

## Runx2 rs59983488 polymorphism in class II malocclusion in the Indonesian subpopulation

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### ABSTRACT

**Background:** Class II malocclusion is one of the main orthodontic issues for patients in seeking treatment. The prevalence of class II malocclusion varies in different populations. Variation in skeletal profile is mainly controlled internally by a regulatory gene. Runt-related transcription factor-2 (Runx2) plays a role in osteoblast differentiation and is highly expressed during development.

**Purpose:** This study aimed to evaluate the relation of regulatory gene variation in the Runx2 promoter with class II malocclusion.

**Methods:** DNA samples were acquired from 95 orthodontic patients in Jakarta, Indonesia, who were divided into two groups: class I skeletal malocclusion (control group) and class II malocclusion. A single nucleotide polymorphism was investigated using the polymerase chain reaction and restriction fragment length polymorphism techniques. The distribution of alleles was assessed using the Hardy-Weinberg test. The relationship between polymorphism and skeletal variation was assessed with the Chi-Square test and logistic regression. **Results:** The frequency distributions of genotypes and alleles were tested for Hardy-Weinberg equilibrium and found to be slightly deviated. There was an equal distribution of G and T alleles throughout class II and class I skeletal malocclusions and the Chi-Square test showed that this relationship was not significant ( $p=0.5$ ). **Conclusion:** Runx2 rs59983488 polymorphism was found in the Indonesian subpopulation; however, an association between Runx2 rs59983488 polymorphism and class II skeletal malocclusion was not found.

**Keywords:** class II; G330T; Indonesia; polymorphism; Runx2; rs59983488

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### INTRODUCTION

Class II skeletal malocclusion is often encountered in orthodontics and is one of the main reasons for patients to seek treatment. Previous studies have shown that the occurrence of class II malocclusion is quite high among populations around the world.<sup>1</sup> A study in Denmark found a 25% occurrence of class II skeletal malocclusion in their population.<sup>2</sup> In Asia, a study with people of Mongoloid ethnicity that showed 33.1% of this population had class II malocclusion<sup>3</sup> and another study in India showed 30.1% class II malocclusion in the population.<sup>1</sup> A study in Singapore with people of Chinese ethnicity showed a 23.1% occurrence of class II malocclusion<sup>4</sup> and 70%

occurrence was found in Iran.<sup>5</sup> Rosenblum<sup>6</sup> found out that in 103 class II skeletal malocclusion cases, 56.3% had maxillary prognathism and only 27% showed mandibular retrognathism.

The differentiation of the osteoblast from its progenitor requires the activity of specific transcription factors which are expressed during development. Runt-related transcription factor-2 (Runx2) is a member Runt family of transcription factors that is essential for skeletal development.<sup>7-9</sup> Runx2 is essential for osteoblast differentiation in endochondral and intramembranous ossification.<sup>10</sup> The Runx gene consists of Runx1, Runx2 and Runx3. Runx2 plays an important role in osteogenesis, cartilage formation, cell migration, vascular bone invasion and tooth formation.<sup>8,11,12</sup> Runx2 is essential

in bone development and chondrogenesis as an upstream controller for several effector genes. Runx2 is required in the preosteoblast maturation process.<sup>10,11,13</sup> Runx2 regulates matrix protein genes and Indian hedgehog transcription factor, an important regulator for chondrogenesis.<sup>11,14</sup> Runx2 is upregulated by bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs) and retinoid acid.

The Runx2 gene is located at 6p21.1 with 250 kb spans. Runx2 consists of two transcription factors, the distal P1 promoter and the proximal P2. Runx2 in osteoblasts was found mainly as transcripts from the P1 promoter. Both promoters are specifically expressed in osteoblast and chondrocytes.<sup>8,11,15,16</sup> Genetic variation of Runx2 has been investigated for its relation to skeletal variation and bone-related disease.<sup>2,17</sup> Single nucleotide polymorphism (SNP) is the most common variation that occurs in the human genome and happens in more than 1% of the population.<sup>18</sup> The association of Runx2 rs59983488 polymorphism with class II malocclusion was inconsistent in previous studies,<sup>16,19</sup> therefore it is important to do further research to determine this relationship in this population. This study aimed to assess the relationship of Runx2 rs59983488 polymorphism to class II skeletal malocclusion in the Indonesian subpopulation.

