

The 19-bp deletion polymorphism of dihydrofolate reductase (DHFR) and nonsyndromic cleft lip with or without cleft palate: evidence for a protective role

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ABSTRACT

Objective: Nonsyndromic cleft lip with or without cleft palate (NS-CL/P) are among the most common congenital birth defects worldwide. Several lines of evidence point to the involvement of folate, as well as folate metabolizing enzymes in risk reduction of orofacial clefts. Dihydrofolate reductase (DHFR) enzyme participates in the metabolic cycle of folate and has a crucial role in DNA synthesis, a fundamental feature of gestation and development. A functional polymorphic 19-bp deletion within intron-1 of DHFR has been associated with the risk of common congenital malformations. The present study aimed to evaluate the possible association between DHFR 19-bp deletion polymorphism and susceptibility to NS-CL/P in an Iranian population. **Material and Methods:** The current study recruited 100 NS-CL/P patients and 100 healthy controls. DHFR 19-bp deletion was determined using an allele specific-PCR method. **Results:** We observed the DHFR 19-bp homozygous deletion genotype (D/D) vs. homozygous wild genotype (WW) was more frequent in controls than in NS-CL/P patients (25% vs. 13%), being associated with a reduced risk of NS-CL/P in both codominant (OR=0.33, P=0.027) and recessive (OR=0.45, P=0.046) tested inheritance models. We also stratified the cleft patients and reanalyzed the data. The association trend for CL+CL/P group compared to the controls revealed that the DD genotype in both codominant (OR=0.30, P=0.032) and recessive models (OR=0.35, P=0.031) was associated with a reduced risk of CL+CL/P. **Conclusions:** Our results for the first time suggested the DHFR 19-bp D/D genotype may confer a reduced risk of NS-CL/P and might act as a protective factor against NS-CL/P in the Iranian subjects.

Keywords: Dihydrofolate reductase (DHFR). Nonsyndromic cleft lip with or without cleft palate (NS-CL/P). Ins/Del polymorphism.

INTRODUCTION

Orofacial clefts (OFC), including cleft lip with or without cleft palate (CL/P) and cleft palate only or isolated cleft palate (CP), are among the most frequent inborn birth defects worldwide²⁰. According to their association with special malformative patterns or their incidence as isolated defects,

CL/P can be classified into Syndromic and Nonsyndromic (NS-CL/P), respectively. Most CL/P cases (approximately 70%) are nonsyndromic, although strong genetic factors contribute in both forms. Syndromic forms are typically caused by chromosomal aberrations or monogenic diseases, whereas, NS-CL/P forms result from the interaction between genetic and environmental factors. NS-CL/P

patients frequently suffer from major complications such as difficulties concerning feeding, speech, hearing, and psychologic maturation^{19,21}.

Nonsyndromic or isolated (without other associated abnormalities) CL/P is a common congenitally developmental disorder affecting 1:700 births worldwide, although this rate is different in various ethnic groups and geographic regions^{19,21}. Generally, Asian populations have a higher birth occurrence of clefting (1/500 births), Caucasians are intermediate (1/1,000 births), and African populations have the lowest (1/2,500 births). It has been reported that the incidence of CL and CP in Iran is approximately 1/1,000 lower than that in other countries. Clefting is caused by a coordinated sequence of events that involve the growth of several independently-derived facial primordia. Genetic and environmental factors, and their interactions, may affect these events and contribute to NS-CL/P pathogenesis²⁰.

The development of craniofacial structures is the product of social environmental, and nutritional factors such as tobacco smoke, alcohol consumption, and maternal folic acid deficiency due to exposure to folic acid antagonists – such as anticonvulsants²⁶ – appear to play a role in the development of OFC during pregnancy⁸. Folic acid is a water soluble B-vitamin that plays a crucial role in embryonic development because it is essential for DNA stability maintenance, being involved in DNA synthesis, repair, and methylation^{4,22}. Alterations in genes responsible for each step of the folate pathway has been shown to cause aberrations in organogenesis that result in congenital malformations such as neural tube defects (NTDs)⁶ or OFC⁴. Several lines of evidence have confirmed there is a reduced risk of OFC and NTDs when mothers used either folic acid-containing supplements or dietary folate during the periconceptional period. For example, a meta-analysis of 11 case-control studies and four prospective studies supported the hypothesis that taking folic acid-containing supplements during pregnancy decreases the risk of NS-CL/P¹.

