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RAPID COMMUNICATION

A Family-Based Analysis of Whether the Functional Promoter Alleles of the Serotonin Transporter Gene *HTT* Affect the Risk for Alcohol Dependence

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A population association between a regulatory variation in the promoter of the serotonin transporter gene (*HTT*) and severe alcohol dependence was recently reported. We analyzed this potential association in a large number of systematically ascertained families in the United States; these families had at least three first-degree relatives who were alcohol-dependent. Analyses focused on individuals defined as alcohol-dependent by criteria from ICD-10 and on subsets of these individuals reporting withdrawal-related symptoms. Application of the transmission disequilibrium test did not provide support for either linkage or association between this functional polymorphism and alcohol dependence; there was no significant bias in the transmission of either allele to the alcohol-dependent offspring. We also report that African Americans differ from Caucasians in allele frequencies for this polymorphism.

Key Words: Serotonin Transporter, Functional Promoter Polymorphism, Alcoholism, Association Study, Linkage Analysis.

THE SEROTONIN (5-hydroxytryptamine; 5-HT) transporter functions in the reuptake of 5-HT into presynaptic neurons, thus helping to terminate serotonergic responses.[1] Serotonin plays a role as a neurotransmitter in many functions. Low levels of platelet imipramine binding, reflecting reduced numbers of serotonin transporters, have been reported in patients with depression.[1,2] The serotonin transporter is a major target for antidepressant drugs, including fluoxetine and imipramine,[1,3,4] which are effective against a wide range of disorders.[5] It is also a target for important drugs of abuse, including cocaine and amphetamines.[3]

The gene encoding the serotonin transporter (called *HTT*, *5-HTT*, or *SLC6A4*) was cloned[6] and mapped to human chromosome 17q11.2.[3,7] Differences in the coding region of the gene have been sought in patients with obsessive-compulsive disorder, unipolar and bipolar depression, and mood disorders, but no coding polymorphisms were found.[8–10] A functional polymorphism (5-HTTLPR) was, however, reported in the *HTT* promoter.[11] The region between bp −1376 and bp −1027 contains 16 tandem repeats of a 20 to 23 bp G+C-rich sequence. Two common forms of this region were found in a Caucasian population: a 528 bp allele with 16 repeats ("long" allele) and a 484 bp allele ("short" allele) with a deletion of 44 bp extending from bp −1255 to bp −1212. The frequency of the 484 bp allele was reported to range from 39.4% to 46.3% in Caucasian populations.[11–16] The 528 bp allele of the *HTT* promoter was 2- to 3-fold more active in driving transcription than the 484 bp allele in transfection experiments.[11,12] Analysis of lymphoblastoid cell lines from individuals of different genotypes showed that the mRNA levels for the endogenous *HTT* gene in cells homozygous for the 528 bp allele was 1.4- to 1.7-fold higher than in cells with either 528 bp/484 bp or 484 bp/484 bp genotypes.[12] 5-HT uptake was twice as high in lymphoblastoid cells from individuals homozygous for the 528 bp allele as in cells from individuals of the other genotypes.[12] This pattern of activity suggests dominance of the 484 bp allele for low expression.

The potential role that this functional polymorphism in *HTT* may play in personality traits and psychiatric diseases has been examined. In one study, the 484 bp variant of the *HTT* promoter was associated with an increase in anxiety-related traits, including the temperament of harm avoidance.[12] However, a study in a different population did not provide any evidence that this polymorphism was associ-

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ated with the anxiety-related temperament of harm avoidance.[14] An association of this promoter polymorphism with susceptibility to affective disorders has been reported, although, in this case, the low activity 484 bp allele appeared to act, at least in part, recessively.[15] A VNTR in the second intron of the *HTT* gene was also reported to be associated with susceptibility to major depression,[17] but the latter finding was not replicated in two different populations[18,19] or in a sample of individuals with bipolar disorder.[20,21] A different polymorphism in the 3′ end of the *HTT* gene did not show linkage with bipolar disorder.[22] The *HTT* promoter polymorphism did not show association with panic disorder.[16]

