ORIGINAL ARTICLE

MTHFR c.677C>T is a risk factor for non-syndromic cleft lip with or without cleft palate in Chile

C Ramirez-Chau¹, R Blanco², A Colombo³, R Pardo⁴, J Suazo¹

¹Instituto de Investigación en Ciencias Odontológicas, Facultad de Odontología, Universidad de Chile, Santiago; ²Programa de Genética Humana, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Santiago; ³Programa de Anatomía y Biología del Desarrollo, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Santiago; ⁴Servicio de Anatomía Patológica, Hospital Clínico de la Universidad de Chile, Santiago; ⁵Sección de Genética, Hospital Clínico Universidad de Chile, Santiago; ⁶Unidad de Neonatología, Hospital Clínico Universidad de Chile, Santiago; ⁷Unidad de Genética, Hospital Dr. Sótero del Río, Santiago, Chile

OBJECTIVE: The functional variant within the 5,10-methylene-tetrahydrofolate reductase (MTHFR) gene c.677C>T, producing alterations in folate metabolism, has been associated with the risk of non-syndromic cleft lip with or without cleft palate (NSCL/P). We assessed this association in a Chilean population using a combined analysis of case–control and case–parent trio samples.

SUBJECTS AND METHODS: Samples of 165 cases and 291 controls and 121 case–parent trios (sharing the cases) were genotyped. Odds ratio (OR) was estimated for case–control (allele and genotype frequency differences), and this result was confirmed by allele transmission distortion in trios. Due to that these samples are not independent, a combined OR was also computed. Maternal genotype effect was additionally evaluated based on a log-linear method.

RESULTS: Borderline but not significant OR (1.28; CI 0.97–1.69) was observed for risk allele (T) in the case–control sample. However, triad sample showed a significant association (OR 1.56; CI 1.09–2.25) which was confirmed by the combined OR (1.37; CI 1.11–1.71). Maternal genotype has been also associated with the phenotype (P = 0.002).

CONCLUSIONS: In contrast to previous reports considering Chilean subjects, our results demonstrated that the offspring and maternal genotypes for MTHFR c.677C>T variant are strongly associated with NSCL/P in this Chilean population.

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Keywords: Non-syndromic cleft lip with or without cleft palate; MTHFR; case–control; case–parent trios

Introduction

Orofacial clefts (OFCs) are among the most common birth defects worldwide. Its prevalence rates have ethnic variations ranging <0.5/1000 live newborns in Africans, 1.5/1000 in Caucasians, and >2/1000 in Asians and Amerindians (Mossey, 2007; IPDTOC Working Group, 2011). In Chile, its prevalence reaches 1.8/1000 (Nazer and Cifuentes, 2014). OFCs are classified as cleft palate only (CPO) and as cleft lip with or without cleft palate (CL/P). Approximately 70% of OFCs are non-syndromic occurring as an isolated condition without any other apparently structural or cognitive abnormality. The remaining 30% are defects found as part of more than 300 recognizable genetic syndromes (Schutte and Murray, 1999). Non-syndromic CL/P (NSCL/P) is a complex genetic disorder showing influences of environmental factors (Jugessur and Murray, 2005). Based on linkage and association analysis, several susceptibility loci have being identified where most of them are involved in maxillofacial development (Blanton et al, 2005).

