VALIDATION OF TOX3 GENE MUTATION AND RISK IDENTIFICATION IN NON-SYNDROMIC CLEFT LIP AND PALATE DEFORMITIES IN MALAY PATIENTS

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LIST OF SYMBOLS, ABBREVIATIONS OR NOMENCLATURE

CL/P	Cleft lip and/or palate
CD	Cleft disorders
UCLP	Unilateral cleft lip and palate
BCLP	Bilateral cleft lip and palate
UCLA	Unilateral cleft lip and alveolus
CLAP	Cleft lip and alveolus and/or palate
SCL/P	Syndromic cleft lip and/or palate
NSCL/P	Non syndromic cleft lip and/or palate
µl	Microlitre
TOX3	TOX high mobility group box family member 3
WHO	The World Health Organization

ABSTRAK

Pendahuluan : Sumbing orofasial merupakan anomali kongenital pada bibir dan/atau lelangit. Ini boleh berlaku dengan sindrom atau tanpa sindrom. Sumbing bibir dan/atau lelangit tanpa sindrom merupakan anomali yang melibatkan kecacatan fizikal sahaja. Beberapa faktor genetik dan persekitaran telah dikaji untuk mengenal pasti kaitannya dengan sumbing bibir dan/atau lelangit tanpa sindrom.

Objektif : Kajian ini telah dijalankan untuk mengenal pasti peranan mutasi gen baharu iaitu *TOX3* gen dengan sumbing bibir dan/atau lelangit tanpa sindrom dan kaitannya dengan faktor persekitaran yang mungkin menyumbang kepada peningkatan pesakit sumbing bibir dan/atau lelangit tanpa sindrom.

Metodologi : Kajian ini merupakan kajian kes kontrol yang dijalankan di Hospital Universiti Sains Malaysia. Seramai lima puluh orang pesakit dan lima puluh orang individu sihat berbangsa Melayu yang tidak mempunyai sejarah penyakit sumbing dalam keluarga telah menyertai kajian ini. Sampel and darah diambil untuk kajian genetik variasi nombor salinan *TOX3* gen dan proforma pesakit untuk mengenal pasti kaitan faktor persekitaran.

Keputusan : Keputusan kajian menunjukkan skor median bagi salinan nombor *TOX3* gen adalah rendah pada pesakit sumbing bibir dan/atau lelangit tanpa sindrom berbanding kontrol tetapi tidak mencapai tahap signifikan. Hanya satu faktor daripada faktor persekitaran iaitu usia ibu mengandung yang mempunyai kaitan signifikan dengan mutasi pada *TOX3* dalam kalangan pesakit sumbing bibir dan/atau lelangit tanpa sindrom (p=0.043). Risiko kecacatan sumbing meningkat dengan meningkatnya usia ibu mengandung. Pemboleh ubah yang lain tidak menunjukkan keputusan yang signifikan dengan *TOX3* namun skor agak rendah dikesan pada pesakit sumbing bibir dan/atau lelangit tanpa sindrom berbanding kontrol pada faktor

sosioekonomi yang rendah, pelajaran ibu yang rendah, sejarah keluarga yang mempunyai kecacatan sumbing dan merokok.

Kesimpulan : Gen TOX3 tidak menunjukkan perbezaan yang signifikan antara pesakit sumbing bibir dan/atau lelangit tanpa sindrom dan kontrol. Usia ibu mengandung sahaja yang mempunyai kaitan yang signifikan dengan mutasi gen TOX3 yang menyebabkan sumbing bibir dan/atau lelangit tanpa sindrom. Oleh itu, kajian ini boleh dijalankan terhadap saiz sampel yang lebih besar dalam kalangan orang Melayu.

ABSTRACT

Introduction: Orofacial cleft is a congenital anomaly of lip and/or palate that can be syndromic or non-syndromic. Non syndromic cleft lip and/or palate (NSCL/P) is an anomaly with a physical deformity alone. Several genetic and environmental factors have been studied to find their association with NSCL/P.

