Research

Association of IRF6 and rs22355371 polymorphism mutation with risk of non-syndromic orofacial cleft

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ABSTRACT

Background: Non-syndromic orofacial cleft (NSOFC) is a congenital abnormality characterized by incomplete division of the oral and nasal cavities, with no other abnormalities present. NSOFC is the result of disruptions in the normal biomolecular processes of craniofacial development. Many genes have been linked to NSOFC, one of them is IRF6 gene. **Purpose:** To investigate the association of IRF6 gene and incident of rs22355371 mutation, with the risk of developing NSOFC. **Method:** A cross-sectional comparative study, conducted in DR M Djamil General Hospital Padang Indonesia from September 2024 to November 2024 involving 13 lip or palate tissues from patient with NSOFC, and 13 maxillary crest from controls. Expression of IRF6 was examined with real time polymerase chain reaction (RT PCR) and the segment of rs22355371 was examined with Sanger sequencing. **Result:** IRF6 gene expression was shown to have a median value of 129.3 in NSOFC patients, and 252.5 in controls. This indicated that IRF6 gene expressions were lower in NSOFC patients. The Mann Whitney test was performed had it can be concluded that there is no statistically significant relation between IRF6 gene expression between NSOFC patients and controls with a p value = 0.174 ($p > 0.05$). **Conclusion:** Compared to controls, NSOFC patients had decreased levels of IRF6 gene expression and we found rs22355371 polymorphism either in NSOFC patient or controls.

Keywords: non-syndromic orofacial cleft, IRF6, rs22355371

ABSTRAK

Latar belakang: *Celah orofasial non-sindromik (CONS) merupakan kelainan bawaan yang ditandai dengan pemisahan rongga mulut dan rongga hidung yang tidak sempurna, tanpa adanya kelainan lain. Celah orofasial ini merupakan akibat dari gangguan proses biomolekuler perkembangan kraniofasial. Banyak gen yang telah dikaitkan dengan kejadian ini, salah satunya adalah gen IRF6.* ***Tujuan:*** *Mengetahui hubungan gen IRF6 dan kejadian mutasi rs22355371, dengan risiko kejadian CONS.* ***Metode:*** *Penelitian komparatif potong lintang, dilakukan di RSUD DR M Djamil Padang, Indonesia dari bulan September 2024 sampai dengan November 2024, yang melibatkan 13 jaringan bibir atau lelangit penderita CONS, dan 13 krista maksilaris sebagai kontrol. Ekspresi IRF6 diperiksa dengan* RT PCR*, dan rs22355371 diperiksa dengan* Sanger sequencing*.* ***Hasil:*** *Ekspresi gen IRF6 terbukti memiliki nilai median 129,3 pada pasien CONS, dan 252,5 pada kontrol. Hal ini menunjukkan bahwa ekspresi gen IRF6 lebih rendah pada pasien CONS. Uji* Mann Whitney *dilakukan dan dapat disimpulkan bahwa tidak ada hubungan yang signifikan secara statistik antara ekspresi gen IRF6 antara pasien CONS dan kontrol dengan nilai p=0,174 (*p > 0,05*).* ***Kesimpulan:*** *Dibandingkan dengan*

kontrol, pasien CONS memiliki tingkat ekspresi gen IRF6 yang lebih rendah, dan kami menemukan mutasi pada polimorfisme rs22355371 pada pasien CONS maupun kontrol.

Kata kunci: *celah orofasial non-sindormik,* IRF6, rs22355371

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INTRODUCTION

Orofacial cleft (OFC) are considered to be among the most common structural birth defects, and arise from the incorrect fusion of tissues in the upper lip, palate (the upper mouth), or in both areas, during craniofacial development.[1] Non-syndromic OFC is a congenital defect defined by the partial separation of the oral and nasal cavities, absent of any additional abnormalities.[2] Clefts can be unilateral, bilateral, complete or incomplete and may affect lip only (CL), the palate only (CPO), or both (CL/P).[3] The occurrence of OFC differs based on geographical factors, ethnicity, and economic status, averaging 1 in 700 newborns or between 0.5 and 2.6 per 1,000 live births.[1] Variations in the occurrence of NSOFC among different ethnic groups and populations further validate the role of genetics in this occurrence.[2]

