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RESEARCH ARTICLE

Analysis of *FOXE1* rs3758249, *IRF6* rs2235375, *MTRR* A66G in Non-syndromic Cleft Lip and Palate among Indonesian Deutero-Malay Population

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Abstract

Non-syndromic cleft lip and palate (NS-CLP) is one of the most common orofacial malformations, with an incidence of 1 in 700 live births worldwide. This study aimed to determine the risk factor for NS-CLP among the Indonesian Deutero-Malay population by analyzing the *FOXE1* rs3758249, *IRF6* rs2235375, and *MTRR* A66G polymorphisms. It is a case-control study, using 50 samples of NS-CLP patients and 50 samples of control (for *FOXE1* rs3758249 and *MTRR* A66G), 30 samples of NS-CLP patients, and 30 samples of control (for *IRF6* rs2235375). After DNA was extracted, polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLPs) were performed by using restriction enzymes of Mscl (*FOXE1* rs3758249) and Nde1 (*MTRR* A66G), and sequencing was performed for *IRF6* rs2235375. The study was done in the Molecular Genetic Laboratory, Faculty of Dentistry Universitas Padjadjaran Bandung, from September 2023 to January 2024. The chi-square test was used with the exact Fisher's alternatives. The results showed that in *FOXE1* 3758249, A allele (mutant) was found more in control (OR=0.744, p>0.05), in *MTRR* A66G, G allele (mutant) was found more in NS-CLP (OR=1.267, p>0.05) meanwhile in *IRF6* rs2235375, G allele (mutant) (OR=1.710, p>0.05) was found more in NS-CLP. This study concluded that *FOXE1* rs 3758249, *IRF6* rs2235375, and *MTRR* A66G genes were not the risk factor for NS-CLP in the Indonesian Deutero-Malay population.

Keywords: Deutero-Malay, *FOXE1* rs3758249, Indonesian, *IRF6* rs2235375, *MTRR* A66G, non-syndromic cleft lip and palate

Introduction

Cleft lip and palate (CLP) is a congenital orofacial malformation caused by the failure of the upper lip and palate to fuse.[1] Clinically, three main types of CLP include cleft lip (CL), which is a cleft that occurs only on the lip; cleft palate (CP), which is an aperture that occurs on the palate; and cleft lip and palate (CLP), which is a cleft that occurs on both the lip and palate.[2] CLP can be divided into syndromic (S) and non-syndromic (NS) depending on whether any other syndromes accompany CLP.[2]

The NS-CLP is thought to be influenced by genetic and environmental factors (multifactorial).[3] Genetic factors include polymorphisms in specific genes that are also associated with mechanisms that predispose to CLP.[2] Environmental factors can be influenced by alcohol consumption and smoking during pregnancy. In contrast, genetic factors occur when the patient's parents directly inherit the condition.[4]

Approximately 70% of CLP cases are NS-CLP.[5] The highest prevalence of NS-CLP is in Asian populations, followed by people of Western European descent and Africans.[6] Based on a study in West Java, Indonesia, from 2011 to 2015, using data collected from patients who received treatment at hospitals in that period, there were 1,596 patients with oral aperture, and it was found that NS-CLP patients had the highest percentage,

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reaching 50.53% from all cases. Furthermore, NS-CP was found in 25.05% of patients, while NS-CL occurred in approximately 24.42% of patients.[7]

The forkhead box E1 (*FOXE1*) gene is thought to be one gene involved in the development of human craniofacial clefts; it is a transcription factor involved in the growth of the thyroid gland and the formation of the craniofacial epithelium. *FOXE1* involvement has been reported in conditions such as congenital hypothyroidism, Van der Woude syndrome, Pierre Robin syndrome, and CLP. Several studies found a significant association between NS-CLP and single nucleotide polymorphisms (SNPs) or polymorphism of *FOXE1* rs3758249. Based on a study in the Northeast region of China, it was concluded that the *FOXE1* gene has a close relationship with NS-CLP.[8] The interferon regulatory factor 6 (*IRF6*) gene is involved in the immune response. It has functions in cell signaling, craniofacial morphogenesis, epithelial cell proliferation, and differentiation. It encodes for a transcription factor that regulates the expression of interferons and other genes necessary for the immune system and wound healing.[9] Polymorphisms in the *IRF6* gene can cause epidermal hyperproliferation, leading to impaired fusion in various lip and palate formation processes. These polymorphisms fail terminal differentiation (proliferation of epidermis or epithelium differentiating into mesenchyme) and multiple epithelial adhesions, leading to CLP. Studies in different populations, such as Europe and China, have identified the *IRF6* gene as one of the genes predisposing to NS-CLP and as a candidate gene that consistently exerts a significant influence on the incidence of NS-CLP, including *IRF6* rs2235375.[10] Methionine synthase reductase (*MTRR*) gene polymorphisms are also associated with the incidence of NS-CLP and being involved in folic acid metabolism, especially *MTRR* rs1801394 A66G.[11] A study in the Chinese population showed that the *MTRR* A66G gene was thought to be associated with an increased risk of NS-CLP.[12] However, a study in Turkey's population showed that *MTRR* A66G was not a risk factor for the incidence of NS-CLP.[13]

