Exome Sequencing Analysis of Rare Autosomal Recessive Disorders

by

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Abstract

Since the human genome project was completed in 2003, extraordinary progress has been made in the field of genomics with the development of new sequencing technologies and the widespread introduction of next generation sequencing (NGS). The application of NGS initiated a new era in genomics by massively increasing the number and diversity of the sequenced genomes at lower cost. Human Molecular Genetics has greatly benefited from the use of NGS-based strategies to identify human disease genes. In this thesis, I investigated the application of genetic techniques to investigate the molecular basis of autosomal recessively inherited disorders of unknown etiology. A range of disease phenotypes, including oligodontia and fetal akinesia/multiple pterygium syndrome (FA/MPS), were investigated in patient cohorts that included many cases with parental consanguinity. Using an autozygosity linkage analysis-based approach and Sanger sequencing of candidate genes resulted in the identification germline *RYR1* mutations in FA/MPS. Subsequently, using exome sequencing techniques, the molecular basis of FA/MPS was further elucidated by the identification of germline mutations in *RYR1, NEB, CHRNG, CHRNA1 and TPM2*. The application of NGS in genetically heterogeneous disorders such as fetal akinesia/multiple pterygium syndrome can enable better and less expensive molecular diagnostic services aimed at specific mutation spectra, though more extensive sequencing can lead to the identification of larger numbers of variants of uncertain significance.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

Adenine	A
Autosomal dominant	AD
Autosomal recessive	AR
basepair	bp
Clinical Exome	CE
Clinical Exome Sequencing	CES
comparative genomic hybridization	CGH
copy number aberrations	CNAs
copy number variations	CNVs
cytosine	C
deoxy nucleoside triphosphates	dNTPs
Deoxyribonucleic Acid	DNA
dideoxy nucleotides	ddNTPs
Escobar Variant multiple pterygium syndrome	EVMPS
Fetal Akinesia	FA
Fetal Akinesia Deformation Sequence	FADS
fluorescent in situ hybridization	FISH
guanine	G
Human Genome Project	HGP
kilobase pair	kbp
lethal multiple pterygium syndrome	LMPS
megabase pair	Mbp
microlitre	µl
Minor Allele Frequency	MAF

Multiple Pteregium Syndrome	MPS
millilitre	ml
nanogram	ng
Next generation sequencing	NGS
Oligodontia	OD
polymerase chain reaction	PCR
Ribonucleic Acid	RNA
Single Nucleotide Polymorphism	SNP
Tris-borate/EDTA	TBE
ultraviolet	UV
untranslated region	UTR
Whole Exome sequencing	WES

Chapter one: Introduction

1.1 The basics of Inheritance

Knowledge towards understanding the basics of genetics and inheritance has been developed during the past 150 years. The way of passing on the traits from the parents to the offspring was not known until George Mendel discovered these basic principles through his work conducted between 1856 and 1863 using pea plants. He had chosen the peas due to their several distinctive varieties, and quick reproduction in order to observe the traits in offspring. Mendel found that the inheritance of certain traits such as height, colour, shape etc. follow particular patterns. Mendel postulated that alleles are inherited in pairs (one from each parent), and furthermore that certain traits are inherited in a dominant manner while some are recessive which could be hidden in one generation and appear in subsequent ones. He also stated that the inheritance of one trait is not influenced by the inheritance of another. From his results, he derived three main theories of inheritance; these are now known as the law of segregation, the law of independent assortment and the law of dominance (Mendel & Bateson 1865). These laws became the foundation of modern genetics. According to Mendel's principles, single gene disorders can be classified into four major groups based on their mode of inheritance; autosomal recessive disorders, autosomal dominant disorders, X-linked inheritance and Y-linked inheritance. Table 1.1 shows all types of inheritance which based on Mendel's principles.

Table 1.1 The basic modes inheritance for single-gene inherited diseases

Pattern of inheritance	Description	Examples
Autosomal dominant	One mutated allele can cause the disease usually one parent is affected. If a parent is affected each child of a pregnancy has 1:2 chance of being affected Appears in every generation - vertical transmission (though *de novo* cases can occur)	Huntington disease Marfan syndrome
Autosomal recessive	Two mutated alleles can cause the disease Parents are usually unaffected heterozygous mutation carriers Each pregnancy has 1:4 chance of being affected	cystic fibrosis sickle cell disease
X-linked dominant	In females, a mutation in one of the two alleles can cause the disease. In males, they only have one X chromosome, therefore a mutation in only this allele can cause the disease. Affected males mostly have more severe symptoms of the disorder than females no male-to-male transmission	Rett syndrome
X-linked recessive	In males one mutated allele is sufficient to cause the condition while in females both X chromosomes should be mutated in order to cause the disease. Consequently, females are usually unaffected carriers No male-to-male transmission but all daughters of an affected male will be mutation carriers.	Haemophilia Fabry disease
Y-linked	Only males are affected Affected males pass the mutated gene to all their sons but to none of their daughters	Y chromosome infertility, some cases of Swyer syndrome
Mitochondrial	Also known as maternal inheritance; only females can pass on the disease to offspring but both male and female can be affected It appears in every generation-vertical transmission	Leber hereditary optic neuropathy (LHON)

1.1.1 Autosomal Recessive Disorders

Autosomal recessive (AR) disorders manifest when the child inherits two mutated alleles (one allele from each unaffected parent). Each child born to healthy carrier parents has a 25% chance of being wild-type, 50% chance to be a heterozygous mutation carrier and 25% chance to be homozygous affected. The affected child may harbour homozygous alleles at the disease locus or be a compound heterozygote for two different mutations on separate alleles (Table 1.1). Known examples of these disorders include hemochromatosis (incidence 1:400), cystic fibrosis (incidence 1:2,500), phenylketonuria (incidence 1: 5,000) spinal muscular atrophy (1: 10,000). Cystic fibrosis (CF), is an autosomal recessive disorder that affects the pulmonary and digestive systems of the patient and is characterized by body secretion of sticky thick mucus in the lungs and airways of affected individuals instead of normal thinner mucus. Around 70,000 individuals are affected with this disorder worldwide (Cutting, 2015). It is caused by bialleleic mutations of the *CFTR* gene, and the severity of the disease is quite variable.

Offspring of consanguineous unions are more likely to exhibit a recessive disorder than offspring of unrelated couples because consanguineous parents share genes from at least one common ancestor. As such, if it carries the same allele mutation, the child will be at high risk of inheriting both mutated copies and therefore become homozygously affected. The significance of consanguinity will be discussed in further detail later in this chapter. Most of the cases studied in my project were consanguineous families who had autosomal recessive diseases, and therefore the gene and pathogenic mutations were mostly expected to be homozygous. However, there is still a possibility that in some cases despite the family being consanguineous, that disease might be caused by compound heterozygous mutations or even a non-autosomal recessive manner such as autosomal dominant disorders that are caused by de novo mutation and so could result in an affected child with normal parents

1.1.2 Autosomal Dominant Disorders

. Autosomal dominant (AD) mutations were previously reported in the phenotypes studied within this project. As described above (Table 1.1), a single mutated allele will be sufficient to cause the disease in this type of inheritance. If one parent is heterozygous for a dominant mutation, there is a 50% chance of the offspring being affected. In rare circumstances both parents may be carriers for the same dominant mutation, and in such cases, there will be a 75% chance that the child will be affected by either inheriting one mutated allele (50% chance) or by inheriting both mutated alleles (25% chance), and in this case the disease is expected to be very severe. There will be only a 25% chance that the child will inherit two wild type alleles and be healthy.

A child might be affected by a dominantly inherited disorder despite having healthy non-carrier healthy parents. This can occur if there is a *de novo* mutation or if there is germline mosaicism in the parents in which the mutation will occur in some of the sperm or egg cells but not the somatic cells. Germline mosaicism can be observed with any inheritance pattern, but it is most commonly seen with autosomal dominant and X-linked disorders and the pedigree will resemble the autosomal recessive pattern of inheritance (Figure 1.1). A child who inherits a mutation from the mosaic parent will be affected with the disorder and will not show mosaicism. An example of this is the heterozygous *de novo* mutation (p.R954L) which was detected in *KIF21A* in two siblings who were diagnosed with congenital fibrosis of the extraocular muscles, whereas both parents were homozygous for the wild allele with apparent recessive inheritance. Further mutational screening revealed another heterozygous variation in another area of the gene in the father and in both affected siblings while the mother was normal. As a result, the authors suggested that the p.R954L mutation arose from the father haplotype who is thought to be a germline mosaic for the mutation (Khan et al., 2010).

Figure 1.1 A pedigree represents germline mosaicism in the father of two affected children

1.2 Main approaches to study genetic disorders

Genetic diseases can be classified traditionally into three major types. First, monogenic disorders caused by mutations in a single gene which can be inherited in one of the above Mendelian patterns (Table 1.1) depending on the disease and the gene involved (e.g. Phenylketonuria and cystic fibrosis). Secondly, polygenic and/or multifactorial disorders which result from mutations in multiples genes or sometimes caused by a combination between genetic mutations and environmental factors, with these disorders not usually following any specific pattern of inheritance. Examples of these disorders are diabetes mellitus, schizophrenia and heart diseases. Third, chromosomal disorders that either caused by a loss or gain of part of (or an entire)

chromosome copy or may be by rearrangement of part of the chromosome such as by translocation and/or inversion.

Over the past century-and-a-half, many methods and techniques have been developed to identify the genetic causes which underlie these disorders in order to help in the diagnostic process and also improve the treatment approach. Three major historical approaches have been established for studying genetic and genomic changes in the laboratory. First, is cytogenetics (which analyse the chromosomal changes). Second, is molecular genetics (which can study the structure and the function of genes at molecular level), or thirdly, it can be a combination of these two approaches that can be referred as molecular cytogenetics. In this project, all investigations carried out fell under the umbrella of molecular genetics, however, I will describe briefly how cytogenetics and molecular cytogenetics can be used.

1.2.1 Cytogenetics

1.2.1a Conventional cytogenetics

Karyotyping is the conventional cytogenetic method used to check a patient's set of chromosomes and test the numerical changes, such as the loss or gain of an entire chromosome or part of it under the microscope as well as structural changes. The technique was developed by Tjio and Levan more than 50 years ago. They were the researchers who first discovered the correct number of human chromosomes as being 46 (Tjio and Levan, 1956). An example of the chromosomal genetic disorders is Down syndrome which is characterized by the presence of an extra copy of chromosome 21 (trisomy 21). This method is also efficient in diagnosing some mental disorders, developmental delay, congenital abnormalities and recurrent miscarriages which are mainly caused by similar abnormalities in the chromosomes.

The most common method for karyotyping is the G-banding technique which is based on the application of Giemsa dye on the metaphase chromosomes in order to give non-fluorescent permanent staining for the chromosome which can thus be visualised under a standard microscope. The main disadvantages of standard cytogenetics is that it takes long time and also has limited resolution. It can only detects large structural and chromosomal aberrations ranging from 5-10 Mb in size (Riegel, 2014) . In order to overcome this limitation, the molecular cytogenetic approach was developed.

1.2.1b Molecular cytogenetics

Three major molecular cytogenetic approaches are used to identify and analyse chromosomal abnormalities: fluorescent in situ hybridization (FISH), comparative genomic hybridization (CGH) and SNP-genotyping arrays. FISH is a powerful technique that utilises a fluorescent probe to bind to a specific chromosomal region to enable the region to be visualised and localized with fluorescence microscopy in a metaphase or interphase stage. It is useful in karyotyping to detect the changes in chromosomes (e.g. copy number variation). Also, it is a sensitive and specific method for detecting abnormalities at a resolution up to few kilobases (kb) such as translocations, aneuploidy, deletions, inversions, or amplifications. CGH is a high-throughput technique developed in the 1990s and utilised for the analysis of the whole genome to identify copy number variations/aberrations (CNVs/CNAs) that cannot be detected by conventional karyotyping or targeted FISH studies, however it is very helpful in the genetic diagnosis of cancer patients. Two DNA samples (tumour and normal) are used and compared to observe any difference between them (Michels et al., 2007, Martin and Warburton, 2015). One of the main advantages of CGH is that it can be applied effectively to discover novel genetic

changes, as it does not really require prior knowledge of the chromosome imbalance that is involved (Speicher & Carter et al.2005).

In addition, SNP arrays is a further molecular cytogenetic technique that can provide high resolution copy number data. Recently SNP arrays have been used to detect copy number variants in the human genome by utilising >946,000 probes with an average inter-marker distance of 680 base pairs. Also, SNP arrays can detect copy-neutral loss of heterozygosity/uniparental disomy (UPD), which cannot be detected by conventional cytogenetics or FISH techniques (Bentley et al., 2008, Mardis et al., 2009)

1.2.2 Molecular Genetics

Mutations at the DNA level can be inherited from the parents or might be acquired at some point of the person's life. They arise in many different forms, including single nucleotide substitution, as both insertions and deletions. Based on their impact, they can either cause no effect, alter the gene product, or prevent the gene from functioning properly or completely. Loss of function mutations mostly cause harmful effects although on some occasions they can be beneficial (Loewe, 2008)

1.2.2a Gene identification approaches:

To identify these molecular changes, two main approaches have been developed over the years. Firstly, functional cloning which is a method that entirely based on the available data about the gene function in order to determine the suspected causative gene without the need to know the genomic location of the gene. The second approach is the positional cloning method. Unlike the first method, a specific region of interest is located in the chromosome and the causative gene is then identified within the located candidate region without knowledge of the gene function. In

practice, using the two methods together has been found to be more efficient and useful rather than relying only on just one approach (Deloukas et al., 1998).

1.2.2a. i Candidate Gene Approach

This approach was previously known as the functional cloning approach. It requires a detailed prior knowledge of a particular disease phenotype including the pathophysiology of the disease. As such it is mainly based on the assessment of the association of a particular candidate gene that is thought to be relevant to the disease. The major difficulty with this approach is that, before selecting the potential candidate gene, the researcher should have adequate knowledge and understanding of the mechanism which underlies the studied disease and its pathophysiology such as the gene function, tissue expression pattern, role in known developmental pathways, homologies to other genes, and/or animal models. In contrast, one of the advantages of this method that it does not require the study of large families with both affected and unaffected individuals, but can be performed on small families with only parents and probands or it can even be carried out on unrelated patients and control subjects in some occasions. Furthermore, this approach can be effective for identifying the genes underlying common and complex disorders where the risk associated with any chosen candidate gene is quite small (Kwon and Goate, 2000a, Risch and Merikangas, 1996).

Large number of disease-causing genes have been identified using this method, for example the identification of phenylalanine hydroxylase *(PAH)* that causes phenylketonuria (DiLella et al., 1987). This method was the main approach for identifying the causing genes before the development of genetic mapping.

However, it is still very helpful today especially when it is combined with the linkage mapping to identify the disease-causing gene amongst the huge number of genes provided by highly

advanced techniques such as next generation techniques. In fact, in the latter scenario, the researcher is dependent on this strategy to identify both the mutations and the causative genes.

By performing a linkage analysis prior to whole exome sequencing, the region of interest can be narrowed down to a smaller size. After identifying many suspected variants in different candidate genes, the list of variants can be filtered according their genetic function and according to any role of the candidate genes may play in the biological process, and also in terms of any previous studies on animal models. If the identified genes had any role in the biological pathway, then it is more likely to select further genes from the same pathway that may lead to specific disease or related conditions. For instance, the identification of several genes to be a part of the RAS-MAPK signal transduction pathway (*PTPN11, SOS1, RAF1, KRAS, HRAS, BRAF, MEK1,* and *MEK2*) with the pathway recognised to be responsible for causing a variety of genetic syndromes (Noonan syndrome, LEOPARD syndrome, Costello syndrome, and Cardiofaciocutaneous syndrome) that all have overlapping clinical phenotypes (Weismann et al., 2005). Recently, an analysis of mouse mutants with some genes knocked out using systematic mutagenesis programs has greatly aided the approach of functional cloning.

Furthermore, the completion of the mouse genome project was a very significant advance (Waterston et al., 2002) due to the high genetic similarities between human and mouse genomes. In this way, genetic disease-causing mutations in mice have been hypothesised to reflect similar diseases in humans. It has also been found that the combination of linkage mapping and a candidate gene approach is the most successful method of identifying disease genes (Kwon and Goate, 2000b).

1.2.2a.ii Positional cloning

Positional cloning studies are used to identify the location of the disease-causing candidate genes in Mendelian disorders. The method requires families with multiple affected individuals and genetic markers of known chromosomal locations to perform linkage analysis from which to define a candidate segment on a chromosome (locus) which contains the disease-causing gene (Wicking and Williamson, 1991). Having pinpointed a candidate interval region, mutational screening for the selected candidate genes within the interval is performed.

In the last decade, The Human Genome Project greatly helped this approach (Lander et al., 2001, Venter et al., 2001) as it provided physical and genetic maps for millions of polymorphisms and sequence repeats on the chromosome. In addition, it provided a comprehensive sequence analysis, which has collectively led to detailed maps of genes of known or unknown function throughout the human genome. In addition, the public databases provided by genome browsers such as UCSC, NCBI, and Ensembl include detailed information about a massive number of the genes and polymorphic markers that have been mapped throughout the whole genome and the maps. In Mendelian disorders, linkage analysis can achieve the direct mapping of any candidate genes to locate the candidate genetic segment on the chromosome. Then, from this localized segment, the candidate gene itself will be selected according to the available information about the gene activity, tissue expression, biological process and any previous work on animal models related to this candidate gene.

The highly-advanced genotyping and sequencing project played a significant role in speeding up the process of gene mapping and identification considerably. Nevertheless, many genes are still not identified although more than 15,000 genes involved in human diseases are present on OMIM.

1.3 Haplotype and genetic markers

A haplotype is a group of adjacent alleles or DNA sequences (genes or polymorphisms) which tend to be inherited together and can thus be used as a helpful tool in genetic mapping and population studies. Genetic mapping depends on the behaviour of chromosomes at the time of meiosis because this is when the linked region containing the candidate genes is passed along as a block unit unless they are separated by crossing over between paired homologous chromosome that results in swapping genetic material, with this process acting as the source of genetic recombination. The closer the genes occur on the same chromosome, the higher the chance that they will be inherited as a single unit, because crossing over between two linked genes is infrequent (Morgan, 1911). Recombination fraction is defined as the genetic distance between two separate loci, and usually loci separated by recombination in 1% of meiosis are defined as being 1centiMorgan (cM) distant. This genetic distance is different from physical distance (measured in bp, kb, or Mb of DNA). In addition, some chromosome regions have a higher frequency of cross over than others. The order of loci should be similar in genetic and physical maps, but the spacing may be different, and on average 1cM corresponds to 1Mb, although this can be variable across the genome. Linkage between two loci is considered to be present when recombination events occur less than 50% of the time, and results in a recombination fraction of <0.5 (Ott and Bhat, 1999) which consequently means that the loci are located on the same chromosome and lie close to each other. Genetic mapping in humans thus looks at a large number of loci and types them for genetic markers.

Different genetic markers were developed over the years and have been used in genetic mapping studies including allozymes, Random Amplified Polymorphic DNA (RAPDs), Restriction Fragment Length Polymorphisms (RFLPs), Amplified Fragment Length Polymorphisms

(AFLPs), Sequence-Tagged Sites (STSs), microsatellites and SNPs. In practice, each type of markers presents slightly different advantages and disadvantages. For selecting the best genetic markers to use in any linkage mapping studies, three main characteristics need to be considered: (i) high polymorphic rate of the genetic marker, (ii) the spreading across the genome or chromosomal region that contains the candidate gene with good coverage (iii) the genotyping error rate should be relatively small. (Ball et al., 2010) All these features can be found with single nucleotide polymorphisms which make them along with microsatellites the most used markers in genetic mapping studies. Both SNPs and microsatellite markers have been utilised in my project.

Microsatellite markers are short tandemly repeated DNA sequences from 2-5 bases with a high polymorphic rate (e.g. [CA] repeats 40 times in a sequence). An analysis of microsatellites can then be performed using polymerase chain reaction (PCR) by amplification of the region which contains the microsatellites using fluorescently-tagged primers followed by fragment analysis. Microsatellites occur on average nearly every 30,000 bases throughout the human genome (Stallings et al., 1991). Practically speaking, these are found to be very informative and successful in many mapping projects but they do have high error rates compared to SNPs (Ball et al., 2010). Because of advances in high-throughput sequencing technologies and bioinformatics, SNPs have been increasingly used in genetic mapping studies in Mendelian monogenic, multifactorial diseases and the detection of copy number variants (CNVs). These are distributed throughout the whole human genome with an average presence of one polymorphism each 300 nucleotides , which means that the human genome contains nearly 10 million SNPs (Salisbury et al., 2003). SNPs are relatively less informative than microsatellites because they are less

polymorphic but in contrast they are significantly more abundant across the human genome with a far less error rate.

1.4 Genetic Mapping projects

In the recent years, human genome sequencing projects such as the international HapMap Consortium facilitated the use of SNP markers in mapping studies by providing around 11 million polymorphisms across the genome. The International HapMap is one of the biggest established mapping projects which has had a great impact in the design and analysis of genome-wide association studies. Using 270 individuals originating from different ethnic populations, the project successfully provided the mapping locations of more than one million SNPs. This achievement was later named as Phase I of the project and the result was published in 2005. In Phase II of the HapMap Project and with genotyping of same individuals, they found a further 2.1 million SNPs. Based on phase I and II, the HapMap project thus contains a catalogue of nearly 3.1 million SNPs in total which means one polymorphism occurs every 1kbp nucleotide (International HapMap et al., 2007). These 3.1 million SNPs represent nearly one third of all the estimated 10 million commonly published SNPs (MAF≥0.05) in the whole assembled human genome (International HapMap et al., 2007). As a result, genome wide scans using SNP markers can be done utilizing high throughput methods such as ('SNP-chips'), which provide high resolution mapping information. The first applied SNP chip array was (GeneChip® 10K Xba Array) and it could scan more than 10,000 SNP markers in a single experiment (Affymetrix Inc, Santa Clara, CA). This 10K chip was subsequently successfully used in many mapping projects (Janecke et al., 2004, Gissen et al., 2004). Also, Affymetrix has developed the SNP array techniques and produced upgraded array chips including 100K, and 500K which genotype more than 100,000 and 500,000 SNP markers respectively. More recently, they released SNP Array

6.0 that contains more than 900,000 SNPs and similar number of markers to detect copy number variation.

Many genetic mapping and linkage studies have been established over the last three decades and these have become a crucial part of the genetic maps and the gene identification approach by providing a high-density framework of genetic markers across the genome with known positions for both the known genes and the markers. An example of these developed maps is the Généthon map which mainly focused on the microsatellite markers (Weissenbach et al., 1992, Dib et al., 1996). Another microsatellite map was constructed by genotyping nearly 8,000 markers based on the analysis of eight large, three-generation families (Broman et al., 1998). In 2002, a paper was published describing a genetic mapping project which was undertaken in Reykjavik, Iceland by genotyping 869 individuals from 146 families. The study provided a defined map of 5,136 polymorphic microsatellite markers. Based on this particular map framework, around 2 million further SNPs have been located as well (Kong et al., 2002). These genetic maps can be very helpful in performing linkage studies and identifying genes in specific diseases and aiding the assembly of known DNA sequence for the human genome project.

1.5 Consanguinity

In clinical genetics, consanguinity is described as a union between two related individuals such as first and second cousins, with an inbreeding coefficient factor (F) not less than 0.0156 (Bittles, 2001) where (F) measures the probability of passing on identical copies of the alleles from the related parents to their offspring, so that they become homozygous for that allele and are identical by descent (IBD). F thus relates to the risk for the child to be homozygous by descent for a particular genetic sequence. In contrast, the coefficient of relationship (R) is related to the

consanguineous couple themselves and measures how much genetic components they would expected to share by descent from a common ancestor (Table 1.2) The closer the biological relationship between couples, the greater is the likelihood of their child being homozygous alleles/mutations for the recessive genes implicated in disease.

Table 1.2 The degree of consanguinity and the chance of passing the genetic component

Relationships between degrees of consanguinity and the effect on F and R and consequential risk of autosomal recessive disease in offspring (Adapted from Young, 1999)

Relationship	Degree of Relationships	Shared genes (R)	Inbreeding factor (F)
First degree	Parent-child Siblings	½	¼
Second degree	Half siblings Uncle-niece Aunt-nephew	¼	1/8
Third degree	First cousins Half uncle-niece Half aunt-nephew	1/8	1/16
Fourth degree	First cousins once removed Half first cousins	1/16	1/32
Fifth degree	Second cousins	1/32	1/64

1.5.1 Global Prevalence of consanguinity

It is estimated globally that at least 20% of the human population live in communities with a preference for consanguineous marriage, and that nearly 15% of children across the world have consanguineous parents (Shami et al., 1990). In a number of specific communities and

populations, there is a preference towards consanguineous marriage and this has led to an increased expression of autosomal recessive disorders among these communities because the offspring is at a higher risk of inheriting homozygous recessive gene mutations from a common ancestor (Bittles et al., 1991, Bittles and Black, 2010, Hamamy et al., 2011). In contrast, no significant associations have been indicated in the genetic studies between the consanguineous marriages and the diseases inherited either in autosomal dominant conditions or for X-linked recessive conditions (Hamamy et al., 2007). Consanguineous marriage has been reported with high rate in some countries such as India, Pakistan Turkey, Arabian Gulf countries and in North Africa in varying rates (Figure 1.2) (Hamamy et al., 2007, Hamamy et al., 2011).

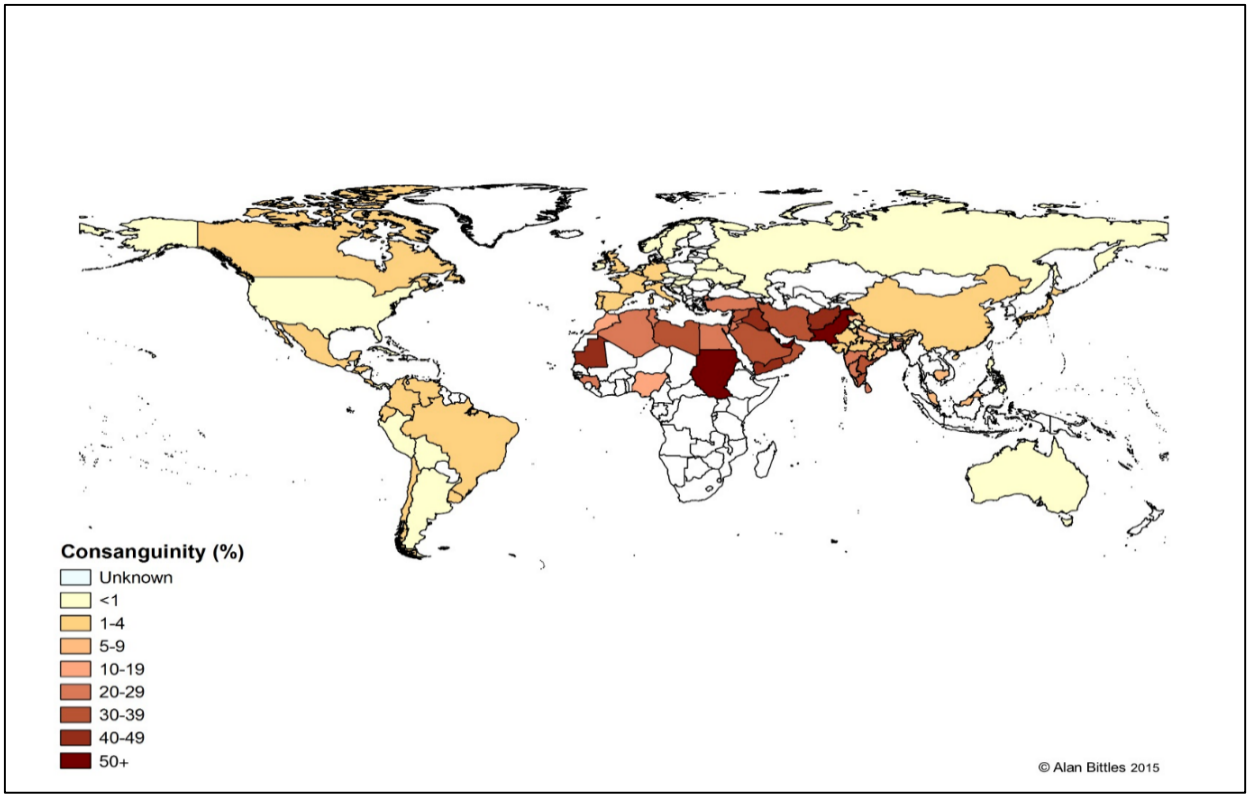


Figure 1.2 A map showing the prevalence of global consanguinity

(from consang.net resource by Allan Bittles, 2015)

Though most of the communities with high rate of consanguinity are of Islamic background, there is no evidence that Islam or the prophet Mohammed encouraged marriage between relatives, in fact some Muslim scholars rather discouraged it. Therefore, it appears that the practice of consanguineous marriage is mainly cultural with the relatives wanting to strengthen their family ties and avoid the uncertainty of health or financial situations of a spouse coming from outside the family. In addition, economic factors could have become another reason for the increased average of blood relatives' marriages, especially in communities where dowry payments are the norm, thus these costs being effectively reduced (Bittles, 2001, Modell and Darr, 2002). In such communities, the early marriage is also quite common so the couples quite possibly would have more children with a consequentially increased chance of autosomal

recessive disorders occurring. The effects of consanguineous marriage have been widely reported in many studies amongst different populations. The observed outcomes have included increased infant morbidity and mortality rates, as well as of congenital abnormalities, learning difficulties, blindness, cardiovascular diseases and neuromuscular disorders. One study review involved 38 studies from several populations in different countries and summarized an average increase of infant mortality by 4.4% amongst the children of first cousins couples compared with unrelated controls. Also, the stillbirths rates amongst infants of consanguineous couples were found to be slightly higher while the birth defects rate was nearly 2–3% higher compared to controls (Bittles and Neel, 1994).

1.5.2 Consanguinity prevalence in Arabian countries

In most Arab populations, there is a high rate of consanguineous marriage (e.g. first cousin marriages) which has been reported in many studies across these countries (Figure 1.3). This has had a significant effect in the increase of inherited recessive diseases. For example, the rate of consanguinity represents between 35-50% of all marriages in Jordan (Hamamy et al., 2007, Tadmouri et al., 2009).

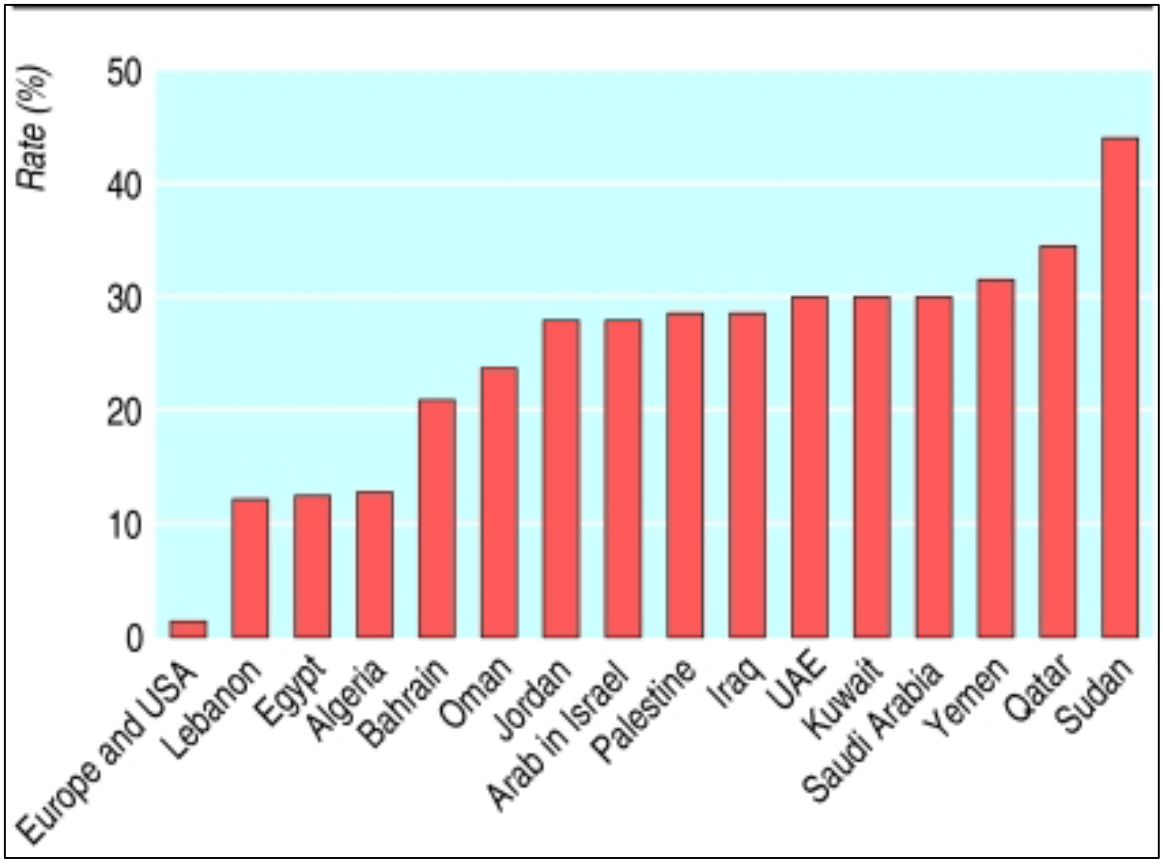


Figure 1.3 diagram shows the rate of first cousin marriages in Arab countries

Image taken as displayed in the paper (Al-Gazali et al., 2006)

In Saudi Arabia, a study screened 16,638 families from 13 different regions across the country. They found that the overall national prevalence was significantly high (56%) and first cousin mating (third degree relatives) was the most common form of relationship. In further analysis, consanguinity was found to be higher in the rural areas (59.5%) compared to (54.5%) within urban areas which indicate a slightly lower preference among urban people. Surprisingly, the rapid improvement and civilization over the last three decades in the country as a result of the oil boom have not significantly affected this high rate of consanguinity (El-Mouzan et al., 2007, El Mouzan et al., 2008). According to many researchers, consanguineous marriage is a significant

factor in increasing the prevalence of autosomal recessive genetic disorders within Arab populations and consanguinity is suggested as a reason of the high postnatal mortality rate amongst offspring of consanguineous unions, compared to offspring of non-consanguineous parents (Hamamy et al., 2007, Tadmouri et al., 2009).

A study focused on investigating the role of consanguinity in pregnancy outcomes involved 4498 pregnant women in Saudi Arabia and identified that the rate of consanguinity was 54.3% including 31.4% of first cousins and 22.9% of other relatives. This very high rate of consanguineous marriages in the Saudi population could be due to the role of the family in arranging the marriages as well as to the social and cultural influences which try to keep the family together. In the results, the high frequency of deleterious pregnancy outcome was noted among the consanguineous compared to those of the non-consanguineous marriages. The rates of the most severe outcomes such as perinatal deaths, infant deaths and neonatal deaths were highly significant among the consanguineous with 62%, 60.3% and 57.9% respectively, and collectively was 5% higher the non-related marriages. Furthermore, a higher average of the other outcomes such as abortions, still births, prematurity, live births and low weight were reported when compared to the non-consanguineous marriages (Wong and Anokute, 1990)

1.5.3 Effect of high consanguinity within Pakistani British in Birmingham

In the UK, consanguineous marriage is very common for communities of Pakistani, Bangladeshi and Middle Eastern origin, some Indian groups, Irish travellers and some refugee groups (Bittles, 2001, Modell and Darr, 2002). A study conducted in Birmingham UK reported that consanguinity was recorded at 0.4% of the North European couples compared to a highly significant rate (69%) of related Pakistani British couples with the vast majority (57%) of these marriages being first cousins. It was further reported that 7.9% of British Pakistani children are

born with genetic defects compared to only 4.3 % affected children of North European couples. The frequency of recessive disorders was 3.0–3.3% amongst the British Pakistani children, ~10 times greater than the North European children (0.28%) (Bundey and Alam, 1993) (figure 1.4).

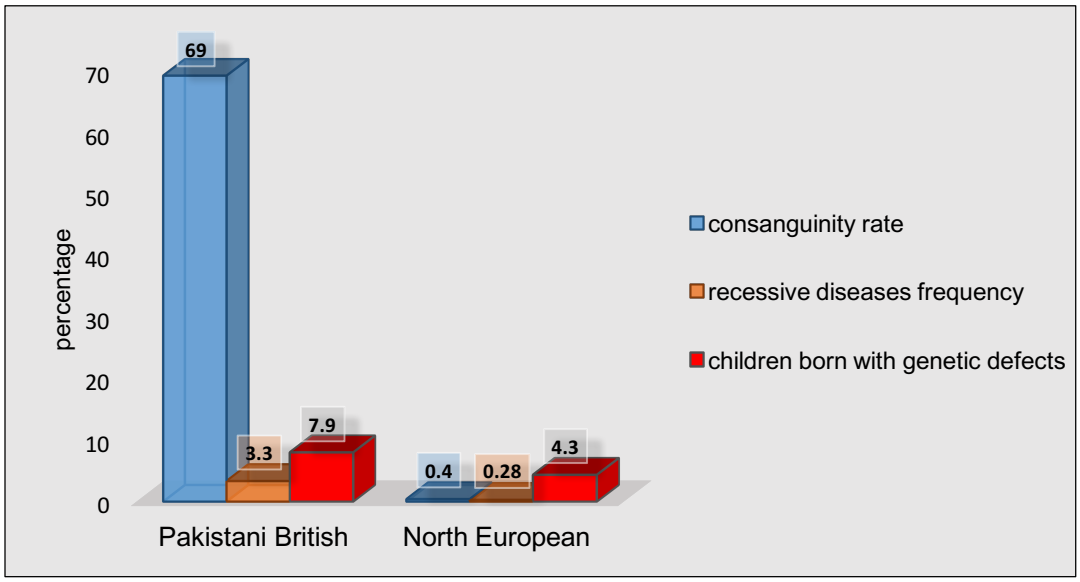


Figure 1.4 The role of high consanguinity in causing recessive diseases amongst Pakistani British community in Birmingham-UK (Bundey and Alam, 1993)

Furthermore, in a study recruited between 2007 and 2011'the Born in Bradford study' investigated a cohort of 13,776 babies and their families and identified that 1922 (37 %) of 5127 babies of Pakistani British had first-cousin parents (Bhopal et al., 2014). Based on the born Bradford study (Sheridan et al. 2013), questionnaire data was obtained from the mothers of those children affected with at least one anomaly. In their results, the risk for congenital anomaly was doubled compared to the children of British origin; it was 6 % of the offspring of first-cousin parents and 5 % of other related couples (Sheridan et al., 2013).

Because of the reported significant impact of consanguinity in causing genetic recessive disorders, it was strongly suggested that it might be preferable to offer a genetic counselling unit

for any related couples who intend to get married rather than simply attempting to convince them to avoid it. This suggestion was made due to the high traditional preference of consanguinity amongst these societies as previously explained. Such genetic counselling would include carrier testing for at-risk couples prior to their marriage. Also, a prenatal test would be suggested to detect any abnormality or severe disorders of the fetus during the first trimester of the pregnancy such as thalassaemia disorder. This test can be typically dome by chorionic villus sampling in the first trimester for severe disorders (Darr and Modell 1988)

1.6 Autozygosity mapping

1.6.1 Background about the technique

The homozygous locus which is inherited from both parents is called autozygous, and the method of identifying this locus is known as autozygosity mapping (Mueller and Bishop, 1993). The technique is found to be very effective in searching for homozygous regions by descent in consanguineous families affected by autosomal recessive disorders (Figure 1.5). The advances in genetic mapping techniques (in particular genotyping SNP assays) have greatly helped in implementing the homozygosity mapping technique for analysing autosomal recessive disorders in consanguineous families (Lander and Botstein, 1987). Though the parental consanguinity is considered as the main cause of the homozygosity, however, several other mechanisms might be involved such as linkage disequilibrium (LD) in a population which could result in a homozygous chromosomal segment. This is not autozygous however and is more likely be shorter. Heterozygous deletion of one chromosome is another cause of apparent homozygosity but it would be in very much shorter segments. Furthermore, homozygosity mapping has been applied on more complex disorders (i.e. schizophrenia), so the success of the strategy can be

seen to have been extended not only for examining monogenic but also for the identification of rare genomic variants in complex traits (Broman and Weber, 1999).

