IDENTIFICATION OF NOVEL MUTATIONS IN NON-SYNDROMIC SKELETAL MANDIBULAR PROGNATHISM IN MALAY SUBJECTS: INSIGHTS FROM WHOLE EXOME SEQUENCING AND INHERITANCE PATTERN

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uL	microliter
°C	celcius
%	percent
≥	greater than or equal to

LIST OF ABBREVIATIONS

ADL	Advanced Diagnostic Lab
AMDI	Advanced Medical and Dental Institute
ASCII	American Standard Code for Information Interchange
AT	Adenine-Thymine
BAM	Binary Alignment Map
bp	base pairs
CCB	Centre for Chemical Biology
CNV	Copy Number Variation
dbSNP	Single Nucleotide Polymorphism Database
DNA	Deoxyribonucleic Acid
etc.	et cetera (and the other things)
GC	Guanine-Cytosine
GO	Gene Ontology
i.e.	id est (that is)
InDel	Insertion and Deletion
ml	mililiter
N	Nucleotide
NCBI	National Centre for Biotechnology Information
NGS	Next-generation Sequencing
OMIM	Online Mendelian Inheritance of Man
PCR	Polymerase Chain Reaction
Qphred	Phred Quality Score
RNA	Ribonucleic Acid
ROK	Rho kinase
SNP	Single Nucleotide Polymorphism
SNV	Single Nucleotide Variant
USM	Universiti Sains Malaysia
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing

LIST OF APPENDICES

MENGENALPASTI MUTASI NOVEL DALAM SKELETAL PROGNATHISME MANDIBULAR BUKAN SINDROMIK DALAM SUBJEK MELAYU: PENEMUAN DARIPADA JUJUKAN EKSOM KESELURUHAN DAN CORAK PEWARISAN

ABSTRAK

Prognathisme mandibular adalah manifestasi asas rangka Kelas III. Ia dicirikan oleh *overjet* terbalik pada gigi anterior dan profil wajah yang cekung, menyebabkan masalah estetik, gigitan tidak selari, dan masalah pertuturan. Faktor genetik dipercayai menjadi punca utama, namun asas genetik bagi prognathisme mandibular bukan sindromik dalam populasi Melayu masih belum diterokai secara meluas. Masalahnya terletak pada pelbagai lokus genetik, gen calon, dan pola pewarisan yang berbeza dalam kalangan populasi, yang menunjukkan bahawa setiap kumpulan etnik mungkin memiliki mutasi dan pola pewarisan tersendiri berkaitan prognathisme mandibular. Kajian ini bertujuan untuk menemui mutasi baharu, termasuk gen dan lokus, serta menentukan pola pewarisan prognathisme mandibular bukan sindromik dalam subjek Melayu. Satu kohort individu Melayu Malaysia yang disahkan memiliki salasilah tiga generasi Melayu dan menghadiri Klinik Pergigian AMDI telah direkrut. Lima keluarga Melayu dengan prognathisme mandibular telah dikenal pasti, menghasilkan 11 subjek kes. Selain itu, 11 pesakit Melayu dengan corak rangka Kelas I dipilih sebagai kawalan, menjadikan keseluruhan peserta berjumlah 22 orang. Diagnosis prognathisme mandibular disahkan melalui radiograf sefalometrik lateral. Penjujukan eksom keseluruhan (WES) mengenal pasti polimorfisme nukleotida tunggal (SNP) jenis missense dalam sepuluh gen, termasuk tiga gen yang telah diketahui sebelumnya

(*C1orf167, DUSP6, COL2A1*) dan tujuh gen baharu (*ZNF17, GEM, CYP26B1, LYSMD4, KAZALD1, FYCO1, CCDC110*). Penjujukan Sanger mengesahkan mutasi ini, dan enam SNP missense baharu yang berkaitan dengan prognathisme mandibular bukan sindromik dalam subjek Melayu telah dikenal pasti: *ZNF17* (rs2014827) pada lokus 19q13.43, *GEM* (rs2170363) pada lokus 8q22.1, *CYP26B1* (rs2241057) pada lokus 2p13.2, *LYSMD4* (rs72760587) pada lokus 15q26.3, *KAZALD1* (rs807037) pada lokus 10q24.31, dan *FYCO1* (rs117543659) pada lokus 3p21.31. Selain itu, SNP dalam *C1orf167, COL2A1*, dan *DUSP6* diperhatikan dalam kedua-dua subjek kes dan kawalan, yang mungkin mencerminkan penetrans tidak lengkap, menunjukkan bahawa varian ini tidak semestinya menjadi penyebab langsung prognathisme mandibular. Analisis salasilah dalam satu keluarga terjejas menunjukkan corak pewarisan dominan autosom. Penemuan ini mencadangkan kewujudan penanda genetik unik untuk prognathisme mandibular bukan sindromik dalam subjek Melayu, dengan enam SNP jenis missense baharu yang berpotensi berfungsi sebagai penanda diagnostik. Penyelidikan lanjut diperlukan untuk memahami peranan khusus gen-gen ini dalam perkembangan mandibular.

IDENTIFICATION OF NOVEL MUTATIONS IN NON-SYNDROMIC SKELETAL MANDIBULAR PROGNATHISM IN MALAY SUBJECTS: INSIGHTS FROM WHOLE EXOME SEQUENCING AND INHERITANCE PATTERN

