SHORT REPORT

Association of methylenetetrahydrofolate reductase gene variant *C677T* and folate levels in non-syndromic cleft lip/palate among Sindhi, Pakistani population

Sadia Effendi[1], Samreen Memon[2], Feriha Fatima Khidri[3], Ali Muhammad Waryah[4]

Abstract

The objectives of this study were to determine the association of methylenetetrahydrofolate reductase (MTHFR) gene variant *C677T* with non-syndromic cleft lip/palate (NSCLP) in Pakistani population and compare the maternal serum folic acid levels in NSCLP-affected and healthy group. A comparative cross sectional study was conducted between 2017 and 2019 at Liaquat University of Medical and Health Sciences, Jamshoro. Sixty mother-infant dyads were recruited (n=120), including NSCLP-affected and healthy infants along with their mothers. The *MTHFR C677T* variant exhibited significant association with NSCLP in dominant and over-dominant models. No differences in maternal serum folic acid levels were observed between both the groups; however, the folic acid intake during pre-conception period was associated with decreased risk for NSCLP. Our study suggested that *MTHFR 677 CT* genotype was related with decreased risk for NSCLP in Sindhi, Pakistani, population. Pre-conception folic acid may decrease the risk for oral clefts.

Keywords: Cleft lip, Cleft palate, Folic acid, Gene, *MTHFR*, Variant.

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Introduction

Non-syndromic cleft lip/palate (NSCLP) is a common congenital deformity. Worldwide, approximately 1.7 per 1,000 live births in Asian countries are affected with cleft lip/palate.[1] There is evidence that a multifactorial model comprising genetic inheritance and environmental interaction has a substantial role in the development of NSCLP. Previous studies have proven a link between genetic variations associated with folate metabolism and higher prevalence of cleft lip/palate. Amongst folate metabolism associated genes, methylenetetrahydrofolate reductase (MTHFR) has been linked to NSCLP.[2] *MTHFR* gene is positioned at 1p36.3 and codes for the *MTHFR* enzyme that catalyses the 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate conversion. *MTHFR* rs1801133 (*C677T*) is a common variant in *MTHFR* gene that causes thermolabile enzyme with reduced activity.[3] Defective pathway may lead to methionine deficiency and increase the levels of homocysteine that may cause teratogenicity in the course of embryogenesis.[4] Adequate folate intake (dietary or supplementation) during preconception and prenatal period has been demonstrated to minimise or prevent the vulnerability of NSCLP, however the impact of maternal serum folate levels with susceptibility of orofacial clefts in off-springs is not consistent.[5] Secondly, there is limited literature on the role of genetic variants in NSCLP in Pakistani population. Therefore, the present study was conducted to investigate the association of *MTHFR C677T* variant in infants and maternal serum folic acid levels with the risk of NSCLP.

Methods, Results and Discussion

Following the permission of the Research Ethics Committee (No. LUMHS/REC/-550), the proposed comparative cross sectional study was conducted between 2017 and 2019 at Liaquat University of Medical and Health Sciences (LUMHS), Jamshoro, Sindh, Pakistan. The parents of the recruited infants provided written informed consent. Patients were recruited from the Plastic and Reconstructive Surgery Unit outpatient department via non-probability convenience sampling. A total of 60 mother-infant dyads were recruited comprising (1) NSCLP affected infants and their mothers (30 mother-infant dyads) and (2) mothers with healthy babies (30 mother-infant dyads). Unrelated new born babies up to six months of age of both genders were recruited. Affected infants were examined by plastic surgeons for syndromes and abnormalities. The inclusion criteria were unrelated male or female infants of Pakistani origin and residents, aged new-born to six months, accompanied by their respective mothers. Patients who were diagnosed with NSCLP were included as affected

[1,2]Department of Anatomy, Liaquat University of Medical and Health Sciences, Jamshoro, Pakistan; [3]Department of Biochemistry, Bilawal Medical College, Liaquat University of Medical and Health Sciences, Jamshoro, Pakistan; [4]Department of Molecular Biology and Genetics, Medical Research Centre, Liaquat University of Medical and Health Sciences, Jamshoro, Pakistan.

