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 (P < .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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EROTONIN (5-HYDROXYTRYPTA-
mine) is a neurotransmitter synthe-
sized 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.10 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.3 Using both in vitro and in vivo systems, studies confirmed that TPH2 controls brain serotonin synthesis.17 Later studies further demonstrated that the TPH2 gene is predominantly expressed in the brainstem, especially in the raphe nuclei neurons, in a rodent model19 and in post-mortem brain tissue,20 whereas TPH1 is expressed in the pineal gland and peripheral tissue.3,18 The expression patterns of the 2 proteins were confirmed recently by using monospecific polyclonal antibodies against TPH1 and TPH2.20 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.3,18

The TPH2 polymorphisms were associated with many psychiatric disorders such as early-onset obsessive-compulsive disorder,21 suicide,22,23 attention-deficit/hyperactivity disorder,24,25 and repetitive behaviors in autism.26 In addition, disease-associated haplotypes have been identified for major depression27 and BPD.28 Furthermore, higher levels of TPH2 expression have been found in the raphe nuclei of suicidal patients with major depression28 and in the dorsolateral prefrontal cortex of patients with BPD29 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.28

The discovery of TPH2 has changed ideas about linking the TPH1 polymorphism with a variety of psychiatric diseases15; 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,2,12 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,12 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.12,31,32 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 cell 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 (RE-zol 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 HS09999901-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.
Table 1. Sequence Variants of the Human TPH2 Gene Identified in This Study

<table>
<thead>
<tr>
<th>SNP Name a</th>
<th>Region b</th>
<th>dbSNP ID c</th>
<th>Allele 1</th>
<th>Allele 2</th>
<th>Amino Acid Alteration</th>
</tr>
</thead>
<tbody>
<tr>
<td>T – 703G</td>
<td>5’ Flanking region</td>
<td>rs4570625</td>
<td>0.54</td>
<td>0.46</td>
<td>None</td>
</tr>
<tr>
<td>T – 473A</td>
<td>5’ Flanking region</td>
<td>rs11178997</td>
<td>0.73</td>
<td>0.27</td>
<td>None</td>
</tr>
<tr>
<td>A90G</td>
<td>Exon 1 (5’ UTR)</td>
<td>rs11178998</td>
<td>0.81</td>
<td>0.19</td>
<td>Ser41Tyr</td>
</tr>
<tr>
<td>C2755A</td>
<td>Exon 2</td>
<td>NA</td>
<td>0.97</td>
<td>0.03</td>
<td>None</td>
</tr>
<tr>
<td>C10662T</td>
<td>Intron 4</td>
<td>rs11179003</td>
<td>0.89</td>
<td>0.11</td>
<td>None</td>
</tr>
<tr>
<td>G26270A</td>
<td>Intron 5</td>
<td>NA</td>
<td>0.83</td>
<td>0.17</td>
<td>None</td>
</tr>
<tr>
<td>G33775A</td>
<td>Intron 6</td>
<td>NA</td>
<td>&gt;0.99</td>
<td>&lt;0.01*</td>
<td>None</td>
</tr>
<tr>
<td>G40237A</td>
<td>Exon 7</td>
<td>rs7305115</td>
<td>0.53</td>
<td>0.47</td>
<td>Pro312Pro</td>
</tr>
<tr>
<td>A83610T</td>
<td>Exon 9</td>
<td>rs4290270</td>
<td>0.57</td>
<td>0.43</td>
<td>Ala375Ala</td>
</tr>
<tr>
<td>G93239A</td>
<td>Exon 11 (3’ UTR)</td>
<td>rs17110747</td>
<td>0.81</td>
<td>0.19</td>
<td>None</td>
</tr>
</tbody>
</table>

Abbreviations: dbSNP, official name of the National Center for Biotechnology Information single-nucleotide polymorphism (SNP) database; ID, identification number; NA, not available; TPH2, tryptophan hydroxylase 2 gene; UTR, untranslated region.

a Genomic localizations of SNPs are given in base pairs relative to the transcription start site (position 0).
b The position in the gene is according to the comparison of messenger RNA clone NM_173353 with human chromosome 12 genomic contig.
c Information is from the dbSNP database.33
d Allele frequencies were calculated in a Han Chinese population.
e The SNP G33775A was observed only in a heterozygous individual. The minor allele frequency was less than 1%; thus, it is considered a mutation.

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 heterozygote individual (minor allele frequency, 0.8%), and thus it could be a mutation. Of the rest, 2 of each polymorphism were in the promoter region (T – 703G and T – 473A), the 5’- and 3’-untranslated regions (A90G and G93239A), and the intron regions (C10662T and G26270A); and 3 coding SNPs contained 2 synonymous changes (G40237A and A83610T) and 1 nonsynonymous change (C2755A). To our knowledge, the SNPs C2755A and G26270A have not been reported in any public databases or literature, thus they are new and probably population-specific polymorphisms in Han Chinese.