Objective: This study was conducted to determine the role of a novel TOX3 gene mutation in NSCL/P and its association with the environmental factors that may be contributing to increase in patients with NSCL/P.

Methodology: This was a case-control study conducted in Reconstructive Science Unit, Universiti Sains Malaysia. Fifty Malay patients with NSCL/P and 50 healthy individuals without cleft or family history of cleft disorder as control. Blood samples were obtained from consented patients and controls for genetic study of TOX3 copy number variation, and patients proforma to detect the environmental associated factors.

Results: Our results showed that the median score of TOX3 genes copy number was lower in patients with cleft as compared to the control but not to a significant level. Only one variable of maternal age in environmental factors showed statistically significant association with TOX3 risk mutation in NSCL/P (p=0.043) whereas cleft deformity increases with an increase in mother's age. Other variables showed no significant result, however TOX3 scores were lower in patients than in controls in folic acid deficiency, maternal diabetes, exposure to toxins, positive family history of cleft.

Conclusion: TOX3 gene did not show a significant difference between patients with NSCL/P and control. Maternal age only has significant association with mutated TOX3 that may cause NSCL/P. Hence larger sample size in Malay population can be studied in future.

CHAPTER 1: INTRODUCTION

1.1 Introduction

Congenital anomalies are the developmental defects that begin in intrauterine life (IUL), identified before or after birth. Congenital anomalies may be limited to the physical structure only or may result with functional defect or both. Most of these anomalies appear as a result of any disturbance during the first trimester i.e., from 4^{th} up to 12^{th} week of IUL. The most common causes are genetic, chromosomal abnormalities, harmful medicinal or toxic exposure, any infection during pregnancy, and nutritional deficiencies (Abebe *et al.*, 2021).

1.2 Cleft lip and palate

Cleft lip or/and palate (CL/P) is a congenital disorder of the mouth roof and the upper lips. It is a condition where neonatal are born with incomplete union of the frontonasal and maxillary processes resulting in cleft lip and palate. Three types of cleft disorders (CD) are cleft lip only, cleft palate only, and cleft lip with cleft palate. The incomplete fusion of anatomical structures of lips, hard palate, soft palate, and the alveolar bone can be unilateral or bilateral, left sided or right sided. The anomaly is also classified as primary (if defect is anterior to incisive foramen), or as secondary (if defect is posterior to the incisive foramen).

It can be syndromic (SCL/P) i.e., associated with any syndrome, or it can be non-syndromic (NSCL/P) as a structural deformity only of lip and palate (Panamonta *et al.*, 2017). Around 70% of the CD cases are non-syndromic (Lou *et al.*, 2020). Multiple syndromes are known to involve a cleft as an anomaly. Some of those conditions are Pierre Robin syndrome, Treacher Collins syndrome, Van der Woude syndrome, and stickler syndrome (Hadadi *et al.*, 2017).

Studies have been conducted to rule out the cause of this congenital disorder. Previous studies showed that the defect occurs as a result of various complex effects of genes and environmental on the neonatal during the IUL (4,5). In addition, the child with these malformations faces multiple challenges with speech, hearing, oral intake, and repeated ear infection. It also has a psychological effect on the children. As the child grows, there is difficulty in speech and the aesthetical appearance of the face which require multidisciplinary approach for the treatment. This condition caused low self-esteem and confidence to the child. (Mahamad Irfanulla Khan, Prashanth and Srinath, 2020).

1.3 Geographical distribution

Clefts occur at different rates in different groups due to ethnic, racial, or geographical variances (Apostole, 1986). Asians as well as American Indians are more likely to have cleft lip and palate than other ethnic groups (Yu *et al.*, 2009; Mahamad Irfanulla Khan, Prashanth and Srinath, 2020). One in every 500 births has reported to have cleft lip with or without palate in the Asian population (Apostole, 1986). Difference in prevalence of CD in different geographical regions and ethnic groups can be due to both genetic and environmental factors that impact these deformities like smoking habits, work area, health care facilities etc. Similarly, the non-syndromic cleft lip and/or palate (NSCL/P) form have a wide range of impacts that may be of genetic and environmental aspects. It may be due to gene-gene or gene-environment interaction (Murray, 2002).

