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Identification and analysis of new sequence variants in the human tryptophan hydroxylase (TpH) gene

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The tryptophan hydroxylase (TpH) gene codes for the rate-limiting enzyme in serotonin biosynthesis. It is one of the major candidate genes for psychiatric and behavioral disorders. A polymorphism in TpH intron 7 has been shown to be associated with suicidal attempts, aggressive behavior and psychiatric illnesses. By systematically screening the TpH genomic sequence, we identified and confirmed an earlier report of four variants in the promoter region and localized six new sequence variants, ie two in intron 1b, one in exon 1c, one in intron 8, one in intron 9 and a microsatellite in the 3′ region, 5687 bp downstream of the last exon 11. We analyzed these polymorphisms, as well as the one in intron 7, by Single Strand Conformation Analysis, microsatellite or restriction analysis in a collection of 175 West European Caucasian healthy subjects. The four variants in the promoter region are in complete linkage disequilibrium (frequencies of G-T-G-T and T-C-A-G haplotypes are 0.41 and 0.59, respectively). Deletion of GTT in intron 1b is rare (0.7%) and so not informative. The rarer allele T of intron 1b polymorphism T3792A has a frequency of 0.34 and is in partial linkage disequilibrium with the more common alleles of intron 7, 8 and 9. The polymorphisms of these three introns are in complete linkage disequilibrium and the frequencies of haplotypes A-T-C and C-C-T are 0.36 and 0.64 respectively. We detected 10 different alleles in the microsatellite localized in the 3′ region; allele '194' is in partial linkage disequilibrium with haplotype A-T-C of introns 7, 8, and 9. Analysis of these different polymorphisms will constitute an important tool for future studies between the TpH gene and psychiatric disorders. *Molecular Psychiatry* (2000) **5,** 49–55.

Keywords: tryptophan hydroxylase gene; polymorphism; psychiatric disorders

Introduction

Tryptophan hydroxylase (TpH) catalyses the biopterin-dependent monooxygenation of tryptophan to 5-hydroxytryptophan which is then decarboxylated to form 5-hydroxytryptamine (serotonin).[1] It is the rate-limiting enzyme in the biosynthesis of the neurotransmitter serotonin. TpH gene expression is limited to a few specialized tissues[2] namely mast cells, mononuclear leukocytes, beta-cells of Langerhans islets, intestinal and pancreatic enterochromafin cells, and in the central nervous system (CNS), brainstem raphe neurons and pinealocytes.

Boularand *et al*[3] first described the complete human *TpH* coding sequence and its genomic organization, and analyzed the unusual splicing complexity of the 5′-untranslated region (5′-UTR).[4] The gene spans a region of 29 kb and is composed of 11 exons with, apparently, a single promoter and a single transcriptional initiation site. Several mRNA species are produced by differential splicing of three intron-like regions and of three exons located in the 5′-end, as well as from an alternative splicing site at the 3′-end.[5] The human TpH gene was mapped to 11p15.3-p14 between markers D11S151 and D11S134.[6,7]

The serotonergic system is involved in the modulation of several physiological processes such as thirst, appetite, sleep, memory and reproduction.[8] A decrease in serotonin turnover, indicated by low 5-hydroxyindoleacetic acid (5-HIAA) concentrations in cerebrospinal fluid (CSF), was shown to be associated with behavioral disorders including suicidality,[9] impulsive violence,[10] depression[11] and early-onset alcoholism.[12] Polymorphisms have been described in several genes involved in serotonin metabolism and function: serotonin transporter,[13] serotonin receptors 5-HT1A,[14] 5-HT2A,[15] 5-HT2C[16] and monoamine oxydase A[17] and B.[18] Association studies between some of these sequence variants and behavioral disorders are now performed by many investigators.

