

Review Article

Association between IRF6 rs642961 polymorphism and non-syndromic cleft lip with or without a cleft palate: a systematic review and meta-analysis

Tzu-Hui Lee^{1*}, Tsung-Ta Liu^{2*}, Chih-Wei Sung^{3,4*}, Ju-Chi Ou⁵, Jui-Ju Yeh⁶, Yung-Ting Kuo^{1,7}, Yuan-Hung Wang^{8,9}

Departments of ¹Pediatrics, ⁵Emergency Medicine, ⁶Family Medicine, ⁸Medical Research, Shuang Ho Hospital, Taipei Medical University, New Taipei City 23561, Taiwan; ²Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei, Taiwan; ³Department of Emergency Medicine, National Taiwan University Hospital Hsin-Chu Branch, Hsinchu, Taiwan; ⁴Department of Emergency Medicine, National Taiwan University Hospital, Taipei, Taiwan; ⁷Department of Pediatrics, School of Medicine, College of Medicine, Taipei Medical University, Taipei 11031, Taiwan; ⁹Graduate Institute of Clinical Medicine, College of Medicine, Taipei Medical University, Taipei 11031, Taiwan. *Equal contributors.

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Abstract: Non-syndromic cleft lip with or without a cleft palate (NSCL/P) is one of the most common newborn malformations. Previous studies have reported that genetic variations of interferon regulatory factor 6 (IRF6) polymorphisms are associated with NSCL/P. However, the effect sizes of individual studies still vary. Our present study is a meta-analysis to investigate the association of IRF6 rs642961 polymorphism with NSCL/P. A literature search in PubMed was performed to select eligible literatures including observational studies which evaluated association between IRF6 polymorphisms and NSCL/P. We conducted a systematic review and a meta-analysis on all qualified eight case-control studies that included 1,899 cases and 3,458 controls to investigate the association between NSCL/P and IRF6 rs642961 polymorphism. We found that the A allele had a higher risk of NSCL/P (odds ratio (OR)=1.64, 95% confidence interval (CI)=1.37-1.97) in comparison to the G allele of IRF6 rs642961 polymorphism. The rs642961 A allele in the Asian population had an increased risk of NSCL/P (OR=2.12, 95% CI=1.66-2.71). Under various genetic models, the A allele of IRF6 rs642961 polymorphism in Asian population showed a significantly increased risk of NSCL/P under the dominant model (OR=2.19, 95% CI=1.64-2.91), recessive model (OR=4.49, 95% CI=2.26-8.92), homozygous model (OR=5.86, 95% CI=2.90-11.8), and heterozygous model (OR=1.91, 95% CI=1.42-2.57). Our major findings suggest that association of IRF6 rs642961 polymorphism and NSCL/P is predominant in an Asian population rather than non-Asian populations, which might be useful in clinical diagnoses and treatment of patients with NSCL/P.

Keywords: IRF6, meta-analysis, non-syndromic cleft lip and palate, polymorphism

Introduction

Non-syndromic cleft lip with or without a cleft palate (NSCL/P) is characterized by an incomplete separation between the nasal and oral cavities. It is a birth craniofacial abnormality, the incidence of which varies with geographic origin and ethnicity [1-3]. Prevalence of NSCL/P in Asian and Amerindian populations appear to have the highest frequencies (~2 in 1000 live births), with European populations being intermediate (~1 in 1000 live births), and African populations the lowest (~0.4 in 1000

live births). In Taiwan, the annual rate of incidence of CL/P was approximately 1.48 in 1000 live births with a 2.9% annual decline in the rate [4]. Patients with NSCL/P are vulnerable to speech and communication problems which result in delayed development, low self-confidence, and a poor quality of life representing challenges to clinical care. The etiology of NSCL/P is multifactorial and includes both genetic and environmental components [5]. Exposure to smoking, drugs, infections, and/or nutrient deficiencies may contribute to the risk of NSCL/P [6]. In addition, specific gene muta-

tions may also play a role in the development of NSCL/P. Several studies have identified specific genes associated with NSCL/P, which may shed light on the etiology of the condition and facilitate efforts to prevent this disease [7, 8].

