SIBELE NASCIMENTO DE AQUINO

"CHARACTERIZATION OF SUSCEPTIBILITY POLYMORPHISMS FOR NONSYNDROMIC CLEFT LIP WITH OR WITHOUT CLEFT PALATE"

"CARACTERIZAÇÃO DE POLIMORFISMOS DE SUSCETIBILIDADE ÀS FISSURAS DE LÁBIO OU PALATO NÃO-SINDRÔMICAS"

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Orientador: Prof. Dr. Hercílio Martelli Júnior

"CARACTERIZAÇÃO DE POLIMORFISMOS DE SUSCETIBILIDADE ÀS FISSURAS DE LÁBIO OU PALATO NÃO-SINDRÔMICAS"

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Estomatopatologia da Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas, para obtenção do título de Doutora em Estomatopatologia na área de Patologia.

Doctorate thesis presented to the Stomatatology Postgraduation Programme of the Piracicaba Dentistry School of the University of Campinas to obtain the Ph.D. grade in Stomatology, in Pathology area.

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA E ORIENTADA PELO PROF. DR. HERCÍLIO MARTELLI JÚNIOR

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Guimarães Rosa

RESUMO

Fissuras do lábio ou palato não sindrômicas (FL/PNS) são as anomalias congênitas craniofaciais mais comuns, com prevalência de 1:500-2.500 nascidos vivos. Possuem etiologia complexa, com participação de fatores ambientais e genéticos. Estudos de larga escala genômica (GWAS) descreveram várias regiões cromossômicas e genes candidatos à etiologia das FL/PNS, entretanto poucos foram confirmados em diversas populações, o que pode ser resultado da diferença na composição étnica das populações. Recentemente, GWAS realizados com populações da Europa e da Ásia identificaram polimorfismos de suscetibilidade para FL/PNS nos genes *FGF12*, *VCL*, *CX43*, e nos *loci* 1p36, 2p21, 3p11.1, 8q21.3, 10q25, 13q31.1, 15q22.2 e 17q22. Como o Brasil é composto por uma população miscigenada, torna-se importante confirmar se esses marcadores também mostram suscetibilidade à FL/PNS na população brasileira. O objetivo deste estudo foi avaliar o envolvimento de polimorfismos genéticos que foram descritos como marcadores de risco para o desenvolvimento de FL/PNS em pacientes brasileiros com FL/PNS. Este estudo caso-controle, com uma análise estruturada de acordo com as proporções de ancestralidade de cada individuo, avaliou 16 marcadores polimórficos de suscetibilidade as fissuras orais em 300 pacientes com FL/PNS e 395 indivíduos sem fissura provenientes de Minas Gerais, Brasil e 7 marcadores polimórficos em 505 pacientes com FL/PNS e 594 indivíduos sem fissura, provenientes de Minas Gerais e Bahia, Brasil. Os polimorfismos foram genotipados pelo método de discriminação alélica com sondas fluorescentes. A ancestralidade genômica de cada indivíduo foi determinada pela caracterização de 40 marcadores bialélicos de inserção/deleção (INDELs). A distribuição genotípica de todos os polimorfismos no grupo controle respeitou o equilíbrio de Hardy-Weinberg, exceto para o polimorfimo rs7632427 que foi excluído da análise. Foram observadas associações entre os polimorfismos rs227731, rs742071, rs1873147, rs8001641 e rs7590268 e de um haplótipo formado pelos polimorfismos rs10787760, rs6585429 e rs1871345 do gene *VAX1* com FL/PNS. Após a correção de Bonferroni para múltiplos testes,

foram observadas associações significativas com os polimorfismos rs742071, rs1873147 e rs227731. Entretanto, a frequência dos alelos de risco variou entre as regiões geográficas, de acordo com as proporções de ancestralidade européia e africana. O grupo com maior proporção de marcadores de origem européia mostrou associação com rs227731, enquanto que o grupo com proporção maior de marcadores de origem africana exibiu associação com o polimorfismo rs1873147. A associação significante com rs742071 foi detectada apenas com a combinação de amostras. Em síntese, os resultados demonstram a associação dos polimorfismos localizados na região 1p36 (rs742071), 15q22.2 (rs1873147) e 17q22 (rs227731), com a suscetibilidade genética ao desenvolvimento de FL/PNS na população brasileira, sendo observada uma influência da diversidade populacional nessas associações, uma discreta associação dos polimorfismos rs7590268 (2p21) e rs8001641 (13q31.1) com FL/PNS e uma modesta associação de um haplótipo no gene *VAX1*, sugerindo um efeito com baixa penetrância em FL/PNS.

Palavras chave: Fissura Labial; Fissura Palatina; Polimorfismo de Nucleotídeo Único; Fatores de risco; Heterogeneidade Genética.

ABSTRACT

Nonsyndromic cleft lip with or without palate (NSCL/P) is the most common orofacial birth defect with prevalence of 1:500-2,500 live births. NSCL/P has complex etiology, which is related to environmental and genetic risk factors. Recent genome-wide association (GWAS) studies have been identified a varied of chromosomal loci and candidate genes in association with NSCL/P development, however few of them has been replicated in different populations, which may be related to the differences in the ethnicity of the populations. Previous GWAS with populations from Europe and Asia have identified putative susceptibility markers for NSCL/P in *FGF12*, *VCL*, *CX43* and polymorphism at the regions 1p36, 2p21, 3p11.1, 8q21.3, 10q25, 13q31.1, 15q22.2 and 17q22. As the Brazilian population is one of the most heterogeneous in the world, it is important to confirm the susceptibility of those markers identified in genetically homogenous populations in the Brazilian population. The purpose of this study was to verify the association of single nucleotide polymorphisms, which were identified as NSCL/P risk markers, with NSCL/P in a Brazilian population. We conducted a structured association study conditioned on the individual ancestry proportions to determine the role of 16 polymorphic markers in 300 patients with NSCL/P and 395 controls from Minas Gerais state, Brazil, and 7 polymorphic markers in 505 patients with NSCL/P and 594 controls from Minas Gerais state and Bahia state, Brazil. The polymorphisms were genotyping using the allelic discrimination method with fluorescence probes. The genomic ancestry of each individual was determined by genotyping 40 biallelic short insertion-deletion polymorphic markers (INDELs). The genotype frequencies observed for all studied polymorphisms in the control group did not reveal statistically significant differences compared to those expected under Hardy-Weinberg equilibrium, except for rs7632427 which was excluded from analysis. Associations with NSCL/P were observed with rs227731, rs742071, rs1873147, rs8001641 and rs7590268, and a haplotype formed by *VAX1* rs10787760, rs6585429 and rs1871345 polymorphisms with NSCL/P. After Bonferroni correction for multiple tests, significant associations with NSCL/P were observed

with rs742071, rs1873147 and rs227731. However, the frequency of the risk alleles varied between the geographical regions, according to the proportions of European and African genomic ancestry. The group enriched by European ancestry showed significant association with rs227731, whereas the group with high African ancestry was significantly associated with rs1873147 polymorphism. The significant association with rs742071 was only detected in the combined sample. In conclusion, our results demonstrated the association of the polymorphisms located at 1p36, 15q22.2 and 17q22 with NSCL/P Brazilian population, and that the diversity of the Brazilian population clearly influences the contribution of polymorphisms, a weakly association of the rs7590268 (2p21), rs8001641 (13q31.1) e rs1873147 (15q22.2) and a modest association of a haplotype of *VAX1*, suggesting a low penetrant gene for oral cleft.

Key words: Cleft Lip; Cleft Palate; Polymorphism, Single Nucleotide; Risk factors; Genetic heterogeneity.

SUMÁRIO

INTRODUÇÃO

Fissura do lábio ou palato não-sindrômica

Fissuras do lábio ou palato (FL/P) são caracterizadas por áreas de descontinuidade no lábio ou palato, representando uma das anomalias congênitas mais frequentes nos seres humanos e mais comuns na região craniofacial (Dixon *et al*., 2011; Marazita *et al*., 2012). Cerca de 70% dos casos de FL/P ocorrem de forma não-sindrômica (FL/PNS), ou seja, sem malformações adicionais, e os demais 30% referem-se às associações, em que são observadas desordens mendelianas, cromossômicas, teratogênicas e condições esporádicas. Mais de 400 síndromes já descritas apresentam FL/P como característica fenotípica (Meng *et al*., 2009; Mangold *et al*., 2011). As FL/P são classificadas, baseando-se na região de envolvimento anatômico, em 4 grupos: fissuras pré-forame incisivo ou fissuras labiais (FL), fissuras pós-forame incisivo ou fissuras palatinas (FP), que envolvem o palato mole ou duro, fissuras trans-forame incisivo ou fissuras lábio-palatinas (FLP), que envolvem lábio e palato mole/duro e fissuras raras da face (Spina *et al*., 1972; Gorlin *et al*., 2001).

As FL/PNS afetam aproximadamente 1 em cada 500 a 2000 nascidos vivos, com grande variabilidade de acordo com a origem geográfica, grupos raciais e étnicos, bem como exposição a fatores ambientais de risco. Em geral, as populações asiáticas e ameríndias possuem uma alta prevalência (1:500), as populações européias possuem prevalência intermediária (1:1000) e as menores taxas de prevalência são observadas em africanos e descendentes de africanos (1:2500) (Mossey & Little, 2002; Murthy & Bhaskar, 2009; Mossey & Little, 2009). A frequência de FL/PNS também difere de acordo com o gênero. FLP e FL são mais frequentes no gênero masculino e a FP é mais comum no feminino (Mossey & Little, 2002; Mossey & Little, 2009). Levantamento realizado em Alfenas–MG mostrou prevalência de 1,46 casos de FL/PNS1 para cada 1000 nativivos, com uma maior frequência no gênero masculino (Martelli-Júnior *et al.*, 2006). Um segundo estudo observou maior frequência de FLP (39,68%), seguida por FL (38,09%) e pela FP (22,23%) nos casos avaliados (Martelli-Júnior *et al.*, 2007).

Mais recentemente, num terceiro estudo, observou-se que FP foi mais comum em mulheres, enquanto as FLP e FL foram mais frequentes no gênero masculino. O risco de FL em relação à FP foi 2,19 vezes maior em homens quando comparados às mulheres e o risco de FLP em relação à FP foi 2,78 vezes maior em homens quando comparado às mulheres (Martelli *et al*., 2012).

Apesar de não ser uma causa importante de mortalidade, as FL/PNS são associadas à significativa morbidade. Efeitos sobre a fala, audição e aparência geram resultados adversos sobre a saúde e integração social (Mossey *et al*., 2009). A criança afetada necessita de cuidados multidisciplinares do nascimento até a vida adulta, os quais incluem enfermagem, cirurgia plástica, cirurgia bucomaxilofacial, otorrinolaringologia, fonoaudiologia, aconselhamento genético, psicologia e odontologia (Wehby & Cassell, 2010). Embora a reabilitação seja possível com o atendimento de boa qualidade, as FL/P inevitavelmente constituem um ônus para o indivíduo, a família e a sociedade, com um custo substancial em termos de saúde e serviços relacionados (Dixon *et al*., 2011).

Uma sequência de eventos de alta complexidade, coordenados pela interação entre fatores de transcrição e sinalizadores moleculares juntamente com interações célula-célula e aquisição de polarização celular, é essencial para o desenvolvimento normal da face (Stanier & Moore, 2004). Durante a quarta semana de gestação, células da crista neural provenientes do tubo neural anterior migram para formar a primórdia facial. Dela surge o processo nasal medial e lateral que se fundem ao processo maxilar para formar a parte central do lábio superior, o palato primário e o nariz. O palato secundário começa a ser formado na sexta semana de gestação. Inicialmente, as placas palatinas do palato secundário aparecem como duas extensões no lado interno da maxila, ao longo da superfície lateral da língua. Na nona semana, ambas as placas palatinas sofrem uma rápida transformação horizontal, movendo-se sobre a língua e fusionando-se uma com a outra e com o septo nasal. Em seguida, o mesênquima palatino se diferencia em elementos musculares e ósseos que correlacionam com a posição do palato mole e duro, respectivamente. Na décima semana da

embriogênese, os processos fusionados provenientes do palato primário e secundário e do septo nasal estão completos. Assim, o desenvolvimento do palato secundário em mamíferos divide o espaço oronasal em cavidades oral e nasal, permitindo que a mastigação e a respiração sejam realizadas simultaneamente (Sperber, 2002). Em razão da alta complexidade no desenvolvimento do lábio e palato, ainda não se conhece todos os mecanismos exatos relacionados à falha na coalescência dos processos que dão origem a essas estruturas.

As FL/PNS são etiologicamente heterogêneas. Estudos genéticos e epidemiológicos indicam que há participação de fatores genéticos e ambientais na etiologia desta anomalia congênita, embora sua etiopatogenia permaneça incerta (Dixon *et al*., 2011; Mangold *et al*., 2012). Nos últimos anos, tem ocorrido uma evolução no entendimento dos fatores causais, com a identificação de novas variantes genéticas, de fatores de risco ambientais e de como os fatores de risco ambientais interagem com os fatores genéticos. Este conhecimento deve, eventualmente, resultar em melhor prevenção, tratamento e prognóstico para os indivíduos afetados (Dixon *et al*., 2011; Mangold *et al*., 2012; Kohli & Kohli, 2012).