Moreover, longer homozygous regions can also be caused by long deletions or chromosomal abnormalities such as uniparental disomy, when both copies of a chromosome or part of it are received from only one parent with no copies from the other parent. The efficiency of autozygosity mapping was confirmed in many studies which focused on investigating different disorders that follow in an autosomal recessive manner in consanguineous families. Over the last two decades, many autosomal recessively disorders (including lethal ones) have been studied with great success by utilizing this method of mapping to provide the genetic basis that could lead to identifying the disease-causing developmental genes in these disorders (Aligianis et al., 2005, Morgan et al., 2006b). For instance, the technique successfully mapped the candidate genetic region that causes alkaptonuria disorder (Pollak et al., 1993). Therefore, the autozygosity mapping technique has proved itself to be a very useful approach and a powerful strategy to locate the disease-causing genes in these closed families. However, this method can't be helpful in terms of finding the definite mutation which ultimately has to be achieved by direct gene sequencing.

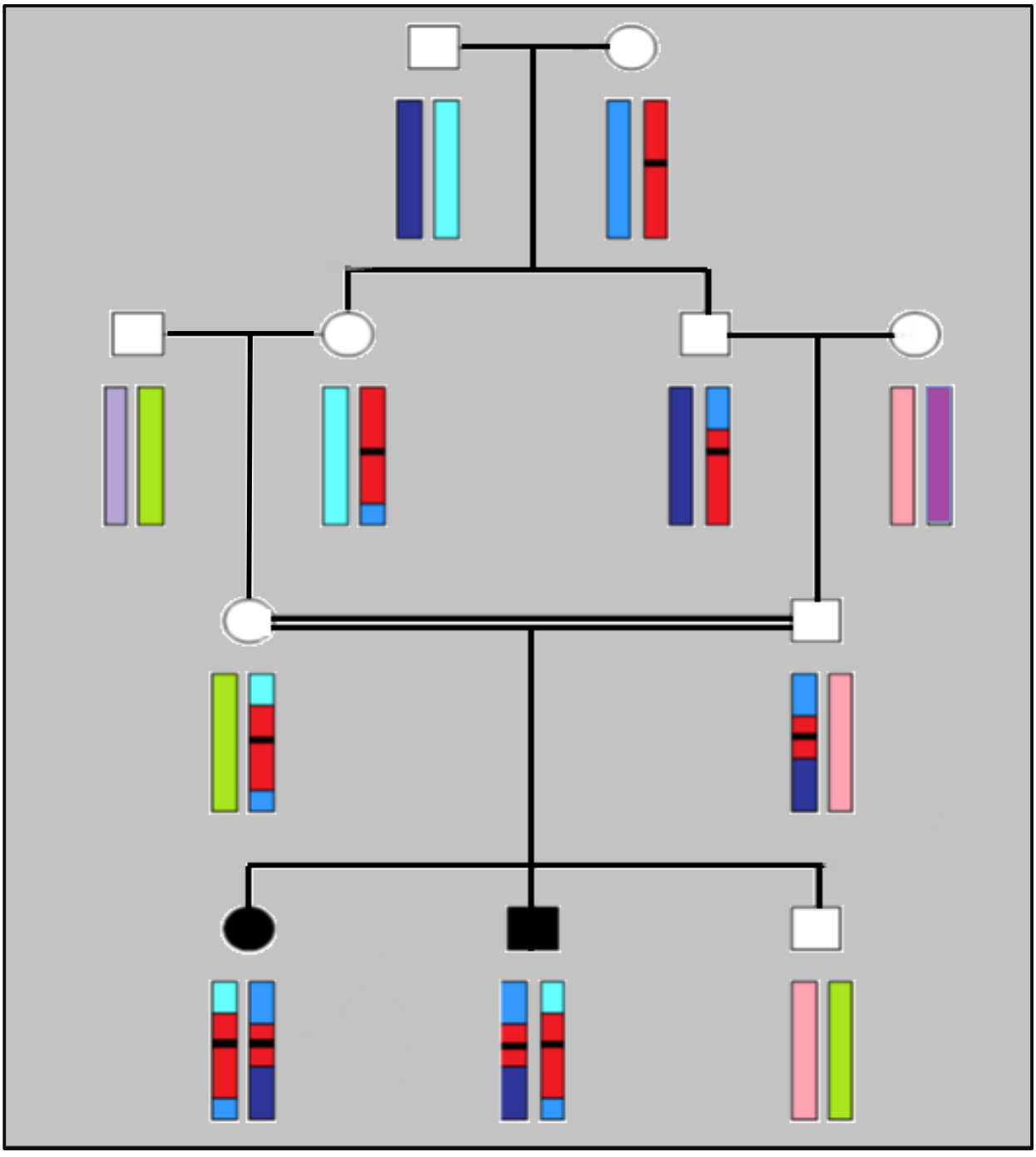


Figure 1.5 The principle of autozygosity mapping

a specific mutation of a disease gene (indicated by the black line) can be passed on from a common ancestor (CA) to offspring and thus the product of a consanguineous marriage, can result in affected offspring (redrawn from thesis by Chirag Patel, 2012)

1.6.2 Advantages / disadvantages of autozygosity mapping

As introduced above, the use of autozygosity mapping and positional candidate gene analysis has shown a high efficiency in identifying the recessive genes of consanguineous families, much more so than in non-consanguineous families. The technique is currently regarded as an efficient and successful approach for localising the disease gene amongst the consanguineous families, even in the presence of locus heterogeneity (Petukhova et al., 2009).

The more affected individuals from a studied consanguineous family, the more efficient the method will be to identify the region of interest that is linked to the disease. As many autosomal recessive disorders are considered very rare among the population, however, it will often be difficult to identify a sufficient number of patients with the same phenotype to perform autozygosity mapping. In the case of a small family size, for example, (i.e. a family with two affected individuals) collaborative projects between several centres would be an alternative solution to obtain more information about the disease genotype. A limitation for the effect of this option in studying some disorders is when there is a 'private mutation' that could only be present in a number of families which would then be difficult to find it in other families even with the same phenotype.

Furthermore, some autosomal recessive diseases in consanguineous families can be caused by compound heterozygous mutations, so in this case the disease locus might not be within a homozygous region at all, and would not be covered by the autozygosity mapping as the technique focuses only on the homozygous regions. Moreover, some rare diseases are also extremely heterogeneous with more than one genetic locus for the disease, so it is also important to identify any previously mapped disease loci in such instances. Linkage studies performed in one large consanguineous family with multiple affected individuals would certainly be more

powerful than using several different families with one or two affected individuals. Once linkage is established in such a large family, other smaller consanguineous families could then be used to investigate the linkage at the same candidate region. Such approaches might help to overcome the problem of locus heterogeneity.

1.7 DNA sequencing

DNA sequencing is referred as the determination of the precise order of nucleotides [Adenine (A), Guanine (G), Cytosine (C), and Thymine (T)] within the DNA molecule/genome. The development of Sanger sequencing has enabled many advances in biology and medicine and improved understanding of the genetic basis of a large number of inherited diseases. The automation of this process also greatly facilitated the completion of the human genome project (Levy et al., 2007). The human genome project was officially started in 1990 and was completed in 2003 with the mapping of nearly 3 billion base pairs at a final cost of approximately $2.7 billion. The Sanger method (First-Generation Sequencing) was the standard method for DNA sequencing since it was originally developed in the 1970s, until the advent of high throughput machines or next generation techniques at the beginning of the last decade. Next generation sequencing greatly reduced the time and the cost of sequencing provided the ability to sequence the entire human genome in a few days with highly reduced costs. These can be considered the two major methods for DNA sequencing.

1.7.1 Sanger Sequencing method

1.7.1a Main principle of the technique

the discovery of double helix structure of DNA was firstly discovered in 1953 by the British scientists Watson and Crick (Watson and Crick, 1953). For their great discovery, they both won

the 1962 Nobel Prize in Medicine. Sanger sequencing was developed by Fredrick Sanger in 1977 (Sanger et al., 1977) who began a new era in molecular biology and it is still used widely today. Until this, the accepted method of sequencing DNA was described by Maxam and Gilbert who first developed the chemical cleavage of DNA for sequencing. After the publication of the Sanger method however, Maxam–Gilbert sequencing became increasingly unfavorable due to the method's complexity, its extensive use hazardous chemicals, and the implicit difficulties with scale-up (Tipu and Shabbir, 2015b). The Sanger technique was based on a chain termination method by using dideoxy nucleotides (ddNTPs) in the presence of a specific primer, DNA polymerase and deoxy nucleoside triphosphates (dNTPs). The ddNTPs lack the hydroxyl group which is required to form a phosphodiester bond between nucleotides causing DNA polymerase to terminate DNA elongation which results in fragmented sequences of DNA with different sizes. The resulting fragments are then separated according to size using gel electrophoresis (Bayes et al., 2012). The used ddNTPs are normally radioactively or fluorescently labeled to enable detection in gel electrophoresis. The reaction is divided into four separate sequencing reactions, each reaction includes the standard deoxynucleotides (dATP, dGTP, dCTP and dTTP). Though this represented a remarkable achievement in developing DNA sequencing the main limitation of this chain-termination method was the non-specific primer binding to the target sequence which affects the accuracy of reading the DNA. Therefore, a new method called Dye-terminator sequencing has been developed more recently. This depends on the labelling of the chain terminator ddNTPs, and sequencing can be done in a single rather than four as used in the labelled-primer method (Tucker et al., 2009)

1.7.1b The automation of the technique

An automated sequencing of Sanger method was further developed by Prober and colleagues. It depends on fluorescent labelling to detect DNA fragments based on the use of fluorescent chain terminators. The method was based on four ddNTPs, each one is attached to a distinct succinyl fluorescein. The DNA fragments are resolved on polyacrylamide gel electrophoresis (PAGE) and then detected by a fluorescent system. Subsequent progress in the technique has resulted in identifying developing colour coding of terminating ddNTPs, allowing running the four ddNTPs in one tube reaction instead of four. Since then, many commercial companies have introduced different automated DNA sequencers. The use of PAGE still has had some limitations such as in the preparation of gels, the use of toxic chemicals, problems of gel loading, thickness and electrophoresis (Prober et al., 1987). In the early 1980s, the Capillary electrophoresis method was successfully developed using high purity fused silica capillaries which hold a sieving medium that allows DNA fragments to be separated based on their molecular size. A laser detector near the end of capillaries enable the detection of fluorescent signals emitted by incorporated labelled ddNTPs which is considered another important step towards the automation of Sanger sequencing. A further acceleration in the automated sequencing instruments was achieved by developing Capillary Array Electrophoresis (CAE) method which is based on the use of laser detector to scan 96 samples one by one across all 96. It only uses 1% of the amount of DNA in each sample to be scanned, however, while the remaining 99% is lost without detection. This limitation was covered by developing a new system that could detect signals from all capillaries simultaneously by the introduction of a sheath flow cuvette. DNA sequencing fragments were run as discrete streams from each capillary within the sheath fluid. A

laser beam focused into the cuvette skims beneath the capillary tips thus scanning all 96 capillaries simultaneously (Tipu and Shabbir, 2015a)

1.7.2 Next generation sequencing (NGS)

Since the discovery of DNA structure in 1953, a lot of rapid advancement in genomic technologies have been made, including the recent NGS techniques to understand the complexity and diversity of human genome in health and disease. One major advancement was the completion of Human Genome Project in 2003. Though this was a huge achievement in the genetic and genomic field, it revealed the need to accelerate the effort to develop more advanced sequencing technologies instead of relying on the limited throughput of Sanger-dye sequencing method. This would thus provide better understanding for the human genome by sequencing more genomes and running bigger databases. Therefore, a great competition was created that could help reducing the high costs of sequencing. In 2005, Life Sciences 454 launched the release of NGS platforms by introducing the 454 genome sequencer (Margulies et al., 2005). This saw a huge advancement in genomic sequencing which brought about a very competitive environment between companies (Goodwin et al., 2016). This competition also resulted in a sharp decrease in the cost of commercial sequencing kits which had a very positive impact on medical research and the associated economic cost in which whole exome sequencing (the coding part of the whole genome) for example might cost currently less than $1000 while the whole genome sequencing costs nearly $1500. This race in share marketing in genome sequencing resulted in a trend in the price falling significantly faster than in Moore's law (Figure 1.6). Moore's law describes the concept that the power of computer technology doubles every two years. This law can also be applied to the progression in the power cand efficiency of NGS.

In fact, the abilities and efficiency of NGS has even overtaken Moor's law in recent years, demonstrating how quickly this field is advancing.

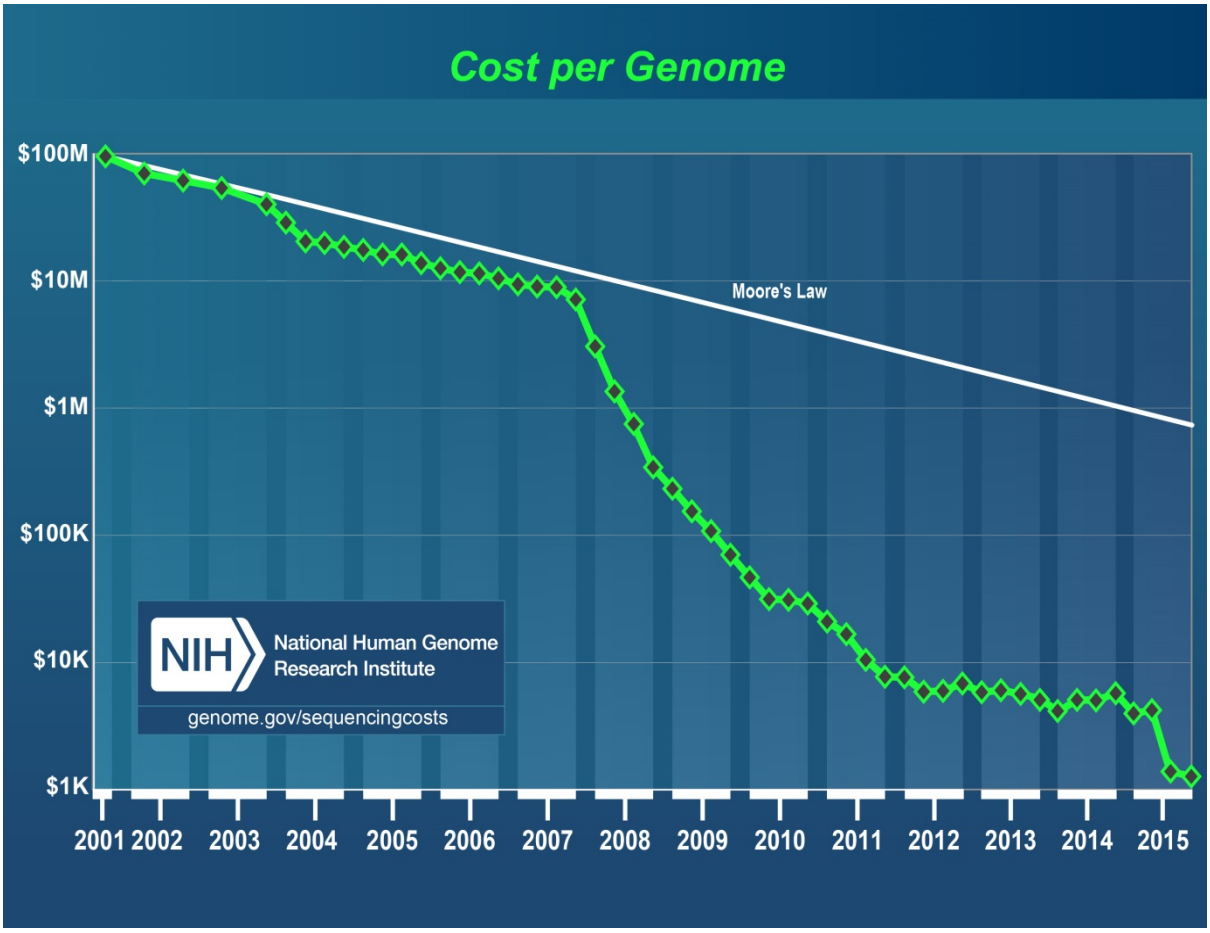


Figure 1.6 Diagram shows the decrease of the cost of genome sequencing in comparison to Moore's law (taken from National Human Genome Research website)

In the UK, a massive genome project was launched in 2012 aiming to sequence 100,000 genomes from a total of 75,000 individuals including 25,000 cancer patients (50,000 genomes; two genomes per patient) and three genomes for 17,000 rare diseases patients (with one genome for the patient and two genomes from healthy relatives. As such, around 17,000 rare disease patients and 33,000 healthy relatives will be studied. The 100K genomes project is planned to be

completed by 2017 with the main project goals to be of benefit for NHS patients and to build up a clinical and research genomic database which could greatly help in the genetic diseases and the gene discoveries (Genomics England) or (Peplow, 2016).

1.7.2.1 Whole Exome sequencing (WES)

1.7.2.1a Background about WES

The Exome represents the coding part of the whole genome and is also known as targeted exome capture. For most genetic disorders, there are numbers of candidate genes that might be linked to the disease and thus the process of investigating every gene and sequencing it individually can be expected to be time consuming and expensive. Exome sequencing as such is a revolutionary advance and a powerful strategy in understanding the genetic basis of Mendelian disorders by identifying the rare causative variants underlying rare mendelian phenotypes and perhaps complex traits as well. Since it was introduced, it has become a standard tool for many genomic researchers (Figure 1.7). It captures the highly interpretable coding region which represents 1% (about 38 Mb) of the whole human genome, so it is significantly less expensive than WGS and is faster to analyse as well. In addition, the majority (85%) of the reported genetic changes have been located in the exome (Wang et al., 2013). Though, non-coding variations cannot be detected, these are less likely to be pathogenic. WES has also been applied to cancer and complex disorders studies. The exome thus represents a highly-enriched region of the genome in which to search for variants with large effect sizes enabling deeper sequencing, which thus permits base calling with higher confidence levels. Furthermore, exome sequencing is more cost-effective compared to the WGS, as a much smaller region is sequenced, which in turn also means that the overall redundancy of information is greatly reduced and will overall be faster to analyse and easier

to manage. These features make exome a favourable option over WGS for studying mendelian inherited diseases (Chilamakuri et al., 2014).

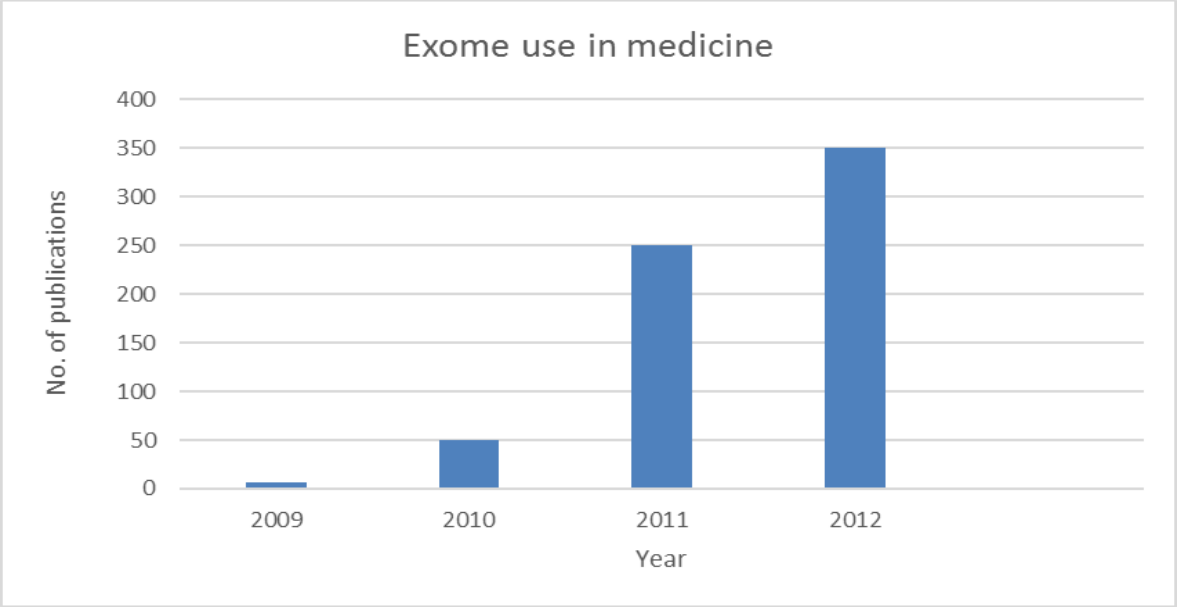


Figure 1.7 the published papers in medical research which applied exome sequencing in the period (2009-2012)

1.7.2.1b Main Principles of Exome Sequencing

In principle, the general concept of next generation sequencing including WES is very similar to the Sanger sequencing method in which DNA polymerase catalyses the incorporation of fluorescently labelled deoxynucleotides (dNTPs) during the extension of a new DNA strand. In each cycle, at the point of incorporation, the nucleotides are detected by a process called fluorophore excitation. The main difference is that NGS can run millions of extension reactions in a massively parallel way rather than sequencing for a single DNA fragment as happens in the case of Sanger sequencing (Illumina 2015). Practically, exome sequencing includes three basic steps: sample preparation, raw data processing, and data interpretation (figure 1.8). Currently, the most commonly used method in this respect is a hybridisation-based capture approach (Rykalina

et al 2014) and the most widely used commercial enrichment kits using this approach include Agilent, NimbleGen and Illumina; and despite the slight differences between each kit, the main principle is almost the same.

1.7.2.1b.i Library preparation and sequencing

Initially, genomic DNA is randomly fragmented into various lengths, followed by adapter ligation at both ends of the target sequence. However, these two steps can be combined into a single step (tagmentation) which is found to create a high efficient library preparation process. Normally a volume of 50ng/µl of genomic DNA should be enough to perform this step because significantly less DNA is lost at the fragmentation step. A massive collection of these adapter-ligated fragments can then finally construct the genomic library. The library is then loaded in a specific cell where fragments are captured by specific oligonucleotides. Each fragment can then be amplified by PCR and hybridised to biotinylated RNA baits, which is designed to target the coding portion of the genome. Using streptavidin coated magnetic beads, biotinylated RNA baits are captured and pulled down while the other unhybridized and intronic regions are washed away. As a result, the library only contains the exomic regions. This captured coding DNA is amplified to produce sequence ready enriched targets (Figure 1.9) (Rykalina et al., 2014). Illumina uses sequencing by synthesis (SBS) technology for generating the exome data. During each single sequencing cycle, a fluorescently labelled dNTP is added to DNA template strand to produce nucleotide label which ultimately terminates polymerization. Following each dNTPs incorporation, a fluorescent image is produced to detect the nucleotide base and then cleaved to be followed by a further incorporation of the next base because all the four reversible terminator-bound dNTPs (A,C,T,G) are present. This makes natural competition by which the bias is minimized and results eventually in highly accurate sequencing reads. A huge quantity of raw

data is produced which is technically received in a complex format, that in turn needs to be highly processed before final analysis and interpretation

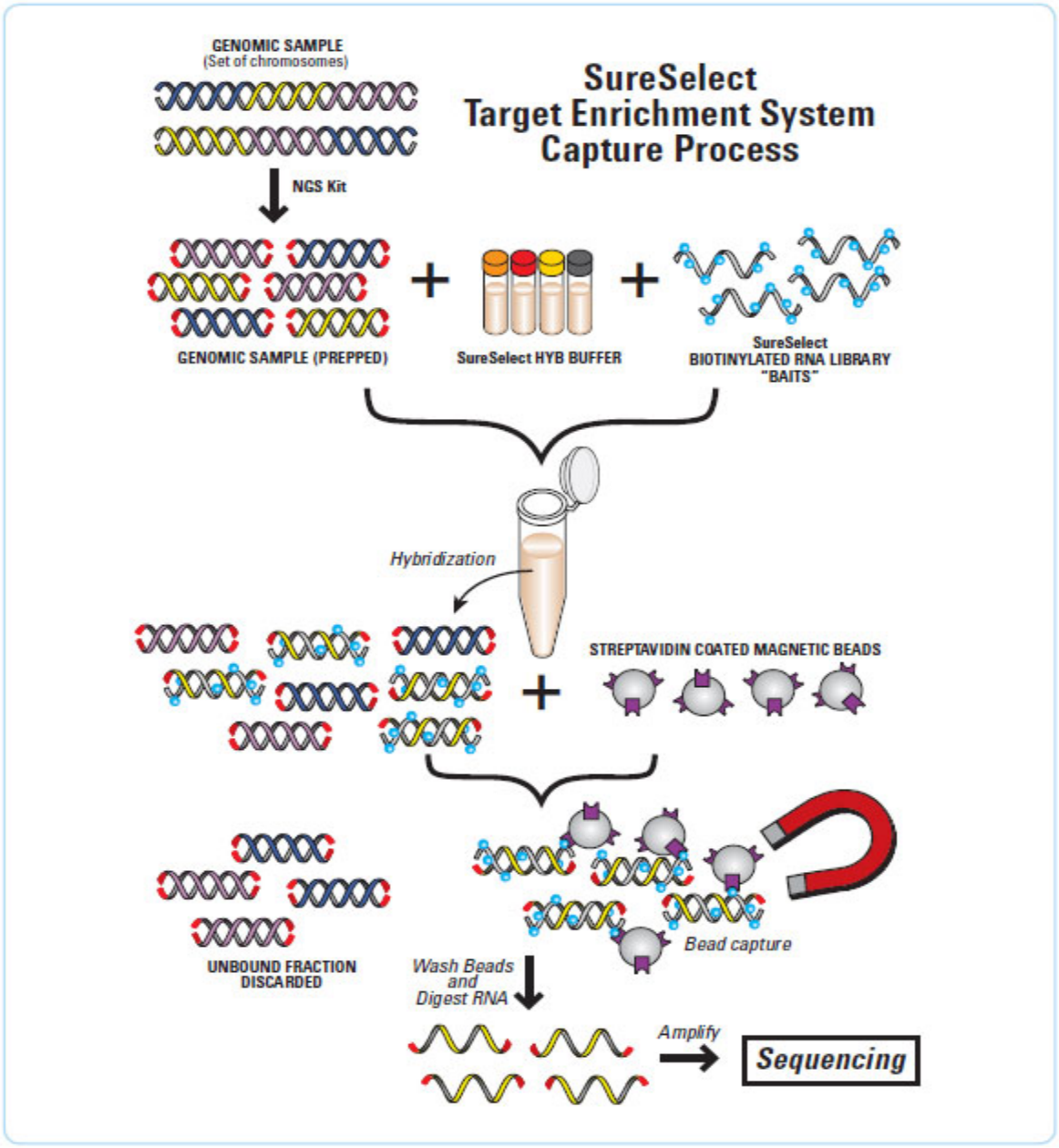


Figure 1.8 The Exome Sequencing Workflow

the diagram summarises the processes of library preparation, exome capture, target enrichment and sequencing; (the image as displayed at http://www.genomics.agilent.com).

1.7.2.1b.ii Raw Data Processing and interpretation

For large-scale data, several key stages need to be done for computationally intensive data processing**.** Most exome sequencing providers are supporting FASTQ format, a text-based format containing the biological sequence. Before aligning the sequence to the reference genome, the quality FASTQ files are checked using a FASTQC tool utilizing a Phred quality score, a common algorithm used to assess the accuracy of the sequence, which is used as a standard quality scoring tool for most commercial sequencing technologies. In addition, further information such as GC content, read length distribution and the amount of sequence duplication can be provided by FASTQC (Bao et al., 2014). The next step is the alignment against the reference genome to identify any sequence variations such as single nucleotide polymorphism (SNP), insertions and deletions (indel) etc. Many computing tools are used for this purpose to map short reads to the reference genome. A common example is Novoalign that is used by most next-generation sequencing (NGS) platforms, which was used in this study. Once they are aligned, SAMtools is used for converting SAMfiles (nearly 20-30Gb) into a compressed binary BAM file format (nearly 4-10Gb). At this stage, the aligned sequences are then checked against any duplicates and a realignment is performed for indels that may have resulted in sequencing slippage. The next step is variant calling and annotation using ANNOVAR, an efficient software that is incorporated to significant genomic databases such as dbSNP, 1000genomes, ClinVar, COSMIC and PolyPhen. Figure 1.9 shows the summary of the general raw data processing step.

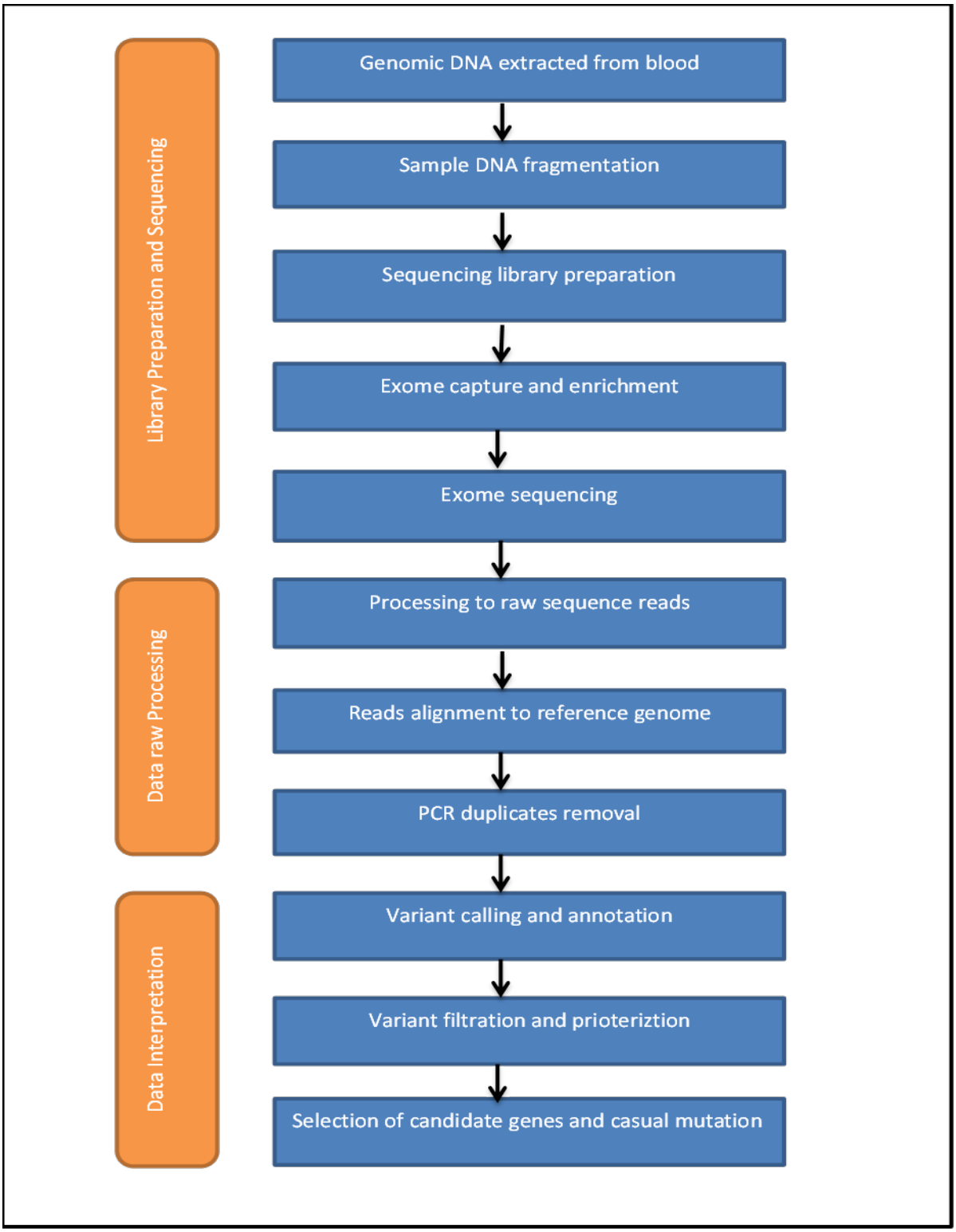


Figure 1.9 Summary of the Main Stages Involved in the Process of Whole Exome Sequencing

1.7.2.1c Major platforms for WES

Currently, there are three main exome capture kit providers: Agilent, NimbleGen and Illumina . The sample preparation methods are highly similar across these different technologies. As explained, Exome capture involves the capture of protein coding regions by hybridization of genomic DNA to biotinylated oligonucleotide probes (baits). The biotinylated DNA or RNA baits are complementary to targeted exons, which are hybridized to genomic fragment libraries. Magnetic streptavidin beads are used to selectively pull-down and enrich baits with bound targeted regions. The major differences between the technologies correspond to the choice of their respective target regions, bait lengths, bait density, molecules used for capture, and genome fragmentation method (Table 1.3).

Table 1.2 Comparison between the main platforms that used for exome sequencing technology

	NimblGen	Agilent	Illumina TruSeq	Illumina Nextera
Probe type	DNA	RNA/DNA	DNA	DNA
Probe size (bp)	55-105	114-126	95	95
Target Size (Mb)	64	50	62	62
Number of target exons	368,146	185,636	201071	201071
Reads remain after filtering (%)	66	71	54	40
Quality of coverage	Very high	Good	good for UTR & miRNAs	good for UTR & miRNAs
Species	Human, mouse & 3 plant species	Human, mouse & 3 plant species	Human	Human

1.7.2.1d the strategy of exome sequencing for Mendelian inherited disorders

Finding rare disease-causing alleles among such a huge number of exome variants is a major challenge when using exome sequencing to identify the novel disease genes in both Mendelian and complex disorders. Exome sequencing typically detects more than 20,000 single nucleotide variants; however, the vast majority (~95%) of these variants are non-pathogenic polymorphisms which are already present in the human public databases. Several factors are included in selecting the effective strategy for identifying the disease-causing rare variants against the exome background. These include: the expected pattern of inheritance according to the family pedigree or population structure; whether a phenotype is supposed to be caused by inherited or *de novo* variation; and the degree of locus heterogeneity for the trait. Therefore, the possible novelty for any suspected rare variants is assessed by filtering against the polymorphism database such as dbSNP and 1000 Genomes Project as well as in-house control databases. This step is expected to eliminate candidate variants down to nearly 2% of the total exome variants for each individual. Further filtering is used to reduce the candidate genes to keep only the high-priority candidates (if not only one) by identifying and selecting those genes circulating in the disease pathway and exclude the others.

This allows for the sequencing of only a modest number of affected individuals, and then applies discrete filtering to the data to reduce the number of candidate genes to a minimum number of high-priority candidates (if not to a single one). This strategy has been effectively applied for rare Mendelian disorders (Bamshad et al., 2011). Some limitations are involved in applying this method, however. First, a small number of pathogenic alleles might be contained within dbSNP which are filtered out by this method. Second, the increase in the number of sequenced exomes and genomes could affect minor allele frequency (MAF) and therefore truly pathogenic variants

that might segregate in the general population at low but appreciable frequencies might be mistakenly eliminated.

1.7.2.2 Targeted Exome Sequencing

As the NGS technology is rapidly advancing and because whole-genome and exome sequencing techniques are still expensive, targeted exome and gene panel capture have been recently developed to assess gene identification and genetic diagnosis. The gene multiple panels can be divided into two main categories i) panels which are designed to cover the associated genes for specific disorders and ii) those that are for wider categories of phenotypes or disorders ranging from nearly 10 genes up to hundreds or even thousands of genes (Zemojtel et al., 2014)

Generally, these targeted panels are characterized by high accuracy and deep coverage. Furthermore, this option is more cost effective compared to the broader approaches. In addition, the data size resulting from these targeted panels is smaller than the more comprehensive approaches such as the whole exome or whole genome sequencing; therefore, the data analysis will be much easier and faster as well. Illumina have developed different panels that target specific regions of the genome in any given sample. These focused panels are designed to cover a select set of genes or gene regions that have been associated with a specific phenotype or even number of phenotypes. A well-known example of these panels is the 'Trusight One' panel which contains more than 4,800 genes involved in the clinical phenotypes associated with inherited human disorders. This panel is characterized by covering the most commonly ordered molecular assays and it was applied in my project to investigate patients diagnosed with multiple pterygium syndrome or foetal akinesia (more details in Chapter 6)

Hence, by selecting this option, the focus has been shifted from the whole exome to enriched regions of the exome/genome that is relevant to human genetic diseases. This interpretable region can thus be referred to as the disease-associated genome (DAG). These multiple genes can be run across many samples in parallel rather than running multiple separate assays in order to both save time and reduce the associated cost. This panel was successfully used in many recent studies that have investigated the Mendelian disorders and it has shown a high diagnostic rate. For instance, in a recent Japanese study, they applied the Trusight One panel to examine 17 families with specific known diagnoses for different mendelian diseases such as Sotos syndrome, and Joubert syndrome to test the efficiency of this panel (Okazaki et al., 2016).

The main disadvantage of this targeted exome approach is that; it can only detect the mutations in genes previously implicated in the studied disease, thus no novel genes can be detected out of those involved genes. Therefore, if a mutation responsible for the examined disease is positioned in a novel gene, this approach cannot successfully expedite genetic diagnosis. In this case, whole exome/genome sequencing would be the better option since it is designed to include all the coding genes (either the previously implicated ones or even the novel ones). In the case of disease caused by chromosomal mutations such as structural rearrangement or large copy number variations the best approach to be applied is still the whole genome sequencing because neither whole exome nor the targeted exome would be able to detect these variants (Gorokhova et al., 2015).

In my project we applied the targeted clinical Trusight One panel to investigate 53 patients who were affected with foetal akinesia because of the high diagnostic rate of this panel as well as the reduced cost compared to the whole exome option as the number of patients in the study was large.

1.7.2.3 Sequencing depth and breadth of coverage

Genomic technologies are developed continuously with the focus on providing a highly qualified sequencing at a lower cost per base so that the high cost of great depth sequencing can be reduced as well. This reduction of the cost per run allows for increasing the number of sequenced exomes/genomes which ultimately increases the statistical power of disease-gene identification. The coverage represents the average number of times for each nucleotide base to be sequenced, while the breadth of coverage is related to the percentage of sequencing a targeted region of interest (Sims et al. 2014). Good coverage is important to eliminate any sequencing errors or false positive that could occur during the sequencing process, therefore, the more the read is sequenced the least error can happen, which leads to more accurate and reliable data. Many studies have focused on investigating the maximum number of variants that can be identified at a low coverage rate to enable researchers to access a highly accurate and efficient degree of sequencing data at the lowest cost. For example, a study published in 2008 identified that sequencing a human genome using short read methodology at a read depth of 15X, resulted in detecting all the homozygous single nucleotide variants while the same number of heterozygous SNVs were detected at a read depth of 33X (Bentley et al., 2008). Recently, it was reported that the read depth of 35X was sufficient to achieve highly covered reads and this has become the standard for many sequencing projects (Sims et al., 2014).

