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Reduced folate carrier 1 (*RFC1*) is associated with cleft of the lip only

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In this report, we have reanalyzed genotyping data in a collection of families from South America based on maternal origin. Genotyping analysis was performed at the Craniofacial Anomalies Research Center at the University of Iowa. These genotypes were derived from genomic DNA samples obtained from blood spots from children born with isolated orofacial clefts in 45 hospitals located in eight countries (Argentina, Bolivia, Brazil, Chile, Ecuador, Paraguay, Uruguay, and Venezuela) collaborating with ECLAMC (Latin American Collaborative Studies of Congenital Malformations) between January 1998 and December 1999. Dried blood samples were sent by regular mail to the Laboratory of Congenital Malformations, Federal University of Rio de Janeiro. Previous findings suggested that mitochondrial haplotype D is more commonly found among cleft cases born in South America. We hypothesized that association of certain genes may depend upon the ethnic origin, as defined by population-specific markers. Therefore, we tested if markers in *MTHFR* (5,10-methylenetetrahydrofolate reductase) and *RFC1* (reduced folate carrier 1) were associated with oral clefts, depending on the maternal origin defined by the mitochondrial haplotype. Transmission distortion of alleles in *MTHFR* C677T and *RFC1* G80A polymorphic variants was tested in 200 mother/affected child pairs taking into consideration maternal origin. *RFC1* variation was over-transmitted to children born with cleft lip only (P = 0.017) carrying mitochondrial DNA haplotypes other than haplotype D. Our results provide a new indication that variation in *RFC1* may contribute to cleft lip only. Future studies should investigate the association between oral clefts and *RFC1* based on more discrete phenotypes.

Key words: Cleft lip and palate; Reduced folate carrier 1; 5,10-Methylenetetrahydrofolate reductase; Folate; Oral clefts; Mitochondrial DNA

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Introduction

Although oral-facial clefts are among the most common congenital defects, their etiology remains largely unknown, with only a few cases associated with identified rare syndromes or secondary to recognized teratogen exposure. Most clefting is non-syndromic (without associated anomalies) and considered secondary to complex gene/gene or gene/environment interactions. We have previously demonstrated that cleft individuals in South America present a higher frequency of Amerindian mitochondrial haplotypes, in particular haplotype D (1). When

this same population was evaluated for genes related to the folate pathway, no association was found between genetic markers in *MTHFR* (5,10-methylenetetrahydrofolate reductase) or *RFC1* (reduced folate carrier 1) and oral clefts (2). Conflicting results have been published regarding the association between cleft lip and palate and *MTHFR* (3-13). Lack of association between markers in *RFC1* and oral clefts has been consistently reported (14-17). However, one study with Filipino families suggested borderline significant nonparametric LOD score results (P = 0.06) for markers in *RFC1* (18).

Isolated cleft lip and palate is more common in Asians and Amerindians, intermediate in Caucasians and less frequent in Africans and African descents (19). Therefore, we hypothesized that some genes that contribute to clefts may have a stronger effect in a particular ethnic group. Our previous work suggested findings that mitochondrial haplotype D is more commonly found among cleft cases born in South America. We hypothesized that association for certain genes may depend upon the ethnic origin, as defined by population-specific markers. Therefore, we tested if markers in *MTHFR* and *RFC1* were associated with oral clefts, depending on the maternal origin defined by the mitochondrial haplotype.

Table 1. Number of cases of cleft lip only (CL) and cleft lip with cleft palate (CLP) according to ECLAMC data from 8 Latin American countries.

Country	Cases	CL	CLP	Unknown
Argentina	80	21	58	1
Bolivia	24	6	17	1
Brazil	27	13	14	0
Chile	24	4	18	2
Ecuador	2	2	0	0
Paraguay	8	1	6	1
Uruguay	7	3	4	0
Venezuela	21	7	12	2
Total	193	57	129	7

Data are reported as number and include 12 cases that were excluded during statistical analysis because they had an underlying syndrome or other major or multiple minor defects, as determined by record review. ECLAMC = Latin American Collaborative Study of Congenital Malformations. Unknown = data not available if patient had only CL or CLP.

