MOLECULAR CHARACTERIZATION OF *LEPR* Q223R SINGLE NUCLEOTIDE POLYMORPHISM (rs1137101) AND SALIVARY LEPTIN LEVELS IN DIFFERENT FACIAL SKELETAL PATTERNS

BY

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ABSTRACT

Leptin hormone regulates several key physiological processes which include signalling for energy homeostasis, endocrine function and bone metabolism and physiology. There is however a gap in the literature regarding leptin's role and its association with the facial skeletal pattern that is heavily related to the dynamic of growth, bone deposition and bone resorption. The role of genetic factors in the growth of the maxilla and the mandible is also still largely being investigated. The Q223R (rs1137101) single nucleotide polymorphism (SNP) of the leptin receptor (*LEPR)* gene has previously been associated with abnormal growth of the maxilla. Limited studies have been done with regards to its association with mandibular growth. The aim of this study therefore is to determine the molecular characterization of the *LEPR* Q223R (rs1137101) SNP and salivary leptin levels in different classes of facial skeletal pattern. A total of 82 participants from Kuantan, Pahang were recruited to participate in this case control study with regards to the examination of the molecular characterization of the *LEPR* Q223R (rs1137101) SNP and a total of 62 participants from Kuantan, Pahang were recruited to participate in the case control study of leptin hormone levels in different classes of facial skeletal pattern. Based on the lateral cephalometric analysis, the subjects were grouped into Class I, II and III facial skeletal patterns, according to Eastman and Wits appraisal. For the genetic analysis, DNA extraction and isolation from the unstimulated saliva samples was done. The samples were then subjected to polymerase chain reaction (PCR) amplification. Subsequently, restriction fragment length polymorphism (RFLP) technique was used for the genotyping analysis using the MspI restriction enzyme. Statistical analysis with the Chi-square (χ^2) test was used to compare genotype and allele frequencies between the different classes of facial skeletal pattern while the Hardy-Weinberg Equilibrium (HWE) was applied to assess distribution of genotype frequency between the classes of facial skeletal pattern. The results of the genetic study indicate no significant association between genotype frequency between the control group (Class I facial skeletal pattern) and Class II (p=0.48) and Class III (p=0.16) facial skeletal pattern. There was also no significant association between allele frequency between the control group (Class I facial skeletal pattern) and Class II (p=0.82) and Class III (p=0.32) facial skeletal pattern. For the leptin hormone analysis, unstimulated saliva samples were taken and purified to undergo leptin enzyme-linked immunosorbent assay (ELISA) analysis to determine the levels of leptin hormone. Statistical analysis using the Kruskal-Wallis test was used to analyse the data obtained. Results of the leptin hormone analysis indicate that there is a significant difference in the median levels of leptin hormone between the control group (Class I facial skeletal pattern) and Class II (p=0.004) and Class III (p=0.003) facial skeletal pattern. In conclusion, for the Malaysian population, there is no significant association between the Q223R (rs1137101) SNP of the *LEPR* gene in different classes of facial skeletal pattern. There is however a significant association between salivary leptin hormone levels between the control group and Class II and Class III facial skeletal pattern. This study suggests that leptin hormone may be used as an indicator to predict the growth of the facial skeletal pattern and to facilitate interceptive orthodontic treatment if necessary. More studies however are required to consolidate the results of both the genetic study and the study of leptin hormone in different classes of facial skeletal pattern.