ABSTRACT

Mandibular prognathism is a Class III skeletal base manifestation. It is characterised by a reverse overjet in the anterior teeth and a concave facial profile, leading to aesthetic concerns, misaligned bite, and speech issues. Genetic factors are believed to be the primary cause, yet the genetic basis of non-syndromic mandibular prognathism in the Malay population remains largely unexplored. The problem lies in the varied genetic loci, candidate genes, and inheritance patterns observed across populations, implying that each ethnic group may possess distinct mutations and inheritance patterns associated with mandibular prognathism. This study aims to discover novel mutations, including the genes and loci, and to determine the inheritance pattern of non-syndromic mandibular prognathism in the Malay subjects. A cohort of ethnically Malaysian Malay individuals with confirmed three-generation Malay lineage attending the AMDI Dental Clinic was recruited. Five Malay families with mandibular prognathism were identified, resulting in 11 case subjects. Additionally, 11 Malay patients with a Class I skeletal pattern were selected as controls, totaling 22 participants. The diagnosis of mandibular prognathism was confirmed through lateral cephalometric radiographs. Whole exome sequencing (WES) identified missense single nucleotide polymorphisms (SNPs) in ten genes, including three previously known (*C1orf167, DUSP6, COL2A1*) and seven novel (*ZNF17, GEM, CYP26B1, LYSMD4, KAZALD1, FYCO1, CCDC110*). Sanger

sequencing validated these mutations, and six novel missense SNPs associated with non-syndromic mandibular prognathism in the Malay subjects were identified: *ZNF17* (rs2014827) at locus 19q13.43, *GEM* (rs2170363) at locus 8q22.1, *CYP26B1* (rs2241057) at locus 2p13.2, *LYSMD4* (rs72760587) at locus 15q26.3, *KAZALD1* (rs807037) at locus 10q24.31, and *FYCO1* (rs117543659) at locus 3p21.31. Additionally, SNPs in *C1orf167*, *COL2A1*, and *DUSP6* were observed in both case and control subjects, which may reflect incomplete penetrance, suggesting that these variants are not necessarily directly causative of mandibular prognathism. Pedigree analysis in one affected family indicated an autosomal dominant inheritance pattern. These findings suggest the presence of unique genetic markers for non-syndromic mandibular prognathism in the Malay subjects, with six novel missense SNPs potentially serving as diagnostic markers. Further research is needed to understand the specific roles of these genes in mandibular development.

CHAPTER 1

INTRODUCTION

1.1 Overview

Mandibular Prognathism (OMIM:176700; Online Mendelian Inheritance of Man, (http://omim.org/entry/176700) is a part of Class III skeletal base manifestations which can occur either because of mandibular prognathism alone, undergrowth of maxilla alone or a combination of both (Cruz, Krieger, Ferreira, Mah, Hartsfield, *et al.*, 2008; Ruslin *et al.*, 2015). Mandibular prognathism is characterised by a negative anterior overjet (Figure 1.1) and a concave facial profile (Figure 1.2) (L. Perillo *et al.*, 2015). Given that mandibular prognathism is a bone development disorder caused by genetic inheritance and ethnic aggregation, genetic factors are probably crucial in its aetiology. The apparent contributions of environmental and genetic factors to the aetiology of non-syndromic mandibular prognathism are ambiguous (A. A. El-Gheriani *et al.*, 2003; F. Xue, Rabie and Luo, 2014). Although the genetic models are distinct, there is a consensus that genetics contribute to determining the presence of mandibular prognathism (A. A. El-Gheriani *et al.*, 2003).

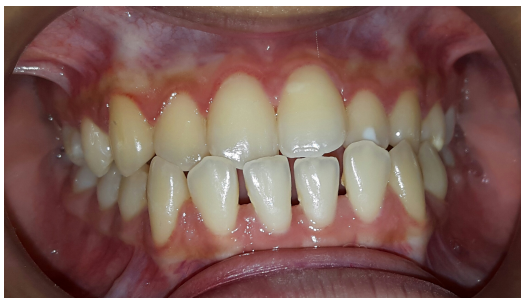

Figure 1.1 Reverse overjet

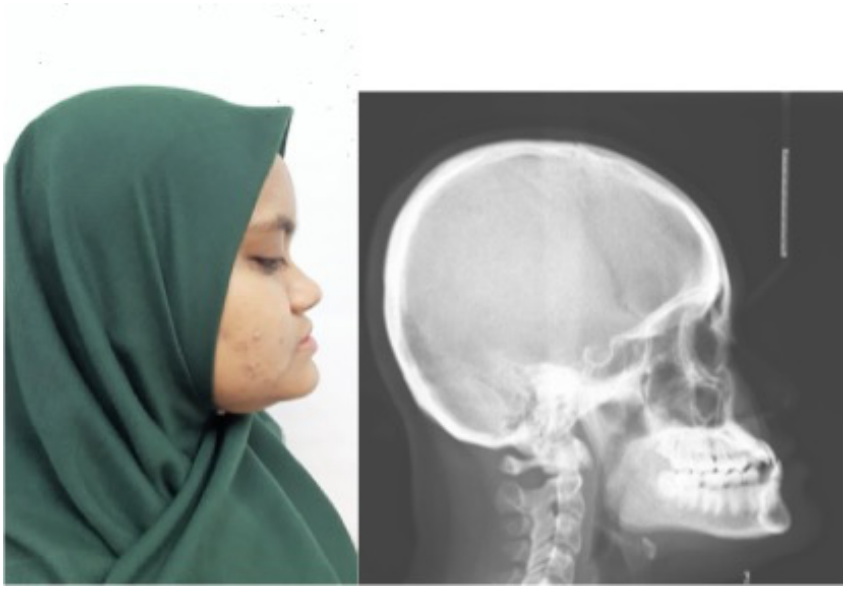

Figure 1.2Concave facial profile

1.2 Background of Study

According to OMIM, mandibular prognathism can present as a systemic disorder like Alpert syndrome or Crouzon syndrome or as a non-syndromic disorder (Chen *et al.*, 2015a). Early childhood typically does not indicate any anomalies. Mandibular prognathism development begins gradually, picks up speed during adolescence, and manifests completely once the body fully develops (Morrill, Baumrind and Miller, 1974).

Patients' social self-confidence may decline due to mandibular prognathism's unappealing facial profile, which may also have major psychological effects. The inability of the lower and upper anterior teeth to establish a bite leads to reduced masticatory efficiency (English, Buschang and Throckmorton, 2002). As a result, there may be gastrointestinal issues and a nutritional deficiency. Speech enunciation issues could also result from the uneven bite of the upper and lower jaws (X. Guan *et al.*, 2015). In patients with mandibular prognathism, the tongue may generally remain in a lower position during swallowing, making it challenging to exert the necessary

pressure against the palate. As a result, a longer time is needed for bolus transfer, and the length of tongue pressure is prolonged (Sakaue *et al.*, 2016).

