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Interferon Regulatory Factor 6 (IRF6) and Gene – Environment Interactions in Non-Syndromic Orofacial Cleft Cases in Saudi Arabia-A Case Control Study

Najlaa M Alamoudi[1*], Heba J Sabbagh[1,2], Nicola P Innes[2], Sherif Edris Ahmed[3], Azeez Butali[4], Eman Abdulbaset Alnamnakani[5], Sari Rabah[6], Mustafa A Hamdan[7], Nasir H Alhamlan[8], Fatma D Abdulhameed[9], Najat M Farsi[1], Ali H Hassan[10] and Peter A Mossey[11]

[1]*Pediatric Dentistry, King Abdulaziz University, Jeddah, Saudi Arabia*
[2]*Paediatric Dentistry, University of Dundee Dental School, Dundee, Scotland, UK*
[3]*Princess Al-Jawhara Al-Brahim Centre of Excellence in Research of Hereditary Disorders, Jeddah, Saudi Arabia*
[4]*Department of Oral Pathology, College of Dentistry, Radiology and Medicine/ Dows Institute for Dental Research, University of Iowa, USA*
[5]*Riyadh Military Hospital, Prince Sultan Military Medical City, Riyadh, Saudi Arabia*
[6]*Surgery Department, King Abdullah bin Abdulaziz University Hospital, Princess Noura University and King Saud bin Abdulaziz University for Health Sciences, Riyadh, Saudi Arabia*
[7]*Plastic Surgery Department, King Saud Medical City, Ministry of Health, Riyadh, Saudi Arabia*
[8]*King Saud bin Abdulaziz University for Health Sciences, Riyadh, Saudi Arabia*
[9]*Pediatric Surgery Department, Madina Maternity and Children's Hospital, Saudi Arabia*
[10]*Orthodontic Department, King Abdulaziz University, Jeddah, Saudi Arabia*
[11]*Division of Oral Health Sciences and WHO Collaborating Centre for Oral Health and Craniofacial*

Abstract

The association between interferon regulatory factor 6 (*IRF6*) and nonsyndromic orofacial cleft (NSOFC) is affected by ethnicity. Also, gene-environment interactions (GEI) may play an important role in its etiology.

Objectives: This case-control study investigated whether *IRF6* gene variants were associated with NSOFC in Saudi Arabian population and whether the gene was affected by maternal environmental exposures.

Methods: We extracted DNA from saliva samples obtained from 171 infant–parent triad cases and 189 matched controls (age, gender, and location) from January 2010–December 2011; this study included a total of 11 referral hospitals in Saudi Arabia. *IRF6* (rs2013162, rs2235375, and rs2235371) polymorphisms were genotyped using restriction-digestion polymerase chain reaction. Data on environmental exposures, for GEI analyses, were collected through questionnaire-led interviews with parents.

Results: We found statistically significant over transmission of the common *IRF6* rs2013162 allele among cleft lip with or without palate CL(P) cases. No associations were found for either of the other two *IRF6* SNPs. Maternal exposure to antipyretics, folic acid, fever, antibiotics, illnesses, common cold/flu, paternal water pipe smoking, stress, x-rays, and/or chemicals could significantly interact with the maternal *IRF6* (rs2013162 and rs2235375) gene variants, affecting the likelihood of having an offspring with NSOFC.

Conclusion: The common allele at *IRF6* rs2013162 was significantly over transmitted among CL(P) cases. This study provides hypotheses for future investigations into genetic and environmental factors and their interaction in the etiology of NSOFC.

Keywords: Interferone regulatory factor 6; Nonsyndromic orofacial cleft; Cleft lip and palate; Gene-environment interactions; aetiology; Cleft lip with or without palate

List of abbreviations: NSOFC: Nonsyndromic Orofacial Cleft; CL(P): Cleft Lip with or without Palate; CP: Isolated Cleft Palate; *IRF6*: Interferon Regulatory Factor 6; GEIs: Gene-Environment Interactions; PCR: Polymerase Chain Reaction; TDT: Transmission Disequilibrium Test; FBAT: Family-Based Association Test

Introduction

Mutations in interferon regulatory factor 6 (*IRF6*), located on 1q32.2, are responsible for the two autosomal dominant orofacial cleft syndromes, Van Der Woude and popliteal pterygium syndrome [1-3]. It was also the first identified nonsyndromic orofacial cleft (NSOFC) susceptibility locus [4] and has been the only candidate gene consistently found to have a significant association with NSOFC across multiple studies in many regions of the world, for example, China and Europe [5-7].

