RESEARCH ARTICLE

Application of high-resolution array platform for genome-wide copy number variation analysis in patients with nonsyndromic cleft lip and palate

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Background: Although more than 14 loci may be involved in the development of nonsyndromic cleft lip and palate (NSCLP), the etiology has not been fully elucidated due to genetic and environmental risk factor interactions. Despite advances in identifying genes associated with the NSCLP development using traditional genetic mapping strategies of candidate genes, genome-wide studies, and epidemiologic and linkage analysis, microarray techniques have become important complementary tools in the search for potential causative oral clefts genes in genetic studies. Microarray hybridization enables scanning of the whole genome and detecting copy number variants (CNVs). Although common benign CNVs are often smaller, with sizes smaller than 20 kb, here we reveal small exonic CNVs based on the importance of the encompassed genes in cleft lip and palate phenotype.

Methods: Microarray hybridization analysis was performed in 15 individuals with NSCLP.

Results: We identified 11 exonic CNVs affecting at least one exon of the candidate genes. Thirteen candidate genes (*COL11A1*-1p21; *IRF6*-1q32.3; *MSX1*-4p16.2; *TERT*-5p15.33; *MIR4457*-5p15.33; *CLPTM1L*-5p15.33; *ESR1*-6q25.1; *GLI3*-7p13; *FGFR*-8p11.23; *TBX1*-22q11.21; *OFD*-Xp22; *PHF8*-Xp11.22; and *FLNA*-Xq28) overlapped with the CNVs identified.

Conclusions: Considering the importance to NSCLP, the microdeletions that encompass *MSX1*, microduplications over *TERT*, *MIR4457*, *CLPTM1L*, and microduplication of *PHF8* have been identified as small CNVs related to sequence variants associated with oral clefts susceptibility. Our findings represent a preliminary study on the clinical significance of small CNVs and their relationship with genes implicated in NSCLP.

KEYWORDS

child, cleft lip and palate, comparative genomic hybridization, DNA Copy Number Variations

1 | INTRODUCTION

Cleft lip and/or palate (CLP) are a category of craniofacial malformations characterized by the presence of spaces or gaps leading to an abnormal upper lip, alveolus, and/or palate, which can result in effects on speech, hearing, appearance, and cognition as well as have adverse long-term outcomes in health and social integration.^{1,2} The nonsyndromic form of CLP (NSCLP) consists of isolated, nonspecific malformations with no other apparent cognitive or structural abnormalities.^{3,4} NSCLP shows a multifactorial etiology with both genetic and environmental contributions and moderate recurrence rates.⁴

Although more than 14 loci may be involved in the development of NSCLP, the precise etiology has not been fully elucidated.⁵ Several chromosomal regions, such as 1q, 2p, 4q, 6p, 14q, 17q, and 19q, were suggested to contain genes associated with genetic susceptibility to NSCLP and some syndromic cases. Almost every year, additional loci are identified and added to the susceptibility list, including more recently 8q21.3, 13q31.1, and 15q22 and 15q13.^{6,7} Potentially implicated genes include growth factors (*TGFA*, *TGFB3*), transcription factors (*MSX1*, *TBX22*, *IRF6*), genes involved in the metabolism of xenobiotics (*CYP1A1*, *GSTM1*, *NAT2*), genes related to nutritional metabolism (*MTHFR*, *RARA*), and genes involved in the immune response (*PVLR1*).^{4,8,9}

The association between candidate genes and environmental factors and their influence on the development of CLP have been widely studied by our group. Specially, the muscle segment homeobox gene 1 (*MSX1*) and genes encoding enzymes involved in folate metabolism, such as methionine synthase (*MTR*), methylenetetrahydrofolate reductase (*MTHFR*), and reduced folate carrier 1 (*RFC1*), have been investigated.^{8,10} Bezerra et al showed a positive association between reduced folic acid levels, alcohol consumption, and the *MTHFR* 677T and 1298C alleles in NSCLP development.¹⁰ Cardoso et al found no evidence that the *MSX1* polymorphisms rs3775261, rs1042484, rs12532, rs6446693, rs4464513, and rs1907998 play a major role in NSCLP.⁸

