CLINICAL REPORT

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Germline Mosaicism of *PHOX2B* Mutation Accounts for Familial Recurrence of Congenital Central Hypoventilation Syndrome (CCHS)

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Congenital central hypoventilation syndrome (CCHS), a rare disorder characterized by alveolar hypoventilation and autonomic dysregulation, is caused by mutations in the *PHOX2B* gene. Most mutations occur de novo, but recent evidence suggests that up to 25% are inherited from asymptomatic parents with somatic mosaicism for these mutations. However, to date, germline mosaicism has not been reported. This report describes a family with recurrence of *PHOX2B* mutation-confirmed CCHS due to germline mosaicism. The first occurrence was a baby girl, noted on day 2 of life to have multiple episodes of apnea, bradycardia, and cyanosis while breathing room air. *PHOX2B* gene testing confirmed the diagnosis of CCHS with a heterozygous polyalanine repeat expansion mutation (PARM); genotype 20/27 (normal 20/20). Both parents tested negative for this mutation using fragment analysis (limit of detection <1%). Upon subsequent pregnancy [paternity confirmed using short tandem repeat (STR) analysis], amniocentesis testing identified the *PHOX2B* 20/27 genotype, confirmed with repeat testing. Elective abortion was performed at 21.5 weeks gestation. Testing of abortus tissue confirmed amniocentesis testing. The *PHOX2B* 20/27 expansion was not observed in a paternal sperm sample. This case represents the first reported family with recurrence of *PHOX2B* mutation-confirmed CCHS without detection of a parental carrier state or mosaicism, confirming the previously hypothesized possibility of germline mosaicism for *PHOX2B* mutations. This is an important finding for genetic counseling of CCHS families, suggesting that even if somatic mosaicism is not detected in parental samples, there is still reason for careful genetic counseling and consideration of prenatal testing during subsequent pregnancies.

Key words: congenital central hypoventilation syndrome; Ondine's Curse; *PHOX2B*; germline mosaicism

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INTRODUCTION

Congenital central hypoventilation syndrome (CCHS) is a disorder of respiratory control with autonomic nervous system dysregulation (ANSD). The symptoms of disordered respiratory control typically manifest in the newborn period with persistent alveolar hypoventilation and impaired ventilatory responses to hypercapnia and hypoxemia during sleep, and to varying degrees during wakefulness. The ANSD, which may also present in the newborn period, and/or become more marked at later ages, includes anatomic disorders (Hirschsprung Disease and tumors of neural crest origin) as well as physiologic dysfunction (diminished pupillary light response, esophageal dysmotility, severe breath-holding spells, reduced basal body temperature, sporadic profuse sweating, lack of perception to dyspnea, altered perception of anxiety, and lack of adequate respiratory and cardiac physiologic responsiveness to the challenges of exercise and environmental stressors) [Weese-Mayer et al., 1992; Paton et al., 1993; Shea et al., 1993a,b,c; Pine et al., 1994;

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Silvestri et al., 1995; Goldberg and Ludwig, 1996; Spengler et al., 1998; Marazita et al., 2001; Weese-Mayer et al., 2001; Faure et al., 2002; O'Brien et al., 2005; Trang et al., 2005; Weese-Mayer et al., 2010].

Mutation of the *PHOX2B* gene is causative in all individuals with CCHS. *PHOX2B* is a homeodomain-containing protein involved in the development of the autonomic nervous system (ANS). Exon 3 of the *PHOX2B* gene normally has a 20 alanine-coding repeat region. The majority of the population has a 20 alanine-coding repeat region on each copy of chromosome 4, thus a *PHOX2B* genotype of 20/20. However, in the majority of patients with CCHS (90–92%) there is a heterozygous expansion of this region from 20 repeats to between 24 and 33 repeats (genotypes of 20/24–20/33) [Amiel et al., 2003; Sasaki et al., 2003; Weese-Mayer et al., 2003; Matera et al., 2004; Horiuchi et al., 2005; Trochet et al., 2005; Berry-Kravis et al., 2006; Repetto et al., 2009]. These expansions are known as polyalanine repeat expansion mutations (PARMs). Remaining individuals with CCHS have either a missense, nonsense, or frameshift mutation in *PHOX2B*, known as non-polyalanine repeat expansion mutation (NPARM; 8–10%), [Amiel et al., 2003; Weese-Mayer et al., 2003; Matera et al., 2004; Trochet et al., 2005; Berry-Kravis et al., 2006; Weese-Mayer et al., 2010] or a microdeletion including all or part of the *PHOX2B* gene (<1%) [Jennings et al., 2012].