Nielsen *et al*[7,19] reported two polymorphisms in intron 7 of the TpH gene (A218C and A779C) using single-strand conformational analysis (SSCA) and PCR-based sequencing. Both variants were in linkage disequilibrium, the rare allele frequency varying between 0.36 and 0.58 depending on the studied population. The authors suggested that, since the variants were

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intronic, it was unlikely that they should alter serotonin biosynthesis, as was confirmed by mRNA analysis.[19] Nielsen *et al*[20] reported a correlation, in Finnish alcoholics, between TpH intron 7 genotype and CSF 5-HIAA concentration, however subsequent studies failed to confirm these positive results.[21] The polymorphism was presented as being associated with a history of suicide attempts in violent offenders. This possible association between TpH intron 7 polymorphisms and behavioral phenotypes was examined in several other studies. Nielsen *et al*[21] reported a significant difference in allele and genotype frequencies in violent offenders with or without a history of suicide attempt. Mann *et al*[22] found a significant difference in allele and genotype frequencies in a sample of depressed patients with or without a history of suicide attempt. New *et al*[23] and Hsu *et al*[24] also reported differences in allele repartition in impulsive-aggressive and alcoholic subjects, respectively. Bellivier *et al*[25] found an association with manic-depressive illness which was not replicated in other studies.[26,27] In a very recent study, Manuck *et al*[28] reported an association between the intron 7 polymorphism and both aggression and anger-related dispositions. The apparent discrepancies between these studies may in fact indicate that the TpH gene is directly involved in the control of impulsivity and/or aggressiveness, behaviors commonly present in psychiatric disorders such as manic-depressive illness and alcoholism.

As a first step in further exploring this hypothesis, we analyzed the already described polymorphisms in the promoter[29] and in intron 7.[20] We searched for new TpH sequence variants. We report in this study the characterization of six new polymorphisms within the TpH gene, and their study by SSCA, microsatellite or restriction analysis. The allele and genotype frequencies and the pattern of linkage disequilibrium were investigated in a collection of 175 European Caucasian healthy controls.

Materials and methods

Subjects
The study collection consisted of 175 French control subjects. The subjects were of West European Caucasian origin for at least two generations and without any history of psychiatric disorders. The 175 control subjects were all interviewed by trained psychiatrists with a French version of the semi-structured Diagnostic Interview for Genetic Studies (DIGS) developed by the NIMH.[30] All subjects gave written informed consent.

DNA extraction and PCR analysis
Genomic DNA was extracted from blood white cells (Nucleon extraction kit, Amersham Life Science, Dubendorf, Switzerland). About 100 ng of genomic DNA was amplified with appropriate oligonucleotides (Table 1) using a Hybaid thermocycler. PCR was performed in a 50-μl reaction mixture containing 20 pmol of each oligonucleotide, 1 or 1.5 mM $MgCl_2$, 200 nmol dNTPs, 2 units of Taq DNA polymerase (Eurobio, Brunschwig, Basel, Switzerland). Optimized PCR conditions for each polymorphic site are detailed in Table 1.

RFLP, SSCP and microsatellite analyses
For restriction length polymorphism analyses, 10 μl of the PCR-amplified products were digested overnight with 5 units of appropriate restriction enzymes (New England Biolabs) (Table 1) in their corresponding digestion buffer, according to the specifications of the manufacturer. The restriction fragments were then separated on 1–2% agarose gels (Standard agarose, Eurobio) depending on the fragment sizes. Following electrophoresis at 100 V, fragments were visualized on an UV transilluminator after ethidium bromide staining. For SSCA, 5 μl of PCR-amplified products were denatured at 95°C for 3 min with 2 μl of loading buffer (15% ficoll, 0.25% bromophenol blue and 0.25% xylene-cyanol), 0.4 μl of 1 M CH_3HgOH, 12.6 μl of 1 × TBE, and chilled on ice before loading. Electrophoresis was performed at 300 V on 4–20% gradient polyacrylamide gels (49:1), 1 mm thick (Hoeffer Pharmacia vertical slab unit) in 1 × TBE buffer at room temperature or at 4°C for different time lengths depending on the product size (Table 1). The single strand conformational polymorphisms (SSCP)[31] were visualized after ethidium bromide staining of the acrylamide gels.