Fogh-Andersen (1942) foi o primeiro a observar, em um estudo populacional, a existência de um componente hereditário associado ao desenvolvimento das FL/PNS. Nas últimas décadas, alguns estudos tem indicado recorrência familial para o desenvolvimento de FL/PNS (Sivertsen *et al*., 2008; Grosen *et al*., 2010) embora um padrão clássico de herança mendeliana não seja identificado (Natsume *et al*., 2000; Vieira *et al*., 2008). Um estudo realizado na Dinamarca observou um aumento de 10 a 32 vezes no risco de recorrência de FL/PNS em parentes de primeiro grau (Grosen *et al*., 2010) e um estudo norueguês, avaliando 4138 crianças com FL/PNS, identificou que o risco de recorrência de fissura em familiares de primeiro grau foi de 32% para FL e 56% para FP (Sivertsen *et al*., 2008).

Em estudos com gêmeos, a taxa de concordância observada de 40 a 60% em gêmeos monozigóticos é muito maior do que a concordância de 3 a 5% identificada em gêmeos dizigóticos (Jugessur *et al*., 2009). Estudo realizado por

Grozen e colaboradores (2010) mostrou um risco maior para fissura em filhos de gêmeos discordantes quanto à presença de fissura, tanto para os descendentes do gêmeo com fissura quanto sem fissura, proporcionando evidência adicional para um componente genético na etiologia das FL/P. Um estudo do nosso grupo, avaliando a incidência familiar de FL/PNS em 185 pacientes, identificou que 35,13% dos indivíduos apresentavam histórico familiar de FL/P, sendo os primos (54,37%) e os irmãos (21,05%) os mais afetados (Martelli *et al.*, 2010). Além disso, a relação entre FL/PNS e consanguinidade é observada em alguns trabalhos (Kanaan *et al*., 2008; Leite & Koifman, 2009; Aquino *et al*., 2011).

Uma variedade de estudos tem sido utilizada para identificar vias e genes envolvidos na etiologia das FL/PNS. Parte dos genes candidatos foi sugerida através de estudos com modelos experimentais em camundongos *knockouts* (Juriloff & Harris, 2008), citogenética (Brewer *et al*., 1999; Higgins *et al*., 2008), estudos de fissura associadas a síndromes mendelianas (Kondo *et al*., 2002; Zucchero *et al*., 2004) e análises de expressão gênica em tecidos embrionários (Mukhopadhyay *et al*., 2004; Gong *et al*., 2005). Em humanos, muitos estudos genéticos utilizando diferentes estratégias, incluindo estudos de ligação, caso-controle ou com trios, sequenciamento direto do DNA e estudos de associação de larga escala genômica (GWAS), que são baseados na comparação de vários polimorfismos comuns entre casos e controles, tem identificado genes e regiões cromossômicas candidatos à etiologia das FL/PNS (Dixon *et al*., 2011;. Kohli & Kohli, 2012; Rahimov *et al*., 2012; Stuppia *et al*., 2012).

O primeiro GWAS realizado com FL/PNS identificou polimorfismos de risco na região 8q24 e no gene *IRF6*, em indivíduos de ascendência européia (Birnbaum *et al*., 2009). A região 8q24 foi confirmada por outro GWAS (Grant *et al.,* 2009). A região 1q32 também foi identificada em associação a FL/PNS (Marazita *et al.,* 2009; Wang *et al.,* 2010). Outros GWAS realizados em diversas populações revelaram polimorfismos situados nos *loci* 18q22 (Grant *et al.,* 2009), e 20q12 (Beaty *et al.,* 2010) como marcadores de suscetibilidade ao desenvolvimento de FL/PNS. Os marcadores na região 1q32, onde o gene *IRF6*

está localizado (Blanton *et al.,* 2005; Scapoli *et al.,* 2005; Park *et al*., 2007;Rahimov *et al.,* 2008; Jugessur *et al.,* 2008; Marazita *et al*., 2009) e 8q24 são os mais consistentes, visto que foram confirmados em algumas populações (Birnbaum *et al.,* 2009; Rojas-Martinez *et al.,* 2010; Mostowska *et al.,* 2010; Murray *et al.,* 2012). Contudo, muitos dos polimorfismos encontrados nestes estudos não foram replicados em diferentes populações, indicando que a contribuição relativa dos genes pode ser variável de acordo com a etnia (Mossey & Little, 2009; Beaty *et al*., 2010; Dixon *et al*., 2011). Estudos prévios na população brasileira confirmaram a associação com o *lócus* 8q24 (Brito *et al*., 2012a; Pinto, 2012), entretanto o envolvimento do gene *IRF6* na nossa população permanece incerto (Paranaíba *et al*., 2010; Brito *et al*., 2012b).

Mangold e colaboradores (2010) identificaram mais duas regiões de susceptibilidade (17q22 e 10q25.3) para FL/PNS. Outro GWAS (Beaty *et al*., 2010) confirmou a associação do *lócus* 10q25.3. Este *lócus* engloba uma região intergênica, que provavelmente apresenta efeitos reguladores em genes adjacentes, especificamente *VAX1*. Um GWAS realizado com 1536 marcadores em 357 genes candidatos para FL/PNS foi realizado em duas populações de origem escandinava e observou associação significante de variantes polimórficas e haplótipos nos genes *FGF12*, *VCL*, *CX43* e *IRF6* com FL (Jugessur *et al*., 2011). Mais recentemente, Ludwing e colaboradores (2012) realizaram a primeira meta-análise a partir de dois GWAS (Beaty *et al*., 2010; Mangold *et al*., 2010) e confirmaram a associação de todos os *loci* já descritos nestes estudos e identificaram seis novos polimorfismos de susceptibilidade às FL/PNS, localizados nas regiões 1p36, 2p21, 3p11.1, 8q21.3, 13q31.1 e 15q22.

A proteína codificada pelo gene *FGF12* (Fator de crescimento dos fibroblastos) é um membro da família fator de crescimento de fibroblastos (*FGF*), localizado no cromossomo 3 (3q28-q29). Os membros da família *FGF* possuem ampla capacidade mitogênica e atividades relacionadas à sobrevivência de células bem como desenvolvimento embrionário, tais como crescimento celular, morfogênese e reparação tecidual (Smallwood *et al.,* 1996; Liu & Chiu, 1997). A

família FGF é composta por moléculas de sinalização altamente conservadas evolutivamente e desempenham papéis importantes no desenvolvimento craniofacial (Nie *et al*., 2006). Vários membros da família *FGF* e seus receptores foram anteriormente implicados em várias doenças humanas, por exemplo, mutações em *FGFR1, 2,* e *3* estão associados com craniossinostose e outras malformações craniofaciais (Pauws & Stanier, 2007).

Embora a exata função de *FGF12* não esteja esclarecida, alguns estudos tem demonstrado uma associação de *FGF12* e câncer de pulmão (Kang *et al*., 2010), carcinoma de esôfago (Chattopadhyay *et al*., 2010), e leucemia (Forconi *et al*., 2008). Um estudo recente observou ainda a expressão deste gene em fases importantes da odontogênese (Kettunen *et al*., 2011). Um estudo com 184 indivíduos afetados por FL identificou sete mutações potenciais causadoras de doenças, incluindo uma mutação nonsense no gene *FGFR1*, uma mutação missense *de novo* em *FGF8*, e outras mutações missense em *FGFR1, FGFR2, FGFR3* (Riley *et al*., 2007). Em seguida, *FGF12* também foi associado à FL em um estudo que avaliou separadamente os tipos de fissuras (Jugessur *et al*., 2009). Outro trabalho mais recente observou a associação de *FGF12* com FL, mas não com FLP e FP, sugerindo uma participação de *FGF12* especificamente em FL, destacando a importância da delineação fenotípica em estudos com FL/PNS (Jugessur *et al*., 2011). Nesse estudo, foi observada uma forte associação do haplótipo A-C-T (dos SNPs rs11717284, rs6790664 e rs1464942, respectivamente) e do haplótipo A-G-G (rs1464942, rs12106855 e rs1875735, respectivamente) relacionados ao gene *FGF12* com FL.

CX43 (*Connexin 43*) é um gene localizado na região 6q22.31 e codifica uma proteína componente de junções que medeiam a difusão de íons e metabólitos entre o citoplasma de células adjacentes. CX43 é expressa no coração, sendo a principal proteína de junções GAP neste órgão, possuindo um papel crucial na contração cardíaca e no desenvolvimento embrionário (Britz-Cunningham *et al*., 1995; Neijssen *et al*., 2005; Roell *et al*., 2007). A proteína CX43 é alvo de várias proteínas quinases que regulam o acoplamento célula-célula no miocárdio.

Mutações em locais críticos relacionados a este regulamento podem induzir alterações funcionais ou de desenvolvimento do coração (Britz-Cunningham *et al*., 1995).

Mutações no gene *CX43* são associadas à displasia oculodentodigital (OMIM #164200), que é caracterizada por alterações no desenvolvimento da face, olhos, membros e da dentição (Richardson *et al*., 2006; Paznekas *et al*., 2009). Anomalias da região craniofacial incluem curtas fendas palpebrais, mandíbula com rebordo alveolar largo, nariz hipoplásico, anomalias dentárias e palato invertido (Paznekas *et al*., 2003). Foi demonstrada uma forte expressão de *CX43* no processo frontonasal de animais, nos arcos branquiais, no processo nasal medial e lateral, nos processos mandibulares e nas prateleiras do palato secundário no momento da fusão (Richardson *et al*., 2004). Consistente com os dados de expressão, FP tem sido relatada em uma proporção de pacientes com displasia oculodentodigital. Além disso, há um estudo recente relatando a associação de *CX43* em duas populações de FL/PNS. Direções opostas de associação foram identificadas com os haplótipos de risco na Noruega e Dinamarca, sendo demonstrada uma associação com a população dinamarquesa (p=0,01) com o haplótipo G-A, nos polimorfismos rs12197797 e rs11961755 (Jugessur *et al*., 2011). Trata-se do primeiro estudo associando este gene a FL/PNS.

O gene *VCL* está localizado na região 10q22.2, possui uma função bem caracterizada como uma proteína do citoesqueleto e contém 22 éxons (Mulligan *et al*., 1992). A proteína vinculina está envolvida na ligação de filamentos de actina a integrinas, um processo que permite às células se anexarem umas às outras e à matriz extracelular (Weller *et al*., 1990). A superexpressão de vinculina resulta na adesão celular aumentada, enquanto que em células com redução na expressão são altamente móveis (Ziegler *et al*., 2006). *VCL* está também implicado nas vias de sinalização envolvidas na apoptose (Subauste *et al*., 2005).

Vinculina e a sua isoforma metavinculina, resultante de *splicing* alternativo, são co-expressas em células musculares e essenciais para a formação adequada dos cardiomiócitos, sendo que mutações missenses em *VCL* estão associadas à

cardiomiopatia (Witt *et al*., 2004; Lee *et al*., 2012). A formação adequada do palato primário requer células aderindo firmemente entre si e à matriz extracelular e a apoptose se faz necessária em fases específicas do desenvolvimento do palato. Como o gene *VCL* é envolvido nesses processos, é plausível que uma função aberrante deste gene possa contribuir para a patogênese das FL/P. Assim como o gene *CX43*, o gene *VCL* representa uma nova associação com FL/PNS. Em estudo realizado por Jugessur e colaboradores (2011), foi verificada uma associação do haplótipo C-A-T (rs10762573, rs2131960 e rs4746172, respectivamente) com FL.

O gene *VAX1* pertence à subfamília *VAX*, está localizado na região 10q25.3, possui 3 éxons e codifica um fator de transcrição homeobox. Genes homeobox são conservados nos vertebrados e geralmente estão envolvimentos na morfogênese e no desenvolvimento do corpo. A proteína codificada pelo *VAX1* desempenha um papel importante no desenvolvimento do prosencéfalo ventral anterior e no sistema visual (Hallonet *et al*., 1999; Mui *et al*., 2005). Animais *knockout* para este gene geralmente morrem ao nascimento e apresentam malformações craniofaciais, incluindo FP (Hallonet *et al*., 1999). Tem sido demonstrado que durante o desenvolvimento do olho, a expressão de *VAX1* é mediada pela expressão de *SHH* (*Sonic hedgehog*) e suprimida pela proteína morfogenética do osso 4 (BMP4) - moléculas de sinalização que também regulam a palatogênese em mamíferos (Zhang *et al*., 2002; Zhao *et al*., 2010). Além disso, variantes polimórficas e as mutações de *BPM4* ter sido associado com o risco de FL bem como fissura submucosa (Lin *et al*., 2008;. Suzuki *et al*., 2009). Interessantemente, o *lócus* 10q25.3 também foi anteriormente associada a defeitos do tubo neural em seres humanos (Rampersaud *et al*., 2005).