Correspondence: Sadia Effendi. e-mail: sadia.effandi@lumhs.edu.pk

ORCID ID. 0000-0001-5391-4322

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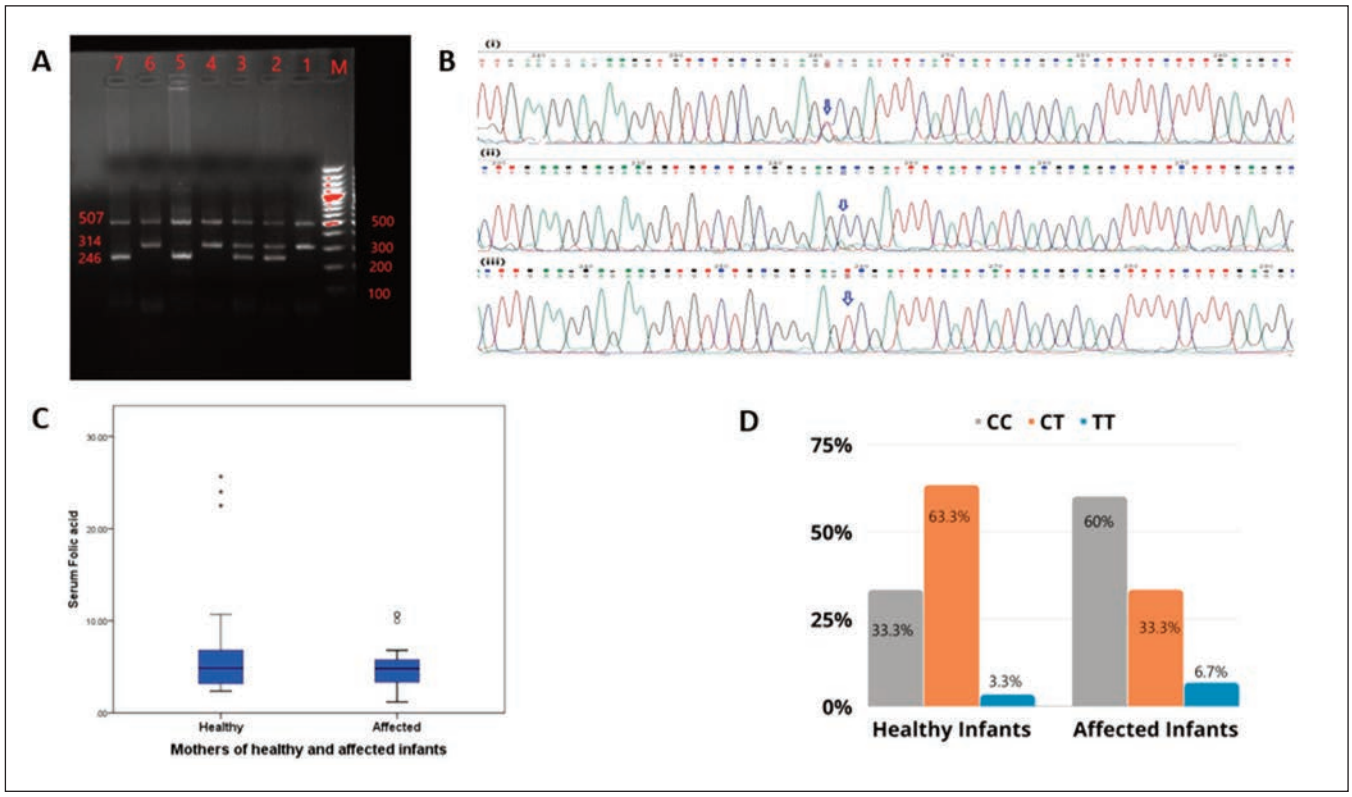

Figure-1: (A) Gel electrophoresis images of PCR products of *MTHFR C677T (rs1801133)* gene variant showing bands in ARMS PCR. *MTHFR C677T* genotypes homozygous CC two bands at 314 and 507 bp (lanes 1, 4, 6), heterozygous CT three bands at 246, 314 and 507 bp (lanes 2, 3) and homozygous TT two bands at 246 and 507 bp (lanes 5, 7). Lane M: Ladder. (B) Chromatograph representing the *MTHFR C677T (rs1801133)* gene variant genotypes (i) heterozygous CT, (ii) homozygous CC and (iii) homozygous TT. (C) Box-and-whisker plot for serum folic acid levels (ng/ml) between mothers of affected and healthy infants. (D) Genotype frequency distribution of *MTHFR C677T* (rs1801133) gene variant among affected and healthy infants.

infants whereas, patients with abnormalities, cardiac disease or syndromes associated with cleft were excluded from the study. The control group included healthy infants with no prior history of congenital malformations, such as NSCLP. Information on folic acid supplementation including dose, frequency, duration, and timings (pre- or post-conception) was inquired from the mothers.

Sample size was determined by using the online available tool calculator.net (https://www.calculator.net/sample-size-calculator), at a 95% confidence level, margin of error 5%, population proportion and population size from 1.7 per 1,000 live births as previously reported in Asian countries.[1] The minimum sample size was calculated to be 26, however it was increased to 30 mother-infant dyads for each group with a total sample size of 120 (60 mother-infant dyads).