The *interferon regulatory factor-6 (IRF6)* gene, located on chromosome 1q32.3-q41, encodes a protein that 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 [9]. In animal models, *IRF6*-knockout mice exhibited abnormal skin, limb, and craniofacial development due to defects in the keratinocyte proliferation-differentiation switch [10]. The *IRF6* gene is involved in the proliferation-differentiation of keratinocytes [11]. In humans, mutations in *IRF6* gene were first identified as an etiologic factor in the autosomal-dominant Van der Woude's syndrome (VWS), which includes NSCL/P along with dental anomalies as well as lip fistulas [12]. Association of NSCL/P with the single-nucleotide polymorphism (SNP) rs642961 in the *IRF6* gene located in chromosomal region 1q32 was confirmed in candidate genes and genome-wide association studies (GWAS) [13, 14].

Several studies have investigated the effects of polymorphisms in the *IRF6* gene (at rs2235371 (G>A) and rs642961 (G>A)) and the 8q24 region (at rs987525 (C>A)) on NSCL/P. The risky A allele disrupts the binding site of the AP-2- α transcription factor and an expression analysis of the mouse localized the enhancer activity to craniofacial and limb structures [15]. Currently, the biological mechanisms of newly identified SNPs of *IRF6* gene remain unclear. Grant et al. conducted a GWAS and identified a locus for non-syndromic cleft lip with or without cleft palate on 8q24 [16]. Beaty et al. replicated the GWAS in Asian population and found that rs987525 polymorphism was another significant SNP [17]. Shi investigated two identified SNPs of *IRF6* gene and three transcription factor AP-2a tag SNPs and observed a significant correlation between the *IRF6* rs642961 polymorphism and NSCL/P [18]. Kousa found that of the several SNPs located within the *IRF6* gene, rs2235371 and rs642961 variants existed in over 30% of the world's population and contributed to the risk

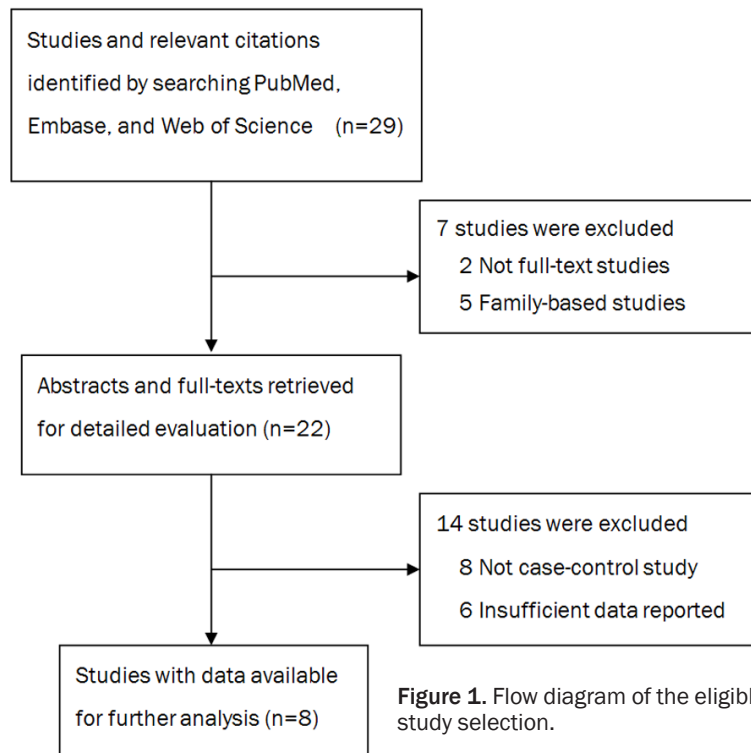
for NSCL/P [19]. Those findings were then further pooled by meta-analyses [20, 21]. However, meta-analyses pooled only allele effects rather than genetic model analyses. There are more pooled studies targeting the *IRF6* rs2235371 polymorphism than those targeting the rs642961 polymorphism. Therefore, we conducted an updated meta-analysis of the association between *IRF6* rs642961 polymorphism and the risk of NSCL/P, aiming to assess both variant effects and possible genetic models.