Mais recentemente, alguns estudos têm investigado a associação da região 10q25 com FL/PNS. Dois GWAS independentes identificaram variantes polimórficas nesta região como marcadores de risco para FL/PNS, com o polimorfimo rs7078160 exibindo maior significância (Mangold *et al*., 2010; Beaty *et al*., 2010). Entretanto, como este *lócus* se encontra em uma região intergênica,

não se sabe se estes marcadores são a causa da associação ou estão em desequilíbrio de ligação com genes adjacentes. Assumindo esta última hipótese, o *VAX1* foi sugerido como o gene candidato mais forte próximo ao *lócus* 10q25.3, já que camundongos *knockout* para este gene exibe FP (Hallonet *et al*., 1999) e há uma síndrome com mutação em *VAX1* que apresenta FLP no seu espectro clínico (Slavotinek *et al*., 2012). Nasser e colaboradores (2012) descreveram recentemente uma análise de sequenciamento do gene *VAX1* em 384 pacientes com FL/PNS e 384 controles e encontraram um grande número de variantes raras, mas nenhuma associação significativa com FL/PNS foi encontrada, embora o número de variantes tenha sido maior no grupo FL/PNS. Neste mesmo estudo, observou-se que seis famílias segregaram as variantes raras, sugerindo que *VAX1* pode ter um efeito na patogênese das FL/PNS de baixa penetrância.

O primeiro relato de associação dos *loci* 1p36, 3p11.1 , 2p21, 13q.31, 8q21.3 e 15q22.2 com FL/PNS surgiu apenas recentemente, com a meta-análise realizada por Ludwing e colaboradores (2012). Genótipos do GWAS conduzido por Beaty e colaboradores (2010) com 666 trios europeus e 795 trios asiáticos foram combinados com dados do GWAS realizado por Mangold e colaboradores (2010), sendo 399 casos de FL/PNS e 1318 controles de origem Européia. A combinação destas duas amostras representa 95% de todos os indivíduos com FL/PNS previamente relatados (Ludwing *et al*., 2012).

O polimorfismo rs7632427, do *lócus* 3p11.1, encontra-se a aproximadamente 3 kb do gene *EPHA3* (*Ephrin receptor A3*). Este gene pertence à subfamília de receptores efrina. *EPH* e receptores têm sido implicados na mediação de eventos do desenvolvimento, em particular no sistema nervoso. Membros da família deste gene são envolvidos na regulação da estrutura celular bem como no contato célula a célula (Himanen *et al*., 2007). No *lócus* 2p21, o polimorfismo rs7590268 foi associado com FL/PNS, com $p=1,1x10^{-5}$. Este polimorfismo está localizado no gene *THADA* (*Thyroid adenoma associated*). Anomalias cromosômicas envolvendo esse gene têm sido observadas em adenomas benignos da tiróide e polimorfismos em *THADA* também têm sido

associados com diabetes tipo 2 (Zeggini *et al*., 2008). Não há relatos da participação destes *loci* no desenvolvimento craniofacial.

O polimorfismo rs8001641 (13q31.1) está localizado numa região intergênica e foi sugerido que este marcador possa exercer algum efeito regulatório em genes próximos, dentre eles o gene *SPRY2* (*Sprouty2*) (Ludwing *et al*., 2012). Mutações pontuais neste gene foram associadas às FL/PNS (Vieira *et al*., 2005) e um estudo realizado com animais deficientes em *SPRY2* exibiram FP, causada por falha na elevação das prateleiras palatinas (Matsumura *et al*., 2011). O polimorfismo rs1873147, do *lócus* 15q22.2 e o polimorfismo rs12543318 (8q21.3), também estão localizados em região intergênica e não há outros estudos mostrando associação destas regiões com FL/PNS.

O polimorfismo rs742071, localizado na região 1p36, pertence a um intron do gene *PAX7* (*paired box 7*). Este gene é um membro da família de fatores de transcrição *PAX*, que desempenham papéis críticos durante o desenvolvimento fetal e no câncer. A função específica do gene *PAX7* é desconhecida, mas especula-se que possua uma função supressora de tumor (Seger *et al*., 2011; Yang *et al*., 2012). *PAX7* já foi funcionalmente implicado no desenvolvimento craniofacial e mutações no gene *PAX3* (MIM 606597) têm sido associadas com a síndrome de Waardenburg, que pode apresentar FL/P como uma das características fenotípicas (Tassabehji *et al*., 1993;. Mansouri *et al*., 1996). Um estudo investigou polimorfismos em genes *PAX* e FL/PNS em trios (pai, mãe e filho afetado por FL/PNS) e identificou um efeito significativo de transmissão materna em duas variantes de *PAX7* e quatro em *PAX3* (Sull *et al*., 2009). No GWAS conduzido por Beaty e colaboradores (2010) além da identificação de outros *loci*, *PAX7* foi fortemente sugestivo de associação com FL/PNS. Nesse mesmo estudo, a análise dos subgrupos asiáticos e europeus revelou um efeito mais forte da região 1p36 em amostras de asiáticos, apesar de que baixos valores de p também foram encontrados na população européia. O polimorfismo rs742071 foi associado pela primeira vez com FL/PNS no estudo recente conduzido por

Ludwing e colaboradores (2012), com p = $7.02x10^{-9}$. Não há estudos que replicaram este marcador em outras populações.

O polimorfismo rs227731 está localizado a 100kb do gene *NOG*. Este gene codifica a proteína *noggin*, que é essencial para o desenvolvimento normal dos ossos e articulações (Zimmerman *et al*., 1996). *Noggin* é uma proteína antagonista extracelular que liga e inativa vários tipos de BMPs (*Bone morphogenetic protein*), incluindo BMP2, BMP4, e BMP7, as quais são expressas no desenvolvimento das prateleiras palatinas (He *et al*., 2010; Krause *et al*., 2011). He e colaboradores (2010) demonstraram que a repressão de BMP via *noggin* é necessária para o desenvolvimento normal de palato. A ausência de *noggin* altera os níveis de BMP, provocando proliferação celular aberrante e morte celular no desenvolvimento do palato. Em animais *knockout* para *NOG*, a integridade do epitélio palatino é interrompida, o que leva a uma fusão anormal do palato com a mandíbula e bloqueia a elevação normal do palato (He *et al*., 2010). Em humanos, *NOG* é o único inibidor de BMPs que tem sido associado a fenótipos anormais (Potti *et al*., 2011). Como o polimorfismo rs227731 está localizado próximo ao gene *NOG*, é possível que este polimorfismo possa exercer algum efeito regulatório neste gene.

Evidência para o envolvimento do *lócus* 17q22 na etiologia das FL/PNS foi originalmente relatado por Mangold *e* colaboradores (2010) em trios de origem européia. No entanto, os estudos de replicação subsequentes realizados em populações da Estonia, populações mesoamericanas e chinesas não confirmaram esses resultados positivos (Nikopensius *et al*., 2009;. Rojas-Martinez *et al*., 2010; Pan *et al*., 2011). Entretanto, Mostowska e colaboradores (2012) replicaram a associação do polimorfismo rs227731 em uma população polonesa e posteriormente, na meta-análse conduzida por Ludwing e colaboradores (2012) foi confirmada a associação deste marcador com FL/PNS.

Após essa breve introdução, o objetivo deste estudo foi validar marcadores polimórficos, previamente identificados em associação às fissuras do lábio ou palato não sindrômicas, contidos nos genes *FGF12*, *VCL*, *CX43*, *VAX1* e nas regiões 1p36 (rs742071), 2p21 (rs7590268), 3p11.1 (rs7632427), 8q21.3

(rs12543318), 13q31.1 (rs8001641), 15q22.2 (rs1873147), 17q22 (rs227731) em populações brasileiras, em uma análise estruturada de acordo com as proporções de ancestralidade de cada individuo.

CAPÍTULO 1

Artigo submetido ao BMC Medical Genetics

Polymorphisms in *FGF12*, *VCL*, *CX43* and *VAX1* in Brazilian patients with nonsyndromic cleft lip with or without cleft palate

Abstract

Background: Nonsyndromic cleft lip with or without cleft palate (NSCL/P) is the most common orofacial birth defect with a wide range prevalence among different populations. Previous association studies with populations from Europe and Asia have identified putative susceptibility markers for NSCL/P in fibroblast growth factor 12 (*FGF12*), vinculin (*VCL*), connexin 43 (*CX43*) and in a region close to the ventral anterior homeobox 1 (*VAX1*) gene. However, there have thus far been no studies of these markers in NSCL/P Brazilian patients, and as the genetic ancestry of the Brazilian population is highly varied, the predisposition to those disease markers can be different.

Methods: Herein we conducted a structured association study conditioned on the individual ancestry proportions to determine the role of 16 polymorphic markers within those genes in 300 patients with NSCL/P and 385 unaffected controls.

Results: None of the alleles and genotypes showed association with NSCL/P, though there was a significant association of the haplotype formed by *VAX1* rs10787760, rs6585429 and rs1871345 polymorphisms with NSCL/P that did not persist Bonferroni correction for multiple tests.

Conclusions: Our results are consistent with a lack of involvement of *FGF12*, *VCL* and *CX43* variants with NSCL/P pathogenesis in Brazilian patients. Furthermore, the higher frequency of a haplotype of *VAX1* with NSCL/P patients suggests a low penetrant gene for oral cleft, and warrants further studies.

Keywords: nonsyndromic cleft lip with or without palate, polymorphism, FGF12, VCL, CX43, VAX1.

Background

Nonsyndromic cleft lip with or without cleft palate (NSCL/P) is the most common human craniofacial defect with a prevalence ranging from 0.36-1.54 per 1,000 live births in Brazil [1,2]. With a population exceeding 190 million people and 3 million babies born every year, NSCL/P is an important problem of public health in Brazil with approximately 4,000 NSCL/P new cases every year. Although the exact environmental and genetic risk factors associated with NSCL/P remains unclear, the understanding of the genetic mechanisms involved in this malformation are evolving [3,4]. To date interferon regulatory factor 6 (*IRF6*) at 1q32.2 and the region 8q24 have been considered the most reliable susceptibility markers for NSCL/P [5-11]. Our previous studies confirmed the association of 8q24 locus with NSCL/P susceptibility in the Brazilian population [12], but the involvement of *IRF6* in NSCL/P pathogenesis is still unclear in Brazilians [13,14]. As result of five centuries of mating between Amerindians, Europeans and sub-Saharan Africans, the Brazilian population displays very high levels of genomic diversity [15], which may have important implications on NSCL/P susceptibility.

Nonsyndromic oral clefts are traditionally divided in cleft lip only (CLO), cleft lip and palate (CLP) and cleft palate only (CPO), however, as there are similarities in both epidemiologic features and embryologic timing for both CLO and CLP, they are considered variants of the same defect and grouped together to form the group cleft lip with or without cleft palate (CL/P). A recent study with 1536 markers in 357 candidate genes for oral clefts was carried out with two Scandinavia-based populations, revealing significant association of CLO with variants in fibroblast growth factor 12 (*FGF12*, MIM 601513), vinculin (*VCL*, MIM 193065), connexin 43 (*CX43*, MIM 121014) and *IRF6* [16]. The haplotype relative risk ranged from 1.47 for *VCL* haplotype in the Norway dataset to 5.49, which was identified for *FGF12* in the Denmark cohort. *FGF12* gene encodes an intracellular non-secretory protein of the large family of FGFs [17]. While the role of the secretory members of the FGF family in the control of the cell growth, differentiation and morphogenesis, which includes craniofacial development, is best known, the functions of the intracellular

members remain partially determined [18]. Intracellular FGFs, including FGF12, contain nuclear localization signals, suggesting a role as transcriptional factors [19]. *VCL* encodes a small actin-bundling protein that has emerging role in the organization of the focal adhesions and adherens junctions [20]. Recent developments advance our understanding of the VCL role on regulation of cell adhesion and motility in both normal development and cancer. Although VCL expression was detected both in vivo during palate formation [21] and in vitro in palatal fibroblast cell cultures [22], the participation of VCL on lip and palate embryogenesis is unknown. *CX43* is one of the 21 members of the homogeneous family of connexin proteins, which are structurally and functionally associated with the formation of the gap junctions [23]. Gap junctions are essential for proper cell homeostasis and have been shown to play important roles in a wide variety of biological and pathological processes [24]. Mutations in *CX43* gene cause oculodentodigital dysplasia, which is characterized among several clinical phenotypes by the presence of cleft lip and/or cleft palate [25,26].

Another risk locus for NSCL/P identified in two large genome-wide association studies is located on chromosome 10q25.3, which encompasses an intergenic region with suggested regulatory effects on adjacent genes, specifically ventral anterior homeobox 1 (*VAX1*) [9,27]. However, the full-sequencing of 384 patients with NSCL/P and 384 controls did not support the association of *VAX1* with NSCL/P [28]. The purpose of the present study was to investigate the contribution of *FGF12*, *VCL*, *CX43* and *VAX1* risk markers with NSCL/P in Brazilian patients through a structured analysis in which the genetic ancestry variation of each individual was taken into account.