Folates/folic acid (FA) metabolism is probably the best example of gene–environmental interaction in NSCL/P etiology (Blakkar et al, 2011). Folates are nutrients involved in the transfer of methyl groups to molecules involved in several biological processes such as DNA methylation, an important epigenetic mechanism of gene expression control (Luccho, 2000; Jones and Takai, 2001). Folates play a pivotal role in the relation between maternal nutrition status and normal early embryonic development (Blakkar et al, 2011). Thus, mothers of cleft cases had a diet poor in folates in comparison with mothers of non-affected children (Figueiredo et al, 2015). Consequently, case mothers have both lower plasma and lower erythrocyte folate levels than control mothers (Vukovic et al, 2007; Munger et al, 2011; Bezerra et al, 2015). In a recent meta-analysis, our group has demonstrated that mothers of NSOFc cases exhibit higher levels of plasma homocysteine than control mothers (Blanco et al, 2016). Moreover, maternal
periconceptional FA supplementation has been extensively demonstrated to be an effective intervention in order to prevent NSCL/P (Badovinac et al, 2007; Butali et al, 2013; Molina-Solana et al, 2013). There are around 50 genes coding products involved in folate transport, metabolism, methylation cycle, DNA and protein methyl group transfer, and nucleotide synthesis where several of them have NSCL/P risk polymorphisms (Franke et al, 2009; Bhaskar et al, 2011; Blanton et al, 2011). The most documented case is the polymorphism c.677C>T (p.Ala222Val) within 5,10-methylenetetrahydrofolate reductase (MTHFR) gene. This enzyme catalyzes a crucial reaction providing both the main circulating form of folates and the availability of methyl groups (Matthews et al, 1998). Genotype TT codes an enzyme with a reduced activity and its carriers show low plasma levels of folate and high levels of homocysteine (Frooss et al, 1995; Molloy et al, 1998). Meta-analyses assessing the risk of NSCL/P associated with this variant showed controversial results. Pooled effects showed increased risk for T allele carriers only in Asians (Zhao et al, 2014). However, multietnic meta-analyses present non-significant results (Luo et al, 2012; Pan et al, 2012). The two meta-analyses assessing maternal genotypes showed significant association for mothers carrying the TT genotype (Luo et al, 2012; Pan et al, 2015). In addition, interaction between maternal MTHFR c.677C>T genotypes and maternal FA intake has been demonstrated in the risk of OFCs (van Rooij et al, 2003).

The contemporary urban Chilean population is mainly the result of the Amerindian Caucasian (Spanish settlers) admixture initiated in the XVI and XVII centuries (Rothhammer et al, 1968). This population shows a gradient among Amerindian admixture, genetic markers, socioeconomic strata, and prevalence of NSCL/P generating a population stratification (Valenzuela, 1988; Palomino et al, 1997). Population-based case–control design, the most used approach for gene mapping in complex traits, may generate spurious results due to the confounding effects of population stratification (Santos et al, 2002). Thus, association evidence using this design needs to be ideally confirmed by alternative methods such as family-based approaches (Kazeem and Farrall, 2005). Based on parental and proband genotypes (case–parent trio design), a preferentially transmitted allele can be detected (Spielman et al, 1993). This method becomes insensitive to population stratification effects by the use of non-transmitted alleles from parents to offspring for a gene-matched pseudocontrol construction (Santos et al, 2002).

The aim of this study was to evaluate the possible role of MTHFR c.677C>T in the expression of NSCL/P in the Chilean population. As reference, the literature shows two previous studies including Chilean OFC subjects and their mothers from the Latin American Collaborative Study of Congenital Malformations (ECLAMC) (Vieira et al, 2005, 2008). In these reports, Chilean samples were grouped with other South American subjects and no associations between this variant and OFCs were detected. For the current work, we performed a two-stage study: (i) a case–control study and (ii) a confirmatory case–parent trio study which also allowed us to detect a maternal genotype effect on NSCL/P risk and its interaction with the child genotype. Due to that samples for each stage are not totally independent, partiality sharing the cases, a correlation between them is generated (Martin and Kaplan, 2000). Therefore, we decided to apply a combined analysis in order to integrate the risk detected in each stage based on the Kazeem and Farrall method (2005) giving a more powerful result than each stage separately (Epstein et al, 2005).

