MUTATION STATUS OF SKELETAL MUSCLE CONTRACTILE GENES (TNNT3 AND TPM2) AMONG MALAY CLUBFOOT PATIENTS: DETERMINATION USING PCR-DNA SEQUENCING ANALYSIS

By

DR MOHAMAD FARIS BIN MD ARIFF

A dissertation submitted in partial fulfilment of the requirements for the degree of Master of Medicine (Orthopedic)

UNIVERSITI SAINS MALAYSIA

2022

ACKNOWLEDGEMENT

First and foremost, Alhamdulillah, I am grateful to Allah The Almighty, for giving me strength, patience and continuous health throughout my research journey.

My highest appreciation to my main supervisor, Associate Prof Dr. Ismail bin Munajat for his guidance and encouragement to accomplish this project. Thank you for teaching me all the way from the beginning and always provide a very positive moral support for me.

I am ineffably indebted to my co-supervisor, Associate Prof. Dr. Sarina Sulong and Dr Syurahbil bin Abdul Halim for their non- stop supervision, continuous support and inspiration. Thank you so much for being very kind, patience and understanding.

I would like to extend my sincere gratitude to Mrs. Nur Shafawati Ab Rajab for her expert technical assistance. Without her passionate participation and input, the lab work and data analysis could not have been successfully conducted.

I am fortunate to have an exceptionally strong support system. Most importantly, I would like to thank my wife, Dr. Emiza Azreen binti Ahmad Suhaimi and my children, Afrina and Dania for being very patience and understanding during my ups and downs. To my extraordinary parents, Haji Md Ariff bin Abas and Hajjah Wan Sepiah Binti Sheikh Ibrahim, thank you so much for the great prayers and strength.

Last but not least, I am thankful to Ministry of Higher Education and Universiti Sains Malaysia for providing me the scholarship to fulfil my career goals and for supporting this study under one of the grants.

Any omission in this brief acknowledgement does not mean lack of gratitude.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS AND SYMBOLS

ABBREVIATIONS

CI	Confidence Interval DNA
D N A	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide Triphosphate
OR	Odds Ratio
PCR	Polymerase Chain Reaction
SD	Standard Deviation
SNP	Single Nucleotide Polymorphism

SYMBOLS

bp	basepairs
mL	milliliter
mM	millimolar
mmol/L	millimoles per litre
ng/mL	nanograms per milliliter nmol/L
n m / L	nanomoles per litre
rpm	revolutions per minute
U	units
µl	microlitre

µM	micromolar
°c	degree Celsius
≥	more or equal than
<	less than

ABSTRAK

Pengenalan

Kaki pengkar adalah salah satu daripada anomali muskuloskeletal kongenital yang paling biasa dengan kejadian 1 dalam 1000 kelahiran. Kaki pengkar idiopatik dan bersebab menunjukkan pengecutan otot betis semasa lahir dan kekal kecil selepas rawatan. Kajian ini dijalankan adalah untuk mencari sebarang mutasi pada genetik yang bertanggungjawab dalam pertumbuhan tisu otot bagi pesakit kaki pengkar tanpa sebab, kerana ia telah dijumpai dalam pesakit kaki pengkar bersebab.

Kaedah Kajian

Kajian ini dijalankan ke atas 33 kanak-kanak kaki pengkar tanpa sebab yang mendapat rawatan di Hospital Universiti Sains Malaysia. Sel pipi diperoleh daripada kanak-kanak itu dan sampel tersebut dihantar ke Pusat Genom Manusia, Universiti Sains Malaysia (USM). DNA diekstrak dari sel pipi dan ketulenan DNA diukur. Tindak balas berantai polimerase (PCR) dijalankan dalam keadaan optimum dan diikuti dengan elektroforesis gel agarose. Penjujukan DNA dilakukan dan sebarang mutasi diperhatikan dan direkodkan. Keputusan untuk penjujukan DNA dianalisis secara statistik.

Keputusan

Dalam kajian kami, tiada mutasi genetik ditemui pada semua peserta kajian untuk TPM2: c.308A>G dan TNNT3: c.187C>A. Oleh itu, perkaitan antara mutasi genetik TPM2: c.308A>G dan TNNT3: c.187C>A dan keterukan kaki pengkar pada pesakit Melayu dengan kaki pengkar

tanpa sebab tidak dapat ditentukan. Tiada analisis statistik dijalankan kerana hasil pembolehubah bersandar (TPM2: c.308A>G dan TNNT3: mutasi c.187C>A) adalah malar (tiada mutasi).

Kesimpulan

Tiada mutasi ditemui pada TPM2: c.308A>G and TNNT3: c.187C>A. Oleh itu, hubung kait antara mutasi gentik dan keterukan kaki pengkar tanpa tidak dapat ditentukan. Walau bagaimanapun, kita tidak boleh menolak sepenuhnya kerana terdapat beberapa lagi gene lain yang bertanggungjawab dalam pertumbuhan dan fungsi otot rangka.