The cause of OFC is intricate, and numerous candidate genes and loci with diverse functions have been demonstrated to play a role in OFC development, highlighting the significant susceptibility of craniofacial developmental pathways. The cause of NSOFC is not completely comprehended, multiple variants in candidate genes have been recognized and associated with the condition.[3] Interferon regulatory factor (IRF) is a homologous protein that regulates interferon transcription (IFN) and is an expression of IFN-induced genes. Interferon regulatory factor 6 (IRF6) plays a vital role in the formation and maintenance of oral periderm, palate adhesion, and palate fusion. The IRF6 genetic variant is strongly associated with non-syndromic CL/P incidence among different populations. Past studies have indicated that out of all the genes believed to play a role in cleft lip and palate, IRF6 stands out as the most consistent candidate. The relationship between SNP IRF6 and susceptibility to non- syndromic cleft lip and palate was found to be consistent in Central European and Han Chinese populations, while the Brazilian population exhibited varied levels of susceptibility in the analyses. No vulnerabilities were seen in the non-Hispanic white and Swedish group studies. This may still be due to differences in the origin of patients and recruitment criteria.[4]

METHOD

This research was a cross-sectional comparative study to examine expression of IRF-6 and rs22355371 polymorphism in NSOFC patients and control, using real-time polymerase chain reaction (RT-PCR), and Sanger sequencing method. All participants were from DR. M. Djamil General Hospital Padang, Indonesia. In total, all participants including 26 individuals: 13 with NSOFC and 13 as controls. This study was conducted in Molecular Biology Laboratory of Health Research Unit of Faculty Medicine Universitas Andalas Padang/DR. M. Djamil General Hospital Padang in September - November 2024.

The materials utilized in this research include either lip or palate tissue from NSOFC patients, and maxillary crest from

patients who were undergoing septoplasty as control. We assigned a code to each sample as PLT for NSOFC patients, and NSL for controls.

Genotyping was performed by isolating DNA isolation by using the primers of forward: 5'GCTCGATCATTAACCCAGG-3' and primer *reverse*: 5'CAGTCATTGGGAGAGAGCT-3' with lengths of about 312 base pairs (bp). Then an examination was carried out to determine a nucleotide. We performed the sequencing technique by PT Genetica Science to analyze polimorphisms IRF6 rs22355371 in the genetic variation. Analyzed nucleotide sequences of NSOFC patients and control with reference nucleotide base sequence. In the meantime, statistical significance was obtained using SPP statistics 26. The PCR results were evaluated utilizing the Mann-Whitney test. Statistical significance was considered for p-values less than 0.05.

RESULT

The general characteristics of this research subjects consisted of gender, age group, history of environtment risk factor, and phenotype of OFC. Table 1 showed the results of subjects characteristics.

Table 2 showed the difference in IRF6 gene expression based on RT PCR examination with a comparative analysis method between NSOFC patients and control. The results obtained a median value of 129.3 for IRF6 gene expression inNSOFC patients, while the median value in control was 252.5. This showed that IRF6 gene expression was lower in NSOFC patients compared to controls. The Mann Whitney test was performed, and it could be concluded that there was no statistically significant relationship between IRF6 gene expression in NSOFC patients and controls with a *p* value = 0.174 (p > 0.05).

Table 1. Subject characteristics

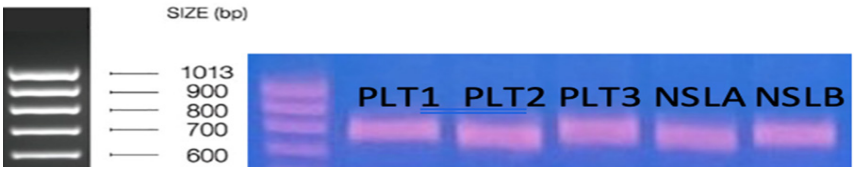
Characteristics	NSOFC (n=13) f (%)	Controls (n=13) f (%)
Gender		
Male	11 (84,6)	11 (84,6)
Female	2 (15,4)	2 (15,4)
Age Group		
0-6 months	1 (7,7)	0 (0,0)
7-12 months	4 (30,8)	0 (0,0)
13-24 months	4 (30,8)	0 (0,0)
25-60 months	2 (15,4)	0 (0,0)
>5-17 years	1 (7,7)	1 (7,7)
≥18 years	1 (7,7)	12 (92,3)
Phenotype		
Cleft lip	3 (23,1)	-
Cleft palate	3 (23,1)	-
Celah lip and palate	7 (53,8)	-
Other risk factor		
History of maternal infection (Yes)	0 (0,0)	0 (0,0)
History of smoking (Yes)	0 (0,0)	0 (0,0)
History of anticonvulsant consumption (Yes)	0 (0,0)	0 (0,0)
History of folic acid consumption (No)	9 (69,2)	0 (0,0)
Hisory of OFC in family (Yes)	8 (61,5)	0 (0,0)