For microsatellite analysis,[32] 8 μl of PCR-amplified products with 2 μl of loading buffer (0.25% bromophenol blue, 0.25% xylene-cyanol, 16% glycerol, 1 mmol EDTA) were loaded on 10 or 15% acrylamide gels (19:1), 1.5 mm thick (Hoeffer Pharmacia vertical slab unit), and electrophoresis was performed in 0.5 × TBE buffer at 250 V for 8 or 11 h. Gels were stained with ethidium bromide and fragments visualized on an UV transilluminator (Table 2).

Sequencing
After purification (Kristal Clean-up Kit, Cambridge Molecular Technologies), PCR-amplification products showing polymorphic patterns were manually sequenced using Thermo Sequenase cycle sequencing kit (Amersham Life Science) and adenosine 5′-γ^{33}P-triphosphate (NEN Life Science) labelling with T4 Kinase according to the manufacturer's protocol (Life Technologies). Electrophoresis was performed on 5% acrylamide (Accugel 19:1, National Diagnostics, Brunschwig), 7 M urea, in 0.5 × TBE buffer at 55 W for 1.5 h. Sequencing gels were dried in a hot vacuum drier and then autoradiographed (Hyperfilm MP, Amersham Life Science) at room temperature.

Statistical analysis
Allele and genotype distributions among our control subjects population were analyzed using the chi-square test. Haplotypes frequencies were estimated using the EH program.[33]

Results

We performed a systematic mutation screening in promoter, exon-intron junctions, and part of the intronic

Table 1 Polymorphisms detected in the genomic sequence of the TpH gene. The nucleotide position indicated for the promoter polymorphisms is relative to the transcription initiation site, to the start of intron 7 for A218C, to the start of exon 9 and exon 10 for T-465C and C-160T, respectively. Appropriate oligonucleotides and PCR conditions are given for the analysis of each variant

Polymorphic site	*TpH region*	*Oligonucleotides sequences*	*Size (bp)*	*PCR conditions*
G-1721T	promoter	5′-GAACTTGGAATAGCCTTCTG-3′ 5′-TGCCAATGGTGAACAGTATG-3′	173 bp	1 mM $MgCl_2$, 57°C annealing
T-1606C	promoter	5′-CTCCTTTGGTTAATTCCTAGG-3′ 5′-GCCTTTCCTTGTAGATCTGG-3′	367 bp	1.5 mM $MgCl_2$, 58°C annealing
G-1067A	promoter	5′-ATGGTACTTACTAGCCTGTG-3′ 5′-CTGTCTCCACAGTTTTGCC-3′	323 bp	1.5 mM $MgCl_2$, 56°C annealing
T-347G	promoter	5′-CTTCGTTATGTGTACAGTCC-3′ 5′-TAGGACTGCAGTGCTTCTC-3′	365 bp	1.5 mM $MgCl_2$, 58°C annealing
2581(-gtt)	intron 1 b	5′-GCATCTCACTATGTTACCC-3′ 5′-AGCAAGATCGTGTCGCTGC-3′	132 bp	1 mM $MgCl_2$, 58°C annealing
T3792A	intron 1 b	5′-CTTGCCTAGATGGATTTGCAG-3′ 5′-AGGGATGGCCTCAGATAAGC-3′	224 bp	1 mM $MgCl_2$, 58°C annealing
T3804C	exon 1 c	5′-CTTGCCTAGATGGATTTGCAG-3′ 5′-AGGGATGGCCTCAGATAAGC-3′	224 bp	1 mM $MgCl_2$, 58°C annealing
A218C	intron 7	5′-TTCAGATCCCTTCTATACCCCAGA-3′ 5′-GGACATGACCTAAGAGTTCATGGC-3′	918 bp	1.5 mM $MgCl_2$, 58°C annealing
T-465C	intron 8	5′-GTAACAGGCAGTTCATTGAC-3′ 5′-CCAAAGACTCTTAGCTGTCC-3′	747 bp	1.5 mM $MgCl_2$, 60°C annealing
C-160T	intron 9	5′-CAAAGCCATGGAGAGGATC-3′ 5′-CTTCCATAATTGGCTCACCTC-3′	163 bp	1.5 mM $MgCl_2$, 59°C annealing
$(CT)_n(CA)_n(CT)_n$	3′region	5′-AATCTCAGGTTGCTGCCTGAC-3′ 5′-GTTTTTCCACAGGACTAGCC-3′	204 bp	1.2 mM $MgCl_2$, 58°C annealing