Blanton et al. [8] confirmed that the association between SNPs at *IRF6* and NSOFC varied between ethnic groups and that there was a need for further evaluation of *IRF6* variations across populations to better determine its role in NSOFC [8].

Gene-environment interactions (GEIs) have been suggested to play an important role in the etiology of NSOFC. Gene-environment interactions are defined as the co-participation of genetic and environmental risk factors in the same causal mechanism to promote disease development [9]. One of the important applications of GEI

***Corresponding author:** Najlaa Alamoudi, Faculty of Dentistry, Pediatric Dentistry, King Abdulaziz University, Jeddah, Saudi Arabia, Tel: 6401000 (22026-20388-22264); E-mail: nalamoudi@kau.edu.sa

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studies is to help public health researchers develop strategies for targeted intervention to allow risk-factor modification based on individual genetic profiles [10].

This study investigated whether (a) *IRF6* was also associated with cleft lip with or without palate (CL(P)) and isolated cleft palate (CP) in Saudi Arabia and (b) there was any association between *IRF6* polymorphisms (rs2013162, rs2235375, and rs2235371) and maternal environmental exposures, especially folic acid supplementation in the etiology of NSOFC.

Methods

Patients

This paper is a part of a series of studies aimed at determining the prevalence and investigating the etiology of NSOFC in Saudi Arabia. Three papers on the prevalence and environmental risk factors related to NSOFC have been published [11-13].

We obtained samples from 11 referral hospitals in three main cities in Saudi Arabia: Riyadh: King Saud Medical City Hospital, King Fahad Medical Cities, Riyadh Armed Hospital, and Riyadh National Guard Hospital; Jeddah: King Fahad Hospital, Abdulaziz University Hospital, Al-Messadia Maternity Hospital, Al-Azizia Maternity Hospital, King Abdulaziz Medical City, and King Fahad Armed Forces Hospital; and Medina: Medina Children and Maternity Hospital. The inclusion criteria for this study population included non-syndromic cleft lip with or without cleft palate or cleft palate children who were 18 months or younger and had been admitted to neonatal, plastic surgery, or orthodontic units, while the controls were healthy, unaffected, age-and gender-matched children recruited from the vaccination clinic. A total of 171 cases and 189 controls (infant–parent triads) were recruited for the study during January 2010–December 31, 2011. Ethical approvals were obtained from King Abdulaziz University Hospital (359-10, 2010), the Ministry of Health (10-079, 2010), and the Institutional Research Review Boards (IRBs) of the military hospitals (429/2011). Parents consented to participate in the study before sample collection.

Methods

Saliva was sampled from both parents and infants using Oragene (500) (for adults) and (575) (for infants). DNA extraction was carried out using the QIAamp DNA Mini Kit (catalog# 51306; Qiagen, Canada) according to the manufacturer's protocol, with modification of the starting quantity of saliva (500 instead of 200 µl). The purity and quantity of extracted DNA were measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Delaware, USA). The DNA quality was also evaluated using agarose gel electrophoresis (1% in 1× TBE buffer).

We used this DNA for subsequent amplification with restriction digestion polymerase chain reaction (PCR), TaqMan® Genotyper Software, and TaqMan® SNP Genotyping Assays. Three polymorphisms at *IRF6* were analyzed: rs2013162, rs2235375, and rs2235371.

To assess GEIs in the etiology of NSOFC, we hypothesized that environmental factors and maternal exposures strengthen or diminish the effect of maternal rare alleles, which in turn affect the risk of having an infant with NSOFC. These factors included maternal medication and supplementation, maternal diseases, maternal stress and parental smoking; we obtained this information through parental interviews. The type of oral cleft was not sub-grouped to CL/P and CP, as the number of cases was not large enough to subdivide or stratify them. Two study designs were used to measure GEI: case-only and case-control study designs.

Statistical analysis

A transmission disequilibrium test (TDT) was measured using the family-based association test (FBAT) and PLINK. PLINK was also used to measure the effect of parents of origin. We compared genotype and allele frequencies among CL(P) and CP cases with those for controls using the chi square test and adjustment with Bonferroni correlation ($P \leq 0.05$) using SPSS. The degree of association between genotype and allele frequencies with NSOFC was estimated by measuring the odds ratio (OR) and respective 95% confidence intervals (95% CI) using an online program found at http://www.quantpsy.org/chisq/chisq.htm.

