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Mutations in the Cyclic Adenosine Monophosphate Response Element of the Tyrosine Hydroxylase Gene

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Tyrosine hydroxylase (TH) deficiency (OMIM 191290) is one cause of early-onset dopa-responsive dystonia. We describe seven cases from five unrelated families with dopa-responsive dystonia and low homovanillic acid in cerebrospinal fluid who were suspected to suffer from TH deficiency. Analysis of part of the TH promoter showed five homozygous and two heterozygous mutations in the highly conserved cyclic adenosine monophosphate response element. Our data suggest that, if no mutations are found in the coding regions of the gene in patients strongly suspected of TH deficiency, the search for pathogenic mutations should be extended to regulatory promoter elements.

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Typically, TH deficiency becomes manifest by the end of the first year and is characterized by ptosis, inexpress-

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sive faces, oculogyric crises, tremor, truncal hypotonia, and limb dystonia, and biochemically by a selectively decreased concentration of homovanillic acid (HVA) in cerebrospinal fluid (CSF). The definite diagnosis, however, depends on the demonstration of mutations in the TH gene. A number of different mutations in the coding sequence of the TH gene have been reported,^{1,2} including a “common” mutation in the Dutch population (c.698G>A; p.Arg233His)³ and a single base pair deletion in exon 3.⁴ In this report, we describe seven cases, clinically suspected to suffer from TH deficiency, with low CSF HVA levels and with a moderate-to-good response to L-dihydroxyphenylalanine (L-dopa) therapy (Table; see supplementary file online for case descriptions).

Patients and Methods

Patients

Clinical features of the patients studied here are described later in the Results.

Cerebrospinal Fluid Analysis

HVA and 5-hydroxy-indole acetic acid (5-HIAA) in CSF of Cases IV-1, IV-2, and V-1 were initially measured in Sydney, Australia. Repeat measurements of Cases IV-1 and IV-2 and initial measurement of Case II-1 were performed in Nijmegen, the Netherlands, according to previously described methods.² CSF of the other patients was analyzed locally according to similar methodology.

Tyrosine Hydroxylase Gene Analysis

Genomic DNA was extracted from leukocytes by standard methods followed by polymerase chain reaction (PCR). The entire coding region of the TH gene was sequenced, including 211bp of the promoter region.⁵ Primers were designed

to include the intron-exon boundaries as described elsewhere⁶ with the exception of modifications in the forward primers for exons 1, 6, 11, 13, and 14 (sequences available on request).

PCR amplifications were performed in a total volume of 50µl on an I-cycler (Bio-Rad, Veenendaal, the Netherlands). Each reaction mixture contained 200ng forward and reverse primer, 500µM of each deoxyribonucleoside triphosphate (dNTP), 50mM tris(hydroxymethyl)aminomethane-HCL buffer (pH 9.2), 16mM (NH₄)₂SO₄, 2.25mM MgCl₂, 2% dimethylsulfoxide, 0.1% Tween 20, 1 unit AmpliTaq DNA Polymerase (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands), and 100ng genomic DNA. The PCR products were purified with the enzymes Exonuclease I and Shrimp Alkaline Phosphatase (Amersham Bioscience, Roosendaal, the Netherlands) and subsequently sequenced on an ABI PRISM 377 automated DNA sequencer using the BigDye Terminator v3.1 Cycle Sequencing Kit, according to the recommendations of the manufacturer (Applied Biosystems).

Mutations in the promoter region were all confirmed by restriction enzyme analysis by using AAT II (recognition site GACGT ↓ C; New England Biolabs, Beverly, MA). Restriction enzyme analysis of the exon 11 PCR fragment was performed with Nla III (recognition site CATG ↓ ; New England Biolabs). Restriction enzyme analysis of the exon 14 PCR fragment was performed with Bsp1407I (recognition site T ↓ GTACA; New England Biolabs).

