DETERMINATION OF DUSP6 GENE MUTATION AND ITS EFFECT ON CRANIOFACIAL MORPHOLOGY AMONG MALAYSIAN MALAY WITH CLASS III MALOCCLUSION PATIENTS ATTENDING AT HOSPITAL UNIVERSITI SAINS MALAYSIA

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by

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LIST OF ABBREVATIONS

A	Adenine
ANB	The difference between angles SNA and SNB
ANOVA	Analysis of variance
ANXA2	Annexin A2
Ar	Articulare
Arg	Arginine
ARHGAP21	Rho GTPase Activating Protein 21
Asn	Asparagine
BLAST	Basic Local Alignment Search Tool
BMP3	Bone Morphogenetic Protein 3
bp	Base pair
C	Cytosine
C	Center of the condyle
Co	Condylion (superior-most point on mandibular condyle)
Co-Gn-B	Angle between Co-Gn and Gn-B
COL2A1	Collagen, type II, alpha 1
ddH_2O	distilled deionized water
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide
DUSP6	Dual specificity protein phosphatases 6
EDTA	Ethylene Diaminete Traacetic Acid
EGF	Epidermal Growth Factor
EPB41	Erythrocyte Membrane Protein Band 4.1
ERK	Extracellular Signal-Regulated Kinases
FGF	Fibroblast Growth Factors
FGFR	Fibroblast Growth Factors Receptor
FLNB	Filamin B, Beta
G	Guanine
G	Center of mandibular symphysis
GH	Growth Hormone
GHR	Growth Hormone Receptor
Gln	Glutamine
Gly	Glycine
Gn	Gnathion (most anteroinferior point on mandibular symphysis)
Go	Gonion (mid-point at angle of mandible)
HCl	Hydrogen chloride
HGF	Hepatocyte Growth Factor
His	Histidine
HOXA2	Homeobox A2
HOXC	Homeobox C Cluster
HREC	Human Research and Ethics Committee
HUSM	Hospital Universiti Sains Malaysia
Ia	3rd generation of patient (female)
Ib	3rd generation of patient (male)
Id	Infradentale (most anterosuperior point on mandibular alveolus)
IGF1	Insulin-like Growth Factor 1
IGH1	Insulin-like Growth Hormone 1

IHH	Indian Hedgehog Homolog
IIa	2nd generation of patient (female)
IIb	2nd generation of patient (male)
IIIa	Sibling of patient (female)
IIIb	Sibling of patient (male)
IIIc	Patient
Ile	Isoleucine
IVa	Niece of patient (female)
JEPeM	Jawatankuasa Etika Penyelidikan (Manusia)
kb	Kilobase
kV	Kilovolt
LTBP2	Latent Transforming Growth Factor Beta Binding Protein 2
M	Midpoint of premaxilla
Me	Menton (inferior-most point on mandibular symphysis)
Met	Methionine
mA	Milliampire
MATN1	Matrilin 1
$MgCl_2$	Magnesium chloride
ml	Milliliter
mM	Milli Mole
MP	Mandibular Prognathism
N	Nasion (frontonasal suture at its most superior point)
NCBI	National Centre for Biotechnology Information
ng	Nanogram
NGF	Nerve Growth Factor
nm	nano meter
ºC	Degree Celicius
OPG	Orthopantomogram
Orange G	Orange Gelb
P	Pogonion (anterior-most point on mandibular symphysis)
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
pH	Numeric scale used to specify the acidity or basicity
Phe	Phenylalanine
Point A	Deepest point at concavity on maxillary alveolar bone
Point B	Deepest point at concavity on mandibular alveolar bone
RNA	Ribonucleic acid
S	Sella (center of sella turcica)
SD	Standard deviation
Ser	Serine
SNA	Sella, Nasion and point A angle
SNB	Sella, Nasion and point B angle
SN-MDP	Angle between mandibular plane to S-N plane
SNPs	Single Nucleotide Polymorphisms
SPSS	Statistical Package For The Social Sciences
T	Thymine
TAE	Tris-Acetate-EDTA
TGFB3	Transforming Growth Factor, Beta 3
TGF-β	Transforming growth factor beta
TGF-β1	Transforming growth factor beta 1

TGF-β2	Transforming growth factor beta 2
TGF-β3	Transforming growth factor beta 3
Thr	Threonine
Tyr	Tyrosine
U	Unit
USM	Universiti Sains Malaysia
UV	Ultra-Violate
V	Volt
Val	Valine
VEGF	Vascular Endothelial Growth Factor
vs	Versus
x	Times
%	Percentage
× g	units of times gravity
µl	Microliter
µM	Micro Mole

PENENTUAN MUTASI GEN DUSP6 DAN KESANNYA KEPADA MORFOLOGI KRANOFASIAL DALAM KALANGAN PESAKIT MELAYU DI MALAYSIA DENGAN MALOKLUSI KELAS III DI HOSPITAL UNIVERSITI SAINS MALAYSIA

ABSTRAK

Maloklusi kelas III adalah sejenis dento-rangka yang diwarisi secara dominan dan progresif secara perlahan-lahan. Maloklusi ini dicirikan oleh pertumbuhan lampau rahang bawah, pertumbuhan maksila yang terbantut atau gabungan kedua-duanya. Etiologi maloklusi kelas III dan peranan gen-gen dalam fenotip ini masih kurang jelas. Mutasi dalam gen Spesifikasi Dual Protein Phosphatases 6 (DUSP6) telah dilaporkan menyebabkan jenis autosom dominan maloklusi kelas III. Objektif utama kajian ini adalah untuk menentukan mutasi gen DUSP6 di dalam tiga generasi kumpulan etnik Melayu di Malaysia yang mempunyai maloklusi kelas III dan untuk menjalankan analisis sefalometrik kumpulan tersebut. Analisis genetik gen DUSP6 telah dijalankan ke atas 30 subjek dengan memilih tiga individu yang mewakili tiga generasi iaitu sepuluh keluarga Melayu di Malaysia yang mempunyai maloklusi kelas III dan 30 orang yang sihat sebagai kumpulan kawalan. Radiograf sefalometrik hanya diperolehi bagi subjek maloklusi kelas III dan sefalometrik linear tentuan awal serta ukuran angular telah dijalankan menggunakan perisian Romexis. Ujian-t dan analisis varian (ANOVA) telah digunakan untuk menganalisis ukuran sefalometrik untuk kedua-dua kumpulan mutasi dan bukan mutasi untuk subjek maloklusi kelas III. Dalam kajian terbaru ini, mutasi salah erti heterozigot c.1094C> T (p. Thr 365 Ile) telah dikenalpasti pada gen DUSP6 dalam tiga orang daripada satu keluarga yang menghidapi maloklusi kelas III namun tiada mutasi ditemui dalam kumpulan

kawalan. Ujian-t menunjukkan perbezaan signifikan dalam ukuran angular bagi pembolehubah Co-Gn-B dan SN-MP dalam kumplan yang memiliki mutasi berbanding dengan kumpulan tiada mutasi. Tambahan itu analisis ANOVA tidak menunjukkan perbezaan yang signifikan untuk semua pembolehubah kecuali dalam sudut yen bagi generasi pertama dan kedua. Kesimpulannya kajian ini telah berjaya mengenalpasi suatu mutasi salah erti pada gen DUSP6 dalam kalangan keluarga Melayu di Malaysia yang mempunyai maloklusi kelas III dan secara sefalometriknya rahang bawah didapati lebih prognatik dari dasar kranial dalam kumpulan yang memiliki mutasi berbanding kumpulan tiada mutasi. Hasil daripada kajian ini telah meluaskan spektrum jenis mutasi bagi maloklusi kelas III dan kepentingan gen DUSP6 dalam morfologi kraniofasial.

DETERMINATION OF DUSP6 GENE MUTATION AND ITS EFFECT ON CRANIOFACIAL MORPHOLOGY AMONG MALAYSIAN MALAY WITH CLASS III MALOCCLUSION ATTENDING HOSPITAL UNIVERSITI SAINS MALAYSIA

ABSTRACT

Class III malocclusion is a dominant inherited, slowly progressive dento-skeletal disharmony. It is characterized by over growth of mandible, stunted growth of maxilla, or a combination of both. The etiology of class III malocclusion and the role of genes in this phenotype remain indistinct. Recently, dual specificity protein phosphatases 6 (DUSP6) gene mutations have been reported to cause autosomal dominant form of class III malocclusion. The main objective of this study was to determine the DUSP6 gene mutation in three generations of Malaysian Malay subjects having class III malocclusion and to conduct their cephalometric analyses. Genetic analyses of DUSP6 gene were carried out in 30 subjects by selecting three individuals representing three generations, respectively, from ten Malaysian Malay families having Class III malocclusion and 30 healthy controls. Cephalometric radiographs were obtained only from class III malocclusion subjects and pre-determined cephalometric linear and angular measurements were performed using Romexis software. t-test and analysis of variance (ANOVA) were used to analyse the cephalometric measurements from both mutation and non-mutation groups of class III malocclusion subjects. In the current study, a heterozygous missense mutation c.1094C>T (p. Thr 365 Ile) was identified in DUSP6 gene in three members of one family with class III malocclusion, whereas no mutation was found in the control group. t-tests showed significant differences in angular measurements Co-Gn-B and

SN-MP variables in mutation group compared to the non-mutation group. Moreover, ANOVA showed no significant differences for all variables except in yen angle of 1st vs 2nd generation. In conclusion, current study successfully identified a missense mutation in DUSP6 gene among one Malaysian Malay family affected by class III malocclusion and cephalometrically found mandible was more prognathic from cranial base in mutation group compared to non-mutation group. The outcome of this study broadened the mutation spectrum of class III malocclusion and the importance of DUSP6 gene in craniofacial morphology.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Class III malocclusion is a dominant inherited slowly progressive dento-skeletal disharmony. It can occur due to either maxillary hypoplasia, or mandibular prognathism or simultaneous occurrence of both.

Etiology of malocclusion can be dental or skeletal in nature. Dental malocclusion is termed as class III malocclusion when mesiobuccal cusp of maxillary molar lies distal to the buccal groove of mandibular molar. This condition can result in an anterior crossbite or underbite. In skeletal class III malocclusion, a discrepancy in jaw relationship leads to similar positioning of teeth as described earlier. This type of malocclusion termed as a true class III malocclusion (Singh *et al.,* 1997).

It is difficult to identify and diagnose class III malocclusion postnatally, until an individual is completely dentate. It appears with a higher incidence in permanent dentition when compared to primary dentition. The antero-posterior discrepancy of jaws is accentuated during the growth period and is fully expressed once the individual reaches age of maturation. Jaw asymmetry leads to a less attractive facial profile which forces patients to seek orthodontic and surgical treatment (Graber *et al.,* 1997).

According to Tweed, class III malocclusion is classified into two categories: Category A and category B. Pseudo class III malocclusion with conventional size of mandible is defined as category A and skeletal class III with large mandible or underdeveloped maxilla is defined as category B (Tweed, 1966).

According to Moyers, pseudo Class III malocclusion is a positional malrelationship with an acquired neuromuscular reflex (Moyers, 1988). Due to the retroclination of maxillary incisors, a functional advancing displacement of mandible occurs and causes a pseudo class III malocclusion.

The aetiology of skeletal class III malocclusion is an interesting topic and there is still much to understand. Environmental and genetic factors play an important role in occurrence of class III malocclusion. Endocrine imbalances, enlarged tonsils, congenital anatomic defects, nasal breathing, pituitary gland disease, habitual protrusion of mandible, and early loss of deciduous incisors are the most common environmental factors associated with class III malocclusion or mandibular prognathism (Angle, 1907; Downs, 1928; Gold, 1949; Monteleone and Duvigneaud, 1963; Pascoe *et al.*, 1960; Rubbrecht, 1939). Positions of the cranial base, maxilla and mandible, any displacement of the lower jaw and the positioning of the temporo-mandibular articulation are also contributing factors which affect the vertical and sagittal relationships of teeth and jaw (Angle, 1907; Gold, 1949; Monteleone and Duvigneaud, 1963; Rabie and Gu, 2000).

To identify the aetiology of any dentofacial characteristic, genetic evaluation is mandatory. Several human and animal studies have been carried out to validate the influence of heredity in the development of class III malocclusion. An animal study on mice established that size of mandible is related to the chromosome number 10 and 11 which corresponds with the regions 12q21 and 2p13 respectively, in human chromosomes. They suggested that, attention should be given on these two chromosomal regions. It might be possible to forecast the size of the mandible of a patient before the cessation of the skeletal growth by searching for the

polymorphisms of these chromosomal regions, whether the outcome would be short or lengthy mandible (Dohmoto *et al.*, 2002).

Since long it has been known that, the class III malocclusion follows the autosomal-dominant mode of inheritance. However, unfortunately few family studies have been conducted relating with class III malocclusion. This phenotype follows the autosomal dominant mode of inheritance and was demonstrated in different studies (El-Gheriani *et al.*, 2003; Cruz *et al.*, 2008). To find out the specific gene or genes responsible for class III malocclusion, limited genome wide family based linkage studies have been conducted (Yamaguchi *et al.*, 2005; Frazier-Bowers *et al.*, 2009; Li *et al.*, 2011).

For identifying the genetic variation, single nucleotide polymorphisms (SNPs) on candidate gene were checked between the case and the control groups. SNPs are the most common hereditary transformations in human beings that affect protein expressions and functions, and they can be related to a disease (Wang and Moult, 2001). Among the dental diseases, malocclusion is very common and it may be suggested that SNPs are the major genetic variations causing malocclusion (Risch and Merikangas, 1996). Recently, one study established a candidate gene DUSP6 (Dual specificity protein phosphatases) for class III malocclusion in an Estonian family. Whole exome sequencing was carried out among affected five siblings from one single family and a rare missense mutation c.545C>T (p.Ser182Phe) was found. This candidate gene spans 4.46 kb of genomic DNA on chromosome 12q22-q23 (Nikopensius *et al.*, 2013).

DUSP6 gene is situated in chromosome 12q22-23 region in human (Furukawa et al., 1998). This gene constitute a huge heterogeneous subcategory of the type I cysteine-based protein-tyrosine phosphatase superfamily. Dual specifity phosphatases (DUSPs) are categorized by their capability to dephosphorylate both tyrosine and

serine/threonine residues. DUSP6 belongs to a class of DUSPs, labelled mitogen kinase phosphatase (MKPs) that dephosphorylate mitogen-activated protein kinase (MAPK) proteins. Extracellular signal regulated kinase (ERK) with specificity distinct from that of individual MKP proteins. MAPK initiation forces facilitate various physiologic processes, including cellular proliferation, differentiation, apoptosis and stress responses (Patterson *et al.,* 2009). Transcriptional initiation of DUSP6 has been assumed to be synchronised by Fibroblast growth factor/Fibroblast growth factor receptor (FGF)/ (FGFR), respectively and MAPK/ERK signaling during major progressions at initial stages of skeletal development. A number of candidate genes within a linkage region on chromosome 12q22-q23 – harboring DUSP6 are associated in the regulation of maxillary or mandibular growth.

A study on mice proved that DUSP6 gene mutation affect the craniofacial development and skull vault. They found that height to length and height to width ratios were significantly larger than those of consistent wild-type control ratios (Li et al., 2007).

Class III malocclusion has been observed to segregate within families. Different pedigree, segregation analysis and linkage analyses studies concluded that gene or genes influence the manifestation of class III malocclusion (Wolff *et al.*, 1993; El-Gheriani *et al.*, 2003; Cruz *et al.*, 2008; Frazier-Bowers *et al.*, 2009).

Genetic and environmental factors are plyaing an important role to determine the craniofacial morphology (Saunders *et al.*, 1980). Studies in craniofacial morphology among close relatives have explained that genetic factors have a significant role in determining the craniofacial morphology (Hunter *et al.*, 1970). With recent advances, clinical genetics has enriched the knowledge regarding genetic predispositions for

craniofacial phenotypes (Coussens and Van Daal, 2005; Yamaguchi *et al.*, 2005; Lee *et al.*, 2006).

1.2 Statement of problem

In order to determine the DUSP6 gene mutation and its effect on craniofacial morphology of class III malocclusion in Malaysian Malay ethnic group, a study is yet to be conducted. If in case an association can be established, testing for these mutations will greatly assist in early screening and timely treatment of class III malocclusion.

1.3 Objectives

1.3.1 General

To determine the DUSP6 gene mutation and its effect on craniofacial morphology with class III malocclusion patients, attending Dental Clinic at Hospital Universiti Sains Malaysia (HUSM).

1.3.2 Specific

a) To determine the DUSP6 gene mutation in patients with class III malocclusion and healthy controls.

b) Compare the cephalometric radiographs between DUSP6 gene mutation and non-mutation groups of class III malocclusion.

1.4 Research questions

a) Is there any DUSP6 gene mutation found in patients with class III malocclusion and/ or healthy controls?

b) Is there any association of cephalometric radiographs in relation with DUSP6 gene mutation and non-mutation groups of class III malocclusion?

1.5 Research hypothesis

a) DUSP6 gene mutation is present in class III malocclusion patients and/ or healthy controls.

b) There is an association of cephalometric radiographs in relation with DUSP6 gene mutation and non-mutation groups of class III malocclusion.

1.6 Null hypothesis

a) There is no DUSP6 gene mutation found in class III malocclusion patients and/ or healthy controls.

b) There is no association of cephalometric radiographs in relation with DUSP6 gene mutation and non-mutation groups of class III malocclusion.

CHAPTER 2

LITERATURE REVIEW

2.1 Class III malocclusion

Angle assumed in his classification of malocclusion that the first permanent molars are constant in relation to jaws, which is associated to the relative sagittal position of mandible and maxilla. When mandibular first permanent molar is more mesially positioned than the maxillary first permanent molar, it is called class III malocclusion (Angle, 1907). In contrast, British Orthodontic Society (1992) announced a classification for malocclusions that grounded on the incisal relationships. In which mandibular incisor edges are positioned forward to the cingulum plateau of maxillary incisors (Williams and Stephens, 1992).

Still, Angle's classification is regularly used due to its simplicity. However, many authors criticized and pointed out due to the vertical and transverse considerations (Van Loon, 1915; Case, 1921). According to Angle's classification of malocclusion, class III malocclusion embraces different dental and skeletal mechanisms that may vary from the perception of normality. Such as, this phenomenon may occur either due to retrusion of maxilla, protrusion of mandible or a blend of both (Graber *et al.*, 2011). Sanborn stated in his study that 33% of the sample with class III malocclusion had maxillary skeletal retrusion with normal mandibule, 45.2% of sample had protruded mandible and 9.5% were perceived combination of both skeletal patterns (Sanborn, 1955). Such skeletal disparity is consequence from growth resentment between maxilla and mandible creates a concave facial profile. Class III malocclusion is of great concern for a fact that many patients having this

malocclusion are treated routinely for orthodontic reasons (Cruz *et al.,* 2008). The concern of development in class III subjects has become important because of the increasing awareness in enhancing treatment timing and planning in dentofacial orthopaedics.

