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

Masoumeh Soleymani 1, Asghar Ebadifar 2, Maryam Khosravi 1, Emran Esmaeilzadeh 3, and Hamid Reza Khorram Khorshid 1,4

1. Genetics Research Center, University of Social Welfare and Rehabilitation Sciences, Tehran, Iran
2. Dentofacial Deformities Research Center, Research Institute of Dental Sciences, Department of Orthodontics, Faculty of Dentistry, Shahid Beheshti University of Medical Sciences, Tehran, Iran
3. Fetal Health Research Center, Hope Generation Foundation, Tehran, Iran
4. Personalized Medicine and Genometabolomics Research Center, Hope Generation Foundation, Tehran, Iran

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.

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 factors 1.

Contribution of genetic factors to the development of cleft lip with or without cleft palate could be proved by the fact that familial recurrence rate is higher than the risk of recurrence in general population 2. 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/P 3. 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 replications 5. Although many susceptibility loci have been suggested by linkage and candidate gene association studies, the vast majority were non replicable across studies 6. 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 GWAS 7. 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 clefts 8. Large studies in different populations have provided the evidence that IRF6 is an important genetic factor in the etiology of NSCL/P 9. The aim of this
study was to evaluate the association between the \textit{IRF6} rs2013162 and rs2235375 SNPs with the risk of NSCL/P in an Iranian population.

\textbf{Materials and Methods}

\textit{Subjects}

This case-control study was performed to determine the potential role of \textit{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 Helsinki. 

\textit{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 \textit{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 MgCl\textsubscript{2}, 1 U Taq DNA polymerase (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 \textit{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 \textit{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 \textit{IRF6} rs2013162 and rs2235375 SNPs were digested with the restriction enzymes of Ddel 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).

\textit{Statistical analysis}

The Chi square ($\chi^2$) 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.

\textbf{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-
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,
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 shelves. In another study, analysis of an isolated chick cDNA revealed that IRF6 levels were elevated in the epithelia covering the frontonasal process, the maxillary primordial, 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 cavity. Bezerra et al. revealed that rs2235371 in this gene is correlated with increased risk of non-syndromic orofacial clefts in Brazilian population. 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 deleterious. 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 clefting.

Huang et al. found strong evidence of over- and under-transmission of the rs2235375 C allele in cleft case-parent trios. There were significant differences in the genotype and allele frequencies of the rs2235375 in the cases and control infants. Analysis of five IRF6 SNPs in Mexican population revealed that the rs2235375 had the strongest association with over-transmission of the allele CL/P in population. 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 population. Furthermore, a new study in Chilean population indicated that C allele of rs2235375 seems to be a risk factor for NSCL/P. 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 allele. In another Chinese population, rs2013162 showed a significant association with NSCL/P. However, another study on Mexican patients revealed that rs2235375 and rs2013162 in IRF6 gene were not associated with NSCL/P.

**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.

**Acknowledgement**

We would like to thank the patients who participated in this study.

**Conflict of Interest**

The authors declare no conflict of interest.