The more costly and time-consuming treatment for mandibular prognathism combines orthodontic treatments with orthognathic surgery. However, as an invasive treatment, orthognathic surgery carries several potential hazards, some of which can be very serious. These risks include hypoesthesia, infection, haemorrhage, tissue damage, and changes to the osteosynthesis systems. Most orthognathic operations may only be performed on skeletally mature patients to ensure adequate diagnosis and prevent additional procedures from treating malocclusion recurrence brought on by post-surgery growth (Gershater *et al.*, 2021).

Current uncertainty regarding the precise aetiology of Class III skeletal pattern makes it challenging to anticipate individual's expected skeletal discrepancy development and therapeutic corrective prognoses (Gershater *et al.*, 2021). The main topics of numerous studies to date have been understanding the genetic variables that contribute to malocclusion and figuring out how these genetic factors might affect how patients respond to orthodontic treatment (F. Xue, Wong and Rabie, 2010).

The majority of research indicates that there is no association between gender and the prevalence of phenotypes, with East Asians having the highest frequency of mandibular prognathism and Caucasians having the lowest. The inheritance pattern of mandibular prognathism is varied, with the most typical inheritance pattern being the dominant trait with incomplete penetrance. According to genetic results from various publications, there may be more than one gene that causes mandibular prognathism. Through genome-wide linkage analyses, several possible chromosomal loci and various candidate genes have been discovered, particularly in Korean (Matrilin-1) (J. Y. Jang *et al.*, 2010), Japanese (PLXNA2 and SSX2IP) (Saito *et al.*, 2017), and

Chinese (FGF12, ADAMTS1, TGFB3, LTBP2, and COL2A1) (X. Guan *et al.*, 2015; Xiong, Li, Cai, Chen, *et al.*, 2017). Most of the loci variants and candidate genes were found to be expressed during mandibular development, which may lead to mandibular prognathism.

1.3 Problem Statement

Various genetic analyses have revealed diverse mandibular prognathism loci, candidate genes, and inheritance patterns, suggesting that every population may have a separate non-syndromic mandibular prognathism mutation. The mandibular prognathism inheritance pattern is varied, with the autosomal dominant and autosomal dominant with incomplete penetrance being the most typical form. The ethnic diversity observed in genetic findings suggests that there might not be a single mandibular prognathism-causing gene. Nevertheless, there have not been many investigations into the genetic causes of mandibular prognathism in the Malay population. This makes it impossible for researchers to pinpoint the genetic cause of mandibular prognathism in the Malay population. According to the findings of these genomic analyses, which also reveal diverse locations, candidate genes, and inheritance patterns for mandibular prognathism, a distinct mutation of non-syndromic mandibular prognathism may exist within each specific population group. Hence, a unique mutation may be involved in causing mandibular prognathism in the Malay population with a distinct inheritance pattern.

1.4 Research Aim

This study aims to investigate novel mutation and inheritance patterns including the genes and loci contributing to skeletal mandibular prognathism development in the Malay subjects.

1.5 Objectives of Study

1.5.1 General Objective

The primary objective of this research is to systematically analyse and characterise novel mutations and inheritance patterns associated with non-syndromic skeletal mandibular prognathism in the Malay subjects.

1.5.2 Specific Objectives

1. To determine whether there are any novel mutations in the Malay subjects that cause non-syndromic skeletal mandibular prognathism.
2. To identify the genetic variant(s), gene(s), and loci, involved in the development of non-syndromic skeletal mandibular prognathism in the Malay subjects.
3. To determine the mode of inheritance of non-syndromic skeletal mandibular prognathism in the Malay subjects.

1.6 Research Questions

1. What type of mutation occurs in non-syndromic skeletal mandibular prognathism in the Malay subjects?
2. What are the variants/genes/loci that cause non-syndromic skeletal mandibular prognathism in the Malay subjects?
3. What is the inheritance pattern of non-syndromic skeletal mandibular prognathism in the Malay population?

1.7 Significance of Study.

This study is expected to yield new findings and knowledge on the occurrence of skeletal mandibular prognathism in the Malay subjects in terms of the type of

mutation, affected loci and genes and inheritance pattern. Once the mutated genes have been identified, it will lead to research and development of suitable gene therapy in this field. The unsightly facial profile caused by mandibular prognathism may diminish patients' social self-assurance and could lead to significant psychological distress. Unravelling the underlying mechanism of genetic mutation will help researchers and clinicians alike improve the current treatment and develop new treatments such as gene therapy and biomarkers specific to this population.

This research paves the way for precision medicine, where dental professionals can accurately forecast a patient's craniofacial growth based on his or her genetic profile. It also offers preliminary perceptions on establishing novel gene-based treatment options for skeletal discrepancy.

CHAPTER 2

LITERATURE REVIEW

2.1 Epidemiology of Mandibular Prognathism

Mandibular prognathism prevalence varies in relation to the population (Li *et al.*, 2010a). Due to varying racial and ethnic backgrounds, impacted ratios and mandibular prognathism prevalence are diverse (Ko *et al.*, 2013). Populations from Southeast Asia, including Chinese and Malaysians, had the greatest prevalence rate of Class III malocclusion, ranging between 12.58% and 26.6%, according to meta-analysis research (Hardy, Cubas and Orellana, 2012). The incidence of Class III was highest among Malaysian Malay among Malaysia's three main ethnic groups, followed by Malaysian Chinese and Indian (Nowrin *et al.*, 2016; Elfseyie, Hassan and Al-Jaf, 2020). However, given that the Angle Classification method was utilized to appraise the occlusal condition, the frequency increased in the Malaysian population mainly because of the dental origin of the malocclusion (Elfseyie, Hassan and Al-Jaf, 2020). It is unknown if the skeletal traits had an impact because the study did not report it. On the other hand, research conducted by (Lim, Ahmad Shafiai and Kamaruddin, 2023) on Malay population found that the prevalence of Class III malocclusion was the lowest (12.6%) compared to Class I malocclusion (41.3%) and Class II malocclusion (46.1%). This study used angular measurement ANB to determine the malocclusion status of the subjects (Lim, Ahmad Shafiai and Kamaruddin, 2023). The ANB angle assesses the anteroposterior relationship between the maxilla and mandible. It is calculated as the difference between the SNA (sella-nasion to point A) and SNB (sella-nasion to point B) angles, using the formula: SNA - SNB = ANB. It was suggested that an individual with a Class II or Class III skeletal pattern may present with Class I malocclusion on

Angle's classification, and vice versa (Lim, Ahmad Shafiai and Kamaruddin, 2023). Figure 2.1 shows the lateral cephalometric tracing and its significant skeletal landmark, where:

- Point A: The deepest point between the anterior nasal spine and prosthion.
- Point B: The deepest point between the infradentale and pogonion.
- Nasion (Na): The deepest midline point at the frontonasal suture.
- Sella (S): Midpoint of the pituitary fossa, or sella turcica.

