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Original Article

Association of RFC1 A80G Gene Polymorphism with Nonsyndromic Cleft Lip and Palate in Hispanics from Venezuela and Guatemala

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Objective: Investigate association of single nucleotide polymorphism rs1051266 on 80th nucleotide of reduced folate carrier 1 (RFC1) gene with non-syndromic cleft lip with or without cleft palate (nsCL±P).

Materials and Methods: A case-control study design was used for this study. Samples of cases (188 individuals with nsCL±P) and controls (157 individuals) were Hispanics from Venezuela and Guatemala. Genomic DNA was extracted from blood spots on filter paper. RFC1-A80G genotypes were established using polymerase chain reaction and polyacrylamide gel electrophoresis.

Results: Statistically significant higher proportion of mutated homozygotes GG and the lower homozygotes AA were found in cases compared to controls (p=.0234). Also, A and G allele frequencies were significantly different (p=.0088). When each location was evaluated separately the highly significant differences were observed only in Guatemalan but not in Venezuelan subsample.

Conclusions: Our study showed that RFC1-A80G variant may contribute to the etiology of nsCL±P. It revealed differences in RFC1-A80G polymorphism frequencies in populations from different locations. A strong association of RFC1-A80G polymorphism with nsCL±P was observed in the population from Guatemala City, Guatemala only. We have shown that lumping together samples from different populations could hide differences in genotype and allele frequencies in individual populations.

Key Words: RFC1; Reduced folate carrier 1 gene; RFC1-A80G polymorphism; Non-syndromic cleft lip and palate; Case-control study

Introduction

Patients with orofacial clefts - cleft lip (CL), cleft lip and palate (CLP), and cleft palate (CP) - require a multidisciplinary treatment including surgical, pediatric, genetic, orthodontic, speech, hearing, and psychological care from birth to completion of growth.[1] The etiology of non-syndromic orofacial clefts is complex and involves both major and minor genetic influences and environmental factors.[2-8] Environmental factors including maternal smoking, alcohol use, and nutritional deficiencies can be co-triggers for development of nonsyndromic cleft anomalies.[6,9] Current efforts in preventing nsCL±

P include a peri-conceptional supplementation with folic acid.[10-18] Tolarova showed that high dose of folic acid can significantly decrease the risk of recurrence of nsCL±P.[10,11] Since the human body obtains folates entirely from dietary sources, Hall recommended that all women of child-bearing age should receive at least 400 micrograms/day of folic acid to reduce the risk of birth defects.[19] Also, Prescott later suggested a potential therapeutic intervention of high dose folic acid supplementation in mothers with a family history of orofacial clefts, particularly, if they were genetically susceptible to a low folate status.[16]

The primary role of folate in humans is to function as a co-factor in one-carbon metabolism that is needed for synthesis of several amino acids (glycine, serine, methionine), nucleotides (purines and thymidine), and epigenetic regulators required for normal tissue growth. Since the reduced folate carrier 1 (RFC1) gene codes for the reduced folate carrier 1 protein, which mediates transport of active form of folic acid (5-methyltetrahydrofolate) from extracellular fluid into the cell, it has been suspected as one of candidate genes for some birth defects. The 80th nucleotide polymorphism (A80G) in exon 2 of RFC-1 is known to abolish a CfoI restriction site in the gene sequence.[20,21] Although there are other reported roles of RFC1 including absorption across intestinal/colonic epithelia, transport across the basolateral membrane of renal proximal tubules, transplacental transport of folates, and folate transport across the blood-brain barrier, its role in transport of folate across the cell membrane making it available for one-carbon metabolism is considered as its main function.[22] According to several studies, children of mothers who had GG mutated genotype of RFC1-A80G and did not take folic acid supplements showed increased risk for neural tube defects (NTD), congenital heart defects (CHD) and Down syndrome when compared to AA wild-type mothers.[23-26] These findings might represent a modest evidence for a gene-nutrient interaction between infant's RFC1 genotype and periconceptional intake of folic acid supplements that can decrease the risk of NTD and CHD. Although the studies on association between maternal folate intake and risk of nonsyndromic clefts yielded conflicting results, RFC1-A80G has been considered as a possible candidate gene variant involved in etiology of nonsyndromic clefts because of recognized etiological similarities between NTD/CHD and nonsyndromic clefts.[18,27,28]

Since variations of prevalence of orofacial clefts in the same ethnicity reflect differences in presence or absence of certain environmental and genetic factors affecting risk of nonsyndromic clefts in different geographic locations, it was always our interest to determine genotype distributions and allele frequencies of candidate genes.[29,30] Therefore, the purpose of this study was to investigate genotype distributions and allele frequencies of RFC1-A80G single nucleotide polymorphism of nsCL±P patients and controls in Hispanics from Cumana, Venezuela, and Guatemala City, Guatemala.

