Molecular Study of Hereditary Progressive Dystonia with Mark Diurnal Fluctuation in Chinese

（　　　　HPD)

　　　　　　　　L-dopa

　　　　1988　　　6　HPD
　　　　　　　Ichinose
　　HPD　　　　　GTP
cyclohydrolase I (GTPCH)

　GTPCH　　　　　　　tetrahydro-
biopterine (BH4)
　　　　　　　　　GTPCH

R249S (747Cù G)　　　　　PHA
　　　　　GTPCH
mRNA

　　　　　　　　　R249S

　　　　　　　（HPD
　）

　　　　　Segawa
GTP cyclohydrolase I

Abstract

GTP cyclohydrolase I (GTPCH), which catalyzes the rate limiting step of tetrahydrobiopterin (BH$_4$) biosynthesis, is associated with two clinically distinctive human diseases. Hyperphenylalaninemia is a severe disease induced by recessive GTPCH mutations, whereas dopa-responsive dystonia (DRD) is a benign disease in which only dominant mutations have been found. We found a homologous (recessive) GTPCH mutation (R249S, 747Cù G) in a DRD patient who had low but measurable GTPCH activity in her PHA-stimulated mononuclear blood cells. Arginine 249 is located at the C-terminus of GTPCH, outside the enzyme catalytic site. The expression of GTPCH mRNA in the patient's mononuclear blood cells was normal. Enzyme specific activity and kinetics of R249S mutant protein expressed in *E. coli* and eukaryotic cells were also normal. However, the amount of R249S mutant protein expressed in transfected cells was lower than the wild-type protein. In pulse-chase experiment, the synthesis of mutant protein was normal, but its stability was decreased. This is the first destabilizing mutation found in the GTPCH gene. Our data raise the possibility that DRD could be either dominantly or recessively inherited. The phenotype of GTPCH deficiency seems to be determined by residual activity, while the inheritance is more likely determined by the mechanism of mutation.

Keywords: Hereditary progressive dystonia with marked diurnal fluctuation, HDP, Segawa, GTP cyclohydrolase I

Introduction

Fib GTP cyclohydrolase I (EC 3.5.4.16) (GTPCH) is responsible for the rate-limiting step of tetrahydrobiopterin (BH$_4$) biosynthesis. BH$_4$ is the cofactor of several

important human enzymes including phenylalanine hydroxylase, tyrosine hydroxylase, tryptophan hydroxylase and nitric oxide synthase. The latter three are involved in the production of bioactive humoral factor dopamine, serotonin and nitric oxide.

GTPCH deficiency is first described in a girl with hyperphenylalaninemia in 1984 (Niederwieser et al. 1984). Neopterin, biopterin, serotonin and dopamine are all very low in her body fluids, and GTPCH activity is not detectable in her liver. Hyperphenylalaninemia induced by GTPCH deficiency is a rare disease, and only 12 cases have been reported from a recent international database (Blau et al. 1996). These patients have progressive mental retardation and seizure. Variable tone and posture ranging from marked hypotonia to opisthotonos are characteristic. Treatment with L-dopa relieves their abnormal tone and seizure effectively, while diet control itself does not.

Dopa-responsive dystonia (DRD), also called hereditary progressive dystonia with marked diurnal fluctuation (HPD) or Segawa disease, is a disease that also shows dramatic response to L-dopa (Nygaard et al. 1988; Segawa et al. 1971). Compared to hyperphenylalaninemia, dystonia is the major symptom in DRD, and patients become symptom-free when they are treated with low dose L-dopa. HPD/DRD was recently mapped to chromosome 14q22.1-q22.2 where human GTPCH was located (Nygaard et al. 1993; Tanaka et al. 1995). GTPCH was then proved to be the gene responsible for DRD (Ichinose et al. 1994).