Materials and methods

Subjects

The sample of NSCL/P was composed by 165 Chilean unrelated cases (35% females) with ages ranging from 0 to 31 years. Sixty-nine percent of them have no family history of OFCs. Ninety-two percent were cleft lip and palate patients and 8% exhibited only a cleft lip. These cases were recruited between 2008 and 2011 after in-depth interviews of at least three family members for family history reconstructions. A careful anamnesis was carried out in order to exclude the effects of teratogenic substances, such as phenytoin, warfarin, and ethanol during pregnancy. These subjects were patients at the following centers: Craniofacial Malformation Unit, School of Dentistry, Universidad de Chile; Cleft Lip/Palate Center, Hospital Exequiel González Cortés; Dental Service, Hospital Roberto del Río; Maxillofacial Service, Hospital San Borja-Arriraran; and Maxillofacial Service, Hospital Sótero del Río (all of them located in the city of Santiago, Chile). For these cases, we also included both biological parents recruiting a sample of 121 case–parent trios. A control group of 291 Chilean individuals was recruited among blood donors from the Blood Bank, Hospital San José (Santiago, Chile), with no family history of OFCs. The gender distribution of controls was 45% females (P = 0.201 for proportion comparison with cases) and ages ranging from 18 to 60 years. Cases and controls belong to the middle–low and low socioeconomic strata which show the highest rates of Amerindian admixture and NSCL/P (Valenzuela, 1988; Palomino et al, 1997). The Institutional Review Board of the School of Medicine, Universidad de Chile, approved our study, and all participants gave their informed consent (Approval # 210-2015, January 12, 2016).

Molecular analysis

A peripheral venous blood sample was extracted in EDTA tubes from each participant. Genomic DNA was purified from white cells according to the method described by Chomczynski and Sacchi (1987). Genotypes for MTHFR c.677C>T (rs1801133) were obtained using a TaqMan SNP genotyping assay (Applied Biosystems, Foster City, CA, USA; ID C_1202883_20) in a real-time PCR system.

Statistical analysis

Genotype and allele frequencies were estimated using simple proportions. Hardy–Weinberg equilibrium for genotype distribution in control and parental samples was estimated by means of a goodness-of-fit chi-square test. For case–control study, the risk associated with MTHFR c.677C>T...
was evaluated computing an odds ratio (OR) with 95% confidence intervals (95% CI). For case–parent sample, allele transmission distortion from heterozygous parents to affected progeny was evaluated performing the transmission/disequilibrium test (TDT, Spielman et al., 1993). All tests were performed in the STATA 12 package. In addition for triads, we apply a log-linear method based on a maximum likelihood evaluating three different hypotheses: a null model hypothesis (a random distribution of genotypes), offspring genotype association, and maternal genotype association and interaction between them. These models were constructed based on the method described by Wilcox et al. (1998). For each model, its maximum likelihood was computed by the LEM software (Vermunt, 1997) using specific scripts designed for each hypothesis (Shi and Weinberg, 2009). A likelihood-ratio test (LRT) was applied by means of 2x (alternative model log-likelihood) with a chi-square with 1 df as the difference in the parameter number between the compared models. Finally, we computed a combined OR (with 95% CI) to obtain an overall view of the two-stage association. This analysis includes a homogeneity test of the individual ORs which distributed as a chi-square with 1 df. Both combined OR and test of homogeneity and their respective significance were performed using the formulas described by Kazeem and Farrall (2005).

Results

For case–control study (stage 1), allele association and genotype association are described in Table 1. Genotypes frequencies among controls were distributed according to Hardy–Weinberg expectations (P = 0.403, data not shown). The risk allele (T) showed a higher frequency among cases than controls, but this difference has a borderline significance (OR 1.28; 95% CI 0.97–1.69). Similar results were observed when the risk associated with TT genotype was evaluated (OR 1.72; 95% CI 0.99–2.97). However, when a recessive model for the T allele was considered (CC + CT vs TT genotypes), a significant association is observed with NSCL/P (OR 1.64; 95% CI 1.01–2.68) (Table 1). Gender-specific association analysis (Table 2) did not find significance at allele or genotype level in any case. However, our results showed a recessive model of risk only for females, in concordance with the total sample, but with confidence intervals including the value 1 (OR 2.15; 95% CI 0.93–4.95). For males, no significant results were detected (Table 2).

For stage 2 (case–parent trio study), genotypes from all parents were distributed in accordance with Hardy–Weinberg equilibrium (P = 0.695). The TDT analysis showed that the T allele was preferentially transmitted from heterozygous parents to affected progeny (75 transmissions vs 48 non-transmissions; OR 1.56; 95% CI 1.09–2.25) (Table 4). We also evaluated the possible effect of the maternal genotype and its interaction with the offspring genotype on the NSCL/P risk. Maternal genotype has influence on the phenotype risk (P = 0.002) as well as the offspring genotype (P = 0.016), corroborating the results of TDT. In addition, a significant result was observed when the interaction between maternal and offspring genotype was assessed (P = 0.0003) (Table 3).

