Global Medical and Health Communication

Online submission: https://ejournal.unisba.ac.id/index.php/gmhc
DOI: https://doi.org/10.29313/gmhc.v12i3.13677

GMHC. 2024;12(3):180–189
pISSN 2301-9123 | eISSN 2460-5441

RESEARCH ARTICLE

Polymorphisms of rs7055763 and rs41307258 in *TBX22* Gene Haplotype as Risk Factors for Non-syndromic Cleft Palate Indonesian Deutero-Malay Population

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Abstract

Non-syndromic cleft palate (NS-CP) is a multifactorial congenital malformation affected by genetic and environmental factors. The incidence of non-syndromic cleft lip with or without cleft palate (NS-CLP) varies considerably between ethnic groups and geographical regions. *TBX22* is a crucial determinant for the formation of intramembranous bone in the posterior hard palate. Therefore, *TBX22* is fundamental to palatogenesis and supports normal palate progress. The rs7055763 and rs41307258 polymorphisms in the *TBX22* gene are associated with risk factors for NS-CP in the Indonesian Deutero-Malay population. In the previous study, NS-CP still needed to be investigated in the Deutero-Malay population. However, there are different races, mainly for the Deutero-Malay population. This study aims to determine whether rs7055763 and rs41307258 polymorphisms in the *TBX22* gene are risk factors for NS-CP in the Deutero-Malay population. This study was conducted in Terpadu Laboratory, Faculty of Dentistry, Universitas Padjadjaran, from February until June 2023. The design of this study was a case-control study. The DNA patient samples were obtained from saliva and whole blood. Moreover, DNA is extracted, and the rs7055763 and rs41307258 segments are analyzed using PCR and Sanger sequencing. PCR data was analyzed by chi-square testing. In this study analysis, polymorphisms of rs7055763 (G>A) and rs41307258 (T>A) in the *TBX22* gene show no significant differences between case and control groups, namely 0.911 and 0.645, respectively. However, the genotype in the rs41307258 shows the p-value as 0.027, indicating substantial differences and the OR is 1.390. In conclusion, the rs7055763 and rs41307258 polymorphisms in the *TBX22* gene do not appear to be risk factors for developing NS-CP in the Indonesian Deutero-Malay population.

Keywords: Indonesian Deutero-Malay, non-syndromic cleft palate, rs41307258, rs7055763, *TBX22*

Introduction

Non-syndromic cleft lip with or without cleft palate non-syndromic (NS-CLP) is a congenital disorder also known as orofacial clefting, comprising disorders such as cleft lip (CL), cleft palate (CP) or cleft lip with or without cleft palate (CLP).[1] Furthermore, CLP is a congenital malformation caused by multifactorial factors. It occurs worldwide with a frequency of about 1 in 700 to 1,000 live births, around 45–50% of cases being CL/CP, 25–30% for cleft lip (CL), and 25% for cleft palate (CP).[1–3] Multifactorial factors include genetic and environmental factors that cause non-syndromic CP.[4] Genetic factors, such as physical, chemical, and biological factors, influence the differentiation, migration, and proliferation of neural crest cells, even causing CP. Environmental factors associated with CP include vitamin intake, diet, access to medications, and lifestyle, such as smoking.[5–7] Ethnic and gender differences in the incidence of NS-CLP contribute to a genetic component in the occurrence of the disorder. Moreover, there is a relationship between specific populations and population variations in the incidence of NS-CLP at birth based on geography. The Asian population has the highest prevalence, followed

Received: 3 May 2024; Revised: 28 July 2024; Accepted: 12 August 2024; Published: 17 December 2024

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by the Caucasian population, and the African population is approximately 1 in 500 births, 1 in 1,000 births, and 1 in 2,500, respectively.[8] In West Java, NS-CLP is around 50.53% for the most significant type of cleft, followed by CL and CP for about 25.5% and 24.42%, respectively.[9]

