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Non-syndromic cleft lip with or without cleft palate in Asian populations: Association analysis on three gene polymorphisms of the folate pathway

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ABSTRACT

Objective: Orofacial clefts (OFCs) are one of the most common birth defects in humans. They are the subject of a number of investigations aimed at elucidating the bases of their complex mode of inheritance involving both genetic and environmental factors. Genes belonging to the folate pathway have been among the most studied. The aim of the investigation was to replicate previous studies reporting evidence of association between polymorphisms of folate related genes and the occurrence of non-syndromic cleft lip with or without cleft palate (NSCL/P), using three independent samples of different ancestry: from Tibet, Bangladesh and Iran, respectively.
Design: Specifically, the polymorphisms rs1801133 of *MTHFR*, rs1801198 of *TCN2*, and rs4920037 of *CBS*, were tested.
Results: A decreased risk of NSCL/P was observed in patients presenting the C677T variant at *MTHFR* gene (relative risk for heterozygotes = 0.53; 95% confidence interval [C.I.] = 0.32–0.87). The investigated polymorphisms mapping at *TCN2* and *CBS* genes did not provide any evidence of association.
Conclusion: Overall, these results indicate that NSCL/P risk factors differ among populations and confirm the importance of testing putative susceptibility variants in different genetic backgrounds.

1. Introduction

Clefts of the lip with or without cleft palate (CL/P), and cleft palate only (CPO) – collectively called orofacial clefts (OFC) – occur in 0.5–3 per 1000 live and stillbirths and represent one of the most common types of major birth defects in humans. OFC pathogenesis is not yet completely understood, but it is acknowledged that both genetic and environmental factors are involved in its multifactorial aetiology. A further distinction is made on the base of the presence or absence of additional defects: syndromic or non-syndromic forms respectively. Over the years, great efforts have been made to attempt to elucidate both genetic contribution and additional factors that play a role in this common orofacial pathology (Leslie & Marazita, 2013). Folate pathway has been extensively studied in the past two decades, since the identification of folic acid as a potential environmental factor in OFC. Even if the association between folate intake and risk of CL/P is still a matter of debate, epidemiologic studies report that a daily folic acid supplementation helps to reduce the risk of orofacial cleft (Tolarova & Harris, 1995; Shaw, Lammer, Wasserman, O'Malley, & Tolarova, 1995; Wilcox et al., 2007). The correlation between a paucity of maternal plasmatic folate caused by the increased foetal need and the increased risk of CL/P is possibly exacerbated by the presence of the C677T common nucleotidic variant at the

Abbreviations: OFC, orofacial cleft; CL/P, cleft lip with or without cleft palate; CPO, cleft palate only; NSCL/P, non-syndromic cleft lip with or without cleft palate; *MTHFR*, methylenetetrahydrofolate reductase; *TCN2*, transcobalamin 2; *CBS*, cystathionine beta-synthase; SNP, single nucleotide polymorphism; TDT, transmission disequilibrium test.

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methylenetetrahydrofolate reductase (*MTHFR*) gene sequence, evidenced by Frosst et al. (1995). The activity of the enzyme encoded by the *MTHFR* gene is crucial for the use of one-carbon unit donors in the homocysteine conversion to methionine and in a number of methylation reactions. For the correct remethylation of homocysteine to methionine, an adequate cobalamin (vitamin B12) intracellular presence is necessary, guaranteed by transcobalamin 2 (encoded by *TCN2* gene), a plasmatic protein able to bind and transport vitamin B12. An alternative way to lower homocysteine plasmatic level is by transsulfuration. This is firstly catalysed by cystathionine beta-synthase (the product of the *CBS* gene), a vitamin B6-dependent lyase responsible for the condensation of homocysteine with serine, the product of which is cystathionine.

Our group previously reported evidence of an increased risk of NSCL/P associated to specific alleles at polymorphic sites for each of the above-mentioned genes: *MTHFR*, *TCN2* and *CBS*, in a sample study representative of the Italian population. Specifically, we observed that the presence of the C677T variant in the *MTHFR* gene in mothers, is responsible for a higher risk of having affected offspring both in familial NSCL/P (Martinelli et al., 2001) and in sporadic cases (Pezzetti et al., 2004). In addition, we found a significant disequilibrium ($P = 0.01$) regarding the transmission of the ancestral allele for the C776G *TCN2* polymorphism in a sample study of 218 familial and sporadic triads (Martinelli et al., 2006). Finally, we evidenced a foetal/maternal interaction, considering the transmission of rs4920037 alleles at *CBS* gene, responsible for an increased risk of clefting (Martinelli, Masiero et al., 2011). Interestingly, two of the considered polymorphisms have an impact on the gene product functionality. In fact, C677T (rs1801133) at *MTHFR* consists in a mutation responsible for the synthesis of a thermolabile enzyme with reduced activity (Kang et al., 1991), while C776G (rs1801198) in *TCN2* seems to be accountable for a reduced proportion of vitamin B12 bound to *TCN2* (Afman, Lievers, van der Put, Trijbels, & Blom, 2002).

