IDENTIFICATION OF MUTATIONS IN GENES COMMONLY ASSOCIATED WITH CHARCOT-MARIE-TOOTH DISEASE IN A MALAYSIAN COHORT AND A SURVEY ON THE MALAYSIAN PERSPECTIVE OF RARE DISORDERS

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DESSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE MASTER OF BIOMEDICAL SCIENCE

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2016

UNIVERSITY OF MALAYA

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ABSTRACT

Charcot-Marie-Tooth (CMT) disease is the most common form of an inherited neuromuscular disorder with an incidence of 1 in 2500. CMT can be classified into demyelinating (CMT1) or axonal (CMT2) subtypes. CMT is typically diagnosed based on clinical and electrophysiological studies, together with genetic testing for mutations in genes commonly associated with CMT. Duplications of the *PMP22* gene is the most common mutation in demyelinating forms of CMT1, followed by point mutations in *GJB1* and *MPZ. MFN2* has been reported as the most commonly associated gene in axonal forms of CMT2. We sought to determine the frequency of mutations in these genes in our Malaysian cohort. A total of 47 CMT probands comprising of demyelinating and axonal forms were screened. *PMP22* duplications or deletions were assessed by the Multiplex Ligation-dependent Probe Amplification technique (MLPA), *MFN2* was analysed by High Resolution Melt (HRM) analysis whilst point mutations in *PMP22*, *GJB1*, *MPZ* and *MFN2* were assessed by PCR and direct sequencing. We found that the frequency of *PMP22* duplications, although most frequent, were fewer than described in other populations, whereas mutations in *GJB1* are much more frequent compared to other studies. In demyelinating forms, mutations in *PMP22* and *GJB1* account for 47% of the cases, while mutations in *MPZ* and *GJB1* were found in 4% of axonal CMT. No mutations were found in 49% of the patients raising the possibility that other rare or novel genes may be involved. Two novel variants were found in *GJB1*, and a combination of bioinformatics analysis including protein prediction and conservation analysis indicated that these may be pathogenic. Expression vectors harbouring the mutated alleles were generated through site-directed-mutagenesis and the cellular expression of the mutant proteins was performed. One of the mutants (P174L) showed altered GJB1 localisation while the second mutation (V74M) did not show any obvious changes. As CMT is a form of a rare disorder, we also conducted a survey to determine

the perception of the Malaysian general public on various issues concerning rare disorders. The survey looked into aspects including types of government assistance and schooling arrangements, and the stigma associated with families with rare disorders. Around two-thirds acknowledged that genetics had a role to play in these diseases and more than half would want to have genetic testing to see if their family were at risk of getting a type of rare disorder. To our knowledge, this is the first study on CMT genetics in Malaysia. For those patients who are positive for mutations, this provides useful information for the clinicians to better understand the phenotype in these patients. For those that are not genetically classified, this study provides the first important step in identifying cases that can be used for further research into the genetic etiology of CMT. Equally important, is understanding the perceptions that the general publics have about rare disorders so that better awareness campaigns can be developed to educate the public and de-stigmatise rare disorders, so that the affected individuals can become more integrated into society.

ABSTRAK

Penyakit Charcot-Marie-Tooth (CMT) adalah sejenis peyakit gangguan saraf yang boleh diwarisi dan merupakan penyakit otot saraf yang paling kerap ditemui dengan angka kejadian 1 dalam 2500 orang. CMT boleh dikelaskan kepada subjenis demyelinating (CMT1) atau axonal (CMT2). CMT biasanya didiagnosis berdasarkan kajian klinikal dan elektrofisiologi, bersama-sama dengan ujian genetik untuk mutasi dalam gen yang biasanya dikaitkan dengan CMT. Duplikasi dalam gen *PMP22* adalah mutasi yang paling biasa ditemui dalam jenis CMT1 yang 'demyelinating', diikuti oleh mutasi titik dalam gen-gen *GJB1* dan *MPZ*. *MFN2* telah dilaporkan sebagai gen yang paling kerap dikaitkan dengan jenis CMT2 'axonal'. Kami berusaha untuk menentukan kekerapan mutasi pada gen-gen yang sering dikaitkan dengan CMT, dalam golongan pesakit CMT kami di Malaysia. Seramai 47 pesakit CMT terdiri daripada jenis demyelinating dan jenis axonal telah disaring. Kejadian duplikasi atau kehilangan gen *PMP22* diuji dengan menggunakan teknik Pelbagai Ikatan Kuar Amplifikasi ('MLPA'), *MFN2* dianalisis dengan Resolusi Lebur Tinggi ('HRM') sementara mutasi titik dalam *PMP22*, *GJB1*, *MPZ* dan *MFN2* diuji melalui kaedah Polimerasi Rantai Reaksi ('PCR'). Kami mendapati bahawa kekerapan duplikasi *PMP22* hampir sama dengan apa yang telah dilaporkan untuk pesakit di seluruh dunia, tetapi mutasi dalam *GJB1* jauh lebih kerap di dalam golongan pesakit kami berbanding dengan negara lain. Bagi jenis CMT 'demyelinating' pula, mutasi di dalam gen-gen *PMP22* dan *GJB1* menyumbang kepada 47% daripada keseluruhan kes, sementara mutasi dalam *MPZ* dan *GJB1* hanya melibatkan sebanyak 4% daripada CMT 'axonal'. Tiada mutasi ditemui dalam 49% daripada pesakit kami, lalu menimbulkan kemungkinan bahawa gen-gen lain yang novel mungkin terlibat dalam pesakit-pesakit ini. Dua varian baru telah ditemui dalam gen *GJB1*, dan gabungan analisis bioinformatik termasuk ramalan protein dan analisis untuk menentukan konservasi menunjukkan bahawa varian-varian ini mungkin

patogenik. Vektor ekspresi yang membawa alel bermutasi direka melalui mutagenesis-diarahkan-tapak dan ekspresi selular bagi protein mutan, telah dilakukan. Salah satu daripada mutasi gen *GJB1* (P174L) menunjukkan perubahan pada lokasi protin GJB1, manakala mutasi kedua (V74M) tidak menunjukkan kesan yang jelas. Disebabkan CMT adalah sejenis Penyakit Jarang Jumpa, kami juga menjalankan satu kajian tinjauan untuk menentukan persepsi orang awam Malaysia terhadap Penyakit Jarang Jumpa. Kaji selidik ini tertumpu kepada dalam beberapa aspek termasuk jenis-jenis bantuan kerajaan dan urusan persekolahan, serta stigma yang dikaitkan dengan keluarga-keluarga yang menghidapi Penyakit Jarang Jumpa. Sekitar dua pertiga mengakui bahawa genetik memainkan peranan dalam Penyakit Jarang Jumpa, dan lebih daripada separuh mahu menjalani ujian genetik untuk melihat jika keluarga mereka berisiko mendapat sejenis penyakit yang jarang berlaku. Setakat pengetahuan kami, ini adalah kajian pertama yang dilakukan untuk menyelidik latar belakang genetik penyakit CMT di Malaysia. Bagi pesakit-pesakit yang positif mutasi, ini memberi maklumat yang berguna kepada para doktor untuk lebih memahami fenotip dalam pesakit-pesakit ini. Bagi mereka yang tidak dapat dikelaskan secara genetik, kajian ini menyediakan langkah permulaan yang penting dalam mengenal pasti kes-kes yang boleh digunakan untuk penyelidikan selanjutnya di dalam etiologi genetik CMT. Tidak kurang pentingnya memahami persepsi orang ramai mengenai Penyakit Jarang Jumpa supaya kempen kesedaran yang lebih baik boleh dijalankan untuk mendidik orang awam dan menghakis tanggapan buruk terhadap Penyakit Jarang Jumpa, agar individu penghidap penyakit terbabit boleh bergaul dan menjadi lebih bersepadu ke dalam masyarakat.

ACKNOWLEDGEMENTS

First and foremost, In the name of Allah, most Gracious, most Merciful, I would like to express my highest respect and deepest gratitude to my supervisors Associate Professor Dr. Nortina Shahrizaila and Dr.Azlina Ahmad Annuar for giving me opportunity to do this project and also their invaluable guidance, motivation and advices throughout the work. I also would like to represent my heartfelt thanks and heartiest appreciation for their ever-lasting patience and willingness in helping me from the initial to the final project and especially keep me always in right track along the writing dissertation.

Special thanks I would like to dedicate to my beloved parents Samulong bin Gunong and Sitti Nona binti Abu Bakar as well as my siblings for their greatest support, encouragement and endless love all these while. Thank you mom for always be patient and thank you dad for your never endless advices whenever I felt give up.

Not forgotten to my housemates Noor Shahirah, Hafiza, Khamdiah and all fellow lab mates Ms. Ching Ai Sze, Ms. Tey Shelisa, Ms. Aroma Agape, Ms. Lim Siew Leng, Ms. Omaira Razali, Ms. Tharani Arumugam, Mr. Ng Jun Bin and Mr. Lim Jia Lun; Thank you very much for all the shared knowledge and the time we have spent learning together on how things work. The friendships, bitter sweet moments and experiences are much precious, and will always be in my treasured. I feel truly fortunate to find very kind friends like you guys. Wish you all the best in the future undertakings.

Last but not least, it would not be successful without Allah who guides me in my everyday life, giving me good health as well as the strength along the way of completing this master project. Alhamdulillah all my highest grateful is only to Him.

To all mentioned above, sincerely, thank you from the deepest of my heart.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percent
(v/v)	Volume per volume
(w/v)	Weight per volume
×	Times/Multiple
°C	Degree Celcius
µg/ml	Micrograms per milliliter
µl	Microliter
µm	Micrometer
µM	Micromolar
260 nm (A260)	Wavelength reading 260 nm
280 nm (A280)	Wavelength reading 280 nm
5’ UTR	5’ Untranslated region
ABI	Applied Biosystems
AD	Autosomal dominant
APS	Ammonium per sulfate
Arg	Arginine
BCA	Bicinchoninic acid
BLAST	“Basic Local Alignment Search Tool”
bp	Base pair
CHN	Congenital Hypomyelinating Neuropathies
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
dNTPs	deoxynucleoside triphosphates

DSN	Dejerine Sottas Neuropathy
EDTA	Ethylenediaminetetraacetic acid
et al.	And Other
FBS	Foetal Bovine Serum
HMSN	Hereditary Motor Sensory Neuropathy
HNPP	Hereditary Neuropathy with Pressure Palsies
HRP	Horseradish Peroxidase
IPN	Inherited Peripheral Neuropathy
IP3	Inositol trisphosphate
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRES	Internal Ribosome Entry Site
kDa	Kilodalton
MCV	Median Conduction Velocities
MLPA	Multiplex Ligation-dependant Probe Amplification
Mm	Millimeter
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
NCV	Nerve Conduction Velocities
Ng	Nanogram
ng/µl	Nanograms per microliter
Nm	Nanometer
PNS	Peripheral Nerve System
RCLB	Red Cell Lysis Buffer
RD	Rare Disorder
SDM	Site-Directed Mutagenesis
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SNPs	Single nucleotide polymorphism
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline and Tween 20
TE	Tris-EDTA
TEMED	N,N,N',N'-Tetramethyl-ethylenediamine
TGS	Tris-Glycine-SDS
™	Trademark
UMMC	University Malaya Medical Centre
USA	United States of America
UV	Ultra violet
UVP	Ultraviolet Products
V	Volts
Vol	Volume
WR	Working Reagent

LIST OF APPENDICES

CHAPTER 1: GENERAL INTRODUCTION

Charcot-Marie-Tooth disease (CMT) is the most common inherited neuromuscular disorder and it is also known as Hereditary Motor and Sensory Neuropathy (HMSN). The disease affects motor and sensory nerves which then impairs muscle function. CMT can be grouped into two major subtypes depending on whether the primary insult is in the axon or myelin. Typical CMT phenotypes include a slow progressive weakness and atrophy primarily in the distal leg muscles which causes foot deformity such as high arched feet (*pes cavus*) as well as wasting of the small muscles of the hands.

The classification of CMT subtypes is important when establishing a diagnosis. Electrophysiological recordings, the pattern of inheritance and genetic analysis are used together to reach a diagnosis. To our knowledge, there have not been any studies investigating CMT genetics in Malaysia and our objective was to investigate the contribution of the commonly associated genes with CMT in our Malaysian cohort. The patients were recruited over a period of 3 years from the University Malaya Medical Centre (UMMC) as well as other medical centres across Malaysia and consisted of 48 probands, with a mixture of CMT subtypes.

We were able to identify mutations in 51% of cases. We identified two previously unreported variants in *GJB1*, and the second part of this thesis was focus on determining whether these variants affected the localisation of the GJB1 protein within the cell. We found that the V74M mutant protein appeared to localise in the same pattern as the wild-type, while the P174L mutant protein did not form any GJB1 plaques at the boundary of neighbouring cells.

We also carried out a study on the perception of the Malaysian public on rare disorders and details of this survey are further described in CHAPTER 5.

In summary, this thesis describes original work on the Malaysian CMT profile, with regards to the phenotypic patterns, the genetic causes and the probable effect of two novel variants identified in our cohort. We have also gained some insight into the Malaysian public's perception of rare disorders which will be useful in planning for future health care awareness campaigns to raise the profile of rare disorders thus ensuring better and earlier treatment and care for affected individuals.

CHAPTER 2: LITERATURE RIVIEW

2.1 Charcot-Marie-Tooth Disease – Historical Perspective

Charcot-Marie-Tooth disease was first described in 1886 when Jean-Marie Charcot and Pierre Marie in Paris, France first discovered an abnormal condition of progressive muscular atrophy. They named the condition "peroneal muscular atrophy". At the same time in London, England, Howard Henry Tooth independently described patients with the same neuropathic symptoms. Therefore in recognition of their joint contributions in identifying the disease, the disease was later named Charcot-Marie-Tooth (CMT) in their honour (Pareyson, Scaioli, & Laura, 2006). CMT is also often referred to as Hereditary Motor and Sensory Neuropathy as it affects the motor and sensory nerves. Currently, CMT is the most common inherited peripheral neuropathy with an estimated prevalence of 1 per 2,500 individuals (Reilly, Murphy, & Laurá, 2011).

2.2 CMT Phenotypes

The classical phenotype of CMT is distal weakness involving distal muscles, predominantly affecting the motor neurons of the lower limbs and foot abnormalities such as high arches or clawed toes, as well as gait abnormalities leading to high steppage gait. Affected individuals may also develop muscle weakness in their hands causing difficulties in fine motor activities such as writing or fastening buttons (Reilly *et al*., 2011). However, the severity in phenotypes vary among patients even between affected family members (Azzedine, Senderek, Rivolta, & Chrast, 2012) and mutations in the same genes can even manifest different phenotypes, for example mutations in *MFN2* are also seen in patients with spasticity or optic atrophy (Züchner & Vance, 2006). The onset of symptoms is typically from the first decade of life and persists into adulthood.

2.3 Inheritance pattern and Nerve Conduction Velocities (NCV)

CMT can be inherited in an autosomal dominant, autosomal recessive and X-linked manner. The autosomal dominant form is the most frequently reported (Szigeti, Nelis, & Lupski, 2006) and in most northern European and US populations, autosomal dominant or X-linked CMT accounts for around 90% of cases and autosomal recessive account for less than 10%. Meanwhile, in countries with a higher rate of consanguineous marriage such as the Mediterranean basin and in the Middle East, autosomal recessive cases account for almost 40% of the cases (Reilly *et al*., 2011).

Together with phenotypic clues and an apparent mode of inheritance, nerve conduction velocities (NCV) are also used as clinical tools in CMT diagnosis. NCVs are normally performed by measuring the upper limb motor conduction velocities of the median or ulnar nerves. Based on NCV classifications, CMT can be further classified into two groups; demyelinating (CMT1) and axonal (CMT2), where CMT1 is characterized as having median conduction velocities (MCVs) of less than 38m/s and CMT2 with MCVs of more than 38m/s (Berger, Niemann, & Suter, 2006). Recently, clinicians have also referred to "intermediate" forms to describe certain cases of CMT that cannot truly be classified as either CMT1 or CMT2 because they have features of both types. Therefore, a new range of NCV values have been proposed - less than 15m/s for very slow, between 15 and 35 m/s for slow, between 35 and 45 m/s for intermediate and more than 45 m/s for normal (Saporta *et al*., 2009) (Shahrizaila *et al*., 2014). This new range of values will help to further categorize the CMT subtypes better.

2.4 Genes associated CMT and classification

In the recent molecular genetics era, genetics has given an added value to classification of CMT. Over 80 genes-associated CMT have been identified and a number are involved in myelin sheath maintenance and axonal function (Timmerman,

Strickland, & Züchner, 2014), (Figure 2.1). Other pathways implicated include those genes involved in housekeeping activities such as amino-acyl tRNA synthetases (*GARS*, *YARS*) (Antonellis *et al*., 2003; Jordanova *et al*., 2006), small heat shock proteins (*HSPB1*, *HSPB8*) (Ismailov *et al*., 2001; Tang *et al*., 2004) and enzymes involved in membrane and transport metabolism (*PRX*, *MTMR2*, *SBF1*, *SBF2*) (Berry, Francis, Kaushal, Moore, & Bhattacharya, 2000; Delague *et al*., 2000; Senderek *et al*., 2003), transcription factors such as *EGR2*, (Warner *et al*., 1998) and mitochondria (*MFN2*, *GDAP1*) (Züchner *et al*., 2004) also contribute to CMT pathogenesis. The complex nature of the disease involving multiple pathways and mechanisms makes CMT a challenging disease to genetically diagnose and treat with drugs.

CMT1 is characterized by a demyelinating, autosomal dominant pattern. Whereas, CMT2 is axonal and mostly autosomal dominant but it can also be inherited in an autosomal recessive manner. Another category identified as a subtype in the recent CMT classification is CMT4 (Table 2.1), which describes autosomal recessive severe neuropathies. CMT1, CMT2 and CMT4 are now the 3 main types used in CMT classification. These are further sub-classified into subtypes depending on the genes involved and the phenotypic presentation such as variable penetrance, early or late onset, the presence of optic atrophy, tremors and severity level. Table 2.1 outlines the various subtypes of CMT and the known genes implicated in each.

CMTX is the X-linked CMT subtype which can be inherited in an autosomal dominant (CMTX1) or autosomal recessive pattern (CMTX5). CMTX1 is caused by mutations in *GJB1* (Gap junction protein, beta 1, 32 kDa) and CMTX5 is caused by mutations in *PRPS1* (Phosphoribosyl pyrophosphate synthetase 1). CMTX1 is one of the most common subtypes but CMTX5 tends to be quite rare.

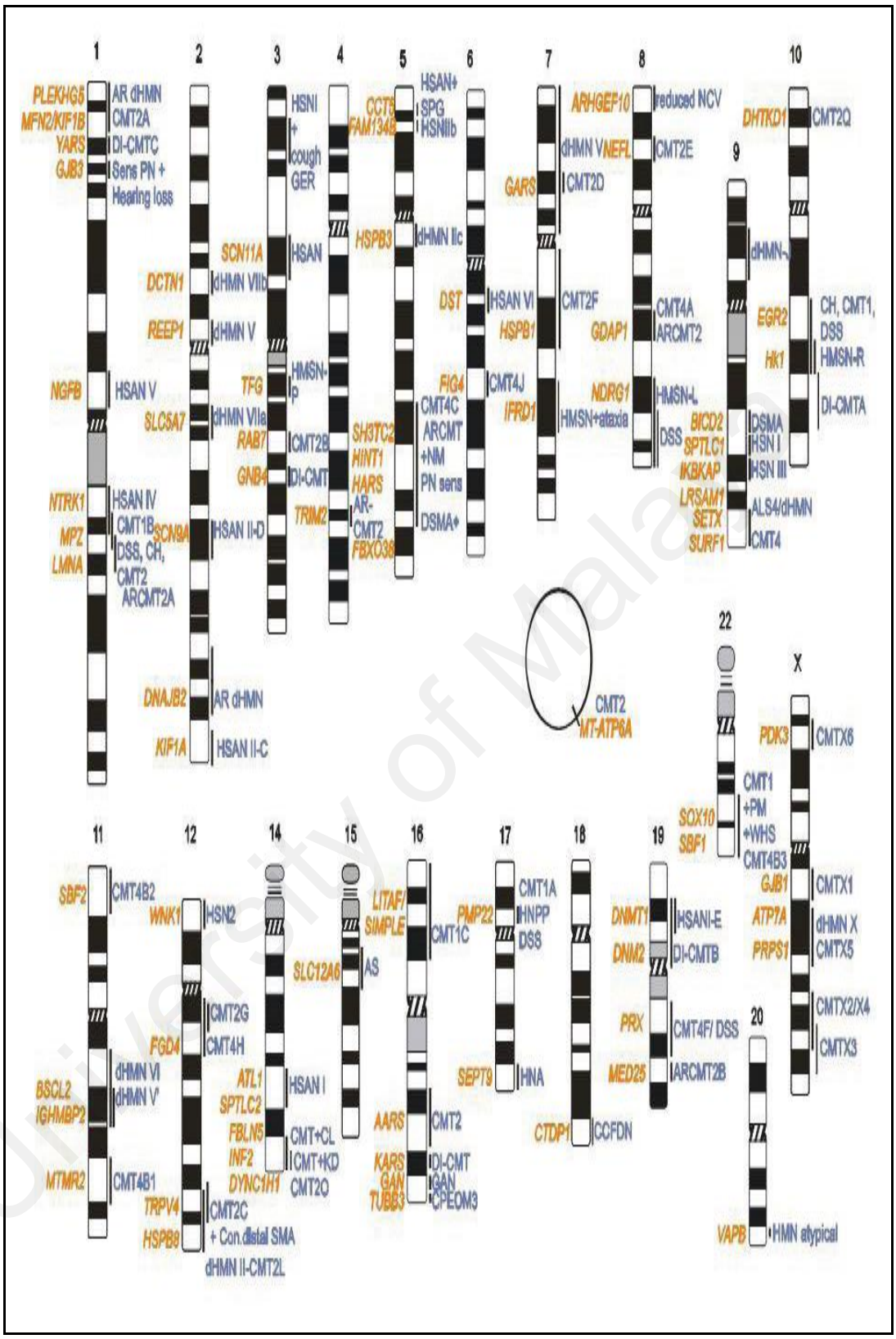


Figure 2.1: 80 genes associated with CMT and the corresponding chromosomes

This figure shows 80 currently known genes (orange symbols) and their corresponding chromosomal loci (vertical bars). The corresponding phenotypes such as optic atrophy, severe sensory, predominant sensory involvement and other are in blue color and can be referred further to in Appendices. Figure adapted from (Timmerman et al., 2014).

Table 2.1: CMT subtypes with corresponding genes

Subtype	Gene	Protein	Frequency	References
Autosomal Dominant				
CMT1A	*PMP22*	Peripheral myelin protein 22	70% of CMT1A (43-50% of total CMT)	Szigeti & Lupski, 2009; Lee JH *et al.*, 2006 Zuchner & Vance., 2006; Reilly and Shy., 2009; Reilly and Shy., 2009; Braathen *et al.*, 2010
CMT1B	*MPZ*	Myelin P0	5-10% of total CMT	
CMT1C	*LITAF*	SIMPLE	<1% Rare	
CMT1D	*EGR2*	Early Growth response protein 2	<1% Rare (reported once in American population and once in Korea)	
CMT1E	*PMP22*	Peripheral myelin protein 22	2.5% of total CMT	
CMT1F	*NEFL*	Neurofilament Light Chain	2% of total CMT(reported 3 cases in Japan, 2 cases in Korea and one case in American populations	
HNPP	*PMP22*	Peripheral myelin protein 22	11% of total CMT	
Autosomal Dominant				
CMT2A1/2	*KIF1Bβ*	Kinesin family member 1B	Rare, once in Japanese population (Review, Lee JH *et al*, 2009)	Zuchner & Vance., 2006; Reilly and Shy., 2009
CMT2A	*MFN2*	Mitofusin 2	20% of CMT2A	
CMT2B	*RAB7*	Ras-related protein Rab-7	Rare	
CMT2C	*TRPV4*	Transient receptor potential cation channel, subfamily V4	Rare	
CMT2D	*GARS*	Glycyl-tRNA synthetase	Rare	

Table 2.1, continued

CMT2E	*NEFL*	Neurofilament Light Chain	2% of total CMT	
CMT2F	*HSPB1*	Heat-Shock Protein B	Rare	
CMT2I	*MPZ*	Myelin P0	Rare	
CMT2J	*MPZ*	Myelin P0	Rare	
CMT2K	*GDAP1*	Ganglioside-induced differentiation protein 1	Rare	
CMT2L	*HSPB8*	Heat-Shock Protein B8	Rare	
Autosomal Recessive				
CMT4A	*GDAP1*	Ganglioside-induced differentiation protein 1	Rare	As reviewed in Zuchner and Vance., 2006; Braathen *et al.*, 2010
CMT4B1	*MTMR2*	Myotubularin-related protein 2	Rare	
CMT4B2	*MTMR13*	Myotubularin-related protein 13	Rare	
CMT4C	*SH3TC2*	SH3 domain and tetratricopeptide repeats 2	Rare	
CMT4D	*NDRG1*	N-myc downstream regulated 1	Rare	
CMT4E	*EGR2*	Early Growth Response 2	Rare	
CMT4F	*PRX*	Periaxin	Rare	
CMT4H	*FGD4*	FRABIN	Rare (reported once in American population)	
CMT4J	*FIG4*	FIG4 homolog	Rare	

Table 2.1, continued

X-linked Dominant				
CMTX1	*GJB1*	Gap junction protein, beta 1, 32 kDa	12% of total CMT 8.8% of CMT1A	Szigeti and Lupski., 2009; Zuchner & Vance., 2006
X-linked Recessive				
CMTX5	*PRPS1*	Phosphoribosyl pyrophosphate synthetase 1	Rare	
Dominant intermediate NCV				
CMTD1A	*unknown*	Unknown	Rare	Zuchner & Vance, 2006
CMTD1B	*DNM2*	Dynamin 2	Rare	
CMTD1C	*YARS*	*Tyrosyl-tRNA synthesis*	Rare	
CMTD1D	*MPZ*	Myelin P0	Rare	

CHN, Congenital Hypomyelinating neuropathy; CMT, Charcot-Marie-Tooth; DSN, Dejerine Sottas neuropathy; HNPP, Hereditary Neuropathy with liability to Pressure Palsies; AD, Autosomal Dominant; AR, Autosomal Recessive; NCV, Nerve Conduction Velocities; Myelin P0, Myelin Protein Zero; Classical or typical CMT phenotype characterised by lower limb motor symptoms (difficulty walking/ foot deformity) beginning in the first two decades accompanied by distal atrophy, weakness and sensory loss, hyporeflexia and frequent foot deformity (*pes cavus*) (Reilly 2011*et al*).

2.5 Hereditary Neuropathy with liability to Pressure Palsy (HNPP)

Hereditary Neuropathy with liability to Pressure Palsy (HNPP) is also a type of Inherited Peripheral Neuropathy (IPN) where patients experience recurrent episodes of nerve palsy or nerve dysfunction at compression sites, also known as entrapment neuropathy. It is a condition caused by direct pressure on a single nerve which may cause pain, tingling, numbness and muscle weakness in the patients and the NCV recordings show a mildly slower conductance. Patients with HNPP have less clinical features compared to patients of CMT1A and the disease usually develops as a painless neuropathy after minor trauma. HNPP is usually caused by a deletion of the same 1.5MB region on chromosome 17 that is duplicated in CMT1A (Reilly *et al*., 2011). In rare cases, frame shift or nonsense mutations could also happen (Berger, Young, & Suter, 2002; Lee & Choi, 2006). HNPP is inherited in an autosomal dominant manner.

2.6 Frequencies of Genes Associated CMT and the Most Common Genes Defects

Published reports have indicated that there are four common genes/genomics rearrangements associated with CMT: *PMP22*, *MPZ, GJB1* and *MFN2*. Even though there were many others genes that had been reported, most of them were found at very low frequencies in discrete populations. Table 2.1 lists all the CMT subtypes and the genes that have been identified under each subtype.

The main objective of this thesis was to investigate the most prevalent genetic mutations in Malaysian CMT patients. As there are over 80 genes associated with CMT, we adopted the strategy whereby only the more commonly associated genes were screened. We reasoned that to capture the genetic aetiology, we should first focus on the

common genes since collectively this would allow us to genetically classify the majority of the demyelinating and axonal forms. The other genes account for only a small portion of the CMT cases as a whole and it would not be economically feasible to be screened as a first-pass approach in our cohort. For cases suggestive of demyelinating CMT, *PMP22* duplication and point mutation is the most common mutation (70% of all CMT1 cases), followed by point mutations in *GJB1* (12%) and *MPZ* (10%). For axonal cases, *MFN2* was screened because it is the most common gene in CMT2 forms, accounting for 20% of total CMT2 cases (Szigeti & Lupski, 2009). Therefore we selected *PMP22*, *MPZ*, *GJB1* and *MFN2* in our panel of genes (Figure 2.2).