Despite advances in identifying genes associated with the NSCLP development using traditional genetic mapping strategies of candidate genes, genomewide studies, animal models, and epidemiologic and linkage analysis,⁸⁻¹³ microarray techniques have become important complementary tools in the search for potential causative CLP genes in genetic studies. These techniques allow investigators to scan the whole genome at once and detect submicroscopic chromosomal imbalances called copy number variants (CNVs).^{5,14,15} These genomic imbalances include insertions, duplications, deletions, inversions, recurring mobile elements, and other rearrangements, now usually defined as abnormalities covering 50 or more base pairs.¹⁶ CNV detection is a strategy to identify and confirm

candidate disease gene regions, which represents a first-tier diagnostic approach for some diseases.^{17,18}

In oral clefts studies, microarray-based comparative genomic hybridization (array-CGH) has shown some CNV-region deletions in NSCLP cases at 6q25.1-25.2 and at 10q26.11-26.13, which are associated with the causative genes estrogen receptor 1 (*ESR1*) and fibroblast growth factor receptor 2 (*FGFR2*).⁵ Also, a deletion in the 1q32.2-q32.3 region, including the entire *IRF6* and 24 other flanking genes, has been linked to cleft palate.¹⁹ More recently, a systematic analysis of CNVs of a large cohort of orofacial cleft patients identified known causative genes for CLP *SATB2* and *MEIS2* and 12 other genes (*DGCR6*, *FGF2*, *FRZB*, *LETM1*, *MAPK3*, *SPRY1*, *THBS1*, *TSHZ1*, *TTC28*, *TULP4*, *WHSC1*, *WHSC2*).¹⁸ This study also reported 34 deleted and 24 duplicated genes associated with novel candidates genes involved in signaling pathways in orofacial development.²⁰ Additionally, according to this study, although the genes *SATB2* and *MEIS2* have been classified as CLP causative genes, they appear to be frequently affected by small deletions in healthy individuals. Therefore, the notion that CNVs are also abundantly present in healthy populations challenges the interpretation of the clinical significance of detected CNVs in patients with cleft and/or lip palate.

For most commonly used arrays, the limit of resolution of ~400 kb throughout the genome provides a reliable ability to identify of pathogenic CNVs, with the median size for presumably benign CNVs being ~200 kb.¹⁸ However, more recent data with higher-density arrays suggest that small (1-30 kb) CNVs could contribute to the risk of certain disorders such as neurodevelopmental disorders (NDDs) and autism spectrum disorders (ASDs).^{21,22}

Thus, considering the increasing use of microarray analysis as a genetic test for some diseases and the challenges in clinical interpretation regarding size distribution of CNVs in the human genome, this study aimed to screen the exonic regions of genes identified through overlapping CNVs in patients with NSCLP from the Brazilian northeast.

2 | MATERIALS AND METHODS

2.1 | Study population

Fifteen patients with NSCLP admitted to the University Hospital Onofre Lopes, in Natal, Rio Grande do Norte, Brazil, from 2012 to 2015 comprised our study population. The patients were evaluated by the Oral Cleft Multidisciplinary Program of the University Hospital, which comprised a group of pediatricians, radiologists, phonoaudiologists, cardiologists, and geneticists. The study was approved by the Research Ethics Committee of University Hospital Onofre Lopes (#714/2008), and informed consent was obtained from all of the adult subjects or the parents or legal guardians of

underage patients. This study is part of Brazil's CranioFacial project, a voluntary, interinstitutional, and multiprofessional research group (<http://www.fcm.unicamp.br/fcm/cranio-face-brasil/projeto-cranio-face-brasil>). Data were collected using a pre-tested form through the CranFlow-Brazilian database on Craniofacial Anomalies/Orofacial Clefts (<https://www.craniofacebrasil.fcm.unicamp.br/cranioface/jsp/login.do>).

2.2 | Genomic DNA extraction

The genomic DNA was isolated from whole blood in tubes containing EDTA using the QIAamp DNA Blood Mini Kit (Qiagen, Chatsworth, CA, USA), following the manufacturer's instructions. The obtained DNA was stored at -20°C until analysis.

