

Association of rs2013162 and rs2235375 Polymorphisms in *IRF6* Gene with Susceptibility to Non-Syndromic Cleft Lip and Palate

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Abstract

Background: Non-syndromic cleft lip occurs by the interaction of environmental and genetic factors. The purpose of the current study was to analyze the association of Single Nucleotide Polymorphisms (SNPs) in *IRF6* and NSCL/P in an Iranian population.

Methods: A group of 105 children with NSCL/P and 185 normal controls were included in the current study. Genotyping of *IRF6* rs2013162 and rs2235375 was performed by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method.

Results: A substantial association of AA and CA genotypes in rs2013162 with the risk of NSCL/P (AA vs. CC; OR=2.36; 95%CI [1.05-5.29], p=0.004; and CA vs. CC; OR=0.47; 95%CI [0.28-0.79], p=0.018) was found. However, there were no important associations between A allele and risk of NSCL/P (p=0.980). According to logistic regression analysis results, subjects with GG genotype and G allele in rs2235375 polymorphism had increased risk of NSCL/P.

Conclusion: The *IRF6* polymorphisms are associated with the susceptibility to NSCL/P in Iranian population.

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Keywords: *IRF6*, Non-syndromic cleft lip and palate, Orofacial clefts, Polymorphism

Introduction

Non-Syndromic Cleft Lip with or without Cleft Palate (NSCL/P) is one of the most common congenital craniofacial abnormalities which is caused by genetic factors alone or by a combination of genetic and environmental factors¹.

Contribution of genetic factors to the development of cleft lip with or without cleft palate could be proved by the fact that familial recurrence risk is higher than the risk of recurrence in general population². Regarding the recent advances in genotyping and sequencing methods, studies have shown a large number of Single Nucleotide Polymorphisms (SNPs) loci affecting susceptibility to NSCL/P³. SNPs are the most common allelic variations of the genome with the frequency of more than 1%⁴.

Among the susceptibility loci identified by Genome-Wide Association Studies (GWAS), the 8q24

region represents the strongest association in different populations of European ancestry, with consistent replications⁵. Although many susceptibility loci have been suggested by linkage and candidate gene association studies, the vast majority were non replicable across studies⁶. A remarkable exception was *IRF6* (interferon regulatory factor 6), in which heterozygous loss-of-function mutations lead to van der Woude syndrome and their association with NSCL/P was confirmed in several GWAS⁷. The *IRF6* gene on chromosome 1q32.3-q41 encodes interferon regulatory factor 6, which is a key element in oral and maxillofacial problems and is one of the candidate genes associated with both syndromic and non-syndromic forms of clefts⁸. Large studies in different populations have provided the evidence that *IRF6* is an important genetic factor in the etiology of NSCL/P⁹. The aim of this

study was to evaluate the association between the *IRF6* rs2013162 and rs2235375 SNPs with the risk of NSCL/P in an Iranian population.

Materials and Methods

Subjects

This case-control study was performed to determine the potential role of *IRF6* rs2013162 and rs2235375 SNPs in developing NSCL/P in an Iranian population. A sample of 105 newborns with NSCL/P and 185 controls without cleft palate were included. A clinical examination was performed to look for dysmorphic features such as lip pits. Cases were excluded from the study if there was evidence of other facial or skeletal malformations, metabolic or neurologic disorders or anomalies of other organ systems. Samples were recruited from Mofid Hospital, a pediatric referral center in Tehran, Iran in 2013-2015. Ethical approval for the study was obtained from the ethics committee of the School of Dentistry, Shahid Beheshti University of Medical Sciences (IR.SBMU.RIDS.REC.1395.195). Informed consent was obtained from all parents in accordance with the Declaration of Helsinki¹⁰.

Genotyping of the candidate SNPs

Genomic DNA was extracted by salting out procedure from 5 ml of peripheral blood samples. The genotyping of the subjects for *IRF6* SNPs was performed by PCR and Restriction Fragment Length Polymorphism (RFLP) methods. The primer sequences and related product sizes are shown in table 1. The PCR amplification was carried out in a reaction mixture containing 10×PCR buffer, 1.5 mM MgCl₂, 1 U Taq DNA poly-

merase (CinnaGen, Iran), 0.2 mM of dNTPs, 5 pmol of each primer, 30 ng template DNA, and sterile distilled water up to 25 μl. Amplification was performed with an initial denaturation step at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, annealing at 60 °C for 40 s and extension at 72 °C for 40 s, and a final extension at 72 °C for 5 min. The *IRF6* rs2013162 minor allele (A) was cut to two fragments of 107 bp and 67 bp, while the C allele (wild type; wt) remained uncut. In addition, the *IRF6* rs2235375 minor allele (G) remained uncut, while the C allele (wt) was cut into two fragments of 137 bp and 26 bp. The PCR products of *IRF6* rs2013162 and rs2235375 SNPs were digested with the restriction enzymes of DdeI and BsrBI at 37 °C overnight, respectively (Figure 1). DNA fragments were subjected to 10% polyacrylamide gel electrophoresis and stained with silver nitrate. The results were confirmed by direct Sanger sequencing (Figure 2).

