Association of Functional Polymorphisms of the Human Tryptophan Hydroxylase 2 Gene With Risk for Bipolar Disorder in Han Chinese

Yi-Mei J. Lin, PhD; Shin-Chih Chao, MD; Tsung-Ming Chen, MS; Te-Jen Lai, MD; Jia-Shing Chen, MS; H. Sunny Sun, PhD

Context: The tryptophan hydroxylase 2 (TPH2) gene encodes the first (also the rate-limiting) enzyme in the serotonin biosynthetic pathway. Despite reports of possible associations between polymorphisms in human TPH2 and many psychiatric disorders, including bipolar disorder (BPD), the functional effect and susceptibility loci of such polymorphisms for BPD have not yet been identified.

Objectives: To examine the association of TPH2 with BPD and to identify the functional variants that may be involved in the pathophysiological development of BPD.

Design, Setting, and Patients: We systematically screened all exons and promoters of the TPH2 gene in Han Chinese subjects to identify sequence variants. Association tests were conducted in 105 cases and 106 control subjects using single-locus, linkage disequilibrium, and haplotype analyses. Two promoter and one exon 2 single-nucleotide polymorphisms were examined for their functional role using a reporter gene system and enzyme activity assay, respectively. Additional statistical analysis was performed to study the interaction between the 2 TPH genes in 205 study participants with TPH1 and TPH2 genotype data.

Results: Significant haplotype association of TPH2 polymorphisms and BPD was identified (∼H11021.001). In addition, allelic alteration of polymorphisms in the promoter region and exon 2 of TPH2 caused noteworthy functional losses in promoter and enzyme activities, respectively, indicating the potential susceptibility loci for BPD. We found that the odds ratio changed from 3.73 of the TAG haplotype to 4.81 or 1.68, depending on the combined effect of both TPH genotypes. These data suggested an interaction between the 2 TPH genes to confer a risk for BPD.

Conclusions: This study supports the involvement of TPH2 in the etiology of BPD, and the functional single-nucleotide polymorphisms identified herein might be the susceptibility loci for BPD. Although the interaction between the 2 TPH genes merits further investigation, our findings suggest that the interactive effect should be considered in future studies of serotonin-related disorders.

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SEROTONIN (5-HYDROXYTRYPTAMINE) is a neurotransmitter synthesized in the raphe nuclei of the brainstem that controls a wide range of physiological events in the central nervous system.1 Accordingly, disruption of serotonergic function has been implicated in the pathogenesis of many psychiatric disorders, including bipolar disorder (BPD).2-4 The biosynthesis of serotonin is initiated by tryptophan hydroxylase (TPH), using tryptophan as a substrate to generate 5-hydroxytryptophan, followed by decarboxylation using aromatic amino acid decarboxylase to produce serotonin.5 Because TPH is the first as well as the rate-limiting enzyme in the synthetic pathway for serotonin, the TPH gene has been considered a candidate for many behavioral and psychiatric traits.6 Two TPH isoforms encoded by different genes have been identified in humans. The first, TPH1, was discovered earlier and was associated with various neuropsychiatric disorders such as manic-depressive illness,7 depression,8,9 suicidality, and alcoholism.10,11 Despite many reports of positive associations, reports finding no association between TPH1 polymorphisms and many neuropsychiatric disorders have also been published12-14; thus, the role of TPH1 in the development of different neural disorders is still inconclusive.

The gene for the second isoform, TPH2, was identified recently because TPH1-knockout mice expressed normal amounts of serotonin in the brain but not in the periphery,5,15 which resulted in a cardiac dys-
function phenotype. The human TPH2 protein shares 71% of its amino acid sequence identity with TPH1; all residues that have important functions for the structural or enzymatic activity of TPH1 are also conserved. Using both in vitro and in vivo systems, studies confirmed that TPH2 controls brain serotonin synthesis. Later studies further demonstrated that the TPH2 gene is predominantly expressed in the brainstem, especially in the raphe nuclei neurons, in a rodent model and in post-mortem brain tissue, whereas TPH1 is expressed in the pineal gland and peripheral tissue. The expression patterns of the 2 proteins were confirmed recently by using monospecific polyclonal antibodies against TPH1 and TPH2. These studies suggested a duality hypothesis for TPH1 and TPH2, namely, that they are responsible for the peripheral and major central nervous system serotonergic effects, respectively.

