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MUTATION IN BRIEF

Parental Origin and Somatic Mosaicism of PHOX2B Mutations in Congenital Central Hypoventilation Syndrome

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Heterozygous polyalanine repeat expansions of PHOX2B have been associated with Congenital Central Hypoventilation Syndrome, a rare neurocristopathy characterized by absence of adequate control of respiration during sleep. Here we report a *PHOX2B* mutational screening in 63 CCHS patients, 58 of whom presenting with poly-A expansions or frameshift, missense and nonsense mutations. To assess a somatic or germline occurrence of poly-A length variations, the relative amounts of mutant and wild type alleles have been quantified in 20 selected CCHS patients presenting with an expansion, and in their parents. Somatic mosaicism was shown in four parents, while no mosaic was found among CCHS patients. Moreover, while co-segregation analysis of the PHOX2B poly-A expansions with selected marker alleles in the same 20 CCHS trios has not demonstrated any parent-of-origin effect of the mutations, it has provided further clues to clarify the molecular mechanism underlying the expansion occurrence. Finally, the segregation of PHOX2B poly-A anomalous tracts within family members has allowed us to exclude tendency of polymorphic variations towards expansion. This strengthens the notion that expanded polyalanine tracts, identified as frequent disease-causing mutations also in other human diseases, are mitotically and meiotically stable. Published 2007 Wiley-Liss, Inc.[†]

KEY WORDS: Congenital Central Hypoventilation Syndrome; polyalanine tract expansions; somatic mosaicism; PHOX2B

INTRODUCTION

Congenital central hypoventilation syndrome (CCHS or Ondine's curse; MIM# 209880), is a rare neurocristopathy characterized by absence of adequate control of respiration with decreased sensitivity to hypoxia and hypercapnia. In particular, children with CCHS show an adequate ventilation while awake but hypoventilate during sleep (Weese-Mayer et al., 1999). CCHS is often associated with other autonomic nervous system

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dysfunctions such as Hirschsprung disease (HSCR) or tumours of neural crest origin (ganglioneuroma, neuroblastoma and ganglioneuroblastoma) (Weese-Mayer et al., 1999; Vanderlaan et al., 2004).

Heterozygous in frame duplications leading to polyalanine (poly-A) expansions of PHOX2B (MIM# 603851) are the most frequent disease-causing mutations in CCHS, while frameshift, missense and nonsense mutations have been detected in a small subset of CCHS patients (Amiel et al., 2003; Sasaki et al., 2003; Weese-Mayer et al., 2003; Matera et al., 2004). A correlation between the length of poly-A expanded stretches and the CCHS phenotype severity has been reported (Weese-Mayer et al., 2003; Matera et al., 2004), with the shortest poly-A expanded tract (+5 alanine residues) found in patients manifesting central hypoventilation in childhood (late onset, LO-CHS) (Matera et al., 2004; Trang et al., 2004). Although most expansion mutations occur *de novo* in CCHS patients, in about 10% of the cases mutations are inherited by an unaffected parent. In addition to incomplete penetrance of PHOX2B mutations, parental somatic mosaicism has also been observed among family members of CCHS probands (Weese-Mayer et al., 2003; Trochet et al., 2005).

Poly-A tracts have been found expanded in other human genes in association with at least nine different congenital disorders, including mental retardation and malformations of the brain, digits and midline structures (Brown and Brown, 2004; Albrecht and Mundlos, 2005). In this light, poly-A tracts expansions are members of a broader category of trinucleotide repeat-associated disorders that includes also polyglutamine (poly-Q) tracts expansions. Differently from poly-Q tracts, poly-A stretches are generally stable, usually coded by imperfect trinucleotide repeats and, with the exception of rare contractions, not present as polymorphic tracts in the human population. These observations have suggested an unequal allelic homologous recombination during meiosis and/or mitosis as the most likely mechanism underlying formation of poly-A tract expansions (Amiel et al., 2004; Brown and Brown, 2004).

Here we report results of a mutational screening of the coding region of the PHOX2B gene carried out in a total of 63 CCHS patients and in most of their parents. Moreover, to understand the molecular mechanism by which these mutations arose, we have investigated occurrence and parental origin of germline poly-A expansions, as well as the possible somatic mosaicism in patients and carrier parents. The hypothesis that polymorphic variations of poly-A tracts are not prone to expansions during transmission has finally been investigated.

MATERIALS AND METHODS

Patients

Sixty-three children affected with CCHS have been collected for several years from different countries, namely Italy (37), Germany (16), The Netherlands (7), Spain (2) and Slovenia (1). Inclusion criteria were based on the accepted definition established through the American Thoracic Society Statement on CCHS (Weese-Mayer et al., 1999) and confirmed in each case by the referring clinician. Blood samples were obtained with informed consent and genomic DNA was extracted from peripheral blood lymphocytes according to standard procedures. A lymphoblastoid cell line was used as DNA source for CCHS patient 9. Other biological samples like buccal cells, skin fibroblasts or hair follicles of CCHS patients and their parents were not available for the PHOX2B analysis.

