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Application of Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) Technique in the Analysis of *MYO1H* Single Nucleotide Polymorphism in Malay Mandibular Prognathism Patients

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ABSTRACT

Genetic studies have reported the association between polymorphism in *MYO1H* with mandibular prognathism. *MYO1H* is found in skeletal muscle sarcomeres and is expressed in the mandibular jaw cartilage signifying its importance during craniofacial development. This study aimed to characterise the genotype and allele of *MYO1H* single nucleotide polymorphism (SNP) (rs3825393) and to associate the SNP with mandibular prognathism in Class III skeletal malocclusion. This was a case-control study, which involved 57 Malay subjects with 30 Class I (control) and 27 Class III skeletal base patients (case). Cephalometric measurements were taken prior to collection of saliva samples. *MYO1H* SNP (rs3825383) was genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Chi-square (χ^2) test was used to compare genotype and allele frequencies between the groups while Hardy-Weinberg Equilibrium (HWE) was applied to assess distribution of genotype frequency in both classes. *MYO1H* SNP (rs3825393) did not yield significant association with mandibular prognathism with $p = 0.33$; OR = 0.66; 95% CI = 0.289~1.518, that was reflected by no significant difference in allele ($p > 0.05$) and genotype ($p > 0.05$) frequency between control and study group. Nevertheless, AA genotype depicted the highest frequency in both groups. The genotype distribution in both groups was in concordance with HWE ($p > 0.05$). Our data showed no association of *MYO1H* SNP (rs3825393) with mandibular prognathism. Interestingly, we observed Allele A representing the major allele in Malay population. Presence of *MYO1H* SNP (rs3825393) was detected in samples analysed. Larger number of samples is required to confirm the involvement of *MYO1H* polymorphisms in mandibular prognathism.

Keywords: *Mandibular prognathism; MYO1H SNP; rs3825393; PCR-RFLP*

INTRODUCTION

Malocclusion is one of the most common dentofacial problems observed worldwide. Malocclusion can be defined as deviation of teeth and dental arches beyond what is normal which can be due to discrepancy between dentoalveolar, skeletal and soft tissue factor (Mtaya *et al.*, 2009; Ghergie *et al.*, 2013). The implications of this condition to an individual's life include aesthetic concern, functional oral disturbances, prone to trauma and increased risk of periodontal disease (Mtaya *et al.*, 2009).

There were six characteristics on 120 casts of normal occlusions of the non-orthodontic patients, which are Class I molar relationship, correct crown angulation, correct crown inclination, no rotation, tight proximal contact and flat occlusal plane (Andrews, 1972; Hassan and Rahimah, 2007). Class III malocclusion is described as the mesiobuccal cusp of maxillary first permanent molar occludes distal to mesiobuccal groove of mandibular first permanent molars (Angle, 1899; Sandeep and Sonia, 2012).

The aetiology of Class III malocclusion can be divided into retrognathic maxilla, prognathic mandible and combination of both conditions. Ngan *et al.* (1997) noted that the highest incidence of Class III malocclusion pattern was normal maxilla with prognathic mandible while only 25% incidence of Class III presented with small maxilla. Excessive mandibular growth can occur due to stimulus created by the constant deviation of condyle from the fossa of the mandible. The aetiology of Class III malocclusion is also related with genetic inheritance among offspring and siblings and with environmental factors, such as habits and mouth breathing as described by Ngan *et al.* (1997).

The prevalence of mandibular prognathism in Caucasians was found to be as low as 1% in contrast with 15% prevalence in the Asian populations (Tassopoulou-Fishell *et al.*, 2012). A study conducted in Malaysia showed higher prevalence of Class III malocclusion in Chinese and Malay in comparison with Indian population (Woon *et al.*, 1989).

Isoforms of myosin heavy chain are needed for the structural and functional integrity of skeletal muscle. Myosin from the superfamily of motor protein will bind to actin and hydrolysed adenosine triphosphate (ATP) to simultaneously move along with actin filaments. Myosin Class I has involved in many motile processes such as translocation of organelle, iron channel gating and reorganisation of cytoskeleton (Arun *et al.*, 2016). Interestingly, the expression of *MYO1H* orthologs in zebrafish model was detected in the mandibular jaw cartilage (Sun *et al.*, 2018). Hence, suggesting the involvement of *MYO1H* during the process of craniofacial development.

