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Genetic screening for IRF6 and GRHL3 in Brazilians with non-syndromic cleft lip/palate

Triagem genética de IRF6 e GRHL3 em brasileiros com fissura lábio/palato não sindrômica

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ABCTRACT
Cleft lip with or without cleft palate (CL/P) is the most frequent craniofacial anomaly. Advances in molecular and quantitative analysis suggests that the etiology is multifactorial of nonsyndromic CL/P (NSCL/P), and provide new opportunities to identify genes and gene-environment interactions relevant to the etiology of this common and representative birth defect. The present study aimed at detecting genetic variants in IRF6 and GRHL3 genes and susceptibility to NSCL/P in West Central and Northern Brazilian populations. We analyzed a set of 80 individuals with NSCL/P from Associação de Combate as Deformidades Faciais, recruited from Midwest and Northern Brazil. We performed Multiplex Ligation-dependent Probe Amplification (P304-B1-IRF6/GRHL3 (Lot B1-0116)) and PCR analysis for confirmation. In the MPLA study exon 4 of GRHL3, show possible alteration. Therefore, we performed a PCR validation of these alterations. The results showed no alteration on these genes (IRF6 and GRHL3) corroborating with previous studies. To our knowledge, this study of both genes is the first in these specific areas of Brazil, analyzing individuals with NSCL/P. Studies have identified a missense variant in the gene grainyhead-like-3 (GRHL3) in cleft palate individuals. The contribution of these genetic variants to NSCL/P susceptibility should be further investigated in different populations and cohorts. Thus, the underlying genetic causes of NSCL/P remain largely unknown.

Keywords: cleft lip, cleft palate, missense mutation, orofacial clefts, polymorphism.

RESUMO
A fissura labial com ou sem fenda palatina (CL / P) é a anomalia craniofacial mais frequente. Os avanços na análise molecular e quantitativa sugerem que a etiologia é multifatorial da CL / P não sindrômica (NSCL / P) e oferece novas oportunidades para identificar genes e interações gene-ambiente relevantes para a etiologia desse defeito de nascimento comum e representativo. O presente

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estudo teve como objetivo detectar variantes genéticas nos genes IRF6 e GRHL3 e suscetibilidade ao NSCL / P em populações da região centro-oeste e norte do Brasil. Analisamos um conjunto de 80 indivíduos com NSCL / P da Associação de Combate como Deformidades Faciais, recrutados no Centro-Oeste e Norte do Brasil. Realizamos a amplificação da sonda multiplexada dependente da ligação (P304-B1-IRF6 / GRHL3 (lote B1-0116)) e análise de PCR para confirmação. No estudo MPLA, o éxon 4 de GRHL3, mostra uma possível alteração. Portanto, realizamos uma validação por PCR dessas alterações. Os resultados não mostraram alteração nesses genes (IRF6 e GRHL3), corroborando com estudos anteriores. Até onde sabemos, este estudo de ambos os genes é o primeiro nessas áreas específicas do Brasil, analisando indivíduos com NSCL / P. Estudos identificaram uma variante missense no gene grainyhead-like-3 (GRHL3) em indivíduos com fissura palatina. A contribuição dessas variantes genéticas para a suscetibilidade ao NSCL / P deve ser investigada em diferentes populações e coortes. Assim, as causas genéticas subjacentes da NSCL / P permanecem amplamente desconhecidas.

Palavras-chave: fenda labial, fenda palatina, mutação missense, fendas orofaciais, polimorfismo.

1 INTRODUCTION

Craniofacial anomalies affect a significant proportion of the global society. The formation of the oral cavity comprises the interaction of different embryological processes, and thus any change or failure in these processes can lead to malformations. Cleft lip with or without cleft palate (CL/P) is the most frequent craniofacial anomaly, corresponding to 25% of all birth defects[1,2]. It occurs in about one in every 1,000 children, and this rate varies considerably according to geographic area and ethnic group. In Brazil, prevalence has been estimated from 1:650 to 1:2,700 live births[3-5]. The registration of cases of birth defects, such as NSCL/P, has no standard because of the lack of an integrated and efficient information system that provides accuracy and reliability of data found in Brazil. In addition, data on the frequency of CL/P may vary according to researcher and country[6].

Approximately 70% of clefts are non-syndromic (NSCL/P), and only 30% have a syndromic form of cleft[7]. The genetics of craniofacial anomalies, particularly of NSCL/P, is complex. The etiologies are many and involve single genes, chromosomal disorders, polygenic interactions, environmental risks, and gene–environment interaction[8-10]. Much has been learned about the genetics of craniofacial anomalies. However, the genetic basis of clefts remains poorly understood despite a number of studies[9-12]. Given the similar phenotype between syndromic and non-syndromic forms of CL/P, causative genes identified in syndromic CL/P are considered promising candidate genes for NSCL/P as well.