2.2 Classification of class III malocclusion

Mostly, class III malocclusion is classified into three types - dental, skeletal and pseudo type (Graber *et al.,* 2011).

2.2.1 Dental features of class III malocclusion

Patient having dental class III malocclusion showing molar relation in class III and incisors may be in edge-to-edge or anterior cross bite. The maxillary arch is narrower and crowded while the mandibular arch is often spaced (Iyyer *et al.,* 2012).

2.2.2 Skeletal features of class III malocclusion

Generally, class III malocclusion is associated with underlying skeletal mal-relationship. Commonly seen skeletal features are-

- A short or retrognathic maxilla
- A long or prognathic mandible
- A combination of both (Graber *et al.,* 2011)

2.2.3 Pseudo type class III malocclusion

Pseudo type class III malocclusion is categorized by presence of premature occlusal contact that causes a habitual forward positioning of the mandible. These patients may exhibit a forward path of closure (Iyyer *et al.*, 2012). Different authors also modified the classification of class III in different ways (Tweed, 1966; Park and Baik, 2001). Park and Baik, (2001) classified Angle's class III malocclusion into three categories based on abnormalities on maxillae.

- Type A: true mandibular prognathism, where the mandible is overgrown but the maxilla is normal
- Type B: characteristics of the overgrown mandible and maxilla along with anterior cross bite
- Type C: indicates a hypoplastic maxilla with anterior cross bite

Moreover, Tweed, (1966) classified class III malocclusion into two categories,

- Category A: Pseudo class III malocclusion with conventional shaped mandible
- Category B: Skeletal class III malocclusion with large mandible or underdeveloped maxilla

2.3 Prevalence of class III malocclusion

The prevalence of Class III malocclusion has been described between 1% (Hill *et al.*, 1959; Emrich *et al.*, 1964) to over 10% (El-Mangoury and Mostafa, 1990), depending on ethnic backgrounds (Emrich *et al.*, 1964), gender (El-Mangoury and Mostafa, 1990; Baccetti *et al.*, 2005) and age (Thilander *et al.*, 2001). It has been reported that approximately 75% of Class III malocclusion cases in male Caucasians

have a skeletal origin and were a result of mandibular prognathism or macrognathia (Staudt and Kiliaridis, 2009). The prevalence of Class III malocclusion among Caucasian people ranges from 0.48% to 4% (Emrich *et al.*, 1964). However, compared to Caucasian people the prevalence of class III malocclusion is higher in Japanese population reaching up to 10% (Nakasima *et al.*, 1986).

Several studies have documented the prevalence of Angle class III malocclusion. However, different population has different proportions (Hill *et al.*, 1959; El-Mangoury and Mostafa, 1990; Staudt and Kiliaridis, 2009). Multiple studies have stated that Asian ethnic groups have a higher prevalence of Angle class III malocclusion than other ethnic groups (Emrich *et al.*, 1964; Woon *et al.*, 1989; Lew *et al.*, 1993; Tang, 1994a; Tang, 1994b; Onyeaso, 2004; Soh *et al.*, 2005). In other populations, the prevalence of class III malocclusion was found between 1-5%, whereas in Chinese and Korean population it increased 9.4 to 19% (Chan, 1974).

Table 2.1 shows the prevalence of class III malocclusion in different studies among different ethnic groups.

2.3.1 Caucasians

Emriche et al., (1964) observed 10,133 Caucasian children that were 6-8 years old and 13,475 children that were 12-14 years old and found that 1% of both groups had class III malocclusion.

2.3.2 Negroes

Altemus, (1959) reviewed 3,289 Negroes between the ages of 12 and 16 years and reported that class III malocclusion was present in 5% of them. Emriche et al., (1964)

also found that 3% of the Negroes surveyed at the age of 12 to 14 years and 2% of the Negroes surveyed at the age of 6-8 years had class III malocclusion. Dacosta, (1998) also found that 2% of 1,028 school children in Northern Nigeria had class III malocclusion. The prevalence of malocclusion was investigated in 245 children from a pastoral community in Kenya and it was found that 5% of them had class III malocclusion (Ng'Ang'A *et al.*, 1993). Similarly 1,601 school going children including 16 different primary schools in Tanzania, aged 12 to 16 years were observed and among them only 2% of children were found having class III malocclusion (Mtaya *et al.*, 2009). In contrast, in another study among Tanzanian's 289 randomly selected primary school children were taken to observe the prevalence of malocclusion and 11% had class III malocclusion (Rwakatema *et al.*, 2006).

2.3.3 Europeans

Perillo et al., (2010) collected 703 samples of 12 years old school children from southern part of Italy to check the prevalence of malocclusion. That study showed 4.3% prevalence of class III malocclusion. Another article documented that 4% of 137 Swedish subjects at 21 years of age, had class III malocclusions (Thilander and Myrberg, 1973). The prevalence of malocclusion was surveyed among 7–15 years old Lithuanian school children and 2.8% had class III malocclusion (Šidlauskas and Lopatienė, 2009).

2.3.4 Asians

2.3.4.1 Chinese

Lew *et al.*, (1993) surveyed 1,050 Chinese school children of age between 12 to 14 years to assess certain occlusal features, both qualitatively and quantitatively. The population was found to have a high incidence of Class III malocclusions (12.6%) compared with Caucasians (5.5%). In addition, 19.9% among 201 Chinese adult showed prevalence of class III malocclusion (Tang, 1994a). They also checked the prevalence of malocclusion among 108 young Chinese individuals and concluded that 14.8% had class III malocclusion (Tang, 1994b).

2.3.4.2 Indian

One Indian study showed that among 3,164 samples (age 6-15 years) only 1.3% had class III malocclusion (Guaba *et al.*, 1998).

2.3.4.3 Malaysian

Woon et al., (1989) surveyed the occlusal relation between three ethnic groups Chinese, Malay and Indian in Malaysia. He found significantly higher prevalence of class III occlusion among the Chinese and Malay ethnic groups compared to the Indian ethnic group in Malaysia. In addition, Soh et al., (2005) also studied Chinese, Indian and Malay ethnic groups and documented the prevalence rate of class III malocclusion were 22.9%, 4.8% and 26.7% respectively.

Table 2. 1 Prevalence of class III malocclusion in different ethnic group

Authors	Year	Ethnic group	Number of samples	Prevalence
Emrich et al	1965	Caucasians	10,133	1%
Altemas LA	1959	American Negro	3,289	5%
Emrich et al	1965	American Negro	##	3%
Dacosta OO	1999	Nigeria	1,028	2%
Ng'ang'a et al	1993	Kenya	245	5%
Mtaya et al	2009	Tanzania	1,601	1.81%
Rwakatema et al	2006	Tanzania	289	19.72%
Perillo et al	2010	Italy	703	4.27%
Thilander B & Myrberg N	1973	Sweden	137	4%
Šidlauskas & Lopatienė	2009	Lithuania	1681	5.62%
Lew et al	1993	Chinese	1,050	12.76%
		Chinese	154	18.18%
Woon et al	1989	Indian	42	0%
		Malay	151	12.58%
Guaba K	1998	India	1532	1.17%
		Chinese	258	22.87%
Soh	2005	Indian	21	4.76%
		Malay	60	26.67%
Tang E	1994	Chinese	201	19.90%

##, Not mentioned in literature; %, percentage.

2.4 Aetiology of class III malocclusion

Class III malocclusion is a multifactorial disease mainly with skeletal involvement. Whereas risk factors such as environment and genetics can manifest on progression of the disease.

2.4.1 Skeletal intervention

The location of the temporomandibular articulation and to some extent displacement of the lower jaw equally disturbs the vertical and sagittal relationships of jaw and teeth of maxilla and mandible (Björk, 1950; Hopkin *et al.*, 1968; Williams and Aarhus, 1986; Kerr and Tenhave, 1988). Size and relative positions of the cranial base, position of the spinal column, foramen magnum (Houston, 1988) and habitual head position (Jacobson, 1989) might also effect the subsequent facial pattern.

2.4.2 Environmental factors

Extensive varieties of environmental factors have been suggested as contributing to the development of class III malocclusion. Hormonal disturbances (Pascoe *et al.*, 1960), trauma and disease, a habit of protruding the mandible, posture, pituitary glandular dysfunction, premature loss of the first molar (Gold, 1949), congenital anatomic defects (Monteleone and Duvigneaud, 1963), enlarged tonsil, difficulty in nasal breathing (Angle, 1907), irregular eruption of permanent incisors or loss of deciduous incisors (Rubbrecht, 1939) are considered main environmental factors contributing to class III malocclusion.

2.4.3 Genetic factors

Class III malocclusion is considered as a developmental problem. Moreover, hereditary or genetic factors play an important role in craniofacial development (Graber *et al.*, 2011). It has already been acknowledged that genes are involved in the guidelines of growth of skeleton (Le Roith and Butler, 1999). The role of genetics in the pathogenesis of class III malocclusion is unravelling gradually. There are around 15 genes suggested to attain polymorphism and they have been related to class III malocclusion (Yamaguchi *et al.*, 2005; Zhou *et al.*, 2005; Frazier-Bowers *et al.*, 2009; Jang *et al.*, 2010; Xue *et al.*, 2010a; Xue *et al.*, 2010b; Li *et al.*, 2011; Nikopensius *et al.*, 2013; Perillo *et al.*, 2015). To yield proper immune response, the hormonal and cellular components of immune system should essentially co-ordinate (Gudmundsson and Agerberth, 1999). Any genetic flaw and/or functional impairment results in a tendency to class III malocclusion (Mossey, 1999).

2.5 Human gene

In living organisms, the molecular element of heredity is called gene. It is accepted by the scientific community that these genes are stretches of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) that code for the body proteins. Human bodies consists of billions of cells. Most of the cells comprise a nucleus with its nuclear membrane. The nucleus contains the hereditary information stored in the form of DNA. The gene is defined as “a locatable region of genomic sequence, corresponding to a unit of inheritance, which is associated with regulatory regions, transcribed regions, and or other functional sequence regions” (Pearson *et al.*, 2006).

2.6 Mutation of gene

A mutation may be demarcated as any change in the genetic make-up of a cell, an organism or a population of cells. Random interaction with the surroundings or because of the normal cellular function natural mutations usually takes place. To maintain the double helix structure of DNA, two base pairs (guanine-cytosine and adenine-thymine) play an important role. If changes occur in single base nucleotide with another nucleotide of the genetic material, then it is called point mutation. It is also called as ‘‘single nucleotide polymorphism’’ (SNP). Point mutation is fixed naturally but sometimes it cannot. Then it can be transferred through generation to generation by inheritance. Commonly by transitioning, comprising the substitution of an adenine–thymine (A–T) pair with a guanine–cytosine (G–C) pair or vice versa (Loos *et al.*, 2005). Point mutation or SNPs occur throughout the human genome and is predicted at every .3-1 kilobases (kb), while other sorts of genetic mutations occur from insertions or deletions (Schork *et al.*, 2000).

2.7 Effects of genetic mutation on disease

Multiple genes and their polymorphisms may collectively have a small overall influence and virtual risk to disease severity and susceptibility. Complex diseases are typically polygenic (Tabor *et al.*, 2002). Clinical exhibition of the altered combination of mutation suggest that genotyping is reliable for forecast of clinical outcome in patients (Wedell *et al.*, 1994).

2.8 Genetic studies and Class III malocclusion

Evidence from previous studies also established that class III malocclusion is strongly influenced by the genetic factors (Nakasima *et al.*, 1986; Nikopensius *et al.*, 2013). Class III malocclusion might develop by polygenic or monogenic mode of inheritance. However, the environmental factors are also responsible for this trait. Few studies have been done to evaluate the quantitative role of heredity in the aetiology of this condition.

Suzuki, (1961) surveyed 1,362 family members from 243 Japanese families and observed that the families who have history of mandibular prognathism, 34.3% of the family member exhibited the trait (Suzuki, 1961). Litton et al., (1970) examined the families of probands with class III malocclusion followed by Angle and found that about 13% of the siblings of probands exhibited the trait which suggested a strong genetic influence in class III malocclusion. Moreover, this study indicated that transmission was in polygenic mode of inheritance. Saunders et al., (1980) studied the similarities in craniofacial dimensions between members of 147 families. By calculating standard product, moment and intraclass correlation coefficients were compared, parents with offsprings and siblings with siblings. The results showed a high level of meaningful co-relations between first-degree relatives, which were compatible with a polygenic theory of inheritance.

Schulze and Wiese, (1965) also mentioned that in case of mandibular prognathism the polygenic mode of inheritance is the transmission medium by studying monozygotic and dizygotic twins. However, a number of study have reported that the genetic transmission follows the monogenic or mendelian pattern of inheritance. Cruz et al., (2008) studied with 2,562 members from 55 families and concluded that

a major gene influenced the expression mandibular prognathism with clear signs of Mendelian inheritance.

A large European noble family study with 409 members from 13 families concluded that a single autosomal dominant gene determines mandibular prognathism (Wolff *et al.*, 1993). El-Gheriani et al., (2003) also concluded after analysing the families in Libya with mandibular prognathism that the inheritance is in the monogenic method.

2.9 Different loci and genes responsible for class III malocclusion

Now-a-days genomewide linkage scan technology can detect several chromosomal regions, which is/are responsible for the mandibular prognathism. However, very few genomewide family based linkage study have been done to determine the specific gene or genes for mandibular prognathism (Table 2.2).

Yamaguchi et al., (2005) identified three chromosomal loci 1p36, 6q25 and 19p13.2, which are susceptible for mandibular prognathism. This study was done on fifty Japanese and forty Korean sibling-pairs. Using permutation of datasets, the Monte-Carlo approximation of Fisher's exact test (Weir, 1990) was done for estimating the different allelic frequency between these Korean and Japanese population. In the linkage region of chromosome 1, D1S2864, D1S234 and D1S2333 allelic frequency of microsatellite markers was found in Korean and Japanese probands (33 each). Japanese population showed linkage in chromosome 9 and 10 and Korean siblings pair showed linkage in chromosome 4. Though commonly linkage pattern is similar between Korean and Japanese population, these differences may occur due to genetic heterogeneity. Nevertheless, the Monte-Carlo approximation of Fisher's exact test showed no statistical significance. Therefore, it can be said that same etiological background exists for mandibular prognathism in these two populations.

Five loci (1p22.1, 3q26.2, 11q22, 12q13.13 and 12q23) were found in Colombian families for class III malocclusion as a suggestive of linkage in another study. IGF1 (Insulin like Growth Factor-1), HOXC (Homeobox C Cluster) and COL2A1 (Collagen, type II, alpha 1) were considered as candidate genes within the chromosome 12q23 region for class III malocclusion. For influencing body size IGF1 plays an important role in both human and mice. HOX counts as a centric gene in vertebrates for craniofacial development. In addition, type II collagen cartilage encoded by COL2A1 gene (Frazier-Bowers *et al.*, 2009). EPB41 and Matrilin 1, cartilage matrix protein (MATN1) were found as plausible genes for the mandibular prognathism on chromosomal locus 1p36 in Chinese and Korean population, respectively (Jang *et al.*, 2010; Xue *et al.*, 2010a).

After investigating 211 cases and 224 controls, EPB41 demonstrated a significant association with mandibular prognathism in Chinese population. The study stated that, EPB41 gene is an important fundamental element of the membranous skeleton of erythrocyte that makes a crucial contribution to the fundamental integrity of the centrosome and mitotic spindle plays a main role in cell division (Conboy, 1993; Huang *et al.*, 2001; Pérez-Ferreiro *et al.*, 2004).

Linkage between the mandibular prognathism and single-nucleotide polymorphisms (SNPs) in MATN1 among 164 (mandibular prognathism) and 132 (normal occlusion) individuals were explored focusing three sequence variants (158 T>C, 7987 G>A, 8572 C>T). Comparing with control 158 T, 7987 G, and 8572 C alleles had a marked hazardous effect for mandibular prognathism. Aforementioned study proposed that for mandibular prognathism, polymorphisms in MATN1 could be used as an indicator (Jang *et al.*, 2010).

A susceptible locus was identified on chromosome 14q24.3-31.2 in Han Chinese population where the candidate genes were TGF-β3 (Transforming growth factor beta 3) and LTBP2 (Latent Transforming Growth Factor Beta Binding Protein 2) (Li *et al.*, 2011). Transforming growth factor beta (TGF-β) superfamily contains TGF-β3 gene. There are three forms of TGF-β having the same construction and in vitro biological activities. They are TGF-β1, TGF-β2 and TGF-β3 (Miyazono *et al.*, 2001). Formation of growth factors and differentiation of bone tissue TGF-β are considered vital for growth. This gene also participates in the growth of oral cleft patients in central European origin (Sassá Benedete *et al.*, 2008) and associates the mineral maturation matrix (Reutter *et al.*, 2008). Therefore, TGF-β3 plays an important role.

LTBP2 plays a functional role in elastic fibres assembly by disturbing the extracellular matrix homeostasis (Saharinen *et al.*, 1999). LTBP2 also contributes in the process of chondrogenic differentiation as found in an *in-vitro* study (Goessler *et al.*, 2005). They suggested that there could be an association of TGF-β superfamily and LTBP2 in mandibular prognathism. Nikopensius *et al.*, (2013) performed whole exome sequencing on five siblings from Estonian family who were affected by class III malocclusion. That study showed that in 12q22-q23 region DUSP6 gene effected the mandibular growth.

Recent studies of craniofacial growth have reported that several genes that encode specific growth factors or other signalling molecules, including Indian hedgehog homolog (IHH), insulin like growth factor-1 (IGF1), and vascular endothelial growth factor (VEGF), and variations in their levels of expression have an important role in the aetiology of Class III malocclusion (Weir, 1990). IGF1 is located at the 12q23 linkage region and represents an excellent candidate gene of biological interest because the GH (Growth Hormone)/ GHR (Growth Hormone Receptor)/ IGF1

system has an essential role in skeletal growth and normal bone metabolism (Sjögren *et al.*, 2000). In addition, other growth factors, including EGF (Endothelial Growth Factor), HGF (Hepatocyte Growth Factor), NGF (Nerve Growth Factor), and PDGF (Platelet-Derived Growth Factor) can activate ERKs during development and in adult tissues and induce the transcription of other members of the DUSP6 family, which could compensate for the lack of DUSP6 in knockout models. However, although various growth factors are capable of inducing DUSP6, there could exist a specific, preferrential relationship between FGF and DUSP6 at the level of transcription (Bermudez *et al.*, 2010). Alternatively, FGF/FGFR signalling could regulate the access of transcription factors to promoter regions of DUSP6 by specific epigenetic mechanisms and modifications of the chromatin, as reported previously for some other genes (Dailey *et al.*, 2005).

