RT-qPCR Gene Expression Analysis on the Irf6 Intron Polymorphism in Oral Epithelium of Non-Syndromic Oral Cleft Risk of Deutero-Malay Sub-Race Indonesian

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Abstract

To analyze the mRNA expression of the IRF6 gene of the oral epithelium of non-syndromic oral cleft risk (NS OC). A group of 198 subjects (136 NSOC and 62 subjects as control) had their blood taken to extract the DNA and RNA. The IRF6 intron polymorphism identified and amplified by PCR, followed by the sequencing method. The IRF6 mRNA expression by qPCR was also analyzed using the Livak method and Mann Whitney analysis. The 136 subjects with NSOC have the PCR product of the DNA band of the IRF6 rs2235373 gene segment is 413 base pairs (bp). These genes are called CT genotype (heterozygous mutant), TT genotype (homozygous mutant), and CC (normal). The qPCR analyses show the expression level of IRF6 mRNA in non-syndromic complete unilateral cleft lip and palate (NS CUCLP) (CT 75%, TT 94%; P<0.001), non-syndromic cleft palate only (NS CPO) (CT 84%, TT 144%-overexpression; p>0.05,), non-syndromic cleft lip only (NS CLO) (CT 96%, TT 0%; P<0.05), and non-syndromic complete bilateral cleft lip and palate (NS CBCLP) (CT 240%, TT, 240%, over-expression; P<0.05). The IRF6 rs2235373 gene can trace the intron polymorphisms in oral epithelium of non-syndromic oral cleft risk. CT and TT genotypes have the same frequency of IRF6 mutation.

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Introduction

Oral cleft (OC) is a common congenital disability estimated to affect 70% or ~1 in 700 individuals worldwide. This is specifically for individuals with non-syndromic (NS) OC with no other apparent cognitive or craniofacial structural abnormalities.[1] The incidence of NSOC involves many factors; genetical and environmental factors are often linked.[2]

Wu-Chou (2013) reported that genetics of NS OC are related to the expression of the gene Interferon Regulatory Factor 6. The *IRF6* gene, located in chromosome 1q32.2 which consists of 9 exons, has been studied as one of the most important genes associated with NS OC and has been believed to be the only gene that has shown a convincing degree of association consistency across studies.[3] This gene was the first factor that was found to be involved in the two autosomal dominant syndromes in clefts lip-palate Van der Woude syndrome (VWS) and pterygium popliteal syndrome (PPS).[4]

The *IRF6* is likely to have a contribution as the risk factor of NSOC, confirmed by the research of Lee (2018) involving several populations and subsequently resumed in both broad genome-wide association studies (GWAS) and meta-analysis.[5] The *IRF6* gene is one of a family of nine IRF genes that code for transcription factors that share a highly conserved helix-turn-helix DNA-binding domain (DBD) and a less conserved C terminal protein-binding Smad-interferon regulatory factor-binding domain (SMIR/IAD) and its function is a crucial determinant to regulate keratinocyte proliferation and differentiation and is essential during orofacial development especially in cell adhesion

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and fusion during lip formation and early palatogenesis.[6] The *IRF6* gene also plays a crucial role in the growth of epithelium that lines the cavities and surfaces of blood vessels and organs throughout the body. Loss of *IRF6* expression during embryonic development has been implicated in VWS, PPS, and NSOC.[7]

The GWAS found the majority of polymorphism of NSOC is intragenic or in non-coding regions, and estimated that 3 to 5 percent of the human DNA will sequence code to produce its protein.[8] The function of many of these polymorphisms in NSOC is unknown, and their role is likely more complicated than coding mutations that can be predicted to damage or alter protein function. In IFR6,[9] IFR6 rs2235373 in the non-coding region is one of the polymorphisms locating in intron 7 (C/T) that associated with NSOC among some different populations worldwide. We are assuming that *IRF6* rs2235373 also correlates with gene expression in mRNA. At the DNA level, the physical position of rs2235373 is at g.20677 C > T, while at the mRNA level, its location is at c.1060 + 37 C > T.[10]