Methods

Sample study

In this case-control study, 300 patients with NSCL/P assisted at the Center for Rehabilitation of Craniofacial Anomalies, Dental School, University of José Rosário Vellano, Brazil and 385 unaffected controls, which were chosen among subjects

admitted as in-patients in the Dental School of the same University with conditions unrelated to clefting disorders, were included. Samples were recruited between 2008 and 2012, and all subjects were born in the study area, South of Minas Gerais State, Brazil. The Center for Rehabilitation of Craniofacial Anomalies of the Dental School, University of Alfenas is the reference hospital for clefting patients living in this area. To confirm the NSCL/P diagnosis, all patients were carefully examined and screened for the presence of associated anomalies or syndromes by the team of the Center for Rehabilitation of Craniofacial Anomalies. Patients with additional congenital malformations (other than NSCL/P), history of consanguinity or with history of familial oral cleft were not included in this study. The nonsyndromic clefts were classified with the incisive foramen as reference, and 105 patients had CLO and 195 had cleft lip and palate (CLP). Control group was composed by healthy subjects without history of congenital malformations or familial history of oral clefting. Written informed consents were obtained and the study carried out with approval of the Human Research Ethics Committee of the University.

Polymorphism selection

Ten single nucleotide polymorphisms (SNPs) previous detected in association with CLO by Jugessur et al. [16], including rs6790664, rs11717284, rs1464942, rs12106855 and rs1875735 in *FGF12*, rs4746172, rs10762573 and rs2131960 in *VCL* and rs11961755 and rs12197797 in *CX43*, were evaluated in this study. In addition, 6 SNPs in *VAX1* with a minor allele frequency (MAF) >0.2 (rs7086344, rs10787760, rs6585429, rs1871345, rs751231 and rs751233) were identified in the International HapMap Project (http://hapmap.ncbi.nlm.nih.gov/) and included in this study. The main features of each polymorphism, including chromosome position, localization within gene, identification of the major and minor alleles and MAF, are described in Table 1.

SNP genotyping and estimation of the genomic ancestry

Genomic DNA was extracted from oral mucosa cells and examined blinded to group status using the TaqMan 5′-exonuclease allelic discrimination assay (Applied Biosystems, Foster City, CA). Genotyping analyses were randomly repeated in 10% of the samples for all polymorphisms. To determine the genomic ancestry of each individual, samples were genotyped for a set of 40 biallelic short insertion/deletion polymorphisms (INDELs) previously validated as informative markers for ancestry [29].

Statistical analysis

Deviation from Hardy-Weinberg equilibrium in control group was assessed through chi-square test. To determine the genomic ancestry of each individual, Structure software was utilized [30] in a model assuming K=3 parental populations based on the tri-hybrid origin of the Brazilian population. Samples with pre-specified population of origin (European, Sub-Saharan African and Amerindian reference populations from Marshfield Clinic Collection) were also incorporated to assist the software in the ancestry estimation. Following ancestry assessment, STRAT was used to test the association, conditioning on the individual ancestry proportions [31]. The odds ratio (OR) and associated 95% confidence intervals (95% CI) were also calculated. Haplotype frequencies and pair-wise linkage disequilibrium (D' and r^2) were estimated using the HaploView software. The Bonferroni correction for multiple comparisons was applied, and the corrected p value of ≤0.003 was considered statistically significant.

Results

The description of study participants' gender and the proportions of ancestry of each group are depicted in Table 2. Initially each sample was genotyped with 40 INDEL markers and the data were analyzed using the Structure program. To assist the software in the estimation of the ancestry, we incorporated reference samples of European, African and Amerindian ancestry from Marshfield Clinic collection.

The average ancestry contributions were estimated at 90% of European, 7.5% of African, and 2.5% of Amerindian in the control group, and in the NSCL/P group was 87.5% of European, 10.7% of African, and 1.8% of Amerindian, revealing no statistical significant differences in the proportions between groups (p=0.32). Supplementary Figure 1 depicts the proportions of the Amerindian, European, and African ancestry of each sample.

Frequencies of the alleles and genotypes of **FGF12, CX43, VCL and VAX1 polymorphisms** structured by genomic ancestry are presented in Table 3. The genotype frequencies observed for all studied polymorphisms in controls did not reveal statistically significant differences compared to those expected under Hardy-Weinberg equilibrium. None of the polymorphisms tested showed association with NSCL/P or its subtypes (CLO and CLP) in this Brazilian case-control cohort **(Table 3).** Further analyses in the dominant and recessive genetic models also revealed no differences in the distribution between groups (Figure 1).

Pair-wise linkage disequilibrium analyses are depicted in Figure 2. One linkage disequilibrium block, which involved rs10787760, rs6585429 and rs1871345, was identified in *VAX1*. Carriers of the *VAX1* G-A-C haplotype (G allele of rs10787760, A allele of rs6585429 and C allele of rs1871345) were found to be more frequent in all NSCL/P groups as compared to controls (Table 4), but those significances did not remain after correction for multiple testing by the conservative Bonferroni procedure. *FGF12* polymorphisms rs11717284 and rs6790664 (D'=0.90 and r^2=0.70), *VCL* rs10762573 and rs2131960 (D'=0.88 and r^2=0.77) and *CX43* polymorphisms (D'=0.97 and r^2=0.94) were in linkage disequilibrium (Figure 2). Interestingly, 3 out of 4 risk haplotypes identified in the study of Jugessur et al. [16] were composed of the major alleles and were the most prevalent in the present cohort (Table 4).

Discussion

Although NSCL/Ps are amongst the most common and distressing congenital defects, the exact genetic and environmental events associated with their

pathogenesis are still unknown. Identifying the causative genetic alterations will have important impacts on genetic counseling, and will lead to a greater understanding of the craniofacial development. In the present study, we evaluated the association of polymorphisms in *FGF12*, *VCL*, *CX43* and *VAX1* with NSCL/P in a representative sample of the Brazilian population through a structured approach. In contrast to the results of Jugessur et al. [16], we have not observed any association of *FGF12*, *VCL* and *CX43* with CLO. No association with CLP or the combination of CLO and CLP (CL±P) was also found. The lack of association observed in our cohort may be related to sample size, therefore modest associations of polymorphisms and oral cleft risk may have been missed. However, the number of CLO samples in this study was quite similar to the Scandinavian datasets (121 CLO from Norway and 76 from Denmark compared with 105 CLO in the current study) that identified significant associations with CLO risk. Furthermore, a frequency of the risk alleles for all polymorphisms in our sample was similar to those observed in the European population (CEU database, Single Nucleotide Polymorphism database-dbSNP). Taken that the frequency of the risk alleles is high in European populations and our sample was enriched by European descents, it is unlikely that these polymorphisms are involved with NSCL/P pathogenesis in Brazilian patients.

Two independent genome-wide scans identified polymorphic variants at region 10q25.3 as risk markers for NSCL/P, with rs7078160 showing the highest significant score [9,27]. Later studies lacked to confirm this association in populations from China [32] and Brazil [33]. As an intergenic region, it is unknown whether markers at 10q25.3 are the cause of the association or are in linkage disequilibrium with adjacent causal variants in the genes. Assuming the later hypothesis, *VAX1* has been suggested to be the strongest candidate near 10q25.3, because *VAX1* knockout mice showed craniofacial malformations including cleft palate and **VAX1** mutation was described in a patient affected by an uncharacterized syndrome with bilateral CLP as one of the clinical features [34,35]. Nasser et al. [28] recently described the sequencing analysis of 384 patients with

NSCL/P and 384 controls of Central European origin and identified a large number of *VAX1* rare variants, but no significant associations with NSCL/P were found. Nevertheless, the authors demonstrated the segregation of the identified *VAX1* rare variants in six NSCL/P structured families, suggesting the *VAX1* may have a low penetrance effect on NSCL/P pathogenesis [28]. Although two of the selected *VAX1* polymorphisms of the present study demonstrated a suggestive protective effect against NSCL/P (odds ratio < 1), none of them was significantly associated with oral clefts. On the other hand, the higher frequency of the G-A-C haplotype in NSCL/P patients suggests that this may be a disease-promoting gene, but there are some limitations in this interpretation. First, the association did not reach significance after Bonferroni correction. Fundamentally, correction for multiple testing is always required when multiple markers (comparisons) are used, correcting for spurious associations. However, Bonferroni correction is especially emblematic with markers in linkage disequilibrium because the alleles are not independent of each other, making the correction too conservative. A less conservative and more realistic procedure is to identify markers or blocks of linkage disequilibrium reducing the number of comparisons. Assuming linkage disequilibrium between markers with $r^2 \geq 0.70$, the number of independent comparisons was reduced to 10, requiring α level of 0.005 to give a 95% probability of correctly concluding not to reject H_0. Nevertheless, the p level of the *VAX1* haplotypes was still beyond the significance level. Second, the frequency of the risk haplotype was relatively low, and to establish more firmly the association of this haplotype with the disease, confirmation with a larger number of samples is necessary.

Conclusion

In summary, our results show a lack of involvement of polymorphisms in *FGF12*, *VCL* and *CX43* with the pathogenesis of NSCL/P in Brazilian patients, and the higher frequency of G-A-C haplotype formed by *VAX1* polymorphisms in NSCL/P suggests that this gene may be involved with the defect. Further efforts are needed

to clarify the relationship between genetic variations of the *VAX1* gene and the development of NSCL/P.

Competing interests

The authors declare that no relationship with industry exists.

Authors' contributions

SNA participated in the design of the study, carried out the molecular genetic studies and drafted the manuscript. ACM carried out the molecular genetic studies and critically revised the manuscript. EB carried out the molecular genetic studies and critically revised the manuscript. HM-J participated in the design of the study, participated in sample collection and critically revised the manuscript. MSOS participated in sample collection and critically revised the manuscript. EG participated in the design of the study and critically revised the manuscript. RDC conceived of the study, participated in its design and coordination, drafted the manuscript and critically revised the manuscript. All authors read and approved the final manuscript.

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Table 1. Characteristics of the single nucleotide polymorphisms of the present study.

	Chromosome position*	Location	Allele	Minor allele frequency*
FGF12 (3q28)				
rs11717284	191925200	Intron	A/t	0.353
rs6790664	191939237	Intron	C/a	0.392
rs1464942	192086644	Intron	T/a	0.253
rs12106855	192346410	Intron	G/a	0.467
rs1875735	192359833	Intron	G/c	0.426
VCL (10q22.2)				
rs10762573	75798148	Intron	C/a	0.398
rs2131960	75831259	Intron	A/c	0.404
rs4746172	75855842	Intron	T/c	0.360
CX43 (6q21-q23.2)				
rs12197797	121763963	Intron	C/g	0.164
rs11961755	121766286	Intron	G/a	0.164
VAX1 (10q26.1)				
rs7086344	118890573	Exon	C/t	0.266
rs10787760	118890693	Exon	G/a	0.483
rs6585429	118893231	Exon	G/a	0.412
rs1871345	118895368	Intron	C/t	0.317
rs751231	118896664	Intron	A/c	0.267
rs751233	118896767	Intron	G/a	0.228

*Reference: http://www.ncbi.nlm.nih.gov

Table 2. Gender distribution and proportions of the European, African and Amerindian ancestry of each group.

	Gender		Ancestry		
	Male	Female	European	African	Amerindian
Control	51.4%	48.6%	90%	7.5%	2.5%
CL±P	55.2%	44.8%	87.5%	10.7%	1.8%
CLO	57%	43%	87%	11.2%	1.8%
CLP	53.3%	46.7%	88.2%	10.1%	1.7%

CL±P: cleft lip with or without cleft palate, CLO: cleft lip only, CLP: cleft lip and palate.

Table 3. Case-control frequencies of the alleles and genotypes of the polymorphisms on *FGF12*, *VCL*, *CX43* and *VAX1*.

