Association between C677T and A1298C MTHFR gene polymorphism and nonsyndromic orofacial clefts in the Turkish population: a case-parent study

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SUMMARY: Semiç-Jusufagiç A, Bircan R, Çelebiler Ö, Erdim M, Akarsu N, Elçioğlu NH. Association between C677T and A1298C MTHFR gene polymorphism and nonsyndromic orofacial clefts in the Turkish population: a case-parent study. Turk J Pediatr 2012; 54: 617-625.

Two common MTHFR gene polymorphisms (C677T and A1298C) have been implicated in the etiology of nonsyndromic cleft lip/palate (nsCL/P). To investigate the genotype association among nsCL/P in the Turkish population, 56 case-parent trios were recruited into the study. Genotype frequencies were compared to two groups of controls from the same population. A total of 46 case-parent trios were included in transmission disequilibrium test (TDT) analysis.

The mothers of the study group had a higher frequency of 677TT genotype, with a three-fold increased risk of having nsCL/P offspring (odds ratio [OR]: 3.14, p=0.03). The combined 677CT/1298AC genotype was also common among these mothers (28%), but it did not reach statistical significance (OR: 2.27, p=0.07). TDT analysis for (C677T) T allele transmission did not reveal a significant association. In conclusion, mothers carrying 677TT genotype or with 677CT/1298AC combined genotype have increased risk of having nsCL/P offspring; therefore, higher periconceptional folic acid supplementation should be advised for decreasing the recurrence risk.

Key words: nonsyndromic cleft lip palate, MTHFR polymorphisms, transmission disequilibrium test.

Nonsyndromic cleft lip with or without palate (nsCL/P) is among the most common congenital orofacial malformation, with the worldwide incidence reported to range between 1/700 to 1/1000 live births[1,2]. nsCL/P follows a multifactorial inheritance model in which both environmental and genetic factors are thought to play a role. A number of environmental factors have been identified as a potential cause of nsCL/P, and the most frequently associated factor has been insufficient maternal folate intake in pregnancy[3]. Maternal folate insufficiency is a well-recognized risk factor for the development of neural tube defects (NTDs), which can be successfully prevented by folate supplementation in the early weeks of pregnancy. Alternatively, any metabolic defect that may cause low serum folate levels may have the same teratogenic effect. A recent meta-analysis examining the effect of maternal folate supplementation agreed on the effectiveness of such intervention on the risk reduction of oral clefts[4].

There is increasing evidence that variations in genes implicated in folate metabolism may play a significant role in the nsCL/P etiology. Among several genes that take part in folate metabolism, the methylenetetrahydrofolate reductase gene (MTHFR) has been the most frequently associated with nsCL/P. The product of the MTHFR gene is an enzyme that catalyzes the methylation of homocysteine amino acid to methionine. Any defect on this pathway can result in methionine deficiency and the accumulation of homocysteine. Whilst methionine is an important precursor in the

DNA and RNA methylation process, high serum homocysteine levels are teratogenic during the embryologic stage[5,6].

Two common single nucleotide polymorphisms (SNPs), C677T (C>T, alanine to valine substitution) and A1298C (A>C, glutamate to alanine), in the MTHFR gene have been linked to NTD[7,8], congenital heart disease[9] and orofacial clefts[10]. Substitution of a single nucleotide causes change in the amino acid sequence, leading to synthesis of thermolabile MTHFR enzyme with reduced activity, elevated plasma homocysteine levels and low folate synthesis[11]. A substantial number of studies have examined the link between the C677T polymorphism and nsCL/P, with contradicting reports from different population backgrounds[10,12-16]. Some studies have suggested that high frequency of maternal T allele and TT genotype is a risk factor for having an offspring with oral clefts[11,17-19]. As a consequence, carriers of TT genotype, who have the lowest MTHFR enzymatic activity, are more susceptible to low dietary folate intake during pregnancy compared to CT and CC genotypes. Moreover, folic acid supplementation in pregnancy has been suggested to reduce the risk of oral clefts in offspring[20]. Similarly, A1298C SNP causes reduced MTHFR enzyme activity, especially when found in homozygote CC form[8]. Although several studies have failed to find an association with the A1298C mutant CC genotype[16,18], Van Rooij et al.[19] found an almost seven-fold increased risk of orofacial clefts for mothers with insufficient periconceptional folic acid intake. In addition, maternal combined heterozygosity (677CT/1298AC genotype) has been suggested as another factor associated with clefts[13].