Table 2. Specific mtDNA lineage frequencies among patients reported in Table 1.

Haplogroups	Number of individuals	Relative frequency
African	4	0.022
European	11	0.059
Amerindian	172	0.919
Total	187	1.0
Amerindian subtypes		
A	13	0.08
B	2	0.002
C	17	0.1
D	140	0.818
Total	172	1.0

Differences in the total number of individuals reported here (187) compared to Table 1 (193) are due to the inability to identify mtDNA haplogroups.

Material and Methods

Samples from this study were drawn from the Latin American Collaborative Study of Congenital Malformations (ECLAMC). ECLAMC has operated since 1967 and utilizes 70 hospitals and volunteer physicians to collect data on approximately 150,000 births per year (4 million since 1967) (20). From January 1998 to June 2000, ECLAMC collected blood spots on filter cards from patients with isolated (non-syndromic) cleft lip with or without cleft palate and their mothers from eight countries in Latin America (Table 1) (21). Samples were obtained after volunteers signed an informed consent form. Patients having a known syndrome or other major or multiple minor defects (N = 12), as determined by record review, were excluded during data analysis. Also excluded from the analysis were the seven patients with cleft type unknown. DNA was extracted from filter card blood spots using modifications of published protocols. Kinetic PCR and molecular beacon assays were performed according to published protocol to test the allelic variants of *MTHFR* C677T and *RFC1* G80A (10).

Cleft lip only and cleft lip with cleft palate cases were evaluated separately and then in combination (cleft lip with or without cleft palate). Mother and proband genotypes were compared to determine the transmitted alleles versus the non-transmitted alleles. The likelihood ratio test (LRT) of Weinberg was applied to detect transmission distortion (22). The major advantage of the LRT over the standard transmission disequilibrium test (23) for parent-child data is that additional pairs are informative (i.e., all mother-child pairs in which the mother is heterozygous), under an assumption that the distribution of paternal alleles is the same as the maternal. Mating types were defined as 0, 1, or 2 indicating none, one, or two copies of the target allele (the more common allele was used as the target allele). This analysis was stratified by mitochondrial haplotype. Table 2 describes the mitochondrial haplotype frequency in the study population.

Results

Individuals with mitochondrial DNA haplotype D were analyzed separately from individuals with other mitochondrial DNA haplotypes. For cleft lip only, the results indicated that there was an association between *RFC1* and risk of cleft lip only among individuals with mitochondrial DNA haplotype other than haplotype D (P = 0.017; Table 3). Individuals with mitochondrial DNA haplotype D did not show association with the *RFC1* alleles (data not shown). Statistically significant differences between these two groups were assessed by the chi-square test (χ^2 = 8.680, P = 0.003). There was also no evidence of any difference between the mitochondrial DNA groups regarding *MTHFR* genotypes.

Table 3. *MTHFR* and *RFC1* LRT results include only individuals with mtDNA haplotypes other than D.

Mating types (mother-father-child)	*MTHFR*			*RFC1*		
	CL	CLP	CL/P	CL	CLP	CL/P
0-?-0	0	0	0	1	3	4
0-?-1	2	3	5	0	3	3
1-?-0	0	1	1	1	1	2
1-?-1	1	7	8	1	2	3
1-?-2	2	7	9	0	3	3
2-?-1	3	6	9	6	3	9
2-?-2	2	9	11	4	16	20
Total	10	33	43*	13	31	11
LRT statistics						
LnL Model II	-15.763	-54.588	-71.485	-19.030	-48.000	-71.183
LnL Model I	-15.571	-54.384	-71.138	-21.901	-47.706	-70.937
χ^2, 1 d.f.	0.383	0.479	0.693	5.742	1.489	0.491
P	0.54	0.49	0.41	0.017	0.22	0.48