PHOX2B mutations are typically de novo, affecting only one member of a family. In a subset of patients (estimated between 5 and 25%) the *PHOX2B* mutation is inherited as a result of low level mosaicism in asymptomatic parents [Weese-Mayer et al., 2003; Berry-Kravis et al., 2006; Parodi et al., 2008; Trochet et al., 2008; Bachetti et al., 2011], with mosaicism ranging from 7 to 50% in somatic cells as determined using blood leukocytes. In a subset of these families, parental mosaicism has resulted in sibling recurrence for CCHS. While germline mosaicism in CCHS has been hypothesized, all families previously reported with sibling recurrence for a CCHS-causing *PHOX2B* mutation have been inherited from a somatic mosaic parent, or in the case of the partially penetrant 24 or 25 repeat expansions, an asymptomatic parent carrying the mutation in the heterozygous state. This clinical report describes a family with recurrence of *PHOX2B* mutation-confirmed CCHS, likely due to germline mosaicism.

CLINICAL REPORT

Patient 1 was a female born at 31 6/7 weeks gestation with birth weight 1,820 g. The mother was 33 years old, married, gravida 1, para 1, RPR serology negative, HIV-negative, Rubella immunized, hepatitis B negative, and GBS negative. Apgar scores at birth were 8 at 1 min and 9 at 5 min. On the second day of life, the infant was noted to have multiple episodes of apnea and bradycardia as well as desaturation events on room air. Caffeine citrate did not change the frequency or duration of her apnea and oxygen desaturation events. Flexible laryngoscopy revealed normal upper airway and normal vocal cord movement. Urine organic acids and blood amino acids were normal. The institution of continuous positive airway pressure showed no improvement, with persistence of her apnea episodes and subsequent need for intubation and mechanical ventilation. Gene testing confirmed the diagnosis with identification of a CCHS-causing *PHOX2B* PARM. She subsequently underwent tracheostomy with transition to a home ventilator. She was noted to have feeding intolerance and had multiple dilated loops of bowel on flat plate abdominal films. A contrast study of colon confirmed the diagnosis of short segment Hirschsprung disease. Initially, she underwent a diverting colostomy; take down upper colostomy was performed at approximately 7 months of age. After undergoing genetic counseling, the parents of patient 1 submitted blood samples for CCHS testing. Results of this testing in somatic cells from both parents confirmed the absence of the *PHOX2B* PARM identified in their daughter. However, upon subsequent pregnancy by the parents of patient 1, amniocentesis testing identified the same *PHOX2B* PARM carried by patient 1. This pregnancy was ended by elective abortion at 21.5 weeks. Abortus tissue was donated for further research. Paternal semen sample was collected to test for presence of the daughter's PARM mutation in germline cells.

MATERIALS AND METHODS

The institutional review board at both Rush University Medical Center and Children's Memorial Hospital (Ann and Robert H. Lurie Children's Hospital) approved the methods of this study.

DNA Extraction

Genomic DNA was isolated from blood, amniocentesis, fetal liver, and semen samples utilizing a Puregene reagent kit (Qiagen; Alameda, CA) according to the manufacturer's instructions.

PHOX2B Targeted Mutation Analysis

Amplification of the region coding for the polyalanine repeat in *PHOX2B* exon 3 was carried out as described previously [Weese-Mayer et al., 2003] with primer pair 5′-CCAGGTCCCAA-TCCCAAC-3′ (forward) and 5′-GAGCCCAGCCTTGTCCAG-3′ (reverse) (methodology and primer set patented, Rush University Medical Center, Chicago, IL; donated patent revenue supports CCHS research). The PCR products were subjected to electrophoresis on a 6% denaturing polyacrylamide gel, and visualized by autoradiography. Allele repeat number was determined by comparison of bands to known size standards for which repeat number had been determined by sequence analysis.