To assess GEIs, two study designs were used: case-only and case-control. In the case-only study design, interactions between maternal SNPs (genotypes and allele) and environmental factors were analyzed by measuring the distribution of maternal genotypes and alleles according to exposure/no-exposure to environmental factors among oral cleft cases. The common allele and null maternal exposure were set as references in our calculation. For the type of drinking water source, tap water with the common allele was set as the reference. Analysis was carried out using the chi square test with SPSS version 16 (SPSS Inc., Chicago, IL, USA), and a P-value of less than 0.05 was considered to indicate significance. Their degrees of association with NSOFC were estimated by measuring the OR and respective 95% CIs. If one cell contained a 0 value, only the P-value was calculated. No analyses were carried out if more than one cell contained a 0 value.

Multi-nominal logistic regression analysis was carried out using SPSS to overcome confounding factors. Factors that showed significant GEIs when analyzed alone in the previous analysis were entered in the logistic regression.

For the case-control design, we compared differences between the frequency of maternal genotypes (homozygous common allele, homozygous rare allele, and heterozygous allele) for different environmental factors using a chi-square test; we adjusted the P-value by Bonferroni correction methods between cases and controls using SPSS. We determined ORs and 95% CIs for factors with P-values ≤ 0.05 by using MedCalc (user-friendly statistical software). For the type of drinking water source, tap water with the common allele was set as a reference. If one cell contained a 0 value, only the P-value was calculated. No analyses were carried out if more than one cell contained a 0 value.

Results

From the 171 cases and 189 controls, 10 NSOFC cases were not examined for sub-phenotype classification, and 16 control fathers refused to provide saliva samples. In addition, genotyping values were not obtained for some samples. This resulted in 120 CL(P) and 33 CP paternal genotypes compared to 158 controls, 127 CL(P) and 34 CP maternal genotypes compared to 185 controls, and 123 CL(P) and 32 CP infant genotypes compared to 178 controls, according to *IRF6* rs2013162 genotype variance. For rs2235375, 122 CL(P) and 33 CP paternal genotypes compared to 169 controls, 127 CL(P) and 34 CP maternal genotypes compared to 189 controls, and 126 CL(P) and 35 CP infant genotypes compared to 188 controls were analyzed. For *IRF6* rs2235371 genotype variance, 120 CL/P and 33 CP paternal genotype compared to 170 controls, 126 CL/P and 33 CP maternal genotypes compared to 189 controls, and 124 CL/P and 35 CP infant genotypes compared to 187 controls were analysed.

Almost all cases (99.4%) and controls (98.8%) were homozygous at *IRF6* rs2235371 for the common CC allele. As the number of rare alleles was negligible TDT using FBAT and PLINK analyses were not carried out for this polymorphism.

Statistically significant over-transmission of the common C allele in CL(P) cases (P= 0.018) was found using FBAT and PLINK analyses. PLINK testing also showed significant reduction of CL(P) risk with the *IRF6* rs2013162 rare A allele (P= 0.018, OR: 0.644) (Table 1). The *IRF6* variant was over-transmitted from the paternal side but this finding was not statistically significant (P= 0.06), (Table 2).

There were no significant differences between cases and controls in the paternal *IRF6* rs2013162 genotype (P= 0.08 for CL(P), P= 0.92 for CP), infant *IRF6* rs2013162 genotype (P= 0.18 for CL(P) and P= 0.32 for CP), and maternal CP *IRF6* rs2013162 genotype (P= 0.187) (Table 3). However, there were significant differences in maternal *IRF6* rs2013162 genotype for CL(P) (P= 0.024) in cases compared to controls.

After chi-square adjustment and Bonferroni correlation were carried out, it was found that the maternal homozygous AA rare allele genotype was significantly more prevalent in controls than in CL(P) (P<0.05). Furthermore, the heterozygous CA genotype was significantly more prevalent in cases than in controls. *IRF6* rs2235375 variants did not show any association with both CL(P) and CP infant–parent triads following either FBAT, PLINK or chi-square testing in the Saudi population.

Gene-environment interactions

The maternal *IRF6* rs2013162 rare homozygous allele genotype (AA) and heterozygous genotype (CA) were compared to the common homozygous allele genotype (CC) in NSOFC cases for environmental factors (Table 4). Mothers who had a homozygous rare allele genotype (AA) and, during their first trimester, had used antipyretics (P= 0.012, OR: 9, 95% CI: 1.62–49.91), experienced pain (P=0.013, OR: 6.57; 95% CI: 1.49–29.01), suffered from depression (P=0.048, OR: 8.11; 95% CI: 1.01–64.84) or who, during the pregestation period, had taken folic acid supplements (P=0.047, OR: 8.22, 95% CI: 1.03– 65.72), were more likely to have an offspring with NSOFC.

