# Investigation Of IRF6 Gene Functional Role In Human Oral Epithelium With Nonsyndromic Orofacial Cleft

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*Abstract*— IRF6 gene association with nonsyndromic orofacial cleft (NS OFC) has been consistently replicated in many distinct geographic areas of the world, but the exact functional role of IRF6 in the NS OFC risk has not been fully identified yet. This study aims to investigate the functional role of IRF6 gene through rs642961 (promoter) and rs2235371 (exon 7) polymorphisms by examining mRNA expression levels from human oral epithelium of NS OFC patients. This study was a crossectional laboratory analitical study and we tested human oral epithelium samples obtained by smear method from 136 individuals with NS OFC and its phenotypes consist of complete unilateral (CU) cleft lip and palate (CLP) (n=42), complete bilateral (CB) CLP (n=34), cleft lip only (CLO) (n=33) and cleft palate only (CPO) (n=27). Both DNA and RNA were extracted according to established protocols. The segment of rs642961 and rs2235371 were evaluated and mRNA expression analysis was performed by real time (RT) PCR. Then, mRNA expression levels were analyzed by Livak method and Mann Whitney test through AA, GA, GG, A*A*, G*A* and G*G* genotypes. There were different mRNA expression levels from all NS OFC phenotypes. The mRNA expression of GA genotype from NS CLO was 0.057 lower than GG genotype ($p<0.001$), where mRNA expression of GA genotype from NC CU CLP was 4.11 higher than GG genotype ($p=0.028$) and the mRNA expression of G*A* genotype from NS CU CLP was 2.46 higher than G*G* genotype ($p=0.048$) where mRNA expression of G*A* genotype from NS CB CLP 0.15 lower than G*G* genotype ($p=0.004$) In conclusion, there are different over and underexpression levels of mRNA expression from both IRF6 polymorphisms among NS OFC phenotypes that can be investigated from human oral epithelium with NS OFC, means that IRF6 has a different functional role in each NS OFC phenotypes.

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## Introduction

Orofacial cleft (OFC) is a common craniofacial malformation with worldwide incidence around 1 in 700 live births[1,2,3,4] OFC is a diverse group of birth defects and can be broadly classified into some phenotypes as cleft palate only (CPO), cleft lip only (CLO) and cleft lip and palate (CLP), or a combined group of cleft lip with or without cleft palate (CL/P). More than 60% of OFC cases have CL/P with the remainder of cases classified as OFC.[2] OFC can occur without any other abnormalities or as part of a constellation of physical or developmental anomalies characterizing a syndrome. Nearly half of CPO and 70% of CL/P cases are isolated or nonsyndromic (NS) as affected individuals have no other physical and developmental anomalies.[5,6,7] Differences in timing and developmental processes of the lip and primary palate from the secondary palate provide evidence for distinguishing CL/P from CPO in the search for genetic causes of clefting. Just as CPO seems etiologically distinct from CL/P, epidemiologic and genetic association data suggest that CLO may be a separate defect from CL/P.[8,9,10,11] Clefts involving the lip are also heterogenous, and may be either unilateral or bilateral, but no mutations or polymophisms have been specifically associated with unilateral or bilateral NS CLO.[4,12] So, it is important to analyze NS OFC separately based on its phenotypes and investigating mRNA expression levels in NS OFC with its phenotypes can enable us to identify distinct expression profiles in the NS OFC phenotypes.

Interferon regulatory factor (*IRF6*) is the poorly understood member of a family of nine transcription factors with highly conserved DNA binding domains and less conserved protein binding domains.[13]*IRF6,* located on chromosome 1q32.3-q41[14], acts as a tumor suppresor in mammary cells[15] and helps to induce keratinocyte differentiation of skin, breast epithelium and oral epithelium.[16] . The observation that *Irf6*-null mice exhibit severe skin defects led to further investigation on the role of *IRF6* in proliferation and differentiation of keratinocytes.[16,17] The *IRF6* gene was expressed in the

ectoderm fusion forming the upper lip, primary palate and secondary palate in human cranifacial structures, although the biological function of *IRF6* during development of the lip and palate in humans remain uncertain.[18]

Mutations in *IRF6* cause Van der Woude (VWS) and popliteal pterygium syndromes (PPS), these mutations typicallly interfere with DNA-binding or protein-protein interactions of this transcription factor and lead to haploinsufficiency of this gene in the affected individual.[13,16] Because VWS syndrome phenocopies NS OFC in 15% of VWS patients, variants that decrease *IRF6* protein levels or result in a less functional *IRF6* allele could be etiologic in NS OFC.[19,20,21,22,23]*IRF6* association with NS OFC has been consistently replicated in other populations.[24,25,26,27,28,29] Thus, the first suggested function for *IRF6* was that its disruption resulted in abnormal palatal development.[16] Expression studies have shown that *Irf6* is detected in epithelial tissues such as skin and the medial edge epithelium (MEE) of the developing palate in mice.[13]