Venous blood samples were collected in ethylenediaminetetraacetic acid (EDTA)- containing tube. Genomic DNA was extracted using inorganic method; blood samples were mixed with Tris EDTA buffer, centrifuged, and the pellet was repeatedly treated until light pink. It was then digested, centrifuged with NaCl, followed by subsequent steps including treatment with isopropanol and 70% ethanol, followed by centrifugation and removal of the supernatant. The DNA pellet was dried, diluted, and stored for analysis. The DNA sequencing was performed at the Molecular Biology and Genetics Laboratory (LUMHS), using Applied Biosystems 3130 Genetic Analyser. Blood sample for folic acid analysis were collected in vacutainers. Serum was separated by centrifugation and serum folic acid measured using Elecsys Folate III kit on Roche modular auto-analyser according to kit manufacturing instructions.

MTHFR C677T (rs1801133) variant genotyping was performed according to the previously described tetra primer amplification refractory mutation system polymerase chain reaction (ARMS PCR) method.[6] Separation of PCR products were observed on a 2% agarose gel (Figure 1A). For quality control assurance 06 samples were randomly selected for Sanger sequencing to confirm the amplicon (Figure 1B).

Data was analysed on SPSS v.23. Mann–Whitney U-test and two-sided Fisher exact test/Chi-square test (where appropriate) were applied for continuous and categorical

Table: Demographic characteristics, folic acid status, genotype/allele frequencies, and association of *MTHFR C677T* variant with non-syndromic cleft lip/palate.

Variables		**Affected** (n=30) n (%)	**Healthy** (n=30) n (%)	**OR (95% CI); *p*-value**
Demographic characteristics				
Age of mother (years)	Median (min-max)	32 (22-45)	31.5 (20-45)	0.33
Age of infant (days)	Median (min-max)	60 (2-135)	60 (15-150)	0.58
Gender	Female	10 (33.3)	13 (43.3)	Ref
	Male	20 (66.7)	17 (56.7)	1.53 (0.54-4.36); 0.42
Family history of orofacial clefts	No	25 (83.3)	28 (93.3)	Ref
	Yes	5 (16.7)	2 (6.7)	2.80 (0.49-15.73); 0.24
Folic acid status among mothers				
Serum folic acid (ng/ml)	Median (min-max)	4.81 (1.18-10.69)	4.86 (2.36-25.66)	0.62
Folic acid status	Normal	15 (50)	20 (66.7)	Ref
	Deficient	15 (50)	10 (33.3)	2.00 (0.70-5.67); 0.19
Folic acid intake	Yes	21 (70)	24 (80)	Ref
	No	09 (30)	06 (20)	1.71 (0.52-5.62); 0.37
*Timing of folic acid supplementation	Post-conception	15 (71.4)	09 (37.5)	Ref
	Pre-conception	06 (28.6)	15 (62.5)	0.24 (0.07-0.84); 0.02
Genetic model/HWE (*p*)				
Alleles	C	46 (77)	39 (65)	Ref
	T	14 (23)	21 (35)	0.57 (0.25-1.26); 0.16
Codominant	CC	18 (60)	10 (33.3)	Ref
	CT	10 (33.3)	19 (63.3)	0.29 (0.10-0.87); 0.027
	TT	2 (6.7)	1 (3.3)	1.11 (0.09-13.84); 0.93
Dominant	CC	18 (60)	10 (33.3)	Ref
	CT-TT	12 (40)	20 (66.7)	0.33 (0.12-0.96); 0.03
Recessive	CC-CT	28 (93.3)	29 (96.7)	Ref
	TT	2 (6.7)	1 (3.3)	2.07 (0.18-24.15); 0.55
Over-dominant	CC-TT	20 (66.7)	11 (36.7)	Ref
	CT	10 (33.3)	19 (63.3)	0.29 (0.10-0.84); 0.01
Log-additive	-	-	-	0.50 (0.20-1.22); 0.12
HWE (*p*)	-	0.63	0.052	-

CI: Confidence interval; HWE: Hardy-Weinberg equilibrium; max: maximum; min: minimum; OR: Odds ratio' *Percentage and proportion from number of mothers with folic acid intake. Bold fonts represent significant *p*-value.

variables, respectively. The association of demographic and folate related variables with NSCLP were determined by odds ratio (OR) and 95% confidence interval (CI). Hardy-Weinberg equilibrium (HWE), genotype/allele frequencies, OR, and 95% CI were estimated on SNPStat software to examine the association of genotypes with affected and healthy infants in various genetic models. P<0.05 was defined as statistically significant.

Table presents the distribution and association of variables with NSCLP between affected and healthy groups. The median age of the mothers and infants presented no difference between both the groups. The youngest affected infant was two days old and the oldest 19.3 weeks (135 days) of age. Male infants were most affected which is in agreement with previous studies;[7] however, no significant difference was observed with respect to gender between affected and healthy groups.