	HWE* (p value)	Control Group (%)	CL±P Group (%)	OR_{allele} (95% CI) p value	CLO Group (%)	OR_{allele} (95% CI) p value	CLP Group (%)	OR_{allele} (95% CI) p value
FGF12								
rs6790664								
Allele (A/C)	0.79	50.5/49.5	50.5/49.5	1.00 (0.80-1.24)	55.3/44.7	0.83 (0.61-1.12)	48/52	1.11 (0.87-1.41)
Genotype (AA/AC/CC)		25.2/50.6/24.2	24.7/51.7/23.6	0.50	28.6/53.4/18	0.28	22.6/50.7/26.7	0.56
rs11717284								
Allele (A/T)	0.42	51.5/48.5	55.5/44.5	0.85 (0.68-1.06)	49/51	1.10 (0.81-1.50)	59/41	0.74 (0.58-0.95)
Genotype (AA/AT/TT)		27.6/47.9/24.5	30.5/50.1/19.4	0.36	22.1/53.9/24	0.22	34.9/48.2/16.9	0.91
rs1464942								
Allele (A/T)	0.06	27.8/72.2	29.6/70.4	0.91 (0.72-1.16)	31/69	0.85 (0.61-1.19)	28.9/71.1	0.94 (0.72-1.24)
Genotype (AA/AT/TT)		9.6/36.4/54	8.7/41.8/49.5	0.74	8.6/44.8/46.6	0.59	8.8/40.2/51	0.50
rs12106855								
Allele (A/G)	0.15	42.1/57.9	40.8/59.2	1.05 (0.85-1.31)	43.3/56.7	0.95 (0.69-1.29)	39.3/60.7	1.12 (0.87-1.43)
Genotype (AA/AG/GG)		19.5/45.2/35.3	17.2/47/35.8	0.23	17.1/52.4/30.5	0.22	17.3/44.1/38.6	0.53
rs1875735								
Allele (C/G)	0.14	46.1/53.9	46.8/53.2	0.97 (0.78-1.20)	44.8/55.2	1.06 (0.77-1.43)	48/52	0.93 (0.72-1.18)
Genotype (CC/CG/GG)		23.1/46/30.9	23.4/47/29.6	0.44	21/47.6/31.4	0.28	24.6/46.7/28.7	0.76
VCL								
rs4746172								
Allele (C/T)	0.89	27.2/72.8	27.2/72.8	1.00 (0.78-1.27)	24.3/75.7	1.16 (0.82-1.66)	28.7/71.3	0.93 (0.71-1.22)
Genotype (CC/CT/TT)		7.5/39.3/53.2	7/40.4/52.6	0.83	2.9/42.8/54.3	0.92	9.2/39/51.8	0.59
rs10762573								
Allele (A/C)	0.46	33.9/66.1	32.8/67.2	1.05 (0.83-1.32)	34.3/65.7	0.98 (0.71-1.35)	32/68	1.09 (0.84-1.42)
Genotype (AA/AC/CC)		10.6/46.5/42.9	12.7/40.2/47.1	0.94	16.2/36.2/47.6	0.57	10.8/42.2/47	0.92
rs2131960								
Allele (A/C)	0.45	62.6/34.4	67.9/32.1	0.90 (0.72-1.13)	68.8/31.2	0.87 (0.62-1.21)	67.5/32.5	0.92 (0.71-1.19)
Genotype (AA/AC/CC)		42.1/46.9/11	47.5/40.8/11.7	0.62	50/37.5/12.5	0.23	46.1/42.6/11.3	0.95

CX43								
rs11961755								
Allele (A/G)	0.73	20.4/79.6	21.9/78.1	0.91 (0.70-1.19)	21.4/78.6	0.94 (0.64-1.36)	22/78	0.90 (0.67-1.22)
Genotype (AA/AG/GG)		4.4/31.9/63.7	4.4/35/60.6	0.68	2.9/37.1/60	0.90	5.2/33.8/61	0.74
rs12197797								
Allele (C/G)	0.70	79/21	79/21	0.99 (0.76-1.29)	78.7/21.3	1.02 (0.70-1.49)	79.3/20.7	0.98 (0.72-1.33)
Genotype (CC/CG/GG)		62.7/32.5/4.8	61.6/35/3.4	0.94	59.2/38.8/2	0.97	62.8/33/4.2	0.94
VAX1								
rs7086344								
Allele (C/T)	0.23	82/18	82.5/17.5	0.96 (0.73-1.27)	82.4/17.6	0.97 (0.65-1.45)	82.6/17.4	0.96 (0.69-1.32)
Genotype (CC/CT/TT)		68/27.8/4.2	69.7/25.7/4.6	0.12	66.7/31.4/1.9	0.63	71.3/22.6/6.1	0.16
rs10787760								
Allele (A/G)	0.11	63.6/36.4	62.7/37.3	1.04 (0.83-1.290	61.9/38.1	1.07 (0.78-1.47)	63/37	1.02 (0.79-131)
Genotype (AA/AG/G)		42.3/42.6/15.1	39.7/46/14.3	0.42	40/43.8/16.2	0.85	39.5/47.1/13.4	0.46
rs6585429								
Allele (A/G)	0.09	26.3/73.7	30.4/69.6	0.82 (0.64-1.04)	32/68	0.75 (0.54-1.06)	29.5/70.5	0.85 (0.65-1.12)
Genotype (AA/AG/GG)		8.6/35.4/56	9.8/41.2/49	0.43	11.6/40.8/47.6	0.46	8.7/41.5/49.8	0.72
rs1871345								
Allele (C/T)	0.06	76/24	75.6/24.4	1.02 (0.79-1.31)	74.3/25.7	1.09 (0.77-1.56)	76.3/23.7	0.98 (0.74-1.31)
Genotype (CC/CT/TT)		59.5/32.9/7.6	56.6/38/5.4	0.96	55.4/37.8/6.8	0.56	57.2/38.1/4.7	0.85
rs751231								
Allele (A/C)	0.34	81.5/18.5	80.1/19.9	1.09 (0.83-1.43)	78.9/21.1	1.18 (0.80-1.72)	80.8/19.2	1.04 (0.76-1.42)
Genotype (AA/AC/CC)		67.1/28.8/4.1	63.3/33.7/3	0.83	61.5/34.6/3.9	0.16	64.3/33.1/2.6	0.91
rs751233								
Allele (A/G)	0.47	17/83	19.5/80.5	0.84 (0.64-1.11)	20.7/79.3	0.78 (0.53-1.15)	18.9/81.1	0.87 (0.64-1.20)
Genotype (AA/AG/GG)		3.4/27.1/69.5	3.7/31.6/64.7	0.58	3.8/33.7/62.5	0.24	3.6/30.6/65.8	0.83

HWE: **Hardy Weinberg equilibrium,** CL±P: cleft lip with or without cleft palate, CLO: cleft lip only, CLP: cleft lip and palate.

Table 4. Distribution of the risk haplotypes.

Tagging Polymorphisms	Candidate risk haplotype*	Control Group	CL±P Group	P value	CLO Group	P value	CLP Group	P value
FGF12 rs11717284, rs6790664, rs1464942	A-C-T	34.9%	35.5%	0.46	32.1%	0.73	37.2%	0.65
FGF12 rs1464942, rs12106855, rs1875753	A-G-G	3.9%	2.7%	0.40	2.4%	0.77	2.9%	0.81
VCL rs10762573, rs2131960, rs4746172	C-A-T	53.5%	56.8%	0.44	58.3%	0.37	55.8%	0.59
CX43 rs12197797, rs11961755	C-G	78.8%	77.6%	0.78	78.1%	0.89	77.4%	0.75
VAX1 rs10787760, rs6585429, rs1871345	G-A-C	2.6%	6.7%	0.026	6.9%	0.19	6.7%	0.033

*Risk haplotypes of *FGF12*, *VCL* and *CX43* were based on Jugessur et al. (2011).
CL±P: cleft lip with or without cleft palate, CLO: cleft lip only, CLP: cleft lip and palate.

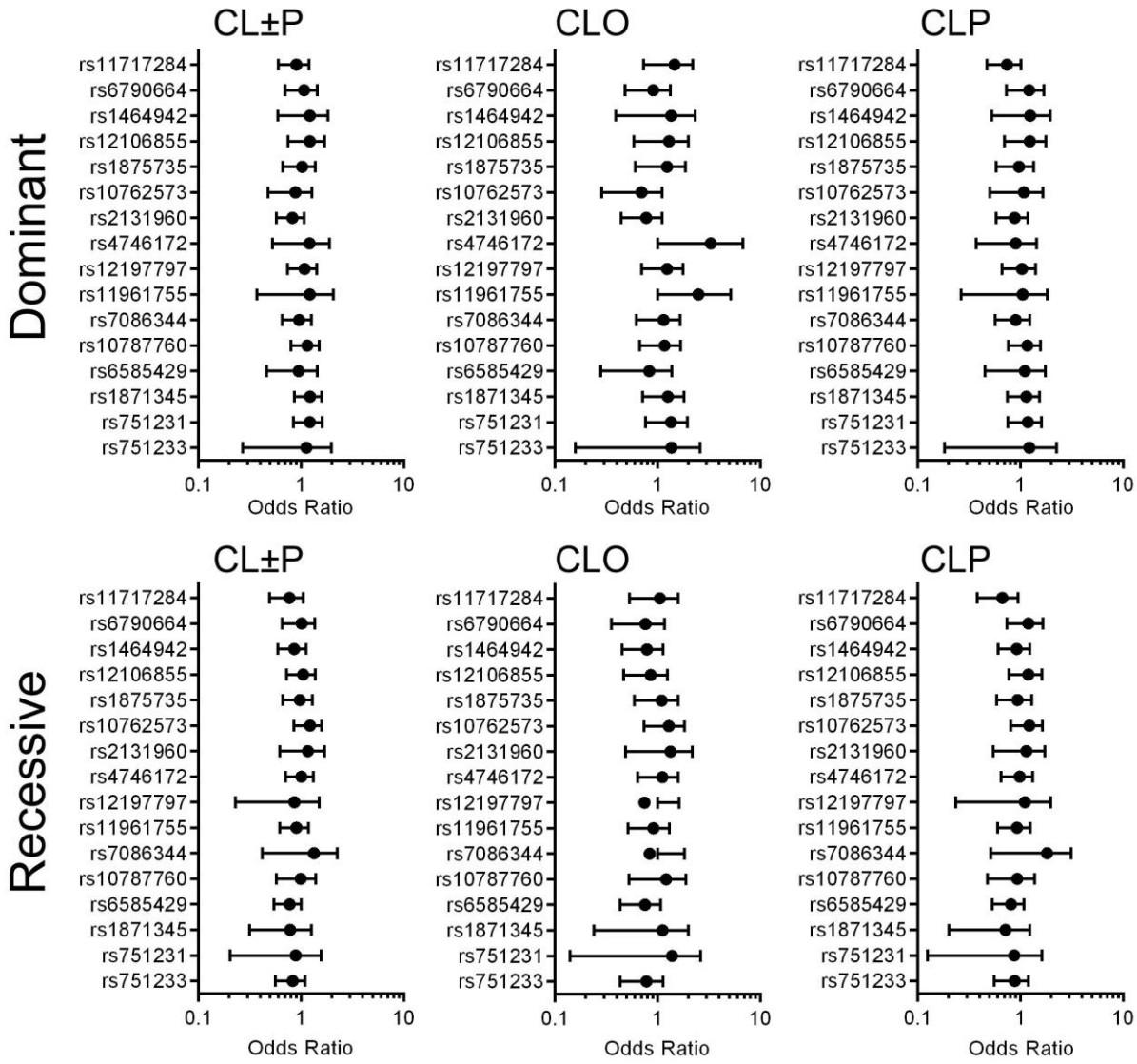


Figure 1. Odds ratio values under the dominant and recessive genetic models. In the dominant analysis, it was assumed that the heterozygote and the rare homozygote have the same disease odds, whereas in the recessive model the rare homozygote has different disease odds compared to the common homozygote and heterozygote. Those analyses did not show significant changes in the odds ratio.

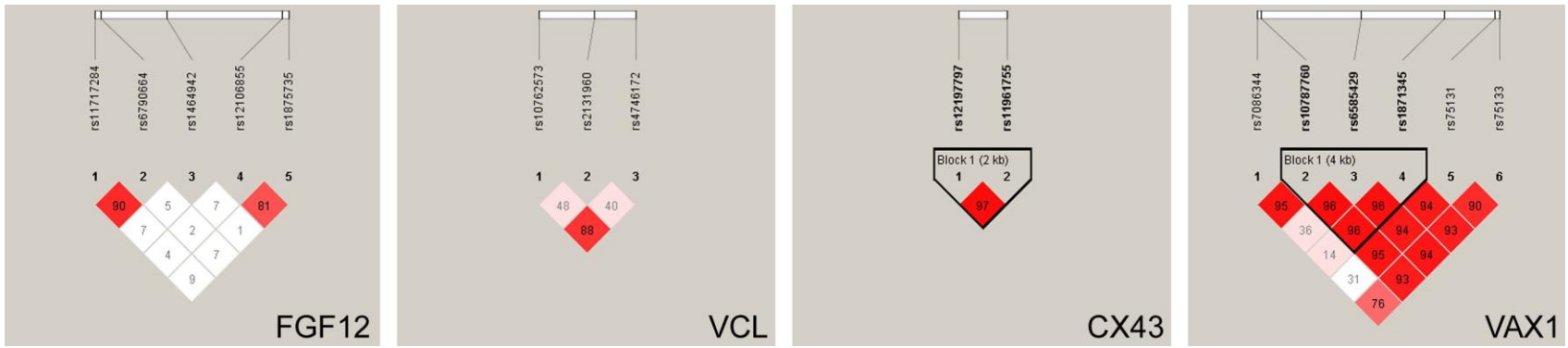
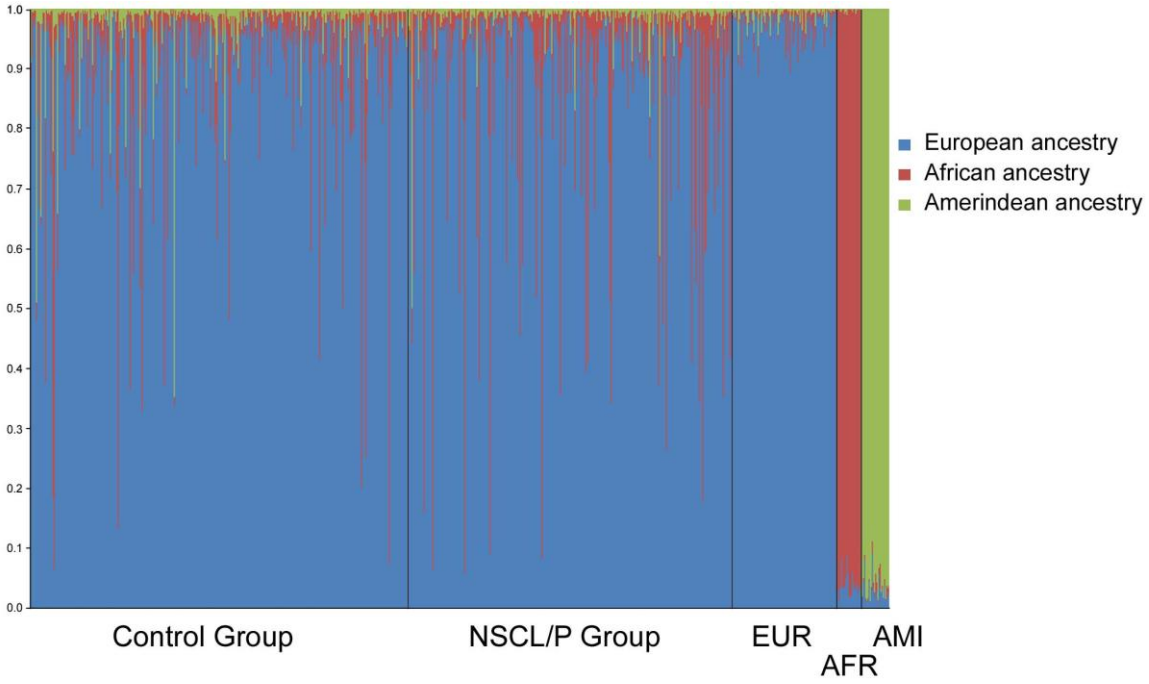


