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ORIGINAL ARTICLE

De novo polyalanine expansion of *PHOX2B* in congenital central hypoventilation syndrome: unequal sister chromatid exchange during paternal gametogenesis

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Abstract The expansion of polyalanine repeats is known to cause at least nine disorders, including congenital central hypoventilation syndrome (CCHS). Unequal crossover has been speculated as the expanding mechanism, in contrast to strand slippage in polyglutamine expansion disorders. We carried out segregation analysis of *PHOX2B* in 13 de novo families with CCHS and found that 6 families were informative regarding a parental origin of polyalanine expansion, with all 6 mutants being of paternal origin. Four of them were also informative regarding a chromosomal event and their mutants were derived from unequal sister chromatid exchange. It is probable that de novo expansion of polyalanine repeats in CCHS results mainly from unequal sister chromatid exchange during spermatogenesis due to the secondary DNA structure of imperfect trinucleotide repeats encoding polyalanine tracts.

Keywords Polyalanine · *PHOX2B* · Congenital central hypoventilation syndrome · Trinucleotide repeats · Unequal sister chromatid exchange

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Introduction

Expansions of trinucleotide repeats are responsible for more than 40 neurological, neuromuscular, and malformational disorders (Pearson et al. 2005). Genetic instability is associated with the expansion of trinucleotide repeats, the mechanisms involved in which have been enthusiastically studied in polyglutamine expansion disorders. Polyglutamine tracts are encoded by perfect CAG repeats, and expanded tracts are unstable during meiosis and mitosis. The major theory explaining CAG repeat expansions involves strand slippage during replication (Sinden et al. 2002). Polyalanine expansion disorders are another family of homopolymer expansion disorders, consisting of at least nine disorders (Amiel et al. 2004; Brown and Brown 2004). Expanded polyalanine tracts are encoded mostly by imperfect GCA, GCG, GCC and GCT repeats and are meiotically and somatically stable. Warren (1997) hypothesized unequal crossover as a causative mechanism for the polyalanine expansion of *HOXD13* in synpolydactyly-1 (SPD1).

One of the polyalanine expansion disorders, congenital central hypoventilation syndrome (CCHS or Ondine's curse; [MIM 209880]), is characterized by failure of the automatic control of breathing during sleep, and is caused by the dominant *PHOX2B* mutation (Amiel et al. 2003; Sasaki et al. 2003; Weese-Mayer et al. 2003). *PHOX2B* encodes a highly conserved homeobox transcription factor of 314 amino acids with two short polyalanine tracts of 9 and 20 residues, respectively. More than 90% of patients carry de novo polyalanine expansion mutations in the polyalanine tracts of 20 residues. Small numbers of patients inherit mutations from affected parents or asymptomatic parents with somatic mosaicism (Weese-Mayer et al. 2004).

In order to determine the molecular basis underlying the de novo polyalanine expansion and the parental origin, we studied the allelic segregation of single nucleotide polymorphisms (SNPs) and deletion insertion polymorphisms (DIPs) in de novo families with CCHS.

Materials and methods

The Ethics Committee of the Yamagata University School of Medicine approved this study. Following written informed consent from the patients' families, peripheral blood was collected from the patients and family members for genomic DNA extraction. We molecularly diagnosed 13 patients (male-to-female ratio, 7:6) with neonatal onset CCHS, as previously described (Sasaski et al. 2003; Horiuchi et al. 2005). We analyzed seven SNPs and two DIPs in *PHOX2B* as follows: two SNPs (rs4608840, rs6811325) in intron 1, one SNP (rs2196822) and two DIPs (rs3038692, rs10614480) in intron 2, and four SNPs (rs17884724, rs6826373, rs11723860, rs10663611) in exon 3 (Fig. 1). In family D, we also analyzed two SNPs (rs16853575, rs16853571) and one DIP (rs3834808) in the 5′-region of *PHOX2B*. They were all within the same haplotype block. For the analysis of SNPs and DIPs, we amplified the PHOX2B genome using three primer sets: 23F: 5′-CCCTGAGGTGCCCACTGAAC-3′ and 11R: 5′-CGAATCCGGGATGGAGGTGA-3′, 11F: 5′-GCGGTAGACTTGGTGGATGTGC-3′ and 20R: 5′-CCTGCCTTGGCTGCCACTTA-3′, 20F: 5′-AGAACCGACAGCGGCTCACA-3′ and 20R. We studied all SNPs and DIPs by sequence determination using amplified products from genomic DNA and after subcloning of PCR-amplified products into the TA cloning vector. The primers employed for sequence determination were designed based on the genomic database (accession number NC_000004.10). Paternity was confirmed using 16 markers provided by the AmpFLSTR identifier kit (PE Applied Biosystems, Foster City, CA).

