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Identification of novel mutations in ASPM and KMT2D Genes responsible for Primary Microcephaly and Kabuki-like syndrome in Palestinian Families

By

Aya Mohammad Awad

In Partial Fulfillment of the Requirements for the Degree of

Master of Science in Biotechnology

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The undersigned hereby certify that they have read and recommend to the Faculty of Scientific Research and Higher Studies at the Palestine Polytechnic University and the Faculty of Science at Bethlehem University for acceptance a thesis entitled:

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Identification of novel mutations in ASPM and KMT2D Genes responsible for Primary Microcephaly and Kabuki-like syndromes in Palestinian Families

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ABSTRACT

Background: Microcephaly is considered a neurodegenerative syndrome, in which the head circumference is at least 2 SD below average which differs according to age and gender. In most cases Microcephaly is associated with mild to severe mental retardation. Microcephaly can be either primary (congenital) or secondary (acquired). On the other hand, Kabuki syndrome is a rare disorder characterized by several congenital abnormalities and intellectual inability. The aim of our study was to identify the genetic causes of both autosomal recessive Primary Microcephaly (MCPH) and kabuki-like syndrome in three related Palestinian families.

Methods: Eleven blood samples were collected from members of the family with microcephaly, 7 blood samples from the first kabuki-like family and 5 samples from the second family with kabuki-like syndrome, and then DNA was extracted. Whole exome sequencing then Sanger sequencing were used to investigate the causative mutation. The mutations detected in the study were screened by Sanger Sequencing in 100 normal controls.

Results: Whole exome sequencing revealed a novel nonsense mutation in Abnormal spindle-like microcephaly-associated protein (ASPM) gene that results in K3228X alteration and which leads to a premature stop codon. As for the kabuki –like syndrome, a missense homozygous mutation (C-T) was detected in Lysine (K)-Specific Methyltransferase 2D (KMT2D) that results in P1912L alteration. Moreover, a De novo 22q11 deletion syndrome was detected in one of affected individual in kabuki family. The 100 controls were negative for both the ASPM and KMT2D variants.

Conclusion: Our results revealed a novel nonsense mutation in ASPM gene that disrupts neuron development and results in primary Microcephaly in human. A missense mutation in KMT2D that may affect the methylation of certain genes important in development and thus leads to

kabuki-like syndrome, and a De novo 22q11 deletion syndrome in a female individual in kabuki family.

Keywords: Primary Microcephaly, kabuki syndrome, ASPM, KMT2D, methylation, neurogenesis, 22q12DS.

اكتشاف طفرات وراثية جديدة في جيني ال ASPM و KMT2D المسؤولين عن متلازمة صغر الرأس ومتلازمة كابوكي

في عائلات فلسطينية

اية محمد عوض

ملخص

خلفية: تعتبر متلازمة صغر الرأس من الأمراض المتعلقة بالجهاز العصبي، والذي يتميز المصابين بها بمحيط رأس أقل من المتوسط بدرجتين على الأقل، ترتبط متلازمة صغر الرأس في أغلب الحالات بوجود تخلف عقلي عند الأشخاص المصابين بهذه المتلازمة والتي من الممكن أن تظهر عند الولادة أو في مراحل لاحقة. من الناحية الأخرى تعتبر متلازمة كابوكي من الأمراض النادرة التي تتميز بوجود عدد من العيوب الخلقية والاضرابات العقلية عند المصابين بها. تهدف هذه الدراسة الى تحديد الأسباب الجينية المسؤولة عن متلازمة صغر الرأس ومتلازمة كابوكي في عائلات فلسطينية.

الطرق المستخدمة: تم جمع 11 عينة دم من العائلة الأولى، 7 عينات من العائلة الثانية، و 5 عينات من العائلة الثالثة. ثم استخراج الحمض النووي DNA. ثم تم استخدام Whole exome sequencing Sanger sequencing للبحث عن الطفرات الجينية. أخيرا، تم اختبار صحة الطفرة باستخدام 200 عينة ضابطة.

النتائج: تم الكشف عن طفرة جينية جديدة في جين ASPM والتي أدت الى ايقاف انتاج البروتين الناتج. وأيضا تم اكتشاف طفرة جينية جديدة في جين KMT2D والتي نتجت في تغيير البروتين الناتج عنه اذ تم استبدال الحمض الأميني برولين في الموقع 1912 الى الحمض الأميني ليوسين. لكن هذه الطفرات كانت غائبة في ال 200 عينة الضابطة.

الاستنتاج: كشفت نتائجنا عن طفرة جديدة في جين ASPM والتي تؤثر على تكوين الخلايا العصبية وبالتالي الاصابة بمتلازمة صغر الرأس. بالإضافة الى اكتشاف طفرة جديدة في جين KMT2D والتي تؤثر على عملية اضافة مجموعة الميثل الى عدد من الجينات المعينة المهمة في تكوين وتطوير الانسان وبالتالي الاصابة بمتلازمة كابوكي.

الكلمات الدالة: ASPM، KMT2D، متلازمة صغر الرأس ،متلازمة كابوكي.

DECLARATION

I declare that the Master Thesis entitled "Identification of novel mutations in ASPM an KMT2D enes responsible for Primary Microcephaly and Kabuki-like syndrome in Palestinian Families" is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

Name and signature: __Aya.Awad _____

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Dedication

I would to dedicate this thesis to my parents, who were always there for me, and whom without their care I couldn't have achieved this.

To Mohammad, my beloved and supportive husband.

And to everyone who has ever supported me.

Acknowledgment

I wish to express my sincere thanks to my supervisor Prof. Moein Kanaan for providing me with all the necessary facilities for the research.

I am also taking this opportunity to thank Prof. Mazin Qumsiyeh for helping in the clinical diagnosis of the patients and taking photos and going with us when visiting the patients families.

I take this opportunity to express gratitude to all of the Hereditary Research Laboratory members Lara , Amal, and Tamara for their help and support.

I also thank my parents for their encouragement, support and attention. And I am grateful to my partner who supported me through this journey.

List of Abbreviations

MCPH	Autosomal Recessive Primary Microcephaly
HC	Head circumference
SD	Standard deviation
NMR	Volumetric nuclear magnetic resonance
NE	Neuroepithelium
NPC	neural progenitor cells
KS	Kabuki syndrome
PGD	Preimplantation genetic diagnosis
H3K4	histone H3 lysine 4
ASPM	Abnormal Spindle-like, Microcephaly-associated
KMT2D	Lysine (K)-Specific Methyltransferase 2D
LCR	low copy repeat
22q11DS	22q11 deletion syndrome

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Chapter1

Introduction

1.1: Autosomal Recessive Primary Microcephaly (MCPH)

Autosomal Recessive Primary Microcephaly is a rare condition correlated with aberrant development, with a head circumference (HC) that is at least 2-3 standard deviations (SD) less than normal individuals of same sex and age[1]. MCPH seems to result from failure of neuronal tissue's normal development, rather than its degradation or regression [2, 3]. Microcephaly is highly linked to mental retardation with no other neurological deficiency [4], People with large degree of microcephaly are more susceptible to have intense mental retardation [5].

Several techniques have been utilized to examine brain size (i.e: volumetric nuclear magnetic resonance (NMR) scan), however, HC stays the most used and suitable one [3].

1.2 Types of microcephaly

Microcephaly is classified either as a primary or secondary microcephaly. Primary microcephaly is a congenital disorder that presents at birth, and is generally linked to stable developmental abnormality and mental retardation that is linked to a genetic defect [6, 7]. Secondary microcephaly shows a continuing neurodegenerative case and is assumed to arise from a brain wound like hypoxic-ischemic injury or a metabolic disorder, in this case the individual is born with a normal HC but the HC lags behind normal growth because of the injury [8-10].

1.3 MCPH Inheritance

MCPH shows an autosomal recessive pattern of inheritance, where both copies of the gene are mutant. Members of MCPH inherit one copy of the mutated gene from each parent, yet, the parents normally don't show any symptoms of the disorder [11].

1.4 MCPH incidence

MCPH is considered a rare disorder with an incidence of 1 in 30,000 in Japan for example [12], and 1 in 25,000 in Holland [13]. However, in communities with consanguineous marriages the incidence is much higher and estimated to be 1/10000 [14]. Moreover, about 43 families with MCPH mutations have been documented in the Middle east , particularly from Saudi Arabia [15]

1.5 Clinical characteristic of MCPH

A) Microcephaly that is clear at birth with a HC that is at least 3 SD less than controls of same sex and age [16].

B) The majority of MCPH patients have ordinary weight, height and normal chromosomes [10, 17], except for patients with mutant microcephalin gene that present short stature and early chromosomal condensation [18].

C) Intellectual disability and mental retardation, that are not correlated to developing cognitive decline or seizure [19].

Woods, C and his colleagues have used ultrasound to examine timing of brain growth reduction in affected fetuses. Until 20 weeks of pregnancy, ordinary head measures were detected, while a reduced HC was found by week 32 [20]. After delivery, HC ranges between -4 to -12 SD. The level of microcephaly for the individual doesn't change through its life, even in a family the HC doesn't differ by more than 2 SD among affected members [21]. Moreover, MCPH patients have small cerebral cortex, as shown using NMR scan [22].

All MCPH patients are mentally retarded, however, it is commonly of a slight severity [21]. Lack of smiling to parents and head control are the first highlights of the syndrome, followed by mild social and moving obstructions then speech delays. In adolescence, MCPH individuals have good balance and movement control, as well as accomplishing well in sports in comparison to other mentally retarded kids. Many can be taught to read, write, and to behave well [1, 21].

1.6: MCPH Etiology

Studies propose that MCPH results from a defect in neurogenic mitosis and not from neural migration or increased apoptosis. MCPH genes are expressed in the neuroepithelium. Brain scans shows a small brain size compared to normal individuals. Brain size is controlled by the

proliferation average and cell death average through neurogenesis. Most neurons arise from neural progenitors, that are the primary cells of the neuroepithelium (NE) lining the brain ventricles [19]. Fly neuroblasts were studied to characterize the accurate mitotic activity of neuronal progenitor cells; symmetrical cell division in which the mitotic spindles are aligned in the neuroepithelium plane, give rise to two neuronal progenitor cells. While asymmetric cell division happens when the mitotic spindles are perpendicularly aligned toward the neuroepithelium and produces one post-mitotic neuron and one progenitor cell [19].

The timing of cell divisions is crucial for the final number of neurons, so, any alteration in its regulation might cause cortical disorders as in Microcephaly [23].

MCPH shows genetic heterogeneity, and till now, twelve loci have been linked to this syndrome [24].

1.7 MCPH genes

Table 1: Summary of identified MCPH genes, location and function.

gene	Locus	Function/ role in MCPH	Reference
Microcephalin	8p23.1	Participates in chromosomal condensation, decreased MCPH1 promotes the production of early born neurons and decreases the late-born neurons. Mutations in this gene have been linked with primary autosomal recessive microcephaly 1 and premature chromosome condensation syndrome	[5, 25]
WDR62	19q13.12	WDR62 manage proliferation and differentiation of neural progenitor cells (NPCs) Mutations of (WDR62) have been distinguished to cause human MCPH	[26]
CDK5RAP2	9q33.2	Vital for linking centrosomes to mitotic spindle poles.	[27]

		The abnormal CDK5RAP2 function can lead to premature decrease in number of neural stem cells and so microcephaly.	
Casc5	15q15.1	CASC5 is part of the kinetochore. It participates in microtubule attachment to chromosome centromeres and in the activation of the spindle checkpoint in mitosis. The CASC5 gene is highly expressed in the areas of cell proliferation surrounding the ventricles through fetal brain development stage.	[28]
ASPM	1q31.3	The ASPM protein product is necessary for normal mitotic spindle function in embryonic neuroblasts .	[22]
CENPJ	13q12.12	Through cell division, the expressed protein plays a structural function in the maintenance of centrosome integrity and normal spindle morphology, and it participates in microtubule disassembly at the centrosome.	[29]
STIL	1p33	STIL encodes for a protein necessary for right mitotic spindle organization and localizes to the mitotic spindle poles during metaphase	[30]
CEP135	4q12	Believed to have a role in maintaining the structure and organization of the centrosome and of microtubules.	[31]
CEP152	15q21.1	It was recognized as a part of mammalian centrosomes	[32]
ZNF335	20q13.12	Important for neural progenitor self-renewal, neurogenesis, and neuronal differentiation.	[33]
PHC1	12p13.31	This gene encodes a member of PRC1 complex, that keeps genes in suppressive state	[34]

CDK6	7q21.11	Encodes an essential protein for cell cycle control and differentiation of different cell types. Moreover, it associates with the centrosome during mitosis	[35]

In this study we will focus on ASPM gene as it is the cause of MCPH in our case study.

