PREVALENCE OF DENTAL ANOMALIES AND GENETIC ABERRATIONS OF NON-SYNDROMIC CLEFT-LIP WITH OR WITHOUT CLEFT PALATE PATIENTS WITH HYPODONTIA

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PREVALENCE OF DENTAL ANOMALIES AND GENETIC ABERRATIONS OF NON-SYNDROMIC CLEFT-LIP WITH OR WITHOUT CLEFT PALATE PATIENTS WITH HYPODONTIA

by

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LIST OF SYMBOLS

%	Percentage
n	Sample size
Z_{α}	Level of confidence
Δ	Delta
$\Delta\Delta CT$	Comparative CT
ΔCT	CT difference
km^2	Square kilometres
kb	Kilobase

LIST OF ABBREVIATIONS

A	Adenine
ABCA4	ATP binding cassette subfamily A member 4
AKAP8	A-kinase anchoring protein 8
AGCC	Affymetrix Gene Chip Command Console
AP2	Apetala 2
Array-CGH or α-CGH	Array-based comparative hybridization
ASCL5	Achaete-Scute Family BHLH Transcription Factor 5
AXIN2	Axis Inhibition Protein 2
BMP	Bone Morphogenetic Protein
BCL/P	Bilateral cleft lip with or without cleft palate
BAC	Bacterial artificial chromosome
BRD4	Bromodomain Containing 4
C	Cytosine
cDNA	Complementary DNA
CDH1	Cadherin 1
CL	Cleft lip
CLP	Cleft lip and palate
ChAS	Chromosome analysis suite
CN	Copy number

CNC	cranial crest
CNVs	copy number variations
CNV	copy number variation
CNAs	Copy number alterations
cnLOH	copy neutral LOH
CNL-LOH	Copy number losses
CMA	Chromosomal microarray analysis
CRKL	CRK Like Proto-Oncogene
CRS	Caudal regression syndrome
CT	Cycle threshold
CI	Confidence interval
DECIPHER	Database of chromosomal imbalance and phenotype in humans using ensemble resources
DI	Dens invaginatus
DNA	Deoxyribonucleic acid
DSBs	Double-strand breaks
DNase1	Deoxyribonuclease1
DNA	Deoxyribonucleic acid
Exons	protein-coding area
EDA	Ectodysplasin-A
EDAR	Ectodysplasin A receptor (EDAR)

TGFB3	Transforming Growth Beta 3
FGF	Fibroblast Growth Factor
FGFR1	Fibroblast Growth Factor Receptor 1
FGFR4	Fibroblast Growth Factor Receptor 4
FGFs	Fibroblast Growth Factors
FGFRs	FGF receptors
FGF3	Fibroblast Growth Factor 3
FGF7	Fibroblast Growth Factor 7
FGF10	Fibroblast Growth Factor 10
FGF18	Fibroblast Growth Factor 18
FGFR	FGF receptor
FGFR2	Fibroblast Growth Factor 2
FHIT	Fragile histidine triad diadenosine triphosphatase
FOSTERS	Fork stalling and template switching
FISH	Fluorescent in situ hybridyzation
FOX13	Forkhead Box I3
FRET	Fluorescence resonance energy transfer
G	Guanine
GSTT1	Glutathione S-transferase theta
GRHL3	Grainy head-like transcription factor 3
HH	Hedgehog

Hospital USM	Hospital Universiti Sains Malaysia
IRF6	Interferon regulatory factor 6
IGF-R	insulin-like growth factor-1
Introns	Non-coding area
IQR	Interquartile range
KRK	Klinik Rawatan Keluarga
LEMD3	Lem-domain containing 3
LUCL/P	Left unilateral cleft lip with or without cleft palate
MAPK	Mitogen-activated protein kinase
MC	Metaphase Cytogenetics
MEE	Medial edge epithelium
MEI	Mobile element insertion
MC	Metaphase Cytogenetics
MGB	Minor groove blinder
mRNA	Messenger RNA
MEGF6	Multiple EGF-like domains 6
MORN1	MORN Repeat Containing 1
NSCL/P	Non-syndromic cleft lip with or without cleft palate
NOS3	Nitric oxide synthase 3
NAHR	Non-allelic homologous recombination
NHEJ	Non-homologous end-joining

NFQ	Nonfluorescent quencher
NI	Non-informative
OFC	Orofacial cleft
PS	Palatal shelves
PAX9	Paired box gene 9
PCR	Polymerase chain reaction
qPCR	Quantitative real-time PCR
RNA	Ribonucleic acid
RQ	Relative quantitation
ROH	Retention of heterozygosity
RUCL/P	Right unilateral cleft lip with or without cleft palate
SATB2	SATB homeobox 2
SCAP	Stem cells from apical papilla
SKI	SKI-Proto Oncogene
SNPs	Single nucleotide polymorphisms
SNP-A	Single nucleotide polymorphism array
SYDE1	Synapse Defective Rho GTPase Homolog 1
STRs	Short tandem repeats
SD	Standard deviation
T	Thymine
TP73	Tumor protein 73

TBX1	T-box protein 1
TGF	Transforming Growth Factor
TSGs	Tumour suppressor genes
TSG	Tumour suppressor gene
UGT2β15	UDP glucuronosyltransferase family 2 member B15
UCLP	Unilateral cleft lip and cleft palate
URCLP	Unilateral right cleft lip and palate
UTRs	Untranslated regions
UPD	Uniparental disomy
VDW	Van der Woude syndrome
WNT3	Wnt Family Member 3
XX	Female chromosome
XY	Male chromosome

LIST OF APPENDICES

PREVALEN ANOMALI PERGIGIAN DAN ABERASI GENETIK BAGI PESAKIT REKAHAN BIBIR DENGAN ATAU TANPA REKAHAN LELANGIT BUKAN SINDROMIK YANG MEMPUNYAI HIPODONSIA

