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The study of association between reduced folate carrier 1 (*RFC1*) polymorphism and non-syndromic cleft lip/palate in Iranian population

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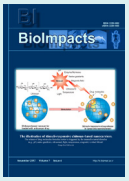

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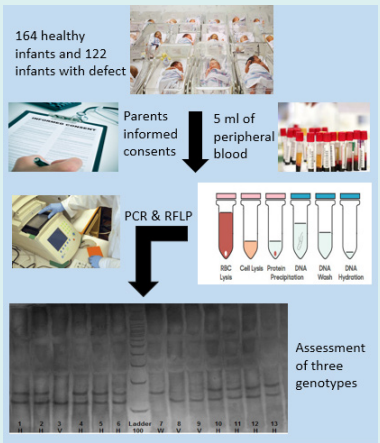
Abstract

Introduction: Cleft lip/palate is one of the most common congenital defects and is supposed to have multifactorial etiology, including a complex interaction between genetics and environment. Reduced folate carrier 1 (*RFC1*) gene takes part in folate transportation within the cells. In this study, the association of A80G polymorphism in the *RFC1* gene with the non-syndromic cleft lip/palate (nsCL/P) was investigated in Iranian infants for the first time.

Methods: In this case-control survey, 122 Iranian infants with nsCL/P and 164 healthy infants were investigated for *RFC1* polymorphism by PCR and RFLP methods. The results were statistically compared with control group, odds ratios with 95% CI were estimated by univariate and multivariate logistic regression model and a P <0.05 was considered statistically significant.



Results: The *RFC1* G allele was significantly higher (P=0.001; OR=7, 95% CI: 4.7-10.2) in the cases (60.3%) compared with the controls (17.9%). Not only the *RFC1* AG genotype was significantly higher (P<0.001; OR=44, 95% CI: 14.6-133) in cases (67.8%) than the controls (27.4%), but also GG genotype (P<0.001; OR=85, 95% CI: 20.5-352) was much higher in cases (26.4%) than the controls (4.3%).

Conclusion: Our study indicated that the *RFC1* (A80G) polymorphism was associated with the nsCL/P in Iranian population. Moreover, 80GG homozygosity was significant in the cases. The presence of G allele can be considered as a risk factor for the nsCL/P. Infants with the GG and AG genotypes were more prone to cleft lip/palate as compared to the AA ones. This finding emphasizes the role of *RFC1* gene and the intracellular levels of folate.

Introduction

Cleft lip with/without cleft palate (CL/P) is one of the most common congenital abnormalities with multifactorial etiology, including environmental and genetic factors.[1] CL/P shows a prevalence of 3.73 per 1000 in Iranian newborns.[2] Genetic factors play a very important role in this disorder because whole genome analysis shows 18 genetic risk loci related to these defects.[3] While the gene identification process for this defect is premature, the steps are rapidly increasing. By the aid of identifying candidate genes of non-syndromic CL/P (nsCL/Ps); not only prediction but also prevention will be possible.[4]

Various studies demonstrate the pathogenicity of folate decreasing in periconceptional period and role of folic acid in preventing neural tube defects.[5,6] Among genes taking part in the folic acid metabolism, the reduced folate carrier

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(*RFC1),* also known as *SLC19A1,* has been shown to be associated with the nsCL/P.[7] Folate is a highly hydrophilic molecule that cannot cross the biological membranes by diffusion solely. This gene have a role as an organic anion exchanger in folic acid absorption by the bi-directional action of transporting 5-methyltetrahydrofolate and thiamine monophosphate.[8,9] 5-methyltetrahydrofolate is the metabolic active version of folate which inserts into cells and constructs intracellular folate concentration.[10]

RFC1 (SLC19A1) gene is located on chromosome 21 (21q22.2-q22.3).[11] *SLC19A1* polymorphism consisting of substitution of adenine to guanine in exon 2 at nucleotide position 80 (A80G), changes the 27th amino acid of protein from histidine (CAC) to arginine (CGC).[12,13]