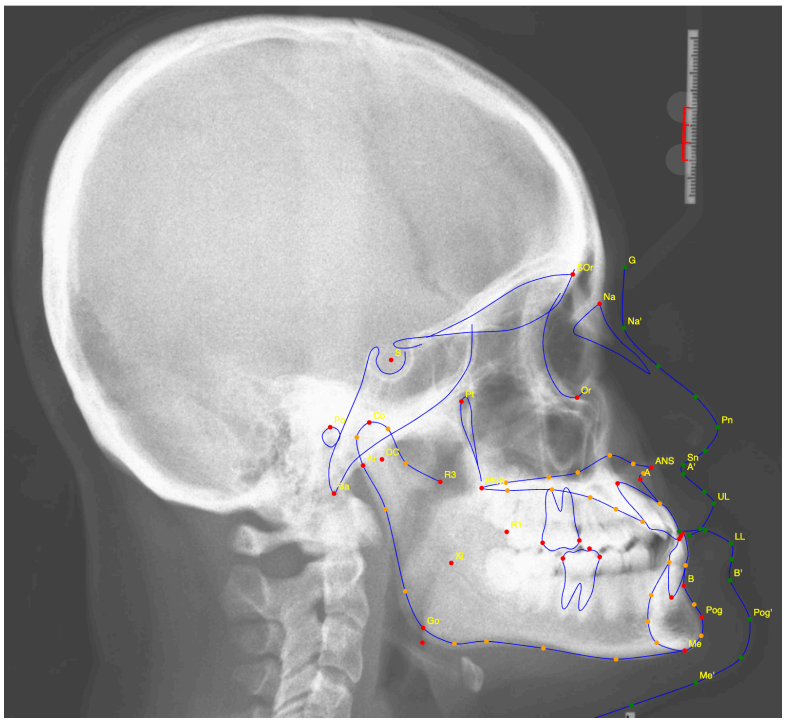

Figure 2.1 Skeletal Landmarks on Cephalometric Tracing

Between 9.48% and 11.38% of Middle Eastern population have mandibular prognathism, compared to an average prevalence rate of 4.88% in European populations (Hardy, Cubas and Orellana, 2012). African groups, on the other hand, had prevalence rates ranging from 1.22% to 19.72%, as opposed to the average incidence of 1.19% for the Indian population (Hardy, Cubas and Orellana, 2012). Additionally, it was asserted in other research that East Asians had a substantially higher prevalence of mandibular prognathism, accounting for 10% of the Japanese population (Kajii *et al.*, 2019a) and

2.1% to 10% in the Chinese group (Chen *et al.*, 2015b). However, it was noted that Caucasians had the lowest mandibular prognathism rates, ranging from 0.48% to 4.3% (Chen *et al.*, 2015b). Figure 2.2 shows a graph representing the prevalence of mandibular prognathism and Class III skeletal pattern from different populations.

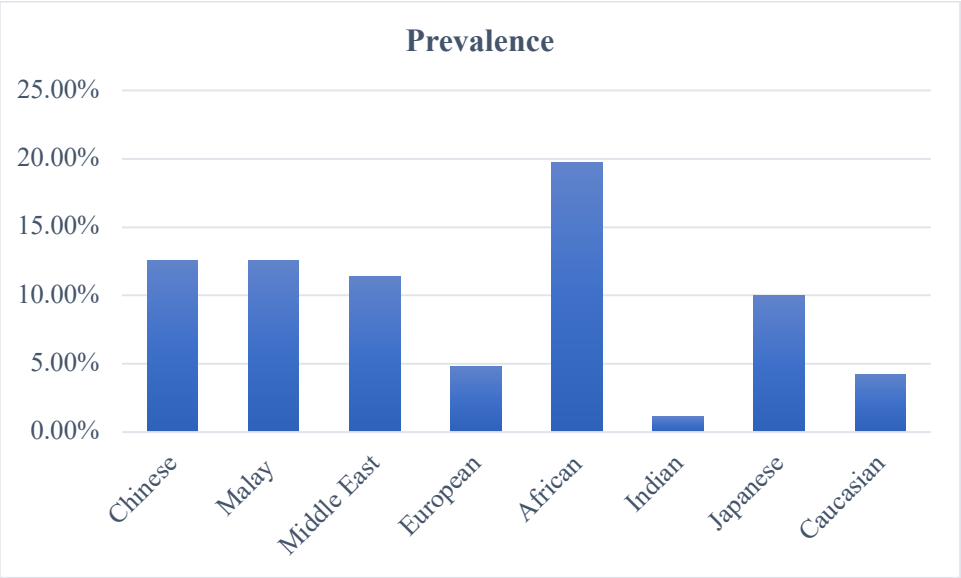

Figure 2.2 Epidemiology of Mandibular Prognathism and Class III Skeletal Pattern reported in different populations

The fact that there is an equal number of affected males and females in the Eastern Mediterranean population research indicates no definite relationship between gender and mandibular prognathism prevalence (Genno *et al.*, 2019a). Other studies likewise claimed no difference in the number of afflicted men and women based on gender (Litton *et al.*, 1970; Q Li *et al.*, 2011). However, a demographic study in Brazil discovered that Brazilian women were more likely than Brazilian men to have mandibular prognathism. These variations have been attributed to a number of reasons, including hormonal variations (which are primarily governed by genetics), developmental or environmental variations, and the placement of genes in the genome (X and Y chromosomes and mitochondrial deoxyribonucleic acid (DNA)) (Cruz, Krieger, Ferreira, Mah, Hartsfield, *et al.*, 2008). A study by (Ruslin *et al.*, 2015) of

Indonesian patients receiving treatment for mandibular prognathism revealed that females were more common than males. This finding on gender do not necessarily imply that women are more likely than men to have mandibular prognathism. This may suggest that women may be significantly more concerned with health and appearance than males and may seek therapy for dentofacial anomalies (Ruslin *et al.*, 2015).

