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Association of BMP4 polymorphisms with non-syndromic cleft lip with or without cleft palate and isolated cleft palate in Latvian and Lithuanian populations

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SUMMARY

Cleft lip with or without cleft palate (CLP and CL, respectively) and isolated cleft palate (CP) represent one of the most common human birth defects, with a prevalence of approximately 1 in 300-2500 depending on the population. Formation of non-syndromic CL/CLP and CP arises from the interaction of environmental and genetic factors. The objective of this study was to investigate the association between the BMP4 gene (encoding bone morphogenetic protein 4) and non-syndromic CL/CLP and CP in order to clarify the role of this gene in the aetiology of the malformation in Latvian and Lithuanian populations. We genotyped three markers of the BMP4 gene (rs17563, rs2071047 and rs1957860) in order to perform single marker and haplotype association analyses for Latvian and Lithuanian non-syndromic CL/CLP and CP patients and controls. Transmission disequilibrium test was also conducted for Latvian and Lithuanian proband-parent trios. The case-control analysis revealed that SNP rs2071047 allele A was associated with a decreased risk of CL/CLP in the Latvian population, which was confirmed by the haplotype analysis. A modest association was detected between SNP rs1957860 and CP in the Lithuanian population, where allele C was associated with a decreased risk of this cleft phenotype, corroborating haplotype analysis data. Our findings support a role of the BMP4 gene in the aetiology of non-syndromic CL/CLP and CP in the studied populations.

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Key words: BMP4 gene, non-syndromic clefts, case-control analysis, haplotype analysis, TDT analysis.

INTRODUCTION

Isolated cleft lip with or without cleft palate (CLP and CL, respectively) and cleft palate (CP) represent one of the most common birth defects worldwide, with a prevalence of approximately 1 in 700 live births in European populations. The aetiology of non-syndromic CL/CLP and CP is determined by a multifactorial model of inheritance in which genetic risk factors of small individual impact may interact with environmental factors (1). The identification of susceptibility genes for this malformation has been the subject of extensive research over the last 70 years. There are many different genetic approaches such as genome - wide and gene linkage scans, association studies, fine mapping, informative mice models and also gene expression studies in mice and human embryonic tissues in order to identify new genes involved in the aetiology of CL/CLP and CP and clarify the roles of previously reported genes. In the last 10 years, several studies have discovered and confirmed

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chromosomal regions such as 1p21-p31, 1q32, 2p13, 3q27-28, 4q21-q26, 8q24, 9q21, 10q25.3, 12p11, 14q21-24, 16q24 and 17q22 (2-7). However, despite the large number of candidate genes investigated, only the *IRF6* gene has shown a convincing degree of consistency across studies, being considered to be responsible for 12-18% of non-syndromic CL/CLP and CP (8). Mutation screening of more than 20 non-syndromic CL/CLP and CP candidate genes showed that only 2–6% of all screened individuals have mutations in genes including *FOXE1*, *GLI2*, *JAG2*, *LHX8*, *MSX1*, *MSX2*, *SATB2*, *SKI*, *SPRY2* and *TBX10* (9, 10). It has been proposed that the FGF signalling pathway may contribute to about 3–5% of non-syndromic CL/CLP and CP cases (11).

The *BMP4* gene (encoding bone morphogenetic protein 4), located at 14q22-q23 in humans, is a member of the transforming growth factor-beta superfamily. Expression studies of bone morphogenetic proteins (BMPs) and their antagonist Noggin in the embryonic chicken face have suggested that BMP signals are important for closure of the upper lip or primary palate. Gain- and loss-of-function experiments showed that BMPs regulate outgrowth and epithelial survival during avian lip fusion (12). Conditional inactivation of Bmp4 in a transgenic mouse line has been found to result in an isolated CL (13). The *BMP4* gene has been suggested as a candidate gene for non-syndromic CL/CLP and CP because of its role in the regulation of skeletal development including cartilage and bone formation during craniofacial and limb development (14). Based on these discoveries, several association studies have been conducted across different populations to identify genetic variants of the *BMP4* gene which could be associated with non-syndromic CL/CLP or CP in humans (16-19).

