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Original Article

Preliminary study of *PAX9* single nucleotide polymorphism (rs8004560) in patients with Class II skeletal base malocclusion contributed by mandibular retrognatism

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Abstract Polymorphism in *PAX9* (rs8004560), a gene responsible for craniofacial and tooth development, is often associated with Class II/Div2 malocclusion. This study aimed to detect the presence of *PAX9* SNP (rs8004560) and to determine its genotype and allele distribution in Class II skeletal base malocclusion, contributed by retrognathic mandible, in the local Malaysian population. The association of *PAX9* SNP (rs8004560) with Class II skeletal base malocclusion was also determined. A case control study was performed on 30 samples; 15 from Class II skeletal base malocclusion, and 15 from Class I skeletal base subject as control. Cephalometric measurements were performed prior to saliva samples collection. Genomic DNA was extracted from unstimulated saliva of all subjects, and the DNA was amplified using specific primers for marker rs8004560, followed by genotyping by sequencing. *SHEsis* online software was used to analyse Hardy-Weinberg Equilibrium (HWE) for cases and controls. Allelic and genotypic frequencies were compared between cases and controls. Significant difference in allele frequency was observed within the group whereby G allele was over-represented in the analysed population (p<0.05). However, when compared between cases and control; there was no significant association between *PAX9* SNP (rs8004560) with Class II skeletal base malocclusion (p=0.56, OR=0.71; 95% CI=0.225-2.246). The distribution of genotype frequency in both groups were consistent with HWE (p>0.05). Although no genetic association between *PAX9* SNP (rs8004560) with Class II skeletal base malocclusion was observed, significant difference in allele frequency observed might provide some indication in the involvement of *PAX9* polymorphism in Class II skeletal base malocclusion contributed by retrognathic mandible. Further research utilising larger sample size will be required in order to determine the role of *PAX9* gene in the aetiology of Class II skeletal base malocclusion observed in the local Malaysian population.

Keywords: Class II malocclusion, *PAX9*, retrognathic mandible, rs8004560 SNP.

Introduction

Malocclusion is the development of a complex trait condition and relationship between both dental arches, in which occlusion has deviated from what is defined as ideal or normal occlusion (Nishio and Huynh, 2016; Patel *et al.*, 2016). Skeletal malocclusion is caused by the distortion of the proper mandibular and/or maxillary growth during foetal development (Joshi *et al.*, 2014). However, malocclusion should not be considered as abnormal or pathological, instead as a variation of occlusion in a continuous multifactorial trait (Patel *et al.*, 2016). According to Angle's classification of malocclusion established since 1890s, the occlusion is based on upper and lower first molar relationship which is categorized into three classes – Class I (neutrocclusion), Class II (distocclusion) and Class III (mesiocclusion) (Angle, 1899). British Standard Institution (1983) classifies the occlusion, according to incisors relationship, into Class I, Class II Division 1, Class II Division 2 and Class III.

Skeletal malocclusion varies among age, race and population. For example, a

study of Western European population reported an average of 60% who had Class I, 35% (Class II) and 5% (Class III) (Proffit *et al.*, 2013). Class II skeletal base malocclusion could be contributed by prognathic maxilla, retrognathic mandible or combination of both. Patients with Class II have retrognathic mandible or reduced mandibular length, causing the mandible to be more retruded than Class I patients (Wall, 2009).