Methods

The methodology and reporting of the present meta-analysis were based on the recommendations of the Cochrane Collaboration, the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement, and the Moose group [22-24]. A systematic search was performed of PubMed, Web of Science, EMBASE, and the Cochrane Libraries to identify all eligible studies. References of all relevant articles were also searched for additional studies. The search strategy included the following key words: "interferon regulatory factor 6 gene", "IRF-6", "polymorphism", "rs642961", "SNP", "NSCL/P", "non-syndromic cleft", "cleft lip", and "cleft palate only (CPO)". Two authors (C.W. Sung and T.H. Lee) performed the literature review and searched independently. The search included papers published by December 2014. Any human population-based association study published in English was included if it also met the following criteria: 1) the outcome of interest was NSCL/P or CPO; and 2) the studied polymorphisms included *IRF6* rs642961 with sufficient data for analysis including the allele/genotype frequency between cases and controls.

Two independent authors extracted the data of eligible studies using a standardized data extraction form. The following terms were extracted: the first author, country of study, ethnicity, source, and number of cases and controls, frequencies of *IRF6* rs642961 genotyping, and year of publication. Ethnic descent was categorized into Asian and non-Asian subgroups. Our study focused on NSCL/P as the outcome of interest, which was diagnosed in accordance with the original studies. The SNP of interest was a polymorphism within the *IRF6* gene (rs642961 G>A). The data was computer-

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ized by two independent authors (T.H. Lee and M.C. Chang) and compared to identify disagreement. Discrepancies were further resolved by discussion with the senior author (Y.H. Wang). The quality of studies was independently assessed using a risk of bias assessment for genetic association studies [25].

Odds ratios (ORs) and 95% confidence intervals (CIs) were used as summary statistics and to indicate the strength of association. The following models were adopted and evaluated: an allele model (A vs. G), homozygote model (A/A vs. G/G), recessive model (A/A vs. G/G), homozygous model (A/A vs. G/G), and heterozygous model (G/A vs. G/G). Heterogeneity was assessed by Cochran's Q test. Statistical heterogeneity was calculated with the I^2 statistic which quantifies the percentage of variation in study results due to heterogeneity rather than to chance.

Pooled ORs and 95% CIs between the *IRF6* rs642961 polymorphism and NSCL/P stratified by ethnicity and disease types were calculated. We adopted the Der Simonian and Laird random effects model. Potential publication bias was examined by Begg's funnel plot, in which the standard error of the OR was plotted against

the OR of the selected outcomes and Egger's test [26]. An asymmetrical funnel plot and a p value of <0.05 suggested possible publication bias. Some of the included studies presented several results using different adjustments for covariates and design elements. We also performed a sensitivity analysis to explore the effects of using differently adjusted results for these studies. All statistical analyses were performed using Software R version 3.2.2 (The R Foundation for Statistical Computing).

Results

The search located 29 relevant studies from Medline by means of the PubMed and Web of Science databases. Among these studies, seven

of them were excluded because of no full text (two studies) or being family-based studies (five studies). The abstract and full text of the remaining 22 studies were retrieved for secondary evaluation. In total, 14 studies were further excluded (eight studies were not case-control and six studies had insufficient data). Reasons for ineligibility are provided in **Figure 1**. Ultimately, the study involved eight case-control studies with totals of 1,899 cases and 3,458 controls. Basic characteristics of eligible studies [14, 15, 18, 27-31] are listed in **Table 1**. The ethnic groups examined by these studies included Caucasian (three studies), Asian (three studies), American (one study), and mixed (one study). Further stratified by disease subtype, five of the studies examined cleft lips with or without a cleft palate (CL/P) while the others examined CL/P and CPO. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was applied for genotyping in all studies. The distribution of *IRF6* rs642961 polymorphism in each study is shown in **Table 2**. Genotype frequencies (G/G, G/A, A/A, and A allele (%)) for both cases and controls are presented. Hardy-Weinberg Equilibrium (HWE) of the controls was confirmed in all studies ($p>0.05$).

