pii: jc-18-00470 https://dx.doi.org/10.5664/jcsm.7688

JCSM
Journal of Clinical Sleep Medicine

CASE REPORTS

A Novel c.676_677insG *PHOX2B* Mutation in Congenital Central Hypoventilation Syndrome

Guodong Ye, PhD[1,2,3,*]; Daxiong Han, PhD[4,*]; Yu Jiang, MS[1,*]; Zengge Wang, MS[1]; Yulin Zhou, PhD[1]; Xinzhu Lin, BS[5]; Weiwei Chen, MS[2]; Maoli Chen, MS[2]; Jianxiong Xu, MS[2]; Yanyan Yang, MS[6]; Qiwei Guo, MS[1]

*[1]United Diagnostic and Research Center for Clinical Genetics, School of Public Health of Xiamen University and Xiamen Maternal and Child Health Hospital, Xiamen, China; [2]Xiamen LifeInt Technology Co., Ltd, Xiamen, China; [3]School of Basic Medical Sciences, Jinan University, Guangzhou, China; [4]School of Pharmaceutical Sciences, Xiamen University, Xiamen, China; [5]Department of Neonatology, Xiamen Maternal and Child Health Hospital, Xiamen, China; [6]Third Institute of Oceanography, State Oceanic Administration, Xiamen, China; *Contributed equally*

Paired-like homeobox (*PHOX*)*2B* is considered to be the causative gene of congenital central hypoventilation syndrome (CCHS), a dominant genetic disorder that results in abnormal central respiratory control with resulting hypoventilation during sleep. In this study, we report a novel c.676_677insG (p.Ala226fs) mutation in a patient with severe CCHS, and we evaluated the function of this mutation. The mutation reduced the translation of the mutant PHOX2B protein and impaired its ability to activate the *PHOX2A* promoter, due to a haploinsufficiency effect. The mutant PHOX2B was able to interact with wildtype PHOX2B, resulting in retention of PHOX2B on the nuclear membrane, which may impair the normal function of the nuclear membrane, and leading to cellular morbidity. Our study provides useful information for the functional studies of PHOX2B and understanding the pathogenesis of CCHS, and thus is beneficial for the prognosis of, genetic counseling for, and development of pharmaceuticals for PHOX2B-associated diseases.

Keywords: congenital central hypoventilation syndrome, dominant negative effect, haploinsufficiency effect, nonpolyalanine repeat mutations, PHOX2B

Citation: Ye G, Han D, Jiang Y, Wang Z, Zhou Y, Lin X, Chen W, Chen M, Xu J, Yang Y, Guo Q. A novel c.676_677insG *PHOX2B* mutation in congenital central hypoventilation syndrome. *J Clin Sleep Med.* 2019;15(3):509–513.

