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Identification of Germline Mosaicism in a Family with 16p11.2 Microdeletion Syndrome

By

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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:

"Identification of Germline Mosaicism in a Family with 16p11.2 Microdeletion Syndrome"

by
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ABSTRACT

Microdeletions and microduplications of chromosome 16p11.2 have been associated with language impairment disorders, intellectual disability, developmental delay and psychiatric disorders. The majority of 16p11.2 microdeletions occurs de novo, whereas most 16p11.2 microduplications are seen in familial cases. A 1.5 month old male has been diagnosed with severe microcephaly and developmental delay. Whole exome sequencing (WES) by Illumina Nextseq500 has revealed his acquisition of a 0.7 Mb deletion at chromosome 16 (16p11.2), involving 42 genes of which 32 are protein-coding genes. Family segregation analysis was performed by SYBR Green quantitative real-time polymerase chain reaction (qPCR) on an ABI 7300 system. In order to measure the fold change in copy number loss the $2^{\wedge-\Delta\Delta Ct*}$ formula was used which normalizes the average DNA content of the 'tested' triplicate Cycle threshold (CT) against the average CT values of the internal *ABL* gene. The results revealed that both of his parents are wild-type (negative) for the microdeletion. Unexpectedly, his affected maternal-aunt has been demonstrated to be a heterozygous carrier (affected) of the same 16p11.2 microdeletion just like the proband. Hence, the proposed hypothesis presumed that this autosomal dominant deletion has been inherited through germ-line mosaicism, which was anticipated to be accompanied by somatic mosaicism within his mother. Therefore, a buccal mucosal cells swab and hair samples were obtained from the mother. DNA was extracted by AutoMate Express DNA extraction system so as to perform a qPCR analysis of the buccal mucosa sample which showed a clear-cut sign of mosaicism in the mother. In conclusion, genetic counseling and pre-implantation genetic diagnosis (PGD) is recommended so as to rule out any risk of recurrence due to the presence of germline mosaicism.

*ΔΔCt = (average Ct value for the tested aberration − average Ct value for *ABL* control) for the tested individual − (average Ct value for the tested aberration − average Ct value for *ABL* control) for a normal individual

اكتشاف نمط فسيفسائي جنسي (Germ-line mosaicism) في عائلة فلسطينية مع متلازمة حذف 16p11.2

جيهان جبرائيل جدعون

ملخص

لقد ارتبط حذف microdeletionوتكرار microduplication الكروموسوم 16p11.2 بالإضطرابات اللغوية والإعاقة العقلية والتأخر في النمو والاضطرابات النفسية. بحيث تحدث غالبية حالات الحذف تلقائياً de novo في 16p11.2بينما معظم حالات الإضافة في 16p11.2 يتم توارثها familial transmission .

لقد تم تشخيص طفل لدى عائلة فلسطينية يبلغ عمره 1.5 شهرًا بصغر محيط الراس microcephaly والتأخر في النمو developmental delay، ولمعرفة أسباب هذا التشوه الخلقي تم عمل فحص whole exome sequencing(WES) بواسطة جهاز Illumina Nextseq 500 الذي كشف عن وجود خلل جيني ألا وهو حذف 0.7 ميجا بايت في الكروموسوم 16p11.2وهذا الحذف يتضمن 42 جينًا منها 32 جين منتج للبروتين protein-coding genes ، ومن ثم تم إجراء فحص تحليلي لكل من الاب والام والخالة family segregationبواسطة فحص Real Time Polymerase Chain Reaction (RT-PCR) على جهاز ABI 7300 - حيث يقوم هذا الفحص بقياس نسب التغير في النسخ الموجودة من الحمض النووي باستخدام صيغة formula $2^{-\Delta\Delta Ct}$- حيث أظهرت نتائج هذا الفحص أن كلا والدي الطفل المصاب غير حاملين لهذا الحذف في عينة الدم المأخوذة.

بشكل غير متوقع، وبعد فحص خالته المصابة بصغر محيط الرأس microcephaly تبين انها تحمل نفس الحذف 16p11.2 deletion وبناء عليه تم بناء الفرضية بأن هذا الحذف الوراثي السائد autosomal dominant disorder قد ورثه الطفل من خلال وجود نمط فسيفسائي جنسي germline mosaicism من والدته والذي من الممكن أن يكون مصحوباً بنمط فسيفسائي جسدي somatic mosaicism ، ولكي يتم التأكد من وجود هذا النمط لدى الوالدة تم الحصول على مسحة من الخلايا المخاطية الشدقية buccal mucosal cells وعينات من الشعر، ومن ثم تم استخراج المادة الوراثية DNA extractionبواسطة جهاز استخراج الحمض النووي Automate Express DNA extraction System وقد أظهر فحص qPCR لعينة الغشاء المخاطي الشدقي علامة واضحة لوجود نمط فسيفسائي في الأم. وأخيرا، يُوصى بالاستشارة الوراثية والتشخيص الجيني قبل الزرع pre-implantation genetic diagnosis (PGD)لاستبعاد أي خطر تكرار كمثل هذه الحالة بسبب وجود هذا النمط لدى الام.

بعد كل هذه الفحوصات تم اكتشاف حالة نادرة الحدوث فلسطينياً الا وهي وجود نمط فسيفسائي جنسي علماً بان هذه الحالة تم توثيقها بعدة دراسات عالميا.

DECLARATION

I declare that the Master Thesis entitled " Identification of Germline Mosaicism in a Family with 16p11.2 Microdeletion Syndrome " 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.

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Dedication

I dedicate this thesis dissertation to my beloved parents Gabriel and Linda, my little sister Anna, my supportive husband Father George, my 4-years old son Andreas, my 5-months old daughter Samantha. I appreciate and cherish your understanding and patience with me as I have withdrawn from university due to high risk pregnancy, but with God's will and the support of my parents I was able to resume my studies.

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This thesis' research wouldn't have been achievable without the help and cooperation of the patients and their families in providing me with the needed information and granting us the permission to access their medical records and see their consultant clinicians.

