Mutation Analysis of the 6-Pyruvoyl-tetrahydropterin Synthase Gene in Chinese Hyperphenylalaninemia Caused by Tetrahydrobiopterin Synthase Deficiency


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Hyperphenylalaninemia (HPA) may be caused by deficiency of phenylalanine hydroxylase or tetrahydrobiopterin (BH₄), the essential cofactor for the aromatic amino acid hydroxylases. 6-Pyruvoyl-tetrahydropterin synthase (PTPS) deficiency is a major cause of BH₄-deficient HPA. In this study, seven single base mutations at nucleotides 73 (C>G), 155 (A>G), 166 (G>A), 209 (T>A), 259 (C>T), 286 (G>A), and 317 (C>T) on PTPS cDNA were detected in Chinese PTPS-deficient HPA by polymerase chain reaction and solid phase DNA sequencing. These nucleotide alterations result in R25G, N52S, V56M, V70D, P87S, D96N, and T106M amino acid substitutions, respectively. The R25G, V56M, V70D, and T106M were novel mutations found in PTPS gene. By analysis of 38 PTPS mutant alleles from 19 unrelated Chinese PTPS-deficient HPA families, the allele frequency of these mutations in Chinese PTPS-deficient HPA were determined to be ~5.3% (R25G), 34.2% (N52S), 7.9% (V56M), 2.6% (V70D), 36.8% (P87S), 7.9% (D96N), and 2.6% (T106M), respectively. Two common mutations, N52S and P87S, were found to account for 71% of the Chinese PTPS mutant alleles. The N52S mutation accounts for 48% of the southern Chinese PTPS mutation, but only one (9%) of the northern Chinese PTPS mutant allele was found to be N52S, which suggested that the N52S mutation might be southern Chinese. Clinically, the V56M mutation was found to associate with the mild form of PTPS deficiency. However, the R25G, N52S, P87S, and D96N were found mainly in the patients with severe clinical symptom. Using polymerase chain reaction-based mutation analysis, a fetus at risk of PTPS deficiency was diagnosed prenatally to be a carrier of N52S mutation. Hum Mutat 11:76–83, 1998. © 1998 Wiley-Liss, Inc.

KEY WORDS: 6-Pyruvoyl-tetrahydropterin synthase; tetrahydrobiopterin-deficient hyperphenylalaninemia; Chinese; mutation analysis; prenatal diagnosis

INTRODUCTION: ANALYZING CHINESE MUTANT ALLELES

Hyperphenylalaninemia (HPA) may be caused by a deficiency of phenylalanine hydroxylase (EC 1.14.16.1) or tetrahydrobiopterin (BH₄), the essential cofactor for the aromatic amino acid hydroxylases. Deficiency of BH₄ may lead to phenylketonuric phenotype with mental retardation as well as severe neurological disorders (Scriver et al., 1995). The BH₄ is synthesized from GTP by GTP cyclohydroylase I (EC 3.5.4.16), 6-pyruvoyl-tetrahydropterin synthase (PTPS; EC 4.6.1.10), and sepiapterin reductase (SR; EC 1.1.1.153) (Duch and Smith, 1991). The PTPS deficiency (MIM 261640) is reported to be the most common form of BH₄-deficient HPA (Blau et al., 1993). The overall incidence of phenylketonuria (PKU) in Caucasian is ~1/10,000, among which ~1–2% are deficient in BH₄ (Scriver et al., 1995). Our