INTRODUCTION

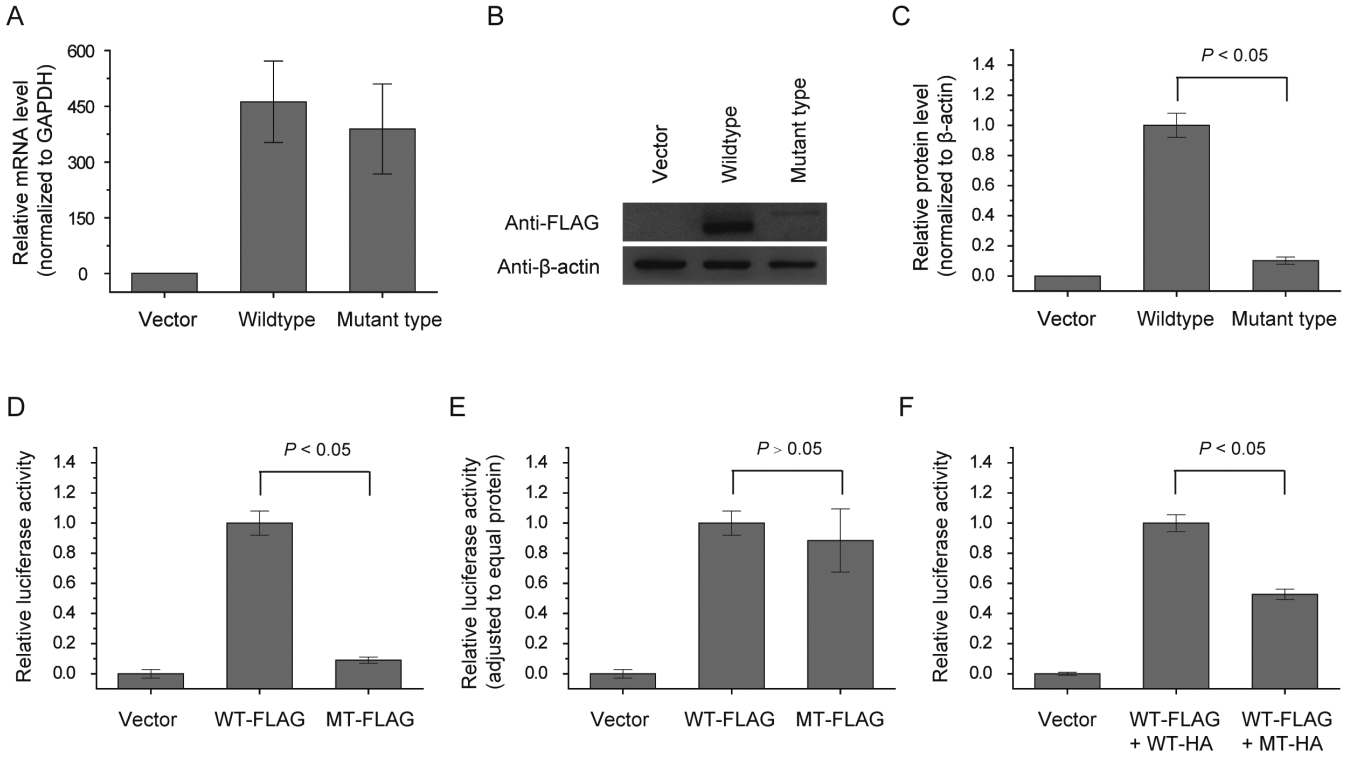
Congenital central hypoventilation syndrome (CCHS; OMIM no. 209880) is a dominant genetic disorder that results in abnormal central respiratory control with resulting hypoventilation during sleep.[1] Approximately 20% and 6% of CCHS cases also have Hirschsprung disease (HSCR) and/or tumors of neural crest origin (neuroblastoma, ganglioneuroblastoma, and ganglioneuroma), respectively.[2] *Paired-like homeobox (PHOX)2B*, which is located on chromosome 4p12, is thought to be the causative gene of CCHS.[3] *PHOX2B* encodes a highly conserved transcription factor that plays a key role in the development of the autonomic nervous system.[3] Human PHOX2B is a 314-amino acid protein that contains a homeodomain and two polyalanine stretches of 9 and 20 residues in the C-terminal domain.[4] Approximately 90% of CCHS cases carry a polyalanine repeat expansion mutation (PARM) within the second polyalanine repeat sequence located in exon 3 of *PHOX2B*, resulting in expansion of the polyalanine repeat from 20 (wildtype) to 25–33.[5] In addition to PARMs, a small number (less than 10%) of CCHS cases carry other mutations (eg, missense, nonsense, and frameshift mutations) in *PHOX2B*, which are termed nonpolyalanine repeat mutations (NPARM).[5] Although the prevalence is much lower, cases of syndromic CCHS with NPARM have a much higher rate of HSCR, a higher likelihood of needing ventilatory support during wakefulness as well as during sleep, and more frequent neural crest tumors, than cases with PARMs, which is almost exclusively associated with isolated CCHS.[6] Based on these findings, NPARM is thought to cause diseases via mechanisms that are distinct from those of PARM, and have a larger and more generalized impact on PHOX2B function. In this regard, the continued identification of CCHS cases with NPARM is great of interest and importance. In this study, we report a novel NPARM in a patient with severe CCHS and an evaluation of the functional role of this mutation.