Case–control (stage 1) and case–parent trio (stage 2) samples partially sharing the case population are not independent. Thus, we decided to apply a combined association analysis. The combined OR expresses an increase in NSCL/P risk for carriers of T allele (OR 1.37; 95% CI 1.11–1.71). This significant result is supported by the evidence of homogeneity between the ORs for each stage (P = 0.388) (Table 4).

Discussion

The aim of the current report was to assess the possible role of MTHFR c.677C>T in the expression of NSCL/P phenotype in a Chilean population, based on two complementary stages: a case–control study (stage 1) and a case–parent trio study (stage 2). We have considered as reference the results of two previous studies from the ECLAMC including lower number of OFC Chilean subjects and analyzed jointly with other samples from South American countries (Vieira et al., 2005, 2008). Both reports did not show association between MTHFR c.677C>T and non-syndromic OFCs. In the current study, for stage 1, a marginal increase in the risk of this OFC was detected at allele (T vs C) or genotype level (TT vs CC) (Table 1). However, we observed that TT genotype carriers vs CC + CT carriers (recessive model) significantly increased NSCL/P risk (Table 1). This result reflects the complexity of the model where two copies of the risk allele could be necessary to increase the phenotype susceptibility. Both positive and negative results of association have been previously described in other Latin American case–control populations (Gaspar et al., 2004; Sőzen et al., 2009; Estandia-Ortega et al., 2014; Bezerra et al., 2015). Multiethnic meta-analyses for genotypes (TT vs CC) present highly similar results to ours showing overall ORs of 1.21 (95% CI 0.97–1.53; Pan et al., 2012) and 1.22 (95% CI 0.97–1.55; Luo et al., 2012). These meta-analyses exhibit a no-significant association tendency with the exception of Asian populations. Consequently, a

| Allele and genotype association for MTHFR c.677C>T and NSCL/P in the Chilean population in cases and controls |
|---|---|---|---|---|
| Frequency | Frequency | OR (95% CI) | P value |
| Allele | (n = 165) | (n = 291) | |
| C | 0.506 | 0.568 | 1.28 (0.97–1.69) | 0.067 |
| T | 0.494 | 0.432 | |
| Genotype | | | |
| CC | 0.267 | 0.309 | |
| CT | 0.479 | 0.519 | 1.07 (0.68–1.68) | 0.769 |
| TT | 0.254 | 0.172 | 1.72 (0.99–2.97) | 0.052 |
| CC + CT vs TT (Recessive model) | 1.64 (1.01–2.68) | 0.034 |
| CC vs CT + TT (Dominant model) | 1.23 (0.78–1.93) | 0.337 |

OR (95% CI), odds ratio with 95% confidence interval.
**Table 2** Allele and genotype association for MTHFR c.677C>T and NSCL/P in the Chilean population in cases and controls by gender

<table>
<thead>
<tr>
<th>Allele</th>
<th>Females</th>
<th></th>
<th>Males</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frequency (n = 58)</td>
<td>Frequency (n = 120)</td>
<td>OR (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td>C</td>
<td>0.509</td>
<td>0.587</td>
<td>1.37 (0.86–2.20)</td>
<td>0.160</td>
</tr>
<tr>
<td>T</td>
<td>0.491</td>
<td>0.413</td>
<td>1.37 (0.86–2.20)</td>
<td>0.160</td>
</tr>
</tbody>
</table>

OR (95% CI), odds ratio with 95% confidence interval.

**Table 3** Maternal and offspring genotype effects and maternal–offspring interaction on the risk associated with MTHFR c.677C>T on NSCL/P in the Chilean population

<table>
<thead>
<tr>
<th>Model</th>
<th>LR χ²</th>
<th>df</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal model vs null model</td>
<td>12.37</td>
<td>2</td>
<td>0.002</td>
</tr>
<tr>
<td>Offspring model vs null model</td>
<td>8.26</td>
<td>2</td>
<td>0.016</td>
</tr>
<tr>
<td>Maternal–offspring interaction vs null model</td>
<td>20.90</td>
<td>4</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

LR χ², likelihood-ratio test; df, degrees of freedom.