PHOX2B mutation screening

The molecular analysis of exon 3 of the PHOX2B gene (GenBank AF117979.1) was carried out by using the protocol already described by Matera et al. (2004). When no mutation was detected in the PHOX2B exon 3, we screened exons 1 and 2 by PCR amplification followed by direct DNA sequencing, as already reported (Matera et al., 2004). Mutations numbering is based on the cDNA sequence (GenBank AF117979.1) with position +1 corresponding to the A residue of the ATG translation initiation codon, which is codon 1.

Analysis of somatic mosaicism

A specific PCR amplification of the PHOX2B polyalanine repeat was set up, in a total volume of 25 µl with primers 22F and 279R, as reported elsewhere (Matera et al., 2004) and 35 cycles at 95°C (45 sec), 60°C (45 sec) and 72°C (45 sec) were run, with a final extension of 20 min. Primer 22F was 5'end-labeled with FAM. One µl of each 1:40 diluted fluorescently-labelled PCR products was mixed to 8.7 µl formamide and 0.3 µl of size marker (ROX 500, Applied Biosystems) and loaded on ABI 3100 DNA automated Sequencer. Data were then analysed by a suitable computer software (GeneMapper, Applied Biosystems). While the position of the peaks indicates the size of the amplified alleles, the area under the peaks is proportional to the amount of fluorescence and therefore represents a reliable estimate of the quantity of the amplified product.

Genomic DNAs of CCHS patients carrying inherited +15bp(+5Ala) or +18bp(+6Ala) were used as control reference samples while testing germ-line or somatic occurrence in patients harbouring *de novo* polyA expansions. Analysis was performed on three independent PCRs from each control, patient and parent sample, and reproducibility of replicates was assessed through the coefficient of variation (CV%). The peaks area ratio between the expanded allele (EA), or the contracted allele (CA), and the normal allele (NA), calculated for each single sample, was correlated to the poly-A tract length. The peaks area ratio did not change when using DNA extracted from lymphoblasts compared to values observed when using lymphocytes (data not shown).

SNPs analysis and identification of informative CCHS trios

From an initial set of 37 trios, 20 proven to be informative with the patient heterozygous for a single nucleotide polymorphism (SNP), close to the PHOX2B polyalanine expansion, whose allele transmission from each parent could be univocally established. SNPs genotyped and conditions used to selectively amplify each allele, were:

(1) rs28647582 (Garcia-Barcelo et al., 2003). Amplification refractory mutation system (ARMS) was performed in a total volume of 25 µl containing 100 ng of genomic DNA, 1 µM of primer ARMS(F) 5'-CTGCCGTATGACCTTGGAGTCC-3' with either ARMS(WT) 5'-ATCGGCCATGGGGCCCTAGGTCCTTAT-3' (for allele A) or ARMS(MUT) 5'-ATCGGCCATGGGGCCCTAGGTCCTTAC-3' (for allele G), 1.5 mM $MgCl_2$, 200 µM of each dNTP, 10% Glycerol and 1.25 U of AmpliTaq Gold (Applied Biosystems). Reactions were run for 35 cycles at 95°C (45 sec), 64°C (45 sec), 72°C (45 sec).

(2) rs17885864 (Matera et al., 2004). Due to its location in exon 3, very close to the poly-A PHOX2B tract, genotypes at this marker (c.870C>A) were deduced after sequencing patient's DNA samples for the presence of PHOX2B mutations.

(3) rs6826373 (Toyota et al., 2004). Amplification was carried out with primers Ph3'UTR(F) 5'-GGCTGCAGAGAAATCTACCG-3' and Ph3'UTR(R) 5'-AACCGTGGCCTCTCTAAACA-3' at the annealing temperature of 60°C and followed by RsaI digestion of the PCR products.

Determination of the parental origin of the *de novo* polyalanine expansion PHOX2B mutations

The parental origin of the PHOX2B mutation in CCHS patients informative for SNP rs17885864 was assessed by amplification followed by DNA sequencing. The parental origin of the chromosomes bearing PHOX2B polyalanine expansion mutations in all other cases was determined by PCR cloning of patients' DNA and successive detection, by either ARMS, restriction enzyme digestion or direct sequencing of the SNP allele in association with the mutation. In each case, the PCR product spanning the polyalanine repeat and the informative SNP was subcloned into the pcR2.1 vector (TOPO TA Cloning Kit, Invitrogen) following manufacture's instructions. PCR products were obtained by using GC-RICH PCR System (Roche) with different conditions for the informative markers: SNP rs28647582 was amplified with primers PH2F and 279R, reported by Matera et al. (2004), at the annealing temperature of 60°C and using an extension time of 10 min; SNP rs6826373, was amplified with primers 22F and Ph3'UTR(R) at the annealing temperature of 64°C and using an extension time of 10 min. Although, only two combinations of SNP alleles with the wild type/mutant alleles should have been expected in each patient DNA, other allelic combinations were present in most cases, owing to PCR-mediated recombination (Cronn et al., 2002).

