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A novel mutation of the human 7-dehydrocholesterol reductase gene reduces enzyme activity in patients with holoprosencephaly

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Abstract

Defects in cholesterol biosynthesis genes are recognized as a leading cause for holoprosencephaly (HPE). Previous reports suggest that mutations of human 7-dehydrocholesterol reductase (*Dhcr7*), which catalyzes the final step of cholesterol biosynthesis, may cause HPE [Clin. Genet. 53 (1998) 155]. To determine whether *Dhcr7* mutations are involved in HPE pathogenesis, we analyzed the sequence of exon 9, which contains both a catalytic domain and a mutational hot spot. We examined 36 prematurely terminated fetuses with HPE at their gestation ages in the range from 21 to 33 weeks by single strand conformation polymorphism analysis and DNA sequencing. A novel missense mutation was identified: G344D. Dhcr7 enzyme assays using overexpressed recombinant mutant proteins revealed altered enzyme activity. Mutant G344D harbored less than 50% of enzyme activity compared with the control. Two previously reported mutations, R404C and G410S, abolished enzyme activity. These results suggest that mutation of the *Dhcr7* gene is involved in HPE pathogenesis.

Keywords: 7-Dehydrocholesterol reductase; Holoprosencephaly; Cholesterol biosynthesis; PCR-SSCP; In vitro mutagenesis; Mutation; DNA sequence; Enzyme activity; Pathogenesis; Korean

Holoprosencephaly (HPE) is an etiologically heterogeneous disorder affecting forebrain and midface development with a relatively high incidence of 1:250 during embryogenesis and 1:16,000 live births. Defects in cholesterol biosynthesis are known to be one of the leading causes of HPE in mammals [1]. It was proposed that mutation of 7-dehydrocholesterol reductase (*Dhcr7*), which catalyzes the final step of cholesterol biosynthesis by converting 7-DHC to cholesterol, may cause HPE. For example, mutation of *Dhcr7* has been shown to be the major defect in Smith–Lemli–Opitz syndrome (SLOS) [2–4] which exhibits variable phenotypes including HPE. Given the need to identify molecular events associated with HPE, we examined the role of the human *Dhcr7* in pathogenesis of HPE in a Korean population. Mutation of *Dhcr7* in either exon 9 or of IVS8-1G→C has been identified with high frequency in SLOS patients [5]. Exon 9 is the most highly conserved region, as determined from protein sequence alignment, and is possibly the essential region for enzyme activity [6]. IVS8-1G→C is the mutation that activates a cryptic upstream splice-site of intron 8, resulting in a 134 bp insertion. Consequently, this insertion causes a frameshift and a premature termination that can produce a truncated Dhcr7 protein lacking 154 amino acids of the Dhcr7 C-terminus [7]. Comparing *Dhcr7* mutational patterns is potentially useful for determining the factors contributing to HPE pathogenesis. Therefore, we screened mutations in *Dhcr7* exon 9 and IVS8-1G→C to determine any correlation between mutations and the functional significance of *Dhcr7* in brain development.

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Prematurely terminated fetuses were primarily screened as they showed the high clinical severity that may indicate deleterious *Dhcr7* alterations, that in turn result in a significant change of Dhcr7 enzyme activity. By this way we may be able to identify a role for *Dhcr7* in HPE pathogenesis. We report here the identification of a novel exon 9 mutation in the Korean population that reduces Dhcr7 enzyme activity. Our results suggest that *Dhcr7* is a candidate gene for HPE pathogenesis.

Materials and methods

Fetuses. Thirty-six cases for HPE were collected from the autopsy files of Asan Medical Center and Seoul National University Hospital, Seoul, Korea, which were recorded during the period between 1991 and 1998. Thirty-six formalin-fixed, paraffin-embedded tissues from prematurely terminated fetuses with HPE were analyzed. The range of gestation ages of the fetuses is from 21 to 33 weeks.