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experiences in newborn screening indicate that the overall incidence of PKU (~1/33,000) among the Chinese population in Taiwan (Hsiao, 1992) is lower than that in the Caucasian population. However, the frequency of BH4 deficiency in southern Chinese HPA (Hsiao et al., 1986, 1990) is much higher than that in the Caucasian HPA (Blau et al., 1996). Recently, the human PTPS cDNA has been isolated (Thöny et al., 1992; Ashida et al., 1993), and the genomic structure has been characterized to contain six exons (Kluge et al., 1996; Liu et al., submitted). A few mutations have been identified from Caucasian (R16C, R25Q, IVS1-3C>G, IVS2-7T>A, V57del, T67M, P87L, 361-374del, K129E, D136V) and Japanese (P87S, D96N, 1114V) PTPS-deficient HPA patients (Ashida et al., 1994; Thöny et al., 1994; Imamura et al., 1995; Opplinger et al., 1995a; Thöny and Blau, 1997). Previously, the P87S (259C>T) and N52S (155A>G) substitutions were identified in the Chinese PTPS-deficient HPA. The P87S mutation was found to be a common mutation in Chinese PTPS-deficient HPA (Liu and Hsiao, 1996). In this study, we confirm that the N52S alteration is a missense mutation. The N52S mutation is also a common mutation in Chinese PTPS-deficient HPA. Five additional mutations, R25G (73C>G), V56M (166G>A), V70D (209T>A), D96N (286G>A), and T106M (317C>T), are also identified by analysis of 38 Chinese PTPS mutant alleles in this study. The R25G, V56M, V70D and T106M alterations are novel mutations found in PTPS gene.

**MATERIALS AND METHODS**

**Patients**

The 19 Chinese PTPS-deficient families (23 patients) studied were not related and the parents of these families were not consanguineous. All the patients were registered in the International Database of BH4 Deficiencies (Blau et al., 1996), except patients P072 and P073. About 80% of the registered Chinese PTPS deficiency by the end of 1996 were included in this study.

Thirteen of the 19 families studied were from Taiwan. Two families were from Shanghai, and one family each was from Beijing, Tianjin, Shandong, and Guangzhou. The ancestry of the patients originating from south and north of Nan Ling were classified as southern Chinese and northern Chinese, respectively. The ancestry of the southern Chinese patients in this study were mainly from Taiwan, Fujian, and Guangdong, the southeastern part of China.

The PTPS deficiency was diagnosed by analysis of urinary pterin (Niederwieser et al., 1982) with a combination test of the oral BH4 administration (Blau, 1988) All the patients showed a decreased biopterin level (<0.003–0.5 mmol/mol creatinine) and a low total biopterin ratio (<0.02–2.8%). B%= (biopterin/ (biopterin + neopterin) x 100%). Most patients showed an elevated neopterin level in the urine. The blood Phe of the patients were elevated, ranging from 360 µM to 2,700 µM, and the activity of their blood dihydropteridine reductase (Arai et al., 1982) for all as within normal reference range (2.8–8.7 U/g hemoglobin). Four hours after oral administration of 7.3–7.6 mg/kg BH4, their blood Phe all decreased to normal. Sixteen of the patients have been described elsewhere (Liu and Hsiao, 1996).

Clinically, the phenotypes of the patients studied were characterized into mild, moderate, and severe forms (Table 1). The classification of the phenotype was based on clinical findings before the supplement of BH4 and neurotransmitters was started. Patients P006 and P059 were very mildly mentally deficient (IQ 75 and 76, respectively) with moderate HPA (Phe: 820 and 710 µM), but without any other clinical symptoms, when they were diagnosed with PTPS deficiency at the ages of 13 and 7 years old, respectively. These patients (P006 and P059) were classified as mild clinical phenotypes. Patient P011 (patient YC) (Table 1) and his sibling YS were detected to be BH4 synthesis deficient HPA with moderate mental retardation (IQ: 65 and 53), moderate HPA (Phe: 720 and 770 µM), and moderate neurological symptoms at 7 years and 5 years of age, respectively (Hsiao et al., 1986). They were classified as moderate clinical phenotypes. Patients P057, P060, P064, P066, P068, P072, and P073 (Table 1) were detected by neonatal screening and treated properly since neonatal stage. Therefore, their clinical phenotype could not be identified.

**Total RNA Isolation and Reverse Transcription-Polymerase Chain Reaction**

Total RNA of skin fibroblasts and lymphoblasts were isolated by Ultraspec RNA reagent (Biotecx Laboratories, Houston, TX). The reverse transcription (RT)-polymerase chain reaction (PCR) and the following sequencing reaction were performed as described elsewhere (Liu and Hsiao, 1996).

