IRF6 rs2235371 as a risk factor for non-syndromic cleft palate only among the Deutero-Malay race in Indonesia and its effect on the *IRF6* mRNA expression level

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Abstract

Background. During the early embryological development of the face, complex orofacial failure results in a non-syndromic cleft lip and palate (NS CLP). The interferon regulatory factor 6 gene (*IRF6*) rs2235371 is a non-synonymous polymorphism that is one of the strong candidate genes associated with NS CLP.

Objectives. The purpose of this study was to determine *IRF6* rs2235371 as a risk factor for NS CLP and its phenotypes, including complete unilateral cleft lip and palate (CUCLP), bilateral cleft lip and palate (BCLP), cleft lip only (CL), and cleft palate only (CP), as well as to examine the effect of the polymorphism on the *IRF6* mRNA expression levels among the Deutero-Malay race in Indonesia.

Material and methods. This study used a case–control design and enrolled 264 samples, including 158 NS CLP cases (42 NS CUCLP, 34 NS BCLP, 33 NS CL, and 49 NS CP) and 106 control subjects. DNA was extracted from venous blood, and then subjected to polymerase chain reaction (PCR) and sequencing. The odds ratio (*OR*) was used to determine the risk factor for NS CLP and its phenotypes. The Livak, Kruskal–Wallis and Mann–Whitney *U* tests were used to determine mRNA expression levels in the oral epithelium, followed by real-time quantitative PCR (RT-qPCR).

Results. Among all of the NS CLP cases, in the NS CP phenotype, *OR* for the A mutant allele and the GA genotype was 2,492 ($p = 0.017$) and 2,114 ($p = 0.048$), respectively. The *IRF6* mRNA expression level of the GA genotype was higher in the NS CP subjects as compared to the GG genotype ($p = 0.031$).

Conclusions. The *IRF6* rs2235371 polymorphism is associated with the NS CP phenotype in Deutero-Malay patients from Indonesia and it affects the *IRF6* mRNA expression level.

Keywords: cleft lip and palate, cleft palate, *IRF6*, rs2235371, *IRF6* mRNA expression level

Introduction

Non-syndromic cleft lip and palate (NS CLP) is caused by the inability of any of the independently formed facial primordia of the orofacial complex to fuse completely during the early embryological development of the face.[1] It is a common congenital defect with a prevalence that varies according to the geographic region, the ethnic origin and the socioeconomic position.[2] The etiology of NS CLP is thought to be multifactorial, with several genetic and environmental factors combined, among which genetic factors need to be primarily analyzed. Non-syndromic cleft lip and palate exhibits complex phenotypes and is indicative of a breakdown in the standard mechanisms of the embryological process.[3] Cleft lip and palate cases are frequently classified into 2 groups, based on anatomical, genetic and embryological findings: those that involve the lip or the palate (CL/P) and those that involve only the palate (CP). Clefts are classified as syndromic or non-syndromic, based on the presence or absence of other physical or developmental anomalies in the affected individuals.[4]

The distinct embryological and pathophysiological mechanisms underlying NS CLP have also been reflected in classifying the condition into primary and secondary palate clefts. The primary palate comprises the maxillary lip, the alveolar process and the hard palate anterior to the incisive foramen.[5] The secondary palate is formed of tissues located posterior to the incisive foramen and extending to the hard and soft palates. Molecular evidence supports etiologic distinctions between the CLP subtypes – cleft lip only (CP) and cleft palate only (CP) – as well as the NS CLP phenotypes.[1]

Cleft lip only and CLP are not included in the CP mechanism, as there is compelling evidence that they are developmentally and genetically distinct from CP in most cases. They should be investigated independently and phenotyping is supposed to shed light on the previously unknown relationships.[6] Additionally, clefts can affect one or both halves of the oral cavity, and are thus classified as unilateral or bilateral clefts.[7] Bilateral cleft lip and palate (BCLP) is the most severe form of NS CLP and is thought to follow a genetic pattern that is distinct from other forms of NS CLP. Being one of the most prevalent anomalies, NS CLP is caused by genetic and environmental factors, neither of which has been fully elucidated due to the complex etiology of the disease.[8]