REPORT OF CASE

The patient was a newborn boy delivered by cesarean section performed for cephalopelvic disproportion and a low baseline fetal heart rate at 38^{+3} weeks of gestation without any other complication during pregnancy. The Apgar scores were 8 (−1 for pale skin, and −1 for tachypnea) at 1 minute and 9 (−1 for tachypnea) at 5 and 10 minutes. Physical examination revealed a pulse rate of 146 beats/min, respiratory rate of 50/min, and body temperature of 36.2°C. The patient was mildly dysmorphic, with a low ear position and a small jaw. The pupils were of equal size and normal appearance. Sluggishness, a slow pupillary light response, tachypnea with shallow breathing, hypotonia, poor Moro reflex, and mildly pale skin were noted.

There were no abnormalities in the heart, brain, or abdomen by ultrasound examination. Similarly, there were no abnormalities in the amino acid and acylcarnitine spectra by mass spectrum examination of blood and urine samples, respectively. Bilateral immature development of the cerebral hemispheres

Figure 1—The c.676_677insG mutation impairs activation of the *PHOX2A* promoter, due to a haploinsufficiency effect.



(A) mRNA levels of wildtype and mutant *PHOX2B*. **(B)** Protein levels of wildtype and mutant PHOX2B. Total cell lysates were analyzed by western blotting. **(C)** Relative protein levels of wildtype and mutant PHOX2B. **(D)** Relative transcriptional activity of the *PHOX2A* reporter promoter in cells expressing wildtype or mutant PHOX2B. **(E)** Relative transcriptional activity of the *PHOX2A* promoter reporter construct in the presence of equal amounts of wildtype or mutant PHOX2B. **(F)** Relative transcriptional activity of the *PHOX2A* reporter promoter construct in the presence of wildtype and mutant PHOX2B (mimicking mutant heterozygosity) and wildtype PHOX2B. The results are the mean ± standard error (error bars) of triplicate experiments.

was noted by brain MRI. A chest x-ray of the bilateral lung field revealed patchy infiltrates, laminated shadows, with inhomogeneous density, and indistinct pulmonary borders suggesting small pleural effusions. However, no pathogen was detected in sputum, gastric aspirate, blood, urine, or stool samples.

Venous blood gas analysis revealed a low partial pressure of oxygen (63.3 mmHg), low oxygen saturation (84.4%), high partial pressure of carbon dioxide (81.1 mmHg), and high total carbon dioxide (31 mmol/L) when on room air. Mechanical-assisted ventilation was required to improve his respiration. Although respiratory assistance was still required on day 8 in the hospital, at the request of the guardians, the patient was compassionately extubated and discharged home. Unfortunately, the patient died while sleeping 1 week after leaving the hospital. An autopsy was not performed according to the family's decision.

Whole exome sequencing revealed a heterozygous c.676_677insG mutation in exon 3 of *PHOX2B*, resulting in an elongated protein of 358 amino acids with an altered C-terminal sequence (p.Ala226fs; **Figure S1**). As a result of this mutation, the 20 alanine residue domain is enriched in glycine and serine residues, and there is an additional seven alanine sequence within the translated portion of the 3′-UTR (**Figure S2**). A low degree mosaicism for this mutation was found in the leukocytes of the patient's asymptomatic mother (**Figure S1A**). Based on the clinical symptoms and genetic findings, the patient was diagnosed with CCHS.

To evaluate the function of c.676_677insG mutation, we constructed four plasmids to express PHOX2B fusion proteins: FLAG-tagged wildtype PHOX2B (WT-FLAG), HA-tagged wildtype PHOX2B (WT-HA), FLAG-tagged mutant PHOX2B (MT-FLAG), and HA-tagged mutant PHOX2B (MT-HA). HeLa cells were used for this functional study of mutant PHOX2B because these cells do not express endogenous PHOX2B.

