Distribution of wingless type 3a (Wnt3a) Rs 752107 gene polymorphism on orofacial cleft patients

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Abstract. Non-syndromic orofacial cleft, including cleft lip with or without cleft on palate (CB +/- CL NS) is the most common disorder resulting from craniofacial development disorder during pregnancy. These developmental abnormalities occur due to interference during the fusion stage or time and position disturbance of the processes and/or palatal shelves. The prevalence of orofacial cleft is around 1:500 - 1:2000 in the world. Wnt3a, which is the genes belonging to the Wnt gene family controls craniofacial development during pregnancy. It involves regulating the development of the middle face and upper lip fusion. Therefore this gene plays an important role as a cause in non-syndromic cleft lip/palate. The relationship of Wnt3a gene polymorphism to the orofacial cleft occurrence in Indonesia remains unclear. To examine the relationship of polymorphism of Wnt3a Rs 752107 genes in orofacial cleft patients in Indonesia. The study samples are raw material stored in the Oral Biology Laboratory of the Faculty of Dentistry, the University of Indonesia in the form of DNA from cleft lip patients. Study on the distribution of genetic variation of wnt3a rs 752107 in 30 samples of CB +/- CL NS patients and 170 control samples without orofacial cleft using Polymerase Chain Reaction (PCR-RFLP) examination with AluI enzyme. Significance of variation difference test using Chi-square test on SPSS 22. Frequency of CC were dominant in both samples (>90%). There was no significant difference between gene polymorphisms of wnt3a rs 752107 in orofacial cleft with control (p>0.05).

1. Introduction
One of the most complex events of the development of the head and neck is control during the embryonic phase. If there were any disturbances during this important period, that is the formation of the face (7-10 weeks in humans), it can lead to the orofacial cleft.[1] Orofacial cleft, also known as cleft lip and palate is a congenital defect in the form of a gap on the lips that can be accompanied with or without a gap in the palate. These developmental abnormalities occur due to interference during the fusion stage or time and position disturbance of the process and or palatal shelves [2]. Orofacial cleft is the most common disorder of craniofacial development during pregnancy [3–5]. The prevalence of orofacial cleft is around 1:500 - 1:2000 in the world. [4,6]. The Asian population has a higher prevalence than the European or Africa [3,4,7]. In Southeast Asia, the estimated prevalence of orofacial cleft is 1.36: 1000 live births, while cleft lip/palate is 1.08: 1000 live birth [8]. Based on Riset Kesehatan Daerah (RISKESDAS) in 2013 performed on infants aged 24-59 months in Indonesia showed a cleft lip prevalence of 0.08%. Many genes are involved in craniofacial development...
during embryogenesis including growth factors (such as: FGFs, TGFs, PDGFs, EGFs, BMPs, and their respective receptors), signaling molecules (eg, WNT family, SHH, and their respective receptors) and transcription factors (eg, MSX, DLX, LHX, PRRX, BARX and their respective receptors).

There are several types of genes in the Wnt gene family: Wnt3, Wnt3A, Wnt5A, Wnt7A, Wnt8A, Wnt9B and Wnt11. Wnt gene family is involved in regulating the development of the middle face and upper lip fusion. Therefore this gene plays an important role as a cause in non-syndromic cleft lip/palate. The expression of Wnt is present on the upper lip and both primary and secondary palate [9]. The Wnt3A gene is one of the genes belonging to the Wnt gene family that controls mesenchymal cells and neural crest cells in the craniofacial process and regulates the palatal fusion in the animal model, so there is a high possibility for this gene to play a role in the development of non-syndromic cleft lip/palate [10]. The wildtype allele of this gene is C while T is the variant, causing 3 possible genotypes- homozygous wildtype CC, homozygous variant TT and heterozygous variant CT. The objective of this study is to examine the relationship of polymorphism of Wnt3a Rs 752107 genes in orofacial cleft patients in Indonesia.