Statistical analysis

In order to identify outlier cases, regression analysis and Mahalanobis distances estimation were carried out with the 'R' statistical package (http://www.R-project.org) and the JMP software (version 5.01), respectively. Sign Test was performed to assign, with >95% confidence, the correct phase to the colonies analysed (http://www.fon.hum.uva.nl/Service/Statistics/Sign_Test.html).

RESULTS

PHOX2B mutations in CCHS patients

We have analysed 63 children affected with CCHS, either isolated or in association with several autonomic nervous system dysfunctions, along with their parents, when available. Thirty-six patients had already been reported, 27 of whom accompanied by detailed clinical descriptions (Matera et al., 2004; Bachetti et al., 2005). In one family, transmission of the disease from an affected mother to her child was observed; and two families showed recurrence in siblings. In addition, our series includes five cases presenting with LO-CHS.

The distribution of PHOX2B mutations in our series of CCHS patients is summarized in Figure 1A. In particular, a PHOX2B heterozygous mutation was identified in 58 of the 63 CCHS patients (92%). In 51 of these 58 CCHS cases (88%), an in frame duplication of 15 to 39 nucleotides within the 20 alanines tract of the protein, leading to an expansion of +5 to +13 alanines, could be demonstrated. The vast majority of these mutations is represented by expansions of 5, 6 and 7 alanines. Frameshift mutations, leading to different aberrant C-terminal of the protein, have been detected in 5 CCHS patients. Four of these mutations have been described elsewhere (Bachetti et al., 2005) while the c.807_825dup mutation, found in a CCHS patient presenting also neuroblastoma, is reported here for the first time. Furthermore, another CCHS patient, whose detailed clinical data were not available, carried a nonsense substitution (c.18T>G) replacing the Tyr6 with a stop codon (p.Y6X), likely leading to haploinsufficiency of the PHOX2B protein. Finally, an expansion of +5 alanines of the PHOX2B gene was found in each of four patients presenting with LO-CHS, while a further LO-CHS case, diagnosed also with HSCR, resulted to carry a missense mutation in the homeodomain (c.419C>A leading to p.A140E).

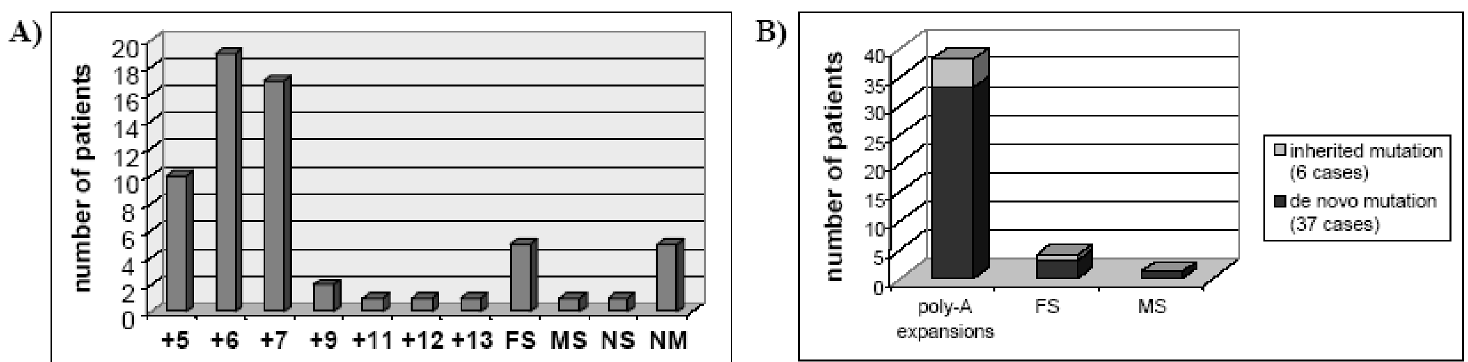

Figure 1. PHOX2B mutations detected so far in a series of 63 CCHS patients. **A:** Frequency of the different mutational types: the length of alanine expansions is described by the plus sign (+) followed by the number of extra residues; FS = five frameshift mutations (c.618delC, p.G207AfsX102; c.866dupG, p.P290SfsX70; c.721_758del, p.A241GfsX106; c.930dupG, p.S311EfsX49 and c.807_825dup, p. G276WfsX90); MS = one missense mutation (c.419C>A leading to p.A140E); NS = one nonsense mutation (c.18T>G leading to p.Y6X); NM = no mutation in the coding region of the PHOX2B gene. GenBank for PHOX2B: AF117979.1. Mutation numbering starts from the A residue of the ATG translation initiation codon (+1 position). **B:** Occurrence of PHOX2B mutations in those 43 CCHS patients whose parents were available for the PHOX2B analysis. FS = frameshift mutations; MS = missense mutations.