Results

We analyzed *PHOX2B* in 13 patients, and found that 3 patients carried 5, 3 patients carried 6, 6 patients carried 7, and 1 patient carried 11 polyalanine expansion mutations (data not shown). Paternity testing indicated that each mutation in all families occurred as a de novo event. To investigate the molecular basis resulting in de novo polyalanine expansion and the parental origin, we determined seven or nine SNPs and two or three DIPs in *PHOX2B* (Fig. 1). As shown in Fig. 2, 6 among 13 families were informative regarding a parental origin, with all six mutants being of paternal origin.

Regarding the type of chromosomal event, de novo polyalanine expansion in each patient of the six families could be explained by unequal crossover or unequal sister chromatid exchange during spermatogenesis. Fathers of families A, B, C, and D were heterozygous for their haplotypes and informative regarding a chromosomal event occurring during spermatogenesis. In family A, the patient (II.1) inherited the 5 polyalanine contracted allele from her mother (I.2) and the 7 polyalanine expanded allele from her father (I.1) (Fig. 2). Haplotypes of upstream and downstream regions of the expanded tract in the patient (II.1) were derived from an identical allele from her father (I.1), indicating that the polyalanine expansion was derived from intrachromosomal rearrangement. This intrachromosomal rearrangement was also observed in each patient of families B, C, and D. During mitosis in the early stages of gametogenesis before meiosis, or during the prophase of meiosis II, intrachromosomal rearrangement is probably caused by unequal sister chromatid exchange due to misalignment of the polyalanine tracts.

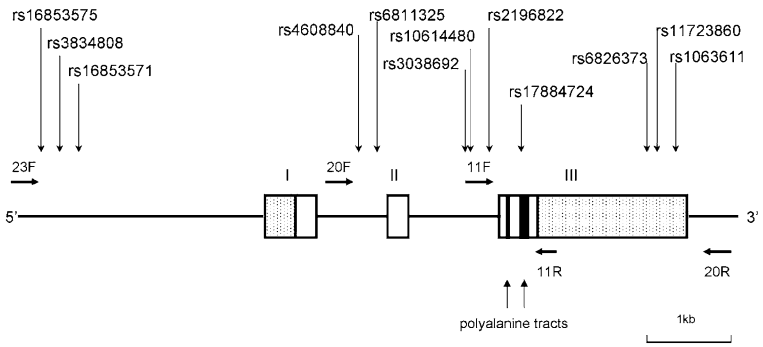

Fig. 1 Structure of the PHOX2B gene, and the sites of single nucleotide polymorphisms (SNPs), deletion insertion polymorphisms (DIPs) and PCR primers. Exons are shown as *boxes* with Roman numerals, and non-coding regions of exons are shown as *hatched boxes*. *Two black boxes* in exon 3 indicate the polyalanine tracts of 9 or 20 residues. The *horizontal arrows* indicate the sites and directions of PCR primers

