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Predisposition of genetic disease by modestly decreased expression of *GCH1* mutant allele

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Abbreviations: DRD, dopa responsive dystonia; SNP, single nucleotide polymorphism

Abstract

Recently it was shown that single nucleotide polymorphisms (SNPs) can explain individual variation because of the small changes of the gene expression level and that the 50% decreased expression of an allele might even lead to predisposition to cancer. In this study, we found that a decreased expression of an allele might cause predisposition to genetic disease. Dopa responsive dystonia (DRD) is a dominant disease caused by mutations in *GCH1* gene. The sequence analysis of the *GCH1* in a patient with typical DRD symptoms revealed two novel missense mutations instead of a single dominant mutation. Family members with either of the mutations did not have any symptoms of DRD. The expression level of a R198W mutant allele decreased to about 50%, suggesting that modestly decreased expression caused by an SNP should lead to predisposition of a genetic disease in susceptible individuals.

Keywords: dystonic disorders; germ-line mutation; point mutation; polymorphism, single nucleotide

Introduction

Dopa-responsive dystonia (DRD) (OMIM 128230) is an autosomal dominant disorder characterized by a fluctuating dystonia which develops during childhood with postural dystonia of lower limbs that is aggravated toward evening and alleviated after sleep. This disorder shows dramatic and sustained responses to low doses of levodopa therapy. DRD gene has been mapped to chromosome 14q21-22, where a lot of mutations in the guanosine 5'-triphosphate cyclohydrolase I gene (*GCH1*) (OMIM 600225) have been discovered in sporadic and familial cases. This gene contains 6 exons coding for the rate-limiting enzyme for the biosynthesis of tetrahydrobiopterin (BH_4) (Figure 1). BH_4 is the cofactor of phenylalanine hydroxylase, tyrosine hydroxylase, tryptophan hydroxylase and NOS. The latter three are involved in the production of dopamine, serotonin and nitric oxide, respectively.

When both *GCH1* was deficient (OMIM 605407), the patients usually presents with neonatal hyperphenylalaninemia and encephalopathy with severe mental retardation, seizures, abnormal muscle tone and movements (Blau *et al*., 1995; Ichinose *et al*., 1995; Furukawa *et al*., 1998; Nardocci *et al*., 2003). However, a patient with homozygous recessive mutation of *GCH1* was reported to have mild symptoms of typical DRD study (Hwu *et al*., 1999), suggesting more complex interplay of *GCH1* muta-

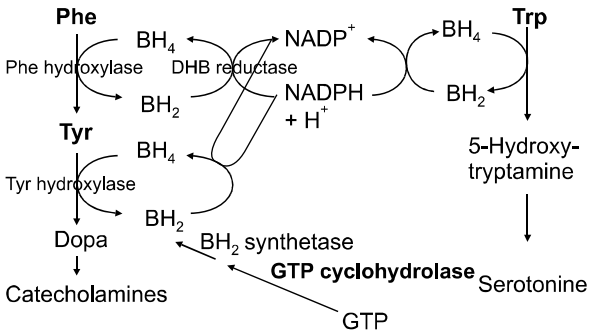

Figure 1. Metabolism of BH_4. GTP is used for the synthesis of BH_4. In the process of the synthesis of BH_4, GTP cyclohydrolase is the first and the rate-limiting step enzyme. DHB or BH_2, dihydrobiopterin; BH_4, tetrahydrobiopterin.

tions.

In this study, a DRD patient with mild symptoms had compound heterozygous mutations in the *GCH1* gene and had no family history of DRD, showing that DRD is induced by recessive *GCH1* mutations. And the expression of one mutant allele was decreased, showing an excellent example that the decreased expression by an SNP might predispose to genetic disease.

Materials and Methods

Genetic analysis

Genomic DNA was extracted from EDTA-anticoagulated peripheral blood of the family members and control samples for the analysis. The procedure was approved by IRB (#7303-80) in Wonkwang University College of Medicine and consent was obtained. Some control DNA samples were purchased at Coriell Institute for Medical Research (New Jersey). All six exons with the splicing junctions of the *GCH1* gene were amplified by PCR using intronic primers as described (Ichinose *et al*.,1994; Hong *et al*., 2001) except for exon 1 and exon 5. For the amplification of *GCH1* exon 1 and exon 5 the following primers were used; Ex1F (GCGTACCTTCCTCAGGTGAC) and Ex1R (TGAGGCAACTCTGGAAACTT) for exon 1: DRD5F (GTCAGACTCTCAAACTTAGCTCCTTATC) and DRD5R (CTTCTAGAGCACCATTATGACGTTAC) for exon 5. Direct sequencing of the PCR products were performed, using an automatic DNA sequencer (Applied Biosystems, Foster City, CA) and the BigDye terminator cycle sequencing kit (Applied Biosystems).