Recently, a study found five missense variants BMP3 (Bone Morphogenetic Protein 3), ANXA2 (Annexin A2), FLNB (Filamin B, Beta), HOXA2 (Homeobox A2), and ARHGAP21 (Rho GTPase Activating Protein 21), which are allied to class III malocclusion in five members of an Italian family. Among them ARHGAP21 showed the missense variants among all individuals with class III malocclusion. Moreover, authors concluded that, ARHGAP21 protein reinforces cell-cell bond. That might regulate the bone morphogenic factors and induce mandibular growth (Perillo *et al.*, 2015).

Growth hormone receptor (GHR) gene was assumed a susceptible gene for class III malocclusion in different populations (Zhou *et al.*, 2005; Kang *et al.*, 2009; Tomoyasu *et al.*, 2009; Bayram *et al.*, 2014).

Evidence from population studies has demonstrated that Class III malocclusion was influenced strongly by genetic factors, and multiple environmental factors have been

shown to affect mandibular growth. If there is a history of skeletal class III malocclusion among family then there is higher chance to develop adverse arch relationship like maxillary undergrowth or mandibular over growth (Alam *et al.*, 2008). According to literature, the prevalence rate of class III malocclusion is high in Asian ethnic groups. Linkage studies and genetic determination would be helpful to find out the exact aetiology of the class III malocclusion.

Table 2. 2: Susceptible loci/ locus found in different populations for class III malocclusion

Author & year	Susceptible loci/ locus	Candidate gene	Ethnicity/ Population
Yamaguchi et al., (2005)	1p36, 6q25, 19p13.2	###	Korean and Japanese
Frazier-Bowers et al., (2009)	1p22.1, 3q26.2, 11q22, 12q13.13, 12q23	IGF1, HOXC and COL2A1	Colombian
Xue et al., (2010b)	1p36	EPB41	Chinese
Jang et al., (2010)	1p36	MATN1	Korean
Li et al., (2011)	14q24.3-31.2	TGFB3 and LTBP2	Han Chinese
Nikopensius et al., (2013)	12q22-q23	DUSP6	Estonian
Perillo et al., (2015)	10p12.1	BMP3, ANXA2, FLNB, HOXA2,ARHGAP21	Italian
Bayram et al., (2014)	5p13.1-p12	GHR	Turkish
Tomoyasu et al., (2009)	5p13.1-p12	GHR	Japanese
Zhou et al., (2005)	5p13.1-p12	GHR	Chinese
Kang et al., (2009)	5p13.1-p12	GHR	Korean

Not mentioned in literature.

2.10 DUSP6

DUSP6 gene is situated in chromosome 12q22-23 region in human body (Furukawa et al., 1998). Genomic analysis of DUSP6 determined that the DUSP6 gene contains 3 exons (Furukawa *et al.,* 1998). Previous *in-vivo* study showed that DUSP6 gene mutation causes higher rates of myocyte proliferation during embryonic and early postnatal development that results in enlarged anatomical structures. Authors stated that DUSP6 synchronises cellular development and survival, hence it directly affects disease receptiveness in adulthood (Maillet *et al.,* 2008).

2.11 DUSP6 gene and associated diseases

Different studies suggested that DUSP6 worked as a tumor suppressor in human pancreatic cancer. The chromosomal location of DUSP6 is one of the frequent regions of allelic loss in pancreatic cancer. Two forms of alternatively spliced transcripts are universally expressed in DUSP6 gene. Reduced expressions of the full-length transcripts were detected in some pancreatic cell lines, which may advise some role of DUSP6 in pancreatic carcinogenesis (Furukawa *et al.,* 2003). Another author recommended that hypermethylation with modification of histone deacetylation showed a significant role in transcriptional suppression of DUSP6 in human pancreatic cancer (Xu *et al.,* 2005).

Animal studies suggested that FGF pathway plays an important role in pathogenesis of congenital scoliosis (CS) (Dequéant *et al.,* 2006, Dequéant *et al.,* 2008). DUSP6 gene has been reflected as one of the key genes in the FGF signal pathways. However, no mutation or new SNP was found in any exons of DUSP6 gene in CS patients among Han Chinese populations (Kotwicki and Grivas, 2012).

Genetic mutations encoding constituents of the FGF pathway are linked with complex modes of congenital hypogonadotropic hypogonadism (CHH) inheritance and perform mainly as providers to an oligogenic genetic architecture underlying CHH. They identified DUSP6 gene mutation in association with CHH and with its anosmia-associated form of Kallmann syndrome (KS) (Miraoui *et al.*, 2013).

2.12 Cephalometric evaluation of class III malocclusion

Class III malocclusion is consider as one of the most complicated and challenging orthodontic problems to diagnose and treat. For orthodontic treatment planning and diagnosis, cephalometric analysis plays an integral role. Patient's cephalometric radiographs were measured with the standard norms and values.

2.12.1 Comparison with normal occlusion

In the Caucasian sample of class III malocclusion patients, investigations were performed to assess the skeletal difference between normal and class III malocclusion. Authors found that Class III patients showed differences in facial morphology in all facial areas examined, when compared with their control peers. The cranial base angle was more acute, the maxilla was shorter and more retrusive, whilst the mandible was longer and more prominent. The proclined upper incisors were as far forward within the face in the Class III group as in the controls, but the retroclined lower teeth were even more labially placed (Battagel, 1993).

Guyer et al., (1986) compared class III skeletal and dental relations to class I norms. The comparative study concluded that the length of posterior cranial base is longer in case of class III malocclusion. The maxilla is usually retrusive in class III subjects,

even the length of class III maxilla is significantly shorter, skeletal position of mandible is protruded in class III malocclusion compared to class I norms, class III maxillary insicors are significantly protrusive, mandibular inscors are retrusive than class I norms.

Both anterior and posterior cranial base are significantly shorter in class III malocclusion than the norms in Syrian population. Moreover, class III maloccluded patients had a tendency of significantly shorter lower anterior facial height and smaller vertical face dimension in the population studied (Mouakeh, 2001).

Saudi population had an increased ANB angle due to retrognathic mandibles and bi-maxillary protrusion which were comparable with European-Americans (Hassan, 2006).

A study among Malaysian Chinese population, Purmal et al., (2013) narrated that generally there is a tendency for maxilla and mandible to be positioned forward in Chinese but the forward mandible gives an impression of class III malocclusion. While compared to class I relation, class III malocclusion exhibits shorter midfacial length, larger mandible, lower anterior facial height, larger facial axis angle and more acute saddle angle (Bahaa *et al.*, 2014).

2.12.2 Compare to the established norm

Generally American and European samples were involved in researches from where standard norms were established for cephalometric analysis (Hwang *et al.*, 2002). Therefore, following these norms for all ethnic groups is not justified, as there are probable ethnic and racial variations.

Different studies show noticeable variances in craniofacial morphology in several ethnicities in cephalometric measurements (Cotton *et al.*, 1951; Nanda and Nanda,

1969; Shalhoub *et al.*, 1987; Paek *et al.*, 1989). Al-Jame et al., (2006) established norm for Kuwait adolescents. He found that Kuwaiti adolescents demonstrated more convex profile with a tendency of reduced chin protrusion, protrusive dentition, and steeper mandibular plane than the established norms.

Class III malocclusion in Korean population occurred due to smaller anterior cranial base and midfacial dimensions. It was intensified by a large and less favourable mandibular morphology when compared to European- American subjects (Singh *et al.*, 1997).

Ishii et al., (2002) executed a study between Japanese and British Caucasian females with class III malocclusion and concluded that Japanese females showing retrusive mid facial components, reduced anterior cranial base, and increased lower anterior facial height associated with obtuse gonial angle paralleled to British Caucasian females. Additionally, Japanese samples showing more proclined upper incisors compare to Caucasians.

For Malaysian Malays, maxilla and mandible had different values than the Caucasian norms. Moreover, they were showing bi-maxillary dental protrusion when compared to the Caucasians (Mohammad *et al.*, 2011).

2.12.3 Variations among ethnicities

Saudi females with class III malocclusions appeared different than Japanese females with class III malocclusion. Saudi females presenting smaller anterior and posterior height, smaller posterior cranial base with cranial base angle, larger anterior cranial base, a retruded chin, a smaller ramus, body and total length of mandible and less retroclined mandibular incisors (Bukhary, 2005).

CHAPTER 3

MATERIALS AND METHODS

3.1 Ethical approval

Ethical approval was obtained from the Human Research and Ethics Committee (HREC), Universiti Sains Malaysia [USM/JEPeM/282.3.(6)] (APPENDIX 1).

3.2 Design of study

This is a case-control study utilizing DNA samples from class III malocclusion subjects and normal healthy individuals as control samples.

3.3 Study population and sample

This research was conducted among Malaysian Malay, which consisted of subjects having class III malocclusion and healthy control samples. Class III subjects were gathered from School of Dental Sciences, Universiti Sains Malaysia (USM). A total of 60 Malay consenting subjects participated in this study. The subjects were distributed into patient and control groups, consisting of 30 patients and 30 controls. Patient group consisted of 10 families with one individual from three consecutive generations. The mean ages for class III malocclusion group were 22.50 (±5.30), 53.50 (±10.06) and 79.20 (±9.35) years old of each generation, respectively. Moreover, healthy controls were chosen with same ethnicity and age between 18 to

29 years old. Random sampling was practiced for both groups based on inclusion and exclusion criteria.

3.3.1 Sample Frame

The sample frame of patient recruitment for this research consisted of patients who gave consent for this research and fulfilled the inclusion and exclusion criteria (APPENDIX 3).

3.3.1.1 Inclusion criteria

3.3.1.1.1 Patient Criteria

i. The selected patients were Malaysian Malay
ii. Age between 14 to 89 years old
iii. Evidence of class III malocclusion based on cephalometric radiographs
iv. Evidence of mandibular prognathism
v. Concave facial profile
vi. At least one generation among three generations was registered in Orthodontic unit, USM for orthodontic treatment
vii. No history of orthodontic treatment before

3.3.1.1.2 Control group criteria

i. Subjects were chosen from undergraduate and postgraduate students from USM with same ethnic group (Malaysian Malay) and age between 18 to 29 years old.

ii. The subjects were healthy controls without any history of class III malocclusion.

iii. Class I normal occlusion.

3.3.1.2 Exclusion criteria

i. Pregnant patients

ii. Patient with cleft lip and palate or other syndromic disease

iii. Inter-racial marriage

3.3.2 Sample size

Based on convenient sampling method ten Malaysian Malay patients with class III malocclusion were selected from the Orthodontic department of USM and three generations of these ten patients were taken based on inclusion - exclusion criterias. Total thirty subjects were selected for the research group and thirty normal healthy individuals were taken as a control group.

3.4 Variables

3.4.1 Dependant variables

The dependent variables involved in class III malocclusion which were

a) Concave profile
b) Cephalometric parameters

3.4.2 Independent variables

The independent variables consisted of

a) Age
b) Gender
c) Ethnic group (Malay)

3.5 Research tools and materials

The equipments and materials used in this research are briefly discussed in (APPENDIX 4).

3.6 Methods

3.6.1 Data collection procedures

Patients with class III malocclusion attending HUSM dental clinics were selected through a dental checkup according to the data collection form (APPENDIX 5). Upon getting convenient patients with class III malocclusion, their three generations were called and ten families were selected (Total 30 subjects) who displayed class III malocclusion in inheritance. Moreover, thirty normal healthy controls from undergraduate and postgraduate students were taken in control group. Cephalometric radiographs were taken only from class III malocclusion subjects and cephalometric landmarks were determined and meassurements were completed using Romexis software (Planmeca, Finland). After analysing the cephalograms, the patients who were diagnosed with skeletal Class III malocclusion were selected by family history, clinical examination, ANB angle and facial profile. The inclusion and exclusion criteria were applied to screen out eligible participants. To minimize selection bias and error, cross-examination of subjects was performed with the help of an experienced and calibrated orthodontist who participated throughout the screening sessions. Participants were informed in details about the research and discussed all their concerns and questions. A written consent was obtained from all participants (One of the parents, either father and/or mother gave written consent for the minor subjects). The recruitment procedures of samples for both patients and control subjects were summarised in Figure 3.1 and Figure 3.2.

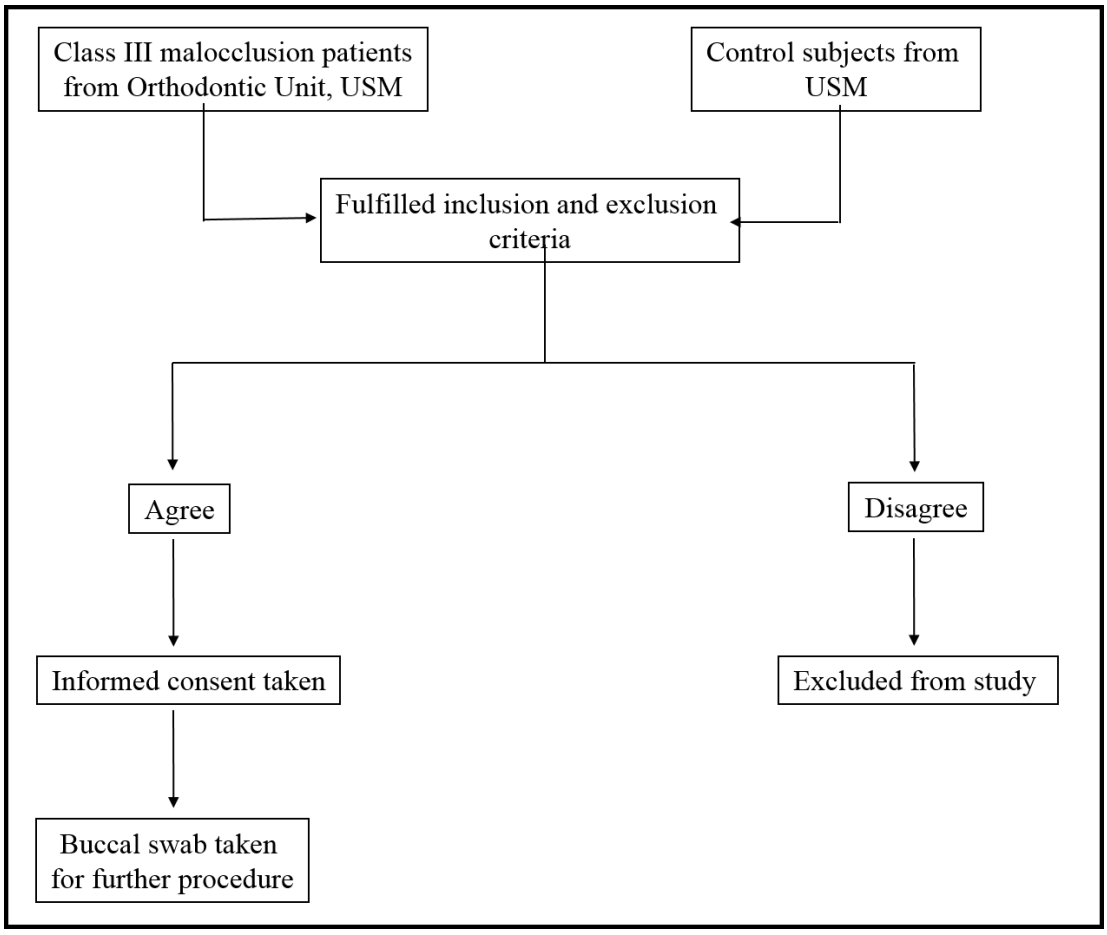

Figure 3. 1 : Flow chart of class III malocclusion patients and control subjects

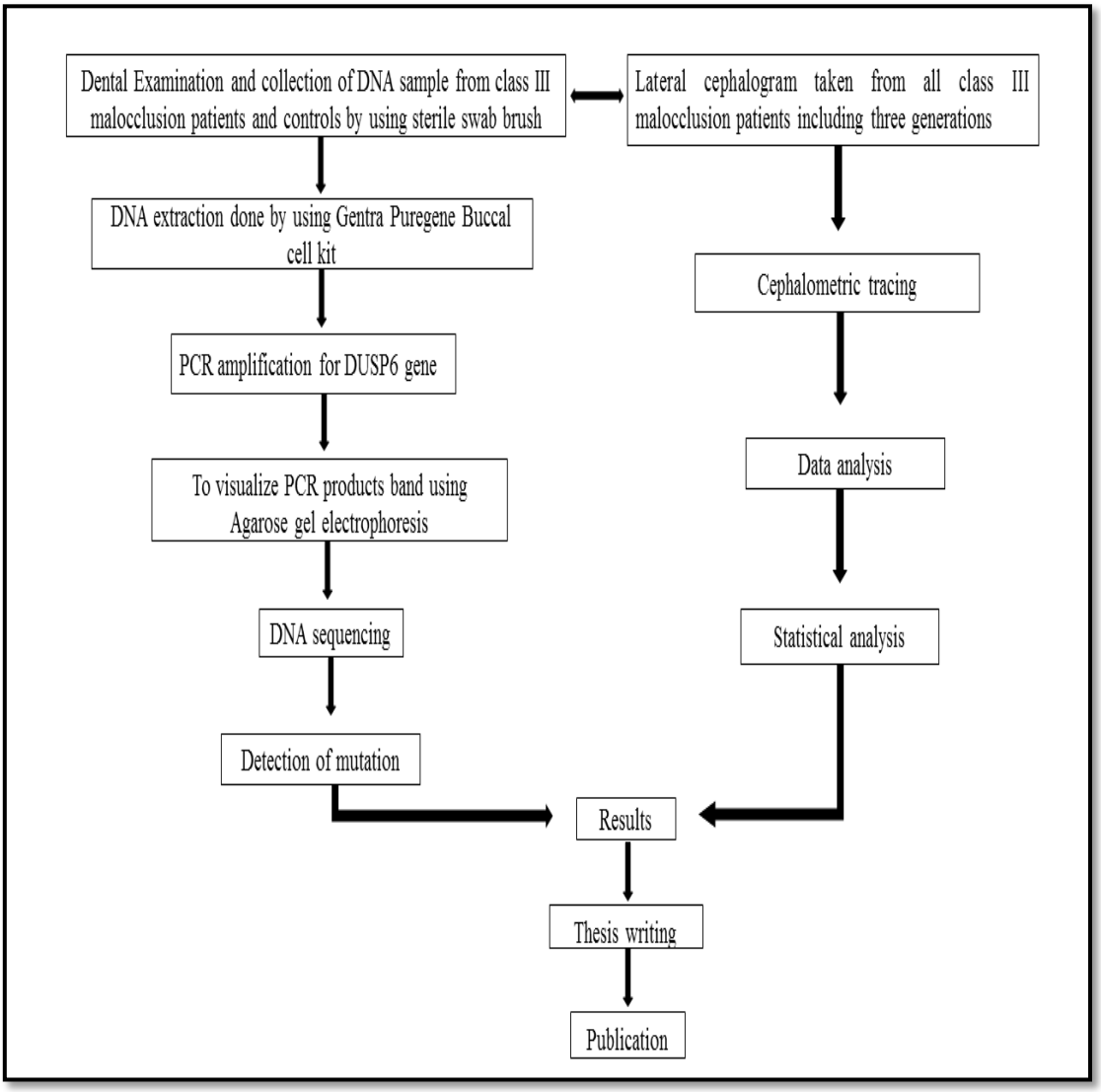

Figure 3. 2 : Flow chart of the study

3.6.2 Preparation of subjects

All the subjects participated (subjects and controls) in this study were instructed not to take any food or drink 30 minutes prior to buccal cell samples collection. Before clinically collecting the swab, all subjects were checked and confirmed that the mouth was empty and clean. The patients and controls were asked to gargle with a plain water/ mouthwash before taking the buccal cell sample.