Abbreviations

ASD	**Autistic Spectrum Disorder**
ADHD	**Attention-Deficit Hyperactivity Disorder**
BMI	**Body Mass Index**
BP	**Break Point**
CT	**Cycle Threshold**
CNV	**Copy Number Variation**
CMA	**Chromosomal Microarray**
DD	**Developmental Delay**
EEG	**Electroencephalogram**
HC	**Head Circumference**
ID	**Intellectual Difficulty**
LCR	**Low Copy Repeats**
MR	**Mental Retardation**
MCA	**Multiple Congenital Anomalies**
NAHR	**Non-Allelic Homologous Recombination**
NDD	**Neurodevelopmental Disorders**
NGS	**Next Generation Sequencing**
PGD	**Preimplantation Genetic Diagnosis**
RT-PCR	**Real Time Polymerase Chain Reaction**
VFSS	**Video Fluoroscopic Swallowing Study**
WES	**Whole Exome Sequencing**

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8.1	Infantile spasms (IS), also called West Syndrome, is a rare type of epilepsy in children. The seizures with this type of epilepsy (called "spasms") look like quick body jerks, sometimes with head drops or extended arms. These seizures are brief, but they can occur in clusters. The figure reveals an electroencephalogram (EEG) test shows a specific type of abnormal chaotic brainwave pattern called hypsarrhythmia, once this pattern is obtained it confirms the diagnosis of infantile spasms. Hypsarrhymatic pattern reveals high amplitude waves and spikes which are randomly appearing and with no topographical distribution identified, also, there is no frequency nor amplitude gradient, indicating a highly disorganized brain activity.	51

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Chapter 1

Introduction

Neurodevelopmental disorders (NDDs) are heterogeneous conditions characterized by the delay in acquiring skills in several developmental domains. Patients may manifest with speech delay, impaired social communication, motor disorders, global developmental delay, intellectual disability, seizures, or autism spectrum disorders (ASDs). Genetic and environmental factors may underlie NDD (Hanson *et al.* 2010).

Genomic intervals that are deleted or duplicated are known as copy number variant regions CNVs, such variants are associated with multiple genomic disorders. Among the most known CNV rearrangements that are associated with neuropsychiatric disorders, language impairment disorders and autism, is the 600Kb CNV (loss and gain) rearrangements that is located within the short arm of chromosome 16, taking place at breakpoints 4 and 5 (BP4-BP5) known as 16p11.2 (deletion/or duplication) with the following genomic locations (Chr16: 29.6- 30.2 Mb). In addition, this CNV has a central core of 600Kb affecting 37 protein-coding genes and it is found at a hot spot location; due to the fact that pericentromeric region of chromosome 16p is rich in segmental duplications with highly similar low copy repeats LCR regions of 143Kb flanking its ends making it prone to genomic instability by unequal crossing over by Non-Allelic Homologous Recombination NAHR during meiosis (Niarchou *et al.* 2019; Tabet *et al.* 2012).

The most clinically used genetic testing which detects large chromosomal abnormalities in NDD's patients is G-banded karyotype. However, this technique has failed in identifying microdeletions and microduplications of chromosomal fragments in some patients with NDDs displaying intellectual disability and normal karyotype (Christian *et al.* 2008). Unlike, chromosomal microarray analysis (CMA) and whole exome sequencing (WES) which were capable of identifying submicroscopic chromosomal abnormalities such as the deletion of chromosome 16p11.2, which was

reported in 0.6% of all patients with ASD (Kumar *et al.* 2009; Shinawi *et al.* 2010; Shiow *et al.* 2009; Steinman *et al.* 2016; Weiss *et al.* 2008).

Several studies have demonstrated the wide range of neurodevelopmental phenotypic manifestations of 16p11.2 microdeletion carriers (Bijlsma *et al.* 2009; Hanson *et al.* 2015; Shinawi *et al.* 2010; Zufferey *et al.* 2012). The main objective of this study was to recruit the family of the proband to identify the genetic bases of his disorder and its mode of inheritance. As in Palestine we have high rate of consanguineous marriages (approximately 40% of all marriages Zawahreh *et al.* 2019), such a scenario increases the rate of autosomal recessive disorders, unlike the present case of the proband which is an autosomal dominant disorder, it has been demonstrated that familial 16p11.2 deletions are rare and such deletions occur de novo as it seems there is a natural selection against them as they reduce the fitness of their carrier (Eichler *et al.* 2008; Weiss *et al.* 2008; Zufferey *et al.* 2012). Two questions further motivated the analyses. First, how come that a dominant autosomal disorder which occurs de novo in the majority of deletion cases appears in a two generation family? Second, why do 16p11.2 deletion carriers show phenotypic expressivity despite carrying the same deletion? This study was conducted to decipher the mode of transmission and genetic heterogeneity underlying this CNV disorder within this Palestinian family and to offer the appropriate genetic counselling for the parents to prevent any risk of recurrence.

Chapter 2

Literature Review

2.1 16p11.2 deletion

The 16p11.2 copy number variation (CNV) has gained the interest of various studies due to its association with autism spectrum disorder (ASD) (Crepel *et al.* 2011). CNVs are hardly detected using conventional cytogenetic techniques. However, next generation sequencing (NGS) techniques have proven their ability to detect human genomic CNVs rearrangements especially in monogenic syndromes such as 16p11.2 microdeletions (Iourov *et al.* 2006; Kaminsky *et al.* 2011). The typical proximal recurrent 16p11.2 loss and gain is usually 600kb occurring at BP4- BP5 breakpoint (BP) which is found at the genomic location from 29.5Mb-30.1Mb on the short arm of chromosome 16 is considered the second most common CNV deletion disorder (OMIM# 611913) (Kaminsky *et al.* 2011), having a population prevalence of approximately 3 in 10,000 individuals (Weiss *et al.* 2008) and 0.4%–0.7% of patients with unexplained intellectual disability (Kaminsky *et al.* 2011). This recurrent deletion must be distinguished from the other atypical and adjacent (distal) recurrent 16p11.2 microdeletion localized at the following genomic region (220 Kb; GRCh37/hg19 chr16:28.74-28.95 Mb) (Barge-Schaapveld *et al.* 2011). The atypical microdeletion is similar to the common 16p11.2 proximal deletion by demonstrating developmental delay, obesity and epilepsy. However, the presence of SH2B1 gene-within the atypical distal 16p11.2 microdeletion- which has role in leptin and insulin- signaling suggesting its association with severe early-onset obesity and congenital abnormalities of the kidney and urinary tract (CAKUT) (Bockukova *et al.* 2010; Tabet *et al.* 2012).