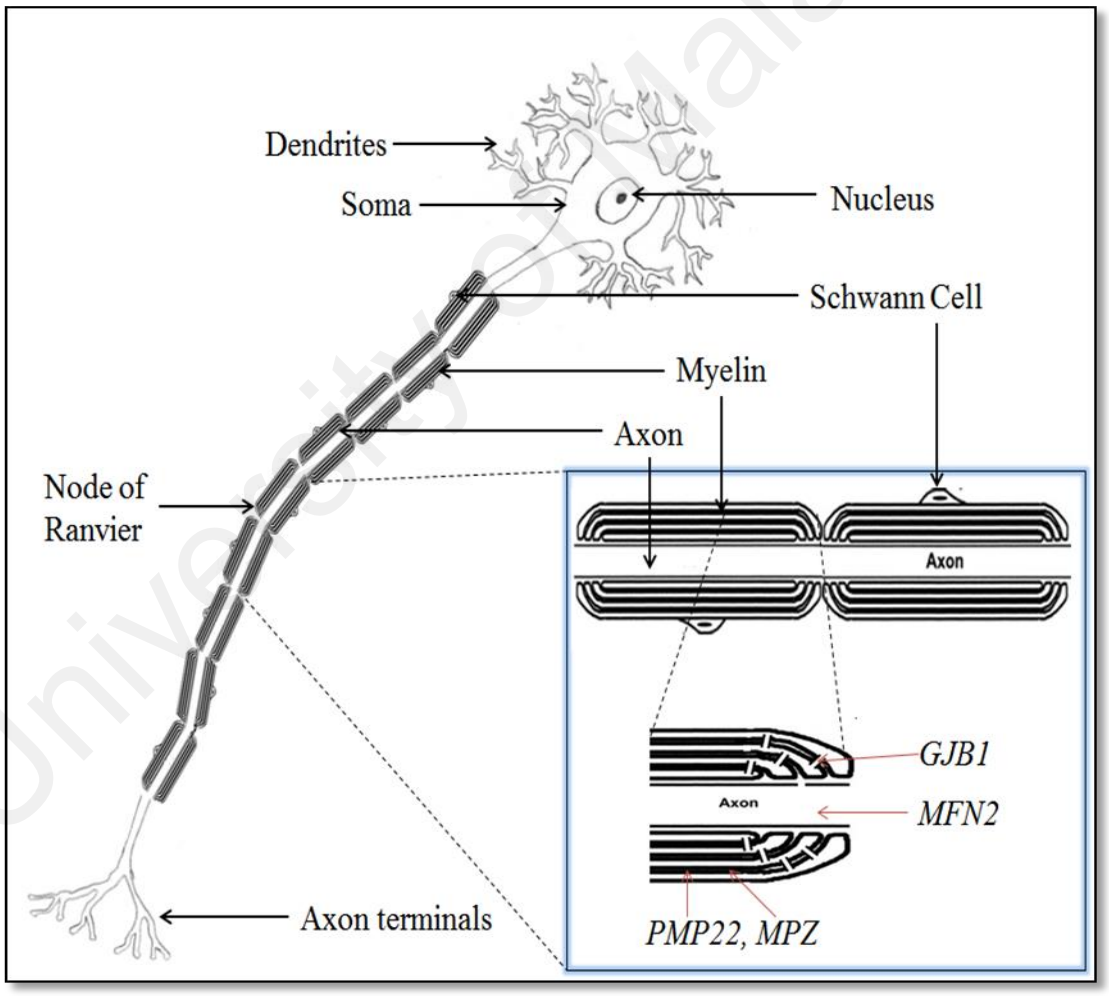


Figure 2.2: Common gene mutations in CMT

Picture was adapted from Sa´ez *et al*, 2003. *PMP22* and *MPZ* are part of the compact myelin and play important roles in myelin structure and stability, while *GJB1* acts as a channel to allow electrical conductance. *MFN2* is involved in mitochondrial membrane fision which is important in axonal transportation.

2.6.1 PMP22

Rapid advances in understanding the genetics of CMT began in 1991 after a 1.5Mb duplicated region containing *PMP22* was identified (Lupski *et al*., 1991), PMP22 is now known to contribute up to 70% of all CMT cases (Krajewski *et al*., 2000). Functionally, *PMP22* codes for peripheral myelin protein 22, an integral membrane protein expressed mainly in Schwann cells and is a major component of compact myelin in the peripheral nervous system (Berger *et al*., 2006). *PMP22* makes up approximately 2–5% of total PNS myelin protein and is thought to be of importance in myelin formation and maintenance (D'Urso, Ehrhardt, & Müller, 1999).

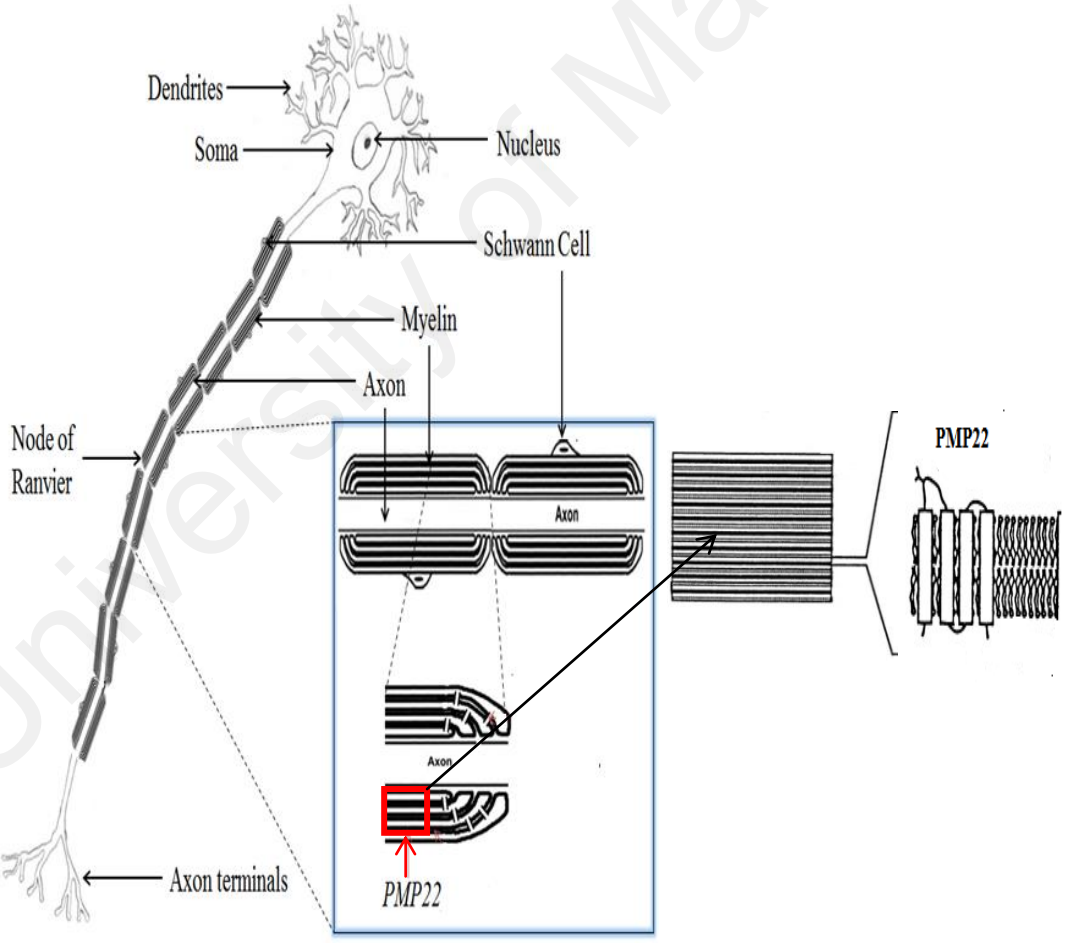


Figure 2.3: The involvement of PMP22 in Schwann cells

PMP22 maintains the structural integrity of the myelin sheath. Picture was adapted from Sa´ez *et al*, 2003.

2.6.2 GJB1

CMTX is the second most frequent subtype of CMT (Ajitsaria, Reilly, & Anderson, 2008) and mutations in *GJB1* account for 12% of these cases. This gap junction protein plays an important role in the transport of small molecules between Schwann cells as well as allowing direct cell-to-cell electrical communications in the nervous system (Lee & Choi, 2006).

The GJB1 channel consists of six individual connexons (hemichannels), and one connexon binds with another connexon on a neighboring cell to form a complete plaque called connexin (Figure 2.4). The pore of a gap junction channel is between 6 and 7Å (Oh *et al*., 1997), and this channel allows the movement of molecules smaller than 1000 Da, such as inorganic ions (Na^+, K1, Ca^{2+}, etc.), cAMP and inositol 1,4,5 trisphosphate (IP3) (Kumar,N.M & Gilula, 1996). Upon depolarisation, the pore opens and allows ion and electrical conductance to pass through. Mutations in *GJB1* can affect the pore properties as well as channel formation like protein bending and docking, which subsequently causes a slower action potential conductance (Kumar, N.M & Gilula, 1996).

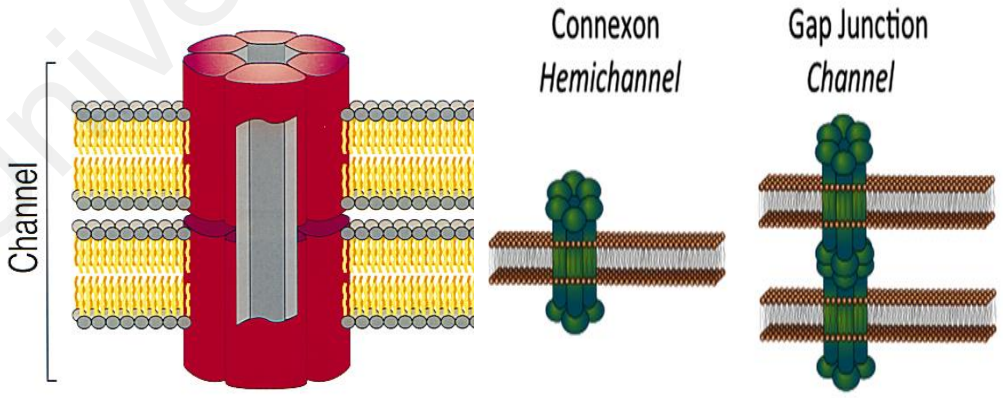


Figure 2.4: GJB1 structuture

Adapted from (Giaume, Leybaert, Naus, & Sáez, 2013; Kumar, N.M & Gilula, 1996). Schematic Drawing of Connexons to form Gap Junction. The channel junction consisting of six connexon subunits. Connexons associate end to end to form a double membrane gap junction channel.

Immunocytochemical evidence suggests that *GJB1* is localized to the incisures of Schmidt–Lanterman and the paranodes of myelinating Schwann cells (Ressot, Gomès, Dautigny, Pham-Dinh, & Bruzzone, 1998; Sáez, Berthoud, Branes, Martinez, & Beyer, 2003).

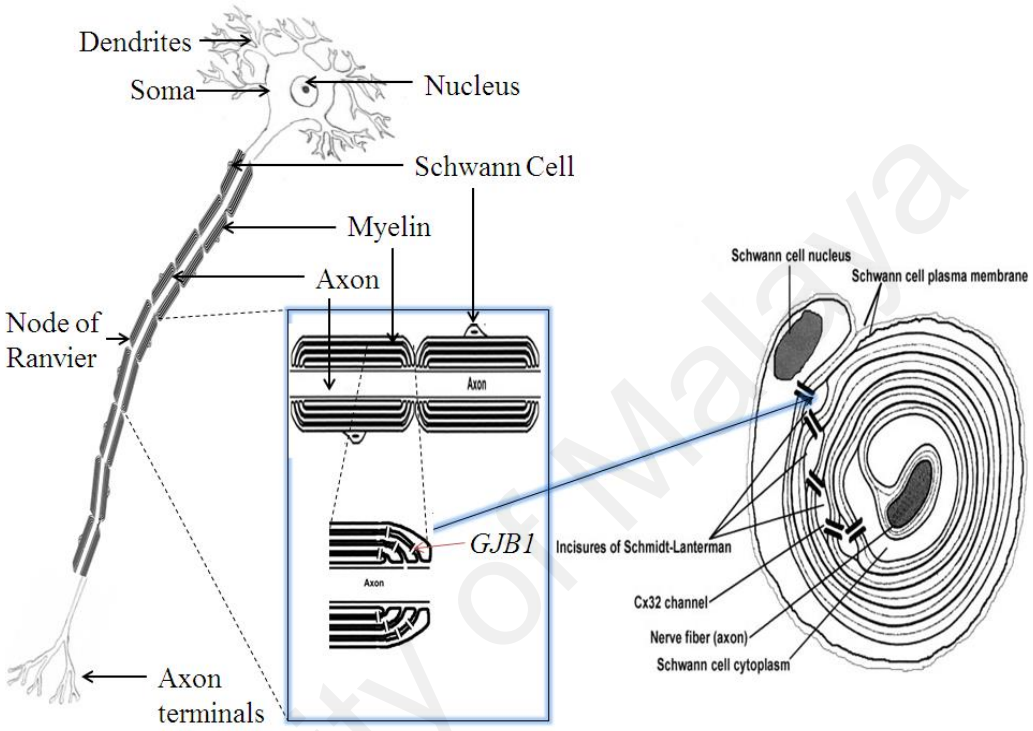


Figure 2.5: The involvement of GJB1 in Schwann cells with a zoomed-in view of incisures of Schmidt-Lanterman region. Picture was adapted from (Sáez *et al*., 2003).

2.6.3 MPZ

MPZ is reported to be the third most common causative gene for autosomal dominant CMT1 (Braathen, Sand, Lobato, Høyer, & Russell, 2011). *MPZ* is highly expressed in myelinating Schwann cells and comprises about 50% of all peripheral myelin proteins. It is necessary for normal myelin structure and formation by holding the myelin membrane compact via extracellular and cytoplasmic domain interactions, forming MPZ-mediated homotypic adhesion (D'Urso *et al*., 1999). Based on a correlation study looking at the genotypes of *MPZ* mutations and the phenotypes in 13 patients as well as

data from the *MPZ* mutation databases, Shy *et al*., 2004 showed that *MPZ* mutations can manifest the disease in two ways. The early onset phenotype is predicted to occur when the *MPZ* mutations cause disruptions to the MPZ tertiary structure which then consequently affect the MPZ mediated adhesion and myelin compaction. Meanwhile the late onset phenotype occurs when there are mutations at the extracellular domain, transmembrane and cytoplasmic domain (specifically at Ser15Phe, Thr95Met) which then affects the axons and causes failure in the Schwann cell-axonal interaction (Shy *et al*., 2004).

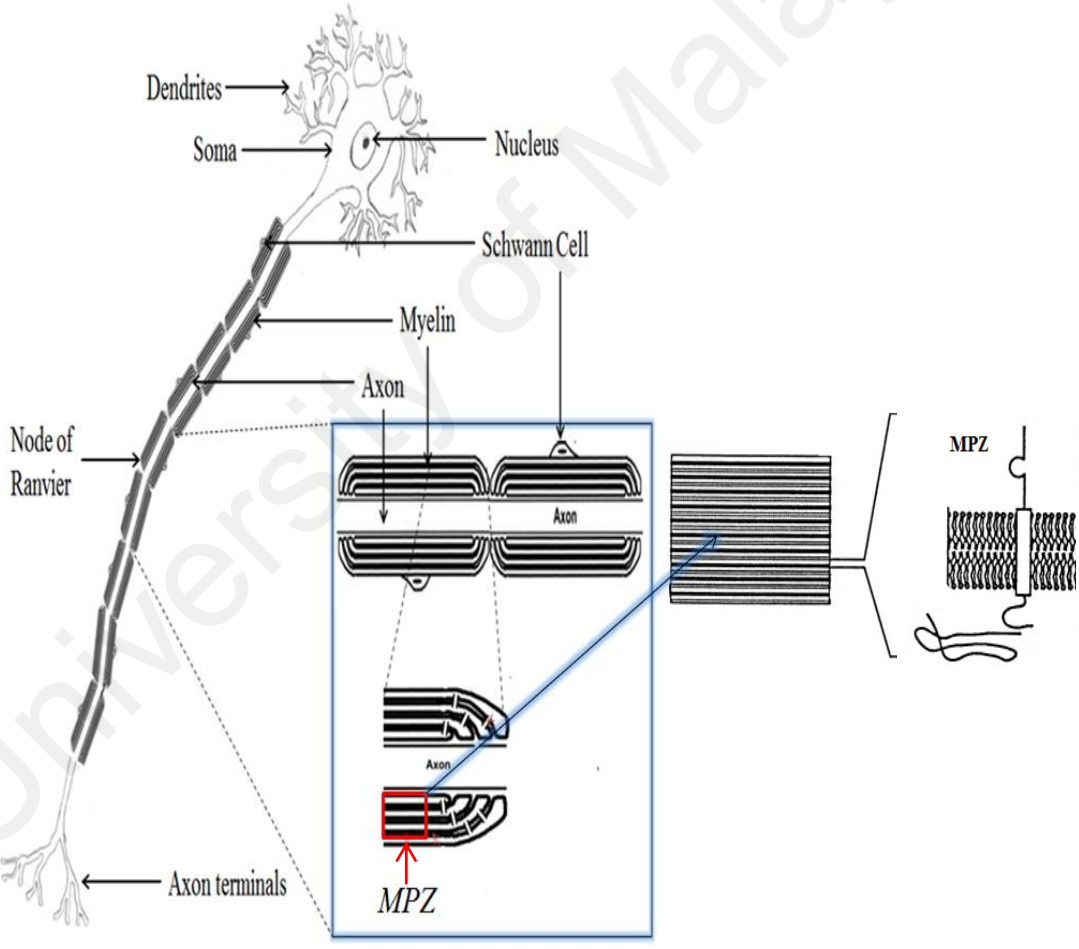


Figure 2.6: The involvement of MPZ in Schwann cells

MPZ maintains the structure of the myelin sheath. Picture was adapted from Sa´ ez *et al*, 2003.

2.6.4 MFN2

We selected MFN2 to screen in our population as it represents about 20% of all autosomal dominant CMT2 cases (Züchner & Vance, 2006). Functionally, *MFN2* encodes the outer mitochondrial membrane protein involved in regulating mitochondrial fusion and metabolism, as well as maintaining membrane potentials (Lee & Choi, 2006). In CMT2A, failure of mitochondrial fusion will reduce mitochondrial mobility which results in the accumulation of dysfunction organelles in the soma of motor neurons. This reduced mobility could lead to insufficient axonal transport of mitochondria, presumably in the extended axons of peripheral nerves (Züchner *et al*., 2004). Damaged mitochondria are also thought to accumulate in the distal axon of sural nerve (Cartoni & Martinou, 2009) disrupting the energy supply along the entire axon.

2.7 Rare Diseases study: a necessity for Malaysia

The last part of this thesis looked into the public perception of rare disorders, of which CMT is one. We were interested in discovering what the public knew about rare disorders and conducted a questionnaire based survey to uncover their view. The results of which are further discussed in CHAPTER 5. Based on the definition by European Organization, Rare Disease is defined as rare when its prevalence was 1 in 2000 people. On the other hand, National Organization for Rare Disease USA says Rare Disease affected less than 200000 people in the population http://www.eurordis.org/about-rare-diseases.

Most of the unfamiliar diseases such as Charcot-Marie-Tooth (CMT) neuropathy is genetic in origin, often chronic and life-threatening. Some may not be fatal but most rare diseases have no cure at the present time (Aymé & Schmidtke, 2007). Thus, rare diseases have an impact on patient's quality of life to various degrees. Living with a rare

disease is an ongoing learning experience for patients and families. Persons and families with rare diseases often share their journal experiences of facing difficulties including public isolation, financial stress and problem to access medical services.

There is no data regarding Rare Disorders published in Malaysia. To our knowledge there is no centralized care system or databases for Rare Disorders in Asia. Since there is a lack of data and knowledge on rare disorders, Malaysians are not likely aware of its impact. Many are unfamiliar with the characteristics of rare diseases. Thus, many especially those from rural areas are likely to refuse health screening or genetic analyses as they do not recognise the need for such measures. Fundamental research on Rare Disorders is important to address the shortcomings in knowledge and awareness of both patients and public. This will also provide the opportunity to improve diagnosis, care and prevention along with enhancing clinical research in our country.

2.8 Objectives of This Study

1. To identify the frequency of mutations in commonly associated genes with Charcot-Marie-Tooth Disease in a Malaysian cohort
2. To investigate the effect of two *GJB1* novel mutations V74M and P174L identified in this cohort
3. To evaluate public knowledge on Rare Disorders in Malaysia

CHAPTER 3: PREVALENCE OF COMMON GENES MUTATIONS IN MALAYSIA

3.1 INTRODUCTION

The objective of this study was to describe the prevalence of mutations in the most commonly associated genes with CMT in a Malaysian cohort of CMT patients. We screened for *PMP22* duplications/deletions, and for point mutations in *PMP22, GJB1, MPZ* for CMT1 and *MFN2* for CMT2. We also screened *GJB1* and *MPZ* when the *MFN2* test was negative.

The epidemiology of CMT in Asian populations is not widely studied. A study on CMT in China reported that the mutation frequency was similar to that reported in the global CMT population, whereby *PMP22* duplication, *MPZ* and *GJB1* mutations were detected in the majority of Chinese CMT1 patients (Song *et al*., 2006). However, limited genetic studies have been done on Indian CMT cases and there are no reports on the Malaysian population (Shahrizaila *et al*., 2014). Below are the reported CMT frequencies reported in other countries.

Table 3.1: CMT prevalence reported in other countries

Country	CMT1A duplication	CMTX	CMT1B	References
China (n= 32)	62.5%	6.3%	3.1%	(Song *et al*., 2006)
Australia (n=224)	61.0%	12.0%	3.1%	(Nicholson, 1999)
Italy (n=170)	57.6%	7.1%	2.3%	(Mostacciuolo *et al*., 2001)
Russia (n=108)	53.7%	7.4%	4.6%	(Mersiyanova *et al*., 2000)
Korea (n=32)	46.8%	6.3%	3.1%	(Choi *et al*., 2004)
Japan (n=128)	31.2%	10.9%	6.2%	(Ikegami *et al*., 1998)
Greece (n=243)	25.9%	4.9%	0.6% (n=172)	(Karadima, Floroskufi, Koutsis, Vassilopoulos, & Panas, 2011)
Turkey (n=64)	15.6%	4.6%	ND	(Bissar-Tadmouri *et al*., 2000)
Norway (n=81)	13.6%	6.2%	1.2%	(Braathen *et al*., 2011)

Given the limited knowledge of CMT genetics in this region of the world and in particular in Malaysia, we sought to investigate this further.

3.2 MATERIALS AND METHODOLOGY

3.2.1 Demographic data of the patients in the CMT cohort

Table 3.2: Demographic data of the cohort

Gender Male = 57% Female = 43%
Age Range in age is between 6 months to 70 years old
Race Malay = 27.66% Chinese = 42.55% Indian = 12.76%
Type of CMT Demyelinating = 26 Entrapment = 4 Axonal = 13 Unclassified = 4 Total number = 47
Patients with family history X linked = 8 Autosomal Dominant = 13 No family history = 24 Consanguineous marriage = 2 (2010CMT003 and 2011CMT015)

3.2.2 Patients

Subjects in this study were recruited from the Neurology Clinic at the University Malaya Medical Centre as well as other centres across Malaysia through a referral basis. The diagnosis was made based on clinical information such as presence of foot deformities, slow progression, distal sensory motor sign and positive family history. Neurophysiological tests were performed to determine the type of neuropathy, either demyelinating or axonal. Consent was obtained from all participants. This study received ethical approval from the University of Malaya Medical Centre Ethics Committee.

3.2.3 DNA Extraction

Five mls of blood were drawn from the subjects into EDTA tubes and the genomic DNAs were extracted from the blood cells using the phenol-chloroform protocol. Red cells were lysed with 40ml of 1X Red Cell Lysis Buffer (RCLB) in 50ml falcon tubes and incubated on ice for 10 minutes. After 10 minutes, the lysed red cells were centrifuged at 3500rpm for 10 minutes at 10°C, and then the supernatant was discarded and pellet re-suspended again with 20ml 1X RCLB to ensure complete lysis. The previous 10 minute ice incubation and centrifugation step was repeated. Then the pellets were resuspended with digestion buffer containing 20µl proteinase K and 400µl lysis buffer prior to incubating the samples overnight at 37°C. The following day, 200µl of 5M NaCl was added and the mix was transferred into 1.5ml eppendorf tubes, and 800µl phenol-chloroform was added. The mix was vortexed vigorously until it appeared milky color and then tubes were centrifuged at 13,000 rpm for 30 minutes at 10°C. The aqueous phase was transferred into a new Eppendorf tube before the DNA was precipitated using 900µl absolute ethanol. The DNA was centrifuged at 13,000rpm again for 5 minutes, after which the supernatant was discarded and 500µl of 70% ethanol added and centrifuged again at 13,000rpm for 3 minutes. The supernatant was discarded and the DNA pellet was dried before it was solubilized with 50 to 100µl sterile Milipore water (depending on the pellet size). The concentration and purity of DNAs were measured by Thermo Scientific NanoDrop 2000 spectrophotometer.

3.2.4 Workflow of the genetic screening

For patients with a demyelinating form of CMT, copy number variation of *PMP22* was first investigated as it is the most common gene mutation causing demyelination and if the patient was negative for the *PMP22* duplication, then point mutation screening was performed for *PMP22*, *GJB1* and *MPZ*. However, if there appeared to be an X-linked pattern of inheritance with no male-to-male transmission, *GJB1* mutation screening was performed first instead of looking at the copy number variation. Only if the *GJB1* test was negative would the sample be tested for *PMP22* and *MPZ*.

If axonal CMT is suspected, then *MFN2* screening was performed first. *MPZ* and *GJB1* were performed when the *MFN2* test was negative. This is because *MPZ* and *GJB1* mutations have also been found in patients with CMT2 which cause axonal defect (Reilly *et al*., 2011).

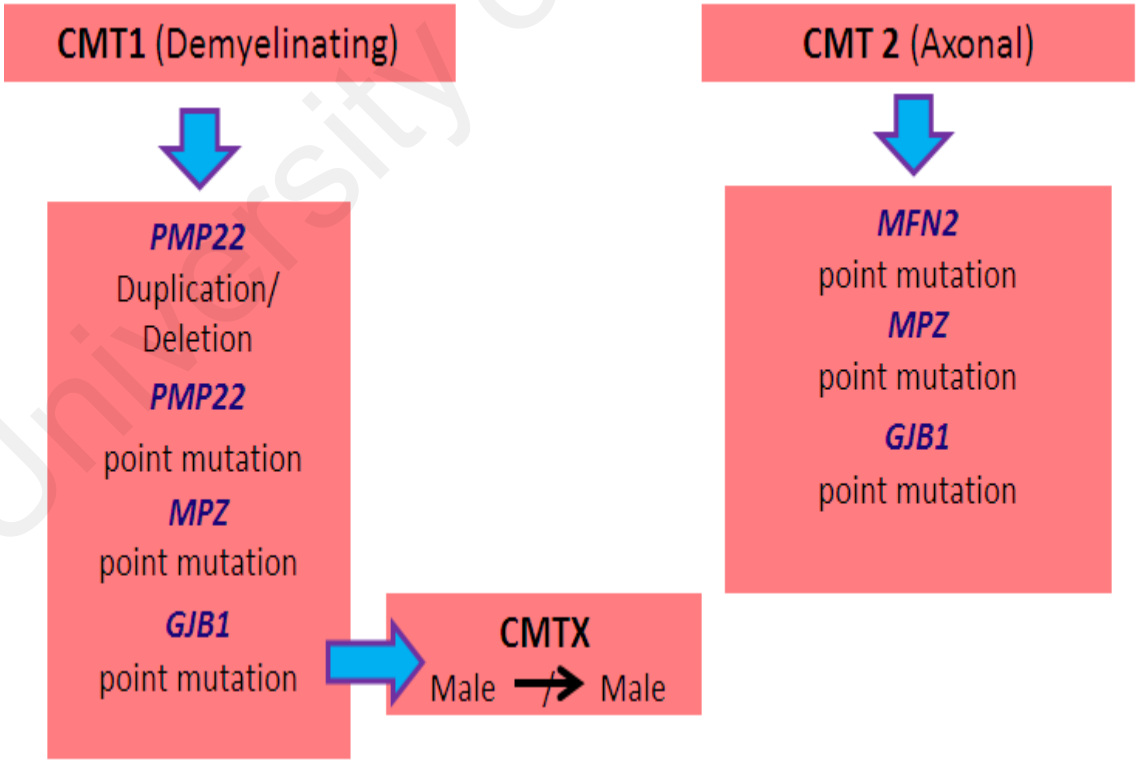


Figure 3.1: Flow chart of the strategy taken for the genetic tests

3.2.5 Genetic Testing of *PMP22* duplication/ deletion by Multiplex Ligation-dependent Probe Amplification

Duplications/deletions of the 1.5Mb region containing the *PMP22* gene was ascertained using the Multiplex Ligation-dependant Probe Amplification (MLPA) technique. The CMT1 MLPA probemix contains probes for the *PMP22*, *COX10* and *TEKT3* genes which are all located in the 1.5Mb region. *COX10* and *TEKT3* were used as internal controls. If the *PMP22* was duplicated, then the two other genes (*COX10* and *TEKT3)* should also show the same duplicated pattern. Probes for each of the five *PMP22* exons were present in the probemix. In addition, this probemix contained several probes just outside the CMT/HNPP region to be used as references to indicate that the duplication/deletion was within the 1.5Mb region. A control individual was included in each reaction to normalise the data from the patient, as well as a positive control (a sample with a known duplication within this locus).