Poly-A PHOX2B length variations are transmitted unchanged

To determine occurrence of the PHOX2B mutations, we have collected data on a total of 86 individuals, belonging to 43 couples of CCHS patients' parents. While 80 parents were homozygous for the wild type allele, confirming that the vast majority of PHOX2B mutations arose *de novo*, six of them resulted heterozygous for the PHOX2B mutation already detected in their affected children (Fig. 1B). Among these latters, we could identify i) one unaffected mother carrying the c.618delC frameshift mutation, already reported by Matera et al., 2004, ii) four unaffected parents transmitting poly-A expansions, namely two +15bp(+5Ala) mutations in individuals 4M and 11F, already reported by Matera et al., 2004, and two +18bp(+6Ala) mutations in novel 59M and 60F cases and, finally, iii) one +15bp(+5Ala) transmitting mother (78M), affected with mild LO-CHS (Table 1). As the expansion size remained unchanged during transmission from affected or unaffected parents to affected children in our CCHS families, we have assumed that poly-A PHOX2B expansions are meiotically stable (Table 1).

In addition, four parents were heterozygous for in-frame polymorphic length variations of the 20-alanine tract: three of them showed polyalanine contractions, one of seven Ala residues (-21bp(-7Ala), case 60M) and two others of five Ala residues (-15bp(-5Ala), cases 28M and 48M), while one unaffected mother (9M), of a patient with a six-alanine expansion, presented a two-alanines expansion (+6bp(+2Ala)), as already reported in the literature in a healthy individual (Toyota et al., 2004). In this case, the occurrence of a further expansion of the maternal two-alanines allele into the six-alanines allele of the patient has been excluded by cloning a PCR amplification product obtained from the PHOX2B genomic sequence of patient 9. This DNA fragment included both the +18bp(+6Ala) mutation and the G allele of the informative marker rs28647582:A>G, this latter inherited from the father and not from the +6bp(+2Ala) carrying mother, who had instead transmitted the A allele.

PHOX2B contracted poly-A stretches do not seem prone to further length decrease during transmission, as exemplified by CCHS patient 60 who inherited unchanged both the contraction variation -21bp(-7Ala) from his mother and the expansion mutation +18bp(+6Ala) from his father (not shown).

Table 1: Polyalanine Length Variations in CCHS Trios and Stability of the Polyalanine Tract Expansions During Transmission of the Mutation From Carrying Parents

CCHS patients	Patient's genotype	Mother's genotype			Father's genotype		
			T	U		T	U
4	wt/+5Ala	4M	+5Ala	wt	4F	wt	wt
9	wt/+6Ala	9M	wt	+2Ala	9F	wt	wt
11	wt/+5Ala	11M	wt	wt	11F	+5Ala	wt
28	wt/+6Ala	28M	wt	-5Ala	28F	wt	wt
48	wt/+7Ala	48M	wt	-5Ala	48F	wt	wt
59	wt/+6Ala	59M	+6Ala	wt	59F	wt	wt
60	-7Ala/+6Ala	60M	-7Ala	wt	60F	+6Ala	wt
78	wt/+5Ala	78M*	+5Ala	wt	78F	wt	wt

"wt" stands for the wild type allele. - or + signs denote the variant alleles characterized by the given number of deleted (-) or inserted (+) nucleotides in the 20Ala-coding segment. T (Transmitted allele); U (Untransmitted allele). * this +5Ala carrying mother has turned to be affected with LO-CHS.