**PCR Amplification of Genomic DNA**

Genomic DNA of blood sample or culture cells were isolated as described (Sambrook et al., 1989). To extract the genomic DNA from a dried blood spot collected on filter paper, a 3 mm spot was punched out from the blood spot. After the protein was fixed on the dried blood spot, the genomic DNA on the spot was extracted by the PCR reaction mixture.
Primer set PTPSB (5’-AGCACCGCAGACAGCGCCGGGAA-3’) and PTPS1A (5’-ATCAGGATGCTGGAGGCCGTCCGAG-3’) were used to amplify exon 1 from genomic DNA by DynaZyme II (Finnzymes Oy, Espoo, Finland) at annealing temperature of 63°C with 4% (v/v) DMSO. Primer set PTPS2B (5’-TTCTGACTCTCCCTTTGGTAGCT-3’) and PTPS2A (5’-GCATTCAACACTGTGTCCGTAAGTT-3’) were used to amplify the exon 2 at annealing temperature of 62°C. Primer set PTPS3B (5’-GTATGTTGCTAACATTGTGCTTGG-3’) and PTPS8 (5’-AACACTTTGTTAGAAGAGGAGGCT-3’) were used to amplify the exon 3 at annealing temperature of 62°C. Primer set PTPS4B (5’-GCACAGTCTCCTGACATTGTTACTG-3’) and PTPS4A (5’-GGAACCTTAGGAGAAGGAGGCT-3’) were used to amplify the exon 4 at annealing temperature of 62°C. Primer set PTPS259B (5’-TAGTGGCTAAGTGATAAGGTGT-3’) and PTPS12 (5’-GAAAATGAAAGTGATTTTAATTTAT-3’) were designed to amplify the exon 5 and exon 6 at annealing temperature of 55°C. The PCR was carried out for 28 cycles with 250 ng of genomic DNA. For genomic DNA extracted from a 3 mm dried blood spot, the PCR was carried out for 35 cycles. The primers PTPSB, PTPS2B, PTPS3B, PTPS4B, and PTPS259B were biotinylated for solid phase DNA sequencing.

DNA Sequencing

For solid phase sequencing, the biotinylated strand of the PCR product was isolated by streptavidin Dynabeads (Dynal, Oslo, Norway) followed by dideoxynucleotide chain-termination reaction using Sequenase version 2.0 (Amersham, Buckinghamshire, UK).

Mutations Surveyed in Normal Population

To survey whether the nucleotide substitutions found in this study can be detected in the normal population, 50 total RNA or genomic DNA samples isolated from apparently healthy unrelated individuals were used. The transitions of 286G>A (D96N) and 317C>T (T106M) in PTPS cDNA obliterate a FokI and BsiHKA I restriction site, respectively. The 73C>G (R25G) and 208T>A (V70D) substitutions create a BsaJI and BsaBI restriction site, respectively. The RT-PCR products were subject to FokI and BsiHKA I (New England Biolabs, Beverly, MA) digestion to detect the D96N and T106M substitutions, respectively. The PCR products of exon 1 and 4 were digested by BsaI and BsaB I (New England Biolabs) to detect the R25G and V70D substitutions, respectively. The digested products were resolved in 2% or 4% agarose gel by electrophoresis. The substitutions of 155A>G (N52S) and 166G>A (V56M) render no change on the restriction site. The PCR products of exon 2 and 3 were subjected to solid phase sequencing to detect the N52S and V56M substitutions.

RESULTS


The RT-PCR products obtained from the total RNA isolated from the fibroblasts or lymphoblasts of the patients were digested by BbvI to screen whether there was P87S mutation and they were sequenced to detect mutation other than N52S and P87S. Two additional mutations at nucleotides 286 and 317 were detected in patients P057 and P059, respectively (Fig. 1A,B). The G>A transition at nucleotide 286 permit an Asp to Asn substitution at codon 96 (D96N). The C>T transition at nucleotide 317 leads to replacement of Thr with Met at codon 106 (T106M). The patient P057 was a compound heterozygotes of D96N and P87S (Table 1). No additional nucleotide change was found in the entire coding region other than the D96N and P87S in the PTPS cDNA of patient P057.

