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Association between IRF6 rs642961 Polymorphism and Nonsyndromic Cleft Lip with or without Cleft Palate Risk in an Iranian Population

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ABSTRACT

Background: The extent of the contribution of rs642961 polymorphism of interferon regulatory factor-6 (IRF6) gene with susceptibility to the syndromic cleft lip with or without cleft palate (NSCL/P) in the Iranian patients is still unknown. Thus, to test the role of IRF6 in NSCL/P susceptibility in an Iranian population, we performed a population based case-control stud.

Methods: One-hundred ten patients with NSCL/P and 110 matched healthy subjects were recruited to this population-based study. The IRF6 rs642961 polymorphism was genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay.

Results: Participants did not differ significantly by age and gender ($P > 0.05$). The AA, AG, and GG genotypes frequencies of the IRF6 rs642961 polymorphism in the NSCL/P cases were 27.3%, 53.6% and 19.1%, respectively while the corresponding frequencies in the healthy subjects were 34.5, 56.4% and 9.1%, respectively. There was a significant association between homozygote mutant genotype (GG) of IRF6 rs642961 polymorphism and increased risk of NSCL/P (OR = 2.360, 95% CI 1.055-5.280, $P = 0.037$).

Conclusion: the current study suggested that IRF6 rs642961 polymorphism might be associated with susceptibility to NSCL/P in an Iranian population. However, well-designed epidemiological studies with larger sample size are needed to further validate our results.

Introduction

Nonsyndromic cleft lip with or without cleft palate (NSCL/P) (MIM 119530) is a common birth defect which based on clinical manifestation divided into cleft lip only (CLO), cleft palate only (CPO), and cleft lip and palate (CLP).^{1,2} NSCL/P incidence is approximately ranging from 1 in 500 to 1 in 2000 live births worldwide, which varied by race or ethnicity. The etiology of NSCL/P is not exactly understood.^{3,4} It is a complex malformation occurred by interaction of genetic and environmental factors in the etiology of this disease occurred by interaction of genetic and environmental factors.⁵⁻⁸ However, influence of genetic factors in implication of NSCL/P may vary by ethnicity. The most systemic environmental risk factors for NSCL/P are maternal exposure to tobacco smoking, alcohol consumption, malnutrition, medicinal drugs, viral infections and environmental pollution.⁹

It was hypothesized that several genes including MTHFR, MSX1, DLX3, TGFB1, TGFB2, TGFB3, BCL3, RARA, and PAX9 in development of NSCL/P have implicated in development of NSCL/P.^{2, 10-13} The interferon regulatory factor-6 (IRF6) gene plays an important role in development of the maxillofacial region. IRF6 mutations may produce a nonfunctional protein leading to haplo-insufficiency, affecting the DNA-binding domain, causing a dominant negative effect, and resulting in severe phenotypes such as Van der Woude syndrome (VWS) (OMIM 119300) and NSCL/P.^{14,15} VWS is a rare autosomal dominant developmental malformation with cardinal signs of CL/P, bilateral midline lower lip pits, cleft lip, and/or cleft palate only (CPO) with dental anomalies and pitted lips.^{16,17}

The human IRF6 gene is located on chromosome 1q32.3-q41 (OMIM#607199) and comprises nine exons. To date, several polymorphisms such as rs2236909, rs2236908, rs2236907, rs2235375, rs2235373, rs2235371 and rs642961 within

IRF6 gene have been suggested to be associated with increased risks to the development of NSCL/P by epidemiological studies. The IRF6 rs642961 polymorphism is located in enhancer region of IRF6 gene.¹⁸ The association of the IRF6 rs642961 polymorphism with susceptibility to NSCL/P has been extensively explored in different populations, but the results of these studies are inconsistent and there are a few studies in the Iranian population. We, therefore, conducted a population based case control study to evaluate the association between IRF6 rs642961 polymorphism and NSCL/P risk in an Iranian population.

Materials and Methods

Study Population: The study protocols were approved by the Institutional Ethics Committee of the Kashan University of Medical Science. Informed consent was obtained from parents of participants prior to any data collection. The patients were recruited who were referred to the Department of Orthodontics, Kashan University of Medical Science, Kashan, Iran. A total of 110 sporadic cases of NSCL/P cases and 110 matched (age and sex) healthy controls were consecutively enrolled in this study between April 2016 and March 2017. Excluding criteria were as follow: family history of orofacial clefts or craniofacial anomalies, dental anomalies, congenital anomalies, learning disabilities, attention deficits, hearing impairment and speech deficits.