ABSTRACT

Introduction

Clubfoot or congenital talipes equinovarus (CTEV) is one of the most common congenital musculoskeletal anomalies and the incidence of 1 in 1000 births. Idiopathic and non-idiopathic clubfeet demonstrate calf muscle hypoplasia at birth which remains small even after corrective treatment of the clubfoot. We conducted this study to look for any skeletal gene mutations in idiopathic CTEV, as it is well described in non-idiopathic CTEV.

Methodology

This study was carried out in 33 idiopathic CTEV children that get their treatment in Hospital Universiti Sains Malaysia. Buccal swab was obtained from the child and the sample was sent to Human Genome Centre, Universiti Sains Malaysia (USM). DNA was extracted and purity was quantified. PCR was conducted in optimum condition and followed by agarose gel electrophoresis. DNA sequencing done and any mutations were observed and recorded. The results for DNA sequencing was analyzed statistically.

Result

In our study, no mutations detected in all the study participants for TPM2: c.308A>G and TNNT3: c.187C>A. Therefore, the association between the TPM2: c.308A>G and TNNT3: c.187C>A gene mutations and the clubfoot severity in Malay patients with idiopathic clubfoot was undetermined. No statistical analysis was conducted since the outcome of the dependent variable (TPM2: c.308A>G and TNNT3: c.187C>A mutations) was constant (no mutation).

Conclusion

No mutation was found in TPM2 and TNNT3 gene, hence the association with the clubfoot severity is undetermined. However, we cannot totally exclude the skeletal muscle genes as here are few more genes that are responsible for development and function of skeletal muscle.

CHAPTER 1: INTRODUCTION

Clubfoot is one of the most common congenital musculoskeletal anomalies and the incidence of 1 in 1000 births. Around 80% of clubfeet occur as an isolated abnormality and are considered idiopathic clubfeet which are also called congenital talipes equinovarus (CTEV). The remaining 20% are commonly associated with a known syndrome, musculoskeletal disorder or chromosomal etiology which are generally called as non-idiopathic clubfeet. It described as the contracted foot will be turned inward toward midline of body.

Patients with clubfoot demonstrate calf muscle hypoplasia at birth which remains small even after corrective treatment of the clubfoot [1,2]. This observation suggests that there might be involvement of skeletal muscle contractile genes which play a role in the muscle growth and development as the child grows. This observation is supported by previous study showing that the genes coding for the components of the contractile muscle complex causes congenital contracture including in clubfoot and distal arthrogryposis [3]. TNNT3 will encode for skeletal troponin-T which is part of troponin complex and is associated with autosomal dominant distal arthrogryposis [4]. TPM2 is responsible to encode for b-tropomyosin that is mainly expressed in skeletal muscle. It is also associated with distal arthrogryposis [5].

Another component of striated skeletal muscle is sarcomeric myosins which is coded by *MYH3* [14]. Therefore, TNNT3, MYH3 and TPM2 are among the genes that have been widely studied before.

The Pirani score is known to be valid and reliable in assessing the severity of clubfoot. It also providing a good forecast about the response of treatment. Four components of clubfoot were assessed (cavus, adductus, varus & equinus) and higher score may indicate the clubfoot is severe and may need higher numbers of casting. By logical, Pirani score of zero would be a corrected clubfoot but in present study, corrected clubfoot Pirani score may or may not be 0 [6,7]. All patients undergo Ponseti method of clubfoot correction where it is a specific method of serial manipulation, casting, and tenotomy of the Achilles tendon [15].

CHAPTER 2: OBJECTIVE OF STUDY

2.1 General

To determine the mutation status of skeletal contractile genes among Malay clubfoot patients and its association with clubfoot severity from birth to adolescence

2.2 Specific

1. To determine the frequency of TPM2, and TNNT3 mutations in Malay patients with idiopathic clubfoot from birth to adolescence.

2. To identify the types of point mutation or variant within the hotspot regions of TPM2 and TNNT3 genes in Malay patient with idiopathic clubfoot.

3. To determine the association between the TPM2 and TNNT3 gene mutations and the clubfoot severity in Malay patient with idiopathic clubfoot.

CHAPTER 3: MANUSCRIPT

3.1 RESEARCH TITLE

Mutation status of skeletal muscle contractile genes (MYH3, TNNT3 and TPM2) among Malay clubfoot patients: Determination using PCR-DNA sequencing analysis

Author: Dr. Mohamad Faris bin Md Ariff (MMC 64291)

Department of Orthopedics,

School of Medical Sciences, Universiti Sains Malaysia.