Although the cost of NGS has rapidly decreased WGS remains a costly option for analysing an individual sample. This disadvantage has made exome sequencing a preferable option for many researchers besides the other advantages already mentioned (such as the short time expended on analysis and less storage requirements). Analysing some data from the 1,000 genomes project showed that the use of WGS with the depth coverage of 20X across 95% of the consensus coding sequence (CCDS) exons required 200Gb of raw input sequencing (YRI and CEU samples) whereas similar percentage of targeted CCDS (~90% of the Agilent capture region and ~85% of the NimbleGen capture region) with similar depth required less than 20Gb of raw input from WES. Therefore, exome sequencing achieves at least the same efficiency as whole genome sequencing with 10-20 folds less raw sequence data (Parla et al., 2011). Solution based hybrid capture enrichment is the most preferred option by many genetic researchers. On the other hand, exome sequencing has few disadvantages that need to be addressed such as uniformity of coverage, target specificity and consistency.

1.8 Summary of genetic testing types and their applications in genetic diagnosis

NGS technology has made different types of tests available for the genetic diagnosis including single-gene tests, gene panel tests, exome sequencing and genome sequencing. Selecting the appropriate test is a big challenge to giving a successful genetic diagnosis. In fact, a review study published in 2015 has critically discussed the approach of selecting the best genetic test for the examined individual. According to the study, single-gene testing is mostly chosen in the case of a minimal locus heterogeneity with a clear clinical diagnosis based on distinctive clinical features. Also, an existing association between the diagnosed disease and a disease-causing gene needs to be already established. Gene panel testing is more cost-effective than a single-gene approach and it should be the suitable test for heterogeneous disorders with otherwise unclear

clinical diagnoses such as disorders with overlapping phenotypes or disorders which share specific manifestations but in which the overall phenotypes are different. Such disorders are generally associated with multiple genes.

Exome Sequencing (ES) and Genome Sequencing (GS) normally selected for the disorders with extreme heterogeneity and *de novo* mutations are the major mutation. Also, it is a good option in the case of a diagnosis which is very difficult to make or when there are at least two possible phenotypes for one patient. In addition, if there is no key phenotypic feature ~~is~~ present at the time of requesting the testing ~~so~~ the real underlying cause of the disease will be very difficult to identify. Examples of these disorders are Autism and Kabuki syndrome. ES was specifically selected in my project for studying foetal akinesia because of the high heterogeneity of the disorder plus because it has many overlapping phenotypes. It has an advantage over the gene panel in that it is less biased regarding which set of genes to test as the latter assumes that the abnormal clinical features are restricted to be associated within the included genes in the panel itself. Factors such as technical limitations involved in NGS technology, the risk of false positive especially for insertion or deletion and the weak coverage for a particular area make both ES or GS are not completely independent methods. Because of this for diagnosis, aCGH and Sanger sequencing are currently required as a complement method to cover the shortcomings of NGS in order to detect the full spectrum of mutations and to validate those findings which are identified by NGS approaches. In this way the differences and difficulties associated with the used technology, test interpretation, clinical significance, and ethical problems, need to be well considered by the clinician who selects the gene test because they ultimately affect the correct order and the diagnosis for the patient (Xue et al., 2015)

1.9 Exome sequencing for the studied disorders in this project

As discussed earlier, exome sequencing is well justified as an efficient approach to identify the genetic causes underlying rare Mendelian disorders. This proven efficiency is supported by a range of genetic evidences. First, many genetic mapping studies that have been applied on the coding part of the genome have successfully identified the candidate genetic region which contain the disease-causing variants of monogenic disorders. Second, the majority of pathogenic variants associated with Mendelian disorders are located within the exome and therefore they are more likely to disrupt the protein function. Third, it has been found that rare coding variants could have pathogenic effect in most rare disorders while the noncoding variants are less likely to have any negative effect even when located in a conservative area. Accordingly, it has become a standard tool to discover genes in these disorders.

A good example for these Mendelian disorders is the autosomal recessive disorders. Typically, the affected individual is most likely a homozygous carrier or in some cases is compound heterozygous with the pathogenic mutation while the parents are heterozygous carriers. Large families with multiple affected siblings compared with healthy individuals are the preferred subjects to carry out the genetic investigation for Mendelian disorders as they provide the opportunity to perform segregation analysis. This technique showed higher efficiency when it has been used in consanguineous families as their offspring are at high risk of carrying homozygous recessive mutations from a common ancestor. In 2009, the technique was applied to investigate Miller syndrome (OMIM263750), an autosomal recessive disorder, successfully identifying the causal variants in the *DHODH gene* (Ng et al., 2010). Furthermore, the technique was also performed to diagnose two affected siblings of a consanguineous Pakistani family diagnosed with congenital oligodontia, another rare autosomal recessive, utilising the SureSelect

Human all Exon 50 Mb kit. In the results, a homozygous stop codon was identified in both individuals in the SMOC2 gene, which encodes the SPARC related modular calcium binding 2 protein (OMIM # 607223) (Alfawaz et al., 2013). Another example for the efficiency of exome sequencing was a work published by Walsh and others at 2010, who applied exome sequencing together with homozygosity mapping in a consanguineous Palestinian family diagnosed with congenital deafness. A pathogenic mutation (p.Arg127*) was identified in the GPSM2 gene and was then reported as a cause of the disease in this particular family (Walsh et al., 2010).

Many other studies that have applied this method have produced promising results for a genetic diagnosis which can provide helpful information to the genetic counselling and diagnosis to both affected and unaffected carriers. Also, these disease-causing genes or disease variants can be used in identifying the risk identified by genetic screening tests such as pre-conceptive and prenatal screening. In addition, it can provide useful information which can be used in treatment approach.

1.10 Aim of the Project:

In my project, I wanted to investigate the role of DNA sequencing in two different examples of rare Autosomal recessive disorders; congenital oligodontia, and lethal multiple pterygium syndrome (LMPS)/Fetal Akinesia. The primary shared aim of my project was to identify disease-causing mutations of these particular phenotypes in view of the frequency of recessive diseases in the Saudi Population Furthermore I was particularly interested in consanguineous families likely to have recessively inherited cases of these phenotypes. Initially, I used autozygosity mapping and Sanger sequencing of single genes and then I proceeded to utilise NGS technique in particularly the targeted enriched methods (whole exome sequencing and clinical exome sequencing) to demonstrate the efficiency of this method in identifying the causing genes which underlie rare mendelian disorders.

Chapter Two: Materials & Methods

2.1 Acquisition of patients

2.1.1 Patients

In this project, I studied two different congenital disorders. In the first year I investigated Oligodontia patients while during the second and third year, I studied patients diagnosed with Fetal Akinesia/ Lethal Multiple Pterygium syndrome. Molecular genetic investigations were undertaken in each disorder in which the underlying genetic causes were unknown yet. In both examined disorders, number of patients from different families most of them were consanguineous have been involved and they were from a variety of ethnic backgrounds (Pakistani, North Indian, Bangladeshi and mixed European descent, Arabs and Turkish). Also few non consanguineous families were also involved. When available, DNA from unaffected family members was studied to check the segregation of interesting genetic variants. All of the families recruited in this project were initially patients seen that had been counseled by the West Midlands Clinical Genetics department. All of the clinical information available about these patients was therefore collected and provided by the West Midlands clinical genetics department

2.1.1.i Congenital Oligodontia

Patients affected with congenital oligodontia from Six different consanguineous families were selected for studying the genetic basis of this disorder. Peripheral blood was obtained from the probands, their parents and, where possible, affected and unaffected siblings. Aa clinical dentist who examined the panoramic radiographs in the Birmingham Dental Hospital confirmed the diagnoses of congenital oligodontia in these patients. Dental examinations revealed congenital oligodontia i.e. six or more missing teeth were reported in one affected individual from each family.. Medical diagnosis was confirmed by a clinical dentist via clinical examinations and panoramic radiographs in Birmingham Dental Hospital.

2.1.1.ii Fetal Akinesia

A total of 66 families that displayed clinical features of either multiple pterygium syndrome or fetal akinesia were selected for molecular genetic studies due to the phenotypic overlap between the two disorders. There was at least one individual in each of 36 families that displayed features consistent with non-syndromic FADS/LMPS without a known underlying genetic cause, while the remaining 30 families were diagnosed with the milder EVMPS phenotype. Consanguinity was recorded in nearly 50% of tested LMPS families and in only 20% of EVMPS families. Clinical information including family history, ethnicity and pregnancy history was collected from the families by clinical collaborators (Dr Julie Vogt, Professor Eamonn Maher and others). Relevant phenotypic information such as brain scans and muscle biopsy results were recorded where available.

2.1.2 Consent and ethics approval

Both studies have been approved by the South Birmingham Research Ethics Committee and the clinical research followed the principles outlined by the Declaration of Helsinki in 1964. An informed consent was obtained by the committee for all the families in all the studies in this project

2.1.3 DNA extractions

All genomic DNAs used in this project were kindly extracted by West Midlands Regional Genetics Molecular Genetics Laboratory mainly from blood samples, using Gentra System's Puregene DNA Purification method according to the manufacturer's instructions which based on salting-out precipitation. After cell lysis, the protein is precipitated, followed by DNA precipitation which is washed then with ethanol to be hydrated. Then, DNA will be ready to store at -80°C.

2.2 Materials:

2.2.1 Chemical Reagents

Reagents	Supplier
100bp DNA ladder	Invitrogen
1 kb DNA ladder	Invitrogen
1X TBE buffer (Tris-borate/EDTA)	Geneflow
Agarose	Bioline
BioMix RT Red	Bioline
CGRich Solution	Roche
DNAse, RNAse free water	Gibco Invitrogen
dNTPs	Roche
EDTA	Fisher Scientific
Ethanol	VWR chemicals
Ethidium Bromide	Sigma
FastStart Taq DNA polymerase	Roche
Genescan-500 LIZ size standard	Applied Biosystems
Hi-Di Formamide	Applied Biosystems
Magnesium Chloride (MgCl2)	Roche
Methanol	VWR chemicals
Micro CLEAN	Web Scientific
Primers	Invitrogen
Water, distilled (dH_2O)	

2.2 Kits

Kit	Supplier
BigDye Terminator Cycle Sequencing Kit V3.1	Applied Biosystems
BigDye 5X Sequencing Buffer	Applied Biosystems
Exosap IT Clean up Kit	Amersham Pharmacia

2.2.3 Other Materials

Material	Supplier
0.1 ml combitips pipette tips	Eppendorf
1.0 ml combitips pipette tips	Eppendorf
5.0 ml combitips pipette tips	Eppendorf

2.3 Molecular Genetic Investigations:

2.3.1 Sanger Sequencing of Candidate genes:

2.3.1a Standard Polymerase Chain Reaction (PCR)

Target DNA was amplified by using PCR in order to give thousands of identical copies (replicates) of a specific DNA sequence. Each standard PCR reaction contained a sample of patient DNA to be amplified, in addition to two oligonucleotide primers that were designed to be complementary to a specific genomic region of interest, Taq polymerase was also included in the reaction. Taq polymerase is isolated from Thermus aquaticus, a bacterium that usually lives in hot springs; its main function in the PCR reaction is to initiate replication, by building each single strand of the target DNA cut by the primers into a new, double-stranded DNA (Chien et al., 1976). In addition to these reaction components, deoxyribonucleic triphosphates (dNTPs) are

also included, as they are the building blocks needed to create a new DNA strand. The dNTPs added consist of an equal mixture of four dinucleotide triphosphates: dATP, dTTP, dCTP and dGTP. Finally, 10X PCR Buffer II and MgCl2 solution are also included in the raction in order to provide a suitable environment and versatility for the reaction.

Based on thermal cycling, each PCR consists of three main steps: denaturation, annealing and finally extension (elongation). Denaturation is achieved by heating the reaction to high temperatures (~95C'), which causes the disruption of the hydrogen bonds that hold the double strands of DNA together, resulting in the formation of single stranded DNA. The reaction temperature is then lowered to 50–65 °C, to allow the primers to anneal to the single-stranded DNA template. The ideal annealing temperature differs for each primer depending on a number of factors, including the CG% content of the primer sequence and the primer length. Taq polymerase enzyme normally works at temperatures of around 72 °C, which allows it to synthesize a new DNA strand which is complementary to the DNA template strand.

2.3.1b Primer Design:

Initially, primers were designed to bind to genomic sequences, which were approximately 20-80 nucleotides upstream or downstream from encoding exons. Primers were were designed to amplify all the coding exons of the targeted genes plus intron-exon boundaries. This was mostly achieved by using primer design programs such as ExonPrimer and Primer3 (https://ihg.gsf.de/ihg/ExonPrimer.html) after obtaining the genomic sequence of the gene of interest from Ensemble browser (http://www.ensembl.org/index.html). The length of the primers designed varied between 18-26bp. The annealing temperature for each primer was calculated manually using the following formula: (Annealing temperature = 60.9 + 0.41 x (%GC) –

600/primer length). Primers were always designed so that the final product size never exceeded 600bp in length, in order to obtain the optimum products for Sanger sequencing. Therefore, large exons (>600bp) were covered with at least two pairs of primers which overlapped to ensure all of the exon could be sequenced clearly. All primer sequences are available in the Appendix.

2.3.1c PCR conditions:

Standard conditions were used to amplify the DNA template in a 25µl master mix reaction. The conditions were as follow:

Reagent	**Volume**
2X BioMix Red*	12.5 µl
Forward primer (5.0pmol)	0.5 µl
Reverse primer (5.0pmol)	0.5 µl
dH2O	10.5 µl
DNA (20ng/µl)	1.0 µl

The BioMix Red contains: taq polymerase, dNTPS, buffer and 1.5mM MgCl2. For primers that contained a high GC content, 5µl of a solution known as 'GC rich solution' was added to the 25µl reaction. This required a reduction of 5µl of water to ensure a final reaction

Reagent	Volume
2X BioMix Red	12.5µl
GC rich solution	5µl
Forward primer (5.0pmol)	0.5µl
Reverse primer (5.0pmol)	0.5µl
dH2O	5.5µl
DNA (20ng/µl)	1µl

After preparing and gently mixing the master mix reaction, the PCR amplification was carried out using a Bio-tetrad or Bio-red thremal cycler using the following standard conditions.

Each PCR reaction performed included a negative control (in which DNA was replaced with dH2O) in order to ensure that no contamination detected in the reaction (Table 2.1a).

2.3.1d Touchdown PCR amplification

For some primer sets, a touchdown PCR had to performed in order to increase the specificity of the region amplified. The reaction steps were identical to the above standard PCR protocol, except the annealing temperature was initially set to a higher temperature (65°C). This initial high temperature increases the specificity of the primers. The temperature is then gradually reduced by 1°C each cycle for 10 cycles until it reaches the calculated optimum annealing temperature, or even a few degrees below, to maximise the yield of primer binding for producing a large amount of specifically amplified DNA (Table 2.1b).

Table 2.1 The designed program of standard PCR steps

This table shows the three main stages involved in PCR amplification; denaturation, annealing and extension plus an initialization step before denaturation and a final extension step after elongation, but these latter two steps are not repeated during the cycle. **A)** the annealing tempreture for the standard PCR is changeable according to the primer CG% by using specific calculation (ranges from 50°C-64°C) and the cycle repeated 35-40 times. **B)** In touchdown PCR, the annealing step started at high temperature and then decreased by 1°C every next cycle (10 times) and finally amplified 55°C for the remaining 25 cycles.

A)

Step	Temperature	Time	
initial denaturation	95°C	5 min	
Denaturation	95°C,	45 sec	**40 cycles**
Annealing	Tm (50°C-64°C)	45sec	
Extension	72°C	1min	
final extension	72°C	5 min.	

B)

Step	Temperature	Time	
initial denaturation	95°C	5 min	
Denaturation	95°C	45 sec	**10 cycles**
Annealing	65°C (-1°C every cycle)	45sec	
Extension	72°C	1min	
Denaturation	95°C,	45 sec	**25 cycles**
Annealing	55°C	45sec	
Extension	72°C	1min	
final extension	72°C	5 min.	

2.3.1e. Gel electrophoresis:

After PCR, the products were checked on a 1.0 % horizontal agarose gel, which separates fragments according to their molecular size. Visualising a single clear band at the correct size ensures that the DNA is successfully amplified without contamination. DNA has a negative charge; therefore, it should runs through the gel towards the positive anode.

To prepare a 1% agarose gel, 2 gm of agarose powder is weighed and added to 200 ml TBE buffer (10X). The mixture is boiled in a microwave for 3 minutes and then cooled under tap water, followed by the addition of 2 µl of ethidium bromide dye. Ethidium bromide is an intercalating substance that reacts with the DNA and enables it to fluorescence under UV light, therefore enabling the bands to be clearly visualized. The prepared gel mixture is poured into the cast, with toothed combs to create wells, and it is left to cool and harden. After cooling, 4µl of loading dye is mixed with 6µl of PCR product. The PCR-loading dye mix is added into the wells of the gel. A DNA size marker (100- bp ladder) is also mixed with 2 µl from loading dye and added to the first well. The gel is then run at 180V for 15-20 minutes. The electrophoresis is stopped before the dye runs off the end of the gel. The gel is then viewed under a UV transilluminator (LKB, UK). The gel can then be photographed using a gel documentation system (CCD camera).

2.3.2 DNA Purification method

2.3.2a Exosap method

Prior to Sanger sequencing, PCR products that have successfully shown a band under the UV light (working DNA) need to be cleaned up. This is done by using ExoSAP; a method used to remove unwanted dNTPs and primers, while maintaining the target sequence.. The ExoSAP kit

contains a variety of components, including Exonuclease I, Antarctic Phosphatase and Antarctic Phosphatase buffer.. In the experiment, for each 4µl of exosap, 6µl of PCR product was added and mixed gently with a pipette. The microtitre plate including the ExoSap-PCR product mix is then run on a tetrad or biometra PCR machine using a specific cleanup program, followed by an incubation step at 37°C for 15 min. Subsequent inactivation of enzymes was achieved by incubation at 80°C for 15 min.

2.3.2b Alternative method of clean-up (microCLEAN)

In most of the experiments in this thesis, the Exo-SAP-IT® PCR product clean up kit was used, however, a different method using a reagent known as microCLEAN was also used in a large number of the sequencing reactions. This was because microCLEAN was much cheaper and it gives the same quality of sequence.

The protocol for microCLEAN involved adding 3µl of PCR product with an equal volume of microCLEAN to a 96-well microtitre plate, and then incubated for few minutes at room temperature and centrifuged then for 40 minutes at 4000RPM. This creates a DNA pellet on the bottom of the well, while the supernatant contains all of the impurities. After centrifuging, the supernatant is removed by centrifuging the plate upside-down on tissue paper at 500RPM for 30 seconds. The slow speed enables the supernatant to be removed while maintaining the pellet at the bottom of the well.

2.3.3 Sanger Sequencing reaction

Sequencing was performed on whole genome amplified (WGA) DNA (acquired using Qiagen REPLI-g kits) in each PCR plate as well as normal DNA controls. All of the fragments were sequenced in both orientations (forward and reverse). Any candidate variants that were identified during analysis were confirmed using original stock DNA samples, in order to avoid

any potentially false positive results that may have occurred using WGA DNA. PCR products were sequenced using a standard BigDye Terminator v3.1 cycle sequencing method on an ABI 3730 automated genetic analyzer (Applied Biosystems). The data was processed by specific sequencing analysis software (i.e. Bioedit and/or mutation surveyor). Sequence traces for each amplified DNA sample was compared to a reference sequence obtained from the ENSEMBL database.

After the clean up step described in sections 2.3.2a and 2.3.2b, a 10µl reaction mixture is prepared with the following:

Two different master mixes are made; one for the forward primer and the other for the reverse one. 4µl of exosap product is added to 6µl of the forward reaction. Another 4µl of the same exosap product is taken and added to the reverse sequencing reaction. The reactions are put into a thermocycler using a PCR program with conditions specific for sequencing. The cycling conditions briefly involve a denaturing step for 3 minutes at 96°C, followed by an additional 30 cycles of 96°C for 30 seconds, 50°C for 15 seconds, and 60°C for 4 minutes.

After the sequencing run is complete, precipitation of the reaction is carried out. 1µl of precipitation buffer (including 250mM EDTA) is added into each well. Following this, 30µl of 100% Ethanol is added to each reaction. The plate is then centrifuged for 20 minutes at 2000rpm, which precipitates the DNA. The plate is then centrifuged upside-down in tissue paper at 400rpm/1minute, in order to remove the ethanol. Following this, 200µl of 70% ethanol is added into each reaction, and again centrifuged for 20 minutes at 4000rpm. The plate is then centrifuged once more, upside-down in tissue paper at 400rpm/1minute. Finally, 10µl of HiDi is

added to each well followed by a denaturing step is performed by heating the plate at 95°C for 5 minutes. Following denaturation, the plate is cooled by placing it on ice, which prevents the products from reannealing. At this point, the plate can be loaded on the ABI 3730 automated sequencer. The sequencing results can then be viewed using BioEdit software and Mutation Surveyor to analyse the sequence.

2.3.4. Mutational screening

Sequence traces from each of the DNA samples analysed was compared to a reference sequence obtained from the ENSEMBL database, using the Mutation Surveyor software. Any variants of interest identified were then confirmed in original stock DNA of the patient.

2.3.5 Linkage Studies

In this project, two main approaches were used to conduct linkage studies: SNP microarrays and microsatellite marker analysis

2.3.5a SNP genotyping:

Autozygosity mapping strategies were utilized to establish disease loci and identify novel disease-causing genes in consanguineous families. To perform this, a genome-wide linkage scan was undertaken in affected children and their unaffected siblings before this PhD started. This was carried out using the Affymetrix 250K Human SNP Array 5.0 (Affymetrix UK Ltd) in order to identify the shared common homozygous regions and/or genomic copy number variants. This SNP array technology allows the simultaneous genotyping of >500,000 single nucleotide polymorphisms (SNPs) for each individual's DNA. This SNP genotyping study was kindly performed by Louise Tee (a research laboratory technician) according to the manufacturer's instructions (Affymetrix GeneChip Human Mapping SNP 5.0 Assay Manual).

In brief, 250ng of genomic DNA was digested with Sty 1 and Nsp 1 restriction enzyme. The Sty and Nsp adaptor molecules were then ligated to the product using a DNA ligase enzyme. For each DNA sample, PCR reactions were set up (Sty-3 reactions and Nsp-4 reactions) using a universal PCR primer. Once amplified, PCR products were run on a 1.5% agarose gel according to molecular size (sizes ranged from 200-1100bp). After that, products were pooled and cleaned up using magnetic beads (Ampure). The amplified DNA was then fragmented into product sizes of 200bp or less in length. Following this step, the amplified products were labelled and underwent a hybridization step to the SNP 5.0 chip (Affymetrix) for 16-18 hours. The DNA arrays were then washed and stained using a fluidics station (Affymetrix) A fluidics station. An Affymetrix GeneChip Scanner 3000 with GCOS 1.3 software was used for scanning the chips. Data analysis was then performed utilizing GCOS v3.0.2 software to derive SNP genotypes, marker order and linear chromosomal location.

2.3.5b Microsatellite Markers:

2.3.5b.i the technique significance and primer design

Linkage analysis using microsatellite markers was, applied during the course of this study with varying conditions. The specific details can be found in the appropriate chapters (Chapters 3 and 4). Microsatellites are di, tri or tetra tandem nucleotide repeats of DNA ranging from few a repeats up to 50 times or more with known location in the genome. A set of polymorphic microsatellite markers with high heterozygosity rates were selected using NCBI, UCSC and Ensembl genome browsers which located within a candidate homozygous region. Linkage analysis was carried out using fluorescently labelled microsatellite markers and an ABI PRISM

3730 DNA Sequencer. For each marker, a mixture of two oligonucleotide primers (Forward and Reverse) had been used in order to amplify the target sequence. The primers are fluorescently labeled (5` HEX, TET or FAM) with different dyes; FAM (blue), HEX (yellow) and TET (green).

2.3.5b.ii PCR amplification

Stock primers for the markers were diluted with dH2O according to the manufacturer's instruction, and working dilutions were prepared at a 1:5 ratio; 20µl of the stock primer added to 80µl of dH2O. A reaction master mix of 10µl was prepared as follow:

Reagent	Volume
Forward primers (2.0pmol)	0.2µl
Reverse primers (2.0pmol)	0.2µl
Biomix	5µl
dH2O	2.6µl
DNA (20ng/µl)	2.0µl

PCR reactions were then carried out according to standard protocols. After running the PCRs on a tetrad, 120µl of water was added to each sample and mixed. A volume of 5µl of LIZ® standard was added to a 1ml tube of HiDi (if only running a half plate, only 2.5µl LIZ® size standard was added to 500µl HiDi), and mixed well. Aliquot 10µl of HiDi/size standard mix into each well in a fresh PCR plate. To the HiDi mix, 1µl of the diluted PCR product was added and mixed well. The plate was then denatured on a PCR block for 5mins at 95ºC and then placed onto ice to snap cool. The plate is then ready to run on the ABI PRISM 3730 DNA Analyzer (Applied Biosystems) under Genescan program (not a sequencing run).

Finally, the data obtained from the microsatellite products can be processed by GENEMAPPER v3.0 software.

2.3.6 Next Generation Sequencing (NGS)

2.3.6a Whole exome sequencing

All whole exome sequencing data was provided by Prof. Eamonn Maher. Agilent SureSelect Whole Exome hybrid capture was used to enrich these fragments for exomic sequences. For each individual exome, nearly 25,000-30,000 variants were detected and have been analyzed and screened using a specific strategy outlined in figure 2.1. In addition to whole exome sequencing, the Trusight One panel kit (covering 4813 genes in total) was also used to acquire data in this project. The reference genome hg18 was used for alignment. The SamTools software was applied to sort through SNPs and small insertion deletions, and sequence variations. These were screened against the 1000 Genomes Project and dbSNP131 to filter out common variants that are already present at high frequencies in dbSNP131 or the 1000 Genomes project database in order to highlight the novel variants which could be pathogenic (Ostergaard et al., 2011). Any variant found on public databases with a frequency of > 1% in the population was considered common, and therefore likely to be benign, and was therefore excluded. Also, synonymous variants that result in the same amino acid product were excluded. The variations considered to be most interesting were nonsense variants, frameshift and splice site variants because they are expected to cause the most damage in the protein function and/or structure. For splicing variants, any variant that was > 2bp from the exon-intron junction was excluded, as these were less likely to affect splicing. This resulted in a list of candidate variants ranging from (10-50 variations). Any novel variants that were present in candidate genes of interest, known to play a role in relevant disease pathways were selected for further analysis. However, we expect one causing gene per

each family. As a supportive tool in selecting candidate genes, prioritization software such as Toppgene software was used, which highlights the most relevant candidate genes according to a number of pathological considerations for each studied disorder.

2.3.6b Assessment of Mutation Pathogenicity

A few important steps have been followed to interpret the probability of pathogenicity of the detected variants. (a) The prediction of possible effects of any amino acid substitution was achieved with specific bioinformatics tools such as PolyPhen-2 and SIFTS tools (e.g. putative missense mutations). These tools mainly predict the consequences of amino acid substitutions based on the levels of conservation of the region affected and whether the change has any occurred within an important functional domain. (b) Identifying if candidate genes are involved in relevant disease-associated pathways. For instance, for a disease that affects bone, a good starting point is to assess the genes involved in bone development and/or additional members of developmental pathways. . This can be achieved using genomic browsers (Ensembl, GeneDistiller, Gene cards, OMIM, PubMed, UCSC and others). (c) Information available from the literature (Mouse Genome Informatics, PubMed), in order to identify if the candidate genes have been associated with similar phenotypes in any animal models. (d) Checking segregation in parents and other family members using Sanger sequencing. (e) Further frequency information in population-based controls for the candidate variants was sought from NHLBI Exome variant server [http://evs.gs.washington.edu/EVS/] if available.

2.3.6c Definitions of the predictive bioinformatics tools

2.3.6c.i Polymorphism Phenotyping v2 (PolyPhen-2)

Polyphen-2 (http://genetics.bwh.harvard.edu/pph2) is a computational analysis tool for

Predicting the pathogenesis of missense variants on proteins. It uses a combination of two parameters; protein structure and protein function, to calculate the score of pathogenicity.Scores range from 0 (predicted to be benign) to 1 (predicted to be probably damaging).

2.3.6c.ii Sorting intolerant from tolerant (SIFT):

SIFT is a further bioinformatical tool (http://sift.jcvi.org/) used as a supportive tool beside Polyphen to predicts whether an amino acid substitution affects protein function or not, based on the degree of conservation of amino acid residues in sequence alignments derived from closely related sequences, collected through PSI-BLAST. It detes whether the variation tolerant or not.

2.3.3c.iii. Splice site prediction by Neural Network

This is an in silico method (http://www.fruitfly.org) to predict the effect of a splice-site mutation on gene splicing and proteins.

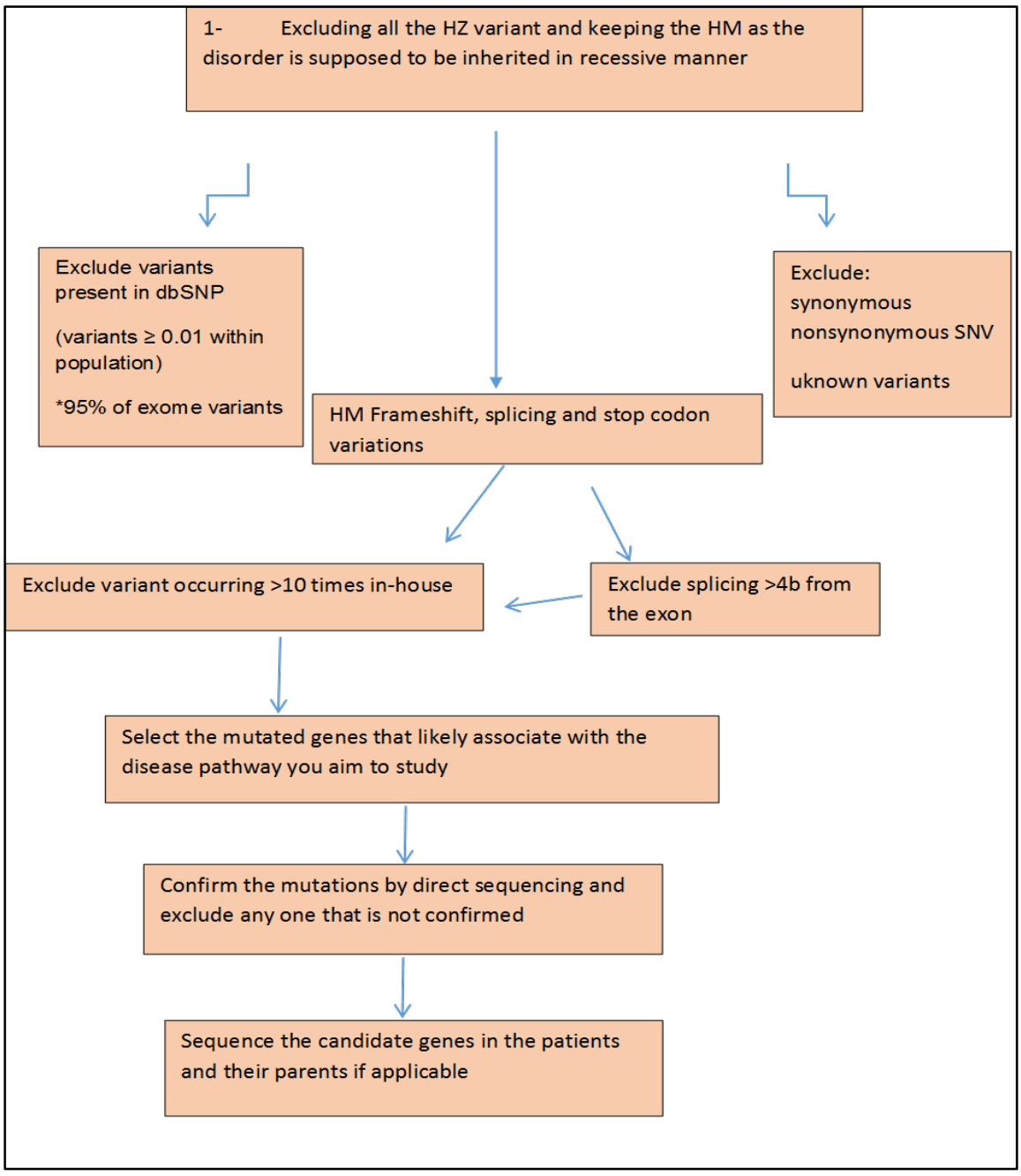


Figure 2.1 the filtering exome strategy to identify the disease causing variants

Table 2.2 Web based resources.

1000 Genomes	http://www.1000genomes.org
dbSNP	http://www.ncbi.nlm.nih.gov./projects/SNP
Ensembl	http://www.ensembl.org
NCBI	http://www.ncbi.nlm.nih.gov
NCBI Nucleotide	http://www.ncbi.nlm.nih.gov/sites/nuccore
UCSC	http://www.genome.ucsc.edu
Gene cards	http://www.genecards.org
PubMed	http://www.ncbi.nlm.nih.gov./pubmed
OMIM	http://www.ncbi.nlm.nih.gov/sites/entrez?db=OMIM
ExonPrimer	http://ihg2.helmholtz-muenchen.de/ihg/ExonPrimer.html
BLAST	http://blast.ncbi.nlm.nih.gov/Blast.cgi
Primer3	http://frodo.wi.mit.edu/primer3
DECIPHER	https://decipher.sanger.ac.uk/application
Mouse Genome Informatics	http://www.informatics.jax.org
Gene Distiller 2	http://www.genedistiller.org
PolyPhen-2	http://genetics.bwh.harvard.edu/pph2
SIFT	http://sift.jcvi.org
Splice Site Prediction by Neural Network	http://www.fruitfly.org/seq_tools/splice.html
Homozygosity Mapper	http://www.homozygositymapper.org

Chapter Three: Molecular Genetic Investigation of inherited Oligodontia

3.1 Disease background

The development of human teeth (known as odontogenesis) is a long, well-organized and complex process. It is regulated by specific interactions between epithelial cells and neural crest-derived mesenchymal tissues (THESLEFF and HURMERINTA, 1981). The process starts prenatally after the first month of embryogenesis and continues through childhood before being completed at some stage in adolescence (18-25 years) when the third molars appear (Aiello et al., 1991, Nieminen, 2009). Congenital teeth agenesis is the most common form of abnormal teeth development in humans and may be caused by inherited disorders or environmental factors such as local trauma, chemical radiation and chemotherapy (De Coster et al., 2009). Genetic disorders of ontogenesis are important to study and are the subject of this Results Chapter.

Based on previous reports, the prevalence of dental agenesis is about 6.76% across different populations (Bozga et al., 2014)

3.1.1 Tooth developmental anomalies

Though much advancement has been made in understanding the developmental basis of tooth formation, the mechanism of dental development and the entire molecular basis of inherited tooth agenesis is still relatively unclear (Cobourne, 2007). Genetic tooth abnormalities normally studied using three main ways. First, the clinical features of the anomaly such as number of teeth, shape, or both. For instance, the presence of extra teeth (hyperdontia), the lack or reduction in the teeth (tooth agenesis), however, the shape of teeth can be abnormal such as taurodontism abnormality (enlargement of the body and pulp of the tooth). Second, determining whether the teeth abnormality is part of another condition (syndromic) or isolated trait (non-syndromic). Third, knowing the mode of inheritance by studying the family pedigree e.g. autosomal dominant or recessive manner (Klein et al., 2013). Clinically, tooth agenesis is classified into three main

numeric teeth developmental abnormalities: i) the loss of one up to six teeth (excluding the third molar) is called as hypodontia (2-10% frequency). ii) Oligodontia is diagnosed when the patient has an agenesis of more than six teeth (excluding the third molar) (0.1-1% frequency) iii) Anodontia, where the patient has complete absence of teeth and this type is extremely rare (Goya et al., 2008).

According to many researchers, it was noted that the prevalence of hypodontia is relatively higher amongst females compared to males , with a ratio of 3:2 and no reasons have been provided yet (Brook, 1975).

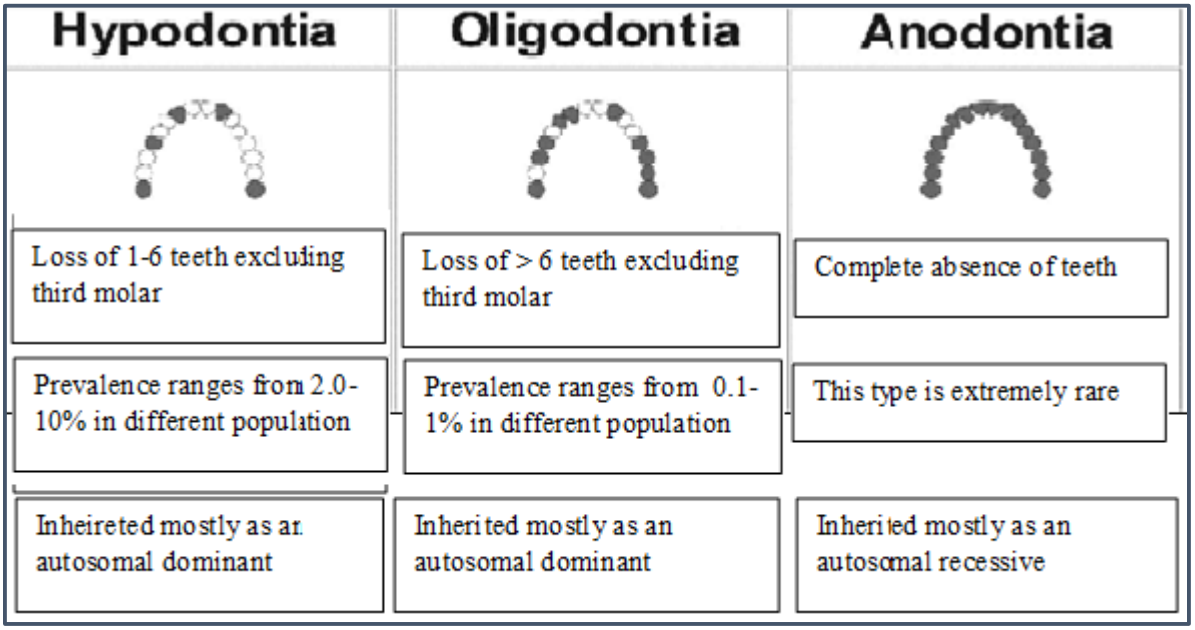


Figure 3.1 The main three phenotypes of tooth agenesis

with their prevalence and the form of inheritance for each different phenotype

Hypodontia can either occur as a part of another genetic disease, or as an isolated non-syndromic feature, and this form is usually familial syndrome which occurs in a family fashion, but it can affect sporadic cases sometimes.). Online Mendelian Inheritance in Man (OMIM) lists more than

60 different syndromic disorders that included hypodontia phenotype as part of other clinical phenotypes spectrum of anomalies. A lot of candidate genes have been identified in both types (Cobourne, 2007, Klein et al., 2013).