The *FOXE1* rs3758249, *IRF6* rs2235375, and *MTRR* A66G gene polymorphisms as risk factors of NS-CLP in the Indonesian Deutero-Malay population, which is the largest population in Indonesia, have never been studied before. Therefore, we are interested in analyzing *FOXE1* rs3758249, *IRF6* rs2235375, and *MTRR* A66G as risk factors of NS-CLP in the Indonesian Deutero-Malay population, which is the largest population in Indonesia.

Methods

This study was done at the Molecular Biology Laboratory, Faculty of Dentistry, Universitas Padjadjaran Bandung, Indonesia, from September 2023 to January 2024. It was approved by the Research Ethics Committee of Universitas Padjadjaran with the number 1341/UN6.KEP/EC/2023.

The samples consist of 50 NS-CLP patients and 50 controls for *FOXE1* rs3758249 and *MTRR* A66G, 30 NS-CLP patients, and 30 controls for *IRF6* rs 2235375. The DNA was isolated from the venous blood of all samples, and then polymerase chain reaction (PCR) was done using primers that include 5'GA TGGTGGTGCCAGGTGA-3 (forward) and 5'GCTTTGAGCGTTTCCACA-3' (reverse) for *FOXE1* rs3758249,[14] 5'AGTTGGCCCAAAACTGAAC3' (forward) and 5'GGCTAGCCAGGAAACAGAAA3' (reverse) for *IRF6* rs2235375, and 5'- GCA AAG GCC CAT CGC AGA AGA CAT-3' (forward) and 5'- GTG AAG ATC TGC AGA AAA TCC ATG TA-3' (reverse) for *MTRR* A66G.[11]

The PCR results were evaluated by using agarose gel electrophoresis. The optimal PCR product for *FOXE1* rs3758249 is a band of 211 basepairs (bp), for *IRF6* rs2235373 is a band of 243 bp, and for *MTRR* A66G is a band of 66 bp. The optimal PCR products were then evaluated by using restriction fragment length polymorphisms (RFLPs), which were digested by restriction enzymes of Msc1 (*FOXE1* rs3758249),[8] and NdeI (*MTRR* A66G).[15] In contrast, the Sanger sequencing method sequenced the optimal PCR product of *IRF6* rs2235375. Allele and genotype frequencies between patients and control subjects were analyzed using the chi-square test, and Fisher's exact test will be used as another alternative.

Results

The optimal PCR products of the *FOXE1* rs3758249, *IRF6* rs2235375, *MTRR* A66G, and