خلاصة البحث

ينظم هرمون اللبتين العديد من العمليات الفسيولوجية الرئيسية التي تشمل إرسال إشارات لتوازن الطاقة ووظيفة الغدد الصماء واستقلاب العظام. ومع ذلك ، هناك فجوة في البحوث المتعلقة بدور اللبتين وارتباطه بنمط الهيكل العظمي للوجه والذي يرتبط ارتباطًا وثيقًا بديناميكية النمو وترسب العظام وارتشاف العظام. لا يزال دور العوامل الوراثية في نمو الفك العلوي والفك السفلي قيد التحقيق إلى حد كبير. البحوث السابقة ربطن تعدد أشكال النوكليوتيدات المفردة (SNP) (rs1137101) لجين مستقبل اللبتين (*LEPR*) بالنمو غير الطبيعي للفك العلوي. لكن لم يتم إجراء أي دراسات سابقة فيما يتعلق بارتباطه بنمو الفك السفلي. الهدف من هذه الدراسة هو تحديد الخصائص الجزيئية لـ *LEPR* Q223R (rs1137101) SNP ومستويات اللبتين اللعابية في فئات مختلفة من نمط الهيكل العظمي للوجه. ضمت هذه الدراسة 82 متطوعا لدراسة هذه الحالة فيما يتعلق بفحص التوصيف الجزيئي لـ *LEPR* Q223R (rs1137101) SNP كما ضمت هذه الدراسة 62 متطوعا للمشاركة في دراسة مستويات هرمون اللبتين في فئات مختلفة من نمط الهيكل العظمي للوجه. بناءً على التحليل الشعاعي السيفالومتري ، تم توزيع المشاركين في أنماط الهيكل العظمي للوجه من الفئة الأولى والفئة الثانية والفئة الثالثة ، وفقًا لتقييم إيستمان وويتس. بالنسبة للتحليل الجيني، تم إجراء استخلاص وعزل الحمض النووي من عينات اللعاب غير المحفزة. ثم خضعت العينات لتضخيم تفاعل البلمرة المتسلسل (PCR) لتحليل التنميط الجيني باستخدام RFLP). بعد ذلك ، تم استخدام تقنية تعدد الأشكال لطول جزء التقييد (PCR) لمقارنة ترددات النمط الجيني والأليل بين Chi-square. تم التحليل الإحصائي باستخدام اختبار MspIإنزيم تقييد لتقييم توزيع تردد النمط HWE) الفئات المختلفة لنمط الهيكل العظمي للوجه. بينما تم تطبيق توازن هاردي-وينبرغ (الجيني بين فئات الهيكل العظمي للوجه. تشير نتائج الدراسة الجينية إلى عدم وجود ارتباط بين تكرار النمط الجيني بين مجموعة التحكم (نمط الهيكل العظمي للوجه من الفئة الأولى) والفئة الثانية (ع = 0.48) والفئة الثالثة (ع = 0.16) لنمط الهيكل العظمي للوجه. لم يكن هناك أيضًا ارتباط كبير بين تردد الأليل بين مجموعة التحكم (نمط الهيكل العظمي للوجه من الفئة الأولى) مع نمط الهيكل العظمي للوجه من الفئة الثانية (ع = 0.82) والفئة الثالثة (ع = 0.32). لتحليل هرمون اللبتين. تم أخذ عينات من اللعاب غير المحفز وتنقيتها للخضوع لتحليل مقايسة الممتز المناعي المرتبط بإنزيم ELISA) اللبتين (Kruskal-Wallis) لتحديد مستويات هرمون اللبتين. تم استخدام التحليل الإحصائي باستخدام اختبار لتحليل البيانات التي تم الحصول عليها. تشير نتائج تحليل هرمون اللبتين إلى وجود اختلاف كبير في المستويات المتوسطة لهرمون اللبتين بين مجموعة التحكم (الفئة الأولى من نمط الهيكل العظمي للوجه) مع نمط الهيكل العظمي للوجه من الفئة الثانية (ع = 0.004) والفئة الثالثة (ع = 0.003). في الختام ، بالنسبة لسكان ماليزيا ، لا يوجد ارتباط كبير بين *LEPR* من جين SNP (rs1137101) Q223R في فئات مختلفة من نمط الهيكل العظمي للوجه. ومع ذلك ، هناك ارتباط كبير بين مستويات هرمون اللبتين اللعابي بين مجموعة التحكم ونمط الهيكل العظمي للوجه من الصنف الثاني والثالث. تشير هذه الدراسة إلى أنه يمكن استخدام هرمون اللبتين كمؤشر للتنبؤ بنمو نمط الهيكل العظمي للوجه ولتسهيل علاج تقويم الأسنان الاعتراضي إذا لزم الأمر. ومع ذلك ، هناك حاجة إلى مزيد من الدراسات لتوحيد نتائج كل من الدراسة الجينية ودراسة هرمون اللبتين في فئات مختلفة من نمط الهيكل العظمي للوجه

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality as a thesis for the degree of Master of Science.

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Assoc. Prof. Dr. Jesni bin Shamsul Shaari

DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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LIST OF ABBREVIATIONS

A	A point
Allele Frequency Aggregator	ALFA
ANB	A point-nasion-B point
AO	A point-functional occlusal plane
B	B point
BMI	Body Mass Index
BO	B-point-functional occlusal plane
bp	Base pair
BSI	British Standard Institute
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FOP	Functional Occlusal Plane
HRP	Horseradish Peroxidase
HWE	Hardy-Weinburg equilibrium
IIUM	International Islamic University Malaysia
IREC	International Islamic University Malaysia Research and Ethics Committee
LEP	Leptin
LEPR	Leptin receptor
N	Nasion
Optical density	OD
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length
S	Sella turcica
SNA	Sella-nasion-A point
SNB	Sella-nasion-B point
SNP	Single nucleotide polymorphism
SV	Spin/vacuum
WHO	World Health Organization