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 G93239A (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' = 1), strong LD for SNPs from T−703G to C2755A was seen only in patients with BPD (D' = 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.

HAPLOTYP 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. The test results are shown in Table 2.

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

<table>
<thead>
<tr>
<th>SNP</th>
<th>Minor Allele, No./Total No. (Frequency)</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>.45</td>
<td>−1.44</td>
</tr>
<tr>
<td>T−473A</td>
<td>41/102 (0.20)</td>
<td>.72</td>
<td>−1.87</td>
</tr>
<tr>
<td>A90G</td>
<td>36/103 (0.17)</td>
<td>.40</td>
<td>−1.28</td>
</tr>
<tr>
<td>C2755A</td>
<td>7/101 (0.03)</td>
<td>.03b,c</td>
<td>3.33b,c</td>
</tr>
<tr>
<td>C10662T</td>
<td>20/92 (0.11)</td>
<td>.71</td>
<td>−1.86</td>
</tr>
<tr>
<td>G93329A</td>
<td>68/101 (0.34)</td>
<td>.07</td>
<td>1.37b,c</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 detected for haplotypes TAG, TAA, and GTG, even after significant differences in distribution between groups were equally distributed between patients and controls, significantly, and the data confirmed the strong LD within this region. Except for the major TTA haplotype, which was located toward the 5′ region of the TPH2 gene. Together, these data suggest that the susceptibility locus for BPD may be different.

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

<table>
<thead>
<tr>
<th>Haplotypesa</th>
<th>Overall (Frequency)</th>
<th>Controls (Frequency)</th>
<th>Patients (Frequency)</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>GTA</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></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).
c Power for the distribution of TAG haplotype is about 98%, with α=.05.
d No OR was calculated for this haplotype for comparison with the patient group.

To show the possible effect of promoter SNPs, we used the Transcription Element Search System (http://www.cbl.ucf.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.

Determination of POU3F2 Binding by Electrophoretic Mobility Shift Assay

To investigate whether the difference in the transcriptional activity observed in reporter gene assays is due to alteration in DNA-protein interaction, we used the electrophoretic mobility shift assay to determine whether the POU3F2 protein can bind to the predicted element of the TPH2 promoter. As shown in Figure 2B, 3 major DNA-protein complexes (C1, C2, and C3) were detected with biotin-labeled probes (lanes 2-9 and 11-18) irrespective of polymorphisms. The presence of the 3 complexes were in agreement with previous reports describing 2 additional proteins that can also bind to the POU3F2 sequence motif.35,36 The addition of anti-POU3F2 antibody but not mouse IgG inhibited POU3F2 binding to the probe (especially the C1 complex), indicating that the DNA-protein complex indeed contains POU3F2. Attenuation of the C1 complex by increasing amounts of

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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-SYSY 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-SYSY 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 humans38 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-SYSY 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.10 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 serotoninergic 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-

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-SYSY 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 −473T and −703T oligonucleotides (lanes 5–6, −473A and −703T, lanes 8–9, −703G and −703T, lanes 10–11, and −703G and −703A, lanes 14–15), and −703G oligonucleotides. Probe increasing amounts of unlabeled −473T (lanes 5–6), −473A (lanes 8–9), −703T (lanes 14–15), and −703G (lanes 17–18) oligonucleotides. Probe incubated in the absence of nuclear protein is shown in lanes 1 and 10. Samples were loaded on a 6% native acrylamide gel. The positions of the complexes C1, C2, and C3 and that of the probe are indicated by solid and open arrows, respectively.

unlabelled probes (lanes 5–6, 8–9, 14–15, and 17–18) further confirmed that the binding is specific. More important, polymorphisms did affect the binding of POU3F2 to its cis element when lanes 2 and 7 and lanes 11 and 16 were compared. When the C1 complex was contrasted, signals were 30% and 40% lower in lanes 7 and 16, respectively. Results of an additional pull-down assay demonstrated both −473T and −703T probes form complexes with POU3F2 in IMR32 cells (supplementary eFigure 2, available on our Web site). These data indicated that the 2 polymorphisms on the TPH2 promoter region can be bound by POU3F2 and that the −473 T-to-A and −703 T-to-G alterations indeed reduce POU3F2 protein binding affinity.
TPH2_41Y proteins were expressed in transfected cells as indicated by Western blot analysis using anti-TPH2 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 immunosorbent assay for serotonin. Experiments were performed using independently prepared plasmid in triplicate. Similar levels of Myc-tagged TPH2_41S and TPH2_41Y proteins were expressed in transfected cells as indicated by Western blot analysis using anti-Myc and anti-actin antibodies. The basal amount of serotonin was first subtracted, and then the data were analyzed using the 2-sample t-test. The means SD serotonin expression of TPH2_41Y-transfected cells was approximately 40% lower than that of TPH2_41S-transfected cells. *P=.002.