DNA Isolation and Genotyping: Genomic DNA was extracted from whole blood collected in EDTA from cases and controls using a commercial DNA Blood Mini Kits (QIAGEN Ltd, Germany) and stored at -20°C until used. The quality of DNA samples was assessed by performing the 0.8% agarose gel electrophoresis. We genotyped the IRF6 rs642961 polymorphism using PCR-RFLP using the following primers: forward: 5'-TGCCAGCTACTCAGCTTGGTTCAT-3' and reverse: 5'-

ATAGAGCATGCTGCCTTCTTCCCA-3'. PCR-RFLP assays were designed for IRF6 rs642961 polymorphism using the NCBI dbSNP database and the free online tools Primer3 (version 0.4.0). PCR was in 25 µL containing 10 µM each primer, 10 µM each dNTPs, 50 µM MgCl₂, 1.5 units Taq polymerase (Invitrogen), and 20 ng/µL genomic DNA. PCR conditions were 94°C for 6 minutes, followed by 35 cycles of 94°C for 30 seconds, 65°C for 30 seconds and 72°C for 1 minute, with a final extension at 72°C for 9 minutes. PCR products were digested with 5 U of BstNI restriction enzyme and then digest products were separated on 3% agarose gel containing 0.5 µg/mL ethidium bromide using gel electrophoresis separation system, and then the gels visualized under UV light on a trans-illuminator. The presence of an A allele divided 276 bp amplified product into unique 246 bp and 30 bp, while the G allele another restriction site which caused 276 bp amplified product was digested into two 213 and 33 bp smaller fragments (Figure 1).

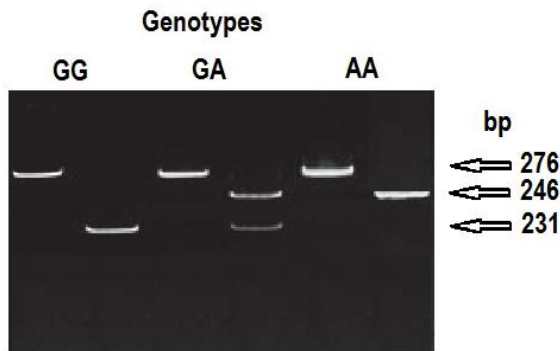


Figure 1. Scheme of electrophoresis of the PCR-RFLP assay for genotyping of IRF6 rs642961 polymorphism

Statistical Analysis: A raw genotyping data for PCR-RFLP assays was input into Excel software of Microsoft Office 2013. Hardy-Weinberg equilibrium (HWE) was tested for IRF6 rs642961 polymorphism among healthy subjects using chi-square test. The genotype and allele frequencies of IRF6 rs642961 polymorphism were compared between NSCL/P cases and the healthy

subjects by the chi-square test. The odds ratio (OR) and 95% confidence interval (CI) were calculated from CMA software for strength of association between IRF6 rs642961 polymorphism and NSCL/P risk. All statistical analysis of data was performed by the Statistical Package for the Social Sciences (SPSS) software package version 19.0 (SPSS, Inc., Chicago, IL, USA), in which a $P < 0.05$ was taken as the level for significance.

Results

Clinical characteristics of patients and controls are presented in Table 1. The mean age of cases and controls at recruitment was 7.41 ± 4.31 and 8.11 ± 6.31 years, respectively. Participants did not differ significantly by age and sex ($P > 0.05$), indicating that matching was appropriate.

Table 1. Characteristics of the NSCL/P cases and controls

	Cases (n = 110)	Control (n = 110)	P
Age (± SD)	7.41 ± 4.31	8.11 ± 6.31	0.631
Gender			
Male	64 (58.2)	60 (54.5)	0.587
Female	46 (41.8)	50 (45.5)	

The frequency and association of IRF6 rs642961 polymorphism with NSCL/P risk are shown in Table 2. The distributions of genotype in the control group for IRF6 rs642961 polymorphism was in agreement with Hardy-Weinberg equilibrium ($P = 0.059$). The AA, AG, and GG genotypes frequencies of the IRF6 rs642961 polymorphism in the NSCL/P cases were 27.3%, 53.6% and 19.1%, respectively while the corresponding frequencies in the healthy subjects were 34.5%, 56.4% and 9.1%, respectively. Moreover, the frequencies of wild (A) and mutant (G) alleles of the IRF6 rs642961 polymorphism in the NSCL/P cases were 54.1% and 45.9%, respectively, while the corresponding frequencies in the healthy subjects were 62.7% and 37.3%, respectively (Table 2).

Table. 2 Genotype frequencies of IRF6 rs642961 polymorphism between NSCL/P and controls.