To maximize sensitivity and to confirm results of targeted mutation analysis at Rush University, samples were transferred to the Molecular Diagnostics Laboratory at Children's Memorial Hospital. Amplification of the region coding for the polyalanine repeat in *PHOX2B* exon 3 was carried out as described previously [Weese-Mayer et al., 2003] with same primer pair used in targeted mutation analysis. The forward primer was labeled with 6-Fam. The PCR reagents and condition are the same as in sequence analysis described previously [Garcia-Barcelo et al., 2003; Weese-Mayer et al., 2003]. The PCR products (232 base pairs for the normal 20 repeat allele) were detected by ABI 3100-Avant genetic analyzer (Applied Biosystems Foster City, CA) with Pop 4 polymer. Rox 500 size standard was used as size marker. Data were analyzed using Gene Mapper software (Applied Biosystems).

Short Tandem Repeat (STR) Analysis

AmpFlSTR Profiler Plus PCR Amplification Kit (Applied Biosystems) was used to confirm the relationship between the parents and offspring. The genomic DNA was amplified in an ABI 9700 thermal cycler (Applied Biosystems) by following the manufacturer's recommendations: 95°C, 11 min one cycle, 94°C, 1 min, 59°C 1 min, 72°C 1 min 28 cycles, hold on 60°C for 45 min and hold on 25°C.

The amplified products were analyzed using capillary electrophoresis, specifically the ABI 3100-Avant Genetic Analyzer (Applied Biosystems), using a 36-cm capillary and the POP4 polymer, according to the manufacturer's recommended protocol. The length of the PCR fragments was determined through the internal size standard Rox 500. Allele designations were assigned using ABI GeneMapper software (Applied Biosystems) by comparison of the sample fragments with those of the allelic ladders supplied with the original kit.

PHOX2B Sequence Analysis

Amplification of the entire *PHOX2B* exon 3 coding region, including the polyalanine repeat, for the sequencing reactions was carried out as described previously with primer pair 5′-ACCCTAACCGGTGCTTTTCT-3′ (forward) and 5′-ACAATAGCCTTGGGCCTACC-3′ (reverse) [Garcia-Barcelo et al., 2003; Weese-Mayer et al., 2003]. The PCR products were sequenced using the same the same sequencing condition as described previously [Garcia-Barcelo et al., 2003; Weese-Mayer et al., 2003] by using ABI's Bigdye V 1.1 kit on ABI's 3130 ×l genetic analyzer (Applied Biosystems). The data were analyzed by Sequencing Analysis 5.1.1 and Autoassembler (Applied Biosystems).

RESULTS

Targeted Mutation Analysis

Upon presentation with the CCHS phenotype, a blood sample from patient 1 was sent to Rush University Medical Center's Molecular Diagnostics Laboratory for testing using the *PHOX2B* targeted mutation analysis. Results of this testing confirmed the diagnosis of CCHS, identifying an expansion of the normal *PHOX2B* polyalanine repeat from 20 alanines to 27 alanines (genotype 20/27). Parental samples were sent and underwent the same testing, revealing the normal *PHOX2B* 20/20 genotype for each parent. Upon subsequent pregnancy by the same father and mother, amniocentesis was performed and analysis of DNA from cultured amniocytes indicated the presence of the 20/27 genotype.

In order to verify the results of the initial analysis using a different detection system, samples were transferred to Children's Memorial Hospital's Molecular Diagnostics Laboratory, and analysis was performed as described. Results confirmed the absence of the CCHS-causing 20/27 genotype in parental samples, and confirmed the presence of this genotype in the amniocentesis sample (Fig. 1). Subsequently, the family underwent elective abortion, and signed consent allowing abortus tissue to be examined. Testing of this tissue confirmed the results of the amniocentesis testing (Fig. 1).