Mutation analysis. Exon 9 of Dhcr7 was screened for mutations by single strand conformation polymorphism (SSCP) analysis. Exon 9 was amplified by two sets of primers. Primer 9-1: 5′-CTTGACC CCTTCCCCCTCG-3′ (9425-9443, GenBank Accession No. AF110060); 5′-GATGTGTAGGAGCACTCGATG-3′ (9639–9619) and primer 9-2: 5′-CTACACATCCGCCGACGGGC-3′ (9631–9650); 5′-AGTAGGGCAGCAGGTGGCC-3′ (9779–9761). PCR was performed at 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min with a final extension of 10 min at 72 °C. The 25 μl reaction mixture contained 1 μg of genomic DNA, 10 pmol primers, 0.2 mM dNTPs, and 1 U *Taq* polymerase (Takara, Japan) in 1× PCR buffer (10 mM Tris, pH 8.3; 50 mM KCl; and 1.5 mM $MgCl_2$). Fifteen microliters of loading dye was added to 5 μl of PCR product (215 bp in size for 9-1 and 146 bp for 9-2), denatured at 97 °C for 5 min, and then cooled on ice. The final product was analyzed on a 20% non-denaturing acrylamide gel for 5 h at 250 V. SSCP analysis was performed at 8.5 °C, with the product from normal genomic DNA run as a control. Gels were stained with ethidium bromide and visualized by UV illumination. For the identification of IVS8-1G→C transversion, restriction fragment length polymorphism (RFLP) was performed as previously described [7]. The PCR product amplified with primer 9-1 was subjected to restriction digestion with AlwNI. The digested product was electrophoresed on a 2.5% agarose gel and visualized by UV illumination after staining with ethidium bromide. Potentially positive products were confirmed by sequencing.

Cloning and DNA sequencing. PCR products were purified using the JETSORB gel extraction kit (Genomed, Bad Oeynhausen, Germany) and cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). Plasmid DNA was extracted from individual clones by alkaline lysis plasmid minipreparation. The inserted PCR fragments of three individual colonies were sequenced for confirmation with both M13 reverse and M13 (−20) forward primers, using an ABI310 DNA sequencer (PE Biosystem).

Site-directed mutagenesis. Appropriate pairs of mutagenic primers (see Table 1) were synthesized and used to generate the mutated construct by PCR, by QuickChange site-directed mutagenesis (Stratagene). Myc-tagged human Dhcr 7 in YEp 351 ADC1 was used as the template for Pfu DNA polymerase (Stratagene). After PCR, wild-type parental plasmid remaining in the PCR product was selectively digested by *Dpn*I (Roche Molecular Biochemicals). The resultant mixture was used to transform chemically competent *Escherichia coli* XL-1 Blue MRF (Stratagene). Desired mutants were sequenced for confirmation with an ABI310 DNA sequencer (PE Biosystem).

Table 1
Mutagenic primer pairs

G344D	5′-TCCTGCTGCTGGACCTGGTGGGCTACTACA-3′
	5′-TAGCCCACCAGGTCCAGCAGCAGGACGCCC-3′
R404C	5′-TGGGGCGTGGCCTGCCACTTCAACTACGTC-3′
	5′-AGTTGAAGTGGCAGGCCACGCCCCAGAACG-3′
G410S	5′-TTCAACTACGTCAGCGACCTGATGGGCAGC-3′
	5′-CCATCAGGTCGCTGACGTAGTTGAAGTGGC-3′

Heterologous expression of mutated myc-tagged human Dhcr7 in Saccharomyces cerevisiae. Mutated human *Dhcr7* cDNAs were subcloned into c-myc-tagged yeast episomal plasmid c-myc-YEp351ADC1 and transformation of *S. cerevisiae* JB811 (*ade2-1 leu2-3*, *112 pep4-3 trp1-289 ura3-52*) was performed as previously described [8]. Cells were harvested at an A_{600} of 1.2 and lysed with glass beads in 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride. For the preparation of microsomes, the lysates were spun for 5 min at 500*g* (4 °C) and the supernatant was pelleted for 45 min at 100,000*g* (4 °C) as described [8]. Immunoblotting with a 9E10 c-myc monoclonal antibody following SDS/polyacrylamide gel electrophoresis was performed as previously described [6]. Protein concentration was determined by the method of Bradford [9] using bovine serum albumin as a standard.

Dhcr7 enzyme assays. A standard assay for Dhcr7 was carried out using 300 μM substrate (7-dehydrocholesterol) that had been suspended in Tween 80 (70:1, detergent:sterol) and 1–2 mg microsomal protein as previously described [10]. For the in vitro inhibition experiments using yeast overexpressed protein, drugs or agents were dissolved in dimethyl sulfoxide (Me_2SO) such that the final concentration of Me_2SO was less than 0.3% (w/v) that of the incubation mixture [11]. The sterol concentration in enzyme assay samples was measured in a Hewlett–Packard gas chromatograph 5890 II (FID, capillary column; SAC-5, 5% diphenyl/95% dimethylsiloxane, 30 m × 0.25 mm, 0.25 μm inner diameter, and flow rate 2.44 ml/min), using 5α-cholestane as a standard [11].