The 16p11.2 microdeletion mode of inheritance is an autosomal dominant. The 16p11.2 microdeletion is a recurrent deletion due to its embedment within a complex hot spot between 99.5% identical segmentally duplicated 147-kb LCR sequence regions of the 16p11.2 which are prone to cross over by NAHR between duplicated sequences (Bochukova *et al.* 2009; Ghebranious *et al.* 2007; Kumar *et al.* 2009; Marshall *et al.* 2008; Weiss *et al.* 2008). The 16p11.2 deletion mechanism of pathogenicity is through autosomal dominant haplo-insufficiency manner. Most of the cases of 16p11.2 microdeletion occur de novo; however, the microdeletion can also be transmitted from mildly affected and unaffected parents to a child, either one of the parents may be a heterozygous carrier of the same 16p11.2 loss (affected), or

may have a germline mosaicism or may have some low-level of somatic mosaicism accompanied by germline mosaicism. As studies by Kumar *et al.* 2009 and Weiss *et al.* 2008 have both reported a case of germline mosaicism.

16p11.2 recurrent microdeletion demonstrates a diverse phenotypic expressivity and extensive clinical phenotypes with multiple symptoms affecting brain and body. The most prominent manifestations of 16p11.2 recurrent microdeletions are severe developmental delay and language impairment disorders. While, the variable phenotypes include mental retardation (MR) (Bijlsma *et al.* 2009; Fernandez *et al.* 2010; Ghebranious *et al.* 2007; Marshall *et al.* 2008; Weiss *et al.* 2008), motor and developmental delay, language impairment, phonological processing disorders, expressive and receptive language disorders (Bijlsma *et al.* 2009; Fernandez *et al.* 2010; Ghebranious *et al.* 2007; Kumar *et al.* 2008; Marshall *et al.* 2008; Steinman *et al.* 2016; Weiss *et al.* 2008; Zufferey *et al.* 2012), autism (Marshall *et al.* 2008; Fernandez *et al.* 2010 Weiss *et al.* 2008), obesity (Maillard *et al.* 2015; Shinawi *et al.* 2010; Walters *et al.* 2010), hyperphagia (Bochukova *et al.* 2010), seizures (Ghebranious *et al.* 2007), macrocephaly (Steinman *et al.* 2016), anxiety disorders (Fernandez *et al.* 2009; Hanson *et al.* 2015; Kumar *et al.* 2008; Niarchou *et al.* 2019; Zufferey *et al.* 2012), sleep disorders (Bijlsma *et al.* 2009; Fernandez *et al.* 2009; Horev et al. 2011), short stature (Bijlsma et al. 2008; Ghebranious et al. 2007; Shen et al. 2010; Weiss *et al.* 2008; Zufferey *et al.* 2012) intellectual disability, over activity, ADHD, epilepsy, hypotonia, aortic valve abnormalities and gastrointestinal problems (Bijlsma *et al.* 2009; Ciuladaite *et al.* 2011; Christian *et al.* 2008; Crepel *et al.* 2011; D'Angelo *et al.* 2016; Ghebranious *et al.* 2007; Hernando *et al.* 2002; Marshall *et al.* 2008; McCarthy *et al.* 2009; Shinawi *et al.* 2010; Shiow *et al.* 2009; Schaaf *et al.* 2011; Tabet *et al.* 2012; Zufferey *et al.* 2012) schizophrenia (McCarthy *et al.* 2009), and severe combined immunodeficiency (Shiow *et al.* 2009).

The effect of 16p11.2 could even extend to normal phenotype depending on the difference in the genetic background of individuals (Eichler *et al.* 2008; Niarchou *et al.* 2019; Shen *et al.* 2010). Individuals with the 16p11.2 microdeletion have a wide range of intellectual abilities, ranging from severe mental retardation to normal intelligence (Ghebranious *et al.* 2007; Bijlsma *et al.* 2009). IQ scores in 16p11.2 microdeletion carriers (carrying an average IQ of 82.7) were 26.8 points lower than non-carrier family members (carrying an average IQ of 109.5) (Blaker- Lee *et al.* 2012; Hanson *et al.* 2015). Familial infantile epilepsy and infantile convulsions with choreoathetosis syndrome (Li *et al.* 2018; Scheffer *et al.* 2012; Zufferey *et*

al. 2012) were detected due to a heterozygous loss-of-function *PRRT2* gene, located within the deleted 16p11.2 regions.

Neuroimaging findings in patients with 16p11.2 microdeletions are mostly nonrecurring abnormalities (Bijlsma *et al.* 2009; Shinawi *et al.* 2010). However, the most noticeable recurrent structural brain abnormality is Chiari I malformation (Owen *et al.* 2018; Schaaf *et al.* 2011; Zufferey *et al.* 2012).

Despite there is not any constant pattern of phenotypes detected in 16p11.2 microdeletion carriers, macrocephaly is the most common feature in microdeletion carriers, however 2% of microdeletion carriers are microcephalic (Steinman *et al.* 2016). Furthermore, craniosynostosis has been detected to occur in 2% of (233) 16p11.2 microdeletion carriers (Ghebranious *et al.* 2007; Zufferey *et al.* 2012). Also, hearing impairments were detected to take place in 11% of 16p11.2 microdeletion carriers. There is not any constant configuration of dysmorphic features detected in individuals with 16p11.2 microdeletion. However, several studies have detected various dysmorphic features (Bijlsma *et al.* 2009; Shinawi *et al.* 2010). Another study by Shiow *et al.* 2009 have reported a case of severe combined immunodeficiency (SCID) in a 16p11.2 microdeletion carrier accompanied with a single-nucleotide variant in *CORO1A* gene on the other allele.