1.8 Histone H3 Lysine 4 (H3K4) Methylation

Expression the genetic information encoded by the DNA depends on a precise series of protein-DNA interactions, and on chromatin structure and epigenetic changes [36]. In the nuclei of eukaryotes, an equal mass of DNA and histone protein is combined together. An octamer of two of each histone types H2A, H2B, H3 and H4 is coiled with DNA Plus some histone isoforms that are rich in lysine residues [37]. The N-terminal tails of neucloeosme histone (H3 and H4) protrude out from the DNA boundary (Figure1) and offer themselves as a base for a set of- post translational alterations such as , ubiquitination, acetylation, phosphorylation, ADP-ribosylation, biotinylation and methylation [38].

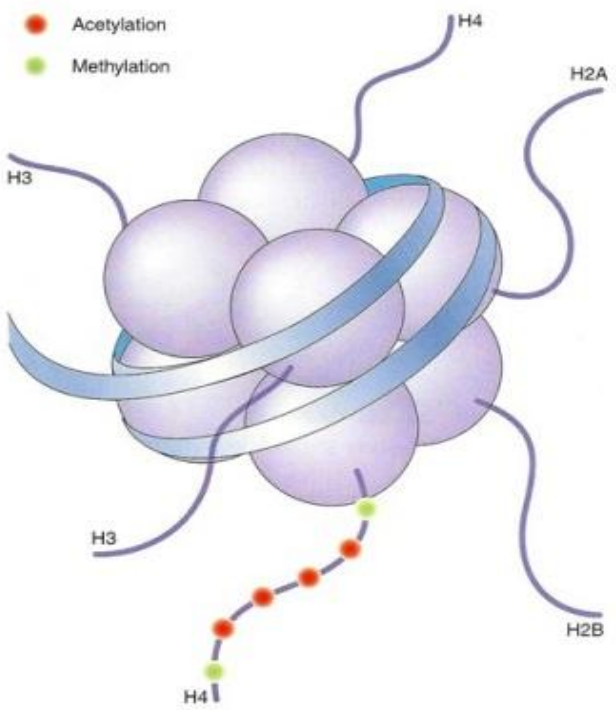


Figure 1. Nucleosome structure. DNA is wrapped around histone core. Histone tails could be subjected to modifications [39].

The existence or absence of specific post- translational alterations, can cause localized condensation or decondensation of the chromatin fiber, respectively. These changes in chromatin structure can either have a positive or negative regulatory influence on DNA-mediated processes like gene regulation, DNA repair and DNA replication [40].

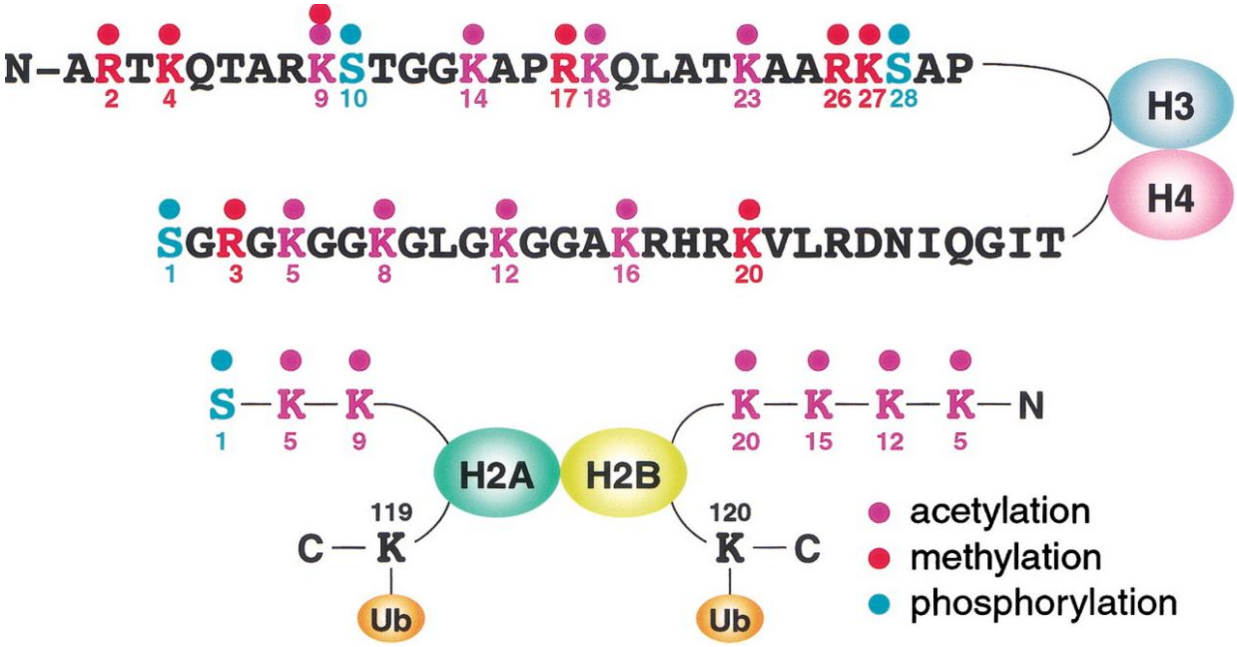


Figure 2. Location of post-translational alterations that happens on the histone tails. These shown alterations include: acetylation (shown in purple), methylation (shown in red) and phosphorilation (shown in blue) [41].

Histone methylation is one of the most studied and described histone modifications. Methylation can happen on either lysine or arginine residues, moreover, these histone methylations are evolutionarily preserved.

Histone methylation unlike acetylation, does not influence the charge of the altered amino acid. Furthermore, every lysine residue can take up to three methyl groups, leading to mono-, di-, and trimethylated lysine states [42]. The site of histone methylation is important; methylation on

histone H3 on lysine 4, 36 and 79 are linked to active transcription of genes, whereas the methylation on histone H3 Lysine9 and lysine 27 and histone H4 Lysine 20 are linked to transcriptional repression of genes [40, 42].

Most of the time, each one of the methyl-lysine states can be detected in the cell at specific times, and have clear localization patterns, proposing that every altered state of lysine has a special biological part and function [41].

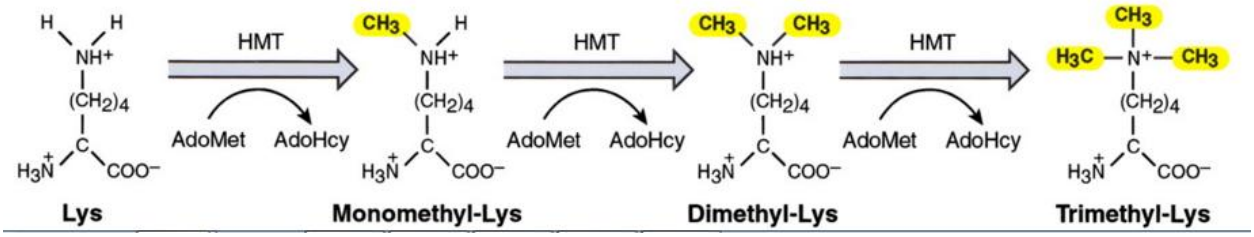


Figure 3. The structure of the following: Lysine residue, mono, di and trimethyl lysine [41].

The major family of enzymes that stimulate histone lysine methylation includes the conserved SET domain. SET domain, is evolutionary preserved in higher eukaryotes and mammals, proteins having multiple SET domain can methylate a specific lysine [41].

In S. cerevisiae, Set1, which is a lysine methyltransferase, is in charge of all the forms of H3-K4 methylation, which means that these forms and their structural patterns should be a result of the management of the enzymatic activity and/or targeting of Set1 [43].

Since histone alterations have been discovered in the 1960s and 1970s, loads of evidence have been gathered indicating the role of certain alterations in developmental gene regulation and DNA repair mechanisms [44].

Although lysine methylation of histones was characterized for the first time in 1964 [45], many years went by without investigating much in the functional importance of this alteration. Lysine methylation may influence DNA conformation affinity, or hydrophobicity [46]. In fact, it was lately revealed that H4 methylation at lysine 20 increased the capability of nucleosomal arrays to

compress and fold in vitro, showing that histone methylation is able to directly impact higher order chromatin structure [47].

In addition, lysine methylation can produce a binding site for proteins which can change the local features of chromatin for transcription [40, 48]

Abnormalities in the histone modifiers have been linked to genetic diseases, like Kleefstra syndrome, Sotos syndrome, Weaver syndrome, and Kabuki syndrome [49]

1.9 Kabuki syndrome

Kabuki syndrome (KS), that is also called Kabuki make-up syndrome or Niikawa–Kuroki syndrome is a rare disorder, caused by heterozygous loss-of-function mutations in one of two genes, KMT2D or KDM6A[50-52]. It was first characterized by Kuroki et al. in 1981 in people who had a distinguishing facial appearance, skeletal abnormality, cardiac and renal distortion, a degree of mental retardation and postnatal growth deficiency [53]. KS is accompanied with multiple modifications in body system, like neurological anomalies, impairment in growth and cardiac problems [54]. Affected individuals can also have seizures, microcephaly, or weak muscle tone (hypotonia) [54].

Two main genes have been recognized to cause KS: lysine-specific methyltransferase 2D(KMT2D) and lysine-specific demethylase 6A (KDM6A). KMT2D is located on chromosome 12 and functions as a methyltransferase that adds a trimethylation mark to H3K4(H3K4me3, an open chromatin mark), while KDM6A is located on chromosome x and functions as a demethylase that takes off trimethylation from histone 3 lysine 27 (H3K27me3, a closed chromatin mark) [51, 52].

KMT2D mutations forms about 55–80% of KS cases, whereas KDM6A mutations account for 9–14% of the patients [51].

1.10 Kabuki syndrome management

In order to handle gastroesophageal reflux, thickened feedings and positioning after meals are recommended. If the patient faces cognitive difficulties, exceptional education methods are required to meet his/her needs. Furthermore, vision and hearing are checked annually.

Prevention procedures are used to avoid any secondary complications, such as using prophylactic antibiotics before or during surgeries or even dental work, especially with those who have certain heart problems [55].

1.10.1 Genetic counseling

Since KS is a multisystem syndrome, patients may need several diagnostic and screening tests, evaluations, consultations, and medical interference at various stages of their lives. [56].

If the mutation causing the disease have been known in an affected family individual, then prenatal diagnosis for KS becomes possible. This is done by extracting DNA from fetal cells gained by amniocentesis (normally done at ~15-18 weeks' gestation) or chorionic villus sampling (normally done at ~10-12 weeks' gestation). Then the DNA is sequenced. [55].

1.10.3 DNA banking

DNA storage can be done for potential future purposes. This is because our recognition of genes , allelic variants, and diseases will be enhanced in the time ahead. So, DNA banking of affected people should be taken into consideration [55].

1.11 The 22q11.2 deletion syndrome (22q11DS)

Chromosomal alterations are noted in at least 200 human genetic diseases as well as in most cancers. Frequent main rearrangements like translocations, inversions and deletions indicate that there might be favored chromosomal positions for such alterations in the human genome [57-60].

Chromosome 22 forms only about 2% of the haploid human genome, [61] but rearrangements of this chromosome are related with numerous malignant diseases and developmental aberrations [62].

