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Association Study of Serotonin Pathway Genes in Attempted Suicide

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Abstract

Epidemiological studies, such as family, twin, and adoption studies, demonstrate the presence of a heritable component to both attempted and completed suicide. Some of this heritability is accounted for by the presence of comorbid psychiatric disorders, but the evidence also indicates that a portion of this heritability is specific to suicidality. The serotonergic system has been studied extensively in this phenotype, but findings have been inconsistent, possibly due to the presence of multiple susceptibility variants and/or gene-gene interactions. In this study, we genotyped 174 tag and coding single nucleotide polymorphisms (SNPs) from 17 genes within the serotonin pathway on 516 subjects with a major mood disorder and a history of a suicide attempt (cases) and 515 healthy controls, with the goal of capturing the common genetic variation across each of these candidate genes. We tested the 174 markers in single-SNP, haplotype, gene-based, and epistasis analyses. While these association analyses identified multiple marginally significant SNPs, haplotypes, genes, and interactions, none of them survived correction for multiple testing. Additional studies, including assessment in larger sample sets and deep resequencing to identify rare causal variants, may be required to fully understand the role that the serotonin pathway plays in suicidal behavior.

Keywords

suicidal behavior; bipolar disorder; major depression

Introduction

Suicidal behavior, which includes both attempted and completed suicide, is a complex phenotype with both genetic and environmental risk factors (Mann et al., 2009). Family,

twin, and adoption studies estimate the heritability of suicidal behavior to be 30-50% (Mann et al., 2009; Brent and Mann, 2005; Brezo et al., 2008). The presence of psychiatric disorders, such as mood disorders and substance abuse, accounts for part of this heritability. Importantly, though, some of the heritability appears to be influenced by an independent factor that is specific to suicidality. This factor has been hypothesized to influence impulsive-aggression, with individuals having both this personality trait and a major mental illness having the greatest risk for suicidal behavior (Brent et al., 2003; Melhem et al., 2007).

Serotonin is a neurotransmitter that affects a range of physiologic functions such as emotion, cognition, sensory processing, motor function, pain, neuroendocrine systems, and circadian rhythm (Lucki, 1998). The serotonergic system was first implicated in the etiology of suicidal behavior by the finding of lowered levels of the serotonin metabolite 5-hydroxyindolacetic acid in the cerebrospinal fluid of suicide attempters (Asberg et al., 1976). Since then, the evidence for the involvement of this neurotransmitter and its pathway in the suicidality phenotype has grown and is now supported by results from multiple lines of investigation, such as postmortem brain analyses and pharmacological studies (Fergusson et al., 2005; Gunnell et al., 2005; Mann et al., 2000; Stockmeier, 2003).

Candidate gene association studies for suicidal behavior have focused primarily on serotonin pathway genes. Of particular interest have been the serotonin transporter gene (*SLC6A4*) and the tryptophan hydroxylase genes (*TPH1* and *TPH2*). However, the results for these genes – as well as for the remaining genes in the serotonin pathway – have been inconsistent, failing to definitively answer whether genetic variation within them is significantly associated with suicidal behavior (Brezo et al., 2008; Bondy et al., 2006; Mann, 2003).

The strong neurobiologic evidence implicating the serotonergic system in suicidality has led us to question whether multiple common risk alleles, as well as interactions between them, may be contributing to the inconsistencies in the genetic studies. In an effort to broadly interrogate the serotonin pathway and to test this hypothesis directly, we genotyped 174 tag and coding SNPs from 17 serotonergic genes and tested the resulting genotype data for evidence of association and epistasis in 516 attempted suicide cases (subjects with a major mood disorder and a history of attempted suicide) and 515 normal controls.

Materials and Methods

Sample

Our sample included 516 cases who were previously ascertained as part of one of three different studies: the Chicago, Hopkins, NIMH Intramural Program (CHIP) bipolar disorder study (Zandi et al., 2007), the Genetics of Recurrent Early-Onset Depression (GenRED) study (Levinson et al., 2003), or the National Institute of Mental Health (NIMH) Genetics Initiative Bipolar Disorder Collaborative study waves 1-4 (Genomic survey of bipolar illness in the NIMH genetics initiative pedigrees: A preliminary report. 1997). Methods for collecting and diagnosing subjects in these studies have been described in detail in the initial study reports. Briefly, all subjects were assessed with either the Diagnostic Interview for Genetic Studies (DIGS) (Nurnberger et al., 1994) or the Schedule for Affective Disorders

and Schizophrenia (SADS) (Endicott and Spitzer, 1978), and family informant data and medical records were obtained. Diagnoses were assigned following a best-estimate procedure according to RDC, DSM-III-R, or DSM-IV criteria. All subjects provided IRB-approved, written, informed consent. To ensure the quality of the phenotypic variables, we used the Bipolar Disorder Phenome Database (Potash et al., 2007), which contains the most up-to-date and accurate phenotypic information on the subjects from the CHIP and NIMH genetic studies.

