Haddad Syndrome with a Germ-Line Mutation in the *PHOX2B* Gene in a Korean Neonate

Chunglyul Baek, M.D., Ji Mi Jung, M.D., Yun-Jung Lim, M.D.*, Ki Hoon Kim, M.D.[†], Han-Wook Yu, M.D.[‡], Gu-Hwan Kim, M.D.[‡], and Mi Lim Chung, M.D.

Departments of Pediatrics, Radiology* and Surgery[†], Haeundae Paik Hospital, Inje University College of Medicine, Pusan, Korea
Medical Genetics Center, Asan Medical Center[‡], Seoul, Korea

ABSTRACT

Congenital central hypoventilation syndrome (CCHS) is a life-threatening disease that primarily manifests as sleep-associated respiratory insufficiency and a markedly impaired ventilatory response to hypercarbia and hypoxemia. Paired-like homeobox 2b (*PHOX2B*) gene mutations are known to cause CCHS. Almost all patients with CCHS are heterozygous for a poly-alanine expansion in *PHOX2B*. However, some patients have other germ-line abnormalities, including missense, nonsense and frame shift mutations. CCHS combined with Hirschsprung disease (Haddad syndrome) is extremely rare. Here, we report the case of a 1-day-old male neonate with recurrent apnea and bowel distension. Genetic analysis showed that he was heterozygous for a germ-line mutation in the *PHOX2B* gene. Only three cases of CCHS including two with Haddad syndrome confirmed by *PHOX2B* gene mutations have been reported in Korea. All of these cases have been heterozygous for a poly-alanine expansion mutation. This is the first report describing Haddad syndrome with a germ-line mutation in the *PHOX2B* gene in a Korean neonate.

Key Words: Congenital central hypoventilation syndrome, Hirschsprung disease, Haddad syndrome

INTRODUCTION

Congenital central hypoventilation syndrome (CCHS) is a life-threatening disorder which primarily manifests as sleep-associated respiratory insufficiency and a markedly impaired ventilator response to hypercarbia and hypoxemia¹⁾. CCHS is often associated with structural and functional impairments of the autonomic nervous system (ANS), including those observed with Hirschsprung disease (HD) and tumors of autonomic neural crest derivatives such as neuroblastoma, ganglioneuroma, and ganglioneuroblastoma^{2,3)}. The combination of CCHS and HD, which is also known as Haddad syndrome, is an extremely rare disorder⁴⁾.

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Correspondence to:

Mi Lim Chung, M.D.

Department of Pediatrics,
Haeundae Paik Hospital, Inje
University College of Medicine
1435, Jwa-dong, Haeundae-gu,
Pusan 612-030, Korea

Tel: +82-51-797-2000

Fax: +82-51-797-1600

E-mail: forevery52@naver.com

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De novo mutations in the paired-like homeobox 2b (*PHOX2B*) gene are associated with CCHS and have been found in over 90% of CCHS cases⁵). Most CCHS patients with *PHOX2B* gene mutations have a heterozygous expansion of poly-alanine (GCN) repeats⁶), whereas only 8% have germ-line mutations, such as missense or frameshift mutations⁷). In the Korean population, only three cases of CCHS including two infants with Haddad syndrome confirmed by testing *PHOX2B* mutations have been reported. All of these cases were heterozygous for a poly-alanine expansion mutation^{8,9}).

Herein, we present the first report of a Korean neonate with Haddad syndrome with a germ-line mutation in the *PHOX2B* gene.

CASE REPORT

A male infant was born by vaginal delivery at 40 and 6/7 weeks of gestation at a private maternity hospital. He was the second child of a 34-year-old Korean mother. The first child was healthy and the mother had no complications and no history of exposure to drugs during pregnancy. There was no family history of congenital anomalies or inherited disorders. The infant's birth weight was 3,660 g. The height and head circumference were 51 cm and 35 cm respectively. All parameters were within the 75th–90th percentile. The apgar score was 6 at 1 min and 8 at 5 mins. At birth, cord compression around the neck and facial cyanosis were observed, but were resolved after routine initial care with oxygen. However, cyanosis was recurrently observed in the hours following birth, and the infant was therefore transferred to our hospital for further evaluation and management.