The MLPA analysis began with the hybridisation of probes on the target sequence in the 1.5 Mb regions on chromosome 17p11.2. After hybridisation and ligation of the probes, the locus was amplified by PCR. Fragment analysis was performed on the ABI 3130xl (Applied Biosystems), whereby the amplicons were separated by capillary gel electrophoresis and the peak area of each amplification product analysed to determine the copy number of that target sequence in the patient compared to controls using the Coffalyser software (Herodež, Zagradišnik, & Vokač, 2005). The Coffalyser.Net software was used to analyse data from the MLPA runs.

For working stocks, DNA of each patient was diluted into 50ng/µl tubes using TE buffer. Each DNA was mixed with 25% glycerol to prevent evaporation during denaturation in the first step of MLPA process.

The MLPA program used was as follows:

Table 3.3.1: Denaturation step in the MLPA reactions

Denaturation	n=1	Thermal cycler conditions
4µl DNA (50ng/µl)	4.0 µl	98°C 20 minutes
25% glycerol	1.0 µl	
Total	5.0 µl	

For the hybridisation master mix – 1.5µl of MLPA buffer and 1.5µl MLPA probemix was added into the tubes containing the denatured DNA and then the MLPA program was continued based on the program below.

Table 3.3.2: Hybridisation reaction in the MLPA reactions

Hybridisation	n=1	Thermal cycler conditions
MLPA probemix	1.5µl	95°C 1 minute
MLPA buffer	1.5µl	60°C 16-18hours
Total	3.0µl	

After overnight hybridisation, a ligation master mix was prepared. Each mix contained 25µl dH_2O water, 3µl ligase-65 buffer A (provided in the kit), 3µl ligase-65 buffer B (provided in the kit), 1µl ligase 65 (provided in the kit) and mixed well by pipetting up and down. Then 32µl of ligase buffer mix was added to each reaction tubes and mixed well by pipetting up and down. The ligation reaction was programmed as follows:

Table 3.3.3: Ligation reaction in the MLPA reactions

Ligation	n=1	Thermal cycler conditions
Distilled water dH_2O	25µl	54°C 15 minutes
ligase-65buffer A	3µl	98°C 5minutes
ligase-65 buffer B	3µl	20°C Hold
ligase 65	1µl	
Total reaction	32µl	

While waiting for the ligation cycle, a PCR master mix was prepared by mixing 7.5 µl dH_2O, 2µl SALSA PCR buffer (provided in the kit) and 0.5µl SALSA Polymerase (provided in the kit). Ten µl of the PCR mix was then added into each tube containing the ligated MLPA product and the PCR program was continued as follows:

Table 3.3.4: PCR reaction in the MLPA reactions

PCR reaction	**n=1**	**Thermal cycler condition**
Distilled water dH2O	7.5µl	54°C 15 minutes 98°C 5minutes 20°C Hold
SALSA PCR buffer	2.0µl	
SALSA Polymerase	0.5µl	
Total reaction	10µl	

To separate the PCR products by capillary electrophoresis, the PCR products were loaded onto an Applied Biosystems ABI-3730XL sequencer. Prior to loading, the PCR products were heating at 86°C for 5 minutes. A size standard, LIZ-500 was added to each sample.

Table 3.3.5: Denaturation prior to loading on the sequencer

Sequencing	**n=1**	**Thermal cycler codition**
Size standard LIZ-500	0.3µl	86°C 5 minutes
Formamide	9.0µl	
PCR product	0.5µl	
Total reaction	10µl	

Data from the sequencer were analysed using the Coffalyser software developed by MRC-Holland (www.mlpa.com). Data generated by the probemix were normalised intra-sample by dividing the peak area of each amplification product by the total area of only the reference probes in this probemix. After that, analysis was performed by comparing the results from the sample with the normalised probe ratio of all reference samples.

3.2.6 Point mutation screening of *PMP22, MPZ, GJB1* by Polymerase Chain Reaction (PCR)

Sets of primers used in PCR amplification were designed by using Primer3 Software, available online. Exons and flanking intronic sequences were amplified by PCR using the Applied Biosystems (ABI) Veriti 96-well Fast Thermal Cyclers. PCR purification was performed using the INtRON BIOTECHNOLOGY kit according to the manufacturer's instructions and then sent to a commercial company for Sanger sequencing. Sequence traces were analyzed using the Sequence Scanner ABI version 1.0. The sequences of the amplicons were compared against published human gene sequences in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) to identify putative mutations.

3.2.6.1 *PMP22* point mutation screening

PMP22 contains 5 exons (ID: NM_000304.3). Below were primers used to amplify the 5 coding exons.

Table 3.4.1: Set of primers used for *PMP22*

Primers	Sense 5'-3'	Antisense 5'-3'
PMP22 E1	TCTCAGGCCACCATGACATA	ATTCCAACACAAATGCACCA
PMP22 E2	GAACCGCTTGTTTTGTTTCC	AACACAGTCCTGAACCAGCA
PMP22 E3	CCTGGGCCTTTCTCCTTC	CTCTGGGCTGAGAAACGTG
PMP22 E4	CTTCTGCTTCTGCTGCCTGT	CATTCTGAGGCCACATCCTT
PMP22 E5	CCAGCAATTGTCAGCATCC	AACAGCAACCCCCACCTC

PCR was performed at an annealing temperature of 60°C.

3.2.6.2 *MPZ* point mutation screening

The *MPZ* coding region consists of 6 exons (ID: NM_000530.6). Below are the set of primers used to amplify the 6 exons.

Table 3.4.2: Set of primers used for *MPZ*

Primers	Sense 5'-3'	Antisense 5'-3'
MPZ E1	AGGCTGCAATTGGTTTTACTGG	TCCTGCTCCTGCTTGTTCTT
MPZ E2	CTTCCTCTGTATCCCTTACTG	CTCCTTAGCCCAAGTTATCT
MPZ E3	TACCCTTTCCAGCCCAAGAT	GCTCCCAGAGCCTGAATAAA
MPZ E4	GGAGTCCTACATCCTCAATGCAG	CCCACCCACTGGAGTAGTCTCCG
MPZ E5	GAAGAGGAAGCTGTGTCCGC	CACATCAGTCACCGAGCGACT
MPZ E6	CTTGGGGCCTAGACAAGATG	TTTTTGAGGCTGGTTCTGCT

PCR was performed at an annealing temperature of 50°C.

3.2.6.3 *GJB1* point mutation screening

GJB1 consists of 2 exons, although the coding region only begins from exon two. The sequence ID: NM_000166.5 was used to refer to the coding sequences on *GJB1* screening.

We designed two sets of primers to cover the whole gene (exon 1 and 2) as well as the 5'UTR region. The reason we also looked at the 5' UTR was because previous reports have shown that pathogenic variants are present at the 5' UTR of *GJB1* (Kabzińska, Kotruchow, Ryniewicz, & Kochański, 2011).

Table 3.4.3: Set of primers used for *GJB1*

Primer Name	Sense	Antisense
GJB1 E2P1	CTATGGCGCCCGACTTTC	GCATAGCCAGGGTAGAGCAG
GJB1 E2P2	AAGAGGCACAAGGTCCACATC	GTAATCCCCAGCAGGCAGAG
GJB1 Promoter	GTTGTTCAGAGCCCCACAAA	GAGCGCCTATCCCTGAGG

PCR was performed at an annealing temperature 60°C for all *GJB1* primer sets.

3.2.7 Novel SNP analysis by Restriction Fragment Length Polymorphism (RFLP) for novel mutation

As mentioned in the introduction, we found two novel mutations in GJB1 in two patients in our cohort. We used the RFLP method to determine whether the variants identified were present in the 100 normal ethnically-matched chromosomes, and in segregation studies involving the relevant family members.

In this study, we used the restriction enzymes, *Mnl1* and *Nde1*. The digested RFLP products were separated on a 1% Super Fine Resolution SFR^{TM} Amresco^{R} acrylamide gel stained with ethidium bromide, and visualized using UV light.

The following reaction components were added in the order indicated:

Table 3.4.4: RFLP reactions

Reaction Mix	**Volume**
PCR reaction mixture	10 µl (~0.1-0.5 µg of DNA)
Water, nuclease-free	16-17 µl
10X recommended buffer for restriction enzyme	2 µl
Restriction enzyme	1-2 µl
Total volume	30 µl

PCR products were incubated at 37°C, overnight.

3.2.8 Pre-screen *MFN2* gene prior to sequencing by High Resolution Melting.

High resolution melt (HRM) analysis is a post-PCR analysis method used to identify genetic variation in nucleic acid sequences. It can be used to identify heteroduplexes based on the change in fluorescent signal as a sample is thermally denatured. When the dsDNA melts into single strands, the dye is released, causing a change in fluorescence. The result is a melt curve profile characteristic of the amplicon which is used to distinguish controls from patient samples (Kennerson *et al*., 2007).

MFN2 has 19 exons but only 17 exons code for the MFN2 protein. The translated sequence begins from exon 3 to exon 19, whereas exon 1 and 2 are in the 5' untranslated region (UTR). We used the HRM method to first pre-screen the exons for potential variants before performing sequencing. Using this methodology was a more efficient and economical approach to screening *MFN2*.

The accession number NM_001127660.1 was used in primer design and analysis of the *MFN2* gene.

Table 3.4.5: Set of primers used to amplify 17 exons of *MFN2* that had been used in HRM

Primers	Sense	Antisense
MFN2 E3*	TAGGTGTTGCTGGGTTCG	ATCTAAACAGGTAAGAGCGGG
MFN2 E4	AGACTTGGGACTGTGGAACTC	AGCCAGGAAGAAAGAAAGGG
MFN2 E5	CAGATACTGGTGGCTTTG	TGTCACAACGGAGGACT
MFN2 E6	CTGGTGGTTCCTCCTCA	TGGTGCCTTCCAGTTTG
MFN2 E7*	TCTGCCTGATGATTTGGTT	CTGGGCGCTTGGGAGAA
MFN2 E8	CTGGGCAGGCAGCTGAT	CCCTCGGGGTTGCATTC
MFN2 E9	CCACCTACACTCACTCT	AAAGGAGGACATCTGTTC
MFN2 E10	AAGTTGTTTCTGGACTAATG	ACA GAATCGCCAGATAC
MFN2 E11	GTGTCCCTGGCAGTGAAA	GTCTCGGCAGCTCTCTC
MFN2 E12	TGCTTAGTCAGACAGGAACAT	TCGGAGTCCAAATCTTCCCA
MFN2 E13	ACTTTGGTCTTCCTTGAT AC	CAGGGGTTGAATCACTTT
MFN2 E14	GCTTCTCTTAACTTCCCTCTT	CCTCCGCATCTGATCATTG
MFN2 E15	GCTTTTCCTCCATTTCTCTT	CACAATGCCCTTGAGGT
MFN2 E16	CCCTCACCCCTCTCATGTTT	CCCACTCCCCGAGCAG
MFN2 E17*	TGGCCCTGGTAGTGATG	CTGCCTAAGGAAGTCCC
MFN2 E18	AACTGGGTCCCTTCTCT	GGAGCCCTAACCTTTGG
MFN2E19*	CCTTGGCGGGTAGTCCTAA	TGGCACTTAGGGCTGGC

However, despite multiple optimisations, there were four sets of primers - indicated with a star (*) in the Table 3.4.5 that were not able to be used in the HRM analysis as they did not give clear melt profiles. Therefore, we performed direct sequencing for these exons.

The HRM reactions were prepared following these procedures;

Table 3.4.6: HRM reaction mix

Components	**Volume for one 20µl reaction**	**Final concentration**	**Acceptable concentration range**
MeltDoctor™ HRM Master Mix	10.0µl	1x	-
Primer Forward (5µM)	1.2µl	0.3 µm	0.2 to 0.5µm
Primer Reverse (5µM)	1.2µl	0.3 µm	0.2 to 0.5µm
Genomic DNA (20ng/µl)	1.0µl	1ng/µl	10pg/µl to 10ng/µl
Deionized water	6.6µl	-	-
Total volume	20.0µl	-	

The HRM reaction was run on an Applied Biosystems 7500 Fast Real-Time PCR System, following the conditions below.

Table 3.4.7: HRM Thermal Cycler parameters

Stage	**Step**	**Temperature**	**Time**	**Ramp rate (7900HT only)**
Holding	Enzyme actiation	95°C	10 min	100%
Cycling (40 cycles)	Denaturation	95°C	15 sec	100%
	Anneal	60°C	1 min	100%
Melt Curve/ Dissociation	Denaturation	95°C	10 sec	100%
	Anneal	60°C	1 min	100%
	High Resolution Melting	95°C	15 sec	1%
	Anneal	60°C	15 sec	100%

3.2.9 *MFN2* point mutation screening-post HRM

If samples showed a shift in melting curves, these select samples were sent for Sanger sequencing to determine if a variant was present within those amplicons.

3.3 RESULTS

3.3.1 Multiplex Ligation-dependent Probe Amplification - copy number of *PMP22* (Demyelinating CMT)

Normal controls were used as reference samples and positive controls (with known duplication/deletions) were also included. In control individuals, this region is present in two copies, but in patients with a *PMP22* duplication, the number of copies will be double that of the controls, while those with a deletion will have fewer than 2 copies.

Results from the MLPA were first obtained as fragment analysis data from the ABI 3730XL sequencer. This data was then analysed using the Coffalyser software. After normalising to the controls, the Coffalyser software generated an excel file indicating whether the sample has a duplication or not. The software calculated the ratio of the peak size and height (from the fragment analysis data) in the patients compared to the controls and represents these ratios as: normal range 0.82-1.27, duplicated 1.50-2.21 and deleted 0.44-0.55.

Typically, the results were very clear but if the ratio fell in-between the cut-off values, the samples were repeated for 3 to 4 times experiments to get an average value and to identify the pattern of ratio.

The initial positive control for the *PMP22* duplication was obtained from our collaborators at the University of Sydney, Australia. In later tests, we used our own positive controls of our own patients who had been confirmed to have the *PMP22* duplication.

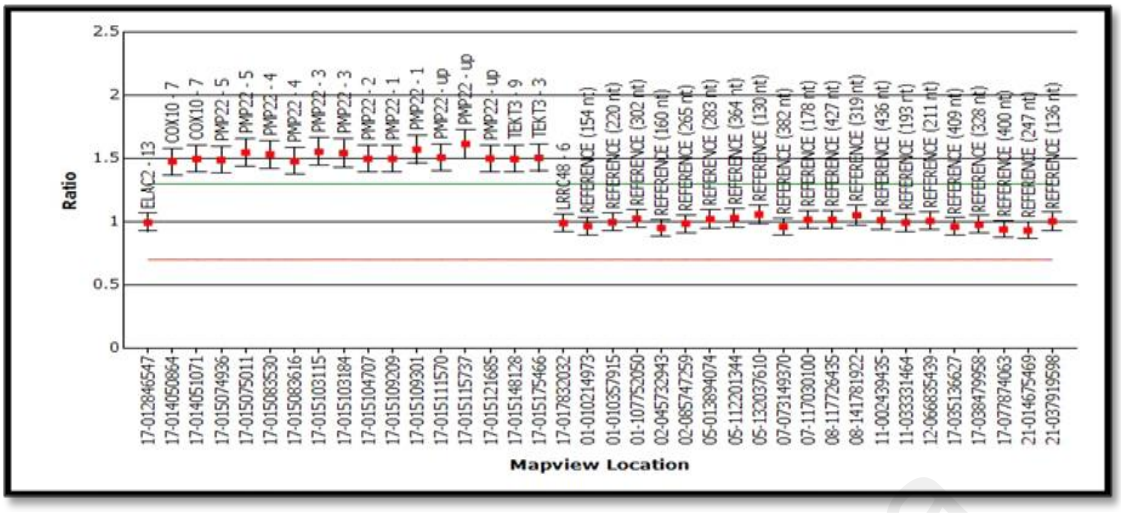


Figure 3.2.1: Ratio chart of the P33-CMT MLPA kit showing a sample with duplications in PMP22

The red dots display the ratios of each probe used in this assay (Figure 3.2.1). The red and green lines at ratio 0.7 and 1.3 indicate the arbitrary borders for loss and gain of function respectively. As seen in this case, the sample has a duplication in the 1.5Mb locus, as the probes within this region lie above the 1.2 cut-off (faint green line), while the other probes outside the 1.5Mb region fall within the normal range (around 1.0). All the probes spanning the *PMP22* gene are within the region that is duplicated, while the control reference genes (for example, *ELAC2* and 'reference 154nt') are not duplicated, as expected.

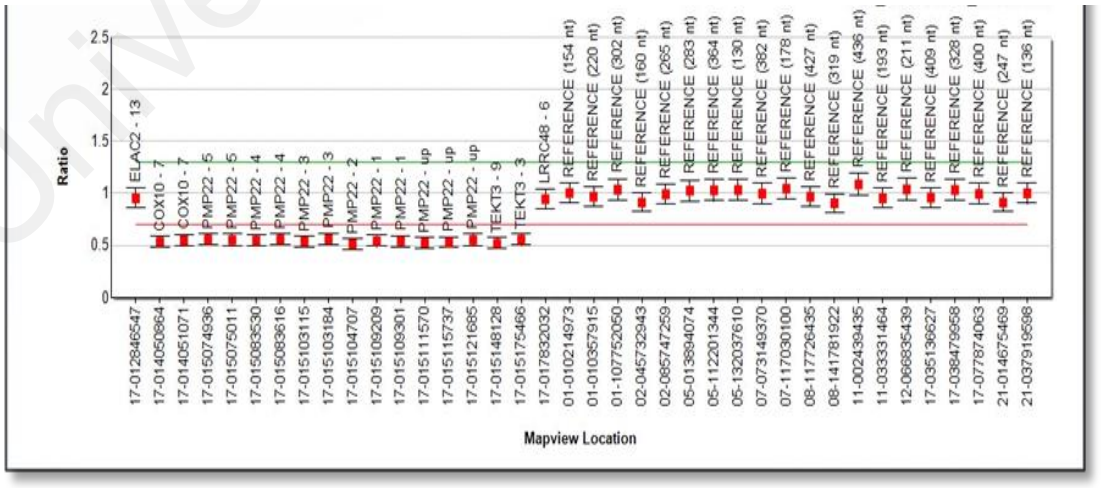


Figure 3.2.2: Ratio chart of the P33-CMT MLPA kit showing a sample with deletion in PMP22

In this case (Figure 3.2.2), the sample has a deletion of the 1.5Mb locus, and the *PMP22* probes within this region lie below the 0.82 lower cut-off (red line), while the other probes outside the 1.5Mb region fall within the normal range (around 1.0).

Out of 26 patients screened for the *PMP22* duplication/deletion, 12 patients were found to be positive for the duplication and 3 patients were detected to have a *PMP22* deletion.

Six patients (2011CMT014, 2011CMT016, 2011CMT022, 2012CMT029, 2013CMT040, 2014CMT046) had family histories of autosomal dominant CMT while the other 6 patients did not (2011CMT023, 2011CMT027, 2012CMT028, 2012CMT034, 2013CMT037, 2013CMT038). All 12 patients had MCV <38m/s and age of onset ranged from the young age of 6 years old to 62 years old. Three patients were detected to have HNPP (2010CMT010, 2011CMT012, 2012CMT030). All of the HNPP patients showed patterns of multiple entrapment sites (which are specific sites along particular peripheral nerves; median across the wrist, ulnar across the elbow and peroneal nerves across the fibula head), a feature indicative of HNPP (Li, Krajewski, Shy, & Lewis, 2002).

Patients with the *PMP22* Duplication

Table 3.5.1: Result summary for the *PMP22* Duplications

No.	Patient ID	#Med/Uln CV (m/s)	Pattern	Family history	Results
1	2011CMT014	18/17	Demyelin	Yes-AD	Dup 17p (*PMP22*)
2	2011CMT016	20/20	Demyelin	Yes-AD	Dup 17p (*PMP22*)
3	2011CMT022	20/21	Demyelin	Yes-AD	Dup 17p (*PMP22*)
4	2011CMT023	17/abs*	Demyelin	None	Dup 17p (*PMP22*)
5	2011CMT027	13/14	Demyelin	None	Dup 17p (*PMP22*)
6	2012CMT028	26/30	Demyelin	None	Dup 17p (*PMP22*)
7	2012CMT029	22/18	Demyelin	Yes-AD	Dup 17p (*PMP22*)
8	2012CMT034	24/23	Demyelin	None	Dup 17p (*PMP22*)
9	2013CMT037	abs/abs*	-	None	Dup 17p (*PMP22*)
10	2013CMT038	11/26	Demyelin	None	Dup 17p (*PMP22*)
11	2013CMT040	20/18	Demyelin	Yes-AD	Dup 17p (*PMP22*)
12	2014CMT046	16/13	Demyelin	Yes-AD	Dup 17p (*PMP22*)

*abs; Absent NCV. Patient's NCV was undetectable
#Med/Uln CV; Median/Ulnar Conduction Velocities (meter/second)
Yes AD; Yes Autosomal Dominant

Patients with the *PMP22* Deletion

Table 3.5.2: Result summary for the *PMP22* Deletions

No.	Patient ID	#Med/Uln CV (m/s)	Pattern	Family history	Results
1	2010CMT010	52/51	Entrap	Yes-AD	Del 17p (*PMP22*)
2	2011CMT012	41/48	Entrap	Yes-AD	Del 17p (*PMP22*)
3	2012CMT030	54/55	Entrap	Yes-AD	Del 17p (*PMP22*)

#Med/Uln CV; Median/Ulnar Conduction Velocities (meter/second)
Yes AD; Yes Autosomal Dominant

No Mutations

Twelve patients diagnosed to have demyelinating CMT were found to be negative for the *PMP22* duplication/deletion. They were then screened for point mutations in *PMP22* and *MPZ*. However, there were no *PMP22* or *MPZ* point mutations in any sample.

Table 3.5.3: Result summary for demyelinating CMT- Negative for all demyelinating test

No.	Patient ID	#Med/Uln CV (m/s)	Pattern	Family history	Results
1	2010CMT002	23/30	Demyelin	None	No mutations detected
2	2010CMT007	28/43	Demyelin	None	No mutations detected
3	2010CMT009	16/abs	Demyelin	None	No mutations detected
4	2010CMT011	25/24	Demyelin	None	No mutations detected
5	2011CMT015	37/37	Demyelin	Cons	No mutations detected
6	2011CMT021	*abs/abs	-	None	No mutations detected
7	2012CMT031	33/abs	Demyelin	Yes-AD	No mutations detected
8	2012CMT032	*abs/abs	-	None	No mutations detected
9	2013CMT039	*abs/abs	-	None	No mutations detected
10	2013CMT041	34/41	Demyelin	None	No mutations detected
11	2014CMT047	19/19	Demyelin	None	No mutations detected
12	2013CMT043	*Entrap	Entrap	None	No mutations detected

*Entrapment: recurrent episodes of nerve dysfunction at compression sites
*abs; Absent NCV. Patient's NCV was undetectable
#Med/Uln CV; Median/Ulnar Conduction Velocities (meter/second)
Yes AD; Yes Autosomal Dominant
Cons; Consanguine marriage

3.3.2 *GJB1* point mutation screening (Demyelinating CMT)

Twenty-four patients showed a demyelinating pattern based on NCV. However, only 7 demyelinating patients clearly had a family history of X-linked CMT. All seven were found to have mutations in *GJB1*.

Table 3.5.4: Result summary of demyelinating GJB1

No	Patient ID	#NCV (m/s)	Pattern	Family history	Results
1	2009CMT001	*Abs/35	Demyelin	Yes-XL	*GJB1*, 5' UTR, -459C>T
2	2010CMT004	28/34	Demyelin	Yes-XL	*GJB1*, c.283G>A, V95M (rs104894821)
3	2011CMT017	38/38	Demyelin	Yes-XL	*GJB1*, c.283G>A, V95M (rs104894821)
4	2012CMT033	*Abs/29	Demyelin	Yes-XL	*GJB1*, c.521 C>T, P174L (novel)
5	2012CMT035	27/34	Demyelin	Yes-XL	*GJB1*, c.220G>A, V74M (novel)
6	2013CMT036	37/43	Demyelin	Yes-XL	*GJB1*, 5' UTR, -459C>T
7	2013CMT042	34/41	Demyelin	Yes-XL	*GJB1*,c.440C>A,Ala147Asp, (CM022790)

*Abs; Absent NCV. Patient's NCV was undetectable
#NCV (m/s); Nerve Conduction Velocities (meter/second)
Yes-XL; Yes X-Linked

Table 3.5.5: Demyelinating *GJB1* results, Electropherogram CMTX

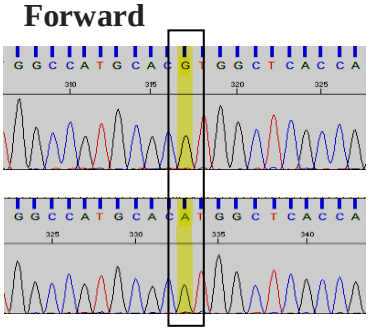
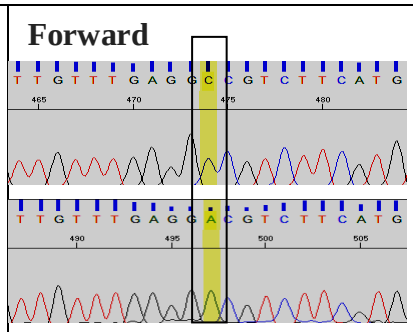
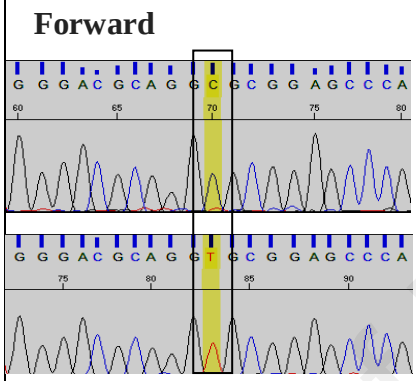
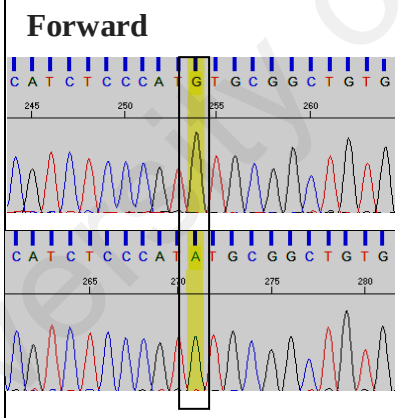
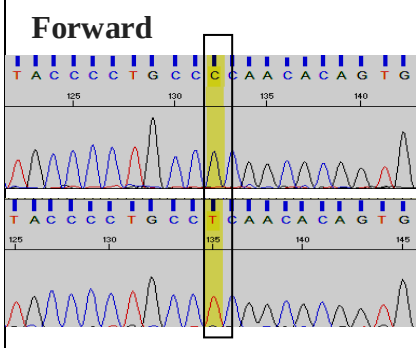
Patient ID	Sequencing Electropherogram	Description
2010CMT004 2011CMT017 SNPs ID: rs104894821	**Forward**  NORMAL PATIENTS c.283G>A, V95M	In the normal individual, at position 283 the normal nucleotide was G but in patients the nucleotide substituted into A and substitutes Valine to Methionine at position 95 in *GJB1*.

Table 3.5.5, continued

13CMT042 SNPs ID: CM022790	 c. 440 C>A , Ala147Asp	In the normal individual, at position 440, the normal nucleotide was C but in our patient the nucleotide was substituted with A. This caused a non-synonymous change of the Alanine residue to Aspartate at position 147 in *GJB1*.
2011CMT001 2013CMT036 SNPs ID: 5' UTR -459C>T	 c.-495C>T	Electropherogram shows sequencing analysis of the patient compared to normal. SNP at position -459 upstream 5' UTR represent changes of nucleotide C to T.
2012CMT035 SNPs ID: Novel mutation (V74M)	 c.220G>A, V74M	A SNP at position 220 of nucleotide sequence, the normal nucleotide was C but in our patient the nucleotide was substituted with A. Mutation changed Valine at position 74 into Methionine in GJB1. This mutation has not been reported before.
2012CMT033 SNPs ID: rs104894821	 c.521C>T, P174L	A SNP at position 521 of nucleotide sequence, the normal nucleotide was C but in our patient the the nucleotide was substituted with T, changing the Proline at position 174 into Leucine. This mutation has not been reported before.