The 22q11.2 deletion syndrome (22q11DS) is a widespread disorder that is also called DiGeorge syndrome (DGS) and velocardiofacial syndrome (VCFS) [63]. This syndrome is produced by the deletion of a small segment of chromosome 22 [64]. 22q11.2DS is the most prevalent micro deletion disorder marked by low copy repeats and the deletion happens near the middle of the chromosome at a specified position 22q11.2 [65].

The latest studies have showed that deletions of chromosome 22q11.2 happen at an elevated frequency, about 1 in 4000 live births [66]. The largest part of such deletions happens de novo (without any parent being affected) suggesting a high degree of mutations in this genomic region ($\sim 2.5 \times 10^{-4}$) [65].

1.12. 22q11DS features and symptoms

The characteristics of this syndrome differ greatly, even between the same family members, and impact several body parts. Features and symptoms can involve congenital heart disease, learning difficulties, and chronic infections because of problems in the T –cell mediated response in the immune system, that might result from thymus absence or from it being underdeveloped [63]. Moreover, 22q11DS is distinguished by diverse neuropsychiatric aspects [64, 67]. Neuropsychiatric characteristic include developmental retardation with variable degree of intellectual inability and diverse psychiatric defects, involving attention loss, anxiety, hyperactivity and autism in early life years, while in teenage years and in the beginning of puberty depression and schizophrenia can appear [67-71].

1.13. 22q11DS Genetics

Around 70-80% of 22q11DS patients lack almost 3 million base pairs on either copy of chromosome 22 in each cell of their body. The deletion happens between two low copy number repeats (LCRs) [63]. Whereas, 15-30% of people with this syndrome have a somewhat smaller deletion of 1.5 Mb in the same part [63]. The rest of patients have a diversity of deletions with one breakpoint in one of the LCRs [63].

The recognition of low copy DNA repeat (LCR) segments on chromosome 22 has produced the assumption that these LCRs could be the cause of the instability of 22q11[72-74]. New evidence argues that these repeat elements are parts of bigger (>100 kb), blocks of duplicated sequence, which is specific for chromosome 22 [74-76]. Copies of these repeat elements have been found at or close to the end points of the regular 3 Mb deletion in 22q11DS [75, 76]. This gave rise to the assumption that homologous recombination between copies of the LCRs on chromosome 22 mediate the deletions correlated with DGS/VCFS, along with other 22 chromosomal rearrangements [76, 77].

1.14 Whole exome sequencing

Since 2005, next-generation sequencing (NGS) technologies are being developed to provide a fast, accurate and cost-effective methods to meet medical sciences and research needs [78].

Exome sequencing- an approach that concentrates only on the protein-coding portion of the genome- provides a rising technology with several advantages [79]. Current improvements in this technology, have aided in revealing genetic disorders with a small number of probands regardless of common genetic heritage, and in the near future, all genes, casual variants and their link to phenotype will be revealed [79].

This approach was used in this study to find MCPH and KS causative mutations in Palestinian families.

CHAPTER 2

Literature Review

2.1 Abnormal spindle-like primary Microcephaly Gene

Abnormal spindle-like primary microcephaly (ASPM) gene, the human orthologue of the Drosophila abnormal spindle gene (asp) [80], is essential for the ordinary performance of mitotic spindles in embryonic neuroblasts [22]. ASPM is located on chromosome 1, band q31 (1q31) [22], composed of 62,567 bp with a 10,906 bp ORF, 28 exons and 3,477 amino acids, and has a recognized microtubule-interacting domain at its N-terminus [81, 82], a 81 isoleucine glutamine motifs acting as a calmodulin binding domain, a calponin homology domain [22, 83, 84], an armadillo-like sequence and a carboxyl terminal area of an unidentified role [22, 85].

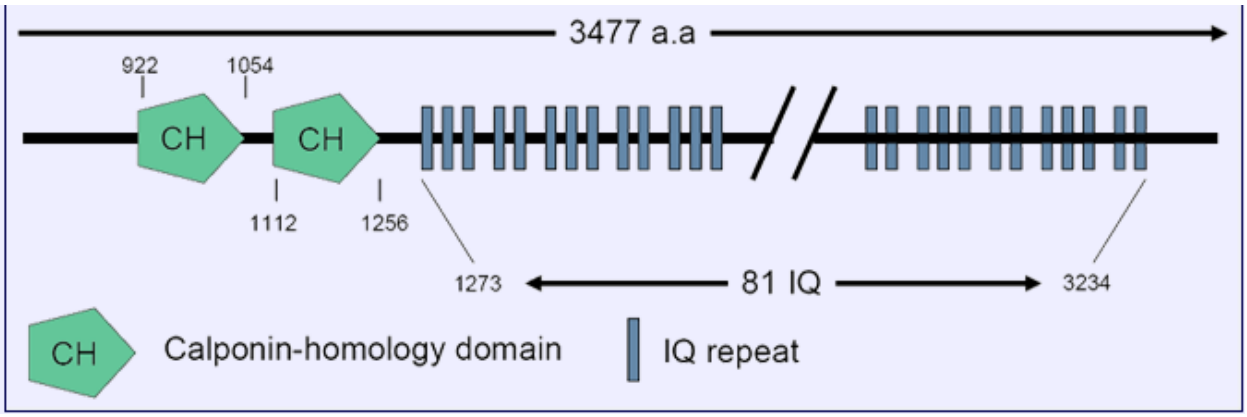


Figure 4. ASPM protein domains. ASPM protein has two calponin homology (CH) domains (Green) , and 81 IQ repeats (Blue). All 81 distinct IQ motifs were observed at position 1273 to 3243. The numbers refer to the amino acid numbers [86].

Imperfect forms of the ASPM gene are linked to autosomal recessive primary microcephaly (MCPH).

2.2 ASPM role

Through the process of neurogenesis, most neurons and glial cells in the neocortex result from the division of neural progenitor cells (NPC) in the neuroepithelial bordering the main cavities of the brain [87]. The NPC have a special fashion of mitotic activity. At first, every symmetrical division raises the number of precursor cells by producing two progenitor cells for each division [88]. Succeeding asymmetric divisions generate one neuron and reproduce one progenitor cell [88]. The division destiny of cells in the mammalian cortex seems to be reliant on the direction of the mitotic spindle and thus the location of the cleavage furrow relative to the apical surface of the neuroepithelium [89]. Glial cells must express proteins that retain this spindle direction and particularly do this during symmetric, proliferative divisions[90].

The Drosophila Asp gene is engaged in spindle microtubule regulation in both mitosis and meiosis [80, 81, 91], and in cytokinesis [92, 93]. The asp mutations in the Drosophila neuroblasts result in metaphase stoppage, causing a decreased CNS development[94]. Moreover, siRNA reduction of asp leads to a serious absence of microtubule focus at spindle poles [95]. On the other hand, the removal of Aspm in mice causes a decrease in neural stem cell proliferation and elevates the possibility that NPCs will go through asymmetric cell division, indicating a decrease in the overall number of progenitor cells generated during brain development [96]. Whereas, human ASPM expression suppression by siRNA suppresses tumour cell proliferation [97].

Overall, The mentioned data propose that Aspm might direct cerebral cortical size by controlling a part of mitotic spindle role that is essential for retaining symmetric, proliferative divisions of the very elongated, polarized NE cells [98], and so permitting the lateral growth of the neocortex [96].

2.3 KMT2D (lysine (K)-specific methyltransferase 2D)

KMT2D (lysine (K)-specific methyltransferase 2D), previously called MLL2 (myeloid/lymphoid or mixed-lineage leukemia 2, also named as ALR/MLL4) [99], is a member of the SET1 family of human SET-domain protein methyltransferase superfamily [100, 101].

KMT2D is a histone methyltransferase that has a vital part in regulating gene transcription [99], it targets histone H3 lysine 4 (H3K4), in which the methylation functions as a gene activation sign [99].

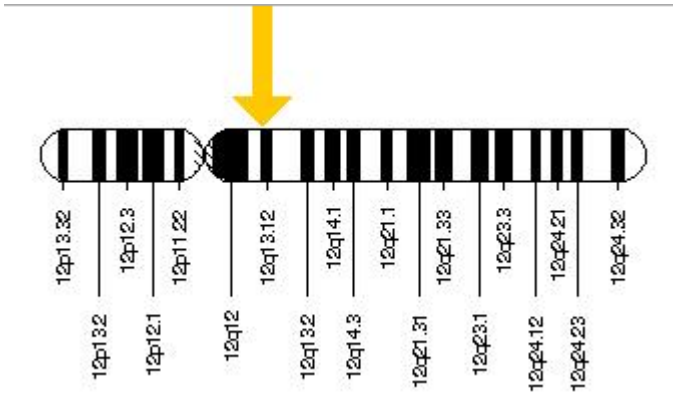


Figure 5. The molecular location of KMT2D. It is located on 12q13.12.

KMT2D is a big protein that is composed of : seven plant homeodomains (PHD, for protein–protein interaction), five LXXLL motifs (for interaction with nuclear receptors), one high-mobility group domain (for binding to DNA with low sequence specificity), one each FYRC and FYRN domains (for heterodimerisation between terminal fragments of MLL) and a single SET domain (for histone lysine methylation) [102].

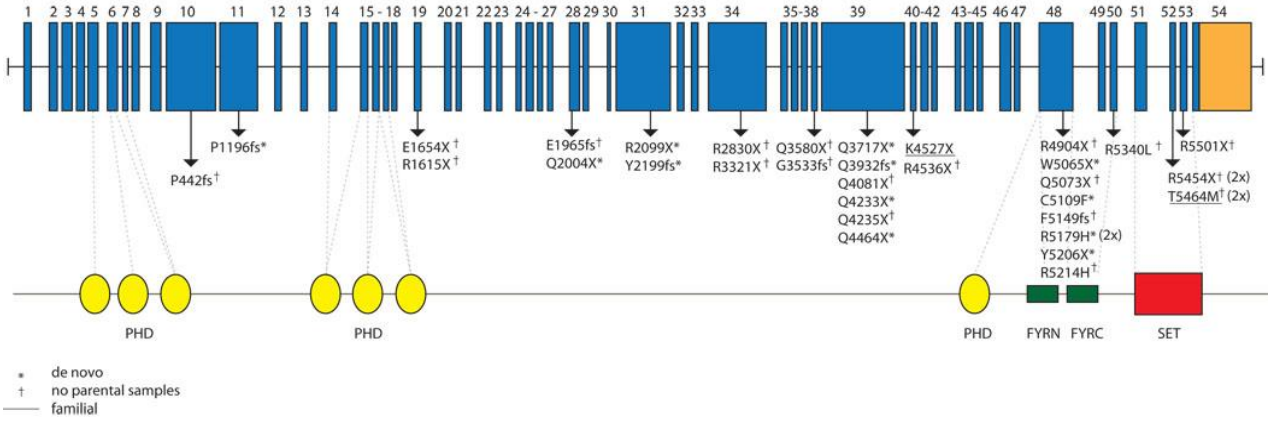


Figure 6. The structure and domains of MLL2, in addition to several MLL2 mutations identified that lead to kabuki syndrome. MLL2 consists of 54 exons that encode untranslated regions (orange) and protein coding sequence (blue) [51]. The arrows point to the positions of 32 mutations detected in 53 families with Kabuki syndrome [51].

2.4 KMT2D domains' function

The plant homeodomain is so called, because it was initially discovered as a Cys4-His-Cys3 motif in the plant homeodomain (PHD) proteins HAT3 in Arabidopsis thaliana and maize ZmHox1a. PHD exist as single finger, but sometimes in a group of two or three, moreover, it can exist together with other domains, like chromodomain and the bromodomain [102]. The PHD domain is able to distinguish methylated lysine residues [103], and it has been shown to have the ability bind to histone H3 tri-methylated on lysine 4 (H3K4me3) [104].