The prevalence of CP affects about 1 in 1,000 live births worldwide.[10] NS-CP incidence varies considerably between ethnic groups and geographical regions.[11] Furthermore, NS-CP refers to any palate cleft located posterior to the incisive foramen, which does not involve the alveolar processus or the lip.[12] In Indonesia, the birth prevalence of orofacial clefts, including NS-CP, is around 0.2%, with a total of 7,500 cases reported each year. This incident highlights the need for special attention in the research and treatment of this disorder in Indonesia, considering the long-term impact on the quality of life of patients and their families.[13] NS-CP influences sufferers are psychological problems caused by feelings of shame. Besides that, NS-CP disorders can also cause eating disorders in sufferers. Furthermore, especially for babies with NS-CP, parents will have difficulty feeding their baby on the first day of birth, so special feeding aids are needed. Another prominent problem that NS-CP sufferers can experience is speech disorders and nasal sounds.[1,14] Besides that, the Deutero-Malay population is one of the ethnic groups in Indonesia consisting of Malay, Makassar, Javanese, Sundanese, Bugis, and Minang people, even if it has a unique genetic background that may influence the frequency and impact of NS-CP.[15–17] Moreover, the genetic factors that influence NS-CP in the Deutero-Malay Indonesian population are rs2235373 and rs2235371 polymorphisms in the *IRF6* gene showed that the rs2235373 polymorphism influences the functional role of the gene, and it is a risk factor for NS-CP incidence.[17–19] Besides that, the various genes that contributed to the occurrence of NS-CP are very diverse, such as interferon regulatory factor 6 (*IRF6*), homeobox gene 1 (*MSX1*), methylenetetrahydrofolate reductase (*MTHFR*), T-box 22 (*TBX22*).[17,18,20–22] The *TBX22* gene is located on the X chromosome, and it is part of the T-box gene family, which plays a vital role in embryonic development, including craniofacial development in NS-CP. Furthermore, genetic and environmental factors showed that about 50% influence NS-CP risk.[23] The genetic and environmental factors include growth, DNA transcription, nutrient metabolism, immunity, and oncogenesis.[22] The *TBX22* gene, which encodes a T-box transcription factor, is a candidate gene associated with NS-CP incidence. *TBX22* gene consists of nine exons at position Xq21.1 and has two promoters, P0 and P1.[24] Furthermore, the P0 promoter contains the rs7055763 and rs41307258 polymorphisms. Single nucleotide polymorphisms (SNPs) can affect gene function and play a role in disease mechanisms, susceptibility to environmental factors, and the increase or inheritance of the risk of certain diseases. Inherited polymorphisms such as rs7055763 and rs41307258 together are referred to as haplotypes.[25,26]

The various studies showed that the mutations in *TBX22* were consistently found in NS-CP patients in Thai, Brazilian, North American, and Indian populations.[24,27,28] The role of the *TBX22* gene in NS-CP has yet to be investigated in the Indonesian Deutero-Malay population.*16–18* Interestingly, previous studies concluded that the *TBX22* gene is significantly associated with NS-CP incidence.[2,24,27] Therefore, this study aims to determine whether the rs7055763 and rs41307258 polymorphism in the *TBX22* gene are risk factors for NS-CP in the Deutero-Malay population.

Methods

This study was conducted in Laboratorium Penelitian Terpadu and Foundation for the CL/CP sufferers in Dentistry of Universitas Padjadjaran from February until June 2023. This study used convenience sampling based on inclusion and exclusion criteria. Furthermore, to be eligible to participate in this study, a participant must be NS-CP without any other abnormalities, willing to be included as a patient subject, while healthy individuals with no relatives who have had a cleft palate in the previous two generations and willing to be included as a control subject. Participants with NS-CP who do not belong to the Deutero-Malay community are excluded. The subjects of this study were 29 patients with NS-CP from the Deutero-Malay population who came to the Cleft Lip and Palate Foundation.

Moreover, 58 patients were selected from healthy individuals without NS-CP and had a family history of NS-CP at least two generations

ago as the control subjects. The sample used in this study was blood or saliva. The sample that forms blood was collected by injection technique, while saliva was taken by gargle. This sampling technique is explained further. First, tie the arm to slow blood flow so that the veins are more clearly visible and the blood sample is easy to take. After that, they cleaned the sampling area with tissue or cotton containing alcohol. Then, a syringe is inserted to take the blood sample, followed by a transfer to the EDTA tube. The saliva sample is conducted by the requirement of the respondents in this study not to eat, drink, gargle, brush their teeth, or smoke for 1 hour before taking the saliva; in addition, the respondents can take a breath, following that is hard coughing.