On admission, neurologic examinations and laboratory findings were normal. Physical examination revealed a cleft palate and a slightly distended abdomen. Initial simple radiographs showed no active lung lesions with normal heart size and a non-specific gaseous bowel distension was noted. The patient required conventional mechanical ventilator care with intubation because of recurrent apnea and desaturation, despite continuous positive airway pressure and a caffeine load. During apnea, there was no evidence of respiratory efforts such as chest wall movement or nasal flaring. Primary apnea was suspected rather than obstructive apnea. On day 2, abdominal distension was aggravated, and simple radiographs showed bowel dilatation without visible distal bowel gas. Abdominal sonography re-

vealed meconium- and gas-filled marked distension of the small bowel in the mid- and lower abdomen with collapse of the terminal ileum. There was no visible bowel gas in the colon, and the caliber was relatively normal, suggesting meconium plug syndrome or ileal atresia. A bedside gastrografin enema was performed as a diagnostic and therapeutic method. A gastrografin contrast enema study showed marked distension of the small bowel, and a relatively small-caliber colon. However, there was no definite transition zone (Figure 1). After the gastrografin enema, meconium was passed and abdominal distension was temporarily relieved. However, on day 5, a diagnostic exploration was performed because of progression of bowel distension and the possibility of mechanical small bowel obstruction, such as ileal atresia. The proximal ileum was distended, and the next segment was narrowed and did not show peristalsis. The entire colon and rectum exhibited a small caliber with meconium packing. Loop ileostomy was performed after evacuation of meconium plugs via enterotomy. Incidental appendectomy was also performed. In the days after the operation, we tried repeatedly to wean the baby from the ventilator but these attempts failed, because the infant experienced recurrent apnea and hypercarbia. Seizure-like movements were observed twice during severe hypercapnic periods. Brain magnetic resonance imaging (MRI) revealed a small amount of intra-ventricular hemorrhage and subdural hemorrhage. Electroencephalography showed no abnormal findings, and the echocardiogram was also normal. Electrocardiography showed sinus bradycardia. Fiberoptic examination revealed mild laryngomalacia. Ophthalmologic examination showed iris depigmentation and mildly dilated

Figure 1. Simple radiograph showing marked distension of the small bowel and a relatively small-sized colon, but no definite transition zone.

pupils. Intravenous respiratory stimulant with caffeine treatment was ineffective. Despite extensive work ups, we could not identify the cause of apnea in the patient. Moreover, there was no response to hypercarbia during weaning attempts, and a biopsy revealed that there were no ganglion cells in the resected appendix, which is consistent with total colonic aganglionosis. Therefore, Haddad syndrome was strongly suspected. Finally, on day 21, a cytogenetic study was performed; this confirmed the presence of a *PHOX2B* mutation, yielding a set of clinical parameters consistent with CCHS.

Tracheostomy was performed due to the necessity for long-term mechanical ventilatory assistance. Enteral nutrition was performed with caution, because the patient passed a large amount of feces if the enteral feeding volume exceeded a certain level. Several audiometric screening tests showed that the patient had hearing failure in both ears. A series of follow-up simple radiographs and abdomen and pelvic sonography demonstrated no abnormal findings, such as mass-like lesions. However, we could not complete detailed examinations for the observed hearing loss or possible presence of a tumor because the parents refused these additional tests.

The infant's body weight was 5.9 kg at 3 months of age (3rd - 10th percentile). He had been hospitalized for 3 months. After educating the parents completely, the infant will be discharged from hospital with a home mechanical ventilator and total parenteral nutrition.