Several candidate genes are associated with an increased risk of NS CLP, including *RARA*, *PAX9*, *MTHFR*, *MSX1*, *BMP4*, *TGFB3*, *TGFA*, and *IRF6*. Interferon regulatory factor 6 (IRF6) is a transcription factor; a mutation in the gene *IRF6* is also known to cause van der Woude syndrome, which is the autosomal dominant condition of CLP with lip pits. *IRF6* is located on 1q32.2, with a highly conserved N-terminal DNA-binding domain (DBD) and a less conserved C-terminal Smad/IRF (SMIR)/interferon association domain (IAD) that has been associated with NS CLP in some populations through *IRF6* polymorphisms.[9] *IRF6* rs2235371 (V274I) is a single nucleotide polymorphism (SNP) in which valine is replaced by isoleucine at position 274 (exon 7) in the *IRF6* SMIR/IAD. It is associated with NS CLP in various populations.[10] Valine found at this location in *IRF6* is highly conserved across species. Previous research hypothesized that *IRF6* rs2235371 might affect the gene function and contribute to NS CLP.[11]

The *IRF6* rs2235371 polymorphism was the first marker in *IRF6* to be associated with NS CLP, most notably in the Asian (particularly East Asian) and South American populations, but not in Europeans, simply because the allele encoding isoleucine is absent or extremely rare in European people, with a maximum frequency of only 2%.[11] In genomic surveys, it was discovered that the polymorphisms affecting transcription and mRNA processing played a significant role in human phenotypic diversity and disease susceptibility. However, the majority of these polymorphisms remain unknown.[12]

We studied the influence of the *IRF6* rs2235371 polymorphism in patients with NS CLP of various phenotypes, such as complete unilateral cleft lip and palate (CUCLP), BCLP, CL, and CP, as well as in control subjects among Indonesians of the Deutero-Malay race. *IRF6* rs2235371 is a non-synonymous polymorphism that alters the amino acid (valine into isoleucine) functionally. A change in the mRNA conformation may affect the protein structure, activity and function, causing disease.[13] Understanding the impact of the polymorphism on the gene function may help discover the causal genes responsible for the phenotypes of NS CLP and other human diseases.

Material and methods

The Faculty of Medicine of Padjadjaran University, Bandung, Indonesia, has granted ethical clearance under ref. No. 395/UN6/C.1.2.3/KEPK/PN/2016. The entire sample was Deutero-Malay, as it is the major race in Indonesia. Only individuals diagnosed with a non-syndromic form of CLP were included in this study. A total of 246 subjects were enrolled; there were 158 NS CLP cases of various phenotypes (NS CUCLP – 42, NS BCLP – 34, NS CL – 33, and NS CP – 49) and 106 healthy controls. The study was conducted at the Molecular Genetic Laboratory of Padjadjaran University/Hasan Sadikin Hospital in Bandung, Indonesia. The subjects were divided into NS CUCLP and NS BCLP groups, as the authors assumed that since they presented different laterality and disease severity, they might have different genetic backgrounds. One of the hypotheses used to explain variable phenotypes in genetic disorders is frequently referred to as a genotype–phenotype correlation, in which the particular genotype is associated with the particular phenotype or the cleft side.[14]

Genotyping assay

DNA was extracted from each subject's venous blood by using DNA extraction kits (Sigma-Aldrich/Merck, Darmstadt, Germany). After obtaining written informed consent, venous blood samples were collected, DNA was extracted and the *IRF6* rs2235371 segment was amplified using polymerase chain reaction (PCR). The PCR analysis was performed using the primers of forward: 5'-CAGGGCTGCCGACTCTTCTA-3' and reverse: 5'-AGGAAAGCAGGAAGGTGAAAGA-3'.[15] The Sanger dideoxy method was used to perform DNA sequencing on *IRF6* rs2235371 in comparison with a standard nucleotide. All nucleotides in those segments were compared to a standard nucleotide in the gene bank, using a sequence alignment program from BioEdit, v. 7.2 (https://bioedit.software.informer.com/).[16] The polymorphism occurs when base G is replaced with A, resulting in 3 genotypes: GG (wild monozygous genotype); GA (heterozygous mutated genotype); and AA (homozygous mutated genotype).

RT-qPCR

After written informed consent was obtained from all subjects, oral epithelial cells from the cleft side were gathered from the NS CP subjects. For control subjects, oral epithelial cells were gathered from the palatal epithelium. All epithelial cells were collected using the smear method, and then stored in small tubes filled with RNA. Total RNA was extracted from those palatal epithelial cells with the TRIzol® reagent (Invitrogen, Waltham, USA) and the concentration was determined using the NanoDrop™ 2000c spectrophotometer (Thermo Scientific, Waltham, USA). Total RNA samples with adequate purity ratios (A260/A280: 1.9–2.1) were then processed with real-time quantitative PCR (RT-qPCR).[17] RNA was converted into cDNA by using an oligo (dT) primer and SuperScript II™ Reverse Transcriptase (Invitrogen). Internally, *GAPDH* was used as a reference gene. The relative mRNA expression of *IRF6* was quantified using RT-qPCR with the SYBR Green™ method (SensiFAST™ SYBR; Bioline USA, Memphis, USA). The primers for *IRF6* were 5'-CGGCATAGCCCTCAACAAGAA-3' and 5'-TCCTTGGTGCCATCATACATCAG-3', and for *GAPDH*, they were 5'-TGCTGAGTATGTCGTGGAG-3' and 5'-GTCTTCTGAGTGGCAGTGAT-3'.[15]