Table 2. Expression of IRF6

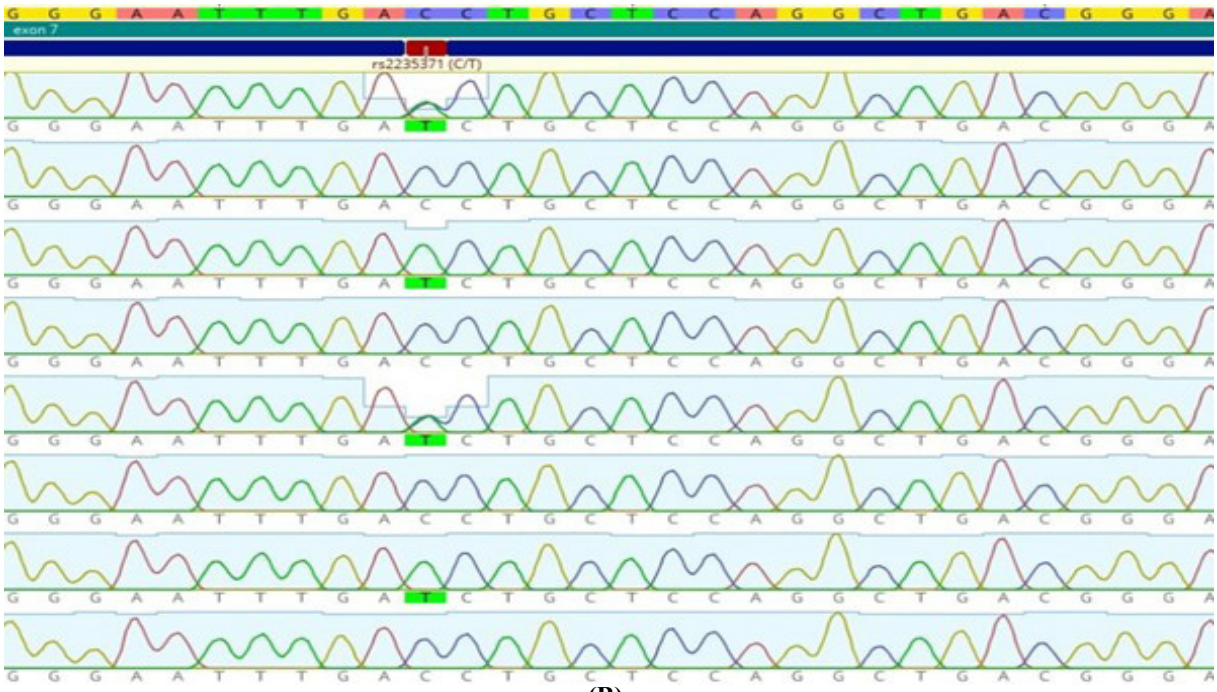
IRF6 expression	N	Median	Min-Max	*p* value
NSOFC	13	129,3	1,0 – 9410,1	0,174
Controls	13	252,5	46,9 – 10846,9	

The genotyping findings from the initial PCR product (indicated a DNA band corresponding to the IRF6 rs22355371 gene region measuruing 757 bp) along with the Sanger sequencing outcomes were displayed in Figure 1. Based on Figure 1, the success of PCR was seen, the amplification result band with an amplicon size of 757 bp was visible. PCR is a series of initial denaturation, advanced denaturation, annealing, elongation/extension and final elongation processes. All PCR product samples were then subjected to PCR sequencing. The sequencing data results were then processed with Genious 11.1.2 software. Respondents who were found to have the CC allele were referred to as wild type, respondents who experienced an allele change from CC to CT were called heterozygous mutants, and those who experienced an allele change from CC to TT were called homozygous mutants. The results of IRF6 gene sequencing SNP rs2235371 (C/T) showed that 6 samples were CC alleles (wild type), 5 samples were CT alleles (heterozygous mutants), and 2 samples were TT alleles (homozygous mutants).