The high rate of early-onset psychiatric disorders and obesity in childhood demonstrates the need for early recognition, diagnosis, and treatment early in development to get better clinical outcomes. Manifestations of the 16p11.2 recurrent microdeletions are highly heterogeneous, and treatment is tailored according to the specific defects and demonstrated phenotypes which are identified in individuals with 16p11.2 microdeletions (Bijlsma *et al.* 2009; Li *et al.* 2013; Owen *et al.* 2018; Zufferey *et al.* 2012). Full developmental evaluation should be performed once the diagnosis of the microdeletion is confirmed; as the earlier the diagnosis is, the better the overall outcome is. Furthermore, in case of seizures a neurologist should be contacted to keep seizure spells at bay. In case of abnormal brain abnormalities, brain MRI shall be performed routinely (Owen *et al.* 2018). Because of the high rate of language impairment, especially the expressive language delays, and other motor delays, speech therapy, occupational therapy and physical therapy should be considered as early as possible (Ghebranious *et al.* 2007; Kumar *et al.* 2009; Zufferey *et al.* 2012). Furthermore, since 16p11.2 microdeletion carriers start to gain weight rapidly once they reach seven years old, healthy eating habits should be followed paying attention to meal size and maintaining an active

lifestyle (Yu *et al.* 2011). Hence, routine clinical checkups are critical for carriers of 16p11.2 microdeletions who are overweight as those individuals may suffer further complications due to their usage of medications used to treat their behavioral and psychiatric problems (Bochukova *et al.* 2010; Fernandez *et al.* 2010; Maillard *et al.* 2015; Tabet *et al.* 2012; Walters *et al.* 2010). Furthermore, a medical geneticist should be regularly consulted to keep the family updated to facilitate long-term monitoring of mental health concerns (Hanson *et al.* 2015).

At the same region of 16p11.2 there is a common 600Kb microduplication of 16p11.2 which has been identified in individuals with developmental delays and/or ASD (Kumar *et al.* 2009; Marshall *et al.* 2008; Weiss *et al.* 2008). The 16p11.2 microduplications have been associated with childhood-onset schizophrenia and bipolar disorder (Weiss *et al.* 2008). Moreover, 16p11.2 microduplication increases the risk of schizophrenia by 14.5 fold (McCarthy *et al.* 2009).

The reciprocal 16p11.2 microdeletions and microduplications CNVs display mirror phenotypes, as microdeletion carriers were found to have increased global and regional brain volumes, macrocephaly and obesity, whereas decreased brain volume, microcephaly and underweight has been associated with 16p11.2 microduplications (Blaker –Lee *et al.* 2012; Golzio *et al.* 2012; Zufferey *et al.* 2012). These opposite phenotypes indicate the presence of dosage sensitive genes lying within the 16p11.2 deleted region. Several studies have revealed that this CNV affects global cognition by lowering IQ value nearly 2 standard deviation SDs in microdeletion carriers while it lowers IQ value by 1 SD in microduplication carriers (Niarchou *et al.* 2019; Zufferey *et al.* 2012). Carriers of the 16p11.2 microdeletions are diagnosed earlier than patients with the mutual microduplications, due to its increased phenotypic severity (Shinawi *et al.* 2010). Additional distinguishing characteristic of the microduplication phenotypic manifestations is tremor that is commonly not noted until school age or later and a trend toward small head size on average. Also, white matter and/or corpus callosum abnormalities and ventricular enlargement are seen most commonly on brain imaging of microduplication carriers.

Moreover, 16p11.2 CNV rearrangement cases do not follow Mendelian rules of inheritance, revealing complex modes of transmission. Most studies have revealed that majority of 16p11.2 microdeletions (71%) take place as de novo unlike the higher incidence of familial cases in the 16p11.2 microduplication carriers which is around (70%), confirming the rarity of familial cases of the microdeletion and revealing that this genomic region is vital for normal health and

reproduction (Crespi *et al.* 2009; Horev *et al.* 2011; Levy *et al.* 2011; Niarchou *et al.* 2019; Walters et al. 2010; Weiss *et al.* 2008). Further implying low fitness of this microdeletion and demonstrating that a negative selection works against its transmission (Eichler *et al.* 2008; Weiss *et al.* 2008; Zufferey *et al.* 2012).

16p11.2 microdeletions are detected by the use of chromosomal microarray (CMA) or by whole-exome sequencing (WES). This microdeletion is submicroscopic thus it cannot be identified by routine analysis of G-banded chromosomes or other conventional cytogenetic banding techniques. Neither fluorescence in- situ hybridization (FISH) analysis nor quantitative polymerase chain reaction (qPCR) analysis could be used in the detection of the deletion in a proband, but rather are used to confirm the existence of the microdeletion. Thus, FISH, qPCR, multiplex ligation-dependent probe amplification (MLPA), and other targeted quantitative methods are being used in family segregation to test parents, siblings and other relatives of a proband who was confirmed of having the 16p11.2 recurrent microdeletion.

2.2 Mosaicism

Since the human body consists of ~10^{14} cells which originally came from a zygote. Therefore, it is presumed that all cells of the human body carry identical genomes. However, the human genome is a dynamic and relatively unstable system, which could lead to the development of intercellular genomic variations leading to mosaicism taking place early during ontology or later in life (Iourov *et al.* 2006). The presence of several different cell populations within the same organism is defined as mosaicism. On one hand, in genetic disorders when the genetic mutation is present in all body cells of an organism, it is considered a constitutional disorder. On the other hand, when a pathogenic variation is found in tissue- specific cell lines and not found in other tissues, one speaks of a mosaic status. Moreover, any monogenic disorder or any autosomal-dominant disorder can occur in mosaic state, leading to an attenuated form of the disorder compared to constitutional disorders (Moog *et al.* 2020). Mosaicism contributes to the wide range of neuropsychiatric diseases associated with the CNVs rearrangements playing a major role in the manifestation of abnormal human brain development (D'Gama *et al.* 2018). It was revealed that upon studying the neurons of postmortem human brains and of human induced pluripotent stem cell (hiPSC) that mosaic CNV detected in the human neurons are plenty (McConnell *et al.* 2013).