One gene that is linked to orofacial clefts is interferon regulatory factor 6 (IRF6, 1q32.2). This gene plays an important role in facial development during the formation of the oral periderm and this regulation is essential for appropriate palatal adhesion. Is among those that have shown a convincing degree of consistency among different studies[12,13]. Mutations in this gene are known to cause two autosomal dominant allelic disorders, the Van der Woude syndrome (VWS) and the popliteal

pterygium syndrome[14,15]. The IRF6 gene has a causal relationship with VWS and is related in 12% of nonsyndromic CL/P (NSCL/P)[16-18]. Mutations in this gene have been reported in patients with NSCL/P[19-21]. Moreover, this gene has great potential to play a role in the etiology of isolated clefts[16,22].

Several studies evaluating single-nucleotide polymorphisms (SNPs) in the IRF6 in individuals with cleft and their families in different populations have found a strong relationship between these polymorphisms and NSCL/P, thus confirming the significant association between the IRF6 gene and the occurrence of cleft[8,14,23]. Mutations in IRF6 have been identified in only 70% of families with VWS. A linkage study on a large Finnish pedigree with VWS identified a novel locus on 1p33–p36 (VWS2) rather than in IRF6 at 1q32–q41, providing further evidence of locus heterogeneity underlying this syndrome[22].

However, in populations genetically heterogeneous witch Brazil the association between NSCL/P and mutations in IRF6 has not been confirmed. Nevertheless, studies investigating IRF6 polymorphisms in different patient populations have given divergent results. These findings are especially discordant in studies with mixed populations, such as those in Brazil, where results have varied according to the geographic region and ethnicity studied[20-23].

Other possibility is the gene grainyhead-like transcription factor 3 (GRHL3, 1p36.11), which is located in VWS2 locus 1p34, has been identified as another VWS causative gene and is considered a novel candidate gene for NSCL/P[1,21]. This gene regulate oral periderm differentiation and patterning of the craniofacial structures. Studies by genome-wide association study (GWA study, or GWAS) and sequencing approaches have indicated a missense variant (rs41268753) in GRHL3 increases the risk for NSCL/P cases in European ancestry[1,10,13-17,20-24].

Studies demonstrated that mutations in both IRF6 and GRHL3 cause almost the same clefting phenotypes. In this study, we investigated the possible contribution between variants in IRF6 and GRHL3 genes and susceptibility to NSCL/P in Midwest and Northern Brazilian populations.

2 MATERIALS AND METHODS

Samples

Dentistry surgeon examined all subjects and diagnoses as NSCL/P based on clinical examination, medical records, and a detailed questionnaire. Blood samples were collected from 80 subjects from Midwest and Northern Brazil and evaluated at the Associação de Combate as Deformidades Faciais. Subjects in the control group were healthy individuals without a family history of orofacial clefts or other major congenital defects. Written informed consent was obtained for all subjects or their parents prior to participation in the study in compliance with the World Medical

Association Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects. The ethical committee of the Federal University of Goiás, Brazil, approved this study (approval number 4382.9115.8.0000.6083).

Molecular analysis

Genomic DNA was isolated from whole blood using a FlexiGene Genomic Purification Kit (Qiagen). The DNA concentration was determined using a NanoDrop 1000 (Thermo Fisher Scientific). DNA samples were analyzed through Multiplex Ligation-Dependent Probe Amplification (MLPA) using the MLPA SALSA kit probemix P304-B1-IRF6 GRHL3 (Lot B1-0116), following the manufacturer's instructions (MRC-Holland). This MLPA assay was designed to detect deletions/duplications of one or more exons of the IRF6 and GRHL3 genes. All runs included DNA from normal controls to calibrate unknown samples. Probe amplification products were run on an ABI-3500 Genetic Analyzer using the GS500 size standard (Applied Biosystems). These data were exported to GeneMarker (Softgenetics) and Coffalyser software (MRC-Holland) for MLPA analysis. In some samples that present doubt results of alteration in MLPA, it was done PCR for validation. The alterations were presented in exon 4 of GRHL3.