Statistical analysis

The Chi square (χ^2) using the SPSS software V 20 (IBM, USA) was performed to compare genotype and allele frequencies in the study groups. $p < 0.05$ were considered statistically significant.

Results

Descriptive analysis showed that in the case group, 49.5% (52/105) and 50.5% (53/105) of the subjects were boys and girls, respectively. In the control group, 54.0% (100/185) and 46.0% (85/185) were boys and girls, respectively. The distribution of sex was not significantly different between the case and control groups ($p = 0.457$). The genotype distribution of rs-

Table 1. Primer sequences used for the genotyping of the *IRF6* polymorphisms

SNPs	Primer sequence (5'→3')	Product size (bp)	Restriction enzyme	Digestion pattern (bp)
rs2013162	CACCTGCGAGCTTGTGTATC CCATCATCCCCACTCACCAT	174	DdeI	C (Wild)=174 A (Variant)=107+67
rs2235375	AAGTAAAGTGAGACTTTATCTTTC TCCCTGGTGACTCATGGGCT	163	BsrBI	C (Wild)=137+26 G (Variant)=163

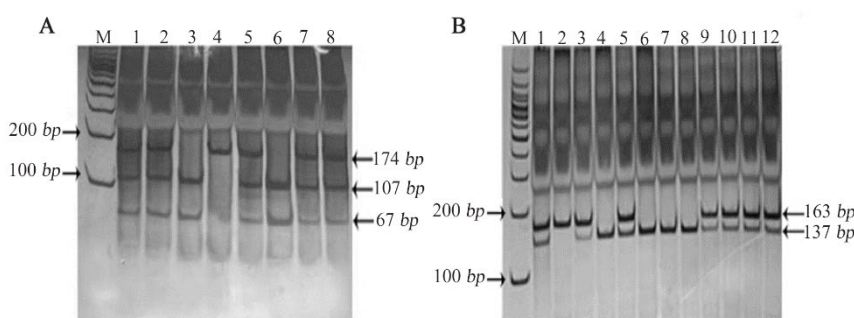


Figure 1. Representative gel pictures of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) results. A) The rs2013162 C/A polymorphism PCR-RFLP result. Lane M; Ladder 100 bp, No. 1,2,5,7 and 8; heterozygote (CA), No. 3; homozygote (AA), No. 4; homozygote (CC), B) The rs2235375 G/A polymorphism PCR-RFLP result. Lane M; Ladder 100 bp, No. 1,3,5,9,10,11 and 12; heterozygote (CG), No. 2; homozygote (GG), No. 4,6,7 and 8; homozygote (CC).

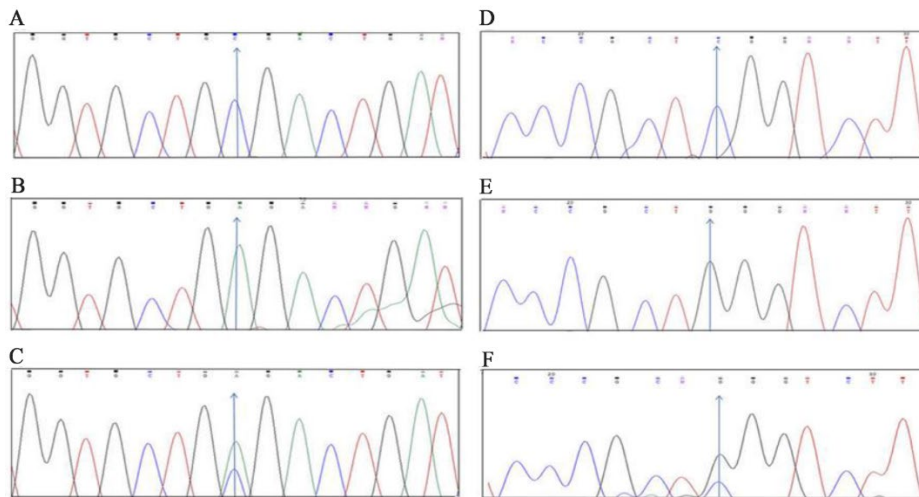


Figure 2. Sequencing results. Polymorphism rs2013162. A) CC homozygote (wild), B) AA homozygote (variant), C) CA heterozygote. Polymorphism rs2235375, D) CC homozygote (wild), E) GG homozygote (variant), F) CG heterozygote.

2013162 and rs2235375 polymorphisms in case and control groups was in agreement with the one predicted via Hardy–Weinberg equilibrium (HWE).