In studying the Chinese PTPS mutations by RT-PCR analysis, a 23-bp deletion extended from nt
164T to 186G (164-186del) at PTPS cDNA was found in both normal and mutant lymphoblasts (data not shown). Starting from this 23-bp deletion, the genomic structure of the PTPS gene was characterized to contain 6 exons by PCR using human genomic DNA as template. This deleted 23-bp fragment was designated as exon 3 (Liu et al., submitted). A G>A transition at nucleotide 166 (exon 3) was found in patients P006, P059, and P072 (Fig. 1C, Table 1). This change causes a Met for Val substitution at codon 56 (V56M). No additional nucleotide change was found other than the T106M and V56M in the PTPS coding region of patient P059.

A C>G transversion in exon 1 (nucleotide 73) was found in two patients P006 and P046 (Fig. 1D, Table 1). This change results in the substitution of Arg for Gly at codon 25 (R25G). No additional nucleotide change other than the R25G and V56M was found in the PTPS coding region of patient P006. In addition to V56M, a T>A transversion at nucleotide 209 (exon 4) was found in patients P072 (Fig. 1E). This change permit a Val to Asp substitution at codon 70 (V70D).

The nucleotide alterations identified in Chinese PTPS-deficient HPA were summarized in Table 1. Except the adopted patient P006, all the other mutations were found to inherit from parents. The families 6, 14, 59, and 72 (Table 1) had two affected siblings in the family. Both of the affected siblings in those families were found to carry the same nucleotide alterations (data not shown).

**Mutations Surveyed in Normal Population**

The 286G>A transition obliterates a Fok I restriction site, the normal allele yielded two fragments (322bp and 275bp), whereas the mutant allele re-
mained as one Fok I-indigestible fragment (597bp) (data not shown). The 317C>T transition obliterates a BsiHKA I restriction site, the normal allele yielded four fragments (309bp, 236bp, 31bp, 21bp), whereas the mutant allele yielded three fragments (545bp, 31bp, 21bp) after BsiHKA I digestion (data not shown). The 73C>G substitutions creates a BsaI restriction site. The 73C>G transversion yielded five fragments (92bp, 78bp, 59bp, 34bp, 13bp) after BsaI digestion, whereas the normal allele yielded four fragments (151bp, 78bp, 34bp, 13bp) (data not shown). The 209T>A substitution yields four fragments (151bp, 78bp, 34bp, 13bp) after BsaI digestion, whereas the normal allele remained as one 260bp fragment (data not shown). To study whether any PTPS gene, were identified to be one of the seven point mutations, namely, R25G, N52S, V56M, V70D, P87S, D96N, and T106M substitutions found in human PTPS gene.

All the 38 mutant alleles but one, which may be not a point mutation in the coding region of the PTPS gene, were identified to be one of the seven point mutations, namely, R25G, N52S, V56M, V70D, P87S, D96N, and T106M (Table 1). The allele frequency of these mutations were found to be ~5.3% (R25G), 34.2% (N52S), 7.9% (V56M), 2.6% (V70D), 36.8% (P87S), 7.9% (D96N), and 2.6% (T106M), respectively, from the 38 mutant alleles studied (Table 2).

Mutant Allele Frequencies in Chinese PTPS-deficient HPA

All the 38 mutant alleles but one, which may be not a point mutation in the coding region of the PTPS gene, were identified to be one of the seven point mutations, namely, R25G, N52S, V56M, V70D, P87S, D96N, and T106M (Table 1). The allele frequency of these mutations were found to be ~5.3% (R25G), 34.2% (N52S), 7.9% (V56M), 2.6% (V70D), 36.8% (P87S), 7.9% (D96N), and 2.6% (T106M), respectively, from the 38 mutant alleles studied (Table 2).