ABSTRAK

Rekahan bibir dengan atau tanpa rekahan lelangit bukan sindromik (NSCL/P) bersama dengan anomali gigi ialah satu keadaan kelainan pada kraniofasial yang biasa berlaku pada manusia dan haiwan. NSCL/P juga dilaporkan mempunyai prevalen anomali gigi yang lebih tinggi berbanding dengan pesakit tanpa rekahan. Kajian ini bertujuan untuk menentukan prevalen anomali pergigian dan mengenal pasti aberasi genetik dalam kalangan NSCL/P menggunakan mikrotatasusunan DNA. Kajian keratan rentas telah dijalankan di Hospital USM dengan pemeriksaan gigi secara klinikal dan diikuti dengan analisis orthopantomogram untuk pengesahan anomali pergigian dalam kalangan NSCL/P dan kanak-kanak tanpa rekahan berumur antara 7 hingga 13 tahun. Sampel air liur diambil daripada subjek untuk pengekstrakan DNA genomik. DNA genomik yang diekstrak daripada 61 NSCL/P dan 20 kanak-kanak tanpa rekahan telah menjalani CytoScan 750K Array untuk mengenalpasti aberasi genetik seperti variasi salinan nombor (CNVs) dan kehilangan heterozigositi (LOH). Untuk CytoScan 750K array, sebanyak 250 ng DNA genomik dicernakan dengan enzim Nsp1 sebelum diligasi dengan adapter. Tindak balas rantai polimerase (PCR) dijalankan menggunakan sepasang primer yang mengenali *adapter*. Empat bahagian produk PCR kemudian digabungkan dan ditulenkan dengan manik magnet diikuti fragmentasi dengan Dnase I kemudian dilabel akhir dengan biotin serta dihibridkan ke dalam tatasusunan CytoScan. Seterusnya, tatasusunan Cytoscan diwarnai dengan

mengunakan GeneChips Fluidic Station sebelum diimbaskan. Data kemudiannya dianalisis secara manual menggunakan perisian chromosome analysis suite (ChAS) untuk memeriksa genom. Analisis data statistik dijalankan dengan menggunakan ujian Mann-Whitney dan Chi-Square dalam perisian IBM SPSS untuk mengesahkan keputusan yang signifikan. Hasil kajian menunjukkan bahawa prevalens (95% CI) anomali berkaitan bilangan gigi (67%:95% CI: 0.540,0.787) adalah lebih tinggi daripada morfologi (8%;95% CI: 0.027, 0.181) dalam kanak-kanak NSCL/P. Enam CNVs yang signifikan telah dikenalpasti iaitu penambahan (1p36.32, 1p36.33, 12q14.3 and 15q26.3) dan pengurangan (3p14.2 dan 4q13.2) dalam NSCL/P dengan pesakit hipodonsia berbanding NSCL/P sahaja. Gen di kawasan ini mengekodkan *TP73*, *SKI*, *LEMD3*, *IGF1R*, *FHIT* and *UGT2β15*. Untuk LOH, kawasan yang paling berulang ditemui pada 1p33-1p32.3, 1q32.2-1q42.13, dan 6p12.1-6p11.1 lokus yang berlaku dalam 13 (16%), 5 (6%) dan 7 (9%) daripada pesakit NSCL/P dan dalam kalangan kanak-kanak tanpa rekahan yang mempunyai hipodonsia. Analisis pengesahan mendedahkan penambahan nombor salinan yang signifikan dalam *TP73*, *SKI*, *IGF1R* dan *LEMD3* dan pengurangan dalam *FHIT* dan *UGT2β15* dalam kanak-kanak NSCL/P dengan hipodontia ($p < 0.005$). Analisa penanda mikrosatelit mendapati perkaitan yang signifikan antara penanda D1S197 (1p36.33-32.3) dalam NSCL/P dengan hipodonsia. Kesimpulannya, kajian ini telah berjaya menentukan prevalen anomali pergigian dan mengenal pasti aberasi genetik di kalangan NSCL/P. Kajian semasa menunjukkan penemuan novel bagi kehilangan nombor salinan pada 3p14.2 dan 4q13.2 yang merangkumi *FHIT* dan *UGT2β15* yang mungkin menyumbang kepada pembentukan NSCL/P dengan hipodonsia. Hasil kajian yang diperolehi ini dijangka akan mempunyai prospek yang penting dalam bidang perawatan dan pencegahan kesihatan mulut.

PREVALENCE OF DENTAL ANOMALIES AND GENETIC ABERRATIONS OF NON-SYNDROMIC CLEFT-LIP WITH OR WITHOUT CLEFT PALATE PATIENTS WITH HYPODONTIA

ABSTRACT

Non-syndromic cleft lip and or without cleft palate (NSCL/P) with dental anomalies are a common craniofacial abnormality in humans and animals. NSCL/P is reported to have a higher prevalence of dental anomalies compared to non-cleft individuals. This study aims to determine the prevalence of dental anomalies and to identify the genetic aberration among NSCL/P children using DNA microarray. A cross-sectional study was carried out at Hospital USM with a clinical oral examination followed by an orthopantomogram analysis among NSCL/P and non-cleft children between the ages of 7 to 13 years old. Saliva was collected from the subjects for genomic DNA extraction. Extracted genomic DNA from 61 NSCL/P and 20 non-cleft were subjected to the CytoScan 750K Array to identify genetic aberrations such as copy number variations (CNVs) and loss of heterozygosity (LOH). For CytoScan 750K Array, 250 ng of genomic DNA were digested with Nsp1, before being ligated to an adapter. Polymerase chain reaction (PCR) using a single pair of primers that recognised the adapter sequence was then performed. All four aliquots of PCR products were then combined and purified with magnetic beads followed by fragmentation with DNase 1 then subsequently end-labeled with biotin and hybridyzed into the CytoScan arrays. Next, the array was stained using GeneChips Fluidics Station 450 before scanning the arrays. The data was then analysed manually using the