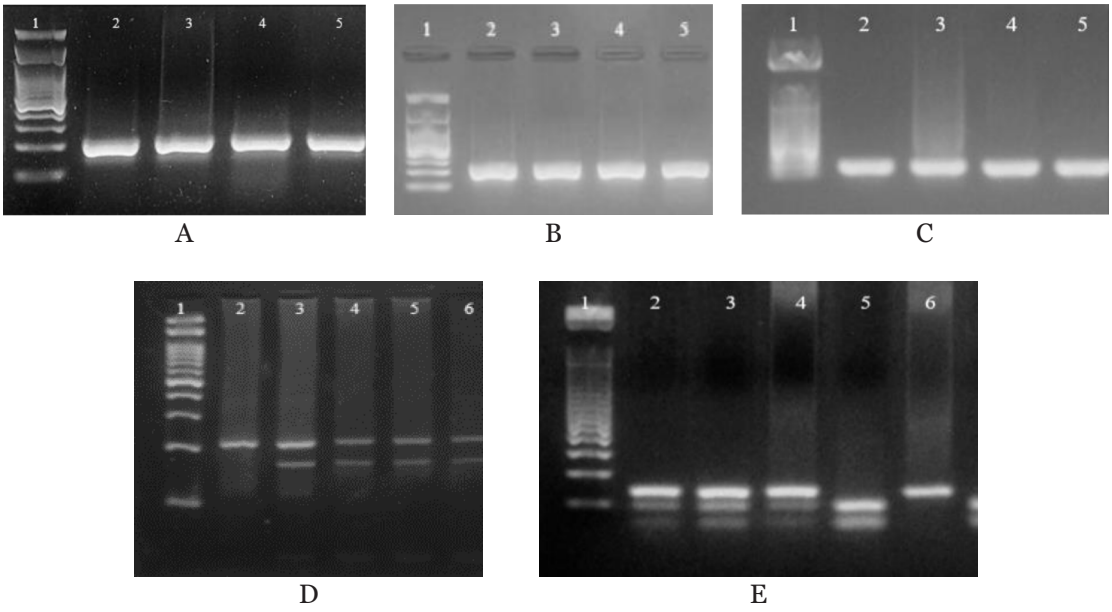

Figure 1 Polymerase Chain Reaction (PCR) Products

Note: (A) PCR product of *FOXE1* rs3758249, lane 2–5 DNA bands of optimal PCR products (211 bp); (B) PCR product of *IRF6* rs2235375, lane 2–5 DNA bands of optimal PCR products (243 bp); (C) PCR product of *MTRR* A66G, line 2–5 DNA bands of PCR products (66 bp); (D) PCR-RFLPs of *FOXE1* rs3758249, lane 2 and 7 GG genotype (homozygous normal), lane 3–6 GA genotype (mutant heterozygote); (E) PCR-RFLPs of *MTRR* A66G, lane 2–4 AG genotype (heterozygous mutant), lane 5 and 7 AA genotype (homozygous normal), lane 6 GG genotype (homozygous mutant)

PCR-RFLPs of *FOXE1* rs3758249 and *MTRR* A66G are shown in Figure 1. In Figure 1D, there were only two genotypes of *FOXE1* rs3758249, including the GG genotype (homozygous normal/wild type; 211 bp) and GA genotype (heterozygous mutant; 211, 163, and 48 bp). In Figure 1E, there were three genotypes of *MTRR* A66G that include AA genotype (homozygous normal/wild type; 44 and 22 bp), AG genotype (mutant heterozygous; 66, 44, and 22 bp), and GG genotype (mutant homozygous; 66 bp). To evaluate the PCR-RFLPs result of *FOXE1* rs3758249, some of the samples were assessed by sequencing the Sanger method from each genotype (Figure 2), but we did not do the sequencing of *MTRR* A66G due to very short base sequences. Sequencing results of *IRF6* rs2235373 are shown in Figure 3, and it shows the CC genotype (homozygous normal/wild type), CG genotype (mutant heterozygous), and GG genotype (mutant homozygous).

The allele and genotype frequencies are described in Tables 1, 2, and 3. The allele and genotype frequencies of all polymorphisms did not show risk factors of NS-CLP. It indicates that *FOXE1* rs3758249, *IRF6* rs2235375, and *MTRR* A66G are not risk factors for NS-CLP

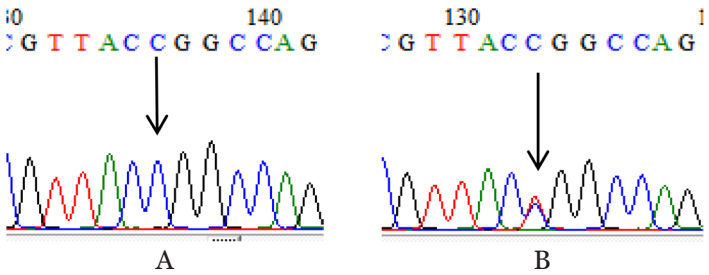

Figure 2 Sequencing Results of *FOXE1* rs3758249

Note: (A) GG genotype (normal homozygous/wild type); (B) GA genotype (mutant heterozygous)