Contrarily, research by (Elfseyie, Hassan and Al-Jaf, 2020) in Malaysian population revealed that males have a higher prevalence of mandibular prognathism. It is thought that the higher occurrence in males may be caused by the fact that males grow more quickly and for a longer period than females (Elfseyie, Hassan and Al-Jaf, 2020). Additionally, another research on the Malay population also revealed male subjects to have a more prevalent Class III skeletal pattern (17.0%) compared to females (11.7%) (Lim, Ahmad Shafiai and Kamaruddin, 2023). Table 2.1 shows the gender differences in the prevalence of mandibular prognathism and Class III skeletal pattern between male and female in different populations.

Table 2.1 Gender Differences in the Prevalence of Mandibular Prognathism and Class III skeletal pattern in different populations

Gender Difference	**Population**
Same	East Mediterranean, Chinese
Females are higher	Brazilian, Indonesian
Males are higher	Malaysian

2.2 Genetic Aspect of Mandibular Prognathism

The variance in malocclusion manifestation may be explained by the interplay of hereditary and environmental variables (Uribe *et al.*, 2013). Although there may be many diverse environmental variables, inherited Class III skeletal components are thought to cause Class III malocclusion (Ko *et al.*, 2013). Numerous genetic theories have also been developed to identify and clarify familial aggregation of mandibular prognathism linked to Class III skeletal base (Abdelhakim A El-Gheriani *et al.*, 2003).

However, these genetic analyses show discrete locations, candidate genes, and mode of inheritance for mandibular prognathism, suggesting the presence of a distinct mutation for non-syndromic mandibular prognathism within each distinct population.

2.2.1 Inheritance Pattern

According to documented data, the mandibular prognathism inheritance pattern is diverse and points to a polygenic transmission model, autosomal dominant inheritance, autosomal recessive inheritance, or dominant inheritance with incomplete penetrance (Li *et al.*, 2010b). Penetrance refers to the proportion of individuals with a specific genotype who display the associated clinical symptoms. When all individuals with the genotype develop symptoms by a certain age, the genotype is considered fully penetrant. In contrast, if fewer individuals express the symptoms, it is described as having reduced or incomplete penetrance (Kingdom and Wright, 2022).

One changed gene causes monogenic inheritance, which follows the Mendelian inheritance pattern (F Xue, Wong and Rabie, 2010). Numerous studies supported the monogenic hypothesis of mandibular prognathism inheritance. Two studies conducted segregation analyses of mandibular prognathism, which involved Libyan and Brazilian populations (Abdelhakim A El-Gheriani *et al.*, 2003; Cruz, Krieger, Ferreira, Mah, Hartsfield Jr, *et al.*, 2008). Autosomal dominant was shown to be the most sparing in a study of the Libyan population (Abdelhakim A El-Gheriani *et al.*, 2003). In contrast, research on the Brazilian group revealed that the mandibular prognathism's predominant mode of inheritance was autosomal dominant, with incomplete penetrance in 65.3% of the pedigrees (Cruz, Krieger, Ferreira, Mah, Hartsfield Jr, *et al.*, 2008). This was additionally supported by investigations into Chinese Han and Hispanic pedigrees, which suggest that autosomal dominant with incomplete penetrance is the most

probable inheritance pattern (Frazier-Bowers *et al.*, 2009a; Uribe *et al.*, 2013). Fifty-one family pedigrees from a study of a community in the Eastern Mediterranean demonstrated that autosomal dominant inheritance was common in most instances (Genno *et al.*, 2019a). Two studies using a visual analysis of the pedigree of the Chinese population also discovered an autosomal dominant form of mandibular prognathism inheritance (Li *et al.*, 2010b; Chen *et al.*, 2015b).

Polygenic or multifactorial inheritance is the inheritance of phenotypic traits that may be connected to two or more susceptibility genes and their interactions with the environment (F Xue, Wong and Rabie, 2010). For instance, research on the Korean population demonstrate that the hereditary predisposition of mandibular prognathism among Korean Class III patients primarily arises not from Mendelian transmission of major genes but rather the aggregation of minor effects from numerous different genes and the influence of environmental factors (Ko *et al.*, 2013). Furthermore, studies conducted on the Brazilian population indicate that 7.3% of the families have sporadic cases, possibly resulting from phenocopies, such as environmental exposure, isolated growth abnormalities, medication usage, or other modes of inheritance like autosomal recessive or new dominant mutations (Cruz, Krieger, Ferreira, Mah, Hartsfield Jr, *et al.*, 2008). Table 2.2 shows the summary of mode of inheritance of mandibular prognathism in different populations.

Table 2.2 Summary of Inheritance Patterns of Mandibular Prognathism reported in different populations

Inheritance Pattern	**Population**
Autosomal Dominant	Libyan, East Mediterranean, Chinese
Autosomal Dominant with Incomplete Penetrance	Brazil, Chinese Han, Hispanic
Polygenic method of transmission	Korean, Brazilian

2.2.2 Susceptible loci and candidate genes

It had been discovered that many potential genes and chromosomal loci were susceptible to mandibular prognathism. It was thought that one of the risk factors for mandibular prognathism is ethnicity (Moreno Uribe and Miller, 2015), and numerous studies were conducted within the Asian population because of the comparatively high prevalence of mandibular prognathism seen in Asian communities. However, because of the variable outcomes of these studies in various ethnic groups, there may be multiple mandibular prognathism-causing genes. Additionally, the small sample size of these researches was seen as a study limitation (Ikuno *et al.*, 2014a; Saito *et al.*, 2017). It was discovered that linkage analysis, which involves statistically analysing the segregation of traits and Single Nucleotide Polymorphisms (SNP) in afflicted families, is the most efficient method for locating loci and genes related to phenotypes. Advanced and more efficient molecular approaches, such as Next Generation Sequencing (NGS), have become widely used. The most recent genome discoveries allow one to pinpoint the sequence variations and regulatory components contributing to mandibular prognathism aetiology. As a result, some recently published articles about the genetic effects of mandibular prognathism are based on the results of Whole Exome Sequencing (WES) or Whole Genome Sequencing (WGS) (Doraczynska-Kowalik *et al.*, 2017). Additionally, the molecular mechanism governing jaw development is not precisely understood (X. Guan *et al.*, 2015).