Hyperphenylalaninemia and DRD differ not only in clinically manifestations but also in inheritance. Hyperphenylalaninemia is a recessive disease, and affected siblings have been found in families. After the cloning of GTPCH gene, two homologous mutations have been found in hyperphenylalaninemia patients (Blau et al. 1995; Ichinose et al. 1995). On the contrary, DRD was believed to be an autosomal dominant disease with low

penetrance and female predominance (Nygaard 1995). All GTPCH mutations previously found in DRD patients showed dominant transmission (Ichinose et al. 1995; Furukawa et al. 1996; Bandmann et al. 1996).

Hyperphenylalaninemia patients (with two mutated GTPCH alleles) have very low GTPCH activities (Niederwieser et al. 1984; Blau et al. 1996). Theoretically, DRD patients (with one mutated and one normal GTPCH alleles) should have 50% GTPCH activity. However, DRD patients usually have below 20% GTPCH activity (Segawa 1996; Ichinose and Nagatsu 1997). DRD mutation may exert dominant-negative effects since GTPCH is a homodecamer (Nar et al. 1995), but some DRD mutations produce truncated proteins that may be unable to join the complex (Hirano 1995). The mechanism of DRD mutation is still not solved.

In this paper, we described a DRD mutation with unusual inheritance and mechanism. The characterization of this mutation by eukaryotic expression system has increased our understanding in the molecular basis of GTPCH deficiencies.roblast growth factors (FGFs) approach to the treatment of genetic disease involving FGFRs.

Materials and Methods:

Description of the patient

The 12-year-old girl with hereditary progressive dystonia with marked diurnal fluctuation (HPD, Segawa syndrome) has been described previously (Hwu et al. 1989; Wang et al. 1994). It was later known that HPD is equal to Dopa-responsive dystonia (DRD). Her symptoms developed when she was 2 years and 8 months old. She had posture dystonia, posture tremor, posture rigidity and neck tilting that were more severe toward the end of a day. She was free of symptoms under the treatment of L-dopa 20 mg/kg/day since the age of 3 years. She

has no family history and both her parents are normal. The parents are not consanguineous. Informed consents were obtained before blood sampling.

Biochemical analyses

Plasma amino acids analyses were performed on a Biotronic LC 3000 amino acids analyzer (Biotronic, Germany). Urinary pterin derivatives were analyzed by HPLC after oxidation (Antonozzi et al. 1988). GTPCH activities of patients were measured in PHA-stimulated mononuclear blood cells, and analyzed by reversed phase HPLC after oxidation and phosphatase treatment (Blau et al. 1985; Werner et al. 1996).

Formate release assay was performed according to the method of Burg and Brown with modifications (Burg and Brown 1968). ^{3}H-GTP (5-20 Ci/mmol) was obtained from Amersham (Amersham, USA), and cold GTP was from Borghinger (Borghinger Manham, USA). A 50 µl reaction mixture containing 45 µl cell extract, 0.1 M Tris-HCl pH 8.0, 2.5 mM EDTA, 1 µCi ^{3}H-GTP and 200 µM GTP was incubated at 37^oC for 30 min with prokaryotic protein or for 1 hr with eukaryotic protein. Eukaryotic protein is stable for at least 3 hours in this assay. Reaction was stopped by 25 µl 4 M HCOOH. After adjusting the volume to 500 µl with water, 0.1 ml (0.2 g/ml) activated charcoal was added to the mixture. After extraction, 0.3 ml of supernatant was subjected to scintillation counting. Protein concentration was determined by Bradford dye reagent kit (Bio-Rad, USA). GTPCH activity was expressed as pmol/mg/hr or pmol/µg/hr. Our method was different from what described by Burg and Brown that cold GTP was added to the reaction mixture. The total concentration of GTP was thus brought closer to the Km of the enzyme to increase the sensitivity of the assay (manuscript in preparation).

PCR and sequencing

Amplification of all six exons of the GTPCH gene was performed as described by Ichinose et al. (Ichinose et al. 1995). PCR products were purified by a QIAEX II gel extraction kit (QIAGEN, Germany). Sequencing was performed by a CirmVent Thermal Cycle Dideoxy DNA sequencing kit (BioLabs, USA). PCR products of exon 6 were cloned to confirm the sequence change. Fnu4HI (BioLab, USA) digestion was performed following the recommendation of the manufacturer. DNA samples from normal, unrelated individuals were used as controls.