Results

Novel Dhcr7 gene mutations in HPE patients in Korea

To identify a role for *Dhcr7* in brain development we attempted to screen mutations of this gene in 36 fetuses with HPE. Mutations in exon 9 and in IVS8-1G→C were extensively screened for these two mutations that have been previously reported as the most frequent alterations of *Dhcr7* [5]. We have identified four cases with abnormal band patterns in SSCP analysis as compared to control. SSCP analysis does not distinguish between functionally insignificant polymorphisms and deleterious mutations nor does it determine the exact molecular nature of the mutation. For this information, direct sequencing was performed. Four novel mutations out of 36 samples were identified. Three silent mutations (cases 8, 10, and 11), and one missense mutation (case 31) that causes a change in an amino acid in Dhcr7 were identified (Fig. 1). Results from the sequence analysis are shown in Fig 2. The three silent mutations are located at codon 325, TTG to TTA (L325L, case 8), codon 329, CCC to CCT (P329P, case 10), and codon 332, CTG to CTA (L332L, case 11). The missense mutation is located at codon 344, GGC to GAC (G344D, case 31) (Fig. 2).

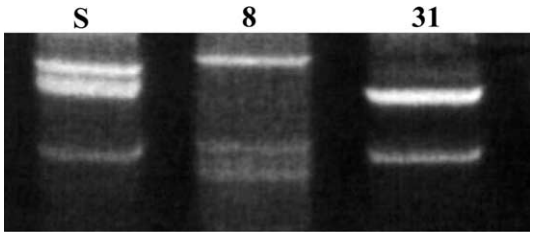

Fig. 1. SSCP analysis of the *Dhcr7* gene, exon 9 in HPE. Normal band pattern is shown in lane S. The band shifts of cases 8 and 31 are shown in each lane as labeled.

The IVS8-1G→C mutation, resulting in a 134 bp insertion sequences by abnormal splicing of exon 9, produces a truncated protein lacking 154 amino acids of the C-terminal sequence of Dhcr7. The transversion mutation of G to C in CAGNNNCTG, an *Alw*NI recognition site, results in CACNNNCTG, which is no longer digested with *Alw*NI. Thus, we can detect this mutation by PCR-based restriction fragmentation length polymorphism (PCR-RFLP). If the *Alw*NI site is mutated the 215 bp undigested product will remain after enzyme digestion while the control will give 185 and 30 bp digested products. None of the 36 cases were mutated at this site (data not shown).

Morphological characteristics of HPE cases

Fetus of case 31 was terminated due to ultrasonographic detection of brain anomaly at 24 weeks of gestation. Pathological diagnosis of case 31 was HPE with facial malformation. Case 31 was the alobar type of HPE, absence of olfactory bulbs, hypotelorism, adrenal enlargement, and mild ischemic neuronal change of spinal cord (Fig. 3).

In vitro expression of Dhcr7 variants and their relative enzymic activities

To determine if these mutations can affect enzyme activity of Dhcr7, c-myc-tagged mutant human *Dhcr7* cDNA was cloned into an expression vector by site-directed mutagenesis as described in Materials and methods. Two previously reported mutations in exon 9

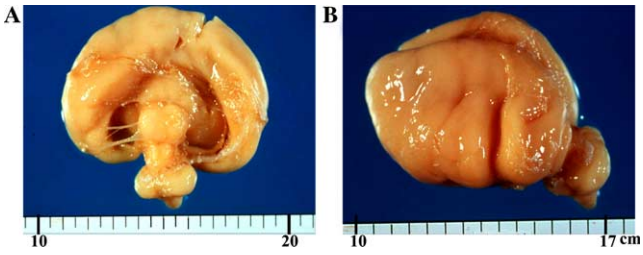

Fig. 3. Alobar holoprosencephaly in 24-week-old fetus (case 31). (A) Posterior view of the brain shows absent interhemispheric fissure and corpus callosum, and a dilated forebrain cavity covered with a thin membrane opened into a posterior sac. Non-cleaved basal ganglia and thalami are exposed. (B) Lateral view shows hypoplastic temporal lobe and cerebellum.