Low serotonergic activity has been suggested to increase vulnerability to alcohol dependence in part by affecting harm avoidance.[23] Thus, genes relating to serotonergic function, including *HTT*, are reasonable candidate genes for involvement with alcoholism. Abstinent alcoholics have been reported to have an enhanced uptake of serotonin into lymphocytes, which express *HTT*.[24] The potential interest in *HTT* was increased by the report that *Alcp2*, a quantitative trait locus for alcohol preference in mice that acts only in females and shows parent-of-origin effects (affecting only females with a C57BL/6 father), maps to a region that contains the mouse serotonin transporter gene *Htt*.[25] A recent population-based association study suggested that the phenotype of severe alcoholism marked by withdrawal seizure or delirium was associated with the 5-HTTLPR promoter polymorphism.[13] There was an excess of 484 bp alleles in the alcoholics, compared with the population controls (nominal $p = 0.049$), due to an excess of the homozygous 484 bp genotype ($p = 0.035$), suggesting a recessive effect,[13] in contrast to the dominant low activity previously reported for that allele.[12] Because the a priori rationale for associating the serotonin transporter with severe withdrawal symptoms is not clear, this marginally significant effect would fall below usual levels of significance if corrected for the testing of multiple alcoholism-related phenotypes.

This article reports the results of a family-based analysis testing the hypothesis that differences in the *HTT* promoter are associated with increased risk for alcohol dependence. Family-based studies avoid the potential problems of population stratification. The sample used in this study, collected by the Collaborative Study on the Genetics of Alcoholism (COGA),[26] consisted of systematically ascertained families with multiple alcoholic members. Families with multiple affected individuals are more likely to have an increased number of genetic risk factors for alcoholism.

SUBJECTS AND METHODS

Ascertainment and Assessment of Subjects

The COGA systematically recruited probands meeting both DSM-III-R criteria for alcohol dependence[27] and the Feighner criteria for definite alcoholism.[28] Probands were excluded if they were intravenous drug users, or if they had AIDS, a severe head injury, or a life-threatening illness that was not alcohol-related. Families were accepted into the study if they included a sibship of at least three and parents were available (or larger sibships if parents were not available). Family history data were used to systematically extend pedigrees through affected first-degree relatives, or to a secondary branch if at least two relatives in that part of the pedigree were affected. All available family members were interviewed using the Semi-Structured Assessment for the Genetics of Alcoholism[29–31] to assess adult lifetime psychiatric status. Major psychiatric disorders, including substance use disorders, were assessed in relatives using a structured family history interview.[32] The study was approved by the appropriate institutional review boards of participating institutions, and informed consent was obtained.

A subset of families in which the proband and at least two first-degree relatives were alcohol-dependent (as determined by personal interview), and at least one parent was unaffected, was chosen for further study. From these families, individuals were chosen for genotyping based on the pedigree structure. Parents were included when DNA was available to allow determination of identity by descent; otherwise, additional family members were genotyped to allow inference of identity by descent.

Genotyping was conducted on 987 individuals selected from 105 pedigrees. Of these, 764 individuals in 86 families were non-Hispanic Caucasians. The genotyped individuals included 267 diagnosed as alcohol-dependent by criteria from ICD-10,[33] 214 of whom were non-Hispanic Caucasians.

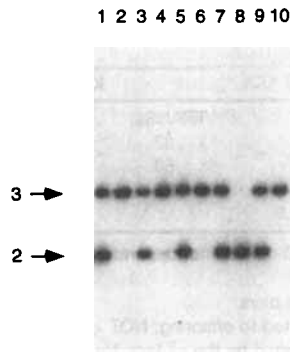

Fig. 1. Detection of the 528 bp and 484 bp alleles. Autoradiogram of a denaturing 6% polyacrylamide gel, showing the 484 bp fragment (allele 2) and the 528 bp fragment (allele 3). Results from 10 individuals are shown. Lanes 1, 3, 5, 7, and 9: heterozygous 2/3 (484 bp/528 bp) genotype; lanes 2, 4, 6, and 10: homozygous 3/3 (528 bp/528 bp) genotype; lane 8: homozygous 2/2 (484 bp/484 bp) genotype.