The cases for this analysis had a history of both mood disorder and suicidality. Mood disorders include bipolar I disorder (BPI), bipolar II disorder (BPII), schizoaffective disorder (SABP), or recurrent major depression (MDDR). Suicidality is defined as a self-reported history of at least one suicide attempt. One case per pedigree was selected from the larger family samples described above. To reduce confounding from population stratification, we included in the analyses only unrelated subjects who identified themselves as Caucasians of European origin.

The 515 control subjects were obtained from the NIMH Genetics Initiative repository (<https://nimhgenetics.org/>). These subjects were originally recruited by Knowledge Networks, Inc. (Menlo Park, CA) from participants in a nationally representative marketing panel (Sanders et al., 2008). Subjects provided informed consent for genetic and clinical information to be used for any medical research, knowing that all samples would be fully anonymized. All control subjects completed an online version of the Composite International Diagnostic Interview-Short Form (CIDI-SF), which diagnoses common mood, anxiety and substance use disorders (Kessler et al., 1998). The CIDI-SF was supplemented by questions about any history of schizophrenia, psychosis or bipolar disorder. Completion of these supplemental questions was required for inclusion in the study.

For the current project, we included only unrelated control subjects who 1) denied a history of major depression, bipolar disorder, psychosis, and schizophrenia; 2) did not meet criteria for alcohol or substance dependence; and 3) were 21 or older at the time of the interview. The CIDI-SF did not specifically address suicide attempts, but subjects were asked in the depression section if they thought frequently about death. We excluded subjects who endorsed this item, since it likely encompasses the construct of suicidality. Our exclusion criteria should have eliminated most of the potential suicide attempters from the control sample since ~89.5% of people who attempt suicide have had a depressive episode or alcohol abuse or dependence (Suominen et al., 1996). Cases and controls were matched for race/ethnicity and sex.

SNP selection and Genotyping

Seventeen genes from the serotonergic pathway were selected for genotyping, including the serotonin transporter (*SLC6A4*), monoamine oxidase A (*MAOA*), tryptophan hydroxylase 1 and 2 (*TPH1* and *TPH2*), and 13 serotonin receptor genes (see Table I for a complete list). We chose genes in the serotonin pathway largely based on a literature review (Brezo et al., 2008; Bondy et al., 2006; Mann, 2003) of the serotonergic system's role in suicide. In addition to the commonly studied candidate genes, we supplemented our list with neuronally expressed genes encoding serotonin receptors.

We used Tagger's pairwise tagging approach (de Bakker et al., 2005) to select tag SNPs that represent common genetic variation across all of the genes (RefSeq transcripts $\pm$ 5kb). Using the CEU sample and the release 22 version of the HapMap database, we required an r^2 of 0.8 and a minor allele frequency (MAF) of at least 0.05. Some additional SNPs were included if they increased coverage, particularly on *TPH2*. The SNP coverage for each gene is described in Table I.

Despite restricting the sample to those subjects who self-reported their race as Caucasian, we remained concerned with the potential for false-positive and or false-negative associations due to the population structure that exists among European-Americans. Rather than existing as a homogeneous group, the genetic variation in European-Americans roughly corresponds to a gradient running from northwest to southeast Europe, with Ashkenazi Jewish ancestry adding an additional source of variation (Price et al., 2008). Following the guidelines described in Price et al, we genotyped an additional 294 ancestry informative markers (AIMs), chosen based on their ability to differentiate the European-American ancestries. We used these AIMs in a principal components analysis to correct for any population stratification within our sample. Genotyping for both the serotonin SNPs and the AIMs markers was conducted at the Center for Inherited Disease Research (CIDR) (<http://www.cidr.jhmi.edu/>) using the Illumina Golden Gate Assay (San Diego, CA).