In the present study, we examined the association between *BMP4* gene markers and non-syndromic CL/CLP and CP to clarify the role of the *BMP4* gene in the aetiology of the malformation in Latvian and Lithuanian populations.

MATERIAL AND METHODS

Subjects

For the case-control study, 354 individuals from Latvia and 218 individuals from Lithuania were analysed. The Latvian data set consisted of 164 non-syndromic CL/CLP and CP patients and 190 unrelated, randomly selected unaffected individuals (130 females, 60 males). Of the non-syndromic CL/CLP and CP cases, 127 had CL/CLP (50 females, 77 males) and 37 had CP (21 females, 16 males). The Lithuanian data set consisted of 91 CL/CLP patients (33 females, 58 males), 28 CP patients (16 females, 12 males) and 99 control individuals (54 females, 45 males). Transmission disequilibrium test was conducted for 65 trios (affected sib with both parents) from the Latvian population (38 CL/CLP, 27 CP) and 115 trios from the Lithuanian population (88 CL/CLP, 27 CP).

For inclusion in the study, there were no restrictions concerning age and gender for patients from Latvia and Lithuania**.** In Latvia, patients and their parents were recruited at the Riga Cleft Lip and Palate Centre, Institute of Stomatology, Rīga Stradiņš University. Clinical geneticists reviewed all the patients and their medical records, and any individual with syndromic CL/CLP/CP, confirmed monogenic syndromes, chromosomal aberrations, associated structural anomalies or mental retardation and adopted individuals were excluded. The Latvian control group consisted of unrelated, unaffected individuals with no family history of CL/CLP/CP. They were randomly selected from the Genome Database of Latvian Population at the Latvian Biomedical Research and Study Centre. As with the patients, no age or gender restrictions were applied. Individuals with syndromic CL/CLP/CP or any confirmed inherited pathology were excluded. In Lithuania, patients and their parents were recruited at the Centre for Medical Genetics, Vilnius University Hospital Santariškių Klinikos in collaboration with the Clinic of Maxillo-facial and Oral Surgery, Institute of Odontology of Vilnius University. All the patients were examined by several team members (clinical geneticists, orthodontists, maxillofacial and oral surgeons). If required, laboratory tests (e.g. cytogenetic, metabolic and molecular testing), X-ray imaging (e.g. cranial, thorax, spine, extremities and panoramic dental X-ray) and other necessary instrumental investigations (brain, heart and visceral organ ultrasound, MRI, etc.) were performed. Patients with confirmed monogenic syndromes or identified causes of teratogenic factors, chromosomal aberrations, associated malformations or mental retardation were excluded. As controls, phenotypically normal unrelated individuals without family history of CL/CLP and CP from six ethnolinguistic groups of Lithuania were selected.

The data collection was performed in accordance with the regulations issued by the Central Medical Ethics Committee of Latvia and the Lithuanian Bioethics Committee. Prior to any research procedure, all participating individuals signed an informed consent form. In the case of patients who

were under 18 years of age, consent was obtained from their parents or legal guardians.

Methods

The genomic DNA of non-syndromic CL/CLP and CP patients and population samples was obtained from venous blood or saliva and extracted according to the established protocol of the phenol-chloroform method with slight modifications (15).

Three *BMP4* gene markers (rs1957860, rs17563 and rs2071047) were selected for study based on recent publications regarding confirmed linkage studies and associations with non-syndromic CL/CLP and CP (16, 18, 20).

Genotyping was performed using TaqMan standard assays (Applied Biosystems, California, USA) on automatic sequence-detection instruments 7500 Real-Time PCR System and ViiA™ 7 Real-Time PCR System (Applied Biosystems). Reactions were carried out under standard conditions as recommended by the manufacturer.