Statistical analysis

Significant differences in the frequency of sequence variants between NS CLP subjects, various NS CLP phenotypes (NS CUCLP, NS BCLP, NS CL, and NS CP) and control subjects were determined using the statistical analysis. The odds ratio (*OR*) was used to identify the risk factor for NS CLP and its phenotypes. Differences in the *IRF6* mRNA expression between the NS CP patients with various genotypes (GG, GA and AA) and controls were then analyzed using the Livak method. Changes in the *IRF6* mRNA expression were determined using the comparative CT method ($2^{-\Delta\Delta Ct}$). The statistical analysis involved the Kruskal–Wallis test, followed by the Mann–Whitney *U* test to compare the groups. The IBM SPSS Statistics for Windows software, v. 23.0 (IBM Corp., Armonk, USA) was used, with a p-value <0.05 considered as statistically significant.

Results

Figure 1 presents the initial PCR product that showed a DNA band of 413 base pairs (bp). After obtaining the initial PCR products of 413 bp, the samples were analyzed with the Sanger dideoxy sequencing method. The sequencing results for all subjects showed the GG, GA and AA genotypes (Fig. 2). Figure 3 shows that among the NC CP subjects, the GA heterozygous mutated genotype presented a higher level of the *IRF6* mRNA expression (overexpression) (36.41 ±85.70) as compared to the GG genotype (3.77 ±23.76) (p = 0.031).

There were no statistically significant differences between the alleles and the genotypes in terms of an increased risk of the disease in NS CLP (in total), NS CUCLP and NS BCLP subjects as compared to controls (Tables 1–3). *IRF6* rs2235371 was not a risk factor for NS CLP in general. *IRF6* rs2235371 was also analyzed

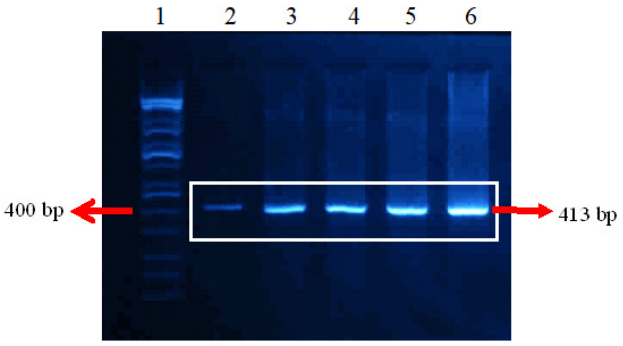

Fig. 1. Initial polymerase chain reaction (PCR) product of the *IRF6* rs2235371 segment

Line 1 – 100 base pairs (bp) ladder; lines 2–6 – initial PCR product.

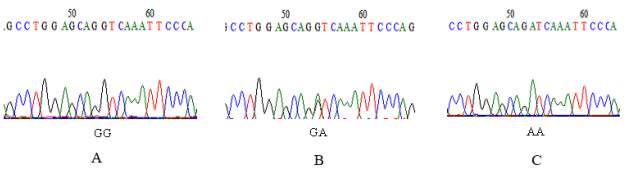

Fig. 2. Sequencing results showing 3 genotypes

A – GG genotype; B – GA genotype; C – AA genotype.

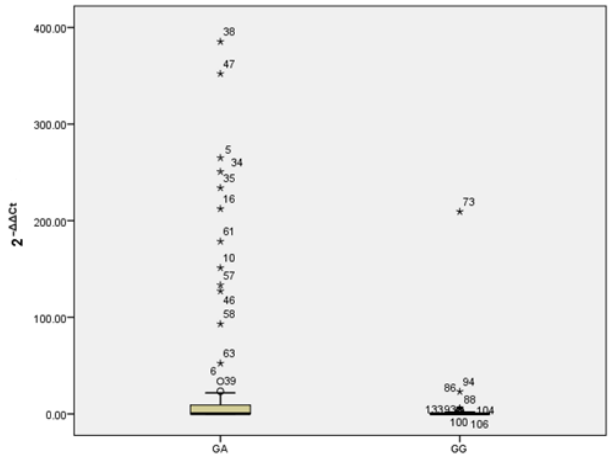

Fig. 3. Significant association between the GA and GG genotypes of the non-syndromic cleft palate only (NS CP) group and the *IRF6* mRNA expression ($2^{-\Delta\Delta Ct}$); the GA genotype shows significant mRNA overexpression as compared to the GG genotype