2.3 | CytoScan HD array

To allow the application of DNA samples in a GeneChip HD CytoScan Array (Affymetrix, Santa Clara, USA), the following processing steps were performed: DNA samples were digested with *NSPI* restriction enzymes, ligated to adapters and to universal primers that recognize the adapter sequences attached to genomic DNA, genomic DNA was amplified by polymerase chain reaction (PCR), resulting DNA fragments were purified using magnetic sensors, the concentration of purified DNA was quantified with a spectrophotometer, purified samples were fragmented with *DNase I* to generate species of 25–125 bp, and fragment size was confirmed by agarose gel electrophoresis. Then, the DNA fragments were end-labeled by the addition of a modified biotinylated base and hybridized to CytoScan Array. The arrays were sequentially washed and stained with a combination of a streptavidin-coupled dye and a biotinylated anti-streptavidin antibody and, finally, the arrays were scanned. All steps rigorously followed the manufacturer's protocol (http://tools.thermofisher.com/content/sfs/manuals/cytoscan_assay_user_manual.pdf). The CytoScan™ HD Array contains greater than 2.4 million markers for copy number and approximately 750 000 genotype-able SNPs, which provide high-resolution copy number, accurate breakpoint estimation, and loss of heterozygosity (LOH) detection.

2.4 | Copy number analysis

After array chip scanning, data files were obtained in CEL format, which were then analyzed using the Chromosome Analysis Suite (ChAS) program (Affymetrix, Santa Clara, USA), in order to examine the genomes.

According to recommendations on diagnostic chromosomal microarray testing, microduplications and microdeletions were selected as relevant genomic regions when gains and losses affected a minimum of 50 and 25 markers, respectively, with a lower limit of resolution of ~ 400 kb. In addition to these parameters, the study analyzed small CNVs (< 30 kb).

The main quality indicators for the GeneChip array HD were the median absolute difference paired (MAPD), SD ripple, and SNP-QC.

The parameters were applied to ≤ 0.25 MAPD, waving SD < 0.12 and ≥ 15.00 for SNP-QC.

All CNVs generated for each patient, as well as total CNVs, were filtrated for the encounter of exonic CNVs with the ExonReducer program. These CNVs were compared with data from reference populations available in public databases, including the Database of Genomic Variants (DGV), which contains benign of polymorphic CNVs identified in healthy controls from previous scientific reports (<http://dgv.tcag.ca/dgv/app/home>). The CNVs were also evaluated based on a list of 61 genes associated with the CLP phenotype (Table S1). This gene list was made from queries in several databases of clinically relevant variants: ECARUCA (<http://www.ecaruca.net>), CAGdb (<http://www.cagdb.org>), combinations of common variants from genome banks and clinically relevant databases: dbVAR (<http://www.ncbi.nlm.nih.gov/dbvar>), Decipher (<http://decipher.sanger.ac.uk>), ISCA CNVS (<https://www.iscaconsortium.org>), databases of genes associated with known diseases: OMIM (<http://www.omim.org>), HGMD, (<http://www.hgmd.cf.ac.uk>), Gene imprint (<http://www.geneimprint.com>), and a review of the scientific literature. The use of the list with candidate genes for performing the array analysis was in order to delimit the search field and to study exonic CNVs that affected only genes associated with phenotype and then to access whether our patients had any changes in these genes. This decision took into account the fact that in a first analysis of all data, a large number of CNVs were generated (2511), but none in particular met traditional criteria to characterize as potential CNV. Then, the small CNVs were analyzed according to the criteria of number of markers and frequency in DGV.

3 | RESULTS

All individuals selected for the study had nonsyndromic cleft lip and palate (NSCLP). Table 1 shows the clinical characteristics of the subjects studied. Most individuals were male, with 67% had unilateral cleft against 33% with bilateral cleft. Approximately 54% of the studied individuals have a family history of oral clefts and 20% of patients' mothers confirmed the use of alcohol during pregnancy. The average age of patients' mothers at conception was 27.8 years. There were reports of gestational diabetes and obesity during pregnancy of 13% and 27%, respectively. There were no reported cases of parental consanguinity.

Microarray analysis (a-GH) of 15 DNA samples from individuals with NSCLP identified 11 small exonic CNVs affecting at least one exon of the candidate genes (Table 2), with eight being microdeletions (Loss) and three being microduplications (Gain). Four microdeletions appeared in more than one patient.