Class III malocclusion has been shown to have association with genetic polymorphisms. Single nucleotide polymorphism (SNP) occurs when there are changes in the nucleotide building blocks. Few markers for SNP in *MYO1H* have been identified to be involved in Class III malocclusion. One of the studies has identified positive association of *MYO1H* SNP (rs10850110) with mandibular prognathism in Caucasian population (Tassopoulou-Fishell *et al.*, 2012). Kajii and Oka (2017) mentioned that genes such as *MATN1* (matrilin 1; cartilage matrix protein), *HSPG2* (heparin sulfate proteoglycan 2) and *ALP* (alkaline phosphatase) also have suggestive linkage to mandibular prognathism. Other genes have also been identified to play a positive role in the aetiology of mandibular prognathism (Doraczynska-Kowalik *et al.*, 2017).

Previously, a preliminary study looking at the *MYO1H* SNP (rs10850110) in local samples has been done by sequencing technique analysis (Yahya *et al.*, 2018). However, no association was detected which might be due to small number of samples. Recent

study has shown that rs3825393 which is another known SNP marker in *MYO1H* was associated with mandibular retrognathism in Indian population (Arun *et al.*, 2016).

Since the incidence of Class III malocclusion is higher in Malaysian population, we focused the analysis on Class III skeletal malocclusion cases in Malay patients as majority of orthodontic patients attending our clinic are Malays (Ismail *et al.*, 2017). In the current study, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique was used to detect the presence of SNP (rs3825393) in Class III malocclusion in Malay patients. We aimed to detect the presence of *MYO1H* SNP (rs3825393) and to determine its genotype and allele distribution in Class III skeletal malocclusion in order to associate *MYO1H* SNP (rs3825393) with mandibular prognathism.

MATERIALS AND METHODS

The research conducted was a case-control study of active orthodontic patients from Orthodontics Department, Kulliyyah of Dentistry, International Islamic University Malaysia (IIUM). Ethical approval was obtained from IIUM Research Ethics Committee (IIUM/504/14/11/2/IREC 2019-012) prior to the initiation of the study, dated 11 February 2019.

Patient Selection

The subjects involved were active orthodontic patients from Orthodontics Department, Kulliyyah of Dentistry, IIUM. They were selected based on assessment that comprised clinical examination and evaluation of clinical and radiographical records. Clinical records include study models, cephalometric tracings, and photographs. Eastman and Wits cephalometric analyses were conducted to distinguish the subjects between controls versus sample group. Informed written consent was also obtained from all subjects.

A total of 57 Malay patients, both sexes, aged 15 to 28 years were included in this study, whereby 27 subjects were having Class III skeletal base (case; with mandibular prognathism) and another 30 subjects were from Class I skeletal base (control; without mandibular prognathism). The samples selected were based on the inclusion and exclusion criteria specified as follows; inclusion criteria for Class III skeletal base samples (cases) are cephalometric value ANB <1°, negative Wits appraisal, has concave profile, SNA of normal value (81 ± 3) while inclusion criteria for Class I skeletal base (controls) are fit and healthy and has Class I skeletal base. Subjects having craniofacial deformity including cleft lip and palate, endocrinological problem, anomalies in tooth number, morphology and eruption were excluded from both the groups.

Cephalometric Analysis

Eastman analysis

SNA angle was used to assess the relationship of maxilla to the cranial base. The mean value in Malay patients was 81° ± 3°, in which value larger than 84° indicates prognathic maxilla and while value less than 78° shows retrognathic maxilla. SNB angle was used in assessing the mandibular relationship to the cranial base. The mean value is 78° ± 3°, and a value less than 75° indicates mandibular retrognathism, while larger than 81° indicates mandibular prognathism.

ANB angle is the relationship of mandible to maxilla, calculated by subtracting the value of SNA-SNB. Mean value is 3° ± 2°, increase in value indicates Class II tendency, while decrease in value shows Class III tendency.

Wits analysis

Wits appraisal is a measurement in assessing antero-posterior jaw disharmony. It is measured by projecting point A and point B to the functional occlusal plane and

AB difference is calculated. The average value is 0–2 mm. Negative value indicates Class III skeletal discrepancy meanwhile Class II skeletal discrepancy will show larger value.