16150 Kota Bharu, Kelantan.

Co-researchers

Dr. Ismail bin Munajat (MMC 34576)

Prof. Madya Dr. Sarina binti Sulong

Dr. Syurahbil bin Abdul Halim (MMC 61881)

3.2 ABSTRACT

Introduction

Clubfoot or congenital talipus equinovarus (CTEV) is one of the most common congenital musculoskeletal anomalies and the incidence of 1 in 1000 births. Idiopathic and non-idiopathic clubfeet demonstrate calf muscle hypoplasia at birth which remains small even after corrective treatment of the clubfoot. We conducted this study to look for any skeletal gene mutations in idiopathic CTEV, as it is well described in non-idiopathic CTEV.

Methodology

This study was carried out in 33 idiopathic CTEV children that get their treatment in Hospital Universiti Sains Malaysia. Buccal swab was obtained from the child and the sample was sent to Human Genome Centre, Universiti Sains Malaysia (USM). DNA was extracted and purity was quantified. PCR was conducted in optimum condition and followed by agarose gel electrophoresis. DNA sequencing was done, and any mutations was observed and recorded. The results for DNA sequencing was analyzed statistically.

Result

In our study, no mutations detected in all the study participants for TPM2: c.308A>G and TNNT3: c.187C>A. Therefore, the association between the TPM2: c.308A>G and TNNT3: c.187C>A gene mutations and the clubfoot severity in Malay patients with idiopathic clubfoot was undetermined. No statistical analysis was conducted since the outcome of the dependent variable (TPM2: c.308A>G and TNNT3: c.187C>A mutations) was constant (no mutation).

Conclusion

No mutation was found in TPM2 and TNNT3 gene, hence the association with the clubfoot severity is undetermined. However, we cannot totally exclude the skeletal muscle genes as here are few more genes that are responsible for development and function of skeletal muscle.

3.3 INTRODUCTION

Clubfoot is one of the most common congenital musculoskeletal anomalies and the incidence of 1 in 1000 births. Around 80% of clubfeet occur as an isolated abnormality and are considered idiopathic clubfeet which are also called congenital talipes equinovarus (CTEV). The remaining 20% are commonly associated with a known syndrome, musculoskeletal disorder or chromosomal etiology which are generally called as non-idiopathic clubfeet. It described as the contracted foot will be turned inward toward midline of body.

Patients with clubfoot demonstrate calf muscle hypoplasia at birth which remains small even after corrective treatment of the clubfoot [1,2]. This observation suggests that there might be involvement of skeletal muscle contractile genes which play a role in the muscle growth and development as the child grows. This observation is supported by previous studiy showing that the genes coding for the components of the contractile muscle complex causes congenital contracture including in clubfoot and distal arthrogryposis [3]. TNNT3 will encode for skeletal troponin-T which is part of troponin complex and is associated with autosomal dominant distal arthrogryposis [4]. TPM2 is responsible to encode for b-tropomyosin that is mainly expressed in skeletal muscle. It is also associated with distal arthrogryposis [5].

Another component of striated skeletal muscle is sarcomeric myosins which is coded by *MYH3* [14]. Therefore, TNNT3, MYH3 and TPM2 are among the genes that have been widely studied before.

The Pirani score is known to be valid and reliable in assessing the severity of clubfoot. It also providing a good forecast about the response of treatment. Four components of clubfoot were assessed (cavus, adductus, varus & equinus) and higher score may indicate the clubfoot is severe and may need higher numbers of casting. By logical, Pirani score of zero would be a corrected clubfoot but in present study, corrected clubfoot Pirani score may or may not be 0 [6,7]. All patients undergo Ponseti method of clubfoot correction where it is a specific method of serial manipulation, casting, and tenotomy of the Achilles tendon [15].

3.4 MATERIAL AND METHOD

3.4.1 Study Design

This case-control study was approved by Human Research Ethics Committee USM (USM/JEPeM/20120688). After explaining the objectives and potential benefits of the study, informed consent was obtained from each participant. The study subjects were recruited from the orthopedic specialist clinic at Hospital Universiti Sains Malaysia, Kelantan while experimental analyses were carried out at Human Genome Centre, School of Medical Sciences, Universiti Sains Malaysia, Kelantan.

3.4.2 Sample Size Calculation

$$n = \frac{Z^2_{\alpha/2} p(1-p)}{d^2}$$

n= the sample size

Z (α/2) = the standard normal coefficient, typically 1.96 for 95% CI

p = the value of the proportion as a decimal percent

d = the desired precision level expressed as half of the maximum acceptable confidence interval width

The estimated sample size for objective 1 was estimated and calculated using formula above and was based on study by Gurnett et al [6] with p=0.2 and d=0.1 will therefore show the estimated sample size of 61.

3.4.3 Inclusion Criteria

The study subject was taken from Malay ethnicity, which define as person who professes the religion of Islam, habitually speaks the Malay language, and conforms to Malay custom. The subject age from birth until late adolescent (14 years old) for both male and female subject. The subject also diagnosed to have idiopathic type of clubfoot either unilateral or bilateral clubfoot.

3.4.4 Exclusion Criteria

Those subject with other foot deformity apart from clubfoot and known genetic syndrome in family were excluded from this study.