Variants observer in MLPA was genotyped by PCR amplification. Each reaction was conducted using 1 µg of DNA in a final volume of 20 µL. Primers for GRHL3 were designed to amplify the exon 4 of all isoforms (sense-AACTCCTTGTTTGAGAGCATTCA; antisense-GCATCGACTCCTGTGGGTC). β-actin gene served as internal controls (sense-AGAGCTACGAGCTGCCTGAC; antisense-AGCACTGTGTTGGCGTACAG). PCR reactions were incubated 1 cycle at 94°C for 5 min, followed by 35 amplification cycles of 95°C for 30s, 60°C for 30s, and 72°C for 30s, and a final extension at 72°C for 7 min. PCR products were sent agarosis gels. The exon 4 variant of GRHL3 showed in MLPA were screened in 06 individuals with NSCL/P and your parents.

3 RESULTS

We screened 80 individuals with NSCL/P, and this group was composed of 44 males and 36 females. Among this group, 45 had cleft lip and palate, 32 had cleft lip, and 3 had cleft palate. All 80 NSCL/P DNA samples were examined by the MLPA technique that revealed six non-related individuals with microduplication on exon 4 of the GRHL3 gene in both software analyses of Genemarker and Coffalyser. We send a mail to the MLPA kit's manufacturer for related this founds. They suggest PCR assay these samples and your parents for confirmation. PCR reactions for

validation these results were not found in any alterations. The results showed no alteration on these genes (IRF6 and GRHL3) corroborating with previous studies.

4 DISCUSSION

We searched for microdeletion or microduplication in a group of individuals with NSCL/P from Midwest and Northern Brazil. We observed this group in an attempt to link them to ethnic and socio-economic factors in the region. However, miscegenation was shown to be high, and heterogeneous socioeconomic factors, detected a positive association between these factors and CL/P. In the present work, we performed MLPA screening to identify variants of IRF6 and GRHL3 gene in the etiology of NSCL/P in a Brazilian cohort. It was observed supposed alterations on exon 4 of the GRHL3 gene in six unrelated individuals from Northern Brazil, but not confirmed by PCR analysis. These families had no history of recurrence, but the information obtained was only in the family core. Wu-Chou et al.[22] analyzed 80 NSCL/P individuals using the SALSA MLPA P304-A1 kit, which analyzed the IRF6 gene only. These authors found no deletion or duplication in the study group. In our study, we used the SALSA MLPA that included the GRHL3 gene (P304-B1 IRF6-GRHL3). We did not find any variation in IRF6 and GRHL3 genes, consistent with Wu-Chou et al.[22]. Other studies using the same MLPA kit were not found. The SALSA MLPA Probemix P304 IRF6-GRHL3 was discontinued for manufacturing by MCR-Holland® (Amsterdam, Netherlands).

Mutations of the VWS causative gene IRF6 (1q32-q41 VWS1 locus) were identified in NSCL/P and considered as the first gene related to clefts. In another VWS2 locus (1p34), mutations in GRHL3 were identified in VWS patients without IRF6 mutations. Therefore, GRHL3 is recognized as a novel causative gene of VWS and is a promising candidate gene for NSCL/P as well[1,13-17,20-24]. GRHL3 may be associated with the risk of NSCL/P, which awaits verification across different ethnic populations.

Leslie et al.[10] performed a GWAS on NSCL/P and discovered a genome-wide significant association with a missense variant in GRHL3 (p.Thr454Met [c.1361C>T]; rs41268753) and replicated the result in an independent sample of case and control subjects. This missense mutation (p.Thr454Met) in GRHL3 that is associated with cleft palate only was first identified in Europeans, but other rare variants of GRHL3 influence heritability for clefts in Africans[24].

GRHL3 mutations with deleterious and pathogenic effects identified in VWS families are rare and therefore seem unlikely to be prevalent among large populations. However, common genetic polymorphisms are considered to contribute to NSCL/P[13-17]. Sequencing analysis revealed truncating GRHL3 mutations, including two that were *de novo* in four families; all nine individuals harboring mutations had NSCL/P[1]. GRHL3 polymorphic variants (SNPs rs10903078, rs41268753, and

rs4648975) are associated with NSCL/P in the Brazilian population. The authors performed a case–control study stratified by the subtypes of oral clefts taking into consideration the intense ancestry miscegenation of the Brazilian population[1].

On the basis of these results, we observed that some studies showed the involvement of the GRHL3 gene in causing oral clefts. Our group of individuals with NSCL/P from Northern Brazil inhabit a small island, where the incidence of oral clefts has been reported to be high. Many of these individuals live in locations that are difficult to access on the island and do not seek medical care. Thus, a high rate of consanguineous marriages or recurrence may occur in the family. In the present study among the individuals that showed variation in MLPA, but not confirmed by PCR, no history of recurrence was found, but the parents did not know for certain. The contribution of GRHL3 genetic variants to NSCL/P susceptibility should be further investigated in different populations and cohorts. Further investigations are warranted to investigate the influence of GRHL3 variants between diferents haplotypes.

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