In Malaysia, a study conducted on 526 patients showed that 43% of the cases were of male patients while female percentage was 57%. Focusing on the type of CD, 77.8% had cleft lip and palate, followed by 13.5% with cleft palate only and then only 8.7% with cleft lip. Out of total patients, 57.2% had unilateral, and 42.8% had bilateral CD. Moreover, in those with unilateral CD, 57.2% , 32.7% had left sided while 24.5% had right sided CD (Ali *et al.*,

2015). Another study on the three ethnic groups of Malaysia showed Malays with the highest frequency, followed by Chinese and then Indians (Yousif Ali Shah, Ali Mirani and Amin Sahito, 2018).

1.4 Environmental factors

Multiple studies have been conducted previously to rule out the cause of CD. Environmental factors have been studied which seem to have an impact on CD. Although no one particular factor can be labelled as the exact cause of NSCL/P. However, studies have highlighted various environmental factors that may be related to personal habits or due to the working environment. Irradiations are also thought to be one of the factors to cause CLAP. But due to the complex nature of the deformity, some studies turn out to be inconclusive.

1.4.1 Smoking

A non-stoppable habit of smoking can harm a person actively or passively. Active smoking is when the person affected smokes himself while in passive, the person affected inhaled the smoke exhaled by a smoker. Exposure of foetus to active or passive smoking has been reported to be an established risk for CL/P (Grewal *et al.*, 2008). Cigarettes are loaded with more than 7000 toxic substances which are potentially harmful. Various studies as well as meta-analyses have indicated an increased risk of 2-20% due to maternal smoking(Grewal *et al.*, 2008; Martelli *et al.*, 2015; Panamonta *et al.*, 2017; Yang *et al.*, 2022).The direct interact of these toxins with the foetal tissue may induce hypoxia due to impaired angiogenesis and nicotine-mediated vasoconstriction, resulting in incomplete fusion of the palate as has been demonstrated in animal models (Vieira and Dattilo, 2018). Also, genetic vulnerability in detoxification genes elevates exposure of the foetus to alcohol-related CD.

1.4.2 Alcohol consumption

High dose alcohol consumption on regular basis during pregnancy have showed to be linked with higher incidence of CL/P (Grewal *et al.*, 2008; Kurita *et al.*, 2021; Lee *et al.*, 2021). A study also showed 3.4 fold increase in risk of CD due to alcohol consumption (Park-Wyllie *et al.*, 2000). This is due to the fact that ethanol exposure in IUL causes neural crest cell lysis resulting in gene alteration/mutation (Azimian Zavareh *et al.*, 2022).

1.4.3 Maternal health

Since the early 1900s, poor maternal diet has been considered as a contributor of oral clefts. Low concentrations of folate, zinc and vitamin B in the liver significantly contributes to the increased risk of CD (McKinney *et al.*, 2013). A study conducted on showed that mothers with folic acid deficiency have 10 folds times more chances of having a baby with CD (Van Rooij *et al.*, 2003). Use of polished rice as a staple food in Asians also increases vitamin-6 deficiency enhancing the risk of NSCL/P deformity (Munger *et al.*, 2004). Pregnancy complications have also been linked to higher risks of CD. Maternal diabetes has shown to have 1.352 times higher chances of causing CD (Spilson, Kim and Chung, 2001). This is due to the alterations in glycaemic control to be the cause for structural birth defects in mothers with gestational diabetes (Izedonmwen, Cunningham and Macfarlane, 2015). Similarly, gestational hypertension disorders are also considered one of the causes of CD. A study showed significance association of pre-eclampsia with increased risk of CD (Kutbi, 2014). However, another study in China could not observe any association between gestational hypertension and CD (An *et al.*, 2022).