KMT2D (MLL2) is considered a main player in nuclear receptor-mediated gene activation and in hormone signaling, by functioning as a nuclear receptor (NR) coactivator. The interaction happens when the LXXLL motif (NR box) interacts with the AF2 domain of the lignad NR. In particular, MLL2 physically connects with estrogen receptor-alpha (ERα), a significant participant in estrogen-mediated gene activation, through its LXXLL motifs in the existence of estrogen [101].

The phenylalanine/ tyrosine-rich domain C-terminal (FYRC) and N-terminal (FYRN) regions are not well characterized [105]. MLL have to be proteolytically processed by taspase1, which cuts the protein between the FYRN and FYRC areas [106]. The N-terminal and C-terminal

segments stay connected after proteolysis, clearly as a consequence of an association between the FYRN and FYRC areas. Interestingly, the FYRN and FYRC motifs in KMT2D (MLL2) are very juxtaposed [107].

The SET domain of MLL2 provides powerfull histone 3 lysine 4 methyltransferase activity and is essential in the epigenetic control of active chromatin status [108]. Nearly all recognized MLL2 variants in Kabuki cases are expected to cut the polypeptide chain before the SET domain is translated [109].

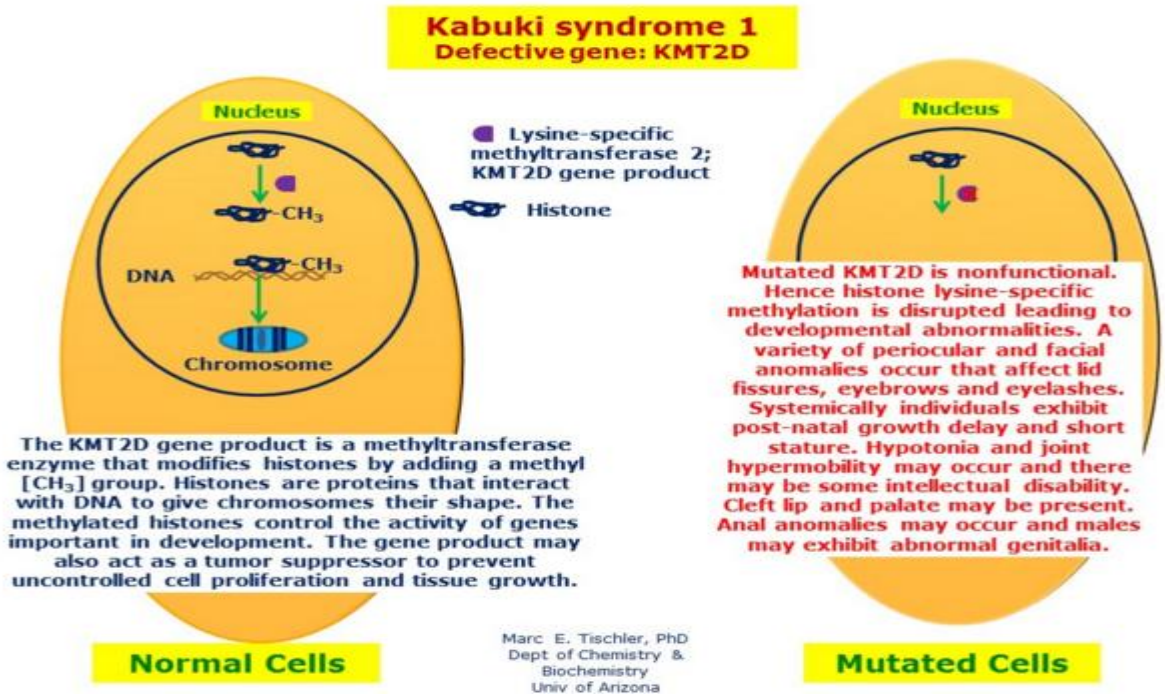


Figure7. Normal vs mutated KMT2D function in cells. The mutated product of the KMT2D disrupts methylation of histone proteins that affect gene action [110].

CHAPTER 3

Problem statements and objectives

Autosomal recessive primary microcephaly is a neurodevelopmental disorder that is present at birth[4]. MCPH causes mild to severe mental retardation and developmental delays [4, 8]. Before the recognition of MCPH genes, MCPH prevalence was estimated to be 1/30,000 in Japan, and 1/2,000,000 in Scotland, and 1/250,000 in Holland [12, 13, 111]. Newer incident studies haven't been done yet [20], but, MCPH incidence in populations where consanguineous marriages happen is much more [112, 113].

Whereas, kabuki syndrome is a rare congenital disorder with different symptoms and signs that can include mental retardation, odd facial features, intellectual disabilities [50] and microcephaly in some cases [114]. Since KS is a multisystem syndrome, patients may need several diagnostic tests, evaluation and counseling at various phases in their lives [56]

There has been some documentation of some MCPH and KS cases in Palestine but not one that addresses causes or prevalence. This is the first study to highlight the genetic and molecular causes of these conditions in Palestine, which is important due to high consanguineous marriage rate.

The study aims to achieve the following goals:

1. Perform whole exome sequencing to detect the causative mutations in Palestinian families with MCPH and KS.
2. Perform mutation validation and segregation in families using Sanger sequencing.
3. Carry out 100 control sample genotyping to exclude any chance that the mutations are normal SNPs in the Palestinian population.

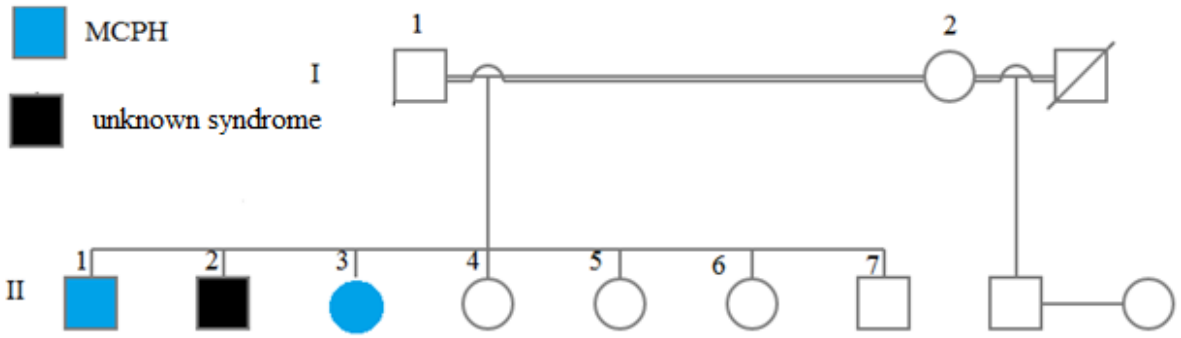
CHAPTER 4

Materials and methods

4.1 Family declaring

Three Families from Hebron were examined and their HC measurements were taken and confirmed to have microcephaly and Kabuki-like syndromes Prof. Mazin Qumsiyeh. Families pedigrees were constructed through the information obtained from interviewing the families.

A)



B)

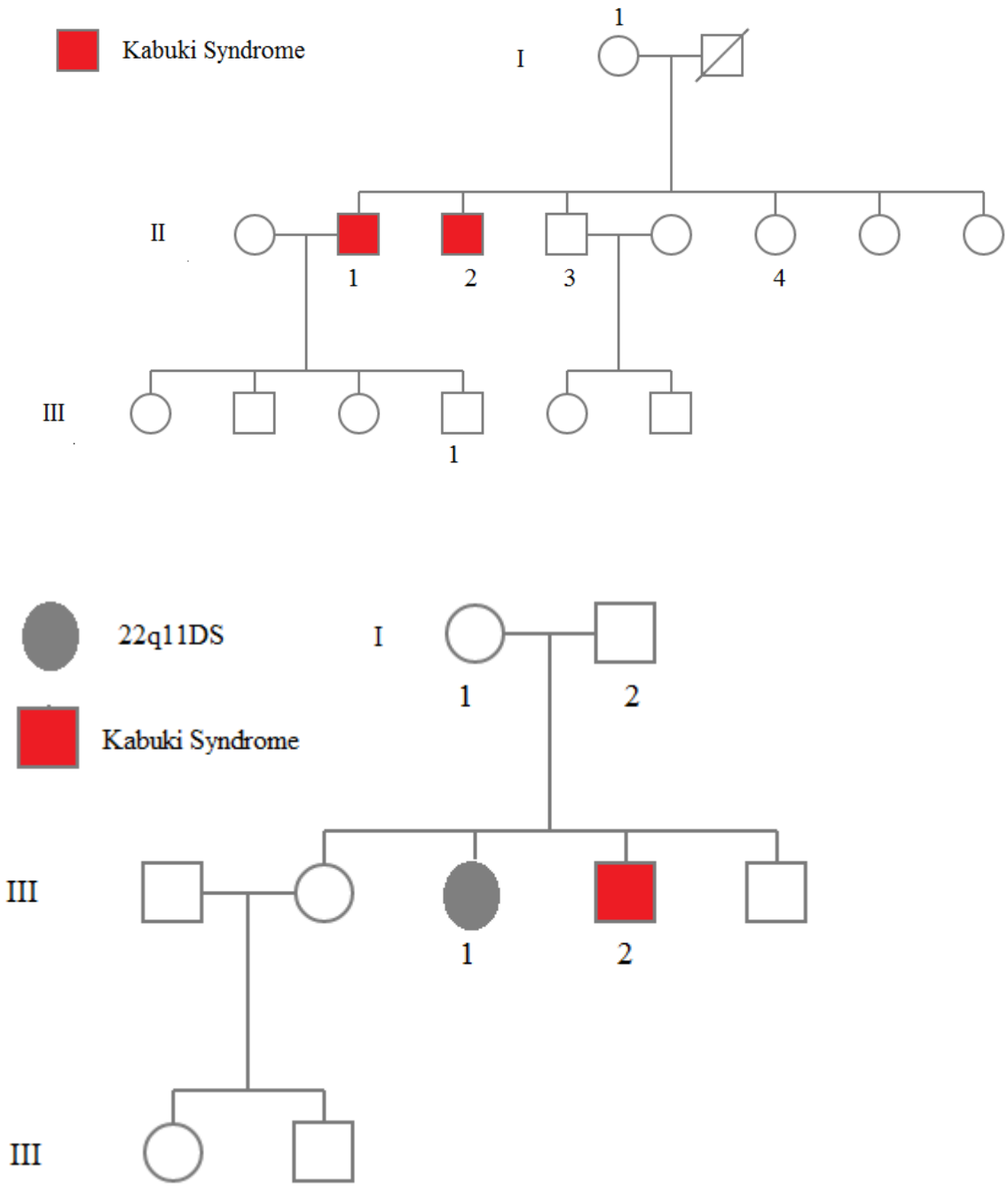


Figure 8. show the pedigree of three branches of family GL. A) Pedigree of family 1 with autosomal Recessive Primary Microcephaly. B) Pedigree of family 2 with autosomal Recessive Kabuki Syndrome. C) Pedigree of family 3 with an autosomal Recessive Kabuki Syndrome and 22q11DS.

Figure 8 shows pedigrees of three families. Family 1 that exhibits MCPH patients, and families 2, 3 have the KS patients. Both syndromes display an autosomal recessive way of inheritance. The family members were fully informed of the study and signed a consent form according to the guidelines of Bethlehem University IRB Committee. 11 members of family 1 participated in this study, 7 from family 2 and 5 from family 3.

DNA was extracted from 5ml of peripheral blood of the individuals QIAGEN kit to be used in HD chips in microarray.

4.2. Isolation of DNA by Flexi Gene DNA kit

About 10 ml blood of the family members was collected in a sterile EDTA vacutainer tubes. DNA was isolated according to the Flexi Gene DNA kit form QIAGEN (cat#51206).

4.3 Affymetrix Microarry with cytoscan HD chips

The High- Density cytoscan HD array was used in addition to the Affymetrix chromosome analysis Suite (CHAs) software to define a possible candidate homologous area.
30 candidate genes resulted from the microarray, two of them were selected for direct sequencing using Sanger sequencing using primers that were designed at the Hereditary Research Lab/Bethlehem University but they were negative for any mutation. These genes are; SEPSECS gene and RBPJ gene.

