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. 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. 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.
for cleft lip with or without cleft palate (CL/P) and cleft palate only (CPO), respectively. 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 1. Characteristics of investigated SNPs.

| Gene   | dbsNP IDa | Genomic positionb | Location | Allelesc | Residue change | MAF  |
|--------|-----------|--------------------|----------|----------|----------------|------|
| MTHFR  | rs1801133 | chr1:11796321      | Exon 5   | C/T      | A [Ala] → V [Val] | 0.38 |
| TCN2   | rs1801198 | chr22:30615623     | Exon 6   | G/G      | P [Pro] → R [Arg] | 0.19 |
| CBS    | rs4920037 | chr21:43061781     | Intron 1 | G/A      | –              | 0.19 |

SNP: single nucleotide polymorphism; MAF: minor allele frequency.

aNCBI-SNP database accession numbers.
bUCSC Genome Browser on Human December 2013 (GRCh38/hg38) Assembly.
cMajor allele first.
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 $\chi^2$ test with a significant threshold of $P < 0.05$.

Association analysis was performed on a Microsoft Windows platform by the PLINK v1.07 software.\textsuperscript{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.\textsuperscript{14} The odds ratio (OR) was used to evaluate the level of association; this corresponds to the ratio $M_1/M_2$, where $M_1$ denotes the number of transmissions of the reference allele from heterozygous parents to affected offspring and $M_2$ the number of times that the allele was not transmitted.\textsuperscript{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.

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       | 41    | 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) |

\textsuperscript{a}Major allele first.

\textsuperscript{b}Number of times the minor allele was transmitted from heterozygous parents.

\textsuperscript{c}Number of times the minor allele was untransmitted from heterozygous parents.

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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{18–20} Recently, Wahl et al.\textsuperscript{21} tried to elucidate the mechanism of protection acted by folic acid during palate development, in a \textit{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.\textsuperscript{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.\textsuperscript{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.

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