The TPH2 polymorphisms were associated with many psychiatric disorders such as early-onset obsessive-compulsive disorder, suicide, attention-deficit/hyperactivity disorder, and repetitive behaviors in autism. In addition, disease-associated haplotypes have been identified for major depression and BPD. Furthermore, higher levels of TPH2 expression have been found in the raphe nuclei of suicidal patients with major depression and in the dorsolateral prefrontal cortex of patients with BPD than in control subjects without psychiatric disorders. Results from these studies strongly support the involvement of the TPH2 gene in the development of affective disorders. Because of the lack of potentially functional single-nucleotide polymorphisms (SNPs) in these studies, the polymorphisms that represent susceptibility loci of the TPH2 have not yet been identified.

The discovery of TPH2 has changed ideas about linking the TPH1 polymorphism with a variety of psychiatric diseases; however, the question of whether the 2 proteins are regulated independently—and thus do not interact—remains unanswered. Because the previous study results concerning TPH1 association with BPD were inconsistent, we propose that the TPH1 gene alone has little or no effect on the etiology of BPD, but it may interact with the TPH2 gene to influence BPD development. To test this hypothesis, the present study aimed to examine the effect of the TPH2 gene on the etiology of BPD in 105 patients with bipolar I disorder and 106 matched controls who had been previously involved in our TPH1 association study, and to combine both data sets for further statistical analysis. Results from our study confirm the important role of the TPH2 gene in the etiology of BPD. Moreover, we have identified common variants of the TPH2 gene that exhibit significant loss in TPH functions. Finally, our data provide statistical evidence, for the first time, that the interaction between these 2 TPH genes might confer the risk for BPD.

**METHODS**

**SUBJECTS**

The patients were all Han Chinese subjects recruited for a series of studies that began in 1998 and sought to examine the association of genes involved in the serotonin system and the etiology of BPD. A detailed description of the participants is available in the supplementary “Methods” section on our Web site (http://140.116.6.160/SUNLab/data/TPH2/).

**POLYMORPHISM SCREENING**

Sequences in the coding and up to 1 kilobase (kb) of the 5′ regulatory regions of the human TPH2 gene were screened using the direct sequencing method. Polymerase chain reaction (PCR) primers were designed to generate fragments to cover 11 exons from the main transcript (GenBank accession number, NM_173353) and 2 alternatively spliced exons from the transcript (GenBank accession number, AK094614) plus 1 kb upstream from the transcription start site. The primers used for polymorphism screening are available in supplementary eTable 1 on our Web site.

**MARKER SELECTION AND GENOTYPING ASSAYS**

Markers selected for further investigation were based on criteria provided in the supplementary “Methods” section available on our Web site. The TaqMan Custom SNP Genotyping Assay by Design system (Applied Biosystems, Foster City, California) was applied for genotyping in all participants in this study. Primers and probes designed for this study and genotyping data for the 6 markers investigated in all participants are given in the supplementary eTable 2 and eTable 3, respectively, available on our Web site.

**QUANTIFICATION OF BRAIN TPH**

**Cell Culture and Human Brain**

**Complementary DNA Panel**

Two human neuroblastoma cell lines, IMR-32 and SH-SY5Y (American Type Culture Collection, Manassas, Virginia), were grown in appropriate media. The endogenous serotonin amounts in IMR-32 and SH-SY5Y cells were measured as 2.9 and 6.2 ng/mL, respectively. In addition, the expressions of TPH2 and Pou domain class 3 transcription factor 2 (POU3F2) in these cells lines were confirmed by quantitative reverse transcription PCR (Q–RT-PCR) and Western blot analyses (supplementary eFigure 1, available on our Web site), respectively.

Using Q–RT-PCR, 8 complementary DNA libraries made from various sections of the human brain (BioChain Institute, Hayward, California) were used to assay the relative messenger RNA (mRNA) expression level of the TPH1 and TPH2 genes.