Detection of the R59G and R198W mutations in genomic DNA

Genomic DNAs were used for the detection of the R59G and R198W mutations. For the detection of the R59G mutation, the amplified product of the *GCH1* exon 1 was digested with *Msp*I. For the detection of the R198W mutation, allele-specific PCR method was used by using two T-specific (Ut: AGCAATCACGGAAGCCTAGT) or C-specific (Uc: AGCAATCACGGAAGCCTAGC) forward primers and common reverse primer (L: AAGGCAGATGCAGACTTACG). Amplification was performed for 35 cycles with the following conditions: at 94°C for 30 s, 52°C for 30 s and 72°C for 1 min.

Determination of the expression ratio of mutant alleles

RNAs from mononuclear cells were extracted by TRIzol (Invitogene, Carlsbad, CA), and Single-stranded cDNA was synthesized using the total RNA and the Superscript Kit (Invitrogene). The partial *GCH1* cDNAs were amplified by cCGH1F (GTCGCGTACCTTCCTCAGGTGACTC) and cCGH1R (ACCGGACAGACAGACAATGCTACTG) The amplified *GCH1* cDNAs were used for the single base extension reaction with the following primers: CGH1SBE1 (GCTCGTTATCCTCCTCGCTGC) for R59G mutation site comparison and CGH1SBE2 (TGCTGTAGAAATCACGGAAGCCTTG) for R198W mutation site comparison. For the single base extension reaction, SNaPshot Ready Reaction Mix (Applied Biosystems) was used as described previously (Shin *et al*., 2005) with slight modification by using 1 μl purified PCR product and 2 pmole of SBE-primer mix of CGH1SBE1 and CGH1SBE2 with the following condition: 30 cycles of 96°C for 10 s, 55°C for 5 s, and 60°C for 30 s. The single base–extended samples were treated with 1 unit of shrimp alkaline phosphatase (Roche) at 37°C for 1 h, followed by inactivation at 75°C for 15 min. The resulting 1.0 μl dephosphorylated samples were mixed with 15 μl of Hi-Di formamide, and analyzed on an ABI Prism (Applied Biosystems) automatic sequencer.

Results

A male child (10 years old) has had a problem of fluctuating lower limb dystonia worsening toward evening since 8 years old. He showed pes equinovarus deformity on walking. His phenylalanine level (54.1 mM) and tyrosine level (69.2 mM) were within normal range. MRI and dopamine transporter PET scan was also normal. Treatment with L-dopa (125 mg/day) brought complete remission of his symptom.

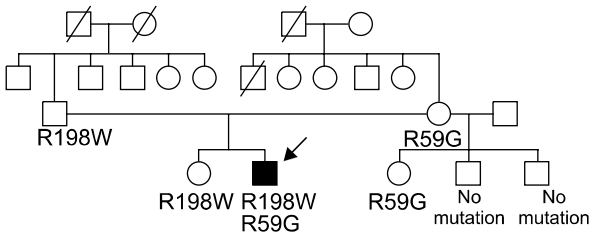

Figure 2. Family pedigree of a dopa-responsive dystonia patient. The probend had two different mutations in *GCH1*. The patient's father and elder sister had R198W mutation. Mother and another sister had R59G mutation. No other relatives except the patient had DRD symptoms.

His parents and relatives had no such symptoms, and his parents were not a consanguineous couple (Figure 2), suggesting a familial *TH* recessive mutation(s) or a newly acquired *GCH1* mutation (s). The whole exons and their boundaries of *TH* sequence were amplified from the patient's gDNA and the sequence was determined. No mutation in *TH* was found (data not shown). Without *TH* mutation, one dominant mutation was expected from the patient's *CGH1* sequence. However, two novel point mutations (R59G and R198W) in *CGH1* were found (Figure 3A). The C at the position 175 in exon 1 of the *GCH1* gene was changed to G in the R59G mutation, and the C at the position 592 in exon 5 was changed to T in the R198W mutation. His mother carried the R59G mutation and his father had the R198W. The patient's sisters with either of R59G or R198W had no symptoms of DRD, and his uncle with R198W mutation had no symptom. For the separate genetic test, PCR-RFLP (Figure 3B) and the allele-specific PCR methods were established in the family (Figure 3C). The presence of R59G or R198W mutations was tested in 50 healthy controls and no mutation was found.