Changes in the development of craniofacial part are strongly believed to be associated with the environmental and genetic predisposition (Prescott and Malcolm, 2002). Alterations in genes which are important during the process of craniofacial development have been associated with the incidence of craniofacial abnormalities. Paired box 9 gene (*PAX9* gene) located at chromosome 14 (locus 14q13.3) is a gene family which is responsible for tooth as well as skeletal development (Ghergie *et al.*, 2013). *PAX9* gene regulates cell proliferation, migration and determination in multiple neural crest-derived lineages, such as cardiac, sensory, and enteric neural crest, pigment cells, glia, craniofacial skeleton and teeth, or in organs developing in close relationship with the neural crest such as the thymus and parathyroids (Monsoro-Burq, 2015). *PAX9* gene is a protein encoding gene that encodes the transcription factor that is important for craniofacial and dental development and is expressed in the developing facial processes and influences the formation of lower face (Seo *et al.*, 2013; Krivicka-Uzkurele and Pilmane, 2016). *PAX9* gene has 4 exons which are highly conserved in human being (Kavitha *et al.*, 2010). In human, mutated *PAX9* gene is frequently associated with oligodontia or hypodontia as well as Class II/Division 2 malocclusion (Ghergie *et al.*, 2013). Animal studies found that mutated or absence of *PAX9* gene resulted in poor skeletal development and odontogenesis with lack of coronoid process formation (Peters *et al.*, 1998; Nakatomi *et al.*, 2010). This particular gene was highly expressed at the region of pharyngeal pouches, mesenchyme of nasal processes, maxillary and mandibular arches as well as at the area of developing tooth buds' formation hence supporting the importance of *PAX9* in craniofacial, tooth and skeletal development (Peters *et al.*, 1998).

Single nucleotide polymorphism (SNP) is a variation in a single nucleotide that occurs at a specific position in the genome with incidence rate more than 1% (Keats and Sherman, 2013). Polymorphism in *PAX9* gene, SNP marker rs8004560, has been suggested to have an association with Class II/Division 2 malocclusion with hypodontia (Wall, 2009). Previous study had reported an association between *PAX9* SNP (rs8004560) with Class I malocclusion patients (Ghergie *et al.*, 2013). The SNP marker (rs8004560) which is located in the intron region can influence the proper splicing region and consequently the phenotypic sequences.

There has been very limited study performed to look into the genetic association of certain genes with Class II skeletal base malocclusion. Previous researchers reported the mutation in *PAX9* gene as the one responsible for the occurrence of hypodontia, oligodontia or dental agenesis (Frazier-Bowers *et al.*, 2002; Vieira *et al.*, 2004), and is associated with Class II malocclusion rather than other types of malocclusion (Wall, 2009; Morford *et al*., 2010). Therefore, this preliminary study was conducted to ascertain whether *PAX9* SNP (rs8004560) is presence in patients with Class II skeletal base malocclusion contributed by retrognathic mandible without dental anomalies in the local Malaysian population. The result obtained from this study may be used as a baseline for further genetic research to develop proper treatment approaches for the Class II skeletal base malocclusion patient in the future.

Materials and methods

Patients' selection

A total of 30 subjects were enrolled in this study. Fifteen subjects with Class II skeletal base and fifteen subjects with Class I skeletal base that act as control group were recruited from Orthodontic Department of Kulliyyah of Dentistry, International Islamic University Malaysia (IIUM). All subjects were fit and healthy, without craniofacial deformity including cleft lip and palate,

endocrinological problem and anomalies in tooth number, morphology and eruption. Ethical approval was obtained from the IIUM Research Ethic Committee (IREC) (reference number: IIUM/504/14/11/2/IREC 772), and consent was taken prior to samples collection.

Cephalometric analysis

Identified subjects underwent comprehensive clinical examination by one of the researchers at IIUM Specialist Clinic to ensure no anomalies in tooth number, morphology and eruption. Lateral cephalometric radiograph was taken as a record for cephalometric analysis. Eastman analysis (Bhatia and Leighton, 1993), measuring sella-nasion-A-point angle (SNA), sella-nasion-B point angle (SNB), A point-nasion-B point (ANB) and the Wits appraisal (AoBo) were executed (Jacobson, 2003). SNA angle was used to assess the position of maxilla to cranial base whilst SNB angle was used to determine the position of mandible to the cranial base. ANB angle was used to establish the relationship between maxilla and mandible. The Wits appraisal was used to further confirm the antero-posterior occlusal disharmony. For Class II skeletal base subjects, their cephalometric value indicative of skeletal base Class II (ANB more than 4°) with positive Wits appraisal (more than 2 mm), convex profile and to ensure the contributing factor of Class II skeletal base was retrogantic mandible and not prognathic maxilla, the SNA of all subjects were within normal (81 ± 3), and SNB indicative of retrognathic mandible (SNB less than 78°). For Class I control subjects, they must have a Class I skeletal base (SNA: 81±3º, ANB: 2º-4º), and Wits appraisal (-2 mm to +2 mm).

