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Short communication

Sequence, splice site and population frequency distribution analyses of the polymorphic human tryptophan hydroxylase intron 7

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Abstract

A human tryptophan hydroxylase intron seven polymorphism previously associated with low CSF 5-HIAA and suicidal behavior was sequenced and characterized for its potential role in TPH pre-mRNA splicing. Two polymorphic sites were identified: A218C and A779C. The 779A allelic frequency in various populations ranged from 0.43 to 0.61 and was in strong linkage disequilibrium with the A218C site. A218C provides a site for restriction fragment length polymorphism analysis. TPH mRNA was reverse-transcribed and sequenced. No aberrant splice products from the 779A or 779G TPH genes were detected nor were any other polymorphic nucleotides found.

Keywords: Tryptophan hydroxylase; Polymorphism; Serotonin; Polymerase chain reaction; Intron; Splicing; Linkage disequilibrium; Reverse transcription

Naturally occurring genetic variants of genes involved in neurotransmitter biosynthesis, catabolism or response may have profound effects on behavior. Genetic factors account for a significant portion of the inheritance of complex behavioral differences [28]. In extreme situations, the role of genetic factors is overt (e.g. the HPRT gene and Lesch-Nyhan syndrome [5]). However, many of the genetic factors influencing behavior are likely to act in more subtle fashion.

Polymorphisms have been identified in a large number of genes that may influence behavior. Several of the genes involved in serotonin function exhibit genetic variation. These include the serotonin receptors 5-HT_{1A} [19], 5-$HT_{1D\alpha}$ [25], 5-$HT_{1D\beta}$ [16], 5-HT_{2A} [26], 5-HT_{2C} [17] and 5-HT_7 [12] and the enzymes cyclohydrolase [14] and monoamine oxidase A [4] and the serotonin transporter [23]. The variant serotonin transporter gene associates with a susceptibility to major depression. Several of the receptor variants have been shown to modify the pharmacological properties of the receptor (e.g. 5-HT_{2A} [26]). Other mutations introduce non-sense codons as occurs in the monoamine oxidase A mutation found in a Dutch kindred to yield a truncated peptide [4]. This X-linked mutation resulted in a behavioral syndrome in males.

Because TPH is the rate-limiting enzyme in the biosynthesis of serotonin in the raphe neurons of the brain [7], we had hypothesized that variants in TPH may influence serotonin turnover. In a cohort of impulsive Finnish alcoholics, cerebrospinal fluid 5-hydroxyinoleacetic acid (CSF 5-HIAA), a metabolite of serotonin, was found to associate with a TPH intron 7 polymorphism [21]. Furthermore, since low CSF 5-HIAA is associated with behaviors characterized by deficient impulse control, such as suicidality [1,29], we also tested for association of the intron 7 polymorphism to suicidal behavior. A positive association was observed both to suicidal behavior and to the number of suicide attempts [21].

To characterize the intron 7 polymorphism, the polymorphic alleles identified by SSCP analysis [20,22] were amplified by PCR from DNA isolated from EBV-transformed lymphoblasts of UU and LL homozygous subjects. As shown in Fig. 1, two polymorphisms were identified in intron 7 and these were A218C and A779C. The TPH 779A and TPH 779C correspond to the U and L TPH

