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Original Article

Analysis of MTHFR Gene C.677C>T and C.1298A>C Polymorphisms in Iranian Patients with Non-Syndromic Cleft Lip and Palate

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Abstract

Background: Non-syndromic cleft lip with or without cleft palate (nsCL/P) is one of the most common congenital abnormalities of the orofacial region with a multifactorial etiology. The present study aimed to investigate the association of two common polymorphisms of methylenetetrahydrofolate reductase (MTHFR) gene (c.677C>T and c.1298A>C) with the occurrence of nsCL/P in an Iranian population.

Methods: Forty-five nsCL/P patients, 43 mothers of patients, and 101 unrelated controls participated in the present study. Analysis of c.677C>T and c.1298A>C polymorphisms in MTHFR gene was conducted using polymerase chain reaction and restriction enzyme digestions.

Results: There was no statistical difference in genotype and allele frequencies for c.677C>T variants between patients or their mothers and the control group. However, differences in the frequencies of alleles and genotypes of c.1298A>C polymorphism were statistically significant between patients and control group (P=0.01 for alleles and P=0.005 for genotypes). The odds ratios (OR) for the CC versus AA homozygotes were 6.1 (95% CI 1.8-20.5) and 4.2 (95% CI 1.1-15.4), in patients and mothers, respectively.

Conclusions: We found no association between genetic polymorphism of MTHFR c.677C>T and the risk of nsCL/P in the population studied. Yet the results suggested that c.1298A>C polymorphism of MTHFR gene may be a risk factor for the occurrence of nsCL/P in the Iranian population.

Keywords: MTHFR, Folic acid, Methylenetetrahydrofolate reductase, Cleft Lip, Cleft palate, Non-syndromic cleft

Introduction

Non-syndromic cleft lip with or without cleft palate (nsCL/P) is one of the most common congenital defects, with an occurrence rate of about 1/500 to 1/2500 in different populations worldwide (1,2). The incidence of cleft lip and palate varies by ethnic origin and socioeconomic status (2). A multifactorial etiology of genetic and environmental factors likely contributes to the risk of oral clefts (3-5). Decreased incidence of orofacial clefts has been found in offspring of

mothers who have received prenatal multivitamin supplementations (6-8). Deficiency of dietary folic acid during embryonic development has been suggested as a candidate environmental factor in the etiology of non-syndromic CL/P, but the results of different studies are controversial (6, 9-11). Moreover, it has been proposed that variations in the genes encoding enzymes of the folate metabolism pathway might play a role in the susceptibility to orofacial clefts. 5,10-methylenetetrahydrofolate reductase (MTHFR, MIM 236250) which is localized to chromosomal region 1p36.3, is important for a chemical reaction converting 5,10 methylenetetrahydrofolate to 5-methyl tetrahydrofolate. The latter compound is the major circulatory form of folic acid and the carbon donor for the conversion of amino acid homocysteine to methionine (3, 4, 12).

MTHFR c.677C>T polymorphism (rs1801133), which causes alanine (A) to valine (V) substitution, was the first common variant identified for this gene. The TT genotype results in lower enzymatic activity and higher thermolability than CT and CC genotypes (3,12-14). Another common polymorphism of MTHFR, c.1298A>C (rs1801131), results in glutamic acid to alanine substitution. This variant is also associated with decreased catalytic activity of the enzyme, but to a lesser extent than c.677C>T (14,15).

Since its identification, c.677C>T polymorphism of MTHFR gene has been related with many conditions and disorders including migraine, smoking behavior, vascular diseases and neural tube defects (13, 16-19). Nevertheless, findings of studies on the association between maternal or infant MTHFR polymorphism and the increased risk of oral clefts in different populations are controversial (20-26). Furthermore, no previous study has investigated the frequencies of common MTHFR polymorphisms in Iranian cleft patients or their parents. Therefore we carried out an investigation to determine the association between two common MTHFR polymorphisms (c.677C>T and c.1298A>C) and the risk of non-syndromic cleft lip and palate in an Iranian population.

Materials and Methods

The study sample consisted of 45 non-syndromic CL/P patients, 43 mothers of patients and 101 control subjects. All cases were recruited between 2010-2012 from the Cleft lip and Palate Clinic of Mashhad Dental School, Sheikh Pediatric Hospital and Ghaem Medical Center in Mashhad, northeast of Iran. In order to exclude the syndromic variants of CL/P, all patients were examined by a clinical geneticist for the presence of any associated physical or developmental abnormalities. CL/P was the only disorder affecting participants. In addition, all patients and their mothers were asked questions about the medical history and factors suspected to be related to clefting such as specific drugs, tobacco or alcohol consumption during the pregnancy.

Control subjects were healthy blood donors from northeast of Iran, with no family history of CL/P who enrolled the study at the same period. The cleft patients and control subjects were from the same ethnic origin. This investigation was approved by the Ethics Committee of Mashhad University of Medical Sciences with the code number 900030. After the written informed consent was obtained, blood samples were collected from the participants.