Genetic analysis

For the genotyping, 5 ml of unstimulated saliva were obtained from each patient. Genomic DNA (gDNA) was extracted from each sample using GeneAll® Exgene™ Kit (Biotech, Korea). The DNA concentration was measured using NanoDrop 1000 Spectrophotometer. The fragment of *PAX9* gene (595 bp; locus 14q13.3) containing the rs8004560 SNP was amplified by PCR using the primer sequence; forward 5'-GTCTATAACCTCTAACTGCCA-3' and reverse 5'-GACCAACTTCATCACACTGAC-3' (Ghergie *et al*., 2013).

The PCR mixture (25µl) contained: 0.5 µM of each primer (Sigma, Singapore), PCR buffer (containing 2 mM MgCl2), 0.2 mM of each dNTP, 1.25 U of EasyTaq® DNA Polymerase (TransBionovo, China), and gDNA accordingly (100-200 ng/ul). Standard amplification conditions were: initial denaturation at 95ºC for 4 min, followed by 35 cycles (94ºC for 30 sec, 56.8ºC for 30 sec, 72ºC for 1 min) and final extension at 72ºC for 10min. The PCR products were purified using the GeneAll® Expin™ Kit (Biotech, Korea). The purified PCR products were sent for sequencing. Then, the sequencing result of purified PCR products was further observed using electropherogram.

Statistical analysis

After the genotype and allele of each subject have been identified, the result was then statistically analysed by non-parametric test using Chi-square for intra-correlation observation. *SHEsis* online software was used to determine the genetic association and to assess Hardy-Weinberg equilibrium in each group. The genotype and allele distribution were compared between the Class II skeletal base subjects (contributed by retrognathic mandible) and Class I skeletal base subjects (control). A *p*-value of less than 0.05 was considered as statistically significant.

Results

Demographic and clinical data analysis

The demographic data and the mean values of cephalometric data were shown in Table 1. Majority of our samples were Malay (86.7%) while others were Chinese (13.3%). The age group of our 30 samples ranged from 12-26 years old with mean age for Class II skeletal base (case subjects) is 19.8 years while Class I skeletal base (control subjects) is 24.4 years. The percentage of male and female subjects was equal as 50%. Based on cephalometric analysis, it shows that the subjects selected were within different classes of skeletal base as the mean value for ANB for Class I and Class II skeletal base subjects were

within the specified range. Additionally, it shows Class II skeletal base subjects were having normal position of maxilla to cranial base as mean value of SNA is within the normal range and reduced SNB indicative that all Class II subjects has retrognathic mandible.

Genetic analysis

The PCR product was electrophoresed to check for the presence of amplified DNA at 595 bp. Once confirmed, the PCR product was purified and sent for sequencing. The sequencing result was further observed using electropherogram to detect the presence of homozygous (GG/AA) or heterozygous (AG) allele and the polymorphic position of both nucleotides (Fig. 1). Then, the genotype and allele distribution of *PAX9* SNP marker (rs8004560) were determined.

The distribution of genotype and allele of each subject from control and case groups were statistically analysed by non-parametric test using Chi-square to identify the relationship within each group (Table 2). Within the group, a significant difference in genotype (p=0.041) and allele frequency (p=0.003) was observed in Class II skeletal base subjects while only allele frequency was observed to demonstrate significant different in the control group (p=0.028). Overall, G allele of marker rs8004560 was over-represented in both classes while A allele was under-represented. However, when compared between cases and control; there was no significant association between *PAX9* SNP (rs8004560) with Class II skeletal base malocclusion (p=0.56, OR=0.71; 95% CI=0.225-2.246). The distribution of genotype frequency in both groups were consistent with HWE (p>0.05) (Table 3).