The maternal *IRF6* rs2013162 rare A allele was compared to the common C allele in NSOFC cases in relation to different environmental factors (Table 5). Mothers who had a rare A allele and were using antipyretics in the first trimester (OR: 2.34; 95% CI: 1.12–4.9) or folic acid supplements during the pregestation period (OR: 2.57 and 95% CI: 1.23–5.37) were significantly more likely to have infants with NSOFC.

Type of NSOFC	Allele	afreq	fam#	P-value
***IRF6* rs2013162**				
CL(P)	**C**	0.696	79	0.018**
	A	0.304	79	0.018**
CP	**C**	0.623	21	0.564
	A	0.377	21	0.564
***IRF6* rs2235375**				
CL(P)	C*	0.634	69	0.441
	G	0.366	69	0.441
CP	C*	0.636	20	0.577
	G	0.364	20	0.577

Note: **Significant relationship P≤0.05; afreq: Estimating allele frequencies; fam#: Number of families

Table 1: Transmission Disequilibrium test (TDT) results for *IRF6* rs2013162 and rs2235375 variants among NSOFC infant-parental triads and its sub-phenotypes (CL(P) and CP) using FBAT analysis.

NSOFC	Transmitted/ Untransmitted minor allele	P-value	OR	A:U_PAR	P-value	Combined statistics P-value
***IRF6* rs2013162**						
CL(P)	47/73	0.018**	0.644	1:02	0.564	0.015**
CP	12/15	0.564	0.8	00:00	NA	0.257
***IRF6* rs2235375**						
CL(P)	50/58	0.441	0.862	00:00	NA	0.441
CP	16/13	0.578	1.23	00:00	NA	0.257
Parent of origin effect						
	T:U_PAT	Paternal P-value	T:U_MAT	Maternal P-value	POO Z	POO P
***IRF6* rs2013162**						
CL(P)	24.5:39.5	0.061	22.5:33.5	0.142	-0.212	0.832
CP	05:07	0.564	07:08	0.796	-0.26	0.795
***IRF6* rs2235375**						
CL(P)	50/58	0.441	0.862	00:00	NA	0.441
CP	16/13	0.578	1.23	00:00	NA	0.257

Note: A:U PAR: Parental discordance count; POO: Parents of Origin; T:U PAT: Paternal transmitted: un-transmitted counts; T:U MAT: Maternal transmitted: untransmitted counts; **Significant relationship P ≤ 0.05

Table 2: Testing *IRF6* rs2013162 and rs2235375 alleles for transmission disequilibrium and parent of origin using PLINK analysis for NSOFC infant-parental triads and its sub-phenotypes (CL(P) and CP).

Mothers who had a rare A allele and experienced fever during the 3-month pregestation period (OR: 0.28, 95% CI: 0.09–0.81), underwent X-ray (OR: 0.19, 95% CI: 0.07–0.5) or ingested iron (OR: 0.57, 95% CI: 0.33–0.97) during the first trimester, or who drank tap water as their main drinking water source rather than bottled water (P= 0.043, OR: 0.51, 95% CI: 0.26–0.98), were significantly less likely to have infants with NSOFC.

Mothers who had a rare homozygous allele genotype (AA) for rs2235375, were using folic acid supplements during the pregestation period (P=0.045, OR: 0.23, 95% CI: 0.05–0.97), or complained of stress (P=0.016, OR: 0.3, 95% CI: 0.1–0.8) were significantly less likely to have infants with NSOFC.

For the case-control study approach for the three maternal *IRF6* rs2013162 genotypes (Supplementary table S1), statistically significant case and control difference in the maternal homozygous rare allele genotype (AA) frequency for mothers using folic acid supplementation was found. Significantly more control mothers used folic acid supplements in the first trimester compared to mothers with NSOFC.

We found significantly higher maternal homozygous common allele genotype (CC) rs2235375 frequencies in cases compared to controls in maternal illness in the pre-gestation and first trimester periods, flu/common cold infection in the pre-gestation period, fever in the pre-gestation period, and for maternal folic acid supplementation.