Somatic mosaicism in affected and unaffected PHOX2B mutation carriers

We have approached the possible occurrence of somatic mosaicism for PHOX2B mutations by quantitatively estimating the ratio between mutant and normal alleles, in 20 informative CCHS patients, five transmitting parents and three mothers with a poly-A tract contraction. The reproducibility of the three replicates was satisfactory, with the coefficient of variation (CV%) lower than 15%, ranging from 1.3% to 11.3% in 28 out of the 31 patients. Though the PCR amplification protocol used has been set up to optimize the efficiency in raising products from expanded and normal alleles, the amplified alleles, having a higher GC content, have confirmed a lower chance to be amplified than the normal alleles, as already reported (Matera et al., 2004). Therefore, as shown in Figure 2A, a significant change in the ratio of expanded/contracted to normal allele peaks area could be observed in the presence of length variations in the polyalanine tracts analysed. The correlation on the whole data was $r=-0.5586$ ($p=0.0011$). However, since a few remarkable values seemed very distant from the dot distribution (Figure 2A), we used a step by step procedure to identify outlier cases. In particular, we recalculated the linear regression, each time by progressively discarding data not included in the predicted interval at 95% level of confidence, until no variation in the slope could be detected. This procedure allowed us to define five cases (indicated in Figure 2B by a blue cross) as outliers and the correlation became highly significant ($r=-0.969$, $p=1.838*10^{-15}$) when outlier values were not included in the analysis. The final equation of the regression line was: ratio of allele peak area (Y)=1.0134 -0.0392 * poly-A length variation (X) ($P<0.0001$, both intercept and coefficient). The same results were obtained discarding outliers by preliminary Mahalanobis distances estimation followed by regression analysis (data not shown).

Based on values perfectly fitting the area of the curve describing the strict inverse correlation between the peak area ratios and the length of PHOX2B polyalanine tract, the hypothesis of somatic mosaicism has been excluded for the corresponding individuals. Consequently, this has allowed us to assume a germline origin for expansion mutations in all the CCHS patients and some of the parents tested. On the contrary, somatic mosaicism was likely in two parents with expanded (+6Ala) alleles (59M and 60F) and two parents with contracted (-5Ala) alleles (28M and 48M) (outlier cases, see blue crosses below the curve in Figure 2B).

The CCHS patient's father 11F, showing a +5Ala expansion and classified as an outlier case (see blue crosses above the curve in Figure 2B), has surprisingly shown a ratio between expanded and normal allele peaks area much higher than those estimated for other +5Ala samples. Based on a SnaPshot ddNTP primer extension analysis performed to evaluate the presence of extra copies of the expanded allele, we have concluded that the apparently distorted (high) ratio between expanded and normal allele peak area observed for individual 11F was likely due to a PCR artefact (data not shown).

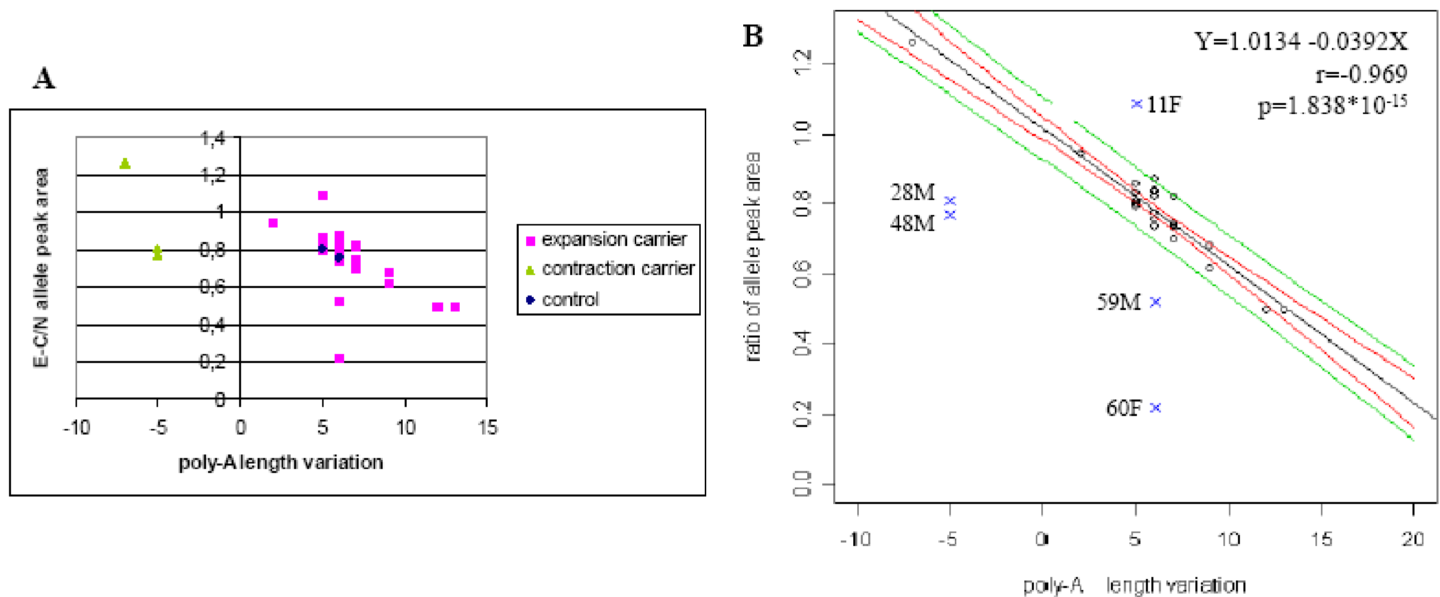

Figure 2. Correlation between length of the PHOX2B polyalanine tract and ratio of expanded/contracted to normal allele peaks area. **A:** The size of expansions and contractions is reported on the X-axis by plus (+) and minus (-) signs respectively, followed by the number of alanines involved. Allele peaks area ratio is represented on the Y-axis. **B:** A stepwise-like regression procedure (see text) has allowed us to define five cases as outliers (blue crosses) and to obtain a highly significant correlation. Confidence interval at 95% level for the mean value (red) and 95% prediction interval for individual observations (green) are reported.