Several associations have demonstrated the relationship between polymorphism of *RFC1* gene (Fig. 1) and the risk of nsCL/P, conotruncal heart defects, Down syndrome,[15] spina bifida,[16] neural tube defects,[17] and lymphoblastic leukemia.[18]

Some previous researches have suggested the association between the nsCL/P and G allele in the *RFC1* gene, thus it could be a candidate gene for the nsCL/P.[19-22]

In 2004, Schultz et al examined fifteen candidate regions for their relationships to nsCL/P. Analysis of 126 Filipino cases and 218 controls revealed a borderline significant association between *RFC1* gene and nsCL/P.[23] Wang et al in a Chinese population,[20] Girardi et al in Italian population[21] and Lakkakula et al in south Indian population[22] indicated that the *RFC1* gene variant increases occurrence risk of nsCL/P. However, this is contrary to the studies which were done by Shaw et al in Californian infants,[24] Pei et al in Chinese population[25] and Mostowska et al in Polish population,[26] demonstrating the role of race and genetic differences in different populations. The literature review of association studies of *RFC1* polymorphism and nsCL/P have been conflicting.[6,16,19-23,25,27] Numerous researches evaluated the correlation between *RFC1* gene polymorphism and ns CL/P but there is no evidence of Iranian population examined. The main aim of the present case-control study was to investigate the association between *RFC1* gene polymorphism (A80G) and the risk of ns CL/P in Iranian population for the first time.

Materials and Methods

Subjects

In this survey, samples were recruited from Mofid Pediatric hospital in Tehran, Iran, from 2013 to 2016. A sample of 122 newborns with a primary diagnosis of ns CL/P was collected. Control group consisting 164 Iranian newborns, simulated to cases regarding socioeconomic, traits and age, were gathered and their blood samples were collected. Cases that had other facial or skeletal anomalies (such as bifida uvula and lip pits), conotruncal heart defects, anomalies of the other organs or metabolic disorders and mother's history of methotrexate consumption in pregnancy were excluded.

Genotyping

Five mL of peripheral blood of infants were gathered in the tube containing 200 µL of 0.5 M EDTA and stored at -80°C, then DNA was extracted using simple salting out method.[28]

Genotyping of the *RFC1* (rs1051266, A80G) polymorphism was accomplished by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) methods with Hha1 enzyme (Fermentas, Germany).[29,30] The sequences are displayed in Table 1.

Concisely, a total volume of 25 µL including 30 ng of genomic DNA, 5 µM of each primer, 1 µL dNTPs mix (Fermentas, Germany), 2.5 µL 10×buffer and 0.5 U of Taq DNA polymerase (Fermentas, Germany) with 1.5 mM $MgCl_2$ was formulated in the 0.5 mL microtube for amplification of the objective sequences. Amplification conditions began with an inceptive denaturation step of 4 minutes at 95°C, proceeded by 33 cycles of 45 seconds denaturation (94°C), 30 seconds annealing (60°C) and 40 seconds extension (72°C), terminated by an ultimate extension for 5 minutes (72°C) and eventually chilling to 4°C. The PCR product of the rs1051266 polymorphism was broken down with the IUHha1 restriction enzymes at 37°C overnight (New England BioLabs, Beverly, USA). All PCR products were subjected to 8% polyacrylamide gel electrophoresis then colored by silver nitrate. The model of restriction fragments for Hha1 is shown in Table 1. All genotyping was executed blinded.

Statistical analysis

Statistical analyses were accomplished using SPSS version 11.5 (SPSS Inc, Chicago, USA). Chi-square ($\chi 2$) test was used to compare the allele frequencies between the study groups. Odds Ratios with 95% confidence Interval were estimated by univariate and multivariate (sex, mother age, parents smoking, folic acid consumption during pregnancy and other CL/P in the family) logistic regression model. The $P<0.05$ was considered for statistically significant results.