in the NS CUCLP phenotype and it turned out that *IRF6* rs2235371 was not a risk factor for NS CUCLP. As far as the NS BCLP phenotype is concerned, which is the most severe type of NS CLP, *IRF6* rs2235371 proved not to be a risk factor for NS BCLP as well. Table 4 shows that *IRF6* rs2235371 was not a risk factor for the NS CL phenotype as the most straightforward type of NS CLP. The AA genotype was not found in the NS CL phenotype subjects. However, in the case of the NS CP phenotype, it was shown that *IRF6* rs2235371 was a risk factor for NS CP, especially in the presence of the A mutant allele and the GA heterozygous mutated genotype, as the results were statistically significant (Table 5). Table 6 shows the influence of the GA genotype on the *IRF6* mRNA expression level among the NS CP subjects. The epithelial cells were taken from the oral mucosa, and then evaluated by means of RT-qPCR. The GA heterozygous mutated genotype as a risk factor for NS CP showed a statistically significant difference in terms of *IRF6* mRNA expression as compared to the normal GG genotype (Table 6). We did not analyze the *IRF6* mRNA expression levels for the AA genotype, since this genotype did not prove to be the risk factor associated with NS CP. Also, as *IRF6* rs2235371 was a risk factor for NS CP, especially in the case of the A allele and the GA genotype, we only compared the *IRF6* mRNA expression in the NS CP phenotype with the controls.

Table 1. Allele and genotype frequency of nucleotides G and A from *IRF6* rs2235371 in the non-syndromic cleft lip and palate (NS CLP) (in total) subjects

Allele/Genotype	NS CLP *n* (%)	Control *n* (%)	χ^2	*p*-value	*OR*	95% *CI*
G	229 (72.47)	157 (74.06)	0.000	1.000	1.046	0.385–2.841
A	87 (27.53)	55 (25.94)	0.180	0.671	1.149	0.701–1.882
GG	81 (51.27)	58 (54.72)	0.180	0.671	0.871	0.531–1.426
GA	67 (42.41)	41 (38.68)	0.226	0.634	1.167	0.706–1.929
AA	10 (6.33)	7 (6.60)	0.000	1.000	0.956	0.352–2.595

n – number; *OR* – odds ratio; *CI* – confidence interval; G – wild type allele; A – mutant allele; GG – wild monozygous genotype; GA – heterozygous mutated genotype; AA – homozygous mutated genotype.

Table 2. Allele and genotype frequency of nucleotides G and A from *IRF6* rs2235371 in the non-syndromic complete unilateral cleft lip and palate (NS CUCLP) subjects

Allele/Genotype	NS CUCLP *n* (%)	Control *n* (%)	χ^2	*p*-value	*OR*	95% *CI*
G	56 (66.67)	157 (74.06)	0.000	1.000	0.919	0.226–3.736
A	28 (33.33)	55 (25.94)	1.904	0.168	1.777	0.860–3.670
GG	17 (40.48)	58 (54.72)	1.904	0.168	0.563	0.273–1.162
GA	22 (52.38)	41 (38.68)	1.784	0.182	1.744	0.848–3.585
AA	3 (7.14)	7 (6.60)	0.000	1.000	1.088	0.268–4.422

Table 3. Allele and genotype frequency of nucleotides G and A from *IRF6* rs2235371 in the non-syndromic bilateral cleft lip and palate (NS BCLP) subjects

Allele/Genotype	NS BCLP *n* (%)	Control *n* (%)	χ^2	*p*-value	*OR*	95% *CI*
G	56 (82.35)	157 (74.06)	0.000	1.000	1.131	0.224–5.724
A	12 (17.65)	55 (25.94)	2.058	0.151	0.503	0.219–1.156
GG	24 (70.59)	58 (54.72)	2.058	0.151	1.986	0.865–4.559
GA	8 (23.53)	41 (38.68)	1.974	0.160	0.488	0.202–1.180
AA	2 (5.88)	7 (6.60)	0.000	1.000	0.884	0.175–4.472