Moreover, mosaicism may be the hidden secret behind incomplete penetrance and the wide range of clinical phenotypes observed in 16p11.2 CNV rearrangement syndrome, ASD and

other neuropsychiatric disorders including schizophrenia and autism. Another feature of mosaicism related to human diseases is its ability to be tissue-specific (Iourov *et al.* 2006). Several studies have identified germline mosaicism in parents by the use of WES analysis which demonstrated that 6.8% of mistakenly presumed de novo mutations are in reality transmitted by parental germline mosaic carriers (Iossifov *et al.* 2012).

However, mosaic individuals are prone to transmit the disorder once married and thus prenatal genetic counselling is vital to acquire the necessary information about the nature of the disorder, its mode of inheritance, the manifestation of the phenotypes and serious attempt must be made to distinguish germline mosaicism from somatic mosaicism. Furthermore, genetic counselling faces the challenge of correctly predicting the phenotype of the individual due to the heterogeneous nature of the 16p11.2 microdeletions (Glessner *et al.* 2009). Genetic counseling should take into consideration the clear genotype-phenotype correlation between cognitive disability and the microdeletion as well as the fact that the degree of cognitive disability is relative to the family background (Hanson *et al.* 2015). Preimplantation genetic testing offers an opportunity for families in which the 16p11.2 recurrent microdeletion has been identified to have a normal offspring.

Mosaic diseases often manifest themselves in the skin and brain due to being tissue- specific disorders. Thus, when seemingly “unaffected” parents are tested negative for the deletion identified in their affected child, it suggests a de novo case. However, since most molecular methods use DNA extracted from blood, the obtained negative result in parents opens up the potential possibility for the existence of parental mosaicism (Dehainault *et al.* 2016). To detect a mosaic disorder, the presence of germline mosaicism (egg and sperm are affected by the mosaic status) should be determined so as to rule out any risk of recurrence of the same deletion in future children. Since, detection of mosaicism in blood samples is rarely successful, thus hair samples, buccal mucosal cells and sperm cells are tested. However, female germline DNA is difficult to access. Hence, determining the presence of female germline mosaicism will be confirmed if the deletion is transmitted from “asymptomatic” or mildly affected mosaic mother to her affected offspring (Youssoufian *et al.* 2002).

It would be of great importance to demonstrate whether the mosaic deletion has taken place pre- or post- zygotic. As on one hand, a pre-zygotic deletion transferred from seemingly unaffected mosaic parent presumes a recurrence risk requiring clinical follow-up and genetic

testing. On the other hand, a post-zygotic deletion means the presence of a somatic mosaicism solely releasing parents and siblings from prenatal diagnosis (D'Gama *et al.* 2018).

2.3 16p11.2 Genes and Disease Involvement

It has been estimated that up to 12% of human genes are located within CNV-prone regions (Shiow *et al.* 2009). CNV rearrangements especially recurrent losses at 16p11.2 are highly associated with several phenotypes by interfering with the function of haploinsufficient genes in this region, or affecting genes found at the breakpoints or revealing recessive mutations on the other allele (Shinow *et al.* 2009). Furthermore, the structural brain abnormalities, aberrant synaptic connectivity and neurocognitive disorders detected in pathological and imaging analysis of 16p11.2 deletion carriers (Owen *et al*. 2018) reveal the tight association of 16p11.2 deletions with impaired brain function, demonstrating the importance of gene dosage in 16p11.2 BP4-BP5 CNV deleted region.

The 16p11.2 deleted region is dense with genes which are highly expressed in the brain and not only affect the nervous system but rather the body size too. Within the nervous system, the directional flow of information is attained through the axon-dendrite neuronal polarity. The capacity of neurons to receive and transmit electrical signals "information" in the brain, is achievable through polarization of axon and dendrites (Li *et al.* 2013). Thus, any defect in neural polarity leads to a group of neuropsychiatric disorders including autism, epilepsy and mental retardation. Several studies have found that once 16p11.2 region is deleted or duplicated, susceptibility to neuropsychiatric disorders increases (Kumar *et al.* 2009; Marshall *et al.* 2008; Weiss *et al.* 2008). Since, autism is a neuronal polarity defect, and autism is associated with 16p11.2 deletion and duplication. Thus, one or more genes within the 16p11.2 region may have a role in regulating neuronal polarity.

Several studies have defined the genes whose expression is altered when lost or duplicated as 'dosage sensors' genes (Blaker- Lee *et al.* 2012; Ghebranious *et al.* 2007). A study by Zoghbi and Bear (2012) demonstrated that "optimal synaptic function occurs within a limited two copies range" and any misbalance from this limited range of two copies by either an increase or decrease of region will lead to the manifestation of a specific phenotype. Furthermore, the zebrafish and mice were used in several studies as model organisms to discover human 16p11.2 dosage-sensitive genes and their function. Since the same genetic pathways are conserved in mammals and fish, the phenotypes obtained when knocking out the 16p11.2 homologs were similar to 16p11.2 deletion disorder in humans, especially since this CNV has mirror

phenotypes that are revealed early during development. Besides, upon the addition of the human genes they were able to substitute their knocked-out homologs which revealed an evolutionary conservation (Blaker –Lee *et al.* 2012; Golzio *et al.* 2012).