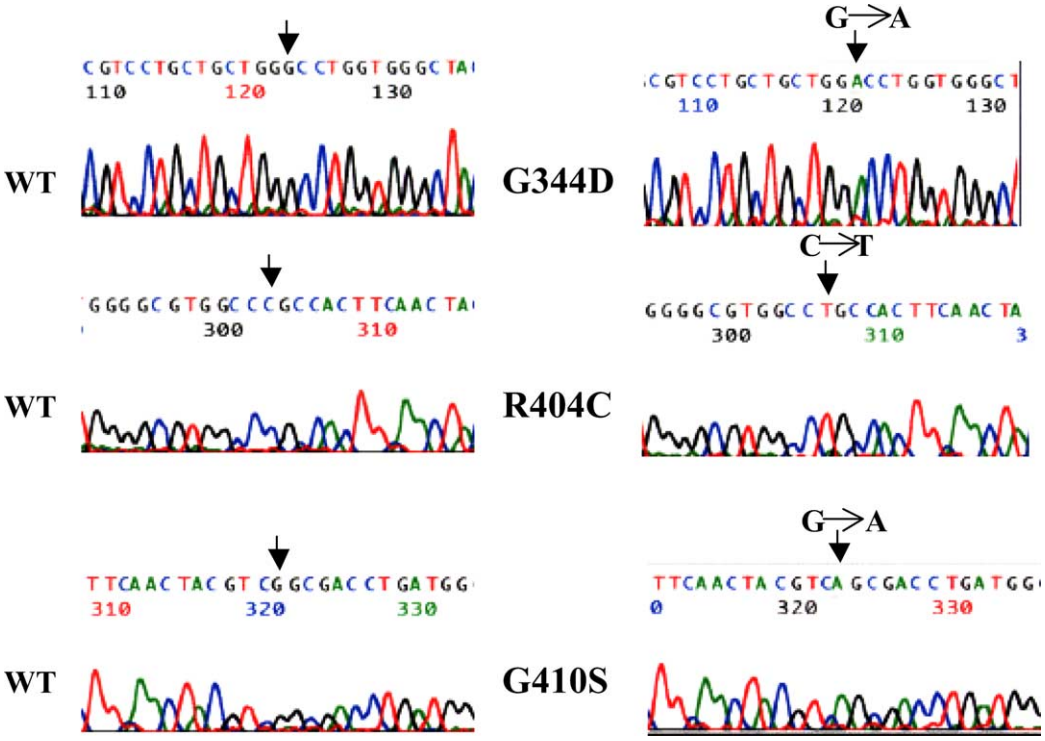

Fig. 2. Sequence analysis of the *Dhcr7*, exon 9. A novel mutation in codon 344, GGC to GAC (G344D) and the previously reported mutations R404C (CGC to TGC) and G410S (GGC to AGC) are shown.

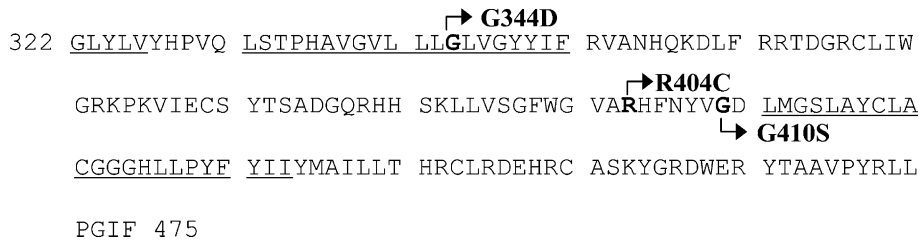

Fig. 4. Exon 9 of human Dhcr7 amino acid sequence and the mutation sites. The putative transmembrane segments are underlined. Three mutation sites are shown in arrows. GenBank accession number of human Dhcr7 is AF034544.

[1210 (C/T; R404C) and 1228 (G/A; G410S)], and a new mutation [1031 (G/A; G344D)] were ectopically expressed (Fig. 4). To determine whether cloned mutant *Dhcr7* was expressed, Western blot analysis was performed using c-myc monoclonal antibody (Fig. 5A). As anticipated, all the cloned *Dhcr7* were well expressed as expected sizes.

To determine whether mutant Dhcr7 proteins were functional, enzyme activity of each was measured and compared to that of wild-type Dhcr7 protein (Fig. 5B). After normalization of variant expression levels, each enzyme activity was graphed (Fig. 5B). The mock control, R404C and G410S, whose mutations have been frequently identified in SLOS patients, did not exhibit activity [5,12]. G344D showed less than 50% activity compared to wild-type control.