1.4.4 Medicinal and chemical toxicity

Consumption of medications like corticosteroids or antiepileptic agents during pregnancy can have also shown association with CD (Xiao *et al.*, 2017). According to a data in 2014,

CD and steroids have an association of 1.0 95%CI, 0.7-1.4 (Skuladottir *et al.*, 2014). Similarly, exposure to toxic chemicals at the work environment like the herbicides and insecticides have also shown positive association with the CD in epidemiologic studies (Johnson *et al.*, 2014; Suhl *et al.*, 2017).

1.4.5 Maternal age at pregnancy

Maternal age and its association with cleft disorders has been studied for a long time but still no decisive. According to the World Health Organization (WHO), extreme maternal age of below 20 and above 35 are associated with higher incidence of CD (Morris, 2009; *Birth defects*, 2022). A study on Nigerian population showed higher risk of CD in children born to mother between age of 25-36 as compared to much younger mothers (James *et al.*, 2020). In contrast, another study highlighted increased risk of CD with both higher maternal or parental age (Bille *et al.*, 2005).

1.4.6 Socioeconomic status and education

According to the WHO report of 2022, the rate of severe birth defects is higher in low- and middle-income countries reaching up to 94%. This could be due to poor access to nutritional requirements (e.g., iodine, folate intake), heath facilities, high infection rate (rubella, syphilis, Zika), or high alcohol consumption. Maternal demographics for example low income, low education and biological factors may also be associated with CD (Lebby, Tan and Brown, 2010). In contrast, one study showed higher incidence of CD in children born to mothers with a higher level of education (Thomas, 2018).

1.5 Genetic factors

The term gene refers to a fundamental unit of inheritance found in chromosomes. An entire DNA sequence is required for the production of a functioning polypeptide or RNA sequence. A change or mutation in one or more genes may hinder their proper functioning.

Certain genes that control the detoxification pathway may increase the risk of CL/P as a result of any mutation. Since 1989, various genetic techniques, including linkage and candidate gene investigations, have been explored to uncover genes and pathways underlying NSCL/P (van Rooij *et al.*, 2019).

Human genetic research has shown that individuals with CL/P have a diverse genetic origin (Mahamad Irfanulla Khan, Prashanth and Srinath, 2020). Transforming Growth Factor-Alpha (*TGFA*) is considered the first gene to be recognized for association with NSCL/P (Ardinger *et al.*, 1989). This study was later supported by many similar studies indicating string link between mutated TGF alpha and NSCLP (Hwang *et al.*, 1995; Machida *et al.*, 1999; Vieira, 2006). Further studies were conducted to reveal the involvement of more genes in the NSCLAP like Transforming growth factor-beta 2 and 3 (*TGFB2, TGFB 3*), interferon regulatory factor-6 (*IRF6*), muscle-segment homeobox 1 (*MSX1*), muscle-segment homebox 2 (*MSX2),* T-box transcription factor-22 (*TBX22*), SMT3 suppressor of MIF two 3 homolog 1 (*SUMO1*), special AT-rich sequence binding protein 2 (*SATB2*), and methylene tetra hydro folate reductase (MTHFR). NSCLP is considered to be heterogeneous, with chromosome regions at certain loci claimed to have a link to NSCL/P like 1q, 2p, 4q, 6p, 14q, 17q, and 19q (Carinci *et al.*, 2007),(Egbunah, 2022).

Multiple studies have been conducted to recognize the pathogenic linkage of NSCL/P and the genes, however, the data available for NSCL/P in Malaysia is scarce especially in Malay population. Previously, five potential genes were identified from a large extended family group to recognize linkage evidence in the Malay community using microarray analysis (Mohamad Shah *et al.*, 2016). Further study was conducted to detect copy number variation on the specific large extended family that exhibited positive linkage evidence and eventually found 1 known gene: Special AT-rich sequence-binding protein 2 (*SATB2*). Also, two novel genes were also detected in the study; TOX High Mobility Group Box Family Member 3

(*TOX3*) and alpha chain of type XX1 collagen (*COL21A1*), where *TOX3* copy number was detected from a family which had shown suggestive linkage on 16q12.1 region through genome- wide linkage analysis. Who had low copy number calculated (CNC)< 1.50 (CNC = 1.36) for *TOX3* compared to normal controls CNC > 2.50. (Mohamad Shah *et al.*, 2019).