Analytic Methods

We used PLINK (<http://pngu.mgh.harvard.edu/~purcell/plink/>) to perform SNP quality control (Purcell et al., 2007). This consisted of dropping SNPs with minor allele frequency 1%, missing data rate 5%, or Hardy Weinberg equilibrium (HWE) p-value $< 10^{-6}$ in the control population. We also tested whether missing data rates differed between cases and controls. We used the program Haploview (Barrett et al., 2005) to evaluate pair-wise r^2 between SNPs and to determine the haplotype-block structure for each gene (Gabriel et al., 2002). Finally, we ran a principal components analysis using the software program EIGENSTRAT (Price et al., 2006) on the AIMs described above. Although this analysis suggested that population stratification in our sample was negligible, we identified two principal components that we included as a covariate in the main association analysis to examine whether they impacted our results (Supplementary Figure 1).

For our main analysis, we tested single SNPs using PLINK's case/control association test, which estimates odds ratios based on allele counts. We additionally examined logistic regression models, which allow for the inclusion of covariates. We adjusted these models for age at interview, which differed between the two sample sets (40.9 in the cases, 52.9 in the controls; $t = 13.0$, $p < 0.001$). Although cases and controls were matched on gender and ethnicity, we also included terms for sex and two principal components. These additional terms (age, sex, and principal components) did not significantly alter the results; therefore, we reported only the results from the case/control association test without the covariates. For this single SNP analysis, we required a p-value of 0.00029 ($0.05 \div 174$, representing a Bonferroni correction for the 174 markers) for an association to be considered significant.

In addition to the single SNP analysis described above, we also tested haplotypes for association with suicidality. We analyzed haplotypes of two and three-locus combinations as

defined by a sliding window approach. For example, a three-locus sliding haplotype examines SNP configurations 1-2-3, 2-3-4, 3-4-5, etc. This approach captures all two-SNP and three-SNP haplotypes across each gene in the dataset. For this analysis, SNPs were ordered according to their physical map locations (NCBI Build 36).

Additionally, we explored a multi-locus approach as implemented in PLINK to test gene-based SNP sets. This set-based approach summarizes the effect of the most significant tag SNPs across a gene and accounts for the LD between markers by dropping SNPs that exceed a user-specified r^2 value with a SNP that has already been selected for inclusion in the analysis, making the SNPs chosen for analysis independent of each other. The set-based statistic is calculated as the mean of the single SNP statistics (odds ratios in this case). Empirical p-values for each gene are generated based on a phenotype permutation procedure. We utilized the default parameters for this analysis: $r^2 = 0.5$, p-value = 0.05, max number of SNPs = 5.

Finally, we tested for pairwise multiplicative SNP-SNP interactions (174 SNPs $\times$ 174 SNPs) using the fast-epistasis routine in PLINK (Purcell et al., 2007). This test takes a 3-by-3 table of all possible genotype combinations across the two loci and twice collapses it into a 2-by-2 table of allele categories such that the allele represents the unit of analysis. Odds ratios for the association between the alleles at the two loci and their standard errors are estimated separately for cases and controls. A test statistic is then calculated based on a z-score of the differences between the two odds ratios. This is an efficient method that can be used with large-scale case-control association studies, and it has been shown to correlate highly ($r = 0.995$) with a more computationally intensive logistic regression approach in which a multiplicative interaction term between the two loci is tested.

We then used a permutation procedure to determine if the interactions were significant in the context of multiple testing. To do this, we randomly permuted the case-control labels to generate 1000 datasets (that maintained the original genotypes for each individual) and re-ran the epistasis test. The proportion of tests using the permuted datasets that reached a higher level of significance than what we observed in the original dataset comprised the empirical p-value to estimate the level of significance of our results.

Power

For the association (single SNP) analysis, we estimated there was 80% power in our sample of 516 cases and 515 controls to detect an association of moderate effect (odds ratio = 1.55) according to the genetic power calculator program QUANTO (Gauderman, 2002). This estimation assumes an additive model, an attempted suicide rate of 5%, $\alpha = 0.00029$ ($0.05 \div 174$, representing a Bonferroni correction for the 174 markers), and a MAF of 0.25 (average MAF for our SNPs = 0.23).

Results

Our 516 attempted suicide cases with mood disorders came from three studies: GenRED (242), NIMH-BP (201), and CHIP (73). Most cases were diagnosed with MDDR (46.7%) or BPI (47.3%). Other diagnoses included BPPII (2.9%) and SABP (3.1%). Minor allele

frequencies in our SNPs ranged from 2.9% to 50%. All SNPs were in HWE ($p=0.0083$ to 1.00) in the control subjects. Missing data rates for the SNPs ranged from 0% to 1.36%. There was no difference in the missing data rates between cases and controls ($p=0.11$ to 1.00). The genes we have analyzed in the serotonin pathway are described in Table I and depicted in Figure 1.