Table 2 Typing method for each analyzed polymorphic site. The G-1721T polymorphism was analyzed by direct sequencing. Allele frequencies and χ^2 and *P* values for Hardy–Weinberg equilibrium are given for the 175 control subjects analyzed. These values are not given for polymorphism T3804C which was found in only one allele of one subject.* For allele frequencies of the 3′ region microsatellite, refer to the Results section

Polymorphic site	*Restriction site*	*SSCP/microsatellite conditions*	*Allele frequencies*	*H-W equilibrium*	
				χ^2	*P value*
G-1721T	–	–	⎫		
T-1606C	–	SSCP, 25°C, 180 min migration	0.41/0.59	0.45	0.79
G-1067A	*Dra*II				
T-347G	*Msl*II		⎭		
2581(-gtt)	–	SSCP, 25°C, 180 min migration 15% acrylamide gel, 11 h migration	0.007/0.993	–	–
T3792A	*Sfc*I	SSCP, 4°C, 90 min migration	0.34/0.66	0.45	0.79
T3804C	*Acl*I	SSCP, 4°C, 90 min migration	0.001/0.999	–	–
A218C	*Bfa*I		0.36/0.64	1.63	0.44
T-465C	*Nla*III		0.36/0.64	1.51	0.47
C-160T	–	SSCP, 4°C, 145 min migration	0.36/0.64	1.74	0.41
$(CT)_n(CA)_n(CT)_n$	–	10% acrylamide gel, 8 h migration	*	–	–

sequences of the TpH gene. With this approach, 10 different polymorphisms were analyzed, ie four previously described[29] in the promoter region (G-1721T; T-1606C; G-1067A; T-347G), two in intron 1b (2581 (-gtt); T3792A), one in exon 1c (T3804C), one in intron 8 (T-465C), one in intron 9 (C-160T) and one in the 3′ region [$(CT)_n(CA)_n(CT)_n$] (Figure 1).

Due to its functional importance, the promoter region was completely explored through analysis of short PCR overlapping fragments. We identified four polymorphisms previously reported, but not described by Rotondo *et al*.[29] In the promoter region, sequencing of one PCR fragment showing different SSCP patterns revealed polymorphisms G-1721T and T-1606C. We then designed oligonucleotides flanking each variant for their individual analysis. The G-1721T variant did not correspond to a restriction site and we failed in establishing SSCA conditions to reveal this variant. We