2. Material and methods

2.1. Samples
The study samples are raw material stored in Oral Biology Laboratory of Faculty of Dentistry, Universitas Indonesia in the form of DNA from cleft lip patients or non-syndromic cleft and from 30 subjects and also from 170 healthy individuals without cleft from Indonesian population. The extraction and storage procedure was done following the methods according to Auerkari et al. The study received the approval from the Ethical Committee of Research, Faculty of Dentistry, Universitas Indonesia (Protocol Number: 01560615).

2.2. Genotyping
Identification of genes is done on the DNA extracts from orofacial cleft group and control group to find out whether the gene in question is present or not. The stage of genetic identification begins with polymerase chain reaction (PCR) and electrophoresis, followed by restriction fragment length polymorphism (RFLP), subsequently electroforesed and visualized with gel-doc.

2.3. Polymerase Chain Reaction (PCR)
Genes of wnt3a rs 752107 were analyzed using forward and reverse primers. Sequence from forward primer is 5’-AGCAGGACTCCCACCTAAAC-3’, while the reverse primer sequence is 5’-GCCTCATCCACCATAAAACC-3’. We identified the gene using Tag PCR mastermix (Bioline). Composition of master mix reaction is 19.5 µL reactant consist of 10 µL of mix (KAPA), 1 µL of primers forward (10 pmol/reaction), 1 µL of primers reverse (10 pmol/reaction), 7.5 µL dDH2O, and 0.5 µL DNA template. PCR condition used was pre-denatured at 94°C for 4 minutes for 1 cycle followed by denaturation at 94°C for 45 seconds, annealing at 60, 5°C for 45 seconds, and extension at 72°C for 45 seconds for 35 cycles. Lastly, 1 cycle of final extension at 72°C for 7 minutes was carried on and then stored at 4°C under infinity time. The PCR results will show one band of fragments on 466 base pairs (pb)

2.4. Electrophoresis of PCR Result
Using a 1.5% agarose gel prepared from a mixture of 1.5 grams of agarose powder and added 100 ml of TAE 1x solution, electrophoresis under 80 volts, 50 minutes, 400A was done. The electrophoresis results are documented using doc gel (Bio-rad).
2.5. Restriction Fragment Length Polymorphism (RFLP)
Restriction enzyme used in RFLP is AluI. The enzyme mixture was made by adding 0.5 µL of the enzyme (according to the calculation on the kit), 1 µL buffer, and 11.5 µL ddH2O so that the total enzyme mixture to be incorporated into each PCR tube was 13 µL. Then incubated for 16 hours in the water bath at 37°C to maximize the working of cutting enzymes. After being incubated for 16 hours, the enzyme is inactivated with thermoblock (Biotech) at the temperature of 65°C for 20 minutes.

3. Results of study
The sample used was 30 CB +/- CL NS patients (15%) and 170 patients whom not CB +/- CL NS (control) (85%).

3.1. Restriction fragment length polymorphism
The restriction enzyme AluI will cut on the palindrome region in a specific sequence and will produce certain fragments. If there are 2 cutting fragments at 310bp and 156bp it represents C allele (wildtype), while for T alleles (polymorphic) it will unveil 292bp, 130bp and 44bp fragments. In homozygous wildtype (CC) genotypes, it will show 2 bands at 156bp and 310bp, for heterozygous (CT) genotypes will be seen 2 or 3 bands that are mixed pieces of C allele and T allele. For homozygous variant (TT) genotypes will be only seen T allele cuts, but in this study of all un-visualized samples of samples having homozygous variant (TT) genotypes.

Figure 1. RFLP result of CC Genotype (lane 1, 2, 3)
The distribution of frequency and percentage of genotype and allele of wnt3a rs 752107 gene polymorphism in orofacial cleft and control patients can be seen in Table 1. The p value from statistical test with chi square and odds ratio (OR) of wnt3a rs gene allele is also shown.

