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Genetic Variants in Folate and Cobalamin Metabolism-Related Genes in Nonsyndromic Cleft Lip and/or Palate

Márcia Waltrick-Zambuzzi[2], Patricia Nivoloni Tannure[1], Thays Cristine dos Santos Vieira[2], Leonardo Santos Antunes[2], Fábio Lourenço Romano[3], Willian Fernando Zambuzzi[4], José Mauro Granjeiro[2,5], Erika Calvano Küchler[3]

[1]Dental School, Universidade Veiga de Almeida, Rio de Janeiro, RJ, Brazil
[2]Clinical Research Unit, Antonio Pedro University Hospital, UFF - Universidade Federal Fluminense, Niterói, RJ, Brazil
[3]Department of Pediatric Clinic, School of Dentistry of Ribeirão Preto, USP - University of São Paulo, Ribeirão Preto, SP, Brazil
[4]Departament of Chemistry and Biochemistry, UNESP Universidade Estadual Paulista, Botucatu, SP, Brazil
[5]Bioengineering Program, INMETRO – Instituto Nacional de Metrologia Qualidade e Tecnologia, Xerém, RJ, Brazil

Correspondence: Erika Calvano Küchler, Avenida do Café, s/n, Monte Alegre, 14040-904, Ribeirão Preto, SP, Brasil. Tel: +55-16-3315-3995. e-mail: erikacalvano@gmail.com

The aim of this study was to evaluate the association of the polymorphisms in *TCN2* (rs1801198) gene and in *MTRR* (rs1801394) gene with nonsyndromic cleft lip and/or palate (NSCL/P) in a Brazilian population. Genomic DNA was extracted from buccal cells. The polymorphisms in *TCN2* (rs1801198) and *MTRR* (rs1801394) genes were genotyped by carrying out real-time PCR and Taqman assay. Chi-square test was used to determine the association between genotype and allele frequencies with NSCL/P and NSCL/P subgroups (cleft lip only, cleft lip and palate, and cleft palate only). Eight hundred and sixty seven unrelated individuals (401 cases with NSCL/P and 466 individuals without cleft) were evaluated. Genotype distributions of *TCN2* and *MTRR* polymorphisms were in Hardy-Weinberg equilibrium. The *TCN2* polymorphic genotype GG was identified in 16.7% of the NSCL/P group and in 14.1% of the non-cleft group (p>0.05). Similarly, the frequency of *MTRR* genotype (GG) was similar in NSCL/P group (15.5%) and control group (17.8%) (p>0.05). Multivariate analysis showed an association between *MTRR* and the subgroup that the mother smoked during pregnancy (p=0.039). Our findings did not demonstrate an association between *TCN2* polymorphisms and NSCL/P, however suggests an association between *MTRR* and NSCL/P etiology

Key Words: cleft lip and/or palate, vitamin B12, folic acid, MTRR gene, TCN2 gene, genetic polymorphisms, subphenotypes.

Introduction

Nonsyndromic cleft lip and/or palate (NSCL/P) is one of the most common congenital anomalies in humans and has an impact in the oral health. NSCL/P is a multifactorial condition and many factors, such as ethnical background, geographic origin, socioeconomic status, genetics and environmental factors are involved (1).

Environmental factors, such as vitamin supplementation at preconception period, have been shown to contribute in the prevention of many birth defects, especially NSCL/P (2). Folate (folic acid) and cobalamin (vitamin B12) participate in the methylation cycle and act as cofactors in DNA and RNA biosynthesis, playing an essential role in cell differentiation and tissue growth as well as during embryogenesis (3). Studies using animal models have demonstrated a specific involvement of folate and cobalamin during palatogenesis (4).

