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Association of Single-Nucleotide Polymorphisms of *MAFB* Gene with Nonsyndromic Cleft Lip with or without Cleft Palate in Kinh Vietnamese Patients

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Abstract

Background Cleft lip with or without palate (CL/P) is the most common orofacial birth defect. Single-nucleotide polymorphisms (SNPs) in *MAFB* gene (V-Maf avian musculoaponeurotic fibrosarcoma oncogene homolog B) were identified as susceptible to this defect in a genome-wide association study. To further evaluate its role in this birth defect, we conducted this study with the aim of identifying allele frequencies, genotype frequencies, and association of SNPs rs13041247, rs6065259, and rs6072081 of *MAFB* gene with nonsyndromic cleft lip/palate (NCL/P) in Kinh Vietnamese patients.

Methods We performed case–control study involved 79 patients with NCL/P and 77 healthy controls. DNAs were extracted from participants' saliva and tetra-amplification refractory mutation system polymerase chain reaction (tetra-ARMS PCR) was used for genotyping SNPs.

Results SNPs of *MAFB* gene were genotyped using the Tetra-ARMS PCR method. We found that genotype CT of rs13041247 was associated with an increased risk of NCL/P in Kinh Vietnamese (odds ratio$_{TCTT}$ [OR$_{TC/TT}$] = 1.63, 95% confidence interval [CI] = 0.83–3.19, $p = 0.17$). The G allele genotypes of SNP rs6072081 increase high risk for the malformation, statistically significant result (OR$_{GG/AA}$ = 7.06, 95% CI = 2.13–23.42, $p < 0.001$). There is no clear association between rs6065259 and CL/P (OR$_{AA/GG}$ = 0.75, 95% CI = 0.22–2.50, $p = 0.32$; OR$_{AG/GG}$ = 1.53, 95% CI = 0.79–2.97, $p = 0.32$). When the patients were divided into the phenotypic subgroups, there was a similar significant trend between the patients and controls for all SNPs.

Conclusions Our study provides further evidence of role of *MAFB* gene variations with NCL/P defect in Kinh Vietnamese.

Keywords
- cleft lip
- MAFB
- SNPs
- Vietnamese

Introduction

Cleft lip with or without palate (CL/P) is the most common deformity in maxillofacial region, with incidence ranging from 1/700 to 1/1000 live births worldwide.[1,2] The incidence of this malformation varies among different populations, the highest incidence in Asian and American population with 1/500 births, followed by European population is ~1/1,000, while African population has the lowest incidence, around 1/2,000 births.[1,3,4] This incidence in Kinh Vietnamese is

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1/1,000. The cause of CL/P is complicated, which results from interactions between multiple genetic and environmental risk factors, but to date only 20% of genetic sensitivity has been identified.[5–7] Although there are more than 400 mendelian disorders associated with oral clefts and CL/P can occur in many malformation syndromes, isolated CL/P (nonsyndrome cleft lip with/without palate) constitutes 70% of all cases.[8] A genome-wide association study by Beaty et al identified gene variants near *MAFB* and *ABCA4* in 2010.[9] After that, replication studies from different populations showed confirming evidence, with families of Asian ancestry giving stronger evidence for association with *MAFB* and *ABCA4*, but in some different studies, there are conflicting results.[9–13] *MAFB* gene encodes the basic leucine zipper transcription factor, which plays an important role in the regulation of hematopoietic cell lines.[14] It also functions as an oncogene, responsible for the transformation of MAF myeloma cells.[15] Expression studies support a role for MAFB in palatal development.[9] Therefore, we conduct this study to evaluate the association between single-nucleotide polymorphisms (SNPs) rs13041247, rs6065259, and rs6072081 of *MAFB* gene reported by Beaty et al with nonsyndromic cleft lip and palate in Kinh Vietnamese.