Polymorphisms in and around *IRF6* have demonstrated consistent association with NS OFC[3,30], with variants rs2235371 and rs642961 most commonly associated in the NS OFC and particularly with rs642961, it is also associated with the severity of NS OFC in Iranian population.[9,19,31.32] The functional role of *IRF6* polymorphism that cause NS OFC has not been identified yet. The V274I variant, rs2235371, in *IRF6* exon 7 is associated with NS OFC,[19] but its role in cleft etiology was not identified. A regulatory single nucleotide polymorphism (SNP) variant, rs642961, in *IRF6* promoter/enhancer was identified with co-segregation of rs2235371, and it demonstrated association with CLO and functional analysis of this regulatory SNP demonstrated that the associated allele/risk allele in rs642961 disrupts an *AP-2α* binding site and *IRF6* enhancer activity.[9,16,32] The regulation of *Irf6* appears to be both at the level of transcription and post translation.[16] It is important to do the determination of a correlation between the level of expression of genes involved in clefting with the genotype at a particular polymorphism. As many polymorphisms associated with NS OFC have no known function, we assumed that the genotype of some of these associated polymorphisms would correlate with the expression level of the genes. Many polymorphisms associated with NS OFC map to promoter or exonic regions. The functions of many of these polymorphisms is unknown, and their role can be predicted to damage or alter protein function. Oral epithelium may be suitable surrogate for functional analysis of promoter and exonic polymorphisms that will improve understanding of many cleft candidate genes and identify targets for clinical treatment and prevention of NS OFC. We chose oral epithelium as our source of investigation due to its availability and also because *IRF6* gene is strongly expressed in oral epithelium. According to our previous study, *IRF6* mRNA expression could be detected from human oral epithelium and there was the mRNA overexpression level in human oral epithelium of NS CBCLP individuals which are 3.61 times higher compared with control individuals ($p = 0.002$).[33] But to get to know the the functional role of the *IRF6* gene, we did the investigation of it through the polymorphisms of rs642961 and rs2235371 from oral epithelium of NS OFC patients. This study may serve as an initial approach towards gaining different molecular process in the development of NS OFC with its phenotypes.

## Materials and Methods

All the subjects of this study were from Deuteromalay subrace as the majority of the race in Indonesia and only those individuals determined to have non syndromic form of CLP were included in this study. In totally, all subjects include 136 individuals consist of CU CLP (n=42), CB CLP (n=34), CLO (n=33) and CPO (n=27). This study is done in Molecular Biology Laboratory, *Unit Penelitian Kesehatan* (UPK) Faculty of Medicine Universitas Padjadjaran Bandung/ Hasan Sadikin Hospital Bandung.

### Sample collection, DNA and RNA isolation

For determining rs642961 and rs2235371, after written informed consent was obtain from each individuals 2 ml peripheral venous blood were collected for all subjects, anticoagulated by 0.5% EDTA (pH 8.0), and then stored at-$4^0$C. DNA was extracted from venous blood of each subjects using DNA isolation kit from Phamacia, then 200 ng of DNA template was using for Polymerase Chain Reaction (PCR) step.

For mRNA expression analysis, epithelial cells were collected by smear method from palatal mucosa and it was obtained by 15 to 20 times of smear and stored in small tube consist of RNA later. Total RNA were extracted from those palatal epithelial by using Trizol Reagent (Invitrogen, USA) and the concentration was measured by using nano drop to ensure that there were RNA concentration obtained optimally.

### Genotyping

**PCR.** For rs642961, PCR was performed by using the primers of forward: 5'-AAAGGCCTGAAGTAATACCCCCG-3' and reverse : 5'-CGGCTCAATCAGGGTCAAGGAT-3'[34] and for rs2235371, PCR was performed by using the primers of forward: 5'-CAGGGCTGCCGACTCTTCTA-3' and reverse : 5'-AGGAAAGCAGGAAGGTGAAAGA-3'. PCR procedure was as follows: $94^0$C initial denaturation for 5 min, followed by 35 cycles of $94^0$C degeneration for 45 s, $62^0$C annealing for 45 s and $72^0$C extension for 50 s; $72^0$C final extension for 5 min and reduced to $4^0$C saved.

**Restriction Fragment Length Polymorphisms (RFLPs).**PCR products for rs642961 were then digested with *Msp*I restriction enzyme at $37^0$C. (pan)All digested PCR products were separated into channels on a 1.5% agarose gel containing ethidium bromide in an electrophoretic chamber. The results were observed in an ultraviolet transilluminator. The polymorphism is the form of substitution of base G into Ato create 3 genotypes : GG

(normal genotype), GA (heterozygous genotype) and AA (homozygous mutant genotype).

***DNA Sequencing.*** DNA sequencing covering *IRF6*rs2235371 was performed by using dideoxy Sanger method. From the sequencing result from whole samples, all nucleotide in those segment compared with normal nucleotide in gene bank by using sequence alignment program from BioEdit. The polymorphism is the form of substitution of base C into Tto create 3 genotypes : A*A* (homozygous mutant genotype), G*A* (heterozygous genotype) and G*G* (normal genotype).

**RT PCR detection of mRNA expression from *IRF6***

**RT PCR**

Level of mRNA expression was determined by quantitative real time (RT) PCR. The RNA was converted to cDNA by using oligo(dT) primer and Supercript II (Invitrogen). Relative gene expression quantitation for *IRF6*, using *GAPDH* as an internal reference gene, was measured by real-time quantitative RT PCR based on the SYBR-Green method. The primers for *IRF6* were 5'CGGCATAGCCCTCAACAAGAA-3'and 5'-TCCTTGGTGCCATCATACATCAG-3'; and for *GAPDH* were 5'-TGCTGAGTATGTCGTGGAG-3' and 5'-GTCTTCTGAGTGGCAGTGAT3', respectively.[34]

**mRNA expression analysis dan statistical analysis**

*IRF6* mRNA expression changes analyzed between genotypes (AA, GA, GG/rs642961; A*A*, G*A*, G*G*/rs2235371) from total NS OFC, also its phenotypes by Livak method that was determined by using the comparative CT method ($2^{-\Delta\Delta Ct}$)[35] and statistical analysis were by Mann Whitney test to compare groups and a P value < 0.05 was considered statistically significant.