Salahshourifar (2012) reported that the *IRF6* polymorphism leads to an increase in the risk of NSOC and its phenotype, so it is crucial to analyze mRNA expression levels among the genotypes caused by *IRF6* polymorphisms because mRNA polymorphism level can reveal more about the functional role of the gene. The syndromic factor of VWS considers the variability in the phenotypic expression that has an impact on a range of locations, from lip pits alone to bilateral cleft lip and palate.[11] We assume that in NSOC, there is a genotype-phenotype correlation also, which means that every phenotype of NSOC has a different pattern of mRNA expression level in each genotype of a polymorphism. During the postnatal period, *IRF6* is still expressed strongly in oral epithelium.[12] For this study, we assumed that oral palatal epithelium could be analyzed to reveal the functional role of *IRF6* through its intron polymorphism effects on mRNA expression levels, especially in NSOC phenotypes. The expression of *IRF6* rs2235373 as an *IRF6* intron polymorphism in the NS subjects tends to have CU CLP, CPO, CLO, and CB CLP as the predictor of non-syndromic oral cleft risk.

Materials and methods

The ethical clearance was approved by the Medical Faculty, Universitas Padjadjaran, Bandung Indonesia No.reg. 0516040339. This cross-sectional laboratory analytical study collected the venous blood and oral palatal epithelium as the resource of DNA extraction. The subjects of this study were all in the Deutero-malay sub-race Indonesian population in West Java, Indonesia. The 198 subjects had non-syndromic status of CLP consisting of 136 NSOC including CUCLP (n=42), CPO (n=27), CLO (n=33), CB CLP (n=34) and 62 were normal subjects.

DNA Extraction, PCR Amplification, and DNA Sequencing

The DNA was extracted from venous blood of each subject using a DNA isolation kit from Pharmacia, then 200 ng of DNA template used for polymerase chain reaction (PCR) steps in 34 cycles that consisted of: early denaturation 95 ^{o}C 3', second denaturation 95 ^{o}C 30'', annealing 63 0C 30'', and extension 72 ^{o}C 1'. The PCR analysis was used to analyze the intron polymorphism of *IRF6* rs2235373, located in intron 7. PCR was performed by using the primers of forward: 5'-CAGGGCTGCCGACTCTTCTA-3' and reverse: 5'-AGGAAAGCAGGAAGGTGAAAGA-3'.[13] The DNA extracted from venous blood of each subjects using DNA isolation kit from Pharmacia, then 200 ng of DNA template used for Polymerase Chain Reaction (PCR) steps in 34 cycles consist of, early denaturation 95^{o}C 3', second denaturation 95^{o}C 30'', annealing 63 0C 30'', and extension 72^{o}C 1'. The PCR study we used intron polymorphism of *IRF6* rs2235373, located in intron 7. PCR performed by using the primers of forwarding: 5'-CAGGGCTGCCGACTCTTCTA-3' and reverse: 5'-AGGAAAGCAGGAAGGTGAAAGA-3'.

DNA sequencing covering *IRF6* rs2235373 was performed by using the dideoxy or Sanger method. The polymorphism in the form of substitution of base C into T was identified and created three genotypes: TT (homozygous mutant genotype), CT (heterozygous genotype), and CC (normal genotype). After identifying the homozygous mutant genotype and heterozygous genotype used to sequence the PCR product (fragment rs2235373), then 50 mL (of what substance) was optimized based on the principle

of Sanger method. The DNA target is the single strand that added the nucleotides in mixing (dATP, dGTP,dCTP dan dTTP). All of the nucleotides were given the labels to express the fluorescence in 4 different colors when they were added to the DNA polymerase. The results of sequencing in form the chromatograph or electromyograph were the color curve as the sequence of DNA. After the identification of polymorphism, then mRNA expression level from all subjects was determined by RT PCR detection.