Chromosome Analysis Suite (ChAS) software to examine the genome. Statistical data analysis was performed using Mann-Whitney and Chi-Square test in the IBM SPSS software to confirm the significant results. The results showed that the prevalence (95% CI) of anomalies related to the number of teeth (67%:95% CI: 0.540,0.787) was higher than the morphology (8%;95% CI: 0.027, 0.181) in the NSCL/P children. The highest dental anomalies was hypodontia among NSCL/P and non-cleft children. Six significant CNVs were identified including gains (12q14.3, 15q26.3, 1p36.32, and 1p36.33) and losses (3p14.2 and 4q13.2) in the NSCL/P with hypodontia patients compared to the NSCL/P only. The genes encoded in these regions are *LEMD3*, *IGF1R*, *TP73*, *SKI*, *FHIT*, and *UGT2β15*. For LOH, the most recurrent regions were found at 1p33-1p32.3, 1q32.2-1q42.13, and 6p12.1-6p11.1 loci occurred in 13 (16%), 5 (6%) and 7 (9%) among NSCL/P and non-cleft with hypodontia children, respectively. Validation analysis revealed a significant copy number gain in *TP73*, *SKI*, *IGF1R* and *LEMD3* and loss in *FHIT* and *UGT2β15* in NSCL/P children with hypodontia ($p < 0.005$). Microsatellite markers analysis found a significant association between D1S197 (1p36.33-32.3) markers in NSCL/P with hypodontia. In conclusion, the present study has successfully determined the prevalence of dental anomalies and identified the genetic aberrations among NSCL/P. The current study shows a novel finding of copy number loss at 3p14.2 and 4q13.2 that includes *FHIT* and *UGT2β15* which may contribute to the formation of NSCL/P with hypodontia. These results have an immerse prospect in the promising field of individualized preventive oral health care.

CHAPTER 1

INTRODUCTION

1.1 Research background

Nonsyndromic cleft lip with or without cleft palate (NSCL/P) is one of the most common congenital disabilities worldwide that affects the lip and palate (Mohamad Shah *et al*., 2019). The incidence of clefts varies among populations through the ethnic, race and geographical differences (Brito *et al*., 2011). The worldwide incidence of NSCL/P may occur in up to one in every 600 newborns, which means patients birth every 2.5 minutes (Monlleó and Gil-da-Silva-Lopes, 2006).

The incidence in the Asian population is twice higher than in the Black population (Murray, 2002). In Malaysia, the cleft occurrence rate was 1.24 in 1000 live births (Abumustafa *et al*., 2019). The etiology of NSCL/P is polygenic and multifactorial, involving various genetic and environmental factors (Bender, 2000). The environmental factors contributed include maternal smoking, alcohol consumption, folic acid deficiency, and medication during pregnancy (Murray, 2002; Lacerda *et al*., 2021). It has been reported that patients with cleft lip and palate (CLP) exhibit a higher frequency of dental anomalies than non-cleft subjects (Camporesi *et al*., 2010). Dental anomalies are craniofacial aberrations of the jaw and mouth's teeth, bones, and tissues in the form of function or position (Reddy and Rao, 2012). Teeth and jaw abnormalities are common in children with CLP. The development of embryonic teeth and CLP closely relates to timing and anatomic position (Rahman *et*

al., 2004). Dental anomalies are more common in c patients than in people who do not have a CLP (Dixon, 1968). Dentitional anomalies can be classified according to their number, size, shape, structural, developmental, and positional characteristics (Akcam *et al.*, 2010; Camporesi *et al*., 2010). The most frequent dental anomaly in cleft children is hypodontia, followed by supernumerary tooth (Camporesi *et al*., 2010). The co-occurrence of cleft lip with or without cleft palate (CL/P) and hypodontia were reported frequently in humans, which may be parallel to the sharing or similar developmental mechanisms and genetic risk (Milien *et al*., 2016).

NSCL/P and hypodontia are the most common congenital disabilities in the craniofacial and dentofacial regions with complex aetiology involving multiple genetic factors, environmental factors, and gene-environment interactions (Leslie and Marazita, 2013). Huda *et al*. (2021) stated that missing teeth or hypodontia were significantly associated with all orofacial clefts (OFCs) while the supernumerary teeth were only significantly related to both CLP. Both conditions led to significant life-long complications that require extensive multidisciplinary treatments and represent severe psychosocial and economic burdens for their families and society (Mossey *et al*., 2009).

According to the American Cleft Palate and Craniofacial Association recommendations, all individuals with craniofacial anomalies including CL/P should be managed by an interdisciplinary team of specialists (Watted *et al*., 2020). These specialists include audiologist, medical imaging, genetic counsellor, neurologist, reconstructive surgery, oral and maxillofacial surgery, orthodontics, paediatric medicine, social worker, speech-language pathologist, and psychologist (Watted *et al*.,

2020). Dental anomalies have direct consequences in terms of physical appearance and emotional and functional impact on affected individuals. Avoiding poor living habits, chemical factors, age, and poor health status during pregnancy is a key to normal craniofacial and dentofacial development (Lu *et al.*, 2019). Martelli *et al*. (2015) revealed a significant association between maternal smoking and the presence of cleft. Increased risks from maternal smoking during the periconceptional period suggest that genes in specific metabolic pathways may play a role in developing NSCL/P (Huda *et al.*, 2021). Markers in the GSTT1 (glutathione S-transferase theta) or NOS3 (nitric oxide synthase 3) genes, in particular, appear to influence the risk of NSCL/P in the presence of maternal smoking (Shi *et al.*, 2007). The development of primary and permanent dentitions may be affected equally because of OFCs. Furthermore, dentition on the affected cleft side is frequently compromised (Camporesi *et al*., 2010).

Tooth and craniofacial development are complex processes that require interaction between epithelial and mesenchymal signals aided by signalling molecules and genetic pathways (Jussila and Thesleff, 2012; Santosh and Jones, 2014). Several signalling factors including those from the *WNT*, *FGF*, *BMP*, and *HH* families, are involved in the mesenchymal signalling interaction during tooth and craniofacial development (Al-Ani *et al.*, 2017; Phan *et al.*, 2016). A change in one or more signalling pathways caused by environmental factors (smoking, alcohol consumption, chemotherapy, trauma, radiotherapy, and infection) or genetic factors may result in abnormal tooth development and orofacial cleft (Phan *et al.*, 2016). Hypodontia in CL/P patients may significantly impact the patient's quality of life in terms of functional decrease, emotional distress, and social prosperity (Ghazali *et al*., 2021).

Various genetic approaches have been used to identify genes and genomic loci contributing to CL/P and hypodontia, including animal model expression studies, candidate gene sequencing, and genome-wide association study (GWAS). Recently, the role of chromosomal deformities has been studied in genetic defects and diseases. CNVs and LOH act by altering gene expression, disrupting gene sequences, and changing gene dosage (Feuk *et al.*, 2006).

