



ORIGINAL ARTICLE

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[☆]

Venkatesh Babu Gurramkonda^a, Altaf Hussain Syed^b, Jyotsna Murthy^b,
Bhaskar V.K.S. Lakkakula^{a,c,*}

^a Sri Ramachandra University, Department of Biomedical Sciences, Chennai, India

^b Sri Ramachandra University, Department of Plastic Surgery, Chennai, India

^c Sickle Cell Institute Chhattisgarh, Raipur, India

Received 29 March 2017; accepted 28 May 2017

KEYWORDS

IRF6;
Orofacial clefts;
NSCL/P;
SNP

Abstract

Introduction: Transcription factors are very diverse family of proteins involved in activating or repressing the transcription of a gene at a given time. Several studies using animal models demonstrated the role of transcription factor genes in craniofacial development.

Objective: We aimed to investigate the association of IRF6 intron-6 polymorphism in the non-syndromic cleft lip with or without Palate in a south Indian population.

Methods: 173 unrelated nonsyndromic cleft lip with or without Palate patients and 176 controls without clefts patients were genotyped for IRF6 rs2235375 variant by allele-specific amplification using the KASPar single nucleotide polymorphism genotyping system. The association between interferon regulatory factor-6 gene intron-6 dbSNP208032210:g.G>C (rs2235375) single nucleotide polymorphism and non-syndromic cleft lip with or without palate risk was investigated by chi-square test.

Results: There were significant differences in genotype or allele frequencies of rs2235375 single nucleotide polymorphism between controls and cases with non-syndromic cleft lip with or without palate. IRF6 rs2235375 variant was significantly associated with increased risk of

[☆] Please cite this article as: 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. 2017. <http://dx.doi.org/10.1016/j.bjorl.2017.05.011>

* Corresponding author.

E-mail: lvksbhaskar@gmail.com (B.V. Lakkakula).

Peer Review under the responsibility of Associação Brasileira de Otorrinolaringologia e Cirurgia Cérvico-Facial.

non-syndromic cleft lip with or without palate in co-dominant, dominant (OR: 1.19; 95% CI 1.03–2.51; $p=0.034$) and allelic models (OR: 1.40; 95% CI 1.04–1.90; $p=0.028$). When subset analysis was applied significantly increased risk was observed in cleft palate only group (OR dominant: 4.33; 95% CI 1.44–12.97; $p=0.005$).

Conclusion: These results suggest that IRF6 rs2235375 SNP play a major role in the pathogenesis and risk of developing non-syndromic cleft lip with or without palate.

© 2017 Associação Brasileira de Otorrinolaringologia e Cirurgia Cérvico-Facial. Published by Elsevier Editora Ltda. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

PALAVRAS-CHAVE

IRF6;
Fendas orofaciais;
NSCL/P;
SNP

Polimorfismo de nucleotídeo único do gene IRF6 rs2235375 está associado com fenda palatina isolada não síndrômica, mas não com fenda labial com ou sem palato em população do sul da Índia

Resumo

Introdução: Fatores de transcrição são famílias de proteínas muito diversas envolvidas na ativação ou repressão da transcrição de um gene, em um determinado momento. Vários estudos usando modelos animais demonstraram o papel dos genes do fator de transcrição no desenvolvimento craniofacial.

Objetivo: Nosso objetivo foi investigar a associação do polimorfismo IRF6 intron-6 na Fenda Labial Não-sindrômica com ou sem fenda palatina em uma população do sul da Índia.

Método: 173 pacientes não-relacionados com fenda labial não-sindrômica com ou sem fenda palatina e 176 controles sem fendas foram genotipados para a variante IRF6 rs2235375 por amplificação alelo-específica utilizando o sistema de genotipagem KASPar Single Nucleotide Polymorphism. A associação entre o Fator 6 Regulatório do Interferon (IRF6) intron-6 dbSNP208032210:g.G>C (rs2235375) single nucleotide polymorphism e o risco de fenda labial não-sindrômica com ou sem fenda palatina foi investigado pelo teste qui-quadrado.