According to the UCSC Browser (http://genome.ucsc.edu; hg 19), 16p11.2 microdeletion region contains the following thirty- two 32 protein- coding genes: (ALDOA,ASPHD1,BOLA2B,C16orf54,C16orf92,CDIPT,CORO1A,DOC2A,FAM57B,GDPD3,HIRIP3,INO80E,KCTD13,KIF22,MAPK3,MAZ,MVP,PAGR1,PPP4C,PRRT2,QPRT,SEZ6L2,SLX1A,SLX1B,SPN,SULT1A3,SULT1A4,TAOK2,TBX6,TMEM219,YPEL3,ZG16) of which 22 genes are expressed in the developing human fetal nervous system (Ciuladaite *et al.* 2011; Li *et al.* 2013). Moreover, within the 16p11.2 BP4-BP5 locus there is a high expression of brain- specific genes which are crucial for nervous system development – affecting brain morphology, eye development, axonal organization, and motor response (Blaker- Lee *et al.* 2012; Tabet *et al.* 2012). Transcriptome profiling had revealed that genes at 16p11.2 rearrangement location are associated with haplo-insufficiency by revealing a dosage-sensitivity pattern (Arbogast *et al.* 2016; Blaker-Lee *et al.* 2012; Kumar *et al.* 2009; Li *et al.* 2013; Marshall *et al.* 2008; Weiss *et al.* 2008). Furthermore, several studies have classified the region of 16p11. 2 as a novel gene domain which is tightly associated with intellectual disability, neural developmental disorders and abnormal brain function (Arbogast *et al.* 2016; Blaker- Lee *et al.* 2012; Li *et al.* 2013; Weiss *et al.* 2008).

2.3.1 Genotype-Phenotype Correlations

Several studies have displayed that there is a strong association between several candidate genes (*Kif22, Aldoa, SEZ6L, QPRT, PRRT2, KCTD13, MAPK3, DOC2A MAZ and TBX*) and neurodevelopmental/seizure disorders demonstrated in 16p11.2 microdeletion carriers; confirming that genes within this 16p11.2 BP4-BP5 CNV rearrangement region are essential for brain function in animal models such as mice and zebrafish other than humans further endorsing the correlation of genotype with the manifestation of specific phenotypes expressed in 16p11.2 microdeletion carriers (Blaker –Lee *et al.* 2012; Golzio *et al.* 2012).

Kif22 gene (OMIM: 603213) encodes a microtubule associated motor protein and a DNA binding molecule which is important in chromosomal alignment and aggregation during anaphase and mediates synaptic vesicles transportation in the synapses (Blaker-Lee *et al.* 2012; Li *et al.* 2013). It was found that *Kif22* has a role in axonal branching upon screening the 16p11.2 homologs region in *Drosophila*. Although it has not been related to brain function

disorders, but it was suggested that *Kif22* is needed for neural progenitor formation, as well as a heterozygous mutation of *Kif22* has been associated with epilepsy (Blaker- Lee *et al.* 2012). Moreover, *Kif22* has a role in the transport of synaptic vesicles to the axon and of transmitter receptors to the dendrite (Li *et al.* 2013). Thus, *Kif22* is essential for the establishment of neuronal polarity.

Aldoa gene (OMIM: 103850) encodes a protein product that is a glycolytic enzyme which reversibly converts fructose-1,6- bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. *Aldoa* gene is critical, since homozygous null mutations have never been identified. However, when *Aldoa* expression is reduced it has been accompanied with mental retardation, hemolytic anemia, microcephaly and language delay (Blaker- Lee *et al.* 2012). While, overexpression of *Aldoa* gene has been detected in schizophrenic and depressed patients, associating this gene with neuropsychiatric disorders. It has been suggested that *Aldoa* has a role in 16p11.2 phenotypes, since in children with autism the mitochondrial citric acid cycle was dysregulated.

16p11.2 deletions are accompanied by seizures, thus the most probable gene causing seizures is the Seizure Related 6 Homolog Like 2 (*SEZ6L2*) gene (OMIM: 616667) which is homologous to the mouse *sez6* gene, which is strictly expressed in spinal cords and brains of mouse embryos and related to seizure disorders (Arbogast *et al.* 2016; Blaker- Lee *et al.* 2012, Kumar *et al.* 2009). *Sez6* has also a role in regulating excitatory synapse branching, as *sez6*-deficient mice have developed neurological and behavioral aberrations, displaying impairment of excitatory postsynaptic currents, memory impairment and motor developmental delays (Ciuladaite *et al.* 2011; Kumar *et al.* 2009). It has been revealed that *SEZ6L2* has high specific expression within the central nervous system, and it has been associated with neurodevelopmental disorders such as autism and motor coordination delay (Ciuladaite *et al.* 2011; Crepel *et al.* 2011; Kumar *et al.* 2009; Miyazaki *et al.* 2006; Shinawi *et al.* 2010).

Quinolinate phosphoribosyltransferase (*QPRT*) gene (OMIM: 606248) encodes a vital enzyme which is required for the breakdown of quinolinate which is a strong endogenous exitotoxin harmful to neuron (Marshall *et al.* 2008; Ghebranious *et al.* 2007). It has been found that heterozygosity of *QPRT* leads to the elevation of quinolinate levels in the brain which has been associated with pathogenesis of epilepsy in humans. Hence, the haploinsufficiency in one or both of *SEZ6L2* and *QPRT* genes may underlie behind the basis of seizure disorders in 16p11.2 CNV carriers (Ghebranious *et al.* 2007; Shinawi *et al.* 2010).

Within the 16p11.2 region there is proline-rich transmembrane protein 2 (*PRRT2*) gene (OMIM: 614386) that its heterozygous loss of function causes autosomal dominant infantile-onset seizure disorders, paroxysmal neurological disorders as well as benign familial infantile epilepsy (BFIE) and infantile convulsions choreoathetosis (ICCA) syndrome, febrile convulsions and epileptic seizures, some of these phenotypes have been demonstrated in 16p11.2 deletion carriers (Blaker- Lee *et al.* 2012; Kumar *et al.* 2009; Li *et al.* 2018; Scheffer *et al.* 2012; Steinman *et al.* 2016; Vlaskamp *et al.* 2018).