Table 4. Allele and genotype frequency of nucleotides G and A from *IRF6* rs2235371 in the non-syndromic cleft lip only (NS CL) subjects

Allele/Genotype	NS CL *n* (%)	Control *n* (%)	χ^2	*p*-value	*OR*	95% *CI*
G	57 (86.36)	157 (74.06)	1.122	0.290	–	–
A	9 (13.64)	55 (25.94)	2.671	0.102	0.453	0.192–1.067
GG	24 (72.73)	58 (54.72)	2.671	0.102	2.207	0.937–5.196
GA	9 (27.27)	41 (38.68)	0.969	0.325	0.595	0.252–1.405
AA	–	7 (6.60)	1.122	0.290	–	–

Table 5. Allele and genotype frequency of nucleotides G and A from *IRF6* rs2235371 in the non-syndromic cleft palate only (NS CP) subjects

Allele/Genotype	NS CP *n* (%)	Control *n* (%)	χ^2	*p*-value	*OR*	95% *CI*
G	60 (61.22)	157 (74.06)	0.209	0.648	0.622	0.187–2.069
A	38 (38.78)	55 (25.94)	5.684	0.017*	2.492	1.226–5.064
GG	16 (32.65)	58 (54.72)	5.684	0.017*	0.401	0.197–0.815
GA	28 (57.14)	41 (38.68)	3.908	0.048*	2.114	1.063–4.205
AA	5 (10.20)	7 (6.60)	0.209	0.648	1.607	0.483–5.343

* statistically significant.

Table 6. Comparison of the *IRF6* mRNA expression between the GA and GG genotypes from the non-syndromic cleft palate only (NS CP) group ($2^{-\Delta\Delta Ct}$)

Genotype	*M* ±*SD*	*Me*	Range	*p*-value
GA	36.42 ±85.70	0.27	0.00–385.34	0.031*
GG	3.77 ±23.79	0.08	0.00–209.38	

M – mean; *SD* – standard deviation; *Me* – median; * statistically significant.

Tables 5,6 and Fig. 3 report only the NS CP subjects to present significant results in the case of the A allele (*OR*: 2.492; 95% confidence interval (*CI*): 1.226–5.064; $p = 0.017$) and the GA genotype (*OR*: 2.114; 95% *CI*: 1.063–4.205); $p = 0.048$). The subjects with NS CP and the controls who were carrying the GA genotype presented a higher level of the *IRF6* mRNA expression (overexpression) (36.41 ±85.70) as compared to the subjects with the GG genotype (3.77 ±23.76) ($p = 0.031$)

The allelic frequency of the G normal allele and the A mutant allele, and the frequency of the GG, GA and AA genotypes among the NS CLP subjects, as well as for each phenotype (NS CUCLP, NS BCLP, NS CL, and NS CP), and control subjects were compared using the χ^2 test (Tables 1–5).

Discussion

The *IRF6* gene is located on chromosome 1q32.2 and consists of 9 exons. It is involved in epidermal differentiation, as mice lacking *IRF6* have a hyperproliferative epidermis that fails to differentiate terminally. *IRF6* controls the transition between proliferation and differentiation. The loss of the gene function or a mutation/polymorphism results in the incomplete fusion of palatal shelves due to failure in the differentiation of the palatal medial edge epithelial (MEE) cells through the epithelial–mesenchymal transformation (EMT).[18]

IRF6 rs2235371 is a polymorphism in the exon region caused by exonic changes that are translated into protein. Polymorphisms in the exon region can be either non-synonymous (protein-changing) or synonymous (non-protein-changing). A non-synonymous polymorphism alters the amino acid sequence within the protein structure and affects the normal protein function as a transcription factor. We assumed that *IRF6* rs2235371 would change protein conformation and disrupt the gene functionally. Hence, failure in the terminal differentiation of MEE cells would occur, leading to an undifferentiated hyperproliferative epidermis.[19]

Numerous studies have demonstrated that *IRF6* polymorphisms may play a significant role in CL, implying that patients with CL may be more susceptible to genetic changes in *IRF6* than patients with CLP or CP. Additionally, this evidence supports the hypothesis that CL is genetically and developmentally distinct from CLP and CP.[1] This finding contrasts with our present study, which found no significant association between *IRF6* rs2235371 and CL. In contrast to some previous studies, our current study discovered a significant association between *IRF6* rs2235371 and CP. Previous studies have not completely defined the association of *IRF6* rs2235371 with NS CLP phenotypes.