3.6.3 Buccal cell collection

Buccal cell samples were used in this study. This sampling technique has been used in many genotyping researches as it was considered a non-invasive technique (London *et al.*, 2001; King *et al.*, 2002). The buccal cells were collected by using buccal cell collecting stick. The sticks were sealed with sterile plastic cover. The collection end of the buccal cell stick was consisted of a soft brush. Participants were requested to open the mouth wide. Then, buccal cell sticks were rubbed on the buccal mucosa for 10 times on both left and right inner cheeks (Figure 3.3). Buccal cell brush sticks were taken out safely and dispensed into a 1.5 ml micro-centrifuge tube filled with 300µl cell lysis solution. Handle of the buccal cell sticks were cut using sterile scissors until the soft brush dispensed into the cell lysis solution and the detached head was placed in the tube. DNA samples were then extracted from the buccal cell sticks within one week of collection.

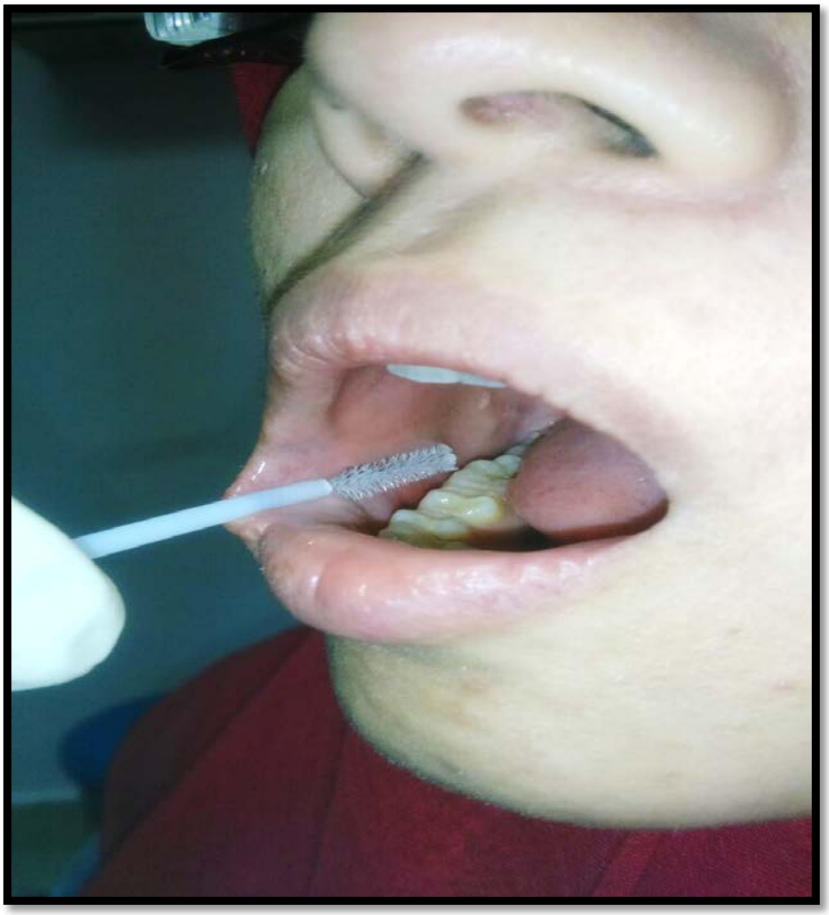

Figure 3. 3 : Collection of buccal cell using sterile buccal cell collecting brush

3.6.4 DNA extraction from buccal cell

The sterile 1.5 ml micro-centrifuge tubes with buccal cell stick were used for DNA extraction by incubating them at 65º C for at least 15 minutes (Figure: 3.4). Collection brushes were removed from the cell lysis solution and scraped on the sides of the tubes to recover as much liquid as possible. RNase A solution (1.5µl) was added into the DNA containing tubes and mixed by inverting the tubes for 25 times. After that the tubes were incubated for 15 minutes at 37º C followed by incubating them for 1 minute over ice to cool down the samples. Protein precipitation solution (100µl) was added and vortexed vigorously for 20 seconds at high speed. Tubes were incubated over ice for 5 minutes and further centrifuged for 3 minutes at 14000×g speed. After centrifuging, tight pellets of precipitated proteins were settled down in the tubes. The supernatant was poured into a new micro-centrifuge tube (1.5 ml) and 300µl isopropanol and 0.5µl Glycogen Solution was added. They were mixed by inverting them for 50 times followed by centrifugation for 5 minutes at 14000×g speed. DNA formed pelletes settled down in the tubes. The supernatant was discarded and tubes were dried by inverting on a clean piece of absorbent paper. Seventy percent (70%) ethanol (300µl) was added into the tubes containing DNA pellete and inverted several times to wash the DNA pellets followed by centrifuging for 1 minute at 14000×g speed. Again, supernatant was discarded and tubes were dried by inverting on a clean piece of absorbent paper. The tubes were dried for 5 minutes. After that, DNA Hydration Solution (100µl) was added followed by vortexing for 5 seconds at the medium speed. DNA containing tube was incubated at 65º C for 1 hour. After 1 hour, the tube was placed on gentle agitation machine for overnight at room temperature (Figure: 3.5). Then tubes were briefly centrifuged for 30 seconds and the DNA sample was preserved at -20º C until further procedures.

Figure 3. 4 : Tubes with the buccal cell brush stick for incubating at 65º C

Figure 3. 5 : Incubation the tubes overnight in agiation machine

3.6.5 Primer Design

DUSP6 gene has 3 exons and primers were designed manually for these 3 exons using www.ncbi.nlm.nih.gov. At first, the whole nucleotide sequence of DUSP6 gene was obtained from national centre for biotechnology information (NCBI). All 3 exons were marked and 21-23 nucleotides were chosen from intial part of each exon reffered to as forward primer and 21-23 nucleotides were chosen from end part of each exon reffered to as reverse primer. The specificity of these primers to the DUSP6 gene were confirmed using the 'Basic Local Alignment Search Tool (BLAST)' program available online at http://www.ncbi.nlm.nih.gov/blast (Table 3.1).

Table 3.1: List of designed primers used in this study

Exon	Primers	Sequence	Size (bp)
1	Forward	5´-TTGAGAGCTAAGATGTGCCAA-3´	889
	Reverse	5´-GTAAGGCGAGGCGGAATTAAA-3´	
2	Forward	5´-TTAAACTCTATGAATGGCTAGG-3´	722
	Reverse	5´-AGGATGCTTGTGGTGTTTCTT-3´	
3	Forward	5´-TATCTATACAGCATGTCCTGTT-3´	667
	Reverse	5´-GATACATTTTCTGCTGCTTGTA-3´	

bp, base pair.

3.6.6 Polymerase Chain Reaction (PCR) preparation

All the surfaces and pipettes including hand gloves were cleaned with 70% alcohol to avoid DNA contamination. The reactions were always run on ice in container box.

Three sets of primers were designed to amplify DUSP6 gene at exon 1, 2 and 3. The sequences of each primer were shown in Table 3.1.

After preparation, 0.2 ml micro centrifuged tubes were marked for PCR. PCR was performed in a total volume of 50µl containing-25µl master mix (reaction buffer, Taq 0.06U/µl DNA Polymerase, 3mM $MgCl_2$ and 400µM of each dNTPs), 4µl forward and reverse primers, 4µl DNA samples and 13µl distilled water for Exon 1. For exon 2, 25µl master mix, 3µl forward and reverse primers, 2µl DNA samples and 17µl distilled water. For exon 3, 25µl master mix, 4µl forward and reverse primers, 3µl DNA samples and 14µl distilled water (Table 3.2). Then, PCR sample containing tubes were centrifuged for 15 seconds. According to the specific reaction as mentioned above, each DUSP6 gene exons were amplified by PCR master cycler (Eppendorf, Germany) (Figure 3.6). PCR was carried out with an initial 5 minutes denaturation at 95ºC, followed by 30 cycles of 95ºC for 30 seconds, 56ºC for 40 seconds, 72ºC for 1 minute and a final extension period at 10 minutes following 30 cycles (Figure 3.7). After reaction end, the PCR product containing tubes were taken for gel electrophoresis.

Table 3.2: List of components in PCR product for DUSP6 gene amplification

Materials	**Exon 1**	**Exon 2**	**Exon 3**
Master Mix	25 µl	25 µl	25 µl
Forward Primer	4 µl	3 µl	4µl
Reverse Primer	4 µl	3 µl	4 µl
DNA sample	4 µl	2µl	3 µl
Distilled Water	13 µl	17 µl	14µl
Total	50 µl		

PCR, Polymerase Chain Reaction; DUSP6, Dual Specificity Protein Phosphatases 6; µl, micro litre

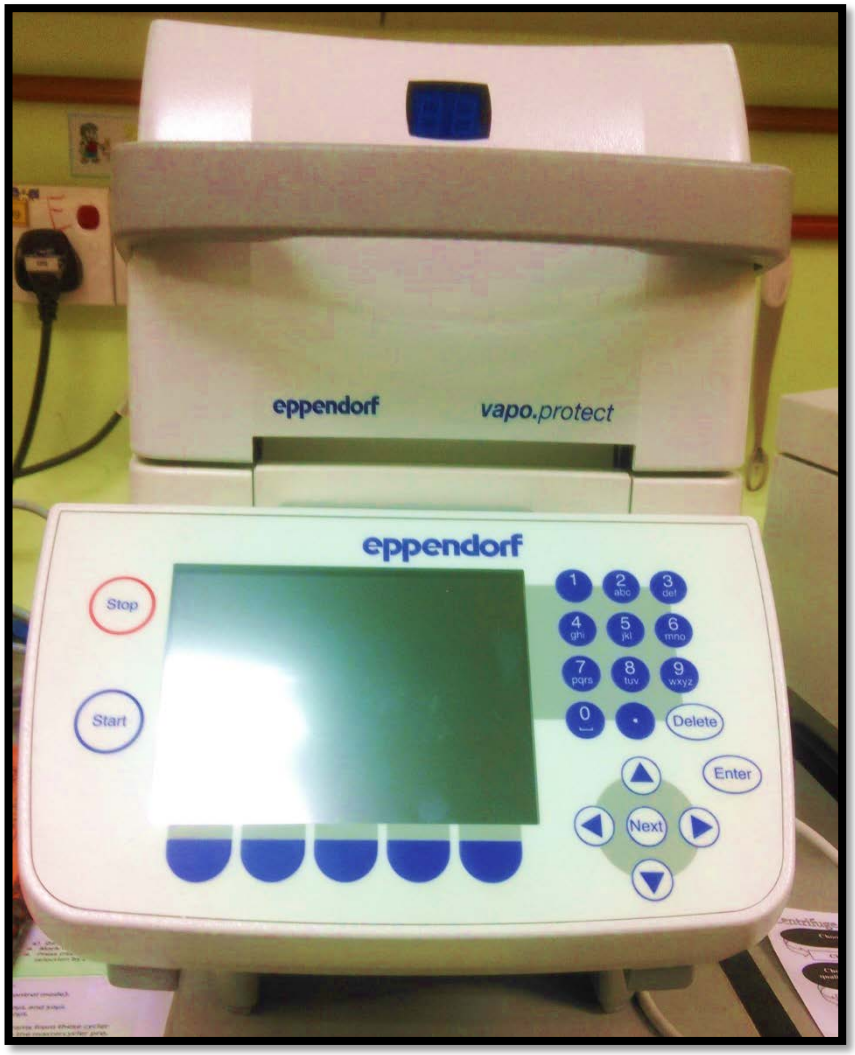

Figure 3. 6 : PCR master cycler (Eppendorf, Germany)

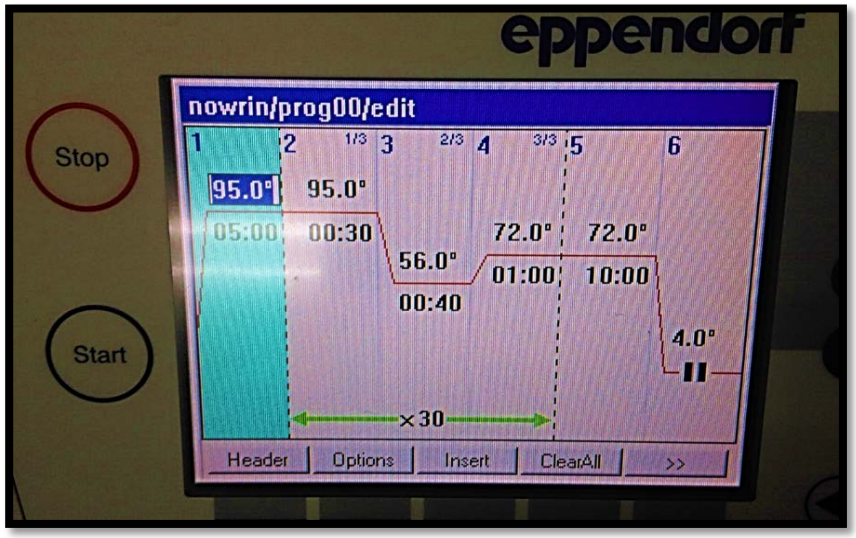

Figure 3. 7 : PCR cycles

3.6.7 Agarose gel Electrophoresis for DUSP6 gene

The presence of DNA in the PCR products was checked by means of 1% agarose gel electrophoresis.

3.6.7.1 Gel preparation

For preparing 1% Agarose gel, 0.25 gm of Agarose powder were measured by using A&D GR-200 lab balance (A & D company, Japan) and mixed with 25ml of 1x TAE buffer in a beaker. Sybr safe (Invitrogen, USA) (2.5 µl) were also taken into the beaker and mixed together then heated in microwave till the agarose powder had fully dissolved. The gel was poured into the gel tray and comb was fixed on it. The tray was kept for one hour to solidify the gel (Figure 3.8)

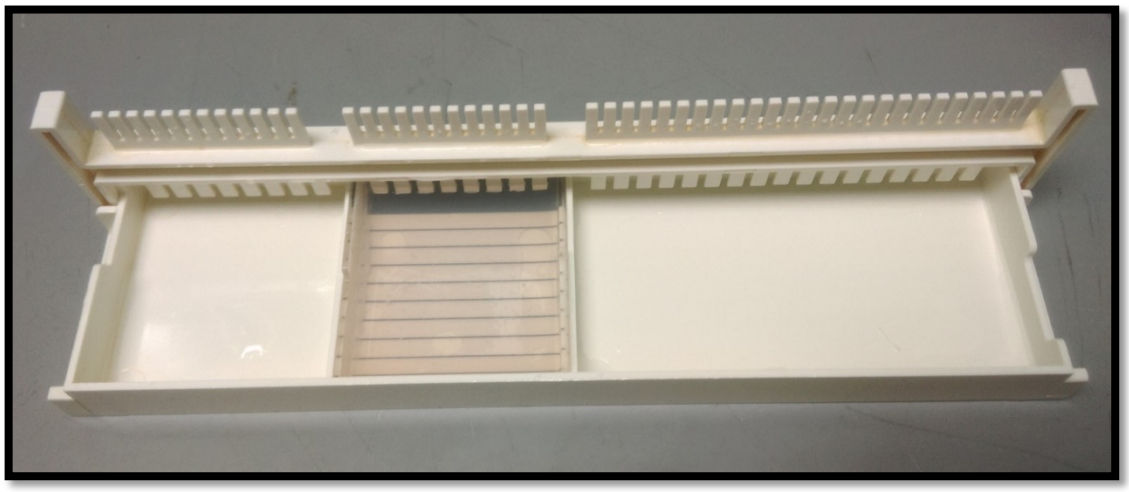

Figure 3. 8 : Making of Agarose gel

3.6.7.2 Electrophoresis

The Agarose gel was dipped into the 10x TAE buffer. Each wells were loaded carefully with mixure of 5µl of PCR product and 2µl of 6x loading dye. And at least one well was loaded with 3µl of DNA ladder (100 bp). The mixture loaded into the wells after mixing thoroughly in a piece of parafilm (Pechiney, USA). During loading the wells, sterile tips were used. Electrophoresis was ran at 70 volt for 45 minutes.

3.6.7.3 Product Visualization

Image analyzer of UV- transilluminator was used to check the presence or absence and the bp size of specific bands of the PCR products (Figure 3.9). Upon getting the desired size of band PCR products were sent to the First Base Laboratory (Selangor, Malaysia) for purification and sequencing to confirm the results.