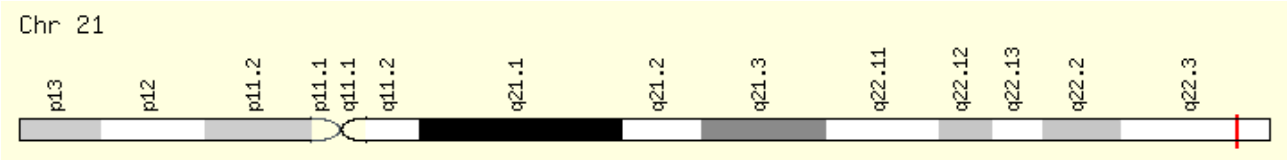

Fig. 1. *RFC1* Gene in genomic location.[14]

Table 1. Primer sequences and their PCR product sizes, restriction enzymes, and RFLP fragments for the *RFC1* rs1051266 A/G polymorphism

SNPs	Primer sequence (5'→3)	Product size	RFLP Fragments (bp)	Cuts positions
RFC1 (rs1051266 A/G)	F: AGCGTCACCTTCGTCCC	230	A allele:162+68	162
	R: TCCCGCGTGAAGTTCTTG		G allele=125+37+68	125, 162

Results

Cases consisted of 122 Iranian infants with cleft lip with or without cleft palate and 164 healthy infants as the control group. The nsCL/P samples consisted of 70 (57%) males and 52 (43%) females. The distributions of genotypes in the entire population, and in female and male subsets, using chi-square showed they were in Hardy-Weinberg equilibrium. The dispersal of genotypes and allele frequencies of the *RFC1* (A80G) polymorphism are presented in Table 2.

Results of this study demonstrated significant differences in allele frequency and genotype distribution of *RFC1* polymorphism between the case and control groups. The *RFC1* G allele was significantly higher (P=0.001; OR=7, 95% CI: 4.7-10.2) in the cases (60.3%) compared with the control group (17.9%).

According to univariate logistic regression analysis, not only the *RFC1* AG genotype was significantly higher ($P<0.001$; OR=44, 95% CI: 14.6-133) in case group (67.8%) than the control group (27.4%), but also GG genotype ($P<0.001$; OR=85, 95% CI: 20.5-352) was much higher in case group (26.4%) than the control group (4.3%). Therefore, AG and GG genotypes are risk factors for CL/P. But, by adjustment the effect of genotypes by sex, mother age, parents smoking, folic acid consumption during pregnancy and other CL/P in family, AG and GG genotypes were also significantly associated with CL/P with multivariate logistic regression. Also, the frequency of homozygosity for G allele in the cases is much greater than controls. This study showed the association ofA80G polymorphism in the *RFC1* gene (SLC19A1), with nsCL/P in Iranian infants for the first time.

Discussion

Reduced folate carrier1 (RFC1) is one of the enzymes in the metabolism of folic acid which transports the active form of folic acid (5-methyltetrahydrofolate) into cells.[10] A change of A allele to G allele results in a mutant protein that can affect enzymatic activity and has a potential role as a risk factor for homocysteinemia, Down Syndrome, cardiovascular diseases,[31,32] neural tube defects and nonsyndromic cleft lip/palate,[21] in contrast to conflicting literature.

Cleft lip with or without cleft palate (CL/P) is one of the most common congenital abnormalities with multifactorial etiology including environmental and genetic factors. Among genes taking part in folic acid metabolism, RFC1, also known as SLC19A1, has been shown associated with nsCL/P.[6] Individuals carrying a specific polymorphism of SLC19A1 (G 80A) have lower levels of folate. As folate transport across cell membranes is mediated in part by RFC1v (Fig. 2 and 3),[9] variants within this gene may influence nsCL/P risk via an effect on folate and/or homocysteine levels.

