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ORIGINAL ARTICLE

Obstructive sleep apnea syndrome (OSAS) in children with Class III malocclusion: involvement of the *PHOX2B* gene

Anna Maria Lavezzi · Valentina Casale · Roberta Oneda · Silvia Gioventù · Luigi Matturri · Giampietro Farronato

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Abstract
Purpose The aim of this study is to provide new molecular approaches to the children with obstructive sleep apnea syndrome by evaluating the possible involvement of the *PHOX2B* gene, notoriously associated to congenital central hypoventilation syndrome (CCHS), in Class III malocclusion.
Methods Fifty subjects with Class III malocclusion, aged from 8 to 14 years, and with history of sleep apneic episodes, and 20 age-matched controls were submitted to genomic DNA examination from oral cells to specifically analyze the PHOX2B genotype.
Results Point "silent" mutations affecting different nucleotides of the *PHOX2B* gene were observed in 32 % of patients with Class III malocclusion and never in controls (0 %).
Conclusion The genetic data obtained in this study in children with Class III malocclusion and sleep-related breathing disorders provide new information useful to the genetic characterization of this pathology. The *PHOX2B* gene silent mutations can lead to structural and functional modification of their product providing to a group of children with Class III malocclusion similar features to those of CCHS (sleep apnea episodes and craniofacial malformations).

Keywords Class III malocclusion · *PHOX2B* gene · CCHS · Children · OSAS

A. M. Lavezzi (✉) · V. Casale · R. Oneda · S. Gioventù · L. Matturri · G. Farronato
Department of Biomedical, Surgical and Dental Sciences, "Lino Rossi" Research Center for the Study and Prevention of Unexpected Perinatal Death and SIDS, University of Milan, Via della Commenda, 19-20122 Milan, Italy
e-mail: anna.lavezzi@unimi.it

Introduction

Obstructive sleep apnea syndrome (OSAS), characterized by recurrent episodes of upper airway closure disrupting normal ventilation during sleep, is a common problem in children, with an estimated incidence of 1 % to 3 % [1–5]. Frequently, its occurrence is the result of various craniofacial anatomic defects (such as mandibular deficiency, bimaxillary retrusion, increased mandibular plane angle and inferior displacement of the hyoid bone) that cause an abnormal dental occlusion and sleep respiratory disorders [6–10].

A typical dysmorphic facies with broad, flat, rectangular appearance, reminiscent of the craniofacial morphology in OSAS, associated to alveolar hypoventilation and altered response to hypoxemia and/or hypercarbia, has been observed among children with congenital central hypoventilation syndrome (CCHS) [11, 12].

Children with CCHS are heterozygous for a polyalanine expansion mutation in the second polyalanine repeat residue (exon 3) of *PHOX2B*, a homeobox gene expressed during the development of the neural crest in the dorsal rhombencephalon, the specific region that gives rise to the facial structures [13, 14]. In the CCHS genotype, the affected allele has 25–33 repeats of the polyalanine sequence, with the normal allele having 20 repeats [11, 15–17].

On the basis of a possible likeness between OSAS and CCHS in young people, the goal of this study was to focus, in particular, on Class III malocclusion, a dysmorphic craniofacial phenotype characterized by mandibular prognathism and maxillary deficiency associated to sleep-disordered breathing that, despite occurring least frequently in comparison with Class I and Class II (relative to mandibular deficiency) [18], is recognized with a significant genetic component [19–22].

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The studies performed up to now on molecular biology have proposed different candidate genes, with both polygenic and monogenic inheritance, that could contribute to the Class III phenotype [19].

Our aim was to provide new approaches to uncovering the genetic etiology of this specific craniofacial pattern by evaluating the possible involvement of the *PHOX2B* gene, the main factor associated to CCHS. Thus, we examined the genomic DNA in a wide cohort of children with skeletal Class III malocclusion and sleep-disordered breathing, in order to specifically analyze the *PHOX2B* genotype and highlight alterations of its expression.

Methods

Patients

The study sample consisted in 50 subjects with a clinical diagnosis of Class III malocclusion, 25 males and 25 females, aged 8 to 14 years (mean age, 10.5±2.1 years). All subjects had a history of disturbed sleep characterized by recurrent apneic periods with habitual snoring. The patients' parents were asked to complete a questionnaire specific for children, adapted and translated for the Italian speaking population from the "Brouillette questionnaire" [23], for information about both daily and nightly OSAS symptoms.

Orthodontic treatments

The patients were treated with two band type rapid palatal expanders (placed on the first permanent maxillary molars) and with a Delaire facemask [24].