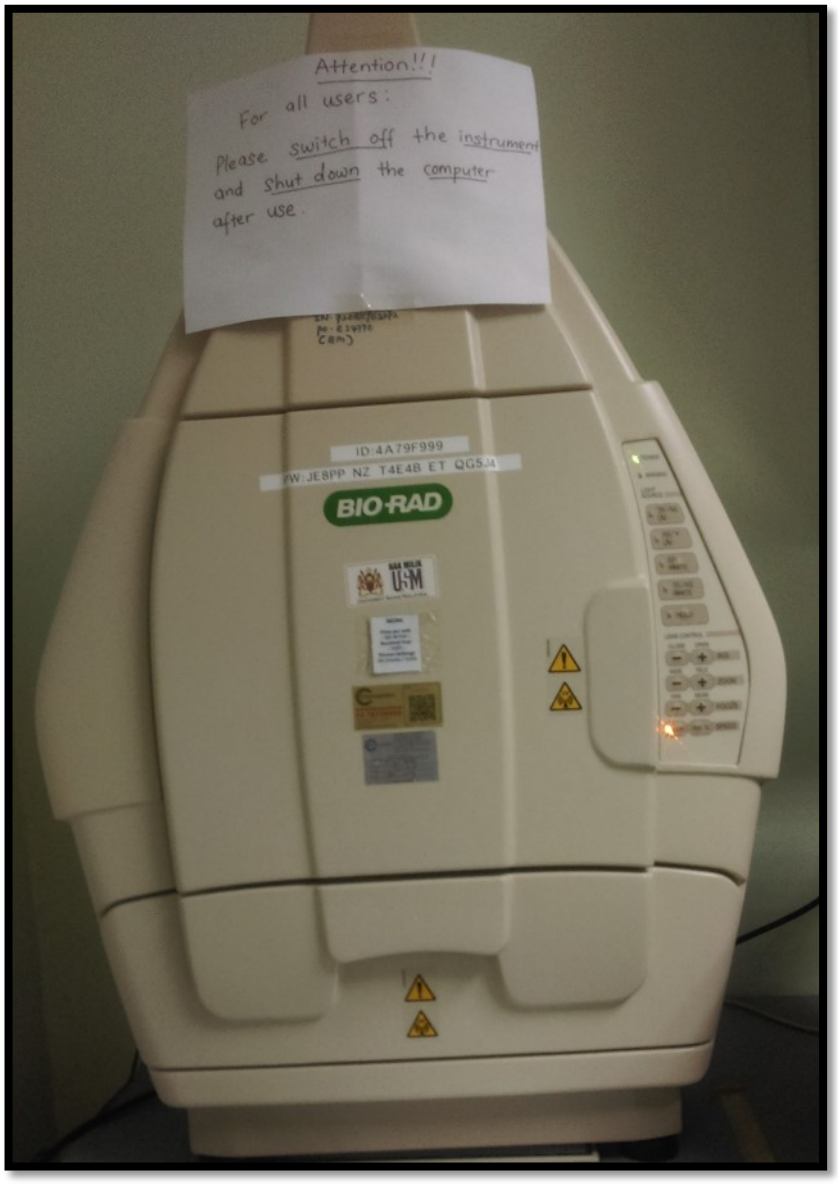

Figure 3. 9: UV-transilluminator machine (Bio-Rad, USA)

3.6.8 Purification and sequencing

PCR product purification and exon sequencing was carried out in the First Base Laboratory (Selangor, Malaysia). However, a brief discussion of this procedure given bellow:

QiaQuick PCR cleanup columns from Qiagen were used to clean up the PCR products. Then PCR reactions were ran on an agarose gel to assure purity. Then a sequencing reaction was performed on pure PCR products which was similar to a PCR reaction. Only one primer was used for a sequencing reaction (PCR reactions use two primers, forward and reverse, resulting in a double stranded amplicon). After PCR end, the tubes were placed in the sequence analyser machine. Laser detection of flurochromosomes and computational sequence analysis were displayed on the monitor. Finally, those sequenced data was sent by electronic mail to the researcher.

3.7 Cephalometric analysis

Initial lateral cephalograms of 10 families with class III malocclusion including their 3 consecutive generations were obtained to check for the skeletal morphology. For lateral cephalogram, Gendex Orthoralix 9200, Italy X-ray machine was used with the exposure of 70kV 6mA 0.80 seconds. Radiographs were obtained at a radiograph unit in USM. The patients were instructed to stand in upright position, teeth were in occlusion, infraorbital meatus line were parallel with the floor, lips were in relaxed position and ear rods were placed in external auditory meatus while the radiographs were taken. Lateral cephalograms were traced using Romexis^{TM} software 2.3.1.R (Planmeca, Finland) and cephalometric reference points were determined (Table

3.3). Therefore, all structures were scaled to an equivalent size, tabulated and compared (APPENDIX 5 & 6).

3.7.1 Glossary of measurements

Different linear and angular measurements were performed to check the skeletal relationships of both groups (Table 3.4).

Table 3.3: Cephalometric landmarks used in the current study

Variavle	Description
S	Sella (center of sella turcica)
N	Nasion (frontonasal suture at its most superior point)
Point A	Deepest point at concavity on maxillary alveolar bone
Point B	Deepest point at concavity on mandibular alveolar bone
Functional occclusal plane	Line passing through the occlusion of molars and premolars
M	Midpoint of premaxilla
C	Center of the condyle
G	Center of mandibular symphysis
Co	Condylion (superior-most point on mandibular condyle)
Ar	Articulare (intersection of dorsal contour of condylar head and posterior cranial base)
Go	Gonion (mid-point at angle of mandible)
Me	Menton (inferior-most point on mandibular symphysis)
Gn	Gnathion (most anteroinferior point on mandibular symphysis)
P	Pogonion (anterior-most point on mandibular symphysis)
Id	Infradentale (most anterosuperior point on mandibular alveolus)

Table 3.4: Linear and Angular cephalometric measurements used in current study

Variables	Definition
SNA	Assessment of the anteroposterior position of the maxilla with regards to the cranial base which formed by the intersection of lines S-N and N-A planes
SNB	For assessment of the anteroposterior position of the mandible with regards to the cranial base which formed by the intersection of lines S-N and N-B planes
ANB	SNA – SNB (The difference between angles SNA and SNB, as providing an evaluation of the anteroposterior relationship between the maxillary and mandibular apical bases)
Wits appraisal	horizontal distance between two lines (AO and BO) drawn perpendicularly from point A and point B to functional occlusal plane)
W angle	A perpendicular line is drawn from point M to S-G line. W angle is angle between this perpendicular line and M-G line.
Beta angle	C-B line joins center of condyle and point B. A perpendicular line is drawn from point A to C-B line angle. Beta angle is angle between this perpendicular line and C-B line
YEN angle	angle between M-G line and S-M line
Length of mandible (Co-Gn) (mm)	A line is measured from the condylion to anatomic gnathion.
Gn - B	Supramentale length. (A line is measured from the gnathion to point B)
Co - Go	A line is measured from the condylion to Gonion
Ar - Go	A line is measured from the Articulare to Gonion
Id - M	A line is measured from the Infradentale to Midpoint of premaxilla
Id - Gn - Go	An angle between Infradentale, Gnathion and Gonion
Id - M - Go	An angle between Infradentale, Midpoint of premaxill and Gonion
Id - Gn - Co	An angle between Infradentale, Gnathion and condylion
SN - MP	Angle between mandibular plane to S-N plane to determine the divergence of mandible to cranial base.
FA (Facial Angle)	The intersection of the Frankfort horizontal and the facial plane (N-Pog) to measure the degree of retrusion or protrusion of the lower jaw.
GA (Gonial angle)	Angle between Ar-Go-Me to determine the divergence of mandibular base

3.8 Control of Error

Dalhberg's formula (Springate, 2012) was applied to analyse the error in measurements:

$$ME = \sqrt{\sum} (x1-x2)2 / 2n$$

Here, x1 determined first measurement, x2 determined second measurement and n is the number of repeated records (Houston, 1983). Twenty percent (20%) of the radiographs from each generation were traced and digitized 2nd time after one month interval.

3.9 Statistical analysis

The data were analyzed statistically using IBM SPSS Statistics Version 22.0 (Chicago, USA) with confidence level set at 95% ($P < 0.05$) to test for significance. Student t-test was conducted for each variable to test for significant changes among mutation group and non-mutation group of class III malocclusion. Analysis of variance (ANOVA) was applied to evaluate the difference in the values for three generations of class III malocclusion.

CHAPTER 4

RESULTS

4.1 PCR amplification results

Amplification of exon 1, 2 and 3 of DUSP6 gene was successfully carried out with the specific primers designed for each exon. After optimization of PCR products, amplification of each exon of DUSP6 gene was performed in a total of 30 subjects with class III malocclusion and 30 controls. PCR products were visualized in 1% agarose gel (Figure 4.1, 4.2 and 4.3). Size of exon 1, exon 2 and exon 3 are 889 bp, 667 bp and 722 bp, respectively.

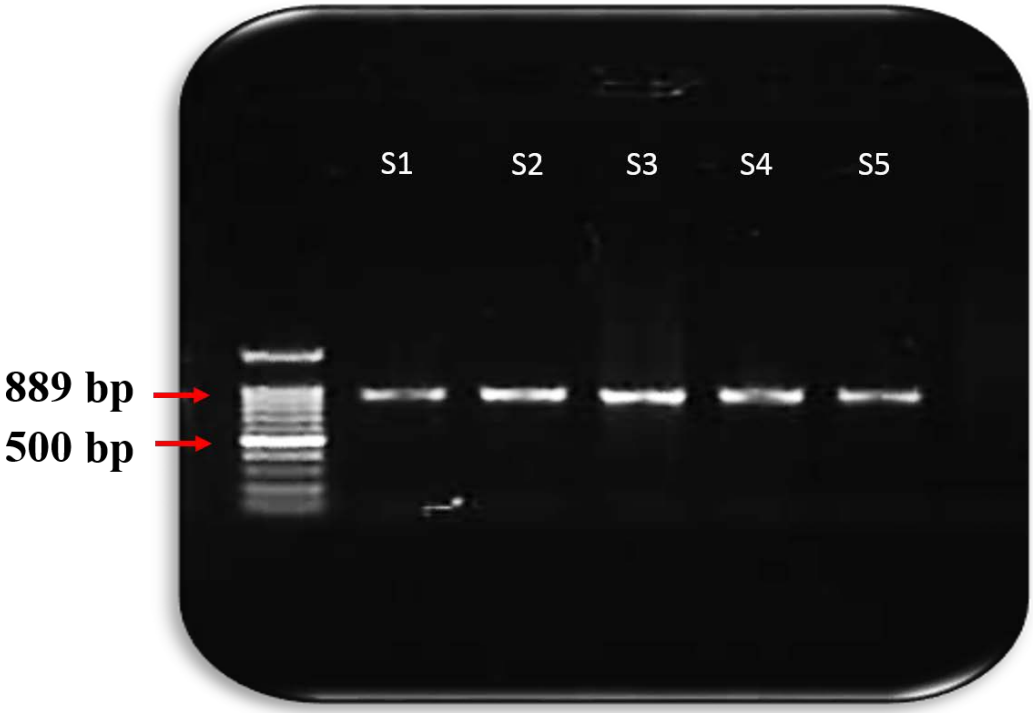

Figure 4. 1 : Some samples of the gel electrophoresis picture of the DUSP6 genes PCR product of exon 1

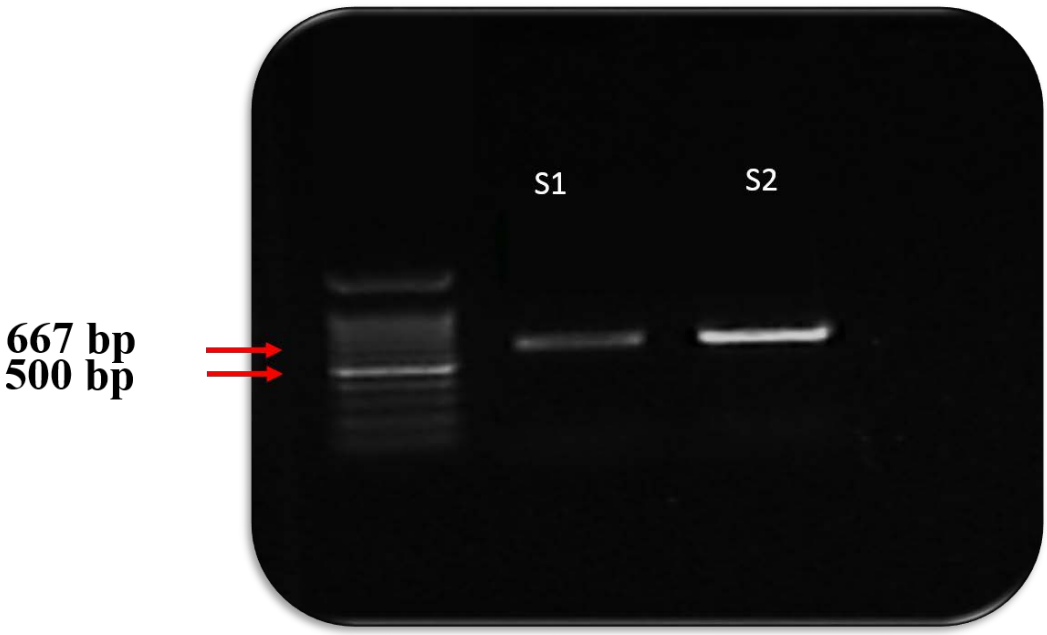

Figure 4. 2 : Some samples of the gel electrophoresis picture of the DUSP6 genes PCR product of exon 2

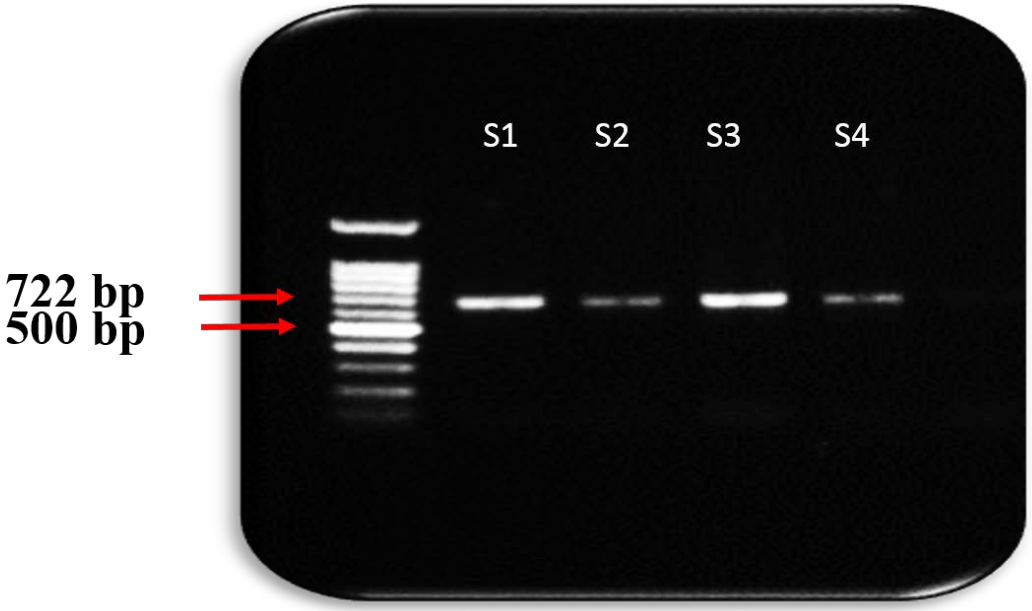

Figure 4. 3 : Some samples of the gel electrophoresis picture of the DUSP6 genes PCR product of exon 3

4.2 Sequencing results of DUSP6 gene exons screening

The results of sequencing were confirmed with the reference sequence using BLAST (Basic Local Alignment Search Tool) of the NCBI (National Centre for Biotechnology Information).

4.2.1 Class III malocclusion Patients

A missense mutation was found in exon 3 with DUSP6 gene in three members of a single Malaysian Malay family. Mutation was detected in amino acid position (Thr 365 Ile) where amino acid Threonine change to Isoleucine in nucleotide position c.1094 C> T in exon 3 (Table 4.1, Figure 4.4). The rest of the families with class III malocclusion showed no mutation in exon 3. There was no mutation found in exon 1 and exon 2 among all subjects with class III malocclusion.

4.2.2 Control group

There was no mutation found in DUSP6 gene among the control group (Figure 4.5).

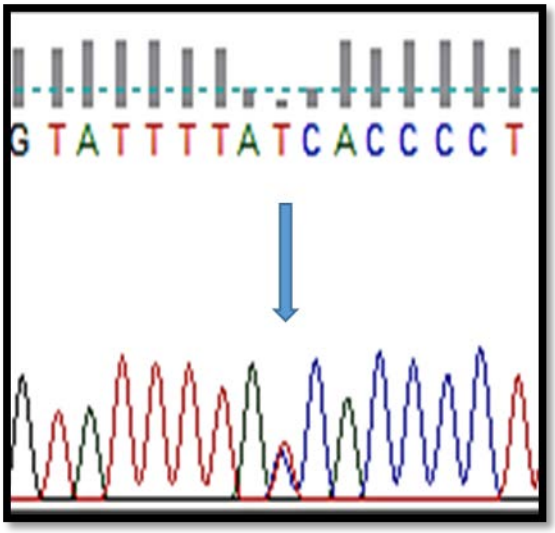

Figure 4. 4: Partial DNA sequences of exon 3 of DUSP6 gene (Test subjects)

Sequence analysis of missense mutation in DUSP6 gene for three family members showing same mutation at amino acid position (Thr 365 Ile) where amino acid Threonine change to Isoleucine in nucleotide position c.1094 C> T

Table 4.1: DUSP6 gene mutations in our patients with class III malocclusion

Family member no.(n)	**Exon location**	**Nucleotide change**	**Mutation type**	**Protein change**
3	Exon 3	c.1904 C>T	Missense	Threonine 365 histidine

C>T, cytocine chang to thymine.

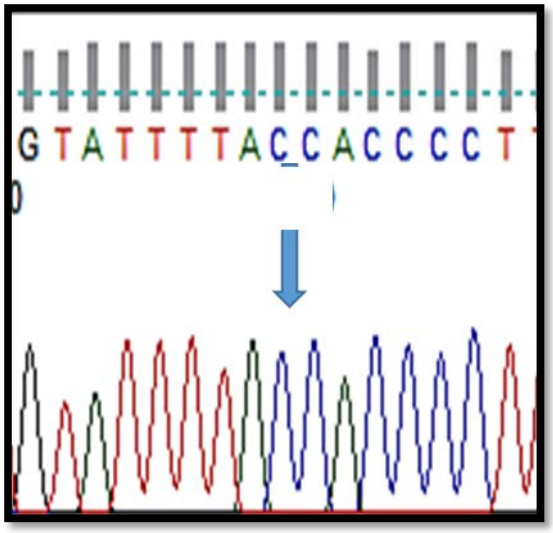

Figure 4. 5: Partial DNA sequences of exon 3 of DUSP6 gene (Controls)

Showing no double pick in nucleotide position 1094

4.3 Cephalometric analysis result

4.3.1 Control of Error

The reproducibility of the measurements were evaluated by relating the data taken at one-month interval. To test the level of miscalculation involved in the current study, lateral cephalometric radiographs from each group were randomly selected and measurements were repeated at one-month interval. Dahlberg's formula have been applied to control the method-error, which did not surpass 0.2 mm for the linear variables, 0.66 degree for the angular variables. The combined error for any of the variables was small and was considered to be within satisfactory limits (Houston, 1983).