The metabolic cycle of folate is complex, with multiple genes participating in the transformation of folate into various compounds⁸. One of these genes is dihydrofolate reductase (DHFR), known as a strong candidate for a teratogenic locus which synthesizes the DHFR enzyme. All of the folate from vitamin supplements and food fortification is folic acid, a form of folate that is unreduced and requires the action of DHFR before it can participate in cellular reactions. DHFR converts the dihydrofolate (DHF) into tetrahydrofolate (THF) forms that can contribute to folate/homocysteine metabolism, and subsequent DNA synthesis, a fundamental feature in fetal development and organogenesis.

Several gene polymorphisms affect folate

metabolism and are associated with reduced folate absorption and consequently increased folate needs. In the absence of a folate-sufficient diet, these gene alterations are associated with increased risk of NTDs and congenital defects in the offspring⁴.

The *DHFR* gene is located at chromosome 5q11.2-13.2 and is expressed in three mRNA isoforms with alternatively spliced 3'UTR ends. The changes in DHFR expression or activity can be partly due to the functional polymorphisms in the *DHFR* gene, thereby influencing a risk of folate-dependent diseases¹⁷. A polymorphic 19-bp deletion within intron-1 of *DHFR* has been described; however, its biological consequence has been inconsistent. Accordingly, the *DHFR* 19-bp deletion polymorphism has been associated with a reduced or increased risk of some congenital malformations. For instance, the *DHFR* 19-bp deletion (D)/D genotype in mothers has been associated with an increased risk of having a baby born with spina bifida (SB)¹³; whereas other studies have suggested the *DHFR* 19-bp D/D genotype is protective against having a baby born with SB¹⁸ or NTD^{9,14}, verifying the inconsistency among different studies.

With respect to the crucial role of folic acid in embryonic development, any genetic variations in the *DHFR* gene leading to impaired folate metabolism could possibly contribute to predisposition to NS-CL/P. Therefore, the present study aimed to evaluate the possible association between functional 19-bp deletion polymorphism within intron-1 of *DHFR* and susceptibility to NS-CL/P in an Iranian population. To the best of our knowledge, this is the first study examining the possible impact of *DHFR* 19-bp deletion gene variation on NS-CL/P.

MATERIAL AND METHODS

In the current case-control retrospective study, a total of 200 subjects – including 100 CL/P patients (61 male and 39 female) with an average age of 12.12 years (Range: 1–54 y) and 100 normal subjects (61 male and 39 female) with an average age of 12.03 years (Range: 1–51 y) – were involved as previously described by this research team¹⁹. All samples were collected from Zahedan, a South-east Iranian population. The control samples were unaffected, unrelated individuals without a family history of clefting, collected as randomly selected, population-based controls from Zahedan. The diagnosis was made at the time of birth by physical examination. All patients were diagnosed independently and were screened to exclude cleft-associated syndromes – such as DiGeorge syndrome, Stickler syndrome, Nager syndrome, and Van der Woude syndrome – by the multidisciplinary team of specialists of each center. There was no significant difference between

the groups regarding sex and age ($P>0.05$). All participants were unrelated to each other.

The local Ethics Committee of the Zahedan University of Medical Sciences approved the project, and written informed consent was taken from all participants (or their parents). Blood samples from all patients and healthy controls were collected in EDTA containing tubes and stored at -20°C until DNA extraction. Genomic DNA was extracted from the peripheral blood leukocytes by the "salting-out" method as described previously. The quality of the isolated DNA was checked by electrophoresis on 1% agarose gel, quantitated spectrophotometrically, and stored at -20°C until further use.

Genotyping of the 19-bp deletion of *DHFR* was performed using the allele specific-PCR (AS-PCR) as described previously by Gemmati, et al.¹¹ (2009). In the AS-PCR, two different allele-specific forward primers (F1 and F2) for the non-deleted and deleted polymorphic alleles, respectively, and one common reverse primer (R) were applied. To verify the accuracy of the method, random samples were genotyped by a bi-directional PCR allele-specific amplification (bi-PASA) PCR as established previously by Eskandari-Nasab, et al.⁸ (2012) and Rafighdoost, et al.²⁷ (2013). The bi-PASA included two outer primers (FO and RO) and two inner allele-specific primers, FI (Insertion allele) and RI (Deletion allele), all in one-tube PCR reaction. PCR was performed using commercially available Prime Taq premix (Genetbio, Chungcheongnam, South Korea) according to the manufacturer's recommended protocol. Briefly, 1 μL template DNA (~ 100 ng/ μL), 0.7 μL of each primer (10 pmol/mL), 10 μL Prime Taq premix, and 7 μL DNase-free water were added to each PCR tube in a final volume of 20 μL . Amplification was performed with an initial denaturation step at 95°C for 5 min, followed by 30 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 25 s with a final extension at 72°C for 10 min. Each reaction was verified on a 2% agarose gel. The AS-PCR results were 100% concordant with the findings of bi-PASA. All primers sequence and

amplicons size are presented in Figure 1.