Genotyping and Data Checking

Genotyping of the 5-HTTLPR polymorphism[11] was performed as described by Edenberg and Reynolds.[34] Briefly, the polymerase chain reaction was conducted with one radioactively labeled primer in the presence of a mixture of dGTP and 7-deaza-2′-dGTP, and an aliquot of the product was electrophoresed on 6% denaturing polyacrylamide gels. This method was found to be a great improvement over the originally reported method[11,12]; it is robust and produced easily distinguishable alleles (Fig. 1).

A specially constructed database, GeneMaster, was used to check for alleles in an individual not present in his or her parents, and discrepancies were re-examined. Markers were further tested for Mendelian inheritance using CRI-MAP[35] and the USERM13 option of the MENDEL suite of programs.[36] Each family with an identified noninheritance was reviewed. If data remained incompatible after review, the genotypic data from one or more individuals incompatible with the rest of the family were removed (three genotypes total). The individuals determining allele sizes and reviewing potential discrepancies were blind to diagnostic data.

Table 1. TDT on Multiple Affected Individuals Per Family

	Affected model					
	ICD-10		ICD-10+W1*		ICD-10+W2*	
Allele†	TRANS‡	NOT‡	TRANS	NOT	TRANS	NOT
484 bp	62	69	52	62	31	39
528 bp	69	62	62	52	39	31
Total	131	131	114	114	70	70
p value§	0.54		0.35		0.34	

* ICD-10+W1 = ICD-10 alcohol-dependent with at least one withdrawal symptom; ICD-10+W2 = ICD-10 alcohol-dependent with at least two withdrawal symptoms.

† Allele sizes in base pairs.

‡ TRANS = transmitted to offspring; NOT = not transmitted.

§ *p* value was calculated by the χ^2 test. No corrections for multiple testing have been made.

Statistical Methods

HTTLPR allele frequencies were estimated from the data using maximum likelihood methods developed by Boehnke,[36] as implemented in the program USERM13. The families were stratified based on their predominant racial group; allele frequencies in the non-Hispanic Caucasian and African-American families were calculated separately. Genotypes of some individuals for whom data were missing were inferred, when that could be done unambiguously from the complete genotyped pedigree, using the program UNKNOWN, part of the LINKAGE package.[37] Inference was done on the full dataset before any trios were selected and involved only 3% of the individuals in the pedigrees; nine of these individuals were parents in trios used for analysis, and none was an offspring in a trio.

Our primary analysis used the transmission disequilibrium test (TDT) for unequal transmission of alleles from heterozygous parents to their affected offspring,[38–40] calculated using the ETDT computer package of Sham and Curtis.[41] Under the null hypothesis (that there is no linkage), data from related individuals in a pedigree are independent, so the TDT can be applied to each affected individual as a test for linkage.[40] When a single affected individual from each pedigree is used, the TDT is a valid test of linkage disequilibrium.[40] For the latter test, a reduced dataset consisting of a single affected individual from each family was produced. To increase power, the selection of the single affected offspring in each pedigree was based on heterozygosity of the parental genotypes for the 5-HTTLPR polymorphism.[42] The choice of trios was made without regard to the genotype of the offspring and should not bias the tests performed, because a heterozygous parent could pass either allele to the child. If two or more individuals in a family had equally informative parents (e.g., were siblings), the individual with the lower (arbitrary) identification number was chosen; these choices were made without knowledge of the offspring's genotype.

The previous report of an association was with alcoholics with a history of withdrawal seizure or delirium.[13] We first tested affected individuals as defined by the ICD-10 definition of alcohol dependence,[31] because that was the definition of alcoholic used in the previous report.[13] To determine whether a more severely physiologically affected subgroup showed linkage or association, two additional models of affected status were examined. The first model included individuals who meet the ICD-10 criteria and in addition have at least one symptom of withdrawal; the second model included individuals who meet the ICD-10 criteria and in addition have at least two symptoms of withdrawal. Withdrawal-related symptoms were delirium tremens, shakes, needing a drink in the morning, and relief drinking or relief medication.