Previous studies also found several genomic loci and genes candidates underlying the co-occurrence of two congenital disabilities, including 1q21-q25, 1q32, 2q31.2-q33.2, 4p16.3, 8q24, 16q22, Axis Inhibition Protein 2 (*AXIN2*), Cadherin 1 (*CDH1*), Interferon regulatory factor 6 (*IRF6*), *MSX1*, Paired box gene 9 (*PAX9*), *TGFα, TGFβ3,* and SATB homeobox 2 (*SATB2*) (Schinzel and Schmid, 1980; Wong *et al.*, 1999; Rifai *et al*., 2010; Maas *et al*., 2008; Yildirim *et al*., 2012; Letra *et al*., 2009; Ariadne Letra *et al*., 2012; van den Boogaard *et al*., 2000). Several transcription factors, signalling molecules, and genes control this process (Huda *et al.*, 2021). Variations in the regulation process of these transcription factors and gene mutations can change the deoxyribonucleic acid (DNA) sequence, thus accounting for many congenital abnormalities of orofacial regions (Huda *et al*., 2021)

Copy number variation has recently been identified as a significant cause of variation in the structural genome involving duplications and sequence deletion (Redon *et al.,* 2006). A previous study found copy number variations (CNVs) in patients with syndromic CL/P and hypodontia such as ectodermal dysplasia syndrome, Wolf Hirschhorn syndrome, and DiGeorge syndrome (Phan *et al*., 2016). However, the CNV and related genes for nonsyndromic cleft lip with or without cleft palate

(NSCL/P) with hypodontia remained with limited significance. The causative genes and genomic loci of hypodontia among syndromic CL/P might also lead to the development of nonsyndromic CL/P. Therefore, a genome-wide copy number analysis was conducted to identify the contribution of CNV in the development of NSCL/P with hypodontia.

1.2 Study rationale

NSCL/P have a higher prevalence of dental anomalies compared to the non-cleft participants. The co-occurrence between dental irregularities and the presence of OFCs is poorly understood. Therefore, this study assessed the prevalence of the highest dental abnormalities (hypodontia) among NSCL/P and non-cleft children. Identifying genes and genomic regions underpinning craniofacial disorders have relied heavily on chromosomal anomalies such as microdeletions, microduplications, and translocations. It has now been widely recognised that genetic aberrations represent an essential feature of NSCL/P and hypodontia. Somatic copy number alterations (CNAs) and loss of heterozygosity (LOH) are known may cause NSCL/P with hypodontia. However, not much is known about CNAs and LOH presence and genomic loci in orofacial cleft and hypodontia in Malaysia.

Moreover, previous technologies with the absence of simultaneous screening of CNAs and LOH have failed to appreciate the significance of copy-neutral LOH (cnLOH) and LOH accompanying CNVs in NSCL/P with hypodontia. Hence, this study also aims to employ the latest SNP array technology for concurrent profiling of CNAs and LOH in NSCL/P with hypodontia. The CytoScan 750K array was used to

identify recurrent and significant CNVs and LOH in all samples including NSCL/P and non-cleft with or without hypodontia. In addition, the selected genes in the region of the significant copy number gain and losses undergo the validation process using TaqMan copy number variation analysis. This validation analysis investigated the copy number of the gene of interest and was normalised to an endogenous reference gene known to be present in two copies in a diploid genome among NSCL/P and non-cleft with hypodontia. This study also evaluated the associations between significant CNVs of selected markers and hypodontia in NSCL/P. Lastly, the chosen markers undergo LOH screening using microsatellite markers analysis. The analysis of microsatellite markers involved PCR amplification of the microsatellite loci at recurrent selected markers using a fluorescently labelled primer that flank the repeated sequence. The labelled PCR products were then analysed by fragment analysis to separate the size.

1.3 Objectives

1.3.1 General objective

To study dental anomalies and to identify novel genetic aberration in Non-syndromic cleft lip with or without cleft palate (NSCL/P) patients compared to non-cleft children

1.3.2 Specific objectives

1. To determine the prevalence and association of different dental anomalies (morphology and number of teeth) among NSCL/P patients compared to non-cleft children

2. To characterise copy number variation (CNV) in NSCL/P and non-cleft children with or without hypodontia

3. To characterise loss of heterozygosity (LOH) in NSCL/P and non-cleft children with or without hypodontia

1.4 Research questions

1. What is the prevalence of dental anomalies among NSCL/P patients?
2. Compared to the non-cleft patients, what is the prevalence of different types of dental anomalies (morphology and number of teeth) among NSCL/P patients?

3. What are the characteristics of genetic alteration (CNVs) and loss of heterozygosity) using the SNP array in the NSCL/P with or without hypodontia?

4. What types of significant candidates' genes of the CNVs among the NSCL/P with or without Hypodontia?

1.5 Research hypothesis

1. There is a significant association between validated candidate genes among NSCL/P patients with hypodontia.

CHAPTER 2

LITERATURE REVIEW

2.1 Cleft lip with or without cleft palate (CL/P)

Craniofacial congenital disabilities are the fourth most common congenital anomaly in newborns (Merritt, 2005). Clefts are divided into isolated cleft palate only (CPO) and CL/P, which are congenital disabilities that affect the facial and oral structures (Máris *et al*., 2011). The failure of lip sections to join together early in the fetus's development causes a cleft lip (Kummer, 2008). A cleft palate also happens when the root of the mouth does not fuse appropriately throughout prenatal development, resulting in a significant gap between the oral and nasal cavities (Kummer, 2008). Clefts can be unilateral, bilateral, complete, or incomplete, and they can disturb the lip only, the palate, or both (Merritt, 2005).

CL/P can happen as an isolated (non-syndromic) or in combination with other inherited abnormalities known as syndromic (Máris *et al*., 2011). A previous study discovered that approximately 70% of CL/P cases are non-syndromic clefts with no congenital deformations (Lasota, 2021). CL/P generates a significant financial strain on the family (Zhang *et al*., 2018). Sequential therapies for non-syndromic cleft lip with or without cleft palate (NSCL/P) children typically take several years. CL/P is the most recurrent craniofacial malformations in humans by generating a severe public health burden and imposing significant health care and financial demands on individuals, families, and society (Mossey *et al*., 2009).