**Results**

From total NS OFC and its phenotypes, AA, GA, GG (rs642961) and A*A*, G*A* and G*G* (rs2235371) genotypes have been identified. The value of mRNA expressions from all of polymorphisms were compared with normal genotypes. Table 1 revealed relative quantification of mRNA expression *IRF6*rs642961 between GA and AA genotypes compared to GG genotype (normal genotype) and table 2 revealed relative quantification of mRNA expression *IRF6*rs2235371 between G*A* and A*A* genotypes compared to G*G* genotype (normal genotype). Both of these analysis were by Livak mehod.

**Table 1 Relative quantification of mRNA expression *IRF6* rs642961 between GA genotype, AA genotype compared to GG genotype**

| Total NS OFC | | | | | | |
|---|---|---|---|---|---|---|
| **Sample** | **Ct *IRF6*** | **Ct *GADPH*** | **ΔCt GG (Ct *IRF6* - Ct *GAPDH*)** | **ΔCt GA (Ct *IRF6* - Ct *GAPDH*)** | **ΔΔCt (ΔCt GA-ΔCt GG)** | **Expression value $2^{-\Delta\Delta Ct}$** |
| **GG genotype** | 30.49 | 26.45 | 4.04 | | 0.28 | 0.82 |
| **GA genotype** | 31.19 | 26.87 | | 4.32 | | |
| **Sample** | **Ct *IRF6*** | **Ct *GADPH*** | **ΔCt GG (Ct *IRF6* - Ct *GAPDH*)** | **ΔCt GA (Ct *IRF6* - Ct *GAPDH*)** | **ΔΔCt (ΔCt GA-ΔCt GG)** | **Expression value $2^{-\Delta\Delta Ct}$** |
| **GG genotype** | 30.49 | 26.45 | 4.04 | | -0.77 | 1.71 |
| **AA genotype** | 29.85 | 26.58 | | 3.27 | | |
| **NS CU CLP** | | | | | | |
| **Sample** | **Ct *IRF6*** | **Ct *GADPH*** | **ΔCt GG (Ct *IRF6* -Ct *GAPDH*)** | **ΔCt GA (Ct *IRF6* -Ct *GAPDH*)** | **ΔΔCt (ΔCt GA-ΔCt GG)** | **Expression value $2^{-\Delta\Delta Ct}$** |
| **GG genotype** | 32.19 | 25.50 | 6.69 | | -2.04 | 4.11 |
| **GA genotype** | 31.49 | 26.84 | | 4.65 | | |
| **Sample** | **Ct *IRF6*** | **Ct *GADPH*** | **ΔCt GG (Ct *IRF6* -Ct *GAPDH*)** | **ΔCt GA (Ct *IRF6* -Ct *GAPDH*)** | **ΔΔCt (ΔCt GA-ΔCt GG)** | **Expression value $2^{-\Delta\Delta Ct}$** |
| **GG genotype** | 32.19 | 25.50 | 6.69 | | -5.,89 | 59.30 |
| **AA genotype** | 28.20 | 27.40 | | 0.80 | | |
| **NS CB CLP** | | | | | | |
| **Sample** | **Ct *IRF6*** | **Ct *GADPH*** | **ΔCt GG (Ct *IRF6* -Ct *GAPDH*)** | **ΔCt GA (Ct *IRF6* -Ct *GAPDH*)** | **ΔΔCt (ΔCt GA-ΔCt GG)** | **Expression value $2^{-\Delta\Delta Ct}$** |
| **GG genotype** | 28.29 | 27.40 | 0.89 | | 2.01 | 0.25 |
| **GA genotype** | 30.39 | 27.49 | | 2.90 | | |
| **Sample** | **Ct *IRF6*** | **Ct *GADPH*** | **ΔCt GG (Ct *IRF6* -Ct *GAPDH*)** | **ΔCt GA (Ct *IRF6* -Ct *GAPDH*)** | **ΔΔCt (ΔCt GA-ΔCt GG)** | **Expression value $2^{-\Delta\Delta Ct}$** |
| **GG genotype** | 28.29 | 27.40 | 0.89 | | 2.82 | 0.142 |
| **AA genotype** | 33.88 | 30.17 | | 3.71 | | |
| **NS CLO** | | | | | | |
| **Sample** | **Ct *IRF6*** | **Ct *GADPH*** | **ΔCt GG (Ct *IRF6* -Ct *GAPDH*)** | **ΔCt GA (Ct *IRF6* -C *GAPDH*)** | | |
| **GG genotype** | 31.56 | 30.76 | 0.80 | | | |
| **GA genotype** | 32.18 | 27.25 | | 4.93 | | |

| Sample | Ct *IRF6* | Ct *GADPH* | ΔCt GG (Ct *IRF6* -Ct *GAPDH*) | ΔCt GA (Ct *IRF6* -Ct *GAPDH*) | ΔΔCt (ΔCt GA-ΔCt GG) | Expression value $2^{-\Delta\Delta Ct}$ |
|---|---|---|---|---|---|---|
| **GG genotype** | 31.56 | 30.76 | 0.80 | | | |
| **AA genotype** | 26.73 | 24.15 | | 2.58 | -1.78 | 3.44 |