LIST OF SYMBOLS

%	Percentage, percent
±	plus-minus
≤	Less or equal to
≥	More or equal to
°	degrees
°C	degrees Celcius
mg	milligram
ml	millilitres
mm	millimeters
rpm	revolutions per minute
xg	times gravity
µL	microlitres

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Genetic association studies have been used to correlate genotype with and individual physical characteristic or disease. In this study, we will be looking at the Leptin Receptor gene (*LEPR*) Q223 Single Nucleotide polymorphism (SNP) and whether the association between genetic variations and phenotype could act as a diagnostic tool for the development of the facial skeletal pattern. There is also a gap in literature with regards to this which we aim to address.

Leptin hormone regulates several key physiological processes which include signaling for energy homeostasis, endocrine function and bone metabolism (La Cava, 2017). Results from various studies indicate that leptin may play a significant role in bone physiology, independent of the central nervous system (Reid et al., 2018).

The function of leptin is exerted through its receptor, the leptin receptor (*LEPR*) (Tartaglia, 1997). Q223R (rs1137101) is a single nucleotide polymorphism (SNP) of the *LEPR* gene (Li et al., 2017). There is a substitution of glutamine to arginine at position 22 or the *LEPR* protein and this is associated with leptin resistance as there is a reduction in leptin binding activity (Quinton et al., 2001).

There is however a gap in the literature regarding leptin's role and its association with the facial skeletal pattern that is heavily related to the dynamic of growth, bone deposition and bone resorption. This prefatory study hopes to provide initial information on leptin in this aspect.

1.2 STATEMENT OF THE PROBLEM

Malocclusion and its treatment can affect physical health in terms of pain (e.g. temporomandibular disorders, and dental/gingival trauma), speech and mastication. Socially, malocclusion and its treatment can affect perceived attractiveness by others, social acceptance and perceived intelligence (Zhang et al., 2006).

While the aetiology of malocclusion is inevitably the result of both an intricate genetic and environmental factor, there is evidence for a significant genetic influence in many dental and occlusal variables. The influence of genetics however varies according to the trait under consideration and in general remains poorly understood (Mossey, 1999b).

This study was designed to examine the relationship between the molecular characterization of rs1137101 polymorphism of the leptin receptor gene *(LEPR*) Q223R and salivary leptin levels in different classes of malocclusion with hopes to provide genetic knowledge that could lead to solutions to overcome the problems mentioned above and possible treatment for malocclusions.

1.3 RESEARCH OBJECTIVES

1.3.1 General Objectives

To determine the molecular characterization of the leptin receptor (*LEPR*) Q223R single nucleotide polymorphism (rs1137101) and salivary leptin levels in different classes of facial skeletal pattern.

1.3.2 Specific Objectives

1. To investigate the relationship between the molecular characterization of the leptin receptor (*LEPR*) Q223R single nucleotide polymorphism (rs1137101) in Class I, Class II and Class III facial skeletal pattern.
2. To investigate the relationship between salivary leptin levels in in Class I, Class II and Class III facial skeletal pattern.

1.4 RESEARCH QUESTIONS

1. Is there any difference in the molecular characterization of *LEPR* Q223R single nucleotide polymorphism (rs1137101) in different classes of facial skeletal pattern?
2. Is there any difference in the salivary leptin hormone levels in different classes of facial skeletal pattern?

1.5 SIGNIFICANCE OF THE STUDY

The specific purpose of this research is to examine the relationship between the molecular characterization of (*LEPR* Q223R) single nucleotide polymorphism (RS1137101) and salivary leptin levels in different classes of facial skeletal pattern.

With these results, we aim to improve the understanding of whether certain gene expressions and leptin hormone can affect the development of different malocclusions.

It is hoped that this current study will be able to produce new information and knowledge in genetics which in turn is a prerequisite for potential for genetic and/or environmental manipulation in orthodontic therapy and to provide a basis on how to possibly prevent and treat malocclusions at the stage of development. As such, this study will provide a key to develop novel treatment approaches in the future and for the benefit of the ummah.

1.6 HYPOTHESIS

1.6.1 Null Hypothesis

There is no difference in the molecular characterization of (*LEPR* Q223R) single nucleotide polymorphism (rs1137101) and salivary leptin levels in different classes of facial skeletal pattern.

1.6.2 Researcher's hypothesis

1. There is a difference in the molecular characterization of (*LEPR* Q223R) single nucleotide polymorphism (rs1137101) in different classes of facial skeletal pattern.
2. There is a difference in the salivary leptin levels in Class II and III facial skeletal pattern compared to the control group, Class I facial skeletal pattern.