Peripheral blood samples were collected in test tubes that contained EDTA. Standard DNA extraction from peripheral blood cells was performed according to Higuchi (26). Briefly, white blood cells were lysed and treated with proteinase K overnight. Following two phenol extractions and three chloroform/butanol extractions, DNA was precipitated with ethanol.

Genotyping was carried out by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), using forward primer 5'-TGAAGGAGAAGGTGTCTGCGG GA-3' and reverse primer 5'-AGGACGGTGCGGTGA-GAGTG-3' for c.677C>T polymorphism, and forward primer 5-GGGAGGAGCTG-ACCAGTG-CAG-3 and reverse primer 5-GGGGTCAGGCCA-GGGGCAG-3 for c.1298A>C polymorphism. PCR was carried out in a volume of 50 μL

containing 10 pmol of each primer, 5 µl of 10X PCR buffer, 10 mM of all four dNTPs, 25 mM MgCl2, 5 U of Taq polymerase and 200 ng of template DNA. The PCR temperature conditions consisted of 5 min at 94 ºC, followed by 30 cycles of 40 s at 94 ºC, 40 s at 58 ºC and 40 s at 72 ºC, with a 5-min elongation step at 72 ºC at the end of the cycles.

Restriction digestion of PCR products for genotyping of MTHFR c.677C>T and c.1298A>C polymorphisms was performed using N*co*I (Metabion) and AfiI (Vivantis) enzymes. Restriction fragments were separated by electrophoresis on 2.5% agarose gel and visualized by ethidium bromide staining. Genotypes were scored 677CC or 1298AA if homozygous for the major allele, 677CT or 1298AC if heterozygous, and 677TT or 1298CC if homozygous for the minor allele (677T or 1298C).

Statistical analysis

Genotypes and allele frequencies in each group (CL/P patients, their mothers and controls) were calculated using descriptive statistics. Standard chi-square test was used to compare the frequencies of genotypes and alleles between the groups. The level of statistical significance was set at $P<0.05$. Odds ratios and 95% confidence intervals (95% CI) were also calculated to estimate the relative risk of different genotypes of MTHFR polymorphisms in affected patients and their mothers.

Results

All samples were typed for two common polymorphisms of MTHFR gene, i.e. c.677C>T and c.1298A>C. Data regarding c.677C>T polymorphism could not be extracted for two patients and one of the mothers group. Furthermore, c.1298A>C genotypes could not be determined for one sample within each group.

Based on the preliminary analysis, c.677C>T and c.1298A>C polymorphisms were both in Hardy-Weinberg equilibrium in all groups. The observed genotype and allele frequencies of the two common MTHFR polymorphisms in the affected subjects, their mothers and controls are reported in Tables 1 and 2.

For the c.677C>T polymorphism, we observed no significant difference in genotype distribution between nsCL/P patients or their mothers and control group (Table 1). Likewise, the frequency of hypomorphic 677T allele was not increased among patients or their mothers as compared with controls (Table 1).

For the c.1298A>C polymorphism, the frequency of CC genotype was slightly higher among mothers of patients than among controls, but the difference was not statistically significant (Table 2). However, the difference in c.1298A>C genotype distribution between patients and controls was statistically significant ($P=0.005$, Table 2). Moreover, there was a statistically significant difference in the frequency of 1298C allele, between the patients and control subjects ($P=0.01$, Table 2). Nonetheless, no significant difference was observed in the frequency of 1298C allele between mothers and control group (Table 2).

Due to the significant results observed for MTHFR c.1298A>C polymorphism, analyses were performed to estimate the risk associated with each of its genotypes.

The results calculated as odds ratios and 95% CI are presented in Table 3. The homozygote CC genotype for the c.1298A>C polymorphism was associated with an increased risk of isolated CL/P in subjects as compared with the AC genotype (odds ratio=5.1 and 95% CI, 1.5-17.3) or AA genotype (odds ratio=6.1 and 95% CI, 1.8-20.5). However, the AC genotype did not increase the risk of nsCL/P in the subjects, compared to the normal AA genotype (Table 3).Furthermore, the mothers' CC genotype was associated with an increased risk of isolated CL/P in their offsprings when compared with the AA genotype (odds ratio=4.2 and 95% CI, 1.1-15.4) (Table 3).

Table 1: Genotype and allele frequencies of MTHFR c.677C>T polymorphism in patients with CL/P, their mothers, and controls

Subject	N	Genotypes			x^2	*P* value[a]	Alleles		*P* value[a]
		CC n (%)	CT n (%)	TT n (%)			C	T	
controls	101	46 (45.5)	41 (40.6)	14 (13.9)	Ref.	Ref.	0.57	0.43	Ref.
mothers	42	22(52.3)	18 (42.9)	2 (4.8)	2.52	0.28	0.74	0.26	0.18
patients	43	20 (46.5)	16 (37.2)	7 (16.3)	0.21	0.89	0.65	0.35	0.90

[a] Chi-square test

Table 2: Genotype and allele frequencies of MTHFR c.1298A>C polymorphism in patients with CL/P, their mothers, and controls

