RESEARCH ARTICLE

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Genetic Contribution for Non-Syndromic Cleft Lip With or Without Cleft Palate (NS CL/P) in Different Regions of Brazil and Implications for Association Studies

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Non-syndromic cleft lip with or without cleft palate (NS CL/P) is a complex disease in which heritability estimates vary widely depending on the population studied. To evaluate the importance of genetic contribution to NS CL/P in the Brazilian population, we conducted a study with 1,042 families from five different locations (Santarém, Fortaleza, Barbalha, Maceió, and Rio de Janeiro). We also evaluated the role of consanguinity and ethnic background. The proportion of familial cases varied significantly across locations, with the highest values found in Santarém (44%) and the lowest in Maceió (23%). Heritability estimates showed a higher genetic contribution to NS CL/P in Barbalha (85%), followed by Santarém (71%), Rio de Janeiro (70%), Fortaleza (64%), and Maceió (45%). Ancestry was not correlated with the occurrence of NS CL/P or with the variability in heritability. Only in Rio de Janeiro was the coefficient of inbreeding significantly larger in NS CL/P families than in the local population. Recurrence risk for the total sample was approximately 1.5–1.6%, varying according to the location studied (0.6–0.7% in Maceió to 2.2–2.8% in Barbalha). Our findings show that the degree of genetic contribution to NS CL/P varies according to the geographic region studied, and this difference cannot be attributed to consanguinity or ancestry. These findings suggest that Barbalha is a promising region for genetic studies. The data presented here will be useful in interpreting results from molecular analyses and show that care must be taken when pooling samples from different populations for association studies.

Key words: non-syndromic cleft lip with or without cleft palate; complex disease; heritability; consanguinity; ancestry contribution; recurrence risk

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INTRODUCTION

Non-syndromic cleft lip with or without cleft palate (NS CL/P) is the most prevalent congenital facial defect, with a large degree of clinical expressivity, ranging from a lip scar to a complete bilateral cleft lip with dental–alveolar and palate involvement, without any other associated malformation [Gorlin et al., 2001]. It is still unclear whether all these subtypes represent the spectrum of clinical

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variability under a common etiological basis or underlie a genetic heterogeneity [Harville et al., 2005; Rahimov et al., 2008].

The worldwide birth prevalence of NS CL/P has been estimated as 1:1,000, but it varies according to ethnicity (African: 0.3:1,000; European: 0.7–1.3:1,000; Chinese: 1.4:1,000; Japanese: 2.1:1,000; Amerindian: 3.6:1,000), geographic origin, and socioeconomic level [Gorlin et al., 2001; Carinci et al., 2007]. In Brazil, birth prevalence estimates for NS CL/P have ranged from 0.28 to 1.54:1,000 [Menegotto and Salzano, 1991a; Loffredo et al., 2001].

A strong genetic basis for NS CL/P has been inferred due to the higher recurrence risk for first-degree relatives of affected subjects, and also to the large proportion of affected subjects with positive family history (about 20–30% [Melnick, 1992; Lie et al., 1994; Carinci et al., 2007]). Twin studies have shown a significantly higher concordance for NS CL/P in monozygotic twins (40–60%) as compared to dizygotic twins (3–5%), further reinforcing the importance of genetic factors in the etiology of NS CL/P [Christensen and Fogh-Andersen, 1993; Jugessur et al., 2009]. A multifactorial inheritance model, with additive effects of genetic and environment factors triggering the malformation, best fits most of the epidemiological findings on NS CL/P [Fraser, 1974; Schutte and Murray, 1999; Gorlin et al., 2001; Grosen et al., 2010].

