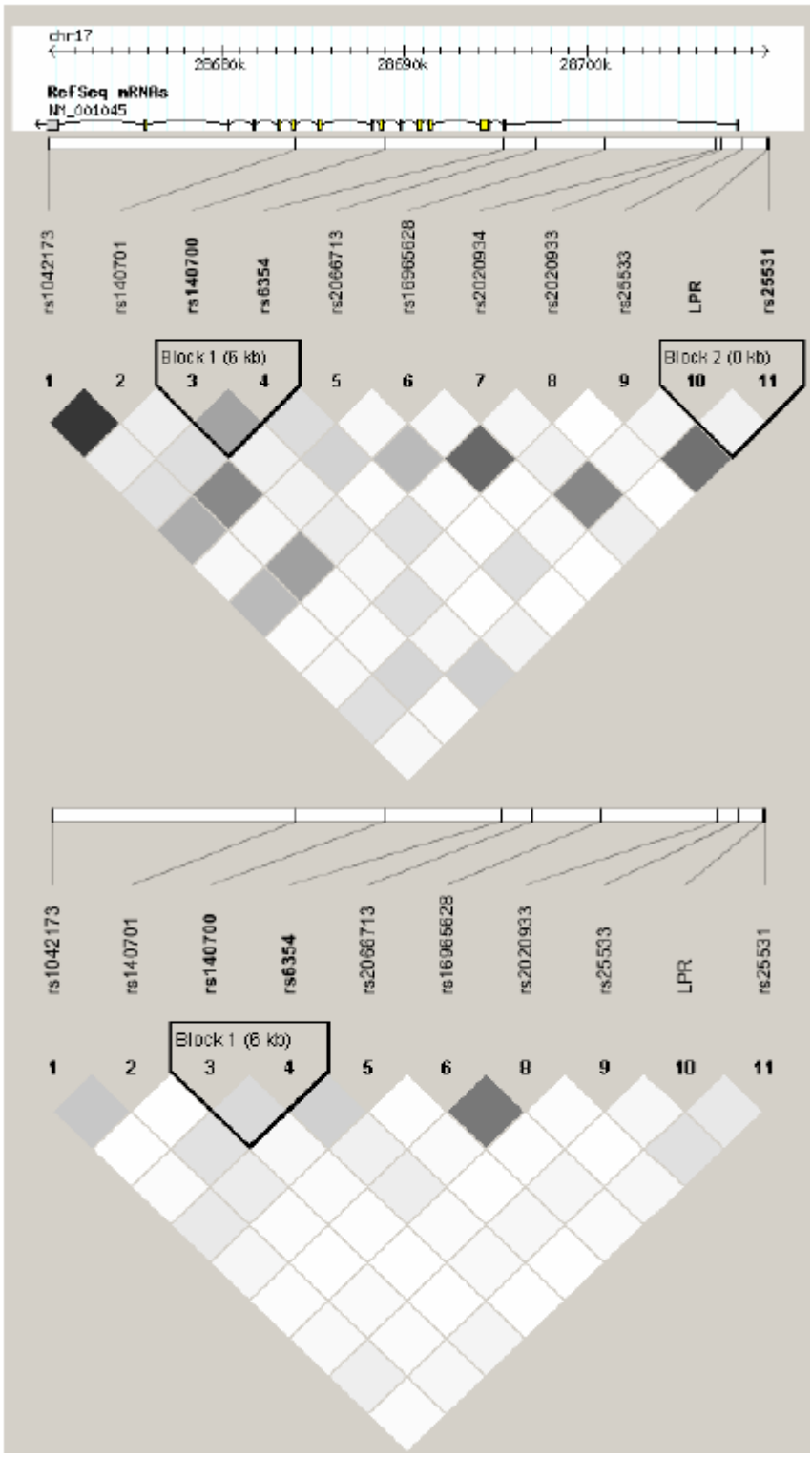

Figure 3.2: Linkage disequilibrium at the SLC6A4 locus. r^2 was estimated in Caucasian (top) and African American (bottom) samples. r^2 is displayed using Haploview, with darker boxes representing larger values of r^2. Haplotype "blocks" are represented using the criteria of Gabriel, et al (22).

3.4 Discussion

We failed to detect association between any of the SNPs within the SLC6A4 and antidepressant response phenotype. Our failure to detect association in a large sample is strong evidence against a role for common variation in this gene as a factor in response to SSRIs. In our primary phenotype, categorical response versus non-response, our results differed with regard to a number of other studies in which associations were found between response and SLC6A4. There may be a number of reasons for this including differences in outcome measures, drugs, ethnicities, and analytical approaches.

For our outcome measures, we used the QIDS-SR. This instrument has been shown to have high correlation (Pearson's correlation = 0.81) with the standard HRSD (13), which has been used in many of the previous studies. Despite this high correlation, however, variability in these measurements may explain the difference in results among the studies, especially if the effect size of any SLC6A4 genotypic effect is modest (23).

All of the studies in Table 3.1 and this study tested for association between SLC6A4 and an SSRI. It would be ideal to compare all of the previous studies to our own. Unfortunately, each of the previous studies differs strikingly from the others, with wide variation in treatment trial design, drug choice, marker choice, outcome measures, and statistical methods in each of the studies reviewed in Table 3.1. For example, the systematic and vigorous dosing strategy employed by STAR*D differs from the forced titrations or flexible dosing approaches used in the other studies, raising the possibility that those studies may be more sensitive to effects due to genotype × dose interactions. Although it is presumed that the SSRIs are equivalent in terms of mechanism, this has not been proven definitively and thus remains a potential explanation for the difference

between studies using different SSRIs. Another difference may involve the inclusion criteria applied to the various studies. The inclusion criteria for STAR*D was $HRSD_{17} \geq 14$, which was somewhat lower than several of the previous studies that used the $HRSD_{17}$ for assessment, suggesting that the STAR*D sample may be less severely affected.

The only previous study that is most directly comparable to ours based on drug is by Arias and colleagues, which investigated only the 5-HTTLPR variant (24). This study used citalopram and defined response as ≥ 50% reduction in $HRSD_{21}$ at 4 weeks and remission as $HRSD_{21} \leq 7$ at 12 weeks. Because this definition is different from ours, we carried out analyses to emulate this approach (≥ 50% reduction in QIDS-SR at 28 days or QIDS-SR ≤ 5 at 84 days) and still found no association between SLC6A4 and treatment response (data not shown). In this context, if the association between this gene and antidepressant response is not consistently found in samples with adequate power, the previous findings are likely spurious because of small sample sizes and low prior probability of the genetic variants affecting response (25). Finally, using four markers and differing phenotypes, McMahon and colleagues reported no association between SLC6A4 and antidepressant response in the STAR*D data set (26). The same group has recently reported no association to response with the 5-HTTLPR in the same data set, essentially confirming our results presented here (27).

An important issue in case–control association studies of antidepressant response (or association studies in general) is population stratification, which in theory may lead to spurious associations. To correct for potential ethnic stratification, we analyzed each population subgroup separately to test for association. Although methods have been developed for quantitating and correcting for stratification (28,29), previous studies have

shown that self-reported ethnicity closely corresponds with clustering of marker allele frequencies (30). We found dramatic differences in allele frequencies among the ethnic groups for many of the markers used here, with the average allele frequency difference between Caucasians and African Americans of 0.17 (Table 3.4). Because of these differences across the ethnic groups found in our study, ethnicity may explain the differences in results across the previous studies. In a number of the previous studies, ethnicity is not clearly delineated, however (Table 3.1).

A major strength of our study is that we attempted to reduce heterogeneity of the clinical phenotype. Accurate assessment of clinical phenotype is essential in pharmacogenetic studies. This is particularly true with antidepressant therapy, because placebo response rates can be as high as 60% for patients with MDD (31). Previous studies with serotonin pathway gene variants and SSRI antidepressant response have failed to address these concerns. Given the high placebo response rate for many antidepressants, it may prove necessary to control for non-specific responses in pharmacogenetic studies of antidepressant response (32). In this regard, we examined two phenotypes that might better represent those subjects who are responding to the biochemical effects of the medication, "specific" responders and remitters. Our goal for these refined phenotypes was to decrease phenotypic heterogeneity among the responders, possibly introduced by any placebo response. We failed to observe any association to the serotonin transporter using these phenotypes, however.

Another strength of our study is that we more fully interrogated the SLC6A4 gene than previous studies. Our tag SNP approach, using HapMap information, and our own previous dense genotyping of the gene (5) has sufficiently covered the gene. Furthermore,

our in-depth sequencing survey of this gene (8) has provided few useful markers beyond publicly available common SNPs. In addition to single-locus analysis, we used haplotypic analyses to allow us to determine whether combinations of alleles were associated with treatment response. The use of both single-locus and haplotypic association has allowed us to take a more comprehensive look at the role of DNA variation in the serotonin transporter locus in antidepressant treatment response. Thus far, the vast majority of the previous studies on serotonin transporter variants in antidepressant response have focused on single polymorphism associations.

A possible limitation of our study may be our choice of clinical phenotype, that is, antidepressant response, as our primary phenotype. We restricted our analyses to subjects receiving 6 weeks of treatment and required a 50% reduction in symptomology. In doing this, we excluded some 250 subjects; although we believe requiring sufficient medication exposure to reduce placebo response should increase the probability of detecting an association to drug response. Note that the rates of response and remission in our analysis were higher when compared with that of a recent analysis of 2,876 STAR*D subjects, in which subjects with less than 6 weeks of treatment were included (10), suggesting a strong correlation between response and length of treatment. Nevertheless, it might be argued that an intent-to-treat approach may be useful. We have done this for the Caucasian and for the African American subjects who had at least one treatment assessment and found that a single marker, rs140700, resulted in a p value of 0.009 in the Caucasian subsample. In a longitudinal analysis using generalized estimating equations and the raw QIDS-SR scores measured at each treatment assessment, we found results similar to our primary analysis, that is, none of the SNPs had p values < 0.01 for either

race. Thus, the fact that these alternative post hoc approaches resulted in similar results to those reported further support our findings of no association between this gene and antidepressant response.

An additional potential limitation arises because this open study, without placebo control or measure of adherence (i.e., serum level monitoring), might have shown only placebo response and therefore may not provide a valid phenotype for gene finding, although this seems less plausible given the similarity in response and remission rate to controlled studies where compliance is measured.

Another limitation of this report involves its generalizability to the STAR*D sample as a whole. There are significant differences between the sample providing DNA and the 2,087 STAR*D subjects who did not provide DNA for the genetics study. For example, the subject who gave DNA was significantly more likely to 1) come from a primary care clinic (43.5% vs. 34.7%, $p < 0.0001$); 2) be a Caucasian subject and not be an African American subject (78.4% vs. 73.0%, 15.8% vs. 19.2%, $p = 0.0003$); 3) be Hispanic 14.6% vs. 11.2%, $p = 0.006$) ; 4) be married (42.6% vs. 39.9%, $p = 0.003$); 5) have recurrent depression (76.0% vs. 71.5%, $p = 0.002$); 6) be older (42.6 vs. 38.5, $p < 0.0001$); 7) be more educated 13.6 vs. 13.3 years, $p = 0.007$); 8) have more MDEs (6.4 vs. 5.4, $p = 0.003$); and 9) have a longer illness (16.6 vs. 13.6 years, $p < 0.0001$). It is difficult to formulate how these differences, typically of small magnitude, would affect the generalizability of our results. In any case, it must be stated that the results may not be generalizable to subjects who are not inclined to provide DNA samples, who in the STAR*D sample appear to have observable differences with the subjects who provided DNA samples.

Furthermore, the clinical importance of some of these statistically significant differences, which are presumably driven by the large sample, is unknown. For example, the average number of years of education was 13.6 in subjects who gave DNA, which was significantly different from the average of 13.3 years in the subjects who did not provide blood samples. The effect size for this observation of a 0.3 year difference in schooling is minute, so that even if it is unlikely to arise by chance, it would be difficult to imagine a scenario or mechanism through which this would affect attempts to find a genetic association. The difference between primary care and specialty care subjects in STAR*D has been shown to be negligible (33). We also compared baseline QIDS-SR in our sample between specialty and primary care clinics and found no significant difference for all subjects ($p = 0.70$) or for groups stratified by ethnicity (Caucasians $p = 0.30$, African Americans $p = 0.23$), comparable to the findings in an analysis of 2,876 STAR*D subjects (10). The response rates between primary care and specialty clinics were similar for our primary response phenotype (57.8% vs. 60.5%, $p = 0.32$) and for our remission phenotype (53.3% vs. 56.3%, $p = 0.38$). Although the age of onset of MDD in STAR*D subjects has been shown to be related to a number of clinical variables (34), we found that it was not correlated with treatment response.

Finally, we cannot exclude the fact that our results may be false negatives. Given our sample size in the Caucasian sample, we have 80% power to detect a minimal odds ratio of 1.39, assuming 5% significance level, dominant model, and common allele frequency greater than 0.2. Our largest observed odds ratio in the Caucasian sample, given a common allele frequency of at least 0.2, was 1.31 (range 1.02–1.31). To detect

effects of this magnitude or less, we would need to increase our sample size by at least 50%. To date, we have the largest sample collection of patients.

In summary, we have looked in depth at the molecular target of SSRIs, the serotonin transporter, in the largest clinical sample analyzed to date and tested the role of DNA variants within this gene in citalopram response and failed to find any associations using both single loci and haplotypic analyses. At this point, we cannot definitively answer the ultimate question: at what point can we say that the contribution of genotype in SLC6A4 to antidepressant response is negligible? We have not accounted for the possibility of gene–gene or gene–environment interaction. By itself, however, this gene does not affect response to drug in our representative population with citalopram using our outcome assessment. Because it appears serotonin plays an important role in depression, this study may simply suggest that variation within other genes in the serotonin pathway such as enzymes that affect serotonin levels (TPH1,TPH2, MAOA) or the serotonin receptors (5HT2A, 5HT2C) may contribute to SSRI response. Similarly, it is possible that serotonin itself may be part of a cascade of events and any genetically determined variability in antidepressant response may lie elsewhere in the cascade or in another neural system all together.

3.5 Reference List

1. Esposito K and Goodnick P. Predictors of Response in Depression. *Psychiatr. Clin North Am* 2003; 26(2): 353-365

2. Lesch KP and Gutknecht L. Pharmacogenetics of the Serotonin Transporter. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 2005; 29(6): 1062-1073

3. Serretti A and Artioli P. The Pharmacogenomics of Selective Serotonin Reuptake Inhibitors. *Pharmacogenomics. J* 2004; 4(4): 233-244

4. Joyce PR, Mulder RT, Luty SE, McKenzie JM and others. Age-Dependent Antidepressant Pharmacogenomics: Polymorphisms of the Serotonin Transporter and G Protein Beta3 Subunit As Predictors of Response to Fluoxetine and Nortriptyline. *Int. J Neuropsychopharmacol.* 2003; 6(4): 339-346

5. Peters EJ, Slager SL, McGrath PJ, Knowles JA and others. Investigation of Serotonin-Related Genes in Antidepressant Response. *Mol Psychiatry* 2004; 9(9): 879-889

6. Serretti A, Artioli P, and Quartesan R. Pharmacogenetics in the Treatment of Depression: Pharmacodynamic Studies. *Pharmacogenet. Genomics* 2005; 15(2): 61-67

7. Smits KM, Smits LJ, Schouten JS, Stelma FF and others. Influence of SERTPR and STin2 in the Serotonin Transporter Gene on the Effect of Selective Serotonin Reuptake Inhibitors in Depression: a Systematic Review. *Molecular Psychiatry* 2004; 9(5): 433-441

8. Kraft JB, Slager SL, McGrath PJ, and Hamilton SP. Sequence Analysis of the Serotonin Transporter and Associations With Antidepressant Response. *Biol Psychiatry* 2005; 58(5): 374-381

9. Rush AJ, Fava M, Wisniewski SR, Lavori PW and others. Sequenced Treatment Alternatives to Relieve Depression (STAR*D): Rationale and Design. *Controlled Clinical Trials* 2004; 25(1): 119-142

10. Trivedi MH, Rush AJ, Wisniewski SR, Nierenberg AA and others. Evaluation of Outcomes With Citalopram for Depression Using Measurement-Based Care in STAR*D: Implications for Clinical Practice. *American Journal of Psychiatry* 2006; 163(1): 28-40

11. Fava M, Rush AJ, Trivedi MH, Nierenberg AA and others. Background and Rationale for the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) Study. *Psychiatr. Clin North Am* 2003; 26(2): 457-94, x

12. Zimmerman M and Mattia JI. The Reliability and Validity of a Screening Questionnaire for 13 DSM-IV Axis I Disorders (the Psychiatric Diagnostic Screening Questionnaire) in Psychiatric Outpatients. *J Clin. Psychiatry* 1999; 60(10): 677-683

13. Rush AJ, Trivedi MH, Ibrahim HM, Carmody TJ and others. The 16-Item Quick Inventory of Depressive Symptomatology (QIDS), Clinician Rating (QIDS-C), and Self-Report (QIDS-SR): a Psychometric Evaluation in Patients With Chronic Major Depression. *Biological Psychiatry* 2003; 54(5): 573-583

14. Carlson CS, Eberle MA, Rieder MJ, Yi Q and others. Selecting a Maximally Informative Set of Single-Nucleotide Polymorphisms for Association Analyses Using Linkage Disequilibrium. *Am. J Hum. Genet.* 2004; 74(1): 106-120

15. Bradley BJ, Chambers KE, and Vigilant L. Accurate DNA-Based Sex Identification of Apes Using Non-Invasive Samples. *Conservation Genetics* 2001; 2(2): 179-181

16. Rozen S and Skaletsky HJ. Primer3 on the WWW for General Users and for Biologist Programmers. 2000; 365-386

17. Ross DC, Quitkin FM, and Klein DF. A Typological Model for Estimation of Drug and Placebo Effects in Depression. *J Clin. Psychopharmacol.* 2002; 22(4): 414-418

18. Quitkin FM, Rabkin JD, Markowitz JM, Stewart JW and others. Use of Pattern Analysis to Identify True Drug Response. A Replication. *Archives of General Psychiatry* 1987; 44(3): 259-264

19. Stewart JW, Quitkin FM, McGrath PJ, Amsterdam J and others. Use of Pattern Analysis to Predict Differential Relapse of Remitted Patients With Major Depression During 1 Year of Treatment With Fluoxetine or Placebo. *Arch. Gen. Psychiatry* 1998; 55(4): 334-343

20. Freidlin B, Zheng G, Li Z, and Gastwirth JL. Trend Tests for Case-Control Studies of Genetic Markers: Power, Sample Size and Robustness. *Hum Hered.* 2002; 53(3): 146-152

21. Schaid DJ, Rowland CM, Tines DE, Jacobson RM and others. Score Tests for Association Between Traits and Haplotypes When Linkage Phase Is Ambiguous. *Am J Hum. Genet.* 2002; 70(2): 425-434

22. Gabriel SB, Schaffner SF, Nguyen H, Moore JM and others. The Structure of Haplotype Blocks in the Human Genome. *Science* 2002; 296(5576): 2225-2229

23. Ioannidis JPA. Why Most Published Research Findings Are False. *PLoS Medicine* 2005; 2(8):

24. Arias B, Catalan R, Gasto C, Gutierrez B and others. 5-HTTLPR Polymorphism of the Serotonin Transporter Gene Predicts Non-Remission in Major Depression

Patients Treated With Citalopram in a 12-Weeks Follow Up Study. *J Clin Psychopharmacol.* 2003; 23(6): 563-567

25. Wacholder S, Chanock S, Garcia-Closas M, El ghormli L and others. Assessing the Probability That a Positive Report Is False: An Approach for Molecular Epidemiology Studies. *JNCI Cancer Spectrum* 2004; 96(6): 434-442

26. McMahon FJ, Buervenich S, Charney D, Lipsky R and others. Variation in the Gene Encoding the Serotonin 2A Receptor Is Associated With Outcome of Antidepressant Treatment. *Am J Hum Genet* 2006; 78(5): 804-814

27. Hu XZ, Lipsky RH, Zhu G, Akhtar LA and others. Serotonin Transporter Promoter Gain-of-Function Genotypes Are Linked to Obsessive-Compulsive Disorder. *The American Journal of Human Genetics* 2006; 78(5): 815-826

28. Bacanu SA, Devlin B, and Roeder K. The Power of Genomic Control. *Am J Hum. Genet* 2000; 66(6): 1933-1944

29. Pritchard JK and Rosenberg NA. Use of Unlinked Genetic Markers to Detect Population Stratification in Association Studies. *Am J Hum. Genet.* 1999; 65(1): 220-228

30. Tang H, Quertermous T, Rodriguez B, Kardia SL and others. Genetic Structure, Self-Identified Race/Ethnicity, and Confounding in Case-Control Association Studies. *Am J Hum Genet* 2005; 76(2): 268-275

31. Walsh BT, Seidman SN, Sysko R, and Gould M. Placebo Response in Studies of Major Depression: Variable, Substantial, and Growing. *JAMA* 2002; 287(14): 1840-1847

32. Licinio J and Wong ML. Pharmacogenomics in Psychiatry: Clinical Issues to Be Considered. *Mol Psychiatry* 2005; 10(7): 615

33. Gaynes BN, Rush AJ, Trivedi M, Wisniewski SR and others. A Direct Comparison of Presenting Characteristics of Depressed Outpatients From Primary Vs. Specialty Care Settings: Preliminary Findings From the STAR*D Clinical Trial. *General Hospital Psychiatry* 2005; 27(2): 87-96

34. Zisook S, Rush AJ, Albala A, Alpert J and others. Factors That Differentiate Early Vs. Later Onset of Major Depression Disorder. *Psychiatry Research* 2004; 129(2): 127-140

CHAPTER 4

SEQUENCE ANALYSIS AND VARIANT DISCOVERY IN THE FIFTH EWING VARIANT (*FEV*) GENE FOR ASSOCIATION WITH RESPONSE TO CITALOPRAM[†]

4.1 Introduction

As detailed in Chapter 1, a major theory for the mechanism of action of SSRI's involves the products of serotonin pathway genes. Having already investigated a critical gene in the serotonin pathway, the serotonin transporter (see chapters 2 and 3), we sought examine other genes that may play a role in antidepressant response. Recent work using a murine model system has pointed us to the transcription factor FEV (Fifth Ewing Variant) which has been reported to control transcription of a number of genes within the serotonin system. Work done by Deneris and colleagues using the murine homolog of FEV, Pet-1, has shown that this gene is expressed exclusively in serotonergic neurons earlier than the serotonin specific markers SLC6A4 or TPH and that expression of crucial serotonergic genes such as tryptophan hydroxalase, monamine oxidase A, and the serotonin transporter are all disrupted by obliterating Pet-1 expression (1,2).

Studies done on FEV using postmortem brain tissue has revealed that FEV mRNA is robustly and exclusively expressed in the major serotonin-containing cell groups of the dorsal and median raphe nuclei (3,4). The only investigation into variation within the FEV locus was done using a case/control SIDS population consisting of 96 cases of African American and Caucasian ethnicity and 96 ethnically matched controls.

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They describe 3 variants in or around exon 3 (rs452985, rs860573, & rs2301296) that were not associated with SIDS. They also describe a previously unknown polymorphism IVS2-191_190insA (rs35898226) within intron 2 of FEV that was associated with increased risk of SIDS (5).

As an upstream transcription factor that appears to exert control over the expression of a number of previously reported risk factors for depression or antidepressant response and given the central role of the serotonin system in mediating the effects of selective serotonin reuptake inhibitors, such as citalopram, we hypothesized that the Pet-1/FEV locus would be necessary for the antidepressant effects of SSRIs.

In this Chapter, I describe the results of mutation screening in the FEV gene, as well as the results of an association study between DNA variants in this gene and antidepressant response. Finally, I will present data showing the effect of deletion of this gene in mice on antidepressant responsiveness. My goal was to enrich the marker set that we genotyped with potentially novel SNPs from the study population, as well as to identify SNPs with possible functional relevance to the antidepressant effects of SSRI's.

4.2 Materials and Methods

4.2.1 STAR*D Study Population

The study population consisted of the subjects who consented to give DNA from the STAR*D antidepressant trial, as reviewed in Chapter 3. Within each ethnic group, and gender, we randomly split our subjects *a priori* into a discovery and validation sample set. Due to heterogeneity within our self-identified "White" subjects, as uncovered via the *structure* analysis described later (Chapter 7), all analyses were split

into three racial subgroups: White, non-Hispanic; White, Hispanic; and African American. Other self-reported race classes were not analyzed.

4.2.2 Gene Sequence and Polymerase Chain Reaction Primers

Genomic and complementary DNA sequences were obtained from GenBank (accession number NM_017521), and primers were designed with Primer3 software (6) and manufactured by Invitrogen (Carlsbad, California). These products were designed to span exons and include flanking intronic sequence at the 5′ and 3′ ends. Six primer pairs were designed to screen 7.9 kb of sequence, including all 3 exons (NM_017521) and 2 highly conserved areas in the promoter region of the gene (Table 4.1).

4.2.3 Sample DNA Analysis

DNA was quantified using the Quant-IT DNA Assay Kit, Broad Range (Molecular Probes, Eugene, OR, USA) and fluorescence read on the VICTOR2 1420 Multilabel Counter (PerkinElmer Life Sciences, Boston, MA, USA). Gender was verified by the use of a PCR based assay of the sexually dimorphic amelogenin locus (7).

Table 4.1: SNPs Identified in FEV Through Direct Sequencing

Location	Forward Primer	Reverse Primer	Coding	Noncoding (Exonic)	Noncoding (Intronic)	Position*	Variant	SNP Name	Coding	# Of Chromosomes (MAF)	dbSNP
5' Region 1	tagacctcactaggaccctctcctt	tactgtcaaaactggcattctcaaa	---	---	621						
5' Region 2	cagcagtccaagccactactaatttt	cttatcgtcccatcgcaataaagt	---	---	755	220,053,138	C → T	FEV02		8 (4.21%)	
						220,053,231	G → A	FEV01		3 (1.58%)	
						220,053,009	C → T	FEV06		1 (0.53%)	
						220,052,835	G → T	FEV07		1 (0.53%)	
						220,053,226	T → C	FEV08		1 (0.53%)	
Exon 1	gttggagactttattgcgatgg	gaaacccagagctctctatctgc	52	343	186	220,052,385	ΔG	FEV04		4 (2.11%)	rs3835980
						220,052,630	G → T	FEV03		4 (2.11%)	
						220,052,483	G → C	FEV15		1 (0.53%)	
						220,052,172	G → A	FEV09		1 (0.53%)	
						220,052,268	C → G	FEV10		1 (0.53%)	
						220,052,105	C → T	FEV11		1 (0.53%)	
Exon 2	tcctgacttgggctctatgg	aaaaccaagtgactgtcttccc	75	---	356	220,051,630	G → A	FEV12		1 (0.53%)	rs364565
Exon 3	ccagcactcttccccagttc	taaactctggattagaggacggttg	582	588	131	220,048,208	C → A	FEV05		10 (5.26%)	
	gcctctccaaactcaacctcat	ctctccctgctttcccctaac				220,048,211	C → T	FEV13		1 (0.53%)	
						220,048,761	A → T	FEV14		1 (0.53%)	

Table 4.1: Results for variant discovery sequencing in FEV within a subset of the STAR*D sample set. SNPs previously identified and present in dbSNP at time of study have rs# identifiers attached. *Position in reference to NCBI Build 34 of the Human genome.

4.2.4 DNA Amplification

For the randomly chosen subset of 96 samples to be sequenced, samples were amplified using polymerase chain reaction (PCR) in 5 microliters (μL) reactions containing 500 nM of the forward and reverse primers (Table 4.1), 5 ng genomic DNA template, 300 μM dNTPs (Roche, Indianapolis, IN, USA), 1 M anhydrous betaine (Acros Organics, Geel, Belgium), 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2 mM $MgCl_2$, and 0.25 units (U) Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). Samples were cycled using a touchdown protocol at 94°C for 3 min, followed by seven cycles of 94°C for 30 s, 65-59°C for 30 s (decreased by 1°C intervals per cycle), and 72°C for 30 s, followed by 38 cycles of 90°C for 30 s, 58°C for 30 s, and 72°C for 30 s, with a final 10 min at 72°C. All reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, California) in 384-well plates (MJ Research, Waltham, Massachusetts).

4.2.5 DNA Sequencing

Before direct sequencing, the excess primers and deoxynucleotides in the polymerase chain reaction (PCR) products were then degraded by adding a 5 μL solution of 1 unit of shrimp alkaline phosphatase (Roche, Indianapolis, Indiana), 0.5 units of *Escherichia coli* Exonuclease I (USB, Cleveland, Ohio), 5 mmol/L $MgCl_2$, and 50 mmol/L Tris-HCl (pH 8.5). The mixture was incubated at 37°C for 90 min, followed by deactivation for 15 min at 95°C. Sequencing reactions were performed with BigDye v3.1 (Applied Biosystems, Foster City, California) chemistry at a 1/16th scale in 5 μL total volume containing 1 μL template (approximately 25 ng), 2.5 pmol primer, 0.75 μL Applied Biosystems 5× buffer, 0.5 μL BigDye v3.1, and 1.75 μL water. The reactions

were cycled at 96°C for 3 min, followed by 25 cycles at 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min. Reactions were then purified with Montage MultiScreen-SEQ (Millipore, Bedford, Massachusetts) plates and the Hamilton Microlab 4200 (Hamilton, Reno, Nevada) 96-probe liquid robotic system. Samples were analyzed on a Prism 3730xl DNA Analyzer (Applied Biosystems), and analysis of mutations was performed with Mutation Surveyor v2.30 software (SoftGenetics, State College, Pennsylvania). All variants were submitted to dbSNP. With this sample set, the probability that we would detect variants at a minor allele frequency of 1% was 85.2%, and >99.9% for variants with minor allele frequencies of >5%.

4.2.6 Marker Selection

To provide adequate coverage of the entire FEV locus, five tagging SNPs were chosen based on the HapMap data for this genomic region, which contained approximately 20 common SNPs (HapMap build 16c.1, June 2005). In addition, four markers from our variant discovery effort were also included in this study, for a total of 9 markers (Table 4.2). The distribution of markers at the FEV locus is schematized in Figure 4.1.

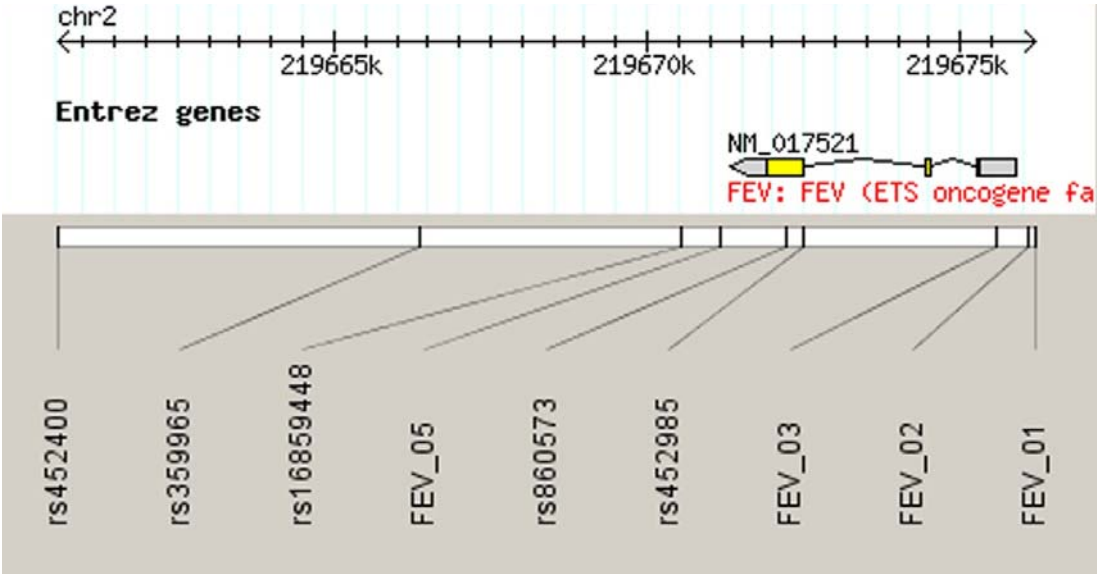

Figure 4.1: Schematic showing the genomic layout for the FEV locus and the relative locations of the 9 SNPs investigated for association to citalopram response in the STAR*D sample. Yellow regions in the gene are coding portions while the blocks represent exons and the lines introns.