Materials and Methods

1. Collection of specimens

A case-control study design was chosen for this study. The samples of cases (individuals affected with nsCL±P) and controls (unaffected individuals from the same location and the same hospital with no family history of nsCL±P) came from two locations: Cumana, Venezuela, and Guatemala City, Guatemal. The case sample consisted of 188 individuals affected with nsCL±P (93 from Venezuela and 95 from Guatemala). The control sample consisted of 157 control individuals (81

Table 1. Sample characteristics of cases and controls

		Venezuela		Guatemala		Total	
		n	%	n	%	n	%
nsCL	Unilateral	18	19.4	17	17.9	35	18.6
	Bilateral	3	3.2	1	1.1	4	2.1
	Total	21	25.6	18	19.0	39	20.7
nsCLP	Unilateral	49	52.7	56	58.9	105	55.9
	Bilateral	23	24.7	21	22.1	44	23.4
	Total	72	77.4	77	81.0	149	79.3
Total nsCL±P		93	100.0	95	100.0	188	100.0
Controls		81	100.0	76	100.0	157	100.0

from Venezuela and 76 from Guatemala). From Venezuela, the case sample consisted of 52 males and 41 females and control sample of 21 males and 60 females. From Guatemala, the case sample consisted of 66 males and 29 females and control sample of 20 males and 56 females (Table 1). They were collected during Rotaplast medical missions to Cumana in 2001, 2002, and 2004 and to Guatemala City in 2001, 2002, and 2003.[31] Every individual (parent or legal guardian if cases were minors) participating in this study was informed in detail (in their mother language) about the study and signed Informed Consent Form. Isolation of genomic DNA and analysis for RFC1-A80G polymorphism were done in the Craniofacial Genetics Laboratory, Arthur A. Dugoni School of Dentistry, University of the Pacific, San Francisco, USA.

2. Isolation of DNA

Genomic DNAs of cases and controls were isolated using Chelex-100 molecular biology grade resin (Bio-Rad, Hercules, CA, USA) from dried venous blood spots by the method of Polski.[32] After an individual blood spot was cut into smaller pieces using sterile scissors and dropped into a sterile 1.5 ml plastic tube, 1.0 ml of sterile DNase- and RNase-free water was added and the tube was incubated at room temperature for 30 minutes. During the incubation, the tube was vortexed every 5 minutes. After spinning at 10,000 rpm for 3 minutes, the supernatant was discarded. After addition of 200 μl 1% suspension of Chelex-100 (Bio-Rad), the tube was incubated at 56˚C for 30 minutes. The tube was then vortexed and incubated at 95˚C for 8 minutes. The tube was again vortexed, spun at 10,000 rpm for 3 minutes, and then stored at 4˚C until ready for DNA amplification by Polymerase Chain Reaction (PCR).

3. Polymerase chain reaction (PCR)

A fragment of DNA containing A80G single nucleotide polymorphism of RFC1 (rs1051266) was amplified by PCR. Forward primer sequence (AGCGGTGGAGAAGCAGGT) and reverse primer sequence (TAGGGGGTGATGAAGCTCT) were used for amplification.

For each specimen, 5 μl of isolated DNA solution was added to 45 μl PCR reaction mixture that consisted of 52.5% sterile DNase- and RNase-free water, 20% 5X Taq Master, 10% 10X Taq Buffer, 2% 10 mM dNTP, 2.5% forward primer and 2.5% reverse primer specific for the RFC1 80 gene sequence and 0.5% Taq DNA polymerase (5 units/μl, Eppendorf, Hamburg, Germany). The total of 40 PCR cycles (start at 95˚C for 15 seconds, switch to 61˚C for 30 seconds, and finish at 72˚C for 15 seconds)

were performed using a Mastercycler (Eppendorf, Hamburg, Germany). Following PCR, the presence of amplified DNA in each sample was confirmed using agarose gel electrophoresis.