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Table 1. Basic characteristics of studies included in the present meta-analysis

First author	Year	Country	Ethnicity	Sample size (case/control)	Disease subtypes [#]	Genotyping method
Rahimov	2008	Denmark and Norway	Caucasian	513/1245	CL/P and CPO	PCR-RFLP
Birnbaum	2009	Germany and other countries	Caucasian	460/951	CL/P	PCR-RFLP
Pan	2010	China	Asian	134/115	CL/P and CPO	PCR-RFLP
Mostowska	2010	Poland	Caucasian	165/565	CL/P	PCR-RFLP
Paranaiba	2010	Brazil	Mixed	228/126	CL/P and CPO	PCR-RFLP
Ren	2010	China	Asian	89/200	CL/P	PCR-RFLP
Shi	2011	China	Asian	173/156	CL/P	PCR-RFLP
Larrabee	2011	Honduran	American	137/100	CL/P	PCR-RFLP

[#]CL/P: cleft lip with or without cleft palate; CPO: cleft palate only.

Table 2. The distribution of IRF6 rs642961 polymorphism among studies included in the meta-analysis

First author	Year	Genotype frequencies for cases				Genotype frequencies for controls				HWE (P-value)
		G/G	G/A	A/A	A allele (%)	G/G	G/A	A/A	A allele (%)	
Rahimov	2008	273	202	38	27.0	758	427	60	22.0	0.55
Birnbaum	2009	237	189	34	27.9	622	283	46	19.7	0.39
Pan	2010	70	58	6	26.0	81	33	1	15.0	0.86
Mostowska	2010	82	74	9	28.0	349	184	32	22.0	0.78
Paranaiba	2010	174	50	4	12.7	93	30	3	14.3	0.65
Ren	2010	38	36	15	37.0	136	56	8	18.0	0.89
Shi	2011	100	62	11	24.0	111	43	2	15.0	0.68
Larrabee	2011	93	40	4	17.8	66	30	4	18.7	0.82

Forest plots shown in **Figure 2** illustrate the association between IRF6 rs642961 polymorphism and NSCL/P. Notably, two studies (Paranaiba et al., 2010 and Larrabee et al., 2011) were removed from the following analysis because of the ethnicity involved. American or mixed populations are difficult to categorize into Asian or Caucasian subgroups. Additionally, results of the two studies presented different orientations from the others ($OR > 1$ vs. $OR < 1$), therefore the results could not be pooled. To avoid potential interference, the two studies were excluded. The IR6 rs642961 polymorphism was significantly related to an increased risk of NSCL/P in the A allele versus G allele model ($OR = 1.64$, 95% $CI = 1.37-1.97$, $p_{\text{heterogeneity}} = 0.02$), dominant model (A/A+G/A vs. G/G) ($OR = 1.74$, 95% $CI = 1.45-2.10$, $p_{\text{heterogeneity}} = 0.11$), and recessive model (A/A vs. G/A+G/G) ($OR = 1.74$, 95% $CI = 1.26-2.97$, $p_{\text{heterogeneity}} = 0.08$). **Table 3** summarizes pooled ORs and 95% CIs between the IRF6 rs642961 polymorphism and NSCL/P after stratification by ethnicity and disease types. The risk of NSCL/P in Asians was significantly higher than that in non-Asian

populations especially in the recessive model ($OR = 4.49$, 95% $CI = 2.26-8.92$, $p_{\text{heterogeneity}} = 0.98$) and homozygous model (A/A vs. G/G) ($OR = 5.86$, 95% $CI = 2.90-11.8$, $p_{\text{heterogeneity}} = 0.95$). After stratifying by disease types, we also found that the risk of NSCLP in CL/P was higher than that in CL/P and CPO in individual models.