3.3.3 RFLP and family study for patients with unreported SNPs

For the novel mutation (V74M) that was identified in patient 2012CMT035, DNA from the mother and uncle were obtained to investigate whether it was a *de novo* mutation

Table 3.5.6: RFLP information for V74M

Patients	Restriction Enzyme	Recognition site	Product size
2012CMT035 V74M	*Nde1*	5'...C A↓T A T G...3' 3'...G T A T↑A C...5'	PCR product = 561bp. After digestion it produced 298bp and 263bp

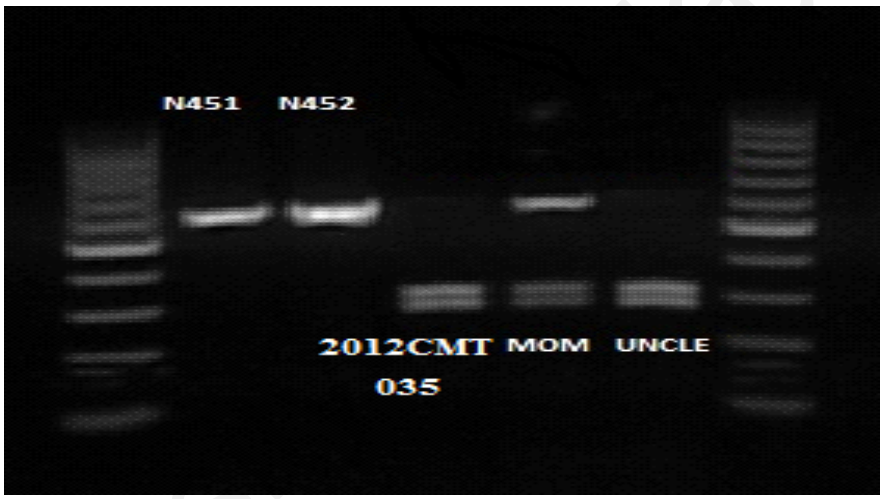


Figure 3.3.1: Gel electrophoresis of RFLP, 2012CMT035's relatives

In Figure 3.3.1, N451 and N452 are normal controls. The patient 2012CMT035 shows two bands indicating the variant is present. As *GJB1* is on the X chromosome, if his mother was a carrier, then she would have three bands, one for the wild type X-chromosome and 2 bands for the X-chromosome carrying the variant. The results here show that she is a carrier. The uncle also shared the same mutation as the patient. Therefore this is not a *de novo* mutation. We further screened this variant in 100 normal chromosomes.

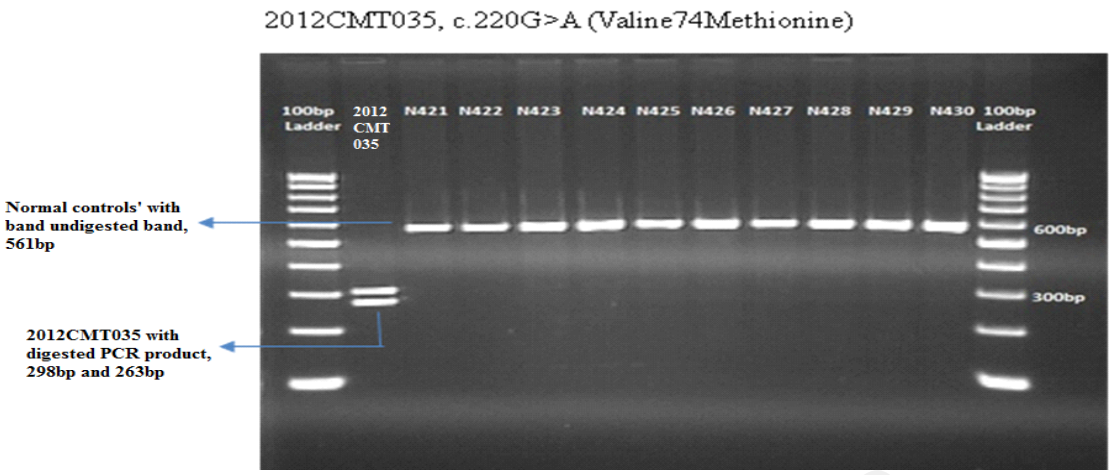


Figure 3.3.2: Gel electrophoresis of RFLP, 2012CMT035

In Figure 3.3.2, patient 2012CMT035 showed two bands but all 100 normal chromosomes (N421-N430 shown in the gel are representative normal controls) had bands at the 561bp mark, therefore none of the normal controls had the variant present in 2012CMT035. The father of this patient did not consent to being screened and in any case was unaffected.

Whereas, for novel mutation (P174L) in patient 2012CMT033, further family investigations was unable to be performed as none of the family members consented to having their DNA analysed. However we also screened the SNP in 100 normal chromosomes and none of the normal chromosomes showed the variant that was present in 2012CMT033.

Table 3.5.7: RFLP information for P174L

Patients	Restriction Enzyme	Recognition site	Product size
2012CMT033 P174L	*Mnl1*	5'...C C T C (N)↓...3' 3'...G G A G (N)↑...5'	PCR product= 110 bp. After digestion, the products = 92bp and 18bp.

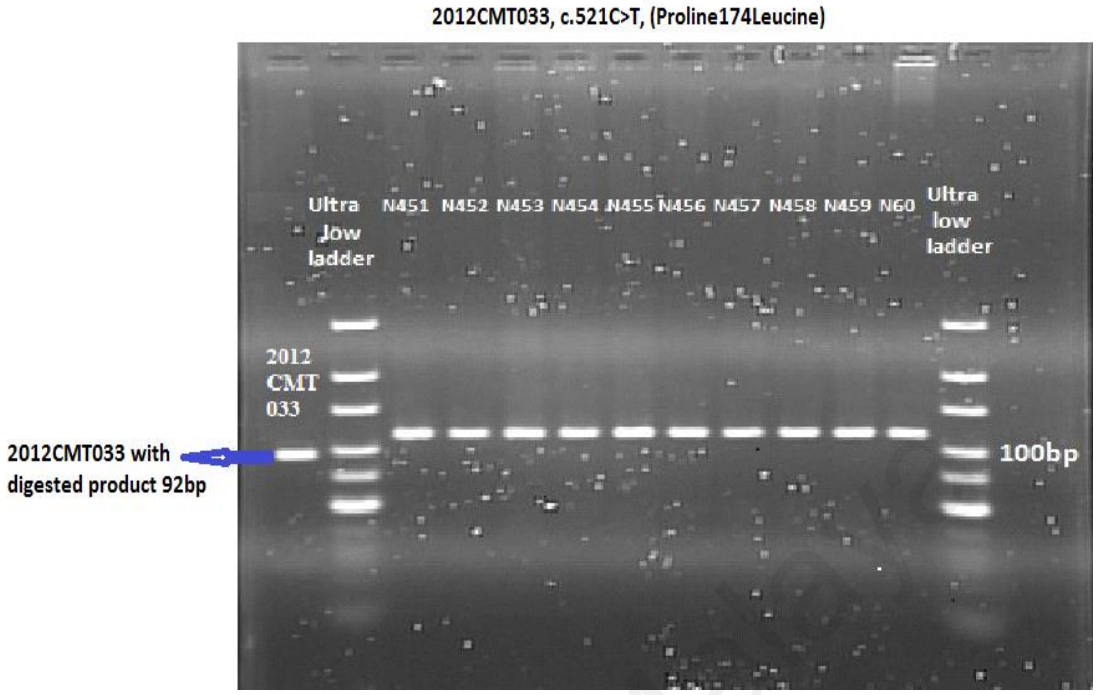


Figure 3.3.3: Gel electrophoresis of RFLP, 2012CMT033

In Figure 3.3.3, patient 2012CMT033 showed 92bp band. N451-N460 represent normal controls, and all 100 normal chromosomes did not show any band at the 92bp mark. The 18bp band is difficult to visualise in the gel due to its small size.

3.3.4 *MFN2*, CMT2A (Axonal CMT)

Among the 13 patients who showed an axonal NCV pattern, 11 patients were screened for *MFN2*, however they were all negative. Two patients were found to have mutations in *MPZ* and *GJB1*, and these patients had a clear family history of X-linked CMT.

No MFN2 mutations were found in the 11 patients presenting with CMT2. Only intronic and synonymous SNPs were found. Below is one of the tested exons that had synonymous changes indicating that we were able to identify shifts in melt curves, but none of the shifts were due to non-synonymous variants.

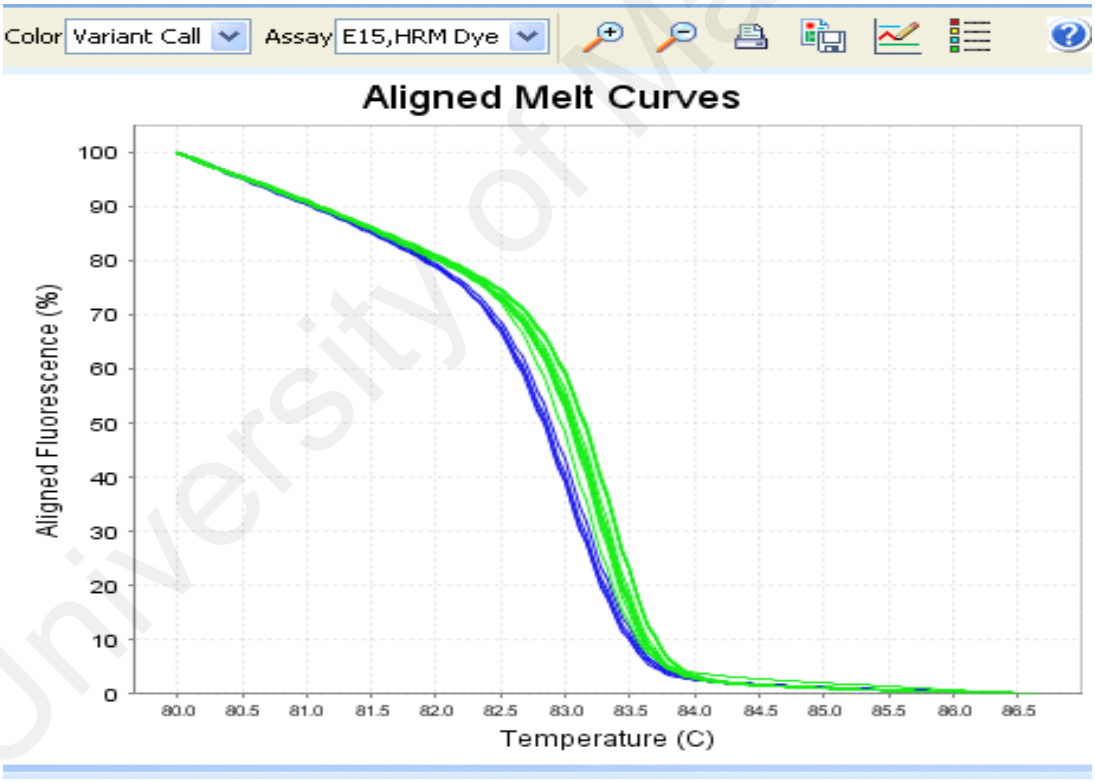


Figure 3.4.1: *MFN2* Alignment Melt Curve

Figure 3.4.1 shows the aligned melt curve for *MFN2* exon 15. There were two distinct melt curves indicating that there were two sequences within this amplicon. The blue belongs to a patient sample and the green belongs to normal controls.

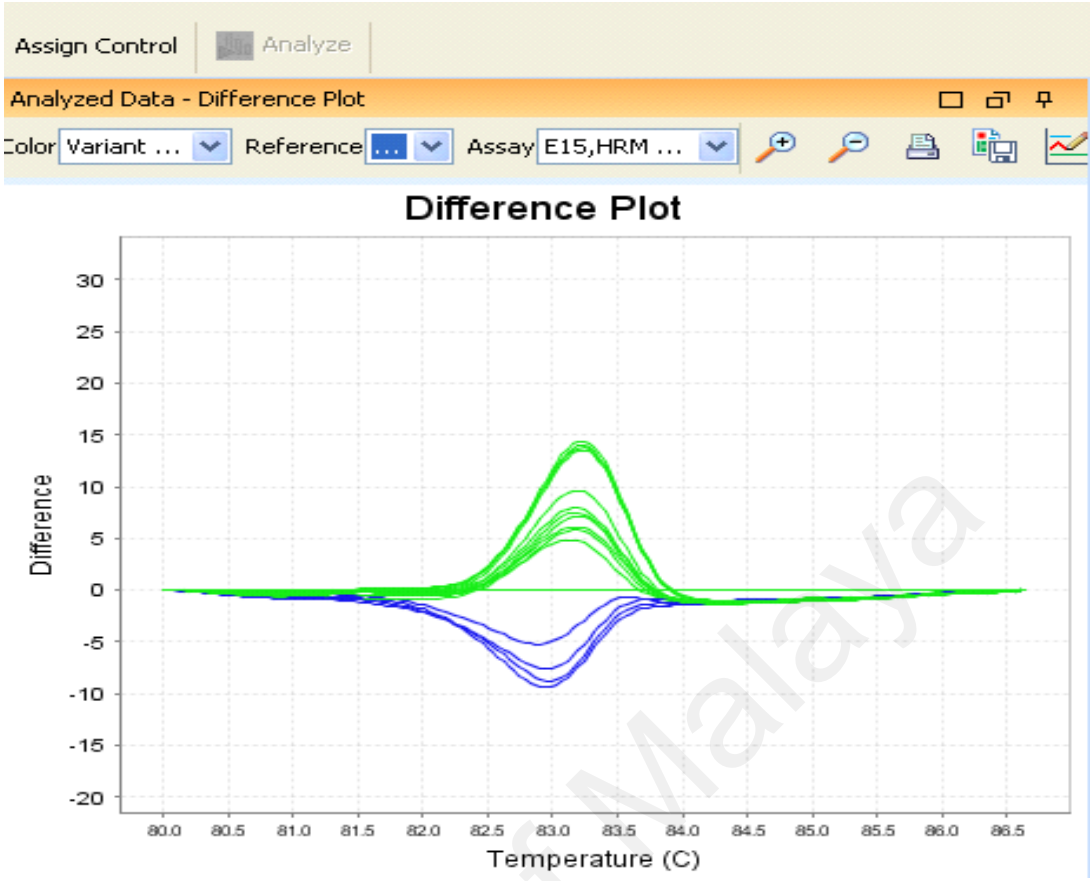


Figure 3.4.2: Differential plots for *MFN2* exon 15

The data shown in Figure 3.4.2 is represented in a differential plot in 3.4.1. The blue curve is the patient sample, and the green is the normal controls. As with the aligned melt curve, there is a distinct curve profile separate from the controls, indicating a difference in the sequence. Any amplicon within *MFN2* that showed a similar profile like shown above (distinct from the controls) was sent for Sanger sequencing.

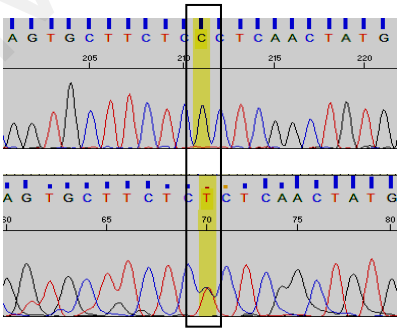
Of all the candidate amplicons screened in *MFN2*, all were synonymous changes with reported SNP IDs, suggesting that the variants were common within the population.

Table 3.6.1: Result summary of Axonal-Negative for all tests

No.	Patient ID	#Med/Uln CV (m/s)	Pattern	Family history	Results
1	2010CMT003	46/46	Axonal	Cons	rs2236056, rs1042842
2	2010CMT006	47/50	Axonal	None	rs2236056, rs41278626, rs 6680984, rs2236057, rs6680984, rs7550536, rs77262016, rs1042842
3	2010CMT008	38/48	Axonal	Yes-ND	No variants found
4	2011CMT013	56/54	Axonal	None	rs2236056, rs2236057, rs7550536, rs1042842
5	2011CMT018	54/57	Axonal	None	rs1042837 rs1042842
6	2011CMT019	42/49	Axonal	None	rs7550536, rs1042842, rs2236056, rs2236057
7	2011CMT020	55/50	Axonal	Yes-AD	rs2236056, rs2236057 rs7550536 , rs1042842
8	2011CMT024	43/54	Axonal	None	rs1042842
9	2011CMT025	57/55	Axonal	None	rs2236056, rs7550536, rs1042842
10	2013CMT044	34/35.2	Axonal	Yes-AD	rs1042842
11	2013CMT045	*NA	Axonal	Yes-AD	No variants found

#MCV (m/s); Median/Ulnar Conduction Velocities (meter/second)
Yes-AD; Autosomal Dominant
* NA= Not Available

Table 3.6.2: Axonal; Electropherogram *MFN2*

Patient ID	Sequencing Electropherogram	Description
MFN2 Axonal SNPs ID rs1042837	**Forward**  NORMAL PATIENTS TCC>TCT	Electropherogram shows sequencing results of a patient compared to a normal. The variant had been reported as rs1042837, and it is a synonymous mutation.

Electropherogram showing the sequence of the rs1042837 variant, identified through HRM. This variant is shown as an example of the confirmation that was used to identify the actual variant present in the amplicons with altered melt curves.

3.3.5 *GJB1*, CMTX (Axonal)

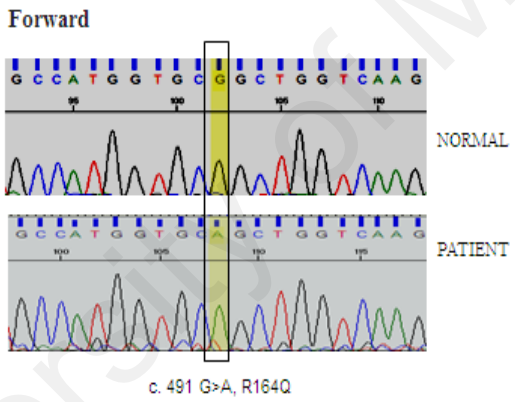
Out of 13 axonal cases, one patient (2011CMT026) had a point mutation in *GJB1*. It has been reported as CM970669 (c.491G>A, R164Q).

Table 3.6.3: Result summary for *GJB1* Axonal

No.	Patient ID	#Med/Uln CV (m/s)	Pattern	Family history	Results
1	2011CMT026	46/42	Axonal	Yes-XL	*GJB1*, c.491G>A, R164Q, (CM970669)

#MCV (m/s); Median/Ulnar Conduction Velocities (meter/second)
Yes-XL; X-Linked

Table 3.6.4: Axonal; Electropherogram *GJB1*

Patient ID	Sequencing Electropherogram	Description
2011CMT026 (*GJB1* Axonal) SNPs ID: CM970669		In the normal individual, at position 491, the normal nucleotide was G but in our patient the nucleotide was substituted with T. This caused a non-synonymous change of the Arginine residue to Glutamine at position 164.

3.3.6 *MPZ*, CMT1B (Axonal)

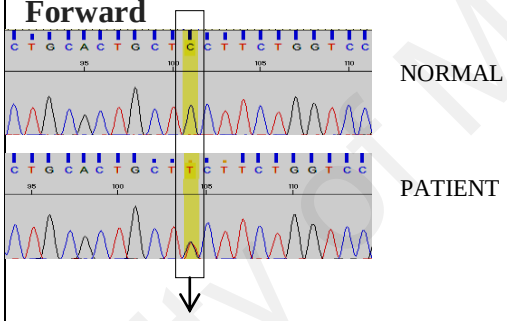
One patient, (2010CMT005) had a point mutation in *MPZ*, and has been reported as CM013408 (c.152C>T, Ser51Phe).

Table 3.6.5: Result summary for *MPZ*

No.	Patient ID	#Med/Uln CV (m/s)	Pattern	Family history	Results
1	2010CMT005	44/45	Axonal	None	*MPZ*, c.152C>T, S51F (*CM013408)

#MCV (m/s); Median/Ulnar Conduction Velocities (meter/second)

Table 3.6.6: Axonal; Electropherogram *MPZ*

Patient ID	Sequencing Electropherogram	Description
2010CMT005 *MPZ* Axonal SNPs ID CM013408	**Forward**  	In the normal individual, at position 152 of the normal allele is a C. However, in our patient the nucleotide was substituted with T showing a heterozygous change. This substituted Serine at position 51 with Phenylalanine

3.3.7 Summary of the Results

Table 3.7: Result summary for the total cohort

Patient ID	Pattern	Family history	Gene Test	Results	Total cases
2011CMT014	Demyelin	Yes-AD	*PMP22* Duplication	Dup 17p (*PMP22*)	12 cases of *PMP22* Duplication
2011CMT016	Demyelin	Yes-AD	*PMP22* Duplication	Dup 17p (*PMP22*)	
2011CMT022	Demyelin	Yes-AD	*PMP22* Duplication	Dup 17p (*PMP22*)	
2011CMT023	Demyelin	None	*PMP22* Duplication	Dup 17p (*PMP22*)	
2011CMT027	Demyelin	None	*PMP22* Duplication	Dup 17p (*PMP22*)	
2012CMT028	Demyelin	None	*PMP22* Duplication	Dup 17p (*PMP22*)	
2012CMT029	Demyelin	Yes-AD	*PMP22* Duplication	Dup 17p (*PMP22*)	
2012CMT034	Demyelin	None	*PMP22* Duplication	Dup 17p (*PMP22*)	
2013CMT037	-	None	*PMP22* Duplication	Dup 17p (*PMP22*)	
2013CMT038	Demyelin	None	*PMP22* Duplication	Dup 17p (*PMP22*)	
2013CMT040	Demyelin	Yes-AD	*PMP22* Duplication	Dup 17p (*PMP22*)	
2013CMT046	Demyelin	Yes-AD	*PMP22* Duplication	Dup 17p (*PMP22*)	
2010CMT010	*Entrapment	Yes-AD	*PMP22* Deletion	Del 17p (*PMP22*)	3 cases of *PMP22* Deletion
2011CMT012	*Entrapment	Yes-AD	*PMP22* Deletion	Del 17p (*PMP22*)	
2012CMT030	*Entrapment	Yes-AD	*PMP22* Deletion	Del 17p *(PMP22)*	
2009CMT001	Demyelin	Yes-XL	*GJB1*	*GJB1*, 5' UTR, -459C>T	7 cases of *GJB1* Demyelination.
2010CMT004	Demyelin	Yes-XL	*GJB1*	*GJB1*, c.283G>A, V95M (rs104894821)	
2012CMT033	Demyelin	Yes-XL	*GJB1*	*GJB1*, c.521 C>T, P174L (novel)	

Table 3.7, continued

2012CMT035	Demyelin	Yes-XL	*GJB1*	*GJB1*, c.220G>A, V74M (novel)	
2013CMT036	Demyelin	Yes-XL	*GJB1*	*GJB1*, 5' UTR, -459C>T	
2011CMT017	Demyelin	Yes-XL	*GJB1*	*GJB1*, c.283G>A, V95M (rs104894821)	
2013CMT042	Demyelin	Yes-XL	*GJB1*	*GJB1*,c.440C>A,Ala147Asp,(CM022790)	
2010CMT002	Demyelin	None	*PMP22*/*GJB1*/*MPZ*	Negative *PMP22*, *MPZ*, *GJB1*	12 Demyelinating cases were negative for all CMT1 test (including 1 HNPP negative deletion)
2010CMT007	Demyelin	None	*PMP22*/*GJB1*/*MPZ*	Negative *PMP22*, *MPZ*, *GJB1*	
2010CMT009	Demyelin	None	*PMP22*/*GJB1*/*MPZ*	Negative *PMP22*, *MPZ*, *GJB1*	
2010CMT011	Demyelin	None	*PMP22*/*GJB1*/*MPZ*	Negative *PMP22*, *MPZ*, *GJB1*	
2011CMT015	Demyelin	Cons	*PMP22*/*GJB1*/*MPZ*	Negative *PMP22*, *MPZ*, *GJB1*	
2011CMT021	-	None	*PMP22*/*GJB1*/*MPZ*	Negative *PMP22*, *MPZ*, *GJB1*	
2012CMT031	Demyelin	Yes-AD	*PMP22*/*GJB1*/*MPZ*	Negative *PMP22*, *MPZ*, *GJB1*	
2012CMT032	-	None	*PMP22*/*GJB1*/*MPZ*	Negative *PMP22*, *MPZ*, *GJB1*	
2013CMT039	-	None	*PMP22*/*GJB1*/*MPZ*	Negative *PMP22*, *MPZ*, *GJB1*	
2013CMT041	Demyelin	None	*PMP22*/*GJB1*/*MPZ*	Negative *PMP22*, *MPZ*, *GJB1*	
2014CMT047	Demyelin	None	*PMP22*/*GJB1*/*MPZ*	Negative *PMP22*, *MPZ*, *GJB1*	
2013CMT043	*Entrapment	None	*PMP22* Deletion	Negative *PMP22*, *MPZ*, *GJB1*	
2011CMT026	Axonal	Yes-XL	*GJB1*	*GJB1*, c. 491 G>A, R164Q, (CM970669)	1 case *GJB1* Axonal
2010CMT005	Axonal	None	*MPZ*	*MPZ*, c.152C>T, S51F (*CM013408)	1 case *MPZ* Axonal

Table 3.7, continued

2010CMT003	Axonal	Cons	*MFN2*/*GJB1*/*MPZ*	rs2236056, rs1042842	11 axonal cases were negative for all CMT2 test. (Mainly *MFN2*)
2010CMT006	Axonal	None	*MFN2*/*GJB1*/*MPZ*	rs2236056, rs41278626, rs 6680984, rs2236057, rs6680984, rs7550536, rs77262016, rs1042842	
2010CMT008	Axonal	Yes-ND	*MFN2*/*GJB1*/*MPZ*	No variants found	
2011CMT013	Axonal	None	*MFN2*/*GJB1*/*MPZ*	rs2236056, rs2236057, rs7550536, rs1042842	
2011CMT018	Axonal	None	*MFN2*/*GJB1*/*MPZ*	rs1042837 rs1042842	
2011CMT019	Axonal	None	*MFN2/GJB1/MPZ*	rs7550536, rs1042842, rs2236056, rs2236057	
2011CMT020	Axonal	Yes-AD	*MFN2/GJB1/MPZ*	rs2236056, rs2236057 rs7550536 , rs1042842	
2011CMT024	Axonal	None	*MFN2/GJB1/MPZ*	rs1042842	
2011CMT025	Axonal	None	*MFN2/GJB1/MPZ*	rs2236056, rs7550536, rs1042842	
2013CMT044	Axonal	Cons	*MFN2/GJB1/MPZ*	rs1042842	
2013CMT045	Axonal	None	*MFN2/GJB1/MPZ*	No variants found	

*Entrapment: recurrent episodes of nerve dysfunction at compression sites
*abs; Absent NCV. Patient's NCV was undetectable
[#]Med/Uln CV; Median/Ulnar Conduction Velocities (meter/second)
Yes AD; Yes Autosomal Dominant
Yes-XL; Yes X-Linked

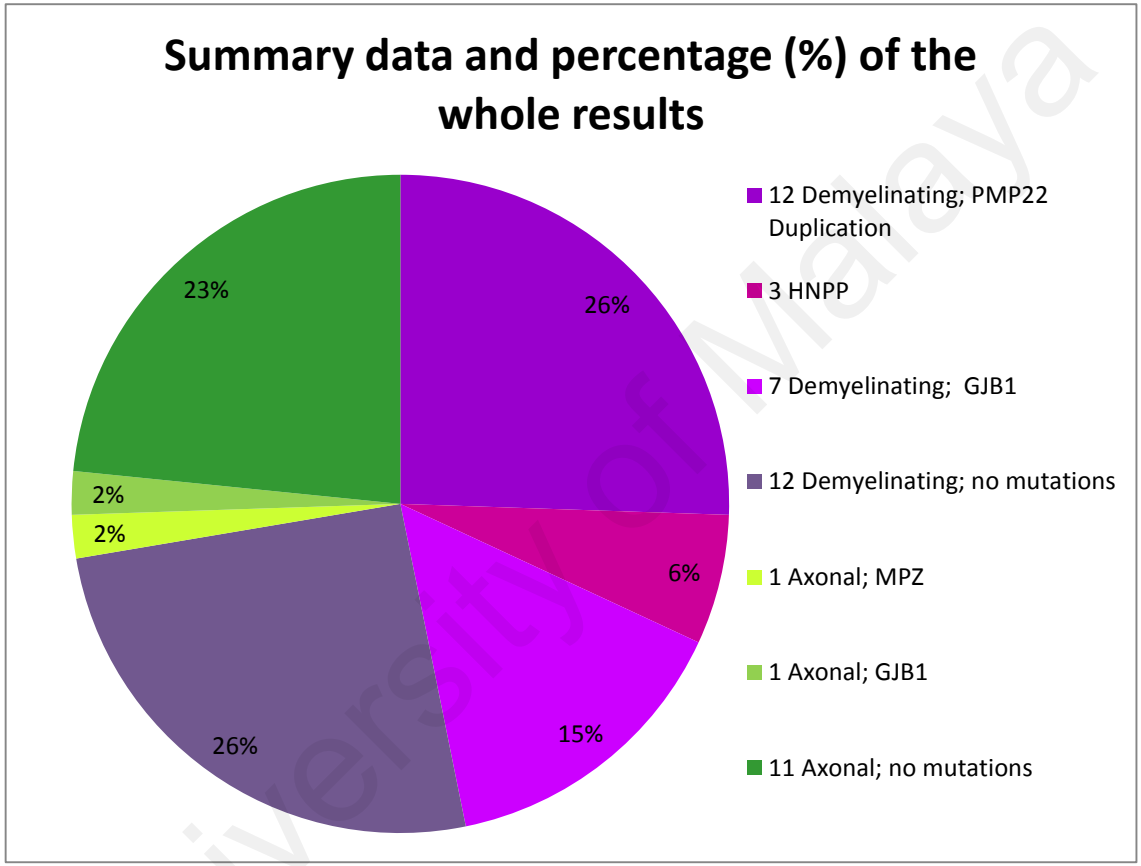


Figure 3.5: Results Summary

3.4 DISCUSSION

3.4.1 PMP22 duplication/ deletion by Multiplex Ligation-dependent Probe Amplification (MLPA)

CMT1A caused by duplications in *PMP22* has been reported in many ethnic groups as the most frequent CMT subtype. In this study, 12 patients were confirmed to have the *PMP22* duplication, making up 26% of the total CMT cohort. Four patients had a positive family history whereas in 6 patients, there was no apparent family history suggesting a possible *de novo* mutation. In many previous reported cases, *PMP22* duplication can arise as a *de novo* mutation in 10% of cases (Blair, Nash, Gordon, & Nicholson, 1996). Out of the 6 probands with no apparent family history, we were only able to test additional family members in one patient (2011CMT027) whereby a *de novo* mutation was observed. The families of the other patients did not consent to DNA analysis.