SNP	Non-Hispanic Caucasians				Hispanic Caucasians				African Americans			
	Phenotype #1	*Phenotype #2*	*Phenotype #3*	*Phenotype #4*	*Phenotype #1*	*Phenotype #2*	*Phenotype #3*	*Phenotype #4*	*Phenotype #1*	*Phenotype #2*	*Phenotype #3*	*Phenotype #4*
RS452400	0.044	----	----	----	----	----	----	----	0.044	----	0.024	----
RS359965	0.013	0.013	0.039	0.026	----	----	----	----	----	----	0.029	----
RS16859448	----	0.030	----	----	----	----	----	0.007	----	----	----	----
FEV05	----	----	----	----	----	----	----	----	----	----	----	----
RS860573	0.050	0.044	----	----	----	----	----	----	----	----	----	0.024
RS452985	0.012	0.011	0.034	0.024	----	----	----	----	----	----	----	----
FEV03	0.010	0.012	0.007	----	----	----	----	----	----	----	----	----
FEV02	0.028	0.022	0.024	----	----	----	----	----	----	0.029	----	----
FEV01	----	----	----	----	----	----	----	----	0.027	0.010	----	0.021

Table 4.2: Association results for the 9 SNPs investigated in the FEV locus. Phenotype #1 corresponds to Resp42 (responders versus non-responders), Phenotype #2 corresponds to Remit (remitters versus non-responders), Phenotype #3 corresponds to Spec.Resp (specific responders versus non-responders), & Phenotype #4 corresponds to Tolerant (tolerant versus intolerant). Results are broken down by ethnic group.

4.2.7 Polymorphism Genotyping

Markers FEV01, FEV02, rs452985, rs452400, rs860573, and rs16859448 were all genotyped on a multiplex genotyping platform called SNPlex (Applied Biosystems, Foster City, CA). Markers FEV05 & rs359965 were genotyped using a 5' nuclease assay (Taqman).

The remaining SNP, FEV03, was genotyped via restriction length fragment analysis (RFLP) due to complications with both the multiplex genotyping platform as well as Taqman. The minor allele introduces an *Nla* III digestion site which was exploited for genotyping. Following PCR amplification, samples were then digested in a 10 μl reaction containing 1 μl 10X Buffer 4 (New England Biolabs, Ipswich, MA, USA), 5 μl PCR product, 0.1 μl 100X BSA, and 1 unit of *Nla* III enzyme (New England Biolabs). The reaction was incubated at 37°C for 90 minutes followed by heat inactivation at 80°C for 15 min. Samples were then separated on a 3% NuSieve (Cambrex Corporation, East Rutherford, NJ, USA) agarose gel at 150V for 1 hour and genotyped based on the presence of cut DNA of predicted sizes.

4.2.8 Statistical Analysis

Four phenotypic comparisons were made, based on the results from the response pattern analysis described previously (Chapter 2). The comparisons made were 1) all responders (specific and non-specific) versus non-responders (Resp42); 2) specific responders versus non-responders (Spec.Resp.); 3) remitters versus non-responders (Remit) and 4) tolerant versus intolerant (Tolerant). Single-point association tests were performed by logistic regression with the statistical package R 1.6.1 (8). Alleles were coded as 0, 1, or 2, corresponding to the presence of 0, 1, or 2 copies of the rare allele.

This coding scheme was chosen because of its robustness to departure from the true additive genetic model and departures from Hardy-Weinberg equilibrium (9).

To take full advantage of the power of our sample, we also analyzed the data using self-reported ancestry as a covariate in an attempt to adjust for population stratification. Single-locus association tests were performed by logistic regression using self-reported ancestry as a covariate with the statistical package R 1.6.1.

4.2.9 Animal Care and Background

The animal experiments described in this chapter were carried out by Miles Berger in the laboratory of Laurence Tecott at UCSF. Pet-1 KO and wild type control littermates were obtained from heterozygote matings. For these crosses, the original Pet-1 null allele (Hendricks et al, 2003) was backcrossed twice onto C57bl6/j background; thus all experimental animals contained approximately 87.5% C57Bl6/j genomic background and approximately 12.5% 129Sv background.

Adult Pet-1 KO and wild type control littermates were maintained in low profile microisolator cages with four to five animals per cage. All animals had *ad libitum* access to food and water, and cages were changed biweekly. All other animal care was performed in accord with NIH guidelines and the Institutional Animal Care and Use Committee (IACUC) of the University of California, San Francisco.

4.2.10 Tail Suspension Test

Adult Pet-1 KO and wild type control littermates were injected intraperitoneally with 10 mg/kg citalopram dissolved in saline or control solution 30 minutes prior to tail suspension testing, and housed individually in fresh cages until testing. Tail suspension testing was performed as described (10) with slight modifications (11).

In brief, mice were attached by their tails with a one inch by half inch piece of duck tape to an 8 inch long wire suspended inside of a large box (two feet by one foot by one foot). The mouse was placed facing the inside of the box so there were no other visual cues available it, and a camcorder was placed five feet away to record the animal's behavior during the six minute test. The experimenter left the room immediately after attaching the animal's tail to the wire to avoid any human interference with the test. All video footage was scored for immobility time by a blinded observer. At least 8 mice were included in each treatment group.

4.3 Results

4.3.1 Sequencing Results

We screened 709 bp of exonic and 2,980 bp of intronic or flanking sequence, totaling 3.7 kb in each of 96 samples. This translates to 355 kb screened in our population.

4.3.2 SNP Detection

Our effort yielded 15 variants in the FEV locus, comprising 14 SNPs and 1 insertion/deletion. Of these, 13 were newly discovered variants, none of which were found in exons and 5 of which were found in upstream promoter regions (Table 4.1). Five of the 15 were singletons, and only 1 had a MAF greater than 5%. For our follow up genotyping, we concentrated on 4 of the 5 "common" SNPs found ignoring FEV04 (rs3835980) which had previously been reported in the dbSNP database.

4.3.3 Single Marker Association

In our primary phenotypic comparisons, we tested nine SNPs found in or around the FEV locus. Stratifying by the three ethnic groups, we tested for association by using an additive model and found marginal significance ($p < 0.05$) for eight of the nine markers looked at. Only the marker FEV05 did not show marginal association with any of the 4 phenotypes in any of the 3 ethnic groups. It should be noted that none of these associations meet a corrected p-value given the number of multiple comparisons we are considering. It would be expected that with a p-value threshold of $p < 0.05$, we would observe 5% of tests to be positive by chance alone. However, we find that taking all 4 phenotypes and 3 ethnicities into account, nearly 25% or 5 times as many as expected are positive. This is even more striking when considering the most highly powered subgroup of white, non-Hispanic which comprises nearly 80% of our sample. In that subgroup, 19 of the 36 tests (53%) show association at $p < 0.05$ level. This is more than ten times that expected by chance but the p values are all marginal ($0.007 < p < 0.05$). These variants showed no significant deviation from Hardy-Weinberg equilibrium and the observed associations do not seem to be due to LD as the average r^2 values for each ethnic group were less than 0.4

4.3.4 Single Marker Association with Self-Reported Ancestry as Covariate

Using our four primary phenotypic comparisons, we tested nine SNPs found in or around the FEV locus using self-reported ancestry as a covariate within the logistic regression model. As seen in Table 4.3, several markers are associated with the three related response phenotypes (#1-3) at levels that exceed Bonferroni correction ($p < 0.0056$). The associated SNPs had dominant odds ratios between 1.25 and 2.22. The

tolerance phenotype is largely negative for markers in the FEV which could be explained by reduced power due to the relatively low numbers of intolerant subjects or may possibly be an indication that this gene does not play a role in the tolerance of citalopram.

4.3.5 Linkage Disequilibrium

We estimated LD between all markers in the FEV region by using D′ and r^2. LD patterns using markers within the gene are shown in Table 4.4. Graphical representations for each ethnic subgroup are shown in Figures 4.2, 4.3, & 4.4. Mean LD using the r^2 metric was 0.31 for Non-Hispanic Caucasians, 0.22 for Hispanic Caucasians, and 0.13 for African Americans. These values are too low to explain the large number of positive results within this gene being due to inter-relatedness between the markers being tested.

4.3.6 Behavioral Effects of FEV in Murine Model System

In collaboration with Miles Berger in the laboratory of Laurence Tecott, the effect of citalopram treatment on mice deleted for the murine orthologue of FEV, Pet-1, was evaluated using a well established mouse model of antidepressant action, the tail suspension test (12).

To further examine the role of the FEV locus in antidepressant response, we measured response to citalopram in mice deficient in Pet-1, the murine homologue of FEV. We treated Pet-1 KO and wild type control animals with citalopram and then measured their immobility time in the tail suspension test. We observed an approximate 50% decrease in immobility time in the tail suspension test in wild type animals treated with citalopram versus placebo, but no change in immobility time in Pet-1 KO animals treated with citalopram versus placebo ($p < 0.01$) (Figure 4.5).

SNP	Entire STAR*D Sample			
	Phenotype #1	*Phenotype #2*	*Phenotype #3*	*Phenotype #4*
RS452400	0.002	0.004	0.003	0.135
RS359965	0.002	0.001	0.006	0.139
RS16859448	0.080	0.111	0.047	0.027
FEV05	0.921	0.373	0.038	0.675
RS860573	0.015	0.003	0.027	0.035
RS452985	0.010	0.004	0.016	0.064
FEV03	0.009	0.012	0.013	0.110
FEV02	0.004	0.001	0.005	0.430
FEV01	0.011	0.006	0.064	0.481

Table 4.3: Association results for the 9 SNPs investigated in the FEV locus using self-reported ancestry as a covariate in the analysis. Phenotype #1 corresponds to Resp42 (responders versus non-responders), Phenotype #2 corresponds to Remit (remitters versus non-responders), Phenotype #3 corresponds to Spec.Resp (specific responders versus non-responders), & Phenotype #4 corresponds to Tolerant (tolerant versus intolerant).

A.

	RS452400	RS359965	RS16859448	FEV05	RS860573	RS452985	FEV03	FEV02	FEV01
RS452400	NA	0.79	0.57	0.42	0.86	0.86	0.55	0.74	0.75
RS359965	0.35	NA	0.02	0.47	0.97	0.98	0.76	0.95	0.96
RS16859448	0.16	0	NA	1	0.02	0	0.07	0.08	0.04
FEV05	0	0	0.01	NA	1	0.48	0.48	0.49	0.5
RS860573	0.36	0.83	0	0	NA	0.97	0.66	0.88	0.94
RS452985	0.37	0.85	0	0	0.93	NA	0.69	0.89	0.92
FEV03	0.14	0.47	0	0.01	0.4	0.43	NA	0.89	0.96
FEV02	0.2	0.59	0	0.01	0.57	0.58	0.64	NA	0.98
FEV01	0.17	0.5	0	0.01	0.54	0.52	0.62	0.8	NA

B.

	RS452400	RS359965	RS16859448	FEV05	RS860573	RS452985	FEV03	FEV02	FEV01
RS452400	NA	0.71	0.25	1	0.78	0.82	0.3	0.47	0.62
RS359965	0.34	NA	1	1	0.89	1	0.64	0.75	0.88
RS16859448	0.01	0.03	NA	1	1	1	0.49	1	0.09
FEV05	0	0	0.02	NA	1	1	1	1	1
RS860573	0.35	0.69	0.03	0	NA	0.94	0.46	0.69	0.76
RS452985	0.38	0.82	0.03	0	0.85	NA	0.53	0.56	0.76
FEV03	0.05	0.32	0.01	0	0.19	0.26	NA	0.56	1
FEV02	0.11	0.41	0.02	0	0.4	0.28	0.3	NA	1
FEV01	0.1	0.29	0	0	0.25	0.27	0.49	0.52	NA

C.

	RS452400	RS359965	RS16859448	FEV05	RS860573	RS452985	FEV03	FEV02	FEV01
RS452400	NA	0.84	0.85	0.62	0.39	0.88	0.13	0.05	0
RS359965	0.49	NA	0.96	0.45	0.72	0.96	0.6	0.21	0.2
RS16859448	0.2	0.17	NA	0.9	0.65	0.9	0.75	0.96	0.8
FEV05	0.02	0.01	0.12	NA	0.72	0.52	0.17	0.47	0
RS860573	0.13	0.31	0.14	0.02	NA	1	0.43	0.59	0.9
RS452985	0.4	0.68	0.11	0.01	0.43	NA	0.51	0.05	0.18
FEV03	0	0.08	0.02	0.01	0.03	0.08	NA	0.53	0.51
FEV02	0	0.04	0.18	0.01	0.21	0	0.07	NA	0.92
FEV01	0	0.01	0.04	0	0.18	0.02	0.17	0.3	NA

Table 4.4: Linkage disequilibrium patterns within the FEV locus. D' values on upper diagonal and r^2 values on lower diagonal. Non-Hispanic Caucasian, Hispanic Caucasians, & African Americans are shown separately in A, B, & C, respectively.

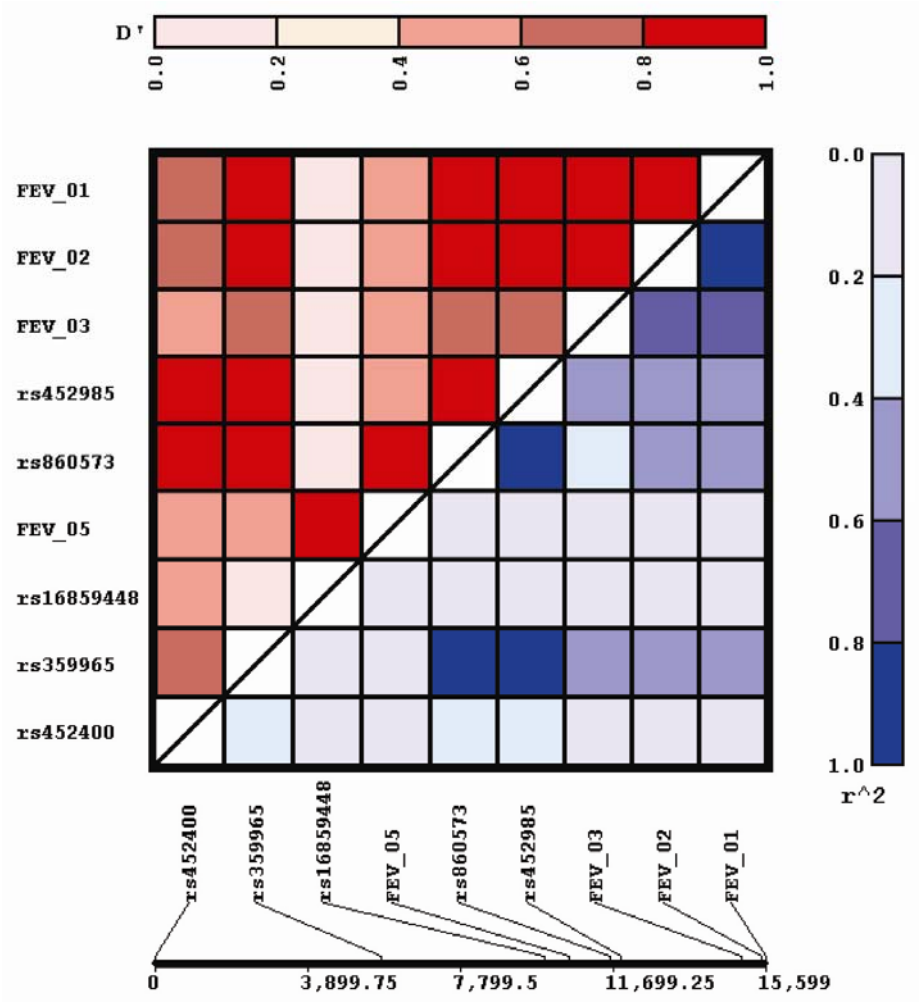

Figure 4.2: Schematic showing the linkage disequilibrium patterns within the FEV locus for the 9 SNPs investigated for association to citalopram response in the STAR*D sample. LD values based on the D' metric are shown in red in the upper diagonal and the r^2 metric is shown in blue in the lower diagonal. This figure shows only the non-Hispanic Caucasian individuals.

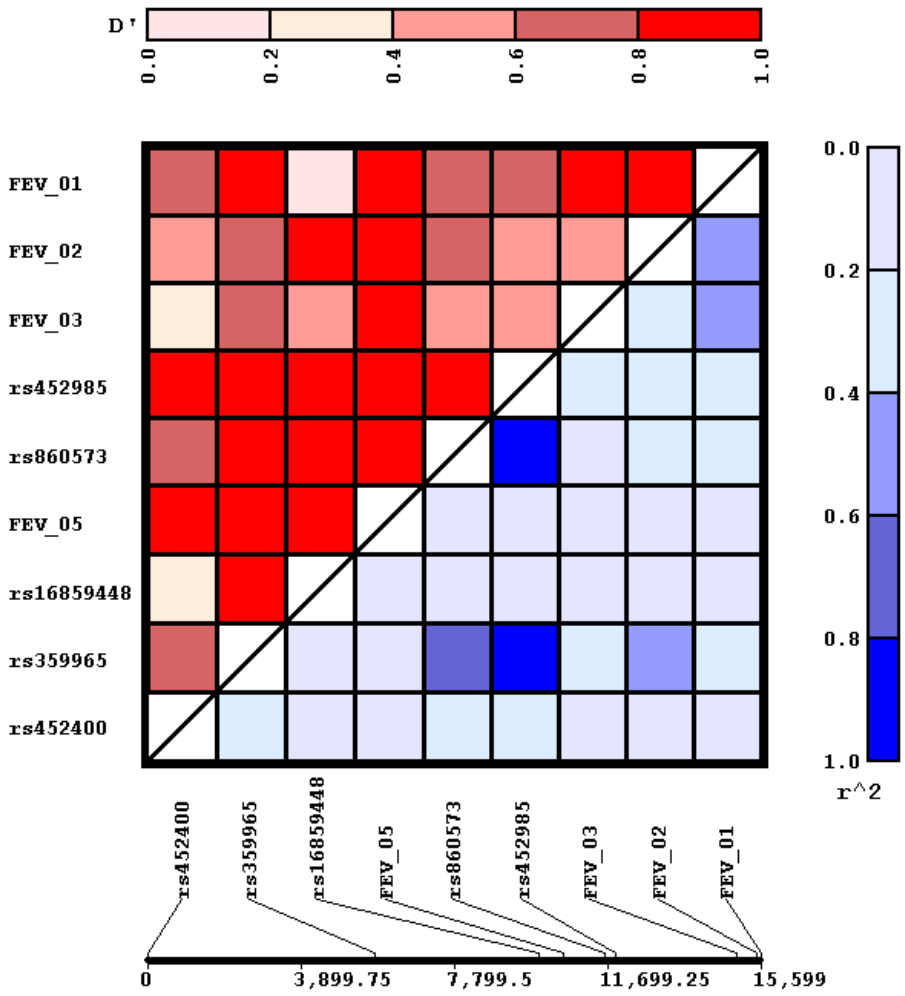

Figure 4.3: Schematic showing the linkage disequilibrium patterns within the FEV locus for the 9 SNPs investigated for association to citalopram response in the STAR*D sample. LD values based on the D' metric are shown in red in the upper diagonal and the r^2 metric is shown in blue in the lower diagonal. This figure shows only the Hispanic Caucasian individuals.

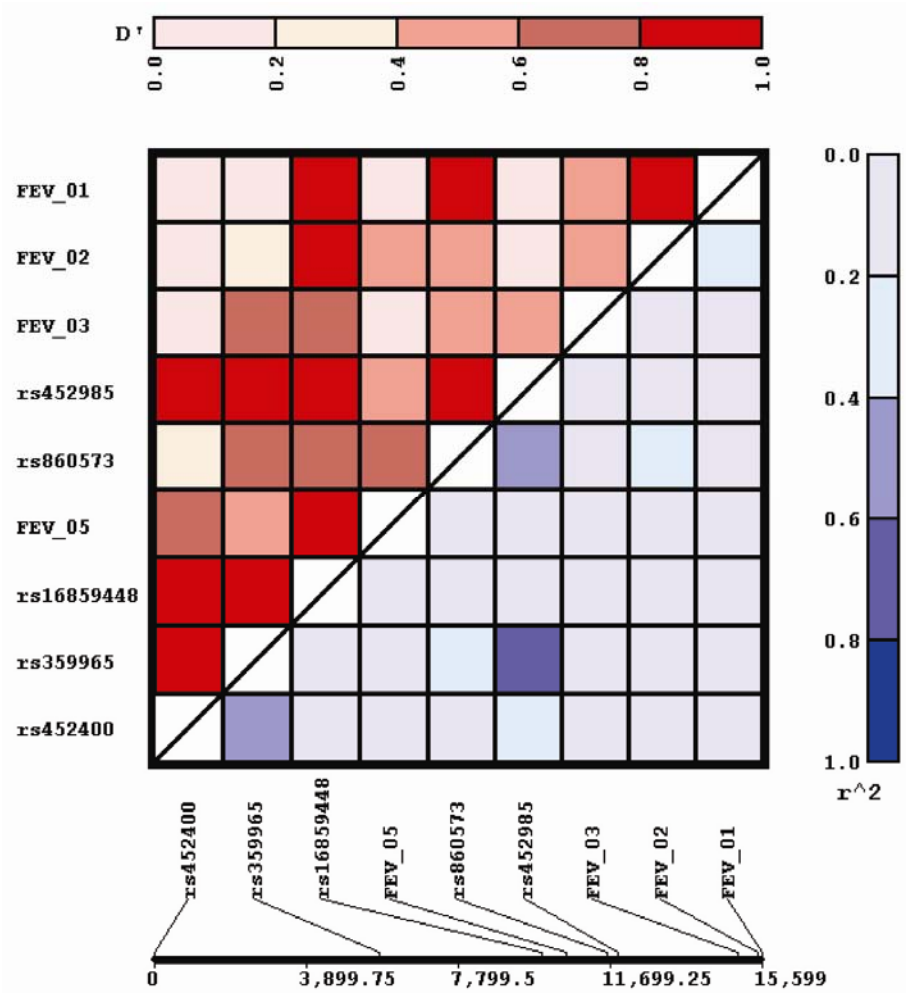

Figure 4.4: Schematic showing the linkage disequilibrium patterns within the FEV locus for the 9 SNPs investigated for association to citalopram response in the STAR*D sample. LD values based on the D' metric are shown in red in the upper diagonal and the r^2 metric is shown in blue in the lower diagonal. This figure shows only the African American individual.

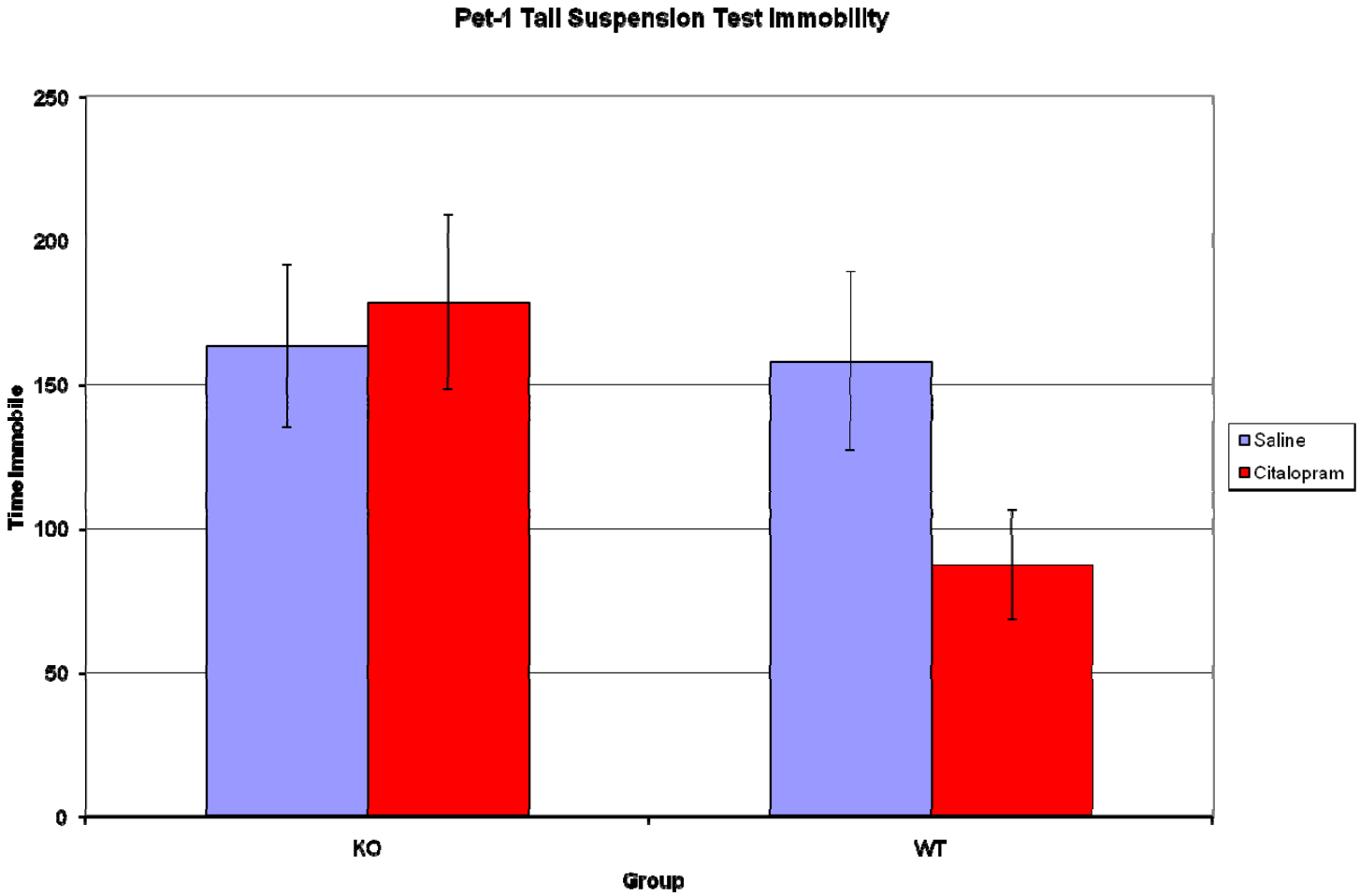

Figure 4.5: Results of the tail suspension test measuring the effects of citalopram on both wild type control animals and Pet-1 KO animals. At least 8 mice were used in each treatment group. Significance for the wild type control group is $p < 0.01$. KO, knock-out; WT, wild type. Animals were treated with saline or citalopram.

4.4 Discussion

In this study, we sought to test whether DNA variation in the candidate gene FEV is associated with clinical response to SSRI treatment. To accomplish this we used known HAPMAP genotype data and our complete exon resequencing information to select tagging SNPs within this gene and then examined FEV in a large population taking citalopram. Our analysis showed several different markers associated with a number of related response phenotypes (Table 4.2) with significance values less than $p = 0.05$. These associations are at best considered marginal given the large number of statistical tests that were performed. While we cannot say for sure that variation at this locus appears to significantly influence patient response to citalopram, our genetic data and behavioral/functional tests suggest further study.

In our primary phenotypes, we found modest evidence of association with SNPs across multiple phenotypes within a given ethnic subgroup. For example, significant associations were observed for SNPs rs359965 and rs452985 for all 4 phenotypes within the non-Hispanic Caucasian group and SNP FEV01 was significant for every phenotype except specific response in the African American group. There were also SNPs which are significant for one phenotype across multiple ethnic populations. For example, SNP rs452400 shows positive association for the Resp42 phenotype in both the non-Hispanic Caucasian and African American groups. In almost all cases, these findings cannot be accounted for in terms of LD between markers. For example, rs452400 is associated with Resp42 phenotype in African Americans ($p < 0.05$) while 2 markers (rs16859448 and rs452985) are in high LD (r^2 values of 0.85 & 0.88, respectively) yet show no evidence of association (p values of 0.27 & 0.31, respectively) (Figure 4.4).

Using self-reported ancestry as a covariate allowed us to analyze the entire STAR*D sample correcting for population stratification but without the associated loss of power incurred when doing stratified analysis. We found several independent markers associated with our related response phenotypes of response, remission, and specificity of response at a level exceeding a Bonferroni correction for the number of tests performed. These associations within the FEV locus provide evidence that this gene plays a role in citalopram response within the STAR*D clinical sample.

Our mouse work shows that the murine homologue of FEV (Pet-1) is crucial for antidepressant behavioral effects of citalopram in the tail suspension test. It is not yet known if this effect is due to direct interaction of FEV with citalopram or through regulation of other downstream effectors that are direct or indirect targets of the drug. Association data suggest a role for variation in the gene itself, and future work should focus on interaction between variants in this gene and other serotonin pathway components. Taken together, these findings in humans and mice suggest that the FEV/Pet-1 locus may be an important genomic locus for antidepressant responsivity in mammals.

Although the results of this study are promising, they are also subject to limitations. A limitation in any case–control association study is confounding based on population stratification. We attempted to control for population stratification in this study by analyzing the data within self-identified ethnic groups, as this has been shown to correlate well with marker allele frequencies (13). The interpretation of our association data also necessitates consideration of multiple testing. The correlation between phenotypes tested, as well as the observed correlation of marker-to-marker relationships,

makes traditional corrections for multiple comparisons inappropriately conservative. We have thus left our significance tests uncorrected. Also, while we have attempted to capture the majority of common variation within these genes, current genotyping costs prohibit complete ascertainment of all variants. Soon, advances in DNA sequencing technology will make possible deeper resequencing of genes associated with phenotypes of interest. Future investigation of FEV in antidepressant response might include complete resequencing of the FEV locus, including the entirety of introns, as well as flanking sequences that may harbor regulatory sequences.

In summary, we have made an in-depth investigation into the role of DNA variants within the gene FEV for association to citalopram response in a large patient population. Using single locus tests, we observed a number of nominal associations between several markers and a number of inter-related phenotypes. When using self-reported ancestry as a covariate in the analysis, several of the polymorphisms we interrogated appear to be associated with citalopram response in the STAR*D population. Given the results of our association study and our behavioral/functional data suggesting the importance of Pet-1 (FEV) in citalopram action, this gene may warrant further investigation. Given that little is known about exactly how SSRIs exert their antidepressant effects *in vivo*, interrogation of DNA variation in other neuronal pathways or across the entire genome may be needed to clarify the picture.