The rs2235371 in *IRF6* is a missense mutation in the protein binding domain (PBD) or SMIR/IAD of *IRF6*. In contrast to what has been observed in the African and European populations, this SNP is highly polymorphic in the Asian population. However, since it is located in PBD or SMIR/IAD, the function of *IRF6* rs2235371 cannot be fully explained.[20] According to a previous study, only one mutation, p.Phe375Ser, is located in the core of PBD and significantly affects the domain folding and function.[21] The transition

from a more considerable hydrophilic residue to a minor hydrophilic residue eliminates many of the stabilizing hydrophobic interactions of the phenylalanine side chain.[21]

In Japan, China, Vietnam, Philippines, South America, and Iran, but not in Europe or India, *IRF6* rs2235371 has been observed to be significantly associated with NS CLP.[22] A study conducted by Park et al. revealed that while *IRF6* rs2235371 was not a causal polymorphism for NS CLP, it appeared to interact and be in linkage disequilibrium (LD) with other *IRF6* polymorphisms that were causal polymorphisms for NS CLP.[10] This was also observed in the Mexican population.[10] A study by Tang et al. revealed that the association between *IRF6* rs2235371 and NS CLP had been extensively studied in Chinese people, with mixed results.[23] Similar to our findings, the A allele of *IRF6* rs2235371 might increase the risk of developing CP in Chinese subjects.[23] In comparison, another study conducted in China discovered that the A allele was more prevalent in controls and appeared to be protective according to the haplotype analysis; this type of analysis is usually used to investigate various population phenomena, including migration and immigration rates, the strength of LD, and population relatedness.[24] Zucchero et al. found that the carriers of the G allele (Val274) of *IRF6* rs2235371 were at higher risk of the recurrence of NS CLP.[25] They established strong evidence for excessive valine allele transmission from the parent to the affected child in CLP families.[25] In comparison, Suazo et al. reported that the C allele of rs2235375 from the *IRF6* gene seemed to be a risk factor for NS CLP in the Chilean population.[26] Furthermore, a genetic variant of *IRF6* might be a protective factor against NS CLP, while the *Rsa1* gene variant (the A allele) could be considered the risk factor associated with the development of NS CLP.[27] Individuals heterozygous for this specific polymorphism (the GA genotype) are at lower risk of the recurrence of CLP than those who are homozygous for the G allele (the GG genotype).[27] The disparate findings may result from diverse ethnic origins, environmental differences, anthropological diversity, different research methods, and the complex genetic etiology of the disease.[28]

According to our present findings, *IRF6* rs2235371 appears to play a functional role in the development of the secondary palate, as it was a risk factor for NS CP in both the A allele (*OR*: 2.492; 95% *CI*: 1.226–5.064); $p = 0.017$) and the GA genotype (*OR*: 2.114; 95% *CI*: 1.063–4.205; $p = 0.048$) groups. However, since the precise biochemical role of *IRF6* rs2235371 in the etiology of NS CP is unknown, additional experimental studies are necessary to establish a direct functional link between *IRF6* and NS CP. *IRF6* mutant mice develop a hyperproliferative epidermis that fails to differentiate terminally, resulting in incomplete soft tissue fusion, indicating that *IRF6* is the critical determinant of the keratinocyte proliferation–differentiation switch.[29] Thus, future experiments may focus on the role of the *IRF6* exon region as a coding region in the proliferation–differentiation switch of keratinocytes during the development of the secondary palate, and whether such exonic changes cause protein damage or dysfunction. Mutations in the *IRF6* gene have been identified as the cause of van der Woude syndrome and mutations in the poliovirus receptor-related 1 gene (*PVRL1*) have been identified as the cause of autosomal recessive ectodermal dysplasia syndrome, which is associated with clefting.[30]

The level of mRNA expression can be considered an intermediate phenotype, and correlating the polymorphism and the mRNA expression level is critical for elucidating the physiological traits associated with human diseases.[31] Polymorphisms in coding genes, particularly non-synonymous polymorphisms, such as *IRF6* rs2235371, can significantly affect protein regulation as compared to non-coding genes, as they alter the structure and function of the resulting peptide due to the altered chemical and physical properties of the altered forms of mRNA secondary structure. However, the effect of non-synonymous polymorphisms on protein expression via mRNA structural modification is essentially unknown.[32]

The *IRF6* rs2235371 (c.820G>A) polymorphism in the exon region may alter the amino acid sequence translated into the protein structure, and thus affect the normal function of the transcription factor.[33] Such exonic changes may also influence mRNA stability, thereby affecting the translational efficiency of the gene. However, the functional significance of *IRF6* rs2235371, which considerably affects the *IRF6* mRNA expression level when associated with the NS CP phenotype, has not been defined. We assumed that *IRF6* rs2235371 would change protein conformation and disrupt the gene functionally, which was confirmed in this study by the observed overexpression of the gene. Thus, MEE cells could not reach terminal differentiation, which resulted in an undifferentiated hyperproliferative epidermis.