Three out of four patients with entrapment neuropathies were found to have deletions in *PMP22* in keeping with HNPP, accounting for 6% of the total cohort. Similar deletions were detected in the parents of two patients (2010CMT010 and 2011CMT012) supporting an autosomal dominant pattern of inheritance.

Patients with the demyelinating form but with a normal *PMP22* copy number were also screened for point mutations in *PMP22*. However none of them were detected to have point mutations.

3.4.2 *MPZ* point mutation

In this cohort, 2010CMT005 was the only patient found to have an *MPZ* point mutation (Ser51Phe). This mutation is located at the extracellular domain, and many studies on IPNs have shown that mutations that disrupt the extracellular domain are

pathogenic (Mandich *et al*., 2009). This mutation has been reported previously in a family with two affected members diagnosed with CMT1B (Young et al., 2001).

It has been suggested that late onset neuropathy with prominent axonal loss (CMT2) is associated with alterations in Schwann cell–axon interactions (Mandich et al., 2009). The patient with the *MPZ* mutation did have a later onset of disease and motor velocities in the 40-45 m/s range, therefore supporting this hypothesis.

3.4.3 *GJB1* point mutations

All of the patients with *GJB1* mutations had the age of onset in the first two decades of life. Male CMTX patients usually have a more severe phenotype compared to the females, and the affected CMTX men in this study had slow motor NCVs which is less than 38m/s whereas an affected female, 2011CMT026 had an intermediate motor NCVs >40 m/sec.

In this cohort, 7 patients with demyelinating and 1 with axonal form had *GJB1* mutations, representing 17% of the whole group. Six *GJB1* mutations were residing in the exons, of which two were novel. We also found two unrelated CMTX patients sharing a -459C>T point mutation in the 5'UTR. Although the patients were unrelated, they are from the same ethnic group, which may suggest that there could be an ethnic specific prevalence of this variant.

Kabzinska and his colleagues in 2011 reported two pathogenic mutations; (c.–529T>C) and (c.–459C>T) in the non-coding region disrupts two important regulatory elements in 5'UTR of *GJB1* gene, (c.–529T>C) known to affect transcription factor SOX10 binding site, whereas, (c.–459C>T) is known to disrupt the region responsible for initiation of translation (Internal Ribosome Entry Site; IRES) (Beauvais, Furby, & Latour, 2006). The ethnicity of their patients were not comprehensively described.

The two novel variants identified in this study were Valine74Methionine, V74M and Proline174Leucine, P74L. The V74M mutation segregates with the phenotype in the family as explained in Section 3.3.3: RFLP and family study for patients with unreported SNPs. Both novel mutations affect amino acids residing in the extracellular domain of GJB1. These mutations are discussed in greater detail in CHAPTER 4.

3.4.4 *MFN2* screening

No patients were found to have *MFN2* mutations even though there were 12 patients with axonal CMT. Only synonymous changes and intronic SNPs were detected. Refer to Table 3.6.1: Result summary of Axonal-Negative for all tests.

3.5 CONCLUSIONS

Data generated from this study suggest some possible differences in the Malaysian CMT profile in comparison with other populations. As outlined in Figure 3.5, we found that mutations in *PMP22* although the most common, accounted for only 23.4%, whereas *GJB1* accounted for 17% in the Malaysian population. Malaysian Population we did not find any mutations in 49% of our cases.

CHAPTER 4: FUNCTIONAL STUDY ON NOVEL MUTATIONS

4.1 INTRODUCTION

In CHAPTER 1, we described two novel mutations (V74M and P174L) in *GJB1*. In this chapter, we sought to investigate the effect of these mutations on the function of the protein.

So far, more than 400 mutations have been reported throughout the entire *GJB1* coding sequence and a complete list can be obtained at http://www.molgen.ua.ac.be/CMTMutations/DataSource/MutByGene.cfm (Kleopa, 2011). Some are listed in Table 4.1 below.

4.1.1 Functional Study of GJB1

To date, many investigations on the function of GJB1 have been carried out using transgenic animals and mammalian cell lines. Using *Xenopus laevis* oocytes, the mechanism by which gap junctions are formed at the cell membrane was studied and these are named as GJB1 'plaques' (Dahl, Werner, Levine, & Rabadan-Diehl, 1992). Further experiments revealed that these gap junctions allowed electrical conductance upon depolarization as well as allowing the transport of small molecules <1000 Da to pass between cells (Kleopa, Abrams, & Scherer, 2012). So, when studying *GJB1* mutations, many papers have examined the effect of mutations on the location of these plaques and on electrical conductivity.

A study on eight *GJB1* mutations (I30N, M34T, V35M, V38M, G12S, P87A, E102G and *D*111–116) which were located at the transmembrane and intracellular domains showed that these mutations could reduce the current transduction between the cells (Oh *et al*., 1997), and this effect was similarly seen by Ressot and colleagues with eleven *GJB1* mutations (R22G, R22P, L56F, L90H, V95M, E102G, Deletion (Del) 111–116,

P172S, E208K, Y211stop, and R220X) (Ressot *et al*., 1998). A comprehensive study by Yum and colleagues 2002 looked at the localisation of GJB1 and they were able to map the effect of mutations along the entire GJB1 on the localisation of these GJB1 plaques (more details below and in Table 4.1).

As seen in Table 4.1 the mutations can be found throughout the different domains but there were no particular hotspots described. In addition, 5'UTR variants have also been reported, and these are discussed below.

4.1.2 5'UTR variants

The presence of mutations at 5'UTR sites often raise the question of whether they affect the transcription of the gene as they may be located within the promoter site. For *GJB1*, several studies have shown that this may be the case with two common mutations (529T>C and–459C>T). The –529T>C mutation alters the putative transcription factor SOX10 binding site and affects transcription efficiency (Bondurand *et al*., 2001). Meanwhile, for the –459C>T variant, it was found to abolish the internal ribosome entry site (IRES) and reduce the level of protein translation (Hudder & Werner, 2000). In our study, we found two patients (2009CMT001 and 2013CMT036, refer CHAPTER 3, Table 3.5.4: Result summary of demyelinating GJB1) who carry the -459C>T mutation, and we predict that the effect may be as what has been described previously by Hudder and Werner, 2000.

4.1.3 Coding region

Mutations in the coding region which include missense, nonsense (premature stop codon), deletions, insertions, and frame-shift mutations have been found in every domain of the GJB1 protein. Although there are no particular mutation hotspots, it is thought that the extracellular domain contains potentially '*vulnerable'* amino acids

which are critical for the docking and assembly of GJB1 hemi channels and the final stages of opening channel (Dahl *et al*., 1992). These '*vulnerable*' amino acids are thought to be six highly conserved cysteine residues (Dahl *et al*., 1992) which are highly conserved in all vertebrates. By introducing mutations of these six cysteine residues, they found the docking and opening of the channels were affected, which lead to an absolute loss of function. As a note, there were no patients with mutations in any of these six cysteines in our cohort.

There also appears to be another site that is highly conserved, which is the Proline residue located at the position 87 in the second transmembrane domain. It is thought to be an important residue for protein bending to form the channel and mutations can also affect channel function (Ri *et al*., 1999).

We highlight the impact of mutations at the extracellular domain because the novel mutations (V74M and P174L) found in this study are also located at the extracellular domain. One of the novel mutations that we found (P174L) is located just beside a cysteine residue on the extracellular domain. The V74M mutation is located at extracellular region but it is closer to a proline residue known to be involved in protein bending.

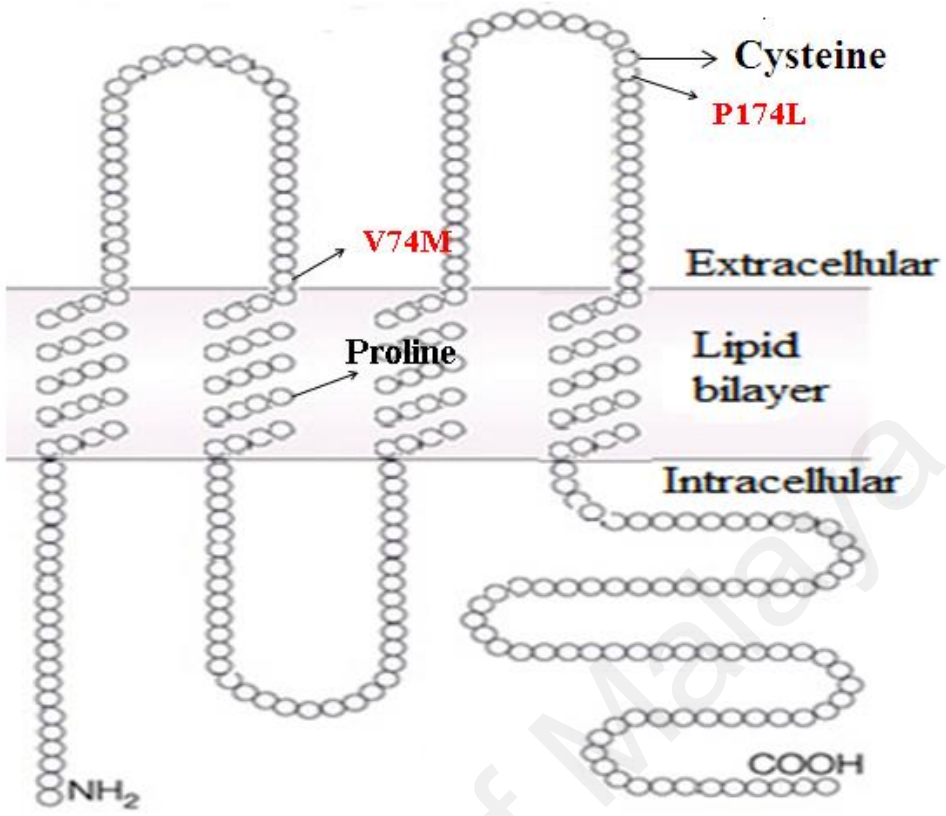


Figure 4.1: Schematic shows the position of the novel mutations, V74M and P174L

A comprehensive study performed by Yum and colleagues analysed 38 different *GJB1* mutations located in different domains and found that there were a number of different phenotypes including trafficking defects, abnormal gap junctions or gap junctions with abnormal biophysical properties (Yum, Kleopa, Shumas, & Scherer, 2002), Table 4.1. Interestingly not all mutations appear to have a clear pathogenic effect as they retain the ability to form GJB1 plaques and are functionally competent as indicated through electrical cell conductance recordings.

There is some evidence to suggest that it may not be the location of the affected amino acids *per se,* but that the properties of the mutant amino acid may have more of an effect. For example, the N205I mutation result in the retention of GJB1 in the ER,

but the N205S mutation allows the protein to reach the cell membrane. Meanwhile, at amino acid position 34, multiple possible effects are seen depending on the substituted amino acid (M34K - GJB1 retained at the ER; M34T - GJB1 retained in the Golgi apparatus; M34I and M34V – normal localization), (Yum *et al*., 2002).

Mutations at the carboxyl terminal do not appear to have as much a deleterious effect as those in the extracellular domain. The majority of carboxyl terminal mutations still show normal GJB1 plaques, however some evidence indicates that the mutations still show a reduction in current transduction (Castro, Gómez-Hernandez, Silander, & Barrio, 1999). Mutations that truncate the protein (e.g. C217stop, R220stop, R265stop, S281stop, C280stop and S281stop) can still form GJB1 plaques with almost normal levels of junctional conductance (Abrams, Oh, Ri, & Bargiello, 2000; Castro et al., 1999).

However, mutations at the carboxyl region can still lead to some defects, as seen with the F235C mutation which has been reported to be associated with a severe CMT phenotype, where the electrophysiological studies showed abnormalities in the electrical transduction even though it presented a normal localization and trafficking of the mutant protein in cell culture (Liang *et al*., 2005).

Several missense mutations resulted in a failure to form functional gap junction and retained in the Golgi or endoplasmic reticulum, and some show a reduction in current conductance (G12S, R22G, R22P, R22X, S26L, I30N, M34K, M34T, M34I, M34V, V35M, V37M, V38M, A40V, R75W, R75Q, R75P, R75W, L90H, H94Q, V139M, R142W, G199R, N205I, C53S, T55I, C60F Y65C, L156R, R164W, P172R, P172S, S182T, E186K, V95M, R107W, E208K, E208L, Y211X, I213V, R215W, C217X, R220X). (Castro *et al*., 1999; Oh *et al*., 1997; Ressot *et al*., 1998; Wang *et al*., 2004; Yum *et al*., 2002). Refer to Table 4.1.

Figure 4.2 shows the mutations in *GJB1* as reported up to 2002 (Yum *et al*., 2002).

However the 5'UTR variants are not shown in the figure, but are listed in Table 4.1.

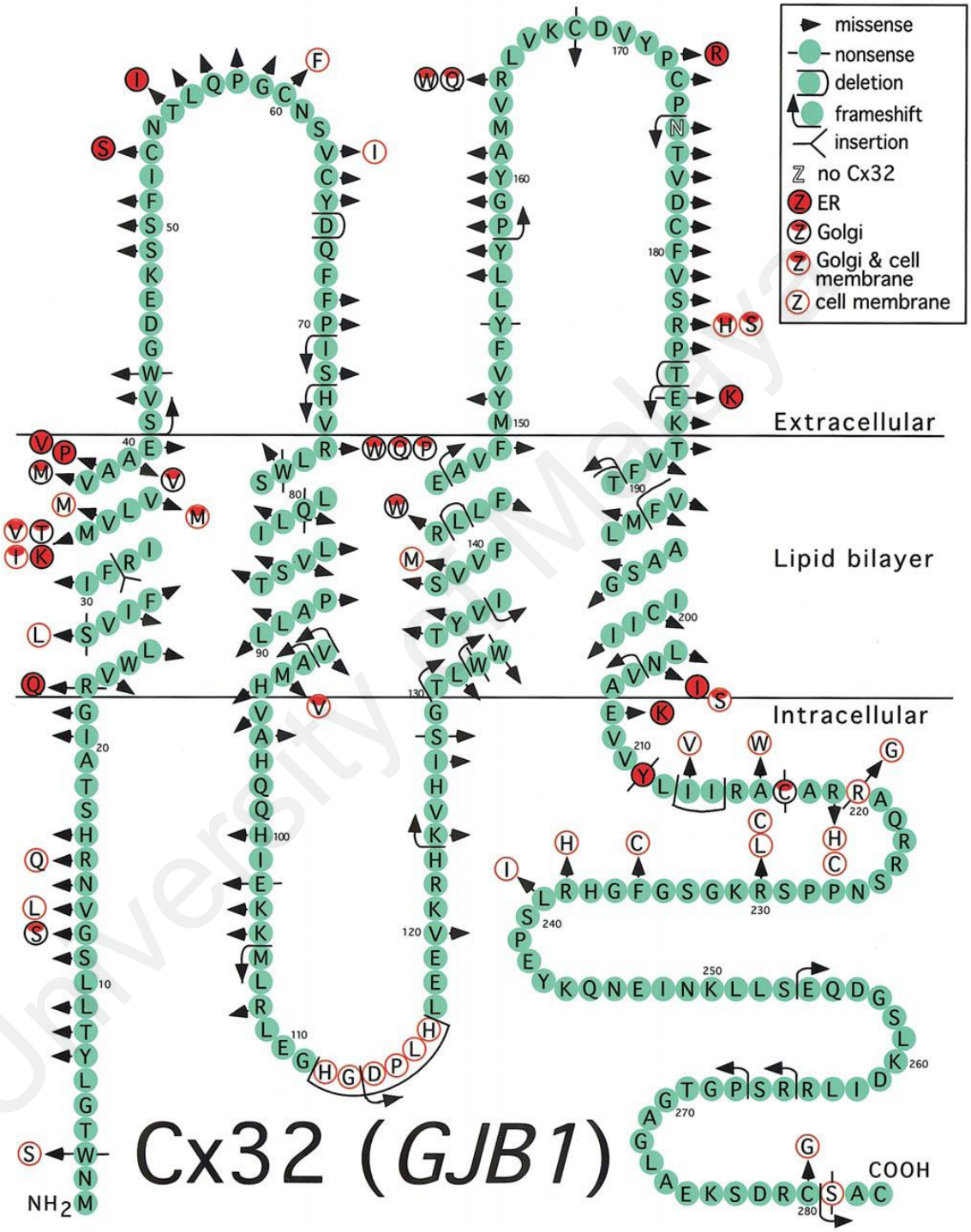


Figure 4.2: GJB1 protein structure with some reported mutations.

Figure shows GJB1 protein structure with some reported mutations. GJB1 is composed of 2 extracellular domains, 4 transmembrane domains, 1 intracellular loop, as well as an amino- and a carboxy-terminal cytoplasmic tail. Adapted from Yum *et al*, 2002.

Some of the reported variants and their effect on GJB1 function are listed in Table 4.1

Table 4.1: Some of reported variants and the effects on the GJB1 function.

Amino acids positions (domain)	**SNPs (variants)**	**Effect**	**References**
5'UTR	c.–529 T>C c.–529 T>G c.–527 G>C c.–458 G>A c.–459 C>T c.–373 G>A c.–215 G>A	CMTX phenotype	As reviewed by Kabinzska *et al*, 2011
Intracellular N-terminal 1-21	G12S	Failed to form gap junction	Wang *et al*, 2004 Oh *et al*, 1997
	V13L	Normal gap junction	Wang *et al*, 2004
	R15Q	Normal gap junction	Wang *et al*, 2004
Trans-membrane Domain TM1-TM4 22-40aa 75-94aa 130-149aa 188-207aa	R22Q	Normal gap junction	Wang *et al*, 2004
	R22G	Failed to form gap junction	Ionasescu *et al*, 1996
	R22P	Failed to form gap junction	Ressot *et al*, 1998
	R22X	Failed to form gap junction	Ionasescu *et al*, 1996
	S26L	Reduction in the permeability	Becigo *et al*, 2006
	I30N I30N	Normal gap junction Reduction in the permeability	Wang *et al* 2004 Oh *et al*, 1997
	M34K	ER	Kleopas *et al*, 2002
	M34T M34T M34I	Golgi Normal gap junction Golgi but forming gap junction-like plaques	Kleopas *et al*, 2002 Tan *et al*, 1996 Kleopas *et al*, 2002
	M34V	Golgi but forming gap junction-like plaques	Kleopas *et al*, 2002
	V35M V35M	Golgi Normal gap junction	Kleopas *et al*, 2002 Wang *et al*, 2004
	V37M	Golgi but forming gap junction-like plaques	Kleopas *et al*, 2002
	V38M	Golgi	Kleopas *et al*, 2002
	A40V	Golgi	Kleopas *et al*, 2002
	R75W	Failed to form gap junction	Sargiannidou *et al*, 2009
	R75Q	Golgi	Kleopas *et al*, 2002
	R75P	Golgi	Kleopas *et al*, 2002
	R75W	Golgi	Kleopas *et al*, 2002
	L90H	Failed to form gap junction	Ressot *et al*, 1998
	H94Q	Failed to form gap junction	Bone *et al*, 1997
	W133R	Normal gap junction	Wang *et al*, 2004

Table 4.1, continued

	V139M	Failed to form gap junction	Omori *et al*, 1996
	R142W	Failed to form gap junction	Bruzone *et al*, 1994
	G199R	Failed to form gap junction	Wang *et al*, 2004
	N205S	Normal gap junction	Wang *et al*, 2004
	N205I	ER	Kleopas *et al*, 2002
Extracellular Domain 41-74aa 150-187aa	C53S	Failed to form gap junction	Yoshimura *et al*, 1998
	T55I	Failed to form gap junction	Sargiannidou *et al*, 2009
	L56F	Normal gap junction	Latour *et al*,1996
	C60F	Failed to form gap junction	Omori *et al*, 1996
	V63I	Normal gap junction	Wang *et al*, 2004
	Y65C	Failed to form gap junction	Wang *et al*, 2004
	L156R	Failed to form gap junction	Wang *et al*, 2004
	P158A	Normal gap junction	Wang *et al*, 2004
	R164W	Failed to form gap junction	Wang *et al*, 2004
	P172S	Normal gap junction	Wang *et al*, 2004
	P172R	Failed to form gap junction	Yoshimura *et al*, 1998
	P172S	Failed to form gap junction	Ressot *et al*, 1998
	S182T	Localized in the cell membrane despite impairing ability to form functional gap junctions	Wang *et al*, 2004
	E186K	Failed to form gap junction	Bruzzone *et al*,
Intracellular Domain 95-129aa	V95M	Failed to form gap junction	Wang *et al*, 2004
	E102G	Normal gap junction	Oh *et al*, 1997
	R107W	Failed to form gap junction	Wang *et al*, 2004
	Del 111–116	Reduction in the permeability	Becigo *et al*, 2006
Carboxyl Terminal 208-283aa	E208K	Failed to form gap junction	Wang *et al*, 2004
	E208L	Failed to form gap junction	Ressot *et al*, 1998
	Y211x	Reticulum Endoplasmic	Kleopas *et al*, 2002
	Y211x	Localized in the cell membrane despite impairing ability to form functional gap junctions	Wang *et al*, 2004
	Y211x	Failed to form gap junction	Ressot *et al*, 1998
	I213V	Golgi	Kleopas *et al*, 2002
	R215W	Failed to form gap junction	Omori *et al*, 1996
	C217x	Golgi	Kleopas *et al*, 2002
	R219C	Normal gap junction	Kleopas *et al*, 2002
	R219H	Normal gap junction	Kleopas *et al*, 2002
	R220G	Normal gap junction	Kleopas *et al*, 2002
	R220X	Reduction in the permeability	Becigo *et al*, 2006
	R230C	Normal gap junction	Kleopas *et al*, 2002

Table 4.1, continued

	R230L	Normal gap junction	Kleopas *et al*, 2002
	R238H	Normal gap junction	Kleopas *et al*, 2002
	L239I	Normal gap junction	Kleopas *et al*, 2002
	R265X	Normal gap junction	Castro *et al*, 1999
	C280G	Normal gap junction	Castro *et al*, 1999
	S281x S281x	Normal gap junction Normal gap junction	Kleopas *et al*, 2002 Castro *et al*, 1999

In our cohort, there were eight mutations which were found throughout the GJB1 domain as well as in the 5'UTR (Figure 4.3 below).

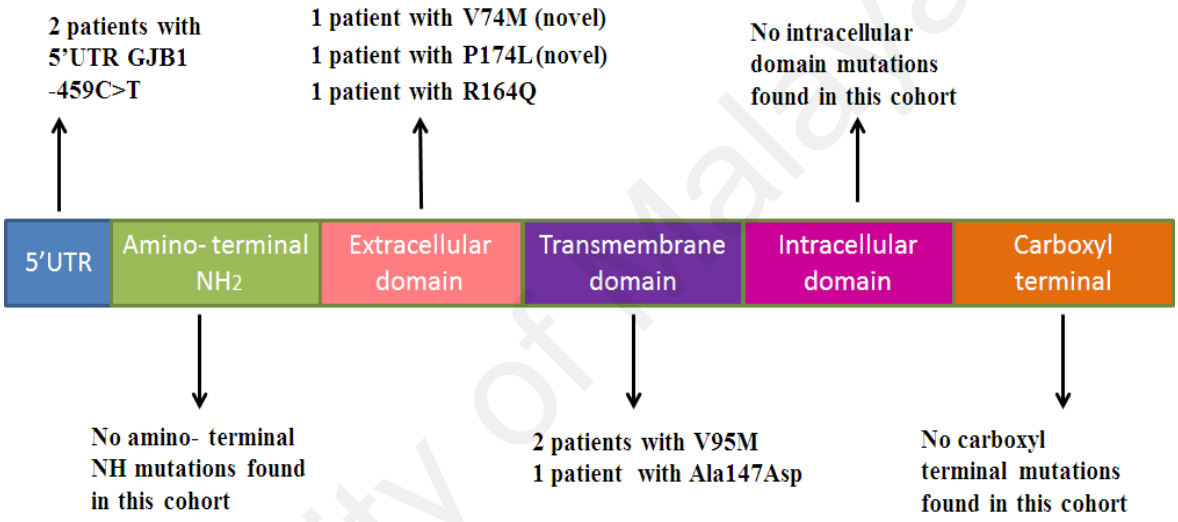


Figure 4.3: Positions of *GJB1* mutations that were found in this cohort in GJB1 domains.

The phenotypes of the CMT1X patients with the V74M and P174L mutations have typical CMT1X phenotypes with electrophysiological data with features of both demyelination and axonal neuropathy. The nerve studies of the patient with P174L mutation was worse with unrecordable median potentials and slow ulnar velocities. However, in comparison to the patient with V74M mutation, this patient was much older and thus the differences in neurophysiology changes are likely to reflect disease duration and age effects rather than a more damaging effect of the mutation.

To further investigate the effect of the mutations, we prepared clones carrying the GJB1 wild type sequence and the two mutations, and transfected them into HEK293 cell lines.

4.2 MATERIALS AND METHODS: General method of research study

CONSERVATION OF AMINO ACIDS & BIOINFORMATICS ANALYSIS

To determine the conservation of the normal amino acid across species, bioinformatics tools were used to assess reasonability of the extended functional study.

SITE-DIRECTED MUTAGENESIS

Mutant constructs of P174L and V74M were cloned into GJB1-tagged GFP plasmids

CELL CULTURE, TRANSFECTIONS AND IMMUNOFLOURESCENCE

Different cDNA constructs (wild type GJB1, V74M and P174L) was transfected into HEK293 cells to determine the level of expression and protein localization.

WESTERN BLOTTING

Western blotting was used to validate the expression of GJB1 of each construct (wild type, V74M and P174L)

4.2.1 Conservation of the amino acid bioinformatics analysis

The regions flanking the mutated sites were checked for the conservation of the amino acids affected, by aligning the sequence with sequences from many different species using the UCSC website (https://genome.ucsc.edu/).

To predict the effect of the unknown variants, several online softwares were used: SNAP (Screening for Non-acceptable Polymorphisms), Polyphen2 and SIFT (Sorting Intolerant From Tolerant). Sequences of GJB1 with the mutated amino acids were inserted into the software and the results of damaging levels presented accordingly based on software prediction.

SNAP is a computational method that uses protein information such as secondary structure, conservation and solvent accessibility in order to make predictions regarding the effect of variants within that sequence. The software will predict if the variants will cause "neutral effects" in the sense that the resulting point-mutated protein does not affect the protein function, or they are "non-neutral" in that the variation has an effect (Bromberg & Rost, 2007). This software is available at http://www.rostlab.org/services/SNAP.

SIFT prediction is a mathematical computation based on the degree of evolutionary conservation of amino acids in sequence alignments derived from closely related sequences, collected through PSI-BLAST (Position-Specific Iterated-Basic Local Alignment Search Tool). SIFT predictions will be grouped as ‘damaging’: the substitution is predicted to affect protein function, or ‘tolerated': the substitution is predicted to be functionally neutral, and the predictions are given a score of 0 to 1: the closer to 0, the more damaging the effect (Kumar, P., Henikoff, & Ng, 2009).

PolyPhen-2 (Polymorphism Phenotyping v2) also uses multiple alignments of vertebrate genomes with the human genome to predict the effect of variation to a conserved amino acid. The output of the PolyPhen-2 prediction pipeline is a prediction of probably damaging, possibly damaging, or benign, along with a numerical score ranging from 0.0 (benign) to 1.0 (damaging) (Adzhubei et al., 2010).

4.2.2 Site-Directed-Mutagenesis

4.2.2.1 Creation of a Mutagenesis on Normal Construct

Desired mutations were created by site-directed mutagenesis using the wild type GJB1 construct as the starting material. The following primer sets were designed as mutagenic primers in order to introduce the specific site of mutations.

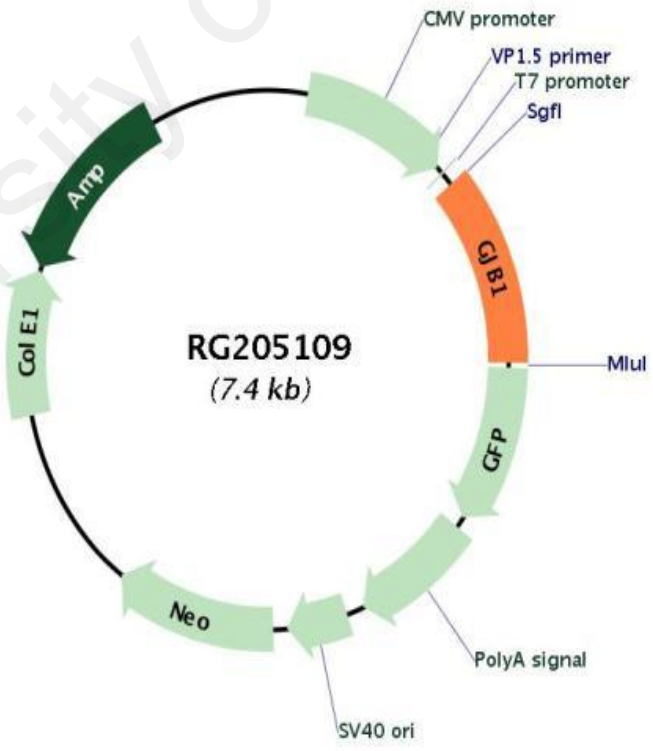


Figure 4.4: Schematic of GJB1 cDNA construct

Table 4.2.1: Sets of primers used to create targeted mutations

Primer Name	Primer Sequence (5'=>3')
V74M (c.220G>A) Forward	5'CTTCCCCATCTCCCATATGCGGCTGTGGTC 3'
V74M (c.220G>A) Reverse	5'GACCACAGCCGCATATGGGAGATGGGGAAG 3'
P174L (c.521 C>T) Forward	5' CGTCTACCCCTGCCTCAACACAGTGGACTG 3'
P174L (c.521 C>T) Reverse	5' CAGTCCACTGTGTTGAGGCAGGGGTAGACG 3'

Site-Directed-Mutagenesis (SDM) was performed according to the manufacturer's protocol where sample reactions were prepared as indicated in Table 4.2.2 below.