4.5 Reference List

1. Hendricks TJ, Fyodorov DV, Wegman LJ, Lelutiu NB and others. Pet-1 ETS Gene Plays a Critical Role in 5-HT Neuron Development and Is Required for Normal Anxiety-Like and Aggressive Behavior. *Neuron* 2003; 37(2): 233-247

2. Hendricks T, Francis N, Fyodorov D, and Deneris ES. The ETS Domain Factor Pet-1 Is an Early and Precise Marker of Central Serotonin Neurons and Interacts With a Conserved Element in Serotonergic Genes. *J. Neurosci.* 1999; 19(23): 10348-10356

3. Iyo AH, Porter B, Deneris ES, and Austin MC. Regional Distribution and Cellular Localization of the ETS-Domain Transcription Factor, FEV, MRNA in the Human Postmortem Brain. *Synapse* 2005; 57(4): 223-228

4. Maurer P, Rorive S, de Kerchove dA, Schiffmann SN and others. The Ets Transcription Factor Fev Is Specifically Expressed in the Human Central Serotonergic Neurons. *Neurosci. Lett.* 2004; 357(3): 215-218

5. Rand CM, Berry-Kravis EM, Zhou L, Fan W and others. Sudden Infant Death Syndrome: Rare Mutation in the Serotonin System FEV Gene. *Pediatr. Res.* 2007; 62(2): 180-182

6. Rozen S and Skaletsky HJ. Primer3 on the WWW for General Users and for Biologist Programmers. 2000; 365-386

7. Bradley BJ, Chambers KE, and Vigilant L. Accurate DNA-Based Sex Identification of Apes Using Non-Invasive Samples. *Conservation Genetics* 2001; 2(2): 179-181

8. Ihaka R and Gentleman R. R: A Language for Data Analysis and Graphics. *J Comput Graph Stat* 1996; 5(3): 299-314

9. Freidlin B, Zheng G, Li Z, and Gastwirth JL. Trend Tests for Case-Control Studies of Genetic Markers: Power, Sample Size and Robustness. *Hum Hered.* 2002; 53(3): 146-152

10. Heisler LK, Chu HM, Brennan TJ, Danao JA and others. Elevated Anxiety and Antidepressant-Like Responses in Serotonin 5-HT1A Receptor Mutant Mice. *Proc. Natl. Acad. Sci. U. S. A* 1998; 95(25): 15049-15054

11. Yoshikawa T, Watanabe A, Ishitsuka Y, Nakaya A and others. Identification of Multiple Genetic Loci Linked to the Propensity for "Behavioral Despair" in Mice. *Genome Research* 2002; 12(3): 357-366

12. Cryan JF, Mombereau C, and Vassout A. The Tail Suspension Test As a Model for Assessing Antidepressant Activity: Review of Pharmacological and Genetic Studies in Mice. *Neurosci. Biobehav. Rev.* 2005; 29(4-5): 571-625

13. Tang H, Quertermous T, Rodriguez B, Kardia SL and others. Genetic Structure, Self-Identified Race/Ethnicity, and Confounding in Case-Control Association Studies. *Am J Hum Genet* 2005; 76(2): 268-275

CHAPTER 5

LINKAGE DISEQUILIBRIUM MAPPING OF VARIANTS IN CANDIDATE GENES FOR ASSOCIATION WITH RESPONSE TO CITALOPRAM

5.1 Introduction

A prominent theme to the work described in previous chapters involves investigation of the role of various candidate genes in determining antidepressant response. I have previously discussed the findings of many of our previously conceived candidate genes (Chapters 2-4). In this chapter, I will now focus on candidate genes identified from the publications of other labs and represent our efforts to replicate those observations with regard to citalopram response within the STAR*D population.

Work published by Svennigsson et al. in a Science article in 2006, showed that a protein called p11 (S100A10) interacted with the serotonin 1B receptor and increased localization of 5-HT_{1B} receptors at the cell surface (1). It was also shown that overexpression of p11 increased 5-HT_{1B} receptor function and recapitulated certain behaviors seen after antidepressant treatment in mice. Further, adding to the allure of p11 expression appeared to be modulated by antidepressant treatment (2). As described earlier (Chapter 1), we had previously been interested in the transcription factor AP-2 due to the interaction with an associated SNP in the promoter region of the serotonin transporter. There is support in the literature that helped to elevate AP-2 as a major candidate gene. Among the most instrumental was work from Damberg and colleagues showing that brainstem levels and activity of several isoforms of AP-2 were changed after treatment with antidepressants and that levels of AP-2 correlated with monoamine turnover in the

rat brain (3-5). Given the findings described above we elected to investigate four candidate genes for association to antidepressant response, p11 (S100A10) and 5HT1B, as well as two isoforms of AP-2 (AP-2β & AP-2δ).

In this study, we sought to conduct a more definitive study by tagging variation within these genes and genotyping these variants in a large sample of subjects with major depression whose response to treatment had been carefully characterized. This sample was collected in the course of Sequenced Treatment Alternatives to Relieve Depression (STAR*D), which has been described previously (Chapter 3).

5.2 Materials and Methods

5.2.1 STAR*D Study Population

The study population consisted of the subjects who consented to give DNA from the STAR*D antidepressant trial, as reviewed elsewhere (see Chapter 3). To limit heterogeneity within our analyses, the analyses for the S100A10 & HTR1B loci were done by splitting samples into two self-identified racial subgroups: White and African American. Due to heterogeneity within our self-identified "White" subjects, as uncovered via the *structure* analysis described later (Chapter 7), the analyses for TFAP2β & TFAP2δ were split into three racial subgroups: White, non-Hispanic; White, Hispanic; and African American. Other self-reported race classes were not analyzed due to very small sample sizes, and thus diminished power to detect association.

Gene	dbSNP Identifier	Assay Method	Location	Position
HTR1B	rs6298	TAQMAN	6q14.1b	78,168,588
HTR1B	rs130058	TAQMAN	6q14.1b	78,168,877
HTR1B	rs11568817	TAQMAN	6q14.1b	78,168,978
S100A10	rs6678672	TAQMAN	1q21.3b	149,171,591
S100A10	rs1873311	TAQMAN	1q21.3b	149,178,804
TFAP2B	rs9367415	SNPLEX	6p12.3a	50,828,883
TFAP2B	rs2143081	SNPLEX	6p12.3a	50,829,670
TFAP2B	rs2272903	SNPLEX	6p12.3a	50,833,407
TFAP2B	rs2076309	SNPLEX	6p12.3a	50,836,237
TFAP2B	rs2817399	TAQMAN	6p12.3a	50,851,357
TFAP2B	rs2817407	SNPLEX	6p12.3a	50,853,073
TFAP2B	rs1569777	SNPLEX	6p12.3a	50,855,581
TFAP2B	rs2245173	SNPLEX	6p12.3a	50,857,376
TFAP2B	rs2817419	SNPLEX	6p12.3a	50,859,742
TFAP2B	rs2817420	SNPLEX	6p12.3a	50,860,168
TFAP2B	rs2817421	SNPLEX	6p12.3a	50,863,913
TFAP2D	rs11961359	SNPLEX	6p12.3a	50,726,739
TFAP2D	rs760899	SNPLEX	6p12.3a	50,740,443
TFAP2D	rs9381890	SNPLEX	6p12.3a	50,741,533
TFAP2D	rs9395616	SNPLEX	6p12.3a	50,747,310
TFAP2D	rs2235497	SNPLEX	6p12.3a	50,749,910
TFAP2D	rs9367409	SNPLEX	6p12.3a	50,751,064
TFAP2D	rs9369971	SNPLEX	6p12.3a	50,758,719
TFAP2D	rs6928472	SNPLEX	6p12.3a	50,759,344
TFAP2D	rs2235495	TAQMAN	6p12.3a	50,774,776
TFAP2D	rs9349557	SNPLEX	6p12.3a	50,780,012

Table 5.1: List of candidate genes investigated and tagSNPS genotyped within each locus as well as method of genotyping. All data based on HapMap build 34 of the genome and dbSNP buil 124 including HapMap Phase II data.

	HTR1B	*S100A10*	*TFAP2B/D*
Location	6q14.1b	1q21.3b	6p12.3a
HapMap Build	HG34/dbSNP124	HG34/dbSNP124	HG34/dbSNP124
Start Position	78,167,530	149,171,000	50,715,964
Ending Position	78,169,200	149,183,800	50,865,963
Size of Region	1.67 kb	12.8 kb	150 kb
# SNPs in Region	9	18	197
MAF >5% CEU	5	13	96
MAF >5% YRI	3	13	102
tagSNPs for CEU	3	4	14
tagSNPs for YRI	2	5	17
tagSNPs for STAR*D	***3***	***2***	***21***

Table 5.2: List of regions investigated and breakdown of SNPs in the region. Number of tagSNPS genotyped within each locus are listed as "*tagSNPs for STAR*D*". Represents HapMap Phase II data.

5.2.2 Marker Selection

To provide adequate coverage of the all four candidate genes, tagging SNPs were chosen based on the HapMap data for each genomic region (HapMap build 19, October 2005, NCBI B34 assembly, dbSNP b124) using the method of Carlson et al. (6). Detailed information on the genes and tagging SNPs selected are listed in Table 5.1 and linkage disequilibrium (LD) values for the SNPs selected are described in Tables 5.3 through 5.6. Due to the diverse ethnic makeup within the STAR*D population, we chose to select tagSNPs based on both the HapMap CEU and the YRI populations to ensure adequate coverage within such a diverse sample.

5.2.3 Polymorphism Genotyping

For the eight tagSNPs genotyped using 5' exonuclease fluorescence (Taqman) assays (Table 5.1), 5 µl reactions containing 10ng of dried genomic DNA template, 2.5 µl of Universal Taqman PCR Master Mix (Applied Biosystems), 0.085 µl of 20X Taqman assay probe (Applied Biosystems), and 2.42 µl of sterile H_20 were cycled at 95°C for 10 minutes, followed by 40 cycles of 92°C for 15 seconds and 60°C for 1 minute. Reaction fluorescence was read and genotypes were scored on an ABI 7900HT Sequence Detection System (Applied Biosystems). All other tagSNPs were genotyped on a multiplex genotyping platform called SNPlex (Applied Biosystems, Foster City, CA).

5.2.4 Statistical Analysis

Three phenotypic comparisons were made, based on the results from the response pattern analysis described previously (Chapter 2). The comparisons made were 1) all responders (specific and non-specific) versus non-responders (Resp42); 2) specific responders versus non-specific responders (Specific); and 3) remitters versus non-

responders (Remit). Single-point association tests were performed by logistic regression with the statistical package R 1.6.1 (7). Alleles were coded as 0, 1, or 2, corresponding to the presence of 0, 1, or 2 copies of the rare allele. This coding scheme was chosen because of its robustness to departure from the true additive genetic model (8). Haploview was used to estimate linkage disequilibrium (LD) across regions of the genes.

5.3 Results

5.3.1 Single Marker Association

In our primary phenotypic comparisons, we tested five SNPs in the regions of the S100A10 and HTR1B genes (S100A10=2 SNPs / HTR1B=3 SNPs). Stratifying by the two ethnic groups, Caucasian and African American, we tested for association by using an additive model and found marginal significance ($p < 0.05$) for one marker (rs130058) in HTR1B gene when looking in the entire African American sample using the specific response phenotype (Table 5.7). Those markers with nominal associations ($p < 0.1$) within S100A10 & HTR1B did not meet even modest corrections for multiple corrections.

In our primary phenotypic comparisons, we tested twenty-one SNPs found in the TFAP2β & TFAP2δ gene cluster. Stratifying by the three ethnic groups, White, non-Hispanic; White, Hispanic; and African American, we tested for association by using an additive model and found marginal significance ($p < 0.05$) for four markers (rs2245173, rs2817420, rs9381890, & rs2235495) occurring in different ethnic groups as well as within different phenotypes (Table 5.8). Those markers with nominal associations within

the TFAP2β & TFAP2δ gene cluster did not meet even modest corrections for multiple corrections.

5.3.2 Linkage Disequilibrium

As expected, the tagSNPs selected for each gene had relatively low levels of linkage disequilibrium as this was used as selection criteria. Also of interest was that the efficiency of tagSNPs was vastly different for the different genes depending on the density of genotyped SNPs within the HapMap Phase II data (Table 5.2). HTR1B required 3 tagSNPs while S100A10 needed only 2 tagSNPs. TFAP2B & TFAP2D are located within a 150kb block and tagSNPs were chose to tag this block containing both genes with 11 tagSNPs falling within the TFAP2β locus and 10 in the TFAP2δ gene for a total of 21.

A.

	RS11568817	RS130058	RS6298
RS11568817		1	1
RS130058	0.54		1
RS6298	0.33	0.18	

B.

	RS11568817	RS130058	RS6298
RS11568817		1	1
RS130058	0.3		1
RS6298	0.06	0.02	

Table 5.3: Linkage disequilibrium patterns within the HTR1B locus. D' values on upper diagonal shaded in red and pairwise r^2 values for each SNP pair on lower diagonal shaded in blue. Caucasian & African Americans are shown separately in A & B, respectively. Levels of shading indicate strength of LD with darker colors indicated stronger LD.

A.

	RS1873311	RS6678672
RS1873311		0.97
RS6678672	0.04	

B.

	RS1873311	RS6678672
RS1873311		1
RS6678672	0.03	

Table 5.4: Linkage disequilibrium patterns within the S100A10 locus. D' values on upper diagonal shaded in red and r^2 values on lower diagonal shaded in blue. Caucasian & African Americans are shown separately in A & B, respectively. Levels of shading indicate strength of LD with darker colors indicated stronger LD.

A.

	RS9367415	RS2143081	RS2272903	RS2076309	RS2817399	RS2817407	RS1569777	RS2245173	RS2817419	RS2817420	RS2817421
RS9367415		0.99	0.96	0.99	0.95	0.83	0.9	0.96	0.77	0.65	0.65
RS2143081	0.62		0.72	0.98	0.98	1	0.61	0.95	0.78	0.65	0.69
RS2272903	0.07	0.06		0.98	0.91	0.88	1	1	0.89	0.77	0.84
RS2076309	0.64	0.94	0.1		0.98	1	0.9	0.97	0.83	0.74	0.73
RS2817399	0.59	0.92	0.09	0.95		0.13	0.79	0.97	0.83	0.72	0.74
RS2817407	0.03	0.03	0.13	0.03	0		0.98	0.04	0.2	0.73	0.29
RS1569777	0.06	0.04	0.94	0.09	0.07	0.15		1	0.82	0.61	0.85
RS2245173	0.07	0.11	0.02	0.11	0.11	0	0.02		1	1	1
RS2817419	0.11	0.18	0.29	0.2	0.2	0	0.27	0.05		0.94	0.98
RS2817420	0.05	0.07	0.02	0.09	0.09	0.06	0.01	0.03	0.51		0.99
RS2817421	0.04	0.08	0.02	0.09	0.09	0.01	0.02	0.03	0.53	0.95	

B.

	RS9367415	RS2143081	RS2272903	RS2076309	RS2817399	RS2817407	RS1569777	RS2245173	RS2817419	RS2817420	RS2817421
RS9367415		0.99	1	0.97	0.9	0.25	1	1	0.91	0.61	0.69
RS2143081	0.64		0.7	0.96	0.94	0.86	0.64	1	0.84	0.47	0.51
RS2272903	0.06	0.04		1	0.6	0.48	0.96	0.7	0.79	0.28	0.67
RS2076309	0.63	0.92	0.09		0.93	0.86	1	1	0.89	0.74	0.77
RS2817399	0.55	0.86	0.03	0.84		0.42	0.76	0.81	0.88	0.62	0.58
RS2817407	0.01	0.05	0.05	0.05	0.01		0.49	0.04	0.58	0.32	0.06
RS1569777	0.06	0.04	0.88	0.09	0.05	0.05		1	0.87	0.01	0.34
RS2245173	0.03	0.04	0.01	0.04	0.03	0	0.01		1	0.9	0.8
RS2817419	0.08	0.11	0.36	0.12	0.12	0	0.42	0.02		0.97	1
RS2817420	0.02	0.01	0	0.03	0.02	0.03	0	0.01	0.38		1
RS2817421	0.02	0.02	0.01	0.03	0.02	0	0	0.01	0.38	0.95	

C.

	RS9367415	RS2143081	RS2272903	RS2076309	RS2817399	RS2817407	RS1569777	RS2245173	RS2817419	RS2817420	RS2817421
RS9367415		0.81	0.76	0.93	0.81	0	0.01	0.6	0.79	0.13	0.14
RS2143081	0.29		0.84	0.95	0.87	0.67	0.35	0.93	0.78	0.14	0.42
RS2272903	0.04	0.1		1	0.03	1	0.75	0.47	0.37	0.68	1
RS2076309	0.35	0.83	0.16		0.92	0.87	0.86	0.92	0.92	0.75	1
RS2817399	0.17	0.43	0	0.53		0.91	0.91	0.9	0.89	0.79	0.78
RS2817407	0	0.26	0.08	0.39	0.27		1	1	0.93	0.18	0.65
RS1569777	0	0.01	0.38	0.08	0.14	0.05		1	0.96	0.18	0.03
RS2245173	0.01	0.07	0.02	0.07	0.11	0.05	0.06		1	0.52	0.16
RS2817419	0.05	0.1	0.12	0.15	0.23	0.08	0.54	0.11		0.91	1
RS2817420	0	0	0.01	0.01	0.02	0	0	0	0.07		1
RS2817421	0	0	0.01	0.01	0.01	0	0	0	0.08	0.86	

Table 5.5: Linkage disequilibrium patterns within the TFAP2β locus. D' values on upper diagonal shaded in red and r^2 values on lower diagonal shaded in blue. Non-Hispanic Caucasian, Hispanic Caucasian, & African Americans are shown separately in A, B, & C, respectively. Levels of shading indicate strength of LD with darker colors indicated stronger LD.

A.

	RS11961359	RS760899	RS9381890	RS9395616	RS2235497	RS9367409	RS9369971	RS6928472	RS2235495	RS9349557
RS11961359		0.99	0.99	0.99	0.99	0.99	0.98	0.96	0.98	0.97
RS760899	0.54		0.98	0.99	1	0.99	0.96	0.97	0.99	0.85
RS9381890	0.98	0.54		0.99	0.98	1	0.98	0.95	0.99	0.97
RS9395616	0.97	0.55	0.97		0.99	0.99	0.97	0.96	0.98	0.97
RS2235497	0.54	0.99	0.54	0.54		0.99	0.96	0.95	0.99	0.85
RS9367409	0.97	0.54	0.98	0.97	0.54		0.98	0.97	0.99	0.98
RS9369971	0.94	0.52	0.94	0.94	0.52	0.94		0.93	0.99	0.96
RS6928472	0.19	0.35	0.19	0.19	0.34	0.19	0.18		0.96	0.76
RS2235495	0.92	0.52	0.93	0.92	0.52	0.95	0.92	0.18		0.99
RS9349557	0.56	0.68	0.55	0.55	0.68	0.55	0.55	0.2	0.54	

B.

	RS11961359	RS760899	RS9381890	RS9395616	RS2235497	RS9367409	RS9369971	RS6928472	RS2235495	RS9349557
RS11961359		0.97	0.97	0.99	0.94	0.94	0.96	1	1	0.94
RS760899	0.58		1	0.97	0.98	0.98	0.88	1	1	0.94
RS9381890	0.95	0.61		0.97	0.97	0.94	0.95	1	1	0.93
RS9395616	0.9	0.63	0.87		0.94	0.99	0.95	1	1	0.93
RS2235497	0.55	0.96	0.58	0.59		0.98	0.87	0.92	1	0.93
RS9367409	0.85	0.61	0.86	0.92	0.61		0.97	0.85	1	0.94
RS9369971	0.82	0.54	0.78	0.86	0.53	0.85		0.6	0.99	0.88
RS6928472	0.07	0.11	0.07	0.07	0.09	0.05	0.03		1	1
RS2235495	0.9	0.55	0.9	0.83	0.56	0.88	0.76	0.06		0.98
RS9349557	0.64	0.77	0.61	0.67	0.75	0.65	0.63	0.09	0.63	

C.

	RS11961359	RS760899	RS9381890	RS9395616	RS2235497	RS9367409	RS9369971	RS6928472	RS2235495	RS9349557
RS11961359		1	1	1	1	0.97	0.97	0.46	0.88	0.95
RS760899	0.11		1	1	0.99	1	0.91	0.97	1	0.98
RS9381890	0.62	0.17		0.49	1	0.52	0.51	0.75	0.81	0.46
RS9395616	0.25	0.42	0.1		1	0.93	0.8	1	1	0.75
RS2235497	0.09	0.82	0.14	0.35		1	0.94	0.93	1	0.98
RS9367409	0.28	0.35	0.13	0.73	0.3		0.85	1	1	0.83
RS9369971	0.27	0.31	0.12	0.56	0.27	0.69		0.78	1	0.92
RS6928472	0.01	0.24	0.02	0.1	0.26	0.09	0.06		1	0.93
RS2235495	0.7	0.12	0.45	0.28	0.1	0.34	0.32	0.03		1
RS9349557	0.25	0.36	0.1	0.5	0.3	0.65	0.84	0.08	0.32	

Table 5.6: Linkage disequilibrium patterns within the TFAP2δ locus. D' values on upper diagonal shaded in red and r^2 values on lower diagonal shaded in blue. Non-Hispanic Caucasian, Hispanic Caucasian, & African Americans are shown separately in A, B, & C, respectively. Levels of shading indicate strength of LD with darker colors indicated stronger LD.

5.4 Discussion

In this study, we sought to test whether DNA variation within several interesting candidate genes from the literature was associated with clinical response to citalopram treatment using the STAR*D sample. To accomplish this we used known HAPMAP genotype data to select tagSNPs within these four genes and then examined these genes in a large population taking the SSRI citalopram. Our analysis showed several different markers associated with a number of related response phenotypes (Table 5.7 & Table 5.8) with significance values less than $p = 0.05$. These associations are at best considered marginal given the large number of statistical tests that were performed. While we cannot say for sure that variation at these loci does not appear to significantly influence patient response to citalopram, our genetic data based upon a LD mapping, tagSNP approach suggests that variation within these genes do not significantly contribute to an individual's response to citalopram.

A limitation in any case–control association study is confounding based on population stratification. We attempted to control for population stratification in this study by analyzing the data within self-identified ethnic groups, as this has been shown to correlate well with marker allele frequencies (7). The interpretation of our association data also necessitates consideration of multiple testing. The correlation between phenotypes tested, as well as the observed correlation of marker-to-marker relationships, makes traditional corrections for multiple comparisons inappropriately conservative. We have thus left our significance tests uncorrected.

In summary, using a tagSNP approach, we have made an effort to investigate the role of genetic variation in four genes (HTR1B, S100A10, TFAP2β, and TFAP2δ) with

regards to their effect on response to citalopram in a large patient population. Using single locus tests, we observed a number of nominal associations between several markers and a number of inter-related phenotypes. None of the polymorphisms we interrogated appear to be strongly associated with citalopram response in the STAR*D population. Furthermore, we were unable to find genetic evidence to validate previous assertions in the literature that these genes play a role in depression or antidepressant response. Given the largely negative results that we have found using a candidate gene approach and that little is known about exactly how SSRIs exert their antidepressant effects in vivo, we will, in the future, employ a systematic approach to looking at variation across the entire genome to help elucidate genetic determinants of antidepressant response.

Gene	SNP	Caucasians			African Americans		
		Phenotype #1	*Phenotype #2*	*Phenotype #3*	*Phenotype #1*	*Phenotype #2*	*Phenotype #3*
HTR1B	rs6298	---	---	0.053	---	---	---
	rs130058	---	---	---	---	---	0.021
	rs11568817	---	---	---	---	---	0.065
S100A10	rs6678672	---	---	---	---	---	0.084
	rs1873311	0.071	---	---	---	---	---

Table 5.7: Single locus analysis for the HTR1B & S100A10 loci. Results are additive model uncorrected p values from logistic regression analysis. Values are reported for all values p <0.1, all other non-significant results listed as "---". Results are broken down based on self-reported ancestry categories of Caucasian & African American. Phenotypes: #1-Resp42, #2-Remit, and #3-Specific Response.

Gene	SNP	Non-Hispanic Caucasians			Hispanic Caucasians			African Americans		
		Phenotype #1	*Phenotype #2*	*Phenotype #3*	*Phenotype #1*	*Phenotype #2*	*Phenotype #3*	*Phenotype #1*	*Phenotype #2*	*Phenotype #3*
TFAP2B	rs9367415	----	----	----	----	----	----	----	----	----
	rs2143081	----	----	----	----	----	----	----	----	----
	rs2272903	----	----	----	----	----	----	----	----	----
	rs2076309	----	----	----	----	----	----	----	----	----
	rs2817399	----	----	----	----	----	----	----	----	----
	rs2817407	0.079	0.083	----	----	----	----	----	----	----
	rs1569777	----	----	----	----	----	----	----	----	----
	rs2245173	----	----	0.011	0.029	0.011	----	----	----	----
	rs2817419	----	----	----	----	----	----	----	----	----
	rs2817420	----	----	----	----	----	----	----	0.018	----
	rs2817421	----	----	----	----	----	----	----	----	----
TFAP2D	rs1923523	----	----	----	----	----	----	----	----	----
	rs11961359	0.076	----	----	----	----	----	----	----	----
	rs760899	----	----	----	----	----	----	----	----	----
	rs9381890	0.034	----	----	----	----	----	----	----	----
	rs9395616	----	----	----	----	----	----	----	----	----
	rs2235497	----	----	----	----	----	----	----	----	----
	rs9367409	----	----	----	----	----	----	----	----	----
	rs9369971	0.075	----	----	----	----	----	----	----	----
	rs6928472	----	----	----	----	----	----	----	----	----
	rs2235495	----	----	----	----	----	0.027	----	----	----
	rs9349557	----	----	----	----	----	----	----	----	----

Table 5.8: Single locus analysis for the TFAP2β & TFAP2δ loci. Results are additive model uncorrected p values from logistic regression analysis. Values are reported for all values p <0.1, all other results listed as "---". Results are broken down based on self-reported ancestry categories of Non-Hispanic Caucasian, Hispanic Caucasian, & African American. Phenotypes: #1-Resp42, #2-Remit, and #3-Specific Response.

2.5 Reference List

1. Svenningsson P, Chergui K, Rachleff I, Flajolet M and others. Alterations in 5-HT1B Receptor Function by P11 in Depression-Like States. *Science* 2006; 311(5757): 77-80

2. Svenningsson P and Greengard P. P11 (S100A10)--an Inducible Adaptor Protein That Modulates Neuronal Functions. *Curr. Opin. Pharmacol.* 2007; 7(1): 27-32

3. Damberg M, Eller M, Tonissaar M, Oreland L and others. Levels of Transcription Factors AP-2[Alpha] and AP-2[Beta] in the Brainstem Are Correlated to Monoamine Turnover in the Rat Forebrain. *Neuroscience Letters* 2001; 313(1-2): 102-104

4. Berggard C, Damberg M, and Oreland L. Chronic Citalopram Treatment Induces Time-Dependent Changes in the Expression and DNA-Binding Activity of Transcription Factor AP-2 in Rat Brain. *European Neuropsychopharmacology* 2003; 13(1): 11-17

5. Damberg M, Ekblom J, and Oreland L. Chronic Pharmacological Treatment With Certain Antidepressants Alters the Expression and DNA-Binding Activity of Transcription Factor AP-2. *Life Sci.* 2000; 68(6): 669-678

6. Carlson CS, Eberle MA, Rieder MJ, Yi Q and others. Selecting a Maximally Informative Set of Single-Nucleotide Polymorphisms for Association Analyses Using Linkage Disequilibrium. *Am. J Hum. Genet.* 2004; 74(1): 106-120

7. Ihaka R and Gentleman R. R: A Language for Data Analysis and Graphics. *J Comput Graph Stat* 1996; 5(3): 299-314

8. Freidlin B, Zheng G, Li Z, and Gastwirth JL. Trend Tests for Case-Control Studies of Genetic Markers: Power, Sample Size and Robustness. *Hum Hered.* 2002; 53(3): 146-152

CHAPTER 6

GENE-CENTRIC ANALYSIS REVEALS MARKERS ASSOCIATED WITH RESPONSE TO CITALOPRAM IN A LARGE CLINICAL SAMPLE WITH MDD[†]

6.1 Introduction

As described in previous chapters, major depression is among the most common psychiatric disorders and treatment with selective serotonin reuptake inhibitors (SSRIs) is a mainstay in the treatment of MDD (1). There is prominent heterogeneity in response to antidepressants (2), which is thought to be at least partly under genetic control (3,4).

To date, studies looking for genetic loci affecting antidepressant response have been inconclusive in small samples (5,6). However, recent studies designed to be much more powerful (7,8) have generally failed to discover variants within known candidate genes strongly believed to influence antidepressant response based on a presumed understanding of the mechanism of action of antidepressant drugs.

The general lack of success of candidate gene studies, even in large samples, is not unique to psychiatric genetics and has led to the development of technologies that enable scientists to interrogate large number of variants from across the genome independent of assumed biological pathways or mechanisms of action. Such studies, whether genome-wide or targeted, are becoming increasingly popular in many areas with highly significantly associated variants being discovered for complex genetic disorders (9-17). Many of these studies have identified loci that were not uncovered during candidate gene experiments.

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Large-scale studies, due to the extent of hypothesis testing, require validation (18), and study design has been a focus of much discussion recently as investigators seek strategies to evaluate the validity and generalizability of their findings (19). The most common design analyzes all genotypic data without a replication stage. The primary disadvantages of this approach are the larger cost and the lack of a validation step. A variation on this approach is to work with other research teams to validate findings, as has been done recently in type 2 diabetes (20-22).

Another approach involves split-sample or two-stage designs (23), in which SNP genotyping is done on one portion of the sample initially and then positive findings within this first group are then genotyped in the rest of the samples to validate the initial associations. The primary disadvantages of this approach are a diminution of power and the lower likelihood of weak genetic effects replicating, although it might be the best available option for singular samples. It has been suggested recently that an alternative to a split-sample design would be to genotype a fraction of a sample set, followed by genotyping of those SNPs showing association in the remainder of the samples. A joint analysis of all samples genotyped for this smaller set of markers is then carried out. This approach obtains the cost savings of a two-stage design, while generally retaining the power of a one-stage approach (24), with the attendant lack of validation.

The selection criteria for determining which markers to carry on to the second stage of genotyping have been discussed far less but may be of equal importance. The most straightforward strategy would be to select a priori those markers that meet the chosen level of significance for the validation stage. One way to enhance the likelihood of choosing high value SNPs for validation is to preferentially weight SNPs based on

prior information, such as when the SNP occurs in a linkage peak or known candidate gene for the phenotype (25,26).

Finally, it may be useful to select SNPs that represent a larger genetic effect despite showing a lower level of statistical significance (i.e., having a lower p value). With the large samples used for many current studies, it is likely that there will be markers with dramatically small p values coming from the discovery stage. Many of these will have negligible effect sizes and therefore the clinical implications of such variants are not immediately clear. It thus may be of great interest to find SNPs that suggest a substantial contribution to risk, and still show an adequate level of significance using association statistics. Undoubtedly, this approach would result in not considering SNPs that are highly associated with a trait, but with a small effect. Another hazard of choosing such markers involves enrichment for low frequency variants, for which allele frequency differences between cases and controls may be unreliable, thus leading to spuriously large odds ratios.

As described in previous chapters, we are determining if common DNA variants contribute to antidepressant response status in a large clinical sample (N=1,914) taking the SSRI citalopram (Celexa). Here, we report an analysis of 40,113 gene-based SNPs, including 20,000 non-synonymous SNPs, in a two-stage design (27). We additionally sought to test if using both significance levels and a threshold effect size (e.g., odds ratio $\geq$2.0) as criteria for SNP selection for stage-two genotyping would be a successful approach to discovering clinically relevant variants associated with antidepressant response in our whole genome study.

6.2 Materials and Methods

6.2.1 Study Design

We used a two stage design to identify associated variants and validate our findings. We selected 962 subjects for the first stage (discovery) and 952 subjects for our second stage (validation). These subjects were selected by random sampling controlling for gender, race, and Resp42 phenotype, thus assuring comparability of the discovery and validation samples for the most meaningful characteristics. All markers with trend test p ≤ 0.001 and with a dominant OR ≥ 2.0 were selected for genotyping in the second stage. It might be argued that power is sacrificed by not using a single stage design using all samples for analysis, or a two-stage design with joint analysis (25). While it is true that our approach sacrifices power, these other approaches do not provide true replication of association findings. Since we are aware of no other sample of comparable size that exists now or for the foreseeable future, we believe we must sacrifice some power for the ability to assess the validity of our findings.

6.2.2 STAR*D Study Sample

The study population consisted of the subjects who consented to give DNA from the STAR*D antidepressant trial, as reviewed elsewhere (see Chapter 3). To limit heterogeneity within our analyses, the analyses were done by splitting samples into two self-identified racial subgroups: White and African American. Due to heterogeneity within our self-identified "White" subject, as uncovered via the *structure* analysis described later (Chapter 7), the analyses were also ran splitting STAR*D into three racial

subgroups: White, non-Hispanic; White, Hispanic; and African American. Other self-reported race classes were not analyzed due to small sample sizes.