This research showed a description of the IRF6 gene polymorphism SNP rs2235371, from 13 NSOFC patients. It was found that there were more in the mutant group, namely 7 patients (53.8%) compared to the wild type group, namely 6 patients (46.2%), while from 5 controls, it was found that there were more in the mutant group too, namely 3 respondents (60%) compared to the wild type group, namely 2 respondents (40.0%).



(A)



(B)

Figure 1. (A) Initial PCR product of IRF6 rs22355371; (B) Alignment of sequencing result

DISCUSSION

Orofacial cleft (OFC) represents the most prevalent congenital deformity of the face, emerging in roughly one out of every 600 deliveries annually, and is observed more often in males compared to females.[5] In this study, it was found that most of NSOFC patients were male, namely 11 respondents (84.6%). This was in line with research conducted by the Palate Cleft Foundation in West Java, which found more male patients than female patients.[6] Another study on 186 NSOFC patients was dominated by males, namely 119 respondents and females 67. The prevalence of OFC in males was about twice that of females, whereas the prevalence of cleft lip CL was reported to be about two-thirds higher in females.

This difference might be associated with the stage of craniofacial development between female and male embryos. It is explained that the shift of the palatal shelf from vertical to horizontal in female embryos occurs approximately half a week later than in male embryos, which might increase the risk of cleft formation.[7,8]

Most of the subjects in this study were in the 7-12 months and 13-24 months which consisted of 4 patients (30.8%). While previous study found that there were 90 respondents aged <1 year, 53 respondents aged 1-6 months, 65 respondents aged 6-12 months, 28 respondents aged 1-5 years, and 19 respondents aged 6-12 years.[9] Another study investigating the incidence of NSOFC reported that 1,104 subjects (24.2%) were aged 3–6 months, 405 subjects (8.9%) were aged 6–12 months, 780 subjects (17.1%) were aged >1–2 years, 995 subjects (21.8%) were aged 2–5 years, 485 subjects (10.6%) were aged 5–10 years, and 816 subjects (17.9%) were aged >10 years.[10]

Orofacial clefts (OFCs) are generally classified into several types, including cleft lip (CL), cleft palate (CP), and cleft lip and palate (CL/P).[11] Based on the results of this study, the most common phenotype identified was CL/P, occurring in 7 patients (53.8%),

followed by CL in 3 patients (23.1%) and CP in 3 patients (23.1%). These findings are consistent with the study by Nahas et al.[12] which reported that 42.1% of respondents had CL/P, while 25.6% had CP. Several other studies had also reported that CL/P was the most frequently observed type of orofacial cleft. Similarly, a study conducted by Riana et al.[6] at the Cleft Lip and Palate Center, Faculty of Medicine, Universitas Muhammadiyah Malang, also reported that CL/P is the most common form of orofacial cleft.

In this study, 8 NSOFC patients (61.5%) had a family history of orofacial clefts. A previous study investigating the relationship between family history and NSOFC reported that 21.8% of patients had first-degree relatives with orofacial clefts, while 24.8% had second-degree relatives with the condition, and the remaining 53.4% had no family history of OFC.[12] The differences in proportions observed across studies might be related to variations in the populations studied, as well as differences in study methodologies. Another study reported that the CL/P group had the highest proportion of positive family history (36%). This finding suggested that a positive family history might be more common in more complex cleft phenotypes. Therefore, in families of individuals with more complex forms of OFC, the likelihood of identifying other affected relatives might be higher compared to families of patients with less complex cleft types.[13]

In this study, there were 9 respondents (69.2%) mothers of NSOFC patients who did not consume folic acid since early pregnancy. Haryadi et al.[14] conducted a study on 19 pregnant women found significant results between consumption of folic acid supplements in early pregnancy and the incidence of orofacial clefts in Kupang City with a *p* value=0.00. The OR value of 20.0 showed that pregnant women who did not consume folic acid supplements in early pregnancy had a risk of giving birth to children with orofacial clefts 20.0 times higher than those who consumed folic acid supplements during early pregnancy had a 20-fold higher risk of giving birth to children with orofacial clefts compared with those who consumed folic acid supplements.