Table 4.2.2: Master mix of Site-Directed Mutagenesis reactions

Reagent	Volume
10x reaction buffer	5µl
10-100ng od dsDNA template	5µl
125ng of nucleotide primer, Forward	1.25µl
125ng of nucleotide primer, Reverse	1.25µl
dNTP mix	1µl
Quick solution reagent	11.5µl
ddH2O to final volume of 50µl	34µl
QuickChange Lightning Enzyme	1µl

Once the site-directed mutagenesis reactions were prepared, they were placed into thermocyclers to allow the process to take place.

Table 4.2.3: Cycling parameters for the Quick Change Lightning Site-Directed Mutagenesis

Segment	Cycles	Temperature	Time
1	1	95°C	2 minutes
2	18	95°C	20 seconds
		60°C	10 seconds
		68°C	7 minutes
3	1	68°C	5minutes

Two µl of *Dpn I* restriction enzyme was added directly to the post-PCR amplification reactions. Each reaction was mixed gently and thoroughly by pipetting solution up and down several times. The reaction mixtures were then spun down and immediately incubated at 37°C for 5 minutes to digest parental (the non-mutated) supercoiled dsDNA.

4.2.2.2 Transformation, Grow and plasmid extraction

The mutant constructs from step above were transformed into XL10-Gold ultracompetent cells provided with the QuickChange Lightning Site-Directed Mutagenesis Kit (Catalog#210518). The transformants were then spread on LB-ampicillin agar plates containing 80ug/ml X-gal and 20uM IPTG. The transformation plates were incubated at 37°C for >16 hours. After 16 hours, plasmids were extracted using the INtRON BIOTECHNOLOGY plasmid extraction kit. The purity and concentration of DNA were determined by Nanodrop.

4.2.2.3 DNA Sequencing

PCR and DNA sequencing was performed after SDM to confirm whether the SDM was successful. Table 4.2.4 shows the primer set used to confirm the presence of the desired mutation at the targeted site. As it covers the entire coding region of GJB1, it was also used to validate that the constructs did not contain any other mutations other than the desired specific one (V74M or P174L).

Table 4.2.4: Set of primers used to verify Site-Directed Mutagenesis was successful

Primer name	**Primer sequence (5'=>3')**	**Annealing Temperature**
SDM Validation primer FORWARD	5'GGATCCGGTACCGAGGAG 3'	60°C
SDM Validation primer REVERSE	5'CTCTCGTCGCTCTCCATCTC 3'	60°C

4.2.3 Cell Culture and Transfection

4.2.3.1 Type of Cell Lines and Cultivation of Cell Lines

HEK293 cells were used for the transfection experiments as they do not produce endogenous GJB1 proteins, therefore any GJB1 protein that are visualized in the cells would be from the transfected constructs.

The HEK293 cell line was cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA), supplemented with 10.0% (v/v) Foetal Bovine Serum (FBS) (Kansas, USA) and 5% (v/v) penicillin streptomycin (Gibco, USA). All cells were grown at 37^{o}C in a 95% humidified incubator (ESCO Cell culture, Esco Micro Pte. Ltd) with 5.0% $CO_{2.}$

The cells were passaged when 80-90% confluence was observed. The used media was discarded then the cells were washed with 1× PBS (Gibco, USA), to remove any residual serum that could inactivate trypsin activity. After PBS was removed, 1 ml of the trypsin solution (Gibco, USA) was added to the flask. The culture flask was then incubated at 37^{o}C for 10 min to allow the detachment of cells from the culture flask (SPL Life Science, KOREA) surface. Then, 6 ml of appropriate growth medium was added to inactivate trypsin activity with the ratio 1:3 (1= trypsin; 3= growth medium) and further pipetted into a 15.0 ml Falcon tube. Trypsinized cells were then centrifuged at 1500 rpm for 7 min, and the supernatant was discarded. The cell pellet was re-suspended in 8 ml of fresh growth medium and split into prepared culture flasks or 6 well plates for further use.

4.2.3.2 Cell Counting

Ten µl of cell suspension was mixed with 10.0µl of 0.08% (v/v) trypan blue (Merck, Germany) dye solution. The solution was then transferred to a haemocytometer

counting chamber. The number of cells in each of the four square grid corners was counted at 100× magnification (Nikon light microscope), and the average number of cells was obtained. Each square grid represents a 0.1 mm^3 or 10^{-4} ml volume, and the concentration of cell was calculated according to the formula:

$$\frac{\text{(Total number of cells counted)}}{\text{(Numbers of chamber counted)}} \times \text{(Dilution factor)} \times 10^3 = Z$$

$$\frac{\text{Number of cells wanted}}{Z} \times 1000\mu l = ?\ \mu l \text{ to put on wells.}$$

4.2.3.3 Transfection

Prior to transfection, cells were divided into 6 well plates. Each plate contains 200,000 cells. Transfection was performed with Lipofectamin® 2000 Transfection Reagent (Invitrogen, USA) kit. The normal and mutant constructs were diluted with Opti–MEM® I Reduced Serum Medium (Gibco, USA) and incubated for 15 minutes at 25℃. Similarly, Lipofectamin® 2000 Transfection Reagent (Invitrogen, USA) also diluted with Opti–MEM® I Reduced Serum Medium (Gibco, USA) and incubated for 15 minutes at 25℃. The diluted DNA and diluted Lipofectamin were then combined at a ratio of 1:1 and incubated for another 10 minutes. HEK293 cells (approximately 80% confluent) were washed with Opti-MEM then incubated with the Lipofectamin/DNA mix overnight at 37℃. After 24 hours, the media was removed and cells were given another wash with 1X PBS before being stained with DAPI (Invitrogen, USA) at room temperature for 7 minutes. Then the cells were fixed with -20℃ absolute ethanol for 10 minutes before being mounted with the ProLong Gold® antifade reagent mounting medium (Life Technologies, USA) on a glass microscope slide.

4.2.3.4 Cell evaluation

The cell morphology and protein localization was captured using the High Resolution Upright Compound Leica DM6000b Microscope.

4.2.4 Western Blotting

4.2.4.1 Protein extraction and sample preparation

For protein analysis, cells were extracted using the RIPA buffer containing 150mM NaCl (Merck), 50mM Tris-HCl pH8.0 (Fisher Scientific), 1% Triton X-100 (AMRESCO, UK), 0.5% sodium deoxycholate (Sigma Aldrich) and 0.1% SDS with 1:1000 inhibitor cocktail (AMRESCO, UK). Cells were washed once with cold PBS and harvested using 100 µl per well of RIPA buffer followed by incubation on ice for 5 minutes. After incubation, cells were scrapped from the bottom of the wells and triturated with fine tipped glass pipettes. Lysed cells were then spun down at 10 000rpm, 10 minutes at 4℃. Supernatant was kept in -20℃ freezer until used.

The protein concentrations of the cell lysates were determined using the Pierce® BCA Protein Assay Kit (Thermo Scientific, USA). From 2mg/mL Albumin Standard, a set of diluted standard albumin samples (9 series with 0 to 400µl volume of diluent) were prepared. BCA Working Reagent (WR) was then prepared based on the following formula (#standards + #unknown) x (#replicates) x (volume of WR per samples) = total volume WR required. For our work, (9 standards + 3 samples) x (2 replicates) × (2mL) = 48mL WR. Each of the dilution series was combined with WR reagent with a ratio of 50:1 (5ml dilution series + 100µl of WR). Twenty-five µl of each standard and tested sample replicates were pipetted into a microplate well and then 200µl of the WR was added into each well and mixed thoroughly on a plate shaker for 30 seconds. The plate was then covered and in being placed in a plate reader. Measurement was performed at or close to 562nm absorbance. A final volume of 20.0µl cell lysate was mixed with 7µl Laemmli loading buffer (AMRESCO) for dye tracking. All samples were then boiled at 95℃ for 5minutes and loaded into the SDS-PAGE.

4.2.4.2 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE) were prepared to fractionate the extracted protein following the transfection of GJB1 cDNA. Ten % (w/v) resolving and 5% (w/v) stacking gels were prepared (as mentioned in Table 4.3 below) to separate proteins ranging in size between 10-260 kDa. One mini-gel with a dimension of 18cm x 0.75mm was prepared by clipping glass plates (BioRad, CA, USA) together on a casting tray (BioRad, CA, USA). The resolving gel solution was loaded until the space between the glasses was ¾ full and allowed to polymerize for 30 minutes. The top of the resolving gel was carefully covered with dH_2O to prevent dehydration. When polymerization was complete, the dH_2O was removed using Kim wipes (Kimberly, Clark, Canada) and the 5% (w/v) stacking gel was loaded until 100% of the glass plates was filled. A10-well gel comb with 0.75mm thickness was inserted into stacking gel and the gel was allowed to polymerize for 30 minutes. The gel was removed from the holding tray and transferred into a Mini PROTEAN® Tetra Cell gel tank (BioRad, CA, USA), and gel comb was gently removed. The inner portion of the gel tank was filled with 1 x Tris-Glycine-SDS (TGS) running buffer (BioRad, CA, USA), until the whole surface of gel and the outer portion was filled to about 50% of the tank depth with 1x TGS buffer. Before the samples were loaded, some running buffer was pipetted into each well to remove any traces of unpolymerized gel and remove the bubbles that form in between the gel. A total 20µl of each protein sample were loaded into each well. Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific, USA) was loaded as markers. Gel electrophoresis was performed by running the gel at 80V with free flowing current for about 20 minutes using a power supply (BioRad, CA, USA) to allow the samples to align before entering the resolving gel followed by 110V with free flowing current for 60 minutes to resolve the protein samples.

Table 4.3: Stacking gel and resolving gel were prepared with the desired percentage

Reagents	10% Resolving Gel	5% Stacking gel 3ml
H_20	4.0 ml	2.1 ml
30% acrylamide	3.3 ml	0.5 ml
1.0 M Tris (pH6.8)	-	0.38 ml
1.5M Tris (pH8.8)	2.5 ml	-
10% SDS	0.1 ml	0.03 ml
10% ammonium persulfate (APS)	0.1 ml	0.03 ml
TEMED	0.008 ml	0.008 ml

*Note: 10% APS was prepared fresh each time

4.2.4.3 Western blotting

Once the SDS-PAGE was completed, the resolving gel containing separated protein, nitrocellulose membrane Invitrolon™ PVDF (Invitrogen, CA, USA) and filter paper (Thermo Scientific, USA) was soaked in transfer buffer for 10 minutes. A transfer 'sandwich' consisting of the resolving gel, Invitrolon™ PVDF membrane (Invitrogen, CA, USA) and filter paper (Thermo Scientific, USA) was then prepared and placed in the Mini Trans Blot® Cell tank. A blotting roller was used to force out the presence of air bubbles between each layer of sandwich. Transfer of proteins to membrane was at 350mA with free flowing voltage for 90 minutes. The membrane was then incubated for 1h at 37°C under agitation in blocking buffer to prevent non-specific background binding of the primary and secondary antibodies. The membrane was then incubated in a primary antibody against the cytoplasmic tail of carboxyl terminal of GJB1 (mouse polyclonal anti-Cx32 at 1:500, Santa Cruz; INC) at a dilution of 1:200 in skimmed milk blocking buffer overnight at 4°C. The following day, the membrane was washed three times for 5 minutes each time with TBST buffer. A secondary antibody (HRP labelled goat anti-mouse) diluted in 1% TBST at a 1:1000 dilution was added to the membrane

and the membrane was agitated for 1 hour. The membrane was then washed again three times with 1x TBST buffer for 5 minutes.

4.2.4.4 Chemiluminescence Detection

The UVP Imager 3 (Brand, UK) gel doc was used for chemiluminescence. The detection of any bound antibody was conducted by adding 1:1 of Luminata™ Crescendo Western HRP Substrate (Millipore, Billerica, MA).

4.3 RESULTS

4.3.1 Amino acid conservation

As described in CHAPTER 2 (refer to section 3.3.3.1), RFLP analysis showed these novel variants were absent in 100 normal control chromosomes suggesting that perhaps these variants may be specific to the disease. When we compared the amino acids at those locations across several species, we found that the wild type amino acids were conserved at a high degree from primates (Rhesus Monkey) to zebrafish. This further suggests that they are important amino acids for the protein to function effectively.

Conservation of amino acid among species, V74M

The V74 residue is conserved from humans to zebrafish but not in chicken and *Xenopus.*

Species	Amino acids sequences
Homo sapiens (Human)	F P I S H V R L W S L Q L I L V S
Macaca mulatta (Rhesus Monkey)	F P I S H V R L W S L Q L I L V S
Mus musculus (Mouse)	F P I S H V R L W S L Q L I L V S
Canis lupus familiaris (Dog)	F P VS H V R L W S L Q L I F V S
Loxodonta africana (Elephant)	F P I S H V R L W S L Q L I L V S
Monodelphis domestica (Opossum)	F P I S H V R L W A L Q L I L V S
Gallus gallus (Chicken)	F P I S H I R L W A L Q L I L V T
Xenopus tropicalis (X-tropicalis)	F P I S H I R L W A L Q L I I V S
Danio rerio (Zebrafish)	F P V S I V R F W C L Q L I F V S

Figure 4.5.1: Amino acid conservation of Valine at position 74 of amino acid sequence (only a partial protein sequence is shown)

Conservation of amino acid across species, P174L

The Proline amino acid at position 174 is highly conserved from human to zebrafish.

Species	Amino acids sequences
Homo sapiens (Human)	K C D V Y P C P N T V D C F V S R
Macaca mulatta (Rhesus Monkey)	K C D V Y P C P N T V D C F V S R
Mus musculus (Mouse)	K C E A F P C P N L V D C F V S R
Canis lupus familiaris (Dog)	K C E A Y P C P N T V D C F V S R
Loxodonta africana (Elephant)	K C E A Y P C P N T V D C F V S R
Monodelphis domestica (Opossum)	K C D S Y P C P N T V D C F V S R
Gallus gallus (Chicken)	K C E A Y P C P N T V D C F V S R
Xenopus tropicalis (X-tropicalis)	K C D A Y P C P N T V D C F V S R
Danio rerio (Zebrafish)	K C E Q W P C P N L V D C F V S R

Figure 4.5.2: Amino acid conservation of Proline at position 174 of amino acid sequence (only a partial protein sequence is shown).

4.3.2 Bioinformatics Prediction Software

To further examine the possible effects of the substitutions, protein prediction software was used to predict the pathogenicity of the amino acids. The analysis indicated that these substitutions are unlikely to be benign and are predicted to be 'non-neutral' with a damaging effect (Table 4.4).

Table 4.4: Prediction results from the various bioinformatics software

Substitution	SNP type	Software	Score	Prediction
GJB1, V74M c.220G>A	Non-synonymous	SNAP	N/A	NON-NEUTRAL
		Polyphen2	0.958	DAMAGING
		SIFT	0	DAMAGING
GJB1, P174L c.521C>T	Non-synonymous	SNAP	N/A	NON-NEUTRAL
		Polyphen2	1	DAMAGING
		SIFT	0	DAMAGING

4.3.3 Site-Directed Mutagenesis

In order to analyse the potential effects of the mutations, a commercial wild type cDNA construct was obtained and site-directed mutagenesis (SDM) was performed to introduce these mutations in separate constructs, V74M and P174L separately, as per described in the Methods section 4.2.2

The constructs were sequenced to confirm that the mutations had been successfully introduced, and analysis showed that both targeted mutations were specifically created in the whole GJB1 coding sequence (Figure 4.6.1 and 4.6.2 show the electropherogram of a region flanking the mutant sites).

4.3.3.1 Electropherogram of V74M

Figure 4.6.1 below shows an electopherogram to confirm the wild type allele G has been changed to A, therefore changing the wild type amino acid from Valine to Methionine.

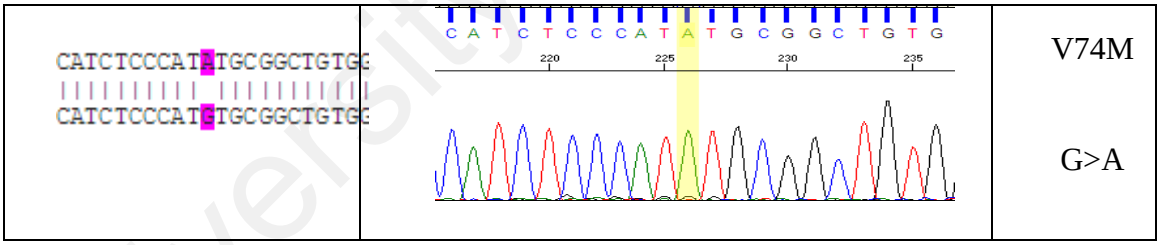


Figure 4.6.1: Electropherogram of V74M

4.3.3.2 Electropherogram of P174L

Figure 4.6.2 below shows electopherogram to confirm the wild type allele C has been changed to T, therefore changing the wild type amino acid from Proline to Leucine.

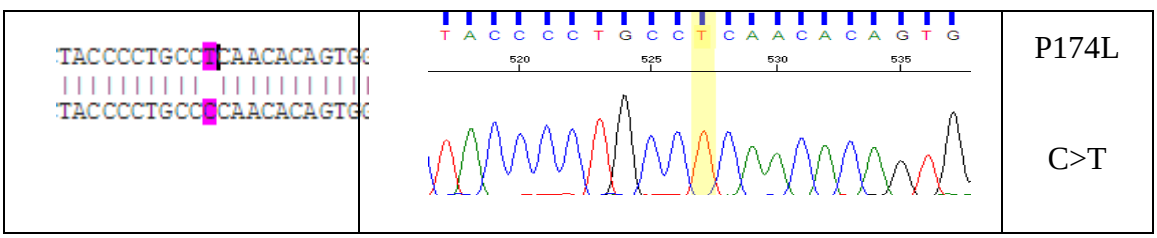


Figure 4.6.2: Electropherogram of P174L

4.3.4 Western Blotting

Below is the western blot that shows GJB1 was expressed for all the constructs.

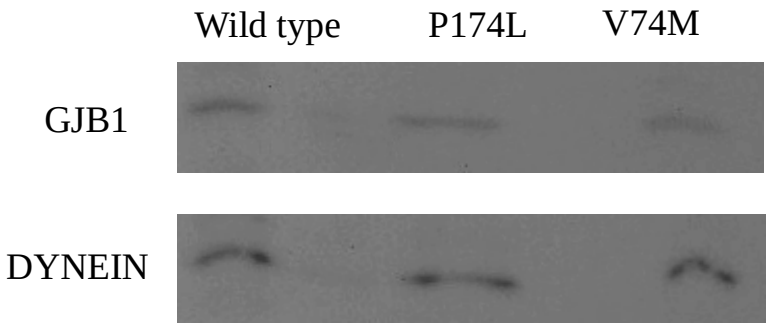


Figure 4.7 Western blot for the protein expression for wild type, V74M and P174L

Densitometric analysis showed a slight reduction in expression in the mutant constructs compared to the wild type construct. The mean intensity levels of each GJB1 and Dynein band was measured by using UVP densitometry software on UVP BioSpectrum Imaging System machine and the relative expression levels were determined as shown in the Table 4.5.

Table 4.5: Densitometric analysis

Cdna	GJB1	Dyenin	Total density	Normalisation
Wild type GJB1	4.3426E+005	4.9150E+005	8.8354E-001	1
P174L	1.8641E+005	5.8316E+005	3.1965E-001	0.833210838
V74M	2.7533E+005	6.2500E+005	4.4053E-001	0.589962059

4.3.5 Localization of GJB1 plaques among the different construct

By immunofluorescence, GJB1 plaques were observed in the wild type and for one of the mutations, V74M. However, in the P174L there were no obvious plaques seen (Figure 4.8).

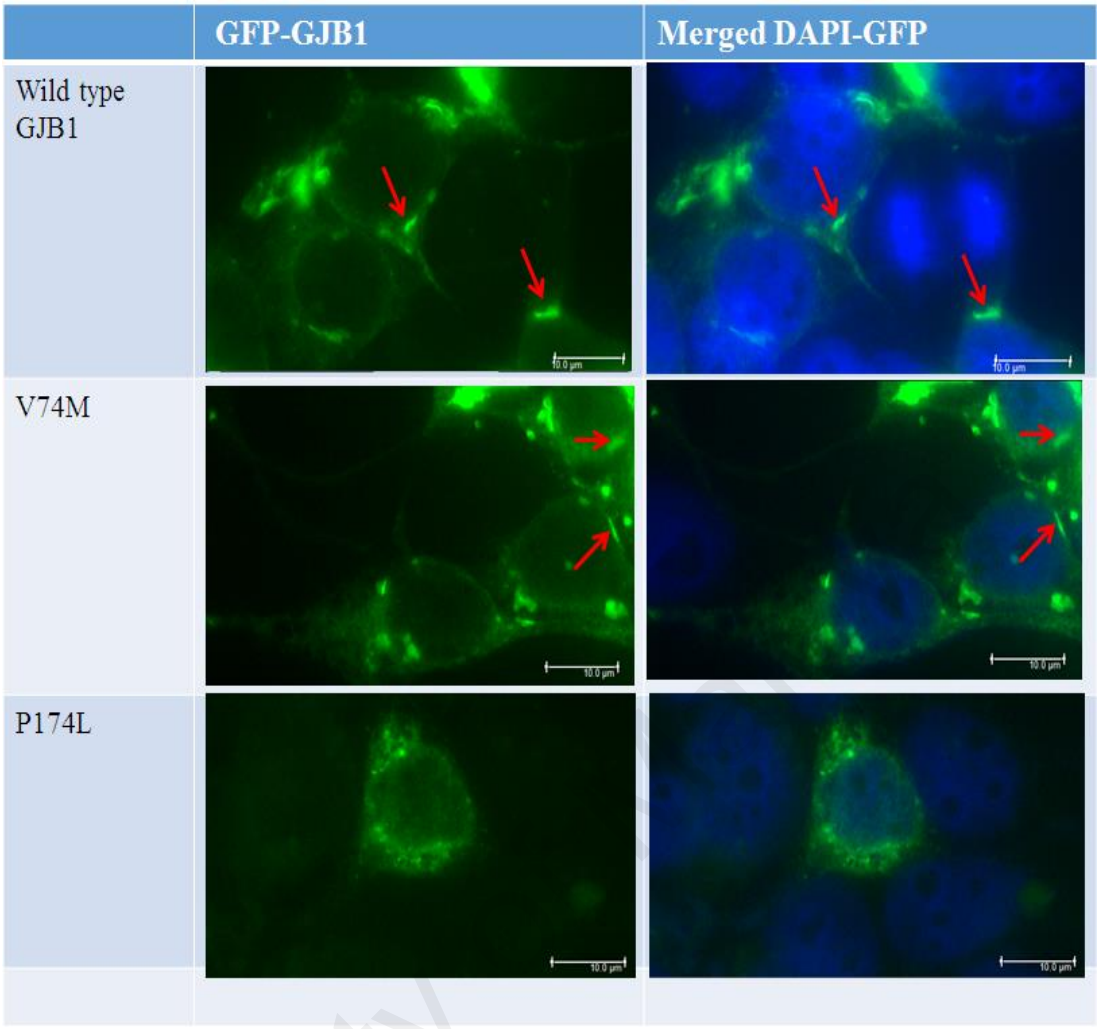


Figure 4.8: Localization of CMTX mutants.

These are digital images of transfected HEK293 cells of wild type construct, V74M and P174L. Scale bar, 10 _m. – An additional panel of pictures is shown in Supplement 3 of the Appendix.

It was evident that the GJB1 plaques were clearly visible in the V74M cells. However, it was much more difficult to see GJB1 staining in the P174L cells. Under the microscopic parameters that we used to take images of the wild type and mutant V74M cells, it was not possible to see any staining for the P174L cells. Therefore, in order to see the staining we had to increase the microscope gain and exposure settings to a much higher level before we were able to take a picture of the P174L. This indicated that it was either expressed at low levels or diffusely within the cell. We tested the expression levels of V74M and P174L in the cells by western blot, and the western blot indicated that the expression of the P174L-GJB1 protein appears to be expressed at a level

comparable with the wild type and the V74M-GJB1 proteins, therefore suggesting that the issue is not with the level of expression but with the pattern of localisation. We repeated the transfections of P174L three times and with different batches of cDNAs, and with each condition, the staining was very faint. It is likely to be faint as the GJB1 proteins are not localizing at one spot as seen in the wild type and V74M-GJB1 cells which are bright and punctate as they localize at particular spots along the cell membrane. In the P174L cells, the staining appears more scattered and diffuse.

It was not possible to perform any electrophysiological recordings, so we were not able to confirm the abnormal localization pattern with abnormal conductivity, but it is clear from the patient's NCV values that there are obvious abnormalities in the conduction along the patient's nerves.

4.4 DISCUSSION

Here we report our findings on the V74M and P174L mutations in *GJB1*. The V74M and P174L mutations are located at the extracellular domain which is an important domain for docking the GJB1 protein. Data from conservation analysis suggested that these were likely to be functionally important residues.

We were able to observe GJB1 plaques in the V74M cells, which appeared to be as bright and as numerous as the wild-type plaques. There were many GJB1 mutations that are also able to form GJB1 plaques at the membrane cell but when it comes to electrophysiological recordings, they showed reduction in electrical conductance (Oh *et al*, 1997; Ressot *et al*, 1998). We were unable to perform electrophysiological recordings for the cells as the equipment was not available. The effect on the electrical conductance can be potentially looked into further for the V74M cells.

In contrast, cells carrying the P174L mutation did not appear to form obvious GJB1 plaques, and the staining was diffuse and faint. Future work could include co-staining with a Golgi or ER marker to see whether or not the P174L-GJB1 protein is localising more within Golgi or ER. By analysing the pattern of staining of published mutants in the literature, it appears that when GJB1 is retained in the Golgi, it looks clumped at one location, while in cases of ER retention, the staining appears more ring-like around the periphery of the cells.

We then compared the pattern of staining in our P174L cells, and it appears more of an ER retention-like pattern rather than a Golgi-like pattern as there are no obvious clumps of plaques and more diffusely organised. A neighbouring mutation that has been reported, P172, failed to form GJB1 plaques and there was abnormal electrical transduction recorded (Abram *et al*, 2009).

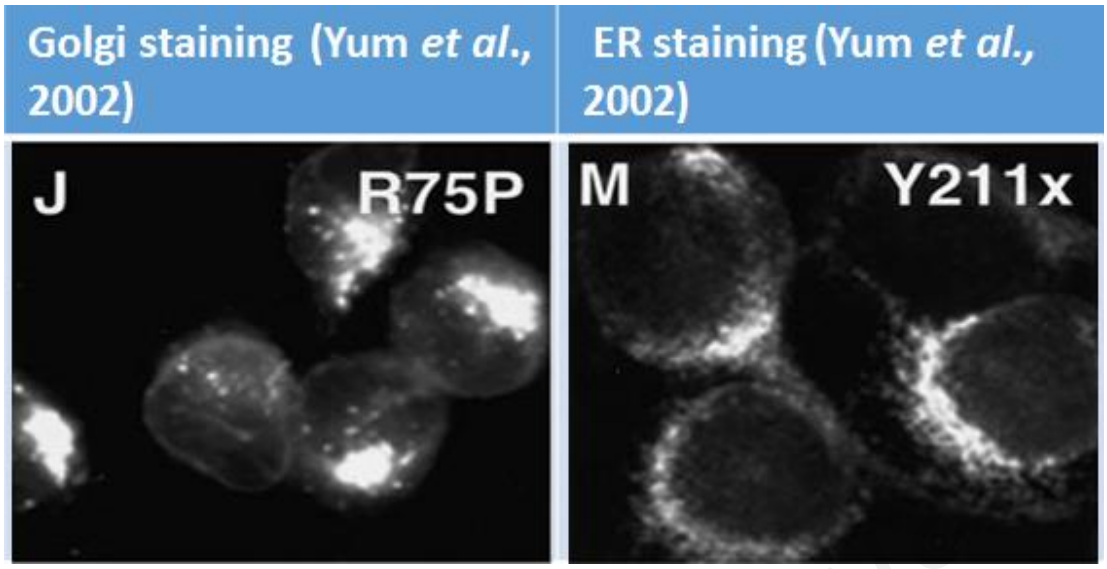


Figure 4.9: Localization of CMTX mutants in the ER and Golgi pattern. Scale bar, 10 µm as stated in the paper. Picture adapted from Yum *et al*, 2002.