6.2.3 Polymorphism Genotyping

Approximately 40,000 markers were genotyped by ParAllele Biosciences (South San Francisco, CA) using Molecular Inversion Probes (MIP), so named because the oligonucleotide probe central to the process undergoes a unimolecular rearrangement from a molecule that cannot be amplified, into a molecule that can be amplified. This rearrangement is mediated by hybridization to genomic DNA and an enzymatic "gap fill" process that occurs in an allele-specific manner. The resulting circularized probe can be separated from cross-reacted or unreacted probes by a simple exonuclease digestion and then amplified using PCR probes sites common to all MIPs (36). Of the 40,113 assays, 593 were duplicate assays and 39,635 passed genotyping quality control. Ten HapMap samples were typed from 2-7 times as external controls, and 5 STAR*D samples were genotyped in duplicate. Duplicate genotyping showed repeatability of 99.92%. SNP and amelogenin data identified one X0 female and one XXY male. 37 of the 45 SNPs genotyped on the validation set of samples were genotyped using the SNPlex multiplex genotyping platform (Applied Biosystems, Foster City, CA) with 7 remaining SNPs genotyped using a 5' nuclease assay (Taqman) and 1 SNP genotyped with restriction length fragment analysis (RFLP) due to assay failure with both SNPlex and Taqman (Table 6.1).

6.2.4 Marker Information & Selection

The 40,113 markers genotyped for our study were not chosen to cover the genome, and are spaced approximately one marker per 72kb (Figure 6.1). SNPs were

chosen by ParAllele to a) represent common variation in genes, and b) represent available known non-synonymous variants. The initial experimental design was to add approximately 60,000 additional markers to provide a higher density of tagging SNPs in genes, with little coverage of non-genic regions. As discussed in the next chapter, this approach was supplanted by a larger-scale effort.

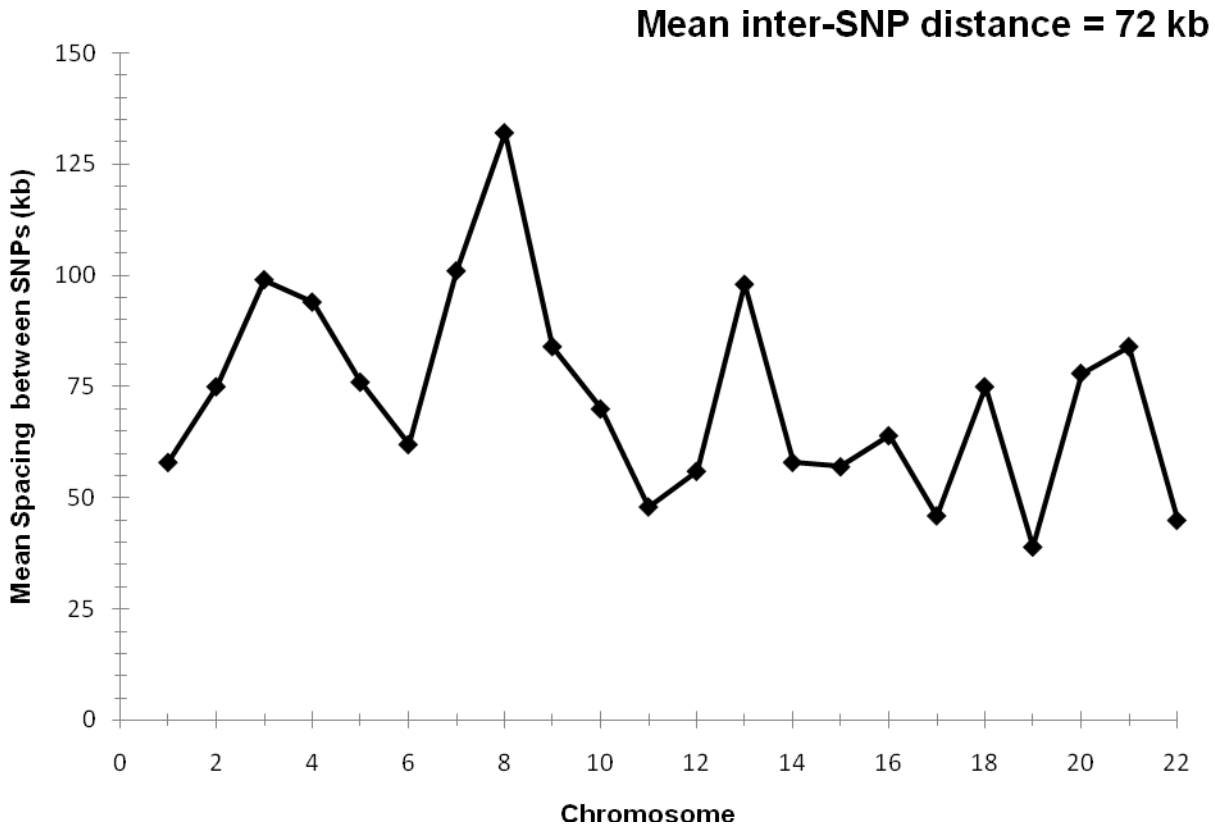


Figure 6.1: Figure showing mean inter-SNP distances broken down by chromosome. Average inter-SNP distance across all chromosomes was approximately 72 kb.

Marker	Gene	cSNP	Chrom	Position	Assay Platform	Race	Discovery Samples					
							MAF (NR)	MAF (R)	Additive Model P-value	Dominant OR	Lower CI	Upper CI
rs400625	SLC37A1	NO	1	43306.339	SNPlex	CA	32.9%	43.0%	7.52E-05	2.03	1.50	2.76
rs11559316	EBNA1BP2	NS	3	184408.11	Taqman	CA	2.5%	0.2%	5.62E-05	0.09	0.02	0.40
rs6804951	MCF2L2, KIAA0861	NO	5	5515.607	Taqman	CA	3.6%	8.9%	0.000197	2.58	1.49	4.47
rs3806873	KIAA0947, CR749441	NS	7	119527.6	SNPlex	CA	5.5%	1.6%	7.29E-05	0.26	0.13	0.51
rs1990428	KCND2	NO	9	134557.47	SNPlex	CA	1.0%	4.1%	0.000190	4.23	1.76	10.14
rs9615	RXRA	NS	15	76138.076	SNPlex	CA	1.8%	5.2%	0.000391	3.17	1.57	6.40
rs735663	TBC1D2B	NO	19	6932.763	SNPlex	CA	2.8%	6.9%	0.000286	2.87	1.60	5.16
rs2035970	EMR4	NO	21	42874.864	Taqman	CA	8.5%	14.7%	0.000418	2.19	1.48	3.23
rs4667001	C2orf10	NO	1	11782.334	SNPlex	AA	77.1%	92.4%	0.000430	4.30	0.47	39.45
rs333993	OSBPL6	NS	1	47429.209	SNPlex	AA	29.9%	48.6%	0.000634	3.17	1.56	6.46
rs1537514	MTHFR	NO	1	47459.465	SNPlex	AA	2.3%	14.4%	7.82E-05	8.35	2.36	29.56
rs2023549	CRIM1	NS	1	47479.934	SNPlex	AA	22.9%	40.5%	0.000886	2.44	1.25	4.76
rs1366842	C2orf10	NO	1	86724.95	SNPlex	AA	77.1%	91.8%	0.000739	4.36	0.48	40.00
rs3821161	CRIM1	NO	1	180562.13	SNPlex	AA	16.0%	32.4%	0.000730	3.19	1.61	6.33
rs360063	TMEM63A, EPHX1	NO	1	200899.33	SNPlex	AA	27.1%	11.5%	0.000271	0.30	0.15	0.61
rs3795573	GOLT1A	NS	1	222343.04	SNPlex	AA	17.6%	34.5%	0.000905	2.89	1.47	5.71
rs3002652	RGL1	NS	2	36541.919	SNPlex	AA	16.0%	32.4%	0.000730	3.19	1.61	6.33
rs2231580	CLCA4	NO	2	36591.446	Taqman	AA	5.7%	19.9%	0.000395	4.98	1.99	12.44
rs3125630	STIL	NO	2	170000.84	SNPlex	AA	20.0%	6.8%	0.000656	0.27	0.12	0.61
rs3122619	STIL	NO	2	178969.54	SNPlex	AA	19.7%	6.8%	0.000680	0.27	0.12	0.62
rs2758735	STIL	NO	2	185627.25	SNPlex	AA	19.7%	6.8%	0.000793	0.27	0.12	0.63
rs915034	NR5A1	NO	2	185627.75	SNPlex	AA	19.3%	41.9%	0.000110	2.64	1.35	5.18
rs915033	NR5A1	NO	2	201948.5	SNPlex	AA	20.0%	41.8%	0.000214	2.73	1.39	5.38
rs6716834	LRP2	NO	3	3100.337	SNPlex	AA	52.1%	29.2%	5.33E-05	0.29	0.14	0.60
rs901273	CA12	NO	3	16302.859	SNPlex	AA	24.3%	8.1%	0.000233	0.24	0.11	0.54
rs1482294	TMEM132D	NO	5	43332.789	SNPlex	AA	40.1%	60.8%	0.000694	3.73	1.68	8.28
rs3825181	SLC15A4	NO	5	95116.957	Taqman	AA	31.3%	50.0%	0.000912	2.32	1.15	4.66
rs9302	PWP1	NO	5	125724.04	SNPlex	AA	71.5%	52.7%	0.000328	0.39	0.13	1.16
rs17759513	OR10S1	NO	5	141000.01	SNPlex	AA	19.3%	6.8%	0.000578	0.25	0.11	0.58
rs920722	FDX1	NS	6	117832.14	SNPlex	AA	9.0%	24.3%	0.000225	4.25	1.97	9.18
rs2282654	CALCB	NO	7	28629.929	SNPlex	AA	44.4%	22.3%	6.98E-06	0.19	0.09	0.40
rs7896781	C10orf52	NS	9	1046.959	SNPlex	AA	15.0%	2.7%	0.000111	0.14	0.05	0.45
rs3769823	CASP8	NO	9	124324.51	SNPlex	AA	52.9%	33.6%	0.000776	0.32	0.15	0.68
rs2416933	NR5A1	NS	9	124325.08	SNPlex	AA	20.3%	40.0%	0.000820	2.49	1.26	4.93
rs1894473	ASCC2	NO	9	124325.11	SNPlex	AA	1.4%	9.9%	0.000870	8.35	1.82	38.29
rs17641078	DMRT2	NO	10	27295.757	SNPlex	AA	12.1%	2.1%	0.000513	0.14	0.04	0.52
rs886750	CREB5	NO	11	15055.719	SNPlex	AA	43.1%	23.0%	0.000167	0.36	0.18	0.70
rs1998206	ROS1	NO	11	109831.75	SNPlex	AA	21.4%	6.9%	0.000469	0.27	0.12	0.62
rs11742646	C5orf16, FCHSD1	NO	11	123353.42	SNPlex	AA	22.1%	8.3%	0.000830	0.25	0.11	0.57
rs11241887	LOC389319	NO	12	106609.38	RFLP	AA	5.1%	18.6%	0.000776	3.99	1.57	10.13
rs34899	RHOBTB3	NS	12	127804.14	Taqman	AA	29.3%	50.0%	0.000627	2.19	1.10	4.34
rs3733768	HMGCS1	NS	12	128235.67	SNPlex	AA	45.1%	23.6%	4.38E-05	0.21	0.10	0.43
rs842274	OXNAD1, MGC15763	NO	15	61465.019	SNPlex	AA	20.6%	38.0%	0.000760	2.33	1.18	4.60
rs340812	IL5RA	NS	21	42549.655	SNPlex	AA	36.8%	16.2%	5.73E-05	0.27	0.14	0.54
rs7281684	ABCG1	NS	22	28545.755	Taqman	AA	12.9%	2.1%	0.000606	0.15	0.04	0.55

Table 6.1: List of 45 SNPs meeting significance and effect size thresholds ($p \leq 0.001$ & $OR \geq 2.0$) in the discovery subset of the STAR*D sample. SNPs colored pink indicate that they were found to be significant in the Caucasian subgroup while blue colored SNPs were found to be significantly associated in the African-American subgroup.

6.2.5 Statistical Analysis

The frequency distributions of demographic and clinical variables were examined. To control for any potential population stratification, we stratified our analyses by self-reported ethnicity: Caucasian and African-American. We tested for Hardy-Weinberg equilibrium within each of the ethnic groups, and for these analyses, all subjects from a stratum were used in the analysis since all subjects had depression and the evaluated polymorphisms were not suspected to influence risk of depression. Linkage disequilibrium was estimated using the r^2 metric.

We used unconditional logistic regression analysis to examine associations of genetic polymorphisms and response to citalopram. Each polymorphism was modeled individually as gene-dosage effects in the regression models. This coding scheme was chosen because of its robustness to departure from the true additive genetic model (28). Multivariate regression analysis was used to control for possible confounding by sex, age, education (years of school), months in current major depressive episode (MDE), and years since first MDE. We estimated odds ratios (OR) and 95% confidence intervals (CIs) for the carriers of the minor allele versus non-carriers of the minor allele. In the validation stage, markers were considered significant only if they met a threshold p value ($p \leq 0.006$) and had the same direction of effect as in the discovery stage. The same directionality requirement is important as it will remove those SNPs where the allele associated with improved response switches between the discovery and validation samples but each different alelele remains significantly associated.

As a secondary analysis, we evaluated the combined effect of multiple SNPs on citalopram response. For this analysis we used SNPs significantly associated when

considering the entire dataset ($p \leq 0.001$ in the combined sample). We selected the allele that was more common in the responder group and treated this allele as the protective allele. We then summed up the total number of protective alleles each individual had. This total was modeled as a continuous variable in a logistic regression analysis. One thousand permutations were run to obtain the corrected type I error rate and odds ratio estimate. Statistical tests were performed in SAS version 8.2 or Splus version 6.2.1 statistical packages.

6.3 Results

6.3.1 Two-Stage Primary Analysis

In our two-stage strategy, we genotyped approximately half of our sample for 40,113 SNPs located in or near known or predicted genes, including approximately 20,000 non-synonymous SNPs. We then sought to validate our initial findings by genotyping the second half of our sample for SNPs that met our a priori threshold for statistical significance and effect size. Markers with a trend test $p \leq 0.001$ and with a dominant OR ≥ 2.0 were selected for genotyping in the second stage. Markers that had $p \leq 0.006$ in the validation stage for the same allele were considered to show replication. We found that 45 SNPs met our selection criteria in our discovery population and were therefore genotyped in the remainder of our sample (Table 6.1). Only one SNP in the LRP2 locus (rs6716834) met the criteria for replication, and occurred in the African-American sample. The minor allele frequency for responders was 0.29 in the discovery sample, compared to 0.52 in the non-responders ($p = 5.3 \times 10\text{-}5$, dominant odds ratio = 0.29 [95% confidence interval = 0.14-0.60]). In the validation sample, the responder

minor allele frequency was 0.33, versus 0.53 in non-responders (p = 0.006, dominant odds ratio = 0.41 [95% confidence interval = 0.17-0.94]) (Table 6.2). Combining discovery and validation samples, we observed continuing support for the LRP2 SNP (p = 1.3 x 10-6, dominant odds ratio = 0.33 [95% confidence interval = 0.19-0.58]).

6.3.2 One-Stage Secondary Analysis

As a secondary analysis, we evaluated the additive effect of multiple SNPs on response to citalopram. We used twelve markers that met our discovery stage criteria and then met a relaxed level of significance in the combined dataset ($p \leq 0.001$) (Table 6.2). Within the Caucasian sample, there were five SNPs from five different genes that were used in this analysis. Thus the number of "protective" alleles (i.e., the allele increased the likelihood of response) for each Caucasian individual ranged from 0 to 10. We found a highly significant trend of response with increasing number of protective alleles (permutation $p \leq 0.0001$). That is, for each additional protective allele, the likelihood of response increases 55% (odds ratio = 1.55, 95% confidence interval = 1.37-1.73) (Figure 6.2). Within the African-American group, there were seven SNPs from six genes that were genotyped in stage two. Because, two SNPs (rs915033 and rs915034) were in strong LD (r2 = 0.95) with each other, only rs915033 was used. We also found a significant effect (permutation $p \leq 0.0001$), with an effect size of OR = 2.01 (95% confidence interval = 1.76-2.34) for each additional allele (Figure 6.3). This suggests multiple independent susceptibility loci acting in an additive manner with no real evidence of epistasis, although we have not formally tested for such.

Marker	Gene	cSNP	Chrom	Position	Race	Discovery Samples						All Samples					
						MAF (NR)	MAF (R)	Additive Model P-value	Dominant OR	Lower CI	Upper CI	MAF (NR)	MAF (R)	Additive Model P-value	Dominant OR	Lower CI	Upper CI
rs3806873	KIAA0947, CR749441	NS	5	5515.607	CA	5.52%	1.62%	7.29E-05	0.26	0.13	0.51	4.90%	2.23%	0.000620	0.42	0.26	0.67
rs1990428	KCND2	NO	7	119527.6	CA	1.04%	4.11%	0.000190	4.23	1.76	10.14	1.71%	4.16%	0.000431	2.44	1.41	4.22
rs735663	TBC1D2B	NO	15	76138.076	CA	2.77%	6.90%	0.000286	2.87	1.60	5.16	3.41%	6.21%	0.001267	1.95	1.29	2.95
rs2035970	EMR4	NO	19	6932.763	CA	8.45%	14.69%	0.000418	2.19	1.48	3.23	9.04%	13.25%	0.001309	1.60	1.20	2.14
rs400625	SLC37A1	NO	21	42874.864	CA	32.93%	43.00%	7.52E-05	2.03	1.50	2.76	33.57%	41.19%	0.000122	1.65	1.31	2.08
rs3002652	RGL1	NO	1	180562.13	AA	15.97%	32.43%	0.000730	3.19	1.61	6.33	19.76%	32.42%	0.001191	2.69	1.61	4.48
rs6716834	LRP2	NS	2	170000.84	AA	52.14%	29.17%	5.33E-05	0.29	0.14	0.60	52.52%	30.95%	1.29E-06	0.33	0.19	0.58
rs11241887	LOC389319	NO	5	125724.04	AA	5.15%	18.57%	0.000776	3.99	1.57	10.13	8.33%	19.51%	0.000577	2.77	1.48	5.17
rs915034	NR5A1	NO	9	124324.51	AA	19.29%	41.89%	0.000110	2.64	1.35	5.18	22.54%	39.84%	8.57E-05	2.25	1.36	3.74
rs915033	NR5A1	NO	9	124325.11	AA	20.00%	41.78%	0.000214	2.73	1.39	5.38	24.18%	40.08%	0.000405	2.26	1.36	3.76
rs2282654	CALCB	NO	11	15055.719	AA	44.44%	22.30%	6.98E-06	0.19	0.09	0.40	42.34%	26.17%	2.67E-05	0.32	0.19	0.54
rs9302	PWP1	NO	12	106609.38	AA	71.53%	52.70%	0.000328	0.39	0.13	1.16	68.15%	54.30%	0.000537	0.40	0.17	0.91

Table 6.2A: List of 12 SNPs meeting significance and effect size thresholds ($p \leq 0.001$ & $OR \geq 2.0$) in the discovery subset of the STAR*D sample as well as a significance threshold of $p \leq 0.001$ in all STAR*D samples.

Marker	Gene	cSNP	Chrom	Position	Race	Discovery Samples						Validation Samples					
						MAF (NR)	MAF (R)	Additive Model P-value	Dominant OR	Lower CI	Upper CI	MAF (NR)	MAF (R)	Additive Model P-value	Dominant OR	Lower CI	Upper CI
rs6804951	MCF2L2, KIAA0861	NS	3	184408.11	CA	3.58%	8.91%	0.000197	2.58	1.49	4.47	10.87%	5.59%	0.002210	0.49	0.30	0.80
rs6716834	LRP2	NS	2	170000.84	AA	52.14%	29.17%	5.33E-05	0.29	0.14	0.60	53.06%	33.33%	0.005576	0.41	0.17	0.94

Table 6.2B: List of SNPs meeting significance and effect size thresholds ($p \leq 0.001$ & $OR \geq 2.0$) in the discovery subset of the STAR*D sample as well as a significance threshold of $p \leq 0.006$ in the validation subset of STAR*D. Only rs6716834 in the LRP2 locus is considered to have validated since the OR stays in the same direction, unlike that of rs6804951.

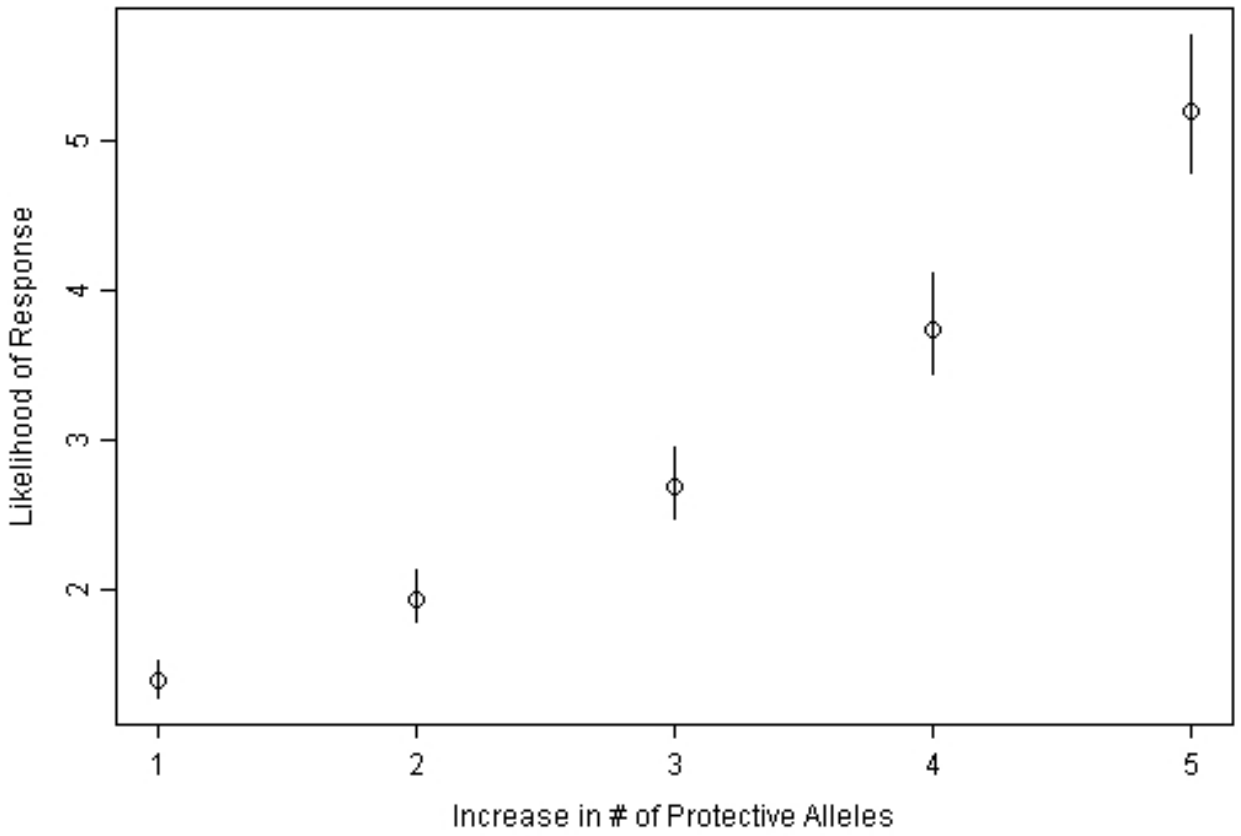


Figure 6.2: Figure showing the Odds Ratio for increasing number of protective alleles for the 5 SNPs associated with citalopram response in the Caucasian racial subgroup.

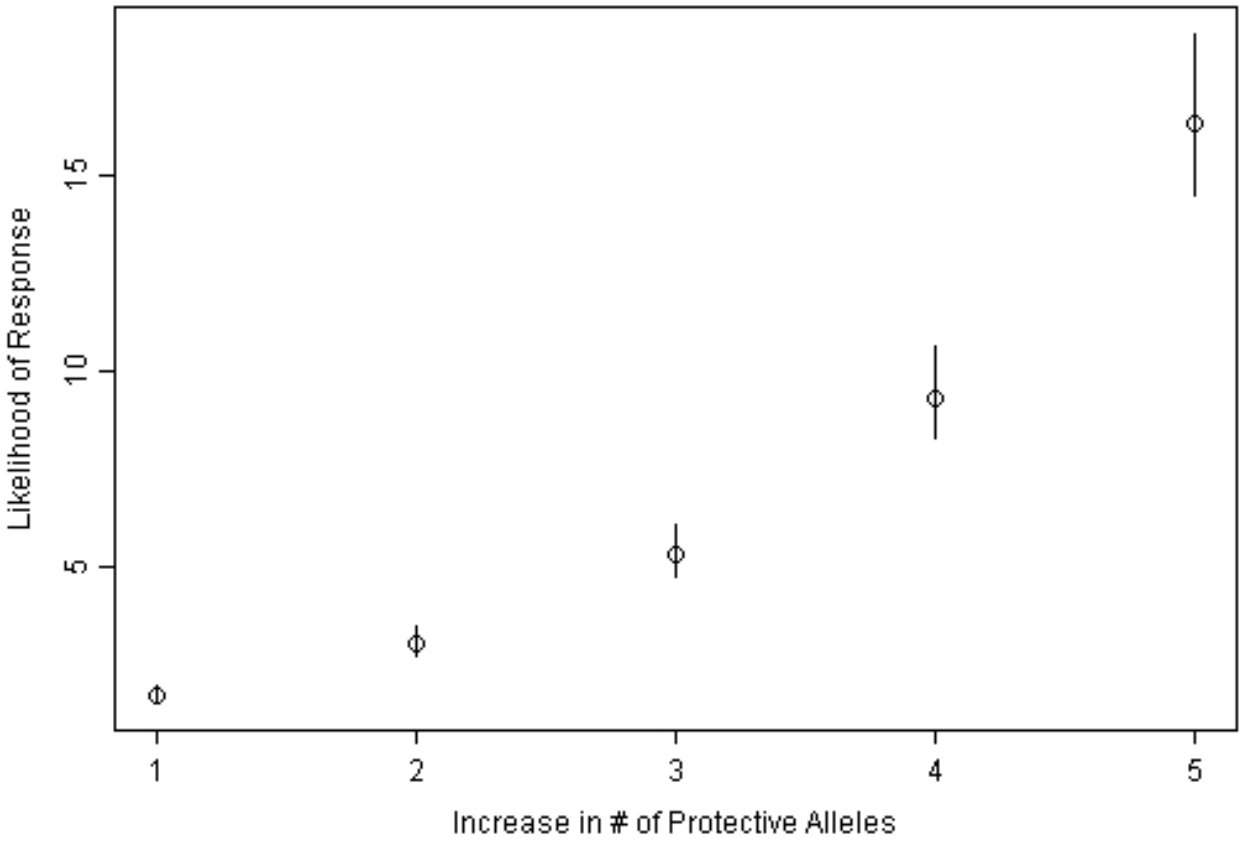


Figure 6.3: Figure showing the Odds Ratio for increasing number of protective alleles for the 6 SNPs associated with citalopram response in the African-American racial subgroup. There were two SNPs in high LD (see text) so only one was used in this analysis. Note difference in y-axis scale with Figure 6.2.

6.4 Discussion

Our 40,113 marker, two-stage design study implicated one SNP in the LRP2 locus with antidepressant response with a relatively large effect size. The dominant odds ratio for the minor allele was 0.29 in the discovery set, and 0.41 in the validation set.

The most likely role for the protein LRP2 in antidepressant response involves its function at the blood brain barrier and possible role in transport of drug into the central nervous system. The SNP associated with antidepressant response within LRP2 is a non-synonymous coding change within the protein changing a serine to an asparagine at amino acid position 83. This is a relatively mild mutation that is predicted to not be a "significant" amino acid substitution and to be well tolerated (score 0.79) as determined by the SIFT algorithm (29-32). This gene is relatively large (~250kb) and contains some 31 non-synonymous SNPs within its 79 exons. DNA variation in the gene has been previously associated with side effects to the chemotherapeutic agent cisplatin, highlighting the role that this protein may play in drug transport (33,34).

Spurious associations due to population stratification within a sample are a major concern for case-control association studies. To correct for potential ethnic stratification, we analyzed both the African American & Caucasian groups separately to test for association. As described above, we also tested for the possible confounding effects of a small subgroup of Hispanics within the Caucasian sample by further dividing the Caucasians on the basis of Hispanic ancestry and found that all markers still remained associated with our phenotype (Table 6.3).

A limitation of our study is that our choice of 40,113 markers does not represent a comprehensive whole genome association study. While approximately half of these

markers were chosen as tag SNPs, the other half of the markers represents coding SNPs within known genes. These 40,113 markers have an average spacing of about 72kb between them which is much larger than what that would be desired for a whole genome study. Even with a non-ideal choice of markers, we were able to discover one marker that was validated within our two-stage design and 11 other markers using a more relaxed threshold. Two of these 12 SNPs were non-synonymous coding changes and minor allele frequencies varied dramatically among the markers (3%-40% MAF). These markers were found to be in several intriguing genes including NR5A1 (a transcription factor involved in steroid and hormone production in response to stress), KCND2 (a potassium ion channel expressed in the brain), and EMR4 (a novel epidermal growth factor expressed in the brain). These results provide us with plausible genes for antidepressant response and provide a picture of what could be uncovered using a larger number of markers designed to more adequately survey the genome. This study will be addressed in detail within Chapter 7.

In summary, we have looked at approximately 40,000 markers throughout the genome in a very large clinical sample using a two-stage "split" sample design to find an association with a single marker (rs6716834) within the LRP2 gene. Although not validated in the two-stage design, we have also implicated 11 other markers in our entire sample found in various loci including some promising genes such as a potassium channel and calcitonin b related peptide. It appears that the LRP2 gene does affect response to drug in our representative population with citalopram using our outcome assessment phenotypes.