Motivated by findings which emerged from analysing our Italian sample, we decided to try to confirm association for the three genes mentioned, investigating three different cohorts of NSCL/P, recruited in Tibet, Bangladesh, and Iran respectively. To our knowledge, no previous studies testing for association between genetic factors and OFC have been carried out with populations from Tibet or Bangladesh, while two epidemiological studies have been published regarding the Iranian population (Golalipour, Kaviany, Qorbani, & Mobasheri, 2012; Taghavi et al., 2012). In one of these, an increased risk of non-syndromic OFC was observed in a case-control study on mothers of affected children, who had assumed 7% less folate during pregnancy, than the control mothers (Taghavi et al., 2012); while in the other study, the association between a lack of folic acid consumption and increased risk of oral clefting was not statistically significant (Golalipour et al., 2012).

2. Materials & methods

2.1. Sample study

For the present investigation, a total of 169 NSCL/P probands were enrolled, in particular 43 patients from Tibet, 61 from Bangladesh, and 65 from Iran. Specifically, probands from Bangladesh belonged to different ethnic groups and tribes. Patients enrolled in Tibet were natives of the Yushu Tibetan Autonomous Prefecture, while Iranian probands belonged to Fars ethnicity from the central region of Iran. In addition 260 of their parents participated in the study. 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 Table 1 is reported a description of NSCL/P probands. After obtaining informed consent, peripheral venous blood samples (10 ml) were collected and conserved in EDTA; DNA was extracted using specific kits (*i.e.* GenElute™ Blood Genomic DNA Kit—Sigma, Milan, Italy) and quantified by a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Table 1
Description of NSCL/P patients.

Ethnicity	Gender		CL			CLP		
	Male	Female	Right	Left	Both	Right	Left	Both
Bangladesh	43	18	5	13		11	19	13
Iran	36	29	1	3		30	30	1
Tibet	30	13	9	13	2	5	11	3

CL, cleft lip.
CLP, cleft lip and palate.

2.1.1. Genotyping

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.

2.1.2. Statistical analysis

Genotypic data were analysed to test different causal scenarios in which the child's own genotype or the mother's one was directly relevant to clefting risk. A log-linear method for the analysis of case-parents triad data, based on maximum likelihood with stratification on parental mating types, was used to test different genetic hypotheses (Wilcox, Weinberg, & Lie, 1998). The method, implemented using specific scripts for the LEM free software (http://www.niehs.nih.gov/research/resources/software/biostatistics/lem/index.cfm), was able to deal with missing parents by using the expectation-maximization algorithm (Weinberg, 1999). Likelihood-ratio tests (LRT) were performed to test for offspring association (child *vs.* null) and for maternal association (mother *vs.* null) as compared with the null hypothesis in which the variant allele, in either a child or a mother, did not alter the risk of NSCL/P. The LRT statistic was obtained by multiplying by two the log-likelihood difference, the larger model minus the log-likelihood of the null hypothesis. The LRT statistic was compared to the chi-squared reference distribution with degrees-of-freedom which were equal to the difference in the number of parameters between the two models.

3. Results

The investigation involved 169 NSCL/P patients and 260 of their parents from three different ethnicities. Three SNPs in different candidate genes were analysed, two of them were suspected to alter the protein activity and directly modulate the risk of NSCL/P. Genotype was obtained from >99% of the samples, no Mendelian error was detected, and genotype frequency among parents was in Hardy–Weinberg equilibrium at each of the investigated SNPs.

A log-linear, likelihood-based method was used to test two hypotheses: (i) if the child genotypes, and (ii) if maternal genotypes may influence the risk of NSCL/P, reported in Table 2 as child *vs.* null, and mother *vs.* null, respectively. The whole sample was analysed, as well as each ethnicity separately. Evidence of association at child level was observed for the *MTHFR* polymorphism in the whole sample ($P < 0.05$). Interestingly, the variant allele (677T) appears to reduce the risk of cleft. The