Genetic Analysis

For genotyping, 5 mL of unstimulated saliva was obtained from all subjects (were asked to spit) and stored in a container at –20 °C until being processed. DNA extraction was done by using GeneAll®Exgene™ Kit (Korea) according to the manufacturer's instructions. The genetic variant rs3825393 of *MYO1H* was amplified using primers as follows: Forward strand 5′-GGCTTACTTCCCTCCCAGAG-3′ and Reverse strand 5′-GAAAGGAAGAAT GCTGTTGCCACAG-3′.

The polymerase chain reaction (PCR) mixture (total volume of 25 µL) consisting of 1.0 µL of each primers, 5 µL of 5× green GoTaq buffer, 2 µL of $MgCl_2$, 0.5 µL of dNTPs, 0.125 µL of Taq polymerase (PROMEGA GoTaq®Flexi DNA Polymerase), 13.375 µL of dH_2O and 2 µL of genomic DNA. The PCR protocol consisted of initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 sec, annealing at 58 °C for 30 sec and extension at 72 °C for 1 min. The final extension was at 72 °C for 5 min. The amplified PCR products were electrophoresed on 2.0% agarose in 1× TBE buffer for 45 min at 125V. To visualise the size of amplified PCR products (302 bp), the gel was observed under UV light using gel documentation system. For RFLP analysis, a master-mix solution (total volume of 25 µL) containing 2.5 µL 10× restriction enzyme buffer, 17 µl dH_2O and 5.0 µL PCR products with addition of 0.5 µL restriction enzyme *Sau96I* (New England BioLab Inc.) was prepared. The mixture solution was incubated overnight in a water bath at 37 °C. Then, the mixture was electrophoresed with the same timing and voltage and was observed under UV light to see the separation of bands (Allele A at 302 bp only; heterozygous for Allele AG at 302 bp and 226 bp; Allele G at 226 bp only).

Statistical Analysis

Direct counting was done to determine allele and genotype frequencies for both classes. Hardy-Weinberg Equilibrium (HWE) was applied to evaluate genotype distribution. Chi-square (χ^2) test was used to assess the association of allele and genotype frequencies of candidate gene polymorphisms *MYO1H* SNP (rs3825393) with mandibular prognathism. SHEsis online software was used to perform the analysis.

RESULTS

The demographic data and cephalometric analysis of all subjects are tabulated in Table 1. There were significant differences of all cephalometric measurements SNB (p = 0.000), ANB (p = 0.000) and Wits (p = 0.000) value between Class I and Class III skeletal base. There was no significant difference in SNA value between Class I and Class III, indicating that the aetiology for the contributing Class III skeletal base is mandibular prognathism and not maxillary retrognathism, as expected in the study.

The extracted DNA was subjected to PCR analysis and the gel electrophoresis was conducted for PCR products. The results then showed a specific band at approximately 302 bp for both Class I and Class III skeletal base. Restriction enzyme analysis from the PCR products showed three distinct bands: 302 bp which indicate AA genotype (homozygous A), 226 bp indicate GG genotype (homozygous G) and combination of both specific band 302 bp and 226 bp indicate AG genotype (heterozygous AG) (Fig. 1).

Table 1 Demographic and cephalometric measurements of malocclusion cases

	Class I (n = 30; control)	Class III (n = 27; case)	
Gender			
Male	13	8	
Female	17	19	
Age in years (mean)	25.1	25.8	
Cephalometric measurements	Mean value		p-value
SNA (°)	81.60	81.556	.905
SNB (°)	78.63	82.852	.000
ANB (°)	2.967	−1.296	.000
Wits (mm)	−.267	−7.630	.000

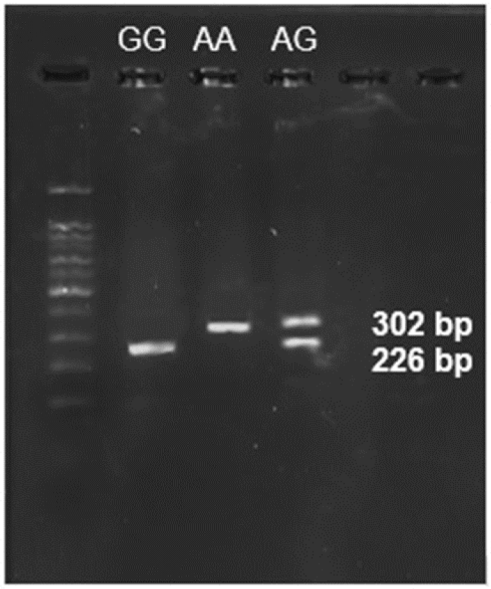

Fig. 1 Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) agarose gel electrophoresis of the rs3825393 polymorphism showing the G/G; homozygous wild-type, A/G; heterozygous, and A/A; homozygous variant genotypes. Lane 1 is 100bp DNA ladder.