Patients and Methods

Study Population

We collected saliva from 79 cases with NCL/P deformity at University Medical Center, Ho Chi Minh City and 77 controls of healthy children without this malformation at Children's Hospital I, Ho Chi Minh City for a case–control study. The control selection ensures independence, suitable for age, gender and residence with disease group. Saliva samples that do not qualify for DNA extraction were excluded. In this study, the case samples ($n = 79$) were divided into two subgroups: the group of children with only cleft lip (CLO, $n = 29$) and the group with cleft lip and cleft palate (CLP, $n = 50$). We assessed the different effect of target SNPs on the different phenotypes of this malformation.

Saliva Collection, DNA Extraction, and Genotyping

Unstimulated saliva samples were collected by tampon absorbing the patient's oral mucosa, then put into a test tube containing 5 mL saline 0.9%, immediately kept on ice and processed within 2 hours. Genomic DNA was extracted using a GeneJet Whole Blood Genomic DNA Purification Mini Kit (ThermoFisher Scientific, Carlsbad, California, United States) according to the manufacturer's instructions. All DNA samples had an A260/A280 ratio of 1.8 to 2.0 and DNA concentration greater than10 ng/mL.

Target SNPs in *MAFB* gene were genotyped using tetra-ARMS PCR technique.[16] For SNP rs13041247, the components include 0.4 µL of 10 mM forward and reverse outer primers, 0.8 µL of 10 mM forward and reverse inner primers, 0.1 µL Takara Taq HS (Takara Bio, Shiga, Japan), 1.5 µL gDNA, 1.5 µL of 2.5 mM dNTP, 1.5 µL of 10x buffer, and water up to 15 µL. The adjusted steps to PCR (conducted on system Mastercyclervapo.protect, Eppendorf) were as follows:

Table 1 The primers for *MAFB* SNP genotyping by tetra-ARMS PCR technique

SNP	Primers	Prime sequence (5′-3′)
rs13041247	Outer forward	TGGCCTAGTCACAGCTTTGG
	Outer reverse	CAGAGAAGACCAGGACTTAG
	Inner forward	TTCTTGTACTTCCTGGCGGC
	Inner reverse	CTCAGAGATATTAAGTGGCA
rs6072081	Outer forward	ATGGATCTAAGACCAGACAG
	Outer reverse	TTGTTGAACCTCCCTAACAG
	Inner forward	GCACTGCGTGTGTGACCGAC
	Inner reverse	ATTTGGTGCTTATTACCTTA
rs6065259	Outer forward	CAACAGCCTGTCTGGTCTTA
	Outer reverse	ATCATTTCATGTGGCGGAGA
	Inner forward	TGATTCAGGCTGCTTGGTGT
	Inner reverse	ATTTGGTGCTTATTACCCTG

Abbreviations: tetra-ARMS PCR, tetra-amplification refractory mutation system polymerase chain reaction; SNP, single-nucleotide polymorphism.

initialization at 98°C for 3 minutes, and followed by 40 cycles of three steps: denaturation at 98°C for 10 seconds; annealing at 56°C for 20 seconds, 72°C for 1 minute; extension at 72° C for 2 minutes. Primer sequence was displayed in ►**Table 1**.

PCR products were analyzed with 1.5% agarose gel electrophoresis in TBE 1X buffer containing ethidium bromide and the genotypes were determined. In SNP rs13041247, 750 bp PCR product was reference fragment, 576 bp when the C allele was present, and 213 bp when the T allele was present (►**Fig. 1A, B**). In SNP rs6072081, 275 bp PCR product was reference fragment, 150 bp when the A allele was present, and 164 bp when the G allele was present. In SNP