Conclusions

The *IRF6* gene variant rs2235371 is considered a risk factor for the NS CP phenotype in the Deutero-Malay race of Indonesian patients. The *IRF6* mRNA expression level in NS CP and control cells reveals that the GA genotype is overexpressed ($p = 0.031$). It demonstrates that the GA genotype may induce *IRF6* mRNA expression alterations. A further genome-wide approach, such as exclusive exon screening in the *IRF6* gene, could be an excellent way to follow up on the findings of the present study, particularly in defining the precise role of this gene in NC CP pathogenesis.

Ethics approval and consent to participate

The study was approved by the institutional Research Ethics Committee (No. of approval 395/UN6/C.1.2.3/KEPK/PN/2016) and all participants provided written informed consent prior to the investigation.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

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References

1. Rahimov F, Jugessur A, Murray JC. Genetics of nonsyndromic orofacial clefts. *Cleft Palate Craniofac J*. 2012;49(1):73–91. doi:10.1597/10-178
2. Impellizzeri A, Giannantoni I, Polimeni A, Barbato E, Galluccio G. Epidemiological characteristic of orofacial clefts and its associated congenital anomalies: Retrospective study. *BMC Oral Health*. 2019;19(1):290. doi:10.1186/s12903-019-0980-5
3. Dixon MJ, Marazita ML, Beaty TH, Murray JC. Cleft lip and palate: Understanding genetic and environmental influences. *Nat Rev Genet*. 2011;12(3):167–178. doi:10.1038/nrg2933
4. Curtis SW, Chang D, Lee MK, et al. The *PAX1* locus at 20p11 is a potential genetic modifier for bilateral cleft lip. *HGG Adv*. 2021;2(2):100025. doi:10.1016/j.xhgg.2021.100025
5. Kosowski TR, Weathers WM, Wolfswinkel EM, Ridgway EB. Cleft palate. *Semin Plast Surg*. 2012;26(4):164–169. doi:10.1055/s-0033-1333883
6. Huang L, Jia Z, Shi Y, et al. Genetic factors define CPO and CLO subtypes of nonsyndromicorofacial cleft. *PLoS Genet*. 2019;15(10):e1008357. doi:10.1371/journal.pgen.1008357
7. de Souza Freitas JA, das Neves LT, Fraga de Almeida AL, et al. Rehabilitative treatment of cleft lip and palate: Experience of the Hospital for Rehabilitation of Craniofacial Anomalies/USP (HRAC/USP) – part 1: overall aspects. *J Appl Oral Sci*. 2012;20(1):9–15. doi:10.1590/s1678-77572012000100003
8. Cobourne MT. The complex genetics of cleft lip and palate. *Eur J Orthod*. 2004;26(1):7–16. doi:10.1093/ejo/26.1.7
9. Zhang M, Huang J, Shi F, et al. Identification of pathogenic variant in a Chinese pedigree affected with non-syndromic cleft lip and palate [in Chinese]. *Zhonghua Yi Xue Yi Chuan Xue Za Zhi*. 2021;38(1):52–55. doi:10.3760/cma.j.cn511374-20200308-00144
10. Park JW, McIntosh I, Hetmanski JB, et al. Association between *IRF6* and non-syndromic cleft lip with or without cleft palate in four populations. *Genet Med*. 2007;9(4):219–227. doi:10.1097/gim.0b013e3180423cca
11. Birnbaum S, Ludwig KU, Reutter H, et al. *IRF6* gene variants in Central European patients with non-syndromic cleft lip with or without cleft palate. *Eur J Oral Sci*. 2009;117(6):766–769. doi:10.1111/j.1600-0722.2009.00680.x
12. Manning KS, Cooper TA. The roles of RNA processing in translating genotype to phenotype. *Nat Rev Mol Cell Biol*. 2017;18(2):102–114. doi:10.1038/nrm.2016.139
13. Zhang Z, Miteva MA, Wang L, Alexov E. Analyzing effects of naturally occurring missense mutations. *Comput Math Methods Med*. 2012;2012:805827. doi:10.1155/2012/805827
14. Goldstein I, Ehrenreich IM. The complex role of genetic background in shaping the effects of spontaneous and induced mutations. *Yeast*. 2021;38(3):187–196. doi:10.1002/yea.3530