The main objective of this study was to investigate the association between nsCL/P and C677T and A1298C polymorphisms in the MTHFR gene among Turkish nsCL/P cases and their parents, as a risk factor for nonsyndromic orofacial clefts.

Material and Methods

Study Population

The subjects for this case-parent triad study were recruited from the interdisciplinary monthly pediatric cleft clinics held within the setting of Plastic and Reconstructive Surgery Department at Marmara University Hospital, İstanbul, Turkey - a tertiary referral center for CL/P patients from all over the country. A total of 66 CL/P cases were approached to take part in the study. Ten cases were excluded at the beginning of the study due to alternative diagnosis of their existing syndromic conditions (ectrodactyly-ectodermal dysplasia-clefting (EEC), ankyloblepharon-ectodermal dysplasia-clefting (AEC), Van der Woude syndrome, Kabuki make-up, oculodentodigital syndrome, Pierre Robin sequence, or if they had other associated anomalies). The study population consisted of 56 cases and their parents. Parents gave written informed consent to take part in the study. The study was approved by the Marmara University Hospital Ethics Committee.

An interviewer-administered demographic and clinical history questionnaire was used to collect data on maternal folic acid supplement intake, smoking, alcohol, antiepileptic drug use, and any perinatal infections and diseases. In addition, the information on family history of nsCL/P and parental consanguinity was collected. A correct diagnosis and the type of nsCL/P were confirmed by detailed physical examination during the child's visit to the clinic. According to the extent of their malformation, children were categorized as having cleft lip with or without palate (CLP) and cleft palate only (CPO). In addition, each child and their parents provided a 5 ml blood sample for DNA extraction.

Laboratory Methods

Blood samples were collected in potassium EDTA tubes and stored at -20°C until further analysis. At the time of analysis, each sample was thawed and processed to provide cell lysates for polymerase chain reaction (PCR) amplification. As previously described, genotyping for C677T and A1298C MTHFR gene mutations was performed by enzymatic restriction digestion of PCR products with Hinf I and Mbo II, respectively[7,8]. DNA fragments were separated and visualized by electrophoresis using ethidium bromide-stained 2% agarose gel.

According the presence or absence of mutant T allele in the C677T region, all cases and their parents were genotyped as TT if homozygous for mutant allele, CT if heterozygous for C677T allele and CC if homozygous for "wild

type" allele. Similarly, cases and parents were genotyped for the A1298C polymorphism as CC homozygous (mutant genotype), AC heterozygous and AA "wild type" homozygous.

Statistical Methods

For comparison of allele and genotype frequencies of C677T and A1289C SNPs, we counted the data on 169 controls from two previous studies conducted among Turkish NTD patients and healthy controls (Akar et al.[21] with 76 [both C677T and 1289A>C genotypes] and Boduroglu et al. [22] with 93 [C677T genotype only] controls). Cases and parents were compared to controls as both single (CLP and CPO groups combined) and separate groups. Statistical analysis was carried out using InStat™ (GraphPad software version 2.02, 1993). Appropriate univariate test (Fisher exact test) was used to investigate the differences in genotype frequencies. To test the association between nsCL/P risk and each of C677T and A1298C polymorphisms, transmission disequilibrium test (TDT) was used. The TDT is a valid test that determines the frequency with which a particular allele is transmitted or non-transmitted from heterozygous parents to their affected offspring[23]. Data obtained from TDT were analyzed by McNemar test of significance using a 2 x 2 contingency table.