**Table 4** Combined analysis for association between MTHFR c.677C>T and NSCL/P in the Chilean population for case–control and case–parent trio samples

<table>
<thead>
<tr>
<th>Study (stage)</th>
<th>OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case–control (1)</td>
<td>1.28 (0.97–1.69)</td>
<td>0.067</td>
</tr>
<tr>
<td>Case–parent trios (2)</td>
<td>1.56 (1.09–2.25)</td>
<td>0.015</td>
</tr>
<tr>
<td>Combined study</td>
<td>1.37 (1.11–1.71)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

*p value for homogeneity test for individual odds ratios 0.388.

In stage 2, we took advantage of the insensibility to population stratification of the case–parent trio design (Santos et al., 2002) in order to confirm stage 1 results. TDT analysis showed that the T allele constitutes a NSCL/P risk factor for the Chilean population (Table 4). Most of the triad studies reported that MTHFR c.677C>T offspring genotypes are not risk factors for NSCL/P, except for two reports (Zhu et al., 2010; de Aguiar et al., 2015) which once again may reflect a genetic heterogeneity phenomenon. To the best our knowledge, there are no systematic reviews assessing these variables.

Stage 1 and 2 results are correlated due to the samples partially share the case population, and therefore, we decided to include a combined analysis (Martin and Kaplan, 2000). Combined OR represents an increase risk of NSCL/P in the offspring carrying the T allele where its significance exceeds around fourfold the triad study and supported by a non-significance homogeneity test (Table 4). Simulation-based studies demonstrated that to add unrelated controls to a sample of case–parent trios significantly increase the test statistical power (Epstein et al., 2005). In conclusion, although we probably detect a spurious association in the case–control study, the offspring T allele is a real risk factor for the appearance of NSCL/P in Chile.

We have also evaluated the maternal genotype effect on the risk of having a NSCL/P offspring as well as the interaction between maternal and child genotype. In comparison with a null model hypothesis of association, both maternal and child genotypes were significantly associated with the offspring phenotype. Consequently, an evidence of interaction between maternal and offspring genotype was founded (Table 3). In contrast to the negative tendency observed for studies assessing case genotypes, multiethnic meta-analyses demonstrated that maternal genotypes of MTHFR c.677C>T are risk factors for NSCL/P (Luo et al., 2012; Pan et al., 2015).

As we previously mentioned, the TT genotype for MTHFR c.677C>T variant encoded an enzyme with a reduced activity impacting the folate/FA metabolism (et al., Froist et al., 1995; Molloy et al., 1998). On the other hand, maternal folate status seems to play a pivotal role during the maxillofacial development (James, 2000).
role in normal early embryonic development (Bhaskar et al., 2011). In humans and animal models, folate/FA deficiencies during early pregnant stages are associated with OFCs in the offspring (Ikeda et al., 2012; Figueiredo et al., 2015). In addition, the interaction between maternal c.677C>T genotypes and maternal FA intake in NSCL/P risk (van Rooij et al., 2003) seems to reflect the influence of the maternal variant in the proper fetal folate bioavailability. Our findings also allow postulating a maternal−offspring genotype interaction on the OFC appearance. Thus, fetal genotype may affect its own circulating folate levels, which is potentiated with a transplacental decrease of maternal folate metabolism genes) remains as a susceptibility factor for spina biﬁda: [521–526].

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Conflicts of interest

All authors declare no conflict of interests.

Author contributions

C. Ramirez-Chau, R. Blanco, and J. Suazo designed the study. R. Blanco and J. Suazo collected the biological sample and patient data. C. Ramirez-Chau and J. Suazo performed genetic and statistical analysis. C. Ramirez-Chau, R. Blanco, A. Colombo, R. Pardo and J. Suazo (i.e. all authors) wrote, revised, and approved the final version of the manuscript.

References