4.4 Exome sequencing

Whole exome sequencing was carried out using Hiseq 2500 sequencing system at King's lab-Department of Medicine, Division of Medical Genetics, University of Washington, Seattle, USA.

4.5 Mutation validation by Sanger sequencing

4.5.1 Polymerase Chain Reaction (PCR)

Forward and reverse primers for ASPM and KMT2D genes were designed at the Hereditary Research Lab/Bethlehem University using primer 3 software.

Table (2): Forward and reverse primers for ASPM and KMT2D genes

Gene	Forward primer	Reverse primers
ASPM	AACACACACACACAGGTAAATTT	TGGAGAGGCTATTCTTGGAGG
KMT2D	GGTAGGAGTCCATTGGGCTG	ACTGTTCCTGGCCATGATCA

4.5.1.1 PCR touchdown protocol

Reaction components:

- 2X PCR ReadyMix (Abgene _Cat# AB-0575-DC-LD) 12.5ul
- Primer F (10pml) 0.5ul
- Primer R (10pml) 0.5ul
- DNA (100ng/ul) 1ul
- DDH_2O 10.5ul

We multiply each fraction by the number of samples that we are doing +1 (for control).

PCR program

Touch down 60 was used for the two genes

Step1: 95 ºC for 4 min.

Step2 (3 cycles): 94 ºC for 30 sec, 68 ºC for 30 sec, 72 ºC for 30 sec

Step3 (3 cycles): 94 ºC for 30 sec, 66 ºC for 30 sec, 72 ºC for 30 sec

Step4 (3 cycles): 94 ºC for 30 sec, 64 ºC for 30 sec, 72 ºC for 30 sec

Step5 (3 cycles): 94 ºC for 30 sec, 62 ºC for 30 sec, 72 ºC for 30 sec

Step6 (35 cycles): 94 ºC for 30 sec, 60 ºC for 30 sec, 72 ºC for 30 sec

Step7: 72 ºC for 5 min, 4 ºC for 10 min.

4.5.2 Electrophoresis of PCR products using agarose gel

1.5% agarose gel containing 0.01% ethidium bromide (Amresco-Cat # E406) was used, prepared using 1X TAE running buffer. 3 µl of PCR product was loaded into the gel , in addition to a DNA size ladder (Thermo Scientific-Cat# SM0241) and run in 1X TAE running buffer at

100V for about 30 minute. DNA fragments were checked and reported using BioRad ultraviolet imaging system.

4.5.3 EXOSAP cleaning for PCR product

Clear PCR product shown on agarose gel were cleaned from the remaining primers and the free nucleotides using EXOSAP cleaning method.

Reaction components:

PCR product	5 µl
Exo Enzyme	0.25 µl
SAP enzyme	0.25 µl
N.F. H2O	1.5 µl

The 7 µl reaction was put in PCR machine at the following program: 37oC for 30min, 80oC for 20min, and 4^{o}C till used.

4.5.4 Sequencing of the purified PCR Product

DNA samples were prepared for sequencing using 2.0µl of cleaned PCR product, 0.2µl of BigDye, 1.5µl of 5X buffer, 0.75µl of 64X buffer, 0.2µl primer (either forward or reverse) , and 11.05µl of dd H2O

Then the mix was run on ABI 3130 DNA sequencer (Applied Biosystem) at the following program:

96^{o}C for 1 min

96^{o}C for 10 sec

50^{o}C for 5sec

60 ^{o}C for 4 min

4 ^{o}C for 10 min

30 cycles

4.5.5 Cleaning of the sequenced PCR using EDTA/Ethanol precipitation method

On each PCR product from the previous step, 60 ul of 100% Ethanol and 5 ul of 125mM EDTA were added. Then, the reactions were incubated at -20° for 20 minutes, centrifugation was done for 30 minutes at 3800 RPM at 4°C. The supernatant was discarded and 100 ul of 70% Ethanol were added to each reaction, and centrifuged again for 20 minutes. After that, the supernatant was discarded again and the samples wells were inverted on tissue paper and centrifuged for 1 minute at 500 RPM.

The samples were then put on 95°C hot plate for 5 minutes to get rid of any residual ethanol, next, 12 ul of Hi-Di Formamide (Applied Biosystems, Cat # 4311320) were added, and the samples were put again at 95°C for 5 minutes. Finally, the reactions were put on ice for 5 minutes before putting in the AB applied biosystem 3130 genetic analyzer (sequencing machine).

Chapter 5

Results

5.1 Clinical Diagnosis and Description

Three families from Halhool/ Hebron were used in this study, the most outstanding feature was the small head of affected, so HC was measured .

5.1.1 First family.

A two generation pedigree for the first family was constructed as shown in (Figure 8.A), the pedigree demonstrates an autosomal recessive inheritance of primary microcephaly.

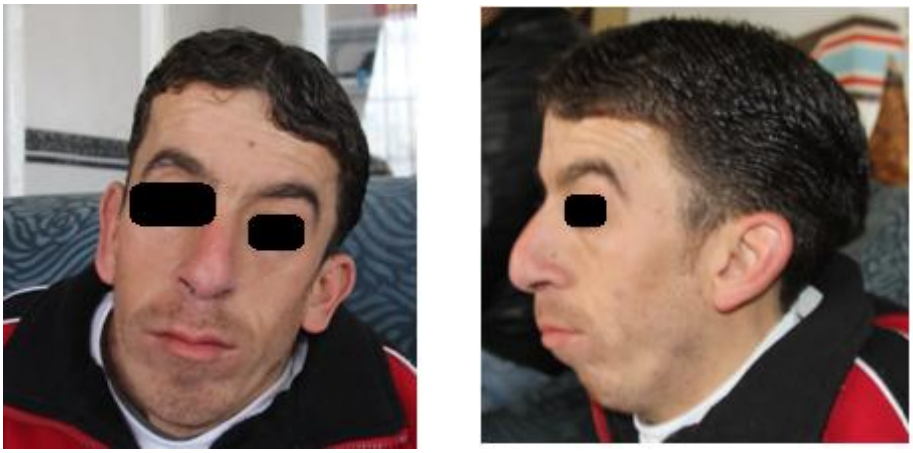
Table 3 illustrates each individual symptom.

Table 3: Family one patient's data.

Patient	Age	HC (cm)	symptoms
II-1	31 years	45 cm	sloping forehead, protruding nose and a second toe clinodactyly, in addition to mental retardation
II-3	17 years	46 cm	high arched palate, developmental delay and mental retardation.

II-2	28 years		No Mental Retardation, no heart, kidney, liver or other problems, no Microcephaly. No delivery problems, No ultrasound or other issues noted by OB/Gyn according to mother. High arched palate Hyperdontia Scoliosis Development delay Narrow chest Flexion abnormalities of upper and lower limbs Upper limbs mobile dexterous, lower limbs shortened hyper flexed narrow palpebral fissures Clinodactyly High nasal arch

Figure 9. II-1 and II-3 MCPH patients.



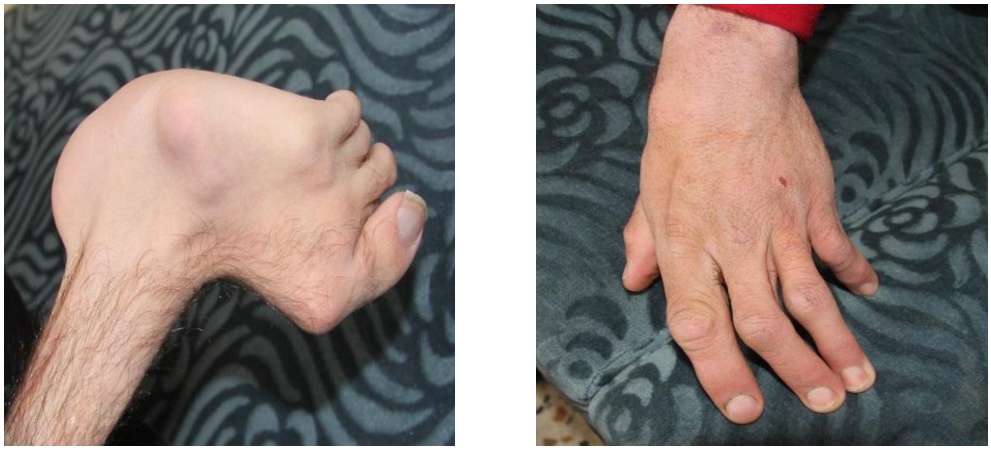


Figure 10. II-2 patient with unknown syndrome. Clinodactyly is obvious in the pictures.

5.1.2 Second family

A three generation pedigree of the second family examined in this study was constructed (Figure8. B). The pedigree demonstrates an autosomal recessive inheritance of Kabuki-like syndrome. Two individuals (II-3 and II-4) were diagnosed as kabuki-like syndrome affected, microcephaly was a symptom of the KS-like patients.

Table (4): KS-like patient's data.

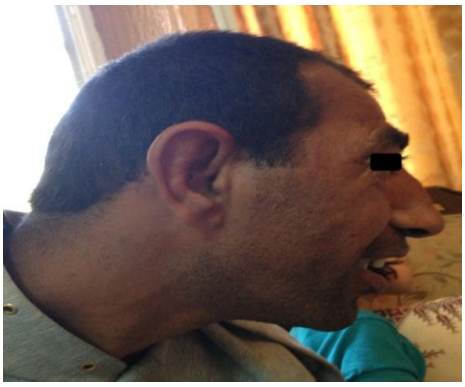
patient	Age	Hc (cm)	symptoms
II-1	45 years	51 cm	Development delay , speech problems, Oligodontia, mental retardation and epilepsy
II-2	42 years	49 cm	Cognitive disability and mental retardation

Figure 11. Patients II-1 and II-2 with Kabuki-like syndrome. Patient (II-1) has a clear oligodontia.

5.1.3 Third family

A two generation pedigree for the third family was constructed as shown in figure (8C).
The pedigree shows an autosomal recessive pattern of inheritance in kabuki-like syndrome.
Member II-3 is a kabuki syndrome affected, whereas, interestingly II-1 had a De novo 2.8 MB deletion, at 22q11.21

A B



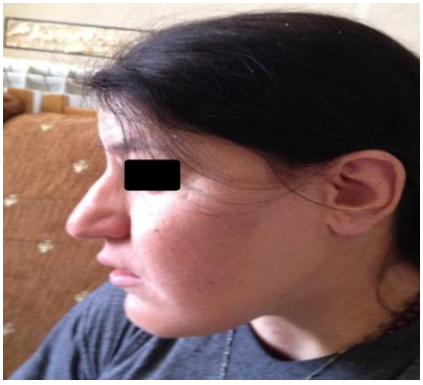


Figure 12. A) An individual with K-like syndrome, patient (II-2). B) An individual with 22q11DS , patient (II-1).

5.2 Exome sequencing results

Targeted exome sequencing was applied for 2 microcephaly affected and for individual II-2 with the unknown syndrome, and one unaffected individuals from family one (Fig 8.A), and it was applied for 3 affected and 3 unaffected individuals in the kabuki-like syndrome families and for the individual (III-2) from family three, (Figure 8.C).

Data revealed one shared variant among the two Microcephaly patients. This variant turned to be A>T substitution mutation in exon 23 of the Abnormal spindle-like microcephaly-associated protein (ASPM) gene. This substitution mutation in one base pair resulted in conversion of the lysine codon at amino acid 3228 to a stop codon (ASPM_K3228X, ch 1 , 197,090,342, A>T) .

As for individual II-2 in family 1, his case wasn't solved because by whole exome we didn't find any related candidate genes.

As for the kabuki-like syndrome patients, data revealed one shared variant among the three kabuki patients. This variant turned to be C>T substitution mutation in exon 26 of Lysine (K)-Specific Methyltransferase 2D (KMT2D) gene. This substitution mutation in one base pair (missense mutation) resulted in a conversion of the proline codon at amino acid 1912 to a codon of the amino acid leucine (KMT2D_P1912L, ch12, 49,042,790, C>T).