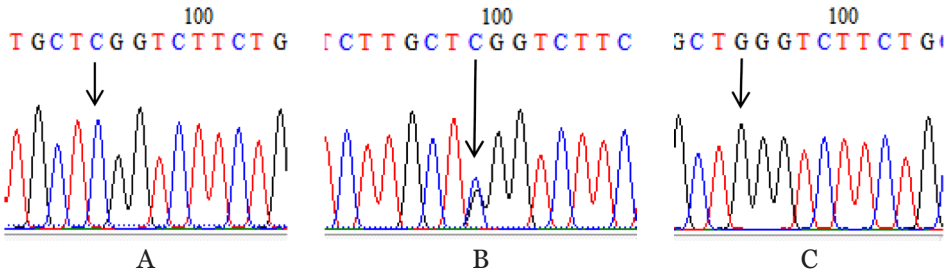

Figure 3 Sanger Sequencing Results of *IRF6* rs2235375
Note: (A) Normal homozygous CC genotype (normal homozygous/wild type); (B) CG genotype (mutant heterozygous); (C) GG genotype (mutant homozygous)

Table 1 Allele and Genotype Frequency of the *FOXE1* rs3758249 among NS-CLP and Control

Allele and Genotype	NS-CLP n=50	Control n=50	χ^2	p	OR	95% CI
G	90	87	0.442	0.506	1.345	0.560–3.228
A	10	13	0.442	0.506	0.744	0.310–1.785
GG	40	37	0.508	0.476	1.405	0.550–3.590
GA	10	13	0.508	0.476	0.712	0.279–1.818
AA	0	0	0	0	0	0–0

Note: OR: odds ratio, CI: confidence interval, G: wild type allele of *FOXE1* rs3758249, A: mutant allele of *FOXE1* rs3758249, significance p<0.05

Table 2 Allele and Genotype Frequency of the *IRF6* rs2235373 among NS-CLP and Control

Allele and Genotype	NS-CLP n=30	Control n=30	χ^2	p	OR	95% CI
C	26	34	2.133	0.144	0.585	0.284–1.204
G	34	26	2.133	0.144	1.710	0.831–3.521
CC	5	11	3.068	0.080	0.345	0.103–1.163
CG	16	12	1.071	0.301	1.714	0.616–4.772
GG	9	7	0.341	0.559	1.408	0.445–4.453

Note: OR: odds ratio, CI: confidence interval, C: wild type allele of *IRF6* rs2235373, G: mutant allele of *IRF6* rs2235373, significance p<0.05

Table 3 Allele and Genotype Distribution of the *MTRR* A66G among NS-CLP and Control

Allele and Genotype	NS-CLP n=50	Control n=50	χ^2	p	OR	95% CI
A	67	72	0.590	0.443	0.790	0.432–1.444
G	33	28	0.590	0.443	1.267	0.693–2.316
AA	22	24	0.161	0.688	0.851	0.387–1.870
AG	23	24	0.040	0.841	0.923	0.421–2.024
GG	5	2	1.425	0.436	2.667	0.492–14.445

Note: OR: odds ratio, CI: confidence interval, A: wild type allele of *MTRR* A66G, G: mutant allele of *MTRR* A66G, significance p<0.05

in the Indonesian Deutero-Malay population. Meanwhile, GA genotype of *FOXE1* rs3758249 (OR=0.712, p=0.476) tend to be a protective factor and no AA genotype was found (Table 1). The G allele (OR=1.710, p=0.144) and CG genotype (OR=1.714, p=0.301) of *IRF6* rs2235373 tend to increase the risk of NS-CLP (Table 2). The GG genotype of *MTRR* A66G tends to be a risk factor for NS-CLP (OR=2.667, p=0.436, Table 3).

Discussion

The *FOXE1* gene is located on chromosome 9q22.q33, consisting of one exon and expressed transiently in the thyroid gland and anterior pituitary, is part of a family of transcription factors containing a DNA-binding forkhead domain that can bind and open chromatin structures.[8,14] Polymorphisms of this gene, especially in the promoter region, have been associated with various thyroid-related etiologies, orofacial cleft including CLP, hypothyroidism (HT), and thyroid cancer, so the *FOXE1* rs3758249 gene has a very important role in the process of embryonic development. *FOXE1* rs3758249 polymorphism is in the form of base G into A substitution in the upstream region.[8,14] There are different results of the risk factors for *FOXE1* rs3758249 in the incidence of NS-CLP in several other populations. A study in Northeastern China showed that *FOXE1* rs3758249 was strongly associated with NS-CLP;[8] strong associations were found in Central Europe and Mayan Mesoamerican.[16] Meanwhile, another study on population in China showed that *FOXE1* rs3758249 was not associated with NS-CLP.[17] In this study, *FOXE1* rs3758249 is also not a risk factor for NS-CLP among the Indonesian Deutero-Malay population, but the GA genotype tends to be a protective factor (OR=0.712, p=0.476, Table 1). Differences in results on the same polymorphisms associated with NS-CLP revealed that the role of the same polymorphisms on NS-CLP depends on different races and geographical statuses.

