MTHFR rs2274976 Polymorphism Is a Risk Marker for Nonsyndromic Cleft Lip with or without Cleft Palate in the Brazilian Population

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Background: Polymorphisms within the MTHFR (rs2274976) and MTHFD1 (rs2236225) genes were previously associated with maternal susceptibility for having an offspring with nonsyndromic cleft lip with or without cleft palate (NSCL/P) in the Brazilian population. However, as the genotypes of the patients with NSCL/P were not evaluated, it is not clear whether the effects are associated with maternal or offspring genotypes. The aim of this study was to evaluate the association of rs2274976 and rs2236225 in the pathogenesis of NSCL/P. **Methods:** By using the TaqMan 5′-exonuclease allelic discrimination assay, the present study genotyped the rs2274976 and rs2236225 polymorphisms in 147 case–parent trios, 181 isolated samples of NSCL/P and 478 healthy controls of the Brazilian population. Transmission disequilibrium test and structured case–control analysis based on the individual ancestry proportions were performed. **Results:** The transmission disequilibrium test showed a significant overtransmission of the rs2274976 A allele ($p = 0.004$), but no preferential parent-of-origin transmission was detected. The structured case–control analysis supported those findings, revealing that the minor A allele of rs2274976 was significantly more frequent in NSCL/P group compared with control group ($p = 0.001$), yielding an odds ratio of 3.46 (95% confidence interval, 2.05–5.85). No association of rs2236225 polymorphism with NSCL/P was observed in both transmission disequilibrium test and case–control analysis. **Conclusion:** The results of the study revealed that the presence of the rs2274976 A allele is a risk marker for the development of NSCL/P in the Brazilian population.

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Introduction

Nonsyndromic cleft lip with or without cleft palate (NSCL/P) is the most common facial birth defect with lifelong distressing consequences for the patient (Rahimov et al., 2012). The prevalence of oral clefts varies among different populations, and its estimated prevalence is between 0.36 and 1.54 per 1000 live births in Brazil (Martelli-Junior et al., 2007; Rodrigues et al., 2009). The risk factors associated with NSCL/P are not completely understood, but there is a clear interaction between genetic and environmental factors in the etiology of this complex defect (Dixon et al., 2011). Some studies have shown a significant association between nutritional deficiency, particularly folate deficiency, and elevated risk of oral clefts (Wilcox et al., 2007; Jia et al., 2011; Kelly et al., 2012), whereas other studies have not (Ray et al., 2003; Little et al., 2008). One possible reason for those ambiguous results may reside on genetic variations that influence absorption, transport and metabolism of folate more than levels of maternal intake or availability to the fetus. In support, population-based studies did not show a reduced risk of oral clefts with dietary folate reinforcement (Ray et al., 2003; López-Camelo et al., 2010; Wehby and Murray, 2010).

In a previous study, we demonstrated that specific polymorphic variants in 5,10-methylenetetrahydrofolate reductase (*MTHFR*) and methylenetetrahydrofolate dehydrogenase 1 (*MTHFD1*) genes, which encode enzymes involved in reactions of oxidation and reduction during folate metabolism, increase the maternal risk for having an offspring with NSCL/P (Bufalino et al., 2010). However, one of the limitations of this study was that genotypes of

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NSCL/P patients were not assessed, not allowing the discrimination between maternal effects from those based on the offspring genotypes. The present study aimed to evaluate whether genetic variants in *MTHFR* (rs2274976) and *MTHFD1* (rs2236225), which were previous associated with maternal risk for NSCL/P, are susceptibility markers of oral clefts in the Brazilian population. By using transmission disequilibrium test (TDT) and case–control analysis structured by the genetic ancestry variation of each individual, the current study confirmed that *MTHFR* rs2274976 polymorphism is a risk factor for the pathogenesis of NSCL/P in the Brazilian population.

Materials and Methods

SAMPLE STUDY

This study recruited patients with NSCL/P from three reference centers for treatment of oral clefts in Brazil: the Center for Rehabilitation of Craniofacial Anomalies, Dental School, University of José Rosário Vellano, Alfenas-Minas Gerais (designated for this study as Center 1), which is located at southeast region of Brazil, the Santo Antonio Hospital, Salvador-Bahia (designated as Center 2), located at northeast region, and the Center for Rehabilitation of Craniofacial Anomalies, Cascavel-Parana (designated as Center 3), which is in south of Brazil. All patients were diagnosed independently and screened for the presence of associated anomalies or syndromes by the multidisciplinary team of specialists of each center. Clefts were classified with the incisive foramen as reference, and patients with cleft palate only (CPO) were excluded. We ended up with 147 NSCL/P trios (1 affected offspring and 2 healthy parents), being 45 patients affected by cleft lip only (CLO) (24 males and 21 females) and 102 cleft lip and palate (CLP) (71 males and 31 females). Eighth four trios were collected in Center 1, 25 trios in Center 2, and 38 in Center 3.