We tested all 174 SNPs in 17 genes for evidence of association (Table II and Supplementary Table I and II). The single SNP analysis identified multiple SNPs with modest evidence for association (best SNP in *HTR7*: rs10509608, $p=0.0074$). Haplotype analyses also identified several genes with modest evidence for association (Supplementary Table III). However, none of the results from the single SNP analysis or the haplotype analysis survived correction for multiple testing.

We also conducted two exploratory analyses aimed at identifying multiple common risk alleles (using the set-based approach) and evidence of epistasis (by testing for SNP-SNP interactions). The set-based analysis utilized a two-stage SNP selection process, based first on significance level ($p\text{-value} < 0.05$) and second on linkage disequilibrium ($r^2 < 0.5$). However, only 11 SNPs in eight genes exceeded the specified significance level, and only one of these eight genes incorporated more than one SNP after accounting for the LD between SNPs (*HTR7*: rs10509608, rs11186299, empirical $p\text{-value} = 0.092$). We also tested for epistasis between the SNPs within all of the 17 genes (Supplementary Table IV). Using this approach, *HTR7* served as an interacting partner in the four most significant interactions (with *HTR2A*, *HTR3A*, and twice with *HTR5A*). However, permutation analyses suggested that these SNP-SNP interaction results could have occurred by chance alone ($p > 0.1$).

Discussion

The serotonergic system has been linked to suicidal behavior through multiple lines of evidence, and this has led researchers to focus on genes within the serotonin pathway in an attempt to identify causal variants that predispose patients to the suicide phenotype. In this study, we analyzed 174 tag and coding SNPs in 17 genes from the serotonin pathway. The results from our investigation of the main (single SNP) and joint (haplotype, set-based, and epistasis) effects of these SNPs included some nominally significant association signals, none of which survived corrections for multiple testing.

In the past, the relatively small sample collections and sparse gene coverage made it difficult to consistently and conclusively demonstrate association between serotonin pathway genes and suicidality. The current project has assembled a sizable number of cases ($N=516$), and has captured on average 89% of the common variation in each of the 17 genes (including alternative transcripts) within the serotonin pathway, permitting us to conduct both primary association analyses and secondary analyses aimed at identifying multiple susceptibility variants within a given gene and at identifying gene-gene interaction within the pathway. Although our results were not statistically significant after appropriate correction, the gene-based and epistasis analyses represent novel contributions to the study of this important pathway in suicidal behavior and serve to highlight the potential complexity that may underlie the role of the serotonergic system in this phenotype.

The samples used in this study were ascertained through three separate initiatives (NIMH, GenRED, and CHIP samples). While combining the sample sets conferred additional power, it also had the potential to introduce heterogeneity into the study. However, we felt that combining these three samples was appropriate since they employed similar recruitment and ascertainment strategies. Furthermore, we compared the SNP allele frequencies using a χ^2 test with two degrees of freedom and found the frequencies to be very similar across studies. All but three SNPs had non-significant ($p>0.05$) χ^2 values while the remaining three SNPs showed only minor differences across samples ($p=0.03-0.003$).

In this study, we conceptualized suicidality as any history of any suicide attempt. However, many alternative definitions of the attempted suicide phenotype exist. It is possible that genetic variation in one or more of these serotonin candidate genes increases the risk for suicidal behavior only in a subset of the samples (for example, in subjects with high-lethality attempts), which would have decreased our ability to identify evidence for association. However, these narrower definitions greatly restricted our sample size, making these analyses cost-prohibitive in terms of power.

Our study should be viewed in light of several limitations. First, while the sample size was considerable, it nonetheless lacked the power to detect common variants of small effect ($OR < 1.55$). Second, the study was designed to test for association with common variants across each of these 17 genes. Thus, we did not directly test for association with rare variants, functional variants, or copy number variants. Third, our decision to focus on the 17 genes themselves and their adjacent genomic sequences may have caused us to miss long-range regulatory elements that could influence the expression of these genes and their impact on the attempted suicide phenotype. Fourth, data on environmental risk factors, such as abuse and other forms of childhood adversity, were not available for the subjects included in this study, so we were unable to test for evidence of gene-environment interaction, the presence of which may have increased the evidence for association.

The Psychiatric GWAS Consortium (PGC) is conducting a large meta-analysis using the attempted suicide phenotype and GWAS sample sets from multiple disorders, such as bipolar disorder, major depression, and schizophrenia (Psychiatric GWAS Consortium Coordinating Committee et al., 2009; Cross-Disorder Phenotype Group of the Psychiatric GWAS Consortium et al., 2009). This analysis is projected to confer dramatically increased power and may provide further evidence for association for genetic variants in the serotonin pathway.