Possible connections between mandibular prognathism and chromosomes 1p36, 6q25, and 19p13.2 were discovered after a genome-wide analysis of 90 affected patients from the Japanese and Korean populations, including 40 Korean sibling pairs and 50 Japanese sibling pairs. Positional candidate genes in the 1p36 locus relevant to the skeletal system include Matrilin-1 (cartilage matrix protein), heparan sulphate

proteoglycan 2, and alkaline phosphatase from the liver, bone, and kidney. These potential candidate genes were proposed to be involved in bone development, possibly leading to mandibular prognathism (Yamaguchi *et al.*, 2005). Two loci, 1q32.2 and 1p22.3, were identified as potential mandibular prognathism susceptibility regions by genome-wide association research on a Japanese cohort, and the candidate genes *SSX2IP* and *PLXNA2* were suggested. According to their theory, *PLXNA2* may lead to excessive mandibular growth. At the same time, *SSX2IP* regulates the activity of synovial tissue in the temporomandibular joint (TMJ), which may produce mandibular prognathism (Ikuno *et al.*, 2014b). Another genome-wide association analysis in a different Japanese population employed microsatellites to identify six mandibular prognathism loci (1p22.3, 1q32.2, 3q23, 6q23.2, 7q11.22, and 15q22.22) as well as the candidate genes *SSX2IP*, *PLXNA2*, *RASA2*, *TCF21*, *CALN1*, and *RORA*. Previous linkage analyses on Japanese patients supported the locus 1p22.3, but the other five loci are novel (Saito *et al.*, 2017). A study using WES in Japanese pedigrees reveals that an uncommon non-synonymous single-nucleotide variant (SNV) of the BEST3 gene may be a candidate for mandibular prognathism (Kajii *et al.*, 2019a).

Following that, studies on the Korean population found that the Matrilin-1 polymorphism haplotype TGC (ht4;158T, 7987G, and 8572C alleles) significantly increased the risk of mandibular prognathism, indicating that Matrilin-1 polymorphisms may act as a genetic susceptibility marker for mandibular prognathism. Matrilin-1 is a non-collagenous protein mainly expressed in cartilage and released by chondrocytes (J. Jang *et al.*, 2010). Additionally, chondrocytes in the TMJ condyle can secrete matrilin-1, especially in arthritic articular mandibular cartilage (Ohno *et al.*, 2003). On the other hand, a study on the Malay population in Malaysia revealed a family with Class III malocclusion to have the heterozygous missense mutation c.1094C>T (p. Thr 365 Ile),

rs370130918 in the DUSP6 gene. According to reports, the DUSP6 gene is one of the critical genes in the FGF/FGFR (FGF Receptor) signalling pathways essential for skeletal growth (Nowrin *et al.*, 2019).

In Chinese populations, mandibular prognathism was linked to several novel susceptibility loci and unique mutations. According to a study by (Xiong, Li, Cai, Chen, *et al.*, 2017), mandibular prognathism was linked to the variants rs79176051 in the fibroblast growth factor (FGF) 12 gene, rs13317 in *FGFR1*, and rs149242678 in *FGF7*. Increased facial height significantly correlates with the previously unreported SNP rs14924267 in *FGF7*. *FGFR1*, which performs various tasks during craniofacial morphogenesis, is found in the craniomaxillofacial skeleton, muscle, palate, teeth, and submandibular salivary gland. Variants in *FGF12* were found to have a significant impact on cleft lip in a recent study (Xiong, Li, Cai and Chen, 2017). A study by (Chen *et al.*, 2015a) also discovered a new heterozygous mutation in *FGF23*. The *FGF23* (c.35C>A) is found in the susceptibility locus of 12pter-p12.3 and is closely related to the mandibular prognathism phenotype. Asp will be substituted for Ala in codon 12 (p.A12D) of the *FGF23* protein as a result of the mutation *FGF23* (c.35C>A). The signal peptide's functionality may be compromised, and the p.A12D mutation may inhibit the secretory activity of *FGF23* (Chen *et al.*, 2015b). A single-nucleotide missense mutation has been found in the ADAMTS1 gene (c. 742I>T), which is thought to be closely associated with mandibular prognathism. However, earlier genome-wide association studies failed to discover this gene (X Guan *et al.*, 2015).

According to a single SNP study in the Chinese Han population, the SNP rs1793953 in the COL2A1 gene displayed a potential relationship with mandibular prognathism. The COL2A1 gene on chromosome 12q13 is crucial for cartilage formation (F Xue, Rabie and Luo, 2014). In addition, a different genome-wide linkage

analysis of the Chinese Han population finds *LTBP2* and *TGFB3* as possible functional genes that are likely implicated in the development of the craniofacial region and potentially associated with mandibular prognathism (Q. Li *et al.*, 2011).

Additionally, (Genno *et al.*, 2019b) observed that no previously identified genes connected to mandibular prognathism matched the genetic analysis of their samples from the East Mediterranean. Rather, they identified three distinct genes (*C1orf167*, *NBPF8*, *NBPF9*) on chromosome 1 that might be connected to mandibular growth and prognathism (Genno *et al.*, 2019b). In a non-parametric linkage investigation of a Hispanic population, three genes, *IGF1*, *HOXC*, and *COL2A1*, on loci (11q22, 12q13.13, and 12q23, respectively), and five loci, 1p22.1, 3q26.2, 11q11, 12q13.13, and 12q23, were found to be candidate genes (Frazier-Bowers *et al.*, 2009b). However, a rare mutation (Gly1121Ser) in the ARHGAP21 gene was shared by every mandibular prognathism individual in the larger family division in the Caucasian population, with nearly complete penetrance. The *ARHGAP21* protein enhances cell-cell adhesions, which may be regulated by bone morphogenetic elements and impact the development of the mandible (L Perillo *et al.*, 2015). All susceptible loci and candidate genes are listed in Table 2.3.