After that, the respondents were asked to gargle with the mouthwash for 10–15 seconds. Then, hold it in the mouth and repeat to gargle 3–5 times. Finally, they collected the fluids in the mouth into a tube and added them to the mixing solution. Shake it till frothy, then wrap it with biohazard plastics. The ethics of this study have been approved by the Research Ethics Committee of Universitas Padjadjaran, number 529/UN6. KEP/EC/2023. Moreover, the materials of this study consist of cell lysis solution containing ten mM Tris-HCL with pH 8.0, 25 mM disodium EDTA, sodium dodecyl sulfate (SDS) 0.5%, K proteinase, RNA-ase, protein precipitation solution (ammonium acetate 5M), absolute isopropanol, cold alcohol (70%), and TE buffer 1X.

The study design is a molecular epidemiology study with an analytical case-control observation. The working method involved DNA isolation from the participant's whole blood and saliva. The first step is to gargle to generate saliva, while an injection procedure is used to collect a whole blood sample. Moreover, the method is based on protocol with references to get the saliva sample and the entire blood.[29–33] First, saliva samples or 300 µl whole blood were placed in a 1.5 ml microtube and centrifuged at around 13,000–16,000 rpm for 30 seconds. Add 900 µl RBC lysis solution for the entire blood samples and incubate for 10 minutes at room temperature. Next, the leucosis pellet was centrifuged at about 13,000–16,000 rpm for 20 seconds, then of the supernatant (repeat this step until the red of the leucosis fades (for the whole blood) and the pellet is thick (for saliva samples)). Subsequently, 300 µl cell lysis solution was added into the microtube containing the cell pellets and homogenized through up and down pipetting. Added 15 µl K proteinase, then homogenize and incubate at 55°C for 30 minutes. After that, add 1,5 µl RNAse, homogenize, and incubate at 37°C for 15 minutes. After adding 100 µl of the protein precipitation solution to the microtube, it was vortexed for 15 seconds and then centrifuged for 3 minutes. Transferred the supernatant to the 1.5 ml new microtube and added 600 µl isopropanol. Next, shake the microtube ten times until the clod is formed. To extract the DNA pellet, centrifuge the microtube for one minute. Following that, the cold 600 µl alcohol (70%) was used to wash the DNA pellet and centrifuged for one minute to remove the alcohol and obtain the DNA pellet. Reverse the tube and let the DNA pellet dry on the tissue until it evaporates. Lastly, 50 µl of TE buffer was added to dissolve the DNA pellet.

In addition, the measurement of DNA fragments in the promoter region of the *TBX22* gene used the polymerase chain reaction (PCR) technique (thermo fisher PCR). Furthermore, the method of this study used primers for the rs7055763 and rs41307258 polymorphisms with lengths of about 125 bp and 556 bp, respectively. The forward and reverse primers of the polymorphisms in the *TBX22* gene were included from the forward primer 5'-3': GAGCTGCCCTGGAGAAATAA and reverse primer 5'-3': AGCACAAGAGAACGTGGTGT. Besides that, electrophoresis was performed to ensure that the PCR products from the amplification had the desired base pair number.

Besides that, sequencing was then carried out to determine a nucleotide. In this study, PT Genetika Science carried out the sequencing technique. Furthermore, a sequencing technique is performed to analyze polymorphisms rs7055763 and rs41307258 in the genetic variation. This begins with the preparation of sequencing reactions using the Sanger dideoxy method to determine the nucleotide sequence. The sequencing results are usually received in files with a .ab1 extension. Several programs must be installed to open and analyze these files. The sequencing results are then completed with chromatograms and nucleotide sequences. Furthermore, chromatograms are represented by the colored lines of the sequence of DNA bases, with adenine bases shown in green, guanine

bases in black, thymine bases in red, and cytosine bases in blue. The polymorphisms of rs7055763 and rs41307258 in patients with non-syndromic cleft palate were observed using sequencing techniques.