Another study detected reduction in the cellular level of fibroblast growth factor (FGF)in CLAP patient tissues. The study highlighted the link between TOX3 mutation and impaired FGF with the deficiency of structural fusion during the pre-developmental stage of IUL. *TOX3* is a 143 kb long nuclear protein with high mobility group (HMG)-box containing 7 exons and controls calcium-dependent transcription (Yuan, Qiu and Ghosh, 2009; Huang *et al.*, 2019; Qian *et al.*, 2019). Two reports have discovered a de novo obliteration on 16q12.1-q12.2 region each containing 17 and 39 genes together with *TOX3* causing severe craniofacial dysmorphism and psychomotor delay on a German patient and abnormal clinical finding of hearing impairment, delayed development, and renal injury on two boys from Japan (Shoukier *et al.*, 2012; Morisada, Nozu and Iijima, 2014). *TOX3* is an indispensable gene that plays a role in lip and palate formation during embryonic development. However, an association between *TOX3* in NSCL/P is still unclear.

1.5.1 Association of consanguinity and cleft disorders

Association between consanguinity and CD is not well demonstrated in previous studies with mixed responses. A study on Israel population showed CD ranging from 57.5% to 80% in intermarriage (Zlotogora *et al.*, 2003). Another study in Palestinians showed that consanguinity rate was 53% and familial clefting rate was 49% (Saeed *et al.*, 2019). Iranian population showed no significant difference between consanguinity and control group (Zandi and Heidari, 2011). Another study revealed that the chances of CD in consanguinity is highest among the first cousins (Silva *et al.*, 2019).

1.6 Objectives

1.6.1 General Objectives

To investigate the role of *TOX3 gene* and enviromental factors associated with NSCL/P formation.

1.6.2 Specific Objectives

1. To determine DNA copy number variation of *TOX3* in NSCL/P patients compared to control.
2. To determine the sociodemographic factors associated with NSCL/P.
3. To determine the effects of environmental factors that may contribute to the mutation in the *TOX3*

CHAPTER 2: MANUSCRIPT

Title Page

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Short title: TOX3 gene mutation in Malay non syndromic cleft deformities

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Abstract

Objective: Orofacial cleft is a congenital anomaly of lip and/or palate that can be syndromic or non-syndromic. Non syndromic cleft lip and/or palate (NSCL/P) is an anomaly with a physical deformity alone. Several genetic and environmental factors have been studied to find their association with NSCL/P. This study was conducted to determine the role of a novel TOX3 gene mutation in NSCL/P and its association with the environmental factors that may be contributing to increase in patients with NSCL/P.

Design: Case-control study

Setting: Reconstructive Science Unit, Universiti Sains Malaysia.

Patients; participants: Fifty patients with NSCL/P and 50 healthy individuals without cleft or family history of cleft disorder as control.

Interventions: Blood samples were obtained from consented patients and controls for genetic study of TOX3 copy number variation, and patients proforma to detect the environmental associated factors.

Results: Our results showed that the median score of TOX3 gene copy number was lower in patients with cleft as compared to the control but not to a significant level. Only one variable of maternal age in environmental factors showed statistically significant association with TOX3 risk mutation in NSCL/P (p=0.043) whereas cleft deformity increases with an increase in mother's age. Other variables showed no significant result however median TOX3 scores were lower in patients than in controls in folic acid deficiency, maternal diabetes, exposure to toxins, positive family history of cleft.

Conclusion: TOX3 genes did not show a significant difference between patients with NSCL/P and control. However, maternal age has a significant association with mutated TOX3 that may

cause NSCL/P. Future studies with a larger sample size of Malay NSCL/P subjects may be able to confirm the findings of this study.

