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 ventilatory response to hypercarbia and hypoxemia. 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. The combination of CCHS and HD, which is also known as Haddad syndrome, is an extremely rare disorder. This is the first report describing Haddad syndrome with a germ-line mutation in the PHOX2B gene in a Korean neonate.

Received: 26 June 2015
Revised: 28 July 2015
Accepted: 29 July 2015
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

Copyright(c) By Korean Society of Neonatology. All right reserved.
This is an Open-Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.
De novo mutations in the paired-like homeobox 2b (PHOX2B) gene are associated with CCHS and have been found in over 90% of CCHS cases. Most CCHS patients with PHOX2B gene mutations have a heterozygous expansion of poly-alanine (GCN) repeats, whereas only 8% have germ-line mutations, such as missense or frameshift mutations. 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.

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 revealed 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.](image-url)
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 20μL, 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 Terminatore 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).

**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,
including HD, and is associated with an increased incidence of neural crest-derived tumors, such as neuroblastoma, ganglion- neuroma and ganglioneuroblastoma1-3). The combination of CCHS and HD, known as Haddad syndrome, was first described by Haddad et al. in 19784). Previous studies have reported that the incidence of HD in CCHS varies from 16% to 50%, whereas the incidence of CCHS in HD is extremely low5-7). Furthermore, HD in CCHS patients is different from usual HD in that there is usually extensive involvement of aganglionosis in a significant number of affected patients and gender balance11). Overall, CCHS and Haddad syndrome are extremely rare, and its early diagnosis requires astute clinical monitoring and intervention.

Before the genetic causes of CCHS were known, diagnosis of CCHS depended on exclusion of all other possible causes of apnea, including pulmonary, cardiac, central, muscular, metabolic and mitochondrial disorders. Currently, however, we have a more comprehensive understanding of the molecular genetics of CCHS. Previous reports of familial cases and concordance in monozygotic twins have suggested a genetic origin of CCHS. In 2003, Amiel et al. first reported mutations in the \textit{PHOX2B} gene in patients with CCHS5). \textit{PHOX2B} maps to chromosome 4p12 and encodes a 314-amino acid paired-like homeobox 2b; CCHS, congenital central hypoventilation syndrome. Partial seq. of \textit{PHOX2B} gene

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{Genetic analysis showed that this patient was heterozygous for c.936dup (T) (p.M313fs) on exon 3 of the \textit{PHOX2B} gene, which is consistent with CCHS. Abbreviations: \textit{PHOX2B}, paired-like homeobox 2b; CCHS, congenital central hypoventilation syndrome.}
\end{figure}

have non-polyalanine repeat expansion mutations (NPARMs), including missense, nonsense and frameshift mutations6,7). Recent data have suggested a relationship between the \textit{PHOX2B} genotype and the CCHS phenotype. Specifically, among poly-alanine 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 \textit{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 PARMs8). Interestingly, Trochet et al. suggested that germ-line alterations in the \textit{PHOX2B} gene including frameshift or missense mutations are associated with a high incidence of HD and may predispose patients to hereditary neuroblastoma1,3). 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 CCHS8-10). Additionally, dysfunctions of brainstem auditory pathways have been observed in infants with CCHS11). However, the relationship between these variable abnormal clinical features and mutations in the \textit{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 genotype.

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 pulmonale. 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 ventilation. However, considering the complications of long-term use of invasive ventilator care, non-invasive ventilation methods including BiPAP and diafragmatic pacing have also been frequently attempted. Long-term prognosis is variable, with mortality rates ranging from 10% to 40%. 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 CCHS.

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 aganglionosis. 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 HD. In 2010, the first case of diagnosis of a Korean neonate with CCHS using PHOX2B gene mutations was reported. 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.

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 repeats and 27 poly-alanine repeats 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 jejunosomy 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.

REFERENCES


6) Matera I, Bachetti T, Puppo F, Di Duca M, Morandi F, Casiraghi GM, et al. PHOX2B mutations and polyalanine expansions...
correlate with the severity of the respiratory phenotype and associated symptoms in both congenital and late onset Central Hypoventilation syndrome. J Med Genet 2004;41:373-80.