Genomic DNA Extraction

Buccal sample was taken from subject using Isohelix swab (Isohelix, UK) and was stabilized using BuccalFix DNA stabilization buffer (Isohelix, UK). DNA was extracted using the BuccalFix Plus DNA Isolation Kit (Isohelix, UK). The buccal samples were extracted according to the BuccalFix Plus DNA Isolation Kit (Isohelix, UK) protocol. Firstly, the water bath was heated on to 56°C. 20 µL of proteinase K was added to the buccal fix tube containing 400 µL of buffer solution and buccal swab. The mixture was mixed well using pulse vortex and incubated at 60°C for 1 hr. The tube was again mixed using pulse vortex. Then, the mixture was transferred to a new 1.5 ml microcentrifuge tube. Later, 400 µL of BP solution was added to the mixture and mixed thoroughly by 15 sec pulse vortexing followed by spinning at 13000 rpm for 10 mins. Then, the supernatant was carefully poured off. Then, 50 µL of Tris- EDTA (TE) solution was added to the tube containing pellet and continued by vortexes and leaved for 5 mins at room temperature. Then, the mixture was spined at 12000 rpm for 15 mins. Then, the supernatant was transferred into new 1.5 ml tube and the tube with pellet was discarded.

50 µL of BLS solution was added to the tube containing supernatant. Then, 100 µL of BP solution also was added to the mixture and continued with vortex. After vortexing, the mixture was centrifuged at 13000 rpm for 10 mins. Then, all the liquid was removed and left pellet. Finally, 50 µL of DNA dehydration buffer was added to the pellet and continued with vortex and was incubated at room temperature for 5 mins. The mixture was stored in -20°C freezer as genomic DNA.

Quantitation of DNA Purity

After performing DNA extraction, the purity and concentration of the extracted DNA will be determined using spectrophotometer (NanoQuant Infinite M200, Tecan Inc., USA). The concentration of DNA ranged from 10.48 - 148.7ng/µl and purity ranged from 1.7-2.

The genomic DNA product was also electrophoresed on 1% agarose gel (Promega, USA) and stained by SybrGreen (Cambrex Bioscience Rockland inc, USA) for determination of the genomic DNA quality. Electrophoresis was performed in 1X TBE buffer for 45 minutes at 100 V. The gel was photographed by UV transillumination.

Designing Primers

Two primers were designed (Table3.1) to amplify p.Q103R (c.308A>G) and p.Arg63Ser (c.187C>A) SNPs of TPM2 and TNNT3 gene using Primer3 software. The designed primers were validated online using Primer Basic Local Alignment Search Tool (BLAST) analysis (http://blast.ncbi.nlm.nih.gov/) to confirm their specificity to the gene region of interest.

The primers that contain both reverse and forward were designed within 20-25 bp in length with GC content of less than 50% and melting temperature ™> 50^{o}C and of not more than 5^{o}C difference from each other. Primer should not be self-complementary to each other to avoid hairpin or primer dimers and no repetitive sequences should be observed in the primers. The PCR products for p.Q103R (c.308A>G) and p.Arg63Ser (c.187C>A) primers were 349 bp and 386 bp respectively.

Mutations	Primers	PCR size	Product
TPM2: p.Q103R (c.308A>G)	F: 5′-AGCCTCTCTGATCCTTATCCAAGG-3′ R: 5′-TCTGAATCCTCAGCGATGTGCTTG-3′	394bp	
TNNT3: p.Arg63Ser (c.187C>A)	F: 5′-CAAGGGAGTCAGGGCTTCTC3′ R: 5′-CCTCTCCTTCTCTGCACGAA3′	386bp	

Table 3.1: primer design for TPM2 and TNNT3

PCR Optimization

In order to get a specific PCR product, the successful of primer annealing was very important. PCR gradient was performed to identify the optimum conditions for the annealing of primers for both targeted area of p.Q103R (c.308A>G) and p.Arg63Ser (c.187C>A). The PCR reaction was performed using 10 duplications from one sample with a total volume of 25 µl reaction mixture in each tube. PCR was performed using Agilent Technologies, SureCycler 8800 thermal cycler. The annealing temperature optimization was carried out in the form of gradient temperature. The temperature range was determined by calculating the average Tm of forward and reverse primers with ± 5^{o}C. Thus, optimization was carried out from 56.0^{o}C to 68.0^{o}C. The optimal annealing temperature for both p.Q103R (c.308A>G) and p.Arg63Ser (c.187C>A) was 56^{o}C and 57^{o}C respectively as the band was most visible with no unspecific bands seen accompanying it

with this annealing temperatures. Time for annealing was 30 seconds each. Dimethyl sulfoxide (DMSO) was added to the PCR mixture to enhance PCR amplification.

Before preparing PCR, the working area was swabbed with 70% alcohol to avoid any contamination.