Moreover, the sequencing results are formed by chromatogram data displayed by Chromas 2.6.5. BioEdit program is used for nucleotide sequence analysis. Furthermore, it analyzes nucleotide sequences of the case and control case samples and compares them with the reference nucleotide base sequence. Meanwhile, statistical significance was obtained using SPSS statistics 26. The PCR data was analyzed using the chi-square test. Statistical significance was assumed for p-values <0.05.

Results

The samples were collected from the blood or saliva forms based on the patient and control subjects; then, DNA extraction was carried out for further PCR analysis. After several optimization attempts, an optimal condition led to single-band PCR products. The overall optimal PCR results of the non-syndromic cleft palate case and control samples were obtained, followed by sequencing techniques to analyze the genetic variations at rs7055763 and rs41307258 polymorphisms. In addition, the PCR products generated in this study for the polymorphisms rs7055763 and rs41307258 in *TBX22* genes had a size of 681 bp.

Figure 1 and Figure 2 show the image of the design primer and the location of rs7055763 and rs41307258 in the *TBX22* gene, respectively, and the electrophoresis image of the *TBX22* PCR products.

The variations in the base sequence of the gene, as depicted by the arrow in Figure 3 and Figure 4, have potential implications. The green-colored curve, indicating homozygous genetic variation, and the single dominant A signal, suggesting a homozygous AA base, could have significant implications. Similarly, the black-colored growth curve marked with the letter G (GG) and the genetic variation in heterozygotes indicated by green and black growth curves with two dominant signals for A and G bases (GA) could also have important implications. These findings, represented in R code, demonstrate the genetic variations of homozygous (AA), wild type (GG), and heterozygous (GA) in the rs7055763 polymorphism, as shown in Figure 3.

Figure 4 presents the genetic variations of homozygous (AA), wild-type (TT), and heterozygous (TA) in the rs41307258 polymorphism with utmost clarity. The change from the T allele, the wild-type allele, to the A allele, which denotes the polymorphic allele, is clearly explained. The green-colored curve, indicating

Figure 1 Design Primer and the Location of rs7055763 and rs41307258 in *TBX22* Gene

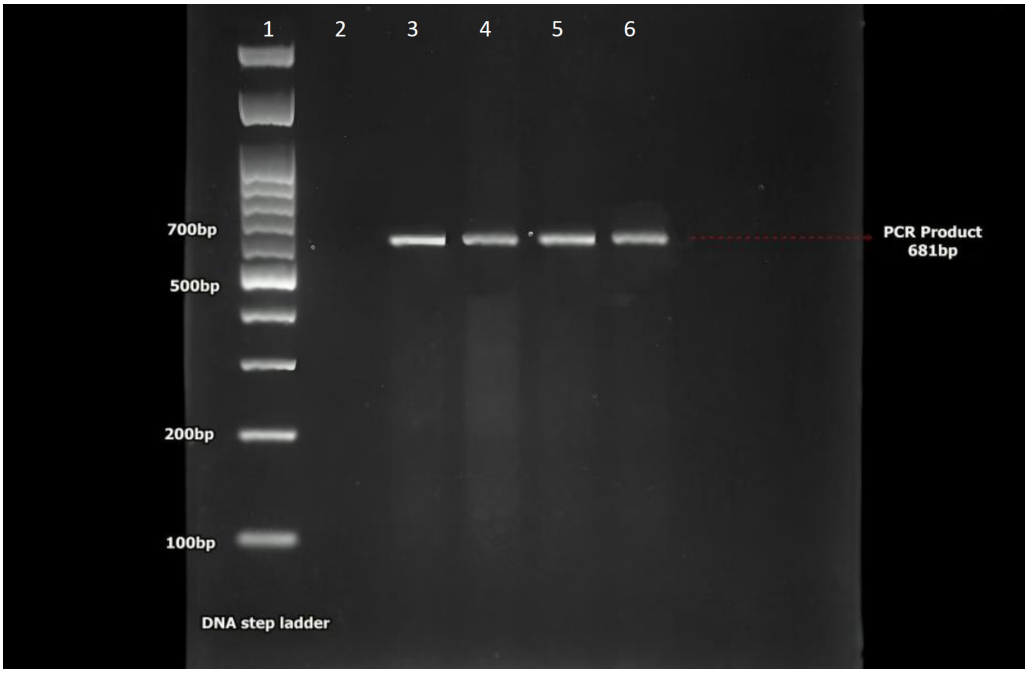

Figure 2 PCR Products of the *TBX22* Gene for rs7055763 and rs41307258
Note: for each track in the number (1) DNA ladder, (2) control negative, (3) PCR products with the size in 681 bp