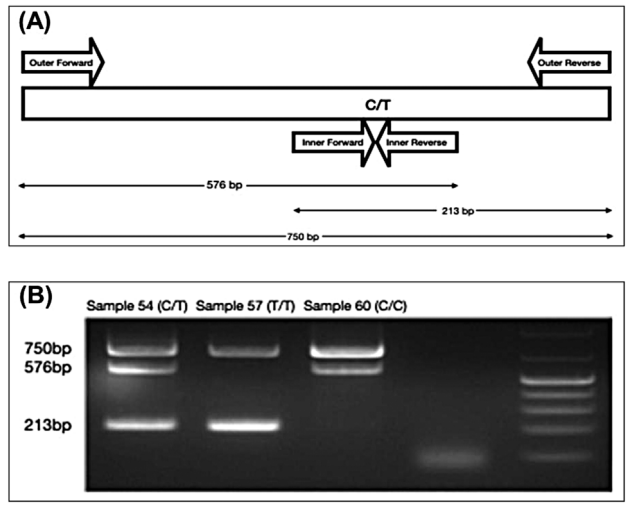

Fig. 1 Schematic illustration of tetra-ARMS PCR assay for SNP rs13041247 genotyping. (**A**) Location of primers and SNP rs13041247. (**B**) Agarose gel electrophoresis image of PCR products. T/T, homozygote for the allele T; C/C, homozygote for the allele C; C/T, heterozygote for the alleles C and T; SNP, single-nucleotide polymorphism; tetra-ARMS PCR, tetra-amplification refractory mutation system polymerase chain reaction.

rs6065259, 717 bp PCR product was reference fragment, 584 bp when the A allele was present, 172 bp when the G allele was present.

Statistical Analysis

We used the chi-squared test to evaluate Hardy–Weinberg equilibrium and difference in allele and genotype frequency between patients and healthy group. Linkage disequilibrium, haplotype analyses, and association analyses of target SNPs were calculated using an online study program.[17] A *p*-value less than 0.05 (two-tailed) was considered statistically significant.

Results

Distribution of Allele and Genotype Frequencies

We genotyped target SNPs by tetra-ARMS PCR technique and calculated allele frequency, genotype frequency in patients with different phenotypic subgroups as well as in controls. Odd ratio (OR) with 95% confidence interval (95% CI) were calculated to determine the association between *MAFB* polymorphisms and NCL/P. The rs13041247 and rs6065259 were in Hardy–Weinberg equilibrium ($P_{case} = 0.45$ and $P_{control} = 0.13$ for rs13041247, $P_{case} = 0.41$ and $P_{control} = 0.3$ for rs6065259),

Table 2 Genotype frequency of *MAFB* tag SNPs between cases and controls with H–W equilibrium

SNP	Genotype	Entire cohort		Cleft lip only		Cleft lip and palate	
		Cases, *n* (%)	Controls, *n* (%)	Cases, *n* (%)	Controls, *n* (%)	Cases, *n* (%)	Controls, *n* (%)
rs13041247	T/T	36 (46)	29 (38)	14 (48)	29 (38)	22 (44)	29 (38)
	C/T	32 (41)	42 (55)	10 (34)	42 (55)	22 (44)	42 (55)
	C/C	11 (14)	6 (8)	5 (17)	6 (8)	6 (12)	6 (8)
	H–W equilibrium *p*-Value	0.45	0.13	0.23	0.13	1.00	0.13
rs6065259	G/G	42 (53)	35 (45)	15 (52)	35 (45)	27 (54)	35 (45)
	G/A	29 (37)	37 (48)	10 (34)	37 (48)	19 (38)	37 (48)
	A/A	8 (10)	5 (6)	4 (14)	5 (6)	4 (8)	5 (6)
	H–W equilibrium *p*-Value	0.41	0.3	0.38	0.3	0.73	0.3
rs6072081	G/G	5 (6)	13 (17)	2 (7)	13 (17)	3 (6)	13 (17)
	G/A	36 (46)	50 (65)	14 (48)	50 (65)	22 (44)	50 (65)
	A/A	38 (48)	14 (18)	13 (45)	14 (18)	25 (50)	14 (18)
	H–W equilibrium *p*-Value	0.43	0.013	0.68	0.013	0.73	0.013

Abbreviations: H–W, Hardy–Weinberg; SNP, single-nucleotide polymorphism.