To predict the pathogenicity of this amino acid substitution, the variant C5735T (P1912L) was run through the PolyPhen 2 http://genetics.bwh.harvard.edu/ggi/pph2/99af37e132cd4442177479bc7962d1eafcff1682/3427396.html) and this mutation was predicted to be probably damaging with a score of 0.997 (sensitivity: 0.41; specificity: 0.98).

5.3 Validation of mutation by Sanger sequencing

In order to validate the results from whole exome sequencing, Sanger sequencing was carried out. The sequencing was done for the affected and the unaffected family members in addition to 100 controls for each mutation. Besides results verification, Sanger sequencing was done to check the mutations segregation in the family branches, and to ensure their absence in the controls.

5.3.1 ASPM mutation

Sanger sequencing has revealed that ASPM (A>T) mutation that causes a nonsense mutation segregates perfectly in an autosomal recessive manner in GL microcephaly family, (Figure13). Thus, all affected individuals were homozygous for the mutation while the unaffected individuals were wild type or heterozygous for the mutation. Moreover, all the 100 controls were wild type. (Figure 14) illustrates Sanger sequencing results for affected and unaffected individuals.

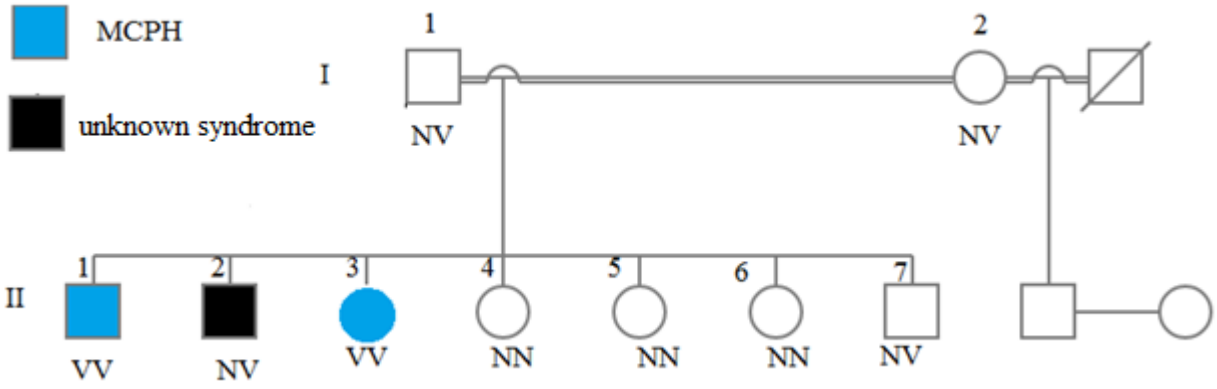
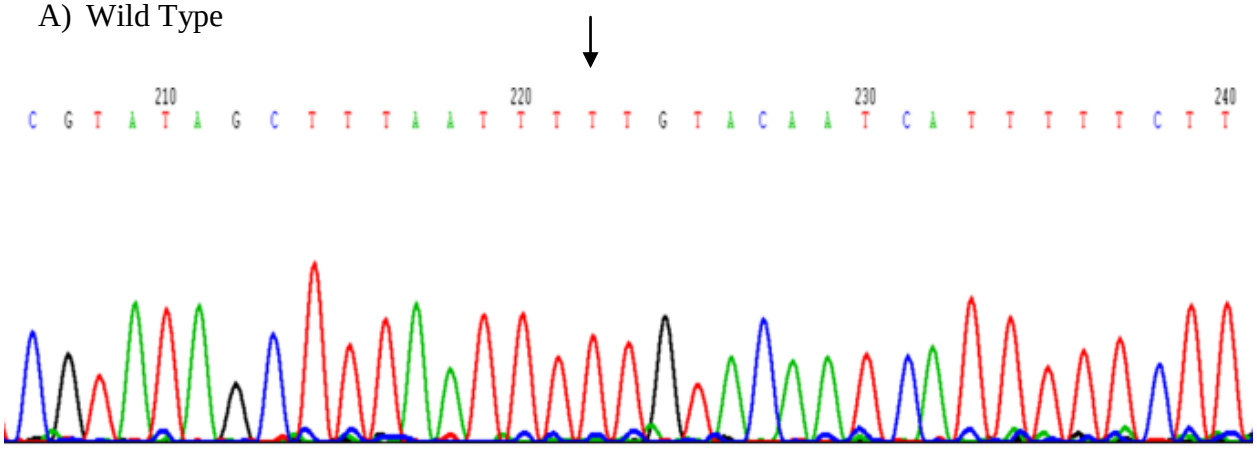
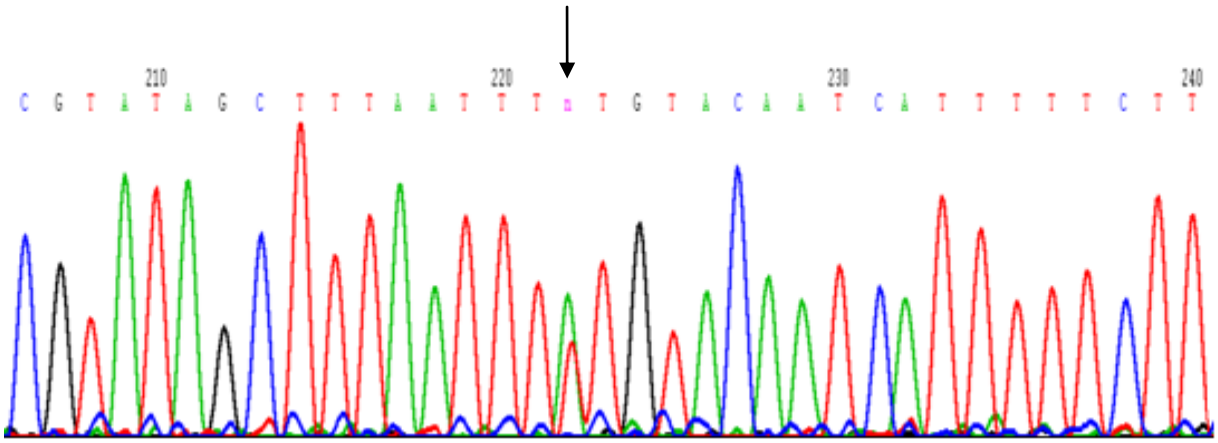


Figure 13. Segregation of the ASPM mutation in the first Family.

A) Wild Type



B) Heterozygous member



C) Homozygous affected

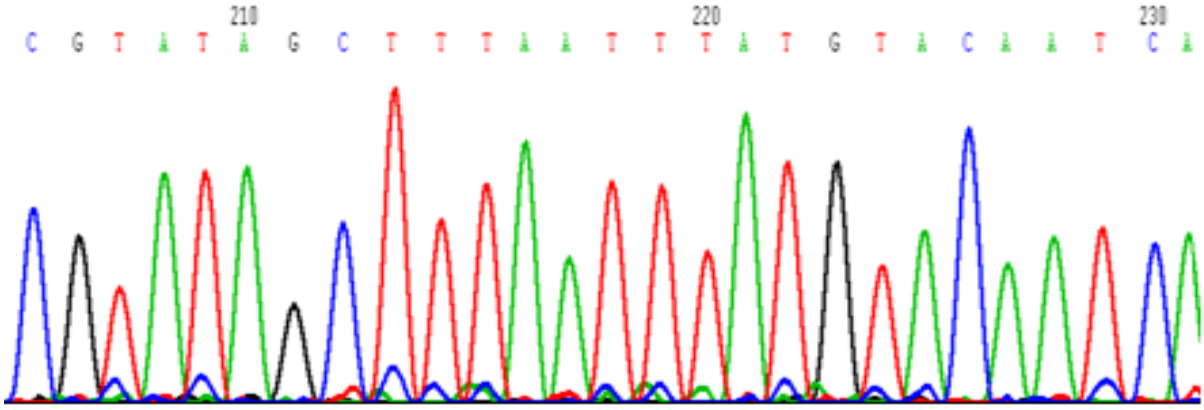


Figure 14. Verification by Sanger sequencing of ASPM nonsense mutation A>T K3228X. Chromatograms of A) wild type. B) Heterozygous C) Homozygous affected for the nonsense substitution of A with T

5.3.2 KMT2D mutation

As for the kabuki-like syndrome, Sanger sequencing has revealed that KMT2D (G>A or C>T) segregate perfectly in an autosomal recessive manner in both the second and third families, (Figure 15 and Fig 16). And so, the affected members were homozygous for the mutation whereas the unaffected individuals were either wild type or heterozygous for the mutation. Furthermore, all the 100 controls were wild type. Figure (17) illustrates Sanger sequencing results for affected and unaffected individuals.

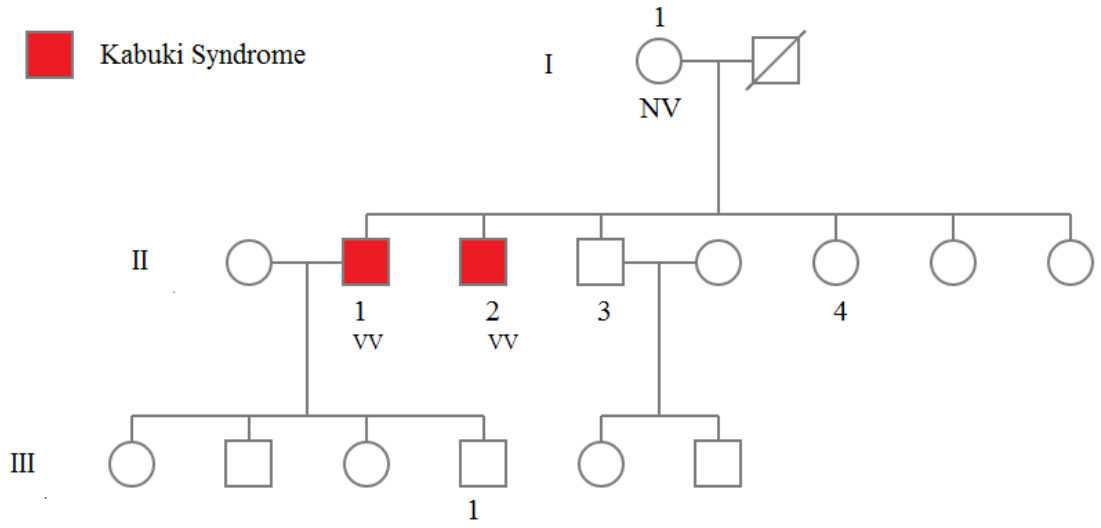


Figure 15. Segregation of the KMT2D mutation in the second family.