4.3.2 Craniofacial morphology changes between mutation and non-mutation group

Mutation group comprised of 3 subjects with class III malocclusion and non-mutation group comprised 27 subjects with class III malocclusion. Table 4.2 shows there are significant differences in Co-Gn-B and SN-MP variables in mutation group. The other variables show no significant differences between the groups. Moreover, almost all variables show larger value in mutation group in comparison to non-muation group. Figure 4.6 is showing representative cephalometric tracing for both mutation and non-mutation group.

Table 4.2: Comparison of mutation and non-mutation group for craniofacial morphology[a]

Variable	Mutation (n=3)		Non-Mutation (n=27)		95% CI		P value
	Mean	SD	Mean	SD	Lower	Upper	
SNA	80.667	4.041	81.248	3.139	-4.586	3.423	0.768
SNB	83.333	3.512	85.313	3.423	-6.254	2.295	0.351
ANB	-2.667	4.509	-4.118	2.140	-1.527	4.428	0.327
Wits	-9.222	2.546	-8.603	2.123	-3.306	2.069	0.641
Beta	45.667	7.638	44.749	4.179	-4.711	6.546	0.741
W	62.000	7.211	59.862	3.505	-2.709	6.986	0.374
Yen	128.000	9.644	134.271	4.575	-12.638	0.095	0.053
Co-Gn	124.667	5.859	116.812	6.334	-0.001	15.709	0.050
Gn-B	17.333	4.041	16.952	2.199	-2.584	3.347	0.794
Co-Go	60.333	3.005	59.607	4.870	-5.212	6.664	0.804
Ar-Go	48.500	6.265	46.685	3.398	-2.770	6.399	0.424
Id-M	33.869	2.868	30.246	3.196	-0.333	7.580	0.071
Co-Gn-B	53.833	6.007	46.880	4.381	1.323	12.584	0.017*
Co-Go-Gn	125.000	5.000	123.832	5.164	-5.256	7.591	0.712
Id-Gn-Go	75.035	3.053	78.446	5.824	-10.481	3.659	0.331
Id-M-Go	79.953	0.931	80.960	5.390	-7.490	5.476	0.753
Id-Gn-Co	53.350	2.090	54.127	5.068	-6.905	5.351	0.797
SN-MDP	36.333	1.528	28.280	2.838	4.606	11.500	0.000*
FA	88.667	2.517	91.221	3.395	-6.717	1.609	0.219
GA	127.833	5.752	128.155	4.154	-5.667	5.024	0.903

[a] Independent t test; *Significant (P <0.05); SD, Standard Deviation; CI, Confidence Interval

4.3.3 Craniofacial morphology changes among three generations

Table 4.3 shows that there are no significant differences among all craniofacial variables except Yen angle. Moreover, table 4.4 shows the differences in the values for 1st generation *vs* 2nd generation, 2nd generation *vs* 3rd generation, and 1st generation *vs* 3rd generation. There are no significant differences among all craniofacial variables except Yen angle in 1st generation *vs* 2nd generation.

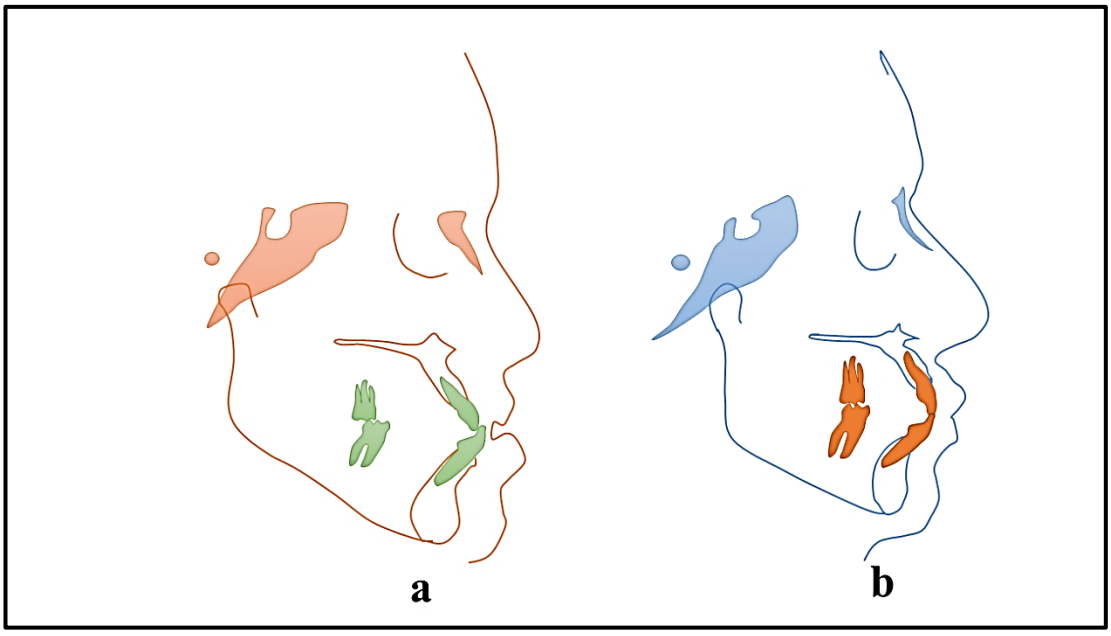

Figure 4. 6: Representative tracing for mutation and non-mutation group

Table 4.3: Linear and angular cephalometric analysis measurement among three generations[a]

Variables	Generations	Mean	95% Confidence Interval		P value
			Lower Bound	Upper Bound	
SNA	1st	80.126	78.136	82.116	0.294
	2nd	81.083	78.648	83.518	
	3rd	82.361	80.0898	84.632	
SNB	1st	85.021	82.8009	87.241	0.503
	2nd	84.247	81.3297	87.165	
	3rd	86.076	83.858	88.294	
ANB	1st	-5.095	-6.918	-3.272	0.158
	2nd	-3.079	-4.589	-1.569	
	3rd	-3.743	-5.356	-2.131	
Wits	1st	-9.231	-11.353	-7.109	0.509
	2nd	-8.099	-9.7084	-6.489	
	3rd	-8.665	-8.665	-8.665	
Beta	1st	46.226	42.385	50.067	0.488
	2nd	43.935	40.294	47.577	
	3rd	44.361	42.666	46.057	
W	1st	60.779	57.349	64.209	0.743
	2nd	60.045	57.388	62.704	
	3rd	59.402	57.078	61.727	
Yen	1st	137.223	134.669	139.776	0.020*
	2nd	130.895	127.953	133.838	
	3rd	132.814	128.297	137.332	
CoGn	1st	120.79	115.113	126.467	0.168
	2nd	115.494	110.122	120.867	
	3rd	116.509	114.892	118.125	
GnB	1st	17.290	15.083	19.497	0.890
	2nd	16.816	15.081	18.551	
	3rd	16.864	15.828	17.899	
CoGo	1st	61.600	57.637	65.563	0.264
	2nd	59.192	55.921	62.463	
	3rd	58.248	55.719	60.777	
ArGo	1st	48.290	44.437	52.143	0.331
	2nd	46.220	44.820	47.620	
	3rd	46.090	44.219	47.961	
IdM	1st	31.620	28.566	34.673	0.478
	2nd	29.828	27.319	32.336	
	3rd	30.376	29.161	31.591	

CoGnB	1st	47.042	42.929	51.154	0.804
	2nd	48.431	45.695	51.168	
	3rd	47.252	43.396	51.108	
CoGoGn	1st	124.888	120.031	129.745	0.575
	2nd	122.563	119.024	126.101	
	3rd	124.396	122.237	126.556	
IdGnGo	1st	79.364	74.455	84.273	0.446
	2nd	78.707	75.169	82.244	
	3rd	76.245	72.597	79.893	
IdMGo	1st	81.431	77.0201	85.841	0.349
	2nd	82.188	79.072	85.304	
	3rd	78.959	75.672	82.247	
IdGnCo	1st	55.257	51.547	58.967	0.487
	2nd	54.259	50.747	57.772	
	3rd	52.630	49.395	55.865	
SNMDP	1st	29.718	26.731	32.705	0.788
	2nd	28.972	26.879	31.064	
	3rd	28.567	25.662	31.473	
FA	1st	90.668	87.986	93.349	0.208
	2nd	89.794	88.191	91.396	
	3rd	92.434	89.799	95.069	
GA	1st	127.696	123.261	132.130	0.540
	2nd	127.331	124.786	129.876	
	3rd	129.342	127.983	130.700	

[a] ANOVA; [*]Significant ($P < 0.05$)

Table 4.4: Linear and angular cephalometric variable changes between generation to generations[a]

Variables	Generations	Mean Difference	95% Confidence Interval		P value
			Lower Bound	Upper Bound	
	1st vs 2nd	-0.957	-4.531	2.617	1.000
SNA	2nd vs 3rd	-1.278	-4.852	2.296	1.000
	3rd vs 1st	2.235	-1.339	5.809	0.366
	1st vs 2nd	0.774	-3.174	4.721	1.000
SNB	2nd vs 3rd	-1.828	-5.776	2.119	0.742
	3rd vs 1st	1.055	-2.893	5.003	1.000
	1st vs 2nd	-2.016	-4.655	0.622	0.185
ANB	2nd vs 3rd	0.665	-1.974	3.303	1.000
	3rd vs 1st	1.352	-1.287	3.990	0.606
	1st vs 2nd	-1.132	-3.586	1.322	0.748
Wits	2nd vs 3rd	0.566	-1.888	3.020	1.000
	3rd vs 1st	0.566	-1.888	3.020	1.000
	1st vs 2nd	2.291	-2.830	7.411	0.791
Beta angle	2nd vs 3rd	-0.426	-5.547	4.694	1.000
	3rd vs 1st	-1.864	-6.985	3.256	1.000
	1st vs 2nd	0.733	-3.802	5.269	1.000
W angle	2nd vs 3rd	0.643	-3.892	5.179	1.000
	3rd vs 1st	-1.377	-5.912	3.159	1.000
	1st vs 2nd	6.328	0.832	11.824	0.020*
Yen angle	2nd vs 3rd	-1.919	-7.415	3.577	1.000
	3rd vs 1st	-4.408	-9.904	1.088	0.151
	1st vs 2nd	5.296	-2.057	12.649	0.231
Co-Gn	2nd vs 3rd	-1.014	-8.367	6.339	1.000
	3rd vs 1st	-4.281	-11.635	3.072	0.446
	1st vs 2nd	0.474	-2.283	3.231	1.000
Gn-B	2nd vs 3rd	-0.048	-2.805	2.709	1.000
	3rd vs 1st	-0.426	-3.183	2.331	1.000
	1st vs 2nd	2.408	-2.869	7.685	0.763
Co-Go	2nd vs 3rd	0.944	-4.333	6.221	1.000
	3rd vs 1st	-3.352	-8.629	1.925	0.350
	1st vs 2nd	2.070	-2.082	6.222	0.642
Ar-Go	2nd vs 3rd	0.130	-4.022	4.282	1.000
	3rd vs 1st	-2.200	-6.352	1.952	0.562
	1st vs 2nd	1.793	-2.016	5.602	0.720
Id-M	2nd vs 3rd	-0.549	-4.358	3.260	1.000
	3rd vs 1st	-1.244	-5.053	2.565	1.000
	1st vs 2nd	-1.390	-7.163	4.384	1.000
Co-Gn-B	2nd vs 3rd	1.179	-4.594	6.953	1.000
	3rd vs 1st	0.210	-5.563	5.983	1.000

Co-Go-Gn	1st vs 2nd	2.325	-3.558	8.208	0.966
	2nd vs 3rd	-1.834	-7.717	4.049	1.000
	3rd vs 1st	-0.492	-6.374	5.391	1.000
Id-Gn-Go	1st vs 2nd	0.657	-5.852	7.167	1.000
	2nd vs 3rd	2.461	-4.048	8.971	1.000
	3rd vs 1st	-3.119	-9.628	3.390	0.696
Id-M-Go	1st vs 2nd	-0.757	-6.581	5.068	1.000
	2nd vs 3rd	3.228	-2.596	9.053	0.506
	3rd vs 1st	-2.471	-8.296	3.353	0.865
Id-Gn-Co	1st vs 2nd	0.997	-4.574	6.568	1.000
	2nd vs 3rd	1.630	-3.942	7.201	1.000
	3rd vs 1st	-2.627	-8.198	2.944	0.718
SN-MDP	1st vs 2nd	0.746	-3.549	5.042	1.000
	2nd vs 3rd	0.404	-3.891	4.700	1.000
	3rd vs 1st	-1.151	-5.446	3.145	1.000
FA	1st vs 2nd	0.874	-2.891	4.640	1.000
	2nd vs 3rd	-2.641	-6.406	1.125	0.254
	3rd vs 1st	1.766	-1.999	5.531	0.725
AC	1st vs 2nd	-2.561	-11.168	6.047	1.000
	2nd vs 3rd	1.197	-7.410	9.805	1.000
	3rd vs 1st	1.363	-7.244	9.971	1.000
GA	1st vs 2nd	0.365	-4.509	5.239	1.000
	2nd vs 3rd	-2.011	-6.885	2.863	0.905
	3rd vs 1st	1.646	-3.228	6.519	1.000

[a] Post Hoc Bonferroni; [*]Significant (P <0.05)

CHAPTER 5

DISCUSSION

Now-a-days, genetic information and technology are playing the most important role in prediction, diagnosis and treatment planning for class III malocclusion. Even though it is frequently mentioned that the skeletal class III malocclusion is an inherited trait but there is a lack of association studies that found concerning genetic contribution. To reveal the genetic influences of dentofacial phenotype, the phenotypic interpretation within and across populations requires tireless efforts.

5.1 Demographic and clinical data

In this study, the genetic analyses were performed for DUSP6 gene in 10 families with their 3 consecutive generations having class III malocclusion in Malaysian Malay subjects and compared with the 30 healthy controls. A missense mutation was determined in exon 3 of DUSP6 gene. This missense mutation c.1094C>T (p. Thr 365 Ile) consequentially results in the replacement of amino acid Threonine to Isoleucine at position number 365. Three members of one family displayed the same mutation at amino acid position (Thr 365 Ile) and presented a concave facial profile, which represents class III malocclusion that was further proved by cephalometric radiographs. Upon analyzing this family, same mutation was found from the Ia, IIa and IIIc subjects (Figure 5.1).

No genetic study has been done yet for assessing class III malocclusion in Malaysian Malay ethnic group. This study represents the first case from Malaysia.

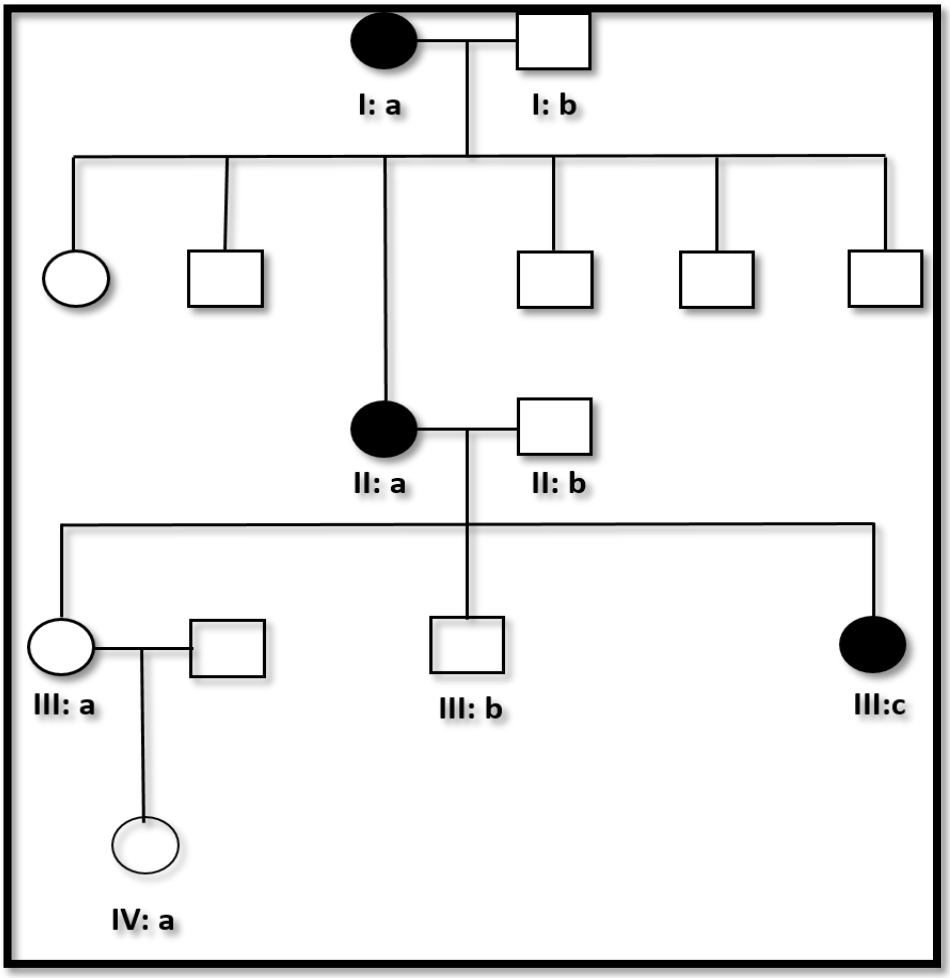

Figure 5.1: Pedigree of a Malaysian family with Class III malocclusion

Symbols: male- squares; females- circles; affected- filled symbol; unaffected- empty symbol. I:a, I:b is 3rd generation of patient, II:a, II:b is 2nd generation of patient, III:a, III:b is sibling of patient, III:c is patient and IV:a is niece of patient

5.2 Genetic mutation with class III malocclusion

Class III malocclusion is one of the most challengeable dentofacial deformities for orthodontists. Many researches were conducted to understand the mechanisms underlying the growth malformation on this phenotype. Moreover, many studies concluded strong genetic influences in the formation of class III malocclusion (Xue *et al.*, 2010a; Nikopensius *et al.*, 2013; Perillo *et al.*, 2015).