We first transfected the WT-FLAG and MT-FLAG plasmids into HeLa cells and examined the amounts of the transcriptional and translational products by qRT-PCR and western blotting, respectively. As shown in **Figure 1A**, transfection and transcription were successfully detected. The amount of the mutant protein was significantly lower than the amount of the wildtype protein (**Figure 1B** and **Figure 1C**). These results suggested that the c.676_677insG mutation impairs the translation of the mutant protein.

In many cases, frameshift mutations decrease the ability of PHOX2B to activate target gene promoters, such as *DBH*, *PHOX2A*, and *TLX2*.[4,7] Therefore, we examined the ability of our mutant protein to activate the promoter of the *PHOX2A* target gene, whose product is expressed in all neurons and is essential for the formation of locus coeruleus.[8] We co-transfected a *PHOX2A* promoter reporter plasmid with a WT-FLAG or MT-FLAG plasmid and measured luciferase activity. As shown in **Figure 1D**, *PHOX2A* promoter activation was

significantly lower in the presence of the mutant protein than in the presence of the wildtype protein. However, after adjusting for equal protein, activation of the *PHOX2A* promoter by the wildtype and mutant proteins was comparable (**Figure 1E**), which suggests that the impaired activation of the *PHOX2A* promoter was due to the reduced amount of mutant protein. Next, we co-transfected wildtype and mutant *PHOX2B*-expressing plasmids to simulate the heterozygous status of the patient. As expected, the activation of the *PHOX2A* promoter was significantly lower for the heterozygote (WT+MT) than for the wildtype (WT+WT; **Figure 1F**). This result suggests that a haploinsufficiency effect is involved in the decreased activation of the *PHOX2A* promoter observed in this case.

Dimerization plays an important role in the normal function of PHOX2B; therefore, heterodimerization of the mutant and wildtype proteins could be the cause of the dominant negative effect on protein function.[9] To examine the potential interaction between the mutant and wildtype proteins, we co-transfected WT-FLAG and MT-HA plasmids into HeLa cells and evaluated their binding by co-immunoprecipitation. Cell extracts were immunoprecipitated with an anti-FLAG antibody and immunoblotted with anti-HA antibodies. As shown in **Figure 2A**, wildtype and mutant PHOX2B do interact.

Lastly, we evaluated the effects of the c.676_677insG mutation on PHOX2B localization. We co-transfected three combinations of plasmids (WT-FLAG+WT-HA, WT-FLAG+MT-HA, and MT-FLAG+MT-HA) into HeLa cells and examined the localization of the FLAG-tagged protein by immunofluorescence using anti-FLAG antibodies. As shown in **Figure 2B** and **Figure 2C**, most of the transfected cells containing the two wildtype constructs were intact, and PHOX2B was localized almost exclusively inside the nucleus, with no evident aggregation. In comparison, various cellular morbidities, such as distortional nuclei and diffusion of PHOX2B in the cytoplasm, were observed in approximately 28% (56/200) of the cells containing two mutant constructs. Moreover, mutant PHOX2B was characteristically localized around the nuclear membrane (**Figure 2D**, **Figure 2E** and **Figure 2F**). Similarly, various cellular morbidities were also observed in approximately 25% (50/200) of cells containing wildtype and mutant constructs, and the wildtype PHOX2B protein showed nuclear membrane localization (**Figure 2G**, **Figure 2H** and **Figure 2I**). These results further confirmed the interaction between wildtype and mutant PHOX2B, and showed that the mutation impairs the cellular localization of both mutant and wildtype PHOX2B due to their interaction. Moreover, the expression of the mutant PHOX2B may be cytotoxic.