Many NS CL/P candidate genes have been identified through different methods, but only a few loci, particularly those recently identified through genome-wide association studies (GWAS), have been consistently corroborated in different populations [Zucchero et al., 2004; Rahimov et al., 2008; Birnbaum et al., 2009a, 2009b; Mangold et al., 2009; Beaty et al., 2010]. One of the reasons underlying this difficulty of replication may be the importance of genetic contribution for the malformation. Genetic contribution to NS CL/P appears to vary according to the population studied, with estimates ranging from as low as 17% in Finland to 84% in Italy [Hu et al., 1982; Calzolari et al., 1988; Tenconi et al., 1988; Stoll et al., 1991; Christensen and Fogh-Andersen, 1993; Nordstrom et al., 1996; Lin et al., 1999]. In South America, genetic contribution to NS CL/P was estimated to be 70–74% [Menegotto and Salzano, 1991b]. However, the degree of genetic contribution can be affected by ethnicity, local environment, consanguinity, and geographical origin. In countries with a recent history of immigration and admixture, such as Brazil, ethnicity might have a strong effect on the genetic contribution to multifactorial diseases as it the case of NS CL/P.

Brazil has an extense territory with significant regional differences in its cultural habits and population origins. The country is divided in five large geographical regions: North, Northeast, Center -West, Southeast, and South. The prevalence of consanguineous marriages, a known risk factor in multifactorial diseases, is very heterogeneous in different regions of Brazil, being more common in the Northeastern part of the country, and increasing in frequency as one travels inland. The North and Southeast regions of Brazil are considered weak inbreeding zones [Salzano and Freire-Maia, 1967].

In this article, we estimate the importance of genetic contribution to NS CL/P susceptibility in Brazilian populations from five locations, in three different geographical regions. We also ascertained whether ethnicity and consanguinity play a role in the etiology of this malformation in our population. This pioneer work will be relevant to future studies that utilize molecular analyses.

PATIENTS AND METHODS

We ascertained NS CL/P families treated under the medical program of a non-governmental organization—Operation Smile (http://www.operationsmile.org/), with the collaboration of local hospitals. Between the years 2007 and 2009, we visited five cities in three different Brazilian regions: Santarém-PA (Northern region-N); Fortaleza-CE, Barbalha-CE, and Maceió-AL (Northeastern region—NE); and Rio de Janeiro-RJ (Southeastern region—SE). The locations of the cities studied are shown in Figure 1.

For each family, a pedigree with at least two ascendant generations of the proband was created, based on a standardized interview of the parents (available on request). We also recorded cleft classification [unilateral or bilateral cleft lip with or without cleft palate (CP)], sex, age, and information about the pregnancy.

We have included only non-syndromic CL/P cases. Patients with only CP were also excluded, as in most of the cases CP constitutes a different genetic entity from NS CL/P [Fraser, 1974; Jehee et al., 2009]. A final sample of 1,042 families was obtained: 121 from Santarém, 331 from Fortaleza, 86 from Barbalha, 188 from Maceió, and 316 from Rio de Janeiro.

Skin color as phenotypic classification for ethnicity correlates poorly with ancestry [Suarez-Kurtz et al., 2007], and therefore, we used molecular methods to determine the ancestral composition of our sample. We genotyped 40 autosomal insertion–deletion ancestry-informative polymorphisms spread across the genome (following the criteria adopted in Bastos-Rodrigues et al. [2006]; primer sequences available on request) through multiplex PCR in

FIG. 1. Locations analyzed in this study: Santarém, in the state of Para-PA (Northern region—N); Fortaleza and Barbalha, in the state of Ceará-CE, and Maceió, in the state of Alagoas-AL (Northeastern region—NE), and Rio de Janeiro, in the state of Rio de Janeiro-RJ (Southeastern region—SE).

602 subjects. Fragment analysis was performed with Gene Mapper software after capillary electrophoresis in ABI 3730 DNA Analyzer (Applied Biosystems). Ancestry contributions of Europeans, West Africans, and Amerindians were calculated with the software Structure 2.3.3 [Pritchard et al., 2000].

Subjects were grouped in familial or non-familial cases, based on the report of at least one other affected member in the family. We did not identify any affected twin pairs. The proportion of affected first-degree relatives (parents and siblings) of the proband was also calculated for each location. Frequencies of familial cases, consanguineous cases, and affected first-degree relatives were compared by chi-squared tests followed by the analysis of adjusted residuals (Haberman's test).