3.1.2 The known causing genes of tooth Agenesis

The early stages of dental development are mostly similar in human and mouse, and the basic findings that discovered by investigating mice models have been confirmed in humans as well. Therefore, studying the dental mice genetics were very helpful approach to understand the mechanism of dental development and the genes involved in the process using several knockouts techniques. By knocking out *PAX9, MSX1, PITX2, GLI2/3, P63* genes, Tooth development was stopped at the bud stage, while when Dlx1/2 gene was knocked out, only maxillary molars were lost (Matalova et al., 2008, Fleischmannova et al., 2008). More than 200 genes have been implicated in tooth development (Jernvall and Thesleff, 2000) but the most common genes that have been associated with dental agenesis are *PAX9*, *MSX1*, *WNT10A* and *AXIN2* genes. Mutations in these particular genes were mostly associated with the familial non-syndromic hypodontia while other genes such as *EDA, EDARADD, NEMO, P63* and others were reported in sybdromic hypodontia (Shimizu and Maeda, 2009). Most disorders of odontogenesis are inherited in an autosomal dominant manner (Larmour et al., 2005) though autosomal recessive and X-linked patterns can exist in some cases (Cobourne, 2007) In this project, I primarily focused on potential autosomal recessive causes within consanguineous families and screened specific associated genes and carried out a linkage analysis to determine potential evidence for novel causing genes.

Table 3.1 Summary of the detected mutations and the included patients identified with non-syndromic Oligodontia

according to a systematic literature review published in 2013 (Ruf et al., 2013a).

Identified gene	Mutations (no)	Patients (n)	References (no)
PAX9	33	93	24
MSX1	12	33	9
EDA	10	51	9
AXIN2	6	17	3
Put/PAX9	1	7	1
EDARADD	1	1	1
NEMO	1	1	1
KRT17	1	1	1

3.1.3 LTBP3 as a possible cause of Oligodontia

Recently, LTBP3, a gene encodes latent transforming growth factor-b (TGF-b) binding protein-3, was reported in a consanguineous Pakistani family with multiple affected members who presented with congenital oligodontia associated with short stature. According to the published paper, both phenotypes appear to be inherited in an autosomal-recessive manner. After the exclusion of MSX1 and PAX9 by direct sequencing, SNP genotyping technique was applied to analyse DNA samples for four affected individuals and one unaffected individual using Affymetrix GeneChip Mapping 500K array that allows genotyping ~260,000 SNPs. In the results, a large homozygous area of between 41.381 and 69.263 Mb (UCSC May 2004) located on chromosome 11 was identified and shared between the four affected individuals but not the unaffected individual. Further analysis using Microsatellite markers for all eight available family

members (mother and seven of her offspring) across this region was carried out which confirmed the shared autozygous region. Then many genes from the identified region were analysed by direct sequencing of genomic DNA based on their known function and expression profiles e.g. *FGF4, FGF19, FIBP, LRP5, EHD1, FOSL1,* and *LTBP3*. Amongst all the analysed genes, they only identified a stop codon variant c.2322C > G, resulting in the nonsense mutation Y744X within LTBP3 gene. However, screening 240 unrelated unaffected individuals of Pakistani origin did not show this mutation (Noor et al., 2009)

3.2 Method

3.2.1 Patients and Clinical assessment

Fourteen cases of non-syndromic oligodontia belonging to six different families were ascertained in order to study the genetic basis, which might cause the disease for each case. All the ascertained families were consanguineous and were all of Pakistani origin (Table 3.2). All of the affected subjects were diagnosed with severe hyopdontia/oligodontia at the time of diagnosis. All patients and their parents gave written informed consent and the study was approved by the Research Ethics Committees.

A Specialist Registrar in Orthodontics (Dr Joyti Vasudev) undertook full dental and medical assessment. A family pedigree was recorded for each case and indicated potential autosomal recessively inherited manner. Blood samples were taken from affected individuals and the available parents, and then sent to the West Midlands Regional Genetics Laboratory in Birmingham Women's Hospital for DNA extraction.

Table 3.2 Oligodontia families, which are included in our study to study their genetic background

Family	Phenotype	Origin	Consanguinity	Affected individuals	available Parents
OD001	NS Oligodontia	Pakistani	Yes	1	mother
OD002	NS Oligodontia	Pakistani	Yes	2	Father
OD003	NS Oligodontia	Pakistani	Yes	1	Mother
OD004	NS Oligodontia	Pakistani	Yes	1	Both
OD005	NS Oligodontia	Pakistani	Yes	1	Both
OD006	NS Oligodontia	Pakistani	Yes	8	Both

Apart from the pedigree of the large family (OD006) in figure 5.1, Dr Joyti Vasudev wrote all the clinical information and the pedigree for each single studied family are available to access in a previous dissertation in 2014.

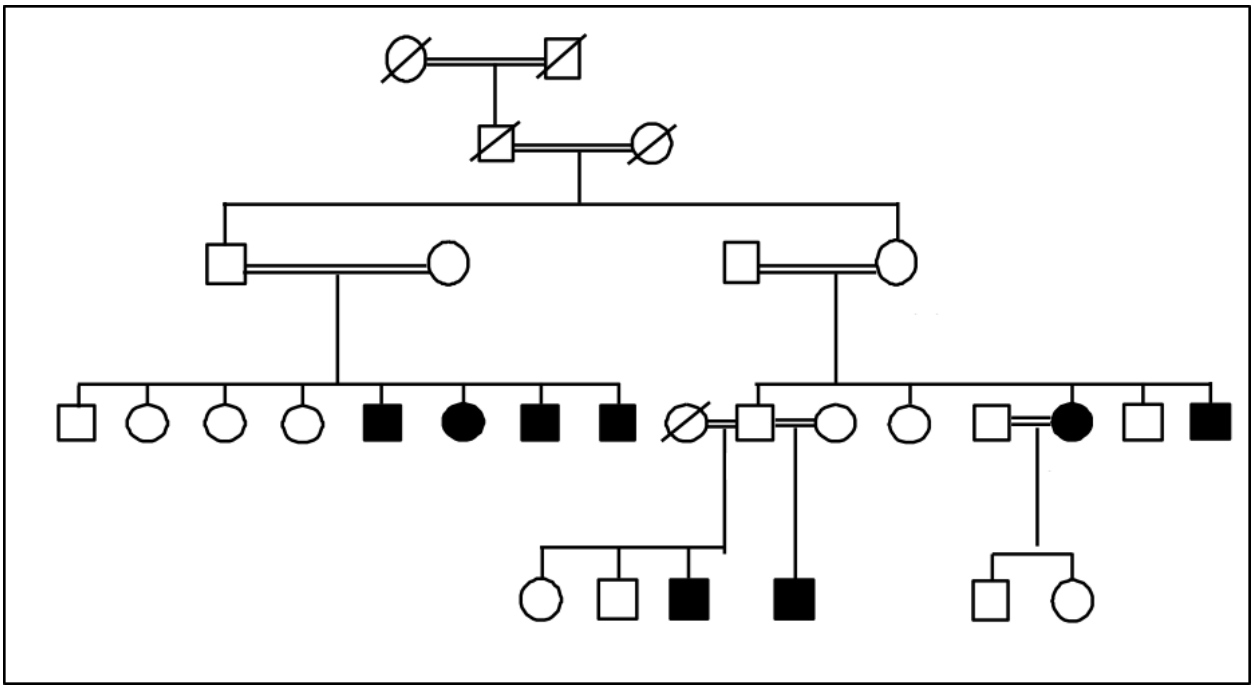


Figure 3.2 the pedigree of members of family OD006

with 8 affected individuals and showing autosomal recessive manner.

3.2.2 Genetic Analysis:

3.2.2a Sequencing the candidate genes:

Three known candidate genes (*MSX1, PAX9* and *WNT10A*) were highly suggested for sequencing based on the literature review that prove their significant role in dental development and their association with non-syndromic oligodontia/severe hypodontia (see Table 3.3). *PAX9* and *MSX1* gene products play a significant role in the development process of teeth and several studies have reported mutations in these genes in patients suffering with hyopdontia/oligodontia (Peters and Balling, 1999) while mutations in the *WNT10A* gene were also reported to be strongly associated with isolated hyopdontia in a further study (van den Boogaard et al., 2012). Though inherited mutations in *MSX1*, *PAX9* and *WNT10A* had previously been associated with

autosomal dominant forms of oligodontia, biallelic mutations in *WNT10A* were subsequently described with more severe disease, and it was decided to investigate whether these three genes might also be implicated in the presumed autosomal recessively inherited forms that present in these consanguineous cases.

Table 3.3 mapping and transcript details of the screened genes (obtained from Ensembl)

Gene	Transcript ID	Location	Number of exons
PAX9	NM_006194	Chr14q13.3	4 coding exons
MSX1	NM_002448	Chr4p16.2	2 coding exons
WNT10A	NM_025216	Chr2q35	4 coding exons

I carried out sequence analysis of all the affected probands and their available unaffected parents to identify causative mutations in these genes. The primers were designed to cover all the coding exons and the exon-intron boundaries for each examined gene (Appendix). For all the genomic DNAs, amplification was done using PCR and the amplified products were sequenced after that using 3730 DNA Analyzer using direct Sanger methodology. Sequencing results were compared to the normal reference gene obtained from human Ensembl database or UCSC genome browsers.

3.2.2b Linkage analysis using microsatellites:

When no mutations could be identified in the selected genes (*MSX1*, *PAX9* and *WNT10A*), I undertook linkage analysis to determine if it was possible to exclude linkage to candidate genes for autosomal recessive forms of oligodontia in OD001-OD005. Thus a set of microsatellite markers close to *LTBP3* were genotyped in all available family members.

Table 3.4 Microsatellite markers used for linkage analysis of the homozygous regions in oligodontia families

Gene/Marker	Location	Start	End	Min size	Max size	color
D11S4191	Chr 11	59756135	59756421	111	135	FAM
D11S4076	Chr 11	61119711	61119869	151	163	FAM
D11S1883	Chr 11	63130300	63130583	224	266	FAM
D11S913	Chr 11	65692737	65693068	220	227	FAM
D11S1889	Chr 11	67069719	67069901	183	207	FAM
D11S4136	Chr 11	69324867	69325101	160	202	FAM

By applying this technique, I aimed to check for any shared homozygosity close to *LTBP3*. If homozygosity was detected, then I would proceed to direct sequencing of *LTBP3*. For each microsatellite marker, a mixture of two oligonucleotide primers (Forward and Reverse) has been used in order to amplify the target sequence. The primers were fluorescently labeled (5` HEX, TET or FAM) and the PCR then was carried out according to standard protocols. Microsatellite products were loaded on ABI PRISM 3730 DNA Analyzer (Applied Biosystems) under the genescan protocol and data was processed by GENEMAPPER v3.0 software.

3.3 Results

3.3.1 Mutation analysis:

Apart from detecting benign polymorphisms (P>0.01) which unlikely to cause the disorder, the screen mutation analysis for the three candidate genes (*MSX1, PAX9 or WNT10A*) did not reveal any candidate mutation or plausible candidate rare variants within the coding regions or at exon–intron junctions in any of the selected genes in the 14 affected individuals or the available

parents. According to these negative findings, we could not confirm any association of these genes in causing oligodontia in these families. The next stage of the project was to undertake linkage analysis to see if linkage to the candidate gene *LTBP3* could be excluded.

3.3.2 Linkage Analysis:

Mapping analysis was conducted for all the oligodontic patients and their parents in order to determine possible linkage of *LTBP3* gene. The analysis was performed with six microsatellite markers which are located across the genomic region on chromosome 11 (59,756,135 to 69,325,101) which containing *LTBP3* gene (from 65,306,030 to 65325699 according to UCSC databases. As shown below (figure 3.3&3.4), there was no evidence of homozygosity by descent linked to the targeted gene. Amongst all the patients of the five families, only one proband of family 5 has showed homozygosity close to the *LTBP3* gene - an ~ 8Mb homozygous region was defined by five microsatellites markers (D11S4191, D11S4076 D11S1883, D11S913 and D11S1889). Though linkage to *LTBP3* could not be excluded in this family it was decided not to proceed with direct sequencing of *LTBP3* as linkage was excluded in the other four families and there remained the possibility that the region of homozygosity in family 5 was a chance finding.

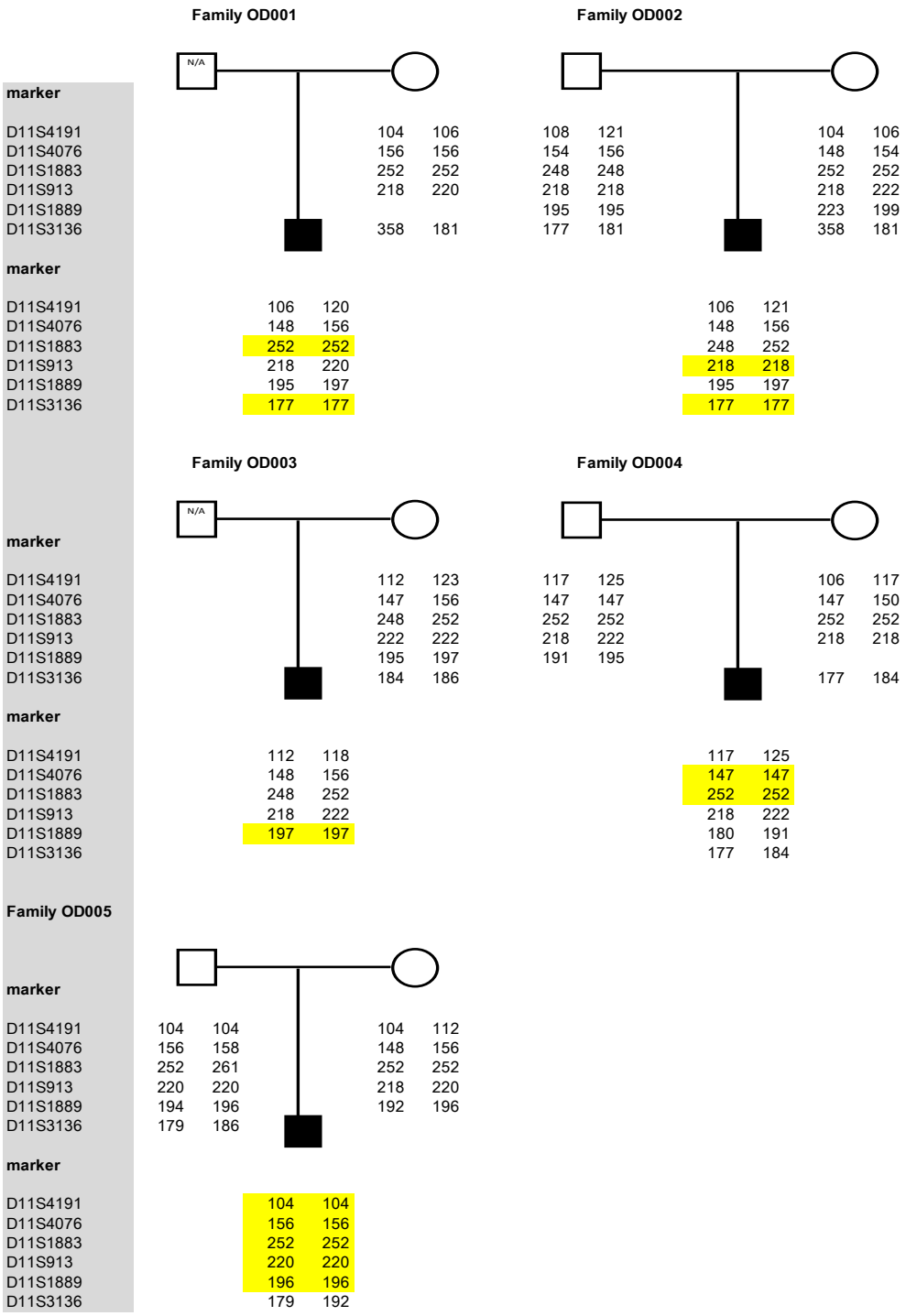


Figure 3.3 Microsatellite analysis for Oligodontia examined families

The affected children are shaded in black. LTBP3 gene is located in the region between 65306030 and 65325699.Microsatellite markers are organised according to their physical distance (MB). Haplotypes for these markers for the 5 examined families are shown and the homozygous region is shaded in yellow. Only this family (OD005) showed homozygosity in 5 out of 6 microsatellites which is the same region the gene is contained.

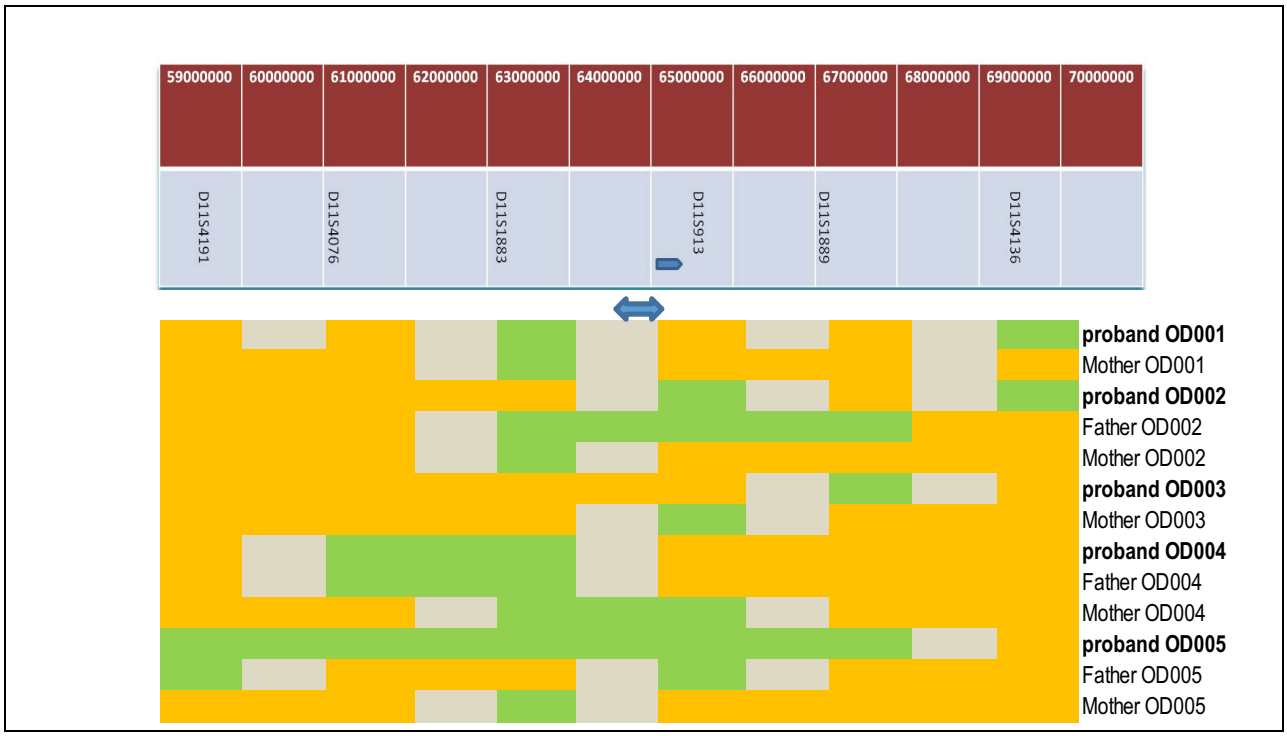


Figure 3.4 Drawings illustrating the autozygous linked region for Oligodontia family OD05

The green coloured regions represent homozygosity; the blue arrow indicate the location of LTBP3 gene. Only subject 4 (Father of F2) and subject 11 (Proband of F5) have shown homozygosity.

3.4.3 Whole Exome Sequencing (WES)

For genetically heterogeneous conditions such as oligodontia analyses by Sanger sequencing of all the candidate genes individually is a time consuming and labour intensive process. I found no evidence of coding sequencing mutations *PAX9, MSX1 and WNT10A* by direct sequencing and excluded *LTBP3* in most cases by microsatellite genotyping due to the absence of homozygous region except in one family (OD005).

Though direct sequencing does not detect exon deletions/duplications it was decided that further analysis of *LTBP3, PAX9, MSX1 and WNT10A* would be less effective than a NGS techniques such as whole exome sequencing (WES). Hence ~3 µg of genomic DNA from one of the

affected individuals of the OD006 family was sent to Kings College London (KCL) for singleton exome sequencing (March 2013).

3.3.3a Whole exome sequencing (WES) at King's College London (KCL)

The scientists at KCL (directed by Professor Michael Simpson) used the 50Mb Agilent SureSelect enrichment kits to target the coding regions of most human genes and then proceeded to NGS. After several months, the raw exome data was available and annotated at KCL using genomic databases, software prediction tools and their own in-house database. The annotation of the variants with respect to genes and transcripts was done by the help of the Annovar tool. They annotated the variants as SNP if it found on the dbSNP or a novel variant if it is not already published and not presents in the in-house genomic database. All the variants that caused no change in the amino acids of the corresponding protein were reported as synonymous and filtered out in the next stage. Also they annotated whether the variant is heterozygous or homozygous. A total of 25,034 variants were identified. All this exome data was provided to me in an excel spreadsheet (Microsoft)

3.3.3b Analysing WES data to identify the causing gene

From the large amount of WES data provided by KCL, I created an Excel file to analyze all the variants using specific filtering strategy. The first step was excluding all the common variants with a high minor allele frequency (MAF). It was reported in many studies that MAF ≥5% is considered common, while MAF between 1-5% is defined as low frequency variants, and when the variant has a MAF <1% will be considered as a rare variant (Genome of the Netherlands, 2014). Also I filtered out all the synonymous variants and (SNPs) that already present in dbSNP131 or the 1000 Genomes project database with a frequency of > 1% in the population

and this highly reduced the number of variants from total 25,034 variants to, only 2,856 variants and I only focused one the nonsynonymous pathogenic variants that might have a significant effect on the protein level such as stop codon, frameshift and splicing site changes. Looking into the rare and functional variants reduced the number from 2856 to 337 variants in 74 genes (Appendix). As the affected proband was from a consanguineous family and because the family pedigree was consistent with autosomal recessive inheritance, I concentrated on the homozygous variants and this reduced the number of variants down to 21. At this stage, the remaining variants were assessed to determine if they were within a gene that had previously been associated with oligodontia, hypodontia or dental abnormalities (e.g. *PAX9 , MSX1 , EDA , AXIN2 , Put/PAX9 1 , EDARADD , NEMO , KRT17* (Ruf et al., 2013b) *WNT10A, IRF6,TGFA, , FGR1 ,EDAR* (Galluccio et al., 2012, Cobourne and Sharpe, 2013), *HYD2 (Ahmad et al., 1998) STHAG5* and *LTBP3*). However none of the variants were within these genes.

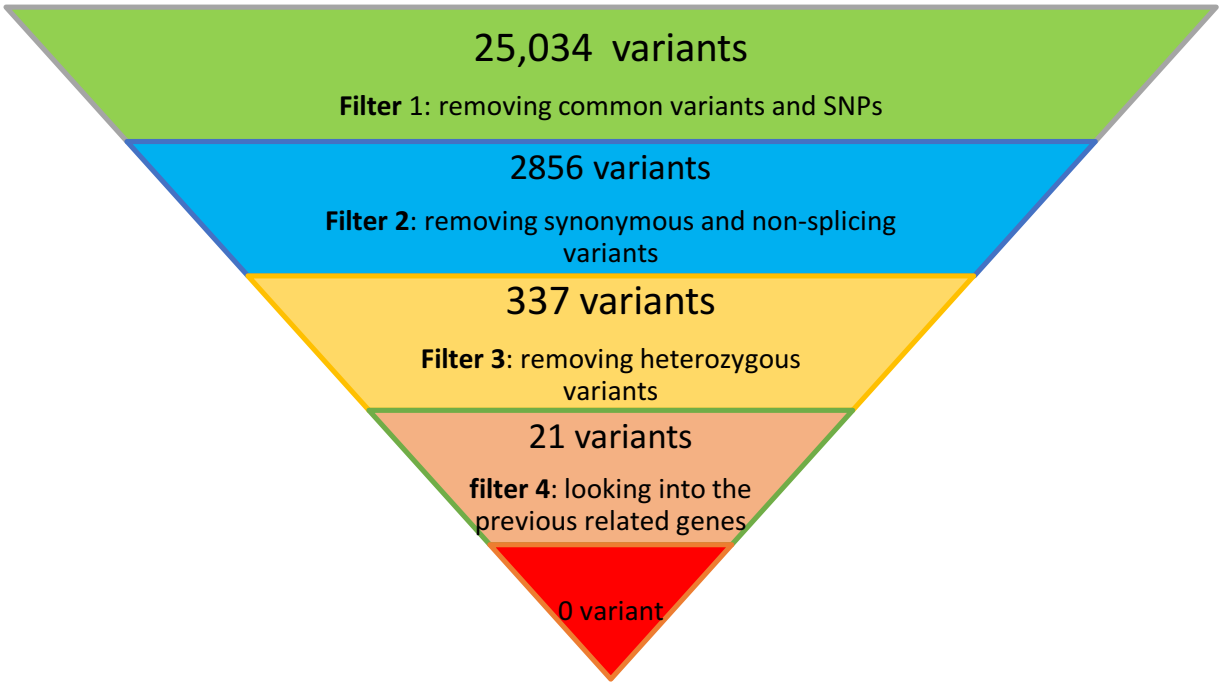


Figure 3.5 Filtering criteria Applied to identify the causing gene from Exome Sequencing Data for Oligodontic patient

3.4 Discussion:

3.4.1 The selection of candidate genes for sequencing

Based on large studies investigating the genetics of tooth agenesis, oligodontia is genetically heterogeneous condition. The marked phenotypic variability in this condition could result from defect in various genes implicated in the disease and also the distribution of mutations within individual genes (i.e. locus and/or allelic heterogeneity). (Mostowska et al., 2003, Hu et al., 1998, Nieminen, 2009).

The *PAX9* gene is a member of the paired box (PAX) family. It contains a paired box domain, an octapeptide and encodes for transcription factors during embryogenesis and fetal development. It maps to chromosome 14q12 and consists of 4 exons. It is believed that PAX9 plays a significant role during the process of odontogenesis by taking part in the signalling interactions between

epithelial cells and mesenchymal cell layers. A published study discovered mutations within PAX9 gene which might cause isolated hypodontia (Das et al., 2003). Also, two more studies identified frameshift and missense mutations amongst patients affected with tooth agenesis and particularly congenital oligodontia (Lammi et al., 2003, Frazier-Bowers et al., 2002). Depending on the clinical phenotypes of the tooth agenesis cases for prioritizing the candidate genes to be sequenced is not a precise method.

MSX1 gene is a member of homeobox family that plays essential role in the progression of many organs during the fetal development, however, the gene located on chromosome 4p16.1 and encodes for MSX1 protein which is expressed in odontogenesis and taking part in inhibiting the transcription and, same as PAX9, it participates in regulating the signalling pathways of odontogenesis (Vieira et al., 2004, Ogawa et al., 2005) . MSX1 has been screened in 92 affected unrelated individuals belonging to 82 nuclear families and resulted in identifying a novel missense mutation which suggested a significant role in causing tooth agenesis acording to the researchers (Lidral and Reising, 2002). Nieminen et al. (2003) analysed MSX1 gene in 8 Finnish patients diagnosed with Wolf-Hirschhorn syndrome. Five of the patients were presenting oligodontia. Using FISH technique, the 5 patients with oligodontia showed a heterozygous deletion in MSX1 gene, while the other three individuals were completely normal. Therefore, they suggested in the conclusion that haploinsufficiency for MSX1 could be as a mechanism that play a significant role in causing a selective tooth agenesis with other factors (Nieminen et al., 2003). Mice homozygous for a deletion in MSX1 gene demonstrate craniofacial and limb anomalies plus decreased development of teeth agenesis whereas the heterozygous *PAX9* mice seems completely normal (Peters et al., 1998, Peters and Balling, 1999). Mice with homozygous deleted *Msx1*exhibit craniofacial abnormalities in cleft palate, and impairment in mandibular and

maxillary alveolar development and also demonstrate malformations in tooth development process in particular during moving from bud to cap stage. In contrast, heterozygous mice for *Msx1* deletion show normal status (Satokata and Maas, 1994).

WNT10A is a member of the WNT gene family. It is mapped to chromosome 2q35and composed of 4 exons. The expression of its protein is mainly expressed in the cell lines of promyelocytic leukaemia and Burkitt's lymphomas. So athough this gene is not as known as *PAX9* and *MSX1* but mutations were detected in 19 individuals (56%) of total 34 unrelated patients of non syndromic tooth agenesis, 8 mutations of them were homozygous, 4 compound heterozygous, and 7 of them were heterozygous for the mutations. Also, it is found that 3% of patients have mutations within *MSX1* and 9% of the patients were found they have mutations in *PAX9* gene (van den Boogaard et al., 2012). Furthermore, in a cohort of 94 families affected with isolated oligodontia, Arzoo and others identified several mutations in 26 affected individuals (27.7%), 17 of them were homozygous while 11 were heterozygous (Arzoo et al., 2014).

3.4.2 Linkage analysis for LTBP3 and the use of WES

Using direct sequencing, no mutations were identified within the coding and splicing regions of *PAX9, MSX1* or *WNT10A* genes except the normal polymorphisms that are unlikely to cause disease. Thus, the cause of oligodontia in the examined families appeared most likely to be in other genes that were either already associated with oligodontia or in novel oligodontia genes (though the possibility of intronic or regulatory mutations in *PAX9, MSX1* or *WNT10A* could not be excluded). After the negative outcome of sequencing the three selected genes, it was decided to evaluate the use of microsatellite genotyping. I applied this strategy for the *LTBP3* gene which had previously been reported as a cause of the disease in a consanguineous Pakistani family that diagnosed with congenital oligodontia and short stature (Noor et al., 2009). However, in most

cases (assuming the proband would be homozygous for a mutation) linkage was excluded - only proband of family OD005 has showed homozygosity that could be linked to *LTBP3* in a ~8Mb region which harbours five microsatellites (D11S4191, D11S4076 D11S1883, D11S913 and D11S1889). In all the other families, no extended region of homozygosity was noted in the genotyped microsatellites, so in conclusion the *LTBP3* gene is unlikely to be a common cause in this cohort of cases. Following the negative results from analysis of *PAX9, MSX1* and *WNT10A* and the exclusion of *LTBP3* (in most cases), it was decided to screen the whole exome by applying NGS techniques due to the high efficiency of WES in finding the rare disease causing mutations in a variety of Mendelian diseases.

In view of the high cost of WES (in 2013) it was planned to sequence a single individual from the largest family and then screen other family members for candidate mutations and then proceed to testing the relevant gene in affected cases from the other five families. After analysing large number of variants provided by exome sequencing using specific filtering criteria to attempt to identify the disease causing variant, none of the known candidate genes appeared to contain a pathogenic mutation. Nevertheless, the cohort of consanguineous families with a common ethnic origin might contain a novel genetic cause for oligodontia or severe hypodontia and we would suggest pursuing WES in additional cases from the cohort.

This study is not the first to fail to find positive linkage between mutations in the known genes tested genes and tooth agenesis. In five unrelated families affected with hypodontia, Nieminen and colleagues did not identify any association of the *MSX1* gene. Also, no mutations were discovered in *MSX1* in other 20 patients with hypodontia (Scarel et al., 2000). Moreover, in a Vietnamese study involving 20 families, *PAX9* and *MSX1* were analyzed but no mutations were identified (Frazier-Bowers et al., 2003). In addition, the small size of the patient cohort could be

considered a major limitation of this study. Therefore, a larger number of affected families is needed in order to be more confident about the contribution of the genes tested to tooth agenesis disorders in individuals of Pakistani descent.

Chapter Four*: Genetic Investigations of FADS/LMPS by Autozygosity Mapping & Gene sequencing

**This chapter contains work published as part of the paper by Mckie et al (2014) (with AlSaedi as joint first author)*

4.1 Introduction:

4.1.1 Clinical background of MPS disorders

Multiple pterygium syndrome (MPS) is a congenital multiple anomaly disorder. MPS is characterised by the presence of skin webs (pterygium) and the lack of muscle movement (akinesia) associated with muscle weakness and joint contractures (arthrogryposis) (Gillin and Pryse-Davis, 1976, Morgan et al., 2006a). Other clinical features of MPS include scoliosis, cystic hygroma, micrognathia, cleft palate, and lung abnormalities (McKie et al., 2014b). MPS is a heterogeneous rare disorder, inherited mainly in an autosomal recessive manner, though autosomal dominant and X-linked inherited cases can also occur (McKeown and Harris, 1988, Tolmie et al., 1987). According to the severity, MPS is divided into two main clinical forms; the milder Escobar multiple pterygium syndrome (EVMPS) [OMIM 26500] and the lethal multiple pterygium syndrome (LMPS) [OMIM 253290]. LMPS is a severe disorder and is fatal either during pregnancy or shortly after birth whereas EVMPS patients can survive into adulthood. There is a clear genetic and phenotypic overlap between Fetal Akinesia Deformation Sequence (FADS [MIM 208150]) and the lethal form of MPS especially with overlapping clinical features and certain genes may cause both disorders (Vogt et al., 2008). FADS is as the classical form of MPS which characterised by most of the common clinical MPS features such as decreased fetal movements, intrauterine growth restriction, craniofacial anomalies, joint contractures and pulmonary hypoplasia. FADS can be caused by environmental factors such as curare exposure and circulating maternal antibodies against the fetal acetylcholine receptor (Michalk et al., 2008) Also, the phenotype can occur as a result of genetic mutations that affect the normal function of the associated organs (Michalk et al., 2008) and this will be discussed with more details in this chapter.

4.1.2 Genetic causes of MPS disorders

Many genes have been identified as potential causes of MPS-related diseases in the literature. However, at the time this work was commenced, the best recognised genetic causes were related to defects in the components of embryonal acetylcholine receptor (AChR). Thus, AChR components are significant cause of recessively or dominantly inherited myasthenic syndromes with variable age of onset and clinical severity. AChR protein is located in the membrane of skeletal muscle cells and it is essential for neuromuscular signalling pathway between axon and muscles which is necessary for the movement in both fetus and adult. During fetal development, AChR consists of two α1, one β1, one δ, and one γ subunit {(*CHRNA1* [MIM 100690], *CHRNB1*[MIM 100710],*CHRND* [MIM 100720], *CHRNG* [MIM 100730] respectively}.

CHRNG gene is naturally expressed until the thirty third week of the pregnancy when it is replaced by the epsilon (ε) subunit, which is produced by the CHRNE gene, to form the adult AChR protein (figure 4.1). All these subunits are clustered in a complex process to produce the signal transduction. However, some other genes are involved in the AChR assembly such as *AGRN, MUSK, DOK7* and *RAPSN* (Sanes and Lichtman, 2001).

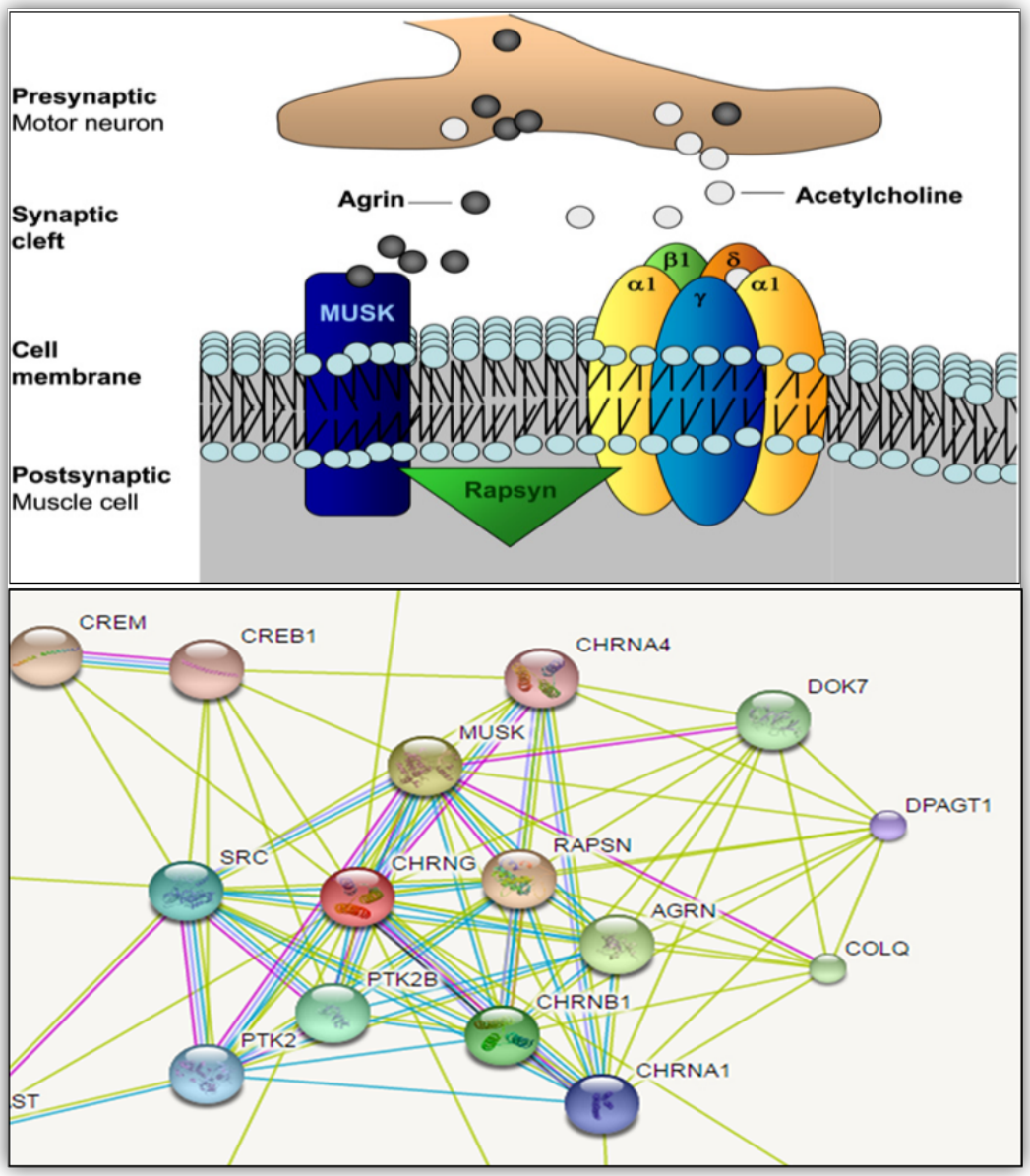


Figure 4.1 drawing shows the structure of AChR and its genetic pathway

Part A) shows the structure of AChR at the postsynaptic membrane in muscle cells that consists of 5 subunits: Two α1, one β1, one δ (2 CHRNA1, CHRNB1, CHRNG). These 4 subunit are always naturally present in the fetus while The fifth subunit ε (CHRNE) is appearing around 33 weeks of gestation in humans when γ subunit expression stops and convert to ε, thereby replacing fetal-type AChR by adult-type AChR. Other contributing proteins in the process of AChR assembling and activation includes agrin (AGRN), muscle skeletal tyrosine kinase (MUSK), however, in part B) a diagram obtained String website shows a network of most of the proteins contribute in the AChR pathway or they have interactions of the AChR componenets.

Mutations in the (*CHRNG*) have been implicated in both FADS and LMPS. Numbers of associated mutations have been identified in the lethal and the Escobar types of MPS. For instance, Hoffmann et al. (2006) identified 8 mutations in CHRNG amongst 7 families diagnosed with Escobar syndrome. In addition, Morgan et al. (2006) was able to find 6 homozygous mutations in 6 families with lethal or Escobar variants (Hoffmann et al., 2006, Morgan et al., 2006a). In addition, genes encoding other components of the AChR complex (e.g. CHRNA1, CHRND, RAPSN, DOK7 and MUSK) that are predicted to cause severe dysfunction of the neuromuscular junction have been described in autosomal recessively inherited LMPS/FADS (Vogt et al., 2008, Michalk et al., 2008, Vogt et al., 2012). Furthermore, many other genes which involved in the neural and skeletal muscle development have been described in some neuromyopathies (e.g. motor neuropathies associated with central or peripheral nervous system disease or muscular dystrophy) that have been associated with a LMPS/FADS phenotype. Also, mutations in genes associated with inherited arthrogryposis have occasionally been linked to EVMPS (e.g. *TPM2, TNNI2, MYH3*) (Ravenscroft et al., 2011, Chong et al., 2015) which are summarized in Table 4.1. Nevertheless, many cases of FADS and MPS do not have a mutation in a known FADS/MPS gene and this is the main reason I undertook further genetic investigations in these conditions.