Subject	N	Genotypes			x^2	*P* value[a]	Alleles		*P* value[a]
		AA n (%)	AC n (%)	CC n (%)			A	C	
controls	100	49 (49.0)	46 (46.0)	5 (5.0)	Ref.	Ref.	0.72	0.28	Ref.
mothers	42	16 (38.1)	19 (45.2)	7 (16.7)	5.53	0.06	0.61	0.39	0.06
patients	44	16 (36.4)	18 (40.9)	10 (22.7)	10.47	0.005*	0.57	0.43	0.01*

[a]Chi-square test; *$P<0.05$

Table 3: Calculated odds ratios with 95% CI of MTHFR c.1298A>C polymorphism in CL/P patients and their mothers compared with controls

	Odds ratios with 95% CI	
c.1298A>C genotypes	CL/P patients	Mothers
CC versus AC	5.1(1.5-17.3)	3.3 (0.9-12.0)
CC versus AA	6.1(1.8-20.5)	4.2 (1.1-15.4)
AC versus AA	0.8 (0.3-1.8)	0.8 (0.3-1.8)

Discussion

The present study was the first investigation on the frequencies of MTHFR c.677C>T and c.1298A>C polymorphisms in Iranian patients with isolated CL/P, their mothers and a group of control subjects. We found no association between genetic polymorphism of MTHFR c.677C>T and the risk of nsCL/P in the population studied. However, differences in the frequencies of alleles and genotypes of c.1298A>C polymorphism were statistically significant between patients and control group (P=0.01 for alleles and P=0.005 for genotypes).

In recent years, investigations have been carried out on the association between c.677C>T polymorphism of folic acid-metabolizing gene MTHFR and the occurrence of oral clefts in different populations. However, the results are inconsistent. Due to the reduced MTHFR activity, this mutation results in the elevation of plasma homocysteine levels and decrease of plasma folate (13). Animal and human investigations have suggested a possible link between folic acid and oral clefts (7,11, 27-29). Martinelli and colleagues (21) found a significantly higher frequency of MTHFR mutation in mothers of patients affected with CL/P compared with controls. They suggested that c.677C>T polymorphism of maternal MTHFR gene could provide a special prenatal environment for the development of CL/P in the embryo (21). Studies conducted by Shotelersuk and Prescott (4,20), also supported this maternal effect theory. Conversely, Blanton et al (23) be-

lieved that this polymorphism is not related to the risk of oral clefts. Likewise, findings of a research from northern Venezuela did not support a causal role for MTHFR variants in the pathogenesis of nsCL/P patients (3). The association between c.1298A>C polymorphism and the risk of nsCL/P has been less investigated in the literature (3,4,12,22).

The frequency of the mutant allele 677T in the control group of our study was 0.43, which is comparable to high frequencies reported for the control populations of Italian and Hispanic origin (22,30). The frequency of the hypomorphic allele 1298C in our controls was 0.28, which is similar to that of Thai and White populations (4,31,32).

Findings of the present study revealed that the allele frequency and genotype distribution of MTHFR c.677C>T polymorphism were not significantly different among non-syndromic CL/P patients or their mothers compared to a control group of Iranian population. These results are consistent with the findings of recent studies from Brazil and north coastal Venezuela (3,24), which showed no evidence for c.677C>T polymorphism as a risk factor for non-syndromic CL/P. Studies conducted by Blanton and colleagues (23,33) showed similar findings about the role of c.677C>T polymorphism in the development of familial or isolated CL/P. Nevertheless, Martinelli, Prescott and Shotelersuk (4,20,21) reported a maternal effect for 677CT or TT genotypes on the increased risk of non-syndromic CL/P. It may be the allelic heterogeneity that leads to the wide variation of odds ratios for a given polymorphism among different populations.

We found a significantly higher frequency of 1298C allele and 1298CC genotype in affected patients compared with control samples. Moreover, the CC genotype for c.1298A>C polymorphism, was associated with an increased risk of isolated CL/P in subjects when compared with the normal AA genotype (odds ratio=6.1 and 95% CI, 1.8-20.5). Additionally, we found a significant effect for the maternal CC genotype on the increased risk of nsCL/P in their off springs. These findings are inconsistent with some previous investigations which studied the association between c.1298A>C polymorphism and oral clefts (3,4,22). The contradictory results could be caused by the diversity of genetic backgrounds and environmental factors in different populations. Country-specific differences in folate supplementation during the prenatal period may also contribute to these conflicting results.

Adequate folate consumption during early pregnancy may compensate for the reduced activity of the MTHFR enzyme and help to decrease the risk of non-syndromic CL/P malformation in the offspring. Therefore the effect of interaction between maternal dietary folate intake and the MTHFR polymorphisms on the risk of oral clefts should be the focus of future studies in the Iranian population.

Conclusion

Our results support the possible involvement of MTHFR c.1298A>C polymorphism, but not the c.677C>T variation, in the development of non-syndromic CL/P. Studies with larger sample sizes which also consider the paternal genotypes are needed to better clarify the role of MTHFR in orofacial clefts. Moreover, the effect of other genes involved in folate metabolism pathway on the risk of oral clefts may be a topic for further studies.

Ethical considerations

Ethical issues (Including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc) have been completely observed by the authors.

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