Parental origin of germline PHOX2B expansions

The genetic analysis of CCHS families has also allowed to establish the parental origin of germline PHOX2B expansions. In particular, we genotyped all the patients and their parents for three SNPs, already described in earlier reports within the PHOX2B gene (Garcia-Barcelo et al., 2003; Matera et al., 2004; Toyota et al., 2004), and selected the informative trios as those in which the patient and one parent resulted heterozygotes for at least one of the three SNPs. The phase of the informative SNPs was determined, with respect to the PHOX2B expansion mutation, by means of different approaches. For patients heterozygous at the rs17885864 (c.870C>A) SNP locus, the allele lying coupled to the poly-A expanded allele was established by amplification and direct DNA sequencing of exon 3. For the other polymorphisms, rs28647582:A>G and rs6826373:G>A, long PCR products were cloned and haplotypes individually genotyped. Because of the possibility of reaction artefacts due to PCR-mediated recombination between the PHOX2B expansion locus and SNPs loci, a remarkably high number of colonies had to be analysed before the correct phase was deduced with a confidence level of $P<0.05$ (Sign Test (Li et al., 2005)). Results for the 20 informative cases have suggested no preferential parental origin, *de novo* poly-A expansions having occurred on the maternal allele in 7 patients and on the paternal allele in 13 patients (Table 2).

Table 2: Parental Origin of the Chromosome Where the Reported *De novo* Expansion Mutations Arose

CCHS Patient	PHOX2B expansion	informative SNP	Parental origin of the expanded allele
2	+ 6Ala	rs28647582:A>G	paternal
6	+ 7Ala	rs28647582:A>G	paternal
7	+ 6Ala	rs28647582:A>G	paternal
9	+ 6Ala	rs28647582:A>G	paternal
14	+ 13Ala	rs6826373:G>A	maternal
16	+ 5Ala	rs6826373:G>A	maternal
18	+ 6Ala	rs17885864:C>A	paternal
23	+ 5Ala	rs17885864:C>A	paternal
28	+ 6Ala	rs28647582:A>G	paternal
30	+ 9Ala	rs17885864:C>A	paternal
37	+ 5Ala	rs28647582:A>G	paternal
39	+ 6Ala	rs17885864:C>A	paternal
48	+ 7Ala	rs28647582:A>G	maternal
50	+ 12Ala	rs6826373:G>A	maternal
52	+ 7Ala	rs28647582:A>G	maternal
55	+ 9Ala	rs28647582:A>G	maternal
58	+ 5Ala	rs28647582:A>G	paternal
62	+ 6Ala	rs28647582:A>G	paternal
63	+ 7Ala	rs28647582:A>G	maternal
69	+ 7Ala	rs28647582:A>G	paternal

DISCUSSION

Congenital central hypoventilation syndrome (CCHS or Ondine's curse) is a rare neurocristopathy characterized by inadequate or absent ventilation during sleep (Weese-Mayer et al., 1999). Heterozygous in frame duplications leading to polyalanine (poly-A) expansions of PHOX2B are the most frequent disease-causing mutations in CCHS, while frameshift, missense and nonsense mutations have been detected in a small subset of CCHS patients (Amiel et al., 2003; Sasaki et al., 2003; Weese-Mayer et al., 2003; Matera et al., 2004). Polyalanine regions have been predicted in roughly 500 human proteins, preferentially found in transcription factors and regarded as flexible spacer elements essential to conformation, protein-protein interactions and/or DNA binding (Amiel et al., 2004). So far, nine of them have been associated with developmental disease phenotypes caused by repeat expansions (Brown and Brown, 2004). Despite the many recent achievements, questions concerning the occurrence and the molecular mechanism(s) underlying poly-A expansions in CCHS still need to be addressed.