## MATERIALS AND METHODS

The research participants were orthodontic patients aged between 20 and 45 years from the University of Indonesia's Dental Hospital. All study participants signed an informed consent document. Ethical clearance of this study was granted by the Ethical Committee, Faculty of Dentistry, University of Indonesia (no. 26/Ethical Approval/FKGUI/VII/2015, Protocol number: 09370515). Age, sex, ethnicity and socioeconomic status were recorded. Malocclusion classification was based on sagittal relationship of Steiner's Analysis measuring Sella-Nasion-A point (SNA), Sella-Nasion-B point (SNB) and A point-Nasion-B Point (ANB). Study participants included 48 class II and 52 class I malocclusion patients. Class I subjects were recruited with SNA 82°±2°, SNB 78°±2°, ANB 2°±2°. Class II subjects were recruited as: class II with mandibular retrusion (SNA 82°±2°, SNB <76°, ANB >4°, Overjet >4 mm), class II with maxillary protrusion (SNA >84°, SNB 78°±2°, ANB >4°, Overjet >4 mm), and class II with maxillary protrusion and mandibular retrusion (SNA >84°, SNB <76°, ANB >4°, Overjet >4 mm). All lateral cephalometric images were traced the by same operator (F.J), who is an experienced orthodontist. To reduce the effect of operator measurement error, the tracing was analysed three separate times at two weeks intervals with single blinding.

Genomic DNA was collected from the peripheral blood of all 95 subjects. DNA extraction was performed by using 3 mL peripheral blood and 9 mL red blood cell lysis solution (1.45 M NH<sub>3</sub>Cl, 5 mM anhydrous EDTA, and 0.1 KHCO<sub>3</sub>, [InstaGene Matrix, Bio-Rad, California,

United State of America]) incubated at room temperature for 10 min. The mixture was then centrifuged at 1500 rpm for 10 min and the supernatant was removed. 2 mL cell lysis solution was added and incubated at 37°C for 60 min. 1.3 ml protein precipitation (PP) solution [Promega, Wisconsin, United State of America] was added and then vortexed and centrifuged at 40°C at 3000 rpm for 5 min. The supernatant was transferred into a new tube containing 2.3 mL isopropanol, and then inverted to dry. This process was repeated until the DNA turned white. After that, the DNA was rehydrated with 200–300 µL Tris-HCl EDTA, incubated in 37°C for 2 h and stored at -20°C.

Runx2 rs59983488 G330T polymorphism was detected by using the polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) method. PCR amplification was done by using the forward primer 5'-AAA GCA AAG GAG GTT GAC CCG-3' and reverse primer 5'-CCC TGC CCT TCT TTC TCT CTC-3' with Perkin Elmer Gene Amp PCR system 9700 [Kumamoto, Japan]. Each reagent contained 10 µL buffer solution [Promega, Wisconsin, United State of America], 2 µL forward and reverse primers, 7.5 µL ddH<sub>2</sub>O, and 0.5 µL genomic DNA. The conditions for this PCR were 94°C for 6 min followed by 35 cycles of the amplification denaturation phase at 94°C for 60 s, the annealing phase at 62°C for 30 s, elongation at 72°C for 30 s and final elongation at 72°C for 5 min.

The PCR product was digested with BSAJ1 enzyme restriction [New England Biolabs (NEB), Massachusetts, United States of America]. The mixture consisted of 1 µL restriction enzyme BSAJ1 (10U/µL), 2 µL enzyme buffer and 18 µL ddH<sub>2</sub>O was added to 10 µL PCR product. The mixture was incubated at 60°C for 4 h and inactivated by incubating at 80°C for 20 min. The restriction products were subjected to electrophoresis on 2% agarose gel [Promega, Wisconsin, United State of America] in 1x TAE buffer solution (0.04 M Tris-acetate, 0.002 M EDTA, pH 8.0) at 80 V, 400 mA, for 60 min, resulting in three possible genotypes: GG, GT and TT. The restriction products were visualised with GelDoc 2000 [Bio-Rad, California, United State of America] and showed qualitative data for the G allele of 205 bp and 20 bp and the T allele of 225 bp.

This study used the Hardy-Weinberg equilibrium to evaluate genetic variation that occurred in the population. Intra-observer reliability was evaluated using the intraclass correlation coefficient (ICC). Chi-Square and logistic regression tests were used to assess the relation of Runx2 rs59983488 polymorphism with class II skeletal malocclusion. Statistical analysis was performed using the Program for Social Science (IBM SPSS) version 22 [Chicago, United State of America].