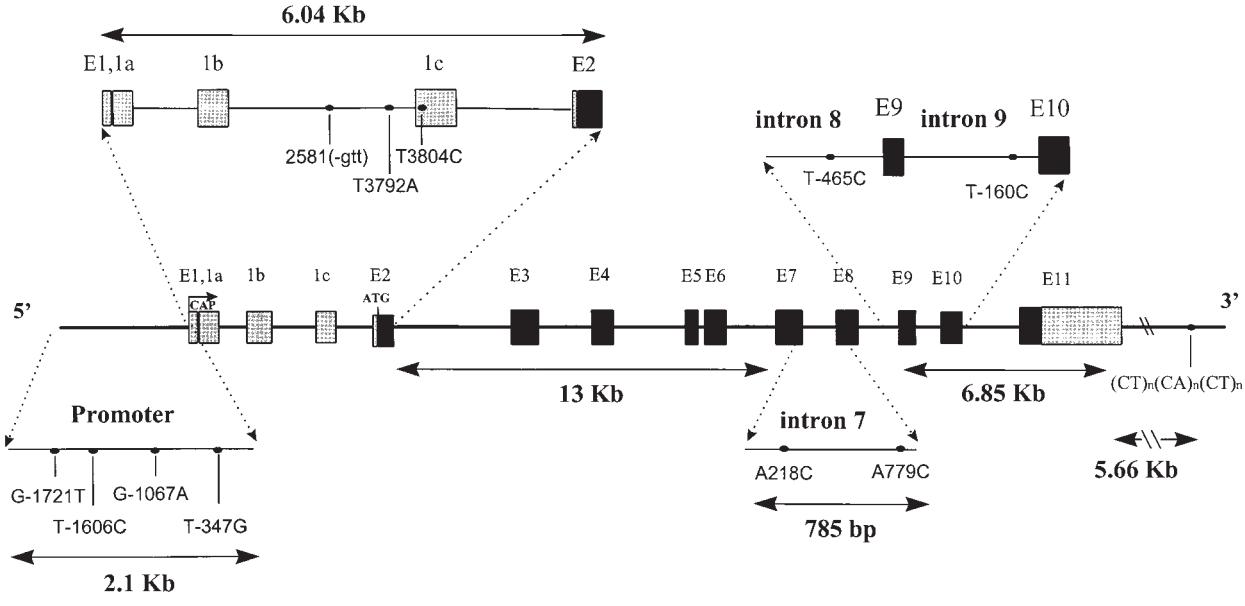

Figure 1 Schematic representation of the human TpH gene illustrating the location of 12 polymorphic sites. Variant A779C was not analyzed in this study. Shaded boxes indicate 5′-non-coding exons, black boxes indicate coding exons. The bent arrow indicates the position of the transcription initiation site. The translation start site is indicated by ATG.

therefore sequenced the corresponding PCR fragment for 12 subjects. The T-1606C polymorphism did not correspond to a restriction site and was screened by SSCP. Polymorphisms G-1067A and T-347G were analyzed by *Dra*II and *Msl*I RFLP, respectively. The typing of the first 100 control subjects for the three promoter polymorphisms T-1606C, G-1067A and T-347G, and sequencing of G-1721T on 12 subjects, confirmed that this region was in complete linkage disequilibrium.[29] We therefore analyzed the promoter region for the 75 remaining subjects, with the T-1606C polymorphism only. The allele frequencies were 0.41 (haplotype 2) and 0.59 (haplotype 1), and the genotypic distributions in Hardy–Weinberg equilibrium (Table 2).

The 5′-UTR containing exons and introns 1a, b and c were also analyzed because of the unusual splicing complexity of this region. Thus we focused on the different splicing junctions as well as the region of a short stretch of (gtt) repeats. Polymorphism 2581(-gtt), corresponding to the deletion of a (gtt) triplet within a stretch of 4 (gtt) in intron 1b, was found by SSCA. We then serially screened all the subjects by microsatellite gel analysis because of the size difference due to this deletion. This polymorphism was found to be very rare (allele frequencies: 0.007 and 0.993) and in Hardy–Weinberg equilibrium. The other intron 1b polymorphism, T3792A, three nucleotides distant from exon 1c, was also discovered by SSCA, sequenced on 12 subjects, and screened by both *Sfc*I RFLP and SSCP for all the subjects. Its allele frequencies were found to be 0.34 and 0.66, with genotypic distributions in Hardy–Weinberg equilibrium. One of the subjects screened for this variant showed a very particular SSCA pattern which was not observed in any other subject (data not shown). We sequenced the corresponding PCR fragment and found that this subject was heterozygous for another variant, T3804C, 12 nucleotides distant from T3792A, and within exon 1c. This polymorphism constituted an *Acl*I restriction site.