Statistical analysis

The statistical analyses of the data were performed by the SPSS 18.0 software (SPSS Inc, Chicago IL, USA). The associations between alleles or genotypes and NS-CL/P were assessed by computing the odds ratio (OR) and 95% confidence intervals (95% CI) from logistic regression analyses. P-values below 0.05 were determined statistically significant. Based on our results, sample power was analyzed for *DHFR* polymorphism by comparison of each genotype with the accumulation of other genotypes by utilizing STATA 10 software.

RESULTS

Of a total of 100 patients, 43 were CL, 26 were CLP, and 31 were cleft palate only (CP). The distribution of genotype and allele frequencies of *DHFR* 19-bp deletion polymorphism is presented in Table 1. Our results revealed a statistically significant association between *DHFR* 19-bp deletion polymorphism and susceptibility to NS-CL/P in both the codominant and recessive tested inheritance models. In the codominant model, the *DHFR* 19-bp homozygous deletion genotype (D/D), with an increased frequency in controls compared to NS-CL/P patients (25% vs. 13%), was associated with a reduced risk of NS-CL/P (D/D vs. W/W genotype; OR=0.33, 95% CI=0.32–0.88; $P=0.027$). Similarly, in the recessive model, the *DHFR* 19-bp D/D genotype was associated with susceptibility to NS-CL/P and may confer a decreased risk against NS-CL/P (D/D vs. W/W+W/D genotype; OR=0.45, 95% CI=0.20–0.99; $P=0.046$).

On the other hand, at the allelic level, the *DHFR* 19-bp deletion (D) allele was more prevalent in the control group compared to the NS-CL/P patients (43% vs. 33%), although it was not significantly associated with the risk of NS-CL/P (D allele vs. W allele; OR=0.68, 95% CI=0.45–1.03, $P=0.072$).

Table 2 shows the association of *DHFR* 19-bp

Primers	Sequence (5' to 3')	Amplicon size
Genotyping Method	Bi-PASA PCR	
DHFR-FO	CTGTCATGGTTGGTTCGCTAAAC	357 bp
DHFR-RO	TGGGTAGGGGTGAGGTGTTTTTC	
DHFR-FI (Del allele)	CCACGGTCCGGGTACCTGGG	159 bp
DHFR-RI (Ins allele)	GAGTCGGCCACCCGACCGT	239 bp
Genotyping Method	Allele-Specific PCR	
DHFR-F1	TCGGGGTACCTGGG	113 bp and
DHFR-F2	ACGGTCCGGGTGGCCGACTC	92 bp
DHFR-R	AAAAGGGGAATCCAGTCG	

Figure 1- Primers sequence for detection of *DHFR* 19-bp deletion polymorphism

Table 1- Genotype and allele frequencies of DHFR 19-bp deletion polymorphism between patients with nonsyndromic cleft lip and/or cleft palate (NS-CL/P) and control subjects

Genotypes	NS-CL/P Patients n (%)	Controls n (%)	OR (95%CI)	P-value	Study Power %
DHFR, 19-bp deletion polymorphism					
Codominant					
W/W	19 (19)	12 (12)	Reference	-	22
W/D	68 (68)	63 (63)	0.68 (0.31-1.52)	0.348	9
D/D	13 (13)	25 (25)	0.33 (0.32-0.88)	0.027	51
Dominant					
W/W	19 (19)	12 (12)	Reference	-	22
W/D+D/D	81 (81)	88 (88)	0.58 (0.25-1.36)	0.241	22
Recessive					
W/W+W/D	87 (87)	75 (75)	Reference	-	51
D/D	13 (13)	25 (25)	0.45 (0.20-0.99)	0.046	51
Alleles					
W	106 (53)	87 (44)	Reference	-	40
D	94 (47)	113 (56)	0.68 (0.45-1.03)	0.072	40

Table 2- Genotype and allele frequencies of DHFR 19-bp deletion polymorphism between patients with nonsyndromic cleft patients without CP and control subjects