Transcobalamin II is a protein responsible for cobalamin transporting to the cells at tissues (5-8) and it is encoded by *TCN2* gene. Metabolically, just after cellular uptake, cobalamin participates as a cofactor in so many biochemical pathways, including that responsible by homocysteine (Hcy) metabolism, involving methionine, a recognized methyl donor (9). This reaction is regulated by two enzymes, methionine synthase (MTR) and methionine synthase reductase (*MTRR*), encoded by the *MTRR* gene (10).

Polymorphisms in *TCN2* and *MTRR* might alter cell metabolism during embryonic development. A previous study has proposed that *TCN2* 776C>G polymorphism may be functionally associated with NSCL/P (11). The G allele is associated with lower circulating concentrations of transcobalamin (12). In addition, other studies suggested that *MTRR* 66A>G polymorphism is a risk factor for neural tube defects (13) and NSCL/P (14). This variant leads to the amino acid change of isoleucine to methionine at position 22 (15). The G allele produces an enzyme with less affinity for the substrate (16). Although some studies have evaluated the association between periconception use of multi-vitamin with NSCL/P (17,18) only few have investigated the folate and cobalamin pathway polymorphisms in the etiology of NSCL/P.

Therefore, it is possible that polymorphisms in *TCN2* and *MTRR* genes are involved in the NSCL/P etiology. We may hypothesize that these polymorphisms act alone or in a gene/gene interaction or gene/environmental interaction. Thus, the present study aimed to evaluate the association between NSCL/P with polymorphisms in *TCN2* and *MTRR* genes and to evaluate the interaction between these two polymorphisms with environmental factors.

Material and Methods

Subjects

The NSCL/P group was ascertained through a public

hospital specialized on orofacial cleft rehabilitation (Hospital Nossa Senhora do Loreto), Rio de Janeiro State, Brazil. Syndromic cleft were excluded. Also, to reduce possible etiological heterogeneity, we excluded those patients with clefts with additional unspecified multiple malformations.

The non-cleft group consisted of unrelated subjects, with no familiar history of NSCL/P that sought for a dental treatment at the Pediatric dentistry Clinic at Federal University of Rio de Janeiro (UFRJ). Both institutions are located in the city of Rio de Janeiro, geographically positioned at southeast of Brazil. Both institutions are located within 10 km of each other, in the same city of Rio de Janeiro. The control group was selected to ensure it matched the cleft group in age, gender, and geographic distribution.

The examiners collected all data from individuals born with or without clefts since September/2009 to September/2011. All individuals or parents/legal guardians answered a questionnaire about demographic characteristics; positive family history of cleft and mothers habits (smoke and alcohol ingestion during pregnancy).

Local Research and Ethics Committee (Protocol Number 113/09) approved this study. All participating individuals or parents/legal guardians allowed participation in this study by signing an informed consent.

Determination of Cleft Types

The determination of cleft types was based on clinical examination. Cases were classified in cleft lip only, cleft lip and palate and cleft palate only. Based on cleft laterality, cases were also divided in left, right or bilateral.

DNA Samples and Genotyping

Genomic DNA was extracted from oral cells by the previously reported method (19). Genetic polymorphisms in the *TCN2* gene (rs1801198) and in the *MTRR* gene (rs1801394) were genotyped by real-time polymerase chain reactions using the Taqman method by Agilent Technologies (Stratagene Mx3005P). All reagents and assays were supplied from Applied Biosystems (Foster City, CA, USA). Markers informations are included in Table 1.

Table 1. Details on the studied genetic markers

Gene	Gene name	SNP ID	DNA change[a]	Protein effect	Location	Alelles[b]	MAF[c]
TCN2	Transcobalamin II	rs1801198	776C>G	Arg259Pro	Chr22	[C/G]	0.45
MTRR	Methionine synthase reductase	rs1801394	66A>G	Met22Ile	Chr5	[A/G]	0.42

Note: [a]Ref. seq.: *TCN2*:c.M60396.1; *MTRR*:c.NM_002454.1.[b] In bold letters are minor allele count
[c] Obtained from ENTREZ SNP database (http://www.ncbi.nlm.nih.gov/sites/entrez).