The distribution of allele and genotype of both classes was statistically analysed using χ^2 test using SHEsis as shown in Table 2. Meanwhile Table 3 exhibits genotype frequency of rs3825393 which revealed no significant difference in both Class I and Class III skeletal base according to HWE ($p > 0.5$) that was applied to observe the distribution of genotype frequency in Class I and Class III skeletal base (Class I: AA: 0.600, AG: 0.333, GG: 0.067; A: 0.767, G: 0.233; Class III: AA: 0.481, AG: 0.407, GG: 0.111; A: 0.685, G: 0.315). This HWE indicated that no deviation occurred in case and control groups and could further be analysed for association of rs3825393 with mandibular prognathism development.

For single association analysis of rs3825393 with allele frequency (Table 4), there was also no significant difference between both classes although Allele A was more frequently observed compared to Allele G with 68.5% and 76.7% in case and control group, respectively. Hence, it can be concluded that there was no association between SNP (rs3825393) with Class III skeletal base ($p = 0.33$; OR = 0.66). In our study, Allele A is considered as the major allele while the Allele G is considered as minor allele in our dataset based on the frequency distribution.

Table 2 Genotype and allele distribution of *MYO1H* (SNP rs3825393)

CLASS (n)	Genotype			Allele	
	AG	GG	AA	A	G
CLASS I (control) *n* = 30	10	2	18	46	14
		HWE ($p = 0.71$)			
CLASS III (case) *n* = 27	11	3	13	37	17
		HWE ($p = 0.77$)			
CLASS I, III	21	5	31	83	31
		$p = 0.899$		$p = 0.953$	

Table 3 Genotype distribution and frequency of rs3825393 with malocclusion

Class of Malocclusion	Genotype AA (% freq)	Genotype AG (% freq)	Genotype GG (% freq)
Class III (Case; *n* = 27)	13 (0.481)	11 (0.407)	3 (0.111)
HWE test for case: $\chi^2 = 0.083599$, df = 1, Fisher's *p* is 0.772491; HWE ($p > 0.05$)			
Class I (Control; *n*= 30)	18 (0.600)	10 (0.333)	2 (0.067)
HWE test for control: $\chi^2 = 0.140041$, df = 1, Fisher's *p* is 0.708256; HWE ($p > 0.05$)			

Table 4 Single association analysis of rs3825393 with allele frequency

Class of Malocclusion	Allele A (freq)	Allele G (freq)
Class III (Case; *n* = 27)	37 (0.685)	17 (0.315)
Class I (Control; *n*= 30)	46 (0.767)	14 (0.233)
Odds ratio = 0.662404 %95 CI = [0.289047~1.518021]		
Fisher's *p*-value is 0.328985 ($p > 0.05$)		

DISCUSSION

SNP occurs when there are changes in the nucleotide building blocks and can be detected using a simple laboratory technique, PCR-RFLP. This straightforward technique allows amplification of genomic sequence to rapidly identify point mutations. This is particularly beneficial in small basic research studies of complex genetic diseases (Ota *et al.*, 2007).

Over the last two decades, many different methods have been developed for SNP genotyping which includes PCR technique, hybridisation, allele-specific PCR, primer extension, oligonucleotide ligation, direct DNA sequencing and endonuclease cleavage after amplification of the subjected genomic region by PCR. Among the mentioned techniques, the last technique was chosen for this particular study, also known as the PCR-RFLP technique. The advantages of PCR-RFLP technique are simple and inexpensive. However, the limitation of this protocol is when the target SNP sequence has too many recognition sites for a single restriction enzyme to digest (Ota *et al.*, 2007). The restriction enzyme *Sau96I* originating from *E-coli* recognises the sequence that has cutting site NG*GNCC (N = any nucleotide).