Resultados: Houve diferenças significativas nas frequências de genótipos ou alelos do rs2235375 SNP entre controles e casos com fenda labial não-sindrômica com ou sem fenda palatina. A variante IRF6 rs2235375 foi significativamente associada ao aumento do risco de fenda labial Não-sindrômica com ou sem fenda palatina em modelos codominantes, dominantes (OR: 1,19; IC 95%: 1,03-2,51; $p=0,034$) e alélicos (OR: 1,40; IC 95%: 1,04-1,90; $p=0,028$). Quando a análise do subgrupo foi aplicada, um risco significativamente aumentado foi observado no grupo Fenda Palatina Isolada (OR dominante: 4,33; IC95%: 1,44-12,97; $p=0,005$).

Conclusões: Esses resultados sugerem que o IRF6 rs2235375 single nucleotide polymorphism desempenha um papel importante na patogênese e risco de desenvolvimento de fenda labial não-sindrômica com ou sem fenda palatina.

© 2017 Associação Brasileira de Otorrinolaringologia e Cirurgia Cérvico-Facial. Publicado por Elsevier Editora Ltda. Este é um artigo Open Access sob uma licença CC BY (<http://creativecommons.org/licenses/by/4.0/>).

Introduction

Transcription factors are very diverse family of protein involved in activating or repressing the transcription of a gene at a given time. During development transcription factors are responsible for dictating the fate of individual cells. Several lines of evidence have demonstrated the role of transcription factor genes in craniofacial development and also the variants in these genes played a crucial role in the aetiology of NSCL/P.^{1,2} Interferon regulatory factor-6 (IRF6) is a transcription factor that codes helix-turn-helix DNA binding domain and less conserved protein binding domain. In humans, nine IRFs were reported and the amino acid sequence composition analysis showed 89% similarity between IRF6 and IRF5, which plays a role in interferon activation and tumour suppression.³ In situ hybridization of mouse embryos demonstrated that IRF6 is highly expressed

in the medial edges of the paired palatal shelves immediately before, and during, their fusion. IRF6 expression was also detected in hair follicles, palatal rugae, tooth germ, thyroglossal duct, external genitalia and on the skin throughout the body.⁴ Recent research has shown that IRF6 mutant mice exhibit a hyper-proliferative epidermis that fails to undergo terminal differentiation, which leads to multiple epithelial adhesions that can occlude the oral cavity and result in cleft palate.⁵

Interferon regulatory factor-6 (IRF6) is located in chromosome 1q32.2 position and is one of the candidate genes associated with both syndromic and non-syndromic forms of clefts.^{6–8} Further, several GWAS studies identified IRF6 as an associated locus for oral clefts.^{9,10} This gene contains 10 exons and among them exon 1, 2, and 10 are noncoding. IRF6 encodes protein contains total 517 amino acids and contains an N-terminal winged-helix DNA-binding domain

and a C-terminal SMIR (Smad-interferon regulatory factor-binding) domain. Studies on humans showed that common alleles in IRF6 were associated with NSCL/P in different populations.¹¹

Our previous study indicated that Haplotypes of two IRF6 gene polymorphisms are associated with NSCL/P.¹² In this study, we extend our previous work to evaluate the association between IRF6 Intron-6 dbSNP208032210:g.G>C (rs2235375) single nucleotide polymorphism with non-syndromic cleft lip with or without palate risk in south Indian Population.

Methods

Institution Ethics Committee of Sri Ramachandra University, Chennai, India, has approved the study design (Ref: IEC-NI/II/OCT/25/60 dated 12.01.2012). Informed consent was collected from parent or legal guardian when the subject was a minor. The study was comprised of 176 NSCL/P cases (77 female and 99 male) and 173 controls (77 female and 96 male). Study participants were recruited from Sri Ramachandra cleft and craniofacial centre, Sri Ramachandra University, Chennai, India. Two surgeons independently assessed cleft phenotype and cases with mental retardation or any other anomalies were excluded from the study. Of the 176 NSCL/P cases, 104 have cleft lip with cleft palate (CL/P; 76 unilateral and 28 bilateral), 40 have Cleft Lip Only (CLO) and 29 have cleft palate only (CPO). Both CLO and CPO cases are unilateral and none of the patients have affected relatives. Age and gender matched subjects without family history of clefting were recruited as controls. From each subjects 3 mL peripheral blood sample was collected and DNA was extracted using a standard procedure.¹³ Genotyping of the IRF6 rs2235375 SNP was performed by KBioscience by using KASPar chemistry.^{14,15} SNP genotyping through KASPar chemistry involves competitive allele-specific PCR using FRET quencher cassette oligos (Table 1). Based on the fluorescence obtained, allele call data were viewed graphically as a scatter plot using the SNPViewer (<http://www.lgcgenomics.com>). Hardy-Weinberg Equilibrium (HWE) was assessed in both cases and controls groups by using chi-square test. Allele frequencies were estimated by the gene counting method.¹⁶

Table 1 Primers used for genotyping IRF6 rs2235375.