All analysed markers were tested for Hardy-Weinberg equilibrium in controls and affected individuals using Pearson's chi-square test with one degree of freedom. Allele frequency differences between non-syndromic CL/CLP and CP patients and control subjects were compared for each marker using the standard chi-square test with one degree of freedom. Allelic odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using the standard chi-square test, assuming a multiplicative model. The level of statistical significance was

Table 1. Case-control analysis of the association of BMP4 SNPs with CL/CLP and CP in Latvian and Lithuanian populations

Chr*	Gene	SNP^	Location	Alleles#	MAF**		p-value	OR^^	95% CI##	p-value adjusted
					Cases	Controls				
LATVIA										
CL/CLP										
14	*BMP4*	rs17563	34580722	G/A	0.349	0.446	0.018	0.67	0.476–0.933	0.053
14	*BMP4*	rs2071047	34581611	G/A	0.303	0.408	0.009	0.63	0.446–0.891	0.026
14	*BMP4*	rs1957860	34592555	T/C	0.429	0.446	0.679	0.93	0.672–1.296	1
CP										
14	*BMP4*	rs17563	34580722	G/A	0.386	0.446	0.354	0.78	0.463–1.318	1
14	*BMP4*	rs2071047	34581611	G/A	0.386	0.408	0.732	0.91	0.540–1.541	1
14	*BMP4*	rs1957860	34592555	T/C	0.529	0.446	0.202	1.40	0.836–2.328	0.606
LITHUANIA										
CL/CLP										
14	*BMP4*	rs17563	34580722	G/A	0.418	0.389	0.569	1.13	0.747–1.698	1
14	*BMP4*	rs2071047	34581611	G/A	0.399	0.338	0.224	1.30	0.852–1.975	0.673
14	*BMP4*	rs1957860	34592555	T/C	0.429	0.485	0.271	0.80	0.531–1.195	0.814
CP										
14	*BMP4*	rs17563	34580722	G/A	0.518	0.389	0.084	1.67	0.929–3.066	0.252
14	*BMP4*	rs2071047	34581611	G/A	0.463	0.338	0.092	1.69	0.915–3.104	0.276
14	*BMP4*	rs1957860	34592555	T/C	0.304	0.485	0.016	0.46	0.246–0.873	0.048
LATVIA and LITHUANIA										
CL/CLP										
14	*BMP4*	rs17563	34580722	G/A	0.380	0.426	0.147	0.83	0.638–1.070	0.441
14	*BMP4*	rs2071047	34581611	G/A	0.344	0.383	0.203	0.84	0.647–1.097	0.608
14	*BMP4*	rs1957860	34592555	T/C	0.426	0.459	0.291	0.87	0.675–1.125	0.873
CP										
14	*BMP4*	rs17563	34580722	G/A	0.444	0.426	0.718	1.08	0.727–1.590	1
14	*BMP4*	rs2071047	34581611	G/A	0.419	0.383	0.457	1.16	0.783–1.724	1
14	*BMP4*	rs1957860	34592555	T/C	0.427	0.459	0.518	0.88	0.593–1.301	1

* Chr – chromosome;
^ SNP – single nucleotide polymorphism;
# Major allele is listed first;
** MAF – minor allele frequency;
^^ OR – odds ratio;
95% CI – 95% confidence interval.

set at α=0.05 for nominal association. Haplotype analysis was performed with the standard chi-square test using the sliding windows approach. PLINK software (21) was used to perform the case-control comparisons and haplotype analysis and also to test for transmission distortions in the proband-parent triads. Bonferroni correction was applied for multiple testing.

Table 2. Case-control analysis of the association of BMP4 haplotypes with CL/CLP in Latvian population

Haplotype	SNP 1	SNP 2	SNP 3	Frequency		p-value
				Cases	Controls	
	rs17563	rs2071047	rs1957860			
WIN1	A	A	C	0.058	0.115	0.018
WIN1	G	G	T	0.290	0.236	0.134
WIN1	A	A	T	0.247	0.297	0.182
WIN1	G	G	C	0.366	0.325	0.296
WIN1	A	G	T	0.039	0.028	0.461
	rs17563	rs2071047				
WIN1	A	A	*	0.303	0.408	0.009
WIN1	G	G	*	0.651	0.554	0.018
WIN1	A	G	*	0.046	0.038	0.621
	rs2071047	rs1957860				
WIN2	A	C	*	0.057	0.114	0.017
WIN2	G	T	*	0.325	0.261	0.086
WIN2	A	T	*	0.246	0.294	0.200
WIN2	G	C	*	0.372	0.332	0.307

WIN1 – sliding window 1; WIN2 – sliding window 2.