In addition, this study enrolled other 65 CLO and 116 CLP, totalizing 181 isolated cases of NSCL/P (101 males and 80 females), and 478 control samples (194 males and 284 females) that were collected from unrelated healthy subjects of the studied areas. Control patients did not have physical or psychiatric diseases, history of congenital malformations or familial history of orofacial clefts. There was no overlapping between the samples used in the TDT and in the case–control study, neither with the samples previously studies by us (Bufalino et al., 2010). The study was approved by the ethics review board of each of the centers or hospital affiliated with the collaborative study. Written informed consent was obtained from the parents or guardians and/or the participants.

GENOTYPING AND ESTIMATION OF THE GENOMIC ANCESTRY

The genomic DNA was isolated from oral mucosa cells using a salting out protocol. Genotyping of rs2274976 in *MTHFR* and rs2236225 in *MTHFD1* were carried out in the StepOnePlus Real-Time PCR platform (Applied Biosystems) using the TaqMan 5′-exonuclease allelic discrimination assays obtained from Applied Biosystems through their assay-on-demand service. Genotyping analyses were randomly repeated in 10% of the samples for both polymorphisms. To determine the genomic ancestry of each individual, samples were genotyped for a set of 40 biallelic short insertion/deletion polymorphisms previously validated as ancestry informative markers (Bastos-Rodrigues et al., 2006).

STATISTICAL ANALYSIS

The TDT based on FBAT software (family based association test) was used to test for genotype–phenotype association, evaluating the differential pattern of excess allele transmission from heterozygous normal parents to affected children. Parent-of-origin effects were examined as described by Suazo et al. (2010). For the case–control analysis, deviation from Hardy-Weinberg equilibrium was assessed through chi-square test. To determine the genomic ancestry of each individual, Structure software was used (Falush et al., 2007) in a model assuming $K = 3$ parental populations based on the tri-hybrid origin of the Brazilian population. Samples with prespecified 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 (Pritchard et al., 2000). The genotypes were analyzed under unrestricted, dominant, and recessive genetic models. The odds ratio associated with 95% confidence interval was also calculated. The p value of ≤ 0.05 was considered statistically significant.

Results

Although the minor allele frequency of the *MTHFR* rs2274976 polymorphism was low (minor allele frequency = 0.041) in the 147 Brazilian NSCL/P trios (32 trios were informative), TDT analysis showed a significant overtransmission of the A allele ($p = 0.004$; Table 1). The transmission of the A allele from the mother occurred more frequently than from the father, but it did not reach a significant level (Table 2). For the *MTHFD1* rs2236225 polymorphism, TDT analysis revealed no preferential transmission of the alleles (Table 1), and the segregation did not occur preferentially from the mother or father to the children with NSCL/P (Table 2).

For the case–control analysis structured by genomic ancestry, the distributions of the alleles and genotypes of polymorphisms are presented in Table 3. The average ancestry contributions were estimated at 87.5% of European, 10.5% of African and 2% of Amerindian in the control group, and in the NSCL/P group was 81.3% of European, 17% of African and 1.7% of Amerindian, revealing no statistical significant differences in the proportions

TABLE 1. *Transmission Disequilibrium Test (TDT) of MTHFR rs2274976 and MTHFD1 rs2236225 Polymorphisms in 147 Case–Parent Trios.*

	rs2274976	rs2236225
MAF	0.041	0.449
Genotype (GG/GA/AA)		
Proband (%)	87.4/12.6/0	29.1/48.7/22.2
Father (%)	89.9/10.1/0	32.6/39.7/27.7
Mother (%)	95/5/0	31.6/54.2/14.2
T/NT	24/8	78/31
p value	0.004	0.34

MAF, minor allele frequency; T/NT, transmission/nontransmission counts.