Primers ^a	Sequence ^b
Allele 1 (FAM)	5'-GTAAGTGAGACTTTATCTTTCTTGCTG-3'
Allele 2 (HEX)	5'-GTAAGTGAGACTTTATCTTTCTTGCTC-3'
Common reverse	5'-GAAAGCAGGACAGGAAAGAGTCTATAATA-3'

^a Primers corresponding to different alleles were labelled with FAM and HEX fluorescent dyes (KBiosciences).

^b Polymorphic bases are underlined.

Comparison of genotype and allele frequencies among cases and the control groups were analyzed by the chi-square test. Odds ratio and 95% confidence intervals were calculated using wild type genotypes or allele as reference group.

Results

The distribution of the IRF6 rs2235375 variant genotypes and alleles in both cases and NSCL/P groups are presented in Table 2. The proportions of genotypes were 30.1% GG, 49.7% GC, 20.2% CC in cases and 40.9% GG, 44.3% GC, 14.8% CC in controls. The C allele frequency was 45.1% in cases and 36.9% in controls. The frequencies of IRF6 rs2235375 genotype was distributed according to the Hardy-Weinberg equilibrium among the controls ($p=0.519$). Significant difference in allele frequencies were found between control and the NSCL/P groups (Table 3). OR and 95% CI were calculated to assess the relative risk of oral clefts by comparing genotype frequencies of cases and controls in co-dominant, dominant as well as in allelic models (Table 3). Significantly increased NSCL/P risk was found for homozygous genotype (CC vs. GG; OR=1.86; 95% CI 1.0–3.47; $p=0.047$). Increased NSCL/P risk was also found under dominant (GC+CC vs. GG; OR=1.19; 95% CI 1.03–2.51; $p=0.034$) and allelic models (C vs. G; OR=1.40; 95% CI 1.04–1.90; $p=0.028$). In subgroup analysis, IRF6 rs2235375 variant showed significantly increased risk in CPO under three different models (Table 3).

Table 2 Genotype distribution and allele frequencies of the IRF6 rs2235375 SNP in cleft lip and palate.

	Control (%)	Overall clefts (%)	CL/P (%)	CPO (%)
<i>Genotype distribution</i>				
GG	72 (40.9)	52 (30.1)	48 (33.3)	4 (13.8)
GC	78 (44.3)	86 (49.7)	69 (47.9)	17 (58.6)
CC	26 (14.8)	35 (20.2)	27 (18.8)	8 (27.6)
<i>Allele frequency</i>				
G allele	222 (63.1)	190 (54.9)	165 (57.3)	25 (43.1)
C allele	130 (36.9)	156 (45.1)	123 (42.7)	33 (56.9)
<i>Test for HWE</i>				
Chi-square	0.417	0.003	0.062	0.996
<i>p</i> -value	0.519	0.959	0.803	0.318

CL/P, cleft lip with cleft palate; CPO, cleft palate only; HWE, Hardy-Weinberg equilibrium.

Table 3 Results of association tests with *IRF6* rs2235375 SNP in cleft lip and palate.

<i>IRF6</i> rs2235375	OR (95% CI)	p-value
<i>Overall clefts</i>		
GG	Reference	0.085 ^a
GC	1.53 (0.95–2.44)	0.077
CC	1.86 (1.00–3.47)	0.047
GC + CC vs. GG	1.19 (1.03–2.51)	0.034
G allele	Reference	
C allele	1.40 (1.04–1.90)	0.028
<i>CL/P</i>		
GG	Reference	0.334 ^a
GC	1.33 (0.81–2.16)	0.225
CC	1.56 (0.81–2.99)	0.18
GC + CC vs. GG	1.38 (0.88–2.19)	0.163
G allele	Reference	
C allele	1.27 (0.93–1.75)	0.137
<i>CPO</i>		
GG	Reference	0.014 ^a
GC	3.92 (1.26–12.21)	0.021
CC	5.54 (1.54–19.95)	0.004
GC + CC vs. GG	4.33 (1.44–12.97)	0.005
G allele	Reference	
C allele	2.25 (1.28–3.96)	0.003

IRF6, interferon regulatory factor-6; OR, odds ratio; CI, confidence interval.