**NS CPO**

| Sample | Ct *IRF6* | Ct *GADPH* | ΔCt GG (Ct *IRF6* -Ct *GAPDH*) | ΔCt GA (Ct *IRF6* -Ct *GAPDH*) | ΔΔCt (ΔCt GA-ΔCt GG) | Expression value $2^{-\Delta\Delta Ct}$ |
|---|---|---|---|---|---|---|
| **GG genotype** | 29.91 | 22.15 | 7.76 | | | |
| **GA genotype** | 30.71 | 25.88 | | 4.83 | -2.93 | 7.62 |

| Sample | Ct *IRF6* | Ct *GADPH* | ΔCt GG (Ct *IRF6* -Ct *GAPDH*) | ΔCt GA (Ct *IRF6* -Ct *GAPDH*) | ΔΔCt (ΔCt GA-ΔCt GG) | Expression value $2^{-\Delta\Delta Ct}$ |
|---|---|---|---|---|---|---|
| **GG genotype** | 29.91 | 22.15 | 7.76 | | | |
| **AA genotype** | 30.60 | 24.59 | | 6.01 | -1.75 | 3.36 |

**Table 2 Relative quantification of mRNA expression *IRF6* rs2235371 between G*A* genotype, A*A* genotype compared to G*G* genotype**

**Total NS OFC**

| Sample | Ct *IRF6* | Ct *GADPH* | ΔCt G*G* (Ct *IRF6* -Ct *GAPDH*) | ΔCt G*A* (Ct *IRF6* -Ct *GAPDH*) | ΔΔCt (ΔCt G*A*-ΔCt G*G*) | Expression value $2^{-\Delta\Delta Ct}$ |
|---|---|---|---|---|---|---|
| **G*G* genotype** | 30.84 | 26.09 | 4.75 | | -0.3 | 1.23 |
| **G*A* genotype** | 31.37 | 26.92 | | 4.45 | | |

| Sample | Ct *IRF6* | Ct *GADPH* | ΔCt G*G* (Ct *IRF6* -Ct *GAPDH*) | ΔCt A*A* (Ct *IRF6* -Ct *GAPDH*) | ΔΔCt (ΔCt A*A*-ΔCt G*G*) | Expression value $2^{-\Delta\Delta Ct}$ |
|---|---|---|---|---|---|---|
| **G*G* genotype** | 30.84 | 20.69 | 4.75 | | 0.47 | 0.72 |
| **A*A* genotype** | 30.13 | 24.91 | | 5.22 | | |

**NS CU CLP**

| Sample | Ct *IRF6* | Ct *GADPH* | ΔCt G*G* (Ct *IRF6* -Ct *GAPDH*) | ΔCt G*A* (Ct *IRF6* -Ct *GAPDH*) | ΔΔCt (ΔCt A*A*-ΔCt G*G*) | Expression value $2^{-\Delta\Delta Ct}$ |
|---|---|---|---|---|---|---|
| **G*G* genotype** | 31.35 | 26.02 | 5.33 | | -1.3 | 2.46 |
| **G*A* genotype** | 31.33 | 27.30 | | 4.03 | | |

| Sample | Ct *IRF6* | Ct *GADPH* | ΔCt G*G* (Ct *IRF6* -Ct *GAPDH*) | ΔCt A*A* (Ct *IRF6* -Ct *GAPDH*) | ΔΔCt (ΔCt A*A*-ΔCt G*G*) | Expression value $2^{-\Delta\Delta Ct}$ |
|---|---|---|---|---|---|---|
| **G*G* genotype** | 31.35 | 26.02 | 5.33 | | 0.13 | 0.91 |
| **A*A* genotype** | 32.85 | 27.39 | | 5.46 | | |

**NS CB CLP**

| Sample | Ct *IRF6* | Ct *GADPH* | ΔCt G*G* (Ct *IRF6* -Ct *GAPDH*) | ΔCt G*A* (Ct *IRF6* -Ct *GAPDH*) | ΔΔCt (ΔCt G*A*-ΔCt G*G*) | Expression value $2^{-\Delta\Delta Ct}$ |
|---|---|---|---|---|---|---|
| **G*G* genotype** | 31.30 | 29.13 | 2.17 | | 2.71 | 0.15 |
| **G*A* genotype** | 31.18 | 26.30 | | 4.88 | | |

| Sample | Ct *IRF6* | Ct *GADPH* | ΔCt G*G* (Ct *IRF6* -Ct *GAPDH*) | ΔCt A*A* (Ct *IRF6* -Ct *GAPDH*) | ΔΔCt (ΔCt A*A*-ΔCt G*G*) | Expression value $2^{-\Delta\Delta Ct}$ |
|---|---|---|---|---|---|---|
| **G*G* genotype** | 31.30 | 29.13 | 2.17 | | 1.32 | 0.40 |
| **A*A* genotype** | 25.37 | 21.88 | | 3.49 | | |

**NS CLO**

| Sample | Ct *IRF6* | Ct *GADPH* | ΔCt G*G* (Ct *IRF6* -Ct *GAPDH*) | ΔCt G*A* (Ct *IRF6* -Ct *GAPDH*) | ΔΔCt (ΔCt G*A*-ΔCt G*G*) | Expression value $2^{-\Delta\Delta Ct}$ |
|---|---|---|---|---|---|---|
| **G*G* genotype** | 31.45 | 27.14 | 4.31 | | 0.55 | 0.68 |
| **G*A* genotype** | 32.17 | 27.31 | | 4.86 | | |