The microdeletion sizes varied from 0.2 kb to 12 kb, with a mean size of 6.1 kb and an average count of approximately 8–20 markers. Considering the relevance of genes involved in orofacial susceptibility, two deletions on chromosome 1 may be presented: a 3.2 kb deletion located at 1p21.1 and a 2.3 kb deletion located at 1q32.2, which encompassed *COL11A1* and *IRF6*, respectively. The 2.3 kb

ID	Gender	Cleft type	Severity of cleft	Family history	Maternal habits
1	M	CLP	Bilateral	Positive	Negative
2	M	CLP	Unilateral	Negative	Negative
3	F	CLP	Bilateral	Positive	Negative
4	M	CLP	Bilateral	Negative	Alcohol
5	M	CLP	Bilateral	Positive	Negative
6	M	CLP	Unilateral	Negative	Negative
7	M	CLP	Unilateral	Positive	Negative
8	M	CLP	Unilateral	Positive	Alcohol
9	F	CLP	Unilateral	Positive	Negative
10	F	CLP	Unilateral	Negative	Negative
11	F	CLP	Unilateral	Negative	Negative
12	M	CLP	Unilateral	Negative	Negative
13	M	CLP	Bilateral	Positive	Smoking/ Alcohol/ Illegal drugs
14	M	CLP	Unilateral	Positive	Negative
15	M	CLP	Unilateral	Negative	Negative

TABLE 1 Clinical characteristics of 15 individuals with nonsyndromic cleft lip and/or palate

CLP, cleft lip and palate; ID, patient identification; M, male; F, female.

deletion on 1q32.2 associated with *IRF6* was described in Database of Genomic Variants (DGV) as variant dgv144e212 and was found in two different patients. The 12.3 kb deletion located at 4p16.2 overlapped with *MSX1*, a gene known to be important in oral cleft development. This microdeletion was identified as the variant esv34196 in the DGV. CNVs that affected *ESR1* and *GLI3* were microdeletions of sizes 5.2 kb and 6.7 kb located at 6q25.1 and 7p14.1, respectively. These deletions were described in the DGV although rare (esv2761004, nsv830844, nsv5717). A 9.8 kb microdeletion located at 22q11.21 was associated with *TBX1*. However, it was well described in the DGV.

The microduplication of 58.2 kb at 5p15.33 and 85.9 kb at Xp11.22 met the parameters for gene duplications, as previously defined for CNV screening. The 5p15.33 duplication covers a region associated with three genes: *TERT*, *MIR4457*, and *CLPTM1L*. This duplication was described in the DGV. The Xp11.22 duplication affects *PHF8*. A 9.0 kb duplication located at 8p11.23 in *FGFR1*, although showing a suboptimal number of markers was described in the DGV only once.

4 | DISCUSSION

The array-CGH technique is an efficient approach for genomewide screening of chromosomal copy number changes and has been shown to be an efficient method to identify submicroscopic gains/duplications and losses/deletions that can serve as informative markers for clinical phenotypes of various disorders, including orofacial clefts.^{15,23,24} Despite this great advance compared with classical cytogenetic analysis, the correct categorization of CNVs is still a challenge. The presence of benign CNVs within the genome, the

continual discovery of novel CNVs, and the insufficient knowledge of the phenotypic effects of most CNVs have led to the classification of many CNVs as being of unknown clinical significance.

Considering that the complex etiology of CLP is not fully understood, the aim of this study was the robust detection of CNVs using a genomic microarray in an attempt to identify chromosomal regions containing causative genes of NSCLP. Although previous studies have reported that common benign CNVs are often smaller in size, with the majority of benign CNVs smaller than 20 kb,^{14,25,26} here we report small exonic CNVs based on the importance of the affected genes in NSCLP, possibly related to sequence variants in the genes involved in genetic susceptibility to oral clefts.

The individual significance of genomewide small CNVs has not been well elucidated in a clinical context. Microarray studies have focused mainly on CNVs > 30 kb because of the presumed limits of reliable calling.²² However, recent studies have shown that rare 1-30 kb exonic CNVs could contribute reliably to the risk of certain disorders, such as NDDs and ASDs.^{21,22} A study in a large cohort of 714 clinically well-characterized patients with NDDs investigating rare exonic CNVs of 1-500 kb revealed that three out of seven true CNVs were 1-10 kb and only eight out of 35 100-500 kb CNVs were considered to be pathogenic, indicating that the highest fraction of pathogenic CNVs occur in the smallest size range.²¹ In an ASD case-control study, a significant increase in the burden of 1-30 kb and 1-10 kb deletions was observed in patients with ASD.²² Small size CNVs, in general, affect only one gene, which, in contrast to larger CNVs, allows easy detection of the associated gene.

