International Journal of Immunopathology & Pharmacology

Molecular Biology in Dentistry- Original Research Article

International Journal of Immunopathology and Pharmacology
Volume 33: 1–5

DOI: 10.1177/2058738419858572
journals.sagepub.com/home/iji
SAGE

Non-syndromic cleft palate: Association analysis on three gene polymorphisms of the folate pathway in Asian and Italian populations

Francesco Carinci[1*], Annalisa Palmieri[2*], Luca Scapoli[2], Francesca Cura[2], Francesco Borelli[3], Paolo Giovanni Morselli[2,4], Nayereh Nouri[5], Hossein Abdali[6], Aldo Bruno Gianni[7,8], Antonio Russillo[7,8], Raffaella Docimo[9] and Marcella Martinelli[2]

Abstract

Periconceptional folic acid supplementation can reduce the risk of inborn malformations, including orofacial clefts. Polymorphisms of MTHFR, TCN2, and CBS folate-related genes seem to modulate the risk of cleft lip with or without cleft palate (CL/P) in some populations. CL/P and cleft palate only (CPO) are different malformations that share several features and possibly etiological causes. In the present investigation, we conducted a family-based, candidate gene association study of non-syndromic CPO. Three single nucleotide polymorphisms, namely, rs1801133 of MTHFR, rs1801198 of TCN2, and rs4920037 of CBS, were investigated in a sample that included 129 Italian and 65 Asian families. No evidence of association between the three genotyped polymorphisms and CPO was found in the Italian and Asian cases, indeed the transmission disequilibrium test did not detect any asymmetry of transmission of alleles. This investigation, although with some limitation, further supports that CL/P and CPO diverge in their genetic background.

Keywords

Asian and Italian populations, cleft palate, folic acid

Date received: 20 September 2018; accepted: 30 May 2019

Introduction

Folate metabolism plays a critical role in embryonic development. The relationship between periconceptional folic acid supplementation and reduced risk for neural tube defects is well established.[1] Neural tube and oral cavity are embryologically related. In fact, the neural crest cells that contribute to the orofacial and tooth morphology originate from the dorsolateral regions of the neural tube.

Evidence supporting a preventive effect for folic acid against birth defects have been reported, but contrasting data have been obtained about clefting.[2] A meta-analysis carried out on 12 case–control studies on folic acid-containing supplement consumption during pregnancy and risk for oral clefts evidenced a combined relative risk of 0.77 and 0.80

[1]Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy
[2]Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Bologna, Italy
[3]Plastic and Reconstructive Surgery Unit, Maugeri Clinical Scientific Institutes, Pavia, Italy
[4]Plastic Surgery Unit, Sant'Orsola Malpighi University Hospital, Bologna, Italy
[5]Department of Genetics and Molecular Biology, Medical School, Isfahan University of Medical Sciences, Isfahan, Iran
[6]Craniofacial and Cleft Research Center, Isfahan University of Medical Sciences, Isfahan, Iran
[7]Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy
[8]Maxillofacial and Dental Unit, Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, Milan, Italy
[9]Department of Clinical Sciences and Translational Medicine, University of Tor Vergata, Rome, Italy

*These authors contributed equally to this work.

Corresponding author:
Luca Scapoli, Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, 40126 Bologna, Italy.
Email: luca.scapoli2@unibo.it

Table 1. Characteristics of investigated SNPs.

Gene	dbSNP ID[a]	Genomic position[b]	Location	Alleles[c]	Residue change	MAF
MTHFR	rs1801133	chr1:11796321	Exon 5	C/T	A [Ala] → V [Val]	0.38
TCN2	rs1801198	chr22:30615623	Exon 6	C/G	P [Pro] → R [Arg]	0.41
CBS	rs4920037	chr21:43061781	Intron 11	G/A	–	0.19

SNP: single nucleotide polymorphism; MAF: minor allele frequency.
[a]NCBI-SNP database accession numbers.
[b]UCSC Genome Browser on Human December 2013 (GRCh38/hg38) Assembly.
[c]Major allele first.