2.2 Epidemiology of cleft lip with or without cleft palate (CL/P)

CL/P affects approximately around one out of every 700 live newborns, with considerable ethnic and geographical variation (Mossey and Modell, 2012). According to the World Health Organization (WHO), the prevalence of orofacial cleft (OFC) at birth varies globally ranging from 3.4-22.9 per 10,000 births for CL/P and 1.3-25.3 per 10,000 births for CPO (Gorlin *et al*., 2001; Mossey and Catilla, 2003). The most common congenital anomaly was orofacial cleft occurring in up to one in every 600 births, implying that a patient is born every 2.5 minutes worldwide (Máris *et al*., 2011). CL/P has a wide range of effects depending on geographic origin and socioeconomic status (Vanderas, 1987). The highest occurrence rates are seen in Native Americans and Asians, while low incidence is found in the black population (Murray, 2002; Murray, 1995; Ankola *et al*., 2005; Das *et al*., 1995). CL/P was observed one in 1,000 births in whites, one in 500 births in Asians and Native Americans, and nearly one of every 2,400 to 2,500 births in people of African heritage. (Kirschner and LaRossa, 2000).

Abumustafa *et al*. (2019) previously reported that the incidence of the cleft in Malaysia was 1.24/1000 live births, 1.94/1000 live births in the Philippines, 1.87-2.07/1000 live births in Singapore, 1.1-1.51/1000 live births in Thailand, and 1.4/1000 live births in Vietnam. CL/P is more frequent among boys while the isolated cleft palate is twice as common among girls and occurs in approximately 0.4 of every 1000 live births in all ethnic groups (Mulliken, 2004). The variation in birth prevalence of oral clefts in different geographic locations is due to sampling type, population, race, and inclusion or exclusion criteria. The study by Shah *et al*. (2018) found that out of

526 CL/P patients in Malaysia, the racial distribution of patients was 88.6% Malays, 8.7% Chinese, 2.5 % Indian, and 0.2% of others.

2.3 Embryological development of the cleft lip with or without cleft palate (CL/P)

Face morphology is developed between the 4th and 10th weeks of pregnancy (Smarius *et al*., 2017). The embryo develops three germ layers (endoderm, mesoderm, and ectoderm) that differentiate into discrete tissues during gastrulation (Muhr and Ackerman, 2021). At 5 weeks' development, while the embryo size is 3 mm long, the ectoderm in the neural plate folds to form a neural tube. The production of neural crest cells in the embryo is required to develop the face and palate during embryonic development (Cordero *et al*., 2011). Ectodermal-derived neural crest cells grow into a unique ectomesenchyme. The ectomesenchyme maintains the formation of the five facial prominences surrounding the primitive oral cavity including the frontonasal prominence and both maxillary and mandibular prominences (Sperber and Sperber, 2013). The frontonasal prominence grows in the brain's midline.

During the 5th week of development, the nasal component of the frontonasal prominence develops into bilateral two ectodermal thickenings and the nasal placodes (Leslie and Marazita, 2013). Each nasal placode invaginates and splits the frontonasal prominence into a lateral and medial nasal process. The lateral nasal prominence, medial nasal prominence, and maxillary prominence merge to form the nose, upper lip, lateral incisor, and primary palate between the 6th and 7th weeks of pregnancy

(Jiang *et al*., 2006). Figures 2.1 and 2.2 show the development of the lip and Caudal vies of the palatal shelves' fusion process, respectively.