For each PCR reaction amplification, the master mix comprises of 10x Pfx amplification buffer, 25 mM $MgCl_2$, 25 mM dNTP mix, 10µM of forward and 10µM of reverse primers, 2.5 units/µl Taq DNA polymerase, 100% DMSO and deionized water (ddH_2O). The PCR master mix was prepared as shown in Table. The mixture was tapped several times, vortexed and centrifuged briefly to ensure it was adequately mixed. A total of 23.5 µl of the master mix was pipetted into 0.2 ml tubes followed by 1.5 µl of DNA templates to make a final volume of 25 µl for each tube. All the tubes were centrifuged to ensure adequate mixture.

The tubes were placed in the Agilent Technologies, SureCycler 8800 thermal cycler PCR machine. For both p.Q103R (c.308A>G) and p.Arg63Ser (c.187C>A) , the PCR cycling programmed started at $95^{o}C$ for 2 minutes to heat-activate the Taq DNA polymerase, followed by 35 cycles of denaturation at $95^{o}C$ for 30 seconds and annealing at $56^{o}C$ and $57^{o}C$ for both p.Q103R (c.308A>G) and p.Arg63Ser (c.187C>A) respectively (Refer table 3.2, 3.3 and 3.4). Lastly final extension was at $72^{o}C$ for 5 minutes.

Reagent Stock	Concentration	Volume (µL)	Final Concentration
ddH_2O		13.4	-
Pfx Amplification Buffer	10X	5	2X
$MgCL_2$	25mM	2	2mM
dNTP	2.5 mM (each)	0.5	0.05 mM (each)
Forward Primer	10 µM	0.8	0.32 µM
Reverse Primer	10 µM	0.8	0.32 µM
DMSO		0.75	
Taq DNA Polymerase	2.5 U/µL	0.25	0.025 U/µL
DNA		1.5	Variable
Total		25	

Table 3.2: Final concentration and volume of reagents used for the Polymerase Chain Reaction master mix for mutation p.Q103R (c.308A>G)

Reagent Stock	Concentration	Volume (µL)	Final Concentration
ddH_2O		14	-
Pfx Amplification Buffer	10X	5	2X
$MgCL_2$	25mM	2	2mM
dNTP	2.5 mM (each)	0.5	0.05 mM (each)
Forward Primer	10 µM	0.5	0.2 µM
Reverse Primer	10 µM	0.5	0.2 µM
DMSO		0.75	
Taq DNA Polymerase	2.5 U/µL	0.25	0.025 U/µL
DNA		1.5	Variable
Total		25	

Table 3.3: Final concentration and volume of reagents used for the Polymerase Chain Reaction master mix for mutation p.Arg63Ser (c.187C>A)

Step	Temp (°C)	Time (sec/min)	Cycles
Initial denaturation	95	2 minutes	1x
Denaturation	95	30 seconds	35X
Annealing	56 (p.Q103R) 57 (p.Arg63Ser)	30 seconds	
Extension	72	30 seconds	
Final extension	72	5 minutes	1x

Table 3.4: The Thermal Cycler conditions of the PCR reactions.

Gel Electrophoresis

The PCR products were visualized by performing agarose gel electrophoresis. The DNA fragments were separated according to their molecular size. The negatively charged nucleic acid molecules move through an agarose matrix with an electric field (electrophoresis). Lighter molecules with lower base pair move faster and migrate further than the bigger molecules with higher base pairs product.

2% Agarose Gel Electrophoresis

2% agarose gel was prepared by initially adding 1.0g of agarose (Vivantis, U.S) to 50 ml of 1X TBE buffer (Tris-Boric Acid-EDTA buffer). The mixture was then heated with microwave oven for about 2-3 minutes. Once the agarose was completely dissolved, it was then immediately poured into the casting apparatus and allowed to cool off.

Ladder/DNA Marker

The 100 bp ladder (Promega Co., Madison, U.S) was used as a marker. It was used to determine DNA size from 100 bp up to 1000bp. This 100 bp ladder was supplied in 10mM Tris-HCI (pH 7.4), 1mM EDTA.

Loading Dye Buffer

Blue/Orange Loading Dye Buffer (Promega Co., Madison, U.S) was used for loading the DNA samples into gel electrophoresis wells and tracking its migration during electrophoresis. This convenient marker dye contained 0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll 400, 10mM Tris-HCl (pH 7.5) and 50mM EDTA (pH 8.0).

Staining Material

SYBR Green 1 Nucleic Acid Gel Stain (Cambrex Bio Sciences Rockland Inc. U.S) was used to stain the double stranded DNA. One part of the stock solution was diluted into 100X with 99 parts of DMSO.