**RNA Isolation and Real-Time Q–RT-PCR**

Total RNA from cultured cells was isolated using a reagent (Rezol C&T; PROtech Technology, Taipei, Taiwan) according to the manufacturer’s protocol. The RNA samples were used for complementary DNA synthesis. We performed Q–RT-PCR of the TPH1, TPH2, and 18S rRNA genes in a sequence detector (ABI PRISM 7900; Applied Biosystems) using the reagents (TaqMan Assay on Demand probe; Applied Biosystems) according to the manufacturer’s protocol, and primer sets (HS00188220_m1, HS00987775_m1, and HS9099901-s1 for TPH1, TPH2, and 18S rRNA, respectively; Applied Biosystems). The relative levels of expression were presented as ΔCt for normalized cycle numbers or as ΔΔCt to further standardize ΔCt in each tissue with ΔCt in the brain.
### Functional Assay of TPH2 5’-Promoter Polymorphisms

Details on the preparation of various reporter constructs and procedures of luciferase assay, electrophoretic mobility shift assay, and pull-down assay are provided in the supplementary “Methods” section available on our Web site.

### Functional Assay of TPH2 Exon 2 C2755A Polymorphism

Detailed procedures for plasmid construction, transfection, and the enzyme activity assay are provided in the supplementary “Methods” section available on our Web site.

### Statistical Analysis

The significance level for all statistical tests was .05. We applied Bonferroni corrections for all multiple tests in the association study. Detailed procedures for the association study, linkage disequilibrium (LD) mapping, haplotype construction, interaction plot, and statistics for functional assays are given in the supplementary “Methods” section available on our Web site.

### Results

### Identification of TPH2 Sequence Variants

We systematically sequenced all functional regions of the human TPH2 gene in a panel of 54 samples and identified 10 single-nucleotide variants. Their positions in relation to the transcription start site of the TPH2 gene and the distribution of alternative alleles in the screening population are summarized in Table 1. The variant G33775A was observed only once in a heterozygous individual. The minor allele frequency was less than 1%; thus, it is considered a mutation.

### Single-Locus Association Analysis

Because this study aimed to identify the association between potentially functional SNPs and BPD, 6 SNPs—T−703G (rs4570625), T−473A (rs11178997), A90G (rs11178998), C2755A, C10662T (rs11179003), and G93329A (rs17110747)—were chosen for further genotyping. All markers were in Hardy-Weinberg equilibrium, except for SNP C2755A in patients who showed significant deviation from Hardy-Weinberg equilibrium (P < .001; available in supplementary eTable 4 on our Web site).

The distributions of alleles for each SNP and the Akaike information criterion values calculated from independent vs dependent models are given in Table 2. A significant difference in allele distribution between patients and controls was obtained for SNP C2755A (P = .03; not significant after Bonferroni correction). Because the Akaike information criterion values of the allele (3.333) and risk estimation (odds ratio [OR], 7.43; 95% confidence interval [CI], 0.91–60.95) were also positive, results from single-locus analysis suggest that BPD disease status might be dependent on the effect of the TPH2 C2755A polymorphism. All other SNPs were not associated with the disease, although few positive Akaike information criterion values were obtained from regression analysis for the few polymorphisms in allelic (Table 2) or genotypic (data not shown) levels.

### LD Mapping

The data from pairwise LD analyses between 6 SNPs in patient and control groups, as represented by the LD co-
efficient (D') values, are given in Figure 1 and supplementary eTable 5 (available on our Web site). As demonstrated in Figure 1, patients and controls had distinctly different patterns of LD. Despite the SNPs from C2755A to G93329A being in complete LD in both groups (D'/H11032, 1), strong LD for SNPs from T−703G to C2755A was seen only in patients with BPD (D'/H11032, 0.87-1). In contrast, complete LD in controls was present only in a shorter region between SNPs T−703G (rs4570625) and T−473A (rs11178997). In addition, the D' values dropped for long-distance SNP pairs (ie, T−703G and G93329A), and the pattern was similar in both groups. Previously Zhou et al. used 15 SNPs covering 106 kb of the TPH2 gene to define 2, 3, and 1 haplotype blocks in US white, African American, and southwestern Native American subjects, respectively. In the present study, we defined 2 LD blocks in Han Chinese, and the boundary defined for block 1 is similar to that for the African American population in Zhou et al. The distinct LD block patterns across populations may suggest different evolutionary paths for these human lineages.

**HAPLOTYPE ASSOCIATION ANALYSIS**

The strong LD between T−703G (rs4570625) to C2755A was present only in patients and not in controls, suggesting a possible disease-related selection, and was further investigated in haplotype association analysis. The SNP C2755A was not used for haplotype construction because of its low population frequency.