for cleft lip with or without cleft palate (CL/P) and cleft palate only (CPO), respectively.[3] Non-syndromic orofacial clefts are complex traits with a strong genetic component that interacts with environmental factors. Folate has long been considered one of these factors. Thus, besides periconceptional folic acid dietary intake and supplementation, a number of genetic variants in folate and methionine metabolism-related genes have been considered as potential susceptibility factors.[4,5] Our studies on Italian population provided evidence that maternal genotype at C677T MTHFR gene, as well as specific alleles of one-carbon metabolic pathway genes, can influence the risk for non-syndromic CL/P (nsCL/P).[6–9] On the contrary, the same three polymorphisms (rs1801133 of MTHFR, rs1801198 of TCN2, and rs4920037 of CBS) investigated in independent samples of different ancestries showed discordant results. In particular, 677T allele at MTHFR gene seemed to have a protective effect at the child level, reducing the risk of cleft in an Asian population sample.[10]

nsCL/P and non-syndromic CPO (nsCPO) are considered distinct but related malformations.[11,12] It is still a matter of debate whether CL/P and CPO share etiological factors. The aim of this study was to verify whether MTHFR, TCN2, and CBS alleles, which were previously found associated with the increased risk of nsCL/P, could be involved in CPO etiology in the Italian population and in a sample of Asian population from Iran, Tibet, and Bangladesh.

Materials and methods

Sample study

This family-based association study was performed with two different cohorts. The first one included 129 unrelated CPO patients and their parents with Italian ancestry, recruited by the Department of Medical and Surgical Sciences of S. Orsola-Malpighi Polyclinic, Bologna University. The second one that included 65 unrelated Asian probands and their parents was composed of 44 families of Fars ethnicity from the central region of Iran, 11 families from Yushu Tibetan Autonomous Prefecture, and 10 from Bangladesh. We have implemented systematic guidelines to collect samples uniformly from different countries. A clinician ascertained the non-syndromic status of all the probands and that their mothers had neither smoked nor used clefting drugs such as phenytoin, warfarin, and ethanol during pregnancy. In the interview, part of the mothers reported folic acid supplementation in late pregnancy, but all of them excluded that supplementation occurred in periconceptional, or in the early weeks of pregnancy. The study was approved by the ethics committees of the Centers involved in the study, and it complied with the Declaration of Helsinki's Ethical Principles for Medical Research Involving Human Subjects. Written informed consent was obtained from all patients and parents before study entry. Among the Italian patients enrolled in the study, 120 (93%) were considered sporadic or non-familial cases, while for the remaining 9 probands (7%) at least one more (second, third, or fourth degree) relative was reported as affected by cleft of the palate. Among Asian cases, 56 (86%) were sporadic, while 9 (14%) familial.

Genotyping

Three polymorphisms of one-carbon metabolic pathway genes were selected for this study, because they can influence the risk for nsCL/P (Table 1).[6–9] Polymorphisms were typed using the TaqMan SNP Genotyping Assay (Assay-On-Demand ID: C_1202883_20 for MTHFR rs1801133, C_325467_10 for TCN2 rs1801198, and C_1605440_1 for CBS rs4920037) on a 7500 Sequence Detection System (Life Technologies, Foster City, CA, USA) following the manufacturer's protocol.

Table 2. Family-based association analysis of folate pathway polymorphisms in nsCPO.

Sample	Gene	SNP ID	Alleles[a]	T[b]	U[c]	TDT *P* value	OR (95% CI)
Italian	MTHFR	rs1801133	C/T	57	58	0.93	0.98 (0.68–1.42)
Italian	TCN2	rs1801198	C/G	61	62	0.93	0.98 (0.69–1.40)
Italian	CBS	rs4920037	G/A	39	33	0.48	1.18 (0.74–1.88)
Asian	MTHFR	rs1801133	C/T	14	12	0.69	1.17 (0.54–2.52)
Asian	TCN2	rs1801198	C/G	22	20	0.76	1.10 (0.60–2.02)
Asian	CBS	rs4920037	G/A	14	14	1.00	1.00 (0.48–2.10)
Whole	MTHFR	rs1801133	C/T	71	70	0.93	1.01 (0.73–1.41)
Whole	TCN2	rs1801198	C/G	83	82	0.94	1.01 (0.75–1.37)
Whole	CBS	rs4920037	G/A	53	47	0.55	1.13 (0.76–1.67)

nsCPO: non-syndromic cleft palate only; SNP: single nucleotide polymorphism; TDT: transmission disequilibrium test; OD: odds ratio; CI: confidence interval.
[a]Major allele first.
[b]Number of times the minor allele was transmitted from heterozygous parents.
[c]Number of times the minor allele was untransmitted from heterozygous parents.

Statistical analysis

Power calculation indicated that the sample size of the study provided a power >80% to detect linkage disequilibrium with mutations that increase two times the relative risk of CPO. The Hardy–Weinberg equilibrium for genotype distribution, in both proband and parent groups, was examined using Pearson's χ^2 test with a significant threshold of $P < 0.05$.