Table 3 Adjusted OR (95% CI) for association between *MAFB* tag SNPs and CL/P with subgroups

SNP	genotype	Cleft lip / palate		Cleft lip only		Cleft lip and palate	
		OR (95% CI)	*p*-Value	OR (95% CI)	*p*-Value	OR (95% CI)	*p*-Value
rs13041247	T/T	1.00 (Ref)	0.17	1.00 (Ref)	0.13	1.00 (Ref)	0.46
	C/T	1.63 (0.83–3.19)		2.03 (0.79–5.19)		1.45 (0.68–3.09)	
	C/C	0.68 (0.22–2.05)		0.58 (0.15–2.23)		0.76 (0.22–2.67)	
	C/T-C/C	1.39 (0.73–2.63)		1.54 (0.65–3.66)		1.30 (0.63–2.68)	
rs6065259	G/G	1.00 (Ref)	0.32	1.00 (Ref)	0.32	1.00 (Ref)	0.53
	G/A	1.53 (0.79–2.97)		1.59 (0.63–4.00)		1.50 (0.71–3.17)	
	A/A	0.75 (0.22–2.50)		0.54 (0.13–2.28)		0.96 (0.24–3.94)	
	G/A-A/A	1.36 (0.73–2.56)		1.29 (0.55–3.02)		1.41 (0.69–2.88)	
rs6072081	A/A	1.00 (Ref)	<0.001	1.00 (Ref)	0.02	1.00 (Ref)	<0.001
	G/A	3.77 (1.78–7.96)		3.32 (1.27–8.66)		4.06 (1.78–9.25)	
	G/G	7.06 (2.13–23.42)		6.04 (1.14–32.04)		7.74 (1.88–31.87)	
	G/A-G/G	4.17 (2.01–8.64)		3.66 (1.44–9.30)		4.50 (2.02–10.03)	

Abbreviations: CI, confidence interval; CL/P, cleft lip with or without palate; OR, odds ratio; SNP, single-nucleotide polymorphism.

Table 4 Linkage disequilibrium results between each pair of loci

	rs6065259	rs13041247
rs6072081	0.146	0.140
	0.812	0.664
	0.655	0.603
	0.429	0.364
	<0.001	<0.001
	156	156
rs6065259	D	0.158
	D'[a]	0.817
	R	0.726
	R^{2}[b]	0.527
	p-Value	<0.001
	N	156

Abbreviations: SNPs, single-nucleotide polymorphisms.
[a]D' represents levels of linkage disequilibrium.
[b]R^2 represents a correlation between two SNPs.

but the other rs6072081 was in disequilibrium with $P_{control} = 0.013$. The result was similar in CLO and CLP subgroups (►**Table 2**). As shown in ►**Table 3**, even though the genotype TC of rs13041247 was associated with a trend of cleft lip and palate in Kinh Vietnamese ($OR_{TC/TT} = 1.63$, 95% CI = 0.83–3.19, $p = 0.17$), this was not statistically significant ($p > 0.05$). The rs6072081 significantly increased risk of the malformation ($OR_{GG/AA} = 7.06$, 95% CI = 2.13–23.42, $p < 0.001$). There was no clear evidence of association between rs6065259 and NCL/P ($OR_{AA/GG} = 0.75$, 95% CI = 0.22–2.50, $p = 0.32$; $OR_{AG/GG} = 1.53$, 95% CI = 0.79–2.97, $p = 0.32$). There was a similar trend between subgroups and controls when the patients were divided into the phenotypic CLO and CLP subgroups for all target SNPs.

Analysis of Haplotype

Analyses of linkage disequilibrium between target SNPs showed that rs13041247, rs6065259, and rs6072081 were weakly pairwise linked ($0.3 < R^2 < 0.6$) (►**Table 4**). We performed haplotype analysis of target SNPs on eight probable haplotype models and found that the risk of developing NCL/P was increased by 2.02 times (OR = 2.02, 95% CI = 1.07–3.84, $p = 0.033$) with haplotype CAG (►**Table 5**). Haplotype TGG increased the risk up to 67.22 times (OR = 67.22, 95% CI = 7.11–635.45, $p < 0.001$). The results obtained were statistically significant with *p*-value < 0.05 and 95% CI > 1.