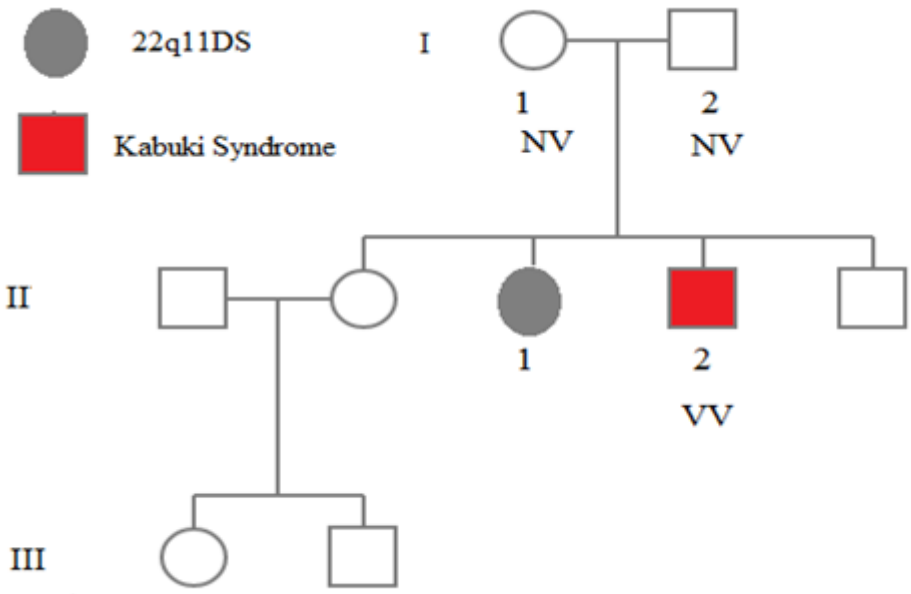
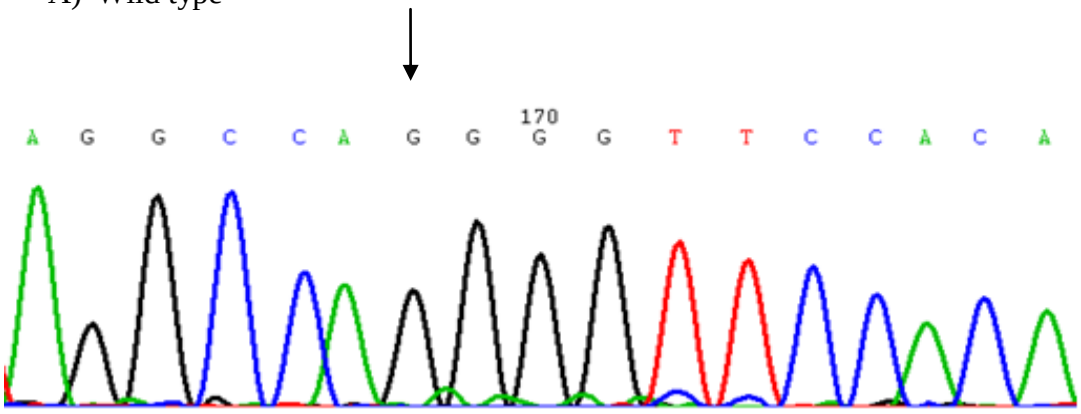
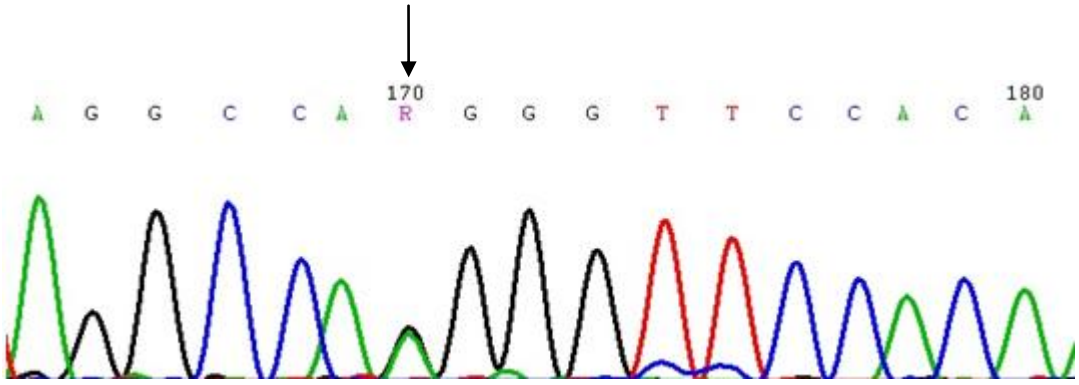


Figure 16. Segregation of the KMT2D mutation in the third family.

A) Wild type



B) Heterozygous individual



C) Homozygous affected

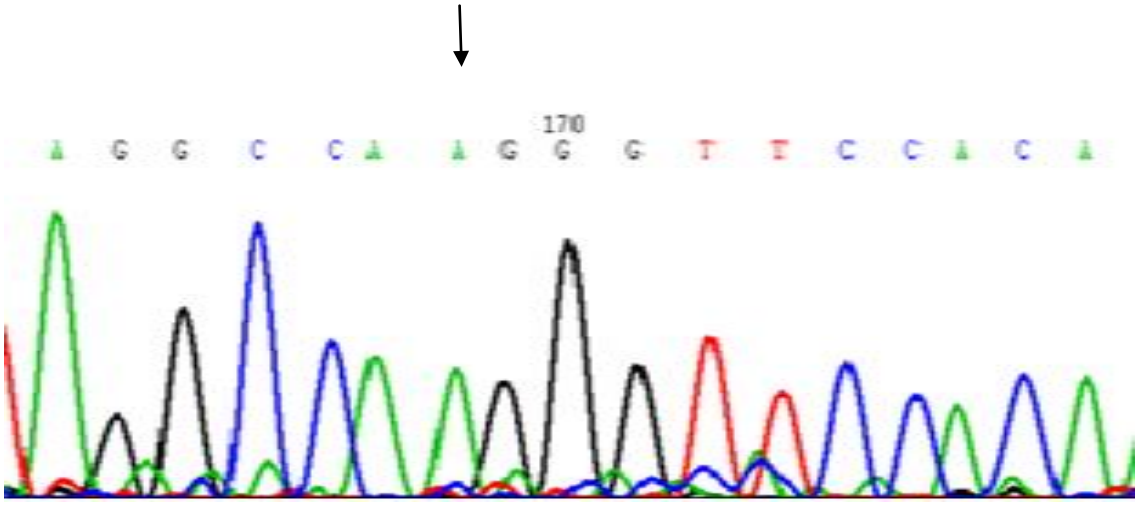


Figure 17. Verification by Sanger sequencing of KMT2D missense mutation G>A (P1912L) A) wild type. B) Heterozygous C) Homozygous affected chromatograms for the missense substitution of G with A.

5.4 Bioinformatic analysis of results

The results have revealed that the microcephaly phenotype resulted from a single base pair substitution mutation that converts the codon of Lysine to a stop codon. When we investigated this amino acid codon by using the UCSC genome browser, we found that it is conserved among the species from human to zebra fish as shown in Figure 18.

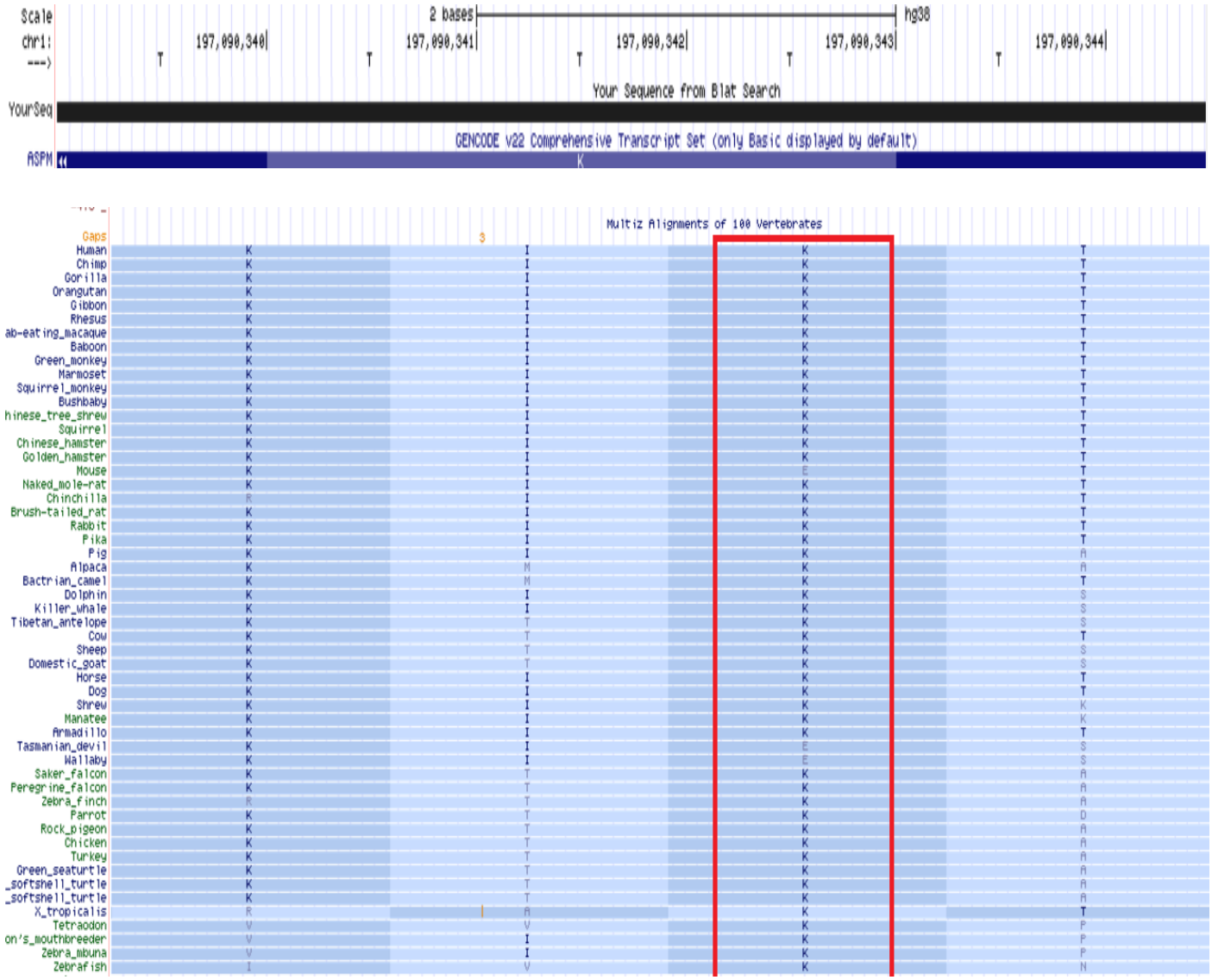


Figure 18. Result analyses by bioinformatic tools. Multiple alignments of ASPM protein for different organisms from human to zebra fish showing the conservation of the mutant site (K3228X).

Moreover, the results have revealed that the kabuki -like syndrome phenotype resulted from a single base pair substitution mutation that converts the codon of the proline at amino acid 1912 to a codon of the amino acid leucine. Using UCSC genome browser, it was found that it is conserved among species from human to lamprey as shown in fig (19B). Polyphen-2 tool was used to predict the potential influence of P1912L substitution on the stability and function of KMT2D (figure 19 A).

It was predicted that the mutation is probably damaging with a score of 0.997 (sensitivity: 0.41; specificity: 0.98), this damage can be because of the differences among the two amino acids. Since, proline is a polar, uncharged, hydrophobic amino acid, whereas, Leucine is non polar amino acid with aliphatic R group.