^a P Value from a chi-square (df = 2).

Discussion

Our findings clearly demonstrate that *IRF6* rs2235375 play a predominant role in the development of NSCL/P. This study provides confirmatory evidence for the variants which have been reported earlier, to be associated with NSCL/P. During the embryonic development, the role of *IRF6* has been identified, but its regulatory function remains unclear.^{5,17} At the time of palate fusion, degradation of the Medial Edge Epithelium (MEE) takes place and increased *IRF6* expression was observed in MEE during this process.^{18,19} Developmental expression patterns of *IRF6* orthologues in mouse and chick revealed presence of *IRF6* expression in the ectoderm fusion forming the upper lip and primary palate in both mouse and chick, but only in the developing secondary palate of the mouse.²⁰

Meta-analysis of 20 published case-control studies demonstrated that the rs2235371 and rs642961 are antagonistic to each other in attributing the risk of NSCL/P.²¹ Allele "A" of a functional polymorphism (rs2235371; 820G > A) contributed to an increased risk of NSCL/P in Chinese population.²² On the contrary, "G" allele was over-transmitted in few studies.¹¹ Few haplotype based analyses confirmed that the rs2235371 was associated with NSCL/P.^{23–25} In contrast to this rs2235371 SNP failed to show an association with oral clefts in some populations.^{26–29} We previously showed that the *IRF6* rs2235371 G allele is over-transmitted in Indian clefts patients, but 820GG genotype contributed to minor risk only.³⁰ There were few association studies available for SNP rs642961 and the results were inconsistent. Significant association between rs642961

and NSCL/P was observed.^{26,31–34} In contrast to the above mentioned studies, no association was reported by several studies.^{29,35–37}

Genome-wide association studies and their follow-up replication studies established *IRF6* as one of the candidate genes for the pathogenesis of NSCL/P.³⁸ The polymorphism analyzed in this study (rs2235375) is located in the intron-6 of *IRF6*. This polymorphism showed the positive association between this variant and NSCL/P in several populations such as Italian,⁷ European-Americans,²⁵ Norwegian,³⁹ Chilean,²⁸ Chinese⁴⁰ and Brazilian.⁴¹

Conclusion

In conclusion, the results of the present study indicate that *IRF6* rs2235375 polymorphism is associated with NSCL/P in South Indian population.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

L.V.K.S. Bhaskar acknowledges funding from the Indian Council of Medical Research (ICMR), Government of India (Project Ref. n^o 56/15/2007-BMS and n^o 45/3/2013-Hum/BMS).

References

1. Britanova O, Depew MJ, Schwark M, Thomas BL, Miletich I, Sharpe P, et al. *Satb2* haploinsufficiency phenocopies 2q32-q33 deletions, whereas loss suggests a fundamental role in the coordination of jaw development. *Am J Hum Genet.* 2006;79:668–78.
2. Park JW, Cai J, McIntosh I, Jabs EW, Fallin MD, Ingersoll R, et al. High throughput SNP and expression analyses of candidate genes for non-syndromic oral clefts. *J Med Genet.* 2006;43:598–608.
3. Barnes BJ, Field AE, Pitha-Rowe PM. Virus-induced heterodimer formation between IRF-5 and IRF-7 modulates assembly of the IFNA enhanceosome in vivo and transcriptional activity of IFNA genes. *J Biol Chem.* 2003;278:16630–41.
4. 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 Genet.* 2002;32:285–9.
5. Richardson LE, Wakley GK, Franklin SH. A quantitative study of the equine soft palate using histomorphometry. *Vet J.* 2006;172:78–85.
6. Rizo M, Spyropoulos MN. Van der Woude syndrome: a review. Cardinal signs, epidemiology, associated features, differential diagnosis, expressivity, genetic counselling and treatment. *Eur J Orthod.* 2004;26:17–24.
7. Scapoli L, Palmieri A, Martinelli M, Pezzetti F, Carinci P, Tognon M, et al. Strong evidence of linkage disequilibrium between polymorphisms at the *IRF6* locus and nonsyndromic cleft lip with or without cleft palate, in an Italian population. *Am J Hum Genet.* 2005;76:180–3.
8. Vieira AR, Avila JR, Daack-Hirsch S, Dragan E, Felix TM, Rahimov F, et al. Medical sequencing of candidate genes for nonsyndromic cleft lip and palate. *PLoS Genet.* 2005;1:e64.
9. Mangold E, Ludwig KU, Nothen MM. Breakthroughs in the genetics of orofacial clefting. *Trends Mol Med.* 2011;17:725–33.