	Cases (n = 110)	Control (n = 110)	OR (95% CI)	P
Genotype				
AA	30 (27.3)	38 (34.5)	Ref.	
AG	59 (53.6)	62 (56.4)	0.896 (0.526-1.524)	0.684
GG	21 (19.1)	10 (9.1)	2.360 (1.055-5.280)	0.037
Allele				
A	119 (54.1)	138 (62.7)	0.700 (0.478-1.025)	0.066
G	101 (45.9)	82 (37.3)	1.428 (0.976-2.090)	0.056
Genetic Models				
Dominant (GG+AG vs. AA)			1.407 (0.792-2.501)	0.244
Recessive (GG vs. AG+AA)			2.360 (1.055-5.280)	0.037

OR: Odds Ratio; CI: Confidence Interval.

Overall, there is a significant association between GG genotype of IRF6 rs642961 polymorphism and NSCL/P risk (OR = 2.360, 95% CI 1.055-5.280, P = 0.037). However, the AA and AG genotypes were not significantly associated with increased risk of NSCL/P (P < 0.05). We also observed a significantly increased risk of NSCL/P risk with IRF6 rs642961 polymorphism under recessive genetic model (GG vs. AG+AA; OR = 2.360; 95% CI, 1.055-5.280; P = 0.037), but not under the dominant genetic model (Table 2).

Discussion

To date, numerous studies have evaluated role of IRF6 rs642961 polymorphism in pathogenesis of NSCL/P. However, the biological mechanisms of IRF6 rs642961 polymorphism in pathogenesis of NSCL/P remain unclear.^{1,19} Thus, we have performed this research based case-control study in an Iranian population to further investigate the correlation between IRF6 rs642961 polymorphism and NSCL/P susceptibility.

In this case-control study we have found that IRF6 rs642961 polymorphism is significantly associated with susceptibility to NSCL/P in an Iranian population. Previously, Kerameddin et al., have observed a significant overrepresentation of a tagSNP haplotype carrying mutant allele of rs642961 polymorphism in the complete bilateral CL/P (most severe sub-phenotype of CL/P). But, they have not found significant association between rs642961 polymorphism and

NSCL/P in an Iranian population.²⁰ Moreover, Brito et al., in a study have detected a significant association between rs642961 and CLO risk in a Brazilian population, but not with risk of NSCL/P.²¹ Nouri et al., in a family-based study found that IRF6 rs642961 not significantly associated with increased risk of NSCL/P among Iranian population.²² According to the Kerameddin et al., Brito et al., and Nouri et al., studies results rs642961 polymorphism more pronounced among CLO than in CLP patients.²⁰⁻²²

Our findings are consistent with previous case-control studies in a sample of northern Chinese²³ and Mexican²⁴ populations. Birnbaum et al., and Rahimov et al., have found that rs642961 was associated with NSCL/P in a Central European population.^{25, 26} Recently, Lee et al., in a meta-analysis of 8 case-control studies with 1,899 cases and 3,458 controls found that IRF6 rs642961 polymorphism was significantly associated with NSCL/P risk in Asian, but not in Caucasians. Our findings supports that the etiologic SNP rs642961 in the upstream enhancer of IRF6 gene play an important role in the formation and maintenance of the periderm and spatiotemporal regulation of appropriate palatal adhesion in early stages of fetus development. Moreover, these findings confirmed that Asians with the IRF6 rs642961 polymorphism had a higher susceptibility NSCL/P than others. However, Paranaiba et al., in a case-control study among 128 patients with NSCL/P and

126 healthy subjects have found that IRF6 rs642961 polymorphism was not associated with NSCL/P pathogenesis in a Brazilian population.

CLP and CLO have been regarded as sub-phenotype of NSCL/P, which differed only by severity. According to the previous epidemiological studies in different ethnicities and current study the IRF6 rs642961 polymorphism might be play different etiologies in development of CLO and NSCL/P. Moreover, CLO and NSCL/P co-occurrence within families is not uncommon, which in turn favors a common etiology for them, which is indicating the possible etiology of CLO might be genetically more homogenous than NSCL/P.²¹

In summary, our results suggested that the IRF6 rs642961 polymorphism might be associated with susceptibility to NSCL/P in the Iranian population. Our findings might be useful in molecular diagnoses and treatment of patients with NSCL/P. More well-designed studies with a larger sample size are warranted to further confirm our findings.

Conclusion

Our results suggested that the IRF6 rs642961 polymorphism might be associated with susceptibility to NSCL/P in the Iranian population. Our findings might be useful in molecular diagnoses and treatment of patients with NSCL/P. More well-designed studies with a larger sample size are warranted to further confirm our findings.

Conflict of Interests

Authors have no conflict of interests.

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