Results

Demographics

There were 36 CLP and 20 CPO cases. The median age of mothers at birth of a child was 26 (16-37) years, with 21/54 mothers (37.5%) reporting not using any vitamin supplement around the time of conception and/or during pregnancy. None of mothers that took vitamin supplement had taken folic acid supplement on its own but rather in a multivitamin tablet form. While 13 mothers (23%) smoked cigarettes, none of them consumed alcohol or had taken any antiepileptic drug in pregnancy.

Parents of 12 children (21%) were in close consanguinity. Of 56 nsCL/P cases, 20 (36%) (mainly CLP, 15/20) reported a positive family history of nsCL/P. Six of the 56 cases (10%) had an affected 1st degree relative with nsCLP in the family. This was distributed as 2 affected mothers, 2 affected fathers and 2 affected siblings.

Comparison of SNP Allele and Genotype Frequencies

Of the overall 56 cases, 54 mothers and 49 fathers provided a blood sample for genotyping. All of the blood samples were successfully genotyped for both C677T and A1298C polymorphisms. The total allele and genotype frequencies for both C677T and A1298C MTHFR gene SNPs are presented in Table I.

C677T Polymorphism

The results of C677T genotype frequencies for both cases and parents are shown in Table I. There was no significant difference in TT (thermolabile variant) genotype frequencies of nsCL/P cases and their fathers compared to

Table I. Allele and Genotype Frequencies for C677T and A1298C MTHFR Gene Polymorphisms Among nsCL/P Cases and Their Parents

	Genotype frequency N (%) C677T			Allele frequency C677T A1298C				Genotype frequency N (%) A1298C		
	CC	CT	TT	C	T	A	C	AA	AC	CC
CL/P cases n=56	25 (45)	28 (50)	3 (5)	0.70	0.30	0.60	0.40	21 (37)	25 (45)	10 (18)
Mothers n=54	16 (30)	27 (50)	11 (20)	0.55	0.45	0.68	0.32	25 (46)	24 (45)	5 (9)
Fathers n=49	27 (55)	21 (43)	1 (2)	0.77	0.23	0.64	0.36	21 (43)	21 (43)	7 (14)
Controls Akar et al.[21] n=76	44 (58)	24 (31.5)	8 (10.5)	0.74	0.26	0.71	0.29	36(47.5)	36(47.5)	4 (5)
Boduroglu et al.[22] n=93	47 (51)	39(42)	7(7.5)	0.72	0.28					

Table II. Frequency of TT Genotype of C677T MTHFR Gene Polymorphism Among nsCL/P Cases and Their Parents

	TT frequency	%	Odds ratio (95% CI)	P value
Cleft lip/palate cases	3/56	5	*0.48 (0.12,1.90) **0.69 (0.17, 2.80)	0.350.74
Mothers	11/54	20	*2.17 (0.80, 5.83) **3.14 (1.13, 8.68)	0.130.03
Fathers	1/49	2	*0.17 (0.02, 1.46) **0.25 (0.03, 2.14)	0.080.26
Control				
*Akar et al.[21]	8/76	10.5		
**Boduroglu et al.[22]	7/93	7.5		

CI: Confidence interval.

controls (Table II). However, we found that TT genotype was significantly more common among mothers compared to control groups. Although comparison of maternal TT genotype frequency with the first group of controls[21] did not reach statistical significance, mothers of CL/P cases tended to carry "mutant" TT genotype more often when compared to controls (*odds ratio [OR] 2.17, 95% confidence interval [CI]: 0.80-5.83, p=0.13). Moreover, the difference in TT genotype frequencies was statistically significant when compared with the second control group[22]([a] OR 3.14, 95% CI: 1.13, 8.68, p=0.03). We observed that out of these 11 mothers with TT genotype, 10 had an offspring with CLP deformity and only 1 of them had a child with CPO (data not shown). Further analysis according to the specific type of nsCL/P malformation (CLP vs CPO), revealed that a significantly higher proportion of mothers with a child having CLP malformation carried TT genotype, increasing their risk of having CLP offspring at least 3 times when compared to control groups from both studies (Table III). There was no difference in "mutant" TT genotype frequency between mothers of CPO offspring and healthy controls (Table III). Also, mothers of nsCL/P cases were at least twice as likely to carry T allele than healthy controls (T allele frequency among mothers vs. controls, respectively: OR [95% CI]: 0.45 vs. 0.26, 2.32[1.37, 3.92] and 0.28, 2.08 [1.27, 3.41], respectively).