MYO1H has been shown to be involved in mandibular development. Hence, any discrepancies (mutation or polymorphism) present in this gene could lead to abnormal mandibular developmental process. *MYO1H* SNP (rs10850110) has been associated with Class III malocclusion cases (Tassopoulou-Fishell *et al.*, 2012; Cruz *et al.*, 2017). Another *MYO1H* SNP variant; rs3825393 located at chromosome 12, has also been shown to be associated with mandibular prognathism and mandibular retrognathism (Arun *et al.*, 2016; Sun *et al.*, 2018). Nonsynonymous variations of rs3825393 involving Allele C (C>T, p.Pro1001Leu) has shown to have increased risk of mandibular prognathism since this allele was associated with increase of SNB, decrease of ANB, Wits appraisal and overjet (Sun *et al.*, 2018).

Our data showed the presence of SNP (rs3825393) of *MYO1H* in both control (normal; Class I skeletal base) and cases (Class III skeletal base) using PCR-RFLP technique. However, there was no association of rs3825393 with Class III skeletal malocclusion observed in Malay population. Previous study by Sun *et al.* (2018) found significant association of Allele C that is the complement for Allele G with mandibular prognathism cases in Chinese population. However, our data could not demonstrate a similar finding, which might be due to the different ethnicity involved in this study.

Interestingly, although studies done by Arun *et al.* (2016) and Sun *et al.* (2018) considered Allele A as mutant allele while Allele G is the normal allele, our data has shown that Allele A represent the major allele frequency while Allele G is minor allele frequency as observed in Malay population. The difference in the allele frequency observed might be due to the difference in population or ethnicity involved in the current study as compared to previous analysis where Sun *et al.* (2018) performed the analysis on a group of Chinese population while Arun *et al.* (2016) conducted their analysis on Indian population. However, based on International HapMap Project (haplotype map), it is shown that our result is quite similar to the study conducted on Japanese population in terms of allele and genotype frequency (www.ncbi.nlm.nih.gov/snp/rs382539). In our study, the allele frequency in Malay population was A = 0.685, G = 0.315 while in Japanese population (HapMap-JPT) it was T = 0.601, C = 0.399. Interesting to note that other Asian populations with close similarity and consistent to our result were Han Chinese population (HapMap-CHB; T = 0.505, C = 0.495) and Kinh Vietnamese population (HapMap-KHV T = 0.525, C = 0.475) in which the distribution of T allele was higher in frequency compared to C allele except in

Chinese Dai population (HapMap-CDX; T = 0.473, C = 0.527).

For genotype frequency, our Malay population showed AA = 0.481, AG = 0.407, GG = 0.111, which was similar to the Japanese population (HapMap-JPT), TT = 0.356, CT = 0.490, CC = 0.154, as compared to Han Chinese population (HapMap-CHB; TT = 0.223, CT = 0.563, CC = 0.214), Chinese Dai population (HapMap-CDX; TT = 0.215, CT = 0.516, CC = 0.269) and Kinh Vietnamese population (HapMap-KHV; TT = 0.293, CT = 0.465, CC = 0.242). Our result detected highest frequency in homozygous AA genotype among other genotypes compared to other Asian populations studied, which had the highest frequency in heterozygous AG genotype.

Thus, these data supported our observation with regard to allele and genotype frequency for *MYO1H* SNP (rs3825393) at least when considering the East Asian populations. According to HWE principle, our results showed value of $p > 0.05$, which indicates that allele and genotype frequencies are, remain constant in both case and control groups hence the absence of evolutionary influences which in concordance with HWE. This value is crucial in assessing the association in case control study.

From this analysis, it can be concluded that Allele A is frequently expressed compared to Allele G in both Class I and Class III skeletal base in Malay population. However, no association could be determined between allele frequency and mandibular prognathism in the current analysis. Larger number of samples is warranted to ascertain the association of rs3825393 with the incidence of mandibular prognathism in the Malay population.

CONCLUSION

The SNP in *MYO1H* with the marker of rs3825393 can be detected and present in the local Malay population exhibited by the allele and genotype of both Class I and Class III skeletal base. Allele A was represented as major allele frequency observed in Malay population while Allele G was considered as the minor allele based on the frequency distribution in this molecular characterisation. However, no significant association between *MYO1H* SNP (rs3825393) and mandibular prognathism was observed. Although PCR-RFLP could provide a rapid screening technique to identify polymorphism in smaller samples as in current study, further studies on larger population size and incorporation of sequencing analysis is however warranted for validation.

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