Response to therapy

In all patients, the treatments promoted a satisfactory correction of the Class III malocclusion with increase of the transversal diameter of the palate, achievement of a more balanced profile and the additional benefit of increasing the size of the upper airway structure with marked reduction in their snoring and respiratory dysfunctions during sleep.

Controls

Twenty children with normal dental occlusion and absence of sleep–apneic episodes were included in this study as controls. They were matched for age, sex and obesity with the patients' group.

Table 1 summarizes the case profiles in this study, indicating the sex distribution, range of ages, body mass index and pathologic features. Figure 1 represents a typical face of a girl with Class III malocclusion.

Ethics and consents

Written informed consent was obtained from all the parents of the study subjects. Ethics approval was given from the institutional ethics committee.

PHOX2B genetic analysis

In all cases (patients and controls), DNA was extracted from oral cells collected by cotton swabs using QIAamp DNA Mini Kit (Qiagen, Inc., Valencia, CA).

To amplify the exon 3 coding region of *PHOX2B*, we used the PCR primer pair 5′-AACCCGGCAAGGGCGGC-3′ (forward) and 5′-CCTGGACAAGGCTGGGCTC-3′ (reverse) [16]. PCR reactions specific for GC reach templates were set up in a total volume of 50 μL containing 100 ng of genomic DNA, 400 μM dNTPs, 1 μM of each primer, 1X of GC-RICH PCR buffer and 1 M GC-RICH resolution solution (GC-RICH PCR System, Roche Molecular Biochemicals, Indianapolis, IN) and 2 U of GC-RICH PCR enzyme mix (Roche). The amplification steps were: 30cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min. The PCR products were analysed by electrophoresis on a 4 % agarose gel.

Where the genotype was normal (20 polyalanine repeats on each allele), *PHOX2B* exon 1, 2 and 3 were amplified as described by Matera et al. [16], in order to obtain three fragments respectively of 320, 260 and 627 bp, and subsequently sequenced. Briefly, standard PCR reactions were set up in a total volume of 50 μL containing 100 ng of genomic DNA, 1 μM of each primer, 1x GeneAmp Buffer II, 2 mM MgCl2, 400 μM of each dNTP and 2 U AmpliTaq Gold polymerase (Life Technologies, Carlsbad, CA, USA).

The fragments of genomic DNA were amplified, purified and subsequently sequenced by cycle sequencing. The amplicons were recovered from the gel and purified with the QIAquick gel extraction kit (QIAGEN), as recommended by the manufacturer. Sequencing reactions were performed with the Thermo Sequenase Cy5.5 Dye Terminator Cycle sequencing kit (Amersham Pharmacia Biotech) and analyzed with the SEQ 4X4 Personal Sequencing System (Amersham Pharmacia Biotech). The genomic DNA sequences obtained were compared with the sequence available in GenBank (accession number NT_022782).

Statistical analysis

The statistical significance of direct comparison between the groups of subjects was determined using analysis of variance.

Table 1 Case profiles of the study

Subjects	Age (years); range, mean value ± SD	Sex		Body mass index[a] (mean value ± SD)	Class III malocclusion (no. of cases)	Obstructive sleep apnea (no. of cases)
		Male	Female			
Patients (n=50)	8–14;10.5±2.1	25	25	18.8±1.5	50	50
Controls (n=20)	8–15;11.1±2.0	10	10	20.0±5.1	0	0

SD standard deviation. [a] Body mass index is a measure of an individual's weight-for-height for estimating body fat. It is defined as the weight in kilograms divided by the square of the height in metres (kilogrammes per square metre)

Statistical calculations were carried out with SPSS statistical software (version 11.0; SPSS, Inc., Chicago, IL, USA). The selected threshold level for statistical significance was $p<0.05$.

Results

Genomic DNA from all patients and controls was analysed: no polyalanine repeat expansion mutations were identified either in the 50 OSAS cases or in the 20 control cases. All individuals were homozygous for the *PHOX2B* 20/20 genotype, indicating the normal number of 20 alanines on both alleles. A mutation screening of the three exons spanning the entire coding region of *PHOX2B* including intron–exon boundaries has been performed by direct DNA sequencing analysis. No changes from the expected DNA sequence were detected in the 20 control cases. In the DNA of 16 of the 50 OSAS patients (32 %), we found four different silent point mutations,[1] one in the exon 1 and three in the exon 3. Precisely, a heterozygous C>T transition has been detected in three OSAS patients. This mutation changed the codon 78 from TAC to TAT, but does not change the amino acids sequence of the protein as both codons codify for tyrosine (Tyr78). Another C>T transition has been detected in two OSAS patients that changed the codon 184 from AGC to AGT but does not determine protein changing (Ser184). In three further OSAS cases, the transversion C>G at codon 213 (from GGC to GGG) that causes no amino acid change (Gly213) has been detected. Finally, in eight OSAS patients, a A>C transversion has been identified: codon 254 changes from GCA to GCC but the protein sequence remains unchanged (Ala254). Table 1 shows all the results obtained from the PHOX2B analysis.