Folic acid deficiency in the first trimester will increase the risk of OFC up to 13 times. It was found that 71% of OFC patients had mothers who experienced folic acid deficiency during pregnancy.[15] Folate and cobalamin deficiencies especially during the first trimester of fetal development, double the risk of developing orofacial clefts.[16] Folic acid is an essential substance for the synthesis of purines and pyrimidines, which are components of DNA and RNA needed in the regulation of gene expression and cell differentiation. Folic acid also functions to protect DNA from damage as its other function is as a re-regulator of toxic waste products of protein metabolism.[17]

In this study, none of the mothers of NSOFC patients had a history of smoking during pregnancy. Alshammary et al.[18] conducted a retrospective study involving 40 mothers of children with orofacial clefts in 2019-2023, and reported that 12.5% of mothers smoked during pregnancy, although the association was not statistically significant. Several studies had consistently reported that maternal smoking during pregnancy was associated with an increased relative risk of orofacial clefts ranging from shown a relative risk of orofacial clefts ranging from 1.3 to 1.5. However, the relationship between maternal smoking and orofacial clefts appeared to be relatively weak when smoking was the only risk factor involved.[16] There is a direct interaction between cigarette products and neonatal tissue, which causes hypoxia caused by impaired angiogenesis and nicotine-mediated vasoconstriction, which has been shown to interfere with palatal fusion in animal models.

Cigarette smoke may directly affect fetal tissues through several biological mechanisms. One proposed mechanism involves fetal hypoxia caused by impaired angiogenesis and nicotine-mediated vasoconstriction, which has been shown in animal models to interfere with palatal fusion. Another hypothesis suggests that maternal smoking may alter DNA methylation patterns in the fetus, potentially affecting the expression of genes involved in the development of craniofacial, and cleft formation.[19]

Reduced oxygenation in fetal tissues may impair the fusion of the lip and palatal processes. In addition, nicotine can modify gene expression and lead to the persistence of epithelial cells in connective tissue regions that normally undergo fusion, thereby contributing to the development of orofacial clefts.[19]

In this study, none of the mothers had a history of infection during pregnancy. However, a study conducted by Adewale et al.[9] reported that 43.7% of mothers of children with orofacial clefts experienced persistent fever during early pregnancy (first trimester). Maternal fever has long been considered a potential risk factor for abnormal fetal development. Since the 1960s, evidence from animal studies has suggested that hyperthermia may have teratogenic effects. Even brief exposure to elevated maternal body temperature has been reported to cause cell death, membrane disruption, vascular damage, and placental infarction, which may increase the risk of structural or functional abnormalities in the fetus.[6] Fever during pregnancy may also interfere with protein synthesis and enzyme production, leading to alterations in essential cellular processes such as cell proliferation, migration, differentiation, and apoptosis.[7] Exposure to maternal fever during the first trimester has been associated with a 40–60% increased risk of congenital anomalies, including orofacial clefts.[20] Furthermore, maternal fever exceeding 40°C during the first 8 weeks of pregnancy has been reported to significantly increase the risk of facial cleft formation in the developing fetus.[9]

In this study, none of the mothers reported using anticonvulsant drugs during pregnancy. Exposure to anticonvulsant drugs during pregnancy had been suggested to increase the risk of congenital abnormalities, and might interfere with fetal growth and development. Sodium valproate is a well-known teratogenic drug and has been recognized as a potential risk factor for congenital and developmental abnormalities in 1972.[21] However, a study conducted by Nahas et al.[22] reported no significant association between maternal anticonvulsant drugs use during pregnancy and the incidence of non-syndromic orofacial clefts.