Figure 2. Linkage disequilibrium plots with the single nucleotide polymorphisms within the genes of this study. The plots were generated using the HaploView software. The numbers in the squares indicate the percentage linkage disequilibrium between a given pair of polymorphisms (**D'** value). Only for *VAX1* gene a clear block formed by rs10787760, rs6585429 and rs1871345 polymorphisms was identified.



Supplementary Figure 1. Genomic proportions of the European, African and Amerindian ancestry in the unaffected control and nonsyndromic cleft lip and/or cleft palate (NSCL/P) groups. Each individual is represented by a single column, and the columns identified as EUR (European), AFR (African), and AMI (Amerindian) represent the parental populations used to assist the structure in estimating ancestry of the admixed individuals.

CAPÍTULO 2

Artigo submetido ao Birth Defects Research Part A

Analysis of Susceptibility Polymorphisms for Nonsyndromic Cleft Lip With or Without Cleft Palate in the Brazilian Population

Abstract

BACKGROUND: Although genome-wide association studies have identified several susceptibility loci for nonsyndromic cleft lip with or without cleft palate (NSCL/P) in populations around the world, in the Brazilian population, which is highly heterogeneous, the role of most loci is unknown. **METHODS:** To determine the association of 7 genome-wide association susceptibility markers in Brazilians with NSCL/P, we conducted a structured association study conditioned on the individual ancestry proportions to evaluate markers at 1p36 (rs742071), 2p21 (rs7590268), 3p11.1 (rs7632427), 8q21.3 (rs12543318), 13q31.1 (rs8001641), 15q22.2 (rs1873147) and 17q22 (rs227731) in 505 patients with NSCL/P and 594 healthy controls recruited from 2 different geographical regions of Brazil. The polymorphisms were genotyped by TaqMan 5′-exonuclease allelic discrimination assay, and each sample was independently typed for 40 biallelic short insertion/deletion markers to characterize the genomic ancestry. **RESULTS:** After Bonferroni correction for multiple tests, significant associations with NSCL/P were observed with rs742071, rs1873147 and rs227731. However, the frequency of the risk alleles varied between the geographical regions, according to the proportions of European and African genomic ancestry. The group enriched by European ancestry showed significant association with rs227731, whereas the group with high African ancestry was significantly associated with rs1873147 polymorphism. The significant association with rs742071 was only detected in the combined sample. **CONCLUSIONS:** The findings of the present study demonstrated that the diversity of the Brazilian population clearly influences the contribution of polymorphisms in the pathogenesis of NSCL/P.

Key words: nonsyndromic cleft lip with or without palate; polymorphism; 1p36; 15q22.2; 17q22.

INTRODUCTION

Nonsyndromic cleft lip with or without cleft palate (NSCL/P) is among the most common congenital defects in all populations worldwide, affecting 1 to 500-2500 live births, but its prevalence shows notable influence of ethnicity (Dixon et al., 2011; Marazita, 2012). A variety of genetic approaches have been employed to identify genes and loci associated with nonsyndromic oral clefting, such as the genome-wide association studies (GWAS), which have identified several susceptibility loci for NSCL/P (Birnbaum et al., 2009; Grant et al., 2009; Beaty et al., 2010; Mangold et al., 2010). The GWAS carried out by Mangold et al. (2010) identified a susceptibility loci for NSCL/P at chromosome 17q22 (rs227731) in triads of European origin, which was later confirmed in NSCL/P populations from Poland (Mostowska et al., 2012) and Honduras (Lennon et al., 2012). Recently, Ludwig et al. (2012) conducted the first genome-wide meta-analysis, confirming the association of 17q22 locus and further identifying 6 new susceptibility regions at 1p36, 2p21, 3p11.1, 8q21.3, 13q31.1 and 15q22.2.

The aim of this study was to verify the association of those NSCL/P susceptibility markers in the Brazilian population. However, as the Brazilian population is the resulted of the genetic admixture of 3 ancestral populations (Europeans, Africans and Amerindians) and displays very high levels of genomic diversity (Pena et al., 2011), it is possible that risk markers identified in genetically homogenous populations are not substantiated in the Brazilian population. Thus, to avoid population stratification bias, the polymorphic markers at regions 1p36 (rs742071), 2p21 (rs7590268), 3p11.1 (rs7632427), 8q21.3 (rs12543318), 13q31.1 (rs8001641), 15q22.2 (rs1873147) and 17q22 (rs227731) were analyzed in a case-control study taking into account the genetic ancestry variation of each individual (structured analysis).

METHODS

Sample Study

This study included 162 patients with cleft lip only (CLO), 343 patients with cleft lip and palate (CLP), totalizing 505 patients with cleft lip with or without cleft palate (CL±P), and 594 healthy individuals (unaffected controls). Patients were available for analysis from Center for Rehabilitation of Craniofacial Anomalies, Dental School, University of José Rosário Vellano, Alfenas-Minas Gerais, Brazil (n=299), which is located at Southeast of Brazil, and from Santo Antonio Hospital, Salvador-Bahia, Brazil (n=206), which is in the Northeast region. All patients were carefully examined and screened for the presence of associated anomalies or syndromes by a team of specialists of each Center, and the clefts were classified with the incisive foramen as reference. Patients with congenital malformations (other than oral cleft), history of consanguinity or with history of familial oral cleft were not included in this study. As control group, healthy individuals without congenital anomalies or history of familial oral clefting were recruited. Control volunteers were born in the studied areas, with 392 individuals from Minas Gerais state and 202 from Bahia state. The samples were obtained between 2008 and 2012. Part of the samples from Center for Rehabilitation of Craniofacial Anomalies was previous described (Brito et al., 2012a). All samples were collected after approval of the Human Research Ethics Committee of the University of Alfenas, and the Research Ethics Committee of the Santo Antonio Hospital. Informed consent was obtained from the patients or legal tutors.

Polymorphism Selection

Seven single nucleotide polymorphisms previous identified in association with NSCL/P, including 1p36 (rs742071), 2p21 (rs7590268), 3p11.1 (rs7632427), 8q21.3 (rs12543318), 13q31.1 (rs8001641), 15q22.2 (rs1873147) and 17q22 (rs227731), were evaluated in this study. The main features of each polymorphism, including chromosome position, localization within gene, identification of the major and minor alleles and minor allele frequency (MAF), are described in Table 1.

Estimation of the Genomic Ancestry and Polymorphism Genotyping

To determine the ancestry of each individual, genomic DNA samples, which were extracted from oral mucosa cells, were genotyped with a set of 40 biallelic short insertion/deletion polymorphisms (INDELs) previously validated as discriminative markers of the Brazilian colonizers (Bastos-Rodrigues et al., 2006). To polymorphism genotyping, we used the TaqMan 5′-exonuclease allelic discrimination assays (Applied Biosystems, Foster City, CA). Genotyping analyses were randomly repeated in 10% of the samples for all polymorphisms.

Statistical Analysis

Deviation from Hardy-Weinberg equilibrium in control group was assessed through chi-square test. To determine the genomic ancestry of each individual, Structure software was utilized (Falush et al., 2007) in a model assuming K=3 parental populations based on the tri-hybrid origin of the Brazilian population. Samples with pre-specified population of origin (European, Sub-Saharan African and Amerindian reference populations from Marshfield Clinic Collection) were incorporated to assist the software in the ancestry estimation. Following ancestry assessment, STRAT was used to test the association, conditioning on the individual ancestry proportions (Pritchard et al., 2000). The odds ratio (OR) and relative risk (RR) associated with 95% confidence intervals (95% CI) were also calculated. Polymorphism interactions were verified with the multifactor dimensionality reduction (MDR) test with a 10-fold cross validation and 1000-fold permutation testing (Hahn et al., 2003). The Bonferroni correction for multiple comparisons was applied, and the corrected p value of ≤0.008 was considered statistically significant.

RESULTS

Table 2 depicts the study participants' gender and the proportions of ancestry of each group. Combining the samples from the 2 Brazilian geographic areas, the average ancestry contributions were estimated at 85% of European, 12.8% of African and 2.2% of Amerindian in the control group and in the CL±P group was 79.8% of European, 18.4% of African and 1.8% of Amerindian, revealing statistical significant differences in the proportions between groups (p=0.03). As expected the European contribution was the most predominant in the populations from both geographic areas, though the frequency of European ancestry was significantly higher in the sample from the Southeast area (Minas Gerais) compared with the sample from the Northeast region (Bahia) (p=0.03), whereas the population from Bahia demonstrated a significantly higher contribution of African ancestry (p=0.002). First we performed the association analysis combining the samples from the 2 Brazilian regions, but as they showed significant differences in the European and African ancestry, the analysis was later carried out separately to identify the influence of the ancestry in the significant associations.

For all polymorphisms, except for rs7632427 polymorphism which was excluded from further analysis, genotype distributions in the control group were consistent with those predicted by the Hardy-Weinberg equilibrium (Table 3). The distributions of the alleles and genotypes of all polymorphisms structured by genomic ancestry are presented in Table 3.

The rs742071 minor T allele was significantly more frequent in patients with CL±P than controls (OR: 1.29, 95% CI: 1.09-1.53, p=0.005). The presence of the T allele increased the relative risk (RR) for oral clefts, since carries of the heterozygous GT genotype showed a RR_{Het} of 1.14 (95% CI: 1.02-1.28) and carries of the TT homozygous genotyped exhibited a RR_{Hom} of 1.28 (95% CI: 1.08-1.52). With relation to cleft type, this polymorphism showed a modest association with CLP group that did not resist to Bonferroni conservative correction for multiple tests (Table 3). Further analyses in the dominant genetic model revealed significant differences between CL±P and controls for rs742071 polymorphism

(OR: 1.44, 95% CI: 1.11-1.87, p=0.0061) (Table 4). A borderline association between CL±P and rs742071 polymorphism (p=0.014) was observed in the sample with the highest level of European ancestry (Southeast region) (Table 5).

The percentage of the C allele of rs227731 polymorphism was found to be significantly higher in the CL±P group as compared to the control group (OR: 1.56, 95% CI: 1.32-1.85, $p=1.7x10^{-6}$) (Table 3). The CC genotype was identified in 20.8% of the control samples (n=124), whereas the CC genotype was found in 32.4% of the CL±P patients (n=164). Patients carrying one copy of the rs227731 C allele (AC heterozygote genotype) showed a RR_{Het} of 1.17 (95% CI: 1.04-1.32), whereas the RR for carries of 2 copies (CC homozygous genotype) was even higher (RR_{Hom}: 1.49, 95% CI: 1.27-1.75). The rs227731 polymorphism demonstrated a significant allelic association with both CLO ($p=1.5x10^{-5}$) and CLP (p=0.004). The significant association of rs227731 polymorphism was observed in both dominant and recessive genetic models of analysis (Table 4). Interestingly, the significant association of the rs227731 polymorphism with CL±P and its isolated clinical forms was only maintained in the population with the highest levels of European ancestry (Tables 5 and 6).