qPCR of *IRF6* gene

Detection of mRNA expression from *IRF6* detected by qPCR that the first steps we are collected of epithelial cells by smear method from the oral palatal mucosa, and it was obtained by 15 to 20 times of smearing and stored in small tube consisting of RNA. Total RNA extracted from those palatal epithelial by using Trizol Reagent (Invitrogen, USA) and the concentration measured by using Nano Drop to ensure that the RNA concentration was obtained optimally. The optimal level is about 1.6 - 2.0 at A260/280. The RNA was converted to cDNA using an oligo (dT) primer and Superscript II (Invitrogen). The GAPDH was used as the reference gene in order to calibrate the level of expression of *IRF6* that was measured by real-time quantitative RT PCR based on the SYBR-Green method and applied to the qPCR running times in 40 cycles that consisted of: early denaturation 94 ^{o}C 15', annealing 60 ^{o}C 1', and extension 68 ^{o}C 2'. The primers for *IRF6* were 5'CGGCATAGCCCTCAACAAGAA-3'and '-TCCTTGGTGCCATCATACATCAG-3'; and for *GAPDH* were 5'-TGCTGAGTATGTCGTGGAG-3' and 5'-GTCTTCTGAGTGGCAGTGAT3', respectively.[14] The gene mutation of TT and CT genotypes of NSOC was analyzed by the Livak method (2-ΔΔCt). [15]

Statistical Analyses

The mRNA expressions of all genotypes in every oral cleft phenotype were compared with controls and also statistical analyses were performing using the Mann Whitney test with a *P*< 0.05 significance level.

Results

The PCR product of the DNA band of the *IRF6* rs2235373 gene segment was 413 base pairs (bp). Sanger sequencing resulted in a homozygous normal of CC genotype, a heterozygous mutant of CT genotype, and a homozygous mutant of TT genotype (Fig.1).

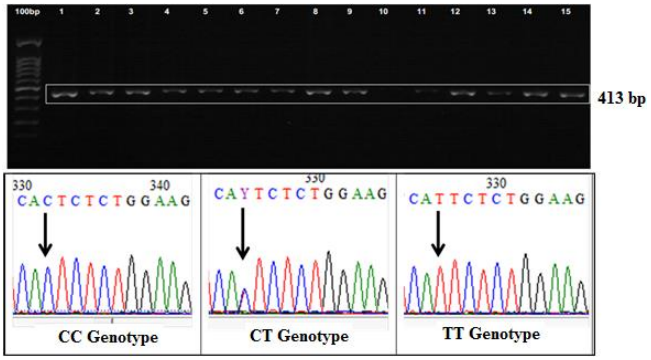

Figure 1. The profile of PCR product (above) and the sequencing product (beneath). The sample PCR products showed the band of gene target (*IRF6* rs2235373), and CT genotype (heterozygous mutants) and TT genotype (homozygous mutant) was the mutation of genes of the subjects of non-syndromic oral cleft risk.