Table 2.3 Susceptible loci and candidate genes related to Mandibular Prognathism in different populations

Susceptible loci and candidate genes that cause mandibular prognathism	**Affected population**
Chromosomes 1p36, 6q25, and 19p13.	Korean and Japanese sibling pairs
Six susceptible loci; 1p22.3, 1q32.2, 3q23, 6q23.2, 7q11.22, and 15q22.22	Japanese
Two loci; 1q32.2 and 1p22.3	
Candidate genes SSX2IP, PLXNA2, RASA2, TCF21, CALN1, and RORA	
A rare non-synonymous single-nucleotide variant (SNV) of the BEST3 gene	
Matrilin-1 polymorphism haplotype TGC (ht4;158T, 7987G, and 8572C alleles)	Korean
A heterozygous missense mutation c.1094C>T (p. Thr 365 Ile) in the DUSP6 gene	Malay
Variant rs79176051 in FGF12, variant rs13317 in FGFR1, and variant rs149242678 in FGF7	Chinese
Mutation of p.A12D in FGF23	
A single-nucleotide missense mutation in ADAMTS1 gene (c. 742I>T)	
SNP rs1793953 in the COL2A1 on chromosome 12q13	Chinese Han
TGFB3 and LTBP2 as potential functional genes	
Three unique genes (C1orf167, NBPF8, NBPF9) on chromosome 1	East Mediterranean
Five loci (1p22.1, 3q26.2, 11q11, 12q13.13, and 12q23) and three candidate genes (IGF1, HOXC, and COL2A1)	Hispanic
A rare variant (Gly1121Ser) in the ARHGAP21 gene	Caucasian

CHAPTER 3

MATERIALS AND METHODS

3.1 Ethics

Ethical approval was obtained from The Human Research Ethics Committee of Universiti Sains Malaysia (USM/JEPeM/20120609) on 1st February 2021 (Appendix A).

3.2 Sample Size Calculation

The sample size was estimated based on (Kajii *et al.*, 2019b). The sample size for this study was calculated based on the need to detect a significant difference in exposure between cases and controls. Prior data indicate that the probability of exposure among controls is 0.50. Assuming that the true probability of exposure among cases is 1.00, a case-control ratio of 1:1 was chosen to maximize study power while conserving resources. Based on these parameters, the study was designed to achieve 80% power with an alpha level of 0.05 to reject the null hypothesis that the exposure rates for cases and controls are equal.

Using standard formulas for sample size calculation in case-control studies:

$$n = \frac{(Z\alpha/2 + Z\beta)2 \times (p1(1 - p1) + p2(1 - p2))}{(p1 - p2)2}$$

Where:

- $p1$ = probability of exposure in cases = 1.00
- $p2$ = probability of exposure in controls = 0.50
- $Z\alpha/2$ = Z-score for a two-sided alpha level of 0.05 ≈ 1.96
- $Z\beta$ = Z-score for power of 80% ≈ 0.84

Substituting these values:

$$n = \frac{(1.96 + 0.84)2 \times (1.00 \times (1 - 1.00) + 0.50 \times (1 - 0.50))}{(1.00 - 0.50)2}$$

Simplifying further:

$$n = \frac{(2.80)2 \times (0 + 0.25)}{0.25}$$

$$n = \frac{7.84 \times 0.25}{0.25} = 7.84$$

The sample size was rounded up to include 11 case subjects and 11 control subjects, resulting in a total of 22 participants. This sample size is sufficient to detect a significant association between exposure and mandibular prognathism in our target population if one exists.

3.3 Study Design and Population Samples

The reference population for this study consisted of Malaysian Malay individuals with a three-generation Malay lineage exhibiting mandibular prognathism without maxillary hypoplasia. The source population included Malay patients with mandibular prognathism, excluding maxillary hypoplasia, who visited the AMDI Dental Clinic during the study period.

3.4 Inclusion and Exclusion Criteria

3.4.1 The inclusion criteria were as follows:

i. Malaysian Malay individuals with at least three generations of documented ancestry.

ii. Age 17 years or older.

iii. Presence of Class III malocclusion.

iv. Reverse overjet or edge to edge incisor relationship.

v. ANB angle less than 1°.

vi. Normal or prognathic maxilla (SNA > 83.7 ± 2.82).

vii. Prognathic mandible (SNB > 84.06).

viii. Family history of mandibular prognathism, including diagnosed siblings or other relatives.

ix. In partially edentulous cases, presence of complete anterior teeth in both upper and lower jaws.

x. Ability to provide informed consent.

3.4.2 The exclusion criteria were as follows:

i. Those with congenital abnormalities, for instance, cleft lip and palate and other craniofacial syndromes.

ii. Patients with a history of facial trauma.

iii. Fully edentulous patients.

iv. History of chemo/radiotherapy in the past.

v. Presence of systemic disorders, for example, heart and endocrine diseases.

3.4.3 The control group criteria were as follows:

i. Malaysian Malay individuals with at least three generations of documented ancestry.

ii. Individuals with a straight facial profile

iii. Overjet between 2 mm to 4 mm

iv. Class I malocclusion

v. ANB angle 2° to 4°

vi. Normal maxillary and mandibular angle in relation to the anterior cranial base

3.5 Method of Sampling

To identify patients with Class III phenotypes, the records of Malay orthodontic patients who attended AMDI Dental Clinic from January 2010 to December 2020 was reviewed, which included the clinical pre-treatment extraoral photographs and pre-treatment lateral cephalograms. Approximately 275 lateral cephalometric and extraoral photographs were retrospectively obtained and analyzed to confirm the presence of Class III skeletal pattern with absence of maxillary hypoplasia among these individuals. Patients with Class III phenotypes were contacted and interviewed through phone calls to confirm their three-generation Malaysian Malay lineage and family history of similar disorders. Patients with a positive family history and confirmed three-generation Malaysian Malay lineage who fulfilled the selection criteria were invited to participate in the genetic study.

