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Further evidence of association of *ABCA4* gene with cleft lip/palate

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Abstract

Nonsyndromic cleft lip with or without cleft palate (CL/P) is a common birth defect with complex etiology. Numerous genes and environmental factors and their interactions are thought to play a role in the susceptibility to CL/P. A recent genome-wide association study with several populations revealed markers in/near *MAFB* and *ABCA4* genes as new susceptibility loci for CL/P. We hypothesized that these genes could also contribute to CL/P in a Brazilian population, hence we evaluated if the associated SNPs in *MAFB* [rs13041247 and rs11696257] and *ABCA4* [rs560426 and rs481931] were associated with CL/P in our case-control dataset. We genotyped 812 Caucasian individuals (400 cases and 412 controls) from Brazil,. Allele frequencies were compared for cases and controls as well as for cleft subgroups and controls. *ABCA4* rs540426 showed strong association with CL/P, unilateral and right CL/P, and bilateral CL/P, whereas SNP rs481931 showed borderline association with CL/P, and bilateral CL/P. No association was found for *MAFB* . Our results support a potential role for *ABCA4* in the etiology of CL/P in individuals from Brazil.

Keywords

cleft lip/cleft palate; association; *ABCA4* gene; SNP

Craniofacial anomalies, and in particular oral-facial clefts, are major human birth defects with a worldwide frequency of 1 in 700 live births and substantial clinical impact. The possible etiologies are many, including single-gene disorders, chromosome aberrations, exposure to teratogens, and sporadic conditions of unknown cause (1). Oral-facial clefts can be further classified as nonsyndromic (isolated) or syndromic based on the presence of other structural anomalies. Approximately 30% of all clefts are associated with one of more than 400 described syndromes (2) while the remaining 70% are isolated defects. It is generally accepted that cleft lip with or without cleft palate (CL/P) and cleft palate only (CPO) are

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CONFLICTS OF INTEREST

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genetically distinct phenotypes. CL/P is more common, affecting 1–2/1000 births and presenting considerable differences in prevalence, with Native Americans and Asians showing the highest rate and Africans the lowest. On the other hand, CPO is less common, with a prevalence of approximately 1/1500–2000 births in Caucasians, less variable among different ethnic backgrounds (3). These observations suggest that the relative contribution of individual susceptibility genes may vary across different populations, thus reinforcing the need of replication of association studies in different populations. Further, numerous lines of evidence now suggest that the phenotypic spectrum of nonsyndromic CL/P is more complex than previously realized and therefore genetic studies should include a more accurate description of the cleft phenotype, such as cleft type and laterality, as well as presence of subclinical phenotypes, such as defects in the orbicularis oris muscle and dental anomalies (4,5).

Several loci and genes - including, but not limited to, *MSX1*, *IRF6*, *CRISPLD2*, *FOXE1*, *AXIN2* , and members of the WNT, FGF, and MMP gene families - have been associated with oral clefts (6–17). Additionally, recent advances in research methodologies have accelerated the discovery of loci conferring susceptibility to isolated CL/P through the use of genome-wide association studies (GWAS). The first three GWAS found strong evidence for association of an intergenic marker (rs987525) in the 8q24 chromosomal region with CL/P (18–20), and this association has been independently validated in additional populations, including a population from Brazil (21). Recently, a third GWAS identified associations with markers in/nearby *ABCA4* and *MAFB* genes, located on chromosomes 1p22.1 and 20q11.1-q13.1, respectively, with CL/P in multiple populations (22). In two subsequent studies, the originally associated SNP in *ABCA4* (rs540026) was associated with increased risk of CL/P in US and South American populations (23, 24) whereas a SNP in *MAFB* (rs13041247) was associated with increased risk of CL/P in Chinese (25). Intriguingly, a study with a Nigerian population did not find evidence of association for either *ABCA4* or *MAFB* genes with CL/P (26).