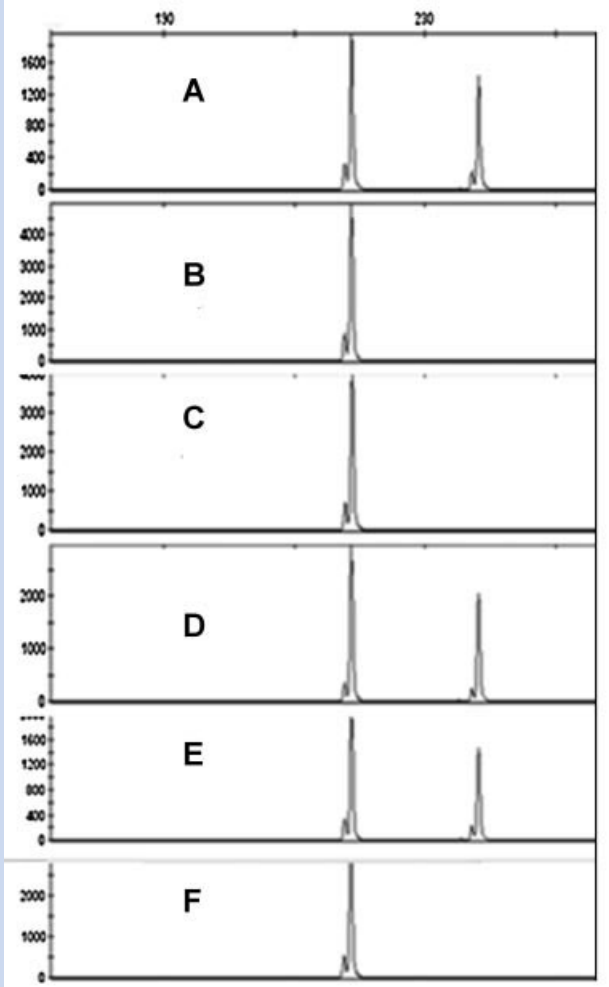

FIG. 1. Targeted mutation analysis of the *PHOX2B* gene in a family presenting with familial recurrence of the 20/27 *PHOX2B* expansion mutation. The first (leftmost) peak in samples A–F represents the 20 alanine-repeat *PHOX2B* allele (wild type allele). The second (rightmost) peak in samples A, D, and E represents the 27 alanine-repeat *PHOX2B* expansion mutation. The CCHS-causing 27 alanine-repeat *PHOX2B* expansion mutation was identified in the CCHS proband (A), and subsequently in prenatal (D) and abortus (E) testing in subsequent pregnancy by the same parents. However, the only allele detected in maternal (B) and paternal (C) somatic cells, and in paternal germline cells (F) is the wild type, 20 alanine-repeat allele, indicating maternal germline mosaicism.

STR Analysis

To verify paternity, samples from patient 1, the amniocentesis, and the father underwent STR analysis. Results confirmed paternity in both samples.

PHOX2B Sequence Analysis

In an attempt to identify the allele and therefore the parent of origin of the *PHOX2B* 27-alanine repeat expansion mutation, sequence

analysis in the region surrounding the expansion was performed. However, no informative variations were identified in this region.

Analysis of Semen Sample

Semen sample was obtained from the father and DNA was extracted as described. This sample underwent *PHOX2B* targeted mutation analysis using both methods described above. No level of the *PHOX2B* 27-alanine repeat expansion mutation could be detected in this sample (Fig. 1).

DISCUSSION

Mutations of the *PHOX2B* gene are disease-defining in CCHS. The majority of these mutations occurs de novo, and are inherited in an autosomal dominant manner. However, recent evidence indicates that a subset is inherited from parents carrying somatic mosaicism for these *PHOX2B* mutations, or heterozygous for one of the less severe mutations that demonstrates incomplete penetrance. Until now, all reported familial cases of CCHS have been inherited from a parent carrier demonstrating incomplete penetrance or somatic mosaicism. Here, we present the first molecularly proven case of familial recurrence of a CCHS-causing *PHOX2B* mutation without detection of the mutation in parental somatic cells, and discuss the implication of this finding.