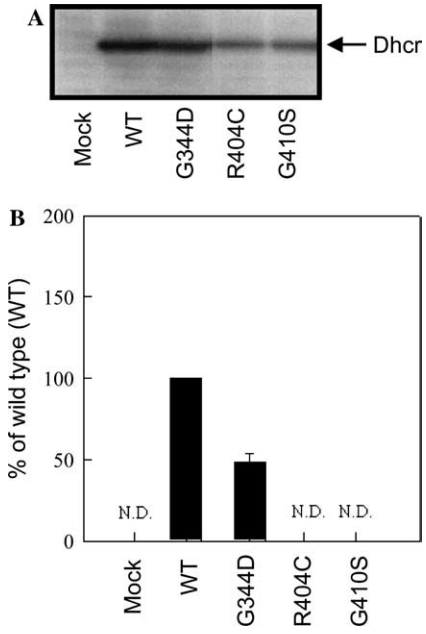

Fig. 5. (A) Western blot analysis of 40 μg of microsomal protein from yeast overexpressing the wild-type *Dhcr7* cDNA (WT) and cDNAs with mutations G344D, R404C, and G410S. The blot was immunostained with 80 ng/ml 9E10 c-myc antibody and analyzed with ECL Western blotting detection system using horseradish peroxidase-conjugated secondary antibody. (B) Enzymic activity of yeast overexpressing wild-type *Dhcr7* cDNA and cDNAs with mutations G344D, R404C, and G410S. Microsomes (0.5 mg) prepared from strains transformed with vector without cDNA (mock). Mutated *Dhcr7* cDNAs were incubated anaerobically for times indicated with DHC in the presence of 2 mM NADPH. Data shown are means ± SD ($n = 3$). N.D. is not detected.

Discussion

Here, we report the first case of molecular characterization of four novel *Dhcr7* mutations in Koreans with HPE demonstrating the existence of genetic variations in *Dhcr7 gene* in the Korean population. Using Dhcr7 enzyme assays, a strong correlation was established between genotypes of *Dhcr7* and their functions. One novel missense mutation showed reduced enzyme activity, suggesting that *Dhcr7* gene mutations are likely involved in the pathogenesis of HPE.

Sonic Hedgehog (*Shh*) is known to be a causative gene for HPE [13,14]. SHH protein undergoes autoproteolytic cleavage into N-terminal and a C-terminal halves, in which cholesterol is attached to the C-terminus of the former to create an active protein [15]. Thus, mutation of *Dhcr7* in HPE may result in the inability of cholesterol to modify SHH, abolishing its function. This hypothesis can further be supported by the observation of rats treated with an inhibitor of Dhcr7 showing a high frequency of HPE [16]. Furthermore, HPE is highly associated with SLOS, a recessive disorder resulting from mutations of *Dhcr7* [17]. In addition, the mutant (case 31) with G344D did not harbor any mutations in exon 1 and exon 2 of *Shh* gene (data not shown).

Each of four cases out of 36 (11%) fetuses was found to be a novel mutation, suggesting the presence of genetic variants in Korean populations. To enhance the efficiency of SSCP analysis we amplified exon 9 in two regions to give a 215 bp (5′ portion) and a 146 bp (3′ portion) PCR product. Interestingly, all the newly found mutations are confined within the 5′ portion of exon 9 (Fig. 4). This can also be attributable to the Korean genetic background. Previous studies with major European populations showed even distribution of mutations throughout exon 9 [5]. The three silent mutations and one missense mutation were located in or close to the eighth transmembrane domain (Fig. 4). SLOS patients with mutations in the transmembrane domain were less severely affected than were patients with null mutations or fourth cytoplasmic domain mutations [5]. In our

enzyme assay the mutant (case 31) with G344D located in the transmembrane domain showed 50% activity compared with wild type whereas previously known mutations R404C and G410S located close to the fourth cytoplasmic domain abolished enzyme activity. These results suggest that mutational genotype correlates with the severity of phenotype.

In the present study with a Korean population we were not able to detect the IVS8-1G→C mutation, though this mutation was found in over 60% of US SLOS patients [7]. Interestingly, most of IVS8-1G→C mutations found in the previous studies were heterozygous and it was suggested that homozygosity might be severe and thus be prenatally lethal [7]. Since we examined the mutations in prenatally aborted fetuses we might have expected to see homozygous mutations of IVS8-G→C if this is as common a mutation in the Korean population as in the US population. However, we found no cases of this mutation, suggesting the presence of genetic variations among populations with ethnic differences. Thus, comparison of ethnic differences is necessary for determining the pathogenesis of disease.

The present study may provide a plausible explanation of the molecular basis for similar phenotypes observed in HPE. Our knowledge of the importance of *Dhcr7* mutations in defects of cholesterol biosynthesis genes affecting brain development is evolving. In addition, we considered *Dhcr7* a candidate HPE gene because of the requirement for cholesterol modification of the SHH protein for proper brain development.

Acknowledgments

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