**Table 2. Genotype and Allele Frequencies of the TPH2 Gene Polymorphisms**

<table>
<thead>
<tr>
<th>SNP</th>
<th>Minor Allele, No./Total No. (Frequency)a</th>
<th>Control Subjects</th>
<th>Patients</th>
<th>P Value</th>
<th>AIC (IM-DM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T−703G</td>
<td>60/95 (0.32)</td>
<td>71/101 (0.35)</td>
<td>.45</td>
<td>−1.44</td>
<td></td>
</tr>
<tr>
<td>T−473A</td>
<td>41/102 (0.20)</td>
<td>44/102 (0.22)</td>
<td>.72</td>
<td>−1.87</td>
<td></td>
</tr>
<tr>
<td>A90G</td>
<td>36/103 (0.17)</td>
<td>42/103 (0.20)</td>
<td>.40</td>
<td>−1.28</td>
<td></td>
</tr>
<tr>
<td>C2755A</td>
<td>7/101 (0.03)</td>
<td>1/104 (0.01)</td>
<td>.03b,c</td>
<td>3.33b,c</td>
<td></td>
</tr>
<tr>
<td>C10662T</td>
<td>20/92 (0.11)</td>
<td>18/93 (0.08)</td>
<td>.71</td>
<td>−1.86</td>
<td></td>
</tr>
<tr>
<td>G93329A</td>
<td>68/101 (0.34)</td>
<td>49/97 (0.25)</td>
<td>.07</td>
<td>1.37b</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: AIC, Akaike information criterion; DM, dependent model; IM, independent model; SNP, single-nucleotide polymorphism; TPH2, tryptophan hydroxylase 2 gene.

a The total number is the number of individuals in each group. The genotyping coverage varied (range, 86%-100%) between different markers, with an average of 92% in all participants.

b Significant difference in the distribution between the case and control groups, AIC (IM−DM) > 0 indicates that the SNP and disease are dependent in the given regression model.

c Odds ratio for major allele vs minor allele, 7.43 (95% confidence interval, 0.91-60.95). Power for the allele distribution of C2755A is about 59% with α = .05.
Three major haplotypes accounted for 76% and 96% of the total haplotypes in the controls and patients, respectively, and the data confirmed the strong LD within this region. Except for the major TTA haplotype, which was equally distributed between patients and controls, significant differences in distribution between groups were detected for haplotypes TAG, TAA, and GTG, even after Bonferroni correction (P<.001). The TAG haplotype was associated with noteworthy risk (OR, 3.73; 95% CI, 1.89-7.36), and the TAA haplotype with significant protective effects (OR, 0.19; 95% CI, 0.08-0.46). Furthermore, P values from an overall comparison of all haplotypes and from permutation tests for disease and gene association were highly significant (P<.001). The statistical analyses for haplotypes constructed with 3 SNPs in the 3' region or 6 SNPs (supplementary eTable 6 and eTable 7, available on our Web site) showed results similar to those in Table 3; therefore, these data strongly imply the involvement of the TPH2 gene in the development of BPD. Previous studies that used 10 SNPs, covering exons 5 to 7,27 and 5 SNPs covering exons 7 to 928 of the TPH2 gene, but that it was in LD with the markers these authors used. Our data independently replicate the results of Harvey et al. Because the potential functional variants present within the haplotype were located from the promoter region to exon 2, our data suggest that the susceptibility locus for BPD may be located toward the 5' region of the TPH2 gene. The previous study by Zhou et al23 suggested that a functional locus for major depression in US white and African American populations might be located between introns 5 and 8 of the TPH2 gene. Together, these data suggested that the TPH2 gene is involved in the development of many psychiatric disorders and that the susceptibility locus responsible for various disorders may be different.