Multinominal logistic regression analysis was carried out to identify significant GEI among NSOFC cases. The homozygous common allele genotype for each SNP included was set as a reference for analysis (Table 6). For maternal rs2013162, the homozygous rare allele genotype (AA) was significantly related to antipyretics in the first trimester (P=0.027, OR: 10.18, 95% CI: 1.31–79.1) and abdominal pain in the first trimester (P=0.031, OR: 7.4, 95% CI: 1.2–45.51) among NSOFC cases. The heterozygous allele genotype (AC) was significantly related to pre-gestation folic acid supplementation (P=0.0167, OR: 6.78, 95% CI: 1.41–33.49) and occurrence of fever pre-gestation (P=0.025, OR: 0.23 and 95% CI: 0.06–0.83) among NSOFC cases.

Discussion

We found a significant over transmission of the common C allele

SNP	Genotype	**CL(P)** (%)	**CP** (%)	**Control** (%)
Paternal *IRF6* rs2013162	CC*	51 (42.5)	16 (48.5)	81 (51.3)
	AA	4 (3.3)	2 (6.1)	11 (7)
	CA	65 (54.2)	15 (45.5)	66 (41.8)
	Total	120	33 (100)	158(100)
	P-value	0.08	0.92	
Maternal *IRF6* rs2013162	CC*	62 (48.8)	14 (41.2)	94 (50.8)
	AA	8 (6.3)	3 (8.8)	28 (15.1)
	CA	57 (44.9)	17 (50)	63 (34.1)
	Total	127 (100)	34 (100)	185 (100)
	P-value	0.024**	0.19	
Infant *IRF6* rs2013162	CC*	67 (54.5)	13 (40.6)	95 (53.4)
	AA	6 (4.9)	3 (9.4)	19 (10.7)
	CA	50 (40.7)	16 (50)	64 (36)
	Total	123 (100)	32 (100)	178 (100)
	P-value	0.18	0.32	
Paternal *IRF6* rs2235375	CC*	16 (13.1)	3 (9.1)	19 (11.2)
	GG	45 (36.9)	11 (33.3)	82 (48.5)
	CG	61 (50)	19 (57.6)	68 (40.2)
	Total	122 (100)	33 (100)	169 (100)
	P-value	0.14	0.18	
Maternal *IRF6* rs2235375	CC*	25 (19.7)	8 (23.5)	41 (21.7)
	GG	54 (42.5)	11 (32.4)	84 (44.4)
	CG	48 (37.8)	15 (44.1)	64 (33.9)
	Total	127 (100)	34 (100)	189 (100)
	P-value	0.76	0.39	
Infant *IRF6* rs2235375	CC*	26 (20.6)	7 (20)	38 (20.2)
	GG	57 (45.2)	13 (37.1)	83 (44.1)
	CG	43 (34.1)	15 (42.9)	67 (35.6)
	Total	126 (100)	35 (100)	188 (100)
	P-value	0.96	0.69	

Note: * The common homozygous allele genotype; **The Chi-square statistic is significant at the 0.05 level; [C] Ten NSOFC cases was not sub-phenotyped

Table 3: Distribution of *IRF6* rs2013162 and rs2235375 infant-parental triad genotypes according to NSOFC phenotypes (CL(P) and CP) and compared to controls. There were missing samples; 23 (8 cases and 15 controls) paternal samples, four maternal control samples and 17 infant samples (6 cases and 11 controls). The phenotype diagnosis of ten NSOFC cases are missing.

at *IRF6* rs2013162 among CL(P) cases and suggest that GEIs may be associated with *IRF6* polymorphisms in the Saudi Arabian population.

The first SNP selected was *IRF6* rs2013162. FBAT showed significant over-transmission of the common C allele in CL(P) cases (P=0.018). In addition, PLINK analysis showed a protective effect and an association between the rare A allele and CL(P) (P=0.018 and OR: 0.644 for CL(P)). A similar finding was reported by Scapoli et al. [14], who detected an over-transmission of the common allele for rs2013162 (P=0.004) and all haplotypes carrying these common alleles among 219 Italian CL(P) trios. This is also similar to the finding reported by Park et al. [15] and Blanton et al. [16]. In addition, the rs2013162 rare allele exhibited a genome-wide significant relationship with CL(P) in a study by Beaty et al. [17]. Stratification of the sample population using Asian ancestry as a factor yielded stronger evidence of association with *IRF6* rs62013162 [17].