Association analysis was performed on a Microsoft Windows platform by the PLINK v1.07 software.[13] Basically, the family-based association analysis was performed by the transmission disequilibrium test (TDT) that examined allele transmission from heterozygous parents to the affected probands.[14] The odds ratio (OR) was used to evaluate the level of association; this corresponds to the ratio M1/M2, where M1 denotes the number of transmissions of the reference allele from heterozygous parents to affected offspring and M2 the number of times that the allele was not transmitted.[15]

Family-based association was analyzed with the whole sample to maximize the power of the study. Then, the Italian and Asian samples were analyzed separately in order to avoid that genetic heterogeneity, or different population structure, mask association signals.

Results

Three candidate gene polymorphisms were investigated for association with nsCPO. Genotype frequencies in patients and parents were distributed according to the Hardy–Weinberg equilibrium law, and no Mendelian errors were detected.

The family-based association study was performed by the TDT. The analysis revealed that alleles of candidate genes were transmitted from heterozygous parents to CPO patients according to Mendelian expectation, indicating no association with the malformation (Table 2). This result was obtained with the entire data set and then confirmed with the Italian and Asian populations, singly.

Discussion

The orofacial shape is determined by several facial prominences that grow, converge, and differentiate during the second–third months of embryo development. The processes that lead to the orofacial morphology are regulated by an intricate network of inductive signals from ectoderm, mesoderm, endoderm, and neural crest in each prominence.[16] A failure of these crucial steps could originate a malformation. An important observation is the awareness that mothers who increase their intake of folic acid can reduce the risk of having a child with a birth defect, such as neural tube defect or cleft palate.[17] How this could happen is still a matter of study. The link between folate and craniofacial development has also been established in several vertebrate models, in which researchers found a correlation between folate deficiency and/or mutations in folate pathway genes and the presence of orofacial defects.[18–20] Recently, Wahl et al.[21] tried to elucidate the mechanism of protection acted by folic acid during palate development, in a *Xenopus* model. The authors

demonstrated that folic acid supplementation can reduce orofacial median clefts by enhancing cell survival or boosting proliferation.

The increasing mass of evidence supporting a critical role of folate in orofacial development and the finding of association between one-carbon metabolic pathway genes and nsCL/P collected so far pose the foundation for an nsCPO investigation. This study aimed to verify the involvement of polymorphisms of three folate-related genes—MTHFR, TCN2, and CBS—in nsCPO etiology. Nevertheless, it was not possible to investigate a potential gene–environment interaction by taking into account of folic acid intake during the periconceptional period. Indeed, dietary intake was not evaluated, while folic acid supplementation in the periconceptional period was excluded. Some mothers reported of folic acid intake during pregnancy, but supplementation began in the second trimester, too late to influence the risk of orofacial cleft in offspring.

In a previous attempt, Mills et al.[22] did not find a significant association with the variant at C677T MTHFR polymorphism in CPO cases, but instead they found support for a maternal effect. In the same investigation, they excluded a role for C667G TCN2 in their sample study. The CBS rs2124459 minor allele was seen associated with a reduced risk of CPO, in both mothers and children of French and Belgian nsCPO cohorts.[23] Since the risk of nsCPO associated with specific variants in genes crucial for the folic acid metabolism was not extensively studied and it differs among ethnic groups, we investigated our cohorts of Italian and Asian CPO cases for three emblematic polymorphisms linked to folate, vitamin B12, and methionine pathways.

Our results did not support the hypothesis of an involvement of the three investigated genes, at least for the three polymorphisms considered. This could be attributable to the small size of the sample study or to its fragmentation. However, our data confirm the difficulty to find common genetic causes for both CL/P and CPO.

Acknowledgements

We are indebted to the families participating in the study for their invaluable contribution, as well as to all the personnel involved in clinical data and specimen collection.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported in part by a grant from the Interethnos-Interplast Association, Italy and in part by the grant number 189087 from deputy for research, Isfahan University of Medical Sciences, Isfahan, Iran.