DISCUSSION

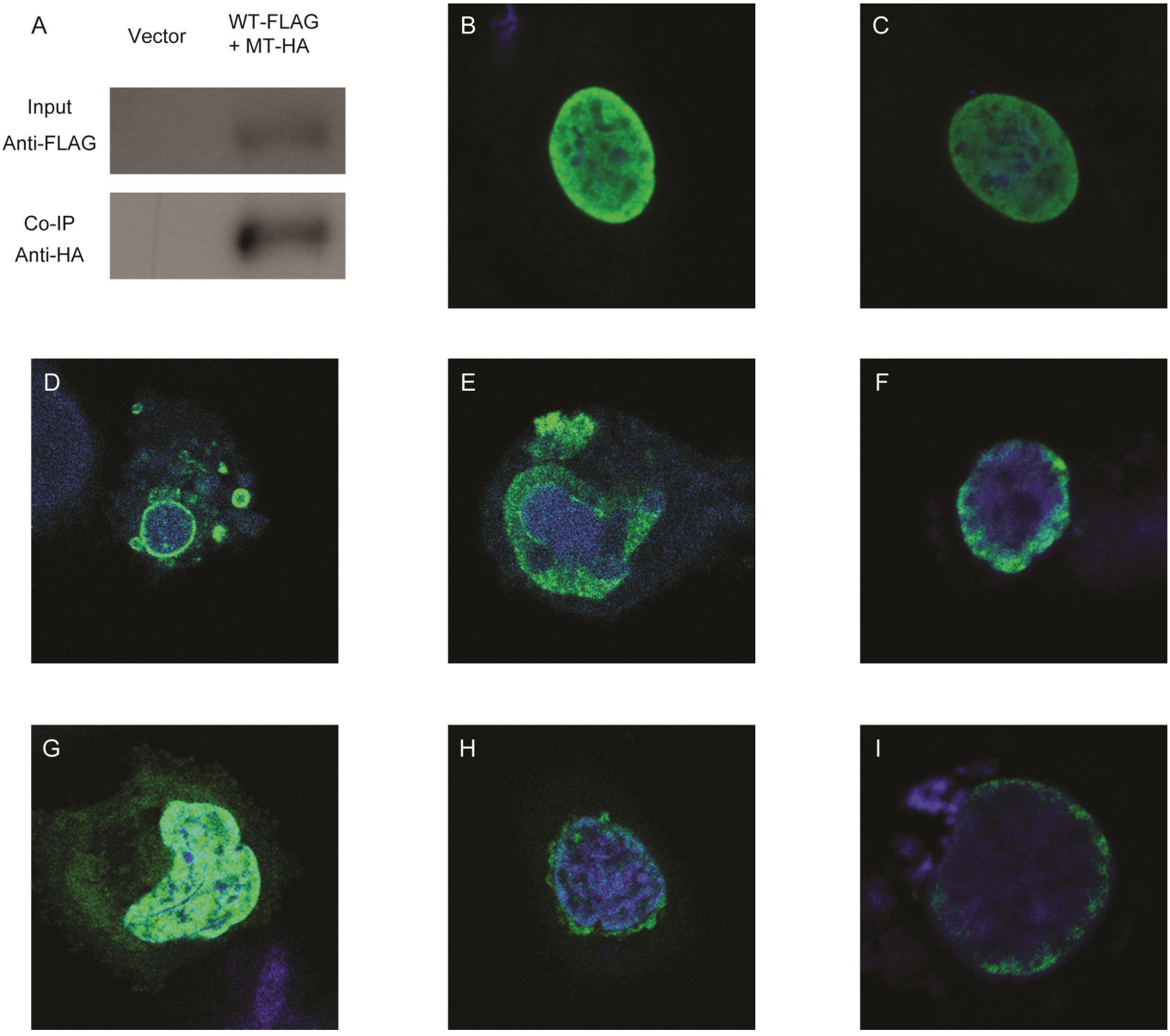
In this study, we reported a novel c.676_677insG (p.Ala226fs) mutation in a patient with severe CCHS. Thus far, 31 frameshift mutations in *PHOX2B* have been reported.[6] Among these, 15 are "frame 2" mutations (which refers to the insertion of one or more triplets [+1 nucleotide] or the deletion of two or more triplets +2 nucleotides) that are associated with syndromic CCHS.[6] HSCR is the most typical manifestation, and it was present in 14/15 CCHS cases with "frame 2" mutations and in all nine CCHS cases with "frame 2" mutations upstream of the 20 polyalanine domains.[6] Notably, unlike other "frame 2" mutations with disrupted 20 polyalanine domains, HSCR was absent in our case (**Figure S1**). Therefore, study of the c.676_677insG mutation could provide useful information regarding the domain functions of PHOX2B and the molecular mechanisms underlying HSCR and CCHS. Unfortunately, unlike HSCR, which is congenital and relatively straightforward to diagnose, we were unable to assess other phenotypes, such as the development of neuroblastoma, in this case due to early lethality.