between groups ($p = 0.10$). The genotype distribution of the 2 polymorphisms did not deviate from expectation based on the Hardy-Weinberg equilibrium ($p = 0.14$ for rs2274976 and $p = 0.25$ for rs2236225). The frequency of the A allele of *MTHFR* rs2274976 polymorphism was significantly higher in the NSCL/P group as compared to the control group, yielding an odds ratio of 3.46 (95% confidence interval, 2.05–5.85; $p = 0.001$). The GA genotype was identified in 4.8% of the control samples, whereas GA genotype was found in 17.2% of the NSCL/P ($p = 2.4 \times 10^{-7}$). The odds ratio for carriers of the A allele (GA and AA genotypes) compared with those with GG genotypes (dominant model of inheritance) was 3.91 (95% confidence interval, 2.24–6.81; $p = 3.6 \times 10^{-7}$). No differences in the distributions of alleles and genotypes between patients with NSCL/P and controls were found for *MTHFD1* rs2236225 polymorphism (Table 3).

Discussion

Products of the folate pathway, as universal donors of carbon, participate of amino acid metabolism, nucleotide synthesis, and formation of primary methylating agents, controlling gene expression and contributing to development and cellular homeostasis (Morrison et al., 1998; Prescott and Malcolm, 2002). Although insufficient folate intake before conception period and during early pregnancy is markedly associated with occurrence of neural tube defects (De-Regil et al., 2010), its impact on NSCL/P is controversial (Wehby and Murray, 2010; Bhaskar et al., 2011). Strong evidence suggested that, as a multifactorial disease, gene-environment interactions are essentials to NSCL/P pathogenesis. In this line of evidence, presence of accepted folate levels may not be the only requirement for NSCL/P prevention, because its metabolism, generating active metabolites, is essential to all folate-requiring reactions (Bhaskar et al., 2011). Indeed, polymorphisms in several genes related to folate transport and metabolism have been shown to alter the disponibility of the active forms

TABLE 2. *Parent-of-Origin Transmission of the Risk Alleles of MTHFR rs2274976 and MTHFD1 rs2236225 Polymorphisms.*

	Risk Allele	Maternal Allele OR (95% CI)/*p* Value	Paternal Allele OR (95% CI)/*p* Value
rs2274976	A	2.42 (0.91-6.40)/0.09	0.36 (0.09-1.51)/0.36
rs2236225	A	1.26 (0.77-2.06)/0.38	1.42 (0.88-2.29)/0.18

OR, odds ration; CI, confidence interval.

of folate. The presence of rs2274976 A variant allele reduces the activity of MTHFR enzyme (Martin et al., 2006), whereas the rs2236225 polymorphism affects MTHFD1 thermostability, diminishing its competence on DNA synthesis (Christensen et al., 2009). As net consequences, the low levels of the folate forms, induced by the rs2274976 and rs2236225 polymorphisms, may induce a global state of genomic hypomethylation, at the expense of pyrimidine synthesis leading to uracil misincorporation and DNA strand breaks.

Given that enzymes involved in folate metabolism play a pivotal role in events related to normal embryogenesis, including craniofacial development, disease-underlying variants of the genes encoding those enzymes may contribute to the etiology of NSCL/P. In a previous study with 29 polymorphic variants in four genes that encode proteins related to folic acid metabolism, we found that two of them (rs2274976 and rs2236225) were associated with maternal susceptibility for having an offspring with NSCL/P. Therefore, the present study was undertaken to determine the susceptibility of those polymorphisms as risk factors for NSCL/P in the Brazilian population, assessing the alleles and genotypes of the affected patients through TDT and case–control structured analysis. As the Brazilian population is result of the genetic admixture of three main ancestral populations (Europeans, Africans, and Amerindians) and displays very high levels of genomic diversity (Pena et al., 2011), we have previously demonstrated that the use of ancestry markers in association studies of ethnically mixed populations (structuration of the samples) is important to avoid interpretation bias (Brito et al., 2012; Bagordakis et al., 2013; de Aquino et al., 2013). In the present study, we confirmed the significant association of the polymorphism rs2274976 with NSCL/P etiology, but not with rs2236225.