The parental origin and somatic mosaicism of PHOX2B mutations have been investigated in 63 patients recruited so far on the basis of CCHS symptoms (Matera et al., 2004). We have first identified heterozygous mutations of the coding region of the PHOX2B gene in 58 patients (92%), 36 of whom previously reported (Matera et al., 2004; Bachetti et al., 2005), with expansions of the 20 alanines tract of exon 3 representing the 88% of CCHS associated mutations, and frameshift, missense and nonsense mutations detected in the remaining 12%. The frameshift mutation c.807_825dup (G276WfsX90), reported here for the first time, changes the 314 amino acids wild type translational frame (frame 1) leading to a 364 amino acids elongated protein (frame 2). Moreover, similarly to other mutations shifting to translational reading frame 2 (i.e. c.866dupG and c.930dupG), a novel seven alanine stretch is created in the C-terminal proteic fragment. Finally, identification of a nonsense mutation in the exon 1 of the gene, probably leading to lack of any PHOX2B product, suggests that haploinsufficiency may cause also the CCHS phenotype and not only Hirschsprung disease, as previously reported (Benailly et al., 2003).

In agreement with this, *in vivo* experiments have shown that mice heterozygous for a targeted phox2b deletion presented sleep-disordered breathing, which partially modelled the key feature of CCHS (Durand et al., 2004).

"De novo" mutations can occur either in any of the parental (maternal or paternal) germ-lines or at any early post-zygotic embryonic stage in the affected individual, thus leading, in this latter case, to mosaicism in the patient. Most PHOX2B defects detected in our patient series were shown to have arisen *de novo* and, since presence of different cell clones for poly-A expansions could be excluded in peripheral lymphocytes, a germline origin of these mutations was assumed in the CCHS patients tested. On the other hand, in five cases (two +5Ala, two +6Ala and the c.618delC frameshift mutations) the mutation was inherited, unchanged, from an unaffected parent. In one case, a +5Ala repeat expansion was transmitted from the affected mother, thus confirming the meiotic stability of these expansions. Moreover, based on the many PCR-colonies we have sequenced, we can also assume that expansions are mitotically stable.

Somatic mosaicism for the +6Ala repeat expansion has been postulated in the present study in two asymptomatic parents, thus confirming data already reported for carriers of expansions larger than 5 alanines (Weese-Mayer et al., 2003; Trochet et al., 2005). On the other hand, none of our asymptomatic carriers of +5Ala is resulted to be a mosaic, implying that in these cases lack of the disease phenotype can be ascribed to reduced penetrance of the mutation. Taken together these data allow to hypothesize that germline expansions larger than 5 alanines are fully penetrant and, consistently, asymptomatic carriers may only be found in association with significant degrees of somatic mosaicism. Therefore, the recurrence risk of healthy individuals carrying a +6Ala repeat can be as high as 50%, depending on the unquantifiable extent of their germline mosaicism, a circumstance which has to be taken into account in genetic counseling to families.

Differently from polymerase slippage, occurring during replication and believed to act in poly-Q expansions, unequal allelic homologous recombination has been regarded for long as the most attractive disease-causing mechanism for poly-A tract expansions (Amiel et al., 2004; Brown and Brown, 2004). However, in mosaic individuals Trochet et al. (2007) have very recently reported only two alleles (wild type and expanded alleles), instead of the three alleles (wild type, contracted and expanded alleles) expected after occurrence of a somatic event of unequal crossing-over (Fig. 3A). Similarly, all the cases we defined as somatic mosaicism did not present any contracted allele, as shown in one individual in Figure 3B, thus suggesting that an alternative mutational mechanism should be considered to explain the origin of these trinucleotide repeats. Observations already reported have raised the question of the mutational model acting in different poly-A tracts containing proteins. In particular, DNA hairpin–induced polymerase slippage has been proposed as the most probable mechanism underlying in-frame changes observed in the poly-A tract of the FOXL2 gene (De Baere et al., 2003). In addition, Chen et al. (2005) have strongly supported the replication slippage as a more plausible mechanism than unequal cross-over for poly-A tract expansions by reasoning that degenerated trinucleotides, typical of poly-A tracts, would reduce the ability of the repeats to form misaligned structures thus strengthening their stability. On the other hand, since repeat instability may involve the formation of unusual DNA structures during DNA replication, repair and recombination, we cannot exclude that trans-acting DNA metabolic proteins, and not only gene-specific cis-elements, do contribute to trinucleotide tracts expansions (Pearson et al., 2005).

Finally, since a paternal origin of the gametes transmitting expansions has preferentially been observed in most poly-Q disorders (Pearson et al., 2005), we have determined the parental origin of poly-A *de novo* expansion mutations. To this end, we have first attempted to exclude the presence of mutations of post-zygotic origin, which are expected to show no preference for arising on the paternal or maternal allele. A germline origin has been shown for all the mutations in our CCHS patients, though very early somatic mutations could not be completely ruled out. Of the 20 trios genotyped in the present study using polymorphic markers suitable to assess the parental source of the poly-A expansion, 13 mutations resulted to have occurred in the paternal chromosome while 7 turned to be originated in the mother. Since no parental expansion bias could be demonstrated in this set of informative CCHS patients, we can conclude that, differently from what reported for other trinucleotide expansion disorders, occurrence of poly-A repeats expansions seems to be independent of processes specific to sperm or oocyte development.