Table 4.1 summary of the reported genes that have been associated with MPS phenotypes in the literature (table is obtained from a review paper by Ravenscroft et al 2011).

Gene	MIM	Mode of inheritance	Disease entry
Genes involved in the neural development			
SMN1	**600354**	**AR**	**FADS**
ERBB3	**190151**	**AR**	**LCCS2**
GLE1	**603371**	**AR**	**LCCS1**
PIP5K1C	**606102**	**AR**	**LCCS3**
Genes function on neuromuscular junction			
CHRNA1	**100690**	**AR**	**FADS**
CHRND	**100720**	**AR**	**FADS**
CHRNG	**100730**	**AR**	**MPS/FADS**
DOK7	**610285**	**AR**	**FADS**
RAPSN	**601592**	**AR**	**FADS**
Genes encoding adult skeletal muscle proteins			
ACTA1	**102610**	**AD**	**FADS**
DMPK	**605377**	**AD**	**FADS**
NEB	**161650**	**AR**	**FADS**
RYR1	**180901**	**AR/AD**	**FADS**
TPM2	**190990**	**AR/AD**	**EVMPS**

4.2 Method

4.2.1 Patients

A total of 66 families with features of non-syndromic FADS/LMPS/EVMPS with unknown underlying genetic cause were selected for molecular genetic studies. Each involved family had at least one affected individual. In 36 families, the clinical phenotype was FADS/LMPS while in the remaining 30, the phenotype was EVMPS. Consanguinity was recorded in 48% of the FADS/LMPS families and 20% of the EVMPS families. The examined families were from various ethnic backgrounds including South Asian (Pakistani and Indian), African, white and mixed race (Table 4.2). My work was a part of collaborative study and whilst Dr. Arthur McKie (a Postdoctoral Research Associate in Prof Maher group) performed analysis of RYR1 in the EVMPS families, I performed *RYR1* analysis in the LMPS/FADS families. In this chapter I will

only include the results of *RYR1* analysis in the LMPS/FADS cases but in the next chapter (Chapter Five), I will describe "Clinical Exome" analysis for both groups of patients.

Each family gave informed consent and the study was approved by the South Birmingham Research Ethics Committee and performed in accordance with the ethical standards in accordance with the Helsinki Declaration in 1964. DNA samples were isolated from the probands, the available parents and other family members by the Regional Molecular Genetics Centres. DNA from unaffected family members was studied to check the segregation of interesting genetic variants. Clinical information such as family history, ethnicity and pregnancy history were collected from the families by clinical collaborators (Dr Julie Vogt, Professor Eamonn Maher and others). Relevant phenotype information such as brain scans and muscle biopsy investigations were recorded where available.

Table 4.2 the available Clinical features of the LMPS/FADS recruited patients

Family number	Phenotype	Ethnicity	Consanguinity
MPS001	LMPS	White	Yes
MPS002	LMPS	South Asian	Yes
MPS003	LMPS	Middle Eastern	Yes
MPS004	LMPS	South Asian	Yes
MPS005	LMPS	South Asian	Yes
MPS006	LMPS	South Asian	Yes
MPS007	LMPS	Not available	No
MPS008	LMPS	White	No
MPS009	LMPS	White	No
MPS010	FADS	Middle Eastern	Yes
MPS011	LMPS	Not recorded	Yes
MPS012	LMPS	North African	Yes
MPS013	LMPS	White	No
MPS014	LMPS	South Asian	Yes
MPS015	LMPS	White	No
MPS016	LMPS	Not available	Yes
MPS017	LMPS	North African	Yes
MPS018	FADS	White	No
MPS019	LMPS	White	No
MPS020	LMPS	White	No
MPS021	LMPS	South Asian	Yes
MPS022	FADS	Middle Eastern	Yes
MPS023	LMPS	Middle Eastern	Yes
MPS024	LMPS	White	No
MPS025	FADS	Mixed race	No
MPS026	FADS	White	No
MPS027	LMPS	Not available	Yes
MPS028	LMPS	Not available	No
MPS029	FADS/LMPS	Not available	No
MPS030	FADS/LMPS	Not available	No
MPS031	LMPS	White	No
MPS032	LMPS	White	No
MPS033	LMPS	White	No

4.2.2 Molecular Genetic analysis:

Before I commenced my PhD studies a candidate locus for LMPS/FADS was mapped to chromosome 19 by other members of the Maher laboratory. The details of these mapping studies are reported briefly below:

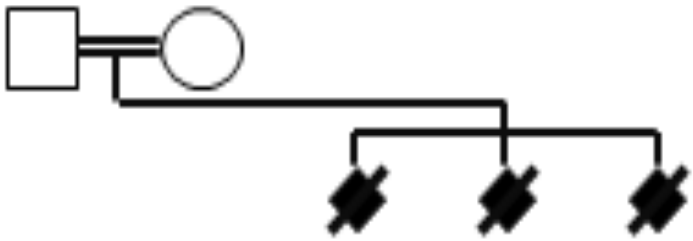
4.2.2a. Autozygosity mapping

4.2.2a.i Genomewide Scan

To identify candidate novel genes utilizing autozygosity mapping techniques, two FADS/MPS patients from a single consanguineous family (MPS001) were investigated to identify regions of homozygosity. Using high-resolution commercial Affymetrix 250K SNP arrays 5.0, genome-wide linkage scan was carried out on DNA from a stored fetal material of the two affected siblings and the scan excluded linkage to known FADS/LMPS genes and resulted in finding a homozygous region of nearly 10 Mb on chromosome 19. This SNP genotyping work was performed by Louise Tee.

4.2.2a.ii Microsatellite marker analysis

From the SNP genotyping results, the defined homozygous region was selected for further analysis using fourteen polymorphic microsatellite markers from the same candidate region to confirm the autozygous region of the three affected fetuses and their parents (Figures 4.2 &4.3). Genotyping of the family with microsatellite markers within the interval on chromosome 19 defined the candidate autozygous region as Chr19; 35108829–44484993bp. A microsatellite marker (D8S373) that mapped within the only other candidate autozygous region >2 Mb was demonstrated to be heterozygous in two affected fetuses and so excluded linkage to chromosome 8 (data not shown).



Marker	Location on Chr 19	Paternal Genotype	Maternal Genotype	Fetus 1 genotype	Fetus 2 Genotype	Fetus 3 Genotype
D19S222	33417730	239-239		239-242	239-242	239-242
D19S433	35108829	199-199	201-203	199-203	199-203	199-203
D19S430	36994291	127-127	127-127	127-127	127-127	127-127
D19S224	41219912	241-255	237-241	241-241	241-241	241-241
Chr19r2-15xAC41	41435658	242-242	242-244	242-242	242-242	242-242
D19S220	43123390	280-285	283-289	280-280	280-280	280-280
D19S228	43181340	203-205	203-205	205-205	205-205	205-205
D19S421	43562946	187-189	187-189	187-187	187-187	187-187
Chr19R2-15Xtat	44221282	164-164	164-175	164-164	164-164	164-164
Chr19r2-22xGT44	44659155	252-252	248-252	252-252	252-252	252-252

Figure 4.2 Genetic Mapping of a consanguineous family (MPS001) using Microsatelites

Area coloured in green showing the shared a common homozygous region of the three affected siblings (F1, F2 &F3) between 28,725,890 - 44,669,155 located on chromosome 19.

Table 4.3 List of Microsatellite markers employed in Mapping MPS001 of chromosome 19

Marker	Genomic position	Source
D19S222	Chr.19-28,725,890-28,726,217 bp	http://rgd.mcw.edu/rgdweb/report/marker/main.html?id=1341900
D19S433	Doesn't map to Chr.19 assembly	http://rgd.mcw.edu/rgdweb/report/marker/main.html?id=1298168
D19S430	Chr.19-32,302,451-32,302,741 bp	http://rgd.mcw.edu/rgdweb/report/marker/main.html?id=1657125
D19S224	Chr.19-35,493,932-35,494,196 bp	http://rgd.mcw.edu/rgdweb/report/marker/main.html?id=1336138
Chr19r2-15xAC41	Chr.19-41,435,658-43,123,394	ftp://ftp.broad.mit.edu/pub/human_STS_releases/
D19S220	cHR.19-34,8798,595-34,879,871 BP	http://rgd.mcw.edu/rgdweb/search/markers.html?term=D19S220&speciesType=1
D19S228	Chr.19-34,937,645-34,937,798 bp	http://rgd.mcw.edu/rgdweb/report/marker/main.html?id=1338234
D19S421	Chr.19-38,871,106-38,871,460 bp	http://rgd.mcw.edu/rgdweb/report/marker/main.html?id=1337182
Chr19R2-15xTAT	Chr.19-44,221,282-44,669,155	ftp://ftp.broad.mit.edu/pub/human_STS_releases/
Chr19r2-22xGT44	Chr.19-44,221,282-44,669,155	ftp://ftp.broad.mit.edu/pub/human_STS_releases/

4.2.2b Candidate gene analysis from autozygosity mapping

4.2.2b.i Candidate gene selection

When I started my PhD studies I inspected the results of the autozygosity mapping in MPS001 and looked for likely candidate LMPS/EVMPS genes from within the chromosome 19 region of homozygosity that contained 345 known or predicted genes. To select the best candidate gene/genes for further genetic analysis, I undertook a literature research for each gene based on the gene function and expression to see whether it had any kind of association with the disease pathway. This procedure involved (a) researching on the genomic databases (OMIM, PubMed, UCSC, Ensemble and others) to find the putative function if available, (b) reading the relative published literature to look for any reported results or linked studies that indicate any role for the

gene relevant to MPS/FADS (c) any information on animal model experiments such as model organism genomic databases (MGI). According to this strategy, *RYR1* gene, mapped to Chromosome 19: 38,433,830-38,587,420 was highlighted to be the best candidate for further analysis to screen any pathogenic mutations in all the studied cases. It is a very large gene (15.3 kb coding sequence) and encodes RYR1 protein which is found mainly in the skeletal muscle and known as skeletal muscle calcium release channel. Mutations within RYR1 found to be implicated in many histological subtypes of congenital myopathies in humans and mouse models. Further details about the clinical associations and the pathogenicity of *RYR1* can be found the Discussion section of this chapter.

4.2.2b.ii RYR1 sequencing:

After selecting RYR1 as the best candidate gene for further analysis, I then proceeded to sequence the entire coding exons of the gene in all the 36 LMPS/FADS families to search for mutations and to establish a frequency figure of any detected mutations. PCR amplification and sequencing was carried out using direct Sanger method for all the genomic DNAs using specific primers that cover the whole RYR1 open reading Frame (ORF), including the coding exons (106 exons) and the exon-intron boundaries. *RYR1* sequencing and mutational screening was undertaken in all LMPS/FADS affected individuals and the segregation of any detected variants was checked in parents and other family members (when available) as well.

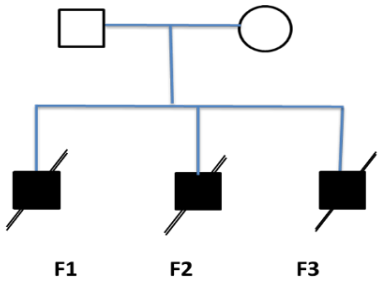
4.2.3 Histopathological investigations:

Our laboratory collaborators in the Department of Pathology, VU University Medical Centre, Amsterdam, Netherlands undertook histopathological analysis in order to assess any role of the *RYR1* mutations. Autopsy tissues of intercostal skeletal muscle were obtained from the two affected fetuses of family MPS001 (12 + 6 and 14 + 0 weeks GA, respectively) and two age-

matched controls (13 + 0 and 13 + 4 weeks GA, respectively). The tissue was taken from the autopsy archive of the VU University Medical Center, and was the only available tissue for both patients. In the experimental details provided by the team, the tissue sections were fixed by formalin and embedded by paraffin and the processing was consistent with the standard protocols (Bancroft and Gamble, 2008)**.** A variety of stains were used including Hematoxylin & Eosin, Gomori trichrome and alizarin red S for calcium and each stain has different use. After heat-induced antigen retrieval in 0.01 M citrate buffer (pH6), immunohistochemical staining was performed with antibodies against desmin (Abcam, 1:500), myosin heavy chain slow (Abcam, 1:100), active caspase 3 (Dako, 1:500), CD3 (Dako, 1:250), CD20 (Dako, 1:50) and CD45 (Dako, 1:100). Immunoreactivity was detected with 3, 3′-diaminobenzidine as chromogen. The photographs for the tissue sections were taken by Leica DM6000B microscope (Leica Microsystems). Omitting primary antibodies yielded no significant staining. The histopathological examinations included an Ultrastructural analysis for all the muscle tissue which fixed in formalin solution. The tissue was deparaffinised in xylene (60 minutes at 70°C), rehydrated,fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), post-fixed in 2% osmium tetroxide and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed in a FEI Technai 12 electron microscope. The pictures were acquired as TIFF files and images were optimized for brightness and contrast using Photoshop, version 7.0 (Adobe systems, San Jose, CA).

4.3 Results

4.3.1 MPS001 family



4.3.1a Clinical assessment of MPS001

Clinical details for the families in this project were provided by Dr. Julie Vogt from West Midlands Regional Genetics Laboratory and other collaborators. MPS001 is a consanguineous family originally from the Netherlands. It had had six pregnancies affected by FADS/MPS. According to the family history, the first pregnancy (F1) was terminated because of increased nuchal translucency of 12 mm, fetal akinesia and a unilateral club foot whereas Proband (F2), presented in the second pregnancy, was terminated at 12+6 weeks of gestation as a consequence of increased nuchal translucency of 9mm, fetal akinesia and joint contractures. There was no intrauterine growth retardation. The post mortem examination evidently showed a cystic hygroma, lung hypoplasia, webbing of both elbows and knees and arthrogryposis. As a result of birth trauma, the calvaria was absent however there were no clear evidence of any skeletal abnormalities. Additionally, the third, fourth, sixth and seventh pregnancy were terminated, because of increased nuchal translucency and fetal akinesia. The fifth pregnancy ended in an early miscarriage.

4.3.1b Molecular findings of MPS001

Sequencing of *RYR1* in an affected fetus (F1) from this family revealed a homozygous *RYR1* nonsense mutation (c.6721C>T; p.Arg2241*) in exon 41 of the gene. Further analysis demonstrated that the other affected sibling (F2) has been identified with the same homozygous change while both parents were heterozygous carriers (Figure 3.2). According to EVS6500-Exome databases, c.6721C>T mutation was previously detected (rs200563280), in the heterozygous state only in one case of 6503 individuals genotypes listed in the exome variant server (http://evs.gs.washington.edu/EVS) but no homozygous genotypes were detected at an average read depth of 49.

Figure 4.3 homozygous RYR1 stop codon detected in MPS0001 family

homozygous stop codon c.6721 (p.Arg2242) was detected in MPS0001 family of two affected sibling (F1 & F2 chromatograms) and both parents were heterozygous for the mutant alleles (3rd & 4th chromatograms).*

4.3.1c Pathological findings of MPS001

The quantitative histopathological examination performed by the Dutch collaborators showed some structural abnormalities in the affected siblings tissues, including fibre loss, increased fibre size variability and increased endomysial spacing with fibrosis (Figure 3A, B). Centrally positioned nuclei were notified in the examined muscle fibres in both patients and controls. In

contrast, thin muscle fibres with intense eosinophilic cytoplasm were seen only in the patients, while the controls were completely normal under the microscope (Figure 3.3 A,B) and this had nothing to do with calcium accumulation according to the specialized team (Figure 3.3 J). Moreover, staining against desmin, an intermediate filament protein which normally exists in muscle, showed no core-like structures (Figure 3.3C,D). Further significant staining against the slow and fast myosin heavy chain was carried out by the group in order to assess fibre type distribution, however, the results showed a notable reduction in the level of myosin slow immunoreactivity, which indicates preferential hypotrophy of type I fibres (Figure 3.3 E-H). Labelling for active caspase 3 excluded apoptotic loss of muscle fibres (Figure 3.3 I). Further fine investigations showed hypotrophy associated with profound myofibrillar disarray and Z-disc loss (Figure 3K,L). The analysis of the spinal cord did not show any loss of motor neurons or other abnormal pathological features in the anterior horns and all the proximal segments of the motor neural system and this confirm that the histopathological findings are mostly myogenic.

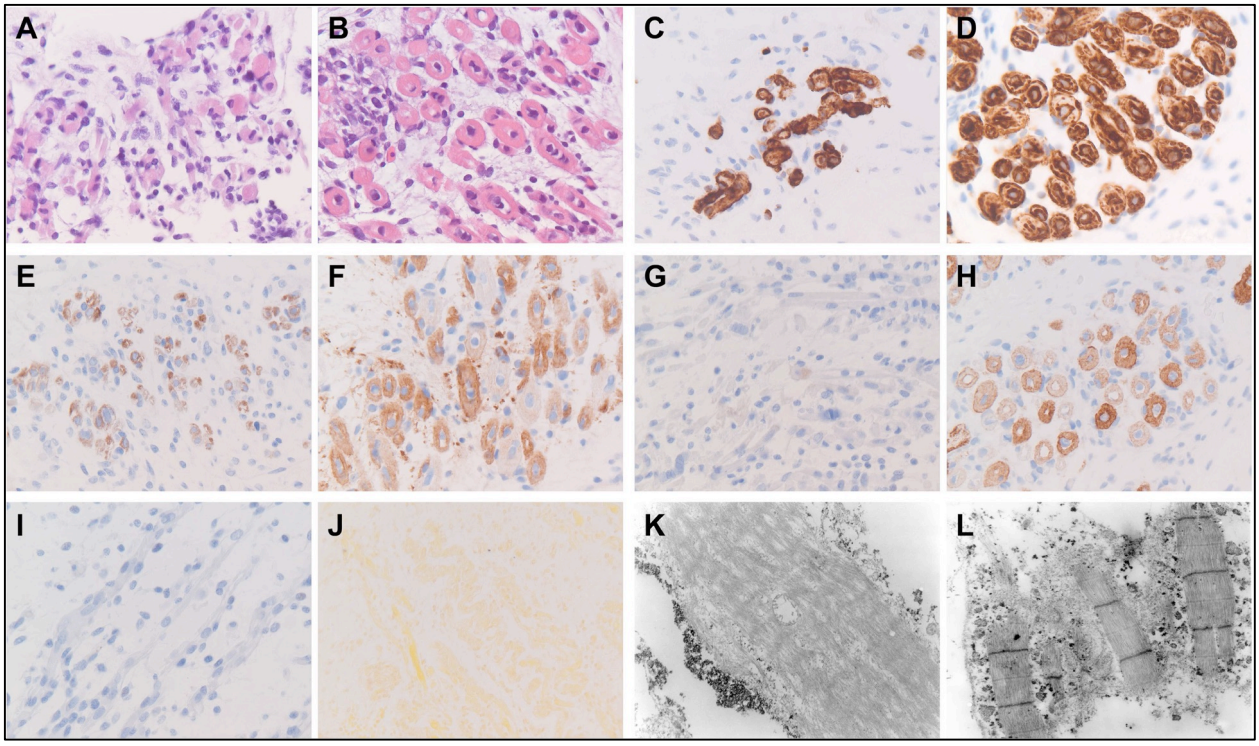
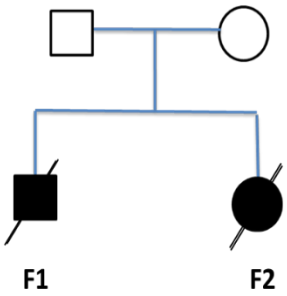


Figure 4.4 : Histological and ultrastructural findings in RYR1-mutant fetal skeletal muscle in MPS001 family.

(A,B) Hematoxilin & Eosin stain shows increased fiber size variability in the RYR1-mutant muscle (A, fetus F1) compared to an age-matched control (B). Some RYR1-mutant fibres have intensely eosinophilic cytoplasm (A, arrows). The nuclei are localized centrally in all fibres, compatible with the gestational age. The perinuclear clear halo present in many fibres is an artefact due to formalin fixation. (C,D) Labelling against desmin reveals a similar pattern of immunoreactivity and no evident core-like structures in both RYR1-mutant (C, fetus F1) and control tissue (D). (E-H) Labelling against the myosin heavy chain fast (E,F) and slow (G,H) shows that the numbers of myosin fast-positive type II fibres is comparable between the patient (E, fetus F2) and the control (F), whereas myosin slow-positive type I fibres are markedly reduced in RYR1-mutant (G, fetus F2) compared to control muscle (H). (I) Labelling for active caspase 3 is negative, excluding apoptosis, also in atrophic RYR1-mutant muscle fibres (fetus F2). (J) Alizarin red S staining shows no detectable accumulation of calcium inside the RYR1-mutant muscle fibres (fetus F1). (K,L) Ultrastructural analysis reveals profound myofibrillar disarray with disappearance of the Z-bands in the RYR1-mutant muscle fibres (K, fetus F2). By contrast, Z-bands are easily detected in control tissue (L). Magnifications: (A-J) 400x; K,L 30000x. (lower panels) (McKie et al., 2014b)

3.3.2 Family MPS002



3.3.2a Clinical assessment of MPS002

In the clinical data of MPS002, the family presented at eighteen weeks of pregnancy with a female fetus with bilateral talipes and fixed flexion of the elbows. There was evidence of fetal hydrops on the ultrasound scan including a large cystic hygroma, subcutaneous oedema, ascites and pleural and pericardial effusions. A cardiac ventriculoseptal defect was also suspected. The pregnancy was terminated at 19 weeks of gestation. Post mortem examination revealed no intrauterine growth retardation, but the fetus was considered to be dysmorphic (protuberant eyes, hypertelorism, a flat nose, low set ears) and had a complete cleft palate. There was fixed flexion of all the limb joints and a fracture of the proximal left humerus. Pterygia were present between the inferior margin of the mandible and the anterior chest wall and across the elbows. The heart was normal. There were no other congenital abnormalities noted. The brain was structurally and histologically normal. The muscle appearance was striking but non-specific. There was variation in myofibre size with larger hyalinised rounded fibres and smaller rounded atrophic fibres with an apparent increase in fibrous tissue. Fast and slow myosin were co-expressed on a proportion of fetal myofibres. Occasional scattered chronic inflammatory cells were confirmed on CD3 and CD20 staining. Additional CD45 staining was interpreted as non-specific. Gomorri trichrome

staining was negative for nemaline rods and ragged red fibres. The couple had a previous intrauterine death at 23 weeks of gestation of a similarly affected male fetus.

4.3.2b Molecular findings of MPS002

Mutation screening in this family revealed a novel in-frame deletion of 27 nucleotides (c.2096-2123del). This was absent from the known genomic database and our in-house database. This 27bp deletion is located in exon 18 and the two affected fetuses of the family (F1&F2) were homozygous for the deletion while both parents were heterozygous carriers (Figure 3.4) (no sequence data for F2 due to the insufficient DNA). This novel deletion was predicted to result in a missense substitution (p.Glu699Asp) followed by a deletion of 9 amino acids; located within the SPRY1 domain of the *RYR1* gene product. All these deleted amino acids were conserved in vertebrate *RYR1* orthologues including zebrafish and 6 of 9 amino acids were conserved in *C.elegans* (Figure 3.4b).

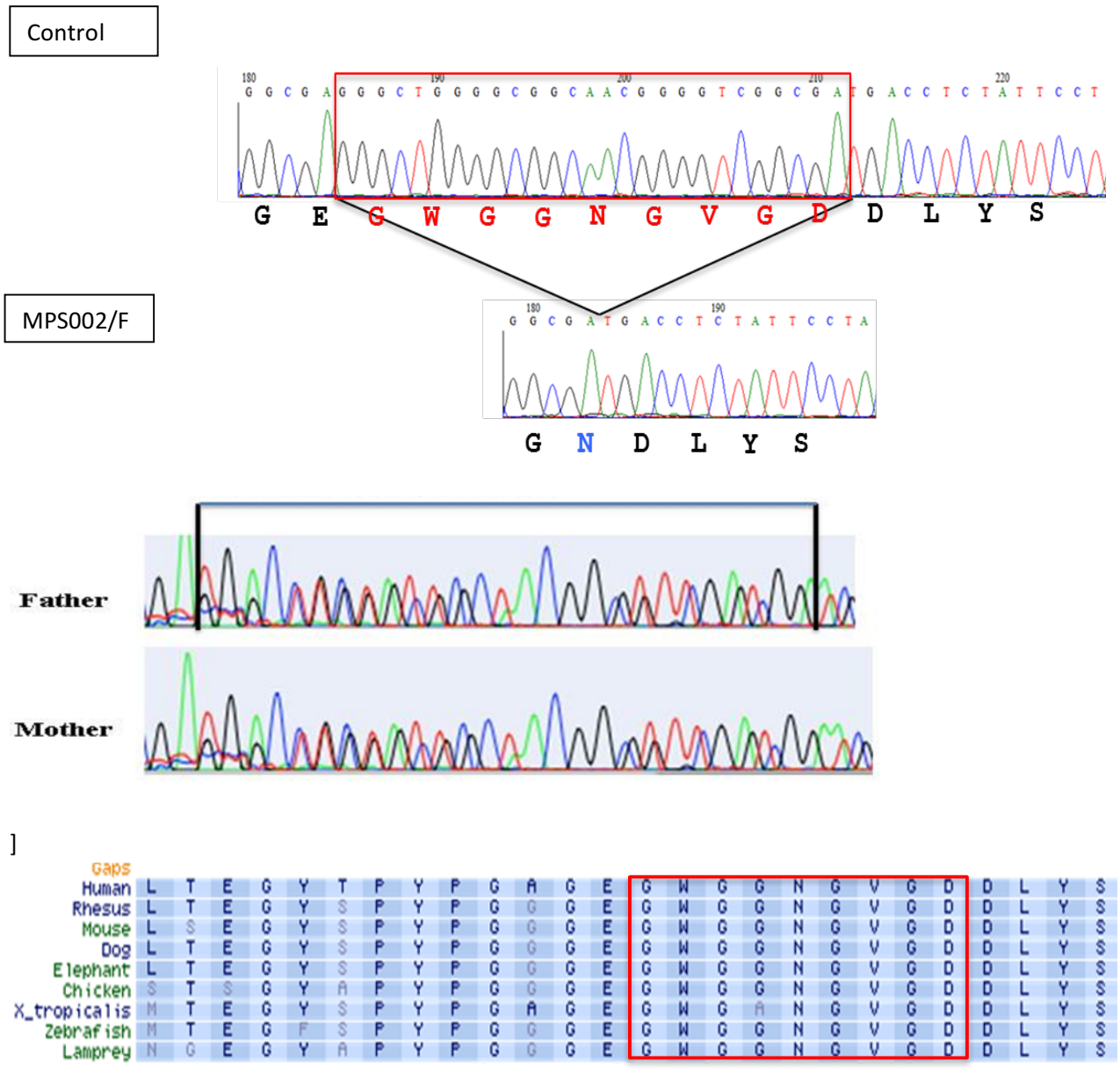
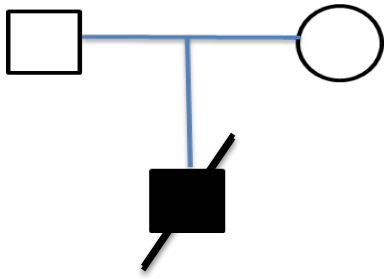


Figure 4.5 A chromatogram homozygous in-frameshift deletion in RYR1

27bp homozygous inframe shift deletion in X18 (c.2096-2123) is clearly shown in the above sequence of one of the affected fetus resulted in result in a missense substitution (p.Glu699Asp) followed by a 9 amino acids deletion compared to the sequence of normal control and this mutation was inherited from herterozygous mutant parents as shown. . the 9 deleted amino acids were all conserved in vertebrate RYR1 orthologues (taken from UCSC)

4.3.3 Family MPS003



4.3.3a Clinical assessment of MPS003

The family presented in the third pregnancy with possible polyhydramnios and reduced fetal movements and joint contractures were detected on ultrasound examination. The fetus had a cystic hygroma, a hydrothorax, a short neck, a kyphosis and a short trunk due to a scoliosis. The pregnancy was terminated at 23 weeks of gestation. Post mortem examination demonstrated no evidence of intrauterine growth retardation but craniofacial anomalies (downslanting palpebral fissures, hypertelorism, a small mouth and high arched palate and low set ears) were noted. There were flexion contractures of the shoulders, elbows, wrists, hips, knees and ankles. There were clenched hands but no finger contractures. There was webbing of the axillae, elbows, knees and groins and rocker bottom feet. There were no CNS abnormalities however there was evidence of a severe congenital myopathy.

4.3.3b Molecular Finings of MPS003:

A homozygous in-frame deletion (c.7039delGAG) was detected in the single affected proband from this consanguineous family of Palestinian origin diagnosed with LMPS. The variation was located within exon 45 (Figure 4.5). It was present in both parents in the heterozygous state and unfortunately, there were no other relatives available for analysis. This deletion variant was not present in >13,000 *RYR1* alleles reported on the exome variant server (http://evs.gs.washington.edu/EVS), but it was described, in the heterozygous state, in affected

members (n=2 and 3) of two unrelated families that presented with malignant hyperthermia. This c.7039delGAG deletion is predicted to result in loss of a glutamic acid residue at codon 2347 in the MH/CCD hotspot region 2 of the *RYR1* gene product (Figure 4.6). This residue is conserved in in zebrafish and although the amino acid sequence around this residue is divergent in *C. elegans*, the glutamic acid is conserved.

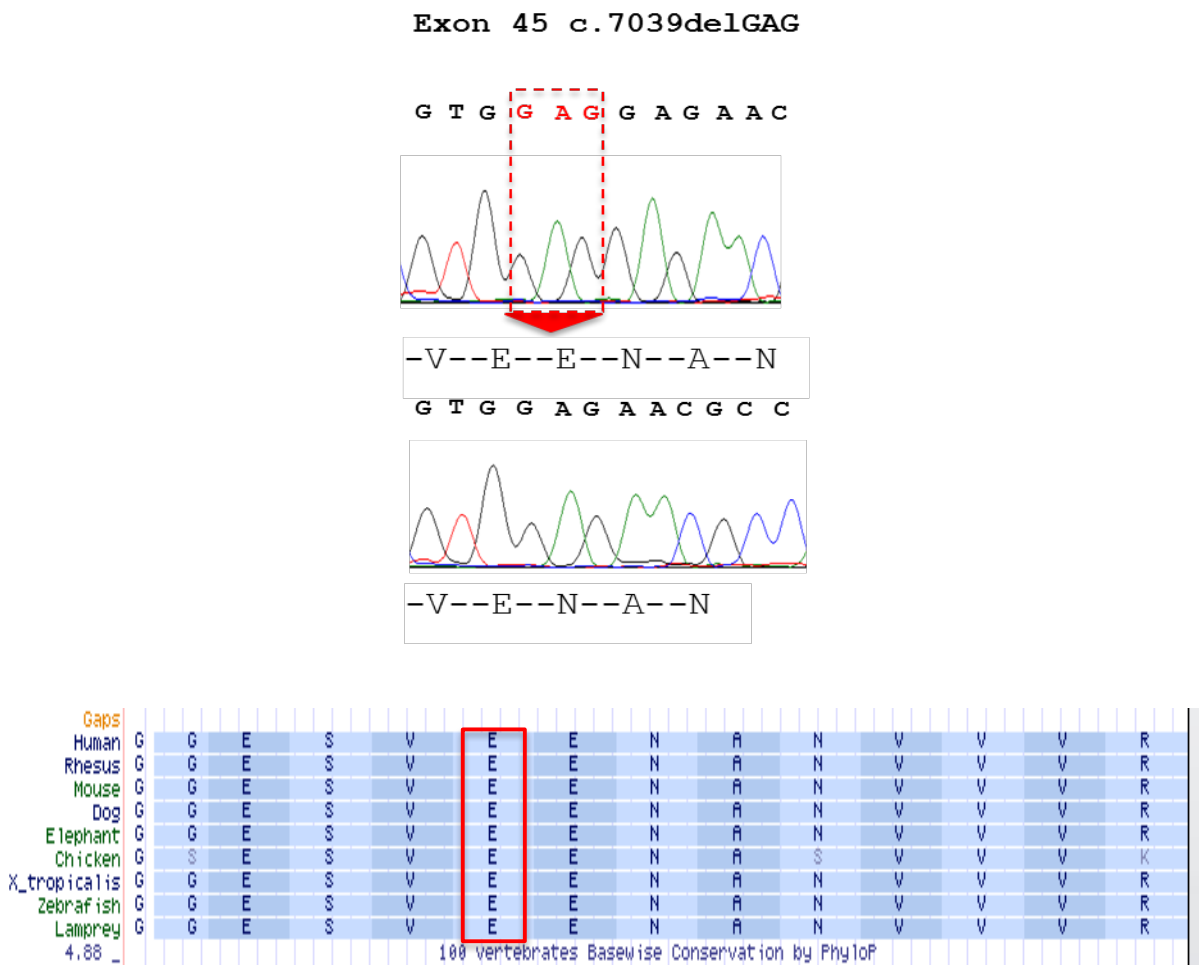


Figure 4.6 homozygous in-frame deletion in X45 (c.7039delGAG)

it was detected within the family (MPS002). As shown, 3bp (GAG) was deleted in the affected proband compared to the normal reference resulting in a missing of glutamic acid (codon 2347) which is conserved in vertebrate and zebrafish.

4.3.3c Pathological findings of MPS003

The muscle analysis (performed by collaborators) for the affected probands demonstrated non-specific abnormalities. The myofibre size had marked variation, and the fibres were large, hyalinised and rounded. In contrast, the atrophic fibres were smaller but rounded with an increased size of the fibrous tissue. Staining against the slow and fast myosin heavy chain showed co-expression in a section of myofibres. CD3 and CD20 staining showed signs of scattered and chronic inflammations of the cells (Figure 4.7)

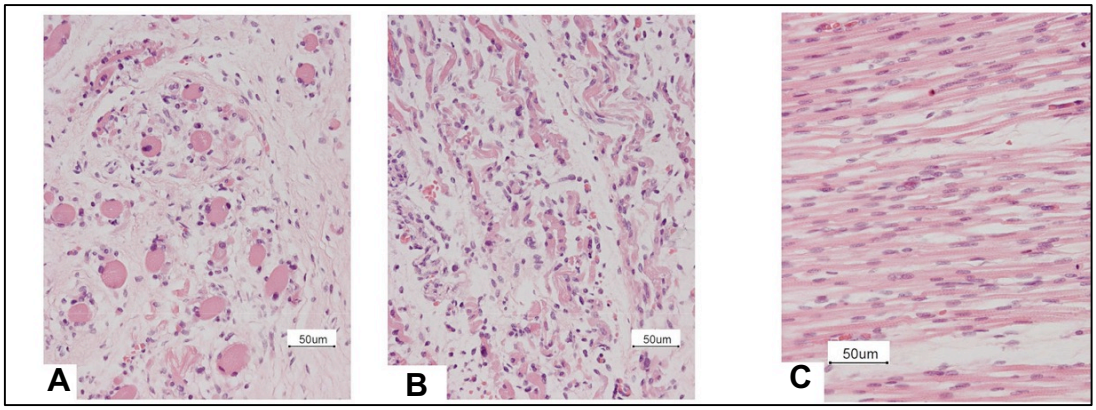


Figure 4.7 histological findings in RYR1-mutant (Family MPS003)

Hematoxilin & Eosin stain of formalin fixed and paraffin embedded psoas muscle shows loss of fibres with increased fibre size variability and mild fibrosis in the RYR1-mutant muscle (A,B) compared to an age-matched control (C).

4.4 Discussion:

4.4.1 Autozygosity mapping analysis

As discussed in the Introduction chapter, autozygosity mapping is regarded as a powerful strategy for investigating the molecular basis for recessively inherited diseases and identifying the causative gene in consanguineous families. Prior to starting my project, this method was applied to a consanguineous family (MPS001 family), with three affected siblings. These studies identified a large region of homozygosity on chromosome 19. However, in MPS001, even after

the candidate region was refined by genotyping with microsatellite markers, the target interval contained a large number of genes (345 genes). I followed a specific prioritization procedure (previously explained) to identify candidate genes and selected *RYR1* as the promising candidate.

4.4.2 Mutational analysis of RYR1

RYR1 is a very large gene (15.3 kb) which composed of 106 exons and encodes a large RYR1 protein (565kDa) that is a key component of the excitation-coupling (EC) process in skeletal muscle. RYR1 is a subtype of Ryanodine receptors (RyRs) which are intracellular calcium (Ca2+) release channels located on the endo/sarcoplasmic reticulum (ER/SR) and represent the major Ca2+ reservoir inside the cell. RyR1 facilitates the rapid and coordinated release of Ca2+ from the sarcoplasmic reticulum stores to initiate the skeletal muscle contraction The excitation-coupling process converts electrical signals/increased Ca2+ levels into mechanical output (i.e. muscle contraction). Ca2+ ions play a significant signalling role in activating the channel and regulating the signal. In this mechanism, plasma membrane is depolarised and cause an activation of L-type voltage-gated calcium channels (Cav). These Cav channels signal RYR1 located on the SR to release Ca2+ to activate muscle contraction (Rios and Brum 1987, Gordon et al. 2000, des Georges et al. 2016)

It is a well-recognized cause of a variety of human congenital myopathies in particular congenital central core disease (CCD; MIM# 117000) and the malignant hyperthermia susceptibility (MHS), congenital skeletal muscle disorder, (MIM# 145600). As these conditions are inherited as an autosomal dominant trait, most reported mutations are heterozygous variants (Brandom et al., 2013, Broman et al., 2011). However, in recent years the further histopathological phenotypes have been associated with RYR1 mutations including multi-minicore disease (MmD),congenital fiber-type disproportion, and centronuclear myopathy

(CNM) and also King Denborough syndrome, a dysmorphic syndrome with associated MHS. Indeed *RYR1* associated congenital myopathies might represent one of the most common forms of congenital myopathy (Amburgey et al., 2013b, D'Arcy et al., 2008). Both dominant and recessive forms of *RYR*1-related congenital myopathies have been reported. In the year 2000, McCarthy and others noted most of RYR1 mutations occurring either within N-terminal region (amino acid residues 35-614) or within the C-terminal region (residues 2163-2458) and the two regions were known later as (MH/CCD region-1 and MH/CCD region-2 respectively)(McCarthy et al., 2000) and they observed that the majority of these clustered mutations were found in patients with autosomal dominant myopathies. In contrast, the mutations associated with autosomal recessively inherited *RYR1* myopathies are widely distributed throughout the whole protein sequence (Amburgey et al., 2013a).

After selection as the best candidate gene, screening for *RYR1* gene mutations in MPS001 revealed a homozygous nonsense mutation (c.6721C>T; p.Arg2241*) in two affected individuals. I then proceeded to screen for *RYR1* mutations in the other LMPS/FADS families in this project. The large size of the gene made this a laborious and time consuming undertaking but mutation analysis in the other families resulted in the detection of a homozygous novel in-frame deletion of 27 nucleotides (c.2096-2123del) in family MPS002 in two affected siblings and also another homozygous in-frame deletion (c.7039delGAG) in the single affected proband from MPS003 family. In conclusion, the screening for mutational changes amongst all the 36 FADS probands resulted in finding three candidate homozygous mutations Table 4.3 (McKie et al., 2014b).