A)

Marker	Gene	cSNP	Chrom	Position	Discovery					Joint				
					Race	MAF (NR)	MAF (R)	Dominant OR	Additive Model P-value	Race	MAF (NR)	MAF (R)	Dominant OR	Additive Model P-value
rs11559316	EBNA1BP2	NS	3	184408.106	CA	2.47%	0.23%	0.09	5.62E-05	CA	1.52%	0.78%	0.51	0.084569
rs1990428	KCND2	NO	9	134557.47	CA	1.04%	4.11%	4.23	0.000190	CA	1.71%	4.16%	2.44	0.000431
rs2035970	EMR4	NO	21	42874.864	CA	8.45%	14.69%	2.19	0.000418	CA	9.04%	13.25%	1.60	0.001309
rs3806873	KIAA0947, CR749441	NS	7	119527.599	CA	5.52%	1.62%	0.26	7.29E-05	CA	4.90%	2.23%	0.42	0.000620
rs400625	SLC37A1	NO	1	43306.339	CA	32.93%	43.00%	2.03	7.52E-05	CA	33.57%	41.19%	1.65	0.000122
rs6804951	MCF2L2, KIAA0861	NO	5	5515.607	CA	3.58%	8.91%	2.58	0.000197	CA	6.78%	7.41%	1.10	0.571581
rs735663	TBC1D2B	NO	19	6932.763	CA	2.77%	6.90%	2.87	0.000286	CA	3.41%	6.21%	1.95	0.001267
rs9615	RXRA	NS	15	76138.076	CA	1.77%	5.22%	3.17	0.000391	CA	3.87%	4.99%	1.34	0.183378

B)

Marker	Gene	cSNP	Chrom	Position	Discovery					Joint				
					Race	MAF (NR)	MAF (R)	Dominant OR	Additive Model P-value	Race	MAF (NR)	MAF (R)	Dominant OR	Additive Model P-value
rs11559316	EBNA1BP2	NS	3	184408.11	CA	2.23%	0.27%	0.115675676	0.000980	CA	1.36%	0.76%	0.55	0.176752
					HCA	3.39%	0.00%	4.40E-07	0.017105	HCA	2.17%	0.93%	0.42	0.306148
rs1990428	KCND2	NO	9	134557.47	CA	0.87%	3.34%	4.06	0.002776	CA	1.35%	3.79%	2.77	0.000711
					HCA	1.72%	9.02%	6.16	0.007608	HCA	3.33%	6.42%	2.06	0.142488
rs2035970	EMR4	NO	21	42874.864	CA	8.93%	14.82%	2.15	0.003043	CA	8.75%	13.26%	1.71	0.001708
					HCA	6.67%	13.79%	2.27	0.064461	HCA	10.33%	13.21%	1.25	0.365420
rs3806873	KIAA0947, CR749441	NS	7	119527.6	CA	2.69%	0.67%	0.24	0.004893	CA	2.88%	0.99%	0.33	0.001324
					HCA	16.38%	7.63%	0.35	0.038941	HCA	13.89%	9.81%	0.63	0.245150
rs400625	SLC37A1	NO	1	43306.339	CA	35.06%	44.47%	2.06	0.000820	CA	34.93%	42.06%	1.63	0.001036
					HCA	24.58%	33.61%	1.60	0.120601	HCA	27.47%	35.78%	1.60	0.083074
rs6804951	MCF2L2, KIAA0861	NO	5	5515.607	CA	4.05%	9.40%	2.45	0.001042	CA	7.64%	7.56%	1.00	0.947197
					HCA	1.82%	5.66%	2.76	0.163399	HCA	2.91%	6.50%	2.00	0.128188
rs735663	TBC1D2B	NO	19	6932.763	CA	2.60%	6.96%	2.89	0.000430	CA	3.55%	6.10%	1.77	0.007186
					HCA	3.45%	6.56%	2.77	0.294431	HCA	2.78%	6.88%	3.43	0.060540
rs9615	RXRA	NS	15	76138.076	CA	2.02%	5.11%	2.71	0.004608	CA	4.38%	4.81%	1.13	0.643448
					HCA	0.85%	5.93%	7.81	0.020259	HCA	1.65%	6.07%	4.06	0.017538

Table 6.3: **A)** List of 8 SNPs meeting significance and effect size thresholds ($p \leq 0.001$ & $OR \geq 2.0$) in the discovery subset of the STAR*D sample within the Caucasian racial subgroup. Also listed are the p values in the entire STAR*D sample. **B)** List of 8 SNPs meeting significance and effect size thresholds ($p \leq 0.001$ & $OR \geq 2.0$) in the discovery subset of the STAR*D sample within the Caucasian racial subgroup. When the Caucasian subgroup was broken down into Non-Hispanic Caucasian (CA) & Hispanic Caucasians (HCA) only 3 of these 8 would have met our original thresholds (colored pink) but the 4 SNPs that were significant in the entire STAR*D without subdividing the Caucasian racial group are also positive within the Non-Hispanic Caucasians (colored blue).

6.5 Reference List

1. Kessler RC, McGonagle KA, Zhao S, Nelson CB and others. Lifetime and 12-Month Prevalence of DSM-III-R Psychiatric Disorders in the United States. Results From the National Comorbidity Survey. *Arch. Gen. Psychiatry* 1994; 51(1): 8-19

2. Esposito K and Goodnick P. Predictors of Response in Depression. *Psychiatr. Clin North Am* 2003; 26(2): 353-365

3. Serretti A, Lilli R, and Smeraldi E. Pharmacogenetics in Affective Disorders. *Eur. J Pharmacol.* 2002; 438(3): 117-128

4. Malhotra AK, Murphy GM, Jr., and Kennedy JL. Pharmacogenetics of Psychotropic Drug Response. *American Journal of Psychiatry* 2004; 161(5): 780-796

5. Kraft JB, Slager SL, McGrath PJ, and Hamilton SP. Sequence Analysis of the Serotonin Transporter and Associations With Antidepressant Response. *Biol Psychiatry* 2005; 58(5): 374-381

6. Binder EB, Salyakina D, Lichtner P, Wochnik GM and others. Polymorphisms in FKBP5 Are Associated With Increased Recurrence of Depressive Episodes and Rapid Response to Antidepressant Treatment. *Nat Genet* 2004; advanced online publication

7. McMahon FJ, Buervenich S, Charney D, Lipsky R and others. Variation in the Gene Encoding the Serotonin 2A Receptor Is Associated With Outcome of Antidepressant Treatment. *Am J Hum Genet* 2006; 78(5): 804-814

8. Kraft JB, Peters EJ, Slager SL, Jenkins GD and others. Analysis of Association Between the Serotonin Transporter and Antidepressant Response in a Large Clinical Sample. *Biol. Psychiatry* 2007; 61(6): 734-742

9. Ozaki K, Ohnishi Y, Iida A, Sekine A and others. Functional SNPs in the Lymphotoxin-Alpha Gene That Are Associated With Susceptibility to Myocardial Infarction. *Nat. Genet.* 2002; 32(4): 650-654

10. Klein RJ, Zeiss C, Chew EY, Tsai JY and others. Complement Factor H Polymorphism in Age-Related Macular Degeneration. *Science* 2005; 1109557

11. Hampe J, Franke A, Rosenstiel P, Till A and others. A Genome-Wide Association Scan of Nonsynonymous SNPs Identifies a Susceptibility Variant for Crohn Disease in ATG16L1. *Nat Genet* 2007; 39(2): 207-211

12. Gudmundsson J, Sulem P, Manolescu A, Amundadottir LT and others. Genome-Wide Association Study Identifies a Second Prostate Cancer Susceptibility Variant at 8q24. *Nat Genet* 2007; 39(5): 631-637

13. Libioulle C, Louis E, Hansoul S, Sandor C and others. Novel Crohn Disease Locus Identified by Genome-Wide Association Maps to a Gene Desert on 5p13.1 and Modulates Expression of PTGER4. *PLoS Genetics* 2007; 3(4): e58

14. Scott LJ, Mohlke KL, Bonnycastle LL, Willer CJ and others. A Genome-Wide Association Study of Type 2 Diabetes in Finns Detects Multiple Susceptibility Variants. *Science* 2007; 316 1341-1345

15. Diabetes Genetics Initiative of Broad Institute of Harvard and MIT, Lund University and Novartis Institutes for BioMedical Research, Saxena R, Voight BF and others. Genome-Wide Association Analysis Identifies Loci for Type 2 Diabetes and Triglyceride Levels. *Science* 2007; 316(5829): 1331-1336

16. Sladek R, Rocheleau G, Rung J, Dina C and others. A Genome-Wide Association Study Identifies Novel Risk Loci for Type 2 Diabetes. *Nature* 2007; 445(7130): 881-885

17. Easton DF, Pooley KA, Dunning AM, Pharoah PDP and others. Genome-Wide Association Study Identifies Novel Breast Cancer Susceptibility Loci. *Nature* 2007; advanced online publication

18. Chanock SJ, Manolio T, Boehnke M, Boerwinkle E and others. Replicating Genotype-Phenotype Associations. *Nature* 2007; 447(7145): 655-660

19. Cardon LR and Bell JI. Association Study Designs for Complex Diseases. *Nat. Rev. Genet.* 2001; 2(2): 91-99

20. Zeggini E, Weedon MN, Lindgren CM, Frayling TM and others. Replication of Genome-Wide Association Signals in UK Samples Reveals Risk Loci for Type 2 Diabetes. *Science* 2007; 316(5829): 1336-1341

21. Scott LJ, Mohlke KL, Bonnycastle LL, Willer CJ and others. A Genome-Wide Association Study of Type 2 Diabetes in Finns Detects Multiple Susceptibility Variants. *Science* 2007; 316 1341-1345

22. Diabetes Genetics Initiative of Broad Institute of Harvard and MIT, Lund University and Novartis Institutes for BioMedical Research, Saxena R, Voight BF and others. Genome-Wide Association Analysis Identifies Loci for Type 2 Diabetes and Triglyceride Levels. *Science* 2007; 316(5829): 1331-1336

23. Satagopan JM, Venkatraman ES, and Begg CB. Two-Stage Designs for Gene-Disease Association Studies With Sample Size Constraints. *Biometrics* 2004; 60(3): 589-597

24. Skol AD, Scott LJ, Abecasis GR, and Boehnke M. Joint Analysis Is More Efficient Than Replication-Based Analysis for Two-Stage Genome-Wide Association Studies. *Nat Genet* 2006; 38(2): 209-213

25. Roeder K, Bacanu SA, Wasserman L, and Devlin B. Using Linkage Genome Scans to Improve Power of Association in Genome Scans. *The American Journal of Human Genetics* 2006; 78(2): 243-252

26. Chen GK and Witte JS. Enriching the Analysis of Genomewide Association Studies With Hierarchical Modeling. *Am J Hum Genet* 2007; 81(2):

27. Satagopan JM and Elston RC. Optimal Two-Stage Genotyping in Population-Based Association Studies. *Genet Epidemiol.* 2003; 25(2): 149-157

28. Freidlin B, Zheng G, Li Z, and Gastwirth JL. Trend Tests for Case-Control Studies of Genetic Markers: Power, Sample Size and Robustness. *Hum Hered.* 2002; 53(3): 146-152

29. Ng PC and Henikoff S. Predicting the Effects of Amino Acid Substitutions on Protein Function. *Annu. Rev. Genomics Hum. Genet.* 2006; 7 61-80

30. Ng PC and Henikoff S. SIFT: Predicting Amino Acid Changes That Affect Protein Function. *Nucleic Acids Res.* 2003; 31(13): 3812-3814

31. Ng PC and Henikoff S. Accounting for Human Polymorphisms Predicted to Affect Protein Function. *Genome Res.* 2002; 12(3): 436-446

32. Ng PC and Henikoff S. Predicting Deleterious Amino Acid Substitutions. *Genome Res.* 2001; 11(5): 863-874

33. Riedemann L, Lanvers C, Deuster D, Peters U and others. Megalin Genetic Polymorphisms and Individual Sensitivity to the Ototoxic Effect of Cisplatin. *Pharmacogenomics. J.* 2008; 8(1): 23-28

34. Kantarci S, Al-Gazali L, Hill RS, Donnai D and others. Mutations in LRP2, Which Encodes the Multiligand Receptor Megalin, Cause Donnai-Barrow and Facio-Oculo-Acoustico-Renal Syndromes. *Nat. Genet.* 2007; 39(8): 957-959

CHAPTER 7

WHOLE GENOME ASSOCIATION STUDY OF RESPONSE TO CITALOPRAM

7.1 Introduction

While candidate gene study designs are often utilized in the investigation of complex diseases, having *a prioi* knowledge of the causative (or even likely causative) candidate genes is often difficult for most phenotypes. Indeed, identification of *new* genes is often the driving force behind complex disease studies. Genome-wide linkage studies have been performed for years, but as discussed in Chapter 1, logistical difficulties arise from collecting families for pharmacogenetic studies. Recent advances in SNP genotyping technology and reduction in costs have made whole genome association (WGA) studies entirely feasible (1). The first reports of WGA studies have appeared in the literature and there have been some great successes such as the CFH gene and macular degeneration (2), however, complexities and questions remain regarding the optimal analysis of WGA data (3).

The obvious strength of being able to assay most of the genes in the human genome is tempered by concerns about multiple testing penalties, population stratification, and the apparent non-replication of many smaller candidate gene association studies. Despite these methodological considerations, large WGA studies are currently progressing with studies such as the Wellcome Trust Case Control Consortium (WTCCC), which genotyped over 16,000 subjects with various common diseases as well as 3,000 control samples, and the Genetic Association Information Network (GAIN) who

are investigating a number of complex disorders. Both of these projects are consortium-based and have pledged to make their raw genotype and clinical data publicly available shortly after it is generated, allowing other investigators to apply different analytical techniques.

We undertook conducting a WGA study using a subset of the STAR*D patient population for which DNA samples were collected, which consists of over 1,900 depressed subjects taking the SSRI citalopram. In order to limit Type I error and reduce overall genotyping costs, we used a two-stage study design (4). In the first stage, we genotyped approximately half of the sample (discovery set, N=964) for 500,567 SNPs distributed across the human genome. The most highly associated SNPs were then genotyped in the second half of the sample (validation set, N=975) to asses if they would replicate the initial association.

Here we report the initial analysis of the WGA data, including descriptions of the genotype data manipulation and quality control checks. Single locus SNP association results are reported for response, specificity of response, remission, and intolerance phenotypes. We analyzed the data using the Cochran-Mantel-Haenszel test to compare across self-reported ethnic strata. While none of the putative associations investigated in the second stage replicated their strong initial associations, as discussed below, this is a very preliminary analysis involving the “lowest hanging fruit” and as such broader, more comprehensive genotyping in the validation sample set needs to be performed.

7.2 Materials and Methods

7.2.1 STAR*D Study Population

The study population consisted of the subjects who consented to give DNA from the STAR*D antidepressant trial, as reviewed elsewhere (see Chapter 3). Within each ethnic group, and gender, we randomly split our subjects *a priori* into a discovery and validation sample set (Table 7.1). The entire discovery and validation sets were genotyped using the WGA platforms. Due to heterogeneity within our self-identified "White" subject, as uncovered via the *structure* analysis described below, all analyses were split into three racial subgroups: White, non-Hispanic; White, Hispanic; and African American. Other self-reported race classes were not analyzed.

7.2.2 Genotyping

The Affymetrix 500K array (5) was used (N=500,567 successful SNPs) to genotype the discovery set, and genotypes were scored using Affymetrix's BRLMM algorithm (6). In addition, the Affymetrix 5.0 array (5) was used (N=500,567 successful SNPs) to genotype the validation set, and genotypes were scored using Affymetrix's BRLMM-P algorithm. Twelve samples were genotyped on both platforms, concordance rate of 99.29%, to ensure compatibility of genotype information between platforms.

	Discovery Set (N = 964)			Validation Set (N = 975)		
	Non-Hispanic Caucasian	*Hispanic Caucasian*	*African American*	*Non-Hispanic Caucasian*	*Hispanic Caucasian*	*African American*
Remitter	334	49	61	256	39	37
Non-Responder	237	59	71	177	34	48
Unclassified	62	13	16	190	50	58
Responder *	396	62	77	292	49	50
Non-Responder *	237	59	71	177	34	48
Unclassified *	2	0	0	154	40	45
Specific Repsonder	286	46	48	197	30	28
Non-Responder	237	59	71	177	34	48
Unclassified	110	16	29	249	59	67
Tolerant	503	91	104	413	61	69
Intolerant	52	7	9	97	25	12
Unclassified	78	23	35	113	37	62

Table 7.1: Sample sizes for the discovery and validation sample sets. Shown are the number of subjects with each phenotype classification for the remission vs. non-responder (first) comparison, the responder vs. non-responder (second) comparison, the specific-responder vs. non-responder (third) comparison, and the tolerant vs. intolerant (last) comparison. Subjects are divided into discovery and validation sets, and further subdivided by self reported race. Unclassified refers to subjects who did not meet our classification criteria. For the intolerance phenotype, the unclassified subjects did not meet the STAR*D algorithm for intolerance or tolerance and were classified as probably intolerant and probably tolerance, these subjects not used in our analyses. * This response phenotype used a 42 day time window as a criterion for determining response.

7.2.3 WGA Quality Control

Raw data files were transferred to us by Affymetrix for the discovery set and generated “in-house” for the validation set. Genotypes were generated using the BRLMM and BRLMM-P algorithms for the discovery and validation sets, respectively. This data was imported along with phenotype data into Progeny 7.0 software. Data was then output in standard linkage format using Progeny and analyzed using PLINK and STATA-MP version 9.

We removed samples from the data for two reasons: low sample call rates (<95%) and samples with self-reported ancestry not falling into the 3 main ethnic groups. Of the 500,568 SNPs that were successfully genotyped, we removed SNPS that: had no chromosomal annotation in dbSNP (121), were duplicated within the panel (1), were on the X chromosome for ease of analysis (10,525), had a call rate less that 95% within the sample set, and had minor allele frequencies less than 0.1% across the sample set. The remaining SNPs were used in the analyses described below. In order to be tested for association to citalopram response phenotypes, SNPs were required to conform to Hardy-Weinberg equilibrium (HWE). SNPs were tested for departure from HWE within each of the 3 racial subgroups and markers with a significant departure from HWE ($p < 0.000001$) in two of the three racial subgroups were excluded from association analysis.

SNP quality control and descriptive statistics were generated using custom files (“WGA_STARD_QC.do”, “MAF_STARD.do”, “WGA_Spacing.do”, written by Jeffrey Kraft) and executed in STATA-MP version 9 or from the program PLINK (7).

7.2.4 Linkage Disequilibrium Analysis

In order to assay the amount of redundancy in the SNP genotype data, SNP binning based on pairwise r^2 values was performed. Within each racial subgroup (White, non-Hispanic, White Hispanic, and African American), pairwise r^2 for each SNP with all other SNPs within a 1 megabase sliding window was calculated. This was process repeated for all the SNPs in the WGA panel. Data was then compiled, any redundancy was removed, and number of proxy SNPs at several r^2 thresholds was calculated. This was performed using "matrix of pairwise LD" function within PLINK and custom scripts, written by Jeffrey Kraft, and executed in STATA-MP version 9.

7.2.5 *Structure* Analysis

In order to assess the levels of genetic heterogeneity in the sample, the MCMC method of Pritchard et al was performed, as implemented in *structure* version 2.0 (8). Using the entire STAR*D sample set and 2,500 random SNPs from the WGA data from across the genome, the algorithm was run using 10,000 burn-ins followed by 100,000 iterations. Several runs were performed assuming from 1 to 4 underlying subpopulations ("K"), and results for each "K" were stable in terms of estimates of alpha, Fst, and proportion ancestry ("Q") for each individual, indicating the algorithm had not inadvertently settled at a local maximum.

7.2.6 Single Locus Analysis

In this report, all association analyses were carried out by stratifying by self-reported ethnicity and then comparing across ethnic strata. We investigated four clinical phenotypes, citalopram response, specificity of response to citalopram, citalopram remission, and intolerance to citalopram. All of these phenotypes have been described in

detail previously for this study population (see section 3.2.7). We used the software PLINK to test each SNP in the WGA panel for association to the four phenotypes within the three ethnic subgroups in each of the subsets (discovery and validation). Only SNPs that passed the QC filters described above were used in the association analysis although these thresholds were applied independently to the discovery and validations subsets resulting in two unique groups of SNPs. We used an allelic model and the Cochran-Mantel-Haenszel (CMH) test to investigate single locus associations across ethnic strata. Dominant (minor allele carrier versus non-carrier) odds ratios for each SNP were also calculated.

We sought to only investigate the most highly associated SNPs within each phenotype. Our strategy was to rank order the p-values using the allelic model in the discovery set, then take the most highly associated 10 SNPs in each phenotype to follow up using the validation set. We then required these follow-up SNPs to have a significance of $p < 0.05$ in the validation sample set in order to declare study-wide significance.

For our post-hoc one-stage analysis, we combined the discovery and validation sets and rank ordered the p values, reporting the top 10 markers for each phenotypic comparison.

7.2.7 SNP x SNP Interaction Analysis

The 10 SNPs with the lowest p-values from the single loci analyses in the entire STAR*D sample were modeled for pair-wise SNP interactions using the epistasis function of PLINK. The epistasis test uses logistic regression and makes a model based on allele dosage for each SNP, A and B, and fits the model: $Y = b0 + b1.A + b2.B + b3.AB + e$, where the test for interaction is based on the coefficient b3. Dominant (minor

allele carrier versus non-carrier) odds ratios for each SNP x SNP interaction were also calculated. These analyses were done on the entire STAR*D sample.

7.3 Results

7.3.1 WGA Data Descriptions and QC

Using our QC filters (described in detail within section 7.2.3) a total of 469,170 SNPs passed in our discovery sample, 435,512 SNPs in our validation set, and 445,260 SNPs in the entire STAR*D sample. Overall, these SNPs had a very high call rate (mean 99.1%).

SNPs were not uniformly distributed across the entire genome. Large gaps exist in centromeric and telomeric regions of some chromosomes. This is due to technical difficulties that arise from assaying those regions which are abundant in repetitive DNA sequences. On average there was a marker every 5.7kb (median 2.5kb), however assay coverage was variable with coverage on the X chromosome markedly lower and no markers present on the Y chromosome (Figure 7.1). As seen in Figure 7.2, the distribution of intermarker distances is largely skewed due to a small number of very large gaps between SNPs (not shown).

Marker minor allele distribution varied between racial subgroups. The Non-Hispanic Caucasian subgroup had a lower average minor allele frequency than either Hispanic Caucasians or the African American subgroup (0.20 / 0.21 / 0.22, respectively), and had fewer SNPs with a minor allele frequency greater than 5% as well (348,467 / 359,487 / 397,909, respectively, see Figure 7.2).

7.3.2 Assessment of Population Structure

As discussed previously, population stratification can lead to confounding in case-control association studies. We ran a *structure* analysis, described in detail in Chapter 1.4.3, on the discovery and validation sample set using 2,500 random SNPs from across the genome (Figure 7.3). The results indicated that a model with 3 genetic subgroups (i.e., K=3) was the best fit for the data. A clear distinction was seen between the self-reported African American and White samples, and the third genetic subpopulation correlated well with Hispanic ancestry.

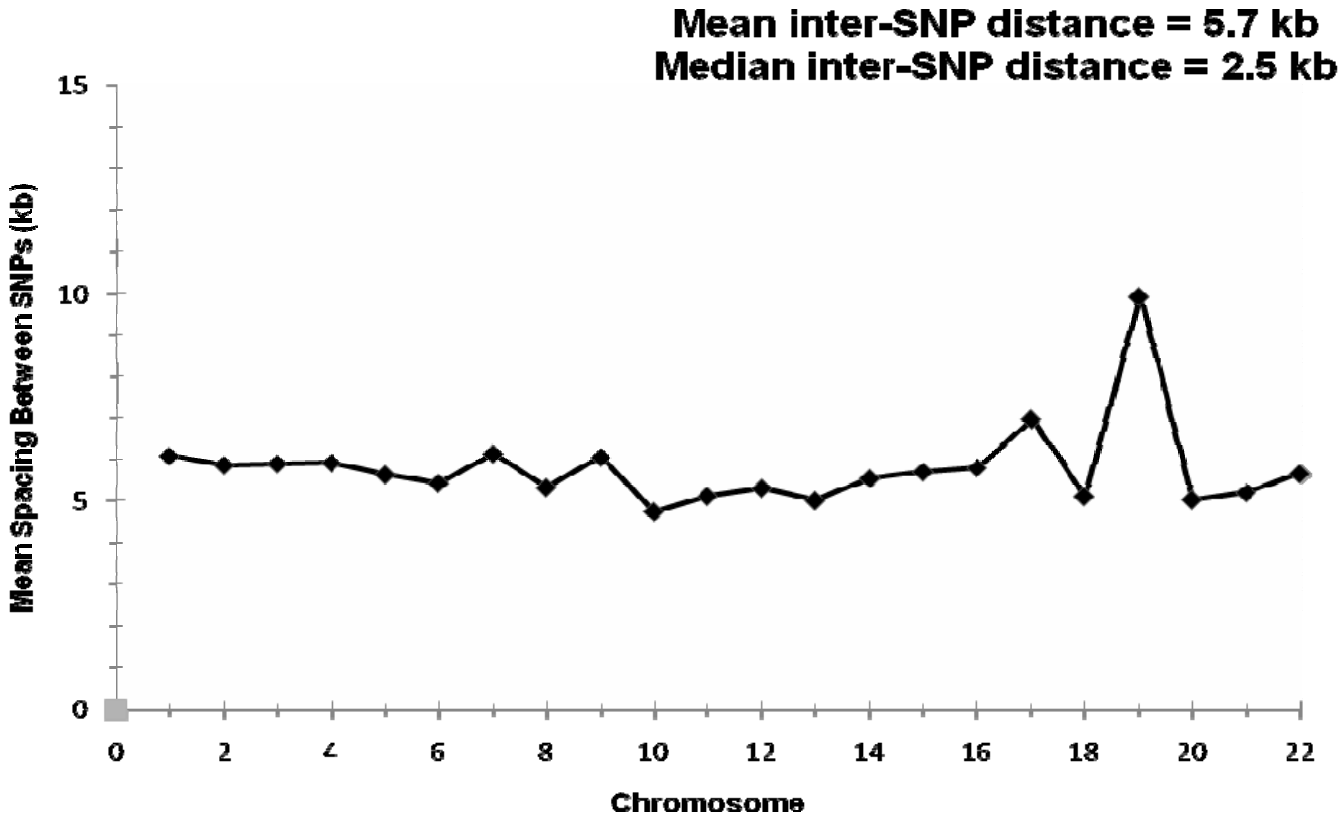


Figure 7.1: Figure showing mean inter-SNP differences broken down by chromosome. Average inter-SNP distance across all 22 autosomes was approximately 5.7 kb while the median inter-SNP distance was much smaller at about 2.5 kb

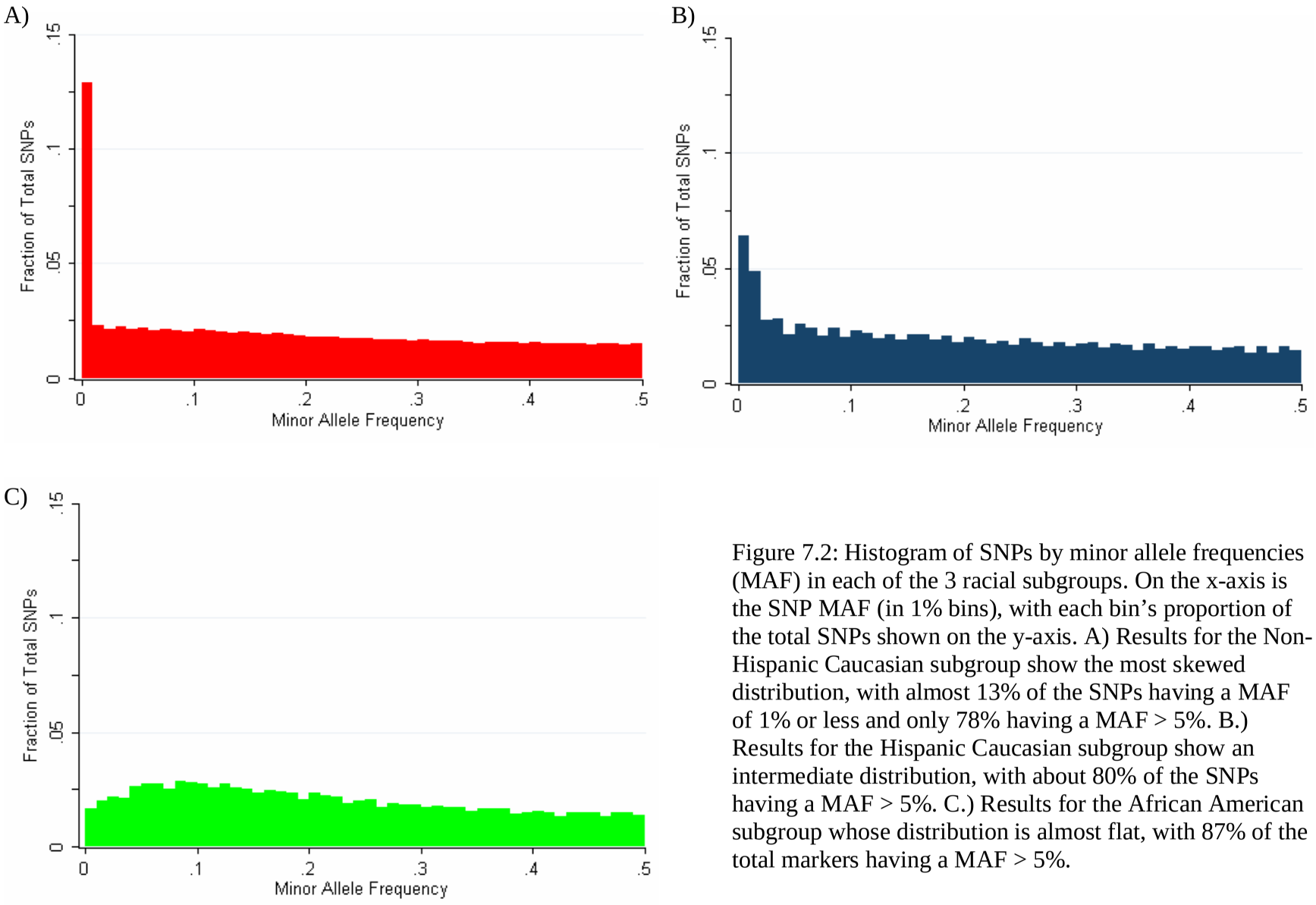


Figure 7.2: Histogram of SNPs by minor allele frequencies (MAF) in each of the 3 racial subgroups. On the x-axis is the SNP MAF (in 1% bins), with each bin's proportion of the total SNPs shown on the y-axis. A) Results for the Non-Hispanic Caucasian subgroup show the most skewed distribution, with almost 13% of the SNPs having a MAF of 1% or less and only 78% having a MAF > 5%. B.) Results for the Hispanic Caucasian subgroup show an intermediate distribution, with about 80% of the SNPs having a MAF > 5%. C.) Results for the African American subgroup whose distribution is almost flat, with 87% of the total markers having a MAF > 5%.

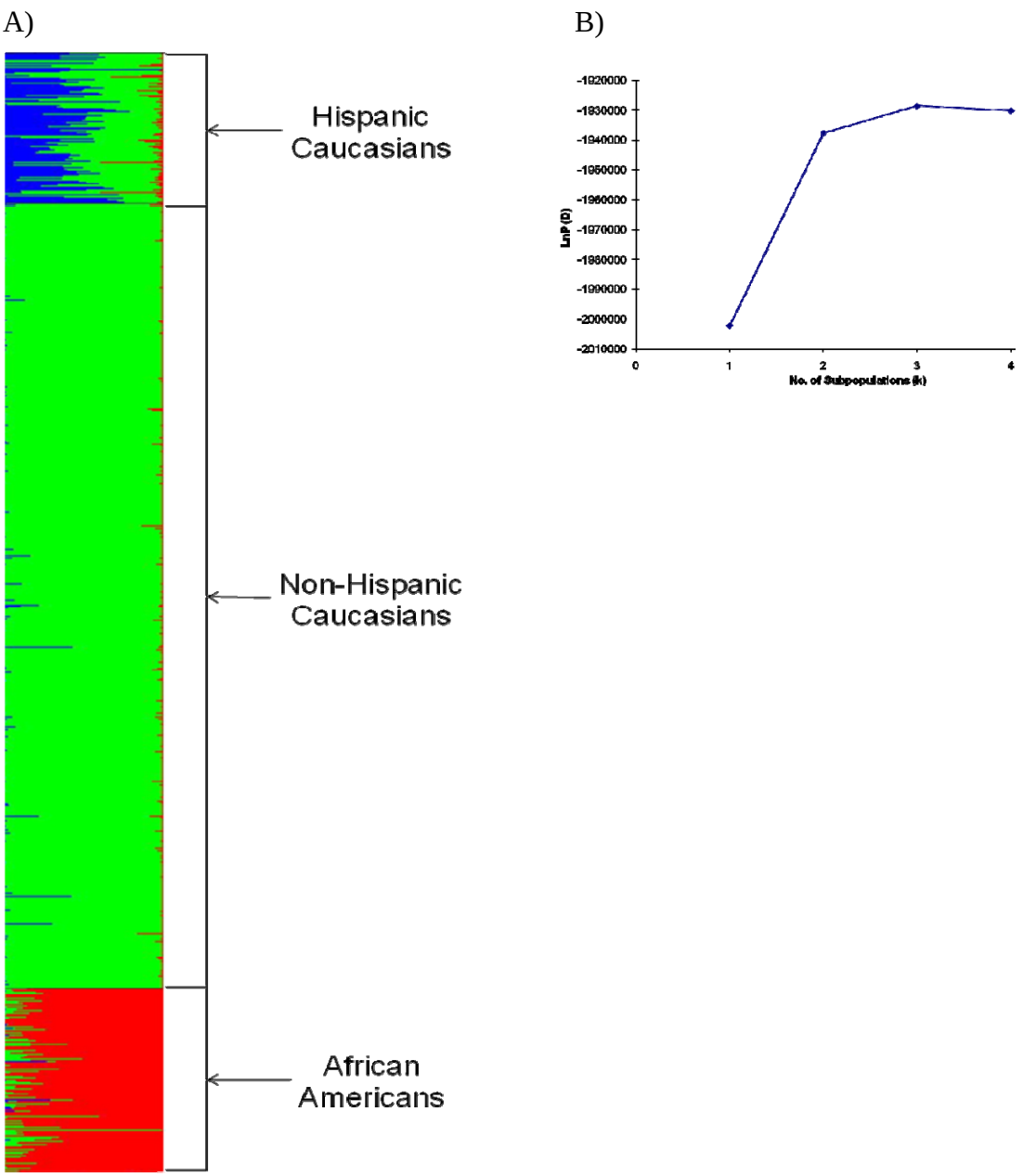


Figure 7.3: Results of *structure* analysis using 2,500 random SNPs in the entire STAR*D sample set. A) Plot shows the percent identity (Q) from the 3 subpopulations for each subject. Subjects are ordered based on self-reported race, as shown to the right of the *structure* plot. B) Graph of the posterior probability of the model at various numbers of historical subpopulations (K). The addition of a 4th subpopulation does not significantly strengthen the model fit, thus K=3 was used.