We also analyzed allele-sharing among sibling pairs affected by alcohol dependence as defined by criteria from ICD-10,[33] or those criteria plus withdrawal symptoms, using the program SIBPAL, part of the S.A.G.E package.[43]

RESULTS

TDTs

We detected two rare variants of the *HTT* promoter, present in only a few families: one shorter than the 484 bp allele and the other longer than the 528 bp allele (data not shown). Because neither of these alleles were present in parents of the individuals analyzed, they played no role in these analyses.

The possible association or linkage of functional polymorphisms in the serotonin transporter gene *HTT* was tested using the TDT. Results from the TDT analysis provided no evidence of unequal allele transmission of either *HTT* allele from heterozygous parents to offspring who were alcohol-dependent as defined by ICD-10 criteria (Table 1). There was, therefore, no evidence for linkage between the *HTT* promoter polymorphism and alcohol dependence. There was no evidence for linkage with the subsets of subjects who in addition to meeting ICD-10 criteria for alcohol dependence reported withdrawal symptoms (Table 1). In all three analyses, there was a very slight excess (not significant) of the 528 bp (long) allele transmitted to the affected offspring. Limiting these analyses to non-Hispanic Caucasians did not affect the conclusions: there was no evidence of unequal allele transmission, and therefore no evidence for linkage (data not shown). The African-American subset was too small to perform meaningful statistical tests.

Restricting the analysis to one affected individual per family reduced the sample size. There was a slight excess of the 528 bp allele transmitted to individuals defined as affected by all three models (Table 2); but in no case was the difference significant. Again, results in the non-Hispanic Caucasian subset were also not significant.

Allele Sharing

We also tested for linkage using nonparametric methods of analysis, examining allele-sharing among affected sibling pairs with the program SIBPAL.[43] There was no significant elevation of allele-sharing among sibling pairs defined as affected by any of the three models: ICD-10, ICD-10 plus at least one withdrawal symptom, and ICD-10 plus at least two withdrawal symptoms (p = 0.36, 0.27, 0.10, respectively). When the sample was restricted to the non-Hispanic Caucasian sibling pairs, the allele-sharing remained not significant.

Table 2. TDT on a Single Affected Individual in Each Family

	Affected model					
	ICD-10		ICD-10+W1*		ICD-10+W2*	
Allele†	TRANS‡	NOT‡	TRANS	NOT	TRANS	NOT
484 bp	28	42	26	41	21	32
528 bp	42	28	41	26	32	21
Total	70	70	67	67	53	53
p value§	0.094		0.067		0.131	

* ICD-10+W1 = ICD-10 alcohol-dependent with at least one withdrawal symptom; ICD-10+W2 = ICD-10 alcohol-dependent with at least two withdrawal symptoms.

† Allele sizes in base pairs.

‡ TRANS = transmitted to offspring; NOT = not transmitted.

§ *p* value was calculated by the χ^2 test. No corrections for multiple testing have been made.

Allele Frequencies

Because our data are from a family sample, the allele frequencies were calculated by maximum likelihood methods.[36] For the non-Hispanic Caucasian subset of families, the frequency of the 484 bp (short) allele was 43.9% (95% confidence interval, 39.9% to 48.0%), and the 528 bp (long) allele was present at 55.8%. The rare variants were present at slightly <0.2% each. The allele frequency in the African-American population was significantly different from that in the Caucasian population. The frequency of the 484 bp allele in the African-American families was only 24.9% (95% confidence interval, 16.4% to 33.4%). The longest allele was present at a frequency of 2.8% among African-Americans, but the shortest allele was not found in these African-American families.

DISCUSSION

A large, family-based sample was used to test for association between the functional *HTT* promoter polymorphism and alcohol dependence. Testing a functional polymorphism in a candidate gene hypothesized to play a role in increasing susceptibility to alcoholism is more robust than testing an anonymous polymorphism, because the latter will at most be in partial linkage disequilibrium with the presumed functional polymorphism. Using family-based methods avoids the problem of false-positive results due to population stratification, by using nontransmitted alleles from the same parents as controls.