Associations that did not persist Bonferroni correction for multiple tests were observed with rs7590268 and CLO (p=0.044) and with both rs8001641 and rs1873147 and CL±P (p=0.048 and p=0.027, respectively) (Table 3). In the dominant genetic model, the rs7590268 polymorphism was significantly associated with the risk of CLO, revealing an OR of 1.60 (95% CI: 1.13-2.28, p=0.008). Furthermore, the association between rs1873147 and nonsyndromic oral clefts in the population with higher levels of African ancestry (Northeast region, Bahia) was strong and resisted to Bonferroni correction (Table 6). Considering only the sample from the Northeast region (Bahia), significant associations between rs1873147 and CL±P (OR: 1.57; 95% CI: 1.17-2.11; p=0.005) and CLO (OR: 1.86; 95% CI: 1.22-2.83; p=0.006) were observed (Table 6). For this polymorphism, the RR_{Hom} for CL±P was 1.64 (95% CI: 1.10-2.43; p=0.005) and the OR under the dominant genetic model was 1.32 (95% CI: 1.08-1.61; p=0.0068).

Potential interactions among the polymorphisms of the present study were carried out with MDR analysis, allowing exhaustive combination search. The 2-factor combination of rs227731 and rs742071 was the only model for prediction of CL±P risk in the combined Southeast and Northeast samples (Table 7). The testing accuracy of this model was 0.58 (p=0.0046; based on 1000-fold permutations). When performing MDR analysis with populations isolated, no significant interactions were observed, but the best 2-factor model for the sample from the Southeast region was the combination of rs227731 and rs742071 (testing accuracy 0.53, p=0.14) and for the Northeast region was the combination of rs1873147 and rs227731 (testing accuracy 0.49, p=0.66).

DISCUSSION

In the last years genome-association studies have provided important information on identification of genetic factors involved in the pathogenesis of NSCL/P. However, as the genetic heterogeneity behind NSCL/P is complex, replication studies are essential to elucidate the significance of susceptibility markers identified in single populations. This is truly important in admixed populations, such as the Brazilian population that demonstrates variable levels of genomic diversity and the contribution of specific polymorphisms identified in large-scale association studies can be misrepresented. This situation can be exemplified with studies that analyzed the association of polymorphisms in interferon regulatory factor 6 (*IRF6*) gene and in region 8q24, the most reliable susceptibility markers for NSCL/P (Rahimov et al., 2008; Birnbaum et al., 2009; Beaty et al., 2010; Blanton et al., 2010a, Blanton et al., 2010b), in Brazilians with NSCL/P. Those studies confirmed the association of 8q24 locus with NSCL/P susceptibility (Brito et al., 2012a; Bagordakis et al., 2013), but the involvement of *IRF6* in NSCL/P pathogenesis is still unclear (Paranaiba et al., 2010; Brito et al., 2012b). Thus, this study analyzed the contribution of 7 polymorphisms characterized as NSCL/P susceptibility markers by GWAS in a representative sample of the Brazilian population through a structured approach. In the present study, we confirmed the

significant association of the polymorphisms rs742071 at 1p36, rs1873147 at 15q22.2 and rs227731 at 17q22 with NSCL/P, and further demonstrated a modest association between rs7590268 (2p21) and rs8001641 (13q31.1) and NSCL/P etiology.

The rs742071 polymorphism resides in an intron of *PAX7* gene at 1p36. Members of the *PAX* family have a crucial role during embryonic development, such mutations in *PAX3* have been associated with Waardenburg syndrome, which contains oral clefting in the phenotypic spectrum (Pingault et al., 2010) and *Pax7* mutant mice revealed malformations in the facial skeletal structures related to neural crest cell proliferation defects (Mansouri et al., 1996). An excess maternal transmission of *PAX7* markers in trios from four populations was previous observed, suggesting that polymorphic variants in *PAX7* may influence the risk of NSCL/P, possibly through imprinting (Sull et al., 2009). Although the GWAS conducted by Beaty et al. (2010) identified positive signals around *PAX7*, it was the meta-analysis performed by Ludwig et al. (2012) that first described the significant association between NSCL/P and the rs742071 polymorphism within *PAX7*. One recent study demonstrated that the *PAX7* rs6659735 trinucleotide polymorphism was significantly associated with oral clefts in patients from United States, and further revealed uncommon missense mutations in *PAX7* gene, suggesting that variants within *PAX7* may contribute to NSCL/P pathogenesis (Butali et al., 2013). Furthermore, Beaty et al. (2013) confirmed the association between NSCL/P and rs742071 in a case-parent study containing 1108 triads from Europe and Philippines. Although a significant association between rs742071 polymorphism and CL±P and its variant CLO was detected in the sample from the Southeast region of Brazil (population with highest European ancestry), was only when we combined the groups that a strong association (which resisted to Bonferroni correction for multiple tests) was observed with CL±P. This sample size issue suggests that *PAX7* rs742071 polymorphism has a modest effect on nonsyndromic oral cleft pathogenesis in the Brazilian patients, and it is possible that the European ancestry is driving this association.

Mangold et al. (2010) identified 2 new loci associated with NSCL/P at 17q22 (rs227731 and rs17760296). For rs227731, the RR_{Hom} was 1.84 (95% CI: 1.32-2.53, $p=1.07x10^{-8}$). In a Polish population, rs227731 polymorphism increased the risk of NSCL/P when analyzed under a dominant model (OR: 1.732, 95% CI: 0.184-2.253, p=0.0044) (Mostowska et al., 2012). The presence of the C risk allele of rs227731 was also significantly associated with NSCL/P in a population from Honduras (Lennon et al., 2012). The recent genome meta-analyses confirmed the association of rs227731 with $p=1.78x10^{-8}$ (Ludwig et al., 2012). In the present study, we observe an increased RR of 1.49 in homozygous (95% CI: 1.27-1.75). However, no support for rs227731 was found in African, Chinese, Estonian or Mesoamerican populations (Rojas-Martinez et al., 2010; Nikopensius et al., 2010; Pan et al., 2011; Weatherley-White et al., 2011), but limited power of the sample can not be excluded as a reason for the lack of association. The rs227731 polymorphism is located in an intergenic region of chromosome 17q22, which may be in linkage disequilibrium with other polymorphisms or have regulatory effects on adjacent genes. This region contains several genes, including *NOG* at ~100 kb. NOGGIN, the protein encoded by *NOG* gene, is a potent antagonist of bone morphogenetic proteins (BMP) with direct effects related to proliferation and apoptosis (Krause et al., 2011). *Nog* transgenic mice demonstrated cleft palate, which was due to alterations in BMP signaling with aberrant cell proliferation and cell death (He et al., 2010). The significant association of rs227731 polymorphism was observed in the group with highest proportions of European ancestry, revealing that the susceptibility of this marker in the Brazilian population is dependent of the ancestry composition of subject.

The rs1873147 polymorphism is located at 15q22.2, an intergenic region that containing important sites with transcription activity (Ludwig et al., 2012). The suggestion of rs1873147 involvement in the pathogenesis of oral clefts was first described by Mangold et al. (2010), and later confirmed by Ludwig et al. (2012). Interestingly, the meta-analysis revealed that this polymorphism is associated with oral clefts in populations from European origin, but not Asian one (Ludwig et al.,

2012). Our results showed that the rs1873147 polymorphism at 15q22.2 is a susceptibility marker for nonsyndromic oral clefts in patients with high percentage of African ancestry. In contrast, a recent study revealed that the presence of the rs1873147 minor allele has a protective effect against NSCL/P in the Chinese Han population (Pan et al., 2013). Taken together, the findings suggest that this polymorphism may have different effects on NSCL/P etiologic depending on specific ancestry.

The results presented here also revealed a modest association of rs7590268 and rs8001641 polymorphisms with NSCL/P in the sample enriched by European ancestry, which disappeared after controlling for multiple comparisons. Further analyses with larger samples and different populations are needed to clarify the underlying role of those polymorphisms in NSCL/P. No significant association with rs12543318 at 8q21.3 was obtained in the examined Brazilian population, demonstrating that they presence may not play an important role in the etiology of the NSCL/P in this highly admixed population.

In summary, our study confirmed the importance of 1p36 (rs742071), 15q22 (rs1873147) and 17p22 (rs227731) loci in the NSCL/P susceptibility in the Brazilian population, and demonstrated a putative and modest association of the rs7590268 and rs8001641 polymorphisms. Furthermore, our findings showed that the diversity of the Brazilian population clearly influences the susceptibility of specific polymorphisms in pathogenesis of NSCL/P. The strength of these associations should be accessed in samples from other countries, particularly in those composed by ancestrally admixed populations, to improving our understanding of the etiology of this complex disease.

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Table 1. Characteristics of the single nucleotide polymorphisms of the present study.

	Chromosome Position*	Location/Gene	Alelle	Minor Allele Frequency*
rs742071 (1p36)	18979874	Intron/*PAX7*	G/t	0.304
rs7590268 (2p21)	43540125	Intron/*THADA*	T/g	0.167
rs7632427 (3p11.1)	89534377	Intergenic	T/c	0.388
rs12543318 (8q21.2)	88868340	Intergenic	A/c	0.391
rs8001641 (13q31.1)	80692811	Intergenic	G/a	0.291
rs1873147 (15q22.2)	63312632	Intergenic	T/c	0.457
rs227731 (17q22)	54773238	Intergenic	A/c	0.488

*Reference: http://www.ncbi.nlm.nih.gov

Table 2. Gender distribution and proportions of the European, African and Amerindian ancestry of each group.

Sample		Gender		Genomic Ancestry		
		Male	Female	European	African	Amerindian
Total	Control	44%	56%	85%	12.8%	2.2%
	CL±P	53.5%	46.5%	79.8%	18.4%	1.8%
	CLO	50%	50%	80.5%	17.9%	1.6%
	CLP	55.7%	44.3%	79.7%	18.4%	1.9%
Minas Gerais	Control	49.6%	50.4%	90%	7.5%	2.5%
(Southeast)	CL±P	55.9%	44.1%	87.5%	10.7%	1.8%
	CLO	54.9%	45.1%	87%	11.2%	1.8%
	CLP	57.3%	42.7%	88.2%	10.1%	1.7%
Bahia	Control	33.2%	66.8%	84.6%	14%	1.4%
(Northeast)	CL±P	50.2%	49.8%	76%	22.2%	1.8%
	CLO	41.6%	58.4%	76.4%	22.2%	1.4%
	CLP	53.6%	46.4%	76.4%	22.1%	1.9%

CL±P: cleft lip with or without cleft palate; CLO: cleft lip only; CLP: cleft lip and palate.

Table 3. Case-control distribution of the alleles and genotypes of the polymorphisms studied for association with oral clefts in the combined Southeast and Northeast samples.

	HWE (p value)	Control (n=594)	CL±P (n=505)	OR_{allele} (95% CI) p value	CLO (n=162)	OR_{allele} (95% CI) p value	CLP (n=343)	OR_{allele} (95% CI) p value
rs742071 (1p36)								
Allele (T/G)	0.27	491/697	481/529	1.29 (1.09-1.53)	158/166	1.35 (1.06-1.73)	323/363	1.26 (1.05-1.53)
Genotype (TT/TG/GG)		95/301/198	106/269/130	0.005	38/82/42	0.08	68/187/88	0.01
rs7590268 (2p21)								
Allele (G/T)	0.34	240/946	234/772	1.19 (0.97-1.46)	82/236	1.37 (1.02-1.83)	152/536	1.12 (0.89-1.41)
Genotype (GG/GT/TT)		28/184/381	23/188/292	0.23	7/68/84	0.044	16/120/208	0.71
rs12543318 (8q21.3)								
Allele (C/A)	0.71	415/763	368/638	1.06 (0.89-1.26)	114/208	1.00 (0.77-1.30)	254/430	1.08 (0.89-1.32)
Genotype (CC/CA/AA)		71/273/245	72/224/207	0.86	25/64/72	0.29	47/160/135	0.63
rs8001641 (13q31.1)								
Allele (A/G)	0.26	465/717	420/590	1.09 (0.92-1.30)	128/194	1.02 (0.79-1.31)	292/396	1.14 (0.94-1.37)
Genotype (AA/AG/GG)		98/269/224	86/248/171	0.048	22/84/55	0.21	64/164/116	0.10
rs1873147 (15q22.2)								
Allele (C/T)	0.45	369/803	371/633	1.27 (1.07-1.52)	123/201	1.33 (1.03-1.72)	248/432	1.25 (1.02-1.52)
Genotype (CC/CT/TT)		62/245/279	63/245/194	0.027	23/77/62	0.06	40/168/132	0.09
rs227731 (17q22)								
Allele (C/A)	0.82	539/645	567/435	1.56 (1.32-1.85)	191/125	1.83 (1.42-2.35)	376/310	1.42 (1.17-1.71)
Genotype (CC/CA/AA)		124/291/177	164/239/98	$1.7x10^{-6}$	58/75/25	$1.5x10^{-5}$	106/164/73	0.004

HWE: Hardy-Weinberg equilibrium. CL±P: cleft lip with or without cleft palate, CLO: cleft lip only, CLP: cleft lip and palate.

Table 4. Analyses in the genetic models of dominance and recessiveness. In the dominant model, it was assumed that the heterozygote and the rare homozygote have the same disease odds, whereas in the recessive model the rare homozygote has different disease odds compared to the common homozygote and heterozygote.

