Accurate, Large-Scale Genotyping of 5HTTLPR and Flanking Single Nucleotide Polymorphisms in an Association Study of Depression, Anxiety, and Personality Measures

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Background: The length polymorphism repeat in the promoter region of the serotonin transporter gene (5HTTLPR) is one of the most studied polymorphisms for association with a range of psychiatric and personality phenotypes. However, the original 5HTTLPR assay is prone to bias toward short allele calling.

Methods: We designed new assays for the 5HTTLPR suitable for large-scale genotyping projects and we genotyped 13 single nucleotide polymorphisms (SNPs) in a 38-kilobase region around the 5HTTLPR, including SNP rs25531, a polymorphism of the 5HTTLPR long allele. Association analysis was conducted for major depression and/or anxiety disorder in unrelated cases ($n = 1161$) and control subjects ($n = 1051$) identified through psychiatric interviews administered to a large population sample of Australian twin families. Participants had been scored for personality traits several years earlier ($n \geq 2643$ unrelated individuals).

Results: We identified a two-SNP haplotype proxy for 5HTTLPR; the CA haplotype of SNPs rs4251417 and rs2020934 is coupled with the short allele of 5HTTLPR ($r^2 = .72$). We found evidence for association ($p = .0062$, after accounting for multiple testing) for SLC6A4 SNPs rs6354 and rs2020936 (positioned in a different linkage disequilibrium [LD] block about 15.5 kb from 5HTTLPR) with anxiety and/or depression and neuroticism, with the strongest association for recurrent depression with onset in young adulthood (odds ratio = 1.55, 95% confidence interval = 1.16–2.06).

Conclusions: The associated SNPs are in the same LD block as the variable number of tandem repeats serotonin transporter intron 2 marker, for which association has previously been reported.

Key Words: 5HTTLPR, anxiety, depression, extraversion, genetic association, harm avoidance, neuroticism, serotonin transporter, SLC6A4, SNP

Serotonergic neurotransmission impacts on a wide range of behaviors, including cognition and emotion (1,2), and drugs targeting serotonin reuptake are clinically effective antidepressants (3). As a result, one of the most studied polymorphisms for association with a broad range of psychiatric and personality phenotypes is the length polymorphism repeat (LPR) in the promoter region of the serotonin transporter gene (5HTT renamed SLC6A4) (5HTTLPR). The 5HTTLPR polymorphism comprises a 43-base pair (bp) (4–8) insertion or deletion (long, "L," with 16 repeat units or short, "S," with 14 repeat units, alleles, respectively). The S allele (frequency in Caucasians ~.45 [9]) reduces transcriptional efficiency, resulting in decreased SLC6A4 expression and function (10). Association studies and subsequent meta-analyses (Table 1) (11–15) have shown conflicting results in support of an association between the S allele and anxiety, depression, and the personality trait neuroticism (a measure of emotional stability that is genetically correlated to both anxiety and depression [16–18]). Conflicting results have dogged candidate gene association studies for many complex disorders, attributable to small sample sizes of both primary and replication studies, heterogenous subject populations, association dependent on environmental conditions such as stressful life events (19), and differing instruments for assessment of phenotypic traits and statistical methods (e.g., [20,21]). However, an additional problem specific to 5HTTLPR relates to the genotyping assay, which has caused considerable bias toward S allele identification (22,23). Furthermore, association may have been compromised by the presence of an A/G single nucleotide polymorphism (SNP), rs25531, that lies within the L allele of 5HTTLPR (5,8); the L allele with the rarer G allele of rs25531 (denoted L_G) is functionally equivalent to the S allele because of changes to the activating protein 2 (AP2) transcription factor binding site altered by this SNP (5,8).

The aim of this study was to investigate the association between the 5HTTLPR polymorphism, including the rs25531 polymorphism, and psychiatric and personality phenotypes in a large cohort. As part of our study, we designed new assays for the 5HTTLPR suitable for large-scale genotyping projects and we genotyped tagging SNPs in a 38-kilobase (kb) region around the 5HTTLPR (Figure 1A) to determine whether any SNP or combination of SNPs could be used as proxies for the difficult and

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Table 1. Summary of Meta-Analyses of Association Studies of 5HTTLPR in Major Depression, Anxiety Disorders, and Neuroticism

Phenotype	Comparison	Association (95% CI)	Number of Studies	Description	Reference
Major Depression	S vs. L	OR = 1.11 (1.04–1.19)	24	3752 cases	(11)
	SS vs. LL	OR = 1.39 (1.21–1.61)		5707 control subjects	
Neuroticism	SS or SL vs. LL	$d = -.22$ (−.44 to −.01)	12	3247 individuals	(12)
Neuroticism	S vs. L	ns: $p = .81$	1[a]	768 individuals selected from the 5% tails of the neuroticism distribution from a population sample of 88,142	(13)
		ns: $p = .47$		4175 selected for extreme neuroticism from population sample of 20,921	
Panic Disorder	S vs. L	OR = .91 (.80–1.03)	10	1025 cases 1568 control subjects	(14)
OCD	SS vs. LL	OR = 1.21 (1.10, 1.45)	13	1242 cases	(15)
	SL vs. LL	OR = .79 (.64, .98)		2203 control subjects	
	S vs. L	OR = 1.01 (.88, 1.03)			
Harm Avoidance	SS or SL vs. LL	$d = -.05$ (−.66 to .56)	13	2521 individuals	(12)

5HTTLPR, length polymorphism repeat (LPR) in the promoter region of the serotonin transporter gene; CI, confidence interval; *d*, standardized mean difference; L, long allele; ns, not significant; OCD, obsessive-compulsive disorder; OR, odds ratio; S, short allele.