A1298C Polymorphism

There were no differences in CC genotype frequencies between each group of parents and control group (Table IV). However, nsCL/P cases were almost 4 times more likely to have CC genotype compared to controls (CC frequency, OR [95%CI]: 18% vs. 5.2%, 3.91 [1.15, 13.22]). A further comparison according to nsCL/P malformation type (CLP or CPO) revealed that CC genotype frequency was significantly higher among CPO cases and their fathers (Table V). Interestingly, mothers of CPO cases had the same CC frequency as the controls (5% vs. 5.2%). There were no statistically significant differences in C allele frequencies between cases or each group of parents compared to controls. However, further analysis according to the nsCL/P malformation type found significantly higher C allele frequency among CPO cases and their fathers (C allele frequency, OR [95%]: cases: 0.50, 2.45 [1.20, 5.00], fathers: 0.55, 3.03 [1.46, 6.28] vs. controls: 0.29).

Combined 677CT/ 1298AC Heterozygosity

Although we found that mothers of CL/P cases carried 677CT/1298AC genotype more frequently compared to controls (677CT/1298AC frequency: 28% vs. 14.5%, OR: 2.27), the results did not reach statistical significance (Table VI).

None of cases or fathers was compound homozygote (677TT/1298CC) for both SNPs. Strikingly, there were 2 mothers who were 677TT/1298CC compound homozygotes, both having offspring with the bilateral CLP.

Transmission Disequilibrium Test Analysis

Blood samples from 47 nsCL/P case/parent triads were available. One case/parent triad was excluded from the analysis as there was inconsistency between case and parents A1298C SNP genotype (both parents had wild type 1298AA genotype while offspring was AC

Table III. Frequency of TT Genotype of C677T MTHFR Gene Polymorphism Among Separated CPO and CLP Groups of Cases and Their Parents

	TT frequency	%	Odds ratio (95% CI)	P value
CPO group				
Cases	0/20	0	*0.19 (0.01, 3.55) **0.28 (0.01, 5.13)	0.190.34
Mothers	1/20	5	*0.44 (0.05, 3.80) **0.64 (0.07, 5.57)	0.681.00
Fathers	0/19	0	*0.20 (0.01, 3.74) **0.29 (0.01, 5.40)	0.350.60
CLP group				
Cases	3/36	8	*0.77 (0.19, 3.10)**1.11 (0.27, 4.58)	1.001.00
Mothers	10/34	29	*3.54 (1.25,10.01)**5.11 (1.76, 14.87)	0.020.002
Fathers	1/30	3	*0.29 (0.03, 2.45)**0.42 (0.04, 3.59)	0.440.67
Control				
*Akar et al.[21]	8/76	10.5		
**Boduroglu et al.[22]	7/93	7.5		

heterozygote). Forty-six case/parent triads were included in the TDT analyses.

TDT analyses were conducted on subjects with heterozygote parents for each of two SNPs separately. There was no statistically significant difference in the number of transmitted and non-transmitted mutant T allele (C677T) between heterozygote parents and their affected children (transmitted vs. non-transmitted allele, 24 vs. 18; TDT χ^2=0.59, McNemar OR: 1.33) (Table VII). However, the analysis in which we examined the frequency of transmission of a second SNP revealed a high frequency of mutant C allele transmission from A1298C heterozygote parents to offspring, with test statistics showing significant linkage disequilibrium (transmitted vs. non-transmitted allele, 29 vs. 8; TDT χ^2=10.81, McNemar OR: 3.62).