In this study, real-time PCR analysis was performed, and the results showed that IRF6 gene expression was lower in patients with orofacial cleft patients compared with individuals without orofacial clefts. These findings were consistent with the study conducted by Kondo et al.[23] which reported higher IRF6 expression levels in individuals without orofacial clefts. The study also suggested that haplo-insuficiency of IRF6 might disrupt normal maxillofacial development and lead to the formation of orofacial clefts, confirming that IRF6 gene plays a key role in lip and palate development, and is also involved in the development of the skin and external genital. Gene expression analysis using lip or palate tissues samples from patients with orofacial cleft has not been widely explored. Therefore, this approach may provide valuable insights into the genes and gene families that interact and contribute to the phenotypic variability observed in the non-syndromic orofacial cleft.[24] The IRF6 gene, located on chromosome 1q32.2, plays an important role in craniofacial development and has been associated with the severity of orofacial clefts. Previous studies had also demonsrated a significant association between

IRF6 and the occurrence of non-syndromic orofacial clefts.[23]

In a study conducted by Ke et al.[25] using rat palatal organ culture, ectopic expression of the IRF6 gene was shown to increase palatal fusion and rescue fusion defects caused by shTGFβ3. The IRF6 gene regulates the switch between cellular proliferation and differentiation, and loss-of-function or mutations or polymorphisms may lead to incomplete fusion of the palatal shelves due to failure of medial epithelial edge (MEE) differentiation. Because IRF6 is strongly expressed in the oral epithelium, tissue samples in this study were obtained from the lip or palate tissues, which were considered keratinized epithelial tissues. This approach was based on the rationale that mRNA expression associated with non-syndromic orofacial clefts could be detected in tissues where the gene is expressed.[24]

The growth of the nose plays a central role in overall facial development. The nasal septum function as a key growth center that generates maxillary traction, directing facial growth forward and downward, and contributing to a sevenfold increase in vertical facial length between the 10th and 40th weeks postconception development. The internal nose develops through expansion of the nasal cavity. As the nasal cavity deepens, the nasal sacs are formed through posterior fusion by the end of the 6th week of embryonic development. The oronasal membrane initially separates the nasal sacs from the oral cavity, and ruptures around the 7th week, creating a communication between the nasal and oral cavities.

The primary palate lies at the base of the primary nasal fossa.[26] Therefore, normal samples were obtained from the maxillary crest, which were considered representative the of IRF6 gene expression in individuals without orofacial clefts. During the postnatal period and adulthood, IRF6 gene expression can also be detected in several tissues, including hair follicles, palatal rugae, tooth buds, thyroglossal ducts, external genitalia, and skin throughout the body. Both palatal development during the fetal period, and wound healing during the postnatal or adult period, require epithelial cell migration and proliferation. Therefore, it is assumed that the functional role of the IRF6 gene may still be detectable during the postnatal or adult period, as IRF6 is involved in epidermal differentiation, an essential process of keratinocyte maturation, and the formation of functional epidermis.[24]

A study conducted by Bezerra et al.[7] using blood samples, reported a significant association between the IRF6 rs2235371 polymorphism and the occurrence of non-syndromic cleft lip (CL). Heterozygous and homozygous variants were identified in 9 CL patients, 19 CL/P patients, and 19 normal individuals.

Similarly, a study conducted by Yegin et al.[27], investigated the association between the IRF6 SNP rs2235371 polymorphism and non-syndromic cleft lip/and or palate in a Turkish population. All analyzed single nucleotide polymorphisms (SNPs) conformed to Hardy–Weinberg equilibrium (HWE). Statistical analysis showed no significant difference in the allele frequencies of rs2235371 between children with NSCLP and the control group (p=0.6026). Similarly, no significant differences were observed when comparing the allele frequencies between mothers of NSCLP children and controls (p=0.3802).

In conclusion, this study demonstrated that IRF6 gene expression was lower in patients with non-syndromic orofacial clefts (NSOFC) compared with controls, although the difference was not statistically significant. The rs2235371 polymorphism of the IRF6 gene was identified in both NSOFC patients and control subjects, indicating the presence of genetic variation in the studied population. These findings suggested that IRF6 might play a role in the molecular mechanisms

underlying craniofacial development and the pathogenesis of non-syndromic orofacial clefts. However, the lack of statistical significance might be related to the limited sample size in this study. Further studies with larger sample sizes and more comprehensive genetic analyses are needed to clarify the role of IRF6 gene expression and polymorphisms in the development of NSOFC.

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