Table 3. Case-control analysis of the association of BMP4 haplotypes with CP in Lithuanian population

Haplotype	SNP 1	SNP 2	SNP 3	Frequency		p-value
				Cases	Controls	
	rs17563	rs2071047	rs1957860			
WIN1	A	A	C	0.104	0.073	0.446
WIN1	A	G	C	0.028	0.030	0.923
WIN1	G	G	C	0.172	0.382	0.003
WIN1	A	A	T	0.358	0.266	0.176
WIN1	A	G	T	0.028	0.020	0.733
WIN1	G	G	T	0.311	0.229	0.214
	rs17563	rs2071047				
WIN1	A	A	*	0.463	0.338	0.092
WIN1	A	G	*	0.056	0.051	0.882
WIN1	G	G	*	0.482	0.482	0.087
	rs2071047	rs1957860				
WIN2	A	T	*	0.105	0.073	0.440
WIN2	G	T	*	0.191	0.412	0.003
WIN2	A	C	*	0.358	0.265	0.183
WIN2	G	C	*	0.346	0.250	0.159

WIN1 – sliding window 1; WIN2 – sliding window 2.

RESULTS

To perform case-control comparisons of the *BMP4* gene, three SNPs were genotyped for 283 patients with non-syndromic CL/CLP/CP and 289 control individuals from the Latvian and Lithuanian populations. Transmission disequilibrium test was conducted for 180 trios from both populations. Following data cleaning, the overall genotype rate was ~99%. All analysed markers were in Hardy-Weinberg equilibrium.

The results of the case-control comparisons with CL/CLP and CP in the Latvian and Lithuanian populations are presented separately and together in Table 1. The strongest association with CL/CLP was found in the Latvian population for SNP rs2071047 (located in intron 4), where allele A was associated with a decreased risk of CL/CLP (p=0.009, OR=0.63, 95% CI=0.446–0.891). The obtained association remained statistically significant after Bonferroni correction (p adj.=0.026). SNP rs17563 (located in exon 5) showed a borderline association with CL/CLP in the Latvian population (p=0.018, OR=0.67, 95% CI=0.476–0.933), which did not remain significant after correction for multiple testing (p adj.=0.053). Allele A

was associated with a decreased risk of CL/CLP. A modest association was detected between SNP rs1957860 and CP in the Lithuanian population, where allele C was associated with a decreased risk of CP (p=0.016, OR=0.46, 95% CI=0.246–0.873; p adj.=0.048). We did not find any associations of the analysed *BMP4* SNPs with CL/CLP or CP when the two populations were combined.

Haplotype-based association analysis was performed to disclose any additional associations of the *BMP4* gene with CL/CLP or CP in the Latvian and Lithuanian populations. Table 2 shows the results of this analysis regarding CL/CLP in the Latvian population. The strongest association was found for haplotype rs17563-rs2071047 (A-A), which was associated with a decreased risk of the CL/CLP phenotype. The haplotype analysis did not reveal any associations with CL/CLP in the Lithuanian population nor when both populations were combined (data not provided).

The strongest association with CP was found in the Lithuanian population for haplotypes rs17563-rs2071047-rs1957860 (G-G-C) and rs2071047-rs1957860 (G-T), which were associated with a decreased risk of CP (Table 3). The haplotype analysis did not uncover any associations with CP in the Latvian population nor when both populations were combined (data not provided).

Transmission disequilibrium test was conducted for Latvian and Lithuanian non-syndromic CL/CLP

Table 4. Transmission distortion results for BMP4 SNPs in Latvian and Lithuanian CL/CLP and CP individuals