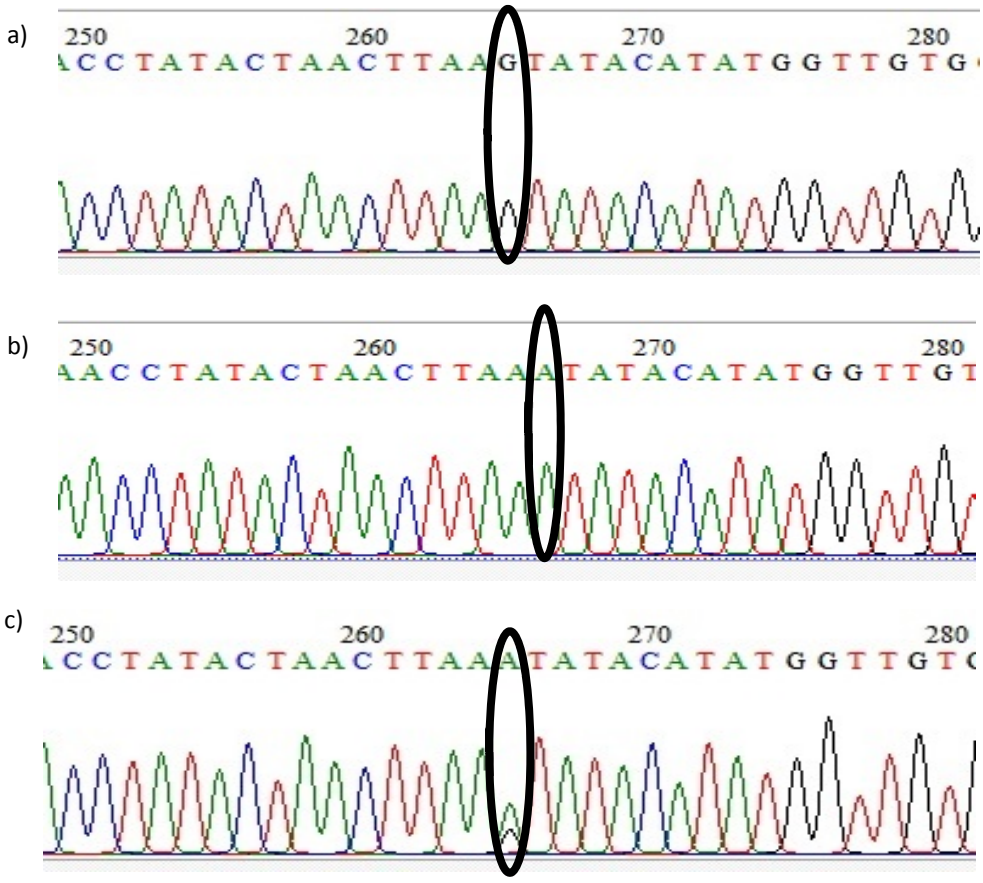

Fig. 1 Electropherograms showing the presence of a) homozygous GG (single peak), b) homozygous AA (single peak) and c) heterozygous AG (double peak) for genotyping of *PAX9* SNP (rs8004560).

Table 1 Demographic and cephalometric analysis of Class I (control) and Class II skeletal base malocclusion samples

			Control (Class I)	Case (Class II)
Demographic	**Gender**	Male	9	6
		Female	6	9
		Total (n)	15 (50%)	15 (50%)
	Race	Malay	15	11
		Chinese	0	4
	Age in years (mean)		24.4	19.8
Cephalometric	*SNA (◦)*		81.9	81.53
	SNB (◦)		78.8	75.07
	ANB (◦)		3.13	6.47
	Wits (AoBo) (mm)		0.23	2.53

Table 2 Intra-correlation analysis of genotype and allele distribution for *PAX9* SNP (rs8004560) analysed by non-parametric test using Chi-square (p<0.05 is considered significant). The table shows the frequency of GG, AG and AA genotype and G and A allele observed in the analysed population

	Genotype			Allele	
	GG	**AG**	**AA**	**G**	**A**
Case (Class II)	**9**	**5**	**1**	**23**	**7**
		p* = 0.041		***p* = 0.003 ***	
Control (Class I)	**8**	**5**	**2**	**21**	**9**
		***p* = 0.165**		***p* = 0.000***	

Allele G is over-represented in the analysed population.