Table 1. Distribution of genotype frequencies and gene polymorphism alleles wnt3a rs 752107 wnt3a rs 752107 gene polymorphism

| Genotype and Allele | Orofacial cleft | Control | p value | Odd Ratio (OR) |
|---------------------|-----------------|---------|---------|----------------|
| CC Genotype         | 30 (100%)       | 162 (95.3%) | 1.471   |                |
| CT Genotype         | -               | 8 (4.7%)   |         |                |
| TT Genotype         | -               | -         |         |                |
| C Allele            | 60 (100%)       | 324 (95.3%) | >0.05   | 0.05           |
| T Allele            | -               | 16 (4.7%)  |         |                |

4. Discussion

Based on the data obtained from 30 samples of orofacial cleft patients and 170 control samples, it can be concluded that there is no variation of genotype and allele of wnt3a Rs 752107 in the orofacial cleft group and there are variations of genotype and allele in the control group. The study on the relationship of polymorphism or gene variation of wnt3a Rs 752107 with non-syndromic cleft lip with or without cleft palate has been done in the Chinese population by Yao T et al using 216 samples of non-syndromic cleft lip and/or palate patients (NSCLP) consisting of 73 cleft palate patients (CP) and 143 patients with cleft lip with or without cleft palate (CL/P).

In a study conducted in the Chinese population, it was found that the majority with a percentage of more than 50% had the homozygous wildtype (CC) genotype while the heterozygous genotypes ranged in the 30% and the rest had the homozygous variant. The homozygous and heterozygote genotypes were more dominant in the NSCLP and control groups, whereas the homozygous variant genotype was more
dominant in the NSCLP group than in the control group. The p-value in this study was 0.013 (p-value <0.05) which means that there is a relationship between gene polymorphisms of wnt3a Rs 752107 with orofacial cleft events in China [10].

From the study by Menezes et al. entitled "Studies with WNT Genes and Non-syndromic Cleft Lip and Palate", 766 samples were used, with details of the NSCLP group of 463 samples and the control group of 303 samples; where the p-value for the gene wnt3a Rs 752107 was 0.36 [11]. The study by Chiquet et al. examined the variation of several wnt genes associated with non-syndromic cleft lip with or without cleft palate using 132 NSCLP for the wnt3a Rs 752107 gene in the European and American population had p=0.00110 [9]. And in Mostowska et al. study entitled "Genotype and haplotype analysis of WNT genes in non-syndromic cleft lip with or without cleft palate", from 210 blood samples, 187 samples were of cleft lip and palate patients and the rest were cleft lip alone. And for the results of chi-square analysis associated with the wnt3a rs 752107 gene, p-value obtained was p=0.686 [12] From these studies, it can be concluded that the most of them showed no significant relationship between genes polymorphism of wnt3a Rs 752107 with orofacial cleft occurrences.[12]

Table 1 shows that in the control group and orofacial cleft group there is a more dominant CC genotype percentage than neither CT or TT genotypes. For the genotype distribution of CT is more dominant in the control group than in the orofacial cleft also in accordance with previous studies conducted in the Chinese population [10]. With the alleged lack of orofacial cleft samples in my study, the TT genotype results in the control group and orofacial cleft were not visualized.

In this study, the relationship of genotype and allele genes of w23a Rs 752107 were analyzed in orofacial cleft and control group. The results showed that there was no significant relationship between polymorphic T allele and orofacial cleft (p> 0.05). In the genotype analysis of wnt3a Rs 752107 gene polymorphism also shows p> 0.05 which means there is no significant relationship between genotype of wnt3a rs 752107 polymorphism genes with orofacial cleft occurrence. Therefore, the research hypothesis states that there is a relationship between gene polymorphism of wnt3a rs 752107 (genotype and allele) with orofacial cleft events were rejected. Thus, it can’t be concluded that the occurrence of wnt3a Rs 752107 gene polymorphism could increase the risk of orofacial cleft in the population in Indonesia.

5. Conclusion
Based on the study, it can be concluded that there is no difference of genotype distribution of gene polymorphism of wnt3a rs752107 in orofacial cleft patients that have 100% homozygous wildtype genotypes and un-visualized heterozygous (CT) and homozygous variant (TT) genotypes and there is no difference of allele distribution of polymorphic gene of wnt3a rs752107 in orofacial cleft patients that had 100% C allele and un-visualized T allele and there is no significant relationship between gene polymorphism of wnt3a rs752107 to orofacial cleft events as chi-square analysis value p>0.05.

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