Publication bias was evaluated by Begg's funnel plot and Egger's test. There was a slight asymmetry in the funnel plot (**Figure 3**) in the dominant model and potential risk of publication bias was assessed via Egger's test ($p = 0.037$). A sensitivity analysis showed that the outcomes did not differ from the main analysis and there was consistency in both the direction and magnitude of the estimates (**Figure 4**). No individual study significantly affected the pooled ORs in either the dominant or recessive model.

Discussion

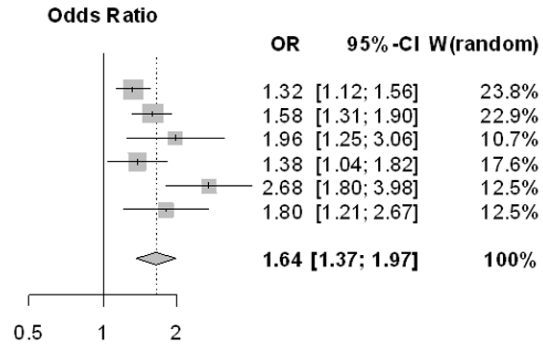
This meta-analysis was undertaken to update information on genetic effects of the rs642961

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A A allele vs. G allele

Study	Experimental		Control	
	Events	Total	Events	Total
Rahimov et al.	278	1026	547	2490
Birnbaum et al.	257	920	375	1902
Pan et al.	70	268	35	230
Mostowska et al.	92	330	248	1130
Ren et al.	66	178	72	400
Shi et al.	84	346	47	312
Random effects model	3068		6464	

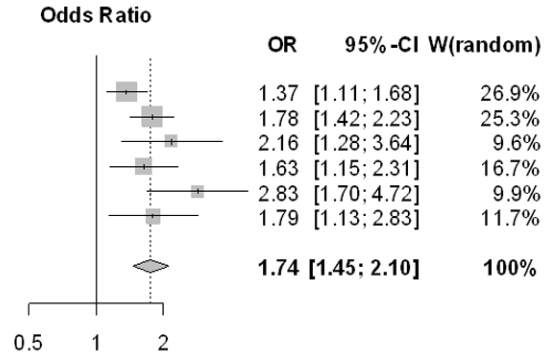
Heterogeneity: I-squared=61.9%, tau-squared=0.0298, p=0.0222



B A/A+G/A vs. G/G (dominant model)

Study	Experimental		Control	
	Events	Total	Events	Total
Rahimov et al.	240	513	487	1245
Birnbaum et al.	223	460	329	951
Pan et al.	64	134	34	115
Mostowska et al.	83	165	216	565
Ren et al.	51	89	64	200
Shi et al.	73	173	45	156
Random effects model	1534		3232	

Heterogeneity: I-squared=44.2%, tau-squared=0.022, p=0.1103



C A/A vs. G/A+G/G (recessive model)

Study	Experimental		Control	
	Events	Total	Events	Total
Rahimov et al.	38	513	60	1245
Birnbaum et al.	34	460	46	951
Pan et al.	6	134	1	115
Mostowska et al.	9	165	32	565
Ren et al.	15	89	8	200
Shi et al.	11	173	2	156
Random effects model	1534		3232	

Heterogeneity: I-squared=49.6%, tau-squared=0.1251, p=0.0773

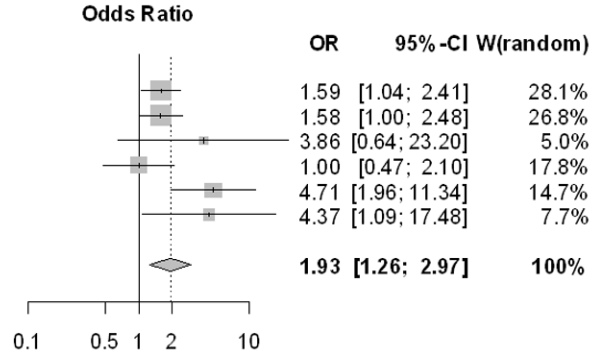


Figure 2. Forest plots illustrate the association between IRF6 rs642961 polymorphism and nonsyndromic cleft lip with or without cleft palate (NSCLP).