Table 3 Association analysis of *PAX9* SNP (rs8004560) with Class II skeletal base malocclusion

Genotype (rs8004560)	Case (Class II) n (freq)	Control (Class I) n (freq)	OR [95% CI]	*p*-value
GG	9 (0.600)	8(0.533)		0.822
AG	5(0.333)	5(0.333)		
AA	1(0.067)	2(0.133)		
	HWE p=0.791	HWE p=0.424		
Allele				
G	23(0.767)	21(0.700)	0.71 [0.225-2.25]	0.559
A	7(0.233)	9(0.300)		

p<0.05 is considered significant in Pearson Chi Square; HWE is Hardy Weinberg Equilibrium.

Discussion

To the best of our knowledge, this is the first study on *PAX9* SNP (rs8004560) in Class II skeletal base (contributed by retrognathic mandible) malocclusion cases of local Malaysian population. Class II subjects were meticulously identified and characterised by having retrognathic mandible with normal range of SNA (normal maxilla). Both Class II samples and control must be without any dental anomalies as previous studies conducted have found that *PAX9* SNP (rs8004560) was present in their subjects with dental anomalies (Wall, 2009; Morford *et al.*, 2010; Seo *et al*., 2013; Patel *et al.*, 2016).

In our study, *PAX9* SNP (rs8004560) was detected in both Class I and Class II skeletal base subjects. The presence of either A allele or G allele was detected in both classes. This result was in accordance to Ghergie *et al.* (2013) and Wall (2009). We found that there was a significant difference in the allele and genotype frequency within the specific group analysed. This might suggest possible genetic interaction between *PAX9* SNP (rs8004560) with skeletal base malocclusion. However, further analysis has shown that there was no association between *PAX9* SNP (rs8004560) and Class II skeletal base malocclusion in the current preliminary study. This might be due to the limited number of samples involved. Larger number of samples will be required to establish this association.

The distribution of genotype frequency in both groups was according to Hardy-Weinberg equilibrium with *p*-value more than 0.05. Our results were similar as Ghergie *et al.* (2013), in which no significant differences in the distribution of genotypes were observed in the samples analysed. In both Class I and Class II skeletal base subjects, majority of the genotype present was homozygous GG genotype, followed by AG genotype while homozygous AA genotype was the least. This was in contrast with Ghergie *et al.* (2013) that shows majority of the genotypes present was heterozygous AG genotype. Differences observed in the genotype distribution might be due to the source of samples analysed since our study only focused on Asian (Malays and Chinese) population while Ghergie *et al.* (2013) utilised Caucasian population (Romanian), and studies by Wall (2009) and Morford *et al.* (2010) were performed in American population. Regarding allele distribution, we found G allele as the major allele present while A allele was less present. This is true since G allele is the ancestral or common allele while A allele is the mutant allele (minor allele frequency) for rs8004560 (Ghergie *et al.*, 2013).

To associate the role and mechanism of *PAX9* SNP within different classes of malocclusion and its influence in the occurrence of Class II skeletal base malocclusion, bigger sample size and stricter variables need to be considered in future study. This is important as it may help in providing useful information to clinicians to provide early intervention by means of gene therapy technology. This could be done probably by correcting the responsible gene for better treatment outcome.

Conclusion

Based on our study, *PAX9* SNP (rs8004560) could be detected in the local Malaysian population and the distribution of genotype and allele could be determined. Nonetheless, there was no association between *PAX9* SNP (rs8004560) and Class II skeletal base malocclusion contributed by mandibular retrognathism observed in the current study. Future study with bigger sample size is recommended to further confirm the genetic association of *PAX9* polymorphism in retrognathic mandible. This preliminary study may help in future research to assess specific function and the effect of *PAX9* SNP (rs8004560) or other related markers in mandibular retrognathism and can enhance treatment planning of maxilla-mandibular discrepancies.

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Declaration

Authors declared no conflict of interest.

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