Our sample consisted of families selected for the presence of multiple alcoholics. Such families are more likely than the general public to carry genetic risk factors for alcoholism. Data presented herein do not show any evidence for linkage or association of the functional promoter polymorphism in the *HTT* gene with alcohol dependence. There was no significant bias in the transmission of either allele to individuals who are alcohol-dependent by ICD-10 criteria, or to the subsets of those individuals who also report withdrawal symptoms. When analyzed using all affected individuals in a family, the TDT is a test for linkage. No evidence for linkage was found. When testing a single affected individual in each family, the TDT is an unbiased test for association that avoids the possibility of erroneous results due to population stratification. Transmission of the two alleles (484 and 528 bp) did not show significant association with any of the three definitions of affected status.

We analyzed the transmission of the functional *HTT* alleles themselves, thereby avoiding assumptions about the mode of action of these alleles (recessive/dominant or additive). The apparent mode of action of the 484 and 528 bp *HTT* promoter alleles is unclear. The reports in which *HTT* mRNA levels, protein levels, or uptake of serotonin were measured showed that the higher activity of the 528 bp allele was recessive: cells with either one or two copies of the 484 bp allele behaved similarly, and significantly differed from cells homozygous for the 528 bp allele.[12,15] In the initial report of association of this polymorphism with anxiety-related traits, individuals with either one or two copies of the 484 bp allele behaved similarly, reinforcing the idea that the 528 bp allele acted recessively.[12] However, a later report by some of the same researchers came to the opposite conclusion: the low-activity 484 bp allele appeared to increase risk for affective disorders in a recessive manner: the odds ratio for the heterozygous 484 bp/528 bp genotype was not significantly different from the controls, but that of the homozygous 484 bp allele was 1.23 (1.02 to 1.49).[15] Likewise, the reported association of the 484 bp allele with severe alcoholism was due to an excess of the homozygous genotype,[13] suggesting recessive action. These contradictory suggestions are both unexpected, because promoter sequences function in *cis* (i.e., they affect the coding region of only the chromosome on which they are located). Thus, one would expect a priori that promoter variants act in a codominant or additive manner. In this confusing situation, analysis of the pattern of allele transmission, as we have done, is preferable.

In the previous report of association of these *HTT* promoter alleles with alcoholism,[13] there was an excess of the 484 bp allele in the alcoholics with severe withdrawal symptoms (0.476 vs. 0.394 for controls, $p = 0.049$). The excess of 484 bp alleles was also present in the larger group of alcoholics with any withdrawal symptom (0.462, $p = 0.035$) and in the full group of alcoholics defined by ICD-10 criteria (0.456, $p = 0.043$). Thus, our analysis of alcoholics defined by ICD-10 criteria, or ICD-10 criteria plus withdrawal symptoms, would be expected to show a similar

association, if the association were real. We saw neither linkage nor association. In fact, we saw a very slight but not significant excess of transmissions of the 528 bp (long) allele (Table 1), the opposite of that found by Sander et al.[13] This suggests that the previous report of a nominally significant association of the *HTT* promoter polymorphism with alcoholism[13] might have been due to chance. It should be noted that analyses of the data of Sander et al.[13] by all three genotypes did not show significant differences ($p > 0.1$ for all three categories of alcoholics noted previously). The only significant genotypic association was found when the 484 bp allele was considered recessive ($p = 0.035$ for alcoholics with severe withdrawal symptoms; $p = 0.047$ for alcoholics with any withdrawal symptoms; neither value corrected for multiple testing).

The frequency of the 484 bp *HTT* allele was 43.9% in the non-Hispanic Caucasians studied herein. This is very similar to the allele frequencies reported for people of European descent (39.4% to 46.3% for control populations reported from Germany, England, Israel, and Italy[11–16]). We found the 484 bp *HTT* allele frequency to be 24.9% in African-American families; there were no previous data on *HTT* allele frequencies in this ethnic group. The significant difference in allele frequencies between African-American and Caucasian populations (confidence intervals do not overlap) raises a caution in population association studies in which there is admixture of different groups. Our study design, using family-based methods, avoids such difficulties.

In summary, we investigated the possible association of a functional polymorphism in the serotonin transporter gene *HTT* with alcohol dependence defined by criteria from ICD-10 or by those criteria plus withdrawal symptoms. Our analyses used robust, family-based methods on a large collection of carefully ascertained families from the United States. We found no evidence of linkage or association of the *HTT* gene with alcohol dependence.

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