3.6 Diagnosing Mandibular Prognathism

Patients underwent initial clinical examinations at AMDI Dental Clinic to diagnose mandibular prognathism. The incisor relationship was assessed. Patients who exhibited mandibular incisor edges positioned anterior to the cingulum plateau of the maxillary incisors were classified as having a Class III malocclusion (British Standards Institution., 1983). The patients' facial profiles were also evaluated. Patients with concave or straight facial profiles indicated a Class III jaw relationship (Stellzig-Eisenhauer, Lux and Schuster, 2002). Patients were subsequently referred for lateral cephalography x-rays to confirm the diagnosis of mandibular prognathism. To ensure precision in diagnosing mandibular prognathism, radiographic images were captured

using Planmeca Romexis® 3.8.3.R (Planmeca, Helsinki, Finland). These digital images were then exported to an online digital cephalometric platform, WEBCEPH™ Version 1.5.0 (AssembleCircle Corp, Gyeonggi-do, Republic of Korea), for analysis (Figure 3.1). Cephalometric values were analysed using the Steiner Analysis specific to the Malay population (Mohammad, Abu Hassan and Hussain, 2011) (Appendix B).

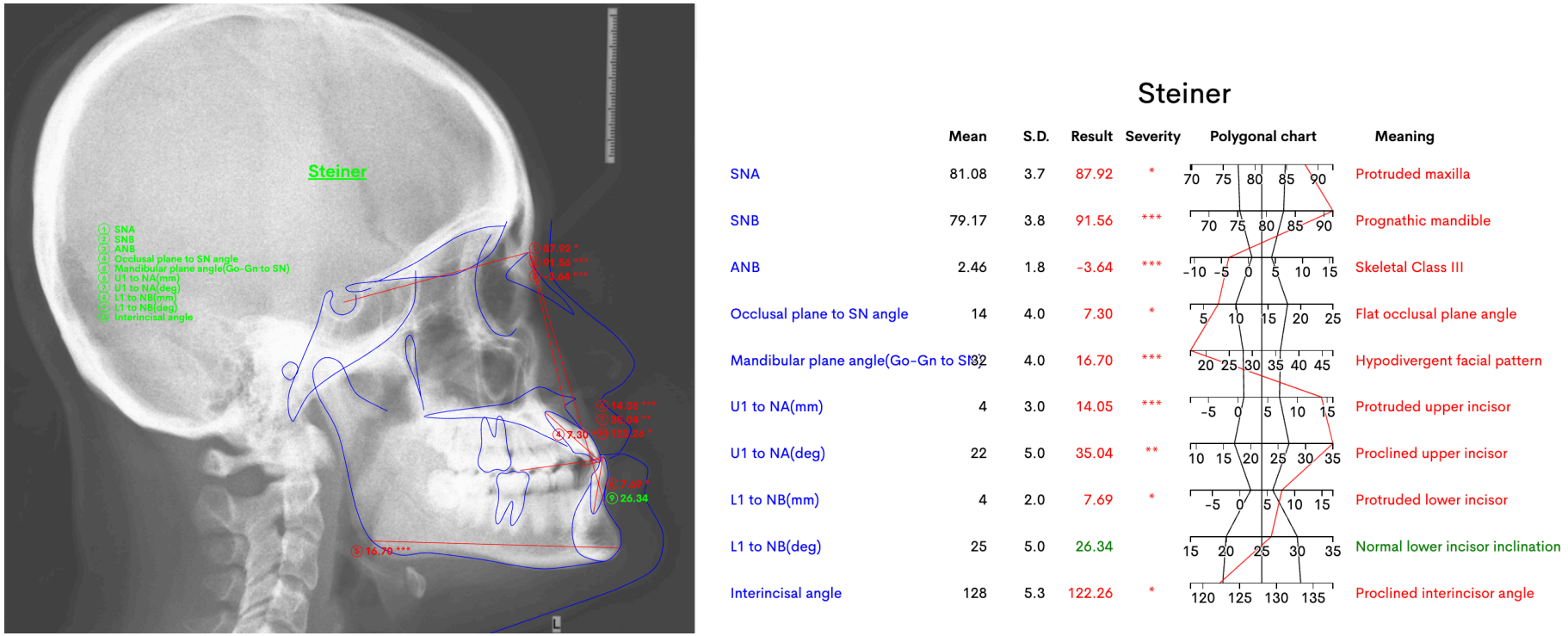

Figure 3.1 A cropped section of a screenshot from the WEBCEPH™ cephalometric analysis webpage

The figure on the left depicts a sample lateral cephalogram tracing from the website. The figure on the right shows the chart derived from Steiner's analysis based on the radiograph.

3.7 DNA Extraction and Quantification

Each individual (case and control) had a total of 6 ml of venous blood drawn to extract genomic DNA. The blood samples were then sent to the Advanced Diagnostic Lab (ADL), USM, for DNA Extraction. Using the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany), genomic DNA will be extract in accordance with the developer's procedure. Before the DNA was extracted, the samples were equilibrated at room temperature. All the relevant tubes were labelled based on the sample names.

Firstly, 20 µl of Qiagen Protease was pipetted into the bottom of a 1.5 microcentrifuge tube. Then, 200 µl of blood was added into the tube. Qiagen protease enzymatically lyse the cells. 200 µl of lysis buffer (AL) was added to the sample and mixed by vortexing for 15 seconds. After that, the samples were incubated in a water bath at 56°C for 10 minutes.

Next, the 1.5 microcentrifuge tube was briefly centrifuged to remove drops from the inside of the lid. 200 µl of Ethanol was added to the sample, followed by vortexing the sample for 15 seconds to mix it. The tube was briefly centrifuged. The mixture was then transferred to the QIAamp spin column (in a 2 ml collection tube) without wetting the rim, and the cap was closed. The mixture was then centrifuged at 8000 rpm for 1 minute.

Next, the spin column was transferred to a new, clean 2 ml collection tube and the old collection tube containing the filtrate was discarded. 500 µl wash buffer (AW1) was added, and the mixture was centrifuged at 8000 rpm for 1 minute.