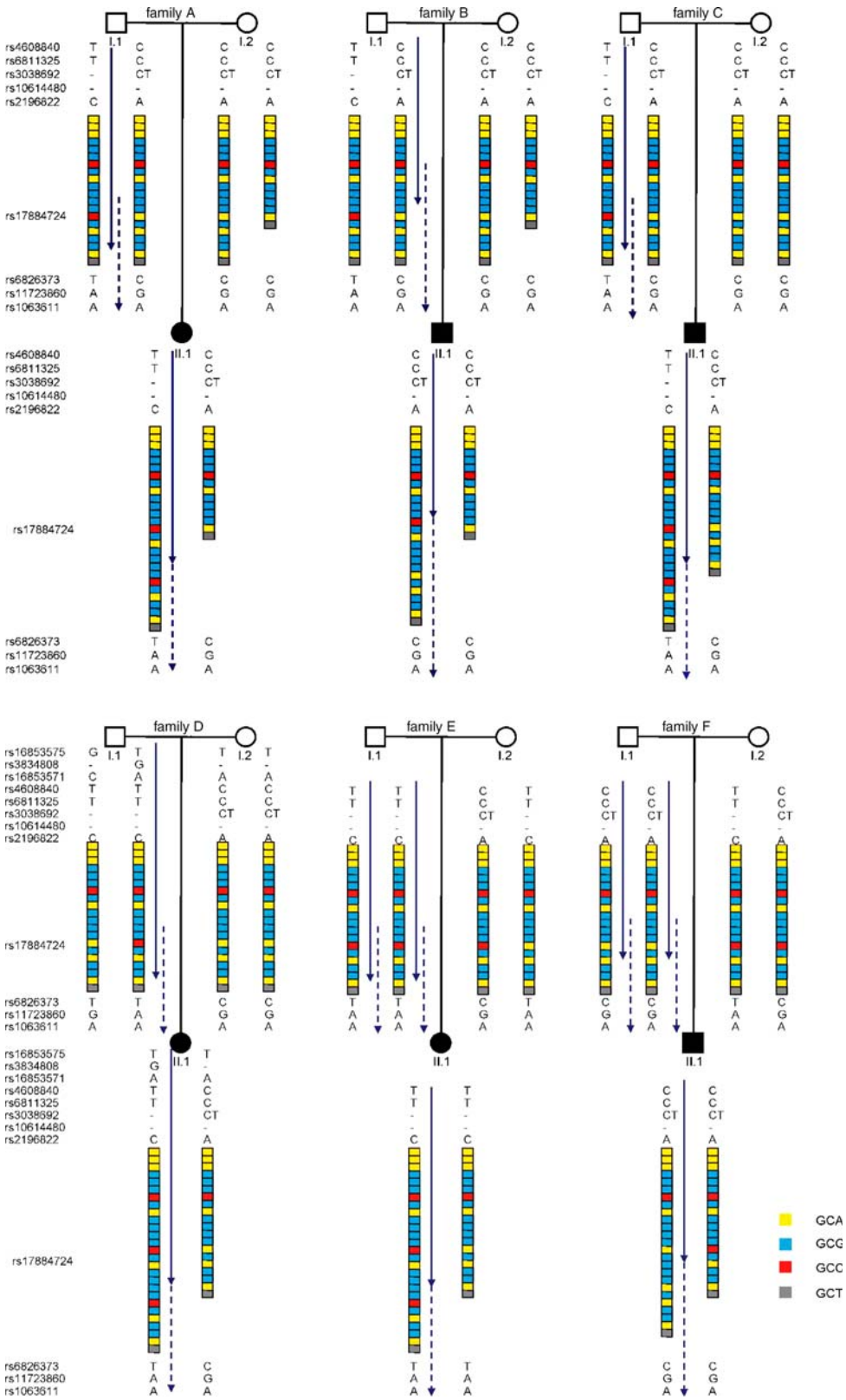

Fig. 2 Postulated mechanism of de novo polyalanine expansion. Six informative families carrying de novo polyalanine expansions are shown. Affected individuals are represented by *solid symbols*. Haplotypes and polyalanine tracts are shown beneath each subject. For each polyalanine tract, the accompanying diagram indicates the codons using a color code. The patients in families A, C, D, and E carried 7, the patient in family B carried 6, and the patient in family F carried 5 polyalanine expansion mutations. The patients in families A and B also carried 5 polyalanine polymorphic contractions inherited from their mothers. The *arrows* indicate breakage and rejoining regions of each chromatid. Haplotype reconstructions of SNPs and DIPs in *PHOX2B* reveal that all six polyalanine expansion mutations were of paternal origin, and the four mutations in the patients of families A, B, C, and D were derived from intrachromosomal rearrangements

Discussion

Polyalanine expansion disorders are a family of homopolymer expansion disorders consisting of at least nine disorders (Amiel et al. 2004; Brown and Brown 2004). Warren (1997) speculated that unequal crossover was a causative mechanism of the polyalanine expansion of *HOXD13* in synpolydactyly. CCHS is one of the polyalanine expansion disorders and most patients carry a de novo 5–13 polyalanine expansion mutation in *PHOX2B*. Our segregation analysis of *PHOX2B* in six informative families indicated that the de novo polyalanine expansion mutation was of paternal origin. Predominant mutation in paternally derived genes was reported in the point mutation of Duchenne muscular dystrophy and achondrodysplasia (Hu et al. 1991; Crow 2000). Paternal expansion bias has also been confirmed in most polyglutamine expansion disorders (Yoon et al. 2003). A major contributory factor to sex differences in mutation rates is likely to be frequent mitotic division during paternal gametogenesis. In addition, other spermatogenic-specific processes, such as stage-specific alterations in the activity of various repair systems, may be associated with this paternally predominant mutation.