Chr*	Gene	SNP^	Location	Alleles#	Transmitted minor allele count	Untransmitted allele count	p-value	OR^^	95% CI##	p-value adjusted
LATVIA										
CL/CLP										
14	*BMP4*	rs17563	34580722	G/A	16	19	0.612	0.84	0.433–1.638	–
14	*BMP4*	rs2071047	34581611	G/A	11	19	0.144	0.58	0.276–1.217	–
14	*BMP4*	rs1957860	34592555	T/C	20	21	0.876	0.95	0.516–1.757	–
CP										
14	*BMP4*	rs17563	34580722	G/A	4	9	0.166	0.45	0.127–1.443	–
14	*BMP4*	rs2071047	34581611	G/A	4	8	0.248	0.50	0.151–1.660	–
14	*BMP4*	rs1957860	34592555	T/C	12	4	0.046	3.00	0.968–9.302	–
LITHUANIA										
CL/CLP										
14	*BMP4*	rs17563	34580722	G/A	47	41	0.522	1.15	0.754–1.743	1
14	*BMP4*	rs2071047	34581611	G/A	45	41	0.666	1.10	0.719–1.676	1
14	*BMP4*	rs1957860	34592555	T/C	39	53	0.144	0.74	0.487–1.113	0.433
CP										
14	*BMP4*	rs17563	34580722	G/A	14	14	1	1.00	0.477–2.098	1
14	*BMP4*	rs2071047	34581611	G/A	13	12	0.842	1.08	0.494–2.374	1
14	*BMP4*	rs1957860	34592555	T/C	8	12	0.371	0.67	0.273–1.631	1
LATVIA and LITHUANIA										
CL/CLP										
14	*BMP4*	rs17563	34580722	G/A	61	59	0.855	1.03	0.723–1.479	1
14	*BMP4*	rs2071047	34581611	G/A	55	59	0.708	0.93	0.646–1.346	1
14	*BMP4*	rs1957860	34592555	T/C	58	72	0.220	0.81	0.570–1.138	0.659
CP										
14	*BMP4*	rs17563	34580722	G/A	18	22	0.527	0.82	0.439–1.525	1
14	*BMP4*	rs2071047	34581611	G/A	17	20	0.622	0.85	0.445–1.623	1
14	*BMP4*	rs1957860	34592555	T/C	20	15	0.398	1.33	0.683–2.604	1

* Chr – chromosome;
^ SNP – single nucleotide polymorphism;
# Major allele is listed first;
^^ OR – odds ratio;
95% CI – 95% confidence interval.

and CP individuals and their parents to identify transmission distortions. We detected a borderline association between SNP rs1957860 (located ~6 kb downstream of the gene) and CP in the Latvian population (p=0.046) (Table 4). No associations were found for any of the analysed markers with CL/CLP.

DISCUSSION

Animal models represent one of the best approaches for the identification of possible candidate genes for any inherited disease. Consequently, amongst others, the *BMP4* gene has been proposed as a candidate gene for non-syndromic CL/CLP and CP from studies using knockout mouse models (13, 14, 22). In order to ascertain the genetic variants associated with a multifactorial disorder, a single marker association analysis is usually performed. Several studies have reported a positive association between the *BMP4* gene and non-syndromic CL/CLP in humans (16-19). A meta-analysis of 13 genome scans identified six regions on five chromosomes with HLODs ≥3.2. One of these regions, 14q21-25, displayed evidence of linkage with non-syndromic CL/CLP (4). Based on this finding, Lin et al. (16) conducted a case-control analysis of *BMP4* gene polymorphisms and detected an association between the 538T/C polymorphism (rs17563) and non-syndromic CL/CLP in the Chinese population. 538C allele carriers were found to be associated with a significantly increased risk of non-syndromic CL/CLP compared with non-carriers (p=0.005) (16). A mutation analysis of the *BMP4* gene showed a significant over-representation of *BMP4* mutations in cases with a range of lip and orbicularis oris muscle defects and an absence of mutations in more than 500 control samples, thus supporting a role for *BMP4* in the pathogenesis of CL/CLP (17). Suazo et al. (18) analysed the association among three *BMP4* SNPs (rs762642, rs2855532 and rs1957860) and non-syndromic CL/CLP in 150 unrelated trios in the Chilean population. Unlike rs1957860-rs762642 haplotypes (T-T (p=0.018) and C-T (p=0.015)), no significant transmission distortions were identified for the individual SNPs. The authors suggested that despite the positive association detected between these haplotypes and non-syndromic clefts, the associated haplotypes probably do not have a functional effect on *BMP4* expression or protein activity, but possibly reflect non-syndromic CL/CLP susceptibility changes which are in linkage disequilibrium with these polymorphisms. These findings support a role for *BMP4* in non-syndromic CL/CLP in the Chilean population (18). A recent study by Araújo et al. (19) showed that the rs17563 polymorphism in the *BMP4* gene was strongly associated with non-syndromic CL/CLP, with allele C having a protective effect against the occurrence of non-syndromic CL/CLP in the Brazilian population. Additionally, an *in silico* test was performed to assess whether the substitution c.538T>C (rs17563) modifies the structure or function of the encoded protein. The test results demonstrated SNP rs17563's neutral character, corroborating the notion of a protective effect of this marker (19). As opposing findings have been reported in the literature (16, 17, 19), *BMP4* gene polymorphisms clearly have different effects in different populations and races.