Statistical Analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS – 16.0; SPSS Inc., Chicago, IL. USA)). In addition, NSCL/P group were analyzed not only as a total group, but also in stratified subgroups: cleft type (cleft lip only, clef lip and palate, and cleft palate only), mothers' smoking and drinking behavior during gestational period; cleft side and cleft completeness. Chi-square determined if NSCL/P or cleft types was preferentially associated with *TCN2* or *MTRR* genotypes and alleles. The binary logistic regression was adjusted for genotype, ethnic groups and mothers' habits (smoke and alcohol ingestion). Gene-gene interactions were also ascertained with binary logistic regression analysis. Differences were considered significant when $p \leq 0.05$. Moreover, the standard chi-square test was used to test for deviation from Hardy-Weinberg equilibrium.

Results

Of 867 individuals included in this study, 401 were NSCL/P (case group) and 466 were non-cleft individuals (control group). The cleft lip and palate was the most common cleft type (67.3%). The characteristics of the studied population are summarized in Table 2. Based on the maternal habits, 18.7% and 13.5% of mothers, respectively, into case group (NSCL/P) and control group (non-cleft individuals) smoked during gestational period. A significant difference was observed (p=0.037) between groups. Alcohol consumption during pregnancy was not different between groups (p=0.080).

Genotype distributions for both polymorphisms were in Hardy–Weinberg equilibrium. There were no significant differences in the allele and genotype distribution of *TCN2* between non-cleft and NSCL/P groups. A lack of association was also observed for cleft types (Table 3). *MTRR* polymorphism distribution between non-cleft and NSCL/P groups is presented in the Table 4.

Alleles distributions were not associated with NSCL/P for in both studied genes (p>0.05).

In the logistic regression analysis adjusted for genotypes and mother smoking during pregnancy, *MTRR* AG genotype was significantly associated (p=0.030) demonstrating an increased risk for NSCL/P (OR=1.439, 95% CI 1.035-2.000).

During logistic regression analysis gene-gene interaction was not observed (p=0.258).

Discussion

A recent meta-analysis reported that folic acid, alone or in combination with vitamins and minerals, reduces birth defects but there are no evidences regarding the effects on NSCL/P prevention (20). In addition

a previous study demonstrated that folate-related gene polymorphisms could be risk factors for NSCL/P (21). Our study analyzed the association of polymorphisms of folate-related gene (*TCN2* and *MTRR*) with NSCL/P in a Brazilian population.

It was not found an association between *TCN2* (776C>G) and NSCL/P. Martinelli et al. (11) investigated the association between NSCL/P and *TCN2* (776C>G) gene in a case-parent triad and they suggested that this polymorphism might be functionally related with NSCL/P. On the other hand, later studies tried to replicate their findings, but could not confirm this association (10).

This inconsistency on different results maybe explained by the ethnical background differences. New studies are necessary to evaluate this association in different populations. In our Brazilian sample group from the southeast of the country, there was no association between *TCN2* gene (776 C>G) and NSCL/P types, neither gene-environmental interaction.

Previous studies investigated *MTRR* gene (66A>G) polymorphism and reported a negative association with NSCL/P (10,11,22,23), although a recent study with Ukrainian individuals observed that the *MTRR* was associated with the NSCL/P risk (14). We found a borderline association when we analyzed the *MTRR* genotypes distribution in NSCL/P versus non-cleft group (p=0.06). It is important to highlight that in our subgroup analysis (according to the cleft type), there was a borderline association only for cleft lip and palate group. This could be explained by the fact that cleft lip with or without palate has a different etiological background than cleft palate only. Our results lead us to hypothesize that *MTRR* (66A>G) polymorphism plays a role in the specific NSCL/P type, the cleft lip with palate phenotype, in which this gene acts with a small effect in clefting establishment.