GTPCH mRNA expression

Total cellular RNA was extracted from PHA-stimulated whole blood. First strand synthesis was performed by AMV reverse transcriptase and oligo dT (Promega, USA). PCR was performed with primer F2 (5'-GCCCCGCAGCGAGGAGGATAAC) and R3 (5'-GACAGACAATGCTACTGGCAGT) as described by Hirano et al. except that 0.5 µCi of α-^{32}P-dCTP was added to the PCR mixture, and the cycle number was decreased to 20 (Hirano et al. 1995). PCR products were digested with Fnu4HI and were separated by a 5% sequencing gel. After electrophoresis, the gel was dried and exposed to a phosphoscreen. Images were analyzed by a Phosphoimager and quantified by the ImageQuant program (Molecular Dynamics, USA). As an internal control, the GAPDH gene product was amplified with primer GAPDH-upper (5'-CCATGGAGAAGGCTGGGG) and GAPDH-lower (5'-CAAAGTTGTCATGGATGACC).

Mutagenesis and construction of plasmids

GTPCH cDNA was kindly provided by Dr. Nagatsu. This cDNA produces functional protein although it lacks a short 5' piece (Ichinos et al. 1995). Mutagenesis was performed by a PCR method with the wild-type cDNA as a template (Vallejo et al. 1994). Two separated PCR reactions were performed with primers F3 / GTP-m747R

(5'-CTCTCATTAG<u>C</u>AGCTGAGCTT, the mutated nucleotide is underlined) and GTP-m747F (5'-AAGCTCAGCT<u>G</u>CTAATGAGAG) / R3. PCR products were then mixed and amplified with F3/R3. The final product was digested with BsrGI, and was used to replace the corresponding fragment in wild-type cDNA. The replaced region was confirmed by sequencing.

Both the wild-type and mutant cDNAs were cloned into pGEX vector (Pharmacia, Sweden) to produce glutathione fusion proteins in *E.coli* JM109. Similar amounts of the wild-type and mutant fusion proteins, determined by both Commasie blue staining and western blot analysis, were subjected to GTP cyclohydrolase I activity measurement.

Eukaryotic expression plasmids CMV-A16-GTPCH, driven by CMV promoter, were constructed by inserting GTPCH cDNAs (NarI/EcoRI fragment) into pCMV-AGP/EBP (replacing its BstBI/ EcoRI fragment) (Lee et al. 1996). This generated an N-terminal AGP/EBP epitope (called A16) to the expressed GTPCH protein. To construct a full length GTPCH cDNA, the 5' end of the cDNA (starting from ATG) was added by PCR. Expression plasmid CMV-GTPCH contains this full-length cDNA.

Production of GTPCH antiserum and western blot analysis

GTPCH cDNA was cloned into pRSET vector, expressed in *E.coli* DE3, and purified by a nickel column. The purified protein was used to immunize rabbits to generate antiserum. This antibody was used in both western blot analysis and immunoprecipitation. Western blot was performed using GTPCH antibody at 1: 1600 dilution or monoclonal antibody A16 (Lee et al. 1996) at 1: 2500 dilution. Secondary antibodies used were HRP-coupled sheep anti-mouse Ig or sheep anti-rabbit Ig

(Amersham, USA) at 1: 1600 dilution. Blots were developed using the ECL detection system (Amersham, USA).

Cells and Transfections

Baby hamster kidney cells (BHK) were cultured in Dulbecco's MEM with 10% FCS. Calcium phosphate precipitation method was used for transfection (Graham and van der Erb, 1973). BHK or HeLa cells in 10 cm or 6 cm dishes were transfected with 10 or 5 μg of the GTPCH plasmid and 1 μg of pRSV-gal. Forty hours after transfection, cells were harvested for GTPCH assay, western blot analysis and β-galactosidase assay. β-galactosidase assay was performed according to the instructions provided by Pharmacia (Pharmacia, Sweden). It was used as a transfection efficiency control.