4. Vertical polyacrylamide gel electrophoresis (PAGE)

Vertical gel electrophoresis apparatus (Fisher Scientific, Pittsburg, PA, USA) was used for detection of single-strand conformational polymorphism patterns[33] characteristic for individual genotypes.

Polyacrylamide gel solution was prepared by mixing 10 mL MDE (Mutation Detection Enhancement) gel monomer solution (Lonza, Inc., Rockland, ME, USA), 2.4 mL 10X TBE buffer, 27.4 mL distilled water, 0.25 mL 10% ammonium persulfate, and 25 μL TEMED. Sample loading buffer was prepared by mixing 2.1 mL formamide, 25 μL M NaoH, 100 μL 0.5 M EDTA, 25 μL 0.02% bromophenol blue/0.02% xylene cyanol dye mixture, and 250 μL distilled water. Three μL of the loading buffer were mixed with 3 μL of each sample's amplified DNA in a 0.2 mL plastic tube. This mixture was heated to 95˚C for 2-3 minutes. Four μL were then loaded into a well in the polyacrylamide gel using a fine tip pipette. A new sterile pipette tip was used for each individual sample. A negative control was run with each batch of samples to confirm absence of foreign DNA contaminants in the amplified DNA. Electrophoresis was carried out at a constant power of 30 W for approximately 4 hours until the bromophenol blue dye band reached approximately 32 cm distance from the start wells. The gel was developed by soaking it first in 0.1% silver nitrate solution for 10 minutes, followed by soaking in a mixture of 1.5% NaOH, 0.01% sodium borohydride, and 0.15% formaldehyde for 20-30 minutes. Finally, the gel was soaked in 0.75% sodium carbonate for 3 minutes and washed in distilled water.

The RFC1 A80G genotypes were determined based on specific configurations of the stained DNA bands (Figure 1). To determine patterns of bands corresponding to RFC1-A80G polymorphism genotypes on the polyacrylamide gel, a complete sequencing of the amplified gene fragment was performed. Once the genotypes were established on the DNA sequence, equivalent patterns were identified on the stained PAGE.

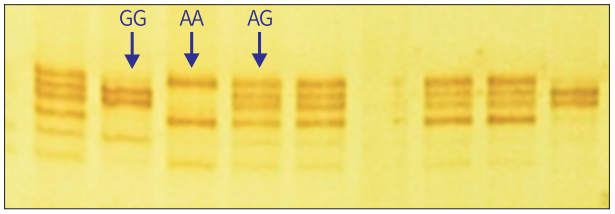

Figure 1. Result of polyacrylamide gel electrophoresis showing patterns characteristic for genotypes of the 80th nucleotide polymorphism (A80G) in reduced folate carrier 1 gene (RFC1). AA means wild-type genotype; AG, heterozygous genotype; GG, mutated genotype.

5. Statistical analysis

Distributions of the homozygous wild-type (AA), heterozygous (AG), and homozygous mutated allele genotypes (GG) and frequencies of A and G alleles for patients with nsCL±P and controls were calculated. Differences between cases and controls and between Cumana and Guatemala samples were compared by Chi-square test.

Results

Proportions of RFC1-A80G genotypes in cases and controls are presented in Table 2.

First, we evaluated samples from both locations together. While in controls almost equal percentages of wild-type AA homozygotes (28.66%) and mutated GG homozygotes (26.12%) were found, the high proportion of mutated homozygotes GG was found in cases (GG 34.21% vs AA 16.84%). These differences in proportions of RFC1-A80G genotypes

Table 2. Proportions of RFC1-A80G genotypes in cases and controls

		AA		AG		GG		Total		Significance
		n	%	n	%	n	%	n	%	
Venezuela	nsCL±P	13	13.98	48	51.61	32	34.41	93	100.00	NS
	Controls	15	18.50	44	54.30	22	27.20	81	100.00	
Guatemala	nsCL±P	19	19.60	45	46.40	33	34.00	97	100.00	χ^2=8.312, p=.01567
	Controls	30	39.50	27	35.50	19	25.00	76	100.00	
Total	nsCL±P	32	16.84	93	48.95	65	34.21	190	100.00	χ^2=7.5096, p=.023405
Total	Controls	45	28.66	71	42.22	41	26.12	157	100.00	

Table 3. Frequencies of G and A alleles of RFC1-A80G gene in cases and controls

		A		G		Total		Significance
		n	%	n	%	n	%	
Venezuela	nsCL±P	74	39.78	112	60.22	186	100.00	NS
	Controls	74	45.68	88	54.32	162	100.00	
Guatemala	nsCL±P	83	42.78	111	57.22	194	100.00	χ^2=7.1235, p=.007608
	Controls	87	57.24	65	42.76	152	100.00	
Total	nsCL±P	157	41.32	223	58.68	380	100.00	χ^2=6.8677, p=.008777
Total	Controls	161	51.27	153	48.73	314	100.00	

between cases and controls were statistically significant (χ^2=7.51, p=.0234).