To determine whether the mutations affect the expression level of *GCH1* mRNA, the expression of the mutated strand relative to the normal was determined with single base extension method. The expression level of the R198W mutant strand was about a half of that from the normal strand (Figure 4) when the relative level was compared to the ratio from genomic DNA in which the ratio between mutant and normal strands should be 1 : 1. This suggests the relative expression level of the R198W mutant strand decrease into a half. However, the expression level of the R59G strand was about the same as that from the normal strand (Figure 4).

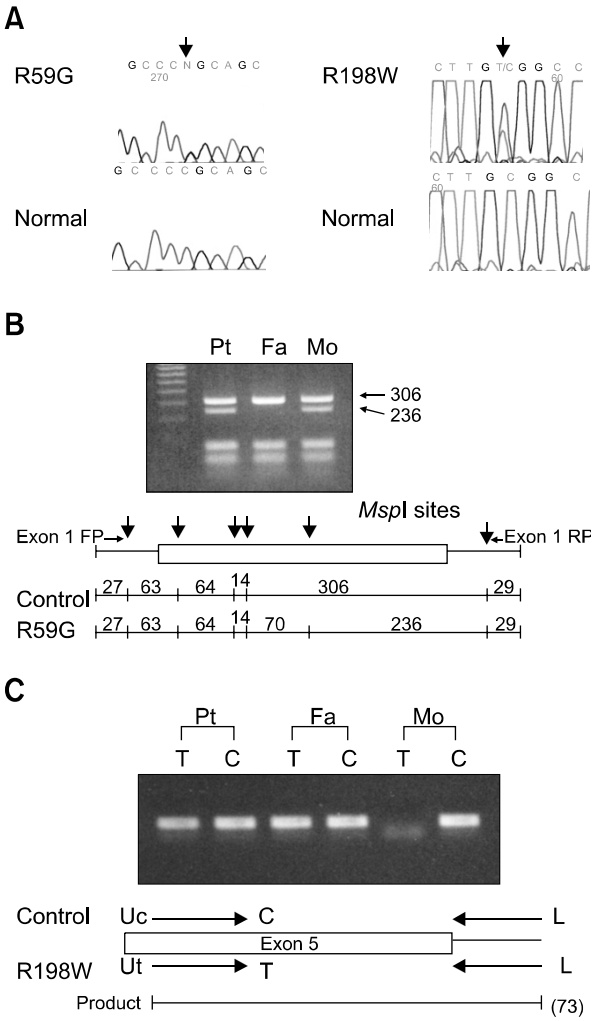

Figure 3. Detection of R59G and R198W mutations. (A) Direct sequencing of PCR products from exon 1 and 5 of *GCH1* was performed. Two novel mutations were found as indicated by arrows. (B) Detection of R59G mutation was checked again by PCR-RFLP method. New *Msp*I site was created in the mutant product, and abnormal 236 bp band appeared on agarose gel electrophoresis. (C) Detection of R198W mutation was checked again by allele-specific PCR method. C or T-specific primers were used for the exon 5 amplification by using the common reverse primer. The patient and his father had abnormal mutant (T) product. The PCR-RFLP and the allele-specific PCR methods were used for the presence of the mutations in control gDNAs.

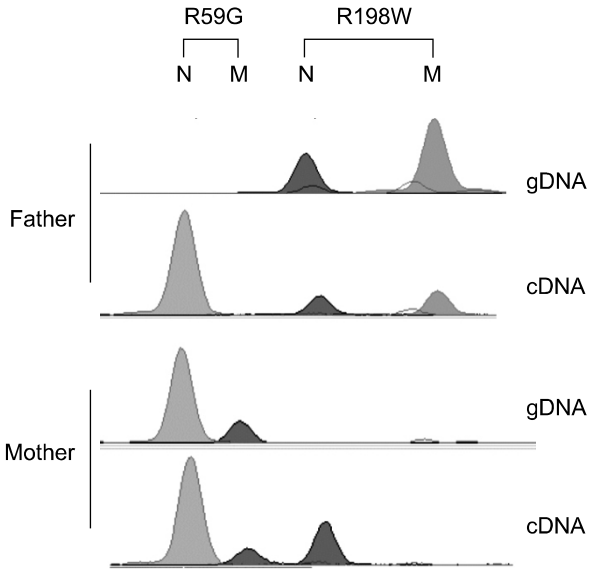

Figure 4. The relative level of the mutant mRNAs in parents' blood cells. Exon 1 and 5 of *GCH1* was amplified and a single base was extended for the quantification of relative ratio between mutant and normal mRNAs. The signal amplitude is not equal among different fluorescent even-though the ratio is the same, so the signal from gDNA was used for the reference. The ratio of mutant (M) and normal (N) for R198W was decreased into about a half in cDNA when compared to the signal ratios of M and N in gDNA (arrow head). But the ratio of M and N for R59G was not changed in cDNA.