In our study, PLINK analysis showed transmission of the *IRF6* rs2013162 variants from the paternal side (P=0.06). Although the relationship was not significant, it agrees with the review by Anderson et al. [18] on male-mediated developmental toxicity; it was suggested mutated DNA were more frequently inherited from fathers than mothers. Brinkworth [19] suggested that the paternal genetic inherence effect occurs through genomic instability or apoptosis suppression of the germ cell. In addition, our study found that maternal gene variants showed significant differences between cases and controls, with the more heterozygous allele genotype in CL(P) cases and the more homozygous rare allele genotype in controls (P=0.024). This suggests a maternal rare allele effect that was not previously described. Ludwig et al. [20], in their Weinberg's log-linear model analysis on *IRF6* gene variants in Central European patients, found no difference in the risk for CL(P) between maternal- and paternal-derived alleles.

The other two *IRF6* SNPs that were analyzed in this study were rs2235375 and rs2235371. Huang et al. [21] in Western China reported an association between the transmission of rs2235375 and rs2235371 (C/T) markers and CL(P). However, our research did not indicate any association between the transmission of rs2235375 and NSOFC. This agrees with a case-control study in a Mexican population where there was no significant difference between CL(P) cases and controls in the frequency of rs2235375 (P=0.08) [22]. Also a case-control triad study by Zhou et al. [23], in a Chinese population, found no significant association between rs2235371 and CL(P) using FBAT analysis.

As the frequency of the rs2235371 rare allele was very low in our study, it was not possible to carry out any analysis. The Ensemble Genome project report stated that the prevalence of the rare T allele in rs2235371 was calculated to be only 2% in a European population compared to 41% in an Asian population [24]. Similarly, in their four-population case-parent trios, Park et al. [15] found that the rare allele frequency of rs2235371 in European Americans was too low to be reported.

Our study did not find any relationship between *IRF6* and CP. This finding is supported by other studies that investigated the relationship between NSOFC and *IRF6* and included CP cases in their sample [6,14,21].

Gene environment interaction

Only a few studies have considered the role of GEIs in the etiology of NSOFC. The factors that were studied were mainly folic acid and vitamin supplements, smoking, and maternal passive smoking. Furthermore, only a small number of gene variants were analyzed [25-28]. In our study, we focused on the interaction between environmental factors and maternal genes to assess any direct maternal effect occurring during pregnancy.

One of the concerns in GEI studies is the study power. False-positive and false-negative outcomes were reported in studies with small sizes [29]. To improve the reliability of our results, two study designs and three statistical analyses were carried out for GEI. Consistency of association between the findings of different study designs in GEI analyses reinforces the potential significance of these factors.

The environmental risk factor that showed a significant interaction with maternal *IRF6* rs2013162 in both study designs and statistical approaches was maternal folic acid supplementation during the pregestation period. This significant association concurs with a population based case-control study in Northern Netherlands that reported "duration of exposure-response effect" increased the risk of cleft lip [30].

In contrast, in the case-control study design, maternal folic acid in the first trimester were significantly more ingested by the controls with homozygous rare AA allele genotype mothers than cases (P= 0.003), indicating a joint protective effect.