10. Beaty TH, Murray JC, Marazita ML, Munger RG, Ruczinski I, Hetmanski JB, et al. A genome-wide association study of cleft lip with and without cleft palate identifies risk variants near MAFB and ABCA4. *Nat Genet.* 2010;42:525–9.
11. Zuccherro 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. *N Engl J Med.* 2004;351:769–80.
12. Gurramkonda VB, Murthy J, Syed AH, Lakkakula BV. Evidence of association between IRF6 polymorphisms and nonsyndromic oral clefts in South Indian population. *Dentistry 3000.* 2013;1:5.
13. Sambrook J, Russell D. *Molecular cloning: a laboratory manual.* Cold Spring Harbor Laboratory Press; 2001.
14. Cuenca J, Aleza P, Navarro L, Ollitrault P. Assignment of SNP allelic configuration in polyploids using competitive allele-specific PCR: application to citrus triploid progeny. *Ann Bot.* 2013;111:731–42.
15. Didenko VV. DNA probes using fluorescence resonance energy transfer (FRET): designs and applications. *Biotechniques.* 2001;31:1106–16, 1118, 1120–1.
16. Ceppellini R, Siniscalco M, Smith CA. The estimation of gene frequencies in a random-mating population. *Ann Hum Genet.* 1955;20:97–115.
17. Ingraham CR, Kinoshita A, Kondo S, Yang B, Sajan S, Trout KJ, et al. Abnormal skin, limb and craniofacial morphogenesis in mice deficient for interferon regulatory factor 6 (*Irf6*). *Nat Genet.* 2006;38:1335–40.
18. Xu X, Han J, Ito Y, Bringas P Jr, Urata MM, Chai Y. Cell autonomous requirement for *Tgfr2* in the disappearance of medial edge epithelium during palatal fusion. *Dev Biol.* 2006;297:238–48.
19. Richardson RJ, Dixon J, Jiang R, Dixon MJ. Integration of IRF6 and Jagged2 signalling is essential for controlling palatal adhesion and fusion competence. *Hum Mol Genet.* 2009;18:2632–42.
20. Knight AS, Schutte BC, Jiang R, Dixon MJ. Developmental expression analysis of the mouse and chick orthologues of IRF6: the gene mutated in Van der Woude syndrome. *Dev Dyn.* 2006;235:1441–7.
21. Wang M, Pan Y, Zhang Z, Wang L. Three polymorphisms in IRF6 and 8q24 are associated with nonsyndromic cleft lip with or without cleft palate: evidence from 20 studies. *Am J Med Genet A.* 2012;158A:3080–6.
22. Tang W, Du X, Feng F, Long J, Lin Y, Li P, et al. Association analysis between the IRF6 G820A polymorphism and nonsyndromic cleft lip and/or cleft palate in a Chinese population. *Cleft Palate Craniofac J.* 2009;46:89–92.
23. Scapoli L, Marchesini J, Martinelli M, Pezzetti F, Carinci F, Palmieri A, et al. Study of folate receptor genes in nonsyndromic familial and sporadic cleft lip with or without cleft palate cases. *Am J Med Genet A.* 2005;132A:302–4.
24. Ghassibe M, Bayet B, Revencu N, Verellen-Dumoulin C, Gillerot Y, Vanwijck R, et al. Interferon regulatory factor-6: a gene predisposing to isolated cleft lip with or without cleft palate in the Belgian population. *Eur J Hum Genet.* 2005;13:1239–42.
25. Blanton SH, Cortez A, Stal S, Mulliken JB, Finnell RH, Hecht JT. Variation in IRF6 contributes to nonsyndromic cleft lip and palate. *Am J Med Genet A.* 2005;137A:259–62.
26. Zhou Q, Li M, Zhu W, Guo J, Wang Y, Li Y, et al. Association between interferon regulatory factor 6 gene polymorphisms and nonsyndromic cleft lip with or without cleft palate in a Chinese population. *Cleft Palate Craniofac J.* 2013;50:570–6.