Mating types defined as 0, 1, and 2, indicating none, one, or two copies of the designated "V" allele. *MTHFR* = 5,10-methylenetetrahydrofolate reductase; *RFC1* = reduced folate carrier 1; CL = cleft lip only; CLP = cleft lip and palate; CL/P = cleft lip with or without cleft palate (CL + CLP); LRT = likelihood ratio test; LnL = natural log of likelihood; Model I = no distortion, only baseline parameters estimated; Model II = transmission distortion (parameter R_1 and R_2 estimated (22)); d.f. = degrees of freedom. *Difference in the total number of non-haplogroup D individuals compared to Table 2 is due to PCR failure. P values were derived from a chi-square table using $\chi^2 = -2\ (LnL_{Model\ II} - LnL_{Model\ I})$.

Discussion

Indians, Africans, and Europeans form the racial background of the American continent. When Europeans discovered America, the Indian population was spread over the entire continent. Spanish and Portuguese settlers distributed themselves fairly well over today's Mexico, Central and South America, except in the far south and the remote interior. The importation of Africans started by the end of the 1400's and lasted for about 400 years (24). The colonization of the new world by the Europeans initially involved men only, and the immigration of European women during the first centuries was insignificant. Admixture analyses performed in Brazil, Chile and Colombia suggest a historical pattern of directional mating that preferentially involved immigrant men and native women (25-30). The LRT analysis performed assumes that the distribution of paternal alleles was the same as the maternal. Although there is strong data supporting a pattern of directional mating during South America's colonization, available data from the eight countries that contributed samples to our studies demonstrate that frequency of random autosomal alleles in unrelated males and females from these countries was comparable, therefore our approach was valid (31-38).

The reanalysis of the *MTHFR* data based on maternal origin confirms our previous results in which no association between *MTHFR* and cleft lip and palate was found (2). On the other hand, it appears that the association between cleft lip only and *RFC1* in South Americans relates to ancestral origin that is independent among individuals with maternal lineage containing the mitochondrial DNA haplotype D. The mitochondrial DNA haplotype D is more frequently found among the Aleuts, Amazonian Amerindians, Andean Amerindians, and Patagonian Amerindians. In Asia, it can be seen more frequently in populations living in the north of Mongolia, far North of Russia, and Korea (39). The mitochondrial DNA haplotype D may identify people where genes other than *RFC1* are acting in the ECLAMC population that predisposes to cleft lip only or is confounded with environmental factors that serve as risk factors for clefts found more commonly in individuals of Amerindian descent.

There is a marked difference in the population origin of the female and male founders in South America (25-30). The association between cleft lip only and *RFC1* in South America may be more easily expressed in individuals carrying mitochondrial DNA Amerindian-specific haplotypes A, B, and/or C and male founders originated in Spain and Portugal.

Although the positive *RFC1* association reported here relates to a small subset of the population studied, our results provide a new indication that variation in *RFC1* may contribute to cleft lip only. With the exception of our previous report (2), none of the previous studies that investi-

gated the possible association between oral clefts and variation in *RFC1* investigated cleft lip only separately from cleft lip with palate (14-17). Future investigations should consider refining data analysis to explore the existent variation in the clinical expression of oral clefts.

Although the biochemical evidence and clinical trial results are nowhere near being consistent and persuasive for a folate effect on oral clefts as they are for a folate effect on neural tube defects, studies such as ours suggest that mutations in genes involved in the folate pathway exist. The relevance of this finding may be dependent on individuals' specific genetic background, in this case, maternal mitochondrial origin.

The mitochondrial DNA haplogroup D in the South American cleft population may be ideal for performing a whole genome linkage disequilibrium screen, because it has theoretically the same 'maternal founder', and might share the same genetic causes of non-syndromic cleft lip and palate. The combination of historical data about population migrations and population-specific DNA variants should be further explored to help understand the heterogeneity underlying non-syndromic cleft lip and palate and other complex traits.

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