## RESULTS

A total of 95 orthodontic patients, consisting of 48 class II skeletal malocclusion cases and 52 class I skeletal

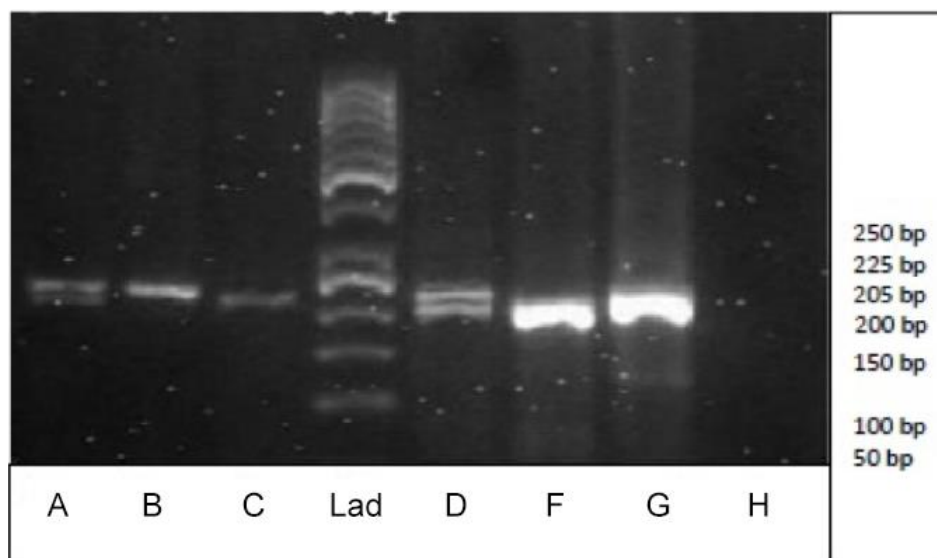
malocclusion cases, were recruited at the Dental Hospital, Faculty of Dentistry, University of Indonesia. The reliability of the cephalometric measurement was good (ICC > 0.820).

Genotyping using the PCR-RFLP methods showed G allele cuts in 205 bp and 20 bp and T allele cuts in 225 bp (Figure 1). Therefore, the GG genotype (wildtype) showed two bands, the GT genotype (heterozygous) showed three bands and the TT genotype (mutant) showed a single band. The distributions of genotypes and alleles are shown in Table 1. This population was tested against Hardy-Weinberg equilibrium and showed a slight deviation ( $p=0.04959$ ). The distributions of the genotype and allele MOI (class I skeletal malocclusion) and MOII (class II skeletal malocclusion) were assessed with the Chi-Square test to identify their relationship within each group and were not found to be significant. The distributions of genotype and allele G and T were equal in class II skeletal malocclusion and the control (class I skeletal malocclusion). A logistic regression test was used and found significant ( $p<0.05$ ) with a slight reduction of the odds ratio 0.908 (95% Confidence Interval :0.51–1.617).

## DISCUSSION

Runx2 rs59983488 shows bases alteration from G to T at -330 in the P1 region. Genotyping using PCR-RFLP methods showed GG genotype cuts in 205 bp and 20 bp, GT genotype cuts in 225 bp, 205 bp and 20 bp, and TT genotype cuts in 225 bp (Figure 1). The 20 bp fragment is hard to see in Figure 1 because traditional agarose gels are most effective in separating DNA fragments between 100 bp and 25 kb. Agarose gel is popular for separating moderate-to large-sized nucleic acids which have a wide range of separations. There are few alternate methods for separating small DNA fragments. Polyacrylamide gel can be used as an alternative since this gel has a higher concentration resulting in better resolution when run vertically.<sup>20</sup> Another method to separate smaller DNA fragments would be to use agarose gel doped with graphene oxide.<sup>21</sup>

The genotype and allotype frequency distributions in this study are slightly deviated from the Hardy-Weinberg equilibrium (HWE) ( $p<0.05$ ). This finding is consistent with previous Runx2 studies in Indonesian and Chinese populations.<sup>22,23</sup> Mokhtar et al.<sup>24</sup> found different results



**Figure 1.** Genotyping Runx2 rs59983488; A and D. GT genotype (225 bp, 205 bp, 20 bp); B and F. TT genotype (225 bp); C. GG genotype (205 bp, 20 bp); Lad. Ladder 50 bp; G. positive control; H. negative control.

**Table 1.** Distributions of genotypes and alleles of Runx2 T330G

Genotype	MOI		MOII		P
	n	%	n	%	
TT	7	13.5	6	13.9	0.831
GT	32	61.5	24	55.9	
GG	13	25	13	30.2	
T allele	46	44.2	36	41.8	
G allele	58	55.8	50	58.2	

MOI: Class I skeletal malocclusion; MOII: Class II skeletal malocclusion.

Chi-Square test,  $p<0.05$  was considered significant. Logistic regression test  $p<0.05$ , OR 0.908, Confidence Interval 95% 0.51–1.617

in the Malaysian population that showed consistency of genotypes and alleles with the HWE. Another study of Runx2 polymorphism in Indonesia has been done in relation to osteoporosis, which had similar findings to ours, but showing a higher deviation in HWE value.<sup>23</sup> This study involved a different population from that study, although they shared the same location, and therefore the populations had similar characteristics. The reason for this deviation was not clear. We suspect it was due to population stratification and a limited number of study participants.<sup>25-27</sup> The population in this study was relatively small – 95 subjects in total, with 13 having the TT genotype (13.7%) and only 6 (0.06%) in the class II skeletal malocclusion group.