The heritability values for NS CL/P were estimated graphically for each location using a diagram as proposed by Professor C.A.B. Smith [Emery, 1986]. The graph correlates the malformation prevalence among first-degree relatives of the probands and in the general population; the obtained heritability is expressed as the proportion of the phenotypic variation attributable to the additive genetic variation. Therefore, high heritability implies high genetic determination. Tests of trend were performed for the average inbreeding coefficient (F), frequency of consanguineous cases, proportion of genetic determination, and frequency of familial cases to verify their association (only positive results are reported here).

When the proband was the offspring of a consanguineous marriage, the family was classified as a consanguineous case and a corresponding F was calculated. For a given type of mating, this coefficient is obtained simply by multiplying by one-half the parent's coefficient of relationship (which in turn reveals the proportion of genes shared by two relatives); a child of a first-cousin mating, for instance, has $F = 1/16$ [Cavalli-Sforza and Bodmer, 1971]. A case was considered consanguineous only when $F \geq 1/64$, that is, offspring of a second-cousin marriage or closer kinships. An average coefficient of inbreeding was calculated for each population by weighing the various F by their corresponding frequencies in the population, including the class $F = 0$ (non-consanguineous marriages [Hedrick, 1986]).

Empiric recurrence risks were calculated for the siblings of affected children. Since all families were ascertained through affected children in sibships, the recurrence risk was obtained from the formula $recurrence\ risk = (nao - nsf)/(nao + nno - nsf)$, where *nao* is the number of affected offspring, *nno* is the number of normal offspring, and *nsf* is the number of sibships or families; $nao - nsf$ is therefore the number of affected children in excess of one per sibship. The correction shown above is essentially the same used for correcting segregation ratios from material collected with bias ascertainment [Emery, 1986]. Recurrence risks for children born to affected mothers or fathers could not be determined in this study due to the small sample size.

RESULTS

We have evaluated 1,042 NS CL/P families ascertained between the years 2007 and 2009 at Operation Smile's programs in five different locations in Brazil: Santarém, Fortaleza, Barbalha, Maceió, and Rio de Janeiro. The proportion of affected males to females was 1.6:1. Most of the cases had cleft lip and palate (82.4%), while the remaining cases (17.6%) had cleft lip only.

After genotyping 40 ancestry-informative markers, we observed that the ancestry contributions of our subjects differ significantly among the regions studied ($P = 0.014$). Fortaleza, Barbalha, and Maceió (NE) presented similar ancestry contributions, whereas Rio de Janeiro (SE) was slightly enriched with African ancestry, compared to the others. Subjects from Santarém (N), on the other hand, presented a higher Amerindian contribution than the other regions (Table I).

To evaluate the genetic contribution to NS CL/P in this sample, we first estimated the frequency of familial cases and the number of affected first-degree relatives of the proband, as shown in Table II. We observed that the proportion of familial cases was significantly different among the five locations ($P < 0.0005$) with the lowest proportions in Maceió (23%) and Rio de Janeiro (24%), and the highest in Santarém (44%), followed by Fortaleza (40%). Comparison of the number of affected first-degree relatives of probands revealed statistically significant differences ($P = 0.02$), for which Barbalha and Maceió contributed the most (Table II). Heritability of NS CL/P, estimated based on these data, revealed a major genetic contribution in Barbalha (85%). Santarém, Rio de Janeiro, and Fortaleza presented intermediate genetic contributions (71%, 70%, and 64%, respectively), whereas Maceió was the location with less genetic contribution to NS CL/P (45%).

The rates of consanguinity among parents of probands were also significantly different among the five studied locations ($P = 0.001$), with the highest values in families from Fortaleza and Barbalha and lower estimates in Santarém, Maceió, and Rio de Janeiro. The same trends were observed for the respective coefficients of inbreeding (Table III). As expected, average F is positively correlated with the frequency of consanguineous cases (correlation coefficient = 0.97; $P < 0.05$) and, from now on, we will refer to F.