If the protein is retained in the Golgi, it will clump in one spot wherase if the protein is retained in the ER, it looks more scatttered around the cells. Based on Figure 4.8, the P174L staining suggests a more scattered, ER pattern of distribution.

4.5 CONCLUSION

V74M mutation managed to be able to form GJB1 plaques while P174L did not perform any obvious one in the cell localization.

CHAPTER 5: PUBLIC KNOWLEDGE AND PERCEPTIONS ON RARE DISORDERS

5.1 INTRODUCTION

Rare Disorders (RD) is a term used to describe clinical disorders that affect a limited number of people. In Europe, the incidence of RD has been estimated to be 1 in 2000 individuals. In Malaysia, RD is estimated to affect around 1 in 3000-4000 individuals, suggesting that approximately 20,000 babies are born annually with some form of RD (Chin, N.F., & Thong, M.K., 2011)

RDs are typically chronic and progressive in nature. These disorders result in debilitating symptoms and are often life-threatening. Due to the severity of these disorders, the quality of life is acutely affected, and the life of the caregivers is also affected.

RDs can be categorized into genetic, chromosomal, environmentally-induced or inborn errors of metabolism. Although each particular disorder may be rare, around 6000 to 8000 RDs have been reported. Therefore, it is essential that the medical community and the public be made more aware of RDs in order to target better treatment and care for affected individuals with the hope of a better outcome.

Although CMT is the second most common neuromuscular disease, its prevalence is low and thus CMT is classified as relatively rare as it affects 1 in 2500 people. Many patients with CMT remain undiagnosed as many general practitioners do not recognize the symptoms and signs of CMT and thus, patients are not always referred to neurologists. This results in a delay in treatment and rehabilitation which may improve their quality of life, especially in preventing injuries from falls and movement complications due to the progressive wasting of the muscles.

The Neurogenetics lab also works closely with the Malaysian Rare Disorders Society (MRDS), a nonprofit organization comprising of families affected with RD and supporters. The society is currently conducting a study with patients and their families to uncover their experience with accuracy and timing of diagnosis, treatment options and governmental/welfare support. Feedback from the families has revealed that there is a lack of urgency towards the various issues they face including stigmatization from the community.

Our objective was to investigate the public perception of RDs to allow for a better understanding of the level of knowledge, awareness as well as misconceptions that may exist within our community. We distributed questionnaires to the general public to investigate their perception of RD, persons living with RD and the level of support that should be made available to this group of patients.

The current study along with that of the MRDS can be used as a leverage to raise the profile of RDs in Malaysia and to lobby for more support from the government. In Europe and Taiwan, RD receives equal attention to that of common diseases, primarily due to a change in public policies that have raised the profile of RDs. There has also been more fundamental research towards understanding the pathogenesis and developing treatment for these disorders. The first European action on developing a program to tackle RD occurred between 1999 and 2009 when RD become one of the priorities in the EU Public Health Program (Aymé & Schmidtke, 2007). Even though it was initiated with the intention of improving knowledge and facilitate access to information about these diseases, individual countries have successfully established a policy and national plan for better healthcare provision. In addition, successful international networking has resulted in standardized clinical activities, information services and medical access for this group of patients. This has also enabled experts in

this field to come together and collaborate in an effort to improve treatment for these diseases. One of these successful collaborations is the development of Orphanet, established jointly by the French Ministry of Health and the National Institute of Health and Medical Research (INSERM). Orphanet is a database of RDs that provides a directory of information on RDs. In the US, there exists databases such as NORD (http://www.rarediseases.org) or GeneReviews/Gene-Tests (http://www.genetests.org) that also provide information about RDs (Aymé & Schmidtke, 2007). In Malaysia, there is no Registry of RD although plans for its establishment have been discussed for many years. We hope that with this study of public perception, we can initiate some of the efforts towards developing similar registries that exist in other countries.

5.2 MATERIALS AND METHODS

A series of questions on RDs were formulated through discussions with the Malaysia Rare Disorder Society (MRDS) and paediatricians at UMMC. Translation from English into the Malay language was verified by native Malay speakers who were also conversant in English utilising appropriate medical terms. A pilot questionnaire was initially validated through 300 respondents at 2012 to 2013 to test the reliability of the questionnaire. Issues with unclear wording and ambiguous answers were addressed and the language was edited for clarity to respondents. The final questionnaire was then distributed to the public in the rural and urban areas throughout Peninsular Malaysia, Sabah and Sarawak and respondents answered the questionnaire through an online survey platform or manually, over a period of November 2013 to January 2015.

5.3 RESULTS

5.3.1 Demographic of the respondents

We received 500 responses and found that the public were more likely to respond manually in printed form, rather than when solicited through online requests. For the manual replies we excluded ones that were incompletely answered and unclear which comprised of around 20% of the total questionnaire which were sent out. The gender of respondents was 62% female and they were also from different ethnicities in Malaysia (refer to Chart 5.3.1). The age range was between 19 to 75 years old. Slightly more than half of respondents did not have any children (57%).

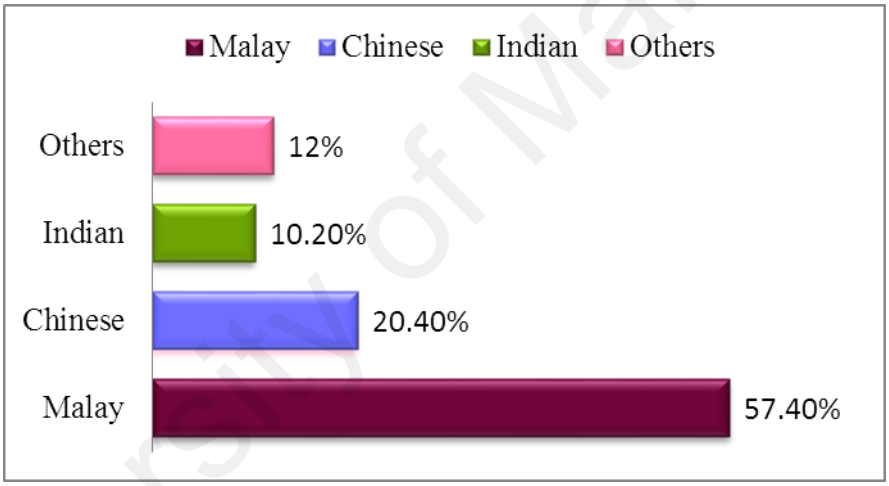


Figure 5.1: The percentage of respondents based on ethnic groups in Malaysia

The participants were from various backgrounds; with around 10% of them being professionals (e.g. managers, executives, architects, lawyers, engineers), 9% were academics, 6% were from the armed forces, 21% were students, 2% worked in public relations, 7% were retirees, 4% were in marketing/sales, 4% ran their own businesses, 8% were in the medical line, 3% in the hospitality field, 1% were social workers, 1% were from the food & beverage sector and 10% were unemployed.

5.3.2 Malaysian Perception on Rare Disease

5.3.2.1 Which of these are Rare Disorders?

The public could correctly identify the listed common diseases to a certain extent. We constructed the questionnaire to also include common diseases as a reference point to determine how well they could differentiate between common and rare disorders. The majority (51%) knew that Down's syndrome was a common disorder. However, they were less certain about Thalassemia where only 40% correctly identified it as a common disorder and 34% classed it as a rare disorder, whereas 11% have never heard of it. This is despite many public awareness campaigns by the government about Thalassemia being a commonly inherited disorder in our population.

In contrast, three out of the four RDs listed - Duchenne Muscular Dystrophy (DMD), Prader-Willi Syndrome (PWS) and Charcot-Marie-Tooth (CMT) were less well recognized. The majority of respondents had never heard of the diseases before (47% DMD, 59% PWS, 62% CMT).

The pattern of response for Achondroplasia was very different, as respondents seemed more aware of the disease with 58% classifying it as rare. Interestingly, compared to other RDs, a large percentage (22%) responded that they thought it was a common disease. An additional 12% were not sure if it was rare or common. We believe it is likely that the greater awareness of Achondroplasia is due to media personalities with this disorder who are featured on TV and movies thus giving an impression that the condition may be more common than it actually is.

We noted that amongst the respondents from medical line background, 40-55% also claimed to have 'never heard'/didn't know whether DMD, PWS, CMT were RDs. The majority of respondents who could not identify RDs were mainly from rural areas. Eighty percent of respondents who were aware of RD were in the younger age group of

19 to 45 years old. Females were also more knowledgeable than males. In the older age group of 55 to 75 years old, those who correctly identified the diseases as rare were retirees living in the urban area.

One might assume that individuals with children may have had more exposure to childhood medical conditions as they are likely to have gone to hospitals or clinics for their children's routine check-ups, and may have seen other children with various disorders compared to respondents without children. However, this did not appear to be the case as the majority of the respondents with children also had a limited knowledge about RD. Seventy-one percent were unable to correctly identify DMD as a RD, 87% were unable to identify PWS as RD, 89% were unable to identify CMT as RD. The exception again was Achondroplasia whereby 61% could correctly identify it as a type of RD.

5.3.2.2 What do you think causes Rare Disorders?

In this section, the respondents could choose more than one answer. The majority of respondents were aware that genetics played a role in causing RD. However, 25% also perceived microbial agents as contributing towards RD. This was followed by environmental factors and social practices. Zero point two percent of the respondents gave their own reasons, which is further explained in the discussion (section 5.4).

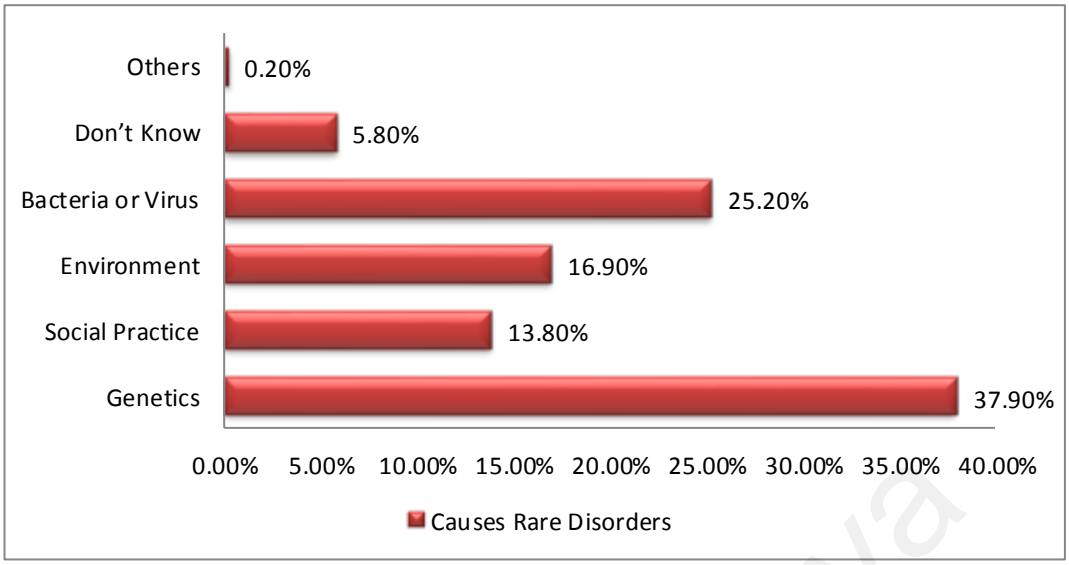


Figure 5.2.1: Chart shows the factors that the respondents thought contributed to RD

5.3.2.3 Is RD transmitted like infectious diseases?

The majority of respondents (66.8%) were aware that RDs could not be transmitted like an infectious disease. Those who chose 'strongly agree' in this section -implying that they believed that RDs were transmissable diseases - had also earlier chosen the option that microbial agents could cause RD (section chart 5.4 above). Taken together, the respondents who chose the 'disagree' or 'strongly disagree' options were in a larger majority (66.8%) than the 'agree' and 'strongly agree' (33.2%) category.

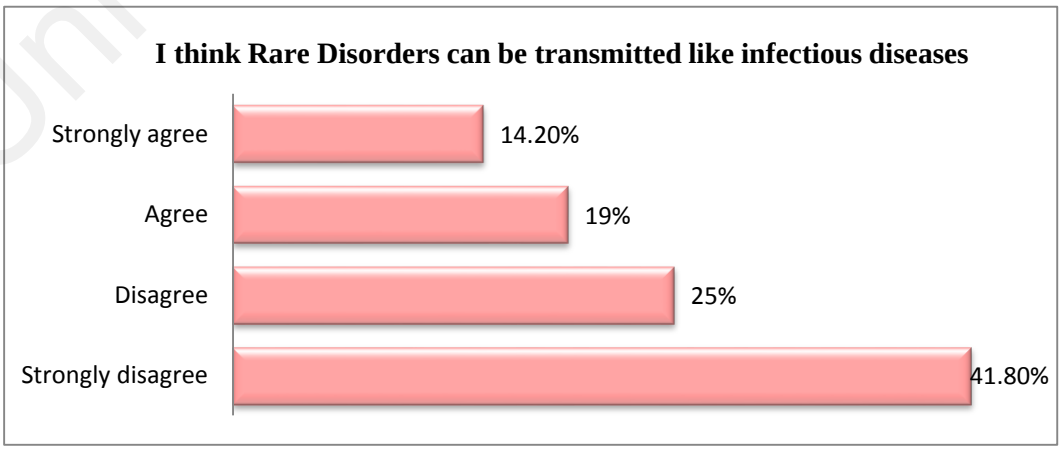


Figure 5.2.2: Chart showed the opinion of respondent whether RD can be transmitted or not.

5.3.3 Social Interaction Involving RD patients in Malaysia

5.3.3.1 Malaysians generally do not discriminate against individuals with rare disorders

When dealing with individuals with RDs in the community, respondents were open-minded about how they would feel and react around them. Only 12.6% claimed that they would feel uncomfortable around individuals with RDs (refer to graph 5.3.1A). This response was reported across both genders, age, ethnicity and place of residence. Of the respondents who felt uncomfortable around people with RD, the majority were from the armed forces, customer services and manual workers.

Although the majority of respondents were accepting of those with RD, they were less inclined to marry someone with a family history of RD with more than 56% in this group choosing the disagree/ strongly disagree (to marry) options (refer to graph 5.3.1B). This fits in with the cultural perceptions that exist within Malaysia and the general stigmatization attitude towards the affected families.

In keeping with the stigmatization culture, 61.4% felt that society would treat them differently if they had a family member with a RD (refer to graph 5.3.1C). More than 70% of respondents would not feel embarrassed if they had a family member with RD (refer to graph 5.3.1D). However, it could be that the responses reflect a cautious attitude as the public may choose not to marry someone with a family history of RD because they are aware of the psychological and financial toll it imparts on families and if given the choice, would likely choose not to have this perceived personal burden.

The 70% who said that they would not feel embarrassed are perhaps reflective of the general attitude of the public that believes people with RDs are not a burden to society (77%), but they are most likely acutely aware that there may be

individuals/communities who may discriminate against the household (refer to graph 5.3.1E)

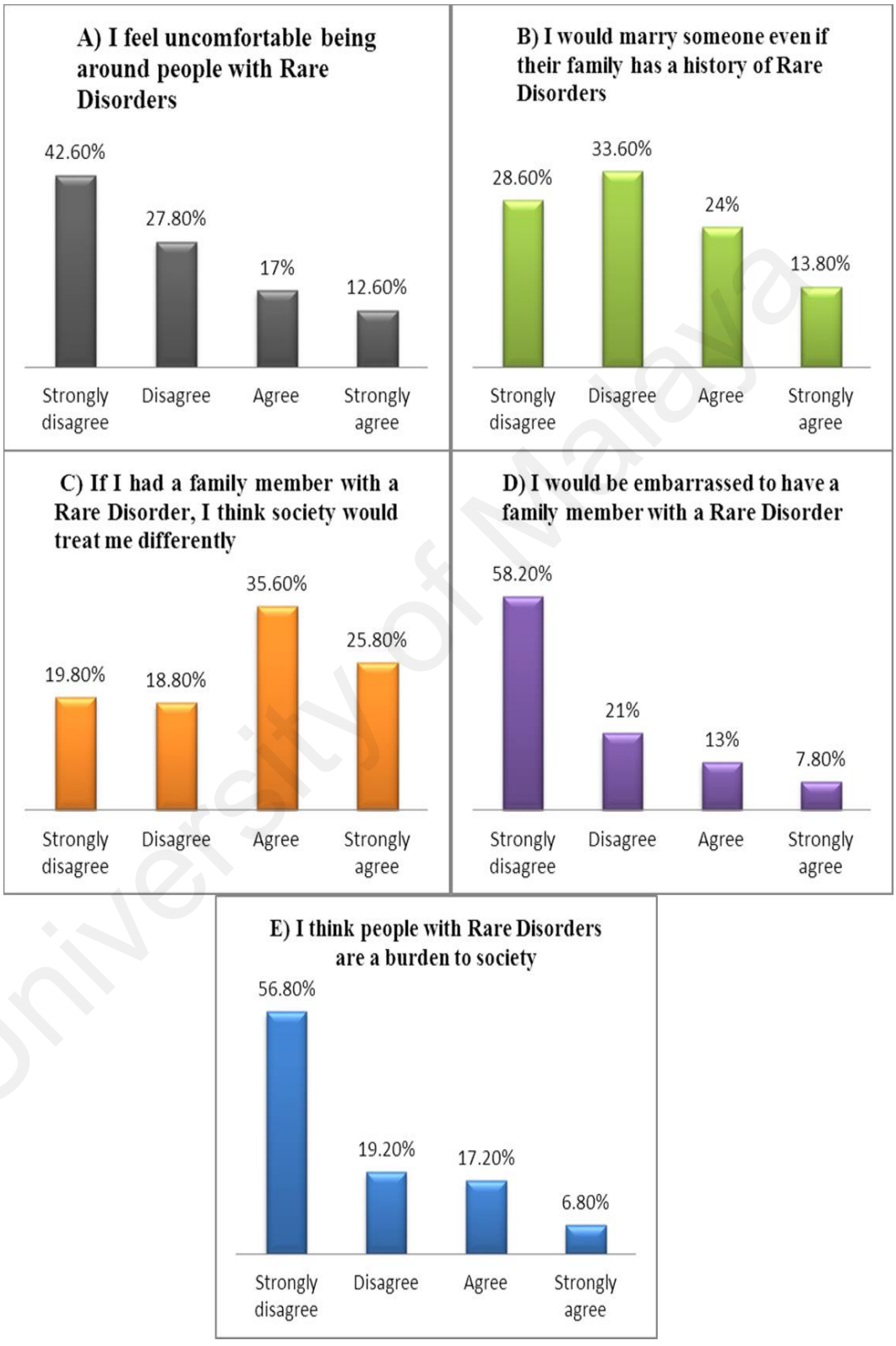


Figure 5.3.1: Chart showed the opinion of the respondents regarding Social Interaction involving RD patients in Malaysia

5.3.3.2 If you saw someone with a strange disease, would you approach them and ask what their condition is?

We included this set of questions due to a particular request from the MRDS. Many families with physically disabled children felt uncomfortable with the stares and prolonged gazes from the members of the public. They would much rather the public asked them directly what the child's condition was so that they could at least make them aware of the disease. Consistent with the experiences of the MRDS members, the majority of respondents (61%) would not approach someone with RD to question their medical condition and the commonest reason is fear of offending the affected individual (Figure 5.3.2). As the Malaysian public is generally quite reserved, this pattern of responses is not unexpected. Those who would approach families with RD were mainly from the medical field, students, academicians, retirees, professional group and non-government organization.

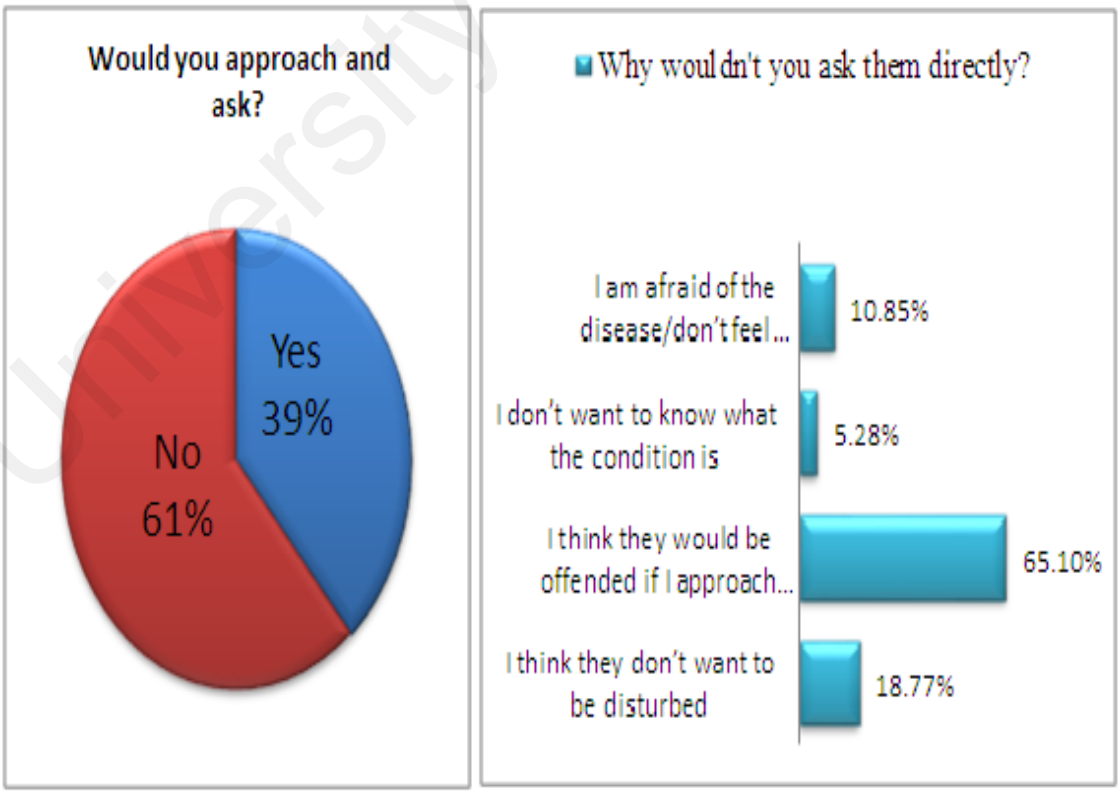


Figure 5.3.2: Chart showed the willingness of the respondents to approach RD patients/people with disabilities

5.3.3.3 Would you employ someone with a Rare Disorder?

Similar to the issue above, we included this question in as the MRDS members wanted to know the general attitude of the public towards hiring people with RDs. A large proportion of individuals with certain types of RD, for example MPS type IV were academically qualified (with university degrees) and often not offered jobs as companies could not accommodate them. This is despite a recent governmental initiative for private companies to have 1% of their workforce comprising of disabled individuals (Khoo, Tiun, & Lee, 2013). It is not enforceable by law and we found that only 50% of respondents would employ RD persons provided they were mentally capable but with the proviso that no changes to the workplace needed to be made whereas 24% would be unwilling to consider employing them. Another 26% of respondents would offer jobs to RD persons even if they have mental and physical disabilities.

5.3.4 Responses of the necessity of Genetic Testing

Eighty-seven percent of the respondents were willing to have genetic testing if their family was at risk of getting a type of RD (Figure 5.4). Of the 13% who would refuse, the most common reason was that they were confident there was no history of genetic diseases in their family and thus testing was not warranted.

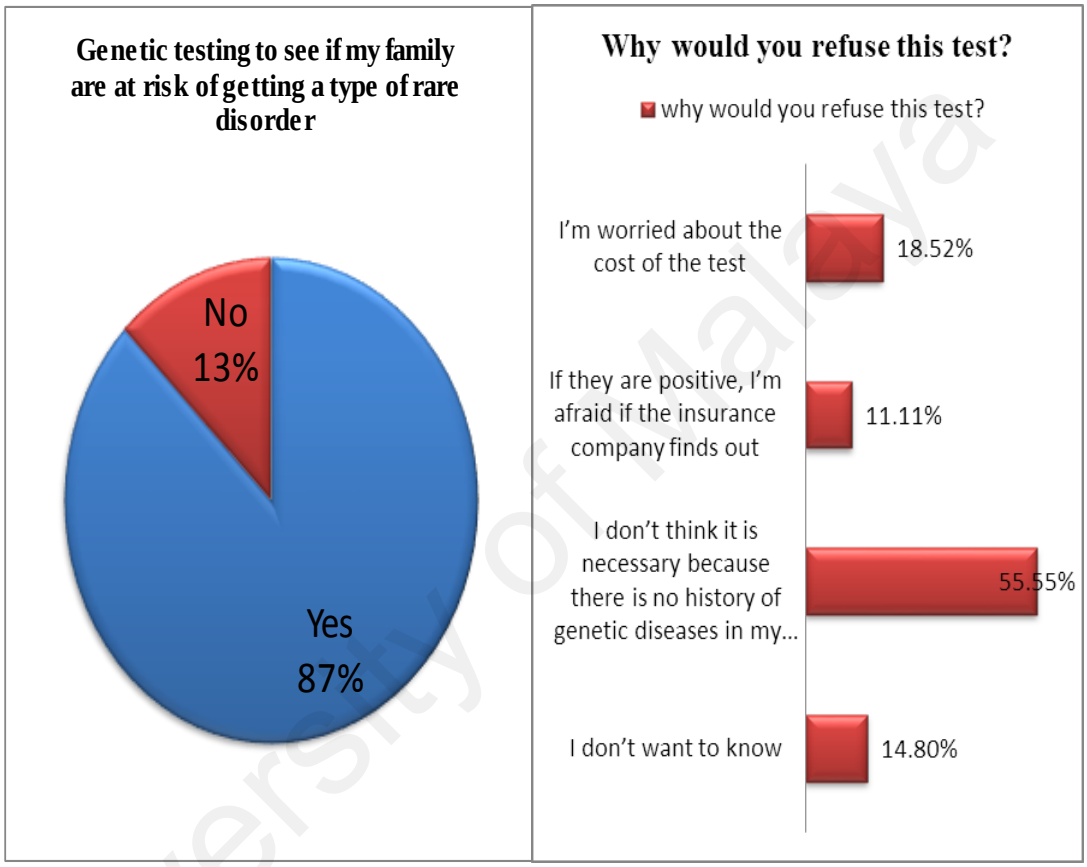


Figure 5.4: Chart showed the opinion of respondent regarding the necessity of genetic testing in family and the reason of reluctant on Genetic Testing.

5.3.5 The involvement of Government

5.3.5.1 What support do you think patients/families with Rare Disorders should get from the government?

A high percentage agreed that people with RDs should receive financial and medical support such as free rehabilitation, discounts for medicine, hospital fees and medical equipment. The public also believed that compassionate leave from employers should be provided to the caregivers. Malaysians also supported special funds allocation to upgrade schools to become more disabled-friendly with easy access and disabled toilet facilities.

Table 5.1: Type of support the respondents felt should be covered by the government

Type of Support	Less important	Important	More important	Most important
Financial (welfare token, tax rebates)	2%	4.2%	25.8%	66%
Medical (discounts for medicine/treatment/hospital fees/ medical equipment, rehabilitation)	0.6%	3.2%	17.6%	78.6%
Extra leave (compassionate leave from employers)	5.2%	14.8%	35.6%	44.4%
Special allocation to upgrade school to become more disabled-friendly (wheelchair accessibility and toilet facilities)?	5.4%	7.4%	26.8%	60.4%

5.3.6 Medical expertise and accessibility in Malaysia

More than 50% of the respondents felt that clinicians were not adequately trained to diagnose RD (Figure 5.5). Thirty percent of respondents from the medical field also claimed the same. The majority also felt that people with RDs did not have easy access to medical treatment.

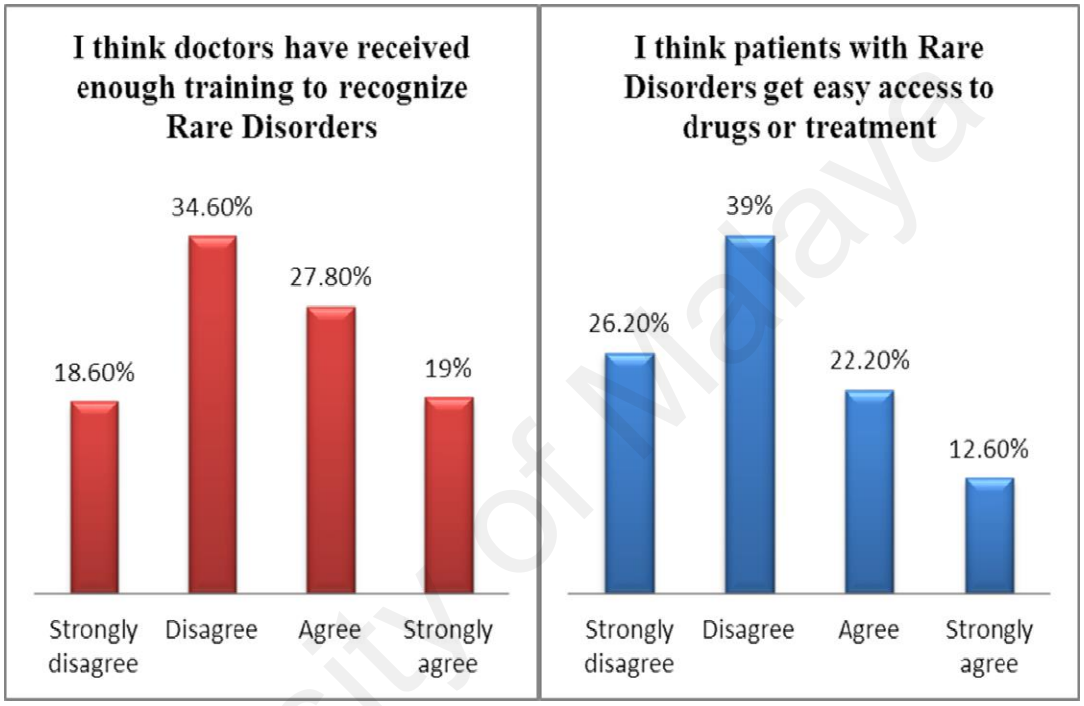


Figure 5.5: Chart showed the opinion of respondent regarding the medical accessibility and the level of clinician expertise in detecting RD.