Keywords: cleft lip, cleft palate, non-syndromic cleft

Introduction

Congenital anomalies are the developmental defects that begin in intrauterine life (IUL), identified before or after birth. Congenital anomalies may be limited to the physical structure only or may result with functional defect or both, as a result of any disturbance during the first trimester i.e., from 4th up to 12th week of IUL. The most common causes are genetic, chromosomal issues, harmful medicinal or toxic exposure, any infection during pregnancy, and nutritional deficiencies [1].

Cleft lip and/or palate (CL/P) is a condition where neonatal are born with incomplete union of the frontonasal and maxillary processes resulting in cleft lip and palate. Three types of cleft disorders (CD) are cleft lip only, cleft palate only, and cleft lip with cleft palate. The CD can be unilateral or bilateral, left sided or right sided, primary (if defect is anterior to incisive foramen), or as secondary (if defect is posterior to the incisive foramen) . It can be syndromic (SCL/P) i.e., associated with any syndrome, or it can be non-syndromic (NSCL/P) as a structural deformity only of lip and palate [2]. Around 70% of the CD cases are non-syndromic [3]. Multiple syndromes are known to involve a cleft as an anomaly. Some of those conditions are Pierre Robin syndrome, Treacher Collins syndrome, Van der Woude syndrome, and stickler syndrome [4].

Studies have been conducted to rule out the cause of this congenital disorder. Previous studies showed that the defect occurs as a result of various complex effects of genes and environmental on the neonatal during the IUL [4,5]. In addition, the child with these malformations faces multiple challenges with speech, hearing, oral intake, and repeated ear

infection. It also has a psychological effect on the children. As the child grows, there is difficulty in speech and the aesthetical appearance of the face which require multidisciplinary approach for the treatment [5].

Clefts occur at different rates in different groups due to ethnic, racial, or geographical variances [6]. Asians as well as American Indians are more likely to have cleft lip and palate than other ethnic groups [5,7]. One in every 500 births has reported to have cleft lip with or without palate in the Asian population [6]. Difference in prevalence of CD in different geographical regions and ethnic groups can be due to both genetic and environmental factors that impact these deformities like smoking habits, work area, health care facilities etc. Similarly, NSCL/P form have a wide range of impacts that may be of genetic and environmental aspects. It may be due to gene-gene or gene-environment interaction [8].

In Malaysia, a study conducted on 526 patients showed that 43%of the cases were of male patients while female percentage was 57 [9]. Another study on the three ethnic groups of Malaysia showed Malays with the highest frequency, followed by Chinese and then Indians [10].

Environmental factors have been studied which seem to have an impact on CD. Although no one particular factor can be labelled as the exact cause of NSCL/P. Exposure of foetus to active or passive smoking has been reported to be an established risk for CL/P [11]. High dose alcohol consumption on regular basis during pregnancy have showed to be linked with higher incidence of CL/P [11–13]. Low concentrations of folate, zinc and vitamin b in the liver significantly contributes to the increased risk of CD [14]. A study conducted on showed that mothers with folic acid deficiency have 10 folds times more chances of having a baby with CD [15]. Consumption of medications like corticosteroids or antiepileptic agents, during pregnancy can have also shown association with CD [16]. Similarly, exposure to toxic

chemicals at the work environment like the herbicides and insecticides have also shown positive association with the CD in epidemiologic studies [17,18]. According to the world health organization (WHO), extreme maternal age of below 20 and above 35 are associated with higher incidences of CD [19,20]. Maternal demographics for example low income, low education and biological factors may also be associated with CD [21].

A change or mutation in one or more genes may hinder their proper functioning. Certain genes that control the detoxification pathway may increase the risk of CL/P as a result of any mutation. Human genetic research has shown that individuals with CL/P have a diverse genetic origin [5]. Transforming Growth Factor-Alpha (*TGFA*) is considered the first gene to be recognized for association with NSCL/P and this study was later supported by many similar studies indicating string link between mutated TGF alpha and NSCLAP [22–25]. Further studies were conducted to reveal the involvement of more genes in the NSCLAP like Transforming growth factor-beta 2 and 3 (*TGFB2, TGFB 3*), interferon regulatory factor-6 (*IRF6*), muscle-segment homeobox 1 (*MSX1*), muscle-segment homebox 2 (*MSX2),* T-box transcription factor-22 (*TBX22*), SMT3 suppressor of MIF two 3 homolog 1 (*SUMO1*), special AT-rich sequence binding protein 2 (*SATB2*), and methylene tetra hydro folate reductase (MTHFR). NSCLAP is considered to be heterogeneous, with chromosome regions at certain loci claimed to have a link to NSCL/P like 1q, 2p, 4q, 6p, 14q, 17q, and 19q [26,27].