To show the possible effect of promoter SNPs, we used the Transcription Element Search System (http://www.cbil.upenn.edu/cgi-bin/tess/tess) to predict whether the genomic positions of these SNPs are associated with any known transcription factors. The Transcription Element Search System prediction showed that SNPs T−703G (rs4570625) and T−473A (rs11178997) were both in the POU3F2 (also known as N-Oct-3) binding sites, and that the base changes at both positions had altered the consensus sequence; therefore, we predicted that it would abolish the binding of POU3F2. We used reporter gene systems with different haplotypes for 2 SNPs to assay promoter activity (Figure 2A). We found that luciferase activities obtained from 3 haplotype constructs containing at least 1 T allele were not significantly different (P=.85 in SH-SY5Y; P=.79 in IMR32) and were consistently more active than the plasmid constructed with the GA haplotype (P=.002 in SH-SY5Y; P=.001 in IMR32). These results suggest that both polymorphisms in the promoter region affect in vitro gene expression.

### Table 3. Haplotype Frequency and Test Statistics Between Patients and Control Subjects

<table>
<thead>
<tr>
<th>Haplotypesa</th>
<th>Overall</th>
<th>Controls</th>
<th>Patients</th>
<th>P Valueb</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTA</td>
<td>182 (0.43)</td>
<td>95 (0.45)</td>
<td>87 (0.42)</td>
<td>.54</td>
<td>0.89 (0.60-1.30)</td>
</tr>
<tr>
<td>GTC</td>
<td>126 (0.30)</td>
<td>52 (0.25)</td>
<td>74 (0.36)</td>
<td>.01</td>
<td>1.70 (1.11-2.59)</td>
</tr>
<tr>
<td>TAG</td>
<td>50 (0.12)</td>
<td>12 (0.06)</td>
<td>38 (0.18)</td>
<td>&lt;.001</td>
<td>3.73 (1.89-7.36)</td>
</tr>
<tr>
<td>TAA</td>
<td>35 (0.08)</td>
<td>29 (0.14)</td>
<td>6 (0.03)</td>
<td>&lt;.001</td>
<td>0.19 (0.08-0.46)</td>
</tr>
<tr>
<td>GTG</td>
<td>17 (0.04)</td>
<td>17 (0.08)</td>
<td>0</td>
<td>&lt;.001</td>
<td>. . .d</td>
</tr>
<tr>
<td>Others</td>
<td>10 (0.02)</td>
<td>7 (0.03)</td>
<td>3 (0.01)</td>
<td>.21</td>
<td>0.43 (0.11-1.68)</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; OR, odds ratio.

a Haplotypes were constructed using the following 3 single-nucleotide polymorphisms, from left to right, T−703G, T−473A, and A90G.

b Total and global P values were <.001; the global P value was calculated using a permutation test (n=1000).

Power for the distribution of TAG haplotype is about 98%, with a=.05.

d No OR was calculated for this haplotype for comparison with the patient group.
Figure 2. Effect of tryptophan hydroxylase 2 gene (TPH2) promoter polymorphism on gene expression in the reporter system. A, Promoter activity of the TPH2 T−703G and T−473A polymorphisms. The human neuroblastoma cell lines SH-SY5Y and IMR-32 were transfected with plasmid constructs containing different haplotype combinations for single-nucleotide polymorphisms (SNPs) T−703G and T−473A. Luciferase activities were measured in luciferase units relative to the empty vector (pGL3-B). Data were normalized to protein concentration (in micrograms) and then analyzed using the 2-sample t test. All assays were performed in triplicate, and the levels of promoter activity are expressed as mean±SD. Although luciferase activities were similar between haplotype TT, TA, and GT transfectants, significant differences were detected for the comparisons between haplotype GA transfectants and the others (P<.002 for SH-SY5Y; P=.001 for IMR-32). *P<.01. B, Electrophoretic mobility shift analysis investigating the −473 T/A and −703 T/G polymorphisms. We incubated 10 µg of nuclear extract from IMR-32 cells with biotin-labeled oligonucleotide probes alone (lanes 2, 7, and 16), in the presence of anti–POU domain class 3 transcription factor 2 (anti-POU3F2) antibody (lanes 3 and 12) or probes alone (lanes 2, 7, 11, and 16), in the presence of anti–Pou domain 3.µg of nuclear extract from IMR-32 cells with biotin-labeled oligonucleotide probes alone (lanes 2, 7, and 16), in the presence of anti–POU domain class 3 transcription factor 2 (anti-POU3F2) antibody (lanes 3 and 12) or probes alone (lanes 2, 7, 11, and 16), in the presence of anti–Pou domain 3.

