

ONLINE MUTATION REPORT

Significant association between *IRF6* 820G→A and non-syndromic cleft lip with or without cleft palate in the Thai population

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Background: Previous data have shown an association between DNA sequence variants in the *IRF6* gene and an increased risk of non-syndromic cleft lip with or without cleft palate (CL/P) in some populations.

Objective: To investigate Thai CL/P patients and relative for a 820G→A polymorphism.

Subjects: 192 CL/P Thai patients, 177 of their mothers, 73 of their fathers, and 278 controls.

Results: There were significant differences in the frequency distributions of both genotypes ($p=0.02$) and alleles ($p=0.04$) among probands as compared with the control group. The odds ratio calculated for the patients having the GG genotype compared with the other two genotypes (GA and AA) was 1.67 (95% confidence interval, 1.13 to 2.47). This pattern is consistent with a recessive effect of the G allele. No association between any of the parents' genotypes and CL/P was found. The *IRF6* 820G→A was responsible for 16.7% of the genetic contribution to CL/P.

Conclusions: The findings confirm that *IRF6* 820G→A is associated with CL/P.

Non-syndromic cleft lip with or without cleft palate (CL/P) (MIM 119530) is the most common craniofacial anomaly. Its birth prevalence ranges from 1/500 to 1/2000, depending on geographical origin, with populations of Asian and native American ancestry having the highest rates and African populations the lowest.¹ The aetiology of CL/P is thought to involve both environmental and genetic factors. The number of causative genetic loci is estimated to be between 2 and 14.²

One gene contributing to CL/P, *IRF6*, has been identified. It was initially targeted for investigation after mutations were detected in the gene in patients with Van der Woude syndrome (OMIM 119300), an autosomal dominant disorder characterised by cleft lip, cleft palate, and pits in the lower lip.^{3,4} Subsequently, two recent studies have suggested that DNA sequence variants associated with *IRF6* are major contributors to CL/P in multiple human populations.^{5,6} To confirm the association, we report on an association study of the *IRF6* 820G→A (V274I) in 192 unrelated CL/P patients of the Thai population.

METHODS

The subjects of this study were 161 sporadic cases of non-syndromic CL/P. In addition, 31 cases were collected with a positive family history, bringing the total families in this study to 192. All subjects were self identified as Thai or Thai-Chinese. These groups are further characterised by type, laterality, severity, and sex, as shown in table 1. We also

Table 1 Characteristics of the patients with non-syndromic cleft lip with or without cleft palate

	Cleft lip only	Cleft lip with cleft palate	Total
No of probands			
Sporadic	57	104	161
Familial	15	16	31
Laterality			
Right side cleft	12	32	44
Left side cleft	49	54	103
Bilateral cleft	11	34	45
Severity			
Complete cleft	45	117	162
Incomplete cleft	27	3	30
Sex			
Male	36	76	112
Female	36	44	80

collected 177 of their mothers, 73 of their fathers, and 278 controls. Fifty three were complete trios. All of the patients were studied under the auspices of the Thai Red Cross, a national charity organisation devoted to providing clinical care for the poor. Subjects were recruited between 2000 and 2004 from 10 centres in Thailand (Nakornratchaseema, Nan, Uthaitanee, Maehongsorn, Trang, Srakaew, Kalasin, Nongkhai, Mahasarakam, and Bangkok). As preoperative evaluations, all patients were screened for the presence of associated anomalies or syndromes by a geneticist (VS), and only those determined to have isolated cleft lip with or without cleft palate were included in the study.

Blood samples for DNA analysis were obtained at the time of blood typing and determination of packed cell volume. The control samples were blood donors, self identified as Thai or Thai-Chinese, with no oral clefts, who denied history of oral clefts in other family members, in Nakornratchaseema, Kalasin, Nongkhai, and Bangkok, collected over the same period.

The study was approved by the institutional review board of the Faculty of Medicine of Chulalongkorn University, and written informed consent was obtained from each person included in the study.