**NS CPO**

| Sample | Ct *IRF6* | Ct *GADPH* | ΔCt G*G* (Ct *IRF6* -Ct *GAPDH*) | ΔCt G*A* (Ct *IRF6* -Ct *GAPDH*) | ΔΔCt (ΔCt G*A*-ΔCt G*G*) | Expression value $2^{-\Delta\Delta Ct}$ |
|---|---|---|---|---|---|---|
| **G*G* genotype** | 29.86 | 23.11 | 6.75 | | -2.74 | 6.68 |
| **G*A* genotype** | 30.79 | 26.78 | | 4.01 | | |

| Sample | Ct *IRF6* | Ct *GADPH* | ΔCt G*G* (Ct *IRF6* -Ct *GAPDH*) | ΔCt G*A* (Ct *IRF6* -Ct *GAPDH*) | ΔΔCt (ΔCt A*A*-ΔCt G*G*) | Expression value $2^{-\Delta\Delta Ct}$ |
|---|---|---|---|---|---|---|
| **G*G* genotype** | 29.86 | 23.11 | 6.75 | | -0.05 | 1.035 |
| **A*A* genotype** | 32.16 | 25.46 | | 6.7 | | |

To evaluate significant mean differences statistically between the $2^{-\Delta\Delta Ct}$ and the group of AA, GA, GG (rs642961) ; A*A*, G*A* and G*G* (rs2235371), Mann Whitney test were performed (table 3 and 4).

**Table 3*IRF6* mRNA expression comparison between GG,GAand AA genotype**

**NS OFC**

| Variable | Group | |
|---|---|---|
| | **GG genotype (n=8)** | **GA genotype** |
| $2^{-\Delta\Delta Ct}$ | | |

| | | |
|---|---|---|
| Mean±Std | 1.87±2.370 | 0.11±0.194 |
| Median | 0.91 | 0.04 |
| Range | 0.02-6.73 | 0.00-0.88 |

| Variable | Group | |
|---|---|---|
| | GG genotype (n=8) | AA genotype (n=7) |
| $2^{-\Delta\Delta Ct}$ | | |
| Mean±Std | 1.87±2.370 | 31.01±53.717 |
| Median | 0.91 | 5.98 |
| Range | 0.02-6.73 | 0.16-147.03 |

**NS CU CLP**

| Variable | Group | |
|---|---|---|
| | GG genotype (n=1) | GA genotype (n=4) |
| $2^{-\Delta\Delta Ct}$ | | |
| Mean±Std | 0.31 | 209.47±46.325 |
| Median | 0.31 | 210.84 |
| Range | 0.31 | 151.17-265.03 |

| Variable | Group | |
|---|---|---|
| | GG genotype(n=7) | AA genotype (n=1) |
| $2^{-\Delta\Delta Ct}$ | | |
| Mean±Std | 0.31 | 5.98 |
| Median | 0.31 | 5.98 |
| Range | 0.31 | 5.98 |

**NS CB CLP**

| Variable | Group | |
|---|---|---|
| | GG genotype (n=2) | GA genotype (n=19) |
| $2^{-\Delta\Delta Ct}$ | | |
| Mean±Std | 594.06±85.308 | 13.21±28.256 |
| Median | 594.06 | 0.57 |
| Range | 533.74-654.39 | 0.00-120.26 |

| Variable | Group | |
|---|---|---|
| | GG genotype (n=2) | AA genotype (n=2) |
| $2^{-\Delta\Delta Ct}$ | | |
| Mean±Std | 594.06±85.308 | 0.04±0.041 |
| Median | 594.06 | 0.04 |
| Range | 533.74-654.39 | 0.01-0.07 |

**NS CLO**

| Variable | Group | |
|---|---|---|
| | GG genotype (n=1) | GA genotype (n=5) |
| $2^{-\Delta\Delta Ct}$ | | |
| Mean±Std | 2048.00 | 0.04±0.022 |
| Median | 2048.00 | 0.03 |
| Range | 2048.00 | 0.01-0.07 |

| Variable | Group | |
|---|---|---|
| | GG genotype (n=1) | AA genotype (n=2) |
| $2^{-\Delta\Delta Ct}$ | | |
| Mean±Std | 2048.00 | 4.44±6.274 |
| Median | 2048.00 | 4.44 |
| Range | 2048.00 | 0.00-8.88 |

**NS CPO**

| Variable | Group | |
|---|---|---|
| | GG genotype (n=2) | GA genotype (n=9) |
| $2^{-\Delta\Delta Ct}$ | | |
| Mean±Std | 0.05±0.050 | 79.87±125.988 |
| Median | 0.05 | 23.42 |
| Range | 0.2-0.9 | 1.15-385.34 |

| Variable | Group | |
|---|---|---|
| | GG genotype (n=2) | AA genotype (n=1) |
| $2^{-\Delta\Delta Ct}$ | | |
| Mean±Std | 0.05±0.050 | 0.16 |
| Median | 0.05 | 0.16 |
| Range | 0.2-0.9 | 0.16 |