**References**

1. Mossey PA, Little J, Munger RG, Dixon MJ, Shaw WC. Cleft lip and palate. The Lancet 2009;374(9703):1773-85.
2. Aldhoree KA, Böhmer AC, Ludwig KU, Esmail AHA, Al-Hebshi NN, Lippke B, et al. Non-syndromic cleft lip with or without cleft palate in arab populations: Genetic analysis of 15 risk loci in a novel case–control sample recruited in Yemen. Birth Defects Res A Clin Mol Teratol 2014;100(4):307-13.
3. Yang J, Yu X, Zhu G, Wang R, Lou S, Zhu W, et al. Integrating GWAS and eQTL to predict genes and pathways for non-syndromic cleft lip with or without palate. Oral Dis 2021;27(7):1747-54.
4. Hassani M, Dehani M, Rafie MZ, Esmaeilzadeh E, Davar S, Pakzad B, et al. Investigation of rs531564 polymorphism in the primary microRNA-124 gene in patients with systemic lupus erythematosus and rheumatoid arthritis: association with disease susceptibility and clinical characteristics. Iran J Allergy Asthma Immunol 2021 Jun 6;20(3):303-13.
5. Ishorst N, Francheschelli P, Böhmer AC, Khan MFJ, Heilmann-Heimbach S, Fricker N, et al. Non-syndromic cleft palate: an association study at GWAS candidate loci in a multiethnic sample. Birth Defects Res 2018;110(10):871-82.
6. Leslie EJ, Marazita ML, editors. Genetics of cleft lip and palate. Am J Med Genet C Semin Med Genet 2013;163C(4):246-58.
7. Mukhopadhyay N, Feingold E, Moreno-Uribé L, Webby G, Valencia-Ramirez LC, Muñeton CPR, et al. Genomewide association study of non-syndromic orofacial clefts in a multiethnic sample of families and controls identifies novel regions. Front Cell Dev Biol 2021;9:621482.
8. Wattanawong K, Rattanasiri S, McEvoy M, Atia J, Thakkinstian A. Association between IRF6 and 8q24 polymorphisms and nonsyndromic cleft lip with or without cleft palate: Systematic review and meta-analysis. Birth Defects Res A Clin Mol Teratol 2016;106(9):773-88.
9. Martinelli M, Palmieri A, Carinci F, Scapoli L. Non-syndromic cleft palate: an overview on human genetic and environmental risk factors. Front Cell Dev Biol 2020;8:592271.
10. Goodyear MD, Krleza-Jeric K, Lemmens T. The declaration of Helsinki. British Medical Journal Publishing Group; 2007.
11. Kondo S, Schutte BC, Richardson RJ, Bjork BC, Knight AS, Watanabe Y, et al. Mutations in IRF6 cause Van der Woude and popliteal pterygium syndromes. Nat Gen 2002;32(2):285-9.
12. Washbourne BJ, Cox TC. Expression profiles of cIRF6, cLHX6 and cLHX7 in the facial primordia suggest specific roles during primary palatogenesis. BMC Dev Biol 2006;6(1):48.
13. Bezerra JF, Silva HPVd, Bortolin RH, Luchessi AD, Ururahy MAG, Loureiro MB, et al. IRF6 polymorphisms in Brazilian patients with non-syndromic cleft lip with or without palate. Braz J Otorhinolaryngol 2020;86:696-702.
14. Neves LT, Dionísio TI, Garbieri TF, Parisi VA, Oliveira FV, Oliveira TM, et al. Novel rare variations in IRF6 in subjects with non-syndromic cleft lip and palate and dental agenesis. Oral Dis 2019;25(1):223-33.
15. Zucchero TM, Cooper ME, Maher BS, Daack-Hirsch S, Nepomuceno B, Ribeiro L, et al. Interferon regulatory factor 6 (IRF6) gene variants and the risk of isolated cleft lip or palate. New Engl J Med 2004;351(8):769-80.
16. Huang Y, Wu J, Ma J, Beatty T, Sull J, Zhu L, et al. Association between IRF6 SNPs and oral clefts in West China. J Dent Res 2009;88(8):715-8.
17. Ibarra-Arce A, García-Álvarez M, Cortés-González D, de Zarate-Alarcón GO, Flores-Peña L, Sánchez-Camacho S, et al. IRF6 polymorphisms in Mexican patients with non-syndromic cleft lip. Meta Gene 2015;4:8-16.
18. Gurramkonda VB, Syed AH, Murthy J, Lakkakula BV. IRF6 rs2235375 single nucleotide polymorphism is associated with isolated non-syndromic cleft palate but not with cleft lip with or without palate in South Indian population. Braz J Otorhinolaryngol 2018;84:473-7.
19. Suazo J, Recabarren AS, Marín NR, Blanco R. Association between IRF6 variants and nonsyndromic cleft lip with or without cleft palate in Chile. Reprod Sci 2020;27(10):1857-62.
20. Xu W, Han W, Lu Y, Feng W, Dai M. Association of single-nucleotide polymorphisms, rs2235371 and rs-2013162, in the IRF6 gene with non-syndromic cleft palate in northeast China. Genet Mol Res 2016;15(3).
21. Lu Y, Liu Q, Xu W, Li Z, Jiang M, Li X, et al. TGFA and IRF6 contribute to the risk of nonsyndromic cleft lip with or without cleft palate in northeast China. PLoS One 2013;8(8):e70754.
22. Velázquez-Aragón JA, Angel AG-d, Alcántara-Ortigoya MA, Reyna-Fabián ME, Estandia-Ortega B. Screening of IRF6 variants in patients subjected to genetic association studies for nonsyndromic cleft lip/palate. Cleft Palate-Craniofac J 2021;58(9):1128-34.