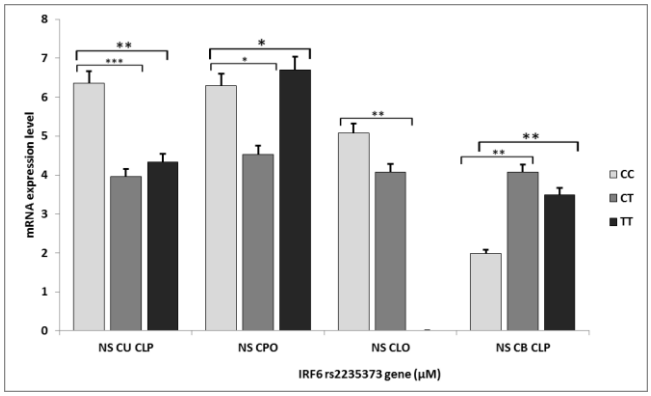

Figure 2. Expression level of *IRF6* mRNA in CT genotype, TT genotype, and non-syndromic oral cleft (CC genotype). NS CU CLP (nonsyndromic complete unilateral cleft lip and palate); NS CPO (nonsyndromic cleft palate only); NS CLO (nonsyndromic cleft lip only); NS CB CLP (nonsyndromic complete bilateral cleft lip and palate). Bar (level expression of genes) Bar error (standard deviation). No significant (*); significant (**) p<0.05; and significant (***) p<0.01. NS CU CLP (non-syndromic complete unilateral cleft lip and palate); NS CPO (non-syndromic cleft palate only); NS CLO (non-syndromic cleft lip only); NS CB CLP (non-syndromic complete bilateral cleft lip and palate).

Figure 2 explains that the level of expression of the *IRF6* gene of the TT genotype (homozygous mutant) in NS CU CLP and NS

CPO cases is higher than the CT genotype (heterozygous mutant) and on the contrary of NS CB CLP. In the NS CPO and NS CU CLP, the gene was over-expressed (P<0.05). These results indicate that the gene from the oral epithelium can be used as a parameter to identify the polymorphism of subjects with non-syndromic oral cleft risk.

Subject	CC	CT			TT		
	Normal	heterozygous mutant	Level Expression	p	homozygous mutant	Level Expression	p
NS CU CLP	32%	24% *	75% *	0.001***	30%	94% *	0.036***
NS CPO	32%	27% *	84% *	0.164	46% **	144% **	0.49
NS CLO	26%	25% *	96% *	0.025***	-	-	-
NS CB CLP	10%	24% **	240% **	0.021***	24% **	240% **	0.01***

Table 1. Distribution and frequency of level expression of mRNA gene of *IRF6* rs2235373 and Mann Whitney analyses.

* Higher than normal; ** Over-expression; *** Significant; NS CU CLP (non-syndromic complete unilateral cleft lip and palate); NS CPO (non-syndromic cleft palate only); NS CLO (non-syndromic cleft lip only); NS CB CLP (non-syndromic complete bilateral cleft lip and palate).

Table 1 shows that the NS CLO subjects had an unexpressed TT genotype (homozygous mutant), but overexpression occurs in subjects NS CPO and NS CB CLP. The CT genotype (heterozygous mutant) only NS CB CLP had over-expression of the *IRF6* gene. Generally, the non-syndromic oral cleft risk subjects are expressed of the *IRF6* gene above 75% (P<0.05), except NS CPO (P>0.05).

Discussion

Analyzing gene expression patterns can lead to the understanding of protein function, biological pathways, and cellular responses. We can better understand specifically the mutations or polymorphisms of the genes that can cause some changes in mRNA expression level that lead to symptoms of disease. The mRNA quantification can be a better method to detect gene expression alteration which is caused by polymorphism and associated with some illnesses or malformations like NSOC. Analyzing mRNA expression level is necessary for a complete understanding of how the cell works. In this study, we determined the *IRF6* rs2235373 as the indicator of gene polymorphism based on the band of the gene target. The symbols of mutation are CT genotype (heterozygous mutant) and TT genotype (homozygous mutant). These polymorphism genes examined through oral palatal epithelium cells with a purpose that these cells could be an oral predictor of NS OC and its phenotypes change.