Deleterious functional effects and underlying mechanisms of diseases caused by genetic variants have not been yet completely understood. Experimental studies on genetic variants are difficult to accomplish in a reasonable timescale due to availability of vast data. To improve knowledge of the relationships between genetic and phenotypic variations, it is important to clarify the molecular basis of hereditary diseases initiated by mutations.

It is expected that the definition of causes responsible for genetic diseases will unravel important clues for the normal function of the genes and the pathobiology of their allied diseases. Development in genetics have only exaggerated the exploration for mutations, especially in complex traits. To reveal the underlying mechanisms, finding the genetic involvement act as a key role. This includes studying the mutants, mapping the mutations, cloning the genes to determine the biochemical processes that are altered in the mutants. Accidental changes of DNA code is termed as mutation. It can lead to missing or malformed protein which could develop into a disease (Cargill *et al.*, 1999).

200,000 nonsynonymous coding variants assumed to be present in human genome (Cargill *et al.*, 1999). Missense mutations occur in the coding regions of the genes

and they lead to a change in the amino acid sequence of protein products. Moreover, these changes lead to dysfunction of the proteins and may result in inherited human diseases (Hamosh *et al.*, 2005).

Genetic variants on gene are claimed to exhibit possible pathogenesis in class III maolocclusion (Nikopensius *et al.*, 2013; Perillo *et al.*, 2015). Different studies used different methods like linkage analyses, whole exome sequencing, polymorphism study or mutational studies to link the genetic association with class III malocclusion (Frazier-Bowers *et al.*, 2009; Li *et al.*, 2011; Xue *et al.*, 2010a; Yamaguchi *et al.*, 2005). Current study has hypothesised detection of mutations in class III malocclusion for the prediction of developing the phenotype with the DUSP6 gene. Until now, only one mutational study has been done in relation with class III malocclusion associated with DUSP6 gene. Whole exome sequencing showed significant association of DUSP6 gene in class III malocclusion in an Estonian family and showed a rare missense mutation on c.545C>T in Exon 2 (p.Ser182Phe) associated with class III malocclusion (Nikopensius *et al.*, 2013). The same missense mutation Ser182Phe was informed before as a responsible variant in case of KS in another study (Miraoui *et al.*, 2013). DUSP6 is a member of the FGF8 synexpression module that determines pleiotropic roles during embryogenesis and in adulthood, and recent studies have delivered sign of an oligogenic model accounting for variable phenotypes in CHH/KS (Sykiotis *et al.*, 2010; Quaynor *et al.*, 2011). These findings can be interpreted as a clue of etiological heterogeneity: the infrequent allele in combination with other (more likely common) alleles constructing different phenotypic anomalies regarding CHH/KS and Class III malocclusion.

Current study was performed on 10 Malaysian Malay families with three generations to detect the mutation in DUSP6 gene in class III malocclusion. A missense mutation was identified in different position on c.1094 C>T in exon 3 (Thr365Ile) in 3 generations of class III malocclusion. This missense variant Thr365Ile of DUSP6 gene exists in NCBI database (rs370130918), however no report had been found on Thr365Ile variants in DUSP6 gene associating it with any disease. Moreover, none of patients that participated in current study demonstrated any KS features, and no pubertal issues were stated during history taking.

More recently, Perillo et al, (2015) found five missense mutations Tyr67Asn, Arg291Gln, Thr381Met, Val327Ile and Gly1121Ser in five different gene BMP3, ANXA2, FLNB, HOXA2, and ARHGAP21 associated with class III malocclusion in a single family living in Italy. However, the genetic variant Gly1121Ser of ARHGAP21 gene was present in all members of the affected family. In current study, we did not find any missense mutation other than Thr365 Ile in three members of a single family.

5.3 Cephalometric study in relation with genetic mutation in class III malocclusion

It has been observed that class III malocclusion is one of the most complex facial disharmony. Moreover, it is proven this malocclusion is primarily due to underlying skeletal problem rather than dental discrepancies (Kapust *et al.*, 1998). True class III malocclusion demonstrates mandibular skeletal protrusion, maxillary skeletal retrusion or a blend of both. Furthermore, a deficient vertical facial height is commonly demonstrated and it can influence the therapeutic and diagnostic verdicts.

Apparently, genetically inherited mandibular morphology can be recognized timely by ontogeny and is perceivable in early childhood (Tollaro *et al.*, 1996).

Nevertheless, the genetic influence on class III malocclusion has been scantly studied to assess the quantitative character of heredity in the development of this condition.

Previous studies on Malaysian Malay ethnic group concluded that etiology of class III malocclusion is identified having a large and prognathic mandible (Mohammad *et al.*, 2011; Bahaa *et al.*, 2014). Therefore, this study was conducted to assess the morphologic characteristics of the craniofacial complex of DUSP6 gene mutation and non-mutation groups in class III malocclusion of Malaysian Malay families.

Vorhies and Adams, (1951) described wiggle or polygon a graph where average norms were plotted in a vertical central line. The minimum and maximum norms used to plot on each side of the central line. Current study used SD in the polygon. Right side of the central line is showing +SD and left side of the central line is showing –SD instead of minimum and maximum reading following a previous study (Hassan, 2006).

For cephalometric tracing, Romexis software (Planmeca, Finland) was used. Rusu *et al.*, (2014) compared three different softwares including romexis and concluded that these computerized cephalometric programmes were reliable, specific and time saving.

According to present study, Co-Gn-B and SN-MDP showed significant differences between mutation group and non-mutation group. Mostly, when a comparison was done between two types of occlusion, the size and shape changes were more obvious

in the symphyseal region. The length of mandible (Co-Gn) was found larger in class III malocclusion subjects when compared to control subjects (Mouakeh, 2001; Alexander *et al.*, 2009; Bahaa *et al.*, 2014). However, another study stated no significant difference in Co-Gn between normal and class III malocclusion and the supramentale length (Gn-B) showed an increased value in class III malocclusion (Singh *et al.*, 1998). As our study comprised of one type of malocclusion, we did not find any significant difference among mutation and non-mutation groups in Co-Gn and Gn-B variables. Conversely, the relation between cranial base and anterior posterior position of mandible is decided by angular measurements. Current study showed that, the angle between Co-Gn and Gn-B showed significant difference in mutation group compare to the non-mutation group. Co-Gn-B angle is more acute in mutation group. Therefore, current study can establish that the mandible is more protruded in mutation group than the non-mutation group.

Other studies focused on genetic association of class III malocclusion with Growth hormone receptor (GHR) gene polymorphism, which contributes the changes in the mandibular ramus height in mandibular prognathism among Korean population (Kang *et al.*, 2009). Likewise, same gene polymorphism has been associated with mandibular height in Chinese population (Zhou *et al.*, 2005). Meanwhile, Co-Gn and lower face height (ANS-Me) were also associated with same gene polymorphism in Turkish population (Bayram *et al.*, 2014).

In addition, normal Japanese population showed significantly greater mandibular ramus length (Co-Go) without GHR variant than who were showing variant in position P56IT (Yamaguchi *et al.*, 2001).

Moreover, angle between S-N and mandibular plane represents the determination of the mandibular deviation from cranial base. Current study shows that the mandible is more deviated from cranial base in mutation group when compared to non-mutation group in class III malocclusion. Rest of the variables did not show any significant differences between mutation group and non-mutation group. Figure 4.12 shows the representative cephalometric tracing of both mutation (a) and non-mutation (b) groups where the mandible of mutation group appears more protruded and deviated than the non-mutation group. Moreover, table 4.2 shows larger value for these two variables compared to the other values in mutation group.

Since long, there has been a controversy regarding skeletal effect of class III malocclusion with age. Guyer et al., (1986) found that, Class III malocclusion shows unique dental and skeletal deviations from an early age (Guyer *et al.*, 1986). However, Bahaa et al, (2014) stated that in class III malocclusion discrepancy develops to become worse with age (Bahaa *et al.*, 2014). In current study, the skeletal relationships were checked in three generations and found almost all variables showing no relation of skeletal changes with age.

CHAPTER 6

CONCLUSIONS, LIMITATIONS AND RECOMMENDATION

The current study was able to unveil a missense mutation in DUSP6 gene in Exon 3. This missense mutation was present in a family, where 3 family members showed the same nucleotide position (c.1094 C> T) mutation which is a hereditary representation. Present study showed that, mutation of DUSP6 gene may cause class III malocclusion in Malaysian Malay population.

In cephalometric analysis, the present results showed that mandibular morphology shows variations in mutation group as compared to the non-mutation group of class III malocclusion. There are significant differences in Co-Gn-B and SN-MP variables in mutation group of class III malocclusion. Almost all variables show increase values in mutation group than the non-mutation group of class III malocclusion. Moreover, analysis of variance suggested that virtually all variables showed no skeletal changes among three generations in mutation and non-mutation group of class III malocclusion.

6.1 Limitation of the study

Current study gives the information about the DUSP6 gene mutation and its role in development of class III malocclusion in Malaysian Malay population. However, the study is limited by some facts.

a) The subjects that participated in this study only include Malay patients and controls from one state of Malaysia. While a diverse gathering of samples might give much better image of the Malay ethnic group, in terms of genetics.

b) For current study, only 10 Malay families were included, whereas a larger sample size would deliver better depth to the current hypotheses and results.

c) As class III malocclusion is mainly related with mandibular prognathism, for cephalometric analysis, current study mainly emphasized on the changes in mandibular morphology and not all the craniofacial structures.

6.2 Recommendation for future study

Current study raises several questions that can be addressed by the future researches.

a) A large sample may give a clear idea about the role of DUSP6 gene and class III malocclusion among Malaysian Malay ethnic group.

b) Collection of samples from different state within Malaysia would provide a generalized illustration of the genetic status for Malay population.

6.3 Clinical recommendations

Orthodontists can benefit from this study by attaining knowledge about patients having class III malocclusion and can possibly inform the patients to conduct early screening of susceptible patients and prevent the disease progression though efficient steps.

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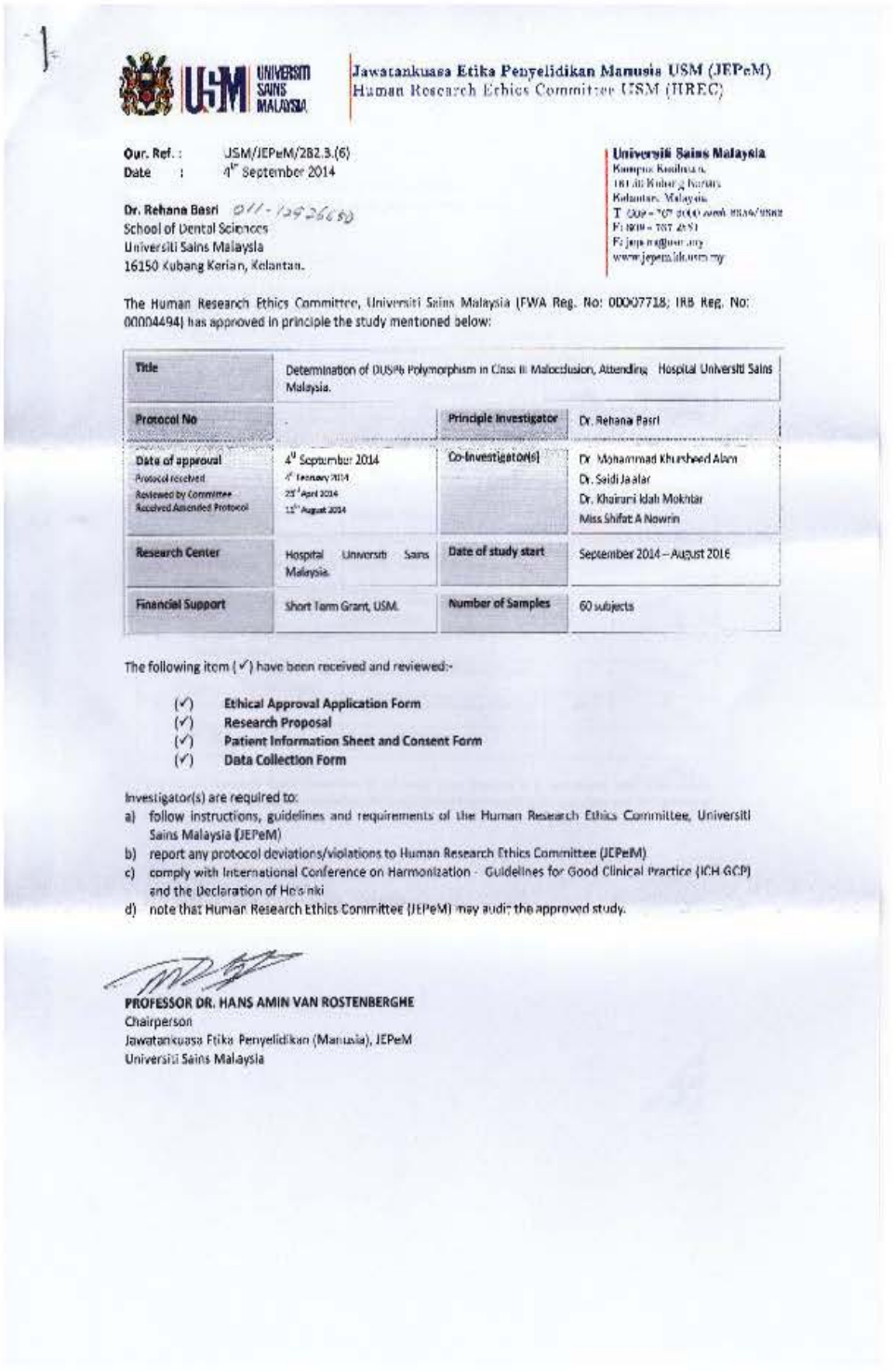
APPENDIX 1



Jawatankuasa Etika Penyelidikan Manusia USM (JEPeM)
Human Research Ethics Committee USM (HREC)

Our. Ref. : JSM/JEPeM/282.3.(6)
Date : 4th September 2014

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The Human Research Ethics Committee, Universiti Sains Malaysia (FWA Reg. No: 00007718; IRB Reg. No: 00004494) has approved in principle the study mentioned below:

Title	Determination of DUSP6 Polymorphism in Class II Malocclusion, Attending Hospital Universiti Sains Malaysia.		
Protocol No	-	**Principle Investigator**	Dr. Rehana Basri
Date of approval Protocol received Reviewed by Committee Received Amended Protocol	4th September 2014 4th February 2014 23rd April 2014 12th August 2014	**Co-Investigator(s)**	Dr. Mohammad Khursheed Alam Dr. Seidi Jaafar Dr. Khairuni Idah Mokhtar Miss Shifat A Nowrin
Research Center	Hospital Universiti Sains Malaysia.	**Date of study start**	September 2014 – August 2016
Financial Support	Short Term Grant, USM.	**Number of Samples**	60 subjects

The following item (✓) have been received and reviewed:-

(✓) **Ethical Approval Application Form**
(✓) **Research Proposal**
(✓) **Patient Information Sheet and Consent Form**
(✓) **Data Collection Form**

Investigator(s) are required to:
a) follow instructions, guidelines and requirements of the Human Research Ethics Committee, Universiti Sains Malaysia (JEPeM)
b) report any protocol deviations/violations to Human Research Ethics Committee (JEPeM)
c) comply with International Conference on Harmonization - Guidelines for Good Clinical Practice (ICH GCP) and the Declaration of Helsinki
d) note that Human Research Ethics Committee (JEPeM) may audit the approved study.

PROFESSOR DR. HANS AMIN VAN ROSTENBERGHE
Chairperson
Jawatankuasa Etika Penyelidikan (Manusia), JEPeM
Universiti Sains Malaysia

APPENDIX 2

IPS/VivaVoce-01/2015

INSTITUTE OF POSTGRADUATE STUDIES
UNIVERSITI SAINS MALAYSIA

SUBMISSION DRAFT COPIES OF THESIS

Section 1 (To be completed by Student)

Fullname : Shibat A Nowsin

Matric No : P-SGM 0006/13(R) Degree Program : Master of Science (Dentistry)

eg : Doctor of Philosophy ,
Master of Science (Botany)

Thesis's Title :

Determination of DUSP6 gene mutation and its effect on craniofacial morphology among class III malocclusion patients attending at Hospital Universiti Sains Malaysia (HUSM)

Translation :

Penentuan DUSP6 mutasi gen dan kesannya terhadap kranio fasial morfologi antara kelas III pesakit malocclusion menghadiri di Hospital Universiti Sains Malaysia (HUSM)

Please insert the Field of Research for the Academic Transcript Records

Field of Research (English) : Orthodontic.

Field of Research (Malay) : Ortodontik

Section 2 : Verification of Turnitin Screening

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16150 KUBANG KERIAN, KELANTAN

* Turnitin report as attached.

1

APPENDIX 3

LAMPIRAN A

MAKLUMAT KAJIAN

Tajuk Kajian: **Penentuan polimorfisme gen *DUSP6* dalam kes maloklusi Kelas III di Hospital Universiti Sains Malaysia (HUSM)**

Nama Penyelidik: Dr Rehana Basri, Dr Khairani Idah binti Mokhtar, Dr Saidi bin Jaafar, Dr Mohammad Khursheed Alam.

No. Pendaftaran MDC :

Dr Rehana Basri – Staff no: **0846/11,**

Dr Khairani Idah binti Mokhtar – Staff no: **0533/08**

Dr Saidi bin Jaafar – Staff no: **1389/08**

Dr Mohammad Khursheed Alam – Staff no: **0473/11**

PENGENALAN

Maloklusi Kelas III merupakan suatu fenotip berkaitan dentofacial yang heterogenus dimana ianya di cirikan oleh pertumbuhan mandibel yang melampau atau pertumbuhan maksila yang perlahan atau kombinasi kedua-duanya. Maloklusi Kelas III boleh berlaku sama ada sebagai sebahagian daripada sindrom atau sebagai sifat terpencil. Fenotip ini boleh di kenalpasti pada usia yang awal dan menjadi semakin ketara dengan tumbesaran individu. Oleh yang demikian, ianya menjadi sebagai salah satu faktor utama yang memaksa pesakit untuk mendapatkan rawatan ortodontik dan pembedahan.