Numbering of coding sequence mutations was according to GenBank reference sequence NM_199292 (TH isoform A) in which the A of the ATG transcription initiation codon is designated position as 1. Numbering of the promoter mutations was defined relative to the ATG initiation codon. According to this numbering, the cyclic adenosine monophosphate response element (CRE) resides between residues -67 and -74 directly upstream of the ATG initiation codon. Previously, a different numbering had been used, defining

Table. Cerebrospinal Fluid Analysis of Neurotransmitter Metabolites Homovanillic Acid and 5-Hydroxy-indole Acetic Acid and Mutations in the Conserved Consensus Sequence of the Cyclic Adenosine Monophosphate Response Element within the Tyrosine Hydroxylase Promoter Region

Case No.	Age (yr)	HVA (nM)	Reference	5-HIAA (nM)	Reference	HVA/5-HIAA	Reference	Consensus Sequence ^a	Mutation ^b
I-1	3	100	211–871	142	105–299	0.7	1.5–3.5	TGACGACA	-69 T>A; homozygous
II-1	7	69	350–550	129	100–225	0.5	1.5–4.0	TGATGTCA	-71 C>T; heterozygous ^c
III-1	11	83	220–560	114	90–237	0.7	1.7–3.7	TGACATCA	-70 G>A; homozygous
III-2	2	319	364–870	301	155–359	1.1	1.5–4.1	TGACATCA	-70 G>A; homozygous
IV-1	0.5	133	478–895	192	231–618	0.7	1.3–3.1	TGACATCA	-70 G>A; homozygous
IV-2	2	159	384–769	138	110–265	1.2	1.8–4.4	TGACATCA	-70 G>A; homozygous
V-1	1.1	100	429–789	200	156–275	0.5	1.6–3.3	TGACATCA	-70 G>A; heterozygous ^c

^aBoldface indicates mutation; wild-type control sequence: TGACGTCA.

^bRelative to the ATG start codon.

^cSecond mutation in Case II-1: c.1159C>A (p. Leu387Met) in exon 11; in Case V-1: c.1475C>A (p.Pro492Leu) in exon 14.

HVA = homovanillic acid; HIAA = 5-hydroxy-indole acetic acid.

the location of the CRE between residues -38 and -45 directly upstream of the transcription initiation site.⁷

Secondary Structure of Protein Prediction

Secondary protein structure prediction was performed using the software developed by the National Institute of Advanced Industrial Science and Technology (Tokyo, Japan; available at: http://mbs.cbrc.jp/papia/cgi/ssp_query.pl?query=seq). The final prediction of protein secondary structure was achieved by combining the results of five different prediction methods ("new joint" method). Conservation of amino acid sequences was performed by using the BLAST software (<http://www.ncbi.nlm.nih.gov/BLAST>).

Results

Conventional sequence analysis of the 14 exons of the TH gene did not demonstrate any mutation in Cases I-1, III-1, III-2, IV-1, and IV-2. Novel mutations were found in exon 11 of the TH gene in Case II-1 (c.1159 C>A transversion), causing an amino acid substitution (p.Leu387Met), and a mutation in exon 14 in Case V-1 (c.1475 C>T, p.Pro492Leu). Both mutations affect amino acids in the TH enzyme that are highly conserved between different species (see Supplementary Table E1). Prediction of the secondary structure demonstrated the replacement of a β -sheet in the wild-type protein to a turn in the mutant p.Leu387Met protein. Similarly, the secondary structure of the protein changes from a random coil into a β -sheet in case of the mutant p.Pro492Leu protein. Finally, these amino acids were conserved in the four human amino acid hydroxylases (see Supplementary Table E1).

Because all seven cases displayed typical clinical features of TH deficiency, combined with a suggestive neurochemical pattern, that is, CSF HVA concentrations less than 50% of the lower reference range in six of seven cases with normal 5-HIAA concentrations, we decided to extend our search for mutations to intron-exon boundaries and part of the promotor region. Novel homozygous mutations were identified in the CRE (TGACGTCA) of the TH promotor in Cases I-1, III-1, III-2, IV-1, and IV-2, as well as heterozygous promotor mutations in Cases II-1 and V-1 (see the Table). Each mutation was confirmed by restriction enzyme analysis, and parents of all cases were heterozygous for one of the pathogenic mutations observed in the index case (Fig). These mutations were not observed in 254 control alleles, suggesting that they are pathogenic.

Discussion

CSF HVA concentrations observed in these seven cases with TH promotor mutations were in the same range, with the exception of Case III-2, as previously described for patients with mutations in the coding exons of the TH gene; that is, levels are usually less than

50% of the lower reference limit.^{4,8} Besides, all cases responded well to low-dose L-dopa treatment.