Discussion

Although dopa-responsive dystonia is usually caused by a dominant mutation in *GCH1*, several recessive mutations in *GCH1* were also reported. The patients with recessive *GCH1* mutations have symptoms of severe mental retardation, seizures, abnormal muscle tone and movements (Blau *et al*., 1995; Ichinose *et al*., 1995; Furukawa *et al*., 1998; Nardocci *et al*., 2003). However, a patient with homozygous mutation of *GCH1* was reported to have mild symptoms of typical DRD (Hwu *et al*., 1999).

In this report, we found two mutations in a patient with mild symptoms of typical DRD and complete response to L-dopa. The patient had no family history of the dystonic symptoms, suggesting another case of DRD patient with recessive mutations. The mutations were not detected in 50 healthy controls, suggesting that the mutations are not just polymorphisms. So far more than 80 different mutations have been described in *GCH1* (www.bh4.org/BH4_Start.asp). However, two recessive mutations from this study are not reported.

In a case of typical DRD patient with homozygous R249S mutation (Hwu *et al*., 1999), the recombinant protein had the same Km value, but the expression level of the mutant R249S *GCH1* in Hela and BHK cells was significantly lower than that of the normal *GCH1* in corresponding cells. But they did not test the level of mRNA directly from blood cell samples to show that the expression level of mutatnt mRNA is different from that from normal strand. So we tested if there was any difference of mRNA expression level between mutant and normal strand in the parents' mRNA. The genomic DNA has the ratio of 1 : 1 of mutant and normal strand, so the test was done along with the gDNA samples as references. In the test, the expression level of R198W mutant decreased into a half compared to that of control strand (Figure 4), suggesting the mutation affects the expression level of mRNA. Our assay will be quite useful for the determination of relative expression level of mutant mRNA and this is the first report that the expression of mutant *GCH1* mRNA strand decreased relative to normal mRNA strand.

In DRD patients with recessive *GCH1* deficiency, there was a spectrum of symptom severity from mild to very severe. In cases of patients with recessive mutations, the activity level might decrease into much less than a half ($< 5\%$) because both alleles are inactivated. This might bring more severe symptoms such as severe mental retardation, seizures, abnormal muscle tone and movements. When the mutations affect the expression level of the enzyme partially (e.g. 50%), the DRD patient with recessive mutations might have typical DRD symptoms as shown in patients with a single dominant mutation. Our result of the decreased mRNA level into a half in a R198W mutant strand should be a typical example.

So far six different autosomal recessive mutations associated with *GCH1* deficiency were reported (Blau *et al*., 1995; Ichinose *et al*., 1995; Furukawa *et al*., 1998; Hirano and Ueno, 1999; Hwu *et al*., 1999; Nardocci *et al*., 2003; www.bh4.org/BH4_Start.asp). Here, we add another recessive case with DRD patient with two novel mutations. In this case, the symptom was mild showing typical DRD symptoms indistinguishable to patients with typical dominant mutations. Also we showed that one of the mechanisms of the symptom in recessive DRD patients might be decreased mRNA level as shown in R198W mutation. However, the *in vitro* activity change by R59G mutation was not confirmed in this study, so the possibility that the patient's DRD symptoms are caused by a dominant mutation of R198W with incomplete penetrance cannot be excluded.

Usually, mutations in genetic disease are known to cause functional changes of the gene products (Hong *et al*., 2000, 2001; Kim *et al*., 2006). Recently, however, it was published that the allelic variation in gene expression is very common in human genome (Lo *et al*., 2003). They showed that 20～50% of human genes are not equally expressed. Instead, their expression was skewed by single nucleotide polymorphisms (SNPs). Small changes in expression level might even affect predisposition to tumorigenesis as published by Yan *et al.* (2002). They showed that the constitutional 50% decreases in expression of one adenomatous polyposis coli tumor suppressor gene (APC) allele can lead to the development of familial adenomatous polyposis. There were few examples showing that small changes in expression result in genetic diseases (Anatonarakis *et al*., 2001). This study provides an excellent example of the predisposition of genetic disease caused by the modestly decreased expression.

Acknowledgements

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