Our study was designed to provide a systematic query of the relationship between suicidal behavior and genetic variation within the 17 serotonergic genes. These analyses do not provide support for the hypothesis that common variants in the serotonin pathway increase the risk for attempted suicide. The continued lack of conclusive findings argues for further analyses of the attempted suicide phenotype in large and densely genotyped samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Diagram of serotonergic neurotransmission highlighting the role of the 17 serotonin candidate genes. 5HT = serotonin

Table 1

Gene Descriptions

Gene	Serotonin Function	Location	Size (+/-5kb)	SNPs Genotyped	Total # SNPs in Gene	% of Gene Captured
HTR1A	receptor	5q11.2-q13	11268	2	4	100%
HTR1B	receptor	6q13	11172	8	16	93%
HTR1D	receptor	1p36.3-p34.3	12834	1	5	80%
HTR1E	receptor	6q14-q15	88987	13	44	90%
HTR2A	receptor	13q14-q21	72662	35	127	84%
HTR2B	receptor	2q36.3-q37.1	26869	4	10	90%
HTR2C	receptor	Xq24	336073	6	192	89%
HTR3A	receptor	11q23.1	25124	11	25	96%
HTR3B	receptor	11q23.1	51694	10	29	93%
HTR4	receptor	5q31-q33	213146	23	174	90%
HTR5A	receptor	7q36.1	24913	7	41	92%
HTR6	receptor	1p36-p35	24275	4	10	90%
HTR7	receptor	10q21-q24	127095	13	79	98%
MAOA	degradation	Xp11.3	100659	4	39	82%
SLC6A4	transport	17q11.1-q12	47809	5	25	84%
TPH1	synthesis	11p15.3-p14	30251	11	25	96%
TPH2	synthesis	12q21.1	103595	17	128	71%

Gene size is based on the RefSeq (hg18) gene definitions +/- 5kb. The total number of SNPs in each gene is based on Haploview, using the CEU population and build 22. Since many of our SNPs are tag SNPs, we were able to capture a higher percentage of the gene through LD than just what was genotyped. We based coverage of these tag SNPs on an $r^2 \geq 0.80$.

Table II

Single-SNP Association Analysis

Gene	Best SNP	MAF cases	MAF controls	OR	95% CI	P	Corrected P
HTR1A	rs6295	0.4738	0.5000	0.90	0.76-1.07	0.2347	1.0000
HTR1B	rs130058	0.3291	0.2854	1.23	1.02-1.48	0.0316	0.9963
HTR1D	rs604030	0.3595	0.3660	0.97	0.81-1.16	0.7580	1.0000
HTR1E	rs10944288	0.3265	0.3502	0.90	0.75-1.08	0.2567	1.0000
HTR2A	rs7984966	0.2733	0.2427	1.17	0.96-1.43	0.1130	1.0000
HTR2B	rs1549339	0.3246	0.3058	1.09	0.91-1.31	0.3586	1.0000
HTR2C	rs10875535	0.06833	0.04649	1.50	1.01-2.24	0.0436	0.9996
HTR3A	rs11604247	0.1047	0.07379	1.47	1.08-2.00	0.0140	0.9135
HTR3B	rs17614942	0.04264	0.06117	0.68	0.46-1.02	0.0579	1.0000
HTR4	rs13166761	0.2946	0.3447	0.79	0.66-0.96	0.0147	0.9245
HTR5A	rs6320	0.2946	0.3165	0.90	0.75-1.09	0.2797	1.0000
HTR6	rs3790756	0.1647	0.1243	1.39	1.09-1.78	0.0090	0.7923
HTR7	rs10509608	0.1647	0.1233	1.40	1.09-1.80	0.0074	0.7248
MAOA	rs3027399	0.08728	0.06717	1.33	0.94-1.87	0.1051	1.0000
SLC6A4	rs12150214	0.1676	0.2019	0.80	0.64-0.99	0.0448	0.9997
TPH1	rs17794760	0.1928	0.1641	1.22	0.97-1.53	0.0882	1.0000
TPH2	rs4430554	0.3804	0.3385	1.20	1.00-1.44	0.0481	0.9998

Odds Ratios (ORs) describe the additive effects of the minor allele such that ORs above 1 represent an increased risk from the minor allele and ORs below 1 represent a protective effect of the minor allele. Asymptotic p-values are based on the t-statistic. Corrected p-values are based on the Bonferroni formula: $P_{\text{corrected}} = 1 - (1 - P_{\text{uncorrected}})^n$, where n = the number of hypotheses tested = 174.