Since associations between the intron 7 polymorphisms and several psychiatric diseases have been reported, we searched for new variants in the flanking regions. We first confirmed by direct sequencing that there were no other polymorphisms in intron 7 than those previously described (A218C and A779C).[19] We then analyzed only the A218C variant by *Bfa*I RFLP, because of the complete linkage disequilibrium reported between both polymorphisms in nearly all the explored populations.[19] The A218C allele frequencies were found to be 0.36 and 0.64 in our collection. Genotypic distributions were in Hardy–Weinberg equilibrium.

As the intron 8 and 9 sequences were not known, we performed PCR-amplification from DNA of 10 different subjects, with oligonucleotides designed at the end of the corresponding exons, and sequenced the obtained fragments. Intron 8 polymorphism (T-465C) constituted a restriction site and was therefore screened by *Nla*III RFLP for all the subjects. Its allele frequencies were found to be 0.36 and 0.64. Intron 9 polymorphism (C-160T) was analyzed by SSCA for all the subjects, with allele frequencies of 0.36 and 0.64. Both genotypic distributions were found to be in Hardy–Weinberg equilibrium.

Through analysis of a newly released sequence of a PAC clone containing the complete genomic sequence of TpH gene (Genbank accession number AC005728), we found a putative microsatellite in the 3′ region, 5657 bp distant from exon 11 [$(CT)_{24}(CA)_{11}(CT)_{10}$]. We PCR-amplified and sequenced this region in 10 different subjects, observing size variations of the repeat. This microsatellite was then serially explored by

microsatellite gel analysis for all the subjects. The size of the corresponding PCR fragment was found to vary as follows, with the respective allelic frequencies: 190 bp (0.29%), 194 bp (33.05%), 196 bp (5.17%), 198 bp (15.23%), 200 bp (2.87%), 202 bp (6.61%), 204 bp (29.6%), 206 bp (5.46%) 208 bp (1.44%) and 216 bp (0.29%).

Linkage disequilibrium was found to be complete between the four promoter variants as well as between intron 7, 8, and 9 variants. At introns 7, 8 and 9 polymorphic sites, the observed haplotypes were A-T-C and C-C-T, respectively. The haplotype A-T-C was in incomplete linkage disequilibrium with the promoter haplotype 1 ($D' = 0.48$), intron 1 b allele A ($D' = 0.49$), and the 194-bp microsatellite allele ($D' = 0.55$). The C-C-T haplotype was only preferentially associated to intron 1 b allele T ($D' = 0.42$).

Discussion

We characterized six new variants and so analyzed 10 polymorphic sites within the genomic sequence of human TpH gene in a population of 175 mentally healthy control subjects. We focused on the non coding sequences since they were more likely to be polymorphic than the coding sequences. Moreover, Nielsen *et al*[19] had already performed a screening of nearly all the coding sequence, by direct cDNA sequencing of several individuals and found no sequence variants.

Two variants were found to be very rare (2581(-gtt) and T3804C), with the rare allele frequency of less than 1% in our population. Individuals carrying these variants do not display any psychological or ethnic particularity. Despite its rarity, it will be of interest to determine the T3804 frequency in samples of psychiatric subjects, since it is the only TpH exonic polymorphism ever reported with another rare variant in exon 10.[34]

We analyzed four promoter polymorphisms. Although we screened the complete reported sequence of the TpH promoter region,[4] we did not find any other polymorphic site. The intron 7 polymorphisms were previously described by Nielsen *et al*[7] and then extensively used for association studies in psychiatric diseases. We scored one of these polymorphisms in our population in order to determine the pattern of linkage disequilibrium with flanking variants. The polymorphic sites we report in intron 1 b, exon 1 c, intron 8, intron 9 and in the 3′ region have never been described and should constitute an important tool for further association and linkage studies.