Discussion

Principal Findings

In this case-parent study, we found a significantly higher proportion of mothers carrying C677T mutant "TT" genotype compared to controls. Although it did not reach statistical significance, we observed a tendency among all these study mothers to be carriers of 677CT/1298AC combined genotype more frequently. Interestingly, A1298C "CC" genotype was at least three times more common among CLP cases than in controls. Moreover, when we assigned children and their parents according to the type of malformation into groups, it was CPO children and their fathers who were more likely to have the A1298C mutant "CC" genotype. In addition, when we looked into the frequency of transmission of mutant allele from parents to the affected nsCL/P, we found significantly higher transmission of mutant allele but only for the A1298C polymorphism.

Meaning of Study

Although this was a comparatively small study, our finding of significantly higher prevalence of TT mutant genotype among mothers is in agreement with findings from previous studies[11,17-19]. Martinelli et al.[17], who studied Italian CLP case-parent trios, observed increased frequency of TT genotype among mothers. Van Rooij et al.[19] found that combination of low maternal dietary folate intake or lack of periconceptional folate supplementation with maternal 677TT genotype increased the risk of having CLP offspring almost six-fold. Moreover, another case-control triad study found that independent of dietary and supplemental folate intake, case mothers with 677TT genotype had significantly lower levels of serum and red blood cell folate compared to controls[24]. As many studies as there are with positive reports, there are at

Table IV. Frequency of CC Genotype of A1298C MTHFR Gene Polymorphism Among nsCL/P Cases and Their Parents

	CC frequency	%	Odds ratio (95% CI)	P value
Cleft lip/palate cases	10/56	18	3.91 (1.15, 13.22)	0.02
Mothers	5/54	9	1.83 (0.47, 7.18)	0.48
Fathers	7/49	14	3.00 (0.82, 10.85)	0.10
Control				
Akar et al.[21]	4/76	5.2		

Table V. Frequency of CC Genotype of A1298C MTHFR Gene Polymorphism Among Separated CPO and CLP Groups of Cases and Their Parents

	CC frequency	%	Odds ratio (95% CI)	P value
CPO group				
Cases	5/20	25	6.00 (1.43, 25.02)	0.01
Mothers	1/20	5	0.94 (0.09, 8.98)	1.00
Fathers	5/19	28	8.30 (2.05, 33.57)	0.003
CLP group				
Cases	5/36	14	2.90 (0.72,11.55)	0.14
Mothers	4/34	12	2.40 (0.56, 10.23)	0.25
Fathers	2/30	6	0.62 (0.06, 5.79)	1.00
Control				
Akar et al.[21]	4/76	5.2		

least as many studies that found no association between MTHFR polymorphisms and nsCL/P[12,14,15,25]. By using a genetically dominant model, a recent meta-analysis of 10 studies that examined the association between CLP (without CPO) and C677T and A1298C polymorphisms failed to demonstrate an increased risk of CLP for those mothers or affected children carrying mutant allele[26]. However, as the authors rightly suggest, the disagreement between studies on the association between MTHFR polymorphisms and nsCL/P is probably due to its multifactorial origin, by which, along with common genetic polymorphisms, other lifestyle, environmental factors and epigenetic mechanisms may influence or modify the disease risk. Moreover, there are suggestions that nsCL/P can be a product of polymorphisms in more than one gene. As an example, a recent study from China reported an increased risk for nsCL/P among children carrying variants for both C677T and transforming growth factor-beta three (TGFB3) polymorphisms[27].

In this study, although we found a significantly higher proportion of mothers carrying the 677TT genotype, TDT analysis did not demonstrate excessive T allele transmission from heterozygous parents to the affected offspring. Although a single study conducted among 139 complete case-parent triads reported significant 677 T allele transmission[28], our findings are in line with other studies which, despite finding a higher frequency of maternal TT genotype, had failed to demonstrate increased T allele transmission among different ethnic populations[11,15,17-19].