DNA was extracted by phenol chloroform extraction protocol and was amplified using the polymerase chain reaction (PCR) with primers IRF6E7F: 5'-AGTGGCCTCCTGAATGCTG-3' and IRF6E7R: 5'-CTTGACCTCCTCCAGACTAA-3', giving a PCR product of 647 bases pairs (bp). Genotyping for the *IRF6* 820G→A polymorphism was carried out by restriction digestion of PCR products with *DpnII* (New England Biolabs, Beverly,

Abbreviations: CL/P, cleft lip/palate

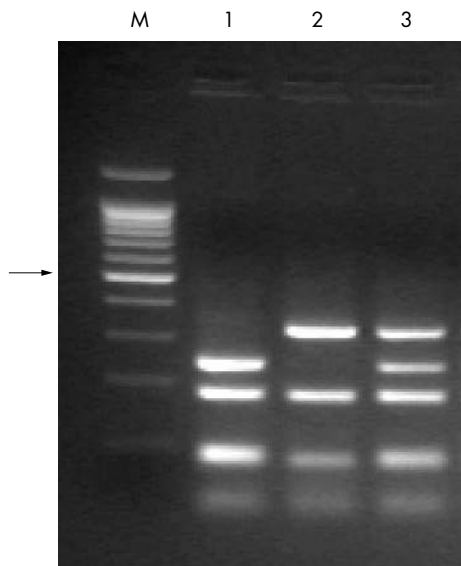


Figure 1 Restriction fragment length polymorphism patterns of *IRF6* 820G→A. Lane M is the 100 bp DNA marker with the arrow head denoting the 500 bp band. Lanes 1, 2, and 3 represent individuals who are homozygous AA, homozygous GG, and heterozygous GA, respectively.

Massachusetts, USA), according to the company's recommendations. *DpnII* digests the G variant to five fragments (322, 177, 80, 35, and 33 bp). The A variant adds another restriction site, causing the 322 bp to be digested into two smaller fragments of 235 and 87 bp (fig 1).

Statistical analysis

Standard χ^2 and p values were calculated by a program available at <http://www.unc.edu/~preacher/chisq/chisq.htm>. Odds ratio and 95% confidence intervals (95% CI) were calculated from the Epi Info 2000 program. The transmission/disequilibrium test (TDT) analysis was carried out on subjects with heterozygous informative parents. Data from families with one parent missing were excluded. The TDT data were analysed using a $k-1/k$ correction (where k is the number of alleles).⁷

Attributable risk

We estimated the attributable risk (AR) for the G allele and the GG genotype according to the formula $AR = \{(P[E/D])(RR-1)\}/RR$, using the odds ratio as an estimate of the relative risk.⁵

RESULTS

The observed frequencies of the 820G and 820A alleles, and the GG, GA, and AA genotypes in affected subjects, their mothers, their fathers, and controls are given in table 2. The observed distribution of genotypes among controls was compared with that expected according to Hardy-Weinberg equilibrium: no difference was found ($\chi^2 = 0.14$, $df = 2$, $p = 0.93$). All genotype frequencies of the patients and their parents also followed Hardy-Weinberg equilibrium (data not shown).

The distributions of genotypes and alleles among patients and their parents were compared with those among controls: differences for both genotypic ($p = 0.02$) and allelic ($p = 0.04$) distributions between patients and controls were found. There were no differences of the distributions between the patients' parents and controls.

Analyses were carried out to estimate the risk associated with each genotype. The results of the GG genotype compared with the other two genotypes, calculated as odds ratios and 95% CI are reported in table 2. A significantly higher frequency of the 820GG genotype was observed in the CL/P patients compared with the controls, with an odds ratio of 1.67 (95% CI, 1.13 to 2.47). The odds ratios in the parental groups were not increased. No association was found with the GA and AA genotypes (data not shown).

TDT analysis was carried out on subjects with heterozygous informative parents, but it showed no evidence for association of CL/P, as shown in table 3.

Table 3 TDT analysis of *IRF6* 820G→A in CL/P patients

Genotype	Transmitted	Untransmitted	χ^{2*}	p Value
G	20	18	0.105	0.874
A	18	20		

* χ^2 were analysed using a $k-1/k$ correction (where k is the number of alleles). Degrees of freedom = [number of rows - 1] × [number of columns - 1] = 1 × 1 = 1.

Table 2 Genotypic and allelic distributions and comparisons of the *IRF6* 820G→A in patients with CL/P, their mothers, their fathers, and controls

	Members of families with CL/P*			
	Patients (n = 192)	Mothers (n = 117)	Fathers (n = 73)	Controls* (n = 278)
Genotypes:				
GG	0.48 (93)	0.39 (46)	0.42 (31)	0.36 (100)
GA	0.38 (72)	0.51 (60)	0.37 (27)	0.49 (137)
AA	0.14 (27)	0.10 (11)	0.21 (15)	0.15 (41)
χ^2 [p value, df = 2]	7.88 [0.02]	2.10 [0.35]	3.74 [0.15]	†
Alleles:				
G	0.67	0.65	0.61	0.61
A	0.33	0.35	0.39	0.39
χ^2 [p value, df = 1]	4.23 [0.04]	1.32 [0.25]	0.01 [0.94]	†
Comparisons of family members and controls (GG v GA/AA)				
Odds ratio	1.67	1.15	1.31	†
95% Confidence interval	1.13 to 2.47	0.72 to 1.84	0.75 to 2.29	†

*The number of subjects is indicated in parentheses.