1.7 LIMITATIONS OF THE STUDY

With regards to the methodology, some of the limitations noted in the study include sample size. A larger sample size would be appropriate to further strengthen the validity of the results obtained from this study as we are able to obtain narrower intervals thus producing more precise results (Hackshaw, 2008). Other than that, the lack of previous research studies done on the topic also provided limitations towards our sample size calculation. However, this further strengthened the need for further research into the topic in order to fill the gap in knowledge in this area.

In terms of the target population, another limitation of this study includes having patients of multiple racial backgrounds in the study which could be a factor in determining the outcome of the results. With a larger sample size, we could split the participants according to race therefore, enabling us to examine the SNP in further detail.

CHAPTER TWO

LITERATURE REVIEW

2.1 MALOCCLUSION

2.1.1 Definition

Malocclusion can be defined as a noticeable deviation from the ideal occlusion that may be considered aesthetically unsatisfactory (Houston et al., 1992). It is one of the most common dental problems in mankind across nations (Zhang et al., 2006). The condition of malocclusion may lead to a distorted facial appearance, limited masticatory function, increased risk of dental trauma and a compromised quality of life (Claudino & Traebert, 2013).

2.1.2 Classification of Malocclusion

Malocclusion can commonly be classified in two ways, namely the Angle classification and the British Standard Institute (BSI) classification. For the purposes of this study, we will be using the BSI classification in which the malocclusion is determined by looking at the relationship of the lower incisors to the upper incisors.

Class I can be defined as the lower incisor edges occluding with or lying immediately below the cingulum plateau of the upper central incisors. Class II malocclusion is when the lower incisor edges lie posterior to the cingulum plateau of the upper incisors. The Class III malocclusion is when the lower incisor edges lie anterior to the cingulum plateau of the upper incisors and when the overjet is reduced or reversed (Barreto, 2020; Mageet, 2016).

2.1.3 Aetiology of Malocclusion

An overview of the aetiology of malocclusion would suggest that it is a result of genetic factors, environmental factors, and a combination of both. There are several factors that can contribute to malocclusion namely, facial skeletal pattern in the transverse, sagittal, and coronal planes; soft tissue factors and dental factors (Littlewood et al., 2019).

2.1.3.1 Aetiological factors contributing to the class II malocclusion

The aetiology of the Class II malocclusion is multifactorial and the factors contributing towards it include the facial skeletal pattern, soft tissues, habits, and dental factors.

With regards to the facial skeletal pattern, the class II facial skeletal pattern presents with mandibular retrognathism, midface protrusion or both. Vertical dysplasia whereby there is a reduced lower facial height and an increased length of the anterior cranial base also is contributory (Shaughnessy & H.Shire, 1988). The decrease of the absolute length of the mandibular body is the second most frequent etiological factor noted in the Class II sample studied by El Hajj et al., 2017.

A more obtuse skull base flexion, in association or not with a greater length of the anterior skull base, can also contribute to the development of Class II division 1 malocclusion (de Almeida et al., 2017a).

The figure below demonstrates the intra-oral view of the Class II skeletal pattern (Figure 2.1).

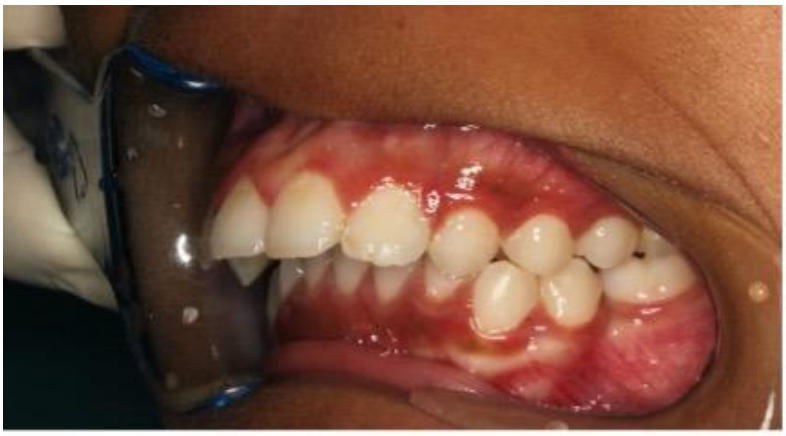

Figure 2.1 The intra-oral view of the Class II skeletal pattern

2.1.3.2 Aetiological factors contributing to the class III malocclusion

The Class III malocclusion occurs when there is a discrepancy between the growth of the maxilla and the mandible coupled together with dentoalveolar and soft tissue compensation (Sanborn, 1955).