The logistic regression analysis suggested that smoke during pregnancy could interact with *MTRR*. Chemical components found in tobacco smoke possibly alter the ability of the cell to store and metabolize folate (24). For this reason, current smoking status affects dietary nutrient intake as well as plasma folate levels (25). Considering that women have a decreased folate and vitamin B12 serum concentrations during pregnancy, we hypothesize that women smokers in gestational period that carrier the polymorphic variant in *MTRR* gene need more vitamin supplementation.

The results of this study suggest that further investigations should be performed in order to confirm the involvement of *MTRR* in the etiology of NSCL/P, maybe by looking for other polymorphic loci on *MTRR* gene. In addition, we recommend that, in the future, the data about mother supplementation during pregnancy should be collected. The absence of these data is an obvious limitation of our study. However, the set of results obtained on this direction will bring some conclusion regarding the knowledge about the *MTRR* involvement in NSCL/P etiology.

In conclusion, the present study did not confirm that the polymorphism rs1801198 in *TCN2* is associated with NSCL/P. However our results suggested that the polymorphism rs1801394 in *MTRR* may be associated with the NSCL/P in a southeast Brazilian population, mainly cleft lip and palate type.

Table 2. Characteristics of the groups

Characteristics	NSCL/P group (n=401)	Non-cleft group (n=466)	p value
Mean age in years (SD)	16.55 (±11.48)	20.39 (±15.94)	0.001*
Sex (%)			
Male	221 (55.1)	211 (45.3)	0.004**
Female	180 (44.9)	255 (54.7)	
Ethnic group (%)			
Caucasian	252 (62.8)	311 (66.7)	0.203**
Black	149 (37.2)	155 (33.3)	
Mothers smoking during pregnancy (%)			
Yes	75 (18.8)	63 (13.6)	0.037**
No	324 (81.2)	401 (86.4)	
Alcohol consumption during pregnancy (%)			
Yes	42 (10.5)	33 (7.1)	0.080**
No	357 (89.5)	430 (92.9)	
Only NSCL/P group			
Cleft Type (%)			
Cleft lip only	71 (17.7)		
Cleft lip and palate	270 (67.3)		
Cleft palate only	60 (15.0)		
Cleft side (%)			
Bilateral cleft	94 (27.6)		
Unilateral left cleft	172 (50.5)		
Unilateral right cleft	75 (21.9)		
Positive family history of cleft (%)			
Familial cases	100 (24.9)		
Sporadic cases	301 (75.1)		

*Note: Student test,** chi-square test p≤0.05; bold forms indicated statistical significance.

Table 3. Frequency of *TCN2* allele and genotype distribution among NSCL/P and non-clefts groups

Subjects	Alleles n (%)		Genotypes n (%)			p value
	C	G	CC	CG	GG	
Non-clefts	557 (63.3)	323 (36.7)	179 (40.7)	199 (45.2)	62 (14.1)	------
Cleft type						
All Clefts	438 (61.0)	280 (39.0)	139 (38.7)	160 (44.6)	60 (16.7)	0.58
Cleft lip only	75 (63.6)	43 (36.4)	25 (42.4)	25 (42.4)	9 (15.2)	0.91
Cleft lip and palate	304 (61.0)	194 (39.0)	94 (37.8)	116 (46.5)	39 (15.7)	0.71
Cleft palate only	59 (57.8)	43 (42.2)	20 (39.2)	19 (37.2)	12 (23.6)	0.18
Cleft side						
Bilateral	113 (62.1)	69 (37.9)	38 (41.8)	37 (40.7)	16 (17.5)	0.61
Unilateral left	189 (63.0)	111 (37.0)	60 (40.0)	69 (46.0)	21 (14.0)	0.99
Unilateral right	77 (57.5)	57 (42.5)	21 (31.3)	35 (52.2)	11 (16.5)	0.35
Subgroups (only NSCL/P)						
Mother smoking during pregnancy						
No	350 (60.2)	232 (39.8)	109 (37.5)	132 (45.4)	50 (17.2)	0.57
Yes	86 (65.2)	46 (34.8)	29 (43.9)	28 (42.5)	9 (13.6)	
Alcohol consumption during pregnancy						
No	395 (61.4)	249 (38.6)	126 (39.1)	143 (44.4)	53 (16.5)	0.85
Yes	41 (58.6)	29 (41.4)	12 (34.3)	17 (48.6)	6 (17.1)	