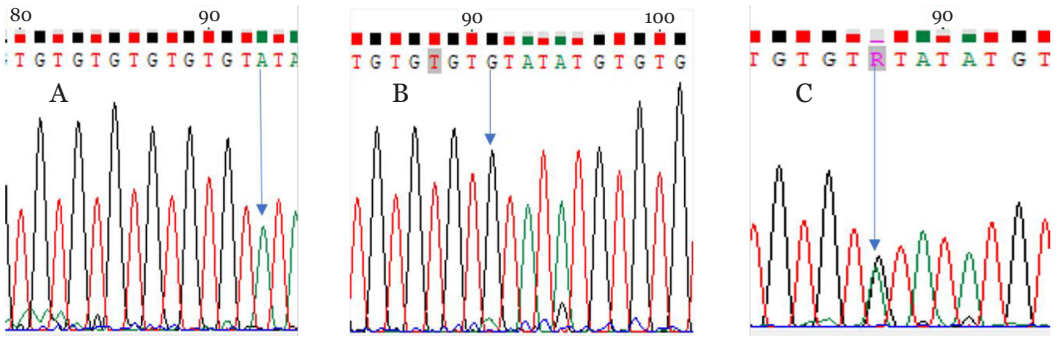

Figure 3 Chromatogram for rs7055763
Note: (A) homozygous (indicated by the green curve), (B) homozygous (indicated by the black curve), (C) heterozygous (indicated by both green and black curves)

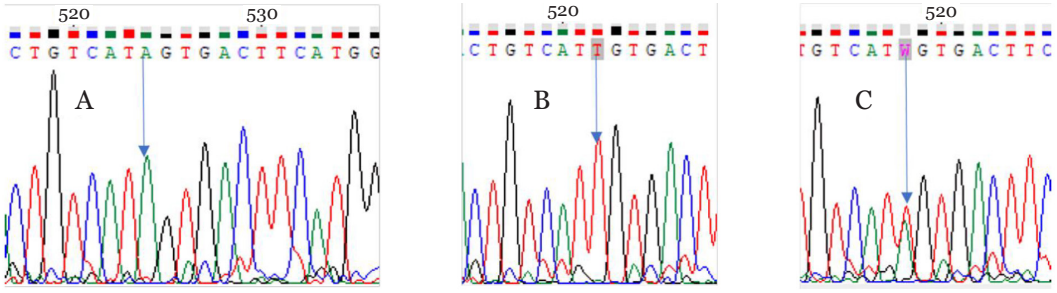

Figure 4 Chromatogram for rs41307258
Note: (A) homozygous (indicated by the green curve), (B) is homozygous (indicated by the red curve), (C) heterozygous (indicated by both green and red curves)

homozygous genetic variation, and the single dominant A signal, suggesting a homozygous AA base, are unmistakable. Similarly, the red-colored growth curve marked with the letter T (TT) and the heterozygous genetic variation indicated by green and red growth curves with two dominant signals for A- and T-bases are presented in a way that leaves no room for doubt. These results are referred to as the A W code, providing a clear understanding of the genetic variations.

Table 1 compares genotypes and alleles of the rs57055763 and rs41307258 polymorphisms in the *TBX22* gene for the case and control group. Our thorough research includes comparing genotypes and alleles of rs57055763 polymorphism in the *TBX22* gene, which showed a p-value of about 0.283 and 0.911, and rs41307258 polymorphism, which showed a p-value of about 0.027 and 0.645. The p-value of comparing haplotypes in the case and control groups of the GT haplotype, GA* haplotype, AT haplotype, and AA* haplotype is 0.984, as summarized in Table 2. The number of cases and controls is 34, 78, and 116 (see Tables 1 and 2), indicating total interaction between each group.