TPH ENZYME ACTIVITY IN C2755 VS A2755 CONSTRUCTS

The novel coding SNP C2755A identified in this study changed the amino acid sequence from serine to tyrosine at peptide position 41. Although this S41Y polymorphism is on the nonpreserved N-terminal region between the human TPH1 and TPH2 proteins, the sequence alignment of TPH2 proteins in 9 species showed that S41 is highly conserved across species from zebrafish to humans (Figure 3A), but not in the mouse and rat. To analyze the substitution effect on TPH2 function, we transfected plasmids containing full-length TPH2 protein–encoding sequences with 2 alternative alleles into SH-SY5Y cells and measured serotonin production in the supernatants of transfected cells using an enzyme-linked immunosorbent assay for serotonin (Figure 3B). Our data demonstrated, with roughly equal recombinant TPH2 proteins in each assay, that the amount of serotonin in SH-SY5Y cells expressing the TPH2_41Y allele was about 36% (range, 30%-42%) lower than cells expressing the TPH2_41S allele (P=.002). Although we did not measure TPH2 enzyme activity, previous studies in humans16 and mice17 indicated a good correlation between serotonin levels measured in the supernatants of transfected cells and the rate of serotonin synthesis in the cells. The levels of serotonin in SH-SY5Y cells can, therefore, serve as indirect data for TPH2 enzyme activities. These data suggest that the TPH2_41Y allele loses the ability to synthesize serotonin.

INTERACTION BETWEEN TPH2 AND TPH1

Expression of mRNAs for TPH1 and TPH2 in the Human Brain

The expression of TPH2 and TPH1 was found to be nonoverlapped in the rat brain18 but coexpressed with different levels using tissue from postmortem human brain.19 To confirm the expression pattern of these 2 proteins in the human brain, we applied nonnormalized complementary DNA panels from human tissue and measured, using Q–RT-PCR, the relative mRNA amount. The measured relative mRNA level was justified with 18S rRNA in each tissue panel and present in a dual y-axis figure (Figure 4). Except in the total brain and cerebellum, where the relative levels of mRNA were similar for TPH1 and TPH2, expression of TPH2 was significantly more abundant in all investigated brain regions, including the amygdala, cerebral cortex, frontal lobes, medulla oblongata (caudal raphe), and pons (rostral raphe). The highest TPH2 mRNA expression was detected in the pons at about 45 times more abundantly than TPH1. These data are consistent with previous reports that TPH2 was the predominant form in the raphe nuclei in the brains of humans19 and rats.18 Results from our study support the overlapped pattern of the 2 TPHs in the human brain and suggest that the 2 genes may be coexpressed in many serotonergic brain regions.

Combined Genotyping Analysis of TPH1 and TPH2

The coexpression of TPH1 and TPH2 proteins in many brain regions but leading to different biological paths sug-
TPH2_41Y proteins were expressed in transfected cells as indicated by Western blot analysis using anti-Myc or with an empty vector. Serotonin levels in SH-SY5Y cells with different constructs were measured in the medium of transfected cells using an enzyme-linked assay. SH-SY5Y cells expressing TPH2_41S. The human neuroblastoma cell line SH-SY5Y was transfected with plasmids constructed with different alleles, and the difference between the 2 subgroups was not significant (P = .22). These data suggested that the effect of TPH2 on BPD etiology can be influenced by the presence of TPH1.

Although TPH2 polymorphisms were associated with many psychiatric disorders, including major depression and BPD, the functional polymorphisms that represent the susceptibility loci of the TPH2 gene have not yet been identified. Our study, which used single-locus analysis, LD mapping, and haplotype analysis, provided additional support for the association of TPH2 with BPD. In addition, we have demonstrated the function of 3 TPH2 polymorphisms, 2 common variants in the promoter region and 1 novel variant in the exon 2, the combination of which may underlie the risk for BPD.