Evaluation of A and G allele frequencies (Table 3) showed also statistically significant differences when cases (A=0.4132, G=0.5868) and controls (A=0.5127, G=0.4873) were compared (χ^2=6.87, p=.0088).

However, when we evaluated each location separately, we observed in the Venezuelan sample a high proportion of mutated GG homozygotes in cases (AA 13.98%, AG 51.61%, GG 34.41%), but a relatively low proportion of wild-type AA homozygotes in controls (AA 18.5%, AG 54.3%, GG 27.2%). There was found no statistically significant difference in proportions of genotypes between cases and controls in the Venezuelan sample. In contrary, the differences in proportions of RFC1-A80G genotypes in cases (AA 19.6%, AG 46.4%, GG 34.0%) and controls (AA 39.5%, AG 35.5%, GG 25.0%) in the Guatemalan sample were highly statistically significant (χ^2=8.31, p=.0157).

Same as for genotypes, there was a highly significant difference observed in A and G allele frequencies of cases and controls in the sample from Guatemala (χ^2=7.12, p=.0076), but no such difference was observed in A and G allele frequencies in the sample from Venezuela.

Discussion

A relationship between supply of folate in mothers' diet and of folic acid in supplements in the beginning of pregnancy and normal embryonic development has been studied for the last several decades and folate deficiency has been associated with neural tube defects, orofacial clefts, congenital heart defects, and other anomalies.

Several studies reported importance of maternal nutrition, specifically a periconceptional intake of folic acid, on reducing the risk of recurrence of nsCL ±P[10,11] and the risk of first occurrence.[6,12,19]

The same attention was focused on genes involved in the folate pathway (e.g., MTHFR, RFC1) and gene-environment interactions.

While the clinical significance of gene variant association studies, currently, lies mainly in the realm of genetic counseling, the ultimate goal is to better understand etiology of nsCL±P and use this knowledge to develop efficient programs for cleft prevention.[34,35] With an increasing number of couples seeking genetic counseling as part of their family planning, knowledge of how specific genes contribute to nsCL±P becomes very important. Determining the RFC1-A80G genotypes of prospective parents may contribute to an overall picture of their risk of having a child with nsCL±P, which is particularly useful to those who have already had a child affected with this condition. If we can show that a specific gene mutation may increase the risk of recurrence of nsCL±P, preventive measures can be targeted towards those individuals who are at the highest risk.

The primary goal of our study was to investigate the association of RFC1-A80G polymorphism with occurrence of nsCL±P in populations of Cumana, Venezuela, and Guatemala City, Guatemala.

Our analysis showed that RFC1-A80G polymorphism was associated with nsCL±P in the sample of Hispanics affected with CL or CLP from Cumana, Venezuela and Guatemala City, Guatemala. Thus, mutation A->G in RFC1 gene on nucleotide 80 may play a role in etiology of nsCL±P in the studied population. Comparison of proportions of genotypes AA, AG, and GG between cases and controls show the higher proportion of mutated homozygotes GG in cases compared to controls (Table 2) and this difference was statistically significant (χ^2=7.51, p=.0234). Also G allele frequency (Table 3) was found higher in cases (0.5868) compared to controls (0.4873) and this difference was also statistically significant (χ^2=6.87, p=.0088).

There is no doubt that larger sample size has many advantages from the statistical point of view. However, we would like to show in this paper the disadvantage of lumping together samples from relatively similar locations. When total sample was analyzed, significant differences in proportions of genotypes as well as in allele frequencies were found, pointing to etiological role of mutation A->G of RFC1 on 80th nucleotide. However, when subsamples of Venezuela and Guatemala were evaluated separately, the statistically significant difference was found only in the subsample from Guatemala.

The good approach, when combining subsamples, is to find out whether they are similar or different in respect to studied variables. When we compared case subsamples, there was no statistically significant difference neither in proportion of genotypes nor in alleles frequencies.