The CRE (TGACGTCA), as well as other regulatory elements in the TH promotor including the activator protein-1 (AP1) and Sp1 sites, and its relative position from the RNA initiation site is highly conserved in the rat, bovine, murine, and human TH genes, suggesting an important function in the regulation of TH transcription.^{9,10} Together with the AP1 site, the CRE site is involved in the Ca²⁺-mediated TH transcription induction.¹¹ Studies in various cell lines show that nucleotide substitutions in the CRE of the TH gene results in significant reduction of the basal transcription. Mutation of the entire eight-nucleotide sequence in mouse cell lines leads to an 80 to 90% reduction in transcriptional activity of the gene.¹² Mutation of any nucleotide within the CRE octamer motif in the rat TH promotor reduced both basal and forskolin-induced expression by 50 to 90%.¹³ Site-directed mutagenesis of -69 T>A in the CRE octamer of the rat TH gene, similar as the mutation observed in Case I-1, results in a reduction of basal TH gene transcription of 78%, which may thus explain the clinical picture of Case I-1.¹³ Site-directed mutagenesis resulted in a 90% reduction of basal expression by a -70 G>T mutation, providing an explanation for the reduced TH activity in Cases III-1, III-2, IV-1, and IV-2, who are homozygous for the mutation at this base pair (-70 G>A). Furthermore, the -71 C>T mutation, as observed in Case II-1, was found to cause a reduction of 90% of basal transcription of the TH promotor.¹³ Residual activity appeared significant in these patients, which likely corresponds to an especially good response to therapy and outcome compared with previously reported patient series.¹⁴ Mutations in the CRE site in several other genes, including human fibronectin,¹⁵ also strongly reduced the basal transcription of the gene, confirming the important role for the CRE site in gene transcription.

We observed the same promotor mutation (-70 G>A) in five cases from five families. Therefore, although TH deficiency is a rare disorder, this mutation may be regarded as relatively common. Because the affected children have different backgrounds (either from Turkish, Lebanese, or Fijian/Indian origin), it is not likely that there is a common founder for these mutations. We have previously identified a relatively common mutation in exon 6 (c.698G>A; p.Arg233His),³ suggesting that certain hotspots for pathogenic mutations exist in the TH gene and promotor.

Mutations in regulatory sites of genes are reported to comprise 1.2% of the total number of known mutations in human genes (Human Gene Mutation Database: <http://www.hgmd.cf.ac.uk/ac/hahaha.php>). Approximately 4,000 genes^{16,17} contain a CRE; but to the best of our knowledge, this is the first report of