Prenatal Diagnosis of PTPS-deficient HPA by Mutation Analysis

The genomic DNA of a fetus at risk of compound heterozygote of N52S and P87S mutations (family 101, Table 1) was isolated from amniocytes at the gestation age of 17 weeks. The exon 5 was amplified with primer set PTPS259B/PTPS12 to detect P87S mutation by BbvI digestion. The exon 2 was amplified with primer set PTPS2B/PTPS2A to detect N52S mutation by DNA sequencing. The results indicated the fetus was a heterozygote of PTPS-deficiency carried a normal allele and the N52S mutation (Fig. 2A,B).

DISCUSSION

In this study, seven missense mutations, R25G, N52S, V56M, V70D, P87S, D96N, and T106M, were identified from 38 Chinese PTPS mutant alleles. The N52S and P87S transitions were reported in our previous study, and the P87S transition was found to be a common PTPS mutation in Chinese (Liu and Hsiao, 1996). In this report, 13 of 38 mutant alleles studied were also identified to have this N52S substitution (Table 1), but none of the apparently normal PTPS alleles showed N52S substitution. These data suggest that N52S alteration is not a polymorphism, but a mutation causing PTPS deficiency. The inheritance of all the mutant alleles were determined except the adopted patient (P006, Table 1). The nucleotide substitutions were tightly cosegregated with the PTPS-deficient alleles in all the other 18 families studied. None of R25G, V56M, V70D, D96N, and T106M alterations was detected in 100 normal PTPS alleles. These results highly indicate that these nucleotide substitutions were not polymorphisms, but mutations that caused PTPS deficiency. The D96N mutation also had been found in a Japanese patient (Opplinger et al., 1995b). To our knowledge, the R25G, V56M, V70D, and T106M substitutions were novel mutations found in human PTPS gene.

A G>A mutation at nucleotide 74 that causes the Gln for Arg replacement at codon 25 (R25Q) had been reported in a Caucasian PTPS-deficient patient (Thöny et al., 1994). The R25G mutation identified in this report and the previously described R25Q mutation occurred within a CpG dinucleotides. Previously, the P87L and P87S substitutions in PTPS cDNA had been found in Caucasian (Opplinger et al., 1995a), Japanese (Imamura et al., 1995), and Chinese (Liu and Hsiao, 1996), respectively. It was speculated that the codon 87 might be a mutational hot spot of PTPS gene (Opplinger et al., 1995b). The R25G and R25Q also occurred across different populations and might indicate that codon 25 was another mutational hot spot of PTPS gene.

The allele frequency of these mutations in Chinese PTPS-deficient HPA were determined to be ~5.3% (R25G), 34.2% (N52S), 7.9% (V56M), 2.6% (V70D), 36.8% (P87S), 7.9% (D96N), and 2.6% (T106M), respectively, by analysis of 38 PTPS mutant alleles from 19 unrelated Chinese PTPS-deficient HPA families. The N52S and P87S mutations account for ~71% of mutant alleles, which indicate that N52S and P87S mutations are common mutations in Chinese PTPS-deficient HPA. As described in our previous report, the P87S mutation could be detected both in the southern and northern Chinese
FIGURE 2. Prenatal diagnosis of a fetus at risk of PTPS deficiency. The genomic DNA of the fetus was isolated from amniocytes and subjected to PCR amplification to detect the P87S and N52S mutations. (a) Exon 5 was amplified by primer set PTPS259B/PTPS12 and digested by BbvI to detect P87S mutation. Lane 1: father of patient P064, lane 2: mother of patient P064, lane 3: patient P064, lane 4: the fetus at risk of PTPS deficiency, lane 5: healthy control, lane M: 1 kb DNA marker, lane U: BbvI -undigested PCR product. (b) Exon 2 was amplified by primer set PTPS2B/PTPS2A and sequenced to detect the N52S mutation. Arrowhead denotes the N52S mutation. Data revealed the fetus was a carrier of N52S mutation.

(Liu and Hsiao, 1996). The P87S mutation accounts for ~36% (9/25) and 45% (5/11) in southern and northern Chinese PTPS-deficient HPA, respectively. The N52S, V56M, and D96N mutations also could be detected both in the southern and northern Chinese mutant alleles. However, the N52S accounts for 48% of the southern Chinese PTPS mutation, but only one (9%) of the northern Chinese PTPS mutant allele was found to be N52S (Table 2). These data suggested that the N52S mutation might be a southern Chinese mutation. Further haplotype analysis will provide information whether these mutations occur as a recurrent mutation or by a founder effect.