7.3.3 Linkage Disequilibrium Analysis

Given the marker density in this study, there was a great amount of LD between the SNPs on our WGA panels, as would be expected. Also, as expected based on population history, the African American subgroup showed less LD on average than the Non-Hispanic Caucasian subgroup and the Hispanic Caucasians displayed an intermediate amount of LD comparatively (Figures 7.4-7.6).

In order to get a sense of the redundancy of the genotype data, which is an important consideration in determining the number of independent tests performed, we ran an r^2 threshold binning approach on the SNPs in the WGA panel. Using a sliding window of 1 million base pairs around the target SNP, the number of proxy SNPs was determined using various thresholds of r^2. These analyses revealed significant redundancy in the SNPs genotyped. In the Non-Hispanic Caucasian subgroup, using an $r^2 = 1.0$ threshold, which means the genotype of one SNP perfectly predicts the genotype of another SNP in all cases, 10% of the SNPs have at least one perfect proxy. At a reduced, but still conservative, threshold of $r^2 = 0.80$ (common threshold for selecting tagSNPs), nearly 60% of the SNPs have at least one good proxy in the dataset (Figure 7.4). Fewer than 200,000 SNPs were correlated at the 0.80 level with no other SNP. In the Hispanic Caucasian and African American subgroups, redundancy was still high, though, as expected, at a level less than in the Non-Hispanic Caucasians (Figures 7.5 & 7.6).

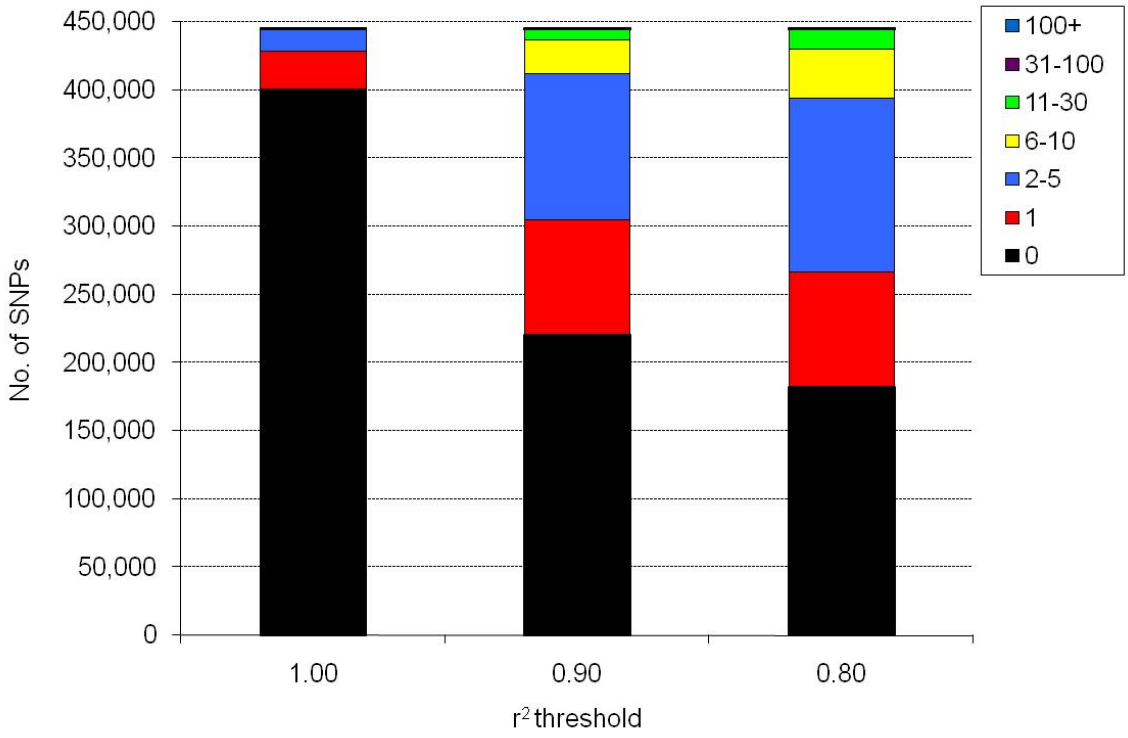


Figure 7.4: Amount of LD and redundancy of SNPs at various r^2 thresholds in the Non-Hispanic Caucasian racial subgroup. All SNPs are shown on the y-axis. Using a sliding window of 1Mb around the target SNP, the number of proxy SNPs (SNPs in LD above the threshold) for each SNP was determined using an r^2 threshold of 1, 0.90 and 0.80 (x-axis).

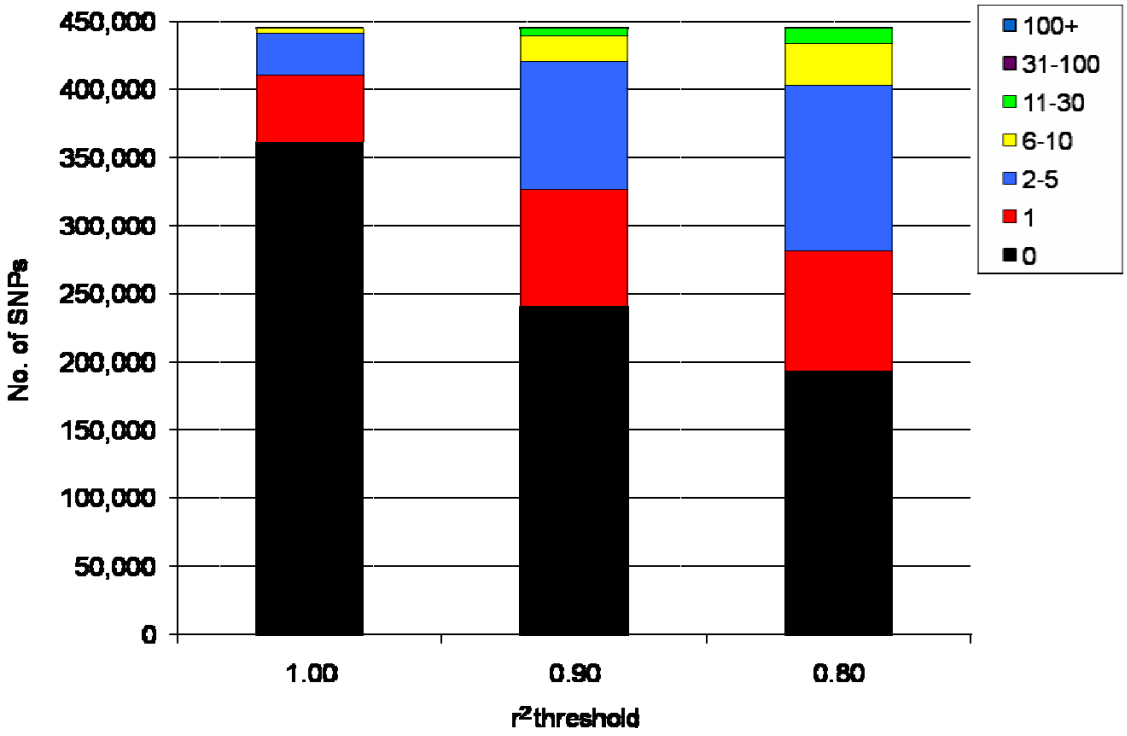


Figure 7.5: Amount of LD and redundancy of SNPs at various r^2 thresholds in the Hispanic Caucasian racial subgroup. All SNPs are shown on the y-axis. Using a sliding window of 1Mb around the target SNP, the number of proxy SNPs (SNPs in LD above the threshold) for each SNP was determined using an r^2 threshold of 1, 0.90 and 0.80 (x-axis).

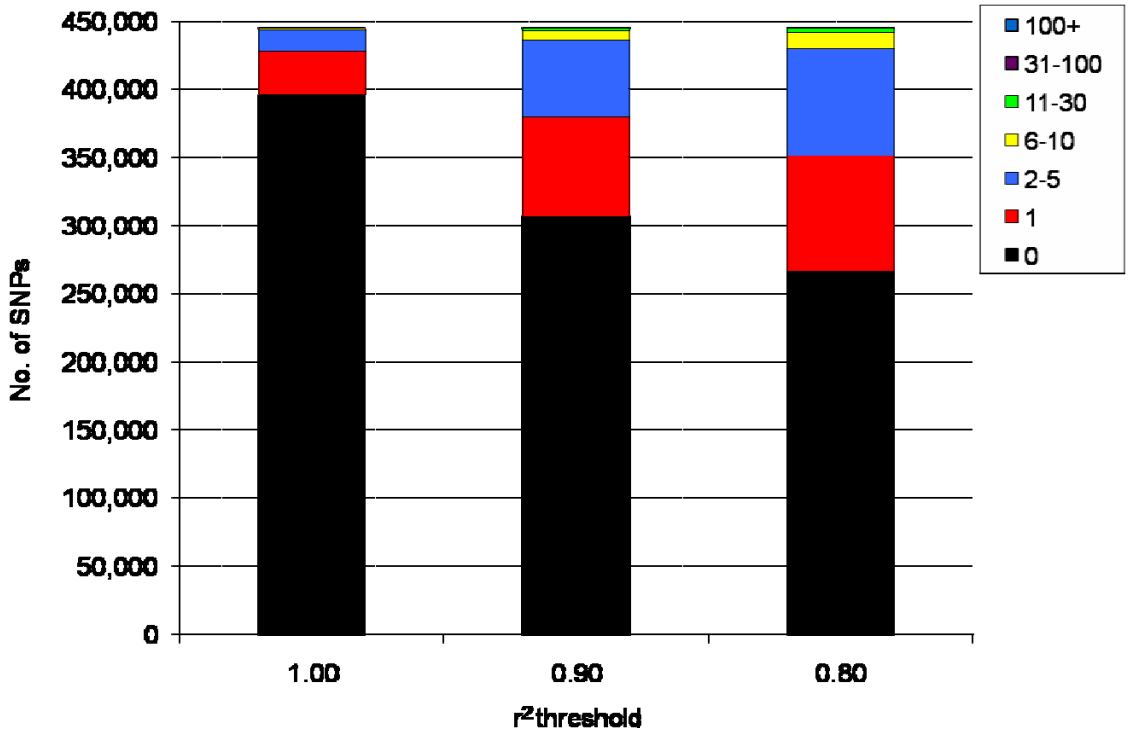


Figure 7.6: Amount of LD and redundancy of SNPs at various r^2 thresholds in the African American racial subgroup. All SNPs are shown on the y-axis. Using a sliding window of 1Mb around the target SNP, the number of proxy SNPs (SNPs in LD above the threshold) for each SNP was determined using an r^2 threshold of 1, 0.90 and 0.80 (x-axis).

7.3.4 Single Locus Association – Discovery Set

We performed four phenotypic comparisons in our subjects as described in detail previously. The results for all the SNPs across the genome for the four phenotypes (remission, response, specificity of response, and tolerance) are shown in Figures 7.7 thru 7.10, respectively. One marker (SNP_A-2024840) was significant in the remission phenotype using a Bonferroni corrected threshold with a significance of 1.01×10^{-7}. Four SNPs (SNP_A-1843477, SNP_A-2242408, SNP_A-1837056, and SNP_A-1842826) showed significance with the tolerance phenotype at levels exceeding a Bonferroni corrected threshold ($p < 1.07 \times 10^{-7}$). For the response and specificity of response phenotypes no markers were significant at the Bonferroni corrected threshold.

The significance values and dominant odds ratios for the top 10 rank ordered SNPs in the discovery set for each of the four phenotypes are listed in Table 7.2. quantile-quantile (QQ) plots for the remission, response, and specificity of response phenotypic comparisons showed no gross inflation of the chi-squared statistics (Figure 7.11A-7.13A). The tolerance phenotype showed systematic inflation which can be indicative of confounding due to population stratification or other factors (Figure 7.14A). Most likely, this is due to the small numbers of genotype counts that accompany this rare phenotype.

7.3.5 Single Locus Association – Validation Set

We genotyped the validation set using the Affymetrix 5.0 platform containing the same 500,568 SNPs as mentioned previously and then looked for replication of the top 10 SNPs from the discovery set in the second phase of the study. For the tolerance phenotype, one SNP (SNP_A-2139836) replicated with a significance value of 0.018. For the remission, response and specificity of response phenotypes, none of the top 10 SNPs

yielded a significant association in the validation sample ($p<0.05$), using the same genotypic model and data coding format as in the discovery set analysis (Table 7.3). Several of the SNPs selected for replication from the discovery set did not meet the QC standards in the validation set and therefore association results are not presented for these few SNPs.

7.3.6 Single Locus Association – Entire STAR*D Sample

Our results from our two stage design were largely negative with only one SNP showing replicated association (in a fairly rare phenotype), therefore, we attempted to analyze our data, post-hoc, as a one-stage study to maximize the power of our clinical sample. This analysis was only possible due to the dense marker genotyping on both sets of STAR*D (discovery and validation). We combined the discovery and validation set genotypes and rank ordered the p values in the combined sample.

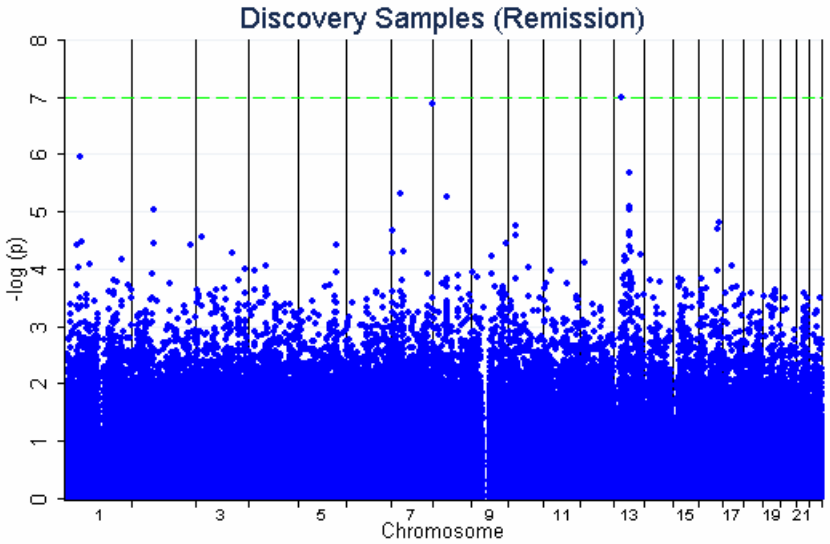
No markers showed association with any of the phenotypic comparisons at levels exceeding a Bonferroni corrected threshold ($p < 1.12x10^{-7}$). The significance values and dominant odds ratios for the top 10 rank ordered SNPs in the entire STAR*D sample for each of the four phenotypes are listed in Table 7.4 and the quantile-quantile (QQ) plots for these phenotypic comparisons showed no gross inflation of the chi-squared statistics with the exception of the tolerance phenotype as seen previously (Figure 7.11B-7.14B).

Our genome-wide results were unable to verify and replicate previously reported associations in the literature with the candidate genes SLC6A4, TPH2, FKBP5, ACE, or GNB3. We did find associations ($p < .01$) with SNPs in HTR2A & GRIK4, which is consistent with reports from the McMahon group working on the STAR*D sample , but we don't consider them significant due to the number of tests performed (Table 7.5).

7.3.7 SNP x SNP Interaction Analysis – Entire STAR*D Sample

The results from our pair-wise SNP interaction analysis were largely negative. All unique pair-wise combinations of the top 10 SNPs for each phenotypic comparison were tested for the associated phenotype. The interaction analysis for the remission phenotype showed only one of the 45 unique interactions had significance $p < 0.05$. The interaction analyses for response and specificity of response phenotypes also showed only one interaction with significance less than 0.05. The interaction analysis for the tolerant phenotype was able to be calculated for only two pair-wise interactions (both non-significant) due to the rarity of this phenotype (i.e. for most pair-wise combinations of SNPs, there were not cases and controls who had the rare allele of both SNPs). Despite the 3 positive interactions, one in each of 3 phenotypes, these results are in line with what would be expected by chance due to the large number of tests and do not demonstrate that significant interaction exists between the most highly associated markers in each phenotype.

A)



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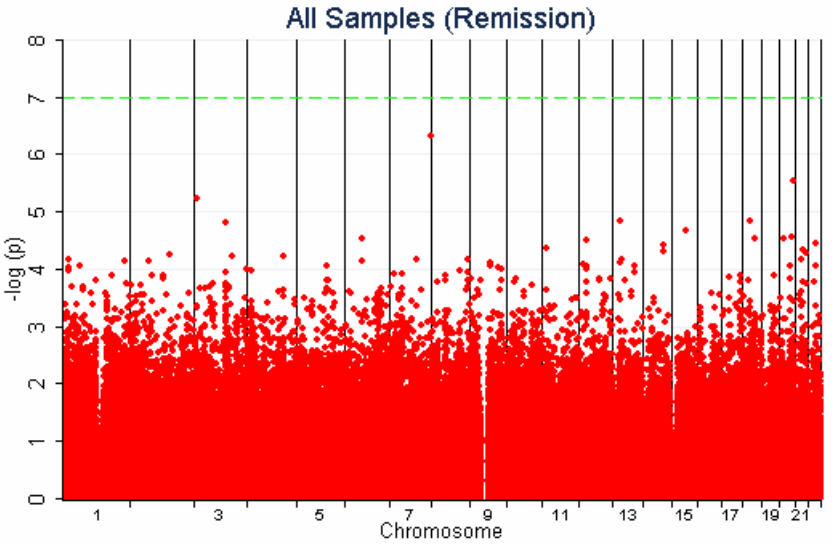
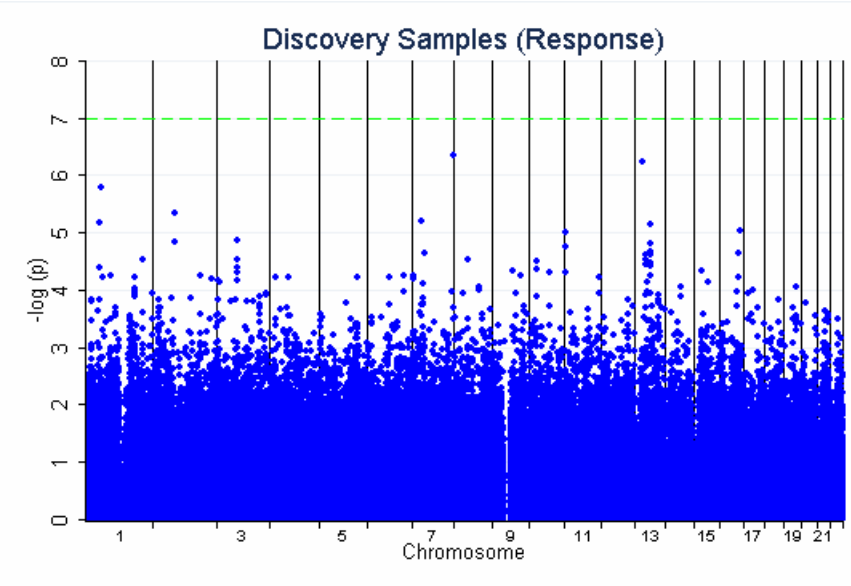


Figure 7.7: Association results of all SNPs with remission phenotype. Each point represents a single marker with chromosomal position (on the x-axis) plotted against the –log of the p value (y-axis). The higher the point resides on the graph, the smaller the p value of the SNP (e.g., -log (p) = 4 when p = 0.0001). The green horizontal dotted line represents the threshold for Bonferroni corrected significance ($1x10^{-7}$). A) Analysis using only the discovery set of STAR*D (blue points). B) Analysis using the entire STAR*D sample (red points).

A)



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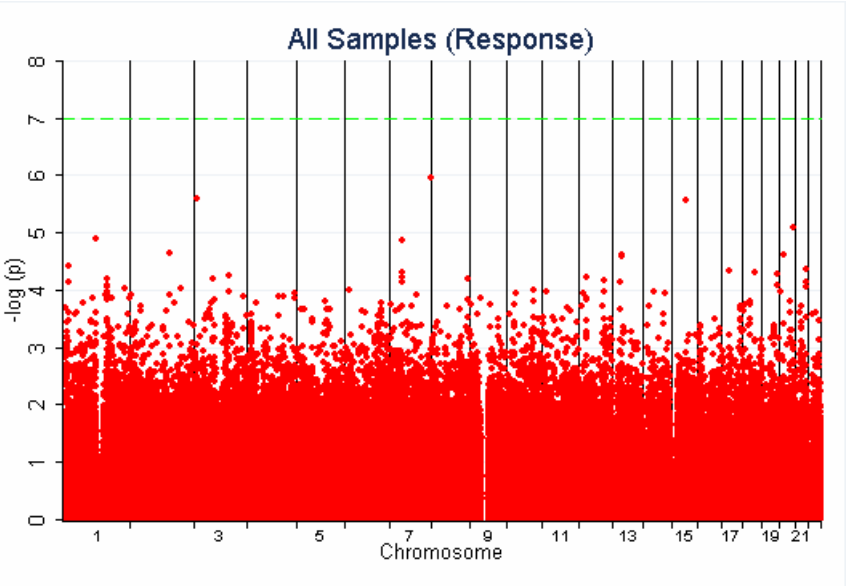
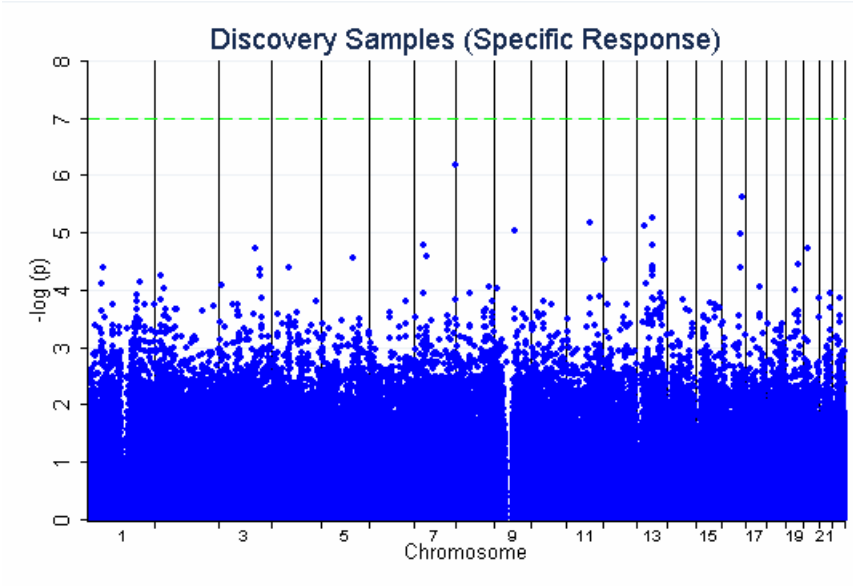


Figure 7.8: Association results of all SNPs with response phenotype. Each point represents a single marker with chromosomal position (on the x-axis) plotted against the –log of the p value (y-axis). The higher the point resides on the graph, the smaller the p value of the SNP (e.g., -log (p) = 4 when p = 0.0001). The green horizontal dotted line represents the threshold for Bonferroni corrected significance ($1x10^{-7}$). A) Analysis using only the discovery set of STAR*D (blue points). B) Analysis using the entire STAR*D sample (red points).

A)



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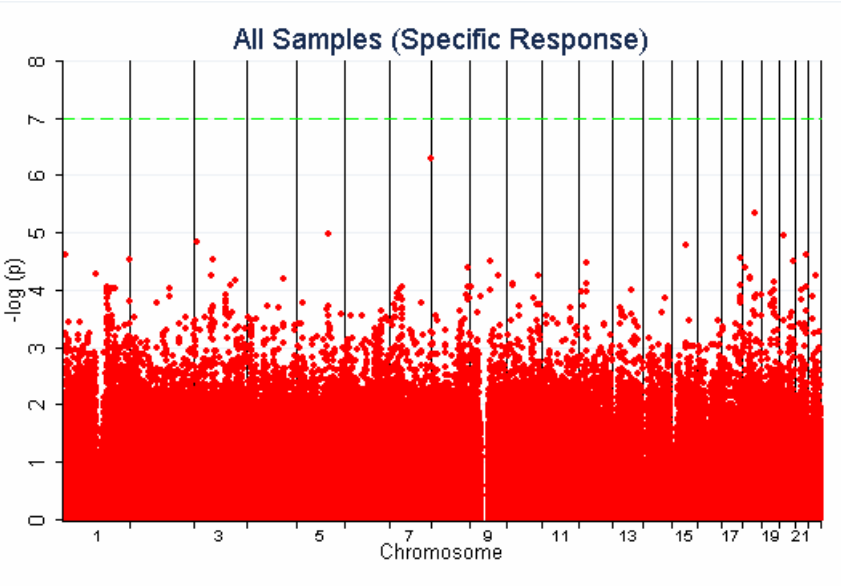
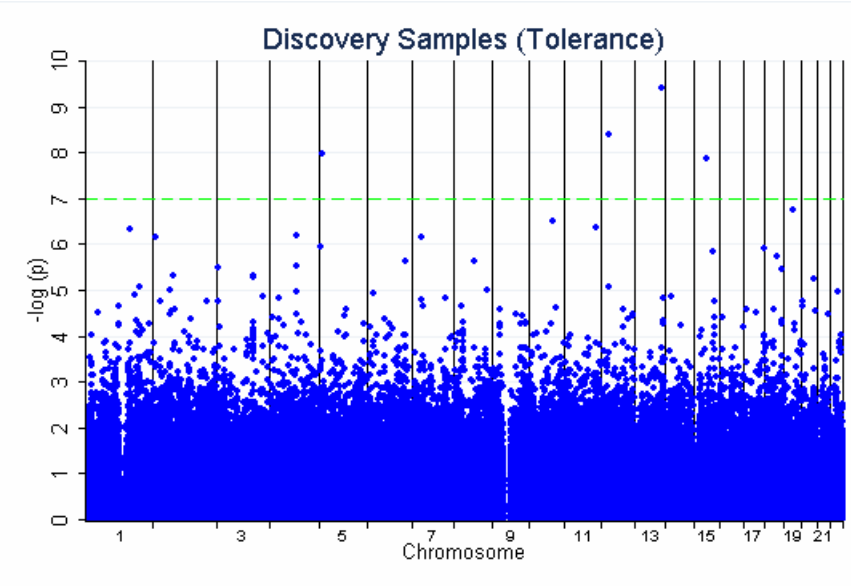


Figure 7.9: Association results of all SNPs with specificity of response phenotype. Each point represents a single marker with chromosomal position (on the x-axis) plotted against the –log of the p value (y-axis). The higher the point resides on the graph, the smaller the p value of the SNP (e.g., -log (p) = 4 when p = 0.0001). The green horizontal dotted line represents the threshold for Bonferroni corrected significance ($1x10^{-7}$). A) Analysis using only the discovery set of STAR*D (blue points). B) Analysis using the entire STAR*D sample (red points).

A)



B)

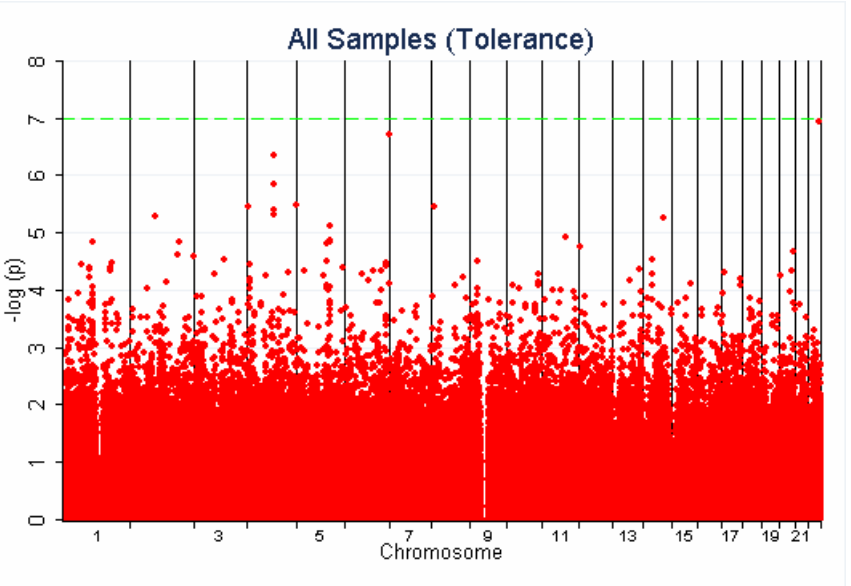
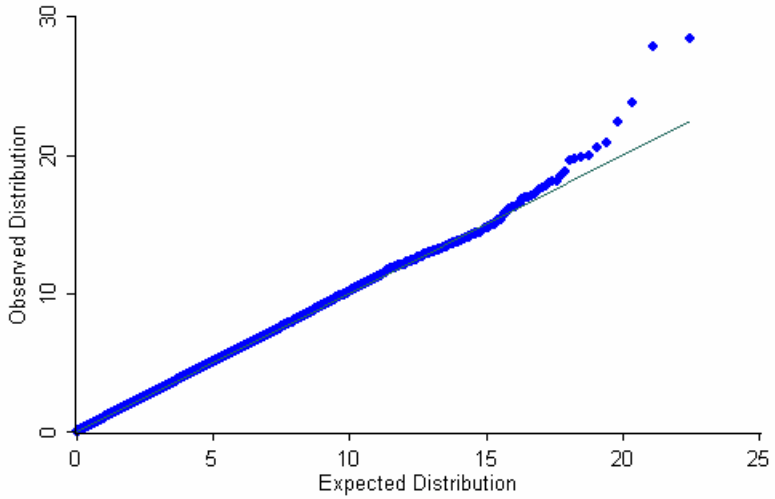


Figure 7.10: Association results of all SNPs with tolerant phenotype. Each point represents a single marker with chromosomal position (on the x-axis) plotted against the –log of the p value (y-axis). The higher the point resides on the graph, the smaller the p value of the SNP (e.g., -log (p) = 4 when p = 0.0001). The green horizontal dotted line represents the threshold for Bonferroni corrected significance ($1x10^{-7}$). A) Analysis using only the discovery set of STAR*D (blue points). B) Analysis using the entire STAR*D sample (red points).