A direct consequence of the c.676_677insG mutation was dramatically reduced expression of the mutant protein (**Figure 1B** and **Figure 1C**), which suggests that a haploinsufficiency effect may be involved in the pathogenic mechanism in this case. This hypothesis was partially confirmed by the results of the *PHOX2A* promoter activation experiment. Several of the frameshift mutations retained the ability to activate the *PHOX2A* promoter.[10] Similarly, the ability to activate the *PHOX2A* promoter was comparable in the presence of equal amounts of the p.Ala226fs mutant and wildtype proteins (**Figure 1E**), suggesting that the mutant protein can still recruit co-activators and function correctly even though homeodomain folding and/or function, which is critical for protein-protein interactions, may be affected. However, when the wildtype and mutant constructs were co-transfected to simulate the heterozygous status of the patient, the ability of the mixed PHOX2B to activate the *PHOX2A* promoter was impaired compared to wildtype PHOX2B (**Figure 1F**), due to the reduced amount of mutant protein, thus indicating that the haploinsufficiency effect results from this mutation.

Dimerization is thought to play an important role in the normal function of PHOX2B, and impaired dimerization of PHOX2B mutants has recently been reported for polyalanine expansion-associated CCHS.[9] According to this study, the dimerization domain encompasses the homeodomain and the c-terminal region of PHOX2B and does not involve the polyalanine domain. Compared with PARM cases, the p.Ala226fs mutant protein contains a wildtype homeodomain but an altered C-terminal region. However, it was able to form heterodimers with wildtype protein (**Figure 2A**), suggesting that the dimerization mechanism for this NPARM differs from that of PARMs.

Dominant-negative effects were previously reported for NPARM PHOX2B, which were due to aberrant protein-protein interactions.[7] Therefore, the formation of heterodimers between the p.Ala226fs mutant and wildtype proteins suggests that a dominant-negative effect may be involved in the pathogenic mechanism in this CCHS case. This hypothesis was confirmed by the results of the immunofluorescent experiment in which wildtype PHOX2B was abnormally co-localized with the mutant PHOX2B around the nuclear membrane in substantial numbers of transfected cells and various cellular morbidities were observed in cells expressing the mutant PHOX2B (**Figure 2**). We speculate that one of the protein structures resulting from the change in the C-terminus of mutant PHOX2B impairs homeodomain-mediated nuclear import and causes retention of the wildtype and mutant PHOX2B on the nuclear membrane, thus impairing

Figure 2—The c.676_677insG mutation impairs the cellular localization of PHOX2B and causes cellular morbidity due to a dominant negative effect.



(A) Co-immunoprecipitation of wildtype and mutant PHOX2B. Representative immunofluorescence images of FLAG-PHOX2B in cells co-transfected with WT-FLAG and WT-HA **(B and C)**, MT-FLAG and MT-HA **(D-F)**, and WT-FLAG and MT-HA **(G-I)**.

the normal function of the nuclear membrane, leading to cellular morbidity. Based on these findings, the c.676_677insG mutation could induce toxic effects, with a dominant-negative pattern, in a range of neuronal cell populations, including the autonomic ganglia, which caused the severe CCHS observed in our patient.