1X TBE Buffer Solution

Tris Boric Acid- EDTA buffer (TBE buffer) was a conductive medium for electrophoresis used for separation of smaller DNA fragments. The 10X TBE buffer stock was prepared by adding 108g of Tris-base (Calbiochem, U.S), 93g EDTA powder (Sigma- Aldrich, U.S) and 55g of Boric acid (Merch, Germany) into 800ml of deionized water (ddH2O) into 1000 ml beaker. The mixture was stirred by using a stirrer until the solution turned colorless. Next, to make a final volume 1000 ml with 10X concentration, 200ml of ddH2O was added. pH meter was used to measure the 10X

TBE buffer stock. Ideallythe pH of the buffer is 8.3. To prepare 1L of 1X TBE buffer, 900ml of distilled water was added to 100 ml of 10X TBE buffer and was then mixed thoroughly.

Agarose Gel Electrophoresis Protocol

The agarose gel was firstly put into the electrophoresis tank that contained 1X TBE buffer. The first well of agarose gel was loaded with 100bp DNA ladder mixture. The mixture composed of 1 µl of 100 bp ladder, 1 µl of 6X loading eye, 1µl 100X SYBR Green and 3 µl of 1X TBE buffer. Next, 1 µl of 6X loading dye, 1 µl SYBR Green and 4 µl of PCR amplicons were mixed into each of the sample and loaded into the well. The agarose gel was then run for 60 minutes at 90V, 400mA. Later, it was visualized under ultraviolet (UV) using UV transilluminator (Wealtec Corp. USA) and the image was captured using the AlphaDigiDoc™ system.

DNA Sequencing

All PCR samples were sent for sequencing (Apical Scientific Sdn. Bhd. Labs, Kuala Lumpur, Malaysia), for visualization of the PCR's nucleotides sequences. The sequencing electropherograms were analyzed using Bioedit software. Through the sequencing results, the nucleotide on the selected SNP can be seen and identified.

Statistical Analysis

Statistical packages for the social sciences (SPSS) version 28 was used for statistical analysis. Group comparisons for continuous and categorical variables were analyzed. P-value <0.05 was considered statistically significant.

3.5 RESULT

Characteristics of Study Participants.

Table 3.5 shows the characteristic of the study participants. The total number of participants in this study was 33. The majority of participants in this study were male, 25 participants (75.8%) and followed by female participants, 8 participants (24.2%) are shown in Figure 3.1.

The mean DNA concentration of the participants' samples was 77.82 ng/µl with a standard deviation (SD) of 43.390 ng/µl and ranged between 20.0 ng/µl and 148.7 ng/µl. Meanwhile, the mean value of the DNA purity of the samples taken was 1.82 with an SD of 0.062 and ranged between 1.70 and 2.00.

We found that the 18 participants were suffering from unilateral clubfoot (54.5%) and 15 were having bilateral clubfoot (45.5%) are shown in Figure 3.2. The average Pirani score was 4.1 (± 1.11) with 3.5 at the 25% percentile, 4.0 at the 50% percentile, and 5.0 at the 75% percentile are shown in Figure 3.3. The number of castings applied to study participants ranged from two to nine for each limb with a mean of 6.8 (± 1.97) and six, seven and eight were the 25%, 50% and 75% percentiles, respectively. The majority number of castings applied was seven (24.4%) followed by nine (22.2%) and eight (19.6%). In addition, the number of participants who underwent the tenotomy was 25 (75.8), while 8 (24.2%) were not subjected to any surgical intervention during the study period.

Meanwhile, we found no TPM2 gene mutation in all the 33 participants in our study. Similar findings were acquired for TNNT3 gene mutation.

Table 3.5 Characteristics of the study participants.

Gender			
Male		25	75.8
Female		8	24.2
DNA Concentration (ng/µl)	77.82 (±43.390)		
DNA Purity	1.820 (±0.062)		
Laterality			
Unilateral clubfoot		18	54.5
Bilateral clubfoot		15	32.6
Pirani Score	4.14 (±1.101)		
Number of castings	6.82 (±1.969)		
Two		2	4.4
Three		2	4.4
Four		3	6.7
Five		2	4.4
Six		6	13.3
Seven		11	24.4
Eight		9	20.0
Nine		10	22.2

Tenotomy		
Yes	25	75.8
No	8	24.2
TPM2 gene mutation		
Yes	0	0.0
No	33	100.0
TNNT3 gene mutation		
Yes	0	0.0
No	33	100.0