Recurrence risk based on the existence of an affected member in the sibship was 1.6% (1.5% if consanguinity is not considered) and

TABLE I. Ancestral Contributions to Patients From Five Regions in Brazil and Total Sample

	Santarém	Fortaleza	Barbalha	Maceió	Rio de Janeiro	Total
Genotyped patients	90	216	60	120	116	602
European	60%	71%	68%	67%	65%	67%
West African	11%	13%	19%	19%	23%	17%
Amerindian	29%	16%	13%	14%	12%	16%

TABLE II. Frequencies of Familial Cases and of Affected First-Degree Relatives and Heritability of Non-Syndromic Cleft Lip With or Without Cleft Palate for Different Regions in Brazil

	Proportion of familial cases	Proportion of affected first-degree relatives	Heritability
Santarém	49/111 = 44%	13/619 = 2.1%	71%
Fortaleza	131/330 = 40%	23/1358 = 1.7%	64%
Barbalha	32/86 = 37%	14/414 = 3.4%	85%
Maceió	42/183 = 23%	8/973 = 0.8%	45%
Rio de Janeiro	75/312 = 24%	30/1492 = 2.0%	70%
Total	329/1022 = 32%	88/4856 = 1.8%	—

TABLE III. Frequencies of Consanguineous Cases and Coefficients of Inbreeding (F) of Affected and General Populations [Salzano and Freire-Maia, 1967] From Five Regions in Brazil

	Proportion of consanguineous cases	F (present study)	F (general population)
Santarém	7/121 = 5.8%	0.002	0.002
Fortaleza	38/331 = 11.5%	0.004	0.004
Barbalha	12/86 = 14.0%	0.004	0.004
Maceió	8/188 = 4.3%	0.001	0.001
Rio de Janeiro	15/316 = 4.8%	0.002	0.001

varied according to the location, with the highest values in Barbalha (2.2% excluding consanguineous cases and 2.9% in the total sample) and the lowest in Maceió (0.7% without consanguineous cases and 0.6% with the total sample; Table IV).

DISCUSSION

The present Brazilian population is the result of centuries of ethnic admixture from three main groups: Europeans, Sub-Saharian Africans, and Amerindians. Because the patterns of immigration flow and interethnic crosses were not equal in different areas of the country, population composition may vary even among geographically close regions [Parra et al., 2003; Pena et al., 2009]. We would also expect a higher contribution of Europeans and Amerindians than Africans to our NS CL/P sample, compared to the overall population, if we take into account the prevalence of NS CL/P in the populations from which Brazilians originate. However, ancestry contributions were similar to previous estimates for the corresponding local control populations (60% European, 12% African, and 28% Amerindian for Santarém (N) and 75% European, 15% African, and 10% Amerindian for Fortaleza, Barbalha, and Maceió (NE); no control data are available for Rio de Janeiro (SE) [Santos et al., 2010]). Although we are aware that the use of 40 markers allows the discrimination of only three ancestries, our data suggest that there is no strong association between ancestry and NS CL/P in our population.

Most studies have supported a genetic contribution to NS CL/P, reporting 20–30% of cases as familial [Schutte and Murray, 1999;

TABLE IV. Number of Sibships and Recurrence Risks (Presented as Number of Affected Sibs/Total Sibs) Excluding and Including Consanguineous Cases in Five Regions in Brazil

	Excluding consanguineous cases		Including consanguineous cases	
	Number of sibships	Recurrence risk (95% CI)	Number of sibships	Recurrence risk (95% CI)
Santarém	114	5/373 = 1.3% (0.4–3.1)	121	5/382 = 1.3% (0.4–3.0)
Fortaleza	290	9/629 = 1.4% (0.7–2.7)	328	13/706 = 1.8% (1.0–3.1)
Barbalha	73	4/184 = 2.2% (0.6–5.5)	87	7/242 = 2.9% (1.2–5.9)
Maceió	164	4/578 = 0.7% (0.2–1.8)	172	4/612 = 0.6% (0.2–1.7)
Rio de Janeiro	261	17/915 = 1.9% (1.1–3.0)	273	19/971 = 2.0% (1.2–3.0)
Total	902	39/2679 = 1.5% (1.0–2.0)	981	48/2913 = 1.6% (1.2–2.2)

CI, confidence intervals.