[1] Silent mutation occurs when the change of a single DNA nucleotide within a protein-coding portion of a gene does not affect the amino acid sequence of a protein. That is possible because most amino acids are codified by more than one triplet of nucleotide bases.

Discussion

It has been reported that infants are particularly vulnerable to obstructive sleep-disordered breathing, including the obstructive sleep apnea syndrome (OSAS), given their upper airway structure and ventilatory control instability [4, 5]. This breathing dysfunction in children can also adversely impact on the autonomic control of the cardiovascular system, leading to increased blood pressure during sleep [25, 26]. An additional exacerbating factor predisposing children toward OSAS is the presence of a Class III malocclusion, a specific craniofacial phenotype clinically heterogeneous being associated with many combinations of skeletal and dental morphological variants, strongly influenced by genetic factors [18].

To date, many investigations have focused on understanding the genetic etiology of Class III malocclusion and on determining how the genetic features might influence the severity of disease and also the response of patients to orthodontic treatment [19–22].

In particular, many population and family pedigree studies have demonstrated a polygenic mode of inheritance as the primary cause of Class III malocclusion [20, 21, 27]. In addition, linkage analyses have suggested the involvement of several regions on chromosome 1 and 12 (precisely the loci 1p22, 1p36, 12q13, 12q23) that might harbor candidate genes relevant to craniofacial development [28, 29].

Also, single mutated genes following the Mendelian pattern of inheritance have been proposed in Class III malocclusion. Xue et al. [19] in particular supported the *EPB41* as a candidate gene that might be involved in susceptibility to mandibular prognathism. An additional candidate of particular biologic interest is the *IGF1* gene, identified in the 12q23 region, known to play an important role in skeletal growth and development in both mice and humans [30, 31]. Significant is the observation of *IGF1* receptors in the fibrous articular surface of the temporomandibular joint condyle [32].

Our genetic and molecular data on *PHOX2B* gene, the main factor involved in CCHS in children with Class III malocclusion and sleep-related breathing disorders, provide new information useful to the genetic characterization of this pathology.

In the present work, that represents the first report in literature concerning the direct genomic DNA analysis in

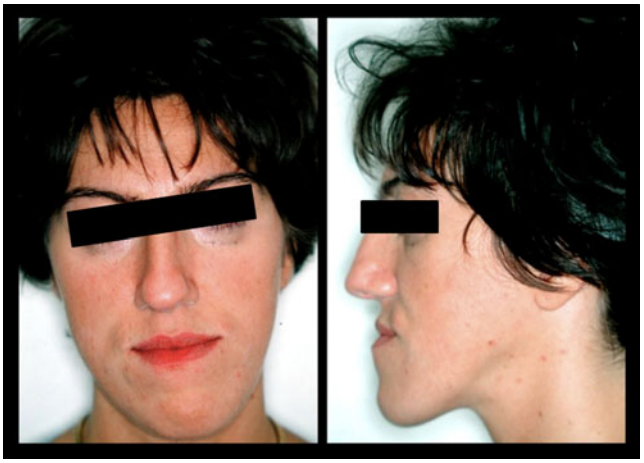

Fig. 1 Frontal and profile view of a case of Class III malocclusion (14-year-old girl)

patients with Class III malocclusion, we observed a significantly high incidence of "silent" point mutations affecting different nucleotides (one in exon 1 and three in exon 3) of the *PHOX2B* gene. (Table 2)

Although silent mutations have largely been assumed to be inconsequential exerting no discernible effect on gene function or phenotype, the identification in our study of DNA sequence variants in the *PHOX2B* gene conferring no change in the encoded amino acid in a wide subset of patients (32 %) and never in controls (0 %), leads us to search for an etiologic meaning.

Our idea is supported by recent literature. A number of studies over the last years have in fact questioned this assumption, asserting that silent mutations can be implicated in diseases [33–36].