[a]Large single study published subsequent to the meta-analysis of neuroticism.

time-consuming 5HTTLPR assay. Our study design allows us to examine association in multiple traits within the same cohort and consistency across independent subcohorts. Within our study sample, we can identify subsets of cases that are predicted to be genetically more homogenous. For example, the relative risk (RR) to first-degree relatives is reported to be higher for recurrent depression (RR ≈ 4–5) (reviewed in [24]) compared with major depression (RR ≈ 2–3). Similarly, although estimates for RR are not available, anxiety comorbid with depression is also considered a genetically more homogeneous group (reviewed in [25]). The optimum balance of sample size versus sample homogeneity cannot be predicted since the true genetic etiology is unknown. However, the availability of a depth of phenotypic information allows us to investigate associations through case subsets.

Methods and Materials

Samples

All participants were adult twins and their families recruited through the Australian Twin Registry. They provided informed consent under study protocols approved by the Queensland Institute of Medical Research and Washington University Human Research Ethics Committees.

During the period 1988 to 1990, study participants from two twin birth cohorts (born 1890–1964 and 1964–1971, respectively) were mailed an extensive Health and Lifestyle Questionnaire (1989 Questionnaire Survey [26,27]). This included the shortened, revised 48-item Eysenck Personality Questionnaire (EPQ) (28) and a short-form, 54-item version of the Temperament and Character Inventory (26,29), the Temperament Personality Questionnaire (TPQ). The EPQ measures four dimensions of personality, including neuroticism and extraversion, and the TPQ measures three dimensions of temperament, including harm avoidance. Between 1992 and 1994, twins from the older cohort ($n = 5995$) (30) were interviewed by telephone using the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA) instrument. The SSAGA is a comprehensive psychiatric interview that was designed to assess psychiatric disorders in adults (31) according to DSM-III-R but subsequently updated to DSM-IV criteria (32) and modified for use as a telephone survey instrument in Australia (SSAGA-OZ). Of these participants, $n = 4597$ have subsequently provided a blood sample (or rarely a buccal or saliva sample) for genotyping (33). Over the period 1996 to 1969, sibling pairs participating in the 1989 Questionnaire Survey that were either concordant or discordant for extreme EPQ neuroticism scores (one sibling in the top or bottom decile, the other sibling in the top or bottom quintile, and allowing inclusion of multiple siblings) were recruited (35–37). Participants ($n = 2456$) completed the shortened Composite International Diagnostic Interview (CIDI) (34), which provides DSM-IV (35) lifetime diagnoses of depression (including recurrent major depression) and anxiety disorders. This extreme discordant and concordant (36) design is a cost-efficient strategy for obtaining an informative dataset for genetic studies (37). Of these participants, 2213 provided DNA samples, plus 837 parents. Full details of the recruitment procedure for the study, including response rates and incidence of DSM-IV diagnoses for anxiety- and depression-related disorders, are given elsewhere (38–40). Finally, between 2001 and 2005, some participants from the earlier studies were re-interviewed using an adaptation of the SSAGA (see [41,42] for details). Participants reported ancestry of all four grandparents, and a small number with two or more grandparents with known non-European ancestry were excluded from the analysis. Selection of individuals used in the association analyses are described in the Association Analysis section below.

Genotyping

Genomic DNA was extracted from blood (or buccal) samples using standard protocols (43). Samples were plated in 384-well plates in two study sample sets comprising 1) monozygotic (MZ) and dizygotic (DZ) twins from the 1992 to 2000 SSAGA interview studies and 2) participants and their parents from the CIDI interview. Some 764 DNA samples are included in both sets, providing an opportunity for extensive quality control checking.

5HTTLPR Assay

The original assay (44) for the 5HTTLPR used polymerase chain reaction (PCR) primers in the nonrepetitive sequences that flanked the 16 repeat elements, which are each comprised of between 19 bp and 23 bp (10,44) (Figure 1B). This assay proved less than ideal: the PCR is difficult because of the very high guanine-cytosine (GC) content and the long length of the PCR products. In pilot studies, we found that these difficulties cause considerable bias toward S allele identification; the L allele signal is weak, so heterozygotes are frequently misscored as S homozygotes (e.g., see Figure 1D of [44]). This observation has also been

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