Table 4.4 Summary of the identified rare RYR1 variants detected in families with LMPS

Family ID	DNA change	Protein change	Genotype	Segregation in family?	Interpretation
MPS001	c.6721C>T	p.Arg2241*	Homozygous	Yes	Pathogenic
MPS002	c.2097_2123del	p.(Glu699_Gly707del)	Homozygous	Yes	Probably Pathogenic
MPS003	c.7042_7044del	p.(Glu2348del))	Homozygous	Yes	Probably Pathogenic

The overall frequency of RYR1-related disease was 8.3% (3/36; 95% CI 0 to 19.5%) in our FADS/LMPS cohort. The case for pathogenicity of the three identified variants is supported by their low frequency in the population and also their absence from large repositories of genetic variation in control individuals, in addition to the co-segregation of the variants within the relevant families and evolutionary conservation of the mutated/ deleted amino acid residues, and finally the location of these variants within conserved domain sites. All these factors are considered very important for the pathogenicity assessment of any causative genetic variants (MacArthur et al., 2014). The novel in-frame deletion of 27 nucleotides (c.2097_2123del p.(Glu699_Gly707del)) detected in Family MPS002 is predicted to result in a missense substitution (p.E699N) followed by a deletion of 9 amino acids (GWGGNGVGD) within the SPRY2 predicted protein-protein interaction motif (Peralvarez-Marin et al., 2011). Previously missense substitutions within or adjacent to this deletion (c.2113G > C; p.Gly705Arg and p.Asp708Asn) have been reported in recessively inherited myopathies (Klein et al., 2012). The exon 45 in-frame deletion (c.7043delGAG) identified in Family MPS003 was predicted to result in loss of a glutamic acid residue at codon 2347. A missense mutation at a nearby residue (p.Arg2355Trp) has been reported in both dominantly and recessively inherited myopathies (Kim et al., 2013) and it is interesting that this deletion was previously described in the heterozygous

state, in two unrelated families with malignant hyperthermia (Sambuughin et al., 2001). p.Glu2347 is contained within the MHS/CCD mutation hotspot in N-terminal region 2 (stippled box Figure 4). Though no history of malignant hyperthermia syndrome was reported in Family MPS003, incomplete penetrance is well recognised in malignant hyperthermia and mutation carriers may not have been exposed to trigger events.

Though my findings further established recessive RYR1 mutations as a cause FADS/LMPS, further work is required to fully establish the precise frequency of RYR1 mutations in FADS/LMPS cohorts and to address how novel missense or in-frame deletions/insertions might be reliably interpreted in a clinical diagnostic setting. It is noted that the identified mutations are located within the mutation hotspot regions (MHS/CCD 1 &2) where most of the previous mutations of RYR1 was located. Also, interestingly, the three mutations also located within RIH domain which is a conserved structure RYR1 protein. The 27bp novel in-frameshift deletion occurred within RIH-1 domain (in the interval 440-643 amino acids) while the nonsense mutation plus the 3-bp in frame deletion occurred in the RIH-2 domain (in the interval 2157-2365 amino acids) and this domain is in fact situated in the second main hotspot of RYR1 mutations (Sorrentino et al., 2000) see the below figure 4.8.

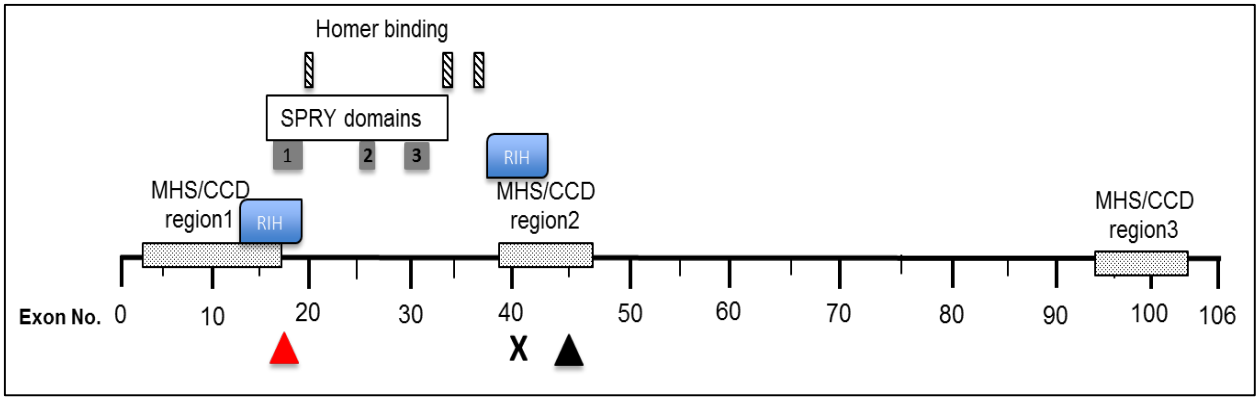


Figure 4.8 A diagram showing the location of the identified mutation within the cDNA of RYR1

nonsense mutation in MPS001 (X) ,27bp in frame deletions in Family MPS002 (red triangle) and 3bp in frame deletion in Family MPS003 (black triangle) in relation to exon structure and RYR1 protein domains. Malignant Hyperthermia/central core disease mutation hot spots shown as stippled boxes, SPRY2 interacting domains 1,2 & 3 as grey boxfibres, RIH domains as blue boxes, Homer binding motifs as hatched boxes.

4.4.3. Genotype-Phenotype correlation

Looking into the genotype-phenotype correlations in both modes of inheritance has provided insights into likely clinical-functional relationships. In a large cohort of RYR1-associated myopathies, dominant mutations were generally associated with milder phenotypes while the recessively inherited cases were mostly associated with severe phenotypes and an earlier age at onset of the disease than in dominantly inherited cases (Klein et al., 2012, Maggi et al., 2013). Although the age the clinical appearance of recessive RYR1 myopathies is quite variable, in a recent series all patients were reportedly presented the clinical features before age 10 years. The vast majority of CCD and the MHS are dominant missense mutations, and in few cases small deletions and duplications while in the recessively inherited *RYR1* phenotypes, they are typically associated with compound heterozygosity for a missense mutation in combination with a nonsense, splice-site or frameshift mutation (Clarke et al., 2010, Jungbluth et al., 2007, Amburgey et al., 2013a). In a cohort studies involved 118 patients with RYR1-associated

recessively inherited myopathies, 61.5% of the cases had a truncating/in frame deletion/splice site mutation in combination with a missense mutation while 38.1% of the cases had two missense mutations (Klein et al., 2012, Maggi et al., 2013, Jungbluth et al., 2007).

In my investigations I identified a homozygous null mutation in LMPS/FADS affected individuals from Family MPS001 and this finding is consistent with a previous study investigating the role of the *RAPSN* gene in FADS/LMPS. The study concluded that homozygosity for a null (frameshift) mutation can cause FADS/LMPS whereas other compound mutations with only a single null mutation can cause a milder phenotype (Vogt et al., 2008) . Also, it is consistent with the observation that in mice *Ryr1* knockout was lethal in the perinatal period due to heart and lung abnormalities (Takeshima et al., 1994). Our finding also agreed with the fact that fetal akinesia caused by RYR1 recessive mutations had earlier onset of the disease in comparison to the dominantly inherited mutations (Klein et al., 2012). In addition, a study reported seven fetuses/infants from six unrelated families affected by central core disease in whom there was a history of fetal akinesia (Romero et al., 2003). In that study: four cases from three families were found to harbour *RYR1* mutations: three cases (from two families) were compound heterozygotes for *RYR1* missense mutations and in one case only a heterozygous missense mutation was detected. Three of the four cases presented at birth and though in one case the fetus died at 32 weeks gestation (following termination of pregnancy after a previously affected sibling). Thus the phenotype in these cases was less severe than in the cases I studied and my findings demonstrated that the association between *RYR1* mutations and fetal akinesia extends to severe early onset lethal FADS and that histopathological evidence of central core disease is not a prerequisite for molecular investigation of RYR1 in fetal akinesia. In a recent review of congenital myopathies treated at a single referral centre, a genetic diagnosis was

established in two-thirds of cases and almost 60% of those with a genetic diagnosis had a RYR1-related myopathy (Maggi et al., 2013).

My findings suggest that RYR1-related neuromuscular disease may be a significant cause of FADS/LMPS. Though recessively inherited RYR1-related myopathies have been associated with certain histopathological subtypes such as minicore, centronuclear and congenital fibre-type disproportion myopathies, *RYR1* mutations may be associated with other histological subtypes or only nonspecific myopathic features. Extrapolating from these observations, it can be suggested that in cases of FADS/LMPS, *RYR1* mutation analysis should be performed as part of a multigene diagnostic strategy (e.g. by second generation sequencing analysis) rather than being specifically targeted to cases with histopathological features that are considered characteristic of a RYR1-associated myopathy. The identification of *RYR1* mutations as a cause of familial LMPS/ fetal akinesia enables accurate reproductive risk prediction and reproductive options including prenatal diagnosis and pre-implantation diagnosis but also might lead to the identification of relatives at risk of malignant hyperthermia.

Chapter Five: the application of CES in MPS disorder

5.1 Introduction

Clinical exome sequencing (CES) is a NGS-based approach that focuses on specific genes/regions which have been associated with disease causing-mutations and reported in the Human Mutation Database (Lee et al., 2014). This targeted exome component represents nearly 25% of the whole exome (~5000 genes). Many users will analyse only a subgroup of genes in a disease specific panel which is updated continuously. The first existence of clinical diagnostic exome panel was in 2011 by Ambry Genetics (Aliso Viejo, CA, USA). Then, Illumina Inc. (San Diego, CA, USA) has designed a new kit for CES that composed of 2800 genes based on Nextera enrichment method (TruSight™ One). However, this particular kit has been developed to contain further 2000 genes (4813 genes in total) which was used in this project.

There are some advantages of the application of clinical exome in the diagnosis of rare inherited disorders. First, it is a cost-effective method compared to the whole exome as it only focuses on the clinical associated genes with deep achievable coverage and less data to be analysed. Second, the number of resulting non-significant variants or variants of uncertain significance is considerably less than the data generated by whole exome/whole genome sequencing. Therefore, analysing the data would be faster and easier with this approach (Klein et al., 2014). Multiple pterygium syndrome (MPS) and its related phenotypes are characterised by marked genetic heterogeneity which makes molecular diagnosis very challenging as many genes could be involved in the disease.

Screening for *RYR1* mutations that I performed in 36 LMPS/FADS families -in the previous chapter- has only resulted in identifying three homozygous mutations in three affected families while the others screened patients remained genetically undiagnosed. Hence, further analysis needed to be done for these families. Furthermore, many other families diagnosed with MPS

subtypes (EVMPS/arthrogryposis etc.) were recruited to this study in order to investigate the genetic causes of these families. Instead of sequencing the candidate genes by Sanger sequencing on an exon-by-exon basis, it was decided that a more efficient approach would be to use a NGS-based sequencing technology.

In collaboration with Dr.Arthur Mckie in Cambridge, I investigated the application of "clinical exome" sequencing (Illumina TruSightOne assay for ~4800 human disease genes) to study the genetic basis of MPS-related phenotypes. I wished to evaluate whether clinical exome sequencing in MPS-related phenotypes (FADS, LMPS, EVMPS, distal arthrogryposis) could provide a more cost-effective molecular genetic analysis strategy for clinical diagnostic testing than the whole exome sequencing (WES) and also whether this analysis might provide a new insight into genotype-phenotype correlations in MPS-related disorders.

5.2 Patients:

Fifty-three patients were recruited in this study. All the cases were clinically diagnosed by MPS-spectrum disorders (LMPS/FADS/EVMPS/arthrogrypposis) and the diagnosis was done by clinicians from across the UK (Table 5.1). All the clinical details and family history for the patients were provided by clinical geneticists (Dr Julie Vogt, Professor Eamonn Maher and others). Also, DNA from parents and unaffected family members was extracted to test the segregation of any candidate mutation. Eight probands out of 53 who underwent clinical exome analysis were known to harbour a mutation in an MPS-related gene (including two cases with RYR1 mutations reported previously in this chapter that have been published) (McKie et al., 2014a) and these were included as positive controls for the study. The remaining 45 cases were genetically undiagnosed though most of them had undergone a variable amount of pre-study genetic analysis either in the NHS diagnostic laboratory or by the Maher research group and had

generally been investigated previously for mutations in *CHRNG, DOK7, RAPSN and RYR1* but no mutations were identified in these genes. The clinical features of the cases without a known molecular diagnosis are summarised in Table 5.1. Autosomal recessive inheritance was assumed in cases with and affected sibling and/or parental consanguinity and autosomal dominant inheritance when there was an affected parent. Isolated cases were listed as sporadic and no assumptions were made regarding inheritance. All families gave written informed consent and the study was approved by the South Birmingham Research Ethics Committee.

Table 5.1 Summary of recruited undiagnosed probands analysed by clinical exome sequencing

Clinical Exome ID	**Phenotype**	**Consanguinity**	**Inheritance**
CE01	LMPS	N	Sporadic
CE02	FADS/LMPS	Y	Autosomal Recessive
CE03	LMPS	Y	Autosomal Recessive
CE04	LMPS	N	Autosomal Recessive
CE05	LMPS	Y	Autosomal Recessive
CE06	LMPS	Y	Autosomal Recessive
CE07	Arthrogryposis	N	Sporadic
CE08	LMPS	Y	Autosomal Recessive
CE09	LMPS	Y	Autosomal Recessive
CE10	LMPS	Y	Autosomal Recessive
CE11	LMPS	Y	Autosomal Recessive
CE12	EVMPS	N	Autosomal Recessive
CE13	EVMPS	N	Sporadic
CE14	Arthrogryposis	N	Sporadic
CE15	EVMPS	N	Sporadic
CE16	LMPS	N	Autosomal Recessive
CE17	Arthrogryposis	N	Autosomal dominant
CE18	Arthrogryposis	N	Sporadic
CE19	EVMPS	N	Sporadic
CE20	EVMPS	Y	Autosomal Recessive
CE21	EVMPS	N	Autosomal Recessive
CE22	LMPS	Y	Autosomal Recessive
CE23	EVMPS	Y	Autosomal Recessive

CE24	LMPS	N	Autosomal Recessive
CE25	LMPS	N	Sporadic
CE26	LMPS	Y	Autosomal Recessive
CE27	Athrogryposis	N	Sporadic
CE28	EVMPS	N	Autosomal Recessive
CE29	EVMPS	N	Sporadic
CE30	EVMPS	N	Sporadic
CE31	LMPS	Y	Autosomal Recessive
CE32	LMPS	Y	Autosomal Recessive
CE33	EVMPS	N	Sporadic
CE34	EVMPS	N	Sporadic
CE35	EVMPS	N	Sporadic
CE36	EVMPS	N	Autosomal Recessive
CE37	LMPS	N	Autosomal Recessive
CE38	LMPS	Y	Autosomal Recessive
CE39	FADS	N	Sporadic
CE40	FADS	N	Sporadic
CE41	LMPS	N	Sporadic
CE42	EVMPS	N	Sporadic
CE43	Arthrogryposis	N	Sporadic
CE44	EVMPS	N	Autosomal dominant
CE45	LMPS	Y	Autosomal Recessive
E46	LMPS	Y	Autosomal Recessive

5.3 Molecular Genetic Analysis:

DNA samples were extracted by West Midlands Regional Genetics Laboratory mainly from blood samples using a standard extraction methodology. The targeted resequencing was performed using Illumina TruSightTM Rapid Capture kit to cover coding sequences from 4813 clinically relevant genes from the TruSightOne panel. In our analysis, 53 probands were analysed (8 were diagnosed with known mutations in MPS candidate genes while 45 without a previous diagnosis). The Trusight One assay was applied first to the 8 diagnosed probands to test the efficiency of the test before proceeding to use it in all other non-diagnosed cases. The panel has successfully detected all the 8 previously known mutations (in CHRNG, LMNA,

RAPSN, RYR1). Then, we decided to use the panel to most of the undiagnosed cases. The panel contains all the required reagents for the amplification, amplicon enrichment, indexing of the samples and the use of NextSeq 500 (Dello Russo et al., 2014). Using Nextera library preparation technology, genomic DNA was fragmented and tagged prior to multiplex pre-enrichment sample pooling, an average 400bp fragment library size is achieved and a final pooled library concentration of 8-12Pm was assembled. For the assessment of the genomic library quality, 2100 Bioanalyzer tools (Agilent Technology) were used. The sequencing was then performed on NGS Illumina's HiSeq2500 analyser employing a pair-end 150-cycle sequencing run. The Trusight One sequencing assay was performed by members of the Stratified Medicine Core Laboratory in Cambridge. I then analysed the clinical exome data to identify candidate mutations. Bioinformatics analysis and annotation of rare genetic variants were performed. Any variant reported >2% heterozygote frequency in 1,000 genomes project and variant exome server data (www.1000genomes.org/ and http://evs.gs.washington.edu/EVS/ respectively) was removed. Sequence data was inspected in two stages: I primarily focused on the gens that present in the clinical diagnostic gene panel (n=39 genes) and then in a wider set of further 47 potential MPS-related genes giving a total of 86 genes that were thought to be of potential relevance to MPS-related phenotypes. Finally, if no candidate mutations were identified, then all rare variants were inspected (Figure 5.1 & Table 5.2).

The variants identified as pathogenic mutations were confirmed by Sanger sequencing in the proband sample and, when available, segregation was checked in DNA from other family members on whole genome amplified DNA (Qiagen REPLI-g kits) and stock DNA. PCR products were sequenced in forward and reverse orientations using a standard sequencing method (BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems®).

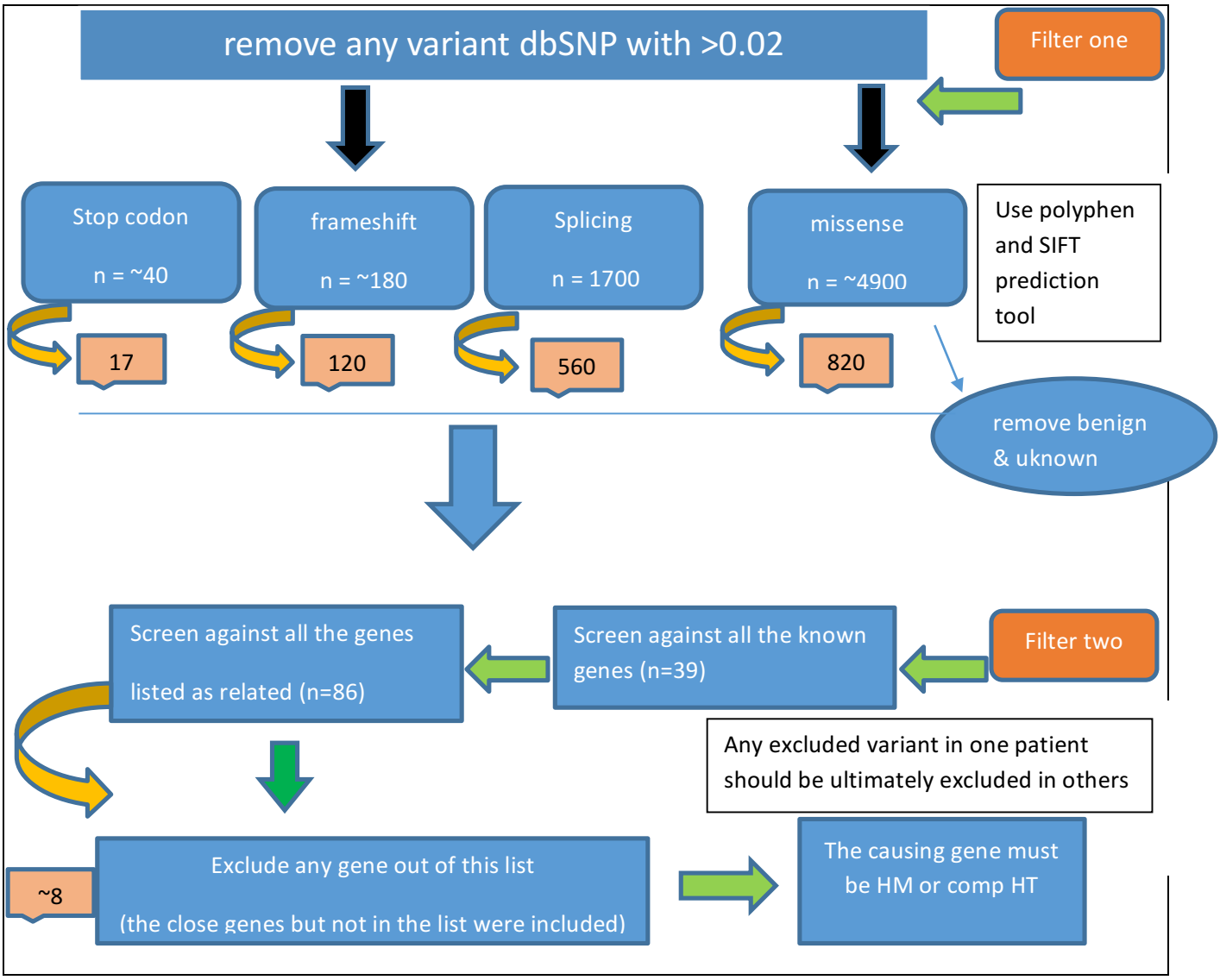


Figure 5.1 An example of the clinical exome file and the filtering steps to find the causing gene

Table 5.2 the list of genes included in the diagnostic and the MPS-related panels

genes included in the clinical diagnostic gene panel (n=39 genes) and the whole MPS-related genes n=86 genes) that were thought to be of potential relevance to MPS-related phenotypes

Panel for Diagnostic Testing (n=39)		**Extended panel (if diagnostic panel failed to demonstrate a mutation) (n=86)**				
ACTA1	MPZ	ACTA1	COG5	GLE1	POMGNT1	SRD5A3
BIN1	MUSK	AGRIN	COG7	IRF6	POMT1	SYNE1
CHRNA1	MYBPC1	ALG1	COG8	KBTBD13	POMT2	TMEM165
CHRNB1	MYH3	ALG11	CRYAB	KLHL40	RAPSN	TNNI2
CHRND	MYH8	ALG12	DAG1	LARGE	RFT1	TNNT3
CHRNG	NEB	ALG2	DDOST	LMNA	RIPK4	TPM2
CNTN1	PIEZO2	ALG3	DMPK	MEGF10	RYR1	TRIM32
DMPK	PIP5K1C	ALG6	DNM2	MGAT2	RYR3	TTN
DNM2	RAPSN	ALG8	DOK7	MOGS	SLC25A19	
DOK7	RIPK4	ALG9	DOLK	MPDU1	SLC35A1	
EGR2	RYR1	ANO5	DPAGT1	MPI	SLC35C1	
ERBB3	SYNE1	BIN1	DPM1	MPZ	CNTN1	
FGFR2	TNNI2	BRAT1	DPM3	MTM1	COG1	
FKBP10	TNNT3	C14orf133	EGR2	MUSK	COG4	
FKRP	TPM2	CFL2	ERBB3	MYBPC1	VPS33B	
FLVCR2	TPM3	CHRNA1	FGFR2	MYH3	PLA2G6	
GBE1	UBA1	CHRNB1	FKBP10	MYH8	PMM2	
GLE1	UTRN	CHRND	FKRP	NEB	UBE1	
IRF6	PLA2G6	CHRNG	FKTN	PIP5K1C	UTRN	
LMNA						

5.4 Results

5.4.1 Detection of known mutations in CHRNG, DOK7, LMNA, RAPSN and RYR1

The Eight previously diagnosed probands had 8 rare pathogenic variants in five genes associated with MPS-related phenotypes. They were analysed by CES method and the analysis resulted in identifying all these variants. Two homozygous *RYR1* mutations (c.6721C > T; p. Arg2241* and c.7043delGAG; p.Glu2347del) (E45 & E46 respectively) associated with LMPS/FADS were detected in two consanguineous families (MPS001 and MPS003) which have been reported earlier in this chapter (McKie et al., 2014a) (Figures 5.2 &5.3). The third detected variants was a

previously characterised mutation in *RAPSN* (homozygous c.264C>A, p.Asn88Lys, associated with congenital myasthenic syndrome (CMS) in sample CE36 (figure 5.4) that shows NGS sequence and no patient's DNA was available to validate this variant by Sanger method . Further two samples contained heterozygous variants in *LMNA* (c.1445G>A, (p.Arg482Gln) and c.357C>T) were confirmed. Two samples contained *CHRNG* mutations: one sample contained two heterozygous frameshift mutations in *CHRNG* (c.458delCinsCA (p.Val154SerfsTer24) and c.753_754delCT (p.Val253AlafsTer44) and another sample was homozygous for one of the frameshift mutation (c.458delCinsCA (p.Val154SerfsTer24). Last exome sample contained a homozygous splice donor site mutation in DOK7 (c.331+1G>T).

Sample – CE45; location- 19:38987106; Gene - RYR1

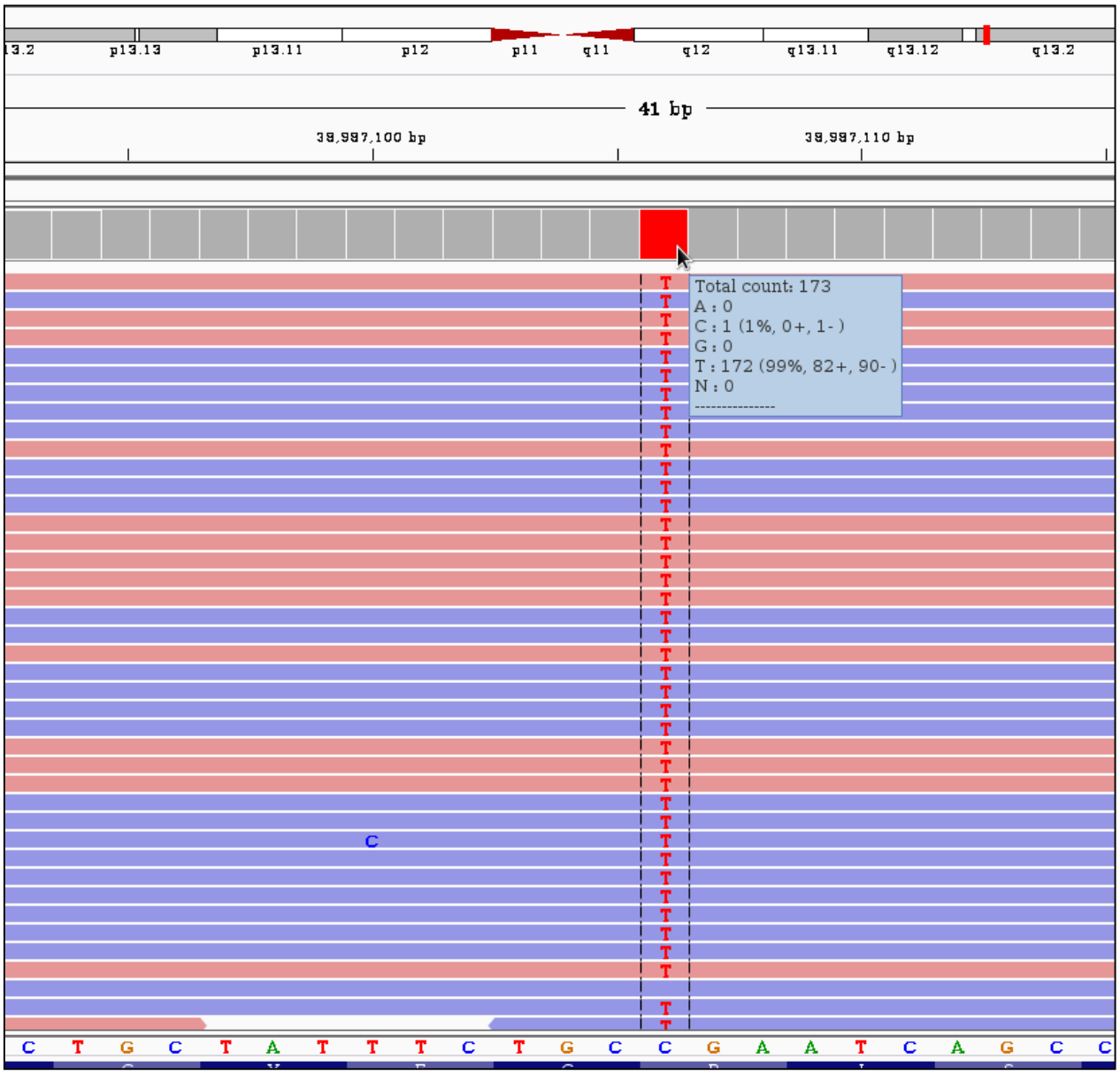


Figure 5.2 Sequence analysis of RYR1 c.6721C >T (p.Arg2241Ter) mutation

Pathogenic nonsense homozygous mutation was identified in Pakistani consanguineous families (MPS001) diagnosed with LMPS phenotype. Homozygous RYR1 c.6721C >T (p.Arg2241Ter) was confirmed by Sanger method in both affected siblings(homozygous) and in the parents in a heterozygous state (figure 4.3) of this thesis (McKie et al 2014)

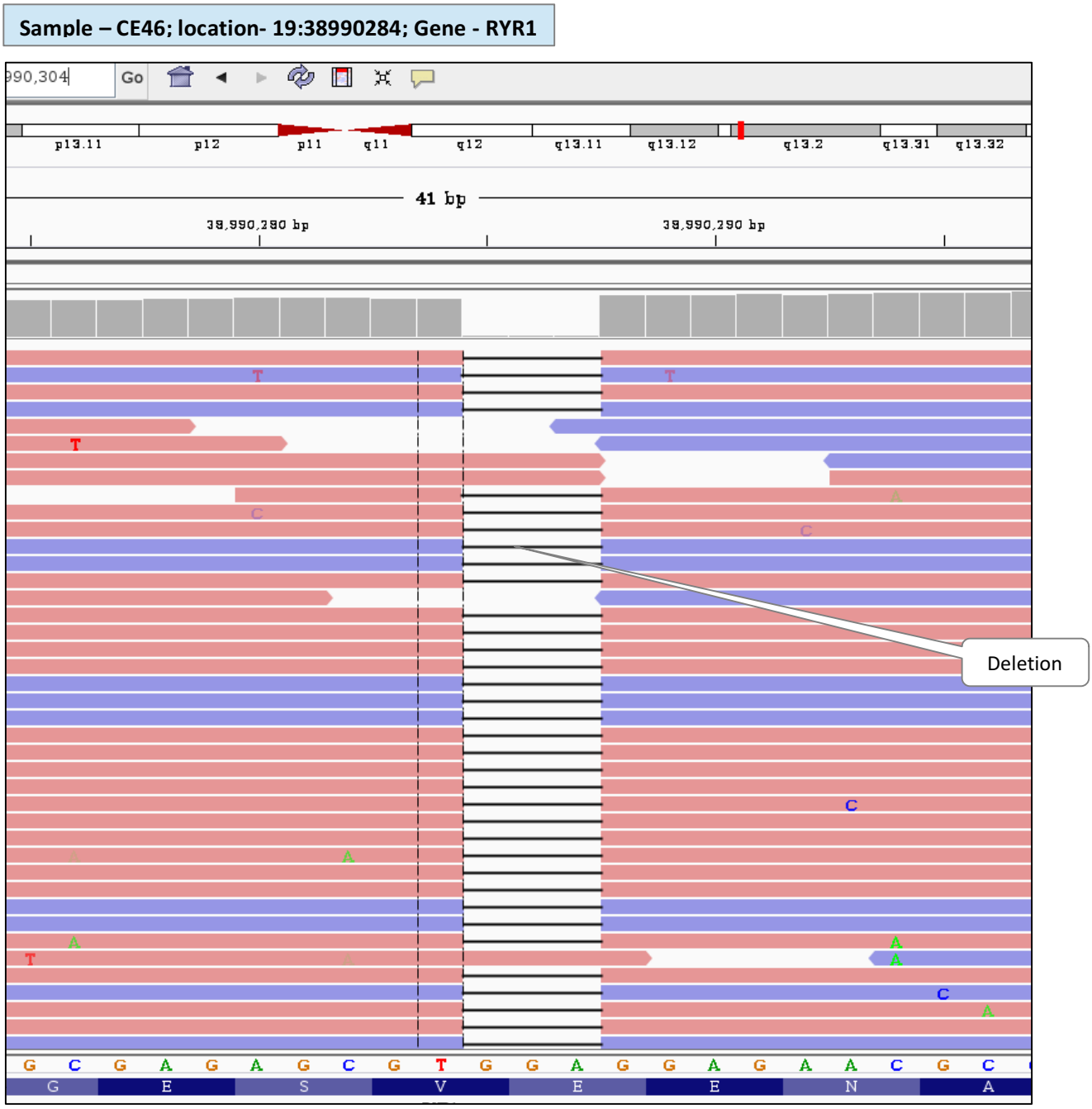


Figure 5.2 Sequence analysis of RYR1 c.7043delGAG (p.Glu2347del)

Pathogenic 27b homozygous deletion was identified in Pakistani consanguineous families (MPS002) diagnosed with LMPS phenotype. Homozygous RYR1 c.7043delGAG (p.Glu2347del) variant was already confirmed by Sanger in figure 4.6 of this thesis (McKie et al 2014)

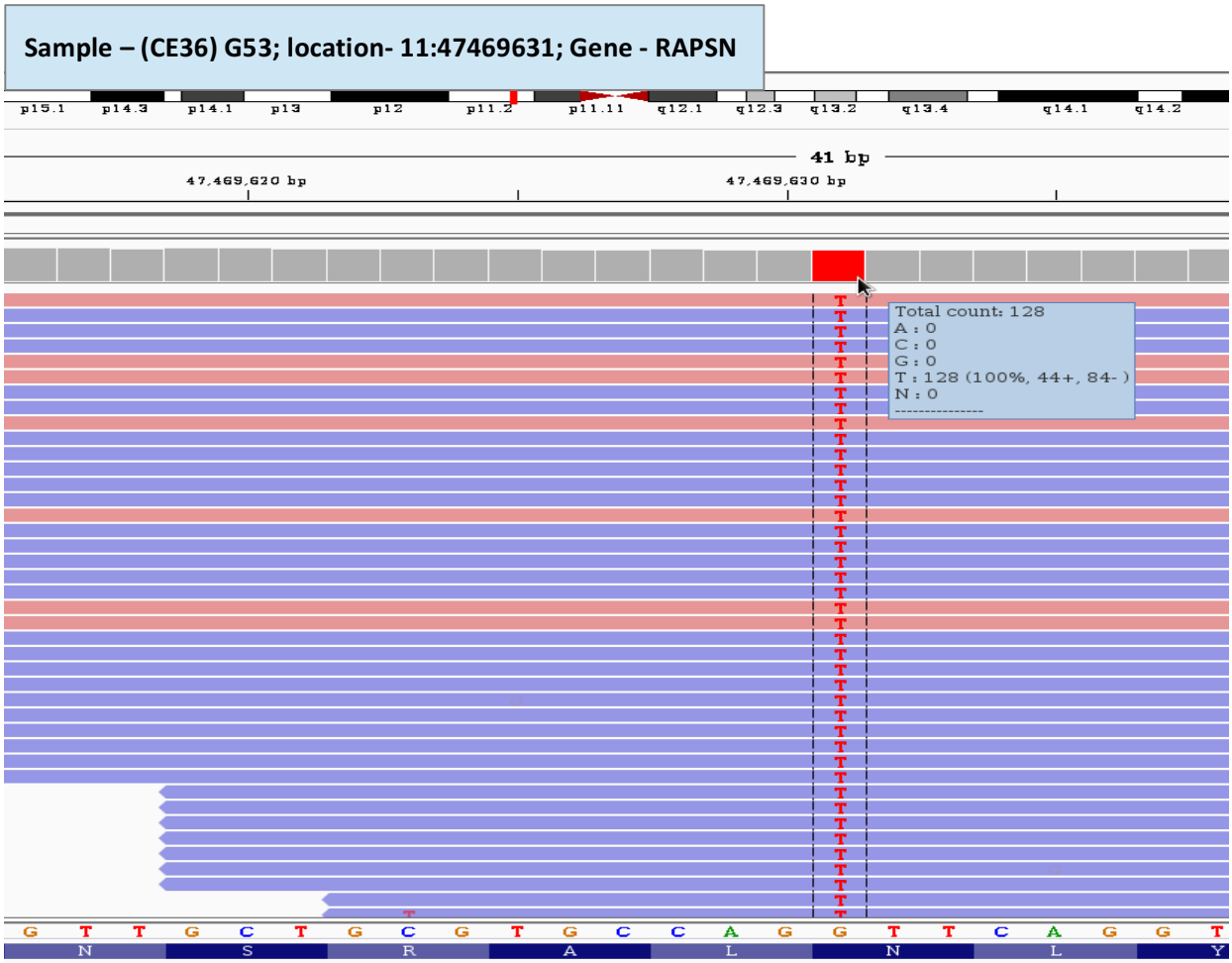


Figure 5.4 Sequence analysis of the variants: RAPSN c.264C>A (p.Asn88Lys)

homozygous missense RAPSN c.264C>A (p.Asn88Lys)was identified in fetus affected with with congenital myasthenic syndrome

5.4.2 Identification of previously uncharacterised mutations by clinical exome analysis

Amongst total 45 undiagnosed analysed patients, 7 cases (15.5 %) have shown 7 potential mutations in five MPS-related disease genes (*CHRNG, CHRNA1, NEB1, RYR1* and *TPM2*). These variants are below: (see Table 5.2).

***CHRNG*:** A pathogenic homozygous missense substitution, (NM_005199.4 *CHRNG* c.715C>T (p.Arg239Cys) was detected in the proband (exome: CE26) from a consanguineous Turkish

family that presented with LMPS and the segregation analysis using Sanger method showed that parents where heterozygous carriers for the mutation (Figures 4.5 & 4.6). This mutation had been previously described in homozygous status in two affected members of two unrelated consanguineous families from Lebanon and Turkey and they were diagnosed with EVMPS and LMPS respectively (Hoffmann et al., 2006).