Our results showed that the frequency of the *IRF6* rs2013162 CA genotype was significantly lower in the cases (36.3%) than the control (59.3%) group ($p=0.004$; OR=0.47; 95% CI=0.28-0.79). On the other hand, the *IRF6* rs2013162 AA ($p=0.018$; OR=2.36; 95% CI=1.05-5.29) was significantly higher in the NSCL/P group (14.7%) compared to the healthy children (3.3%). In addition, the *IRF6* rs2235375 GG ($p=0.040$; OR=2.36; 95%CI=1.05-5.29) was significantly higher in the NSCL/P group (14.7%) compared

to the healthy children (7.6%) and frequency of the *IRF6* rs2235375 G allele in the case group (35.3%) was significantly (0.040) higher than the controls (27.0%) with the odds ratio of 1.47 (95% CI:1.02-2.13). The results are shown in tables 2 and 3.

Discussion

In this study, the association of *IRF6* common SNPs with the risk of NSCL/P in an Iranian population was evaluated. First, it was shown that the distributions of genotype in the control group for both the *IRF6* rs2013162 and rs2235375 polymorphisms were in HWE. Both *IRF6* SNPs, rs2013162 and rs2235375,

Table 2. Genotype and allele frequencies of *IRF6* rs2013162 SNP in cleft lip/palate and control groups

Genotype/allele	Control (n=182)	Case (n=102)	p-value	OR (95% CI)
CC	68 (37.4)	50 (49.0%)		Reference
CA	108 (59.3%)	37 (36.3%)	0.004	0.47 (0.28-0.79)
AA	6 (3.3%)	15 (14.7%)	0.018	2.36 (1.05-5.29)
C	244 (67.0%)	137 (67.2%)		Reference
A	120 (33.0%)	67 (32.8%)	0.980	0.99 (0.69-1.43)

Table 3. Genotype and allele frequencies of *IRF6* rs2235375 SNP in cleft lip/palate and control groups

Genotype/allele	Control (n=185)	Case (n=105)	p-value	OR (95% CI)
CC	99 (53.5%)	45 (44.1%)		Reference
GC	72 (38.9%)	42 (41.2%)	0.350	1.29 (0.76-2.16)
GG	14 (7.6%)	15 (14.7%)	0.040	2.36 (1.05-5.29)
C	270 (73.0%)	132 (64.7%)		Reference
G	100 (27.0%)	72 (35.3%)	0.040	1.47 (1.02-2.13)

were associated with the development of NSCL/P. This study provides additional confirmatory evidence for contribution of the *IRF6* in the etiology of NSCL/P. Regarding the physiological function of *IRF6*, it has been shown that *IRF6* was expressed in the medial edge epithelia of the fusing region of secondary palatal shelves¹¹. In another study, analysis of an isolated chick cDNA revealed that *IRF6* levels were elevated in the epithelia covering the frontonasal process, the maxillary primordium, and nasal pits. *IRF6* expression was also detected in the ectoderm of the leading edges of the developing palatal shelves and in the ridges of the primitive oral cavity¹². Bezerra *et al* revealed that rs2235371 in this gene is correlated with increased risk of non-syndromic orofacial clefts in Brazilian population¹³. On the other hand, with sequencing of *IRF6* in 100 Non-Syndromic Unilateral Cleft Lip and Palate (NSUCLP) patients, ten new and rare missense variations were identified, among which four variations were potentially deleterious¹⁴. The common polymorphisms in *IRF6* account for up to 12% of the total incidences of NSCL/P, which indicates that this gene is strongly associated with orofacial clefting¹⁵. Huang *et al* found strong evidence of over- and under-transmission of the rs2235375 C allele in cleft case-parent trios¹⁶. There were significant differences in the genotype and allele frequencies of the rs2235375 in the cases and control infants¹⁶. Analysis of five *IRF6* SNPs in Mexican population revealed that the rs2235375 had the strongest association with over-transmission of the G allele with CL/P in population¹⁷. *IRF6* rs2235375 variant has also been significantly associated with increased risk of NSCL/P in co-dominant, dominant (OR=1.2), and allelic models (OR=1.4) in Indian population¹⁸. Furthermore, a new study in Chilean population indicated that C allele of rs2235375 seems to be a risk factor for NSCL/P¹⁹. In a recent study by Xu *et al* there was a significant difference in both genotypic and allelic distributions between patients and controls at rs2013162. In addition, case-parent analysis revealed over-transmission of the rs2013162A allele²⁰. In another Chinese population, rs2013162 showed a significant association with NSCL/P²¹. However, another study on Mexican patients revealed that rs2235375 and rs2013162 in *IRF6* gene were not associated with NSCL/P²².

Conclusion

In conclusion, the results of the current study indicated that two *IRF6* SNPs, rs2235375 and rs2013162, were associated with the NSCL/P in Iranian population.

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Conflict of Interest

The authors declare no conflict of interest.

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