Inside the 16p11.2 BP4-BP5 region, there is a smaller deletion of around 118-kb that has been revealed to be segregating with ASD and other neurodevelopmental abnormalities (Crepel *et al.* 2011). Within this 118Kb deleted region there is *KCTD13* gene (Potassium Channel Tetramerization Domain containing 13) (OMIM: 608947) which is a key element behind the neurodevelopmental aberrations of the 16p11.2 microdeletions (Golzio *et al.* 2012). *KCTD13* has a high expression level in the developing brain and it encodes *PDIP1* protein (polymerase delta-interacting protein 1) which cooperates with the proliferating cell nuclear antigen, thus affecting neurogenesis by regulating cell cycle. As a study by Golzio *et al.* 2012 has revealed that overexpression of *KCTD13* in embryos of zebrafish models has led to microcephaly and significant increase in apoptosis of the developing brain and a decrease in proliferation of neuronal progenitor cells, while on the other hand knockdown of *KCTD13* gene at the same locus induced macrocephaly and obvious increase in proliferative cells with no change in apoptosis, revealing the same mirror phenotype of different head size detected in human 16p11.2 CNV losses and gains, such critical changes in head circumference are due to changes in the numbers of mature neurons since *KCTD13* gene has a role in neurogenesis (Golzio *et al.* 2012; Steinman *et al.* 2016).

Mitogen-activated protein kinase (*MAPK3*) (OMIM: 601795) phosphorylates a number of transcription factors which recruits specific genes crucial for synaptic remodeling, maturation and synaptic plasticity during early periods of brain development with high expression in human fetal and adult brains (Ciuladaite *et al.* 2011; Kumar *et al.* 2009). Presynaptic NMDA receptors have a key role in excitatory neurotransmission and synaptic plasticity. Moreover, NMDA receptors were revealed to be involved in long term potentiation (LTP), which is vital for learning and memory and it has been detected to be reduced in *MAPK3* deficient mice displaying hyperactivity, abnormal avoidance behaviors, low LTP, difficulty adapting to change and sleeping abnormalities (Barnby *et al.* 2005; Horev *et al.* 2011; Mazzucchelli *et al.*

2002; Shinawi et al. 2010). While a study by Horev *et al.* 2011 has displayed that size of certain brain structures was different than other brain structures due to the altered gene copy numbers of 16p11.2 loci. Additionally, an increase in the activity of *mTOR* and *MAPK* signaling pathways, which are crucial for synaptogenesis and protein synthesis, was detected in the brains of syndromic ASD patients. Further associating this gene *MAPK3* with learning difficulties found in 16p11.2 microdeletion carriers as the *MAPK* pathway is a key regulator of neural progenitor biogenesis, learning and memory, which was confirmed by other studies where an upregulation of *mTOR* and *MAPK* pathways was detected in post-mortem brains of mild and severe idiopathic ASD patients (Blaker- Lee *et al.* 2012; Rosina *et al.* 2019).

Double C2-like domains Alpha (*Doc2a*) (OMIM: 604567) gene is one of the post-synaptic density genes which is uniquely expressed in brain and neuronal cells, located at synaptic vesicles as a calcium sensor involved in neurotransmission release (Arbogast *et al.* 2016; Blaker- Lee *et al.* 2012; Kumar *et al.* 2009; Li *et al.* 2013; Mazzucchelli *et al.* 2002; Marshall *et al.* 2008; Shinawi *et al.* 2010). It has been shown that *Doc2a* has a role in controlling neural polarity "structure" and synaptic transmission "function" to ease the informational flow by regulating synaptic release when calcium concentration is high in presynaptic terminal. Thus, *Doc2a* regulates the neurotransmission during repetitive synaptic activation which is critical for learning and memory formation. It has been demonstrated that *DOC2A*- heterozygous mutant mice and zebrafish models developed defects in excitatory synaptic transmission, impairing long-term potentiation, hyperactivity, seizures, displaying learning and behavioral issues and demonstrating an abnormal passive avoidance task (Arobogast *et al.* 2016; Kumar *et al.* 2009; Li *et al.* 2013; Mazzucchelli *et al.* 2002; McCammon *et al.* 2017; Sakaguchi *et al.* 1999).

Other studies have revealed the *MAZ* gene (OMIM: 600999) which is highly expressed in brains of humans, especially in mid-frontal cortex and it encodes the regulator gene *MYC* which improves the activity of NMDA receptor subunit type 1 during neuronal differentiation and serotonin pathway which are key elements in long term potentiation which is critical for learning and memory, which further confirms the association of 16p11.2 BP4-BP5 CNVs with language impairment disorders (Barnby *et al.* 2005; Kumar *et al.* 2009; Shinawi *et al.* 2010). Several studies demonstrated that *TBX6* (OMIM: 602427) gene encodes a transcriptional regulator involved in developmental processes, regulating neural crest migration and controlling neural polarity. Thus, *TBX6* haploinsufficiency might be a key element leading to

vertebral and spinal- related abnormalities reported in 16p11.2 CNVs carriers, as has been demonstrated in *TBX6*- deficient mice who displayed rib and vertebral anomalies (Arbogast *et al.* 2016; Blaker- Lee *et al.* 2012; Ciuladaite *et al.* 2011; Li *et al.* 2013; Schaaf *et al.* 2010; Shinawi *et al.* 2010; Zufferey *et al.* 2012).

2.4 Mechanisms underlying Pathogenesis of 16p11.2 Deletion

Several studies have revealed that dosage alternation of one or more genes within the recurrent 16p11.2 deleted region drive the disruption of molecular pathways involved in body mass index and brain volume (Kumar *et al.* 2009; Maillard *et al.* 2015); suggesting that neural circuitry has a role in the pathogenesis of both ASD and obesity as they may share similar molecular and pathological mechanisms leading to the manifestation of wide range of heterogeneous phenotypes of 16p11.2 deletion due to the haploinsufficiency of one or more genes located within the deletion locus (Bijlsma et al. 2009; Steinman *et al.* 2016; Zufferey *et al.* 2012).