In our study, thirteen candidate genes (*COL11A1*, *IRF6*, *MSX1*, *TERT*, *MIR4457*, *CLPTM1L*, *ESR1*, *GLI3*, *FGFR1*, *TBX1*, *OFD1*, *PHF8*, and *FLNA*) overlapped with the CNVs identified in cleft palate patients. Taking into account criteria such as the number of markers

TABLE 2 Exonic CNVs of a-GH analysis in nonsyndromic cleft lip and/or palate studied individuals

Type	Chr	Cytoband	Start (hg19)	End (hg19)	Size (kb)	Marker count	Overlap genes	ID	Qty of descriptions in DGV
Loss	1	p21.1	103448313	103451527	3.214	8	COL11A1	1/2/12	7
	1	q32.2	209975052	209977378	2.326	8	IRF6	1/2	1
	4	p16.2	4863775	4876104	12.329	10	MSX1	1/13	1
	6	q25.1	152127133	152132368	5.235	8	ESR1	15	2
	7	p14.1	42003076	42009807	6.731	20	GLI3	13	1
	22	q11.21	19746066	19755845	9.779	15	TBX1	1	20
	X	p22.2	13781656	13781921	0.265	8	OFD1	14	1
	X	q28	153576560	153577296	0.736	12	FLNA	1/3/69/10/11	7
	5	p15.33	1286129	1344342	58.213	80	TERT, MIR4457, CLPTM1L	13	33
	8	p11.23	38279385	38288451	9.066	28	FGFR1	1	1
Gain	X	p11.22	53973302	54059274	85.972	182	PHF8	7	4

Chr, chromosome; DGV, base genomic data variants; ID, patient identification; Qty, quantity.

and frequency in the DGV, microdeletions that encompass *MSX1*, microduplications of *TERT*, *MIR4457*, *CLPTM1L*, and the microduplication of *PHF8* resulted from analyzing small CNVs. Future microarray studies should focus on the potential for small CNVs to represent small changes in the sequences of genes important in oral cleft susceptibility.

MSX1 is a small muscle segment homeobox gene on chromosome 4p16, consisting of two exons and one intron spanning less than 4300 bp.²⁷ The *MSX1* regulatory protein functions as a transcriptional repressor and is involved in the modulation of craniofacial, limb, and nervous system development.⁸ It has been proposed that mutations in *MSX1* alone could contribute to as many as 2% of total cleft lip and palate cases.²⁸

Previous studies have supported the interaction between environment factors and *MSX1*. Maternal cigarette smoking and alcohol consumption during pregnancy seem to increase the risk of oral clefts resulting from the interaction of such exposures with specific allelic variants of *MSX1*,²⁹ although not confirmed by other studies.³⁰ In our study, a microdeletion in *MSX1* was found in two male patients with bilateral NSCLP and family history of oral clefts. The mother of one of these patients informed cigarette smoking and alcohol consumption during pregnancy. Clinical data from our study, in agreement with reports in the literature, showed a higher frequency of oral clefts in males, with familial recurrence and an association with environmental risk factors such as maternal exposure to tobacco and alcohol.^{3,8,10} These results suggest that the presence of microdeletions found in *MSX1* may act to potentiate the effects of environmental factors associated with and contributing to the multifactorial etiology of oral clefts.

A submicroscopic duplication was identified in a patient at 5p15.33 encompassing two protein-coding genes (cisplatin resistance-related protein 9 (*CLPTM1L*), telomerase reverse transcriptase (*TERT*), and one microRNA gene (*MIR4457*). The patient presented bilateral NSCLP and the patient's mother reported exposure to environmental factors most commonly associated with the etiology of CLP during pregnancy. Again, this microdeletion may have an influence on the environmental effects associated with susceptibility to CLP. The association between *CLPTM1L* and CLP was first described by Izzo et al who detected an approximately 300-kb interstitial microduplication at 5p15.33 in a 10-year-old boy with syndromic CLP and neuro-psychomotor developmental delay.³¹ These authors suggested that owing to a genetic similarity between the proteins encoded by *CLPTM1L* and a CLP-associated transmembrane protein-1 encoded by *CLPTM1* located at 19q13.3, a duplication of the entire gene is likely to lead to increased gene expression, thus triggering overexpression of apoptotic pathways, which may contribute to phenotypical abnormalities, particularly CLP.