Discussion

This study focused on the genetic polymorphisms of the rs7055763 and rs41307258 in the *TBX22*

Table 1 Comparison of Genotype and Allele of the rs7055763 and rs41307258 Polymorphisms in *TBX22* in the NS-CLP Case and Control Groups

Genotype/Allele	Groups		OR (95% CI)	p	χ^2
	Case n=29 (%)	Control n=58 (%)			
Genotype of *TBX22* rs7055763				0.283	2.527
GG	16 (52)	28 (48)	2.171 (0.680–6.933)		
GA	5 (17)	19 (33)	0.362 (0.095–1.384)		
AA	8 (28)	11 (19)	0.786 (0.262–2.357)		
Allele *TBX22* rs7055763	n=58 (%)	n=116 (%)	0.963 (0.499–1.858)	0.911	0.013
G	37 (64)	75 (65)			
A	21 (36)	41 (35)			
Genotype of *TBX22* rs41307258	n=29 (%)	n=58 (%)		0.027*	7.217
TT	20 (69)	31 (53)	10.323 (1.268–84.046)		
TA	1 (3)	16 (28)	0.086 (0.009–0.788)		
AA	8 (28)	11 (19)	0.887 (0.304–2.587)		
Allele *TBX22* rs41307258	n=58 (%)	n=116 (%)	1.175 (0.592–2.332)	0.645	0.213
T	41 (71)	78 (67)			
A	17 (29)	38(33)			

Table 2 Comparison of Haplotypes in the Case and Control Groups

Haplotype	Groups		OR (95% CI)	p	χ^2
	Case n=29 (%)	Control n=58 (%)			
GT	21 (61.8)	47 (60.2)	0 (0.0–0.0)	0.984	0.459
GA*	0 (0.0)	1 (1.3)	0 (0.0–0.0)		
AT	4 (11.7)	2 (2.6)	6.222 (0.972–39.814)		
AA*	9 (26.5)	28 (35.9)	1.390 (0.5599–3.454)		

associated with haplotypes as risk factors for NS-CP incidence in the Indonesian Deutero-Malay population. Dixon et al.[8] found that the highest incidence was in Asian populations. Furthermore, the study by Sjamsudin and Maifara[9] showed that the percentage incidence of CP in the population of West Java between 2011 and 2015 was around 25.05%. Besides that, other studies explained that the CP incidence occurs more frequently in male patients than female patients. In addition, the survey by Pauws et al.[24] revealed that the incidence of CP occurs frequently in female patients because the rs7055763 and rs41307258 polymorphisms are located in promotor and associated with the CP incidence. The other study by Gurramkonda et al.[28] showed that rs7055763 and rs41307258 polymorphisms increased the NS-CP incidence in South India, as indicated by AA haplotypes. Besides that, one-fifth of patients have a family history of CP. In addition, most patients with CP have low socioeconomic status, which is considered an environmental factor10, but ecological factors were not considered in this study.

Another study by Burg et al.[34] demonstrated the association of the *TBX22* gene with the incidence of CP. *TBX22*, a gene that plays a crucial role in palatogenesis, is found to be associated with the development of CP. The rs7055763 and rs41307258 polymorphisms in the *TBX22* gene are located in the promoter region, which is upstream of the transcription start site and is involved in the initiation of the transcription process. This study reveals that the nucleotide base polymorphism of rs7055763 changes from G to A, while the polymorphism of rs41307258 changes from T to A. The interaction between the polymorphisms of rs7055763 G/A (G>A) and rs41307258 T/A (T>A) leads to GT, GA, AT, and AA. Errors in the DNA sequence during the transcription process can alter the structure and function of the resulting product, leading to the appearance of different phenotypic traits in the organism. This provides a clearer understanding of the genetic basis of CP.

Eighty-seven subjects, carefully selected based on the inclusion and exclusion criteria, were part of this study. The results, which revealed all samples had an optimal PCR product labeled with single bands, are significant. The sequencing results of the rs7055763 and rs41307258 polymorphisms in the *TBX22* gene, compared with human nucleotide sequence data in the DNA bank, provide crucial genetic variation. This study's findings shed light on the significant impact of genetic factors on NS-CP incidence in the Indonesian Deutero-Malay population, including the *IRF6* polymorphisms of rs2235373 and rs2235371. The discovery that the rs2235373 polymorphism affects the functional role of the gene and the action, making it a risk factor for the incidence of NS-CP, is particularly enlightening. Additionally, the association of *MTHFR* A1298C rs1801131 with NS-CP in the Indonesian Deutero-Malay population is a significant finding.