†Reference category.

Table 4 Estimation of the attributable risk for the G allele

Group	G allele	A allele	Odds ratio	Attributable risk
Probands	258	126	–	–
Population based	337	219	1.33	16.69

For the comparison of the subjects with CL/P with population based controls, as shown in tables 4 and 5, the estimated attributable risk for the G allele and the GG genotype were 16.69% and 19.47%, respectively.

DISCUSSION

CL/P is a multifactorial disorder caused by a combination of genes and environmental interactions. Each factor may contribute differently to CL/P in different populations.^{5, 8} In the Thai population, we have previously observed a significantly higher frequency of the 677CT/1298AC genotype in the mothers of CL/P patients compared with controls, with an odds ratio of 4.43 (95% CI, 1.33 to 15.10).⁹ The observation was reinforced by other studies in different populations.^{10, 11} In the current study, we have established an *IRF6* genotype in 192 CL/P patients, their parents (177 mothers and 73 fathers), and 278 controls.

The frequency of the 820G allele in our controls was 61%, which is comparable to the frequencies of 58–66% in other east Asian (China and Japan) and southeast Asian populations (Cambodia).⁵ However, the frequency of this variant is very different among ethnic groups, with the frequency of the G allele in African of 100%⁵ and in European of 90–100%.^{3, 5}

When we analysed the genotype of our CL/P patients and their parents, we found the odds ratio calculated for the patients having the GG genotype—compared with the other two genotypes (GA and AA)—was 1.67 (95% CI, 1.13 to 2.47). The GA and AA genotypes were not associated with clefting (data not shown). This pattern is consistent with a previous finding of a recessive effect of the G allele.⁵ Our observation of the recessive effect was further supported by the finding of a significant difference in the frequency distributions of both genotypes ($p=0.02$) and alleles ($p=0.04$) among probands as compared with the control group; the frequencies of the GG genotype and the G allele were increased, and the frequencies of the GA and AA genotypes and the A allele were reduced in CL/P patients as compared with unaffected subjects (table 2). No association between any of the parents' genotypes and CL/P was found. This is different from variation in the *MTHFR* gene, in which the maternal genotype is associated with CL/P, rather than the affected subjects themselves.⁹

Of our 192 CL/P patients, 177 of their mothers, and 73 of their fathers, only 53 were complete trios. Transmission disequilibrium calculation was carried out on subjects with heterozygous informative parents. We found a statistically non-significant trend toward a positive association between cleft lip and the G allele (table 3), consistent with a previous

Table 5 Estimation of the attributable risk for the GG genotype

Group	GG genotype	GA or AA genotypes	Odds ratio	Attributable risk
Probands	93	99	–	–
Population based	100	178	1.67	19.47

study in the Indian and European groups.⁵ The statistical non-significance may be explained by the small numbers of the complete trios, reflecting in the small number of heterozygous informative parents in our studies. Therefore, our findings require additional investigation before they can be extrapolated to a clinical setting.

The estimated attributable risk for the G allele of CL/P of 16.69% for the *IRF6* 820G allele in the Thai population is in accordance with the attributable risk of 11.6% in the Filipino population.⁵ Although the *IRF6* 820A allele is very rare or absent among Europeans (inhibiting the estimation of the attributable risk of the polymorphism), a recent study in an Italian population showed a strong evidence of linkage disequilibrium between polymorphisms at the *IRF6* locus and non-syndromic CL/P.⁶ These findings confirm the contribution of the gene in the aetiology of non-syndromic CL/P in several populations.

The *IRF6* 820G→A is the substitution of an isoleucine for an evolutionarily conserved valine residue at codon 274 in a protein binding domain, termed SMIR (Smad-interferon regulatory factor binding domain).¹² Although the exact functions of *IRF6* are still unknown, previous data suggest it plays a role in the transforming growth factor β signalling pathway.³ The A allele, which is the protective allele, is specific to humans, implying that it was selected for in human evolution.¹³ Owing to the commonness of the G allele in human populations, genetic changes in *IRF6* are the most common known genetic cause of this birth defect.

In summary, our findings confirm that *IRF6* G→A is associated with CL/P. This improves our understanding of the birth defect, has implications for the genetic counselling of families with children who have cleft lip, and provides a lead for identifying other genes or environmental factors linked to CL/P.

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