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    ...GAGCC
1   GTAAGTACTTCTATTTCAGCCAGGAATTCA
31  TCAAATGGTTATTTATAATAATGGCATCTA
61  CCTTATGGGTTCTTTTTTTTTTTTTTTTT
91  TTTGGTGTGCGAGGATTAAATAAATTAGCA
121 CATGTGAAGCATTTAGAATGGTACCTGGCA
151 TGAAATACATGTTCCATGCTCTATATGTGT
181 TAGCCATTATGATTATTAATTGACAACCTA
211 TTAGGTG[A/C]TAGCTGCTATTCTGAGCATAGG
241 GAATGTAACACTGAAAAAAATCAGACACAA
271 ATTTCTGCCTGCATAAAGCTTGTATTCCAG
301 TGGGGGAAACAGATAATAAACAAACAAGTA
331 AATGTATGCAAATGTTGCATCGAGTGGTGT
361 TGAGTCCCATGGAGAAAAATAAAGCTGAGA
391 AAGGGGGATGGAGGAAAGTGTAGGTGGGTG
421 GGAGTGTGTGTGTGTTGCTGTTTTGAAA
451 AGGGTGATCAGGGAAGGCCTTGCTGAGAAG
481 GTGATATCTGAGCAGAGATCTGATTTGGGT
511 GTGTATGTGGTC^^GTTGGGTGTTGGGGGT
541 TGTGGTTTTGGGAGTGGCATGTTGATGTCC
571 ATAGGTATCTGATGTGCACTTATATGTGTG
601 AGTCTGAGTGGCCAAGGTTTTGAACCAAAA
631 TTGTTTCTTTATTTGATTAGTGTCCTTTGT
661 GATCCATTACTAAAGTATTATCACCCGATC
691 ATTAGAATAAAATATTGGATTTCGATTTGA
721 TTGAATGGTTGATTATAAACGTATGTTAAG
751 CACTGCAGCGTGACAAA[C/T]TTGTACCTCT[A/C]T
781 TTCAG AGATA...
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Fig. 1. Sequence of TPH intron seven. Shown is the nucleotide sequence of the human TPH intron 7 with the sequence polymorphisms displayed in bold. The sequence of the 218A/779A (U) allele is presented with the changes in the 218C/779C (L) allele shown below. Exon 7 and 8 sequences are in italics. PCR amplification was performed essentially as in [20] with 100 ng DNA isolated from Epstein-Barr virus immortalized lymphoblastoma cell lines. DNA was amplified by PCR with primers HTHSSCP4 and HTHSSCP5. The DNA product was gel purified, sequenced (Amersham) and was run on a 6% urea-PAGE gel and visualized on an PhosphorImager 400 (Molecular Dynamics) and autoradiography.

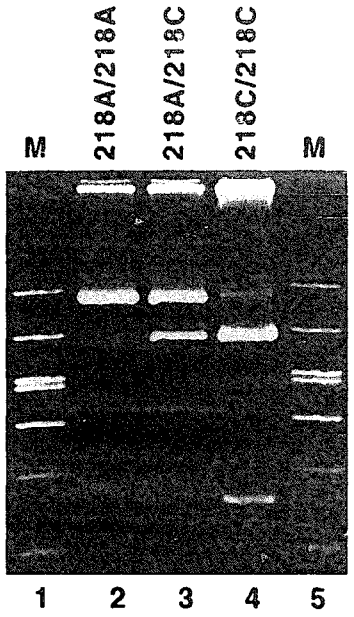

Fig. 2. *Nhe*I RFLP analysis of the polymorphic intron 7. Lymphoblast DNA from subjects was PCR-amplified essentially as in [20], using primer 1: 5′-TTCCATCCGTCCTGTGGCTGGTTA-3′ and primer 2: 5′-TTTGAACAGCCTCCTCTGAAGCGC-3′. The amplified product was digested with *Nhe*I (New England Biolabs) according to manufacturer's directions and electrophoresed on a 2% agarose gel. Samples were visualized by ethidium bromide staining and photography under UV light. DNA in lane 2 is derived from a subject homozygous for the 218C/779C (L) allele, lane 3: heterozygous for the 218/779 (U) alleles, lane 4: homozygous for the 218A/779A (U) allele and lanes 1 and 5 contain DNA markers.

alleles [20], respectively. The A218C polymorphism can be detected by RFLP analysis using the restriction enzymes *Nhe*I (Fig. 2), *Pst*NHI, *Bfa*I, *Mae*I or *Cac*81.