Figure 3. The C2755A polymorphism of the tryptophan hydroxylase 2 gene (TPH2) is highly conserved across species and affects TPH2 protein function. A. Sequence alignment of the N-terminal region of the TPH2 protein across species. Sequences include human (locus H: Homo sapiens: gi:3179563), chimpanzee (Pan troglodytes: gi:55638559), cow (Bos bison: gi:76660723), rat (Rattus norvegicus: gi:27735970), mouse (Mus musculus: gi:27734182), chicken (Gallus gallus: gi:47604924), dog (Canis lupus familiaris: gi:73968761), Takifugu rubripes (Takifugu rubripes: gi:74095887), and zebrafish (Danio rerio: gi:47505885). Sequences were aligned using the Multalin program (available at http://biomut.geneole-po-louise.pr/ll/multalin/multalin.html) with the hierarchical clustering method.1 Numbers indicate positions of amino acids. Red and blue indicate that conservation at a given position is higher than 80% or ranges from 50% to 80%, respectively. The serine residue of TPH2 located at position 83 is conserved across species except in the rodent (arrowhead). B. Serotonin levels in SH-SY5Y cells expressing TPH2_41Y were lower in SH-SY5Y cells expressing TPH2_41S. The human neuroblastoma cell line SH-SY5Y was transfected with plasmids constructed with different TPH2 C2755A alleles 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 immunosorbent assay for serotonin. Experiments were performed using independently prepared plasmid in triplicate. Similar levels of Myc-tagged TPH2_41S and TPH2_41Y proteins were expressed in transfected cells as indicated by Western blot analysis using anti-Myc and anti-actin antibodies. The basal amount of serotonin was first subtracted, and then the data were analyzed using the 2-sample t-test. The means SD serotonin expression of TPH2_41Y-transfected cells was approximately 40% lower than that of TPH2_41S-transfected cells. *P=.002.

Although TPH2 polymorphisms were associated with many psychiatric disorders, including major depression and BPD,21-28 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 reports32,33 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,30 P206S,22 and R441H30 are all present at very low frequencies and are not found in our population. Because the R441H variant identified in the US population30 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→T3G (rs4370625) and T→G3A (rs11178997), are commonly present in many...
A previous study by De Luca et al.\textsuperscript{40} showed abnormal POU3F2 binding and modulate provide evidence that the 2 promoter SNPs can affect POU3F2 binding and modulate POU2 gene expression. A recent publication\textsuperscript{36} reported that a polymorphism on a GABRB3 promoter reduces POU3F2 binding affinity in vitro luciferase reporter assay, we demonstrated that significant loss in promoter activity is indeed associated with the GA haplotype. Gel shift assays indicate a reduction in POU3F2 binding affinity of probes containing the −703T 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 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 may represent the common susceptibility loci of the TPH2 gene.

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.\textsuperscript{41} Furthermore, TPH2 is more soluble than TPH1, with a higher molecular weight and different kinetic properties.\textsuperscript{41} 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.\textsuperscript{42} More important, our data confirm the results of Harvey et al.\textsuperscript{39} 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.\textsuperscript{43} Growing evidence also

**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 rRNA (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>
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<tr>
<td>Controls</td>
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<td>TPH2 haplotype\textsuperscript{a}</td>
<td></td>
</tr>
<tr>
<td>TAG</td>
<td>14</td>
</tr>
<tr>
<td>Non-TAG</td>
<td>396</td>
</tr>
<tr>
<td>P value\textsuperscript{b}</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>OR (95% CI)\textsuperscript{b}</td>
<td>4.81 (2.62-8.83)</td>
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Abbreviations: CI, confidence interval; OR, odds ratio; TPH, tryptophan hydroxylase gene.
\textsuperscript{a}The TPH2 risk haplotype is constructed by −703T, −473A, and 90G.
\textsuperscript{b}Calculated using SAS statistical software (SAS Institute Inc, Cary, North Carolina).
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, but between the serotonin transporter and dopamine transporter for harm avoidance and reward dependence traits, and between prothrombin and factor XII-A for myocardial infarction. The TPH1 and TPH2 proteins are paralogues and have distinct structural and kinetic properties but share common resources for serotonin production. 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. 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, the pons and medulla oblongata) may come from pineal gland projection. 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 interaction area is synthesized by TPH1 or TPH2, respectively. To our knowledge, this is 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 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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Correspondence: H. Sunny Sun, PhD, Institute of Molecular Medicine, National Cheng Kung University Medical College, 1 University Rd, Tainan 70101, Taiwan (hssun@mail.ncku.edu.tw).

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