Latvians and Lithuanians are geographically close and genetic relation between these populations has been confirmed based on a principal component analysis (23). Our case-control analysis found associations between genetic variations in the *BMP4* gene and non-syndromic CL/CLP in the Latvian population and isolated CP in the Lithuanian population. Haplotype analysis showed similar results, reinforcing the results of the case-control association analysis. However, transmission disequilibrium test – conducted to detect any transmission distortions in Latvian and/or Lithuanian trios – produced conflicting results compared to the single marker association analysis. No significant associations were found between the analysed *BMP4* SNPs and CL/CLP, only evidence for an association with isolated CP in the Latvian data set was found.

CL/CLP and CP represent a common birth defect with a very complex and heterogeneous aetiology. A large number of samples and very accurate sample subphenotyping according to cleft type are required to detect genetic markers involved in the aetiology of CL/CLP and CP and to reduce the risk of false positive results and the possibility that the observed association may be due to random chance.

We analysed individual genetic markers in the *BMP4* gene only and not the interaction between this gene and environmental factors, which can also be a very important factor in the development of CL/CLP and CP. Despite the confirmed genetic relatedness between the Latvian and Lithuanian populations, it is possible that the two populations may have inherently distinct genetic susceptibilities to CL/CLP and CP, which could explain the results presented here.

CONCLUSIONS

Our results demonstrate that the *BMP4* gene

could be involved in the development of non-syndromic CL/CLP and CP and that polymorphisms in this gene could have a protective effect concerning the susceptibility for non-syndromic CL/CLP and CP. In general, our results support previous findings regarding the role of the *BMP4* gene in the development of non-syndromic CL/CLP and CP. However, the number of patients and controls analysed here could be too small to determine genetic markers which have functional effects in the development of non-syndromic CL/CLP and CP, which may explain the obtained results and negative association in the combined Latvian and Lithuanian data set.

In conclusion, our findings attest that non-syndromic CL/CLP/CP is a very complex disease, which is not fully understood. Many genes involved in the aetiology of this malformation remain unidentified, only a few of which play a major role in the development of non-syndromic CL/CLP and CP.