The allele-frequency distribution of the A779C in American Indian, Finnish, Korean, Siberian Yakut, Swedish and Taiwanese populations are listed in Table 1. The 779A allele frequency ranged from 0.43 in the Finns to 0.61 in the Siberian Yakuts. Linkage disequilibriums between the A218C and the A779C polymorphic alleles in these populations are listed in Table 2. A218C is in significant linkage disequilibrium with A779C having a normalized linkage disequilibrium ranging from 0.838 to 1.00.

The A779C polymorphism is located in a polypyrimidine stretch immediately upstream of the 3′ acceptor splice site [18]. In pre-mRNA, this polypyrimidine stretch forms a consensus sequence which is recognized by several proteins [30] and is required for proper acceptor site selection [8,10,11]. In eukaryotic introns, a pyrimidine occurs 86% of the time at the −7 position [27], the location of the A779C polymorphism. In fact, several genetic diseases are caused by transversions within this polypyrimidine stretch (reviewed in [15]). Furthermore, purine to pyrimidine transversions within this sequence increase splicing efficiency [8].

To test the hypothesis that the A779C or A218C alters splicing of the TPH pre-mRNA, mature spliced TPH mRNA was analyzed. PolyA mRNA was isolated from EBV-transformed lymphoblasts from subjects homozygous for the intron 7 alleles by hybridization to oligo $(dT)_{25}$ magnetic beads and reverse-transcribed by AMV reverse transcriptase. The resulting cDNA was amplified with primers specific to TPH mRNA. Sequencing of the amplified region revealed no nucleotide difference from the published cDNA [3].

Table 1
TPH intron 7 A779C allele frequencies in various populations

Population	779A allele frequency [a]	Subjects (n)
Italian	0.356	46
Finnish	0.426	812
Swedish	0.430	500
Taiwanese	0.443	122
American Indian	0.468	204
Korean	0.536	56
Siberian Yakut	0.584	42

Genotyping was by SSCP analysis as in [21] or [23].
[a] 779A corresponds to the U allele [21].

The sequencing gel across the exon 7/exon 8 splice junction is shown in Fig. 3. No aberrant splicing products were detected. The sequencing primer utilized sequenced the cDNA from the 5′ to 3′ of the TPH mRNA. If exon skipping had occurred to produce a stable alternatively spliced mRNA, then alternatively spliced TPH mRNAs would have been detected. However, if an aberrantly spliced mRNA was unstable, it may not have been detected.

Many examples of human genetic diseases caused by single base-pair substitutions in introns are known (reviewed in [15]). Transversions of pyrimidines to purines in the 3′ polypyrimidine consensus sequence have been found to cause several hemophilias, thalassaemias and a number of other genetic diseases. Of the two mutations found in intron 7, the A779C polymorphism is in the 3′ polypyrimidine tract. Although substitutions of pyrimidines for purines in the polypyrimidine consensus sequence have been shown to decrease the fidelity of splicing [11], sequencing of TPH cDNA (Fig. 3) revealed no evidence of exon skipping or aberrant splicing.

The A218C site is located in a potential GATA transcription factor-binding site [9]. GATA sites direct hematopoetic and, to a lesser extent, endothelial gene expression [24] by binding the GATA proteins, GATA-1, -2, -3 and -4 [2]. However unlikely, it is possible this GATA-binding motif may influence TPH gene expression.

Sequencing of the TPH cDNA derived from subjects homozygous for the 218A/779A or the 218C/779A alleles revealed no other variants in the TPH coding region. Since only the cDNA sequence of the human TPH gene was known, sequencing of the PCR product of the reverse-transcribed TPH mRNA using nested primers only allowed us to sequence from amino acids 20 to 370. To analyze the 3′ end of the cDNA, PCR-amplified genomic DNA from subjects homozygous for the 218A/779A or the 218C/779A alleles was sequenced. No variants in exon 11 from amino acids 397 to 436 were detected. Perhaps, linked variants in the coding region not sequenced (< 8% of the cDNA), promoter, other introns or the 3′ downstream region of the TPH gene may be involved.