Pulse-chase experiment

4 μg CMV-GTPCH-wt or CMV-GTPCH-R249S was transfected into BHK cells grown in 6 cm culture dishes. Forty hours after transfection, cells were washed twice with PBS, incubated in DMEM-10% FCS without methionine and cysteine for 1 hr, and pulsed with Pro-mix (Amersham, USA) for 30 min. After chasing for 1, 2, 4 or 8 hours, cells were harvested and lysed by RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2.5 mM EDTA, 1 mM PMSF, and 1 μg/ml leupeptin), and were subjected to immunoprecipitation with GTPCH antibody and protein A-sepharose. After washing for 4 to 5 times, samples were separated by SDS-PAGE. Exposure and quantification were done by Image Analyzer (BAS 1500, FUJI).

Results

The patient had GTPCH deficiency

GTPCH activities were measured in PHA-stimulated mononuclear blood cells of

a DRD patient and her parents by HPLC. The patient's cells had low, but measurable GTPCH activity (4.2 pmol/mg/hr, normal 38.4-102.6 pmol/mg/hr) which is compatible with DRD (with residual activity up to 20% of normal) (Segawa 1996, Ichinose and Nagatsu, 1997). GTPCH activity was 7.8 pmol/mg/hr for her mother and 32.4 pmol/mg/hr for her father. Although the reason for her mother's low GTPCH activity is not clear, DRD does affect females more than males. Both the patient and her parents had normal plasma phenylalanine levels (0.018 µmol/ml for patient, 0.020 µmol/ml for her mother, and 0.021 µmol/ml for father, normal 0.01-0.03 µmol/ml). The patient's urinary neopterin level was 0.143 mmol/mol CRE (average of two analyses) (normal 0.1-0.5 mmol/mol), and her urinary neoptein to biopterin ratio was 69.4% (normal 49-85%). Therefore, she had partial GTPCH deficiency with borderline low urinary neopterin level.

Homologous R249S mutation

Genomic DNA was extracted from peripheral blood of the family. Direct sequencing after amplification of all six exons of the GTPCH gene of the patient showed a 747C$\acute{u}$G change in exon 6 which changes amino acid residue 249 from arginine to serine (R249S). This is a homologous mutation that happens on both her GTPCH alleles (Figure 2A). Both of her parents were heterozygous for this mutation (Figure 2A), so this is a recessive mutation. After cloning of the PCR products, ten independent clones were analyzed for each member of the family. Only mutant clones could be found in the patient, but both mutant and wild-type clones were found in parents (Figure 2A). This mutation was searched in normal population by Fnu4HI digestion which specifically cleaves the mutant allele. In 100 unrelated normal controls (200 alleles), no R249S mutation was found (data not shown). No other mutation could be found in the coding region and exon-intron borders of the GTPCH genes in this family.
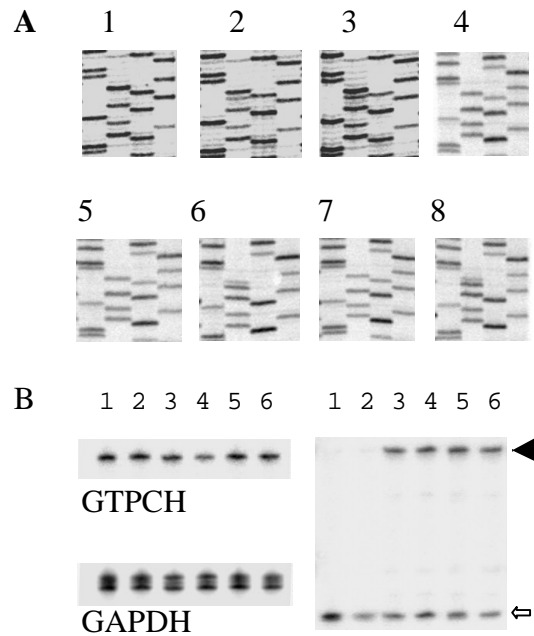