1. DNA isolation and polymerase chain reaction (PCR)

Genomic DNA was isolated from peripheral blood using a QIAmp DNA blood kit (Qiagen, Hilden, Germany). All exons of the *PHOX2B* gene were amplified by performing PCR with five sets of primers (Table 1). Amplification of the exons was performed in 30 cycles, with each cycle consisting of denaturation at

95°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 45 sec. PCR was carried out in reaction volumes of 20 µL, containing 100 ng of genomic DNA template, 1 µM of each primer, 200 µM of each dNTP, 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 1 U of Taq polymerase (Promega, Madison, WI, USA). Subsequently, PCR products were separated on 1.2% agarose gels in the presence of ethidium bromide to verify the size and purity of amplification products.

2. DNA sequence analysis

After verifying that a single specific PCR product was amplified, DNA sequencing was performed using the same primers that were used in PCR, and a BigDye Terminator V3.1 Cycle Sequencing Ready reaction kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Reactions were performed with 30 cycles at 94°C for 20 sec, at 58°C for 20 sec and at 72°C for 30 sec in a PCR machine (C1000 Thermal Cycler, BioRad, Hercules, CA, USA) using 10 ng of PCR product, which was treated with exonuclease I and shrimp alkaline phosphatase (Amersham Pharmacia Biotech, Piscataway, NJ, USA), as template and 10 pmol of appropriate primers. Ethanol precipitation was used to remove incorporated dyes. Electrophoresis and analysis of the reaction mixtures were performed using an ABI 3130xl Genetic analyzer (Applied Biosystems, Foster City, CA, USA). The sequence analyses were carried out using Mutation Surveyor v.3.24 (Softgenetics, PA, USA) and were compared to sequences deposited in RefSeq with the accession number NM_003924.3. To detect expansion of the polyalanine tract, Exon3-II was amplified with PCR and the PCR product was then electrophoresed on a 1.2% agarose gel and subjected to sequence analysis¹⁰.

3. Results of genetic analysis of the *PHOX2B* gene

The baby was heterozygous for c.936dup (T) (p.M313fs) on exon 3 of the *PHOX2B* gene (Figure 2).

Table 1. Sequences of Primers Using Polymerase Chain Reaction*

	Sense (5'-3')	Antisense (5'-3')
Exon1	taaggaggccagttggagg	gcttcctatatacgggcgga
Exon2	ttctctcggaactctctcg	acttcgaattccaccagccg
Exon3-I	atggggaggagggtgttaa	cttgccgggttcgcctc
Exon3-II	atcccaacccaccccc	aagataggacgtgcggaag
Exon3-III	ctgggggccctggacaa	ttaggcctcaatgaaaagc

*Primers were designed with primer3Plus available at <http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/>, using sequences from GenBank accession number of NT_006238.11

DISCUSSION

CCHS or "Ondine's curse" is a life-threatening condition characterized by inadequate control of breathing, especially during sleep, and by markedly impaired responses to severe hypoxemia and hypercapnia. CCHS is observed concurrently with several disorders involving dysregulation of the ANS,

have non-polyalanine repeat expansion mutations (NPARMs), including missense, nonsense and frameshift mutations^{6,7}. Recent data have suggested a relationship between the *PHOX2B* genotype and the CCHS phenotype. Specifically, among polyalanine expansion mutations (PARMs), the size of the polyalanine expansion is associated with the severity of clinical manifestation and prognosis. Longer poly-alanine expansions result in worse respiratory symptoms, a longer R-R interval as measured by Holter monitoring, and a more distinctive facial phenotype⁷. Although, this strong genotype-phenotype interaction is not certain in patients with germ-line *PHOX2B* mutations, a previous report suggested that overall clinical symptoms were severe in most patients carrying long PARMs (26 or more) and NPARMs than in patients with 25 PARMs¹². Interestingly, Trochet et al. suggested that germ-line alterations in the *PHOX2B* gene including frameshift or missense mutations are associated with a high incidence of HD and may predispose patients to hereditary neuroblastoma^{1,3}. Moreover, tumors of the sympathetic nervous system in patients with CCHS tend to be multifocal. In our patient, there was no evidence of ANS malignancy during the study duration; however, close monitoring will be needed throughout the patient's lifetime. Ophthalmologic impairments including abnormal pupillary function and iris abnormalities, such as iris atrophy, iris pigmentation, smooth iris surface and atrophic sphincter, have been reported in patients with CCHS⁶⁻⁸. Additionally, dysfunctions of brainstem auditory pathways have been observed in infants with CCHS¹³. However, the relationship between these variable abnormal clinical features and mutations in the *PHOX2B* gene has not yet been identified.