In conclusion, the analysis of somatic mosaicism reported here in asymptomatic parents with a poly-A length variation has provided both useful clues for genetic counseling and interesting indications on the mutational mechanism leading to polyalanine repeat expansions and contractions. Moreover, we have excluded a preferential parental origin for the germline poly-A expansion mutations. All together, our data represent a first step to define how poly-A expansions occur in human and induce CCHS development.

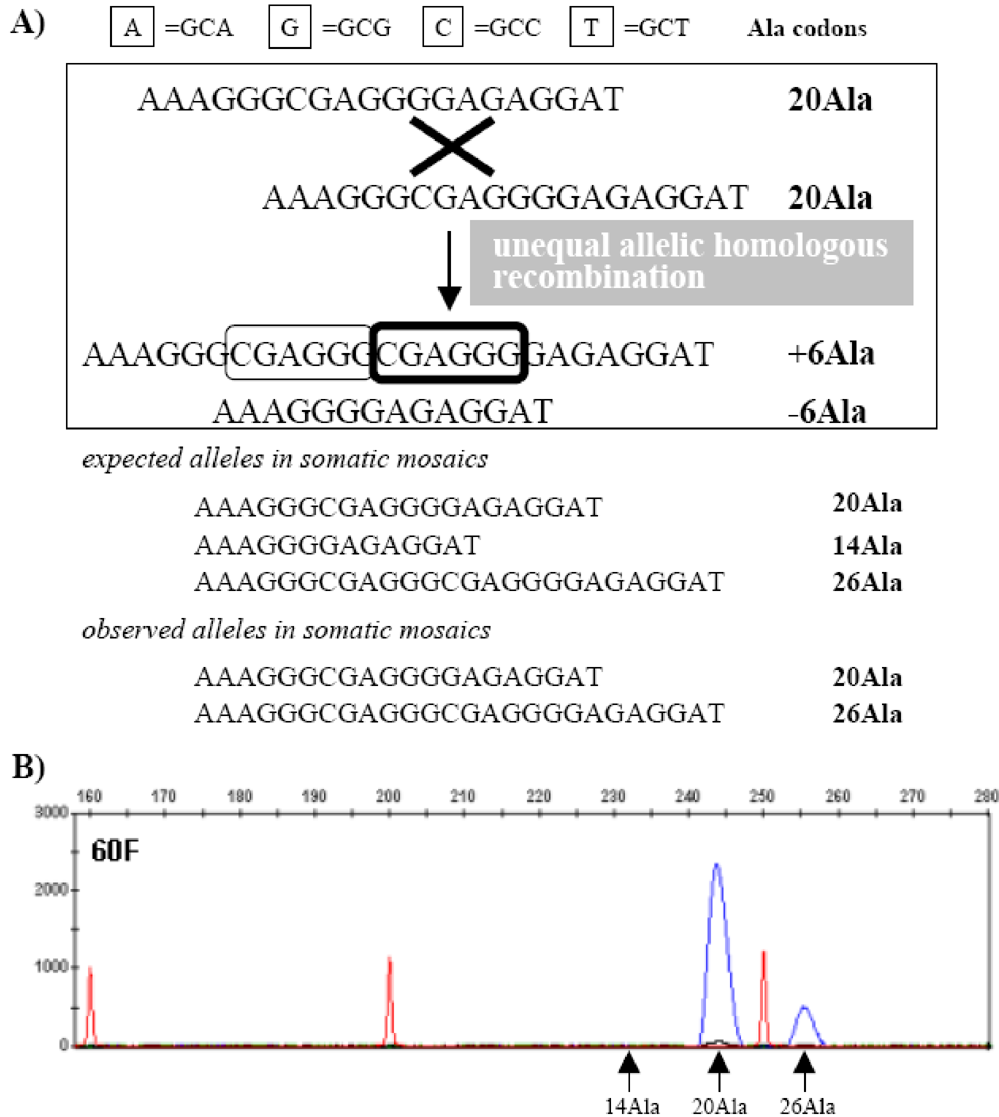

Figure 3. Schematic representation of the PHOX2B alleles **(A)** expected after occurrence of a somatic event of unequal allelic homologous recombination and **(B)** observed in the unaffected father of patient 60. Despite the clear condition of somatic mosaicism for the expanded +18bp(+6 Ala) allele in 60F, no sign of the presence of contracted allele has been found in the genomic DNA extracted from his peripheral blood lymphocytes. Failure to detect the expanded and contracted alleles together, among alleles observed in mosaic individuals, supports a different mechanism generating the poly-A length variations.

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