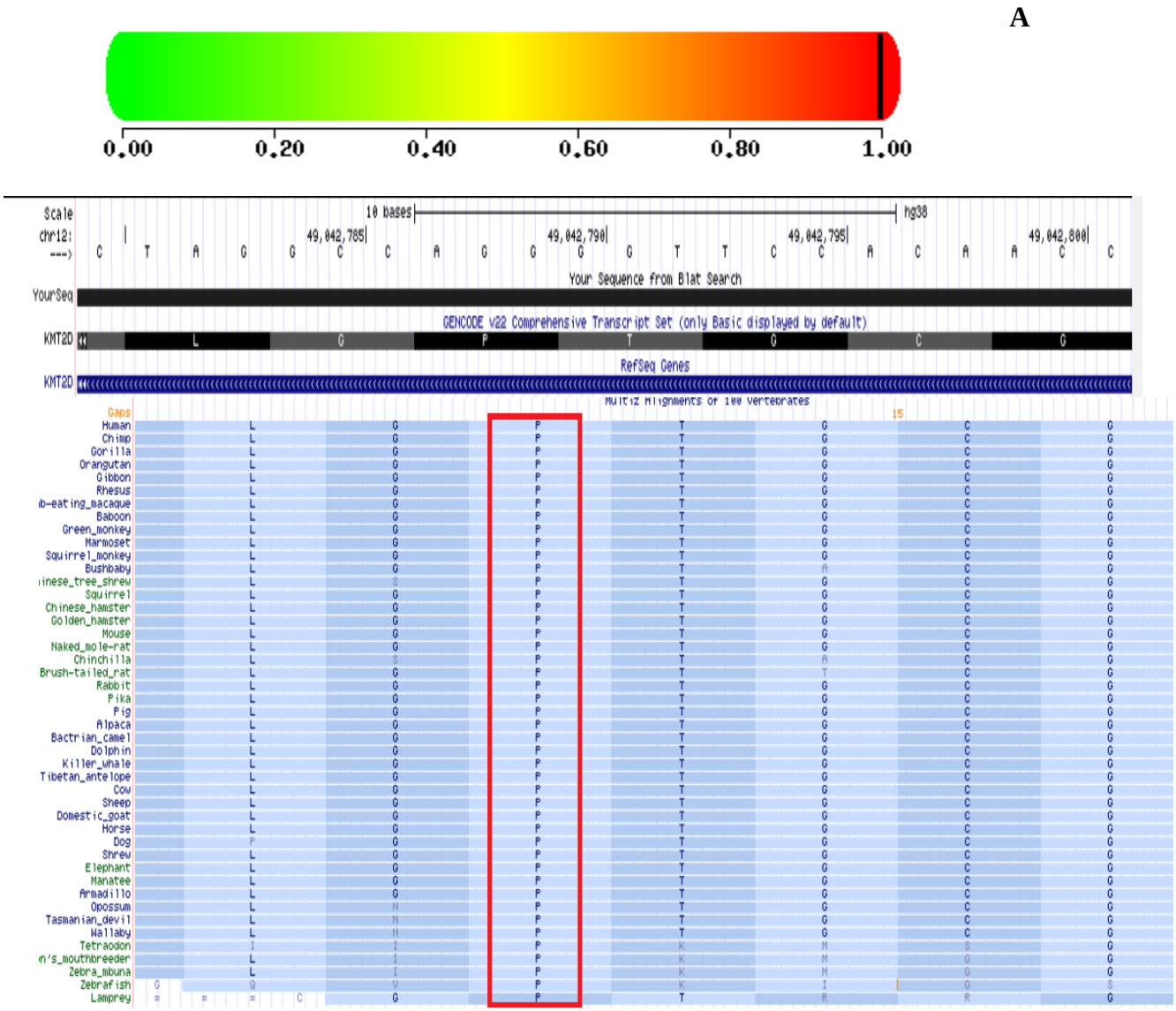


Figure 19. Result analyses by bioinformatic tools. A) The black bar at the red region indicates the mutation is damaging. B) multiple alignments of KMT2D protein for variable organisms from human to lamprey showing the conservation of the mutant site (P1912L)

Chapter 6

Discussion

In this research, a novel nonsense mutation in ASPM gene, a missense mutation in KMT2D gene, and a De novo 2.8 MB deletion at chr22q11.21 were detected using whole exome sequencing.

6.1 ASPM discussion

A novel ASPM mutation (Fig 14) was detected in a Palestinian family (Fig 13) diagnosed with Microcephaly that inherited it in an autosomal recessive way, next, using sanger sequencing the K3228X (stop codon) mutation was proved to segregate with the MCPH phenotype. This non sense mutation is damaging because it causes the mRNA to be degraded before being translated into a functional polypeptide, protein truncation mutations in ASPM are the most frequent cause of primary Microcephaly [22].

The underlying mechanism, by which ASPM causes MCPH, could be argued by the ASPM role in Wnt signaling pathway.

New research results have recognized ASPM as a positive regulator of the Wnt signaling pathway, proposing a possible pathway by which ASPM might control neurogenesis [115].

The mammalian cortex development needs many subprocesses, involving neurons production, neuronal migration, formation and improvement of synaptic connectivity, and the generation of progenitor cells. The Wnt pathway has an important role in initiating anterior–posterior axis of the forebrain [116] , enhancing the proliferation of neural progenitors [117-119], The formation of neural dendrites [120], and axon formation and directing [121].

FoxO activity suppresses the expression of Aspm whilst enhancing the expression of Wnt pathway antagonists in neural progenitor cells, proposing a mechanism to associate Aspm expression with Wnt activity [122]. Moreover, overexpression of ASPM , similar to several Wnt-activating elements, is linked with elevated cell proliferation and tumor development, approving a common impact on proliferation [84, 123, 124]

The ASPM mutation detected in this study happened in the Armadillo-type fold domain, which is a structural domain with an armadillo (ARM)-like fold, composed of a multi-helical fold involving two curved layers of alpha helices organized in a regular right-handed superhelix, in which the repeats that form this structure are organized around a common axis [125]. However, this domain is still of unknown significance in ASPM [126].

6.2 KMT2D discussion

In this study, we have identified a missense mutation in KMT2D gene using exome sequencing. The mutation was found in two Palestinian families (Fig 15, 16) that inherited Kabuki-like syndrome in an autosomal recessive pattern. Then, by using Sanger sequencing the mutation was confirmed and found to be perfectly segregated with kabuki-like syndrome phenotype. This substitution mutation C>T in exon 26 of KMT2D gene is predicted to cause conversion from the polar Proline amino acid to non - polar Leucine amino acid at the site of 1912 KMT2D protein and it was also predicted to be a deleterious mutation (Fig 19A).

On the other hand, this is considered the first reported case of KS inherited in an autosomal recessive way, since the reported cases where inherited in an autosomal dominant pattern.

KMT2D (Mll2) codes for a (H3K4) N-methyltransferase protein that consists of 5,537 amino acids [51]. In particular, KMT2D alternates the lysine residue at the fourth amino acid location of the histone H3 protein, stimulating the transformation of non-methylated H3K4 to monomethylated and, thereafter, dimethylated state [127, 128].

New researches have focused on the communication between MLL family of proteins with other core proteins, which have a role in the epigenetic control of gene transcription and chromatin structure in the initial stages development and in mature organisms[128, 129].

The methylation process can happen only when the MLL protein is connected to a minimal core set which is composed of WDR5, RbBP5, Ash2L, and DPY-30, also named using the phrase WRAD[108, 130]. Particularly, Issaeva et al [108] showed that all SET1-like complexes, along with those that involve KMT2D, have three core proteins (Ash2L, RbBP5, and WDR5) in common which are important for methylation of H3K4. Whereas the majority of studies have

been carried out utilizing MLL1 as a model system, earlier researches have proved that this interaction is applicable to KMT2D (MLL2) too [108].

KMT2D works with KDM6A to adjust the epigenetic regulation of transcriptionally active chromatin. The general role of the complex is to eliminate suppressive epigenetic signals on chromatin via a histone demethylase and to add activating H3K4 methylation signals on chromatin via a histone methyltransferase, that then attracts RNA polymerase II complex [129, 131, 132].

Despite being a field of active study, the majority of genes that are targeted by the KMT2D and KDM6A regulatory pathways are not identified so far. KMT2D and its central complex have the ability to connect to estrogen receptors, that as a result control the transcription of HOX6A in an estrogen-dependent way [133, 134]. Other suggested target genes have involved additional members of the HOX family of genes, and of the S100A genes family [36, 101, 133].

To sum up, the detected KMT2D mutation (P1912L) that resulted in kabuki syndrome leads to various disabilities. Yet, different molecular assays like the knockdown of KMT2D gene functional assays using mouse models are needed to check the consequences of the missense mutation of KMT2D on protein function and how it changes it. Moreover, it would be of importance to study the 3D structure using bioinformatics tools to understand the mutation effect on KMT2D structure.

6.3 22q11DS discussion

A De novo 2.8Mb 22q11.2 deletion syndrome was detected in a female member, patient (II-1) in the third family (Fig 16), who has moderate intellectual and learning disability, cognitive disorder, and characteristic facial features.

The 22q11 area is susceptible to rearrangements causing congenital disorders. A number of LCRs were proven to be existing in chromosome 22q11 [74]. The repeat sequences and very homologous sequences are expected to steady chromosomal pairings and assist crossover incidents. The mechanism causing deletions possibly includes either interchromosomal or intrachromosomal recombination. In the first case, mispairing of repeat or homologous

sequences in sister chromatid happens, whereas in the second case, a loop in the chromatids are formed succeeded by removal of the extended loop [135].

Scientists [77] have speculated that meiotic homologous recombination incidents among LCR22s intermediate these chromosomal rearrangements (Fig 20. A,B). Both of the proximal and distal 3 Mb LCR22s have a size of 250 kb and posses a 200 kb direct repeat that is very homologous [136]. This 200 kb region involves an area composed of five genes or pseudogenes which are surrounded by inverted sub-repeats that contain anonymous genomic sequences .

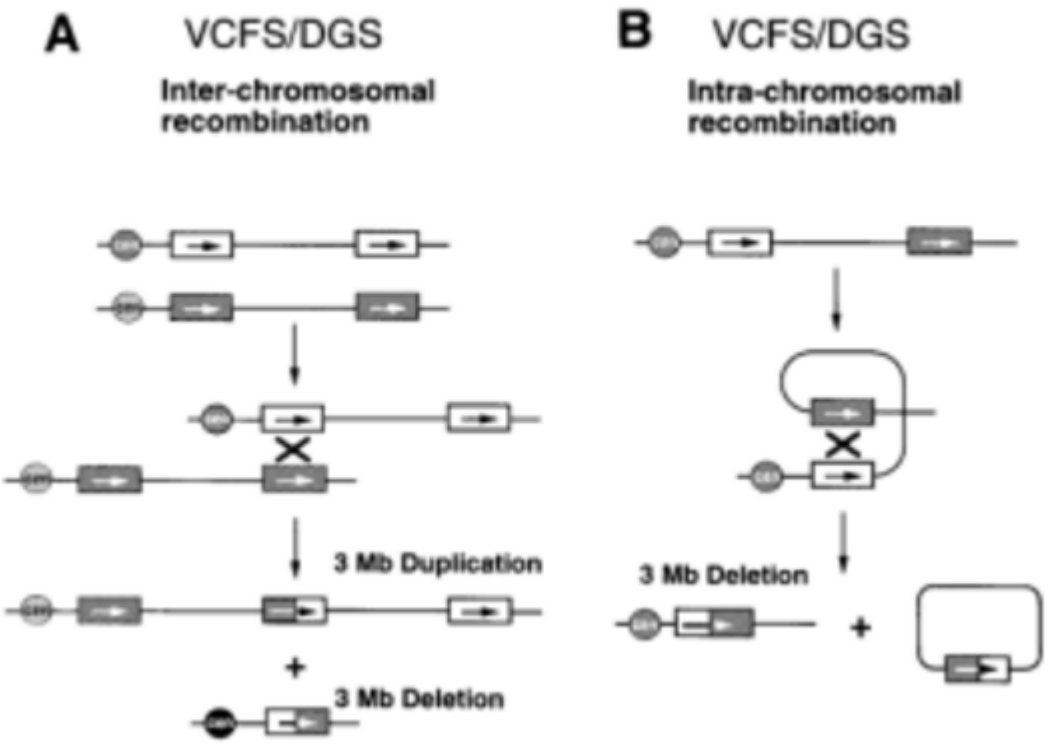


figure 20. Types of homologous recombination incidents engaging the LCR22s. A) Inter-chromosomal recombination among the homologous sequences of two 3MB LCR22s can result in both deletion and duplication of 3Mb. B) Intra-chromosomal recombination that causes removal of excision of an internal loop, leading to a 3 Mb deletion, the other outcome is a speculative round chromosome missing a centromere [77].

It is much likely that the misaligning at the LCR22s happens as a result of them having big areas of homologous sequence. The 200 kb area serves as the means to produce deletion through an intra-chromosomal mechanism, or both deletion and duplication through inter-chromosomal recombination mechanism [137]. However, the majority of deletion cases were due to interchromosomal recombination [75].

In conclusion, The 22q11.2 DS that involves (DGS/VCFS), is the most frequent microdeletion syndrome, with several symptoms and signs. Thus, it is important to have the right diagnosis and prognosis, in order to have the suitable treatment, management and genetic counseling for prolonged and better life.

Chapter 7

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