Due to allelic heterogeneity among populations, in order to validate the findings of genetic association studies, it is necessary to independently attempt to replicate these findings in multiple populations. Hence, to further investigate a possible role for *ABCA4* and *MAFB* in the susceptibility to CL/P in a population from Brazil, we tested the previously associated SNPs in *ABC4* (rs560426 and rs481931), and *MAFB* (rs13041247 and rs11696257) genes (Table 1) for association with CL/P in our case-control dataset.

MATERIALS AND METHODS

Subjects

A convenience sample consisting of 812 unrelated Caucasian individuals was included in this study. Of these, cases comprised 400 individuals (average age 17.3 yr, 252 males, 148 females) with isolated CL/P: 246 with unilateral CL/P and 154 cases with bilateral CL/P whereas 412 individuals without CL/P or family history of CL/P (average age 24.8 yearas, 165 males, 247 females) served as controls. Individuals were recruited at the Hospital of Rehabilitation and Craniofacial Anomalies and Bauru Dental School at the University of São Paulo, Brazil, after signing an informed consent. All individuals were from the

Southeast region of Brazil, who are mostly European descendants from Portugal and Spain and have been suggested as bearing ~99% Caucasian ethnicity (27). Only cases with CL/P were recruited for the study, regardless of cleft side. Cases with cleft palate alone were not included. This study was approved by the University of Sao Paulo and University of Texas Health Science Center Institutional Review Boards.

Sample collection and genotyping

Saliva samples were collected from each individual as source of genomic DNA using Oragene kits (DNA Genotek, Ontario, CA). Genotyping was performed using Taqman chemistry (28) in 5uL reactions and detected on a 7900HT Sequence Detection Instrument (Applied Biosystems, Foster City, CA, USA). Assays and reagents were supplied by Applied Biosystems (Applied Biosystems). For quality control purposes, negative control reactions were performed using no nucleic acid template; positive control reactions included samples of known genotypes. Genotyping was performed blind to sample status.

Statistical Analyses

Power calculations were performed using the Genetic Power Calculator (29) and indicate that the sample size would provide approximately 80% statistical power to detect an associaton with an alpha of 0.05, if the markers selected are in linkage disequilibrium with the causal factor (D'=0.8) and their frequencies are around 20%.

Statistical analyses were performed using PLINK software (v.1.06) (30). For each SNP, We tested for deviation from Hardy-Weinberg equilibrium in cases and controls using a Pearson's chi-square test. Association analyses were performed comparing differences in genotype and allele frequencies for each SNP between CL/P cases and controls, and between unilateral and bilateral CL/P cases and controls. We applied Bonferroni correction for multiple testing considering the number of tests and variables (0.05/7) and P≤0.007 was considered statistically significant.

RESULTS and DISCUSSION

There was no evidence of deviation from Hardy-Weinberg equilibrium for any of the SNPs investigated (data not shown). The results of the association analyses are summarized in Tables 2 and 3. We found evidence of genotypic and allelic association for the SNPs in *ABCA4* and CL/P. SNP rs560426 showed association with CL/P (P=0.0002 for genotype, P=0.00007 for allele), particularly bilateral (P=0.0006 for genotype, P=0.001 for allele) and also unilateral (P=0.004 for genotype, P=0.001 for allele) CL/P. Additional associations were also found for *ABCA4* SNP rs481931 alleles and bilateral (P=0.006) and unilateral (P=0.009) CL/P. We did not find association of *MAFB* SNPs and CL/P in our population (Tables 2 and 3).

Much progress has been made in the identification of putative candidate genes for CL/P. Recent genome-wide association studies (GWAS) have identified several novel loci associated with CL/P (18–20, 22). Nonetheless, to validate the findings of these studies and understand the overall impact of these results in the general population, it is necessary to independently replicate these findings in multiple populations. Our replication study

suggests a role for *ABCA4* and not *MAFB* in this Brazilian population of Caucasian ethnicity. Similarly, YUAN et al. (23) found evidence of association of *ABCA4* with CL/P, whereas *MAFB* presented only nominal association values in their study with non-Hispanic white and Hispanic CL/P families. Moreover, both *ABCA4* SNPs rs560426 and rs481931 showed association in the Hispanics, although the association in the non-hispanic whites was stronger for SNP rs481931.