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Maternal rs2013162				
Environmental factors		**CC* (%)**	**AA (%)**	**CA (%)**
Antipyretic medication 1st trimester X^2=7.67, df=2, P= 0.022** **N=162**	Yes	4 (5.3)	3 (33.3)	9 (11.7)
	No	72 (94.7)	6 (66.7)	68 (88.3)
	P value OR (95% CI)		0.012** 9 (1.62-49.91)	0.164 2.38(0.7-8.1)
Fever pre-gestation X^2=7.37, df=2, P= 0.025** **N=163**	Yes	13 (17.3)	0 (0)	4 (5.2)
	No	62 (82.7)	11 (100)	73 (94.8)
	P value OR (95% CI)		0.277 a	0.025** 0.26 (0.08-0.84)
exposure to X-ray 1st trimester X^2=1.22, df=2, P= 0.544 **N=145**	Yes	23 (31.1)	1 (9.1)	3 (4.9)
	No	46 (62.2)	10 (90.9)	62 (95.4)
	P value OR (95% CI)		0.136 0.2 (0.02-1.66)	<0.001** 0.09 (0.03-0.34)
Folic acid pre-gestation X^2=8.24, df=2,P= 0.016** **N=164**	Yes	2 (2.6)	2 (18.2)	12 (15.6)
	No	74 (97.4)	9 (81.8)	65 (84.4)
	P value OR (95% CI)		0.047** 8.22 (1.03-65.72)	0.014** 6.83 (1.47-31.66)
Depression 1st trimester X^2=5.68, df=2, P= 0.059 **N=163**	Yes	2 (2.7)	2 (18.2)	3 (3.9)
	No	73 (97.3)	9 (81.8)	74(96.1)
	P value OR (95% CI)		0.048** 8.11 (1.01-64.84)	0.673 1.48 (0.24-9.12)
Abdominal pain 1st trimester X^2=7.86, df=2, P= 0.02** **N=162**	Yes	6 (8.0)	4 (36.4)	8 (10.5)
	No	69 (92.0)	7 (63.6)	68 (89.5)
	P value OR (95% CI)		0.013** 6.57 (1.49-29.01)	0.594 1.35 (0.45-4.1)
Maternal *IRF6* rs2235375				
Environmental factors		**CC*** (%)	**GG (%)**	**CG (%)**
Folic acid pre-gestation X^2=4.43, df=2, P= 0.109 **N=164**	Yes	6 (17.6)	3 (4.6)	7 (10.8)
	No	28 (82.4)	62 (95.4)	58 (89.2)
	P value OR (95% CI)		0.045** 0.23 (0.05-0.97)	0.34 0.56 (0.17-1.83)
Mother complains of being under stress X^2=0.69, df=2, P= 0.71 **N=147**	Yes	17 (50)	12 (41.5)	29 (46)
	No	17 (50)	38 (58.5)	34 (54)
	P value OR (95% CI)		0.016** 0.3 (0.1-0.8)	0.709 0.85 (0.37- 1.97)

Note: *Homozygous common allele genotype; **The Chi-square statistic is significant at the 0.05 level; could not analyze because the groups contained zero values

Table 4: Relationship between maternal *IRF6* (rs2013162 and rs2235375) genotypes and different environmental factors, including: maternal illness, medication, stress, supplements, paternal smoking and maternal domestic exposure using case-only study design.

Maternal *IRF6* rs2013162			
Environmental factors		**C* (%)**	**A (%)**
Antipyretic medication 1st trimester **N=324**	Yes	17 (7.4)	15 (15.8)
	No	212 (92.6)	80 (84.2)
	OR (95% CI)	2.34 (1.12-4.9) **	
Fever pre-gestation **N=326**	Yes	30 (13.2)	4 (4)
	No	197 (86.8)	95 (96)
	OR (95% CI)	0.28 (0.09-0.81) **	
Folic acid pre-gestation **N=328**	Yes	16 (7)	16 (16.2)
	No	213 (93)	83 (83.8)
	OR (95% CI)	2.57 (1.23-5.37) **	
Iron 1st trimester **N=326**	Yes	79 (34.8)	23 (23.2)
	No	148 (65.2)	76 (76.8)
	OR (95% CI)	0.57 (0.33-0.97) **	
Maternal exposure to X-ray 1st trimester **N=290**	Yes	49 (24.1)	5 (5.8)
	No	154 (75.9)	82 (94.2)
	OR (95% CI)	0.19 (0.07-0.5) **	

Type of maternal drinking water **N=290**	Tap (reference)	58 (27.9)	14 (17.1)
	Bottle	137 (65.9)	65 (79.3)
	OR (95% CI)	0.51 (0.26-0.98) **	
	Well	13 (6.2)	3 (3.6)
	OR (95% CI)	1.05 (0.26-4.18)	
	Zamzam	0	0
	OR (95% CI)	a	
Maternal IRF6 rs2235375			
Environmental factors		**C* (%)**	**G (%)**
Mother complains of being under stress **N=294**	Yes	63 (48.1)	53 (32.5)
	No	68 (51.9)	110 (67.5)
	OR (95% CI)	0.52 (0.32-0.84) **	
Maternal exposure to X-ray 1st trimester **N=211**	Yes	3 (2.3)	9 (4.6)
	No	13(97.7)	186 (95.4)
	OR (95% CI)	0.21 (0.05-0.87) **	
Consanguinity **N=314**	Yes	66 (50)	112 (61.5)
	No	66 (50)	70 (38.5)
	OR (95% CI)	41.6 (1.02-2.52) **	

Note: *Common allele; **Significant level P≤ 0.05; [a] Not possible to analyse because the groups contain zero values

Table 5: Relationship between maternal *IRF6* (rs2013162 and rs2235375) allele frequency and different environmental factors, including: maternal illness, medication, stress, supplements, paternal smoking and maternal domestic exposure using case-only study design.