15. Pan Y, Ma J, Zhang W, et al. *IRF6* polymorphisms are associated with non-syndromic orofacial clefts in a Chinese Han population. *Am J Med Genet A*. 2010;152A(10):2505–2511. doi:10.1002/ajmg.a.33624
16. Zerbino DR, Achuthan P, Akanni W, et al. Ensembl 2018. *Nucleic Acids Res*. 2018;46(D1):D754–D761. doi:10.1093/nar/gkx1098
17. Ma L, Li Z, Liu K, et al. Association of genetic variants with polypoidal choroidal vasculopathy: A systematic review and updated meta-analysis. *Ophthalmology*. 2015;122(9):1854–1865. doi:10.1016/j.ophtha.2015.05.012
18. Richardson RJ, Dixon J, Malhotra S, et al. *Irf6* is a key determinant of the keratinocyte proliferation–differentiation switch. *Nat Genet*. 2006;38(11):1329–1334. doi:10.1038/ng1894
19. Degen M, Girousi E, Feldmann J, et al. A novel Van der Woude syndrome-causing *IRF6* variant is subject to incomplete non-sense-mediated mRNA decay affecting the phenotype of keratinocytes. *Front Cell Dev Biol*. 2020;8:583115. doi:10.3389/fcell.2020.583115
20. Majewski J, Pastinen T. The study of eQTL variations by RNA-seq: from SNPs to phenotypes. *Trends in Genet*. 2011;27(2):72–79. doi:10.1016/j.tig.2010.10.006
21. Phillips DC, York RL, Mermut O, McCrea KR, Ward RS, Somorjai GA. Side chain, chain length, and sequence effects on amphiphilic peptide adsorption at hydrophobic and hydrophilic surfaces studied by sum-frequency generation vibrational spectroscopy and quartz crystal microbalance. *J Physic Chem C*. 2007;111(1):255–261. doi:10.1021/jp0645263
22. Xia Y, Hu B, Chen J, Zheng L, Song J. Association between the *IRF6* rs2235371 polymorphism and the risk of nonsyndromic cleft lip with or without cleft palate in Chinese Han populations: A meta-analysis. *Arch Oral Biol*. 2017;84:161–168. doi:10.1016/j.archoralbio.2017.09.032
23. Tang W, Du X, Feng F, 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(1):89–92. doi:10.1597/07-131.1
24. Fallin D, Schork NJ. Accuracy of haplotype frequency estimation for biallelic loci, via the expectation–maximization algorithm for unphased diploid genotype data. *Am J Hum Genet*. 2000;67(4):947–959. doi:10.1086/303069
25. Zucchero TM, Cooper ME, Maher BS, et al. Interferon regulatory factor 6 (IRF6) gene variants and the risk of isolated cleft lip or palate. *N Engl J Med*. 2004;351(8):769–780. doi:10.1056/NEJMoa032909
26. 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):1857–1862. doi:10.1007/s43032-020-00203-9
27. Sufiawati I, Maskoen AM, Soeria Soemantri ES. Genetic variation of *IRF6* and *TGFA* genes in an HIV-exposed newborn with non-syndromic cleft lip palate. *Oral Dis*. 2020;26(Suppl 1):165–168. doi:10.1111/odi.13403
28. Mijiti A, Ling W, Guli, Moming A. Association of single-nucleotide polymorphisms in the *IRF6* gene with non-syndromic cleft lip with or without cleft palate in the Xinjiang Uyghur population. *Br J Oral Maxillofac Surg*. 2015;53(3):268–274. doi:10.1016/j.bjoms.2014.12.008
29. Pegelow M, Koillinen H, Magnusson M, 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(1):49–55. doi:10.1597/11-220
30. Cobourne MT. The complex genetics of cleft lip and palate. *Eur J Orthod*. 2004;26(1):7–16. doi:10.1093/ejo/26.1.7
31. Squires KE, Montañez-Miranda C, Pandya RR, Torres MP, Hepler JR. Genetic analysis of rare human variants of regulators of G protein signaling proteins and their role in human physiology and disease. *Pharmacol Rev*. 2018;70(3):446–474. doi:10.1124/pr.117.015354
32. 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(12):3080–3086. doi:10.1002/ajmg.a.35634
33. Stergachis AB, Haugen E, Shafer A, et al. Exonic transcription factor binding directs codon choice and affects protein evolution. *Science*. 2013;342(6164):1367–1372. doi:10.1126/science.1243490