Regarding the type of chromosomal event, our study showed that de novo expansion of polyalanine repeats in CCHS probably results from unequal sister chromatid exchange during spermatogenesis. Unequal sister chromatid exchange is also expected to produce contractions, and four in-frame polyalanine contraction polymorphisms, with 5, 6, 7, and 13 polyalanine contractions (Amiel et al. 2003; Sasaki et al. 2003; Weese-Mayer et al. 2003, 2004; Toyota et al. 2004; Horiuchi et al. 2005), have been reported in healthy control subjects, as seen in the mothers of families A and B. It is worth noting that somatic mosaicism for polyalanine expansion in *PHOX2B* (Weese-Mayer et al. 2003), *ZIC2* (Brown et al. 2001), and *ARX* (Nawara et al. 2006) was observed in several unaffected parents of the patients. This suggests that unequal sister chromatid exchange at the time of mitotic division of the zygote is not so frequent, but is also one of the mechanisms underlying polyalanine expansion. In addition, unequal sister chromatid exchange cannot explain a few mutants in *HOXD13*, *FOXL2* and *PHOX2B* (Brown and Brown 2004; Trochet et al. 2007). In spite of those exceptional mutants, unequal sister chromatid exchange during spermatogenesis is probably the major common mechanism of polyalanine expansion.

Trinucleotide repeat sequences can form slipped-stranded, quadruplex, triplex, and sticky DNAs, and intra-strand hairpins. The formation of these secondary structures is considered to compromise DNA replication, attenuate replication-fork progression, cause transient dissociation and reassociation of the replication-folk, and facilitate misalignment between the template and synthesized DNA strand, resulting in a recombination event (Wells et al. 2005). In contrast to strand slippage in trinucleotide repeats encoding polyglutamine tracts in polyglutamine expansion disorders, trinucleotide repeats encoding polyalanine tracts in CCHS probably results from unequal sister chromatid exchange during spermatogenesis. The difference may be associated with the difference between perfect and imperfect trinucleotide repeats encoding polyglutamine and polyalanine tracts, respectively, in each expansion disorder. Genetic instability constitutes the background of many genetic disorders. The analysis of genetic mechanisms in analogous disorders would help us to understand common mechanisms of genetic rearrangements.

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