No significant association of familial history of cleft/lip palate among study participants was observed. However, it was observed that among the affected infants, one male infant's father, one male infant's brother, and three infants' (two males and one female) first cousins had cleft lip/palate. Two of the healthy infants had distant relatives with cleft lip/palate.

The serum folic acid levels showed no significant difference between affected and healthy groups (Figure 1C). The minimum folic acid level in the affected group was 1.18 ng/ml, whereas the maximum level was 10.69 ng/ml. Among the healthy group, the lowest level was 2.36 ng/ml and the highest level was 25.66 ng/ml. Majority of the mothers had folic acid supplementations in their pregnancies. However, decreased risk for cleft lip/palate was observed with pre-conception folic acid intake (OR=0.24; 95% CI=0.07 – 0.84, *p*=0.02). One-third of the mothers of the affected infants did not take folic acid in their pregnancy and only 20% (6 out of 30) had folic acid supplements in preconception period. Mothers in both groups who had folate supplements were in compliance with dose and duration as prescribed by their consultants.

The genotype distribution of the infant *MTHFR C677T* variant was concordant with HWE in affected and healthy groups (Figure 1D). Overall, the *MTHFR C677T* variant showed association with NSCLP in dominant and over-dominant models. The *MTHFR C677T* variant CT genotype exhibited a 0.29 fold lower risk of NSCLP (CT versus CC: 95% CI=0.10–0.87; *p*=0.027). Lower risk for NSCLP was observed with *MTHFR C677T* variant, in dominant (CT-TT versus CC: OR=0.33, 95% CI=0.12-0.96; p=0.03) and over-dominant (CT versus CC-TT: OR=0.29, 95% CI=0.10-0.84; *p*=0.01) models.

To the best of our knowledge, it is the first study to investigate the role of *MTHFR* variant in NSCLP among Pakistani population. In this study, CT genotype of *MTHFR* gene was associated with decreased risk of NSCLP among

Pakistani infants. Our results are consistent with the study on Moroccan population that noted low association of *MTHFR C677T* with NSCLP.2 In a meta-analysis, Li Q et al5 reported a significant association between *MTHFR C677T* and NSCL/P risk in two genetic models: TT vs CC and recessive model. Pan Y et al[8] observed that *MTHFR C677T* T allele, CT and TT genotypes and CT/TT genetic model among Asian infants increase the risks of NSCLP, whereas recessive genetic model (CT/CC) was found to confer reduced susceptibility to NSCLP. In contrast with these findings, the current study observed CT genotype to be protective, with a 0.29-fold lower probability of possessing an NSCLP in the Pakistani population.

No significant difference in the serum folic acid between the affected group and the healthy group was noted. However, the range of serum folic acid levels in the affected group was lower than healthy group. In accordance with the current study, Ajila V et al[7] and Munger RG[9] did not observe a significant difference in the serum folate concentration between affected and healthy groups. Various interventional studies have suggested that folic acid may protect against the recurrence of orofacial clefts.[10] In a recent meta-analysis folic acid/multivitamin supplementations before or during pregnancy were linked to a lower risk of orofacial clefts. It is suggested that the timing of supplementations had a marked effect on the occurrence of orofacial clefts. Pre-conception supplementations lower the risk of having a child with cleft lip/palate (OR=0.65; 95% CI=0.50–0.86)[7] that is consistent with the results of the current study suggesting decreased risk for oral clefts in mothers using pre-conception folic acid supplementations as compared to post-conception use. Numerous studies showing link between folic acid consumption and clefts evaluated the role of folic acid based on its intake during pre- and post-conception period of pregnancy[7] rather than as a biomarker to diagnose or predict the orofacial cleft status.

The study had limitations, such as being conducted at a single centre. Further studies on other ethnic groups are recommended to enhance the comprehension of the genetic foundation of NSCLP among the Pakistani population.

Conclusion

In conclusion, the current study suggested that MTHFR 677 CT genotype was related with decreased risk for NSCLP in Sindhi, Pakistani population. Serum folic acid levels were not different between affected and healthy groups, though higher range of folic acids were revealed in healthy group. Pre-conceptional folic acid may have a preventive effect on the occurrence and recurrence of orofacial clefts; however, serum folic acid levels alone might not be sufficient to establish the status of orofacial clefts.

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Conflict of interest: None.

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Author Contribution:

SE: Enrolled patients and controls, laboratory experiments, literature review, interpreted results.
SM, AMW: Conceived the study, supervised the laboratory experiments, data verification, and drafting.
FFK: Interpreted the results, statistical analysis, and manuscript writing.
WS: Patient enrolments and performed laboratory experiments.
All authors read and approved the final manuscript.