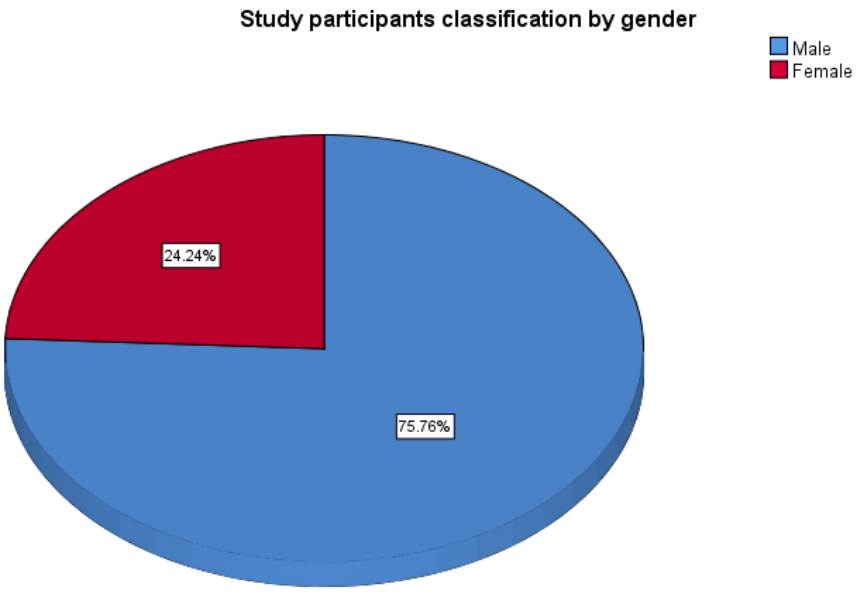

Figure 3.1: distribution of CTEV patient by gender

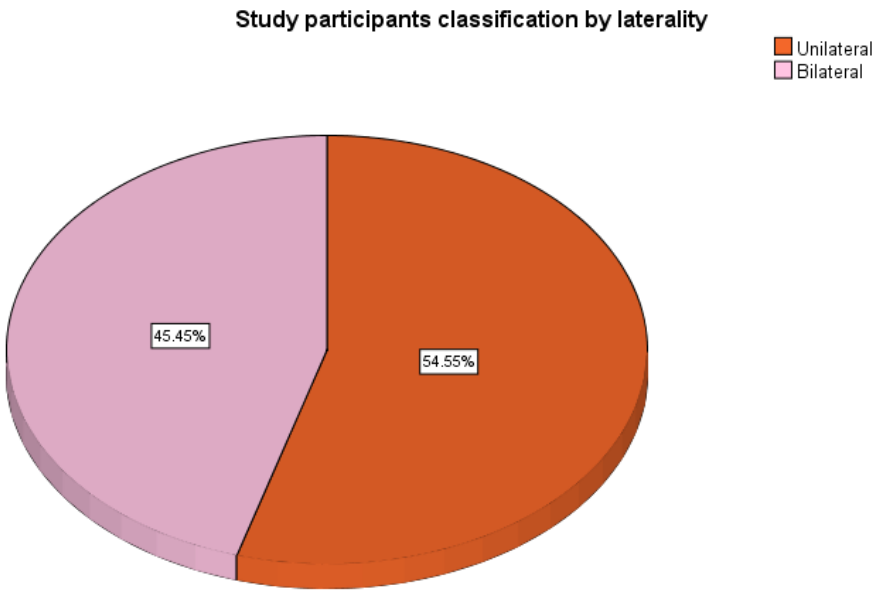

Figure 3.2: distribution of CTEV patient by laterality

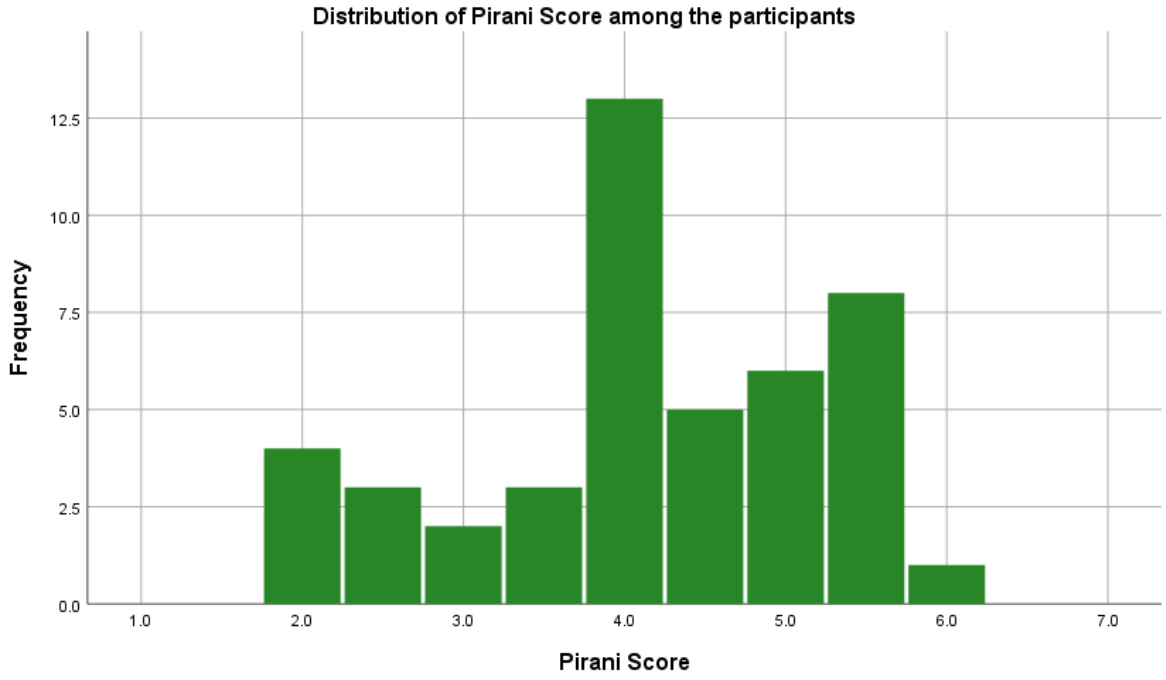

Figure 3.3: distribution of Pirani score among participants

Association Between Participants' Characteristics and Clubfoot Severity, And the Numbers of Casting.