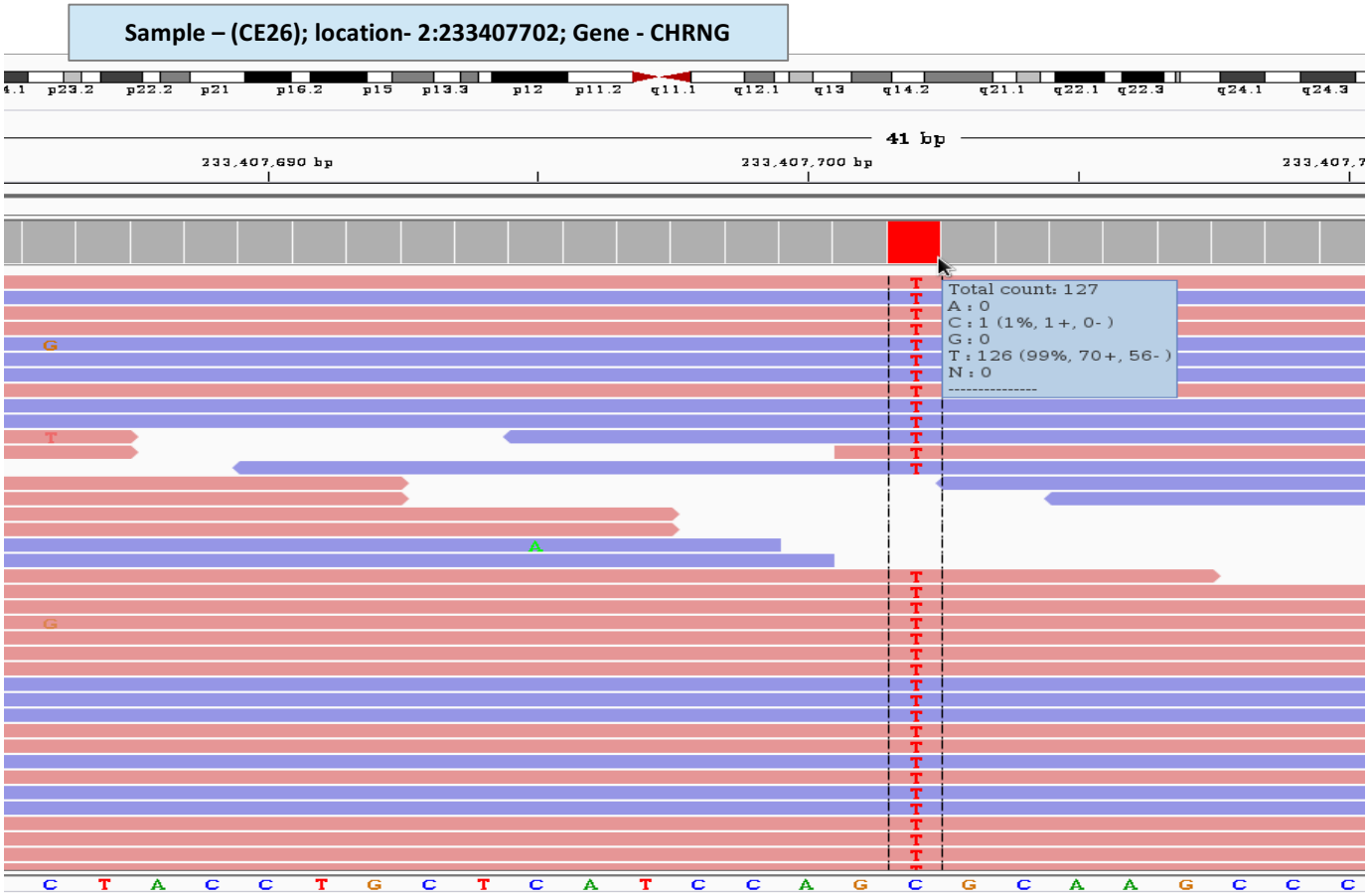


Figure 5.5 Sequence analysis of the rare variant: CHRNG c.715C>T (p.Arg239Cys)

Pathogenic homozygous missense substitution was identified in a Turkish consanguineous family diagnosed with EVMPS. As shown in NGS file (exome: CE26)

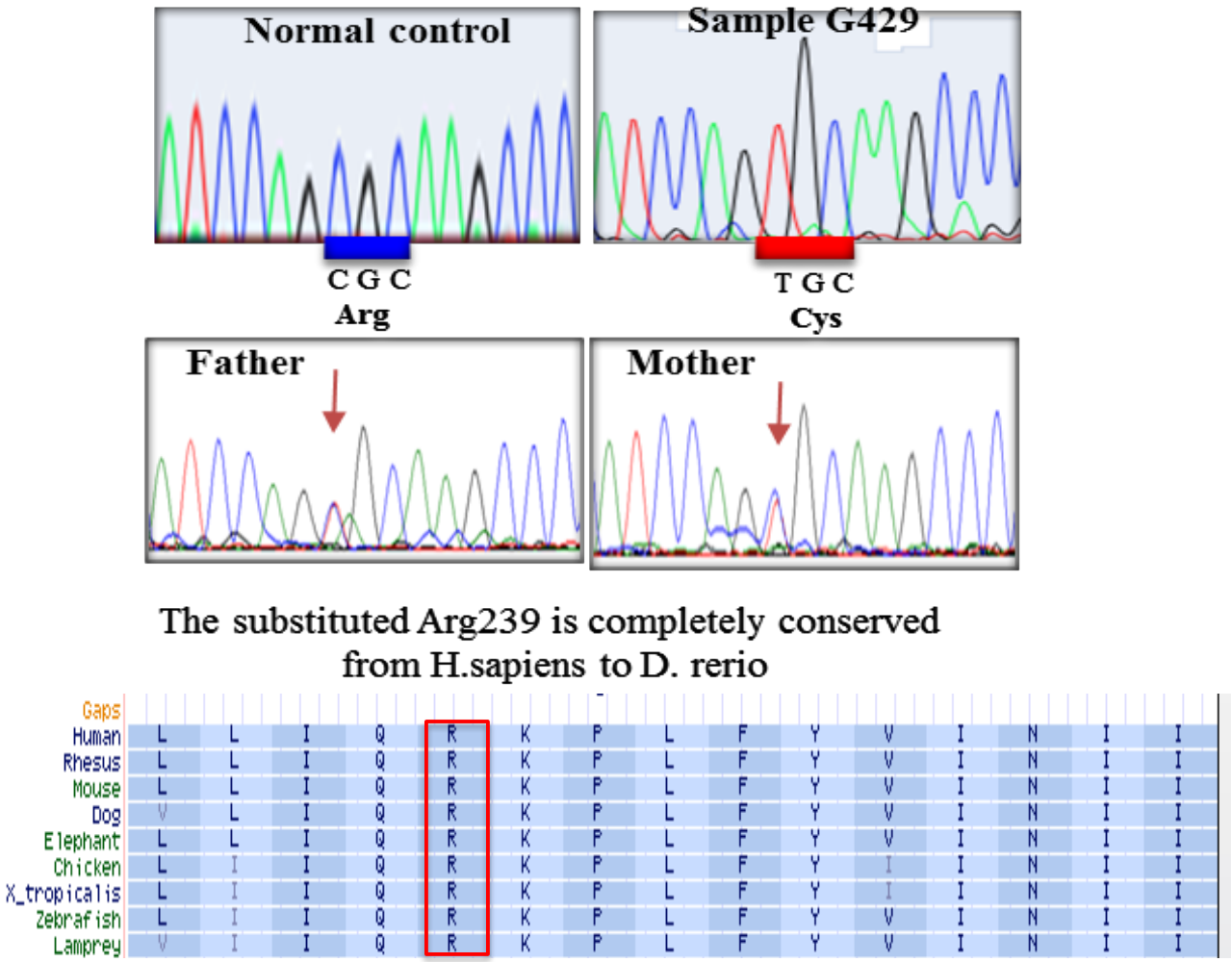


Figure 3.6 Sanger confirmation for the variant: CHRNG c.715C>T (p.Arg239Cys) and the conservative location of the amino acid

This variant was confirmed by Sanger Sequencing. The affected sibling was homozygous for the mutation (NM_005199.4 CHRNG c.715C>T (p.Arg239Cys) while both parents were heterozygous carriers. The amino acid sequence is showing the conservation of the amino acid residue in different species.

***CHRNA1*:**

The second identified mutation was a homozygous insertion (duplication) of 17 nucleotides of CHRNA1 gene (NM_000079.3 *CHRNA1*; c.117_133dupGCGGCCAGTGGAAGACC (p.His45ArgfsTer19) which was detected in a proband (exome: CE22) (Figure 5.7) that was diagnosed with FADS and born to a consanguineous parents from Somalia, resulting in a frameshift mutation with a subsequent premature stop codon (a1.H25RfsX19). This mutation was previously reported in a family of African origin diagnosed with FADS (Michalk et al, 2008). According to the provided clinical history, the male fetus had a severe septated cystic hygroma, early hydrops and fixed flexion deformity of all four limbs detected on antenatal ultrasound scan. Microarray analysis for copy number abnormalities (CNA) was normal. Autopsy was declined. The couple had had three previous male pregnancy losses between 20 and 23 weeks of gestation with cystic hygroma, hydrops and fixed flexion contractures. Prior testing of *CHRNG* and *DOK7* had not identified any pathogenic sequence variants. Confirmation by Sanger sequencing revealed the insertion was a tandem duplication of 17 nucleotides and appropriate segregation was demonstrated in two unaffected siblings and the parents.

Sample – (CE22) location- 2:175624271; Gene - CHRNA1

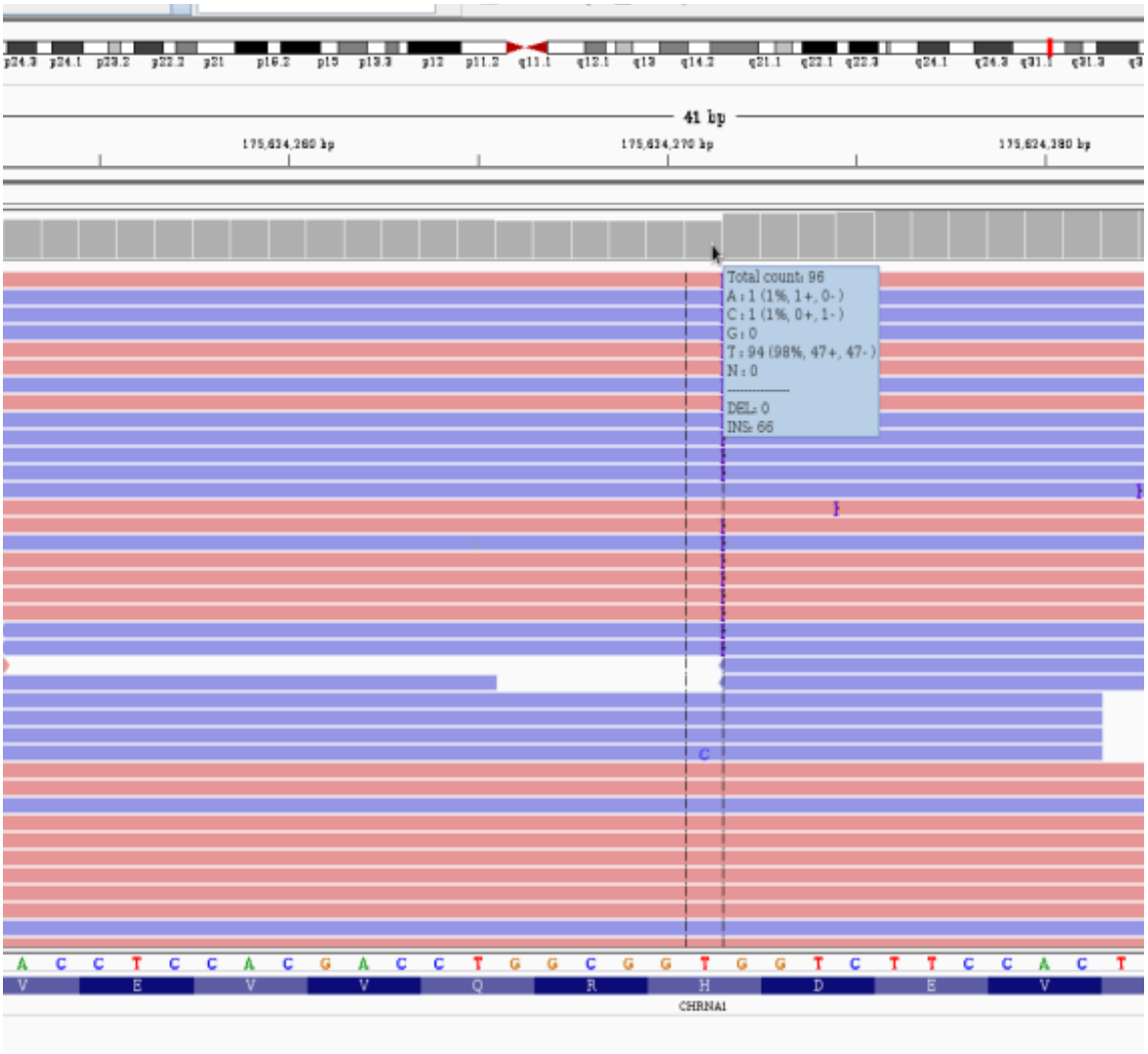


Figure 5.7 Sequence analysis of the rare CHRNA1 variant insertion: p.His45ArgfsTer19

further homozygous CHRNA1 insertion was detetected in a Somalian consanguineous family diagnosed with FADS phenotype. As shown in NGS file (exome: CE22), the variant was homozygous for the insertion;

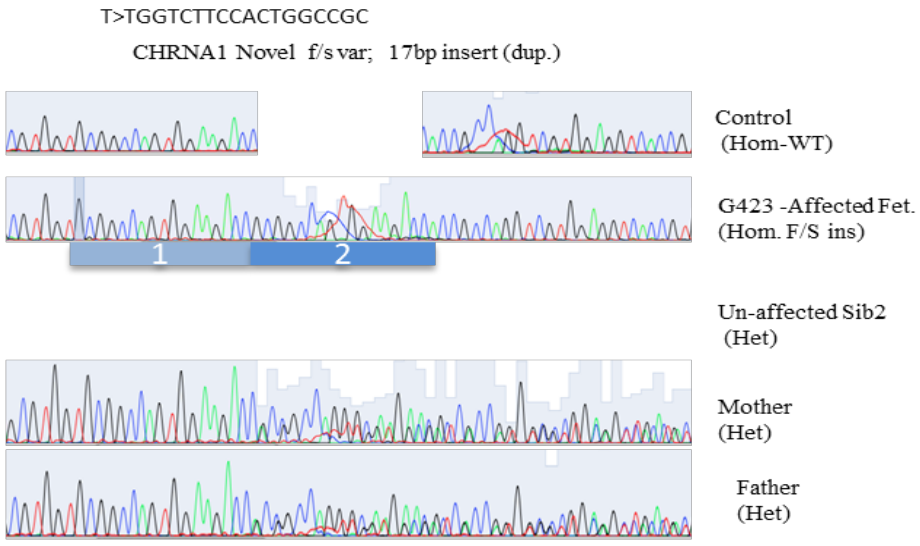


Figure 5.8 Sanger sequencing for the rare CHRNA1 variant insertion: p.His45ArgfsTer19

This insertion; c.117_133dupGCGGCCAGTGGAAGACC (p.His45ArgfsTer19) was confirmed by Sanger Sequencing as shown (by Arthur Mckie). The affected sibling was homozygously deleted while both parents were heterozygous carriers.

RYR1: A novel homozygous nonsense mutation in (Exome:CE08) was identified in RYR1 gene (c.12882G>T; p.E4294*) in a female fetus (F1) affected with LMPS from a consanguineous family (MPS013) of Pakistani origin (Figure 5.9) . Sanger sequencing confirmed this nonsense mutation and showed a segregation in another affected (male) fetus (F2) by detecting it in a homozygous state and in the parents who were heterozygous for the nonsense mutation. This mutation has not been previously reported in any patients with MPS- related syndromes. The proband (F1) had an antenatally detected large cystic hygroma and intrauterine death occurred at 18 weeks of gestation. At post mortem intrauterine growth retardation (IUGR), cystic hygroma, cleft palate, micrognathia, scoliosis of the vertebral column and multiple joint contractures with skin webs at the elbows, axillae and knees were noted. The muscle bulk was significantly

reduced with no muscle identified in the extremities. All the muscles were autolytic with severe degeneration of the fibres and variability in the shape and size of the muscle cells. There was some positive staining of slow and fast myosin in the muscles. PAS and DiPAS stains were negative excluding a glycogen storage disease. Lymphocytic infiltration of the muscle cells was considered suggestive of a possible inflammatory myopathy. The second affected fetus was terminated at 19 weeks following the antenatal detection of a large cystic hygroma. Post mortem examination revealed an affected male fetus with IUGR, a cystic hygroma, cleft palate, flexion contractures of the upper and lower limbs and skin webs at the elbows, groins and knees. There was bilateral talipes and prominent heels. The thoracic cavities were narrow and the ribs appeared shorter than usual. The long bones were gracile. The muscle bulk was significantly reduced and microscopicy revealed degenerative and regenerative changes with very scattered myocytes. There was no slow fibre staining and a few fibres stained with fast myosin. The a-laminin staining was weak and patchy. There was no family history of malignant hyperthermia.

Sample – (CE08); location- 19:39055740; Gene - RYR1

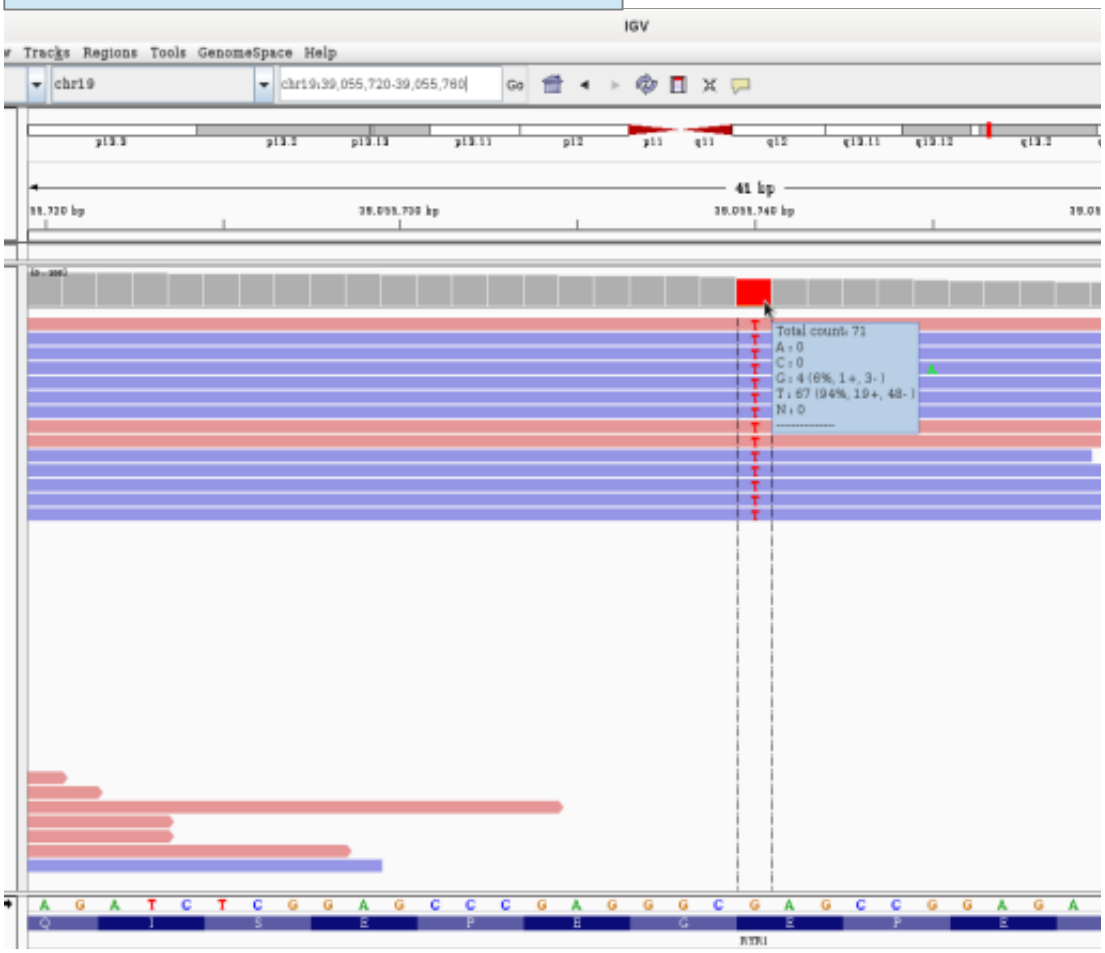


Figure 5.9 Sequence analysis of the rare RYR1 nonsense variant: (c.12882G>T; p.E4294)*

A novel homozygous RYR1 stop codon variation was detected in a Pakistani consanguineous family diagnosed with LMPS phenotype. As shown in NGS file (exome: CE08) the affected female fetus was homozygous for the mutated allele; (c.12882G>T; p.E4294)*

NEB: Another novel variant was detected in *NEB* gene. It was a homozygous null *NEB* nonsense mutation (c.10075G>T; p.Glu3359*) that identified in the proband CE03) and two other affected siblingss of a consanguineous Pakistani couple (MPS058). The children presented LMPS phenotype. The *NEB* mutation created a SpeI RFLP. in the clinical details, the first pregnancy was terminated at 21 weeks' gestation following the detection of a cystic hygroma and fetal akinesia. At post mortem examination the fetus was appropriately grown but a large posterior cystic hygroma, fetal hydrops and pulmonary hypoplasia were noted. There were four limbs contractures and pterygia. The brain and spine appeared normal however the muscle bulk was severely reduced. The fetus appeared dysmorphic with down-slanting eyes, an upturned nasal tip, a small mouth with a high arched palate, micrognathia and low set ears. The muscle was composed of loose connective tissue with some fatty tissue. The surviving muscle fibres showed abundant central nuclei and degenerative and regenerative changes. There were some multi-nucleated fibres and vacuolation was prominent and appeared to contain abundant glycogen. The excess glycogen in the muscles was confirmed on electron microscopy. In the second pregnancy fetal akinesia sequence was detected on ultrasound scan at 13 weeks and there was an intrauterine death at 28/40. At post mortem the male fetus had features of LMPS. There was severely reduced muscle bulk with almost complete lack of muscle fibres in the psoas muscle and diaphragm with severe degeneration of the muscle. There was a large variation in the size of the muscle fibres and groups of multinucleated giant muscle cells containing small vacuoles. There was no evidence of the accumulation of glycogen. In the third pregnancy a cystic hygroma, bilateral pleural effusions and fetal akinesia were detected. There was an intraterine death at 23 weeks. The male fetus had features of LMPS. In addition, there was a bilateral cleft palate and abnormal ossification of the vertebral column. There were contractures

and the vertebral column appeared short with block and hemivertebrae. There was deficient ossification of the sacrum. The muscle contained connective tissue and lacked muscle fibres. There were very thin muscle fibres and very large giant cells with multiple nuclei. There were no vacuolar or PAS positive inclusions.

Maternal Myotonic dystrophy genetic testing was normal and maternal AChRAbs were negative. Karyotyping and genetic testing of PFKM for glycogen storage disease type VII was normal. Prior to CES analysis, no pathogenic sequence variants were detected in *CHRNG, CHRNA1, CHRND, RAPSN*, or *DOK7*.

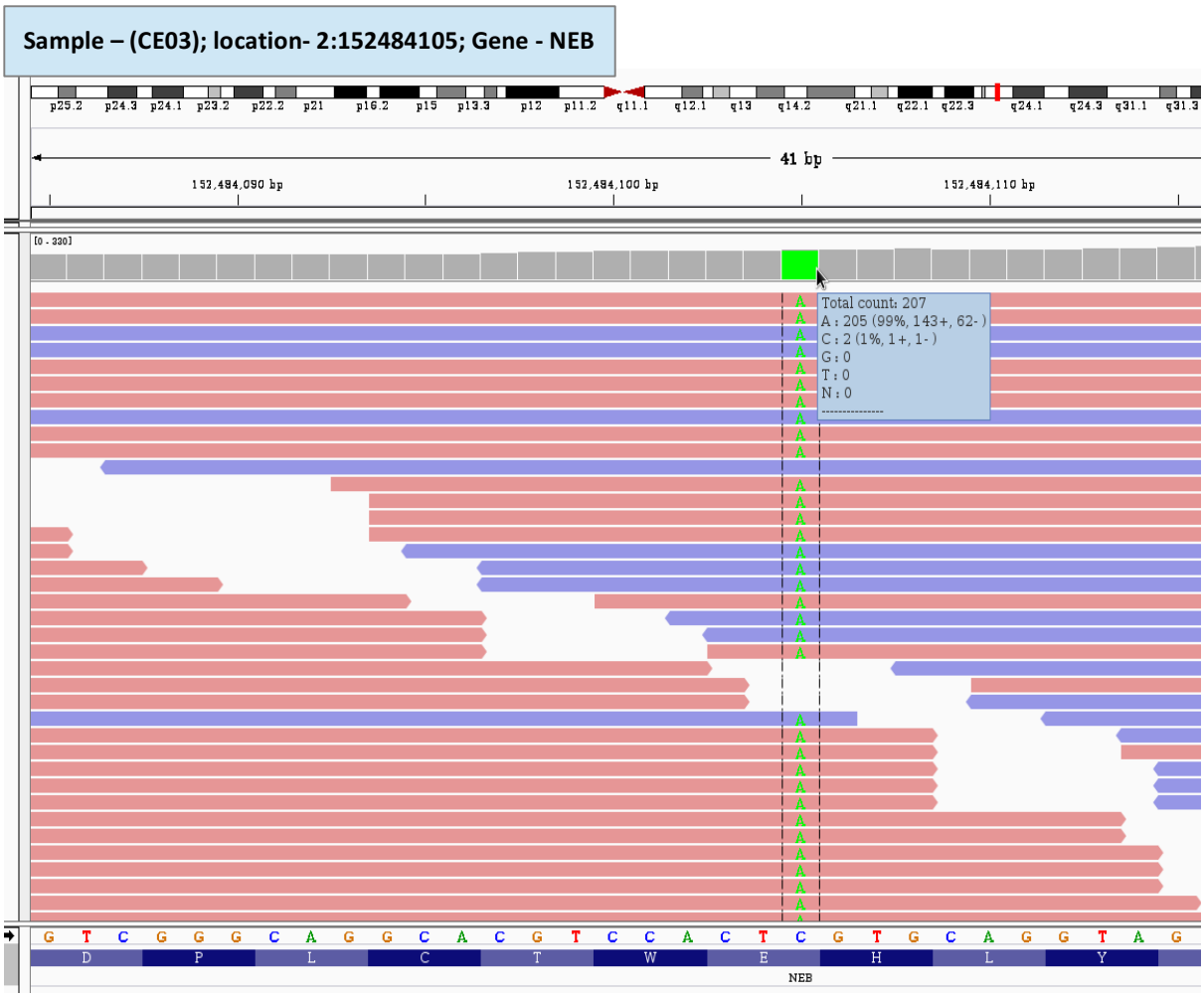


Figure 5.10 Sequence analysis of the rare NEB variant: c.10075G>T; p.Glu3359.*

*Further homozygous NEB nonsense mutation : c.10075G>T; p.Glu3359*was detected in a Pakistani consanguineous family is shown in NGS file (exome: CE03).*

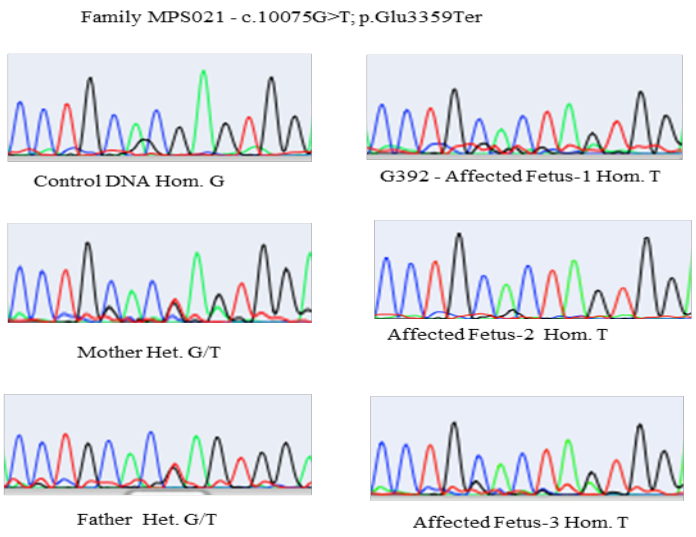


*Figure 5.11 Sanger confirmation of the rare nonsense NEB variant: c.10075G>T; p.Glu3359**

NEB nonsense variant; c.10075G>T was successfully validated by Sanger Sequencing as shown. The affected sibling (CE03) who diagnosed with LMPS phenotype was homozygous for the mutation; c.10075G>T; p.Glu3359. Also, the variation was detected in two other affected siblings in a homozygous state while both parents were heterozygous carriers.*

TPM2: An identical heterozygous *TPM2* missense substitution (ENST00000360958 c.379C>T c.502G>A; p.Arg133Trp) was detected in three apparently unrelated probands CE07, CE18 and CE30) (figures 5.12 &5.13). CE07 and CE18 were diagnosed with Distal Arthrogryposis (DA) whereas CE30 presented EVMPS clinical features. This mutation is located in a highly conserved amino acid residue of TPM2 and has been previously described in 2 female patients diagnosed with DA type 2B and muscle weakness (Tajsharghi et al., 2007)

CE07 was a female patient born to unrelated white parents. From 34 weeks gestation there were concerns about poor growth and reduced fetal movements. The delivery was normal and she was born with a birth weight of 2.944 kg, a length 45 cm and a head circumference 33cm. She was a poor feeder and required readmission after because of weight loss. She had multiple joint contractures. She had removal of a left brachial cyst remnant and required grommets. On

examination she had mild restriction of her wrist movement with ulna deviation and flexion contractures of fingers 2-5, most severe in her third fingers. She had smooth hands with a paucity of palmar creases. Pseudocamptodactyly was not present. She had bilateral congenital talipes equinovarus, slightly worse on the left and full heels. She had full flexion of her knees but lacked 30 degrees of extension. Both hips and spine were normal. Her muscles appeared normal and she had normal power in both quadriceps. Facially she had periorbital fullness of the eyelids, a slightly anteverted nose and a smooth philtrum. She had a small mouth with normal mouth opening. Her palate appeared normal. She had a low posterior hairline. Prior *CHRNG* analysis was normal.

CE18 was a male child of unrelated white parents. Decreased fetal movements, bilateral talipes equinovarus, clenched hands, pleural effusions and polyhydramnios were detected antenatally. He was delivered by Caesarian section at 38 weeks with a birth weight of 3.34 kg and a head circumference was 36 cm. He was self-ventilating but required tube feeds for the first 4 weeks of life. He had multiple joint contractures, a small atrial septal defect and bilateral undescended testis. At the age 5 years his diet is supplemented with high calorie milk. His height and weight are around the 2 percentile. He has Movicol for constipation. He has recurrent infections and tired easily. Psychomotor development was delayed but he was making progress.

CE30 is a male patient was born to non-consanguineous parents originating from South America. Reduced fetal movements were noted towards the end of the pregnancy. His birth weight was 2.83 kg and there were no neonatal problems. He had down-slanting palpebral fissures, hypertelorism and low set ears. He had a low posterior hairline and neck webbing. He had sloping shoulders and contractures of his shoulders, elbows, wrists and fingers, with ulna deviation at the metacarpalphalangeal joints and soft tissue syndactyly of fingers 2-5. He had

contractures of the hips, knees and ankles. He had a mild thoracic kyphoscoliosis and eleven pairs of downward sloping ribs. He required bilateral inguinal hernia repairs and bilateral tendon releases between his 1st and 2nd fingers of both hands and his feet. He also had release of a tongue tie. At the age of 6 he was short with poor muscle bulk. His height was on the 0.4 percentile, his weight below the 0.4 percentile and his head circumference between the 0.4 -2 percentile. There were no other concerns about his health or development. On x-ray there was bilateral forefoot valgus deformities and hindfoot valgus. There was bilateral coxa vara. Previous *CHRNG* analysis was normal.

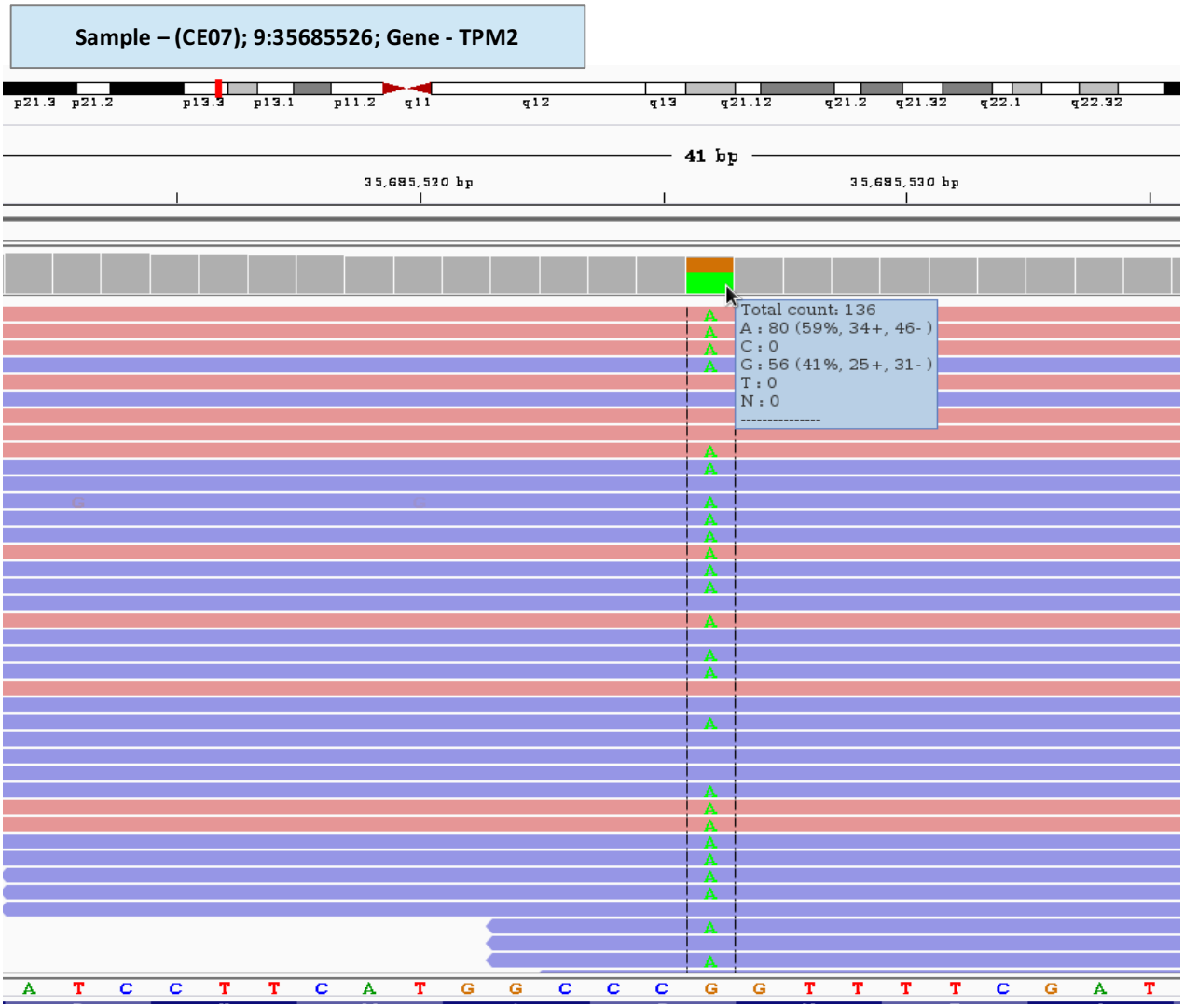


Figure 5.12 Sequence analysis of the rare TPM2 missense variant (c.379C>T c.502G>A; p.Arg133Trp)

NGS detected a heterozygous TPM2 missense variant (c.379C>T c.502G>A; p.Arg133Trp) was detected in three apparently unrelated probands (CE07, CE18 G399, G419 who diagnosed with arthrogryposis and CE30 who diagnosed with EVMPS ; however, this particular attached sequence belongs to CE07 but the others sequences (CE18 & CE30) are exactly the same.

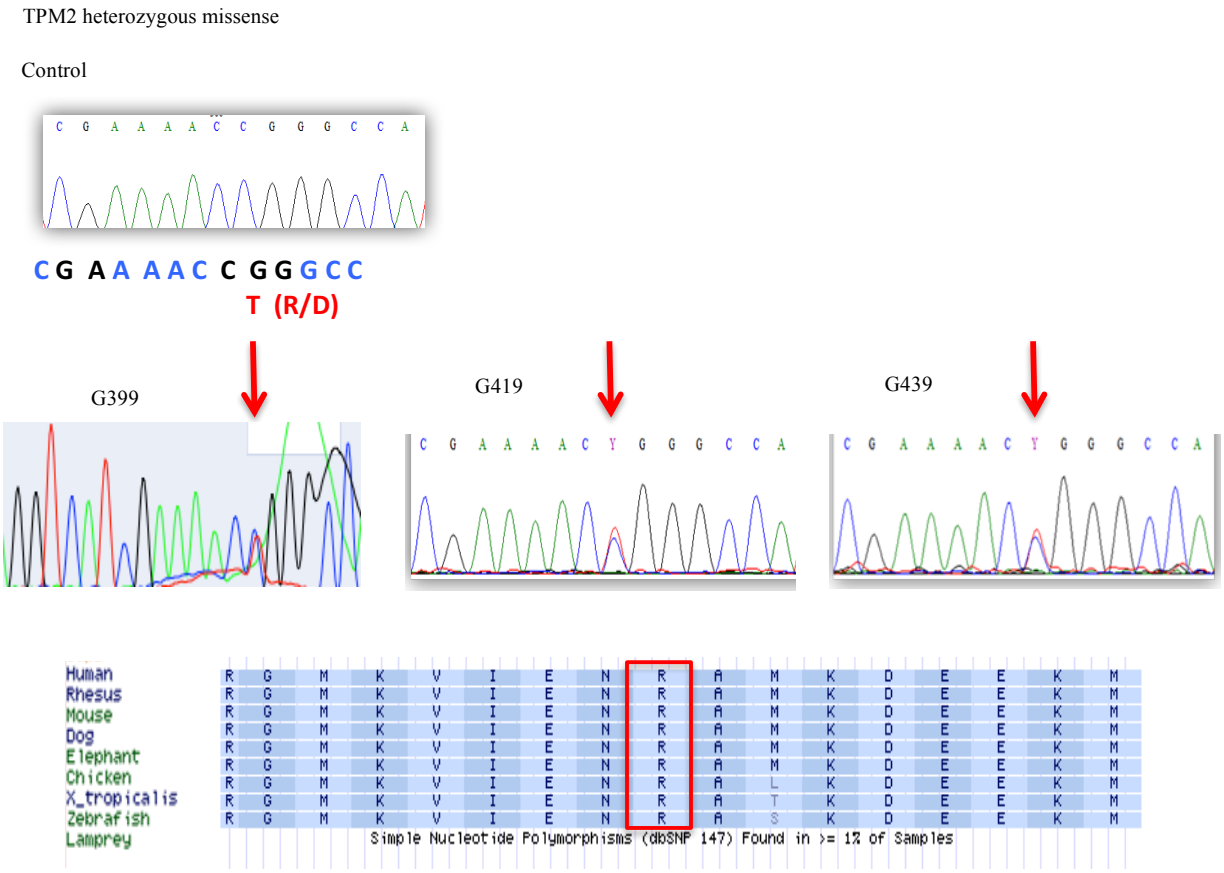


Figure 4 Sanger confirmation of the rare TPM2 missense variant (c.379C>T c.502G>A; p.Arg133Trp)

Figure 5.13: Confirmation of TPM2 missense variant (c.379C>T c.502G>A; p.Arg133Trp)

The variation was detected using NGS clinical exome panel and confirmed by Sanger sequencing. the amino acid sequences shows that mutation is located in a highly conserved amino acid residue of within TPM2 protein (taken from UCSC).

5.4.3 Identification of potential pathogenic mutations by clinical exome analysis

The second type of detected rare genetic variants has been called as potential pathogenic mutations with uncertain significance. Three different candidate mutations were detected; two variations were detected in known MPS-related genes (*NEB, RYR1*) and whilst the third was detected in another MPS-related gene, *RIPK4*. Thus a novel rare homozygous *RIPK4* missense substitution c.481G>C (p.Asp161His) was detected in a fetus (*CE06*) with LMPS from a

consanguineous family of Indian origin. It occurred at a highly-conserved residue and was predicted to be pathogenic by *in silico* analysis (figures 5.14 & 5.15). In addition, two further heterozygous truncating mutations; *NEB* frameshift deletion; c.7523_7526delTCAA (p.Ile2508ThrfsTer14) and *RYR1* nonsense variation c.481G>C (p.Asp161His) were detected. Both known genes (*RYR1 & NEB*) may cause autosomal dominant and autosomal recessively inherited muscle disorders. It could be postulated that the proband might harbour a second mutation *in trans* (e.g. an exon deletion that would not be detected by NGS or that a rare missense variant might be pathogenic).