In conclusion, we report a novel c.676_677insG mutation in *PHOX2B* in a patient with severe CCHS and suggest that functional haploinsufficiency, caused by reduced expression of the mutant protein and cellular toxicity due to a dominant-negative effect, could be responsible for the clinical manifestations. Our study provides useful information for functional studies of PHOX2B and investigation of the pathogenesis of CCHS and HSCR, and thus will be beneficial for the prognosis of, genetic counseling for, and the development of pharmaceuticals for PHOX2B-associated diseases. Further investigations based on more relevant targets other than *PHOX2A* in the PHOX2B functional network are required to reveal the precise pathogenic mechanism of this mutation and to further understand the molecular pathogenesis underlying CCHS and its relevant phenotypes.

REFERENCES

1. Coleman M, Boros SJ, Huseby TL, Brennom WS. Congenital central hypoventilation syndrome. A report of successful experience with bilateral diaphragmatic pacing. *Arch Dis Child.* 1980;55(11):901–903.

2. Berry-Kravis EM, Zhou L, Rand CM, Weese-Mayer DE. Congenital central hypoventilation syndrome: PHOX2B mutations and phenotype. *Am J Respir Crit Care Med.* 2006;174(10):1139–1144.
3. Amiel J, Laudier B, Attie-Bitach T, et al. Polyalanine expansion and frameshift mutations of the paired-like homeobox gene PHOX2B in congenital central hypoventilation syndrome. *Nat Genet.* 2003;33(4):459–461.
4. Adachi M, Browne D, Lewis EJ. Paired-like homeodomain proteins Phox2a/Arix and Phox2b/NBPhox have similar genetic organization and independently regulate dopamine beta-hydroxylase gene transcription. *DNA Cell Biol.* 2000;19(9):539–554.
5. Vanderlaan M, Holbrook CR, Wang M, Tuell A, Gozal D. Epidemiologic survey of 196 patients with congenital central hypoventilation syndrome. *Pediatr Pulmonol.* 2004;37(3):217–229.
6. Di Lascio S, Benfante R, Di Zanni E, et al. Structural and functional differences in PHOX2B frameshift mutations underlie isolated or syndromic congenital central hypoventilation syndrome. *Hum Mutat.* 2018;39(2):219–236.
7. Di Lascio S, Bachetti T, Saba E, Ceccherini I, Benfante R, Fornasari D. Transcriptional dysregulation and impairment of PHOX2B auto-regulatory mechanism induced by polyalanine expansion mutations associated with congenital central hypoventilation syndrome. *Neurobiol Dis.* 2013;50:187–200.
8. Flora A, Lucchetti H, Benfante R, Goridis C, Clementi F, Fornasari D. Sp proteins and Phox2b regulate the expression of the human Phox2a gene. *J Neurosci.* 2001;21(18):7037–7045.
9. Di Lascio S, Belperio D, Benfante R, Fornasari D. Alanine expansions associated with congenital central hypoventilation syndrome impair PHOX2B homeodomain-mediated dimerization and nuclear import. *J Biol Chem.* 2016;291(25):13375–13393.
10. Bachetti T, Matera I, Borghini S, Di Duca M, Ravazzolo R, Ceccherini I. Distinct pathogenetic mechanisms for PHOX2B associated polyalanine expansions and frameshift mutations in congenital central hypoventilation syndrome. *Hum Mol Genet.* 2005;14(13):1815–1824.

SUBMISSION & CORRESPONDENCE INFORMATION

Submitted for publication July 31, 2018
Submitted in final revised form November 30, 2018
Accepted for publication January 7, 2019
Address correspondence to: Qiwei Guo, MS, United Diagnostic and Research Center for Clinical Genetics, School of Public Health of Xiamen University and Xiamen Maternal and Child Health Hospital, Xiamen, China; Email: guoqiwei@xmu.edu.cn; guoqiwei@gmail.com

DISCLOSURE STATEMENT

All authors have seen and approved the manuscript. This work was supported by the National Natural Science Foundation of China (project No. 81572084), and Xiamen Science and Technology Major Project (project No. 3502Z20171006). The authors report no conflicts of interest.