5.3.7 Perspective on the normal government schools and the education system

More than 70% of respondents felt that the existing government school and education system were ill-equipped to cater and educate children with RD (Figure 5.6). The response was universal and did not discriminate between gender, catchment area or whether they were parents or without their own children.

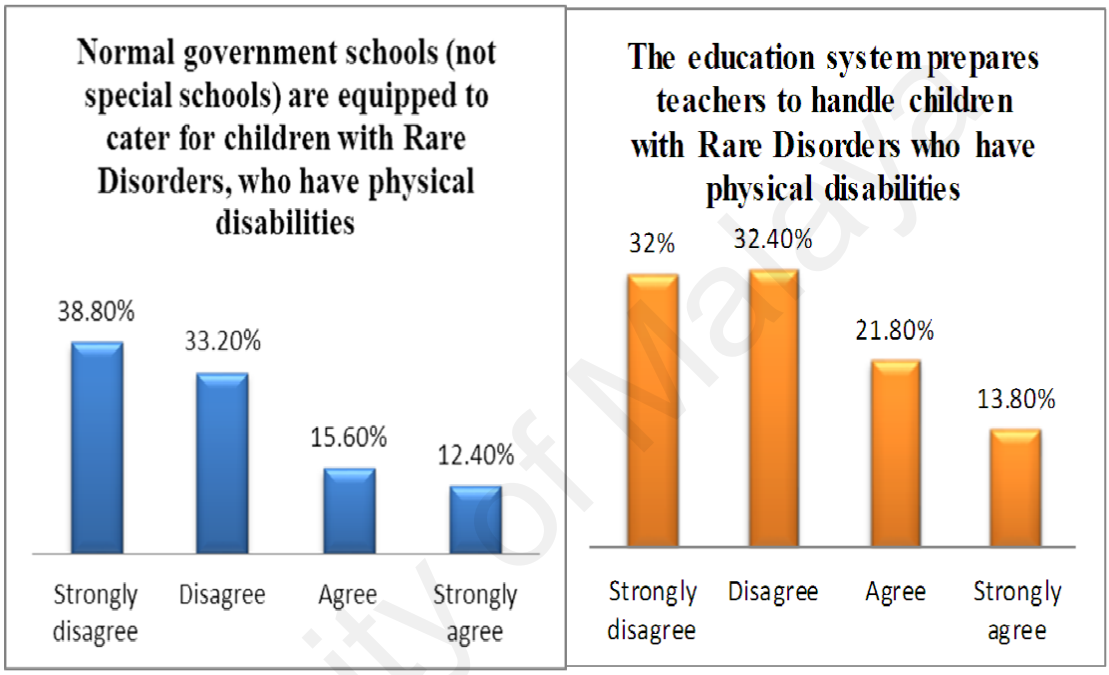


Figure 5.6: Chart showed the opinion of respondents regarding normal government schools and Malaysian education system in relation to handling RD students.

Out of the academics/teachers who responded, 70% of them agreed that the normal government schools in Malaysia were not well equipped for students with RDs and the education system have not trained them to be able to handle students with RDs.

5.3.8 Funds for research should be given into Rare Disorders or into common diseases?

The majority of respondents strongly agreed that more funding should be provided for RD research and not only for research into common diseases (Figure 5.7).

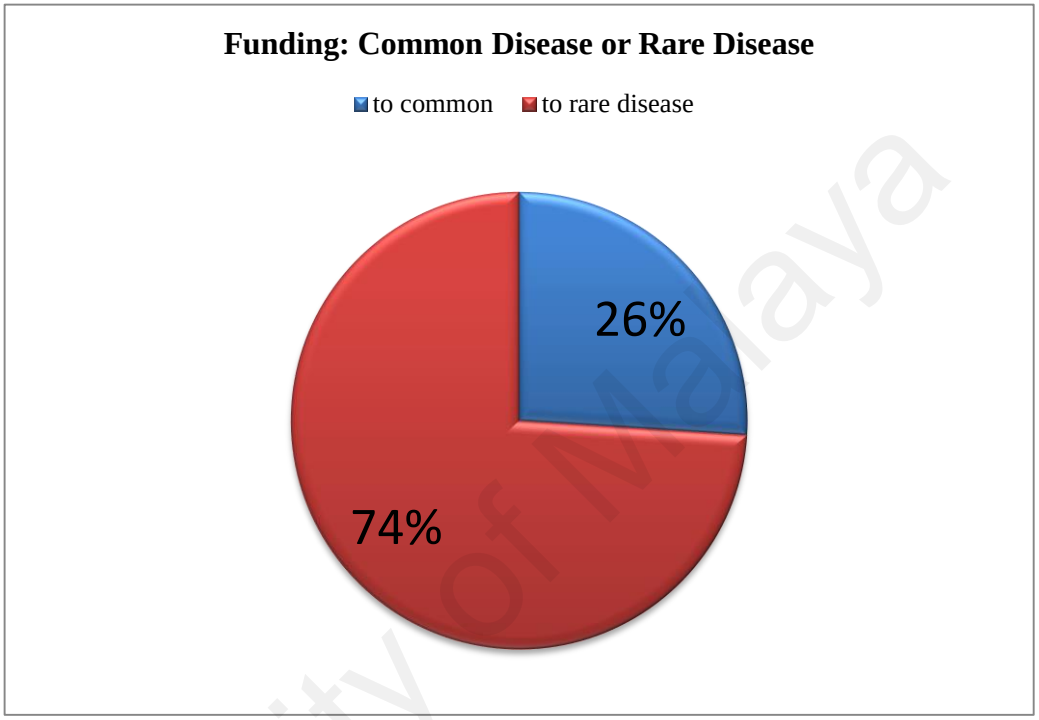


Figure 5.7: Chart showed the opinion for funding research into RD

5.3.9 The role of Media

Do you think Rare Disorders are highlighted sufficiently in the media?

Sixty-seven percent of respondents felt that issues surrounding RD were not sufficiently highlighted in the media (Figure 5.8). Television and social networking were felt to be the most effective means of providing the public with more information.

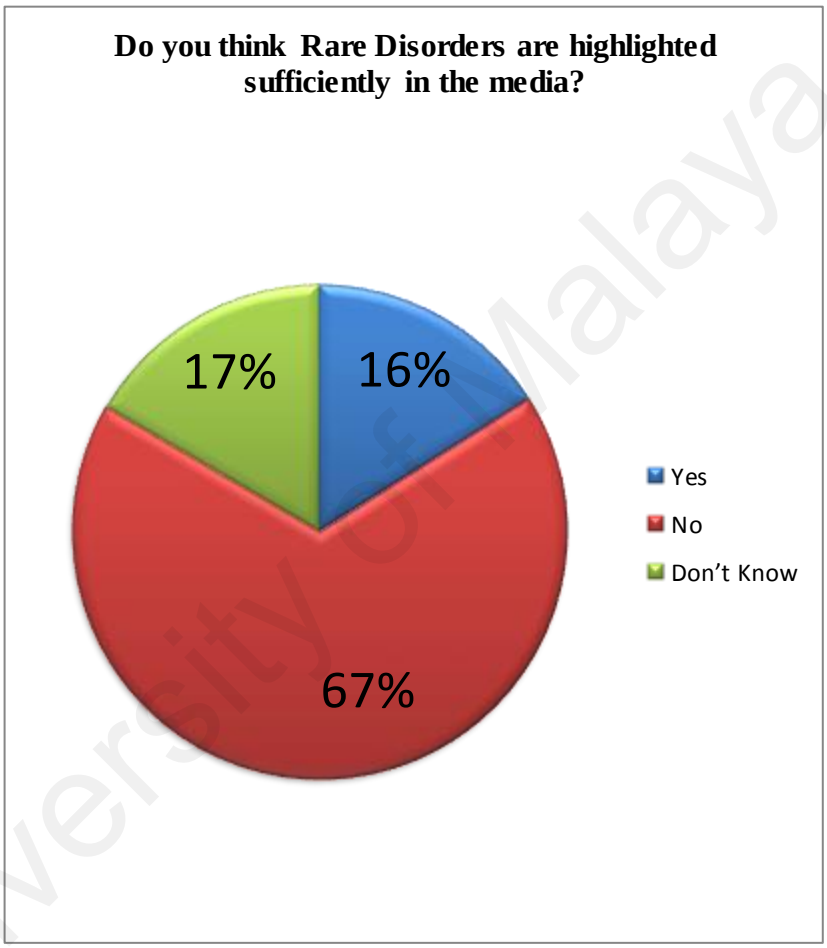


Figure 5.8: Chart showed the opinion on the coverage of RD by media

5.3.9.1 Which medium would be effective channels to give the public information about RD?

Malaysians felt more could be done to raise awareness of RD among the public. TV was still the favourite medium to be used to spread awareness among public (Figure 5.9). In accordance with time, social networking was the second most popular choice followed by campaigns and newspapers.

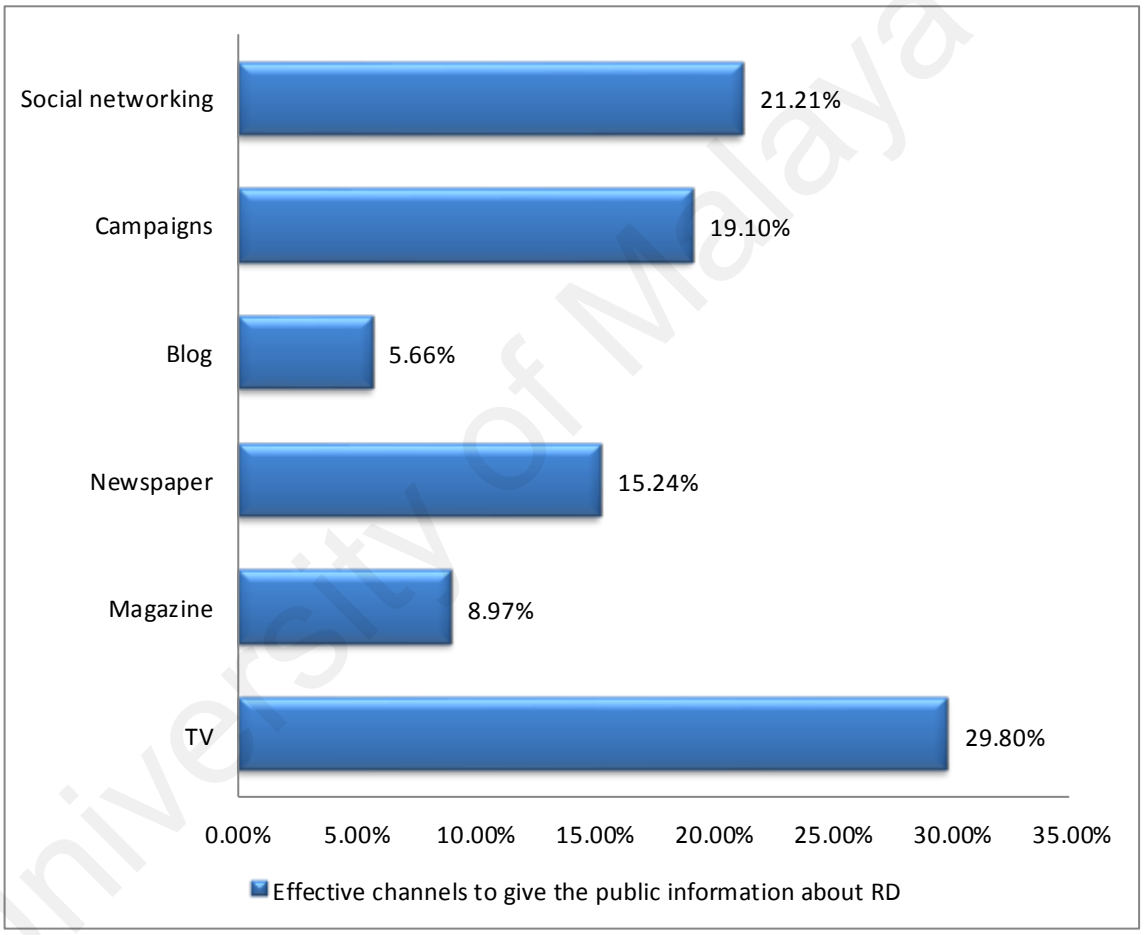


Figure 5.9: Chart showed the channels options that can be used to promote RD to the public.

5.4 DISCUSSION

Malaysians are aware of the definition of RD, but the detailed knowledge was poor. In the current study, we investigated the public perception of RD through questionnaires and received responses from 500 Malaysians from various age groups, ethnicity and social background. The majority of respondents were unable to accurately name a rare disorder. Some mistook infectious diseases like Chikungunya, Leptospirosis, SARS or Ebola as forms of RDs. As they were also given the option of describing the disease when they couldn't remember the name, some described disorders like the "Tree Man" (a recent documentary on local television about an Indonesian man with cutaneous warts), "muscle turning into bone", 'nerve disease' were some of the examples given. A few respondents from urban areas could name certain RD for instance, Amyotrophic Lateral Sclerosis (ALS). Cri Du Chat Syndrome, Crouzon Syndrome, spinocerebellar degeneration and spina bifida were disorders that were named by those from a medical background. Three respondents from the rural area felt that RD was a form of 'black magic'. Although these numbers are low compared to the other cited causes, nonetheless it suggests that misconceptions exist amongst the public which may lead to discrimination towards affected individuals. Of the RDs presented, Achondroplasia was one of the more recognizable conditions and this likely reflects the depiction of actors and reality shows of individuals with Achondroplasia in main stream media, inevitably raising awareness of this condition. In general, the Malaysian public was aware that RDs were caused by genetic abnormalities. However, there remains some confusion as to whether certain infectious disorders such as Ebola and SARS were also forms of RD.

Most Malaysians felt that more could be done for people with RDs and their caregivers in the form of medical treatment, compassionate leave and income. Adaptations to schools to accommodate children with physical disabilities due to RD were felt to be an important form of support. However, many respondents implied that

these children should be sent to 'special schools' i.e those for the disabled so that the 'normal' schools would not need to be modified, despite being told that some children were capable of learning through the normal curriculum. Although this was not a large number, it does indicate some level of community isolation of children with RD as they are categorized as disabled and marginalised into separate schools rather than assimilated into mainstream schools.

There also appears to be a lack of confidence amongst the public of the capabilities of clinicians in diagnosing patients with RDs and managing such patients. More training was felt to be required. It was encouraging to see that 87% of respondents would be open to genetic testing to test for any possible RDs in their family.

The public felt that there should also be more funding towards research in RD instead of common disorders. In line with this, the public felt that RD has not received sufficient attention and more could be done to raise awareness. Most of the respondents over the age of 30 chose TV and radio as the medium to promote RD, whereas, the younger respondents chose social networking.

5.5 CONCLUSION

The current study provided a much needed insight into the Malaysian public perception of RDs. Even though Malaysians have a limited knowledge of RDs, most responded a need for greater awareness as well as better support by government and public in the form of medical care, education, and employment opportunities. It is hoped that our findings will in some measure help to influence public policies and reduce the stigma that currently exists on persons with RD, to allow affected individuals to gradually become more integrated into our society.

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APPENDIX

Supplement 1: List and information of patients

Table 1: Cohort informations

Patient ID	Age of onset	Sex	#Med/uln CV (m/s)	Pattern	Family history	Gene Test
2009CMT001	22	M	*Abs/35	Demyelin	Yes-XL	*GJB1*
2010CMT002	3	M	23/30	Demyelin	None	*PMP22/GJB1/MPZ*
2010CMT003	14	F	46/46	Axonal	Cons	*MFN2/GJB1/MPZ*
2010CMT004	15	M	28/34	Demyelin	Yes-XL	*GJB1*
2010CMT005	61	F	44/45	Axonal	None	*MPZ*
2010CMT006	1	F	47/50	Axonal	None	*MFN2/GJB1/MPZ*
2010CMT007	5	M	28/43	Demyelin	None	*PMP22/GJB1/MPZ*
2010CMT008	69	F	38/48	Axonal	Yes-ND	*MFN2/GJB1/MPZ*
2010CMT009	16	M	16/abs*	Demyelin	None	*PMP22/GJB1/MPZ*
2010CMT010	12	M	52/51	*Entrapment	Yes-AD	*PMP22* Deletion
2010CMT011	40	F	25/24	Demyelin	None	*PMP22/GJB1/MPZ*
2011CMT012	37	F	41/48	*Entrapment	Yes-AD	*PMP22* Deletion
2011CMT013	5	M	56/54	Axonal	None	*MFN2/GJB1/MPZ*
2011CMT014	6	F	18/17	Demyelin	Yes-AD	*PMP22* Duplication
2011CMT015	12	M	37/37	Demyelin	Cons	*PMP22/GJB1/MPZ*
2011CMT016	62	F	20/20	Demyelin	Yes-AD	*PMP22* Duplication
2011CMT017	14	M	38/38	Demyelin	Yes-XL	*GJB1*
2011CMT018	10	M	54/57	Axonal	None	*MFN2/GJB1/MPZ*
2011CMT019	10	M	42/49	Axonal	None	*MFN2/GJB1/MPZ*
2011CMT020	10	F	55/50	Axonal	Yes-AD	*MFN2/GJB1/MPZ*
2011CMT021	6mth	F	*Abs/abs	-	None	*PMP22/GJB1/MPZ*
2011CMT022	28	F	20/21	Demyelin	Yes-AD	*PMP22* Duplication
2011CMT023	61	M	17/abs*	Demyelin	None	*PMP22* Duplication
2011CMT024	7	F	43/54	Axonal	None	*MFN2/GJB1/MPZ*
2011CMT025	10	M	57/55	Axonal	None	*MFN2/GJB1/MPZ*
2011CMT026	4	F	46/42	Axonal	Yes-XL	*GJB1*
2011CMT027	4	F	13/14	Demyelin	None	*PMP22* Duplication
2012CMT028	50	F	26/30	Demyelin	None	*PMP22* Duplication
2012CMT029	48	F	22/18	Demyelin	Yes-AD	*PMP22/GJB1/MPZ*
2012CMT030	16	M	54/55	*Entrapment	Yes-AD	*PMP22* Deletion
2012CMT031	12	M	33/abs*	Demyelin	Yes-AD	*PMP22/GJB1/MPZ*
2012CMT032	7	F	*abs/abs	-	None	*PMP22/GJB1/MPZ*
2012CMT033	17	M	Abs/29	Demyelin	Yes-XL	*GJB1*
2012CMT034	50	F	24/23	Demyelin	None	*PMP22* Duplication

2012CMT035	22	M	27/34	Demyelin	Yes-XL	*GJB1*
2013CMT036	20	M	37/43	Demyelin	Yes-XL	*GJB1*
2013CMT037	25	M	*abs/abs	-	None	*PMP22* Duplication
2013CMT038	12	M	11/26	Demyelin	None	*PMP22* Duplication
2013CMT039	13	M	*abs/abs	-	None	*PMP22/GJB1/MPZ*
2013CMT040	7	F	20/18	Demyelin	Yes-AD	*PMP22* Duplication
2013CMT041	10	F	42/40	Demyelin	None	*PMP22/GJB1/MPZ*
2013CMT042	12	M	34/41	Demyelin	Yes-XL	*GJB1*
2013CMT043	35	M	Entrapment	*Entrapment	None	*PMP22* Deletion
2013CMT044	10	M	34/35.2	Axonal	Yes-AD	*MFN2*/*GJB1*/*MPZ*
2013CMT045	***NA	M	NA	Axonal	Yes-AD	*MFN2*/*GJB1*/*MPZ*
2013CMT046	10	M	16/13	Demyelin	Yes-AD	*PMP22* Duplication
**2014CMT047	NKnown	M	19/19	Demyelin	NA	*PMP22* Duplication
2014CMT048	22	F	40/42	Demyelin	None	*PMP22/GJB1/MPZ*

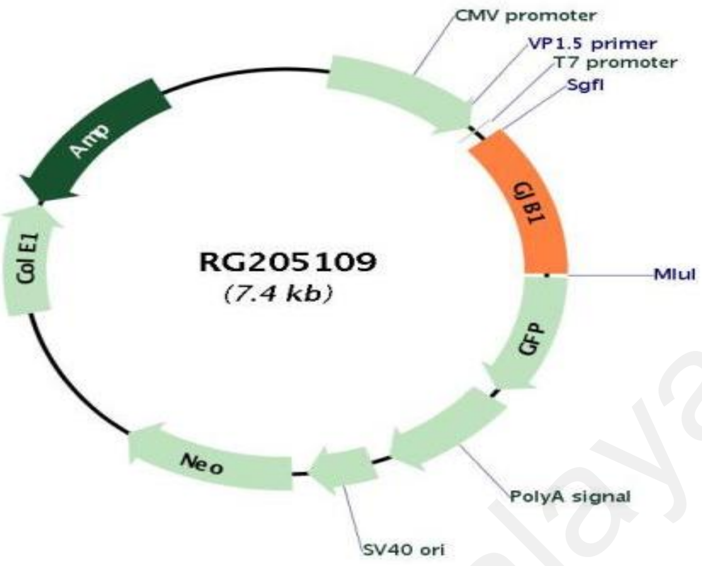
*Entrapment: recurrent episodes of nerve dysfunction at compression sites

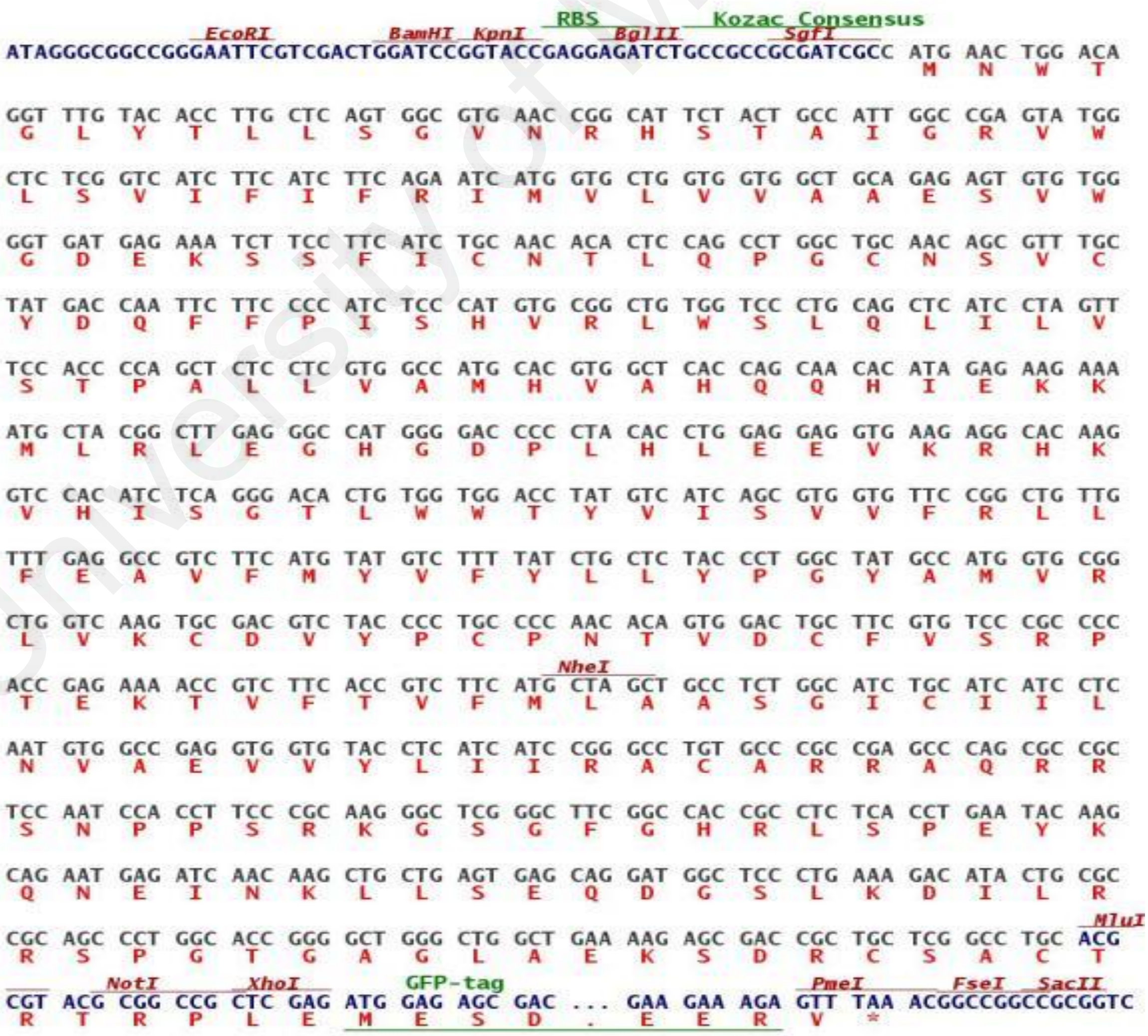
*abs; Absent NCV. Patient's NCV was undetectable

**2014CMT047 presented for the first time at the age of 68. He has 6 month history of distal limb weakness. Examination revealed *pes cavus* and clawed toes, suggesting that his condition is likely to have been present since a young age. However, patient denied any symptoms. Thus, we are unable to confirm a true age of onset or a reliable family history.

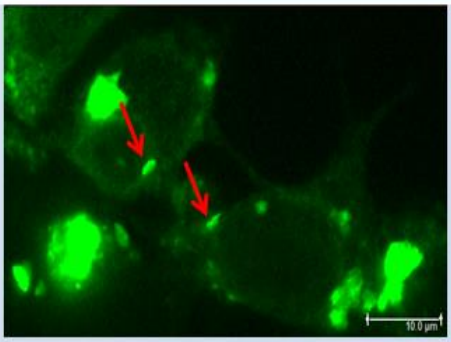
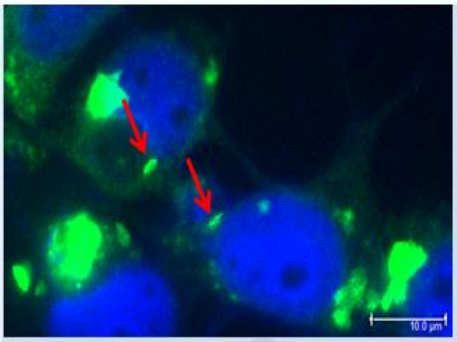
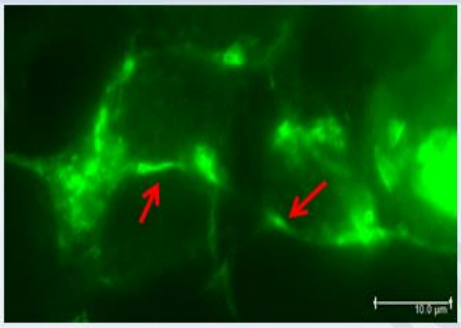
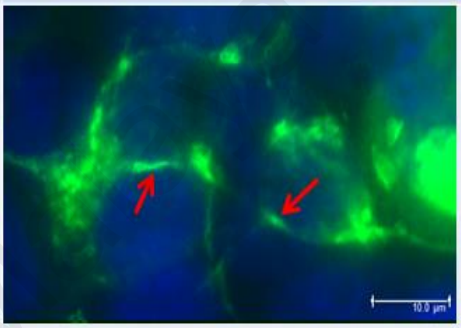
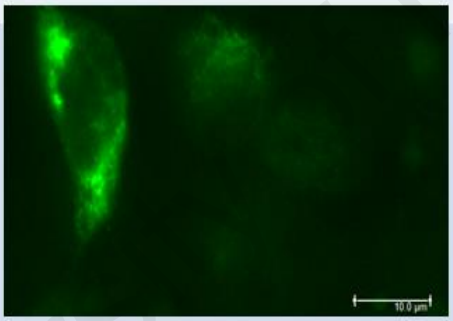
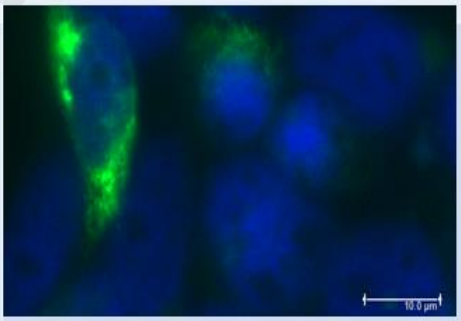
***NA= Not Available

Supplement 2: GJB1 cDNA construct and the sequences





Supplement 3: Another GJB1 localisation picture

	GFP-GJB1	Merged DAPI-GFP
Wild type GJB1		
V74M		
P174L		

Supplement 4: Copy of Questionnaire

Supplement 5: Publication, seminar presentation and conference papers