On the other hand, in the second TDT analysis, we found that A1298C heterozygous parents were at least three-times more likely than not to transmit risk C allele to the affected child. To our knowledge, this is the first study to demonstrate an excessive transmission of C allele from parents to the affected offspring. It could be speculated that due to the high proportion of consanguinity (12/56) among our index parents, we observed a higher A1298C heterozygosity rate, which in turn may have contributed to our positive findings. Unfortunately, due to the small sample size, we could not examine whether there was an excessive transmission of both 677CT and 1298AC variants from heterozygous parents to their offspring.

The results from our study support the

Table VI. MTHFR 677CT/ 1298AC Combined Genotype Frequency Among nsCL/P Cases and Their Parents

	CT/AC	%	Odds ratio (95% CI)	P value
Cleft lip/palate cases(n=56)	13	23	1.78 (0.73, 4.35)	0.25
Mothers(n=54)	15	28	2.27 (0.94, 5.44)	0.07
Fathers (n=49)	7	14	0.98 (0.35, 2.74)	1.00
Control				
Akar et al.[21](n=76)	11	14.5		

Table VII. TDT Analyses Among nsCL/P Cases

SNP	Transmitted	Non-transmitted	χ^2	OR (95% CI)	P value†
C677T					
C	18	24	0.59	1.33 (0.69, 2.56)	0.44
T	24	18			
A1298C					
A	8	29	10.81	3.62 (1.58, 8.59)	0.001
C	29	8			

† McNemar test

hypothesis regarding the importance of maternal folate metabolism during embryonic development. Low dietary folate intake during the first trimester of pregnancy coupled with maternal TT genotype (C677T) can have a damaging effect on the ongoing embryonic organogenesis by decreasing the amount of available MTHFR enzyme, which has a unique function in that it regulates the availability of methyl groups for methylation reactions at the cost of purines and pyrimidines synthesis. DNA methylation, which is reprogrammed during early embryogenesis, is part of the epigenetic code and is a chief regulator of gene expression. MTHFR is especially critical for methionine synthesis. Methionine is fundamental for DNA synthesis and cell growth, and any compromise in its synthesis may reduce the cell proliferation rate[29,30]. Methylenetetrahydrofolate reductase genotype of the affected offspring seems to be another important aspect of the CLP etiopathogenesis, although to a lesser degree, A1298C mutations have been associated with decreased MTHFR enzyme activity, where unborn children with CC genotype may be more vulnerable to 5-methyltetrahydrofolate depletion[8].

On the same principle, maternal combined heterozygosity (677CT/1298AC) may have similar consequences as TT genotype by reducing the levels of MTHFR enzyme. A study of 109 nsCL/P cases found a significantly higher frequency of 677CT/1298AC combined heterozygosity among mothers of nsCL/P cases compared to controls[13]. Another study conducted in 198 subjects, which examined the effect of combined 677CT/1298AC heterozygosity on MTHFR enzyme activity, found significantly higher fasting serum homocysteine levels in those that were combined heterozygotes compared to those that were C677T heterozygotes[31].

As a proof of the concept, there is strong experimental evidence showing that silencing of the MTHFR gene in a murine model can prevent growth and induce apoptosis in murine embryonic palatal mesenchymal cells (MEPM)[29]. Even more important, researchers in that study found that supplementation with folic acid was sufficient to reverse the teratogenic effect of the silenced MTHFR gene. A detailed flow cytometric analysis showed that while MTHFR gene silencing induced G_0/G_1 cell phase, supplementation with folic acid caused transition to G_1/S cell proliferation phase. These findings suggest that nsCL/P prevention strategies should include higher periconceptional folic acid supplementation.

In conclusion, maternal 677TT genotype seems to be an important risk factor for development of nsCL/P among the sample of affected Turkish children. As regards the A1298C polymorphism, excessive transmission of mutant C allele to affected children may be implicated in the etiopathogenesis of nsCL/P. Although this is a small study, this may be an important finding,

as the proposed deficiency in endogenous folate production may be overcome by the relatively simple measure of periconceptional folate supplementation, which could be recommended as a preventative measure for those women deemed at risk.

Acknowledgements

We are grateful to all patients and parents for their cooperation.

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