The ABCA4 gene is located on chromosome 1p22.1 and belongs to a superfamily of transmembrane proteins expressed exclusively in retinal photoreceptors (31, 32). So far, there is little biological evidence to support a role for *ABCA4* in craniofacial morphogenesis, particularly since *Abca4* null mice do not exhibit cleft palate (22, 33). It is possible that the associated variants in *ABCA4* are in linkage disequilibrium with a causal variant located in another gene, and act as indirect surrogates for a true etiologic variant in CL/P cases. Nevertheless, the previous associations of *ABCA4* with CL/P in both GWAS (22, 33) and association studies in different populations (23, 24), including the population of the present study, warrants additional investigations on the possible role for this gene in the etiology of CL/P.

In summary, our results provide additional evidence for the association of *ABCA4* with CL/P in a population from Brazil. Given the genetic heterogeneity across populations, it is important to investigate the association of previously reported genes with CL/P in multiple populations.

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Table 1

Details of the SNPs investigated in this study.

Gene	Chromosome	SNP	SNP Function	Alleles[a]
ABC4	1p22	rs560426	intron	c/T
		rs481931	intron	G/t
MAFB	20q11.2-q13.1	rs13041247	intergenic	c/T
		rs11696257	intergenic	C/t

[a]Minor allele indicated as lower case, according to NCBI dbSNP.

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Table 2

Results of genotypic association analysis between single nucleotide polymorphisms (SNPs) in *ABCA4* and *MAFB* genes and nonsyndromic cleft lip/palate (CL/P).

Gene	SNP		CL/P (n=400)			P-value[b]	CL/P Unilateral (n=246)			P-value[b]	CL/P Bilateral (n=154)			P-value[b]
			Genotype[a]				Genotype[a]				Genotype[a]			
			AA	Aa	aa		AA	Aa	aa		AA	Aa	aa	
ABC4	rs560426	Case/control	116/74	118/203	86/123	**0.0002**	70/74	118/203	55/123	**0.004**	45/74	54/203	31/123	**0.0006**
	rs481931	Case/control	184/154	155/192	44/57	**0.02**	113/154	104/192	29/57	0.15	71/154	49/192	14/57	**0.05**
MAFB	rs13041247	Case/control	182/166	165/180	38/42	0.45	110/166	108/180	25/42	0.83	13/42	55/180	71/166	0.24
	rs11696257	Case/control	185/181	163/185	32/41	0.44	115/181	107/185	22/41	0.78	69/181	54/185	10/41	0.29

[a] Order of genotypes, AA/Aa/aa where a is the minor allele

[b] Fisher exact test, significant if P≤0.05. Undetermined genotypes not included in analysis.

Table 3

Results of allelic association analysis between single nucleotide polymorphisms (SNPs) in *ABCA4* and *MAFB* genes and nonsyndromic cleft lip/palate (CL/P).

Gene	SNP		CL/P		P-value[b]	CL/P Unilateral		P-value[b]	CL/P Bilateral		P-value[b]
			Allele[a]			Allele[a]			Allele[a]		
			A	a		A	a		A	a	
ABC4	rs560426	Case/control	406/351	346/449	**0.00007**	258/351	228/449	**0.001**	144/351	116/449	**0.001**
	rs481931	Case/control	523/500	243/306	0.06	330/500	162/306	**0.009**	191/500	77/306	**0.006**
MAFB	rs13041247	Case/control	529/512	241/264	0.25	328/512	158/264	0.58	197/512	81//264	0.14
	rs11696257	Case/control	533/547	227/267	0.21	337/547	151/267	0.48	192/547	74/267	0.13

[a] Order of alleles, a where a is the minor allele

[b] Fisher exact test, significant if P≤0.05.