In a male patient with unilateral NSCLP and a family history of NSCLP but no exposure to environmental factors, a gain of 85.9 kb at Xp11.22, that encompasses *PHF8*, was observed. Mutations in the human plant homeodomain (PHD) finger protein 8 (*PHF8*) cluster within its JmjC encoding exons and are linked to mental retardation and a cleft lip/palate phenotype.³² *PHF8* protein activity is

related to oxygen availability, and modifications in this gene have been associated with the occurrence of fetal cleft lip due to maternal hypoxia, which can be worsened by maternal hypoxia habits, as smoking and hypertension treatment.³² A microdeletion at Xp11.22 was described in two brothers with ASD, intellectual disability, and CLP.³³ This microduplication, therefore, seems to be an important factor in the genetic etiology of CLP.

In oral clefts studies, however, little is known about the importance of small CNVs detected by genomewide high-resolution CGH for genetic diagnosis and gene discovery. Simioni et al (2015) studied 23 unrelated individuals with CLP considered relevant two CNVs of sizes <300 kb (a duplication involving the *FGFR1* (chromosomal region 8p12) gene and a deletion involving the *TCEB3* (chromosomal region 1p36.11) gene) due important play of these genes in the pathogenesis and development of CLP.¹⁵ Although these CNVs were found in patients with abnormalities associated with CLP, mutations in these genes have been reported in the literature to be associated with nonsyndromic CLP cases.³⁴

It is important to note that there was a high frequency of family history among the studied subjects (eight patients), which reflects the strong familial aggregation peculiar to orofacial clefts. Relatives of cleft cases have a high relative risk compared to the population's baseline risk, and there is a sharp decline in risk to relatives of cases with increasingly distant relationships.³⁵ In this pattern of inheritance, also known as multifactorial threshold model, the probability of sharing identical alleles by descent is constant, be the risk controlled by a gene, some genes or many genes; however, other explanations could potentially result in these same patterns of risk for relatives.³⁶ In this way, the CNVs found in patients with positive family history of CLP may represent the heritability or the proportion of variation in risk attributable to independent, autosomal genes, whose penetrance at any one of these separate risk genes is impossible to estimate.

In nonsyndromic forms of oral clefts, in which there is no involvement of causal genes strongly associated with risk, it is hypothesized that multiple genetic alterations with modest individual effects on risk may be capable of disrupting normal craniofacial development under specific circumstances given by exposure to environmental risk factors.³⁶ Therefore, cumulative effect of microdeletions in the copy number of *MSX1* could represent a mechanism of deregulation in *MSX1* proteins, which are responsible to maintain the growth of the primary palate during mammalian palatogenesis through expression regulation of growth factors such as *Bmp4*.³⁷ In the same direction, the effect of microduplication reported in the chromosome region involving the three genes *TERT*, *MIR4457*, *CLPTM1L* may be responsible for an increase in the expression of these genes, promoting an over-expression of apoptotic pathways during the palate morphogenesis.³¹ And also, the effect of microduplication of *PHF8* may promote a disrupt catalytic of *PHF8* activity, which is histone modifying enzyme oxygen dependent, that under conditions of associated hypoxia may be related with chromatin regulated gene expression including of homeobox genes during the development and fusion of facial prominences.³²

Despite evidence that genomic analysis based on arrays has been a robust tool for CNV screening of patients who are suspected

to harbor chromosomal aberrations, this technique presents difficulties in classification and interpretation of CNVs, particularly in smaller CNVs. Our study was limited by the unavailability of parental DNA to determine the inheritance patterns of the identified CNVs. Therefore, researches with more individuals, using secondary confirmatory testing and with comprehensive bioinformatics analysis, are required to better understand exonic CNVs in the size range of 1-30 kb by array-CGH.

In conclusion, our findings represent a preliminary study on the search for the clinical significance of small CNVs and the relationship between CNVs and genes implicated in the development of nonsyndromic cleft lip and palate. The microdeletions that encompass *MSX1*, microduplications of *TERT*, *MIR4457*, *CLPTM1L*, and the microduplication of *PHF8* are small CNVs, which may be related to changes in the genes involved in genetic susceptibility to oral clefts in Brazilian patients. The combined effect of changes in the small copy number of various genes could represent one of the genetic mechanisms for nonsyndromic forms of oral clefts. However, further studies are encouraged to establish the significance of small CNVs in oral cleft risk.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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