The *IRF6* gene, also known as CLP gene,[18] is located on chromosome 1q32.2-q4 and encodes a member of IRF family and consists of 10 exons, with the start codon in exon three and the stop codon in exon.[8,10,19,20] *IRF6* plays an important role in epidermal development and is helpful for the expression of the leading ectoderm on the palatal shelves before and during primary palate formation. The *IRF6* mediates TGFβ3 in regulating epithelial-mesenchymal transformation (EMT) and apoptosis during palatal fusion. The *IRF6* gene regulates the degradation of Δp63 protein, resulting in the induction of p21 expression and apoptosis of medial edge epithelium (MEE), which are important for palatal fusion.[10] Mutations in *IRF6* result in hyperproliferation of the epidermis, which inhibits the terminal differentiation process so that the MEE in both palatal shelves fail to transform into mesenchyme in the EMT process and palatal shelves fusion does not occur then bring it into CP condition.[21] The study in Western China found an association of *IRF6* rs2235371, rs2013162, and rs2235375 polymorphisms with the incidence of NS-CLP abnormalities.[22] *IRF6* rs2235375 is located in the intron six as base C into G substitution. The study in Mexico detected a significant under-transmission of the common allele C and a significant over-transmission of the allele G for the rs2235375 marker, and the study in South India also detected *IRF6* rs2235375 to have a positive association with NS-CLP.[20,22,23]

Some *IRF6* polymorphisms have been studied among the Deutero-Malay population associated with NS-CP and NS-CLP, with significant results in Indonesia. Those polymorphisms are *IRF6* rs2235371 as a risk factor of NS-CP,[24] *IRF6* rs2013162,[25] and *IRF6* rs642961[26] as the risk factors of NS-CLP. In this study, *IRF6* rs2235375 is not a risk factor for NS-CLP in the Indonesian Deutero-Malay population, but the mutant G allele tended to increase the risk of NS-CLP (OR=1.710, p=0.144). *IRF6* is known to be a CLP gene,[18] but not all polymorphisms in this gene can be associated with the risk of NS-CLP, especially in the Indonesian Deutero-Malay population, suggesting the different roles of each SNPs in the *IRF6* gene.

The *MTRR* gene is located on chromosome 5p15.2-15.3 with a length of about 3.6 kb and encodes 698 amino acids with a molecular weight of about 77 kDa and has 15 exons.[11] The *MTRR* gene is linked to folic acid metabolism. It plays a vital role in homocysteine metabolism.[27] The *MTRR* gene is required for reductive methylation of vitamin B12 and is a gene that encodes the enzyme methionine synthase reductase, which plays a vital role in methionine metabolism and vitamin B12 regeneration.[11] *MTRR* A66G is the most common polymorphism in which adenine

is replaced by guanine, causing the substitution of isoleucine with methionine at codon 22.[11,28] This polymorphism involved in homocysteine metabolism associated explicitly with elevated plasma homocysteine concentration that may increase the predisposition of NS-CLP.[27] Homocysteine is formed from methionine metabolism, homocysteine must undergo methylation to be converted into methionine. Folic acid deficiency can disrupt the process of the folate-methionine reaction, where methionine is needed for protein formation. When there is a disturbance in methionine formation, the process of protein formation can not occur properly, which causes *MTRR* deficiency and brings into hyperhomocysteinemia, which is thought to affect fusion between the medial nasal process and maxillary process, resulting in NS-CLP.[29]

In this study, *MTRR* A66G was not a risk factor associated with NS-CLP. However, the GG genotype tends to increase the risk of NS-CLP among the Indonesian Deutero-Malay population (OR=2.667, p=0.436, Table 3). This result was supported by a study of the population in Turkey, which also showed no significant relationship between *MTRR* A66G gene polymorphism and the incidence of NS-CLP.[11] However, a different result was found in the Chinese population, as there was a significant association between the *MTRR* A66G and the incidence of NS-CLP.[12]

Conclusion

FOXE1 rs 3758249, *IRF6* rs 2235375, and *MTRR* A66G gene are not risk factors for NS-CLP in the Indonesian Deutero-Malay population.

Conflict of Interest

The authors reported no potential conflict of interest.

Acknowledgment

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