Figure 2. Detection of GTPCH R249S mutation. (A) GTPCH exon 6 PCR products were sequenced either directly (panels 1-3) or after cloning (panels 4-8). The pictures are direct sequencing for the patient, her father and mother (panel 1-3), sequencing of a representative clone for the patient (panel 4), mutant and wild-type clones for her mother (panels 5-6), and father (panels 7-8). (B) Quantification of mononuclear cell GTPCH mRNA for the family by RT-PCR. Upper panel shows the PCR products of GTPCH, and lower panel the products of GAPDH. All tests were duplicated. Lanes 1-2, 3-4 and 5-6 represent the patient, her father and mother respectively. (C) Fnu4HI cleavage of RT-PCR products. Lanes 1-2, 3-4 and 5-6 are the results for the patient, her father and mother respectively. The uncut (wild type) products were marked by an arrow, and the cut (mutant) products by an open arrow.

R249S mRNA was expressed normally in peripheral blood

RT-PCR was performed to measure the expression of GTPCH mRNA in PHA-stimulated blood of the family. For better quantification, radioisotope labeling with fewer amplification cycles prevented the

saturation of PCR reactions. The results showed that the expressions of GTPCH mRNA were equal in this family (Figure 2B). GAPDH mRNA expression was used as an internal control. RT-PCR products were futher digested by Fnu4HI which specifically cleaves the mutant allele. As shown in Figure 2C, the amounts of uncut (wild type) and cut (mutant) DNA products were equal for her heterozygous parents. Therefore, GTPCH mRNA expression is not affected by the R249S mutation.

R249S mutant protein expressed in *E.coli* has normal activity

To further characterize this mutation, expression of R249S and wild-type glutathione fusion proteins in *E. coli* was established. The expression levels of recombinant proteins were determined by commasie blue staining and western blot analysis. Enzyme activities in crude bacterial lysates analyzed by HPLC and formate release assay showed that recombinant R249S protein had normal GTPCH specific activity. In kinetic study it also showed normal Vmax and apparent Km.

R249S mutant protein expressed in transfected cells showed normal specific activity but decreased expression levels

Transient expression in cultured cells was also used to characterize the mutant protein because the processing and degradation of over-expressed recombinant proteins in *E. coli* may be abnormal (Laiho et al. 1997). To facilitate the detection of the mutant protein, we added an N-terminal tag (A16 epitope of AGP/EBP) to the cDNAs. This tag does not affect GTPCH function as cells transfected with the wild-type construct showed good GTPCH activities. In the experiments, either CMV-A16-GTPCH-wt or CMV-A16-GTPCH-249, together with pRSV-gal, was transfected into HeLa (Fig 4A, upper panel) and BHK cells (Fig. 4A, lower

panel). Transfection efficiencies monitored by β-galactosidase assay were similar in these experiments. Western blot analysis with A16 monoclonal antibody showed that R249S protein was expressed at a lower level (23% in HeLa, 28% in BHK cells) as compared to the wild-type protein (Figure 4A). GTPCH activity in cells transfected with R249S cDNA was also low. After normalization for protein expression, specific activity of R249S mutant protein was normal. Data from a typical kinetic study was plotted on Fig. 4B.

R249S showed decreased stability

To explore the mechanism of decreased mutant protein expression, a pulse-chase experiment was designed. Full-length GTPCH cDNAs (starting from ATG) were used in this study. As shown in Figure 5A, after a 30-min pulse, wild-type and mutant GTPCH proteins were equally labeled. Control transfection without GTPCH plasmid produced no GTPCH protein (picture not shown). Therefore, the synthetic rate of R249S mutant protein was normal. These newly synthesized GTPCH proteins were chased for up to 8 hr. The amount R249S protein decreased more rapidly than the wild-type protein during the chase period. The quantitative result was shown in Fig. 5B. Half-lifes calculated from Fig. 5B were 8.94 hr for the wild-type and 2.39 hr for the mutant protein. A cross-reacting protein (open arrow, Fig. 5A) with a size slight larger than GTPCH was pretty stable during the experiment. These results indicate that R249S mutation decreases the stability of GTPCH protein.
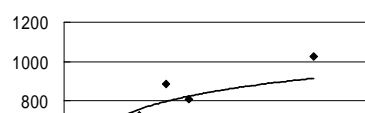