Table 3. The pooled ORs and 95% CI between IRF6 rs642961 polymorphism and NSCLP stratified by ethnicity and disease types

Subgroups	A allele vs. G allele		A/A+G/A vs. G/G (dominant model)		A/A vs. G/A+G/G (recessive model)		A/A vs. G/G (homozygous model)		G/A vs. G/G (heterozygous model)	
	OR (95% CI)	P _h ^b	OR (95% CI)	P _h ^b	OR (95% CI)	P _h ^b	OR (95% CI)	P _h ^b	OR (95% CI)	P _h ^b
Overall (n=6)	1.64 (1.37-1.97)	0.02	1.74 (1.45-2.10)	0.11	1.93 (1.26-2.97)	0.08	2.39 (1.50-3.78)	0.05	1.63 (1.39-1.90)	0.28
Ethnicity										
Asian (n=3)	2.12 (1.66-2.71)	0.34	2.19 (1.64-2.91)	0.43	4.49 (2.26-8.92)	0.98	5.86 (2.90-11.8)	0.95	1.91 (1.42-2.57)	0.59
Non-Asian (n=3)	1.42 (1.27-1.60)	0.36	1.57 (1.32-1.86)	0.24	1.48 (1.11-1.96)	0.53	1.74 (1.30-2.33)	0.05	1.55 (1.27-1.90)	0.17
Disease types ^a										
CL/P (n=4)	1.73 (1.36-2.20)	0.05	1.84 (1.54-2.20)	0.35	2.11 (1.07-4.18)	0.03	2.67 (1.31-5.42)	0.03	1.77 (1.49-2.10)	0.78
CL/P and CPO (n=2)	1.52 (1.05-1.05)	0.02	1.61 (1.05-2.47)	0.11	1.66 (1.11-2.50)	0.08	2.03 (1.01-4.06)	0.05	1.52 (1.02-2.26)	0.14

^aCL/P: cleft lip with or without cleft palate; CPO: cleft palate only. ^bP_h: P value of Q-test for heterogeneity test.