Table 4. Frequency of *MTRR* allele and genotype distribution among NSCL/P and non-clefts groups

Subjects	Alleles n (%)		Genotypes n (%)			p value
	A	G	AA	AG	GG	
Non-clefts	465 (57.9)	337 (42.1)	136 (34.1)	193 (48.1)	72 (17.8)	------
Cleft type						
All Clefts	384 (56.1)	300 (43.9)	95 (27.8)	194 (56.7)	53 (15.5)	0.06
Cleft lip only	66 (56.9)	50 (43.1)	17 (29.3)	32 (55.2)	9 (15.5)	0.60
Cleft lip and palate	260 (55.8)	206 (44.2)	63 (27.0)	134 (57.5)	36 (15.5)	0.07
Cleft palate only	58 (56.9)	44 (43.1)	15 (29.4)	28 (54.9)	8 (15.7)	0.66
Cleft side						
Bilateral	95 (57.9)	69 (42.1)	24 (29.3)	47 (57.3)	11 (13.4)	0.30
Unilateral left	160 (54.8)	132 (45.2)	39 (26.7)	82 (56.2)	25 (17.1)	0.21
Unilateral right	71 (56.3)	55 (43.7)	17 (27.0)	37 (58.7)	9 (14.3)	0.29
Subgroups (only NSCL/P)						
Mother smoking during pregnancy						
No	305 (55.3)	247 (44.7)	77 (27.9)	151 (54.7)	48 (17.4)	0.07
Yes	78 (60.9)	50 (39.1)	18 (28.1)	42 (65.6)	4 (6.3)	
Alcohol consumption during pregnancy						
No	348 (56.7)	266 (43.3)	89 (29.0)	170 (55.4)	48 (15.6)	0.28
Yes	35 (53.0)	31 (47.0)	6 (18.2)	23 (69.7)	4 (12.1)	

Resumo

O objetivo desse estudo foi avaliar a associação entre os polimorfismos no gene *TCN2* (rs1801198) e no gene *MTRR* (rs1801394) com fissura de lábio e/ou palato não sindrômica (NSFL/P) em uma população brasileira. DNA genômico foi extraído de células bucais. Os polimorfismos nos genes *TCN2* (rs1801198) e *MTRR* (rs1801394) foram genotipados através do PCR em tempo real pelo método Taqman. O teste do qui-quadrado foi utilizado para determinar a associação entre a frequência alélica e genotípica e NSFL/P e nos subtipos (fissura de lábio, fissura de lábio com palato e fissura de palato). Oitocentos e sessenta e sete indivíduos não aparentados (401 casos com NSFL/P e 466 indivíduos sem fissura) foram avaliados. A distribuição dos genótipos dos polimorfismos de *TCN2* e *MTRR* estavam em equilíbrio de Hardy-Weinberg. O genótipo polimórfico GG do gene *TCN2* foi identificado em 16,7% do grupo com NSFL/P e em 14,1% do grupo sem fissura (p>0,05). Da mesma forma, a freqüência do genótipo GG do gene *MTRR* foi bastante semelhante entre o grupo com NSFL/P (15,5%) e o grupo controle (17,8%). A análise multivariada mostrou associação entre o gene *MTRR* e o subgrupo que apresentou tabagismo materno durante a gestação (p=0,039). Nossos resultados mostraram que não há associação entre os polimorfismos nos genes *TCN2* e NSFL/P, entretanto sugerem uma associação entre *MTRR* e a etiologia de NSFL/P.

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