This study was executed to determine whether the *RFC1* (rs1051266, A80G) polymorphism has a relationship with increased risk of nsCL/P in Iranian infants consisting 122 cases and 164 controls. The results proved that substitution of A allele by G allele in *RFC1* was associated

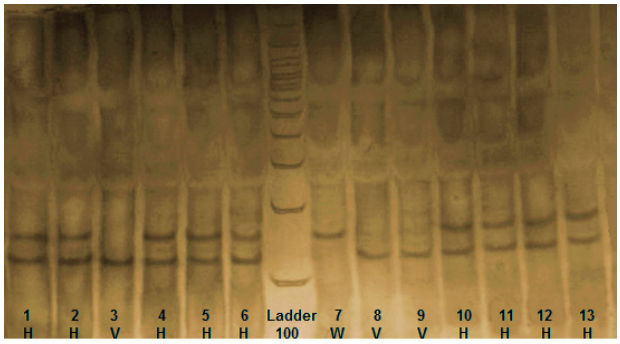

Fig. 2. RFC1 (SLC19A1) RFLP. Three genotypes from nsCL/P cases indicating the wild type (W), heterovariant (H) and homovariant (V). After digestion of PCR product with the restriction enzyme *Hha*1, one specific band of 162 bp was indicated in wild genotype, two specific bands of 162 bp and 125 bp were indicated in heterovariant genotype and one specific band of 125 bp was indicated in homovariant genotype.

Table 2. The genotype and allele frequencies of the RFC1 (A80G) polymorphism in nsCL/P case and control

Variable	Controls, No. (%)	Cases, No. (%)	*P* value	OR (95% CI)
		Allele		
A	269 (82.0)	96 (39.6)	Reference Allele	
G	59 (17.9)	146 (60.3)	0.001	7 (4.7-10.2)
		Genotype		
AA	112 (68.3)	7 (5.8)	Reference Genotype	
AG	45 (27.4)	82 (67.8)	<0.001*	44 (14.6-133)
GG	7 (4.3)	32 (26.4)	<0.001*	85 (20.5-352)
Yes	0 (0)	10(8.2)		

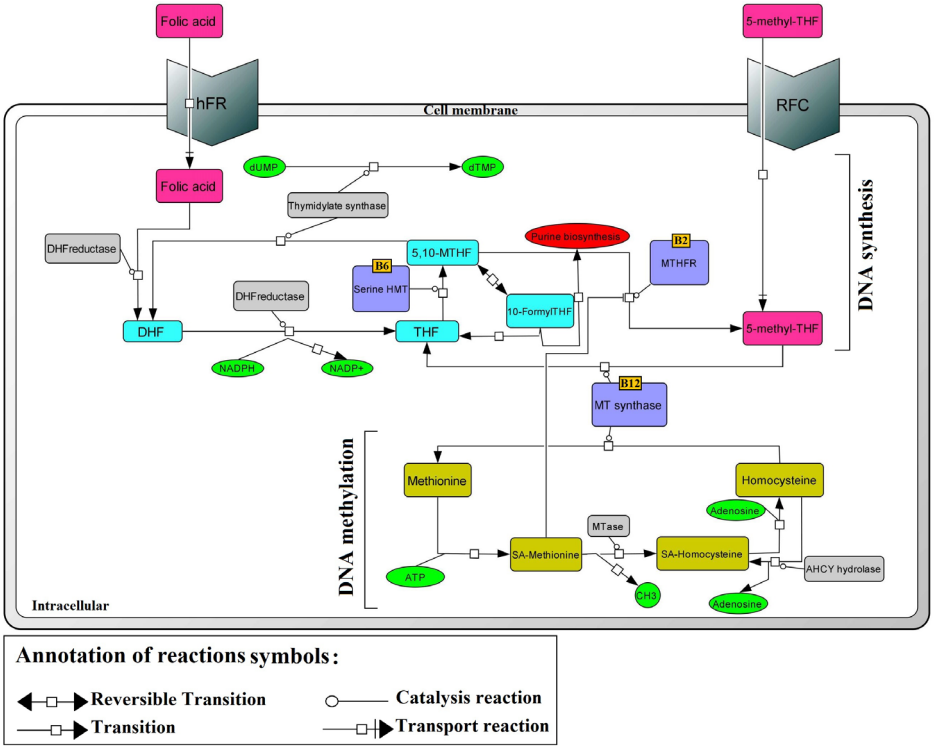

Fig. 3. Schematic diagram of RFC1 in folate metabolism. RFC, reduced folate carrier; hFR, human folate receptor; MTR, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; SHMT, serine hydroxymethlytransferase; TS, thymidylate synthase; THF, tetrahydrofolate; DHF, dihydrofolate; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate.[33]