Environmental factors	**P value OR 95% (CI)**	
Maternal rs2013162[b]		
	AA	**CA**
Antipyretic medication 1st trimester	0.027** 10.18 (1.31-79.1)	0.132 2.74 (0.74 -10.13)
Folic acid pre-gestation	0.351 3.83 (0.23-64.42)	0.017** 6.87 (1.41-33.49)
Maternal exposure to X-ray 1st trimester	a	0.947 1.07 (0.16-7.01)
Fever pre-gestation	a	0.025** 0.23 (0.06-0.83)
Depression in the 1st trimester	0.274 6.22 (0.24-164.39)	0.56 1.96 (0.2-18.98)
Abdominal pain 1st trimester	0.031** 7.4 (1.2-45.51)	0.61 1.36 (0.42-4.38)
Maternal *IRF6* rs2235375[b]		
Environmental factors	**GG**	**CG**
Folic acid pre-gestation	0.051 0.23 (0.05-1)	0.385 0.59 (0.18-1.94)
Mother complains of being under stress	0.508 0.75 (0.32-1.75)	0.76 0.88 (0.38-2.03)

Note: ** Significant value P≤ 0.05. a. No value either because the parameter is set to zero or the value is redundant. b. The reference category is the common homozygous allele genotype CC

Table 6: Multi-nominal logistic regression analysis for case-only study design gene-environmental interaction including significant factors.

Other environmental risk factors were either significant in the case-only study design or in the case-control study designs. In the case-only study design, antipyretic medication and fever showed GEI in both analyses (genotypes and allele analysis). This highlights the importance of future studies to verify the influence of maternal disease symptoms on the function of maternal genes and their effect on the embryonic development.

Moreover, in the case-control study, mothers who were homozygous for the common allele (CC), and who consumed antibiotics during the pregestation period or were exposed to illness and common cold/flu were significantly more likely to have an infant with NSOFC. In addition, paternal waterpipe smoking associated with maternal common allele (CC) is more likely prone to have an infant with NSOFC. This indicates a synergic effect between environmental risk factors and the gene variant that when isolated were found to be associated with an increased risk of oral clefts.

Maternal stress was an environmental risk factor that showed a significant interaction with maternal *IRF6* rs2235375 in both study designs and all GEI statistical approaches. However, the pregestational use of folic acid supplementation and presence of maternal *IRF6* rs2235375 were found to be associated with NSOFC in the case-only study design. This finding was not supported by the Velázquez-Aragón et al. [22] Mexican study. However, these differences may be related to differences in the study setting.

In addition, although association with the multivitamin supplementation interaction with *IRF6* rs2235375 was significant in the case-control analysis (P= 0.026) it was not supported by the Wu et al. [28] population-based study that was carried out in China. In addition,

the number of subjects exposed to multivitamins in our study was small and a significant relationship was only found in one analysis and in the pregestation period.

To draw conclusions regarding definitive GEIs, we need to overcome the limitation of the study sample size. In addition, a GWAS study using a log-linear modeling approach is necessary. However, achieving this number is difficult, particularly because NSOFC is a rare disease and sub-phenotyping is necessary. In addition, there are multiple risk factors that contribute to these diseases [9,30].

There is a great value in preliminary studies that may be underpowered for definitive findings, but that act as instruments for preliminary description of GEI and for generating hypotheses that can then be further tested in studies with adequate power [29]. Accordingly, we expect that our findings will play a role in directing future research to identify possible gene-environment risk factors that may allow prevention of NSOFC through public health strategies.

Conclusion

The differences between our findings and those of previous studies could indicate that the Saudi population has a different genetic etiology of NSOFC compared to other populations worldwide. TDT and PLINK analyses showed that the *IRF6* rs2013162 rare A allele was not a marker for CL(P) risk as the common C allele showed significant transmission in CL(P) cases. Maternal exposure to antipyretics, folic acid, fever, antibiotics, illnesses, common cold/flu paternal waterpipe smoking, stress, and x-rays and/or chemicals could significantly interact with the maternal *IRF6* (rs2013162 and rs2235375) gene variants, affecting the risk of having a child with oral cleft. Because of the genetic heterogeneity of OFC and the evidence for population specific genetic etiology, adequately powered GWAS studies combined with further investigation of environmental factors and GEI are recommended.

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