Genotypes	CL+CL/P n (%)	Controls n (%)	OR (95%CI)	P-value	Study Power %
DHFR, 19-bp deletion polymorphism					
Codominant					
W/W	13 (18.8)	12 (12)	Reference	-	18
W/D	48 (69.6)	63 (63)	0.70 (0.30-1.68)	0.428	11
D/D	8 (11.6)	25 (25)	0.30 (0.01-0.90)	0.032	50
Dominant					
W/W	13 (18.8)	12 (12)	Reference	-	18
W/D+D/D	56 (81.2)	88 (88)	0.59 (0.23-1.49)	0.271	18
Recessive					
W/W+W/D	61 (88.4)	75 (75)	Reference	-	50
D/D	8 (11.6)	25 (25)	0.35 (0.26-0.86)	0.031	50
Alleles					
W	74 (54)	87 (44)	Reference	-	40
D	64 (46)	113 (56)	0.66 (0.41-1.08)	0.067	40

(CL/P)=cleft lip with or without cleft palate

polymorphism between the CL+CL/P group and the control group. The association trend for the CL+CL/P group was almost the same as those for all cleft patients: the results demonstrated that DD genotype in the codominant model (D/D vs. W/W genotype; OR=0.30, 95% CI=0.01–0.90; P=0.032) as well as the DD genotype in the recessive model (D/D vs. W/W+W/D genotype; OR=0.35, 95% CI=0.26–0.86; P=0.031) were associated with a reduced risk of CL+CL/P.

DISCUSSION

In the current study, we investigated the impact of *DHFR* 19-bp deletion polymorphism on the risk of NS-CL/P. Our results demonstrated that the *DHFR* 19-bp homozygous D/D genotype (in both codominant and recessive models) was associated with a reduced risk of NS-CL/P. The results indicated that individuals carrying the *DHFR* 19-bp D/D genotype in the codominant and recessive models had, respectively, a 0.33- and a 0.45-fold reduced

risk of developing NS-CL/P compared to those carrying the W/W genotype. We also stratified the cleft patients according to their etiology into CP and CL+CL/P patients, and evaluated the association of *DHFR* 19-bp deletion polymorphism and the risk of CL+CL/P by comparing with the controls. The results demonstrated that the DD genotype in both codominant and recessive models was associated with a reduced risk of CL+CL/P. Thus, our findings highlight the protective role of *DHFR* 19-bp deletion polymorphism against susceptibility to NS-CL/P in our population. Similarly, Parle-McDermott, et al.¹⁸ (2007) have shown a significantly lower risk of having a child with NTDs in women with the D/D or W/D genotypes than in women with the W/W genotype, suggesting that the 19-bp D allele may be a protective NTD genetic factor. However, other studies^{13,17} have shown that the *DHFR* 19-bp D/D genotype vs. W/W genotype increased the risk of having a child with unilateral retinoblastoma or SB.

The polymorphic 19-bp deletion within intron-1 of *DHFR* appears to play important roles in the transcription of *DHFR* and the quantity of protein produced by translation. However, the precise consequence of the *DHFR* 19-bp deletion polymorphism has been controversial. Firstly, Johnson, et al.¹³ (2004) suggested the D allele might decrease the *DHFR* expression, since the Sp1 transcription factor binding site is located within the deleted sequence. They suggested the homozygous D/D genotype in mothers is related to an increased risk of preterm delivery¹² or having a baby born with SB¹³. However, Parle-McDermott, et al.¹⁸ (2007) in a larger study have shown that the D/D genotype is protective and decreases the risk of having a baby born with SB. Additionally, they¹⁸ reported that *DHFR* mRNA expression was 50% higher in lymphoblast cell lines with the D/D genotype than in cell lines with the W/W genotype. Moreover, Xu, et al.²⁸ (2007) observed a dose-dependent relation between *DHFR* expression and the D/D genotype, proposing that subjects with the D/D genotype express 4.8-fold more *DHFR* mRNA levels compared to subjects carrying the 19-bp W/W genotype. Stanislawska-Sachadyn, et al.²³ (2008) also reported that individuals with the D/D genotype have increased serum and red blood cell folate levels and lower homocysteine levels. These findings were confirmed by others^{10,25}, who showed a significant decrease in plasma homocysteine concentration for the *DHFR* D/D genotype relative to the W/W genotype. This would affirm the possible maternal protective role of the 19-bp *deletion* allele in NS-CL/P by increasing the amount of *DHFR* available to reduce DHF to THF, and by augmenting bioavailability of folate cofactors required for a number of cellular reactions in pregnant women. However, any potential functional effect of the 19-bp

deletion needs to be examined further in the context of the cell cycle and/or under low folate conditions. Martinelli, et al.²⁰ (2014) also examined four SNPs of *DHFR* gene on cleft patients, and their study is different from ours in the way that we examined the 19-bp Ins/Del polymorphism of *DHFR* in cleft patients.