The study by Suphapeetiporn et al.,[27] which showed that the mutation of *TBX22* frequently causes NS-CP in the Thai population, has significant implications. This study aimed to investigate whether the mutations in *TBX22* play a role in the formation of NS-CP in the Thai population, a question of great interest to the medical community. The mutations in the *TBX22* gene were performed in 53 Thai patients unrelated to NS-CP, further engaging the audience. The discovery that the mutations in the *TBX22* gene are responsible for a significant incidence of NS-CP cases in Thailand is a finding of great interest. The study by Pauws et al.,[24] which showed that the rs41307258 polymorphism plays a crucial functional role in Brazil, America, and European populations, also has significant implications. The result showed that the promoter activity of the *TBX22* gene declines by around 50%, leading to the incidence of CP, a finding that will surely pique the audience's interest. Haplotypes containing the rs41307258 promoter are associated with decreased transcriptional activity of *TBX22*, further engaging the audience.

Another study by Fu et al.[10] showed that loss of function mutations in the X-linked *TBX22* promoter disrupts the ETS-1 binding site and causes NS-CP incidence. Furthermore, the incidence of NS-CP is identified by the ÿ73G>A mutation in the X-linked *TBX22* promoter. Therefore, the X-linked *TBX22* promoter mutations can cause CP by disrupting the *TBX22*-ETS-1 pathway. Furthermore, the X chromosome harbors the genomic region of the *TBX22* gene, which is associated with the vital role of transcription factors in mammalian cell differentiation and embryonic development. Gurramkonda et al.[28] reported that the rs7055763 and rs41307258 polymorphisms in the *TBX22*

gene are located in the P0 promoters developing pathogenesis of NS-CP in the Indian population. Furthermore, the result showed that the rs7055763 and rs41307258 polymorphisms had a significant p-value in women with NS-CP, for around 0.034 and 0.022, respectively. Therefore, the two polymorphisms with heterozygous and homozygous variation increased the risk of developing NS-CP in women. However, the rs7055763 and rs41307258 polymorphisms were not significant in men. In the AA haplotype, carrying both mutant alleles (rs7055763 A - rs41307258 A) was significantly associated with NS-CP risk in women but not men. Moreover, the SNPs were not associated with NS-CP risk in men.

This study investigated the role of the rs7055763 and rs41307258 polymorphisms in the *TBX22* gene related to NS-CP incidence in the Indonesian Deutero-Malay population. Moreover, the rs7055763 polymorphism in the *TBX22* gene shows a positive correlation of allele A and genotype AA with the NS-CP phenotype. Besides that, there were more allele A and genotype AA in the NS-CP group than in the control group. However, this result is not statistically significant. At the same time, the rs41307258 polymorphism in the *TBX22* geneshows a positive correlation of allele T and genotype TT with the NS-CP phenotype. In addition, there were more alleles T and genotype TT in the NS-CP group than in the control group. Therefore, this study showed that rs41307258 and rs7055763 polymorphisms in the *TBX22* gene as a risk factor for NS-CP in genotype, allele, and haplotype in the case and control groups show no statistically significant. However, the limitation of this study is that the sample size may influence these results. Besides that, the genotype for the rs41307258 in the *TBX22* gene is statistically significant, as summarized in Table 2. In addition, the odds ratio shows the value is more than 1, indicating potential polymorphisms as risk factors for NS-CP incidence. However, further study is needed to increase the number of case samples in rs7055763 - rs41307258 polymorphisms of the *TBX22* gene, even compared with other polymorphisms for analyzing their interaction.

Conclusion

In the Indonesian Deutero-Malay population, the rs7055763 and rs41307258 polymorphisms in the *TBX22* gene are not risk factors for developing non-syndromic cleft palate.

Conflict of Interest

The authors declared no conflict of interest.

Acknowledgment

The author would like to thank Reny Adiarni Nurhayati and our team at Terpadu Laboratory, Faculty of Dentistry, Universitas Padjadjaran, for their assistance and guidance in preparing this article.

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