IRF6 and nonsyndromic cleft lip with or without a cleft palate

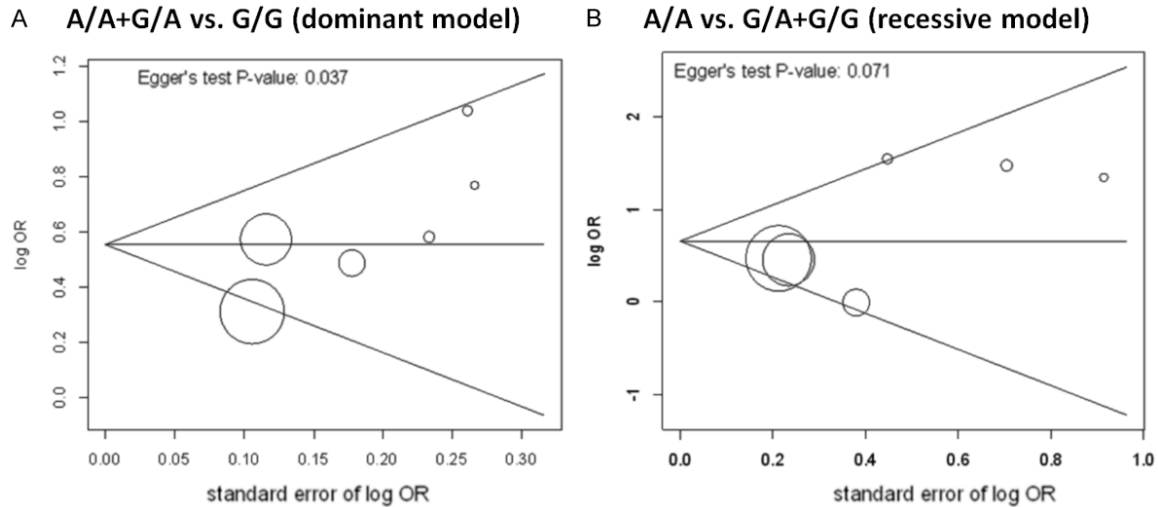


Figure 3. Funnel plot for the examination of the publication bias in the present meta-analysis. Each point represents an eligible study. Log [OR], the natural logarithm of OR. Horizontal line, mean magnitude of effect size.

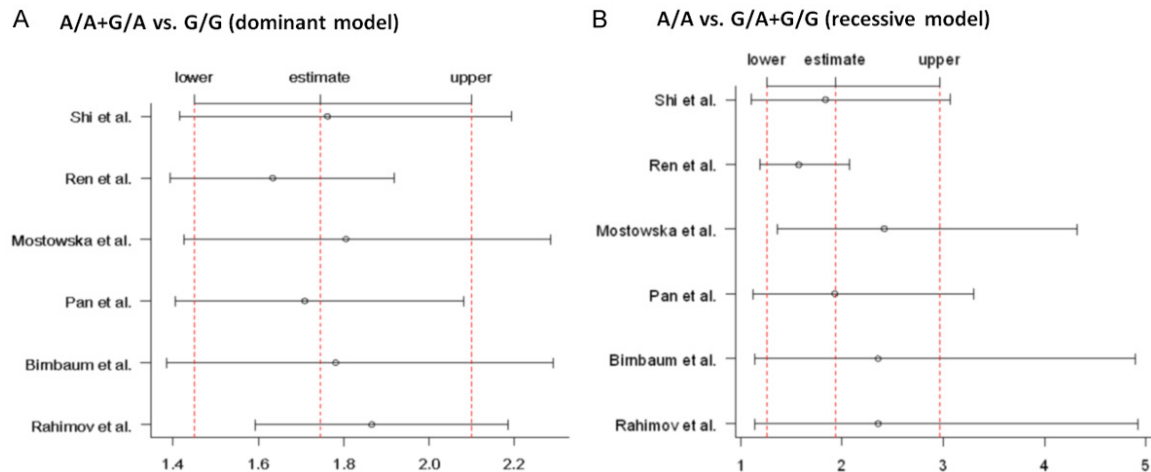


Figure 4. Sensitivity analysis of the summary odds ratio (OR) on the association between IRF6 rs642961 polymorphism and non-syndromic cleft lip with or without cleft palate (NSCLP).

polymorphism in *IRF6* gene on NSCL/P. In total, eight studies were included in the pooling for the *IRF6* rs642961 polymorphism. The A allele of the *IRF6* rs642961 polymorphism showed a significantly increased risk of NSCL/P under the dominant model, recessive model, homozygous model, and heterozygous model. Our findings suggest that the *IRF6* rs642961 polymorphism is more significantly associated with NSCL/P in Asian population than in non-Asian populations.

Our findings are consistent with previous human GWASs [14, 17, 32-36] and animal studies [10, 11]. Evidence from animal studies also supports the role of IRF6 in NSCL/P and con-

firms the association of abnormalities of epithelial differentiation in mice with a mutation in IRF6. This supports a role of IRF6 in the formation and maintenance of the periderm and spatiotemporal regulation of appropriate palatal adhesion. Also, mice with a mutation of IRF6 showed abnormal skin, limb, and craniofacial development which implied a role of IRF6 in keratinocyte proliferation and differentiation of the face and limbs [11]. Additionally, our findings are also consistent with previous meta-analyses of genetic association studies regarding IRF6 rs642961 polymorphisms [20, 21]. We confirmed the previous findings and provide updated information by both expanding the analyses to various models and stratifying the

results by ethnicity and disease types. Although the *IRF6* rs642961 polymorphism was considered in previous meta-analyses for genetic association studies, this analysis was used to further confirm the GWAS. The presence of the A allele at the *IRF6* rs642961 polymorphism site, which alters the binding site of the AP-2a transcription factor, is the true polymorphic allele associated with NSCL/P. The A allele effects were estimated and also pooled genotype effects by ethnicity and disease types were additionally provided from previous evidence.