Phenotype	SNP	Chr.	Allele 1	Allele 2	Discovery Set	
					P-Value	OR (95% CI)
remit	SNP_A-2024840	13	A	G	1.01E-07	0.5 (0.39 - 0.65)
remit	SNP_A-4218787	7	G	A	1.34E-07	1.97 (1.53 - 2.54)
remit	SNP_A-2042516	1	A	G	1.10E-06	2.07 (1.54 - 2.78)
remit	SNP_A-4238877	13	T	A	2.17E-06	1.92 (1.46 - 2.52)
remit	SNP_A-4266303	7	A	C	4.77E-06	1.6 (1.31 - 1.96)
remit	SNP_A-4230512	8	C	T	5.68E-06	3.05 (1.86 - 4.99)
remit	SNP_A-1789669	13	C	T	7.87E-06	1.81 (1.39 - 2.35)
remit	SNP_A-2141865	13	C	G	8.69E-06	1.8 (1.39 - 2.34)
remit	SNP_A-2256177	13	T	C	8.99E-06	1.8 (1.39 - 2.33)
remit	SNP_A-2149893	2	G	A	9.44E-06	0.27 (0.15 - 0.5)
resp42	SNP_A-4218787	7	G	A	4.52E-07	1.88 (1.47 - 2.4)
resp42	SNP_A-2024840	13	A	G	5.68E-07	0.54 (0.43 - 0.69)
resp42	SNP_A-2042516	1	A	G	1.62E-06	2 (1.51 - 2.67)
resp42	SNP_A-2149893	2	G	A	4.43E-06	0.29 (0.17 - 0.51)
resp42	SNP_A-4266303	7	A	C	6.35E-06	1.56 (1.29 - 1.9)
resp42	SNP_A-4227466	1	G	A	6.69E-06	0.57 (0.45 - 0.73)
resp42	SNP_A-4238877	13	T	A	7.23E-06	1.82 (1.4 - 2.37)
resp42	SNP_A-2057977	16	T	G	9.21E-06	0.65 (0.53 - 0.79)
resp42	SNP_A-2297908	11	T	C	9.94E-06	2.18 (1.53 - 3.1)
resp42	SNP_A-2128247	3	G	A	1.36E-05	1.55 (1.27 - 1.89)
spec_resp	SNP_A-4218787	7	G	A	6.68E-07	1.94 (1.49 - 2.53)
spec_resp	SNP_A-2057977	16	T	G	2.44E-06	0.61 (0.49 - 0.75)
spec_resp	SNP_A-4261350	13	G	A	5.41E-06	1.82 (1.4 - 2.36)
spec_resp	SNP_A-1915448	11	T	C	6.63E-06	0.6 (0.49 - 0.75)
spec_resp	SNP_A-2024840	13	A	G	7.78E-06	0.55 (0.42 - 0.72)
spec_resp	SNP_A-1996954	9	T	C	9.46E-06	1.85 (1.41 - 2.44)
spec_resp	SNP_A-4196344	16	C	G	1.06E-05	1.6 (1.3 - 1.98)
spec_resp	SNP_A-4266303	7	A	C	1.63E-05	1.59 (1.29 - 1.95)
spec_resp	SNP_A-4238877	13	T	A	1.68E-05	1.85 (1.39 - 2.45)
spec_resp	SNP_A-2007509	20	T	C	1.84E-05	1.59 (1.28 - 1.96)
tolerant	SNP_A-1843477	13	A	G	4.04E-10	0.07 (0.02 - 0.19)
tolerant	SNP_A-2242408	12	A	G	3.96E-09	N/A
tolerant	SNP_A-1837056	5	A	G	1.03E-08	0.08 (0.03 - 0.22)
tolerant	SNP_A-1842826	15	T	C	1.32E-08	N/A
tolerant	SNP_A-2288013	19	C	T	1.76E-07	0.09 (0.03 - 0.28)
tolerant	SNP_A-4195753	10	C	A	3.15E-07	0.05 (0.01 - 0.24)
tolerant	SNP_A-2095694	11	A	C	4.19E-07	0.03 (0 - 0.27)
tolerant	SNP_A-1943222	1	A	G	4.68E-07	0.2 (0.11 - 0.38)
tolerant	SNP_A-2139836	4	A	G	6.26E-07	0.06 (0.02 - 0.25)
tolerant	SNP_A-1962163	2	T	A	7.05E-07	0.02 (0 - 0.25)

Table 7.2: List of top 10 associated SNPs in the discovery set from each of the four phenotypic comparisons (remit, resp42, spec_resp, & tolerant). “N/A” as the odds ratio indicates the dominant odds ratio cannot be calculated.

A)



B)

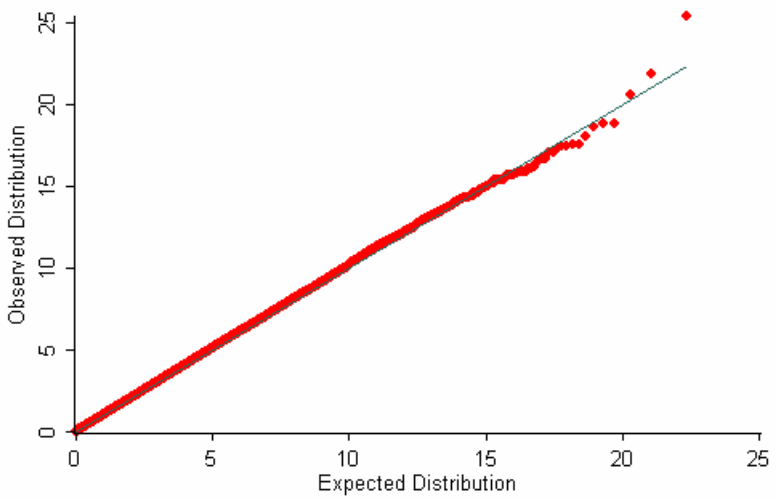
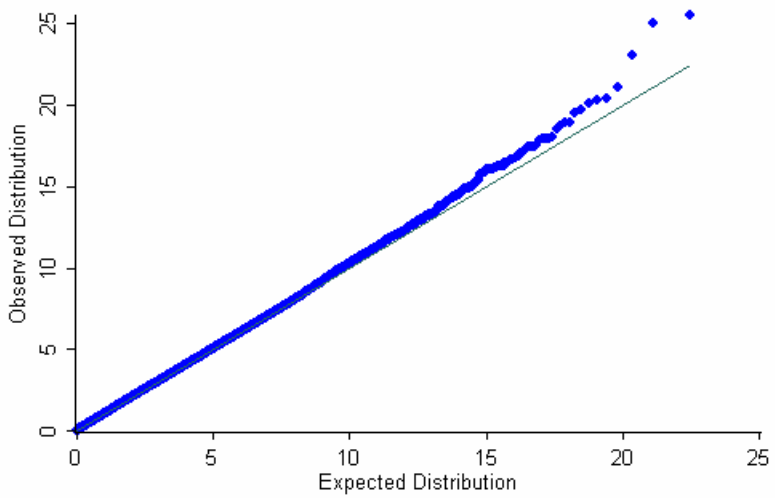


Figure 7.11: Quantile-quantile (QQ) plot showing the expected distribution of chi-squared statistics (x-axis) versus the observed distribution within the data (Y-axis). These plots represent association with the remit phenotype. The line near 45° is the null distribution which most of the data should fit with only the extreme most p-values above the line at the tail as seen. A) Analysis using only the discovery set of STAR*D (blue points). B) Analysis using the entire STAR*D sample (red points).

A)



B)

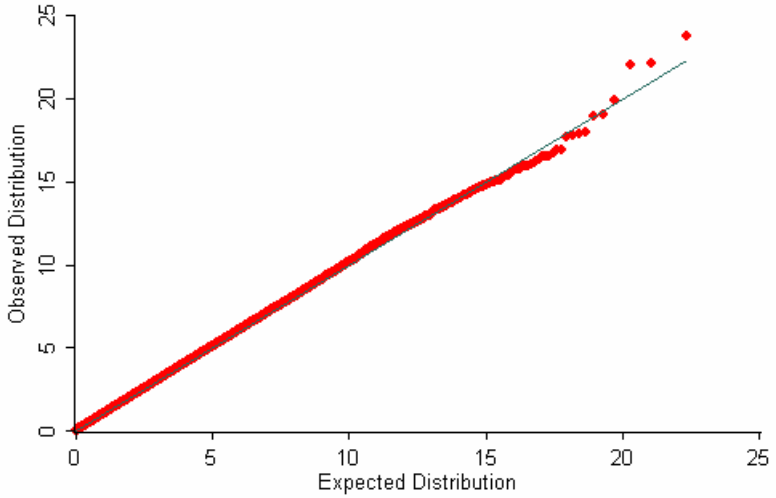
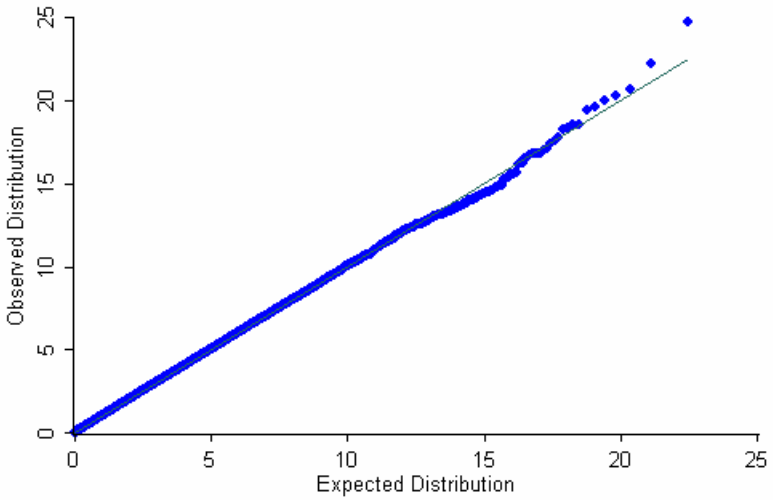


Figure 7.12: Quantile-quantile (QQ) plot showing the expected distribution of chi-squared statistics (x-axis) versus the observed distribution within the data (Y-axis). These plots represent association with the resp42 phenotype. The line near 45° is the null distribution which most of the data should fit with only the extreme most p-values above the line at the tail as seen. A) Analysis using only the discovery set of STAR*D (blue points). B) Analysis using the entire STAR*D sample (red points).

A)



B)

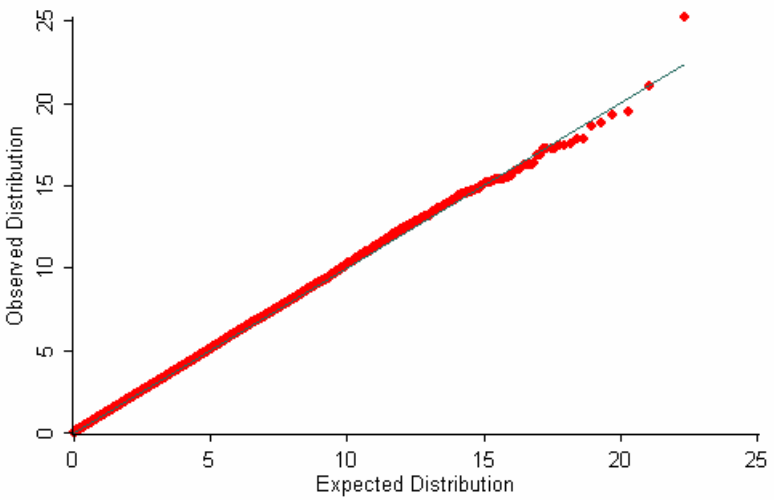
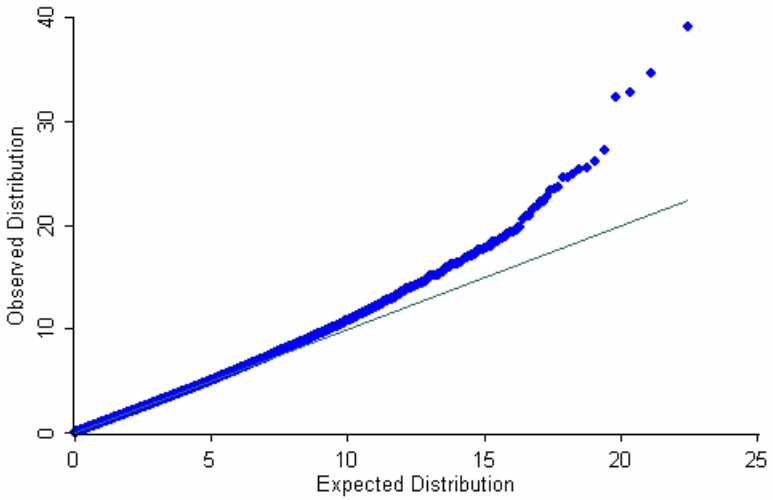


Figure 7.13: Quantile-quantile (QQ) plot showing the expected distribution of chi-squared statistics (x-axis) versus the observed distribution within the data (Y-axis). These plots represent association with the spec_resp phenotype. The line near 45° is the null distribution which most of the data should fit with only the extreme most p-values above the line at the tail as seen. A) Analysis using only the discovery set of STAR*D (blue points). B) Analysis using the entire STAR*D sample (red points).

A)



B)

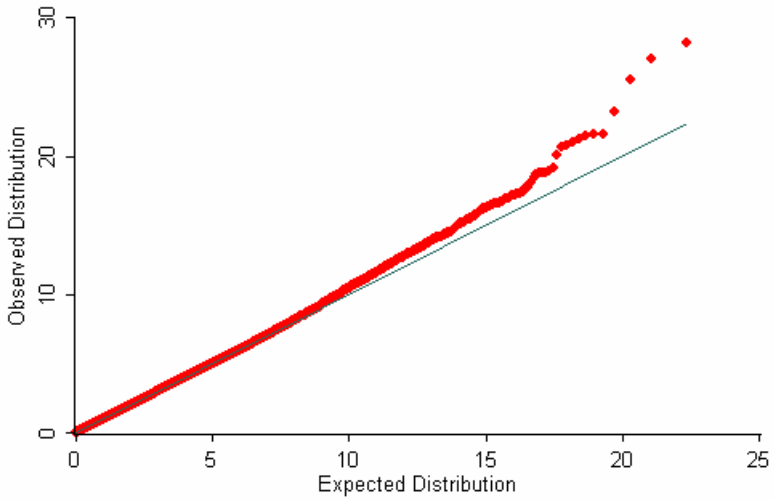


Figure 7.14: Quantile-quantile (QQ) plot showing the expected distribution of chi-squared statistics (x-axis) versus the observed distribution within the data (Y-axis). These plots represent association with the tolerant phenotype. The line at 45° is the null distribution which most of the data should fit, however, this phenotype has gross inflation. A) Analysis using only the discovery set of STAR*D (blue points). B) Analysis using the entire STAR*D sample (red points).

Phenotype	SNP	Chr.	Allele 1	Allele 2	Validation Set	
					P-Value	OR (95% CI)
remit	SNP_A-2024840	13	A	G	0.684	0.94 (0.69 - 1.28)
remit	SNP_A-4218787	7	G	A	0.178	1.24 (0.91 - 1.7)
remit	SNP_A-2042516	1	A	G	0.417	0.87 (0.62 - 1.22)
remit	SNP_A-4238877	13	T	A	-----	-----
remit	SNP_A-4266303	7	A	C	0.827	1.03 (0.81 - 1.31)
remit	SNP_A-4230512	8	C	T	0.747	1.1 (0.61 - 1.98)
remit	SNP_A-1789669	13	C	T	0.515	0.91 (0.67 - 1.22)
remit	SNP_A-2141865	13	C	G	0.493	0.9 (0.67 - 1.21)
remit	SNP_A-2256177	13	T	C	0.572	0.92 (0.68 - 1.24)
remit	SNP_A-2149893	2	G	A	0.473	0.8 (0.43 - 1.47)
resp42	SNP_A-4218787	7	G	A	0.157	1.24 (0.92 - 1.68)
resp42	SNP_A-2024840	13	A	G	0.866	0.97 (0.73 - 1.31)
resp42	SNP_A-2042516	1	A	G	0.184	0.81 (0.59 - 1.11)
resp42	SNP_A-2149893	2	G	A	0.823	0.94 (0.53 - 1.65)
resp42	SNP_A-4266303	7	A	C	0.745	1.04 (0.82 - 1.31)
resp42	SNP_A-4227466	1	G	A	0.833	0.97 (0.71 - 1.32)
resp42	SNP_A-4238877	13	T	A	-----	-----
resp42	SNP_A-2057977	16	T	G	0.453	1.1 (0.86 - 1.39)
resp42	SNP_A-2297908	11	T	C	0.705	1.08 (0.73 - 1.6)
resp42	SNP_A-2128247	3	G	A	0.722	0.96 (0.75 - 1.22)
spec_resp	SNP_A-4218787	7	G	A	0.092	1.33 (0.95 - 1.85)
spec_resp	SNP_A-2057977	16	T	G	0.791	1.04 (0.8 - 1.35)
spec_resp	SNP_A-4261350	13	G	A	-----	-----
spec_resp	SNP_A-1915448	11	T	C	0.745	1.05 (0.79 - 1.39)
spec_resp	SNP_A-2024840	13	A	G	0.778	1.05 (0.76 - 1.45)
spec_resp	SNP_A-1996954	9	T	C	0.165	1.24 (0.91 - 1.69)
spec_resp	SNP_A-4196344	16	C	G	-----	-----
spec_resp	SNP_A-4266303	7	A	C	0.899	1.02 (0.78 - 1.32)
spec_resp	SNP_A-4238877	13	T	A	-----	-----
spec_resp	SNP_A-2007509	20	T	C	-----	-----
tolerant	SNP_A-1843477	13	A	G	0.103	N/A
tolerant	SNP_A-2242408	12	A	G	-----	-----
tolerant	SNP_A-1837056	5	A	G	-----	-----
tolerant	SNP_A-1842826	15	T	C	0.418	2.3 (0.29 - 18.33)
tolerant	SNP_A-2288013	19	C	T	0.595	1.5 (0.33 - 6.72)
tolerant	SNP_A-4195753	10	C	A	0.278	N/A
tolerant	SNP_A-2095694	11	A	C	0.124	N/A
tolerant	SNP_A-1943222	1	A	G	0.777	1.1 (0.57 - 2.15)
tolerant	SNP_A-2139836	4	A	G	0.018	0.17 (0.03 - 0.87)
tolerant	SNP_A-1962163	2	T	A	0.783	0.73 (0.08 - 6.83)

Table 7.3: List of top 10 associated SNPs in the discovery set from each of the four phenotypic comparisons (remit, resp42, spec_resp, & tolerant). Listed are the p-values for the validation set. “N/A” as the odds ratio indicates the dominant odds ratio cannot be calculated. “-----“ indicates SNP failed QC in the validation set but not in the discovery set.

A)

Phenotype	SNP	Chr.	Allele 1	Allele 2	Gene	Entire STAR*D		Discovery Set		Validation Set	
						P-Value	OR (95% CI)	P-Value	OR (95% CI)	P-Value	OR (95% CI)
remit	SNP_A-4218787	7	G	A	UBE3C	4.78E-07	1.65 (1.36 - 2.01)	1.34E-07	1.97(1.53 - 2.54)	1.78E-01	1.24(0.91 - 1.7)
remit	SNP_A-2312802	20	C	A	BMP7	2.91E-06	0.59 (0.48 - 0.74)	1.99E-02	0.72(0.54 - 0.95)	-----	-----
remit	SNP_A-4198575	3	G	A	IQSEC1	5.85E-06	0.44 (0.31 - 0.63)	2.79E-04	0.44(0.28 - 0.69)	5.65E-03	0.43(0.23 - 0.79)
remit	SNP_A-2024840	13	A	G	HMGB1	1.43E-05	0.65 (0.53 - 0.79)	1.01E-07	0.5(0.39 - 0.65)	6.84E-01	0.94(0.69 - 1.28)
remit	SNP_A-1866894	18	A	G	NOL4	1.46E-05	0.7 (0.6 - 0.83)	9.13E-04	0.7(0.57 - 0.87)	4.07E-03	0.69(0.54 - 0.89)
remit	SNP_A-2260830	3	G	A	IQCB1	1.55E-05	0.71 (0.6 - 0.83)	2.85E-02	0.8(0.65 - 0.98)	3.87E-05	0.59(0.46 - 0.76)
remit	SNP_A-2170173	15	C	T	RORA	2.19E-05	1.52 (1.25 - 1.84)	2.81E-04	1.58(1.23 - 2.03)	2.28E-02	1.43(1.05 - 1.95)
remit	SNP_A-2235053	20	T	G	BCAS1	2.83E-05	0.72 (0.62 - 0.84)	5.47E-03	0.75(0.62 - 0.92)	1.11E-03	0.67(0.52 - 0.85)
remit	SNP_A-1937160	18	A	C	WDR7	2.86E-05	1.52 (1.25 - 1.85)	9.09E-04	1.54(1.19 - 1.98)	1.65E-02	1.47(1.07 - 2.01)
remit	SNP_A-2007509	20	T	C	INSM1	2.96E-05	1.4 (1.2 - 1.65)	3.28E-04	1.45(1.19 - 1.78)	-----	-----
resp42	SNP_A-4218787	7	G	A	UBE3C	1.10E-06	1.6 (1.32 - 1.94)	4.52E-07	1.88(1.47 - 2.4)	1.57E-01	1.24(0.92 - 1.68)
resp42	SNP_A-4198575	3	G	A	IQSEC1	2.53E-06	0.45 (0.32 - 0.63)	7.23E-05	0.43(0.28 - 0.66)	9.49E-03	0.48(0.28 - 0.85)
resp42	SNP_A-2170173	15	C	T	RORA	2.73E-06	1.56 (1.3 - 1.89)	7.14E-05	1.63(1.28 - 2.07)	1.16E-02	1.47(1.09 - 1.99)
resp42	SNP_A-2312802	20	C	A	BMP7	7.96E-06	0.62 (0.51 - 0.77)	1.36E-02	0.72(0.55 - 0.93)	-----	-----
resp42	SNP_A-4201095	1	G	T	MAN1A2	1.29E-05	0.62 (0.5 - 0.77)	4.36E-03	0.68(0.52 - 0.89)	4.35E-04	0.52(0.36 - 0.76)
resp42	SNP_A-4212364	7	C	A	IGFBP3	1.35E-05	1.52 (1.26 - 1.83)	4.73E-03	1.41(1.11 - 1.8)	5.43E-04	1.71(1.26 - 2.33)
resp42	SNP_A-4283086	2	T	A	------	2.21E-05	1.41 (1.2 - 1.65)	8.47E-04	1.41(1.15 - 1.73)	1.16E-02	1.39(1.08 - 1.79)
resp42	SNP_A-2007509	20	T	C	INSM1	2.37E-05	1.39 (1.19 - 1.62)	1.63E-04	1.46(1.2 - 1.78)	-----	-----
resp42	SNP_A-4301367	13	A	G	RFC3	2.49E-05	2.34 (1.56 - 3.51)	5.16E-03	2.09(1.23 - 3.54)	1.35E-03	2.74(1.45 - 5.19)
resp42	SNP_A-2311397	13	T	G	RFC3	2.55E-05	2.34 (1.56 - 3.51)	5.26E-03	2.09(1.23 - 3.53)	1.35E-03	2.74(1.45 - 5.19)

Table 7.4: List of top 10 associated SNPs in the entire STAR*D sample from each of the four phenotypic comparisons: A) remit & resp42 B) spec_resp & tolerant. Listed are the p-values for the discovery and validation sets. “N/A” as the odds ratio indicates the dominant odds ratio cannot be calculated. “-----“ indicates SNP failed QC in the validation set but not in the discovery set.

B)

Phenotype	SNP	Chr.	Allele 1	Allele 2	Gene	Entire STAR*D		Discovery Set		Validation Set	
						P-Value	OR (95% CI)	P-Value	OR (95% CI)	P-Value	OR (95% CI)
spec_resp	SNP_A-4218787	7	G	A	UBE3C	5.19E-07	1.69 (1.38 - 2.08)	6.68E-07	1.94(1.49 - 2.53)	9.20E-02	1.33(0.95 - 1.85)
spec_resp	SNP_A-1937160	18	A	C	WDR7	4.41E-06	1.62 (1.32 - 1.99)	8.95E-04	1.56(1.2 - 2.03)	1.58E-03	1.7(1.22 - 2.37)
spec_resp	SNP_A-1983260	5	T	G	SNCAIP	1.04E-05	1.85 (1.4 - 2.43)	2.73E-05	2.16(1.5 - 3.11)	4.64E-02	1.53(1.01 - 2.33)
spec_resp	SNP_A-2007509	20	T	C	INSM1	1.12E-05	1.45 (1.23 - 1.71)	1.84E-05	1.59(1.28 - 1.96)	-----	-----
spec_resp	SNP_A-4198575	3	G	A	IQSEC1	1.43E-05	0.43 (0.29 - 0.63)	8.04E-05	0.38(0.23 - 0.62)	4.22E-02	0.52(0.27 - 0.98)
spec_resp	SNP_A-2170173	15	C	T	RORA	1.65E-05	1.55 (1.27 - 1.9)	1.73E-04	1.63(1.26 - 2.1)	2.86E-02	1.44(1.04 - 2)
spec_resp	SNP_A-2023819	1	G	A	AJAP1	2.40E-05	0.55 (0.41 - 0.73)	2.78E-03	0.59(0.42 - 0.84)	2.31E-03	0.48(0.3 - 0.77)
spec_resp	SNP_A-1970535	21	T	C	SLC37A1	2.42E-05	1.43 (1.21 - 1.69)	1.15E-04	1.52(1.23 - 1.88)	7.05E-02	1.28(0.98 - 1.68)
spec_resp	SNP_A-1847174	17	T	C	NPTX1	2.75E-05	1.56 (1.26 - 1.92)	8.88E-03	1.42(1.09 - 1.86)	4.86E-04	1.81(1.29 - 2.53)
spec_resp	SNP_A-4202268	3	G	T	FOXP1	2.99E-05	0.61 (0.48 - 0.77)	1.84E-04	0.57(0.42 - 0.76)	5.61E-02	0.7(0.48 - 1.01)
tolerant	SNP_A-2245760	22	C	T	SCUBE1	1.12E-07	0.47 (0.36 - 0.63)	1.87E-01	0.58(0.26 - 1.32)	3.14E-02	0.7(0.51 - 0.97)
tolerant	SNP_A-1795580	7	C	T	FAM20C	1.97E-07	0.18 (0.09 - 0.37)	2.17E-02	0.31(0.11 - 0.91)	4.88E-06	0.11(0.04 - 0.32)
tolerant	SNP_A-2139836	4	A	G	CXXC4	4.39E-07	0.1 (0.04 - 0.29)	6.26E-07	0.06(0.02 - 0.25)	1.81E-02	0.17(0.03 - 0.87)
tolerant	SNP_A-2118974	4	T	C	CXXC4	1.44E-06	0.12 (0.04 - 0.32)	3.00E-06	0.08(0.02 - 0.29)	1.81E-02	0.17(0.03 - 0.87)
tolerant	SNP_A-4236225	4	C	T	FRG1	3.38E-06	0.11 (0.04 - 0.33)	2.11E-02	0.16(0.03 - 0.93)	2.55E-04	0.09(0.02 - 0.43)
tolerant	SNP_A-2098107	8	A	G	MSRA	3.43E-06	0.03 (0 - 0.29)	1.30E-04	0.04(0 - 0.46)	9.59E-04	N/A
tolerant	SNP_A-1915683	4	T	C	SORCS2	3.55E-06	0.56 (0.44 - 0.72)	5.50E-01	0.52(0.06 - 4.58)	-----	-----
tolerant	SNP_A-1796662	4	A	G	CXXC4	4.12E-06	0.13 (0.05 - 0.35)	1.11E-05	0.09(0.03 - 0.33)	1.81E-02	0.17(0.03 - 0.87)
tolerant	SNP_A-1978347	4	C	T	CXXC4	4.73E-06	0.15 (0.06 - 0.37)	3.35E-05	0.11(0.03 - 0.37)	1.42E-02	0.2(0.05 - 0.81)
tolerant	SNP_A-1894206	2	C	A	NPAS2	5.29E-06	0.57 (0.44 - 0.73)	8.19E-01	1.28(0.16 - 10.25)	-----	-----

Table 7.4: List of top 10 associated SNPs in the entire STAR*D sample from each of the four phenotypic comparisons: A) remit & resp42 B) spec_resp & tolerant. Listed are the p-values for the discovery and validation sets. “N/A” as the odds ratio indicates the dominant odds ratio cannot be calculated. “-----“ indicates SNP failed QC in the validation set but not in the discovery set.

Phenotype	Gene	Chr.	Start Pos.	Ending Pos.	# of SNPs	Entire STAR*D	
						Min. P-Value	Max. P-Value
remit	SLC6A4	17	25,539,000	25,596,000	7	0.326	0.981
remit	TPH2	12	70,628,000	70,723,000	27	0.078	0.948
remit	HTR2A	13	46,295,000	46,358,000	31	0.001	0.995
remit	FKBP5	6	35,639,000	35,775,000	6	0.238	0.998
remit	ACE	17	58,898,000	58,939,000	4	0.275	0.975
remit	GRIK4	11	120,026,000	120,372,000	85	0.006	0.968
remit	GNB3	12	6,809,000	6,837,000	1	0.782	0.782
resp42	SLC6A4	17	25,539,000	25,596,000	7	0.351	0.983
resp42	TPH2	12	70,628,000	70,723,000	27	0.027	0.982
resp42	HTR2A	13	46,295,000	46,358,000	31	0.002	0.998
resp42	FKBP5	6	35,639,000	35,775,000	6	0.293	0.790
resp42	ACE	17	58,898,000	58,939,000	4	0.236	0.959
resp42	GRIK4	11	120,026,000	120,372,000	85	0.004	0.988
resp42	GNB3	12	6,809,000	6,837,000	1	0.755	0.755

Table 7.5: List of genes that have been previously reported in the literature to be associated with antidepressant response and our association results using our genome-wide data. Highlighted genes have markers showing association at $p < 0.01$ significance. Position data is based on HapMap build 34 of the genome.