The mechanism or how the pathogenesis of *IRF6* mRNA expression could affect the NS OC has not yet been defined. The result of this study reported that the level mutation of *IRF6* is significant in NS OC subjects (Table 1). These results show the same result as previous research which only showed that the expression patterns indicate that *IRF6* is strongly expressed in the developing maxillary process, lower jaw molar, oral epithelium cells, naris, upper jaw incisor, and molar.[16] In epithelial development, buccal epithelial cells of *IRF6* gens required for the regulation of proliferation.[17] Ferone, et al (2012) reported the elimination of the *IRF6* gene in mice has shown defective stratified epidermis, skin, limb, craniofacial development, and gene expression.[18] These analyses have indicated that the primary faults are in the keratinocyte proliferation-differentiation switch.[19] In the adult, *IRF6* expression is still required for the proliferation and differentiation of keratinocyte and epidermis suprabasal layers also errors in *IRF6* can prevent the appearance of a large number of genes directly controlled by *IRF6*.[20]

We are founding the mutation of the TT genotype (homozygous mutant), and the CT genotype (heterozygous mutant) has the overexpressed oral epithelial *IRF6* gene. That result has the similarity of the previous study, the *IRF6* mutant mice, which have a hyper-proliferative epidermis that fails to undergo terminal differentiation, leading to multiple epithelial adhesions that can impede the oral cavity and result in cleft palate.[21] These reports show that the *IRF6* has a role in the process of keratinocyte proliferation and differentiation switch. In line with that, the *IRF6* has a role in the formation of the oral periderm, whose spatiotemporal regulation is essential for ensuring proper palatal adhesion. If the *IRF6* transcription is activated by p63, which underlies several malformation syndromes, including OC.[22] Based on the results of Fig. 1, Fig. 2, and Table 1, we assumed that the mutation of the TT genotype is higher than CT mutation. It is related to the ability of *IRF6* to trace the mutation with two copies of the same allele.[23]

Within the in vivo study, it was reported that the combination of mouse genetics has demonstrated that *IRF6* is a direct target of p63, which underlies several malformation syndromes

that include CLP. The p63 activates *IRF6* transcription via the *IRF6* enhancer element, and variation in this element increases the susceptibility to CLO.[24] In the palatal development of mice embryo, previous research has shown that besides *IRF6,* p63 plays a central role in maintaining cellular proliferation during development.[25] Therefore, the mutation may affect downstream functions such as the processes of keratinocyte proliferation and differentiation and eventually lead to OC.[26] The *IRF6* has been selected as a significant predictor for all forms of OC. DNA polymorphisms are known to be associated with phenotypic variation and may alter gene expression patterns and that it will play a crucial role in disease susceptibility.[27] The evaluation has aligned with our previous studies which explained the effects of mRNA expression changes of *IRF6* rs2235373 associated with NS OC phenotypes that are mixed with healthy controls.[10]

Based on the underlying theory as a result of some previous studies, the mechanism, or how the pathogenesis of *IRF6* mRNA expression could affect the NS OC, has not yet been defined. Early differentiation, ΔNp63 (p63 isoform) promotion of the transcriptional activation of *IRF6*, and the *IRF6* protein, in turn, supports ΔNp63 degradation, which will result in an induction of p21 expression and MEE apoptosis and allow keratinocytes to exit cell cycle and differentiate, [24] which is also crucial to the palatal fusion process. This feedback regulation may play an essential role in controlling the proliferation and differentiation of oral periderm and keratinocytes.[28] During the fetal period, the development of lip and palate, cell cycle arrest (stopping at G0 phase) is associated with a significant increase in the total amount of *IRF6*.[29] The CT genotype in our study has the overexpression of the mRNA level of the *IRF6* gene mainly in NS CU CLP and CLO subjects. These results cannot be explained yet, because we examined it in the postnatal period from oral palatal epithelium, so it cannot be concluded whether it has a connection with the role of *IRF6* during the fetal period, as there was the altered in SNAI2 and TGFβ3.[30]

Conclusions

The conclusion of this studied is the IRF rs2235373 gene can trace the intron polymorphisms in the oral epithelium of non-syndromic oral cleft risk with the higher expression levels. CT and TT genotypes have the same frequency of *IRF6* mutation. Based on the results, the *IRF6* can be the predictor in non-syndromic cleft palate risk.

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Declaration of Interest

The authors report no conflict of interest.

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