	Dominant Model OR (95% CI), p value			Recessive Model OR (95% CI), p value		
	CL±P	CLO	CLP	CL±P	CLO	CLP
rs742071	1.44 (1.11-1.87)	1.43 (0.96-2.11)	1.45 (1.07-1.95)	1.39 (1.03-1.89)	1.61 (1.05-2.46)	1.35 (0.96-1.91)
	0.0061	0.07	0.014	0.035	0.027	0.08
rs7590268	1.29 (1.01-1.65)	1.60 (1.13-2.28)	1.17 (0.89-1.54)	0.97 (0.55-1.70)	0.93 (0.39-2.17)	0.98 (0.52-1.85)
	0.036	0.008	0.25	0.91	0.86	0.96
rs12543318	1.02 (0.80-1.30)	0.88 (0.62-1.25)	1.09 (0.83-1.43)	1.29 (0.91-1.83)	1.42 (0.86-2.32)	1.23 (0.83-1.82)
	0.90	0.53	0.53	0.15	0.16	0.30
rs8001641	1.19 (0.93-1.53)	1.17 (0.81-1.69)	1.20 (0.90-1.58)	1.03 (0.75-1.42)	0.79 (0.48-1.31)	1.15 (0.81-1.63)
	0.18	0.41	0.20	0.84	0.37	0.43
rs1873147	1.44 (1.13-1.84)	1.46 (1.03-2.09)	1.43 (1.09-1.88)	1.21 (0.83-1.76)	1.40 (0.83-2.34)	1.13 (0.74-1.72)
	0.0029	0.034	0.009	0.31	0.19	0.58
rs227731	1.75 (1.32-2.32)	2.27 (1.43-3.60)	1.57 (1.15-2.16)	1.83 (1.40-2.41)	2.19 (1.50-3.20)	1.69 (1.25-2.28)
	8.7×10^{-5}	0.0004	0.004	1×10^{-5}	1.4×10^{-5}	0.0007

CL±P: cleft lip with or without cleft palate, CLO: cleft lip, CLP: cleft lip and palate.

Table 5. Case-control distribution of the alleles and genotypes in the sample from the Southeast geographic region of the Brazil (Minas Gerais state). .

	HWE (p value)	Control (n=395)	CL±P (n=299)	OR_{allele} (95% CI) p value	CLO (n=102)	OR_{allele} (95% CI) p value	CLP (n=197)	OR_{allele} (95% CI) p value
rs742071 (1p36)								
Allele (T/G)	0.92	322/466	295/303	1.41 (1.14-1.75)	107/97	1.59 (1.17-2.17)	188/206	1.32 (1.04-1.68)
Genotype (TT/TG/GG)		66/190/138	70/155/74	0.014	27/53/22	0.031	43/102/52	0.10
rs7590268 (2p21)								
Allele (G/T)	0.53	159/631	146/446	1.30 (1.00-1.67)	54/148	1.45 (1.01-2.07)	92/298	1.22 (0.91-1.64)
Genotype (GG/GT/TT)		18/123/254	15/116/165	0.084	6/42/53	0.061	9/74/112	0.42
rs12543318 (8q21.3)								
Allele (C/A)	0.57	267/517	224/370	1.17 (0.94-1.46)	72/132	1.06 (0.76-1.46)	152/238	1.24 (0.96-1.59)
Genotype (CC/CA/AA)		43/181/168	47/130/120	0.51	16/40/46	0.27	31/90/74	0.24
rs8001641 (13q31.1)								
Allele (A/G)	0.69	318/470	263/333	1.17 (0.94-1.45)	84/120	1.03 (0.75-1.41)	179/213	1.24 (0.97-1.58)
Genotype (AA/AG/GG)		66/186/142	54/155/89	0.027	16/52/34	0.023	38/103/55	0.071
rs1873147 (15q22.2)								
Allele (C/T)	0.32	251/535	204/392	1.11 (0.88-1.40)	69/135	1.09 (0.78-1.51)	135/257	1.12 (0.86-1.45)
Genotype (CC/CT/TT)		44/163/186	29/146/123	0.66	10/49/43	0.63	19/97/80	0.71
rs227731 (17q22)								
Allele (C/A)	0.35	329/461	320/270	1.66 (1.34-2.06)	117/81	2.02 (1.47-2.78)	203/189	1.51 (1.18-1.92)
Genotype (CC/CA/AA)		64/201/130	86/148/61	0.001	31/55/13	0.00001	55/93/48	0.005

HWE: Hardy-Weinberg equilibrium. CL±P: cleft lip with or without cleft palate, CLO: cleft lip only, CLP: cleft lip and palate.

Table 6. Case-control distribution of the alleles and genotypes in the sample from the Northeast geographic region of the Brazil (Bahia state).

	HWE (p value)	Group (n=202)	CL±P (n=211)	OR_{allele} (95% CI) p value	CLO (n=60)	OR_{allele} (95% CI) p value	CLP (n=151)	OR_{allele} (95% CI) p value
rs742071 (1p36)								
Allele (T/G)	0.06	169/231	186/226	1.12 (0.85-1.48)	51/69	1.01 (0.66-1.53)	135/157	1.17 (0.86-1.59)
Genotype (TT/TG/GG)		29/111/60	36/114/56	0.28	11/29/20	0.87	25/85/36	0.29
rs7590268 (2p21)								
Allele (G/T)	0.45	81/315	88/326	1.05 (0.74-1.47)	28/88	1.23 (0.75-2.02)	60/238	0.98 (0.67-1.42)
Genotype (GG/GT/TT)		10/61/127	8/72/127	0.82	1/26/31	0.75	7/46/96	0.80
rs12543318 (8q21.3)								
Allele (C/A)	1.00	148/246	144/268	0.89 (0.67-1.19)	42/76	0.92 (0.59-1.41)	102/192	0.88 (0.64-1.21)
Genotype (CC/CA/AA)		28/92/77	25/94/87	0.84	9/24/26	0.82	16/70/61	0.83
rs8001641 (13q31.1)								
Allele (A/G)	0.16	147/247	157/257	1.02 (0.77-1.36)	44/74	0.99 (0.65-1.53)	113/183	1.04 (0.76-1.41)
Genotype (AA/AG/GG)		32/83/82	32/93/82	0.81	6/32/21	0.70	26/61/61	0.83
rs1873147 (15q22.2)								
Allele (C/T)	1.00	118/268	167/241	1.57 (1.17-2.11)	54/66	1.86 (1.22-2.83)	113/175	1.47 (1.06-2.02)
Genotype (CC/CT/TT)		18/82/93	34/99/71	0.005	13/28/19	0.006	21/71/52	0.069
rs227731 (17q22)								
Allele (C/A)	0.25	210/184	247/165	1.31 (0.99-1.73)	74/44	1.47 (0.96-2.25)	173/121	1.25 (0.92-1.70)
Genotype (CC/CA/AA)		60/90/47	78/91/37	0.12	27/20/12	0.20	51/71/25	0.22

HWE: Hardy-Weinberg equilibrium. CL±P: cleft lip with or without tcleft palate, CLO: cleft lip only, CLP: cleft lip and palate.

Table 7. Prediction risk of NSCL/P based on epistatic interactions among polymorphisms by multifactor dimensionality reduction (MDR) test in the combined Southeast and Northeast samples.

Number of factors	Best model	Testing accuracy	Cross validation consistency	P value[a]
2	rs227731 rs742071	0.58	10/10	0.0046
3	rs227731 rs742071 rs1873147	0.57	9/10	0.11
4	rs227731 rs742071 rs1873147 rs12543318	0.55	7/10	0.25
5	rs227731 rs742071 rs1873147 rs12543318 rs8001641	0.53	8/10	0.43
6	rs227731 rs742071 rs1873147 rs12543318 rs8001641 rs7590268	0.55	8/10	0.24

[a]p value based on 1.000 permutations

CONSIDERAÇÕES GERAIS

No primeiro capítulo deste estudo, verificamos que alguns polimorfismos nos genes *FGF12*, *VCL* e *CX43* não estão associados à FL/PNS na população brasileira. Essa falta de associação poderia ser atribuída ao tamanho da amostra, já que associações mais fracas podem não ser detectadas em amostras pequenas. Entretanto, a nossa amostra é robusta e o poder de detectar associação nos polimorfismos avaliados nesses genes foi alto. A análise de haplótipos mostrou ainda uma maior frequência de um haplótipo formado por três polimorfismos do *VAX1* no grupo caso, indicando que este gene pode estar envolvido na etiopatogênese das FL/PNS. Entretanto, deve-se considerar que a associação do haplótipo de risco não foi mantida após a correção de Bonferroni e a frequência do haplótipo de risco foi baixa.

No segundo capítulo, avaliamos a participação de sete marcadores previamente associados à FL/PNS em GWAS. Numa análise estruturada de acordo com a ancestralidade, três polimorfismos mostraram associação significante, o rs227731 do *lócus* 17q22, rs1873147 da região 15q22.2 e o rs742071 da região 1p36. Embora tenha sido identificado em um GWAS, replicado em dois estudos e identificado novamente em uma meta-análise a partir de dois GWAS, o polimorfismo rs227731 não foi associado em outras quatro populações, indicando que a contribuição relativa dos genes é variável de acordo com a etnia. Interessantemente, a associação significante desse polimorfismo foi direcionada pelo grupo com maior proporção de marcadores de origem europeia, sugerindo que a susceptibilidade deste polimorfismo para FL/PNS na população brasileira é dependente da composição ancestral do indivíduo. O polimorfismo rs742071 foi associado apenas recentemente a FL/PNS em um estudo, tendo sido confirmado posteriormente em trios. Neste polimorfismo, uma fraca associação foi observada com a população com maior proporção de marcadores de origem européia, tendo sido significante (após a correção de Bonferoni) com as duas populações somadas, indicando que o polimorfismo rs742071 do gene *PAX7* possui apenas um modesto efeito em FL/PNS na população brasileira e é possível que a

ancestralidade européia esteja relacionada a essa associação. O polimorfismo rs1873147 foi associado à população brasileira com uma proporção maior de marcadores de origem africana (em comparação ao outro grupo). Um estudo mostrou que esse polimorfimo estava associado à FL/PNS de origem européia, mas não com indivíduos de origem asiática, enquanto que um segundo estudo mostrou que esse polimorfismo possui efeito protetor quanto às FL/PNS, indicando que o polimofismo rs1873147 possui diferentes efeitos na etiologia das fissuras do lábio ou palato não sindrômicas dependendo da ancestralidade.

Foi observada ainda uma associação do polimorfismo rs7590268 com FL e do polimorfismo rs8001841 com FL/PNS, que não foi mantida após a correção de Bonferroni para múltiplos testes. Estes polimorfismos foram recentemente associados às FL/PNS numa meta-análise com populações européias e asiáticas, entretanto na população brasileira observou-se apenas uma associação modesta destes marcadores com FL/PNS.

Nos últimos anos tem ocorrido uma evolução no entendimento dos fatores genéticos relacionados à etiologia das FL/PNS, embora os mecanismos exatos permaneçam incertos. A identificação e replicação de variantes genéticas associadas a esta malformação em diferentes populações, como o presente estudo, possibilitará maior conhecimento dos mecanismos biológicos envolvidos no desenvolvimento craniofacial, o que poderá resultar em melhor orientação genética, prevenção e prognóstico para os indivíduos afetados.

CONCLUSÃO

- Os resultados do presente estudo demonstraram que os polimorfismos e haplótipos avaliados nos genes *FGF12*, *VCL* e *CX43* não estão associados a fissuras do lábio ou palato não sindrômicas.

- Foi observada uma associação de haplótipo formado pelos polimorfismos rs10787760, rs6585429 e rs1871345 do gene *VAX1*, sugerindo que este gene está relacionado a fissuras do lábio ou palato não sindrômicas, porém com baixa penetrância.

- Os polimorfismos rs227731 (17q22), rs1873147 (15q22.2) e rs742071 (1p36) foram significativamente associados a um risco aumentado para o desenvolvimento de fissuras, sendo que a associação do polimorfismo rs227731 foi dirigida pela população com maior proporção de marcadores de origem européia e o polimorfimo rs1873147, pela população com proporção maior de marcadores de origem africana. O polimorfismo rs742071 foi significativamente associado a fissuras do lábio ou palato não sindrômicas apenas com as duas populações combinadas.

- De forma modesta, o polimorfismo rs7590268 em 2p21 foi associado à fissura labial e o polimorfismo rs8001641 em 13q31.1 em 15q22.2 foram associados às fissuras do lábio ou palato não sindrômicas.

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ANEXO 1

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Avaliação de polimorfismos gênicos em pacientes com fissuras lábio-palatinas não-sindrômicas**", protocolo nº 152/2009, dos pesquisadores Ricardo Della Coletta, Lívia Máris Ribeiro Paranaíba e Sibele Nascimento de Aquino, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 28/06/2010.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project "**Polymorphisms in patients with nonsyndromic cleft lip and palate**", register number 152/2009, of Ricardo Della Coletta, Lívia Máris Ribeiro Paranaíba and Sibele Nascimento de Aquino, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 06/28/2010.

Prof. Dr. Pablo Agustin Vargas
Secretário
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.