We have undertaken a systematic approach to screen polymorphisms of the human TPH2 gene in the Han Chinese population. Of the 9 polymorphisms identified in this study, 2 SNPs have not been reported previously, to our knowledge, and thus may represent population-specific polymorphisms in Taiwan. The C2755A polymorphism is the only variant that causes the S41Y substitution. These data are consistent with other reports in which most of the variants identified from the TPH2 gene were in noncoding regions. Results from these studies point out the conserved nature of the human TPH2 gene. In addition, 3 nonsynonymous variants of L36P, P206S, and R441H are all present at very low frequencies and are not found in our population. Because the R441H variant identified in the US population results in approximately 80% loss of function in serotonin production, these data suggest that the S41Y polymorphism identified in our study may also be functional and thus worth further characterization. The 2 promoter SNPs, T−703G (rs4570625) and T−473A (rs11178997), are commonly present in many species, including human, mouse, and zebrafish.
A previous study by De Luca et al showed abnormal POU3F2 binding affinity and modulate gene expression. Gel shift assays indicate a reduction in POU3F2 binding affinity of probes containing the −703G and −473A alleles when compared with the −703T and −473T-containing probes, respectively. These data provide evidence that the 2 promoter SNPs can affect POU3F2 binding and modulate TPH2 gene expression. A recent publication by De Luca et al reported that a polymorphism on a GABRB3 promoter reduces POU3F2 binding affinity and therefore could be a cause for childhood absence epilepsy. Taken together, our data offer additional evidence and suggest that the 2 promoter polymorphisms

Figure 4. Both of the tryptophan hydroxylase genes (TPH1 and TPH2) are expressed in various regions of the human brain. Eight complementary DNA libraries made from various sections of the human brain—total brain, amygdala, cerebellum, cerebral cortex, frontal lobes, hippocampus, medulla oblongata, and pons—were used to assess the relative expression level of TPH1 and TPH2. The expression levels of TPH1 and TPH2 were measured using quantitative real-time polymerase chain reaction with the human brain complementary DNA panel. The relative expression levels were calculated by normalizing the value of TPH1 or TPH2 messenger RNA (mRNA) to the value of 18S control RNA (the ΔCt). The ΔCt in each panel was then standardized using the ΔCt in the brain (the ΔΔCt). The expression levels are represented by 2−ΔΔCt in the dual y-axis, and the axes on the left and right indicate the relative expression levels of TPH1 and TPH2, respectively. In both cases, the expression levels in the brain are equal to 1 and the TPH2 expression is 1.18-fold more abundant than TPH1 in the brain. All measurements were performed in triplicate and analyzed using 1-way analysis of variance. *P<.05, †P<.01.

Table 4. The Combined Distribution of TPH2 Haplotype and TPH1 Allele for Interaction Analysis

<table>
<thead>
<tr>
<th>TPH1 G−346T</th>
<th>Controls</th>
<th>Patients</th>
<th>Controls</th>
<th>Patients</th>
<th>P Valueb</th>
<th>OR (95% CI)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPH2 haplotypea</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAG</td>
<td>14</td>
<td>57</td>
<td>10</td>
<td>17</td>
<td>.08</td>
<td>2.40 (0.90-6.35)</td>
</tr>
<tr>
<td>Non-TAG</td>
<td>396</td>
<td>259</td>
<td>78</td>
<td>79</td>
<td>.32</td>
<td>0.84 (0.59-1.19)</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;.001</td>
<td></td>
<td></td>
<td></td>
<td>.22</td>
<td></td>
</tr>
<tr>
<td>OR (95% CI)b</td>
<td>4.81 (2.62-8.83)</td>
<td>1.68 (0.72-3.89)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; OR, odds ratio; TPH, tryptophan hydroxylase gene.

aThe TPH2 risk haplotype is constructed by −703T, −473A, and 90G.


Although TPH1 and TPH2 are homologous proteins and share an overall 71% of amino acid identity, the N-terminal end and the regulatory region are quite divergent between these 2 proteins. For example, both TPH1 and TPH2 can be phosphorylated by protein kinase A but TPH2 is phosphorylated at Ser19, a site not even present in TPH1. Furthermore, TPH2 is more soluble than TPH1, with a higher molecular weight and different kinetic properties. These data strongly suggest that the diverse region of the TPH2 protein may be important for regulating serotonin production in the central nervous system. The C2755A (S41Y) polymorphism is located on the diverse N-terminal region of human TPH2 proteins, and sequence alignment indicated that the position is highly conserved in the TPH2 proteins across species. Further data using recombinant constructs demonstrated that the TPH2-41Y fusion protein exhibits lower enzyme activity than the TPH2-41S fusion protein. We assume that the reduced enzyme activity is due to a change in the regulatory level, perhaps through the alteration of the 3-dimensional structure in the N-terminal of the TPH2 protein, thus affecting its association with proteins like protein kinase A or 14-3-3. Alternatively, because the S41 residue is predicted on the protein kinase C-binding motif, the polymorphism itself may be an important phosphorylation site for activating TPH2. Thus, a change in this residue might significantly alter the amount of active TPH2 and result in a loss of final serotonin production.