ATTACHMENT B

RESEARCH INFORMATION/ Patient/Subject Information and Consent Form

Research Title: **Determination of DUSP6 polymorphism in class III malocclusion, attending at Hospital Universiti Sains Malaysia (HUSM)**

Researcher's Name: **DR REHANA BASRI, DR KHAIRANI IDAH BINTI MOKHTAR, DR SAIDI BIN JAAFAR, DR MOHAMMAD KHURSHEED ALAM,**

MDC Registration No.:

INTRODUCTION

Class III malocclusion is a heterogeneous dentofacial phenotype that is skeletally characterized by an overgrowth of the mandible (mandibular prognathism) an undergrowth of the maxilla (maxillary deficiency), or a combination of both, and can occur either as part of a syndrome or as an isolated trait. This phenotype can be recognized at an early age and becomes progressively more evident with growth, appearing as one of the main factors that force patients to seek orthodontic and surgical treatment.You are invited to take part voluntarily in a research of genetic study to understand the interaction of DUSP6 gene polymorphisms with malocclusion patients. Single nucleotide polymorphisim (SNPs) are the most frequent genetic mutations in humans, affecting protein expression and functioning, which then cause disease (Wang Z et al. 2001). Malocclusion is a common dental anomaly and from the "common disease, common variant" hypothesis, it can be speculated that SNPs are important genetic components of malocclusion (Risch N et al. 1996). In Nikopensius *et al.* 2013 reported that p.Ser182Phe mutation in *DUSP6* as being attributed to Class III malocclusion in an Estonian family. DUSP6 gene is significantly associated with malocclusion. This variant co-segregated with the disease following an autosomal-dominant mode of inheritance with incomplete penetrance. Gene *DUSP6* – is biologically relevant to craniofacial development, and May responsible for both mandibular prognathism and maxillary deficiency.

Before agreeing to participate in this research study, it is important that you read and understand this form. As i mention earlier that we will do genetic test to you, your parents, and your great grand parents, those have similar symptom in your family.If you participate, you will receive a copy of this form to keep for your records.

Your participation in this study is expected to last up to 1 hour. Up to 60 patients will be participating in this study.

PURPOSE OF THE STUDY

The purpose of this study is to study the association between DUSP6 gene polymorphisms (genetic variation in individuals that affects the physical characteristics- 'phenotype') and malocclusion among patients attending Dental Clinic, Hospital Universiti Sains Malaysia. In Malaysia, there is no information yet regarding this issue.

It is possible that information collected during this study will be use in the future for other possible medical or scientific purposes other than those currently proposed.

APPENDIX 4

Research tools

a) Clinical examination equipment

i. Dental chair

ii. Dental examination and diagnosis set which includes,

- dental mirror
- probe/explorer
- tweezers

iii. Lateral Cephalogram

b) Sterile oral swab stick for the collection of buccal swab

c) Laboratory Equipment

i. Sample storage facility (NuAire, USA)

ii. Centrifuge 5415D (Eppendorf, Germany)

iii. Centrifuge mini spin (Eppendorf, Germany)

iv. PCR master cycler (Eppendorf, Germany)

v. Gel Doc (Bio-Rad, USA)

vi. HVE-25 Autoclave (Hirayama, Japan)

vii. Minigel agarose electrophoresis (Bio-Rad, USA)

viii. Nano drop (Thermofisher, USA)

ix. Pipette (Eppendorf, Germany)

x. Spectrophotometer (BioPhotometer, Eppendorf, Germany)

xi. A&D GR-200 lab balance (A & D company, Japan)

d) IBM SPSS Statistics Version 22.0 (Chicago, USA)

e) Romexis™ software 2.3.1.R (Planmeca, Finland)

Materials

DNA Extraction Reagents

DNA extraction was carried out using Gentra Puregene Buccal Cell Kit for buccal swab (Qiagen, Germany) following the protocol supplied by manufacturer. The DNA extraction kit consists of-

i. Cell lysis solution:

   Cell lysis solutions are detergent-based, buffers and reagent sets that have been optimized for particular cell lysis applications. It was stored at room temperature.

ii. Protein precipitation solution:

   Protein precipitation solution is a high salt buffer that lowers the solubility of proteins. It was stored at room temperature.

iii. DNA hydration solution:

   DNA hydration solution is used to hydrate the extracted DNA. It was stored at room temperature.

iv. Buccal swab collection brushes:

   Buccal collection brushes were used to take the buccal swab from the patients and the controls as well.

v. RNase A solution:

RNase A solution was used to get the RNA free DNA form the buccal swab and stored at 4ºC.

vi. Glycogen solution:

Glycogen solution is a nucleic acid isolation enhancer and stored in -20ºC.

vii. Isopropanol:

Isopropanol was used to precipitate the DNA in the column of extraction kit.

viii. Ethanol:

70% Ethanol (HmbG, Germany) was used to wash the DNA pellet. 70% ethanol was prepared by adding 970 ml of absolute ethanol in 30 ml of distilled water (dH_2O).

Polymerase Chain Reaction (PCR) Reagents

i. PCR Master Mix (First base, Singapore):

PCR master mix consists of reaction buffer, Taq DNA Polymerase 0.06U/µl, 3mM $MgCl_2$ and 400µM of each dNTPs. This pre mix formulation saves time and reduces contamination by reducing the number of pipetting steps required for usual PCR set up. The master mix is optimized for efficient and reproducible PCR. It was stored in -20ºC.

ii. Forward and reverse primer (First Base Laboratories Sdn Bhd, Malaysia):

Three pairs of primers sequences used in this study were designed in order to amplify the target region of DUSP6 gene. Primers were diluted to 10mM from 100mM stock solution by adding distilled water. The stocks and diluted primers were kept at -20ºC.

iii. DNA template:

DNA template for the PCR amplification was obtained from DNA extraction process (Section 3.7.4) using Gentra Puregene Buccal Cell Kit for buccal swab (Qiagen, Germany).

iv. Distilled water:

Distilled water was used to adjust final volume and pH of the master mix.

Electrophoresis Reagents

The reagents that have been used for electrophoresis were -

i. Agarose powder (Invitrogen, USA):

Agarose powder (Invitrogen, USA) was used in this study to prepare the electrophoresis gel and stored at room temperature.

ii. 10X Tris-Acetate-EDTA (TAE) Buffer (First Base, Singapore):

To prepare 1000ml 10xTAE buffer, 10 ml 10xTAE buffer was added with 990ml distilled deionized water (ddH2O). The prepared buffer was kept at room temperature.

iii. 6x Loading dye (First Base, Singapore):

6X loading buffer was used in the electrophoresis process which contain 0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 36%

glycerol, 10mM Tris-HCL (pH 7.4) and 50mM EDTA (pH 8.0). This allows better visual tracking of DNA migration during electrophoresis and stored in -20ºC.

iv. Sybr safe (Invitrogen, USA):

SYBR Safe DNA gel stain is a highly sensitive stain for visualization of DNA in agarose gels. SYBR Safe stain is specifically formulated to be a less hazardous alternative to ethidium bromide that can be used with either blue-light or UV excitation. It was stored in -20ºC.

v. Ladder 100 bp (First Base, Singapore):

DNA ladder (100bp) was used for sizing and approximate quantification of wide range double- stranded DNA on agarose gel. It was supplied in mixtures of 10mM Tris- HCl (pH 8.0) in 1mM EDTA. The ladder is composed of eleven individual DNA fragments (in base pairs) like 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100. It contains two reference bands of 1500 and 500bp for easy orientation. Orange G and xylene cyanol FF are used as tracking dyes. It was stored in -20ºC.

vi. Parafilm (Pechiney, USA):

Flexible, thermoplastic, rolled and waterproof sheet with paper to prevent adhering semi-transparent, almost colourless, odourless, and tasteless. Which adheres quickly and firmly to itself and covering culture tube or flask mouths. It softens enough to make an adhesive bond between papers, cloths, leathers, etc. (Supplied in dispenser cartons). It was stored in room temperature.

APPENDIX 5

Data collection form for patient

REGISTRATION NO:

AGE:

SEX :

RACE:

ADDRESS:

RELIGION:

INFORMEN: Regarding Patient and other Family member affected the same problem (Class III malocclusion).

1. **Yes:** (if yes, mention the relation with patient) .....
2. **No:**

OCCUPATION:

MOLAR RELATION:

CANINE RELATION:

INCISOR RELATION:

OVER BITE:

OVER JET:

MIDLINE:

PROFILE:

APPENDIX 6

Patients	Mandibular morphology	Variables	1st generation	2nd generation	3rd generation
1	Linear	Co-Gn			
		Gn-B			
		Co-Go			
		Ar-Go			
		Id-M			
	Angular	Co-Gn-B			
		Co-Go-Gn			
		Id-Gn-Go			
		Id-M-Go			
		Id-Gn			
		SN - MP			
		FA			
		GA			
2	Linear	Co-Gn			
		Gn-B			
		Co-Go			
		Ar-Go			
		Id-M			
	Angular	Co-Gn-B			
		Co-Go-Gn			
		Id-Gn-Go			
		Id-M-Go			
		Id-Gn			
		SN - MP			
		FA			
		GA			
3	Linear	Co-Gn			
		Gn-B			
		Co-Go			
		Ar-Go			
		Id-M			
	Angular	Co-Gn-B			
		Co-Go-Gn			
		Id-Gn-Go			
		Id-M-Go			
		Id-Gn			
		SN - MP			
		FA			
		GA			
4	Linear	Co-Gn			
		Gn-B			
		Co-Go			
		Ar-Go			
		Id-M			
	Angular	Co-Gn-B			
		Co-Go-Gn			
		Id-Gn-Go			
		Id-M-Go			
		Id-Gn			
		SN - MP			
		FA			
		GA			
6	Linear	Co-Gn			
		Gn-B			
		Co-Go			
		Ar-Go			
		Id-M			
	Angular	Co-Gn-B			
		Co-Go-Gn			
		Id-Gn-Go			
		Id-M-Go			
		Id-Gn			
		SN - MP			
		FA			

APPENDIX 7

No	Family Member (Generation)	Cephalometric Analysis						
		SNA	SNB	ANB	Wits Appraisal	W angle	Beta angle	Yen Angle
1.	1st							
	2nd							
	3rd							
2.	1st							
	2nd							
	3rd							
3.	1st							
	2nd							
	3rd							
4.	1st							
	2nd							
	3rd							
5.	1st							
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8.	1st							
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	3rd							
9.	1st							
	2nd							
	3rd							
10.	1st							
	2nd							
	3rd							
Mutation Family	Ezzaty							
	Father							
	Mother							
	Brother							
	Grand Mother							

LIST OF PUBLICATIONS AND PRESENTATIONS

Article (Published)

1. **Nowrin, SA.**, Khursheed Alam, M., Basri, R. (2015) Genetic effect and prevalence of class iii malocclusion in different population: an overview. *International Journal of Pharma and Bio Sciences*, **6(2),** 910-918.
2. **Nowrin, SA.**, Khursheed Alam, M., Basri, R. (2015). Genetic analysis: Future diagnostic tool in clinical Orthodontics. *Bangladesh Journal of Medical Science*, **14(3),** 310-311.
3. **Nowrin, SA.**, Basri, R., Khursheed Alam, M., Yusa, T., Nakano, J., Jaafar, S., Idah Mokhtar, K., Osuga, N. (2016) Craniofacial morphology of Class III Malocclusion with DUSP6 gene: mutation and non-mutation groups. *The Journal of Hard Tissue Biology*, **25(3),** 247-256.

Articles in Review

1. **Nowrin, SA.**, Basri, R., Khursheed Alam, M., Jaafar, S., Idah Mokhtar, K. Genetic study on DUSP6 gene in Class III Malocclusion subjects in Malaysian Families. *American Journal of Orthodontics and Dentofacial Orthopedics*, 2016.

Abstracts in conference

1. **Shifat A Nowrin**, Rehana Basri, Mohammad Khursheed Alam, Saidi Jaafar, Khairani Idah Binti Mokhtar. A novel mutation in class III malocclusion: A family study. 20th National Conference on Medical and Health Sciences

"Humans and Nature Wellness: Balancing a Healing Act".September 12- 14, 2015.

2. **Shifat A Nowrin**, Rehana Basri, Mohammad Khursheed Alam, Saidi Jaafar, Khairani Idah Binti Mokhtar. Genetic study in Class III Malocclusion. ISAHS International Symposium on Allied Health Sciences. September 5-6, 2015.

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GENETIC EFFECT AND PREVALENCE OF CLASS III MALOCCLUSION IN DIFFERENT POPULATION: AN OVERVIEW

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ABSTRACT

The prime aim of this review is to highlight the genetic effect and prevalence of class III malocclusion in different population. A literature search was conducted. Evidence from previous studies also established that class III malocclusion is strongly influenced by the genetic factors. May be class III malocclusion had developed by polygenic or monogenic mode of inheritance. But the environmental factors also responsible for this trait. Class III malocclusion has been the topic of intension and eager to many researchers. Researchers concluded that diverse combinations of skeletal and dental rudiments are drawn in to produce class III malocclusion. Genome wide linkage scan technology can detect several chromosomal regions, responsible for the mandibular prognathism. However, very few genome wide family based linkage study have been done for the determination of the mandibular prognathism. This article motivated on understanding the genetic influence and the prevalence of class III malocclusion in different population.

KEYWORDS: Class III malocclusion, genetic influence, Prevalence, genetic factor, Genome wide linkage scan technology.

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Brief Communication

Genetic analysis: future diagnostic tool in clinical Orthodontics

Nowrin SA[1], Alam MK[2], Basri R[3]

Orthodontic diagnosis and treatment planning, genetic basis of a skeletal anomaly should think through. The collaboration of various genes has been shown to be the primary cause of an unbalanced malshaped craniofacial structure[1].

Facial profile and structure seems to have a familial trend. From population studies proof gained, that families and twin have shown a genetic factor is responsible for the etiology of malocclusions. A predicted value of more than 25,000 human genes adds contribution to the development of craniofacial structure[2].

With recent technological advances that allow the simultaneous characterization of entire genomes via high throughput genotyping of Single-nucleotide polymorphisms (SNPs)or sequencing of the genome to evaluate human genetic variation, future gene and gene-environment studies of malocclusion can be performed on precisely defined phenotypes. This will provide valuable insights into the etio-pathogenesis underlying malocclusion[3].

Tung Yuen et al.,[4] surveyed class I occlusion with crowding in the Hong Kong Chinese population using MassArray technique for the first time and conclude that there is link of EDA and XEDAR genes in dental crowding in Chinese population (Table 1).

Both genetic and environmental influences play a role in the development of Class II malocclusion. Studies of Class II division 1 patients have shown that this condition is heritable and is consistent with a polygenic mode of inheritance. A polygenic model implies that a number of genes with small additive effects provide genetic predisposition to the phenotypic expression observed in the class II division 2 malocclusion. Gutierrez et al.,[5]analyzed four Colombian families with Class II malocclusion and found the be homozygous for the rare allele in SNP on the Nog gene (Table 1).

Evidence from previous studies also established that class III malocclusion is strongly influenced by the genetic factors. May be class III malocclusion had developed by polygenic or monogenic mode of inheritance. But the environmental factors also responsible for this trait.

Few works has been done to evaluate the quantitative role of heredity in the etiology of this condition. Various studies discussed various susceptible loci and genes for the class III malocclusion. DUSP6, EPB41, IGF1, HOXC, COL2A, TGFB3, LTBP2 (Table 1)are the mostly founded genes for class III malocclusion[6-9].

Genetic analysis is an important tool in clinical Orthodontics. The etiological diversity is the main complicating factor for treatment and diagnosis in various types of malocclusions. But it still need time to be better accepted by dentists and explore the advantages.

Conflict of Interest: None declared

Table 1. Type of malocclusions and related genes in mutation.

Class I malocclusion	Class II malocclusion	Class III malocclusion
EDA	NOG	IGF1, EPB41, TGFB3, COL2A1
XEDAR		LTBP2, HOXC

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AWARD OF BEST ORAL PRESENTATION

1st Place

for the presentation entitled:

A Novel Mutation in Class III Malocclusion: A Family Study

Authors:

Shifat A Nowrin, Rehana Basri, Mohammad Khursheed Alam, Saidi Jaafar, Khairani Idah Binti Mokhtar

at the

20th National Conference on Medical and Health Sciences

"Humans and Nature Wellness: Balancing a Healing Act"

12-14 September 2015
Health Campus, Universiti Sains Malaysia

Organised by:
School of Medical Sciences
Health Campus, Universiti Sains Malaysia

Prof. Dr. Ahmad Sukari Halim
Dean
School of Medical Sciences
Universiti Sains Malaysia

Prof. Dr. Rosline Hassan
Chairman
20th National Conference on Medical and Health Sciences

Genetic study in Class III Malocclusion

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Introduction: Class III malocclusion is a common phenotype associated with genetic inheritance patterns characterized by protrusion of mandible. It may cause due to failure of development of either maxilla or mandible respectively. We hypothesized that, the mandibular or maxillary growth may be standardized by specific locus 12q22-q23 that is genetically linked with class III malocclusion. **Methods:** We observed Malaysian Malay family history in relation with class III malocclusion. Genetic study was performed in 30 subjects of 10 Malaysian families, including their 3 generations and compared the result with 30 controls. **Results:** Two rare heterozygous novel missense mutation 1087 C> T (p. Thr 363 Ile) and 1111 T> C (p. Tyr 371 His) were detected in the Dusp6 gene among these class III malocclusion subjects. **Conclusion:** Analysis of our data directed that DUSP6 gene mutation with class III malocclusion following an autosomal-dominant mode of inheritance. The present work emphasizes that the gene mutation may be linked with the ethnicity of the population. Moreover, further studies are required to clarify the significance of this evidence.

Keyword: Class III malocclusion, DUSP6 gene, Genetic polymorphism, Malaysian family, Mandibular prognathism, Missense mutation

OP18: A NOVEL MUTATION IN CLASS III MALOCCLUSION: A FAMILY STUDY

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Introduction:

Class III malocclusion is a conjoint dento-skeletal phenotype along with significant genetic components. Nevertheless, very few susceptible genes have been identified for this phenotype. Asian races are showing high incidence of Class III malocclusion or mandibular prognathism. Moreover, this ethnicity became a risk factor for this phenotype.

Objective:

To identify the causative gene of DUSP6 mutation in a class III malocclusion.

Methods:

Based on clinical and radiological examination, a Malaysian Malay family including four generations with class III malocclusion was taken for genetic study.

Results:

Two heterozygous novel missense mutations 1087 C> T (p. Thr 363 Ile) and 1111 T> C (p. Tyr 371 His) were identified in DUSP6 gene.

Conclusion:

Analysis of our data concluded that there is a suggestive linkage for class III malocclusion with the DUSP6 gene in Malaysian Malay population. Moreover, this verdict can pooled with previous studies for advance knowledge of the genetic basis of class III malocclusion.