In this study, a family presented with one child, and a subsequent pregnancy with the same CCHS-causing *PHOX2B* 20/27 PARM. As this 20/27 genotype *PHOX2B* mutation is extremely rare, with less than 200 cases identified and reported worldwide since the development of *PHOX2B* testing in 2003, the family and results strongly indicate inheritance of the *PHOX2B* mutation from an asymptomatic parent. Two targeted mutation assays, one with detection by PAGE and the other by capillary electrophoresis, were used in this study to test all samples. Each of these tests has been shown to have an extremely low limit of detection of *PHOX2B* expansion mutation mosaicism (<1% for fragment analysis) [Jennings et al., 2010]. However, despite this low limit of detection, these tests did not identify the 20/27 mutations in either parental somatic (using blood leukocyte) or paternal germline (semen) cells (Fig. 1). While testing for mosaicism in the maternal germline cells was not possible in this study, this appears to be the only plausible explanation for the identified recurrence in this family. Previously, two families have been reported with possible familial recurrence of a CCHS-causing *PHOX2B* mutation, without detection of the mutation in parental somatic cells [Weese-Mayer et al., 1992; Hammel et al., 2009]. However, as the first case in both reported families was deceased prior to the development of *PHOX2B* testing in CCHS, recurrence of the mutation in these siblings could not be determined. Additionally, in one of these families detection of *PHOX2B* mutation was only attempted using sequence analysis [Hammel et al., 2009]. This method of detection has been previously shown to have a worse limit of detection (~20%; therefore ~20% mutated allele concentration necessary for detection to be possible) for *PHOX2B* expansion mutations than targeted mutation analysis (<1% mutated allele concentration necessary for detection to be possible) [Jennings et al., 2010]. Thus it is possible that the expansion mutation identified in the proband is present in a parental sample, but not at a level detectable by the sequencing method used in this study. This report gives more direct support documenting the occurrence of germline mosaicism in this disease.

While identification of germline mosaicism of a CCHS-causing *PHOX2B* mutation was not possible in this study, the results presented here are the strongest reported evidence that germline mosaicism exists in CCHS. It is possible that somatic mosaicism does exist in the maternal or paternal somatic cell line and was not detectable by the methods used here. However, in this case the mosaicism would have to be less than 1% in blood lymphocytes, making the likelihood of recurrence extremely low. It was also not possible in this study to rule out mosaicism in tissues other than blood lymphocytes in the mother, and in blood lymphocytes and semen in the father. It is possible that a low level mosaicism may be detected in skin fibroblast cells, or other tissues, despite not being detected in DNA extracted from whole blood and semen.

These results are extremely important for genetic counseling of CCHS families. Individuals affected with CCHS, and thus carrying the full *PHOX2B* mutation, have a 50% chance of transmitting this mutation and the disease phenotype to each offspring. In unaffected parents carrying mosaicism for a *PHOX2B* mutation, there will be up to a 50% chance of recurrence in any offspring. Recurrence risk will depend on the percentage of mutation present in the maternal or paternal germ line. Previous reports have found somatic mosaicism from levels as low as 7% to levels as high as 50% in parents of children with CCHS [Trochet et al., 2008; Bachetti et al., 2011]. To assess recurrence risk in a family, both parents of children with CCHS should have *PHOX2B* testing completed. As methods using fragment analysis have been shown to have a lower limit of detection of *PHOX2B* mutations than those employing sequence analysis [Jennings et al., 2010], this testing should ideally be completed using fragment analysis. Prenatal testing is available and can be carried out for individuals with or without CCHS who are known germline mutation carriers or recognized as somatic or germline mosaics. Despite negative testing of parents of a CCHS child, germline mosaicism cannot be ruled out and prenatal testing for subsequent pregnancies should be considered. Prenatal testing can allow parents optimal information with which to make an informed decision with a range of possibilities from elective termination to a fully prepared delivery room to optimize the baby's chance for a smooth transition to extrauterine life. Additionally, in these families, it may be advisable to test paternal germline cells, if the family is planning additional offspring, to rule out paternal germline mosaicism. Pre-implantation genetics would be another consideration for family planning.

In summary, the results presented here support the existence of germline mosaicism for *PHOX2B* mutations in families with CCHS. This finding is important to advancing family planning and genetic counseling for families in which a *PHOX2B* mutation-confirmed CCHS case has been identified.

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