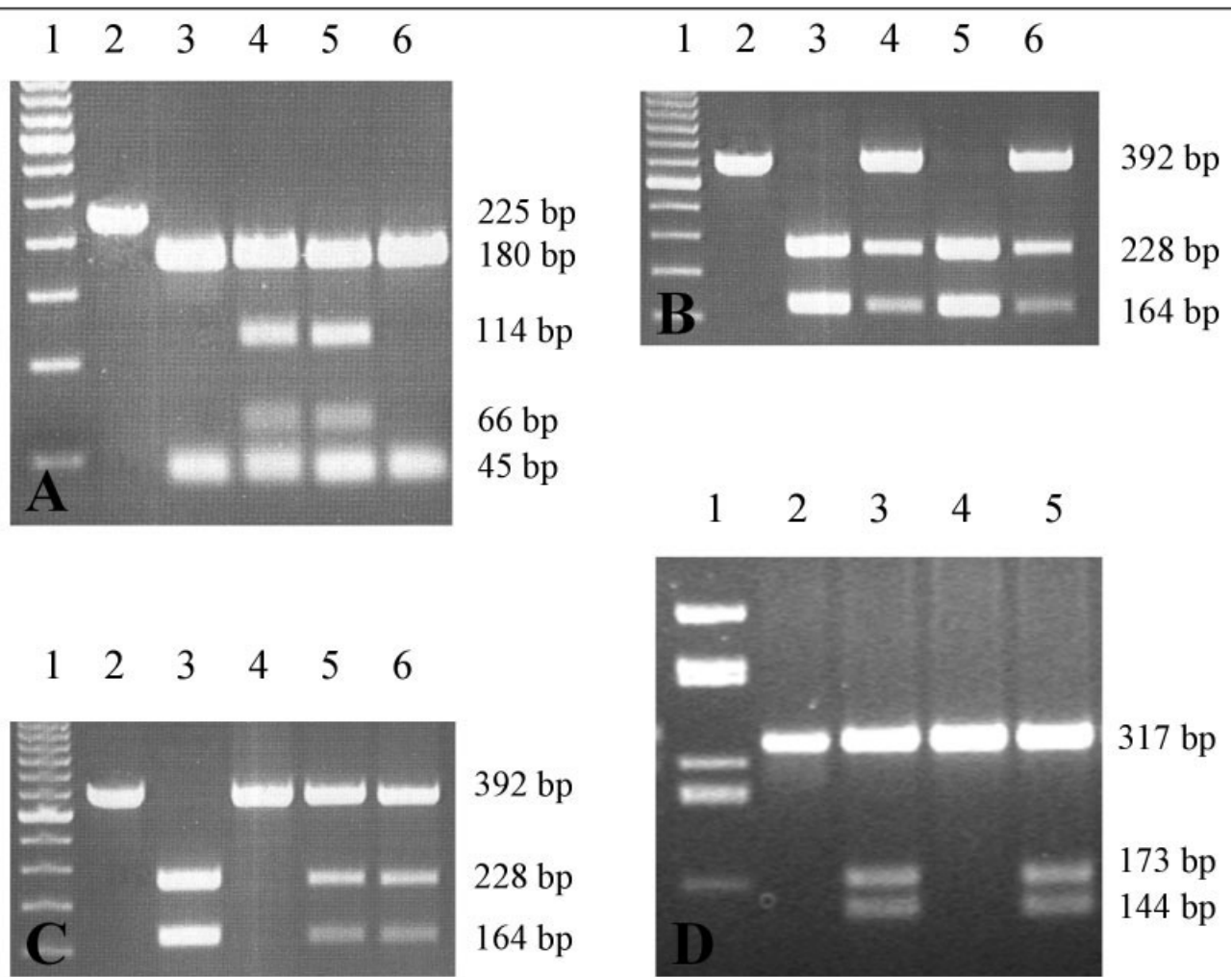


Fig. Restriction enzyme analysis of polymerase chain reaction (PCR) amplicons of Cases II-1 (A, B), I-1 (C), and V-1 (D). (A) Amplimer of tyrosine hydroxylase (TH) exon 11 after digestion with *Nla* III on a 3.5% agarose gel. The undigested PCR product is 225bp long; wild-type amplicon is cut by *Nla*III into fragments of 180 and 45bp; mutant amplicon is digested into fragments of 114, 66, and 45bp. Both the index case (Case II-1) and the father are heterozygous carriers of the 1159 C>A mutation. (B, C) Amplimer of a part of the TH promoter region after digestion with *AAT* II on a 3% agarose gel. The undigested PCR product is 392bp long; wild-type amplicon is cut by *AAT* II into fragments of 228 and 164bp; mutant amplicon remains undigested. (B) Both the index case (Case II-1) and his mother are heterozygous carriers of the -71 C>T mutation. (C) The index case (Case I-1) is a homozygous carrier of the -69 T>A mutation, whereas both his parents are heterozygous carriers of this mutation. (A-C) Lane 1: 50bp marker; lane 2: undigested amplicon; lane 3: wild-type control; lane 4: index case; lane 5: father; lane 6: mother. (D) Amplimer of TH exon 14 after digestion with *Bsp*1407I on a 3.5% agarose gel. The undigested PCR product is 317bp long, wild-type amplicon remains undigested, and mutant amplicon is digested into fragments of 173 and 144bp. Both the index case (Case V-1) and the mother are heterozygous carriers of the 1475C>T mutation. Lane 1: marker; lane 2: undigested amplicon; lane 3: index case; lane 4: father; lane 5: mother.

mutations in the CRE region leading to a human disorder. This suggests that mutations in the CRE of other genes, although not yet identified, may potentially be related to other familial syndromes where mutations in the coding regions of the responsible gene are not identified. We conclude that TH deficiency may be caused by mutations in the CRE of the TH promoter, and if mutations are not found in the coding regions of the TH gene in patients strongly sus-

pected of TH deficiency, the search for pathogenic mutations should be extended to the CRE region.

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