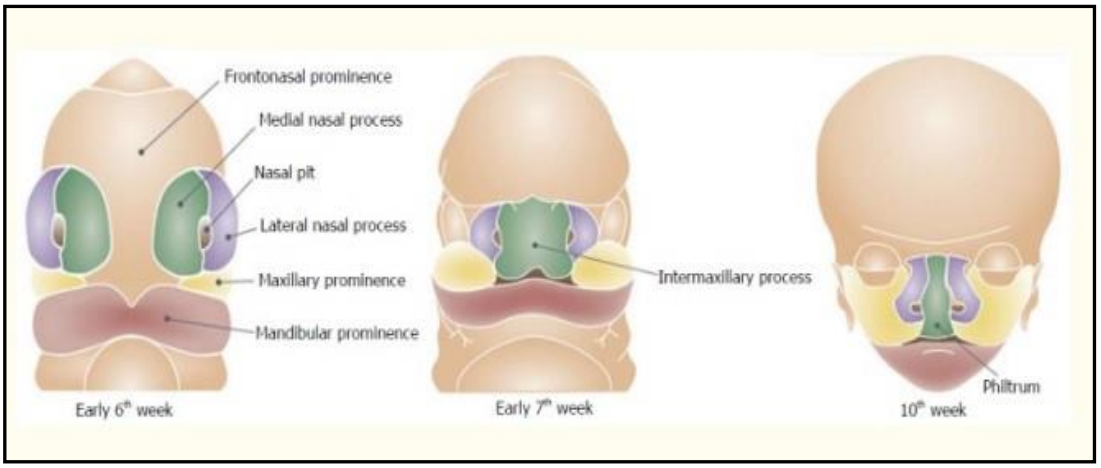

Figure 2.1: Development of the lip. Adapted from Smarius *et al*., 2017

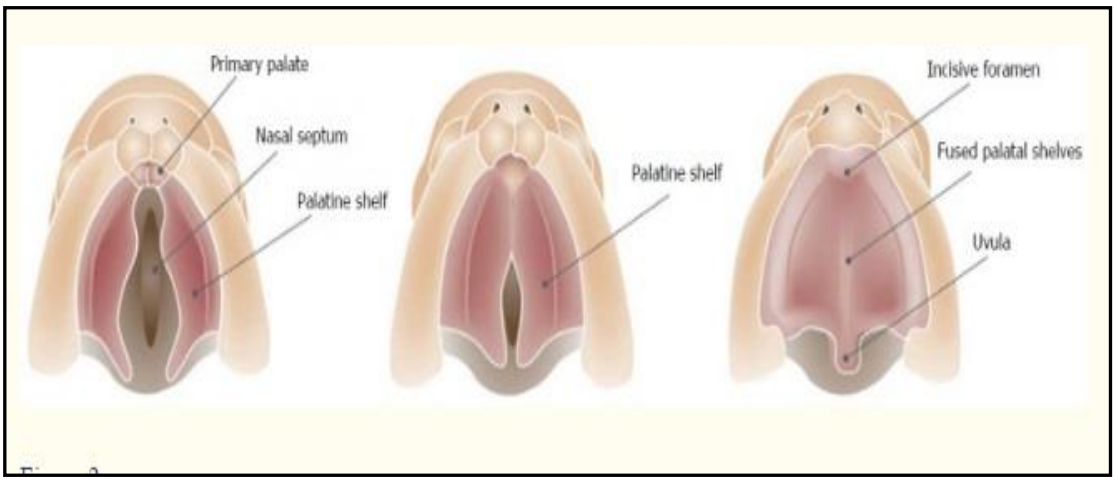

Figure 2.2: Caudal vies of the fusion process of the palatal shelves. Adapted from Smarius *et al*., 2017

CL/P occurs when normal craniofacial growth processes are disrupted (Leslie and Marazita, 2013). A complicated series of activities including cell proliferation, migration, differentiation, and apoptosis are required for proper face development. When these processes fail to mature or fuse, orofacial clefting affects the upper lip, alveolus, philtrum, incisor teeth, and primary palate (Leslie and Marazita, 2013; Smarius *et al*., 2017). The primary palate is the ventral section to the incisive foramen while the secondary palate is dorsal to the incisive foramen. A cleft lip can be unilateral (on either the left or right side) or bilateral (on both sides). A complete unilateral cleft lip is a result of a complete failure of fusion of the medial nasal prominence and maxillary on one side. A partial unilateral cleft lip is the result of partial medial nasal prominence and maxillary prominence on one side. The palate was not altered in this case.

During the 7^{th} week of embryogenesis, the secondary palate typifies by developing two palatal shelves (PS) and growth from the maxillary prominence. PS started in a vertical position accompanying the edge of the tongue and then re-construct to the horizontal position (Nakajima *et al*., 2018). Both PS above the tongue come into contact at the horizontal position and the medial edge epithelium (MEE) at the palatal midline seam fuses. At 12 to 13^{th} week of embryogenesis, the MEE disappeared to complete the palate fusion. The nasal and oral chambers are separated after a successful secondary palate fusion. A cleft palate can form due to a failure at several phases in secondary palate development (Leslie and Marazita, 2013)

2.4 Types of Cleft

Orofacial cleft (OFC) is a diverse range of abnormalities affecting the anatomy of the face and oral cavity that have been classified into three broad categories namely those involving only the lip, those impacting the lip and palate, and those affected only the palate (Leslie and Marazita, 2013). The cleft lip affects mostly soft tissue and extends from the red regions of the lip to the upper lip near the nostrils (Smarius *et al.*, 2017). The cleft lip involves only soft tissue and extends through the red parts of the lip and vermillion border into the upper lip towards the nostrils. The three cleft lip types are incomplete cleft, unilateral complete cleft lip, and bilateral complete cleft lip. An incomplete cleft lip is shown as a small notch in the lip tissue without involving the alveolar ridge. The roof of the mouth development occurs between the 8^{th} and 12^{th} weeks of embryological gestation. Typically, the palate is formed and fused from the anterior and posterior directions. The cleft palate can occur as anterior cleft palate, posterior cleft palate, and complete cleft palate (anterior and posterior).

Anterior cleft palate is present when the lateral palatine process fails to fuse with the primary palate. The clefting of the posterior to the incisive foramen is defined as a cleft of the secondary palate. Submucous cleft palate is a cleft of the hard palate and is covered by mucosa and continuous through the soft palate. Submucous cleft palate may occur in the hard palate only and continue to the open cleft of the soft palate or it may occur as a submucous cleft of the soft palate with or without a notch into the hard palate. The cleft lip and palate affect both the primary and secondary palate. A CLP is present when the cleft lip continues from the foramen incisive further through the sutura palatine in the middle of the palate. A wide range of severity may be

observed. The cleft line may be interrupted by soft (skin or mucosa) bridges, complex (bone) bridges or both corresponding to a diagnosis of an incomplete cleft. Figures 2.3 and 2.4 show the types of clefts.