Paranaiba et al. (2010) and Larrabee et al. (2011) were not included in the pooled analyses because of their inappropriate handling of various populations. Paranaiba et al. suggested an association between NSCL/P and *IRF6* rs642961 polymorphisms in an examined Brazilian population (or mixed population), demonstrating that their presence might not play an important role in the etiology of NSCL/P. Larrabee et al. suggested that rs22-35371 showed an association with CLP, implying a functional role of this polymorphism in a Honduran population (non-Caucasian). As for the rs642961 polymorphism, it was previously reported to have an effect in other populations, suggesting that different populations may be affected. Because of our stratification by Asian and non-Asian populations, these two studies were not included in the final pooled analyses.

NSCL/P is a common birth defect with a complex etiology. Although genetic and environmental factors have been discovered to cause syndromic CLP, the pathogenesis of the more common non-syndromic (isolated) forms remains poorly characterized. Birnbaum and colleagues revealed the first GWAS of NSCL/P in Caucasians and showed that rs987525 was a susceptibility locus [14]. Based on data from prevalence screening, more than 60% of families with VWS had a mutation in *IRF6* gene [37]. *IRF6* mutations are risks contributing to the incidence of oral cleft in German, Swedish, Brazilian, African-American, Caucasian, European, and Asian populations [38] and *IRF6* is a marker of CLP severity [39]. Notably, in our major findings, we first found that Asian individuals with the *IRF6* rs642961 A allele had a significantly higher risk compared to those in non-Asian populations.

Our study has some strengths. We attempted to pool various effects on NSCL/P separately by ethnicity and disease types, if data were available. We also adopted several combinations as genetic models to estimate the association between *IRF6* rs642961 polymorphism and NSCL/P. Admittedly, our study also had some limitations. First, given that we worked with summary data, the lack of sufficient and original data from the reviewed studies confined our further evaluation of potential correlations. Interactions, including gene versus gene, gene versus the environment, and gene versus ethnicity may have modulated the risk of NSCL/P. Second, we could not control confounding effects, although the major source of confounding for genetic studies is population stratification, and we summarized results by ethnic group. Third, in sensitivity analysis by ethnicity and source of control, the sample size was relatively small which may have led to insufficient power to detect actual correlations.

In conclusion, our findings suggest that the A allele of *IRF6* rs642961 polymorphism shows a significantly increased risk of NSCL/P under various genetic models. Among Asian populations, the risk of NSCL/P is more predominant. The association of *IRF6* rs642961 polymorphism within NSCL/P in Asian populations suggests potential genetic heterogeneity of NSCL/P at this locus, which may be a critical phenomenon of different linkage patterns of this polymorphism among different ethnic groups. Clinically, information on genetic polymorphisms in patients with NSCL/P might be useful in diagnosis and treatment. Moreover, there are still polymorphisms other than *IRF6* rs642961 that may affect the risk. The effects of these polymorphisms should be investigated in further studies.

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Disclosure of conflict of interest

None.

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Address correspondence to: Dr. Yuan-Hung Wang, Graduate Institute of Clinical Medicine, College of Medicine Taipei Medical University, 250 Wu-Hsing Street, Taipei 11031, Taiwan. Tel: +886-2-27361661 Ext. 3232; E-mail: 12072@s.tmu.edu.tw; Dr. Yung-Ting Kuo, Department of Pediatrics, Shuang Ho Hospital, Taipei Medical University, 291 Jhongjheng Rd., Jhonghe Dist., New Taipei City 23561, Taiwan. Tel: +886-2-22490088; Fax: +886-2-22490088 Ext. 2507; E-mail: pedkuoyt@s.tmu.edu.tw

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