27. Hering R, Grundmann K. The IRF6 p.274V polymorphism is not a risk factor for isolated cleft lip. *Genet Med.* 2005;7:209 [author reply 09–10].
28. Suazo J, Santos JL, Jara L, Blanco R. Linkage disequilibrium between IRF6 variants and nonsyndromic cleft lip/palate in the Chilean population. *Am J Med Genet A.* 2008;146A:2706–8.
29. Paranaiba LM, Bufalino A, Martelli-Junior H, de Barros LM, Graner E, Coletta RD. Lack of association between IRF6 polymorphisms (rs2235371 and rs642961) and non-syndromic cleft lip and/or palate in a Brazilian population. *Oral Dis.* 2010;16:193–7.
30. Gurramkonda VB, Murthy J, Syed AH, Lakkakula BV. Lack of association between IRF6 polymorphisms and nonsyndromic oral clefts in South Indian population. *Dentistry 3000.* 2013;1:5.
31. Birnbaum S, Ludwig KU, Reutter H, Herms S, Steffens M, Rubini M, et al. Key susceptibility locus for nonsyndromic cleft lip with or without cleft palate on chromosome 8q24. *Nat Genet.* 2009;41:473–7.
32. Shi J, Song T, Jiao X, Qin C, Zhou J. Single-nucleotide polymorphisms (SNPs) of the IRF6 and TFAP2A in non-syndromic cleft lip with or without cleft palate (NSCLP) in a northern Chinese population. *Biochem Biophys Res Commun.* 2011;410:732–6.
33. Pan Y, Ma J, Zhang W, Du Y, Niu Y, Wang M, et al. IRF6 polymorphisms are associated with nonsyndromic orofacial clefts in a Chinese Han population. *Am J Med Genet A.* 2010;152A:2505–11.
34. Mostowska A, Hozyasz KK, Wojcicki P, Biedziak B, Paradowska P, Jagodzinski PP. Association between genetic variants of reported candidate genes or regions and risk of cleft lip with or without cleft palate in the Polish population. *Birth Defects Res A Clin Mol Teratol.* 2010;88:538–45.
35. Larrabee YC, Birkeland AC, Kent DT, Flores C, Su GH, Lee JH, et al. Association of common variants, not rare mutations, in IRF6 with nonsyndromic clefts in a Honduran population. *Laryngoscope.* 2011;121:1756–9.
36. Pegelow M, Koillinen H, Magnusson M, Fransson I, Unneberg P, Kere J, et al. Association and mutation analyses of the IRF6 gene in families with nonsyndromic and syndromic cleft lip and/or cleft palate. *Cleft Palate Craniofac J.* 2014;51:49–55.
37. Brito LA, Bassi CF, Masotti C, Malcher C, Rocha KM, Schlesinger D, et al. IRF6 is a risk factor for nonsyndromic cleft lip in the Brazilian population. *Am J Med Genet A.* 2012;158A:2170–5.
38. Beaty TH, Taub MA, Scott AF, Murray JC, Marazita ML, Schwender H, et al. Confirming genes influencing risk to cleft lip with/without cleft palate in a case-parent trio study. *Hum Genet.* 2013;132:771–81.
39. Jugessur A, Rahimov F, Lie RT, Wilcox AJ, Gjessing HK, Nilsen RM, et al. Genetic variants in IRF6 and the risk of facial clefts: single-marker and haplotype-based analyses in a population-based case-control study of facial clefts in Norway. *Genet Epidemiol.* 2008;32:413–24.
40. Huang Y, Wu J, Ma J, Beaty TH, Sull JW, Zhu L, et al. Association between IRF6 SNPs and oral clefts in West China. *J Dent Res.* 2009;88:715–8.
41. Ibarra-Arce A, Garcia-Alvarez M, Cortes-Gonzalez D, Ortiz de Zarate-Alarcon G, Flores-Pena L, Sanchez-Camacho S, et al. IRF6 polymorphisms in Mexican patients with non-syndromic cleft lip. *Meta Gene.* 2015;4:8–16.