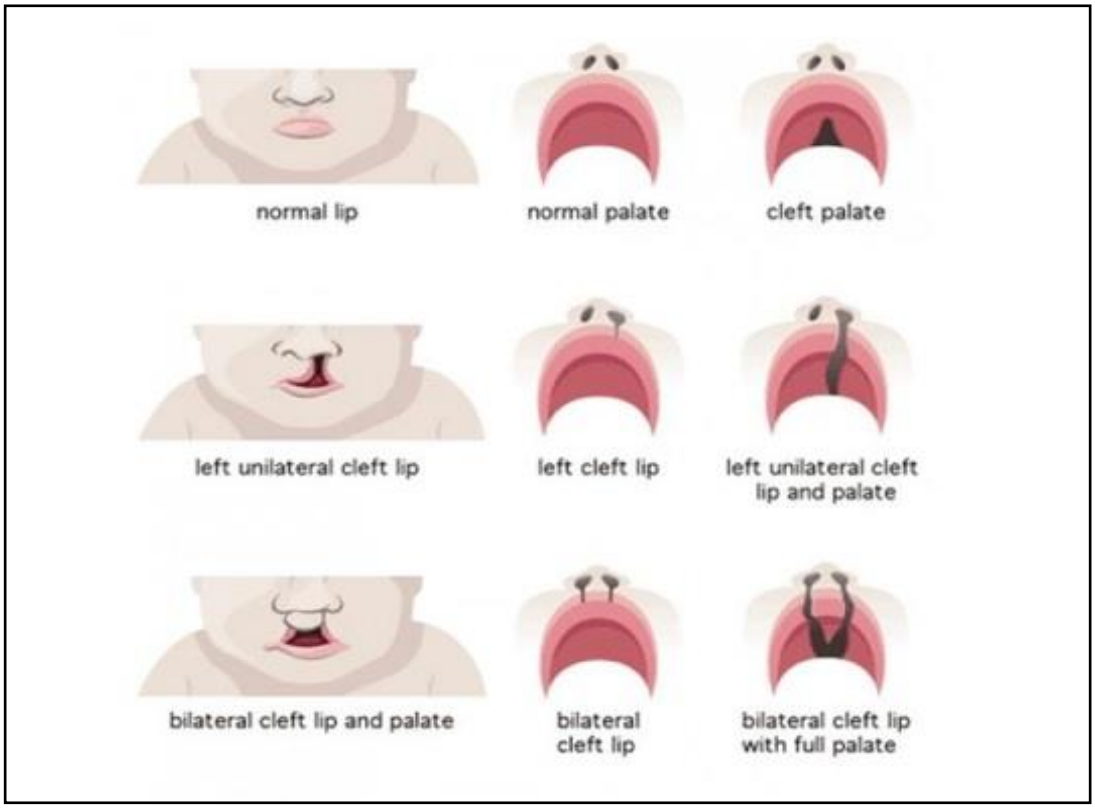

Figure 2.3: Different types of clefts. Adapted from bettersafercare.vic.gov.au /clinicalguidance/neonatal/cleft-lip-and palate-in-neonates

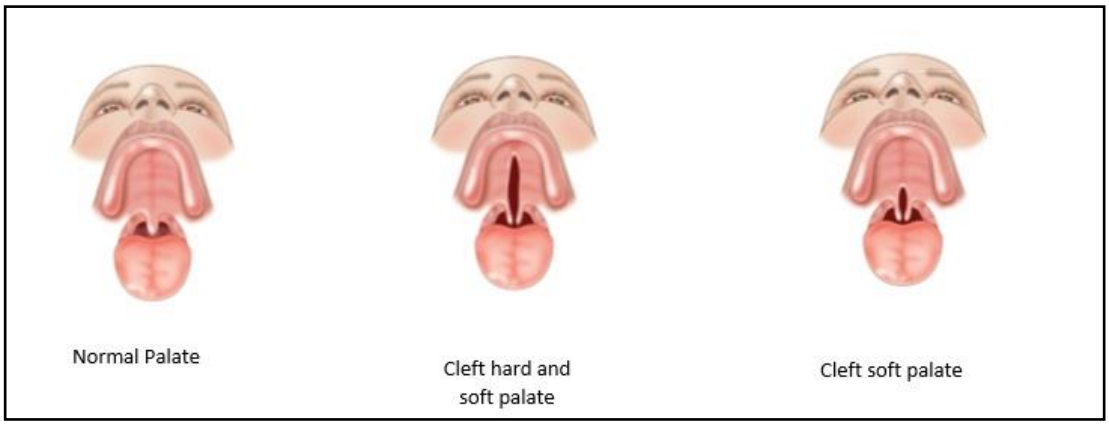

Figure 2.4: Types of cleft palate. Adapted from bettersafercare.vic.gov.au/clinical-guidance/neonatal/cleft-lip-and-palate-in-neonates

2.5 Etiology of non-syndromic cleft lip with or without cleft palate (NSCL/P)

NSCL/P is a complicated, partially heritable congenital disorder involving several genes and complex genetic and environmental variables *(*Dixon *et al*., 2011).

2.5.1 Genetic influences

The genetics of NSCL/P is a growing field of investigation and accelerated discoveries. Although a positive family history increases the risk of NSCL/P, no expected trend of repetition has been observed (Wantia and Rettinger, 2002). A parent who has been diagnosed with NSCL/P has a 3% to 5% chance of having an afflicted kid (Wantia and Rettinger, 2002). Nevertheless, parents who have one affected child have a 40% coincidental of having another cleft child (Sandberg et al., 2002). Fogh-Andersen (1967) reported that genetic factors cause 12 to 20 % of non-syndromic orofacial clefts, and the remaining case is caused by the environment or interaction of genetic and environmental factors. While congenital abnormalities are considered the cause of NSCL/P, no single gene has been identified as the common cause of all clefts (Kerrigan *et al*., 2000).

There are a few candidate genes involved in NSCL/P. The current list of target genes for clefting includes Transforming Growth Factor-Alpha (TGFα), Retinoic Acid Receptor Alpha (RARA), Msh Homeobox 1 (*MSX1*), Methylene-tetrahydrofolate-reductase (*MTHFR*), and Transforming Growth Beta 3 *(TGFB3*) (Murray, 2002). CL/P genetic linkage investigations have been restricted by limiting families and genotyping facilities (Brewer *et al*., 1998). One region at 1p36 has at least three genes of interest

and an increased cleft frequency, including SKI, p73, and MTHFR genes (Shapira *et al*., 1997). Brewer *et al*., (1998) found that the chromosomal deletion on 2q32, 4p16, 4q31, 1q21, 4p16 and 7q34 were significantly associated with cleft lip and palate. The regions associated with cleft were discovered within chromosomes 1q25, 3p21, 4p15, 4q32, and 10p15 (Murray, 2002). Mutation resulting in CL/P can occur on 1q24, 2p, 3p20, 3q, 4q32, 10p15, 17q, 18q, and 21q (Shapira *et al*., 1997). The mutation on certain genes such as the *TGFβ3*, Apetala 2 (*AP2*), and *MSX1* may alter the signalling molecules, transcription factors or growth hormones in the growing prominences of the lip and palate, thereby impairing their natural merging (Bender, 2000).

2.5.2 Environmental factors

Although genes play a significant part in facial morphogenesis, the environment plays an equally important role in modifying genetic consequences. A few triggers may cause cleft development, such as maternal smoking, maternal ingestion of pharmaceuticals such as anticonvulsants phenytoin and benzodiazepines or pesticides (Murray and Schutte, 2004). Smoking throughout the 1st trimester increases the risk of CL/P (Merritt, 2005). Intermittent hypoxia induced by nicotine may affect facial development. Heavier smokers have a higher risk (Bender, 2000). A genetic predisposition (changed transforming growth factor) may increase the dangers of smoke exposure in some people (Merritt, 2005). Eventually, nutrition and cholesterol metabolism increasingly impacted embryonic growth. Folate is thought to be vital in neural tube formation. The discovery that folic acid supplementation can reduce the incidence of neural tube abnormalities is one of the 21st century's significant genetic

public health accomplishments, along with the treatment of phenylketonuria (Williams *et al*., 2002).