There are few studies assessing the role of *MTHFR* rs2274976 polymorphism, and most of them are in disease-specific conditions such as lung cancer (Shi et al., 2005), head and neck cancer (Neumann et al., 2005), early-onset ischemic stroke (Giusti et al., 2010), and Kawasaki disease (Yoon et al., 2011). After our original study demonstrating that the A allele of *MTHFR* rs2274976 is associated with the maternal susceptibility for having an

TABLE 3. *Distribution of the Alleles and Genotypes of rs2274976 in MTHFR and rs2236225 in MTHFD1 in the Control and NSCL/P Groups.*

		Genotype (%)	OR_{allele} (95% CI)		OR_{Het} (95% CI)		OR_{Hom} (95% CI)		OR_{Dom} (95% CI)		OR_{Rec} (95% CI)	
	MAF	**GG/GA/AA**		***p* Value**		***p* Value**		***p* value**		***p* Value**		***p* Value**
rs2274976												
Control	0.028	94.8/4.8/0.4	Reference		Reference		Reference		Reference		Reference	
NSCL/P	0.091	82.2/17.2/0.6	3.46 (2.05-5.85)	0.001	4.11 (2.33–7.28)	2.4×10^{-7}	1.52 (0.14–16.97)	0.73	3.91 (2.24–6.81)	3.6×10^{-7}	2.65 (0.16–42.68)	0.47
rs2236225												
Control	0.400	37.9/44.1/18.0	Reference		Reference		Reference		Reference		Reference	
NSCL/P	0.396	35.2/50.3/14.5	0.98 (0.77-1.26)	0.42	1.22 (0.84–1.79)	0.29	0.87 (0.51–1.47)	0.59	1.12 (0.78–1.60)	0.53	0.77 (0.48–1.25)	0.29

Values of p were based on structured association.
MAF, minor allele frequency; OR, odds ratio; CI, confidence interval.

offspring with NSCL/P (Bufalino et al., 2010), one study evaluated this polymorphism in Hispanic and non-Hispanic white NSCL/P families from United States and did not find association of rs2274976 with NSCL/P (Blanton et al., 2011). The results of the current study suggest that the presence of the *MTHFR* rs2274976 A allele is associated with an increased risk for NSCL/P in the Brazilian population, but further studies with larger cohorts from different populations and taking into account the levels of dietary folate intake are warranted. Interestingly, rs1801133 (C677T) polymorphism in *MTHFR* is the most extensively investigated in NSCL/P in both affected patients and their parents. Although the association between rs1801133 and oral cleft risk is controversial, with some studies showing significant association while others rebutting it (Luo et al., 2012; Butali et al., 2013), the studies with the Brazilian population refuted the association of this polymorphism with both maternal and fetal risk for NSCL/P (Gaspar et al., 1999; Brandalize et al., 2007; Bufalino et al., 2010). Revaluating our published data (Bufalino et al., 2010), we found a linkage equilibrium between rs1801133 and rs2274976 ($D' = 0.10$), revealing that the genotypes present at those loci are independent. In this sense, if the major predisposing allele in *MTHFR* is indeed rs2274976, part of the controversial results can be attributed to population variations in linkage equilibrium/disequilibrium between rs1801133 and rs2274976.

The presence of *MTHFD1* rs2236225 polymorphism has been described as maternal and offspring risk factor for several conditions, including neural tube defects (Etheredge et al., 2012), severe placental abruption and miscarriage (Parle-McDermott et al., 2005a, b), intrauterine growth restriction (Furness et al., 2008), and congenital heart defects in children (Christensen et al., 2009). Furthermore, rs2236225 was associated with decreased risk for lung cancer (Liu et al., 2008), but not in cancers of prostate (Collin et al., 2009), cervix (Mostowska et al., 2011), and ovaries (Pawlik et al., 2012), and the A variant allele was also suggested as a risk factor for early onset Alzheimer's disease (Bi et al., 2010). There are few studies describing the association of rs2236225 polymorphism and maternal risk for NSCL/P, and the results are unclear. Mills et al. (2008) demonstrated an association between CPO and CLP and *MTHFD1* rs2236225 in both cases and case mothers in the Ireland population, but similar studies involving populations from Poland (Mostowska et al., 2006), Norway (Boyles et al., 2008), and Italy (Palmieri et al., 2008) did not support this finding. Taken that the majority of the studies, including the current one, did not find association with NSCL/P, it is unlikely that the polymorphism rs2236225 in *MTHFD1* contributes for the pathogenesis of the NSCL/P.

In closing, the present study demonstrates that the polymorphic variant rs2274976 in *MTHFR* is a susceptibility marker for NSCL/P in the Brazilian population, however,

further investigations with samples from different populations are needed to confirm its global association, improving our understanding of the etiology of this complex disease.

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