In this study, an association between Runx2 rs59983488 polymorphism and class II skeletal malocclusion was not found. The T and G alleles were spread evenly in both groups of class I and class II malocclusion. Küchler et al.<sup>19</sup> found a different result in the Caucasian population. He studied two sites of Runx2 promoter and found an association between rs59983488 polymorphism and maxillary protrusion, although he did not find any association between rs1200425 and either maxillary protrusion or class II malocclusion. The study also found a strong interaction between Runx2 rs59983488 SNP and SMAD6 rs3934908 SNP in the class II malocclusion model.<sup>19</sup> Another study in Malaysia investigated the relation of Runx2 rs6930053 polymorphism with class II malocclusion and found a significant difference in both allele and genotype.<sup>24</sup> Bigger sample size and additional maxillary measurement are recommended to evaluate any association of Runx2 SNP with class II skeletal malocclusion and maxillary growth, and the possible mechanism for this.

Runx2 polymorphism at various sites has been widely investigated in its relation to different phenotypes of skeletal growth (Table 2). Chang et al.<sup>28</sup> reported that there was an association between Runx2 polymorphism and ossification

of the posterior longitudinal ligament. Auerkari et al.<sup>23</sup> and Bustamante et al.<sup>16</sup> reported no association between Runx2 rs59983488 polymorphism and osteoporosis. There were also studies that reported on the relationship between Runx2 polymorphism and non-syndromic cleft lip and palate.<sup>22,29</sup> There are still inconsistencies regarding the relationship of polymorphism in Runx2 with skeletal phenotypes, and further research is needed to assess the role of Runx2 in skeletal growth and development.

The level of Runx2 expression and its activity fluctuate during the developmental process. The activity of the Runx2 protein is regulated by various post-translational modifications, such as phosphorylation, methylation and acetylation or ubiquitination. BMP-2 activity was reported to stimulate Runx2 acetylation.<sup>11</sup> Post-translational regulation by phosphorylation and acetylation is essential for FGF-induced cranial development, and this has been investigated for premature fusion of cranial sutures and the midfacial hypoplastic changes that occur because of lack of growth at the fused sutures. Enhanced stability of Runx2 by post-translational regulation could offer a methodology for bone regeneration therapy.<sup>30</sup> One study shows that epigenetic histone modifications on receptors for vitamin D (VDR) and bone cell differentiation affect the binding of Runx2 to the genome, modify and restrict patterns of gene expression and alter cellular response to the vitamin D hormone.<sup>31</sup> Yan et al.<sup>32</sup> found that METTL3 could directly induce m<sup>6</sup>A methylation of Runx2 mRNA to enhance its cellular stability and indirectly upregulate the cellular level of Runx2 by m<sup>6</sup>A methylation of pre-miR-320. Ling et al.<sup>33</sup> reported that nicotinamide phosphoribosyltransferase (NAMPT) promotes osteogenesis through the epigenetic regulation of Runx2 expression, thus upregulating Runx2 expression. Future studies involving Runx2 post-translational regulation modification should be carried out to investigate its relation with maxillary growth and class II skeletal malocclusion.

**Table 2.** Runx2 polymorphism studies

RS	P	phenotype	Reference
rs967588	C>T	Not significant	
rs16873379	T>C	Significant	
rs3749863	A>C	Not significant	
rs6908650	G>C	Not significant	Ossification posterior longitudinal ligament
rs1321075	A>C	Not significant	
rs1406846	T>A	Significant	
rs2677108	C>T	Significant	
rs59983488	G>T	Not significant	Osteoporosis
deletion	17A>11A	Significant	Bone fracture
rs6930053		Significant	Class II malocclusion
rs545239			
rs1200425		Significant	Non-syndromic cleft lip and palate
rs16873396			
rs1934328		Significant	Non-syndromic cleft lip and palate
rs59983488	G>T	Significant	Maxillary protrusion
rs1200425	G>A	Not significant	
rs59983488	G>T	Not Significant	Osteoporosis
rs59983488	G>T	Not Significant	Class II malocclusion

In conclusion, Runx2 rs59983488 polymorphism was detected in the Indonesian subpopulation. However, a relationship between the Runx2 polymorphism promoter and class II skeletal malocclusion was not found. A larger sample of this population and an additional maxillary component of measurement are recommended to further assess the link between Runx2 rs59983488 polymorphism and class II skeletal malocclusion and maxillary growth.

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