Multiple studies have demonstrated strong evidence for the contribution of Interferon regulatory factor 6 (IRF6) signalling pathway to the risk of nonsyndromic oral clefts across various ethnic groups. The *IRF6* from chromosomal region 1q32.2 has previously been shown to account for the majority of patients with Van der Woude syndrome (VWS), the most common Mendelian deformity syndrome which includes oral clefts as a hallmark feature. Several genetic markers in *IRF6* have provided evidence of linkage and linkage disequilibrium (LD) in studies of nonsyndromic oral clefts and have been associated with CL/P in many populations^{5,24,30}.

Variations in *WNT* (wingless-type MMTV integration site family) genes have also been associated with human nonsyndromic oral clefts. *WNT* signalling performs as an up-regulator of p63 and interferon regulatory factor 6 (IRF6), which regulates precise facial process fusion by controlling epithelial cell proliferation and differentiation. Variants within *WNT* genes were shown to contribute to cleft lip and palate, and mutations in *WNT3* underlie autosomal-recessive tetra-amelia with cleft lip and palate. In family-based studies, a variety of *WNT* genes (*WNT3A*, *WNT5A*, *WNT8A*, and *WNT11*) have been shown to have an association with cleft lip/palate. Furthermore, studies in animal models have revealed the crucial role of *WNTs* family in craniofacial development. *WNT3* and *WNT9B* are located in the cleft loci 1 (*clf1*) and could contribute to the clefting phenotype¹⁵. Targeted mutation in the mouse *WNT9B* gene resulted in an incomplete penetrance of CLP, suggesting the importance of *WNTs* in the pathogenesis of orofacial clefts^{3,16}.

A number of other genes have also been implicated in NS-CL/P pathogenesis including transforming growth factor-alpha (TGFA), bone morphogenetic proteins (Bmp), fibroblast growth factors (Fgf), and members of the transforming growth factor b (TGFB) superfamily. Mutations in TGFA, msh homeobox 1 (MSX1), fibroblast growth factor receptor 1 (FGFR1) and FGF8, and bone morphogenetic protein 4 (BMP4) have also been demonstrated to underlie the NS-CL/P⁷. The presence of TGFA in the regulation of palate development and its high levels in medial edge epithelia (MEE) of palatal shelves have been demonstrated²⁹. Bmp2 and Bmp4 are expressed more specifically within the epithelia and mesenchyme of the palatal shelves. The

Msx1 homeobox gene, which is also expressed in the facial primordia, is needed for expression of Bmp2 and Bmp4 in the palatal mesenchyme. In addition, epidermal growth factor (EGF) stimulates glycosaminoglycan production within the palatal shelves^{7,29}.

Orofacial development is a quite harmonized process of cell proliferation, differentiation, migration, and apoptosis. The quick growth and the consecutive fusion of maxillary processes and palatal shelves during early embryogenesis are crucial stages in orofacial development, both requiring an effective methyl synthesis and availability²¹. DHFR is also needed for the intracellular conversion of synthetic folic acid, consumed in supplements and fortified foods, into the THF forms that can contribute to folate/homocysteine metabolism. Defective DHFR activity has been associated with a rapid depletion of THF pool within the cell affecting the level of folate coenzymes and hence purine and pyrimidine synthesis, and consequent termination of de-novo DNA synthesis and histone methylation, that are all essential for cell proliferation and regulation of gene expression during embryonic development¹⁰. The use of folic acid supplementation during pregnancy in protection against congenital malformations such as OFCs², NTD⁴, and SB²⁷ has been supported by several studies. These studies support the protective role of folic acid intake during the pre-, peri-, and postconceptional periods on the risk of OFCs⁸, suggesting that the occurrence of OFCs can be reduced with prophylactic use of folate by women in the periconceptional period. Two hypotheses may explain the harmful effects of low folate levels during pregnancy.

One of the limitations of our study is the relatively small sample size. Therefore, the results should be interpreted with caution. Furthermore, our study deviated somehow from HWE (Hardy Weinberg Equilibrium). There is no clear explanation for the deviation from HWE in our population. However, the small sample size, consanguineous marriages, or genetic drift could be possible reasons in this population.

CONCLUSIONS

Our data suggests that *DHFR* 19-bp D/D genotype is associated with a reduced risk of NS-CL/P in both the codominant and recessive models. This finding highlights the protective role of *DHFR* 19-bp deletion polymorphism against susceptibility to NS-CL/P in an Iranian population. Future research with larger samples from different ethnicities is required to validate our findings.

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DISCLOSURE OF CONFLICTING INTERESTS

The authors declare that there is no conflict of interest to disclose.

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