Discussion

Saliva sampling for DNA extraction is a less invasive and easier method than taking blood, and tetra-ARMS PCR technique is a low-cost, fast, and reliable method of genotyping that is suitable for developing country conditions. The Hardy-Weinberg equilibrium analysis showed that rs13041247 and rs6065259 had *p*-values greater than 0.05 in both the case and control groups, indicating that these SNPs had a balanced distribution of genotype and allele frequency in the Kinh Vietnamese population. However, with rs6072081, only the disease group was in Hardy–Weinberg equilibrium, but not in the control group (►**Table 2**). We found that there were not statistically significant association between rs13041247 and rs6065259 with the malformation. Only with rs6072081, all genotypes other than G/G increase the risk of birth defect, and there was a similar significant trend in phenotypic subgroups (►**Table 3**). These results were clearly different from those of Mi's, Beaty's, and Pan's, and Yuan's, and Fontoura's reports.[9,18] In Mi's study, rs6065259 was the most important SNP in *MAFB*, followed by rs13041247.[18] In Beaty's study, rs13041247 was the most important SNP and associated with a decreased risk of the birth defect.[9] In Pan's study, rs13041247 CT, CC, and CT/CC were associated with decreased nonsyndromic orofacial clefts susceptibility, compared with rs13041247 TT wide-type homozygote in a Chinese Han population.[12] In Yuan's study, marginal associations were detected for rs13041247 of *MAFB* in the Hispanic dataset, while no association was found for the non-Hispanic white dataset.[13] In Fontoura's study, no association was found between *MAFB* and CL/P in Caucasian individuals from Brazil.[10] This suggests that racial differences may lead to different genetic research results. The results from haplotype model

Table 5 Association between *MAFB* haplotypes (rs13041247-rs6065259-rs6072081) and CL/P with phenotypic subgroups

Haplotype			Cleft lip with/without palate		Cleft lip only		Cleft lip and palate	
			OR (95% CI)	*p*-Value	OR (95% CI)	*p*-Value	OR (95% CI)	*p*-Value
T	G	A	1.00	—	1.00	—	1.00	—
C	A	G	2.02 (1.07–3.84)	0.03	1.98 (0.83–4.72)	0.13	2.14 (1.02–4.51)	0.05
T	G	G	67.22 (7.11–635.45)	<0.001	22.40 (2.35–213.57)	0.01	116×10^{9}	<0.001
C	G	A	0.92 (0.25–3.41)	0.90	0.97 (0.17–5.38)	0.97	0.94 (0.22–4.01)	0.94
C	G	G	2.94 (0.68–12.69)	0.15	2.45 (0.36–16.83)	0.36	3.34 (0.64–17.29)	0.15
T	A	G	0.27 (0.01–8.82)	0.47	0.29 (0.01–7.35)	0.45	0.77 (0.04–16.12)	0.87
C	A	A	1.01 (0.19–5.42)	0.99	0.84 (0.14–4.89)	0.84	1.42 (0.15–13.11)	0.76
T	A	A	8.04 (0.39–165.61)	0.18	2.61 (0.13–51.39)	0.53	—	—

Abbreviations: CI, confidence interval; CL/P, cleft lip with or without palate; OR, odds ratio.

analysis of target SNPs showed a significant increase in risk compared with individual analysis of those. This indicates that SNPs can affect the birth defect following the polygenic genetic model and that risk factor for haplotype analysis was much higher than when assessing the individual effects of each target SNP. There was a similar result when the cases were divided into the phenotypic subgroups (►**Table 5**).

Conclusions:

In conclusion, although our study is limited by sample size, the results provided preliminary evidences of the association of *MAFB* polymorphism with NCL/P in Kinh Vietnamese. The results of the study have contributed to understanding of the genetic factors of maxillofacial congenital malformation in Vietnamese children, as well as facilitating the implementation of molecular biology techniques in the condition of Vietnam. In the future, we will conduct more research on the genetic factors and genetic–environment correlation of the most common orofacial birth defect, which currently only 20% of genetic sensitivity has been identified.

Ethics Statement

The study was reviewed and approved by the Ethics Committee of University of Medicine and Pharmacy at Ho Chi Minh City. Each participant provided informed consent before enrolling in the study.

Conflict of Interest

None declared.

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