ORCID iDs

Francesco Carinci https://orcid.org/0000-0001-9639-6676

Luca Scapoli https://orcid.org/0000-0003-4006-9910

References

1. Wolff T, Witkop CT, Miller T, et al. (2009) Folic acid supplementation for the prevention of neural tube defects: An update of the evidence for the U.S. Preventive Services Task Force. *Annals of Internal Medicine* 150: 632–639.
2. De-Regil LM, Pena-Rosas JP, Fernandez-Gaxiola AC, et al. (2015) Effects and safety of periconceptional oral folate supplementation for preventing birth defects. *The Cochrane Database of Systematic Reviews* 12: CD007950.
3. Badovinac RL, Werler MM, Williams PL, et al. (2007) Folic acid-containing supplement consumption during pregnancy and risk for oral clefts: A meta-analysis. *Birth Defects Research Part A, Clinical and Molecular Teratology* 79(1): 8–15.
4. Marini NJ, Yang W, Asrani K, et al. (2016) Sequence variation in folate pathway genes and risks of human cleft lip with or without cleft palate. *American Journal of Medical Genetics Part A* 170: 2777–2787.
5. Jiang C, Yin N, Zhao Z, et al. (2015) Lack of association between MTHFR, MTR, MTRR, and TCN2 genes and nonsyndromic CL±P in a Chinese population: Case-control study and meta-analysis. *The Cleft Palate-Craniofacial Journal* 52(5): 579–587.
6. Martinelli M, Scapoli L, Pezzetti F, et al. (2001) C677T variant form at the MTHFR gene and CL/P: A risk factor for mothers? *American Journal of Medical Genetics* 98(4): 357–360.
7. Pezzetti F, Martinelli M, Scapoli L, et al. (2004) Maternal MTHFR variant forms increase the risk in offspring of isolated nonsyndromic cleft lip with or without cleft palate. *Human Mutation* 24: 104–105.

8. Martinelli M, Scapoli L, Palmieri A, et al. (2006) Study of four genes belonging to the folate pathway: Transcobalamin 2 is involved in the onset of non-syndromic cleft lip with or without cleft palate. *Human Mutation* 27: 294.
9. Martinelli M, Masiero E, Carinci F, et al. (2011) New evidence for the role of cystathionine beta-synthase in non-syndromic cleft lip with or without cleft palate. *European Journal of Oral Sciences* 119: 193–197.
10. Martinelli M, Girardi A, Cura F, et al. (2016) Non-syndromic cleft lip with or without cleft palate in Asian populations: Association analysis on three gene polymorphisms of the folate pathway. *Archives of Oral Biology* 61: 79–82.
11. Fogh-Andersen P (1942) *Inheritance of Harelip and Cleft Palate*. Copenhagen: Busck.
12. Christensen K and Fogh-Andersen P (1994) Etiological subgroups in non-syndromic isolated cleft palate. A genetic-epidemiological study of 52 Danish birth cohorts. *Clinical Genetics* 46(5): 329–335.
13. Purcell S, Neale B, Todd-Brown K, et al. (2007) PLINK: A tool set for whole-genome association and population-based linkage analyses. *American Journal of Human Genetics* 81(3): 559–575.
14. Spielman RS, McGinnis RE and Ewens WJ (1993) Transmission test for linkage disequilibrium: The insulin gene region and insulin-dependent diabetes mellitus (IDDM). *American Journal of Human Genetics* 52(3): 506–516.
15. Kazeem GR and Farrall M (2005) Integrating case-control and TDT studies. *Annals of Human Genetics* 69(Pt. 3): 329–335.
16. Szabo-Rogers HL, Smithers LE, Yakob W, et al. (2010) New directions in craniofacial morphogenesis. *Developmental Biology* 341(1): 84–94.
17. Wilcox AJ, Lie RT, Solvoll K, et al. (2007) Folic acid supplements and risk of facial clefts: National population based case-control study. *BMJ* 334(7591): 464.
18. Tang LS, Santillano DR, Wlodarczyk BJ, et al. (2005) Role of Folbp1 in the regional regulation of apoptosis and cell proliferation in the developing neural tube and craniofacies. *American Journal of Medical Genetics Part C, Seminars in Medical Genetics* 135C(1): 48–58.
19. Momb J, Lewandowski JP, Bryant JD, et al. (2013) Deletion of Mthfd1l causes embryonic lethality and neural tube and craniofacial defects in mice. *Proceedings of the National Academy of Sciences of the United States of America* 110(2): 549–554.
20. Maldonado E, Lopez Y, Herrera M, et al. (2018) Craniofacial structure alterations of foetuses from folic acid deficient pregnant mice. *Annals of Anatomy* 218: 59–68.
21. Wahl SE, Kennedy AE, Wyatt BH, et al. (2015) The role of folate metabolism in orofacial development and clefting. *Developmental Biology* 405(1): 108–122.
22. Mills JL, Molloy AM, Parle-McDermott A, et al. (2008) Folate-related gene polymorphisms as risk factors for cleft lip and cleft palate. *Birth Defects Research Part A, Clinical and Molecular Teratology* 82(9): 636–643.
23. Goffinet L, Oussalah A, Gueant-Rodriguez RM, et al. (2016) Cystathionine beta-synthase genetic variant rs2124459 is associated with a reduced risk of cleft palate in French and Belgian populations. *Journal of Medical Genetics* 53(12): 828–834.