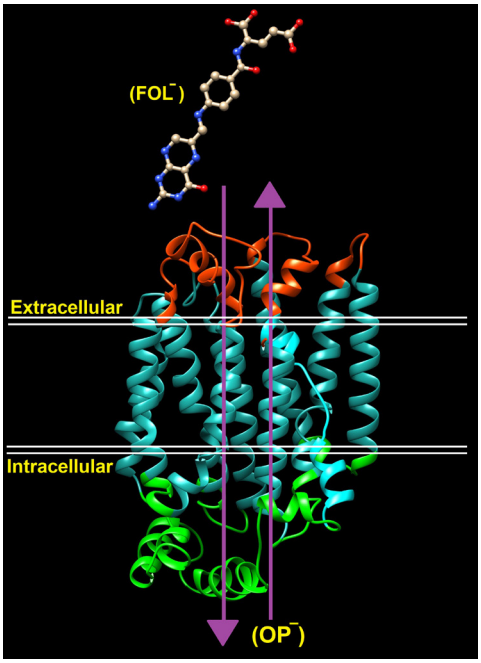

Fig. 4. Overview of folate transporter (RFC (reduced folate carrier/ SLC19A1), organic phos- phate [OP ⁻]).

with nsCL/P in Iranian population. The frequency of *RFC1* AG genotype in cases (67.8%) was nearly 2.5 times more than that of controls (27.4%). The frequency of *RFC1* GG genotype in cases (26.4%) was approximately 6 times more than that of controls (4.3%). The *RFC1* G allele was much higher in the nsCL/P infants (60.3%) compared with control infants (17.9%). These results recommend the possible role of G allele as a risk factor for nsCL/P in the Iranian population with a strong effect.

Chango et al found lower plasma folate and higher homocysteine level in the cases who had GG genotype for *RFC1*[13] and it was an important step for later researches of *RFC1*.

Shaw et al studied the association between *RFC1* (A80G) in Californian population and realized the risk of spina bifida and conotruncal heart defects among infants who had GG genotype of *RFC1* and their mothers did not consume vitamins during pregnancy.[29] These findings demonstrated gene-nutrient relations.

Vieira et al investigated the association of *RFC1* polymorphism with orofacial clefts in Californian population. They found the relation of *RFC1* with cleft lip only.[19] Lakkakula et al in the Indian population evaluated the association between the *RFC1* polymorphism and nsCL/P. Their samples consisted of 142 cases and 141 controls. They concluded that G allele ($P = 0.050$; OR = 1.40, 95% CI: 1.00-1.97) was associated with the nsCL/P.[21] This finding seems to similar to our results based on the population (both Iran and India located at Asia). Therefore, G allele of *RFC1* may be associated with nsCL/P in Asia; nevertheless, more studies should be carried out to address this issue.

Research Highlights

What is current knowledge?

√ Association studies of the RFC1 polymorphism and nsCL/P seem to be a controversial issue.

What is new here?

√ We found a strong association between the RFC1 (A80G) polymorphism and nsCL/P among the Iranian population.

Conclusion

Briefly, our study indicated a strong association between *RFC1* (A80G) polymorphism and nsCL/P in Iranian population. Moreover, AG heterozygosity and GG homozygosity were significant among the Iranian cases. Presence of G allele can be considered as a risk factor for nsCL/P in Iranian infants. By considering the role of *RFC1* in folate pathway and the importance of intracellular folate level, the results are indictable. Because gene-environment interaction and folate's level play important roles in the nsCL/P etiology, further studies with different methods such as intracellular folate measurement should be taken into consideration.

Conflict of interests

All the authors declare that they have no competing interests.

Ethical approval

The survey was approved by Ethics Committee of the Dental Research Center in Shahid Beheshti University for dentofacial deformities (Code No. IR.SBMU.RIDS.REC.1395.195). The survey was also preceded by completing written consent forms by all parents.

Acknowledgments

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