However, when control subsamples were compared, they differed in allele frequencies (χ^2=4.19, p=.0406) and also in genotype proportions (χ^2=9.14, p=.0104). We have no explanation for this difference in controls. However, if we carefully interpret results from our study, we can conclude, that mutation A->G of RFC1 on 80th nucleotide may have a stronger effect in the Guatemalan population.

Although genotype distributions and allele frequencies did not show significant differences between cases and controls in the Venezuelan subsample, it cannot be assumed that presence of the G allele at the 80th nucleotide of RFC1 gene is not associated with risk for nsCL±P in this population, because percentage of GG genotype (34.41% vs. 27.2%) and frequency of G allele (0.6022 vs 0.5432) were much higher in cases than in controls (Tables 2 and 3). However, most likely the effect will not be so strong as in Guatemala.

According to several studies about association of RFC1-A80G with congenital birth defects, Shaw suggested that use of peri-conceptional vitamins containing folic acid could neutralize the increased risk of spina bifida induced by GG homozygosity.[23] A similar association may exist between RFC1-A80G genotype, maternal peri-conceptional intake of folic acid, and occurrence of congenital heart defects.[24,25] Also, in a case-control study by Soghani, results indicated a strong association between RFC1 (A80G) polymorphism and nsCL±P in Iranian population. The frequency of RFC1 AG and GG genotypes in cases was nearly 2.5 and 6 times higher than in controls, respectively.[36] Accordingly, Girardi suggested a role of RFC1 in NONSYNDROMIC CLEFTS etiology in Italian population while Kumari introduced RFC1 80G as a risk factor and RFC1 80A as protective against MTHFR 677T risk allele.[37,38] Other similar studies also showed the suggestive or significant association between RFC1 A80G variant and the risk of nonsyndromic clefts in families from Philippines, South India and China.[39-41] Therefore, the genes involved in the development of the craniofacial structures and other folate pathway genes can be potential candidate genes for nonsyndromic clefts.[27] However, in a later study, Vieira insisted that variation in RFC1 was over-transmitted to patients with cleft lip only carrying mitochondrial DNA haplotypes other than haplotype D. Therefore, they suggested that further studies are needed to investigate the association between nonsyndromic clefts and RFC1 based on more discrete phenotypes.[28]

On the other hand, several other studies reported that there was no evidence of association between nonsyndromic clefts and the RFC1 A80G polymorphism in the Californian population, South American families, Chinese, Brazilian and Polish, respectively.[24,25,27,42,43]

The relationship between RFC1 and other candidate genes such as MTFHR, MSX1, and TGF-B3 may be important in the etiology of nonsyndromic clefts. Since MTHFR catalyzes the rate-limiting step of folic acid biosynthesis and MTHFR-C677T is one of the common folate-related polymorphisms associated with thermolabile MTHFR, Chango studied the plasma folate levels in relation to RFC1-A80G and MTHFR-C677T genotypes. Although there was no significant difference in the plasma folate levels between RFC1-80AA and 80GG when studied alone, individuals who had RFC1-80AA and MTHFR-677CT simultaneously showed higher plasma folate levels than those who had RFC1-80GG and MTHFR-677CT simultaneously.[20]

Each one of these studies, including our present study, contributed to our knowledge, and understanding of the role of RFC1 gene variants in etiology of orofacial clefts. The RFC1 and MTHFR as "folate related" genes are just one piece in the puzzle of genetic background of multifactorial etiology of nonsyndromic clefts.

Conclusion

Our study showed that RFC1-A80G polymorphism might contribute to the etiology of nsCL±P and revealed, that there exist differences in RFC1-A80G variant frequencies in populations of the same ethnicity but different locations. Specifically, while very strong association of RFC1-A80G polymorphism with nsCL±P was observed in Guatemala City, Guatemala, population, it failed to support the same relationship in Cumana, Venezuela, population. Thus, there is always a risk of buried differences that may exist in genetic as well as in environmental factors that play a role in etiology of nsCL±P that should be considered if samples from different populations are combined.

We are learning more clearly in these days that "one size does not fit all" and that different genetic and different environmental factors play role in etiology of nonsyndromic clefts in different populations.[35]

Further studies will be needed to investigate the association between nsCL±P phenotype, RFC1 variants, and polymorphisms of other candidate genes.

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