**Table 4*IRF6* mRNA expression comparison between G*G*,G*A*and A*A *genotype**

**NS OFC**

| Variable | G*G* genotype (n=53) | G*A* genotype (n=16) | P value |
|---|---|---|---|
| $2^{-\Delta\Delta Ct}$ | | | **0.002**** |
| Mean±Std | 267.53±1138.363 | 291.39±251.785 | |
| Median | 3.58 | 242.33 | |
| Range | 0.00-8023.41 | 16.56-885.29 | |

| Variable | G*G* genotype (n=53) | A*A* genotype (n=7) | P value |
|---|---|---|---|
| $2^{-\Delta\Delta Ct}$ | | | **0.029*** |
| Mean±Std | 267.53±1138.363 | 0.55±0.695 | |
| Median | 3.58 | 0.20 | |
| Range | 0.00-8023.41 | 0.06-1.59 | |

**NS GU CLP**

| Variable | G*G* genotype (n=6) | G*A* genotype (n=3) | P value |
|---|---|---|---|
| $2^{-\Delta\Delta Ct}$ | | | **0.028*** |
| Mean±Std | 51.74±84.361 | 209.50±56.982 | |
| Median | 4.97 | 212.30 | |
| Range | 1.18-209.38 | 151.17-265.03 | |

| Variable | G*G* genotype(n=6) | A*A* genotype (n=3) | P value |
|---|---|---|---|
| $2^{-\Delta\Delta Ct}$ | | | **1.000** |
| Mean±Std | 51.74±84.361 | 0.65±0.814 | |
| Median | 4.97 | 0.20 | |
| Range | 1.18-209.38 | 0.16-1.59 | |

**NS CB CLP**

| Variable | G*G* genotype (n=6) | G*A* genotype (n=5) | P value |
|---|---|---|---|
| $2^{-\Delta\Delta Ct}$ | | | **0.010*** |
| Mean±Std | 263.02±213.893 | 0.33±0.660 | |
| Median | 227.86 | 0.03 | |
| Range | 47.84-515.56 | 0.00-1.52 | |

| Variable | G*G* genotype (n=6) | A*A* genotype (n=2) | P value |
|---|---|---|---|
| $2^{-\Delta\Delta Ct}$ | | | **<0.001**** |
| Mean±Std | 263.02±213.893 | 17.90±25.238 | |
| Median | 227.86 | 17.90 | |
| Range | 47.84-515.56 | 0.06-35.75 | |

**NS CLO**

| Variable | G*G* genotype (n=21) | G*A* genotype (n=6) | P value |
|---|---|---|---|
| $2^{-\Delta\Delta Ct}$ | | | **0.002**** |
| Mean±Std | 191.20±515.745 | 0.03±0.029 | |
| Median | 3.38 | 0.04 | |
| Range | 0.00-2048.00 | 0.00-0.07 | |

**NS CPO**

| Variable | G*G* genotype (n=4) | G*A* genotype (n=13) | P value |
|---|---|---|---|
| $2^{-\Delta\Delta Ct}$ | | | **0.036*** |
| Mean±Std | 0.01±0.007 | 69.34±137.471 | |
| Median | 0.01 | 1.14 | |
| Range | 0.00-0.02 | 0.01-385.34 | |

| Variable | G*G* genotype (n=4) | A*A* genotype (n=1) | P value |
|---|---|---|---|
| $2^{-\Delta\Delta Ct}$ | | | **0.328** |
| Mean±Std | 0.01±0.007 | 1.55 | |
| Median | 0.01 | 1.55 | |
| Range | 0.00-0.02 | 1.55 | |

The level of mRNA overexpression (in the form of $2^{-\Delta\Delta Ct}$) of AA, GA compared to GG (rs642961) and A*A*, G*A* compared to G*G* (rs2235371) from total NS OFC and its phenotypes that showed significant association can be seen in figure 1 and 2.

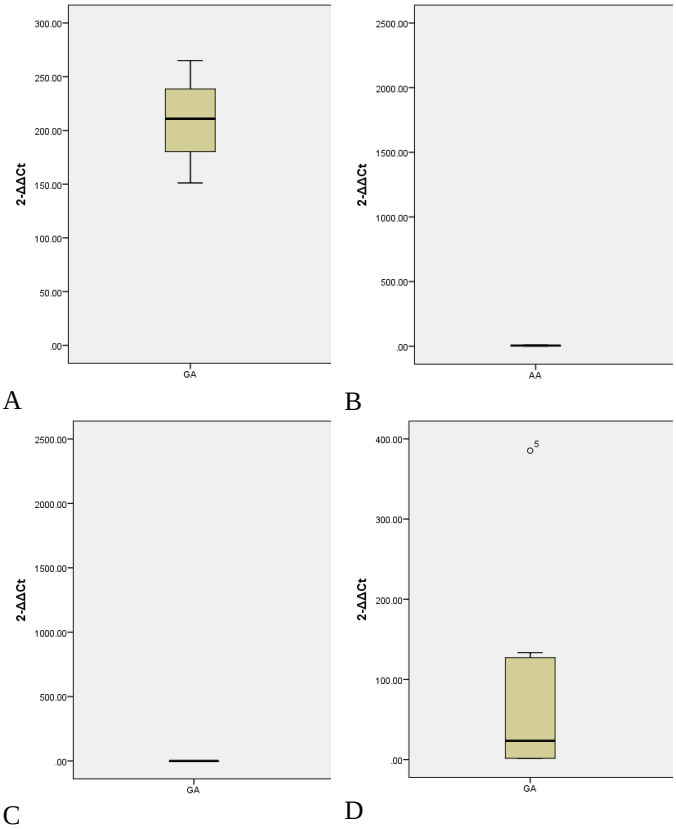


Figure 1 Significant association between GA genotype from NS CU CLP (A), AA genotype from NS CLO (B), GA genotypes from NC CLO (C) and GA genotypes from CPO (D) compared with *IRF6* mRNA expression ($2^{-\Delta\Delta Ct}$). GA genotypes from NS CU CLP and NS CPO show significant mRNA overexpression compared with GG genotype, GA and AA genotypes from NS CLO shows mRNA underexpression compared with GG genotype.