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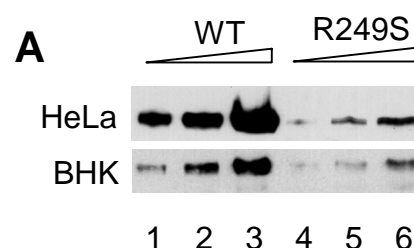
Figure 4. Transient expression of wild-type and R249S GTPCH proteins. (A) Western blot analysis of BHK and HeLa cells transfected with either wild-type or R249S GTPCH cDNAs. 6, 12 or 25 μg of total cell extracts was loaded onto adjacent lanes of a polyacrylamide gel. Western blot analysis was performed using a mouse monoclonal antibody against A16 (AGP/EBP) epitope. Film exposure time was 1 min for BHK protein and 5 min for HeLa protein. (B) A kinetic study of GTPCH activities in transfected BHK cells. Equal amounts of crude lysates were assayed under different substrate concentrations.

Discussion

In this paper, we describe an HPD patient who has a homologous GTPCH mutation. Both her parents are carriers of this mutation, so DRD is recessively inherited in this family. This inheritance pattern is unusual because it was known that dominant GTPCH mutations induced DRD, while recessive mutations induced hyperphenylalaninemia (Nygaard 1995).

GTPCH monomer is composed of a helical N-terminal part, a compact C-terminal domain and a C terminus (Nar et al. 1995). The C-terminal domain contains the enzyme catalytic site. Although R249S is an unconserved change, it is located at the C-terminus of GTPCH. This could explain why R249S mutant protein possesses normal specific enzyme activity. However, R249S decreases the stability of GTPCH. This is the first destabilizing mutation described in the GTPCH gene. Previously, only loss-of-

function mutations or possibly dominant-negative mutations have been found. Supposedly, complete GTPCH deficiency produces hyperphenylalaninemia, while partial deficiency produces DRD. Because R249S mutation results in higher residual enzyme activity than other known mutations, homologous R249S mutation retains enough activity to prevent the development of hyperphenylalaninemia. The inheritance of GTPCH deficiency is more likely determined by the mechanism of mutation.

Recently, mutations affecting protein processing or stability are more recognized as etiologies of genetic diseases. Mutant proteins could be degraded by different mechanisms before they are processed to a mature form. For example, the cystic fibrosis transmembrane conductance regulator (CFTR) F508 mutant protein could act as normal chloride channels, however, it fails to exit the ER and is degraded by the ubiquitin/proteosome pathway (Cheng et al. 1990; Ashkenas and Byers, 1997). We do not know how R249S mutant protein is degraded. The interaction between the wild-type and mutant proteins in GTPCH homodecamer is another issue that may be concerned for other GTPCH mutations.

Currently, more than 14 GTPCH mutations have been found in DRD, and three mutations have been found in hyperphenylalaninemia (Thony and Blau, 1997). In the studies of Ichinose et al, recombinant expression of GTPCH in *E. coli* could not tell the difference between DRD and hyperphenylalaninemia because no mutation gave any activity (Ichinose et al. 1994, 1995). We suggest that these mutations should be tested in eukaryotic expression system. Just before we submit this manuscript, there is a report describing dystonia with motor delay in compound heterozygotes for GTPCH gene mutations (Furukawa et al. 1998). Therefore, GTPCH activities should be measured in dopa-responsive dystonia with recessive inheritance (Segawa and Nomura, 1993; Gorke and Bartholome, 1990) unless the

deficiency of tyrosine hydroxylase could be proved (Knappskog et al. 1995).

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