Trochet et al. suggested that the variable clinical features may be related to the effects of unknown modifier genes, because all the observed variations in the CCHS phenotype cannot be explained solely by the *PHOX2B* genotype¹⁴⁾.

The goal of treatment for CCHS is to ensure adequate oxygenation and ventilation during waking hours and while sleeping, thereby improving long-term neurodevelopmental outcomes by reducing the risk of hypoxic-ischemic insults from chronic hypoxia and cor pulmonale¹⁵⁾. There are several options for adequate oxygenation and ventilation. Tracheostomy with positive pressure ventilation is the most common method for ventilation in infants with CCHS and is guaranteed to secure the airways and provide adequate ventilation¹⁵⁾. However, considering the complications of long-term use of invasive ventilator care, non-invasive ventilation methods including BiPAP and diaphragmatic pacing have also been frequently attempted¹⁶⁾. Long-term prognosis is variable, with mortality rates ranging from 10% to 40%¹⁷⁾. The main causes of death include cor pulmonale, pneumonia and aspiration. Thus, advances in home ventilation technology and monitoring equipment are expected to improve outcomes in infants with CCHS¹⁵⁾.

In 1993, Ahn et al. described the first case of Haddad syndrome in Korea; this case was diagnosed based on clinical manifestations, including hypoventilation primarily during sleep, despite the presence of normal neuromuscular and pulmonary systems and total aganglionosis¹⁸⁾. Since then, four additional cases of CCHS have been reported, all of which were diagnosed as Haddad syndrome on the basis of clinical features with primary hypoventilation and HD¹⁹⁾. In 2010, the first case of diagnosis of a Korean neonate with CCHS using *PHOX2B* gene mutations was reported²⁰⁾. After this, two more genetically confirmed CCHS cases have been reported, both of which were accompanied by HD; these cases were finally diagnosed as Haddad syndrome^{8,9)}. Two infants had experienced apnea and abdominal distension from birth. Extensive testing for apnea and hypoventilation could not explain the clinical manifestations. Genetic analysis showed that both had poly-alanine expansion mutations in the *PHOX2B* gene, and these two cases supported the genotype-phenotype relationship of the *PHOX2B* gene. As mentioned above, in poly-alanine expansion mutations of the *PHOX2B* gene, the numbers of poly-alanine repeats can predict clinical severity. In neonates with advanced Haddad syndrome, studies have reported 26 poly-alanine repeats⁸⁾ and 27 poly-alanine repeats⁹⁾ in the *PHOX2B* gene. All of these patients eventually

required tracheostomy because of prolonged hypoventilation. In the infant with 26 poly-alanine repeats, descending loop colostomy was performed because the transition zone was located in the mid-descending colon, as shown by barium enema. However, in the infant with 27 PARMs, more extensive aganglionosis extending up to the proximal small bowel was observed, requiring proximal jejunostomy and Hickman catheter insertion for prolonged parenteral nutrition.

In summary, in this study, we described the clinical manifestations of a male neonate who was diagnosed with Haddad syndrome, and we discuss the genetic characteristics of the accompanying CCHS. *PHOX2B* genotyping is considered a reliable diagnostic test for CCHS, and a robust predictor of the clinical course of the disease. Considering the rarity of CCHS, and the fact that there are no other definitive diagnostic tools available through laboratory or radiologic tests, *PHOX2B* testing is useful for early diagnosis and appropriate interventions. Moreover, prenatal genetic analysis may provide more comprehensive information about the severity of the disease in affected children in subsequent pregnancies.

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