Although SNPs on the promoter region and exon 2 show significant functional alterations in gene expression and TPH2 activity, respectively, their associations with BPD at single-locus levels are not significant. These results indicate that an association of partial functional loss in each SNP with BPD may be weak, but that the combination of several common variants may have a dramatic effect on the etiology of BPD. Our results support the common disease/common variants hypothesis. More important, our data confirm the results of Harvey et al and suggest that the functional polymorphisms detected in the promoter region and exon 2 might be the susceptibility loci for BPD.

The current conventional wisdom suggests that genes in the same pathway or involved in the same regulatory network tend to have risk interaction. Growing evidence also...
supports the existence of higher-order risk interactions between genes to cause common and complex diseases, for example, the interactions between 5,10-methylene tetrahydrofolate reductase and transcobalamin for spontaneous abortion,\textsuperscript{49} between the serotonin transporter and dopamine transporter for harm avoidance and reward dependence traits,\textsuperscript{46} and between prothrombin and factor XIII-A for myocardial infarction.\textsuperscript{46} The TPH1 and TPH2 proteins are paralogous and have distinct structural and kinetic properties\textsuperscript{11} but share common resources for serotonin production.\textsuperscript{2} The dual functions of serotonin correlated well with the expression levels of these 2 enzymes, in which serotonin production as a precursor for melatonin synthesis (the pineal gland projection area) or for central nervous system neurotransmission (raphe nuclei projection area) is synthesized by TPH1 or TPH2, respectively.\textsuperscript{3}

In the present study, we applied Q–RT-PCR to demonstrate that both TPH1 and TPH2 mRNA are expressed in all brain regions under investigation. Expression of TPH2 is about 20 to 45 times greater than that of TPH1 in the raphe nuclei and about 10 to 12 times greater in the raphe nuclei projecting area. The detection of TPH1 in a few brain regions involved in serotonergic neurotransmission (eg, thepons and medulla oblongata) may come from pineal gland projection.\textsuperscript{37,48} Based on this observation, we propose a competing hypothesis for the 2 TPH proteins. Under normal conditions, serotonergic brain regions predominantly express TPH2 and lead to the generation of the neurotransmitter serotonin in the central nervous system. The existence of a minimal amount of TPH1 that competes with limited cofactors or cosubstrates to generate serotonin for melatonin synthesis can be tolerated. However, when the function of TPH2 in an individual is impaired, ie, gene expression is decreased or enzyme activity is reduced, the competition between the 2 TPHs becomes harmful and significant. The situation turns worse when TPH1 is present with increased activities or activity. This hypothesis can be partially supported by the observation in our study that the OR increased when at-risk haplotypes of TPH2 combined with the TPH1 G346 allele, which exhibits stronger promoter activity.\textsuperscript{10} To our knowledge, the first article to propose the interaction between the 2 TPH genes, and the theory can explain the lack of association in our previous study between TPH1 and BPD\textsuperscript{12}: because the association of TPH1 with BPD is influenced by the genotype of TPH2, examination of TPH1 alone missed the gene effect. Although the model seems reasonable, our data cannot exclude the possibility that the observation may simply indicate that critical behavioral outputs have complex contributions from the brain and periphery; as such, they depend on both genes' actions but not direct molecular interaction. Further experiments are required to confirm the proposed model in vivo biological systems.

Although the results of our genetic analysis were highly significant, the possibility of false-positive findings cannot be totally excluded because of the sampling limitations (ie, small sample size, confounders with other medical conditions, and subject assessment lacked support by structured interviews). However, we have provided additional statistics and functional analyses to support our conclusion. The discovery of TPH2 has changed scientists' views of TPH1 as the candidate gene for a variety of psychiatric diseases. We believe that the present study redefines the role of TPH1 in the development of BPD. Although its effect is indirect, our data suggest that TPH1 can interact with TPH2 to influence the risk for BPD. We propose that the interactive effect between the 2 TPH genes be considered in future studies of serotonin-related disorders.

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