Table 2
Linkage disequilibrium between the TPH intron 7 A779C and the A218C polymorphisms

Population	Δ	D	Haplotypes
Finnish	1.00	0.247 [a]	42
Italian	1.00	0.244 [a]	40
Siberian Yakut	1.00	0.234 [a]	40
Korean	1.00	0.242 [a]	44
Swedish	0.972	0.236 [a]	74
Taiwanese	0.838	0.209 [a]	38

Δ = normalized linkage disequilibrium: $(g1g4 - g2g3)/[(g1 + g3)(g2 + g4)(g1 + g2)(g3 + g4)]^{1/2}$.
D = linkage disequilibrium [6,13,18].
[a] Significantly deviating from linkage equilibrium ($P \leq 0.0001$).

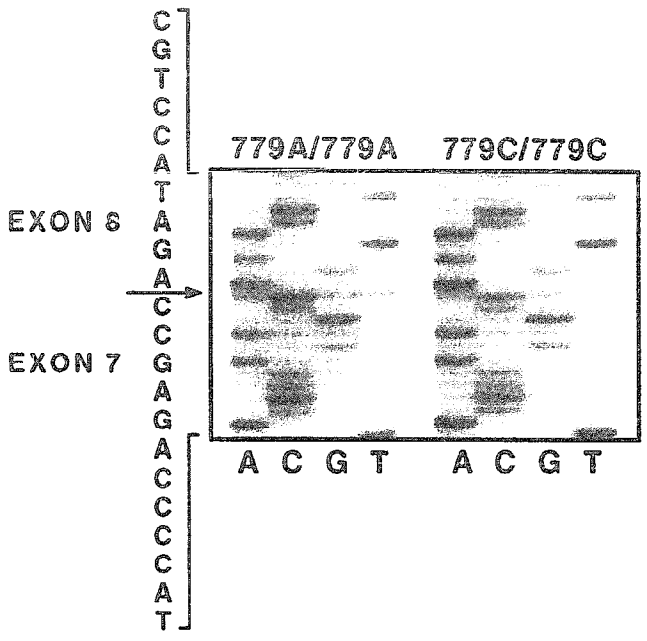

Fig. 3. Sequence of the exon 7/exon 8 splice junction. Sequencing was performed on cDNA made from EBV-transformed lymphoblasts homozygous for the 779A or the 779C allele. mRNA was isolated from transformed lymphocytes using Dynabeads oligo $(dT)_{25}$ beads (Dynal) and Dynabead lysis/binding buffer per manufacturer's instructions. cDNA was synthesized from 2 μg purified mRNA using a cDNA synthesis kit (Boehringer Mannheim), 10 pmol primer AFCDNA1, 5′-AGATACTCGGCTTCCTGCTGACCTT-3′ and AMV reverse transcriptase. 800 ng cDNA was PCR-amplified with 200 nM primers R1, 5′-CTCGCTCGCCCATTATGCTCTTGGTGTCTTTCAGGAT-3′ and R2, 5′-CTGGTTCGGCCCAGGAGAACAAAGACATTCCTTAGA-3′, 200 μM each of dCTP, dGTP, dTTP and dATP, 50 mM KCl, 3.5 mM $MgCl_2$, 0.001% BSA, 10 mM Tris, pH 8.3, and 2 U AmpliTaq polymerase (Perkin Elmer) in 100 μl. Samples were amplified for 30 cycles (1 min at 94°C, 2 min at 56°C and 3 min at 72°C) followed by 7 min at 72°C. The DNA was electrophoresed on a 5% polyacrylaminde and isolated by electroelution. Amplified DNA was cycle-sequenced according to manufacturer's instructions (TAQuenase cycle-sequencing kit, Amersham), using ^{32}P-end-labeled primer A, 5′-AGAGCGTACAGGTTTTTCCA-3′, electrophoresed on a 5% gel and visualized on an PhosphorImager 400 (Molecular Dynamics) and autoradiography.

Further studies are required to identify the nature of the A779C polymorphism association to CSF 5-HIAA and suicidal behavior. Another linked polymorphism nearby the A779C polymorphism may be involved in regulating or altering tryptophan hydroxylase activity.

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