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REFERENCES

1. Rahimov F, Marazita ML, Visel A, Cooper ME, Hitchler MJ, Rubini M, et al. Disruption of an AP-2alpha binding site in an IRF6 enhancer is associated with cleft lip. *Nat Genet* 2008;40:1341-47.
2. Marazita ML, Murray JC, Lidral AC, Arcos-Burgos M, Cooper ME, Goldstein T, et al. Meta-analysis of 13 genome scans reveals multiple cleft lip/palate genes with novel loci on 9q21 and 2q32-35. *Am J Hum Genet* 2004;75:161-73.
3. Riley BM, Schultz RE, Cooper ME, Goldstein-McHenry T, Daack-Hirsch S, Lee KT, et al. A genome-wide linkage scan for cleft lip and cleft palate identifies a novel locus on 8p11-23. *Am J Med Genet A* 2007;15;143A:846-52.
4. Marazita ML, Lidral AC, Murray JC, Field LL, Maher BS, McHenry TG, et al. Genome scan, fine-mapping, and candidate gene analysis of non-syndromic cleft lip with or without cleft palate reveals phenotype-specific differences in linkage and association results. *Hum Hered* 2009;68:151-70.
5. Birnbaum S, Ludwig KU, Reutter H, Herms S, Steffens M, Rubini M, et al. Key susceptibility locus for nonsyndromic cleft lip with or without cleft palate on chromosome 8q24. *Nat Genet* 2009;41:473-77.
6. Mangold E, Reutter H, Birnbaum S, Walier M, Mattheisen M, Henschke H, et al. Genome-wide linkage scan of nonsyndromic orofacial clefting in 91 families of central European origin. *Am J Med Genet A* 2009;149A:2680-94.
7. Mangold E, Ludwig KU, Birnbaum S, Baluardo C, Ferrian M, Herms S, et al. Genome-wide association study identifies two susceptibility loci for nonsyndromic cleft lip with or without cleft palate. *Nat Genet* 2010;42:24-6.
8. Zucchero TM, Cooper ME, Maher BS, Daack-Hirsch S, Nepomuceno B, Ribeiro L, et al. Interferon regulatory factor 6 (IRF6) gene variants and the risk of isolated cleft lip or palate. *N Engl J Med* 2004;351:769-80.
9. Vieira AR, Avila JR, Daack-Hirsch S, Dragan E, Félix TM, Rahimov F, et al. Medical sequencing of candidate genes for nonsyndromic cleft lip and palate. *PLoS Genet* 2005;1:e64.
10. Jezewski PA, Vieira AR, Nishimura C, Ludwig B, Johnson M, O'Brien SE, et al. Complete sequencing shows a role for MSX1 in non-syndromic cleft lip and palate. J *Med Genet* 2003;40:399-407.
11. Riley BM, Mansilla MA, Ma J, Daack-Hirsch S, Maher BS, Raffensperger LM, et al. Impaired FGF signaling contributes to cleft lip and palate. *Proc Natl Acad Sci U S A* 2007;104:4512-17.
12. Ashique AM, Fu K, Richman JM. Endogenous bone morphogenetic proteins regulate outgrowth and epithelial survival during avian lip fusion. *Development* 2002;129:4647-60.
13. Liu W, Sun X, Braut A, Mishina Y, Behringer RR, Mina M, et al. Distinct functions for Bmp signaling in lip and palate fusion in mice. *Development* 2005;132:1453-61.
14. Wan M, Cao X. BMP signaling in skeletal development. *Biochem Biophys Res Commun* 2005;328:651-57.
15. Ausubel I, Frederick M. Current protocols in molecular biology. 1st ed. New York; 1987.
16. Lin JY, Chen YJ, Huang YL, Tang GP, Zhang L, Deng B, et al. Association of bone morphogenetic protein 4 gene polymorphisms with nonsyndromic cleft lip with or without cleft palate in Chinese children. *DNA Cell Biol* 2008;27:601-5.
17. Suzuki S, Marazita ML, Cooper ME, Miwa N, Hing A, Jugessur A et al. Mutations in BMP4 are associated with subepithelial, microform, and overt cleft lip. *Am J Hum Genet* 2009;84:406-11.
18. Suazo J, Santos JL, Jara L, Blanco R. Association between bone morphogenetic protein 4 gene polymorphisms with nonsyndromic cleft lip with or without cleft palate in a chilean population. *DNA Cell Biol* 2010;29:59-64.
19. Araújo TK, Simioni M, Félix TM, de Souza LT, Fontes MÍ, Monlleó IL, et al. Preliminary analysis of the nonsynonymous polymorphism rs17563 in bmp4 gene in brazilian population suggests protection for nonsyndromic cleft lip and palate. *Plast Surg Int* 2012;2012:247104.
20. Chen Q, Wang H, Hetmanski JB, Zhang T, Ruczinski I,

Schwender H, et al. BMP4 Was Associated with NSCL/P in an Asian Population. *PLoS One* 2012;7:e35347.
21. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MAR, Bender D, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 2007;81:559-75.
22. Gong SG and Guo C. Bmp4 gene is expressed at the putative site of fusion in the midfacial region. *Differentation* 2003;71:228-36.
23. Nelis M, Esko T, Mägi R, Zimprich F, Zimprich A, Toncheva D, et al. Genetic structure of Europeans: a view from the North-East. *PLoS One* 2009;4:e5472.

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