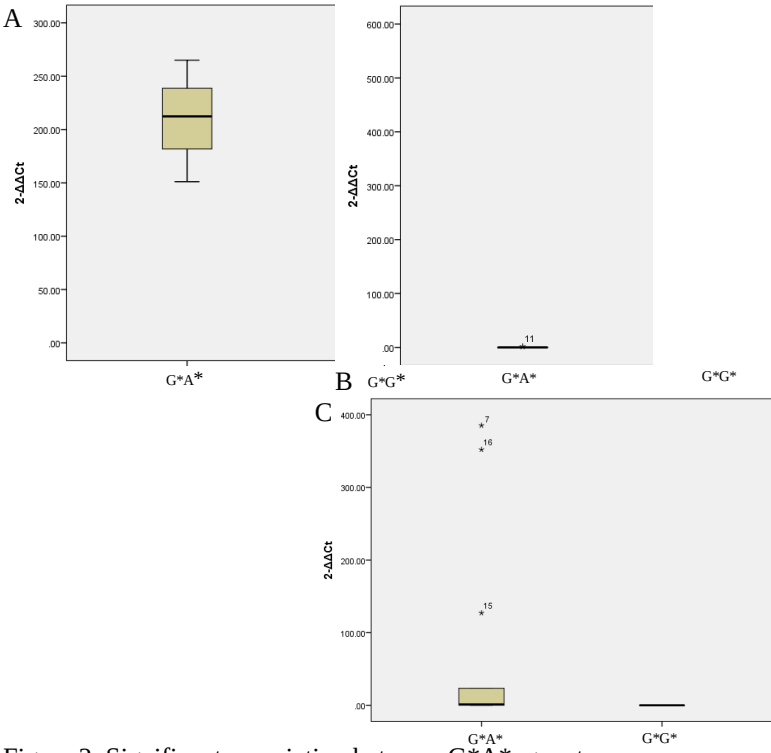


Figure 2 Significant association between G*A* genotype from NS CU CLP (A), G*A* genotype from NS CB CLP (B) and G*A* genotype from NC CPO (C) compared with *IRF6* mRNA expression ($2^{-\Delta\Delta Ct}$). G*A* genotypes from NS CU CLP and NS CPO show significant mRNA overexpression compared with G*G* genotype, G* from NS CB CLP shows mRNA underexpression compared with G*G* genotype..

**Discussion**

Morphogenesis of the face requires transcription factors, signaling molecules, and effector proteins that are essential for tight coordination of cellular behaviors including migration, cells proliferation, differentiation and apoptosis.[11,36] As a transcription factor, there is a multi-species conserved sequence 9.7 kilobases upstream of the *IRF6* start site (*MCS9.7*) that has been reported as a potential enhancer of *IRF6*.[9,16] Expression studies of this highly conserved *MCS9.7* enhancer region showed that it is expressed in orofacial processes early in murine facial development.[9,16] Polymorphism rs642961 is a common variant located in *MCS9.7* which seems to have a functional role in *IRF6* transcriptional activity that related to mRNA expression. The enhancer region of *MCS9.7* contains a conserved binding site for the transcription factor of *TFAP-2* ,as well as binding site for *p63* and *Notch*.[9,16,37,38] Polymorphismrs642961 is a risk allele that may disrupts the *TFAP-2* binding site and this common variant is a risk allele for NS OFC in some populations.[4,9,16,39] *IRF6* is also regulated by *ΔNp63*. There are three *p63* binding sites with the potential to regulate *IRF6*: one in the *MCS9.7* enhancer, one upstream of the transcriptional state site, and one within the first intron.[16,38] In addition, three *IRF6* response elements (ISRE), one of which is the *ΔNp63* binding site

within the *MCS9.7*, increase endogenous transcript.[16,38,40]Biggs et al propose that in human cutaneous keratinocytes, *IRF6* and *p63* act jointly in a positive feedback loop to regulate *IRF*6 transcription, in which *IRF6* replaces *ΔNp63* to keep transcription high in differentiated keratinocytes when *ΔNp63* is downregulated.[16,40] In turn, induction of *IRF6* results in proteasomemediated degradation of *p63*, which limits keratinocyte proliferation[16,40]and allow keratinocyte to exit from the cell cycle to form terminal differentiation and cause epithelial mesenchymal transformation (EMT) and during embryological period it allows fusion of palatal shelves.[40] In the palate, *Irf6* is also part of a regulatory module wherein Pbx proteins control the expression of *Wnt9b* and *Wnt3*, which regulate *p63*, which then binds the *MCS9.7* to regulate *Irf6* expression and proper palatal development.[11,16]