Gorlin et al., 2001; Jugessur et al., 2009]. Considering the five locations together, we found 32% of cases were familial; however, this proportion varied significantly accordingly to the population ascertained, with the highest proportion of familial cases in Santarém (44%) and the lowest in Maceió (23%). Consistent with these findings, heritability analysis revealed a lower genetic contribution in Maceió (45%) than in the other locations. The higher heritability value in Barbalha (85%, even though it did not have the highest proportion of familial cases) is explained by the stronger aggregation of affected first-degree relatives in this location. Although heritability estimated through affected first-degree relatives is not an independent line of evidence in favor of increased genetic etiology from the proportion of familial cases, it appears to more accurately characterize populations with higher contributions from genetic factors. With the exception of Maceió, our heritability values are close to those previously reported from South America (70% [Menegotto and Salzano, 1991b]) or European countries such as Italy and France (81–84% [Calzolari et al., 1988; Tenconi et al., 1988; Stoll et al., 1991]).

Although we observed different F values among the five studied populations, the coefficients of inbreeding estimates obtained in our study are very similar to those found by Salzano and Freire-Maia [1967] in local Brazilian control populations. The one exception is Rio de Janeiro, in which F was twice as high in NS CL/P families as in the local population (Table III). Therefore, it seems that consanguinity is not a major contributing factor for NS CL/P in most of these populations and does not appear to be sufficient to explain the higher heritability in Barbalha.

The recurrence risk for the total sample, whether consanguineous couples were included or excluded, was approximately 1.5–1.6%, which is lower than values estimated for other populations (Italy, 2.4% [Tenconi et al., 1988]; Denmark, 3.5% [Grosen et al., 2010], and Norway, 7.0% [Sivertsen et al., 2008]. The recurrence risk varied according to the ascertained location, with the highest values in Barbalha (2.2–2.8%), which further confirms that genetic factors play a more prominent role in this group.

Although the reasons for the higher degree of genetic contribution to NS CL/P in Barbalha are unknown, these results clearly indicate that this is a promising location to study candidate loci for NS CL/P. On the other hand, the lowest heritability value observed (45% in Maceió) does not seem to be related to differences of ethnic constitution, as no obvious differences were observed in the contribution of European and African ancestries to affected subjects and local population from Maceió and Barbalha (Table I). Environmental factors may have a larger impact in the etiology of NS CL/P in Maceió than in the other locations.

In summary, we have shown that the contribution of genetic factors to NS CL/P varies across different regions of Brazil, and that neither consanguinity nor ancestry is the main factor influencing this finding. However, we cannot rule out that the effect of consanguinity was not evidenced because the F values of the control population used in the analysis might have changed from the time that they were calculated. The recurrence risks obtained for our populations were much lower than those previously reported in other countries, and consanguinity played a small role only in Fortaleza and Barbalha. These results have direct implications for genetic counseling, as they provide more accurate information about the genetic risks for Brazilian families with NS CL/P. These data can provide the basis for delineation of public health research strategies to prioritize regions to identify genetic or environmental factors of susceptibility to NS CL/P. Identification of such factors may in the future contribute to lowering the prevalence of NS CL/P. In most association studies thus far conducted for NS CL/P, the proportion of genetic contribution has not been considered. Our data indicate that at-risk alleles will be underrepresented in populations in which heritability for NS CL/P is lower, compared to those with higher heritability values. This effect should be even more dramatic for alleles that confer only a modest increase in disease susceptibility. Recent genetic studies including GWAS have replicated findings identifying *IRF6*, *MSX1*, and loci in chromosomes 1p22.1, 8q24, 9q21, 10q25.3, 17q22, and 20q12 as susceptibility loci for NS CL/P [Zucchero et al., 2004; Birnbaum et al., 2009b; Mangold et al., 2009; Moreno et al., 2009; Beaty et al., 2010; Ingersoll et al., 2010]. Most of these studies have included very large cohorts of patients and controls. If our assumption is correct, we would expect to find a correlation between positive association for some of these loci and higher heritability. In addition, we would expect that in a group of patients with higher heritability, a smaller sample size will be sufficient to detect the association. We intend to test this hypothesis in the near future. If confirmed, it will indicate that pooling of samples from different populations is not necessarily the best strategy to study the genetics of NS CL/P, and will also represent a significant decrease in the costs of genetic analysis.

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