Phenotype	SNP	Chr.	Distance From Gene	Gene	Relevant Function	Relevant Expression
remit	SNP_A-4218787	7	55kb	UBE3C	------	expressed in brain
remit	SNP_A-2312802	20	100kb	BMP7	Ca++ binding / Wnt signaling regulator	highly expressed in brain
remit	SNP_A-4198575	3	IN	IQSEC1	β-1 integrin / cell adhesion	expressed in brain
remit	SNP_A-2024840	13	25kb	HMGB1	DNA transcription / DNA repair	------
remit	SNP_A-1866894	18	IN	NOL4	RNA binding	highly expressed in brain
remit	SNP_A-2260830	3	15kb	IQCB1	interacts with calmodulin / neural growth	expressed in brain
remit	SNP_A-2170173	15	IN	RORA	circadian rhythm	highly expressed in brain
remit	SNP_A-2235053	20	120kb	BCAS1	------	highly expressed in brain
remit	SNP_A-1937160	18	IN	WDR7	exocytosis of neurotransmitters	highly expressed in brain
remit	SNP_A-2007509	20	25kb	INSM1	Shh signaling regulator	highly expressed in brain
resp42	SNP_A-4218787	7	55kb	UBE3C	------	expressed in brain
resp42	SNP_A-4198575	3	IN	IQSEC1	β-1 integrin / cell adhesion	expressed in brain
resp42	SNP_A-2170173	15	IN	RORA	circadian rhythm	highly expressed in brain
resp42	SNP_A-2312802	20	100kb	BMP7	Ca++ binding / Wnt signaling regulator	highly expressed in brain
resp42	SNP_A-4201095	1	IN	MAN1A2	Ca++ binding	highly expressed in brain
resp42	SNP_A-4212364	7	225kb	IGFBP3	cell growth	expressed in brain
resp42	SNP_A-4283086	2	>500kb	------	------	------
resp42	SNP_A-2007509	20	25kb	INSM1	Shh signaling regulator	highly expressed in brain
resp42	SNP_A-4301367	13	10kb	RFC3	DNA replication / DNA repair	------
resp42	SNP_A-2311397	13	5kb	RFC3	DNA replication / DNA repair	------
spec_resp	SNP_A-4218787	7	55kb	UBE3C	------	expressed in brain
spec_resp	SNP_A-1937160	18	IN	WDR7	exocytosis of neurotransmitters	highly expressed in brain
spec_resp	SNP_A-1983260	5	50kb	SNCAIP	neuronal degradation via α-synuclein	highly expressed in brain
spec_resp	SNP_A-2007509	20	25kb	INSM1	Shh signaling regulator	highly expressed in brain
spec_resp	SNP_A-4198575	3	IN	IQSEC1	β-1 integrin / cell adhesion	expressed in brain
spec_resp	SNP_A-2170173	15	IN	RORA	circadian rhythm	highly expressed in brain
spec_resp	SNP_A-2023819	1	350kb	AJAP1	cell adhesion	------
spec_resp	SNP_A-1970535	21	IN	SLC37A1	sugar transport	------
spec_resp	SNP_A-1847174	17	20kb	NPTX1	Ca++ binding / synaptic transmission	highly expressed in brain
spec_resp	SNP_A-4202268	3	450kb	FOXP1	transcription factor / neural growth	expressed in brain
tolerant	SNP_A-2245760	22	IN	SCUBE1	Ca++ binding / neural growth / platelet function	highly expressed in brain/platelets
tolerant	SNP_A-1795580	7	IN	FAM20C	Ca++ binding	highly expressed in brain
tolerant	SNP_A-2139836	4	350kb	CXXC4	Wnt signaling regulator	highly expressed in brain
tolerant	SNP_A-2118974	4	350kb	CXXC4	Wnt signaling regulator	highly expressed in brain
tolerant	SNP_A-4236225	4	375kb	FRG1	DNA transcription	------
tolerant	SNP_A-2098107	8	15kb	MSRA	oxidative stress / DNA repair	expressed in brain
tolerant	SNP_A-1915683	4	IN	SORCS2	neuropeptide signaling	highly expressed in brain
tolerant	SNP_A-1796662	4	250kb	CXXC4	Wnt signaling regulator	highly expressed in brain
tolerant	SNP_A-1978347	4	300kb	CXXC4	Wnt signaling regulator	highly expressed in brain
tolerant	SNP_A-1894206	2	IN	NPAS2	transcription factor / circadian rhythm / autism	highly expressed in brain

Table 7.6: List of top 10 associated SNPs in the discovery set from each of the four phenotypic comparisons (remit, resp42, spec_resp, & tolerant) along with the closest gene to the associated SNP and that distance away. "-----" indicates either expression of functional data could not be found. Highlighted SNPs indicate they were found in more than one phenotype.

7.4 Discussion

Association studies involving this large number of markers present both unique analytical and computational challenges. In our study, we pursued a limited number (N = 20) of single locus associations in each of four phenotypic comparisons using our validation sample set, only one of which replicated ($p<0.05$) the initial association for either phenotype comparison (SNP_A-2139836). This SNP is intergenic and is 250kb away from the closest known gene, CXXC4, which is a negative regulator of the canonical Wnt signaling pathway and highly expressed in several regions of the brain (9). Since it did meet our criteria for study-wide significance, this variant (which is greater than 250kb from any known gene) deserves follow-up testing in other study populations taking SSRIs.

It has been argued that a joint analysis of the combined discovery and validation sample sets is more powerful than the two-stage replication strategy (10). With this in mind, we analyzed our data post-hoc as a one-stage design. The top hits from this analysis provide several interesting findings with biological plausibility (Table 7.6). Of interest were two genes involved in canonical Wnt signaling and highly expressed in the brain. Five genes that bind calcium and were also highly expressed in the brain also showed association. Finally, the genes NPAS2, implicated in autism studies, and RORA are both involved in circadian rhythm regulation and were among the most associated SNPs. In addition to being largely associated ($p < 1x10^{-4}$) in the entire sample, these markers reside in genes whose functional roles could easily play a role in depression or response to antidepressants.

We originally chose a replication based strategy as we were chiefly interested in the ability of the putatively associated SNPs to consistently show association in different populations. In order for these SNPs reported from either our two-stage or one-stage design to be used in clinical decision making, replication in different populations is essential, as non-replication of putative associations is an unfortunate reality that is all too common with complex genetic phenotypes.

Given that we had an adequate sample size to replicate the initial associations in the validation set, the reason for the general lack of replication is unclear, though there could be several underlying causes. In order to limit Type I error in the screening stage we only attempted to replicate SNPs that were within the 10 most significant p-values in the discovery set. However, only a handful of these associations would survive a Bonferroni correction for multiple comparisons, so there is a reasonable risk that these are false-positive signals in the discovery dataset. It is quite possible that findings that reliably replicate are much further down in the rank ordering. Unfortunately, it's unclear how many independent tests were performed and thus require adjustment, given the high levels of LD seen in the data. Population stratification could also be underlying the lack of replication although we attempted to control for population stratification using self-reported race as a proxy for genetic ancestry. This strategy was guided by a *structure* analysis using 2,500 SNPs in the entire STAR*D population dataset. Analyzing the data using a principal components analysis may better correct for stratification in the sample.

The non-replication could also be due to unknown heterogeneity between the discovery and validation sample sets. This heterogeneity could be a clinical characteristic (e.g., depression subtype) or epistatic DNA variation that was not controlled for in the

sample splitting, but that nonetheless alters the strength of the association. Extending or altering the BRLMM calling algorithm, which is designed to give the highest call rates the data allows, to focus on call quality could be useful and is under development (11).

As mentioned previously, this study was designed to investigate only the most "low hanging fruit", and was not intended to be a comprehensive follow-up of the discovery sample set results. Certainly, additional genetic models need to be tested in the discovery set (genotypic, dominant, recessive, etc.). Also, a larger proportion of follow-up SNPs will need to be investigated. Fortunately, the validation set is already genotyped for the same 500,000 markers as the discovery set and given the two-stage study design, multiple correction penalties for the follow-up of SNPs will be far less than the correction for the entire WGA panels, allowing for more liberal selection criteria. For example, there are 25 SNPs for the remission phenotype meeting p-values of < 0.01 in discovery and validation analyses, as well as having a p-value < 0.005 in the overall analysis. While not striking, it is of note that the signals are in the same direction in the two samples, and perhaps worthy of further consideration.

The statistical sacrifice with the two-stage design is of course a reduction in power. However, even with a split sample, we can capture (and replicate) clinically meaningful effect sizes with reasonable power. With dense marker data for both the discovery and validation sets, a more powerful analysis using a one-stage design is also possible. An FDR based approach may also be worthwhile, given that the actual causative SNPs may not be the most highly associated SNPs and would be missed using the current analysis. Permutation techniques could also be used, but could prove

challenging due to current computational limits, although newer techniques can approximate permutation results more quickly (12).

Alternatively, follow-up SNPs could also be selected using an effect-size (OR) threshold, instead of one based purely on a significance threshold. This approach may be of particular utility in pharmacogenetic studies, where the eventual goal is to develop genetic tests for use in clinical treatment and this would yield the SNPs with the largest clinical effects, regardless of biological meaning. Fine mapping of SNPs in the validation set for regions surrounding the putatively associated SNPs would also be beneficial, since differences in LD patterns across the discovery and validation sample sets could complicate indirect association analysis. In addition to SNP data, the panels used to genotype the discovery sample set yield quantitative hybridization data that can in theory be used to score copy number variations, or CNVs (13). Identifying and testing these CNVs for association to citalopram response would be worthwhile, though the techniques for performing this are still being developed.

For complex genetic diseases, the common disease/common variant hypothesis states that several DNA variants will, in combination, contribute a clinically meaningful risk of having the phenotype. Techniques for uncovering interacting loci are poorly developed, largely due to the computation and statistical burden of the number of tests that can be performed. For instance, performing all pairwise comparisons (SNP x SNP) with our WGA panel would require 1.3×10^{11} statistical tests. With needing to correct for this many tests, sample sizes like the one used in this study have power only to detect unrealistically large interaction effects (14). The correction penalties for more than two SNPs interacting are even more severe. Additionally, as higher level interaction testing is

performed, the number of subjects with the desired allelic combination to be tested is reduced, further decreasing the statistical power. Thus, for powerful interaction analysis, extremely large clinical populations, on the order of tens of thousands of patients, will need to be collected or limited numbers of markers to test in interaction analyses. Our attempt to test for interactions between markers showed no significant interactions in any of the 4 phenotypes tested.

We also investigated previously reported genes (e.g. FKBP5, HTR2A, etc.) in the literature for association using our whole genome data. We were not able to validate any previously reported associations for the genes SLC6A4, TPH2, FKBP5, ACE, & GNB3 in the STAR*D sample using the markers we had genotyped. We did find evidence of association with markers in GRIK4 and HTR2A ($p < 0.01$) but we would dismiss these due to the number of tests being performed.

In summary, here we have presented the preliminary results of a two stage whole genome association study for citalopram response and tolerance using the STAR*D clinical population. While only one of the single locus associations (SNP_A-2139836) in our discovery sample set met our replication criteria in the validation sample set, this SNP is near a quite plausible candidate gene (CXXC4). This gene is an inhibitor of the canonical Wnt signaling pathway, is highly expressed in several regions of the brain, and may play a role in synaptic rearrangement and plasticity. Combining the data together to maximize power led to many strong associations in biologically interesting gene pathways.

It is clear that further analysis is required to comprehensively follow-up the discovery set results and replication of our findings in other populations taking SSRIs will be crucial to understanding which hits are real and clinical meaningful. Finally, given the lack of understanding of citalopram's mechanism of action, gene-agnostic studies such as these may be required to find genetic markers that are informative and predictive of citalopram response or tolerance.

7.5 References

1. Syvanen AC. Toward Genome-Wide SNP Genotyping. *Nat Genet* 2005;

2. Klein RJ, Zeiss C, Chew EY, Tsai JY and others. Complement Factor H Polymorphism in Age-Related Macular Degeneration. *Science* 2005; 308(5720): 385-9

3. Lawrence RW, Evans DM, and Cardon LR. Prospects and Pitfalls in Whole Genome Association Studies. *Philos. Trans. R. Soc. Lond B Biol. Sci.* 2005; 360(1460): 1589-1595

4. Wang H, Thomas DC, Pe'er I, and Stram DO. Optimal Two-Stage Genotyping Designs for Genome-Wide Association Scans. *Genet Epidemiol.* 2006; 30(4): 356-368

5. Kennedy GC, Matsuzaki H, Dong S, Liu WM and others. Large-Scale Genotyping of Complex DNA. *Nat Biotech* 2003; 21(10): 1233-1237

6. Rabbee N and Speed TP. A Genotype Calling Algorithm for Affymetrix SNP Arrays. *Bioinformatics.* 2006; 22(1): 7-12

7. Purcell S, Neale B, Todd-Brown K, Thomas L and others. PLINK: a Tool Set for Whole-Genome Association and Population-Based Linkage Analyses. *Am. J. Hum. Genet.* 2007; 81(3): 559-575

8. Falush D, Stephens M, and Pritchard JK. Inference of Population Structure Using Multilocus Genotype Data: Linked Loci and Correlated Allele Frequencies. *Genetics* 2003; 164(4): 1567-1587

9. Hino S, Kishida S, Michiue T, Fukui A and others. Inhibition of the Wnt Signaling Pathway by Idax, a Novel Dvl-Binding Protein. *Mol. Cell Biol.* 2001; 21(1): 330-342

10. Skol AD, Scott LJ, Abecasis GR, and Boehnke M. Joint Analysis Is More Efficient Than Replication-Based Analysis for Two-Stage Genome-Wide Association Studies. *Nat Genet* 2006; 38(2): 209-213

11. Hua J, Craig DW, Brun M, Webster J and others. SNiPer-HD: Improved Genotype Calling Accuracy by an Expectation-Maximization Algorithm for High-Density SNP Arrays. *Bioinformatics.* 2007; 23(1): 57-63

12. Seaman SR and Muller-Myhsok B. Rapid Simulation of P-Values for Product Methods and Multiple-Testing Adjustment in Association Studies. *Am J Hum Genet* 2005; 76(3): 399-408

13. Komura D, Shen F, Ishikawa S, Fitch KR and others. Genome-Wide Detection of Human Copy Number Variations Using High-Density DNA Oligonucleotide Arrays. *Genome Res.* 2006; 16(12): 1575-1584

14. Evans DM, Marchini J, Morris AP, and Cardon LR. Two-Stage Two-Locus Models in Genome-Wide Association. *PLoS. Genet* 2006; 2(9): e157

CHAPTER 8

SUMMARY AND FUTURE DIRECTIONS

8.1 Summary of Dissertation Work

The overarching goal of this thesis was to find genetic markers associated with antidepressant response that could be used as predictive markers for future antidepressant treatment in patients. The experiments described in the previous chapters have attempted to establish genetic markers that are predictive of a depressed subject's clinical outcome following antidepressant therapy. To accomplish this goal, several complementary genetic approaches have been utilized (outlined in Figure 1.1). Our initial efforts involved a small but highly characterized (N=96) depressed patient population taking the selective serotonin reuptake inhibitor (SSRI) fluoxetine in which we investigated the serotonin transporter, the molecular target of SSRIs. In order to uncover novel SNPs or potentially functional variants, the coding regions, intron-exon boundaries, and conserved non-coding regions were directly screened in all subjects in the fluoxetine population. Within the fluoxetine patient sample, several SNPs in SLC6A4 were associated ($p < 0.05$) with response and response specificity and several previously unknown SNPs were identified, all of which were at low minor allele frequencies (MAF < 0.05) (1).

In an attempt to replicate the initial associations within the serotonin transporter, tagging SNPs were selected to adequately capture variation within the gene and then genotyped in the large STAR*D sample (N=1,953), which had been treated with the SSRI citalopram. None of the variants were associated with citalopram response or response specificity (2). This apparent lack of replication could be due to several factors

including Type I error, differences between the drug's mechanisms of action, or differences between the clinical populations, particularly in terms of genetic heterogeneity.

We then explored variation within the transcription factor FEV as a possible modulator of citalopram response. Using a one-stage study design, several of the variants in FEV that we screened were significantly ($p < 0.05$) associated with citalopram response within the STAR*D population. In collaboration with Miles Berger from the laboratory of Dr. Larry Tecott, we examined a murine model of FEV using mice deficient for the FEV homologue, Pet-1. Using a well accepted model for depression in mice (tail suspension test), we found that citalopram treatment, while effective for wild-type controls, had no effect on the knockout mice for Pet-1. The genetic and functional data provides excellent support for a role for FEV in citalopram response and this gene should be investigated further.

We also investigated variation within a number of other pharmacodynamic candidate genes as possible predictors of citalopram response or tolerance. These candidate genes included the serotonin 1B receptor (5-HT1B), a protein called p11 (S100A10), and two isoforms of the transcription factor AP-2 (AP-2β & AP-2δ) which gained our interest due to functional evidence from our earlier studies of SLC6A4 (1). Unfortunately, none of the variants in any of these 4 candidate genes that we screened were significantly associated ($p < 0.05$) with citalopram response within the STAR*D population. The genetic data suggested these genes do not play a role in citalopram response or tolerance in our sample.

Given the difficulty we and others have had in predicting relevant candidate genes *a priori*, we used a panel of gene-centric markers to investigate a large number of genes using a two-stage study design to genotype over 40,000 SNPs in approximately half of the STAR*D sample. Forty-five of these SNPs were associated with citalopram response using a combination of significance level ($p < 0.001$) and effect size (odds ratio > 2.0) as criteria in the discovery sample set. We attempted to replicate these significantly associated SNPs in the validation sample set. One of these SNPs showed significant ($p < 0.01$) association with response in the validation sample set. Furthermore, in exploratory analysis we looked at the entire data set using a one-stage design and twelve markers were predictive of response to citalopram in the entire STAR*D sample.

In our last analysis, we used a whole genome association (WGA) platform and a two-stage study design to genotype over 500,000 SNPs in the discovery half of the STAR*D sample. Several of these SNPs were associated with citalopram response, specificity of response, remission or tolerance at high significance levels in the discovery sample set. We attempted to replicate significantly associated SNPs within the discovery set at a threshold of $p < 1x10^{-5}$ using the validation sample set. Three SNPs showed evidence of replication in the tolerance phenotype (rs4274851, rs10026406, and rs6959125). However, none of the SNPs showed significant ($p < 0.05$) association with response, specificity of response, or remission in the validation sample set. This non-replication could be due to a variety of factors, including uncorrected population stratification, unknown clinical confounders, or simple Type I error in the discovery set.

This was an attempt to replicate the "low hanging fruit" of the discovery portion of the WGA study. For example, the "truly" associated variants may not have provided

the most extreme estimates of statistical significance, and instead may be represented by more modest, but consistent, measures of significance. An example is the gene HEX6, which was found with modest association independently in the Type II diabetes studies done by the FUSION, WTCCC, and DGI GWAS consortium groups and when the data was combined across studies, this gene becomes quite significant ($p < 1.0x10^{-7}$) (3-5). An unanswered question is how far to pursue findings for replication (e.g., absolute p-value threshold, effect size, significance in more than one subgroup of phenotype).

8.2 Future Directions

The field of human genetics examining complex phenotypic traits has been evolving at a stunning pace since the sequencing of the human genome. Much of the evolution of the field can be traced to rapidly advancing genotyping technologies that increase genotype throughput and reduce genotyping costs. Thanks to these advances, larger scale genotyping, like our WGA study involving 500,000 SNPs, can now be completed in a few weeks. The scale of genotyping will continue to grow, as Affymetrix and Illumina have both already released a 1 million SNP panel, part of which will be designed to detect copy number variants (CNVs). It is now becoming clear that within the next decade, large-scale targeted genome resequencing in large sample sets will become economically feasible. This will allow us to better address complex phenotypes utilizing both common variants and rare variants simultaneously (SNPs, CNVs, microsatellites, etc.) and lead to a better understanding on the role of genetics on complex phenotypes. This is not without pitfalls as a question with both practical and theoretical implications,

however, is will the field be able to interpret such high density genetic data in meaningful ways?

The largest concern for population based association studies has been the risk of confounding due to population stratification. Population stratification is thought to play a role in the non-replication of many association studies, and as study populations become larger the risk of confounding also increases due to recruitment across wider geographic and sub-population distances. Current methods for detecting and correcting for population stratification, such as *structure*, can perform adequately, but for small scale genotyping studies they require additional marker genotyping which can be cost prohibitive and with large scale genotyping data computational power becomes a concern (6). The development of efficient ancestry informative marker (AIM) panels that have maximal allele frequency differences across subpopulations would have great utility in small scale association studies (7-11). The selection of AIMs for distant subpopulations (e.g., Africans and Asians) in order to detect large levels of stratification is fairly straightforward, given the dense marker data available from the HapMap project (12-14).

Recent reports suggest population stratification may have a confounding effect even within isolated populations such as Iceland and Europe, which were both thought to be relatively homogeneous (15,16). Selection of AIMs for more subtle levels of stratification across continental clines will require more large scale genotyping in these subpopulations in order to define their allele frequencies. For WGA studies, and in the future whole genome resequencing studies, the dense amount of marker data available should allow for accurate matching of cases and control based on ancestry and consequently little reduction in statistical power.

Another major hurdle in the development of analytical methods is the issue of multiple testing (17). It is difficult to separate true associations from those caused by chance when performing 500,000 or more single locus tests. When several phenotypes and several populations are examined, the problem obviously is amplified. The problem becomes much larger when searching for interacting SNPs, which are a cornerstone of the common disease common variant hypothesis. False discovery rate (FDR) methods can be used to gauge how many SNPs to move forward in multi-stage studies, and aren't affected by inter-marker relatedness (18). Increases in computational power will allow for permutation based methods to be applied across entire WGA panels, which will take into account inter-marker relatedness. However, given the inherent risk of Type I error from the number of tests and since we often have no *a priori* reason to believe putatively associated SNPs in WGA studies, replication in separate populations will be of the utmost importance. Even this gold standard is problematic, when "independent" populations may differ subtly by phenotypic definition or less subtly by differences in population ancestry or genotyping platform. Statistical limits may make innovative study designs, along with the collection of large clinical populations for replication, the only way to get through the multiple testing concerns in large scale associations studies. There is still room for flexibility in the face of this seeming statistical absolutism. For example, it may be possible to look for patterns of association to variation in networks of genes representing pathways, although the methodology for such investigation has not been adequately developed. Similarly, common sense and curiosity may still have a role in addressing our data. For example, a pattern of low-level association to many uncorrelated variants within a single gene may highlight genes for further examination. Likewise,

modest association in genes with compelling biological connection to the phenotype could generate additional hypothesis testing.

Analytical techniques in the genetics of complex traits have not evolved as quickly as methods for genotyping. For instance, as recently as 10 years ago, it was still unclear whether useful amounts of LD exist in the human genome (19). It is clear now that significant LD extends to useful distances in human populations (20,21). The HapMap project, which was completed (phase II) during the course of this project, allows users to select tagging SNPs from the publicly available dataset of dense markers across the genome, which is an invaluable resource to LD mapping. However, much is still unclear about the most powerful way to utilize LD in association studies. For example, determining association between traits and rare variants is a challenge, as tagging strategies are largely ineffective, and truly massive samples would be required for reliable estimates of association (22).

Currently, the utility of haplotype testing in association studies is still debatable, as some feel it does not add enough additional information to single locus testing to justify the multiple correction penalties (23). A substantial number of methods have been published in the past 5 years that utilize LD in order to select tagging SNPs (24-28). It seems that the most analytically straightforward, based on a threshold for pairwise r^2, has also become the most popular method for selecting tagging SNPs, though other methods appear to be more efficient at reducing genotyping load. It's still unclear exactly how well tagging SNPs will transfer across different human populations, although much research has focused on this question (29,30).

Single nucleotide polymorphisms have been the major type of variation investigated in genetic association studies for the past decade, largely because of their ability to be easily assayed in a cost-effective manner. However, it is becoming increasingly clear that other types of variation, namely copy number variation (CNVs), is common in the genome and may contribute to human phenotypes. Copy number variants take the form of segmental duplications or deletions, and are thought to alter at least as much of the human genome as SNPs (31). Given their sizable changes to the genome, CNVs are reasonably thought to cause considerable differences in expression or function of the genes they encompass, although there is little evidence for this as of yet. Since the majority of genotyping techniques focus on a small area around the SNP of interest (generally less than 100bp), CNVs that encompass interrogated SNPs can have a detrimental and often unknown effect on genotype accuracy and quality. Fortunately, current genotyping methods for WGA studies mostly rely on hybridization to a fixed DNA array, which yields quantitative data that can be scored for copy number variants as well as SNPs (32). A catalog of common CNVs in the human genome and a large, detailed search for common CNVs across the genome similar to the SNP Consortium project is currently being investigated and will greatly aid in the integration of CNVs into WGA studies (33-36).

Large collections of well-phenotyped subjects are crucial to the success of complex genetics in the next decade. Large populations are necessary in order to provide replication of initial findings, to lessen the burden of multiple testing by increasing power, and for studies of interacting SNPs, which is a critical component of the common disease common variant hypothesis of complex diseases. Alternatively, for the common

disease rare variant (CDRV) hypothesis, where individual variants are thought to have large effect sizes but occur very infrequently, large collections of patients will be necessary to find adequate number of subjects carrying the risk variant. There will always be room for more specialized samples, such as from isolated populations, in order to reduce heterogeneity that may interfere with LD mapping. Likewise, informative family-based samples will continue to be useful.

While single investigators typically have the resources to collect on the order of hundreds of patients at best, large government sponsored clinical trials (such as STAR*D) and late phase investigational drug trials can involve large numbers of subjects, but usually not more than two or three thousand. Even with all the resources used to fund such studies, it is becoming clear that sufficiently powered pharmacogenetic studies will require on the order of tens of thousands of patients. For this scale of populations, large consortia will need to be formed, where investigators share subject DNA, phenotype data, and ultimately, credit for any findings. Examples of efforts on a similar scale can be seen with the Type I diabetes genetic consortium or the Welcomme Trust Consortium (37-39).

As with all genetics research, false positives and disappointments are common, and more subjects are always needed. I feel with healthy cooperation among researchers and some luck, in the next decade there will be many examples of the clinical utility of personalized medicine.

8.3 References

1. Kraft JB, Slager SL, McGrath PJ, and Hamilton SP. Sequence Analysis of the Serotonin Transporter and Associations With Antidepressant Response. *Biol Psychiatry* 2005; 58(5): 374-381

2. Kraft JB, Peters EJ, Slager SL, Jenkins GD and others. Analysis of Association Between the Serotonin Transporter and Antidepressant Response in a Large Clinical Sample. *Biological Psychiatry* 2006; In Press

3. Genome-Wide Association Study of 14,000 Cases of Seven Common Diseases and 3,000 Shared Controls. *Nature* 2007; 447(7145): 661-678

4. Salonen JT, Uimari P, Aalto JM, Pirskanen M and others. Type 2 Diabetes Whole-Genome Association Study in Four Populations: the DiaGen Consortium. *American Journal of Human Genetics* 2007; 81(2): 338-345

5. Saxena R, Voight BF, Lyssenko V, Burtt NP and others. Genome-Wide Association Analysis Identifies Loci for Type 2 Diabetes and Triglyceride Levels. *Science* 2007; 316(5829): 1331-1336

6. Falush D, Stephens M, and Pritchard JK. Inference of Population Structure Using Multilocus Genotype Data. Linked Loci and Correlated Allele Frequencies. *Genetics* 2003; 164(4): 1567-1587

7. Price AL, Butler J, Patterson N, Capelli C and others. Discerning the Ancestry of European Americans in Genetic Association Studies. *PLoS. Genet.* 2008; 4(1): e236

8. Price AL, Patterson N, Yu F, Cox DR and others. A Genomewide Admixture Map for Latino Populations. *Am. J. Hum. Genet.* 2007; 80(6): 1024-1036

9. Tian C, Plenge RM, Ransom M, Lee A and others. Analysis and Application of European Genetic Substructure Using 300 K SNP Information. *PLoS. Genet.* 2008; 4(1): e4

10. Tian C, Hinds DA, Shigeta R, Adler SG and others. A Genomewide Single-Nucleotide-Polymorphism Panel for Mexican American Admixture Mapping. *Am. J. Hum. Genet.* 2007; 80(6): 1014-1023

11. Tian C, Hinds DA, Shigeta R, Kittles R and others. A Genomewide Single-Nucleotide-Polymorphism Panel With High Ancestry Information for African American Admixture Mapping. *Am. J. Hum. Genet.* 2006; 79(4): 640-649

12. Choudhry S, Coyle N, Tang H, Salari K and others. Population Stratification Confounds Genetic Association Studies Among Latinos. *Human Genetics* 2006; 118(5): 652-664

13. Seldin MF, Shigeta R, Villoslada P, Selmi C and others. European Population Substructure: Clustering of Northern and Southern Populations. *PLoS. Genet* 2006; 2(9): e143

14. Tian C, Hinds DA, Shigeta R, Kittles R and others. A Genomewide Single-Nucleotide-Polymorphism Panel With High Ancestry Information for African American Admixture Mapping. *Am J Hum Genet* 2006; 79(4): 640-649

15. Helgason A, Yngvadottir B, Hrafnkelsson B, Gulcher J and others. An Icelandic Example of the Impact of Population Structure on Association Studies. *Nat Genet* 2005; 37(1): 90-95

16. Campbell CD, Ogburn EL, Lunetta KL, Lyon HN and others. Demonstrating Stratification in a European American Population. *Nat Genet* 2005; 37(8): 868-872

17. Carlson CS, Eberle MA, Kruglyak L, and Nickerson DA. Mapping Complex Disease Loci in Whole-Genome Association Studies. *Nature* 2004; 429(6990): 446-452

18. Fernando RL, Nettleton D, Southey BR, Dekkers JC and others. Controlling the Proportion of False Positives in Multiple Dependent Tests. *Genetics* 2004; 166(1): 611-619

19. Huttley GA, Smith MW, Carrington M, and O'Brien SJ. A Scan for Linkage Disequilibrium Across the Human Genome. *Genetics* 1999; 152(4): 1711-1722

20. Reich DE, Cargill M, Bolk S, Ireland J and others. Linkage Disequilibrium in the Human Genome. *Nature* 2001; 411(6834): 199-204

21. Ardlie KG, Kruglyak L, and Seielstad M. Patterns of Linkage Disequilibrium in the Human Genome. *Nat. Rev. Genet.* 2002; 3(4): 299-309

22. Zondervan KT and Cardon LR. The Complex Interplay Among Factors That Influence Allelic Association. *Nat. Rev. Genet.* 2004; 5(2): 89-100

23. Clark AG. The Role of Haplotypes in Candidate Gene Studies. *Genet Epidemiol* 2004; 27(4): 321-333

24. Carlson CS, Eberle MA, Rieder MJ, Yi Q and others. Selecting a Maximally Informative Set of Single-Nucleotide Polymorphisms for Association Analyses Using Linkage Disequilibrium. *Am. J Hum. Genet.* 2004; 74(1): 106-120

25. Weale ME, Depondt C, Macdonald SJ, Smith A and others. Selection and Evaluation of Tagging SNPs in the Neuronal-Sodium-Channel Gene SCN1A: Implications for Linkage-Disequilibrium Gene Mapping. *Am J Hum Genet* 2003; 73(3): 551-565

26. Ke X and Cardon LR. Efficient Selective Screening of Haplotype Tag SNPs. *Bioinformatics.* 2003; 19(2): 287-288

27. Stram DO. Tag SNP Selection for Association Studies. *Genet Epidemiol.* 2004;

28. Liu Z, Lin S, and Tan M. Genome-Wide Tagging SNPs With Entropy-Based Monte Carlo Method. *J. Comput. Biol.* 2006; 13(9): 1606-1614

29. De Bakker PI, Burtt NP, Graham RR, Guiducci C and others. Transferability of Tag SNPs in Genetic Association Studies in Multiple Populations. *Nat Genet* 2006; 38(11): 1298-1303

30. Mueller JC, Lohmussaar E, Magi R, Remm M and others. Linkage Disequilibrium Patterns and TagSNP Transferability Among European Populations. *Am J Hum Genet* 2005; 76(3): 387-398

31. Redon R, Ishikawa S, Fitch KR, Feuk L and others. Global Variation in Copy Number in the Human Genome. *Nature* 2006; 444(7118): 444-454

32. Carson AR, Feuk L, Mohammed M, and Scherer SW. Strategies for the Detection of Copy Number and Other Structural Variants in the Human Genome. *Hum Genomics* 2006; 2(6): 403-414

33. Sharp AJ, Locke DP, McGrath SD, Cheng Z and others. Segmental Duplications and Copy-Number Variation in the Human Genome. *Am. J. Hum. Genet.* 2005; 77(1): 78-88

34. Tuzun E, Sharp AJ, Bailey JA, Kaul R and others. Fine-Scale Structural Variation of the Human Genome. *Nat. Genet.* 2005; 37(7): 727-732

35. Freeman JL, Perry GH, Feuk L, Redon R and others. Copy Number Variation: New Insights in Genome Diversity. *Genome Res.* 2006; 16(8): 949-961

36. Conrad DF, Andrews TD, Carter NP, Hurles ME and others. A High-Resolution Survey of Deletion Polymorphism in the Human Genome. *Nat. Genet.* 2006; 38(1): 75-81

37. Rich SS, Concannon P, Erlich H, Julier C and others. The Type 1 Diabetes Genetics Consortium. *Ann. N. Y. Acad. Sci.* 2006; 1079 1-8

38. Samani NJ, Erdmann J, Hall AS, Hengstenberg C and others. Genomewide Association Analysis of Coronary Artery Disease. *N. Engl. J. Med.* 2007; 357(5): 443-453

39. Genome-Wide Association Study of 14,000 Cases of Seven Common Diseases and 3,000 Shared Controls. *Nature* 2007; 447(7145): 661-678

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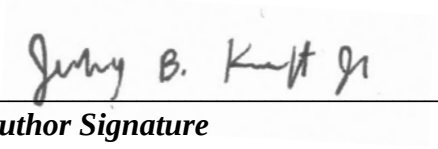

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