The four promoter variants were found to be in complete linkage disequilibrium, as well as the intron 7, 8 and 9 polymorphisms. However we observed partial linkage disequilibrium between the promoter region and the intron 7, 8 and 9 regions. Therefore if a true association between intron 7 polymorphism and a disease is observed, it can be predicted that association with intron 8 and 9 polymorphisms will also be found. It will be of interest to determine whether this putative association is strengthened by the combination with one of the two promoter haplotypes, and/or one allele of the 3′ region microsatellite.

Some of these polymorphisms may have functional effects. Heils *et al*[35] recently reported a polymorphic site in the promoter region of the serotonin transporter gene (5-HTT), correlated to variations in transcriptional activity and protein expression, and accounting for 4–5% of population variation of anxiety-related behavioral traits. TpH promoter transcription regulation was explored through deletion analysis by Boularand *et al*[4] who showed that deletion of the promoter region from −2117 to −204 bp did not significantly modify the transcriptional activity of the corresponding constructs. However, they found a weak tissue-specific repressor located between −724 and −252 bp, the location of one of the promoter polymorphisms (T-347G) reported here. Also by deletion analysis, a very recent study has shown that successive deletions of the promoter region between −732 and −41 led to a significant decrease in promoter activity.[36] We can therefore hypothesize that sequence variations within this region could slightly modify tissue-specific expression of TpH enzyme and affect serotonin biosynthesis.

Another possible mechanism by which some of these variants may influence TpH activity is by interacting with splicing processes. It is estimated that up to 15% of all point mutations occurring in human genetic diseases result in a mRNA splicing defect.[37] One of the polymorphic sites we describe (T379A), is located in intron 1 b, three nucleotides distant from exon 1c, ie position −4, at the 3′ intronic splice site. Their invariant AG dinucleotide at the 3′-intronic acceptor splice site is usually preceded by a pyrimidine residue and a 10–20 nucleotide pyrimidine rich tract binding with U2AF protein of the splicing machinery.[38] Experimental findings have demonstrated an important role for this pyrimidine tract and mutations at positions −7, −8 and −13 have been shown to exert a pathological influence on efficient splicing. We can hypothesize that the T to A variation we described, within the pyrimidine rich tract of intron 1b, may modify binding affinity for splicing proteins and eventually affect exon 1c splicing. Exon 1c is inconstantly present in the TpH mRNA, with the 5′-UTR showing a large diversity of splicing patterns for the exons E1, E1a, E1b and E1c, whereas the coding region is identical in all studied tissues.[4] Although exon 1c is in the 5′-UTR and is inconstantly present in TpH mRNA, a variation within its sequence could affect the transcript stability and possibly have consequences on gene expression.

The intron 8 and 9 polymorphisms are located at positions −465 and −160 relative to exons 9 and 10 respectively and are therefore less good candidates for splicing defects even though intronic mutations that are not in the direct vicinity of an exon can still create splicing defects through cryptic splice sites.[37]

Detection and analysis of the microsatellite found in the 3′ region of TpH gene will be helpful in non parametric studies (Transmission Disequilibrium Test, Haplotype Relative Risk). We detected 10 alleles for this marker which is more informative than biallelic

polymorphism. The higher heterozygosity rate will allow us to construct a more precise haplotype and increase the number of informative transmissions.

Further biological assays will be necessary to determine if one of the polymorphisms affects TpH gene transcription and could represent a functional variant. First, however, it will be necessary to confirm the data suggesting that TpH is involved in psychiatric disorders with high rates of impulsivity and aggressivity. The availability of these new markers spanning the entire TpH gene will certainly be essential for this goal.

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