2.6 Tooth development

Odontogenesis is a complicated process of tooth development that occurs during embryonic development. Tooth development is controlled through complex cellular information processing between the oral epithelium and mesenchyme (Thesleff and Tummers, 2010). The primary teeth begin to form between the 6^{th} and 8^{th} weeks of prenatal development while the permanent teeth are included in the 20^{th} week. The formation of 20 primary teeth and 32 permanent teeth occurs due to an interaction between the oral epithelium cells and the underlying mesenchymal cells. Enamel, dentin, cementum, and periodontium are form during a specific stage of foetal development in a healthy oral environment. The tooth development process is divided into several locations including the initiation, bud, cap, bell, and erupted tooth stages (Figure 2.5).

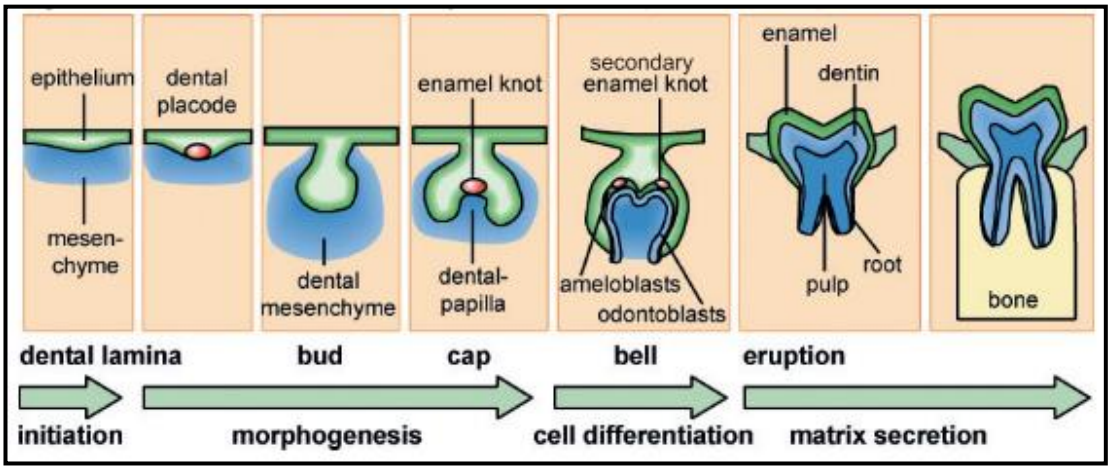

Figure 2.5: The stage of tooth development. Adapted from Järvinen, 2008

2.6.1 Initiation stage

Tooth development begins at the end of the 5th week of pregnancy. The dental lamina is the first morphological structure that was formed. The oral epithelium thickens at the site of future tooth formation called an odontogenic band or stomodeal epithelia. The dental placode comprises of thickened epithelium and neural crest-derived mesenchyme (Jussila and Thesleff, 2010.) The dental placode is the main epithelial structure of tooth formation and the first tooth signalling centre. Disturbances in the initiation stage can cause anodontia and extra teeth.

2.6.2 Bud stage

This stage happens between the 7th and 9th weeks of human pregnancy. The epithelial invagination or folding into the oral ectomesenchyme describes the bud stage. The stellate reticulum, a group of cells located in the centre of the enamel organ, is found in the tooth bud (Waddington, 2009). The suprabasal layer of the oral ectoderm gives rise to stellate reticulum cells. Disruptions in the bud stage can result in the formation of macrodontia and microdontia caused by excessive or insufficient cell proliferation.

2.6.3 Cap stage

The cap stage of tooth development takes place between the 9th and 10th weeks of embryonic development. The progression of oral epithelial growth distinguishes this stage into the mesenchyme. The enamel organ is formed by epithelial cells during the cap stage of development, and the dental papilla is formed by mesenchymal cells. A

dental follicle forms around the two structures (Tucker and Sharpe, 2004). This process is thought to be governed by the primary enamel knot, which serves as a signalling centre for the developing teeth (Vaahtokari *et al*., 1996). The primary enamel knot was created during the transition from the bud to the cap stage and is fully differentiated at the start of the cap stage. Although enamel knot cells do not multiply, they do stimulate the division of epithelial and mesenchymal cells (Catón and Tucker, 2009). At the start of the bell stage, primary enamel knot cells die due to apoptosis and signalling centre silencing.

2.6.4 Bell stage

This stage occurs between the 11^{th} to 12^{th} weeks of human gestation. Cell differentiation occurs at the bell stage of development, and the ameloblasts and odontoblasts are established in the adjacent layer at the site of interaction between the epithelium and mesenchyme. The enamel and dentin of a fully formed tooth are produced by these layers.

2.7 Dental anomalies

Dental anomalies have been classified based on the stage of tooth germ development and the number, morphology, size, and structure. The cleft population has various dental abnormalities in morphological anomalies, delayed tooth development, variation in tooth number, and delayed eruption of permanent maxillary incisors (Rahman *et al*., 2004). These anomalies could be caused by the orofacial cleft itself or a poor surgical outcome. According to a previous study, the prevalence of dental abnormalities in CLP is higher compared to the general population (Al Jamal *et al*., 2010). Many studies have recently focused on the relationship between cleft type and dental anomalies in terms of the teeth number, size, and shape (Al-Kharboush *et al*., 2015). In a previous study, hypodontia was prevalent in 77% of cleft patients (excluding 3rd molar teeth) (Shapira *et al*., 1999). The aetiology of dental anomalies in cleft patients was not fully recognised, but a few researchers believed that a genetic factor plays a prominent role (Lasota, 2021).

2.7.1 Alteration in number of teeth

The alteration in the number of teeth among cleft patients usually occurred due to the disturbance during the initiation of tooth germ (Rohilla, 2017). This aberration can be hypodontia and hyperdontia. Hypodontia is the absence of primary or secondary teeth, excluding the 3rd molars (Rohilla, 2017). It is the most common developmental dental anomaly, and it can be difficult to treat clinically. Hyperdontia can manifest by forming other teeth, which can occur in succession as a deciduous or post permanent