None of the mutations identified in Caucasian PTPS-deficient patients (Thöny and Blau, 1997) were detected in Chinese patients. However, two (P87S, D96N) of the three PTPS mutations reported in Japanese patients (Ashida et al., 1994; Imamura et al., 1995; Thöny and Blau, 1997) were also found in Chinese PTPS-deficient patients. Both of these two mutations could be detected in northern Chinese PTPS-deficient patients (Table 2), which indicated that there might be geographic relationship between Chinese and Japanese PTPS mutations.

The 164-186del (Lys54stop) mutation, previously identified in Caucasian PTPS deficiency (Oppliger et al., 1995a), and a T- to -A transversion at position -7 of the 3' splice acceptor site in intron 2 resulted in the K54X mutation of PTPS cDNA was concluded (Kluge et al., 1996). This 164-186del also could be detected in normal Chinese lymphoblast, fibroblast as well as PTPS-deficient lymphocytes and fibroblasts (data not shown). However, none of our DNA samples from the PTPS-deficient HPA indicated this T- to -A transversion (data not shown). These results suggested that the 164-186del alteration in
PTPS cDNA of most cells may be a result of alternative RNA splicing instead of a mutation causing PTPS deficiency.

The native form of human PTPS was suggested to be a homohexamer (Bürgisser et al., 1994) and accords with the rat PTPS crystallographic works of Nar et al. (1994). According to the proposed three-dimensional structure of rat (Nar et al., 1994) and human PTPS (Oppliger et al., 1995a), the Asn52 involves in the hexamer stabilization by hydrogen bonds between two subunits. Therefore, the change of Asn52 to Ser52 (N52S) in human PTPS might destabilize the PTPS hexamer and cause the functional event. The proposed binding mode by Bürgisser et al. (1994) suggested that the Glu107 and the amide bond between Thr105 and Thr106 (Glu108, Thr106, Thr107 in human) involved in the substrate anchoring by hydrogen bonding, and the Val69 (Val70 in human) lay close to the pocket of active site. The change of Thr106 and Val70 to Met106 (T106M) and Asp70 (V70D), respectively, found in PTPS-deficient patients might interfere with the substrate binding and cause a PTPS-deficient consequence.

Most of the patients studied were the severe form of PTPS deficiency (Table 1). Some of the patients were detected by neonatal screening and were treated with BH4 and neurotransmitters immediately. Therefore, the clinical phenotype of these patients detected by neonatal screening was not determined. According to the mutations identified in this study, the V56M mutation might associate with a mild clinical form of PTPS deficiency. Patient P011 and his affected sibling were breast fed for 1 year and 6 months after birth, respectively. The BH4 supplied by breast feeding during neonatal period also may play a role in improving the outcome of their clinical phenotype. The clinical phenotype of the V70D and T106M mutations could not be determined in this study. Further study is needed to illustrate the correlation between these two mutations and clinical phenotypes.

Currently, prenatal diagnosis of PTPS-deficient HPA is performed at the second trimester by determination of PTPS activity in fetal blood and analysis of neopterin and biopterin quantity in amniotic fluid (Shintaku et al., 1992; Blau et al., 1994; Shintaku et al., 1994). In this study, one fetus at risk of PTPS deficiency was diagnosed to be a N52S carrier at 17 weeks of gestation by PCR-based mutation analysis. The baby was confirmed to be a N52S carrier with normal blood Phe after birth (data not shown). To our knowledge, this is the first report on the prenatal diagnosis of PTPS-deficient HPA by mutation analysis of genomic DNA directly. With the understanding of the mutations in human PTPS gene, molecular analysis may provide an effective aid to prenatal diagnosis by amniocentesis. This PCR-based mutation analysis of genomic DNA may provide early prenatal diagnosis at the first trimester with chorionic villi sampling, which cannot be used for pterins and PTPS activity analysis to provide early prenatal diagnosis.

### ACKNOWLEDGMENTS

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