Kimchi-Sarfaty et al. [33] observed that individuals carrying silent single nucleotide polymorphisms (SNPs) in the *MDR1* gene encoding P-glycoprotein, sometimes revealed altered P-glycoprotein pharmacokinetics. Even if seemingly there is no rational explanation for why silent SNPs might have such effects, especially when no change in P-glycoprotein mRNA and protein expression levels has been observed, they demonstrated that these genetic variants in MDR1 can alter the P-glycoprotein conformation and consequently the protein activity. This study is of immense importance as it demonstrates for the first time that naturally occurring silent mutations can lead to the synthesis of a protein product with the same amino acid sequence but different structural and functional properties.

In addition, Tomita-Mitchell et al. [34] in patients with congenital heart diseases identified ten silent sequence variants of the GATA4 gene, which were not seen in the control population, and provided evidence that these mutations alter the translational kinetics of mRNA, affecting protein folding and consequently its normal activity. The changes of the tridimensional conformation of the proteins could also have a significant effect to therapeutic targets and explain the different responses of individual patients to a certain treatments.

We believe that the silent mutations of the *PHOX2B* gene observed in this study might confer to children with Class III malocclusion a noteworthy susceptibility to OSAS.

The involvement of *PHOX2B* gene in craniofacial phenotypic dysmorphology associated with breathing dysfunctions has been reported by Todd et al. in children and young adults with congenital central hypoventilation syndrome (CCHS) [11]. Precisely they demonstrated altered anthropometric measures, including mandible–face width index, in subjects with severe alveolar dysfunctions caused by a polyalanine expansion mutation in *PHOX2B*. The *PHOX2B* genetic variations here reported prevalently concern the same DNA codons encoding the alanine amino acid, thus showing that sleep-related breathing alterations associated to Class III malocclusion, and CCHS can represent different levels of the same wide pathology modulated by the *PHOX2B* gene.

The *PHOX2B* gene provides instructions for making a protein that acts early in human development, especially active in the neural crest, a group of cells in the early embryo that gives rise to many tissues in the face and skull [13, 14]. Furthermore, several neural crest cells migrate to form parts of the autonomic nervous system, which controls many functions and, above all, breathing [37]. Consequently, *PHOX2B* mutations can cause craniomaxillomandibular alterations and result in disordered breathing, as seen in CCHS and in the Class III phenotype presented in this study.

Table 2 Distribution of mutations of the *PHOX2B* gene in OSAS children with Class III malocclusion compared to controls

PHOX2B silent mutations			OSAS patients (n=50)	Controls (n=20)	P value
Exon	DNA codons	Encoded amino acid			
1	Transition TAC→TAT	Tyrosine (Tyr78)	3	–	
3	Transition AGC→AGT	Serine (Ser184)	2	–	
3	Transversion GGC→GGG	Glycine (Gly213)	3	–	
3	Transversion GCA→GCC	Alanine (Ala254)	8	–	
Total number of mutations			16 (32 %)	0 (0 %)	0.04

Limitations of the study

Despite these important even if preliminary findings, we have identified three key limitations to our study. First, there were a relatively low number of subjects available for the analysis. Second, our subjects were selected as orthodontic cases but we have not considered otolaryngology-related factors, such as adenotonsillar hypertrophy or nose problems that could affect the results. Third, we have included in the control group only children with normal dental occlusion and absence of sleep–apneic episodes. Nevertheless, it would be useful also to consider, in this group, a set of children with only sleep disorders and a normal facial pattern, and vice versa a set of children with dental malocclusion without OSAS, to clear whether the *PHOX2B* gene primarily associates with obstructive sleep apnea or malocclusion.

Conclusions

On the basis of our results, we propose a possible genetic etiology of a subgroup of children with OSAS, all affected by Class III malocclusion. Consequently, we provide new data also to the current knowledge on the gene variants predisposing this specific craniofacial defect, underlining that its recognized clinical complexity and heterogeneity, given the many possible combinations of skeletal and dental morphological variants, is supported by a comparable genetic heterogeneity.

It is therefore advisable to submit each subject with Class III malocclusion to a simple removal of cells from the oral cavity to analyze the *PHOX2B* genotype and then highlight possible congenital associations with obstructive apneas.

Future directions of the research

This paper will undoubtedly stimulate new research in this area and a multicenter effort with recruitment of several hundreds of children, fundamentally directed to define the possible genetic etiology of sleep–apneic conditions, above all of OSAS, a significant problem for children. A further larger study could be addressed to evaluate the possible involvement of the *PHOX2B* gene even in sleep-related breathing disorders in adults.

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