*IRF6* is highly expressed in epithelial cells and is required for regulation of proliferation and differentiation during epithelial development[41,42] and knockdown of the *IRF6* gene in mice has shown abnormal stratified epidermis, skin, limb, and craniofacial development, and gene-expression analyses have indicated that the primary defects in the keratinocyte proliferation-differentiation switch.[43] The effect of rs642961 on *IRF6* expression has been investigated in foreskin keratinocytes and lip tissues from NS OFC patients.[9,16]But there is still no data about the same studies that being investigated in human oral epithelium with NS OFC. Using oral epithelium for functional investigation has several advantages because oral epithelium is available to be got and there is no need to do such invasive way. However, human oral epithelium in post natal period is not an ideal cells still, as it is not available to be collected in developmental period of upper lip, primary palate and secondary palate development. Based on our study result, there were different values in mRNA expression levels between NS OFC phenotypes and also in total NS OFC in human oral epithelium. It means that *IRF6* rs642961 strongly correlated with *IRF6* mRNA expression levels that has different functional roles in between NS OFC phenotypes to be detect from human oral epithelium with NS OFC and its phenotypes. As there were different *IRF6* mRNA over and underexpression caused by rs642961 , the regulation of *IRF6* at the level of transcription also has different mechanism that has not been yet defined. As *IRF6* rs642961 located in promoter/enhancer region, normally, transcription begins when an RNA polymerase binds to promoter sequence on the DNA molecule.The promoter sequence contains the binding motifs for the polymerase and for additional transcription factors or repressors. Clearly, when the motives are affected also the transcriptional activity will be affected. In recent years, researchers have discovered that other DNA sequences, known as enhancer sequences, also play an important part in transcription by providing binding sites for regulatory proteins that affect RNA polymerase activity. Binding of regulatory proteins to an enhancer sequence causes a shift in chromatin structure that either promotes or inhibits RNA polymerase and transcription factor binding.

In NS CU CLP and NS CPO, AA and GA genotypes seemed to cause *IRF6* mRNA overexpression with the highest value is on the AA genotype from NS CPO (7.62 times higher than GG genotype, p=0.036). This result may in contrary with the previous theory that promoter polymorphism can alter its binding with *AP-2α*and its impact is through the decrease of *IRF6* mRNA expression.[44] We assumed that this mRNA overexpression caused by the alteration of such regulations with *TFAP-2* ,as well as binding site for *p63* and *Notch*. The alteration of *p63* degradation in controlling epithelium degradation may also affect the *IRF6* mRNA overexpression. *IRF6* is also a mediator of *TGFβ3* in the regulation of epithelial mesenchymal transformation (EMT) and of apoptosis during EMT-apoptosis pathway.[45] Ectopic expression *IRF6* rescues sh *Tgfß3*-blocked palatal fusion, but whether it also influences the mRNA overexpression has not yet been defined.*IRF6* mRNA overexpression may also cause epidermal hyperproliferation and result terminal differentiation failure. On the other hand, in NS CB CLP and NS CLO, AA and GA genotypes seemed to cause *IRF6* mRNA underexpression with the lowest value is on the GA genotype from NS CLO (0.056 times lower than GG genotype, p<0.001). It could give the influence to the inhibition effect of transcription process. However, mutations in the regulatory sequences (including the promoters) may effect the levels of the expression of the mRNA if the mutation or SNP alter the interaction with transcription factors, co activators and co repressors, thus the mutation or SNP can alter the levels and spacial distribution of the protein encoded by the gene. But our study result was different with study result from Pan et al, where *IRF6* mRNA expression from lip patient revealed overexpression from all patients.[34]

The rs2235371 (820 G>A) is a functional polymorphism replaces a valine by an isoleucine at amino acid position 274 (V274I/non-synonymous substitution) of the SMIR-binding domain of *IRF6* which showed strong evidence of overtransmission of the V allele of an intragenic of *IRF6* polymorphism in Asian and South American populations.[18,19,25,46] The rs 2235371, located in exon 7/coding region, can be as missense mutation also and missense mutation can affect DNA-transcription factors resulting in altering the expression of the corresponding protein. Altering the wild-type protein expression in the compartment where it is designed to function will disrupt the normal cell cycle and in turn may cause disease.[47] Mutations or polymorphisms in coding regions may also greatly impact on the organisms phenotype when they are non-synonymous (changing the amino acid which the codon codes). This will change the peptide sequence of the encoded protein with potential to influence the function of the protein.

In NS CU CLP and NS CPO G*A* genotype seemed to cause mRNA overexpression with the highest value is in NS CPO (G*A* genotype was 6.68 times higher than G*G* genotype, p=0.006). In NS CB CLP G*A* seemed to cause mRNA underexpression (G*A* genotype was 0.15 lower

than G*G* genotype, p=0.004). These results were different with mRNA expression values from rs642961, means that polymorphism in enhancer/promoter region cause different functional role with polymorphism in coding regioin that can reveal different pathogenesis of both polymorphism. That been said, mutations or polymorphisms in the coding region might also influence the expression levels of the mRNA because some regulatory sequences are in coding sequence and also because micro RNA also regular the stability and localisation of mRNA by binding to specific complementary sequences in the mRNA. But when associated with the *IRF6* rs2235371, the functional role of this polymorphism to the *IRF6* mRNA expression still can not be explained yet.

## Conclusion

NS OFC phenotypes that can be investigated from human oral epithelium with NS OFC and there are different over and underexpression levels of mRNA expression from both *IRF6* rs642961 and rs2235371 polymorhisms among NS OFC phenotypes , means that *IRF6* has different functional role in each NS OFC phenotypes.

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