Multiple studies have been conducted to recognize the pathogenic linkage of NSCL/P and the genes, however, the data available for NSCL/P in Malaysia is scarce especially in Malay population.

To the best of our knowledge, no previous study has been conducted to validate *TOX3* mutation on larger sample size and its associated environmental factors to outline recommendation for clinical and public education strategies for reduction of NSCL/P

formation in Malay population. Determining the relative risk of TOX3 mutation to cause NSCL/P based on genetic background and environmental influence will be beneficial for genetic counseling and the development of future preventive measures.

Methodology

This is a case control study among Malays, fifty patients with NSCL/P who underwent cleft lip or palate surgery under Plastic and Reconstructive Surgery unit, Hospital Universiti Sains Malaysia, and fifty volunteer healthy individuals who did not have cleft or a family history of cleft were selected from staff and students of Universiti Sains Malaysia to use as a control.

The selection criteria included all NSCL/P patients who underwent cleft repair by the Plastic and Reconstructive Surgery unit in HUSM. Patients with one minor anomaly were included in the study as it does not interfere with cleft abnormalities such as low-set ears, hypertelorism, clinodactyly and single palmar crease. Control samples were taken from healthy volunteers who did not have cleft or family history with cleft and should not associate with any major congenital anomalies.

Syndromic cleft lip and/or palate patients and non-syndromic with major abnormalities such as heart problem and brain defect were excluded. Healthy individuals were excluded if they exhibited non-syndromic or syndromic cleft lip and/or palate, syndromic diseases, and any major abnormalities. Venous blood samples were collected after getting consent from NSCL/P patients and healthy individual controls in the ethylenediaminetetraacetic acid (EDTA) tube. The blood samples were then stored in the fridge at a temperature of 4ºC or taken as soon as possible for DNA extraction.

Genetic variations and mutation of TOX3 gene study

DNA extraction

DNA was extracted from fresh or stored venous blood using commercially available DNA extraction kit (QIAamp DNA Mini Kit). Extraction was performed with spin protocol based on manufacturer's methods. First, 20 µl of QIAGEN Protease was pipetted into the bottom of a 1.5ml microcentrifuge tube. Then, 200 µl of whole blood sample and 200 µl of Buffer AL were added into the microcentrifuge tube. Then, the contents were mixed by pulse-vortexing for 15 seconds. The tube was incubated at 56°C for 10 minutes with heating block. Next, the microcentrifuge tube was briefly centrifuged to remove the drops from the inside of the lid. Then, 200 µl of absolute ethanol (96%-100%) was added to the mixture and mixed thoroughly by pulse-vortexing for 15 seconds. The tube was briefly centrifuged after the mixing. The mixture was then carefully applied to the QIAamp Mini Spin column and centrifuged at 6000 x *g* for 1 minute. The QIAamp mini spin column was transferred into a clean 2 ml collection tube and the filtrate was discarded. Next, 500 µl of Buffer AW1 was added to the QIAamp Mini Spin column and centrifuged at 6000 x *g* for another 1 minute. The QIAamp Mini spin column was transferred into a new collection tube and the filtrate was discarded. Then, 500 µl Buffer AW2 was added into the spin column and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 minutes. The filtrate was discarded. The QIAamp mini spin column was placed into a clean 1.5 mL microcentrifuge tube. 200 µl Buffer AE was added into the spin column. The tube was left incubated for 1 minute in room temperature before centrifuge at 6000 x *g*. The DNA will be used immediately or stored at -20°C for long term storage.