The aetiology of the Class III malocclusion is also multifactorial and is a result of both hereditary genetic factors together with environmental factors (Zere et al., 2018) , (Kawala et al., 2007). There is sufficient evidence to suggest that genetic influence plays a significant role in mandibular growth (Doraczynska-Kowalik et al., 2017).

It is associated with mandibular prognathism and/or maxillary retrognathism (Staudt & Kiliaridis, 2009), (Ellis & McNamara, 1984). A study by Staudt & Kiliaridis, 2009 concluded that 75% of the Class III malocclusion originated from the facial skeletal pattern due to mandibular prognathism or macrognathia.

Environmental factors also come into play and influence the development of the Class III malocclusion including functional mandibular shifts due to respiratory needs, enlarged tongue size and pharyngeal airway

shape/size, hormonal imbalances such as pituitary adenomas, premature loss of deciduous teeth and congenital anatomical defects such as cleft lip and palate (Zere et al., 2018).

The figure below demonstrates the extra-oral view of the Class III skeletal pattern (Figure 2.2).

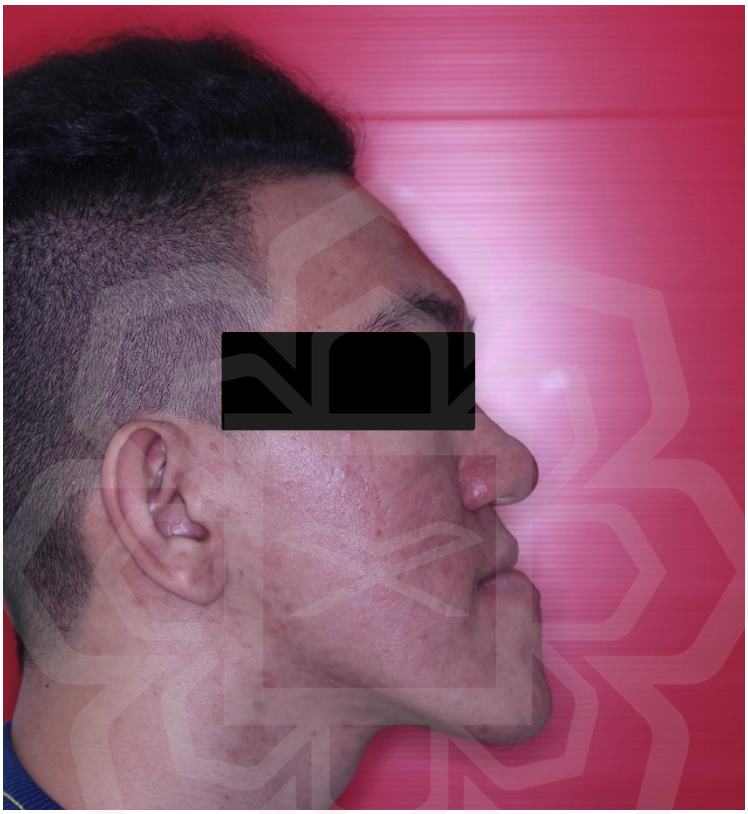

Figure 2.2 The extra-oral view of the Class III facial skeletal pattern

2.2 GENETICS OF MALOCCLUSION

2.2.1 The genetic aspect of Malocclusion

As the facial skeletal pattern is an aetiological factor for Class II and III malocclusions, an understanding the factors that influence skeletal growth is essential. Understanding how genetic factors along with environmental factors affect the pattern of facial skeletal growth is vital to the treatment of malocclusions through orthodontics (Mokhtar et al., 2020).

Mossey, 1999a suggested that although the genetics influencing bone development play a part in the development of the facial skeletal pattern, the genetics involved with neurological, muscular and neuromuscular fields also have an indirect effect on the facial skeletal pattern.

It is therefore beneficial to identify the genetic polymorphisms that may affect the development of the above structures as it may provide useful information to be used as diagnostic techniques for identifying facial skeletal pattern growth which in turn, would influence orthodontic treatment (Mossey, 1999b).

2.2.2 Genes associated with malocclusion

Multiple studies have been done on the relationship of genetics to various malocclusions. Some of them include a study done by Nazirah Yahya et al., 2017 which showed that nucleotide changes in rs10850110 within the *MYO1H* gene observed in mandibular prognathism of local Asian Malay population. A similar study by Cruz et al., 2017 found that *MYO1H* (rs10850110) was associated with an increased risk for the mandibular prognathism phenotype. Conversely, observations in a recent study by