Sample – CE06; location- 21:43171399; Gene - RIPK4

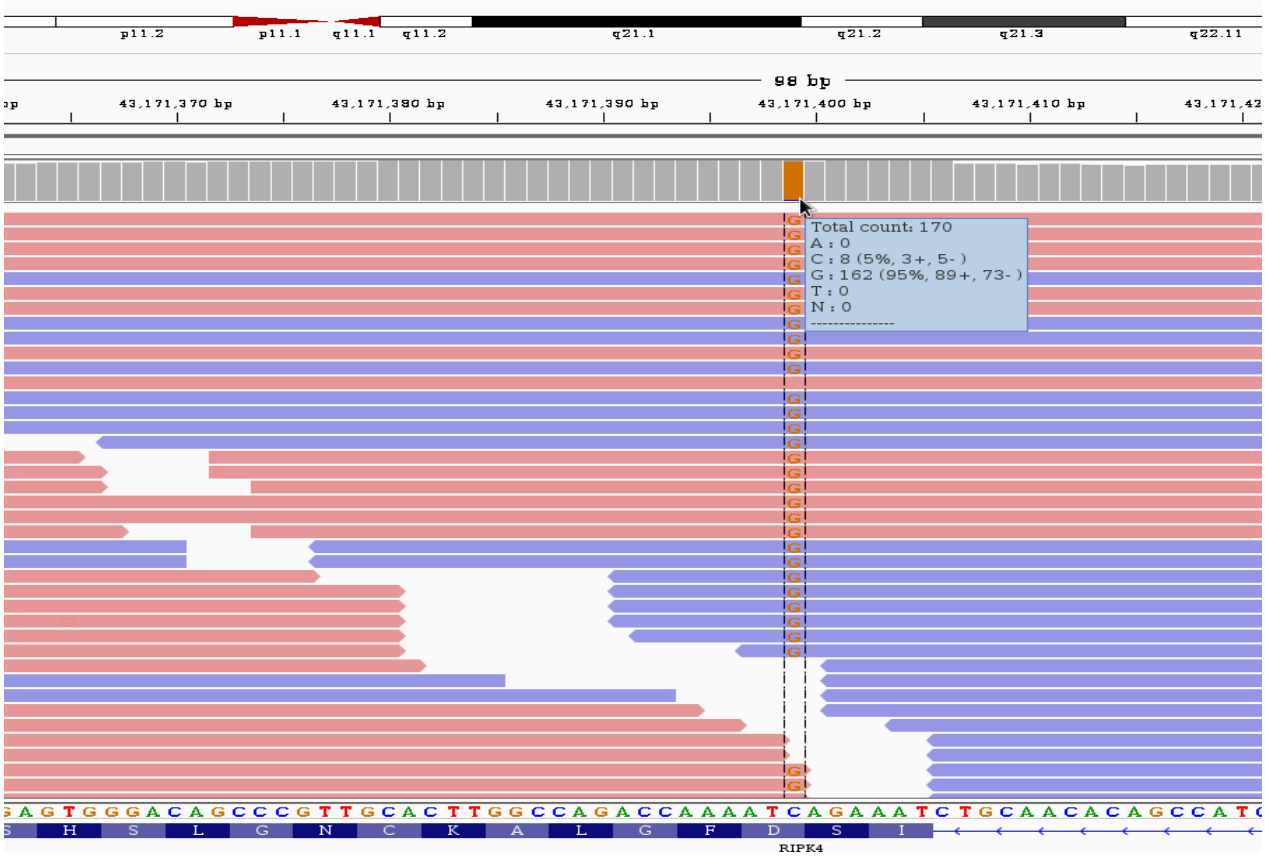


Figure 5.14 Sequence analysis of the rare RIPK4 missense: c.481G>C (p.Asp161His)

A novel homozygous RIPK4 missense variation was detected in Indian consanguineous family and shown in NGS file (exome: CE06)

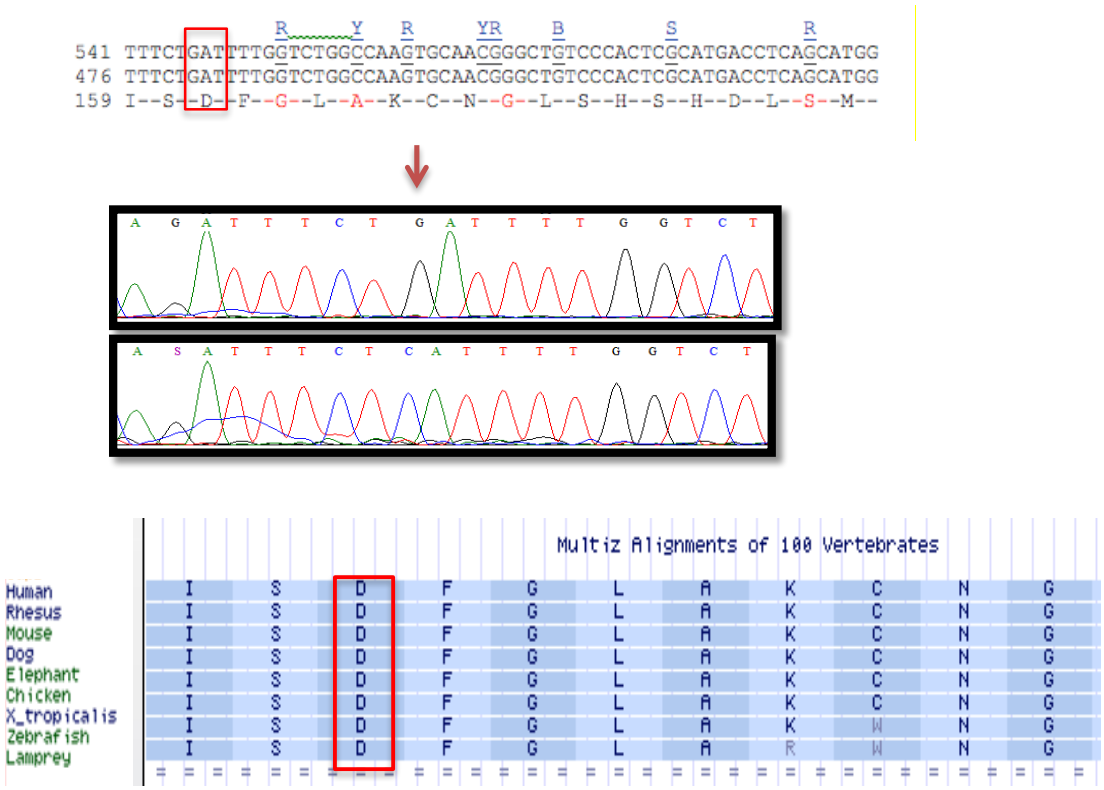


Figure 5.15 Sanger analysis of the rare RIPK4 missense: c.481G>C (p.Asp161His)

The variant was validated by Sanger Sequencing as shown in the chromatogram. The amino acid sequence is showing the conservation of the Arginine amino acid residue in different species (Taken from UCSC).

Table 5.3 *Summary of the detected rare mutations using Clinical Exome Trusight One Panel in different MPS-related genes.*

Gemini number	G439	G419	G399	G392	G402	G423	G429
CES reference	CE30	CE18	CE07	CE03	CE08	CE22	CE26
Gene	*TPM2*	*TPM2*	*TPM2*	*NEB*	*RYR1*	*CHRNA1*	*CHRNG*
Transcript	ENST00000360958	ENST00000360958	ENST00000360958	ENST00000603639	ENST00000355481	ENST00000409542	ENST00003 89494
DNA change	c.379C>T	c.379C>T	c.379C>T	c.10075G>T	c.12751G>T	c.117_133dupGCGGCCAGTGGAAGACC	c.715C>T
Protein change	p.Arg133Trp	p.Arg133Trp	p.Arg133Trp	p.Glu3359Ter	p.Glu4251Ter	p.His45Argfs Ter19	p.Arg239Cys
Protein consequence	Missense	Missense	Missense	Truncation	Truncation	Truncation	Missense
Frequency of alt allele in EVS							
PolyPhen2	Probably damaging (1)	Probably damaging (1)	Probably damaging (1)	-	-	-	Probably damaging (0.998)
SIFT	Deleterious (0.02)	Deleterious (0.02)	Deleterious (0.02)	-	-	-	Deleterious (0)
Genotype	Heterozygous	Heterozygous	Heterozygous	Homozygous	Homozygous	Homozygous	Homozygous
segregation in family	No DNA available	No DNA available	No DNA available	affected siblings homozygous, mother			Parents heterozygous

[redacted]	G439	G419	G399	[redacted]	[redacted]	[redacted]	[redacted]
CES reference	CE30	CE18	CE07	CE03	CE08	CE22	CE26
Gene	*TPM2*	*TPM2*	*TPM2*	*NEB*	*RYR1*	*CHRNA1*	*CHRNG*
Transcript	ENST00000360958	ENST00000360958	ENST00000360958	ENST00000603639	ENST00000355481	ENST00000409542	ENST00000389494
DNA change	c.379C>T	c.379C>T	c.379C>T	c.10075G>T	c.12751G>T	c.117_133dupGCGGCCAGTGGAAGACC	c.715C>T
Protein change	p.Arg133Trp	p.Arg133Trp	p.Arg133Trp	p.Glu3359Ter	p.Glu4251Ter	p.His45ArgfsTer19	p.Arg239Cys
Protein consequence	Missense	Missense	Missense	Truncation	Truncation	Truncation	Missense
PolyPhen2	Probably damaging (1)	Probably damaging (1)	Probably damaging (1)	-	-	-	Probably damaging (0.998)
SIFT	Deleterious (0.02)	Deleterious (0.02)	Deleterious (0.02)	-	-	-	Deleterious (0)
Genotype	Heterozygous	Heterozygous	Heterozygous	Homozygous	Homozygous	Homozygous	Homozygous
segregation in family	No DNA available	No DNA available	No DNA available	affected siblings homozygous, mother			Parents heterozygous

5.5 Discussion

5.5.1 Why we selected CES strategy to investigate MPS disorders?

We undertook "clinical exome sequencing" (CES) of a cohort of families with MPS-related disorders and detected both previously characterised (positive control) and previously unknown mutations in MPS-related disease genes. A major advantage of CES or whole exome sequencing (WES) for diagnostic testing is the ability to cost-effectively sequence large numbers of candidate genes in parallel. Traditionally molecular genetic analysis of MPS-related disorders has been undertaken by sequential analysis of single genes (*CHRNG, DOK7, RAPSN and RYR1*) for most of the cases. Apart from few identified mutation, known MPS genes were not reported as a cause for most cases. According to The United Kingdom Genetic Testing Network (http://ukgtn.nhs.uk/find-a-test/), the cost of single gene sequencing analysis of *CHRNG* or *RAPSN* in a clinical diagnostic laboratory as at least £423 while genetic testing of extremely large genes such as *RYR1* (106 exons) and *NEB* genes (160 exons) is more expensive. Both genes (but not *RAPSN* or *CHRNA1* or *CHRNG*) are included in a panel test of congenital myopathy (n=22 genes) that cost nearly £1300. In contrast, the cost for testing our diagnostic panel of 39 MPS-related genes (including the *RYR1* and *NEB*) in a clinical diagnostic laboratory is £650 (unpublished data). Furthermore, if no mutation is detected in the 39 genes selected for the "MPS clinical diagnostic panel" sequence information for additional candidate genes can be interrogated. WES and WGS offer a wider choice of candidate genes but involves higher costs. Also, the interpretation of large obtained data is difficult. Hence, target gene capture sequencing using gene panels has been recently applied in genetic diagnosis of Mendelian diseases. For instance, in a recent Japanese study, they applied Trusight One panel to investigate the genetic aetiology of 17 families with different mendelian diseases such including Sotos syndrome,

Joubert syndrome and others (Okazaki et al., 2016). According to the study, the overall rate of accurate molecular diagnosis was 35% which was relatively higher than the diagnostic rate of the whole exome option (25%) when it had been applied in 250 patients. Most cases (80%) were children diagnosed with mendelian neurologic conditions (Yang et al., 2013). In very recent study, whole exome sequencing was applied for 52 patients (from 48 kindreds) with clinical presentation of arthrogryposis. Several candidate variants have been detected in known arthrogryposis-associated genes including homozygous variants in 35.4% of probands (e.g. *CHRNG* in 6 subjects and *ECEL1* in 4 subjects). In addition, they identified variants in candidate arthrogryposis-causing genes such as FBN3, MYO9A, pleckstrin and *PSD3 (*Bayram *et al.,* 2016*)*. In contrast, a very recent paper has concluded that WES has a very high rate in detecting the pathogenic variants in heterogeneous disorders such as inherited retinal disease by detecting pathogenic variants (single-nucleotide variants, indels, or structural variants) for 404/722 (56%) individuals (Keren and McKenna, 2016). However, differences in the composition of the patient cohorts studies and the extent of prior genetic testing prevent a direct comparison of the results of CES and WES testing in this study and that reported by Bayram et al (2016). Nevertheless, number of genes that harboured variants in the cohort reported by Bayram et al (2016) were not included in our MPS-related gene panels including candidate or potential arthrogryposis linked genes (*CENPJ, COL6A3, ECEL1, ERCC2, FBN3, GPR126, ICAM1, IDS, LIFR, MYBPC2, MYO9A, MYO18B, POLR3A, PSD3, VPS8*). Resequencing theses implicated genes using Trusight One panel did not reveal any reported mutations. However, ECEL1 was not included in the version of the Illumina TrusightONE gene panel used by us.

Both CES and WES diagnostic strategies for MPS-related disorders are limited by the inability to reliably detect heterozygous exon deletions (or duplications) and failure to detect mutations in

parts of a gene that are not captured by the exome probes (e.g. much of the intronic sequences). Though WES has the potential to identify novel genes for MPS-related phenotypes, the preference is shifted to the application of CES. That is mainly because of economic advantages of restricting the sequencing to known human disease genes and analysing candidate mutations to the genes known to be associated with MPS-related disorders which will result in reducing number of variants of uncertain significance and incidental findings. The overall frequency of pathogenic mutations in our cohort was 16% (7/45) and a further (3/45) 7% of probands had candidate (possible pathogenic) mutations that might, in time, prove to be diagnostic. So, the overall frequency of the detected candidate mutation in this study is 13/45 (29%) of the examined patients which suggest that clinic al exome method (Trusight One) is a successful approach for studying FADS/LMPS/EVMPS phenotypes. Many of our cases had been previously screened for mutations in single candidate genes (e.g. *CHRNG, RAPSN, DOK7, and RYR1*) and the mutation detection rate expected to be higher in a previously unscreened cohort and/or a cohort of exclusively familial cases. Though CES/WES can provide a powerful strategy for the diagnosis of MPS-related disorders, that correct diagnosis requires careful interpretation of variants, determination of the pathogenicity and clinical significant of the variants which is a big challenge.

5.5.2 RIPK4 as a novel potential gene for MPS disorder

The detection of rare missense mutations in known MPS-related genes can pose problems of interpretation if no functional assay is readily available and clinical or histopathological findings are not specific. This was illustrated by the finding of a homozygous rare missense variant (p.Asp161His) in RIPK4 in a fetus with autosomal recessively inherited FADS. Biallelic mutations in *RIPK4* cause the frequently lethal autosomal recessively inherited form of popliteal

pterygium syndrome (Bartsocas-Papas syndrome) which is characterized by marked popliteal pterygium and multiple congenital malformations (Mitchell et al., 2012, Kalay et al., 2012). So, RIPK4 is not previously known to be associated with inherited MPS disorder in particular LMPS. Though the p.Asp161His substitution was predicted to be damaging by *in silico* tools and was not present in the ExAC database, it had not previously been reported in FADS. Review of the clinical data revealed that affected fetus was the second affected pregnancy of a consanguineous couple. Also it was reported that the presence of pterygia (axillae, elbows, groins, knees and there were pterygia between the two lower limbs) and developmental defects (retracted eyelids, hypertelorism, depressed nasal bridge, cleft palate, absent mandible and digits and indeterminate genitalia) led to an independent clinical diagnosis of Bartsocas-Papas syndrome. The congruence of the presumptive clinical and molecular diagnoses supported the case for pathogenicity but familial segregation studies have not been possible because of unavailability of DNA samples.

5.5.3 Genotype/phenotype correlations of *RYR1 and NEB* genes

Previously, others and we have described LPMS/FADS associated with biallelic *RYR1* mutations (Romero et al., 2003, McKie et al., 2014a, Kariminejad et al., 2016) and here we describe few additional candidate variants identified by CES panel. It seems likely that the frequency of this disorder has been underestimated because the large size of *RYR1* gene (15.3 kb coding sequence in 106 exons) which made genetic sequencing and analysis using standard method is expensive and time consuming. As NGS-based diagnostics approaches such as CES or WES were applied more commonly, we expect additional cases of *RYR1*-related FADS/LMPS to be identified. *RYR1* mutations are associated with a range of muscle disease phenotypes including a variety of

histological subtypes of congenital myopathies and malignant hyperthermia (MH) (Manning et al., 1998, McCarthy et al., 2000, Wilmshurst et al., 2010, Ghassemi et al., 2009).

RYR1-associated congenital myopathies may be inherited as autosomal dominant or recessive trait with recessively inherited cases tending to be more severely affected and having an earlier age-at-onset. *RYR1* mutations described in recessively *RYR1*-myopathies have predominantly consisted of a combination of a null mutation with a missense mutation (Amburgey et al., 2013b, Bharucha-Goebel et al., 2013, Klein et al., 2012, Zhou et al., 2007) whereas *RYR1*-associated cases of severe LMPS/FADS appear to be more likely to be associated with biallelic truncating mutations. Though a potential implication of detecting *RYR1*-associated LMPS/FADS is to indirectly identify parents and other mutation carriers at risk of malignant hyperthermia, to date MH not been observed in the cases we have studied and we note that MH-associated *RYR1* mutations are usually missense mutations and cluster in specific protein domains (Brandom et al., 2013, Kim et al., 2013, Broman et al., 2011)

Similar to *RYR1*, mutations in nebulin (*NEB*) have been associated with a variety of congenital myopathies including autosomal recessively inherited nemaline myopathy, one of the most common congenital myopathies (Anderson et al., 2004, Lawlor et al., 2011, Lehtokari et al., 2006). Also, it was implicated, less commonly, with early-onset distal myopathy without nemaline bodies (Wallgren-Pettersson et al., 2007), distal nemaline myopathy (Lehtokari et al., 2011) and childhood distal myopathy with rods and cores (Scoto et al., 2013). In a recent comprehensive review of *NEB* mutations (212 mutations in 159 kindreds), it was noted that 83% of patients were compound heterozygotes and only 2% had a prenatal presentation (in two cases there was a LMPS/FADS phenotype and in one case FADS (Lehtokari et al., 2014) As with *RYR1*-associated FADS/LMPS it appears that *NEB*-associated FADS/LMPS cases mostly have

biallelic truncating mutations suggesting a genotype-phenotype correlation with the most severe mutations causing prenatal presentation (Todd et al., 2015). It is interesting to note that in the three cases with homozygous NEB null mutation (c. 10278C>A; p.Glu3359Ter) described here there were severe muscle abnormalities (the muscle was composed of connective tissue with a severe lack of muscle fibres). Like, *RYR1, NEB* is a very large gene (~26 kb coding sequence and 183 exons) which is not routinely screened and cases of NEB-associated MPS-related disorders have probably been underdiagnosed.

An interesting observation was that we identified multiple probands with heterozygous truncating (or candidate missense) mutations in MPS-related gens (*NEB, RYR1, TTN, COL6A2*). The interpretation of these findings can be challenging as such cases might represent autosomal recessively inherited cases in which the second mutation has not been detected. Conversely it might be that the mutation is acting in a dominant manner or might be contributing to an oligogenic phenotype. In the study of 48 families with arthrogryposis, it was noted that 8 families with a homozygous mutation in an arthrogryposis-associated gene also had a second locus with a homozygous/ compound heterozygous variant in a further candidate gene. These potential diagnostic uncertainties illustrate the complexity of the interpretation of NGS-based approaches that can generate large numbers of potentially relevant genetic variants (Bayram et al., 2016)

5.5.4 TMP2 gene: a further implicated gene in MPS disorder?

Though autosomal recessively inherited forms of MPS are the most commonly recognised, in sporadic cases recessive, dominant and X-linked causes should be considered. We detected an identical heterozygous p. R133W missense substitution in *TPM2* (which encodes beta-tropomyosin) in three sporadic individuals. Two of them were diagnosed with Arthrogryposis

(*CE07, CE18*) while the third proband (CE30) has presented EVMPS phenotype. This particular mutation was initially described in a mother and daughter with distal arthrogryposis type 2B and muscle weakness without progressive muscle wasting (Tajsharghi et al., 2007). Also it was reported in various overlapping muscle phenotypes including nemaline myopathy, cap myopathy, core-rod myopathy, congenital fibre-type disproportion, distal arthrogryposis and trismus-pseudocamptodactyly (Donner *et al.* 2002, Sung *et al.* 2003, Ohlsson et al. 2008, Clarke et al., 2012, Dowling et al., 2012). Although most disease causing *TPM2* mutations are dominant, autosomal recessive *TPM2* mutations have been described in association with EVMPS and a nemaline myopathy on histology (Monnier et al 2009). Interestingly, the three patients we identified with a *TPM2* mutation were referred to our study because of clinical features reminiscent of multiple pterygium syndrome, although there was an absence of joint webbing. The three cases that harboured this mutation in our series demonstrated overlapping but variable features. It has been shown that *TPM2* mutations causing increased calcium sensitivity result in a hypercontractile phenotype with limb and jaw contractures compared to the non hypercontractile mutations (Marttila et al., 2014). However although *in vitro* studies did not demonstrate an increased sensitivity of the contractile proteins to calcium it resulted in reduced myosin–actin interactions causing muscle weakness without muscle wasting (Ochala et al 2007).

5.6 Conclusion

The "MPS spectrum disorders" are genetically heterogeneous and although, in some cases, a diagnosis of a specific primary myopathy, metabolic or neurodevelopmental disorder can be made by clinical and pathological investigations, the underlying aetiology is unknown in the majority of cases (Cox PM et al., 2003). Thus, **e**stablishing the cause of MPS by clinical and

histopathological investigations is challenging because in many cases the histopathological features may be non-specific or autolysis prevents accurate characterisation of muscle pathology. Hence comprehensive molecular genetic analysis can greatly facilitate the diagnostic process. Extensive genetic analysis by CES or WES enables almost all candidate MPS genes to be examined and so CES/WES provide better strategies to determine the relative frequencies of individual genetic causes of MPS than sequential testing of single genes. Thus, our data suggests that after, *CHRNG,* mutations in *RYR1* may be the second most common cause of autosomal recessive LMPS/FADS. As NGS strategies for genetic diagnosis become more readily available mutations in large genes such as *RYR1* and *NEB* are more likely to be identified. Additionally, as the expanded phenotypes that can be associated with mutations in genes such as *TPM2* (i.e. EVMPS as well as distal athrogryposis) become better recognised the proportion of cases of MPS-related disorders with a molecular diagnosis should increase. Testing mutation negative cases for whole exon deletions/duplications should further increase diagnostic rates but, considering that currently a molecular diagnosis is only reached in a minority of cases, it seems likely that further MPS genes remain to be identified.

Chapter Six: General Discussion

6.1 Summary:

This thesis demonstrates the evolution of genetic advances in the field of mutation identification in autosomal recessively inherited diseases; in particular, amongst families with parental consanguinity, progressing from genetic mapping, candidate gene analysis to NGS based approaches. Two main outcomes can be concluded from this thesis and these will be discussed in detail in this chapter. Firstly, the benefit of applying autozygosity mapping in studying the autosomal recessively inherited diseases. Secondly, the power of using NGS techniques in investigating such rare disorders. In this project, NGS was applied as a main investigative tool in an attempt to identify genes associated with a range of different rare autosomal recessive disorders in consanguineous families. Two different "exome strategies" were applied in the project. Whole exome sequencing (WES) was applied to one affected patient of congenital Oligodontia in order to investigate the genetic basis of the disease whilst the clinical exome sequencing (CES/Trusight One) was used for the genetic analysis of MPS disorders. The use of enriched exome panel (Trusight One) for studying MPS disorders has shown good detection rate (31%) in identifying pathogenic variations in known candidate genes. However, applying whole exome sequencing for one patient affected with Oligodontia did not result in identifying any pathogenic variants. Though no mutation was identified by WES in the analysed oligodontia patient, this method is highly suitable for mutation detection in diseases with genetic heterogeneity and for gene discovery in such cases. In the future, a larger WES study of patients with inherited oligodontia.

6.2 Application of Autozygosity mapping for investigating autosomal recessive diseases

Linkage analysis can be used to identify genetic markers linked to disease genes but is particularly powerful in consanguineous families for excluding linkage to candidate genes. This

was illustrated in this project in excluding *LTBP3* gene involvement for oligodontia, in most patients, by analysing microsatellite markers located across the genomic region on chromosome 11 (59,756,135 to 69,325,101) which contain the target LTBP3 gene. However, no evidence of linkage was identified in 4 families and only one proband of family 5 has showed nearly ~8Mb homozygous region that contains LTBP3 gene in five microsatellites markers (D11S4191, D11S4076 D11S1883, D11S913 and D11S1889). Therefore, the gene was not selected to be a candidate for the disease in the majorities of families. Linkage analysis in addition to excluding candidate genes is also used to identify candidate region particularly when studying consanguineous families with multiple affected members with the presence of parents and unaffected members. This was illustrated in my project by analysing two FADS/MPS patients from a single consanguineous family (MPS001) to identify regions of homozygosity. This was carried out using Affymetrix 250K SNP arrays 5.0, genome-wide linkage scan on DNA of the two affected siblings. The SNP genotyping work was performed by Louise Tee (a research laboratory technician) prior to starting my project. After excluding linkage to known FADS/LMPS genes, a candidate homozygous region of nearly10 Mb on chromosome 19 was identified. As the locus was mapped to chromosome 19, the target region was genotyped using fourteen polymorphic microsatellite markers from the same candidate region, over 345 genes were found within the target interval. Amongst these genes, *RYR1* was selected as the best candidate to be sequenced using specific prioritization strategy.

A homozygous null mutation (c.6721C > T) was identified within the *RYR1* gene in two affected siblings of the family MPS001. Then, I proceeded to sequence *RYR1* using Sanger method in a cohort of 36 unrelated probands with FADS/LMPS and revealed two additional homozygous deletions. Thus, the frequency of *RYR1*mutations in the analysed probands with FADS/LMPS

was 8.3%. These findings suggest that *RYR1* gene is a significant cause of FADS/LMPS. Accordingly, it was recommended adding this gene to the diagnostic panel for the disease in the future testing.

As introduced in the introduction chapter, our findings agree with many studies that autozygosity mapping is an effective approach in searching for homozygous regions in particular amongst consanguineous families. However, the method becomes less informative in case of studying single cases with no relatives. Over the past decade, Professor Maher's research group utilised the autozygosity mapping studies as a main approach in studying different rare recessive disorders and they have successfully identified a large number of novel genes including genes for: Achromatopsia (GNAT2) (Aligianis et al., 2002), ARC syndrome (VPS33B, VIPAR) (Gissen et al., 2004; Cullinane et al., 2010), Beckwith-Wiedemann syndrome (NLRP2) (Meyer et al., 2009), Faisalabad histiocytosis/3 Rosai- Dorfman disease (SLC29A3) (Morgan et al., 2010), Fowler syndrome (FLVCR2) (Meyer et al., 2010), Hermansky-Pudlak syndrome (BLOC1S3) (Morgan et al., 2006b), Immunodeficiency syndromes (TRAC) (Morgan et al., 2011), Infantile neuraxonal dystrophy (PLA2G6) (Morgan et al., 2006a), Infantile parkinsonism (DAT) (Kurian et al., 2009), Martsolf syndrome (RAB3GAP2) (Aligianis et al., 2006), Meckel-Gruber syndrome (MKS3) (Smith et al., 2006), Multiple pterygium/Fetal akinesia syndrome (CHRNG, RAPSN, DOK7) (Morgan et al., 2006c; Vogt et al., 2008) Also, many other genes were identified by the help of this efficient method and they can be found in the literature either of the Maher group or of the many other genetic researchers.

6.3 The Application of NGS in investigating rare inherited disorders:

Linkage mapping and gene-by-gene analysis based on Sanger Sequencing in a disease interval is a time consuming and expensive process. Several factors could affect the power of that approach

such as the small size of studied samples or families, reduced penetrance and locus heterogeneity (Bamshad et al., 2011). Exome and NGS sequencing techniques in general have revolutionised human genetics and helped in understanding the genetic basis of many inherited disorders through identifying the pathogenic variants underlying the rare and complex diseases. NGS is regarded as the best tool to elucidate disease causing mutation not only in the large extended families with possibility of performing linkage analysis to identify the candidate genomic region but also can be efficient in sporadic cases to search mainly for *de novo* mutations (Veltman and Brunner, 2012). Exome sequencing became a standard technique because it is more cost-effective compared to WGS as it only captures the highly interpretable coding region which represents 1% (about 38 Mb) of the whole human genome. In addition, it contains the majority (85%) of the reported genetic changes (Wang et al., 2013). Partial exome approach was adopted in our study for investigating MPS disorder by applying an enriched clinical exome (Trusight One) panel whereas whole exome sequencing method was used for investigating one patients who is diagnosed with a severe oligodontia disorder.

In genetically heterogeneous diseases such as MPS, large number of candidate genes is usually involved in the investigation process. Different gene panels can be tested including specific diagnostic panel which might comprise up to 50 genes (e.g. NHS diagnostic panel for MPS=39 genes). In addition, whole exome sequencing (WES) can be applied for the extreme heterogeneous diseases and was used in my project to investigate the genetic causes of congenital oligodontia. In the results, this project has proven the power of clinical exome techniques in investigating the autosomal receive disorders by identifying pathogenic mutations and potential candidate genes that could play a significant role in the genetic basis of MPS disorders.

6.4 Evaluation of Clinical Exome Sequencing for use in MPS diseases/Autosomal recessive

Clinical exome sequencing was applied using the Trusight One panel which screens more than 4,800 genes involved in the clinical phenotypes associated with inherited human disorders. 53 patients were analysed using this clinical panel. 8 analysed patients were already identified with known mutations in MPS-related genes and the clinical exome was used as a confirmatory method for them. 45 recruited patients had no previous genetic diagnosis but they have undergone mutation-screening analysis in number of candidate genes (*CHRNG, DOK7, RAPSN* and *RYR1)* and it was decided to apply clinical exome sequencing to determine if a pathogenic variant could be identified in another candidate gene.

For each individual, the panel detected nearly 14,800 variants in total. For those whose family history and parental consanguinity suggested autosomal recessive inheritance of MPS, I focussed my attention on homozygous variants or compound heterozygous variations while in no-consanguineous cases, single heterozygous were not excluded as the autosomal dominant inheritance is considered. After using specific filtering strategy, the total candidate variants are summarised in table. All 8 previously mutations in *CHRNG, LMNA, RAPSN*, and *RYR1* were successfully detected with 100% accuracy. In the undiagnosed cases, 7 out of 45 cases (~16%) novel mutations in known MPS-related genes (*CHRNG, CHRNA1, NEB1, RYR1* and *TPM2*) within seven different affected patients. Other rare genetic variants which classified as potentially associated with MPS phenotype were identified in 6 probands in five genes; two of them are known genes which already reported with pathogenic mutations (*NEB, RYR1*) and 3 (*RIPK4, TTN, COL6A2*) were less well characterised genes. Therefore, clinical exome sequencing has successfully identified the causing genetic variants in 13 out of 45 total individuals which demonstrate a 29% success rate in identifying associated mutations within

MPS-related phenotypes (FADS, LMPS, EVMPS, distal arthrogryposis) in both consanguineous and non-consanguineous cases including the most pathogenic cases summarised in Table 5.3. The method is a cost-effective strategy for investigating these inherited diseases compared to WES and provided new insights into genotype-phenotype correlations.

These research findings could be helpful in the diagnostic process in the future because many newly detected genes in such research and studies were quickly added to the pipelines of number of designed diagnostic testing. Sometimes, it takes only few months from the publication of a new gene to its incorporation into commercial diagnostic gene panels (Lohmann and Klein, 2014).

6.5 Limitations of applying NGS in investigating rare autosomal recessive disorders:

Due to the huge number of data, interpretation of these detected variants is the biggest challenge for applying the NGS approach in genomic medicine. NGS techniques have some disadvantages that need an improvement in the future such as the risk of false positive findings from sequencing errors and the insufficient coverage for a particular gene or nucleotides that reduce the method specificity. Exome Sequencing approach suffers from an inability to reliably detect heterozygous exonic deletions or duplications and/or rearrangements that occur within the targeted regions of associated genes. Though these changes might be reported in HGMD, they would not be detected due to the limitations exome technology. However, number of alternative techniques can be used to detect these changes including Multiplex Ligation-dependent Probe Amplification (MLPA technique) or high-density cytogenetic technologies (e.g. aCGH) that designed by Oxford Gene Technology and regarded as the most accurate method for detecting CNV. Another approach that can detect these changes is whole genome sequencing (WGS).

Second, there is inadequate and different coverage of the region that contains a causal variant, which could result in missing variants and false positive findings.

For exome technology, the kits can only target exons that already identified, however, as knowledge of all truly protein-coding exons in the genome is still incomplete, so the kit might miss the non-covered exons. A pathogenic variant may be present, but if it is not targeted in analysis it may not be detected depending on the filtering NGS parameters.

Currently, whole genome sequencing can be good alternative approach but is still more expensive than exome sequencing. WGS offers some advantages compared with exome sequencing comprises the unbiased analysis of the genome. First, it allows more reliable detection of structural variants (e.g. CNV) as well as identifying the noncoding variation (Gilissen et al., 2014, Spielmann and Klopocki, 2013). Also, the potential protein-coding regions that have not yet been annotated as genes are included in the genome sequence analysis and it would not face the technical challenges seen in WES such as exon capture and coverage. However, although the costs of WGS only higher than WES 2–4 times but the larger data storage and longer analysis time makes WES a convenient technology To some extent, the results in this project revealed both the strengths and limitations of using WES/CES as a tool to evaluate genes related to rare autosomal recessive conditions.

6.6 The future of human gnomes and national genome projects

One of the limitations of gene identification studies is the rarity of the patients with the disease of interest. In familial diseases, the method can be most successful in large extended families with multiple affected individuals. In addition, it can be a powerful tool to investigate the genetics for sporadic disease in the presence of many affected individuals displaying similar phenotypes. Finding those vulnerable people is very difficult for the rare inherited disorders. However, as genomic medicine continues to progress, many projects could help overcoming this problem by providing big database based on large number of recruited patients. One of these large-scale projects is the UK 100,000 Genomes Project that aims to sequence 100,000 genomes from around 70,000 people including 17,000 rare disease patients and 33,000 healthy relatives. Another global example of genomic projects is the Saudi Genomic Project to sequence 20,000 subjects including healthy people and patients affected with common or rare genetic disorders. One of the main project goal is eliminating the huge burden of recessive genetic disease which is very high in the kingdom (8% of the births) that can be achieved through identifying the causing gene variants that cause these diseases and then providing genetic counselling for those who at risk. Also, if the costs of sequencing genome/exome continue to fall as expected with the accelerating advances in the genomic techniques, similar projects can emerge and support the process of identifying novel genes and associated pathways. Therefore, NGS can be very helpful not only in the prediction/diagnostic process through discovering novel genes but may also be beneficial for the therapeutic approach.

6.7 Comparison of various genetic tests and their applications

NGS technology has made different types of tests available for the genetic diagnosis including single-gene tests, gene panel tests, exome sequencing and genome sequencing. Selecting the

appropriate test is a big challenge to giving a successful genetic diagnosis. In fact, a review study published in 2015 has critically discussed the approach of selecting the best genetic test for the examined individual. According to the study, single-gene testing is mostly chosen in the case of minimal locus heterogeneity with a clear clinical diagnosis based on distinctive clinical features. Also, an existing association between the diagnosed disease and a disease-causing gene needs to be already established. Gene panel testing is more cost-effective than a single-gene approach and it should be the suitable test for heterogeneous disorders with otherwise unclear clinical diagnoses such as disorders with overlapping phenotypes or disorders which share specific manifestations but in which the overall phenotypes are different. Such disorders are generally associated with multiple genes.

Exome Sequencing (ES) and Genome Sequencing (GS) normally selected for the disorders with extreme heterogeneity and *de novo* mutations are the major mutation. Also, it is a good option in the case of a diagnosis which is very difficult to make or when there are at least two possible phenotypes for one patient. In addition, if there are key phenotypic features are not present at the time of requesting the testing the real underlying cause of the disease will be very difficult to identify. Examples of these disorders are Autism and congenital heart disease. ES was specifically selected in my project for studying foetal akinesia because of the high heterogeneity of the disorder plus because it has many overlapping phenotypes. It has an advantage over the gene panel in that it is less biased regarding which set of genes to test as the latter assumes that the abnormal clinical features are restricted to be associated within the included genes in the panel itself (though the use of WES rather CES would have enabled a larger number of genes to be tested). Factors such as technical limitations involved in NGS technology, the risk of false positive especially for insertion/deletion and the weak coverage for a particular area make both

ES or GS are not completely independent methods. Because of this for diagnosis, aCGH and Sanger sequencing are currently required as a complement method to cover the shortcomings of NGS in order to detect the full spectrum of mutations and to validate those findings which are identified by NGS approaches. In this way the differences and difficulties associated with the used technology, test interpretation, clinical significance, and ethical problems, need to be well considered by the clinician who selects the gene test because they ultimately affect the correct order and the diagnosis for the patient (Xue et al., 2015)

6.8 Future Directions:

There are a number of limitations of this project that hopefully directed and improved in further studies in the future. Firstly, familial cases of congenital oligodontia (like many other congenital defects) are a valuable source to undertake genetic research and investigate the genetic basis of the disease. Identification and including more familial cases of oligodontia would facilitate the discovery of the causing genes of the disorder. This could help in performing conventional autozygosity mapping in consanguineous families to identify new candidate regions/genes for the disease or preferably, the method can be combined with WES technique. So, after performing the exome sequencing to identify rare mutations, the identified linked region can be further screened for candidate mutations. Also, large cohort of unrelated control subjects from the same ethnicity of the recruited patients would be suggested in future studies to screen the identified mutations in these subjects. This might be achieved through a collaboration with similar research groups within the UK and internationally. Such comparison would provide a further powerful filter for determining pathogenicity of any candidate variants as well as narrowing down mutations responsible for familial cases in which the absence of the candidate-causing variant

might support the hypothesis that the gene is playing a disease-associated role. Finally, functional studies in appropriate animal models will be helpful to characterise the pathogenicity of mutations in candidate regions/genes by recording any molecular or phenotypical changes and compare it to the human subjects.

In MPS project, for the patients who had no mutations using the use clinical exome method, WES offers better choice as it includes all the coding genes in case the mutations present in genes out of the used panel. However, in case the WES failed to identify the candidate rare variant, moving to the whole genome (WGS) will become the next tool to investigate the genetic basis of the disease as it includes coding and non-coding region of the genome. In addition, it can detect the structural changes such as CNV and large genetic changes that cannot be detected neither by CES or WES.

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