The association between gender and laterality and the number of castings among the study participants was determined using the Mann-Whitney U test. The test showed that the male participants had a higher number of castings overall compared to the female participants (mean rank of 18.60 and 12.00, respectively). However, the association was found not statistically significant (Mann-Whitney U = 60.00, p = 0.098).

Meanwhile, participants with bilateral clubfoot have a higher number of castings compared to unilateral clubfoot participants with a mean rank of 19.43 and 14.97, respectively. However, the association was also found not statistically significant (Mann-Whitney U = 98.50, p = 0.190).

A Spearman's rank-order correlation was run to determine the relationship between the Pirani score and the number of castings. There was a moderate, positive correlation between the Pirani score and the number of castings, which was statistically significant ($r_s = 0.538$, $p < 0.001$)

Association Between Participants' Characteristics and Clubfoot Severity and The Tenotomy Surgery Status.

A Fisher's exact test (FET) was conducted to determine the association between gender and laterality and tenotomy status. Twenty out of 25 (80.0%) male participants underwent the tenotomy surgery while five out of eight (62.5%) underwent the surgery. However, the association was found not statistically significant (p = 0.336).

Meanwhile, 12 out of 18 (66.7%) participants with unilateral clubfoot underwent tenotomy and 13 out of 15 (86.7%) participants with bilateral clubfoot underwent similar surgery. However, the association was found not statistically significant with a p-value of 0.242.

On the other hand, a Kruskal-Wallis test between Pirani score and tenotomy status showed a statistically significant association ($\chi^2(1) = 6.083$, $p = 0.014$). The participants who underwent tenotomy surgery had a higher score compared to those who did not have the surgery in terms of Pirani score with a mean rank of 19.35 and 9.75, respectively.

Genotyping of TPM2: c.308A>G and TNNT3: c.187C>A

All DNA was confirmed by the existence of a band on gel electrophoresis. The concentration and purity of all DNAs were measured and passed using NanoQuant Infinite M200, Tecan Inc., USA. (Figure 3.4)

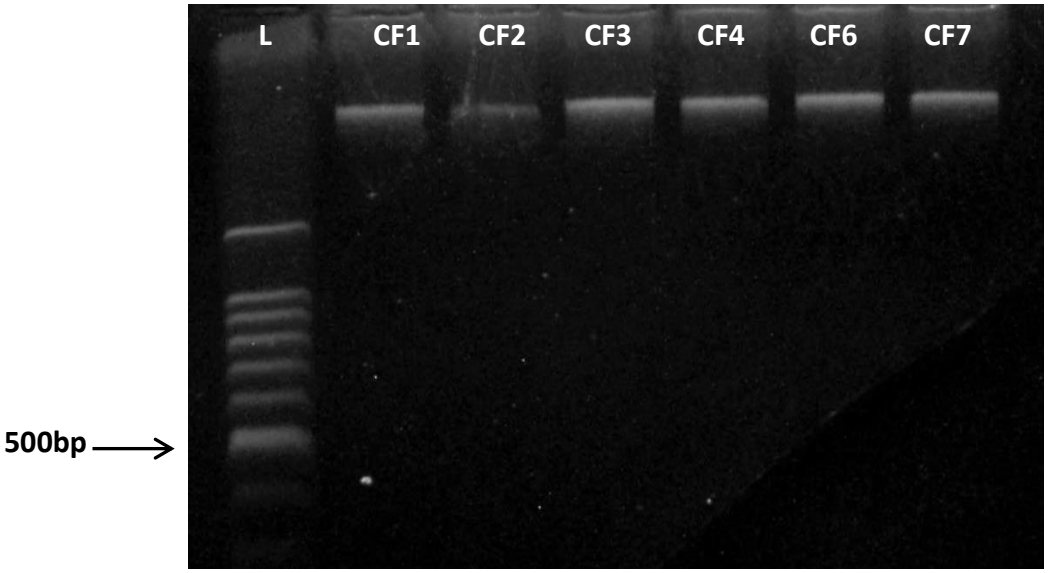

Figure 3.4: Gel picture showing genomic DNA for clubfoot patients visualized on 1% agarose gel. Lanes 1: 100bp marker; Lane 2 to Lane 7: Genomic DNA of clubfoot patients.