Neuroimaging analysis performed by Hinkley *et al.* 2019 has revealed that 16p11.2 CNV BP4-BP5 rearrangement region influences regional brain volume, white matter integrity, and early sensory responses in auditory cortex. In 16p11.2 microdeletion carriers, it was displayed that they suffer from reduced left hemisphere language specialization (Crespi *et al.* 2009). The clinical manifestation of speech and motor delays are linearly correlated to neural activity as it has been revealed that insufficient copy number at the 16p11.2 region leads to excessive neural activity (increase in beta oscillation (12-30Hz) sensory motor cortical rhythm), which is an accurate neurological mark of motor behaviors as any movement of the body is preceded and in synchronous with a decrease in beta rhythms.

Since, the genes in 16p11.2 microdeletion region are required for language laterality, thus two copies of genes located at 16p11.2 location are crucial to have a normal neural activity. Those abnormal neural activities were detected in 16p11.2 deletion carriers (children and adults alike), meaning that abnormal alternation in neural activity and laterality persists way beyond early brain developmental stages. Those results further associate motor and language impairment with increased neural activity leading to impairment in sensorimotor abilities. As several studies have reported delays in linguistic and fine motor skills in 16p11.2 microdeletion carriers (Hanson *et al.* 2015 Bijlsma *et al.* 2009; Shinawi *et al.* 2010; Zufferey *et al.* 2012) as those speech and language defects are motoric; consisting of poor articulation and low speech production. Those abnormal alternations in neural activity are due to haploinsufficiency of copy number repeats at 16p11.2 deleted region and not as a result of motor impairment;

indicating the necessity of acquiring the suitable copy number of genes at 16p11.2 region which is vital for normal neural circuits in the brain to acquire human-specific behaviors (speech and fine motor skills). What further confirm these results is the evolutionary studies of the 16p11.2 region which is highly flanked by duplicated repetitive elements which are human specific. Thus, since 16p11.2 region is unique to human species, then absence of genes within it would impact the complex behaviors of speech and fine motor skills which render our human race to be special (Crespi *et al.* 2009; Eichler *et al.* 2008; Hinkley *et al.* 2019; Horev *et al.* 2011; Shinawi *et al.* 2010; Steinman *et al.* 2016).

Various studies have revealed that structural brain abnormalities (Owen *et al.* 2018) detected by pathological and imaging studies (Hinkley *et al.* 2019) ahead with the abnormal neural connectivity (Li *et al.* 2013) trigger the manifestation of 16p11.2 deletion's phenotypes. In 16p11.2 microdeletion carriers, the most significant feature is a dysmorphic and thick corpus callosum, while in mutual microduplication carriers, a dysmorphic and thin corpus callosum accompanied by enlarged ventricles and a decrease of white matter volume are manifested (Owen *et al.* 2018). Those brain MRI findings could be used as indicator for behavioral defects and help radiologists to recommend genetic screening of individuals revealing those brain structural abnormalities to distinguish 16p11.2 CNV carrier from other neurodevelopmental disorders' individuals.

Chapter 3

Research Methodology and Materials

3.1 Study Subjects

On December 2019, Whole Exome Sequencing (WES) analysis was recommended because a one and half months- old Palestinian male infant affected with severe microcephaly and developmental delay was recruited from Caritas Baby Hospital in Bethlehem, where he has been examined by pediatric neurologist. Blood was withdrawn from both of the parents and the proband and sent to the Research lab of Bethlehem University. During the acquisition of detailed information, it was found out that the maternal aunt had a severe microcephaly case since birth accompanied with language impairment disorder, short stature, severe obesity and serious behavioral issues. Hence, it was arranged that her blood would be withdrawn at Istishari Arab Hospital in Ramallah. All samples were taken after all eligible participants in this research have signed upon an informed consent form provided by Bethlehem University Institutional Review Board. This two generational Palestinian family affected with heterozygous 16p11.2 microdeletion syndrome is the topic of this thesis.

3.2 DNA Extraction, Quantification and Qualification

Approximately 5ml of peripheral blood samples were collected by venipuncture into EDTA-coated tubes. Then, it was poured into a 50ml conical tube and 45 ml of RBCs lysis buffer (8.28g NH4Cl, 0.79g NH4HCO3, 0.2ml of 0.5M EDTA) was placed to lyse the red blood cells within each sample. All tubes were placed on ice for 40 minutes then centrifuged for 10min at 4^{o}C at 2000 rpm. Afterwards, the supernatant was decanted, while the pellet was mixed with another 15ml of RBCs lysis buffer, shaken and centrifuged at the same conditions. Then, the pellet was suspended with 3ml of DNA lysis buffer (25 ml of 1M Tris base, 16.7ml of 3M NaCl and 1ml of 0.5 M EDTA), 100ul of 5mg/ml Proteinase K (Amresco, Cat# E195) and 100ul of 20% Sodium Dodecyl Sulfate SDS (Amresco, Cat # 1328- M112). The tubes were shaken and incubated for 37^{o}C for 48 hours. After incubation, 1 ml of 6M NaCl was added and shaken vigorously, then centrifuged for 20min at 25^{o}C at 3000rpm, the supernatant was transferred into 15 ml conical tube. Theu tubes were centrifuged again at the same conditions and the supernatant was poured into another 15ml conical tube. Around 8ml of Absolute cold Ethanol 100% was added to each sample tube. Then the tube was gently inverted until the DNA threads are detected and made visible as thin white fibers. With a hooked Pasteur pipette the

DNA was collected and washed with 70% Ethanol and air- dried for 3-5 miuntes. Then, the DNA was transferred into screw-capped tubes containing 0.02% Sodium azide (Sigma Aldrich, Cat # S2002) to conserve the DNA and dissolve it for 48 hours then kept at -20^{o}C.

The genomic DNA (gDNA) was quantified by Qubit v.3 Fluorometer (Life technologies, Cat #Q32854) by using the Qubit double strand DNA (dsDNA) High Sensitivity (HS) Assay Kit as indicated by the manufacturer. Size qualification was checked by gel electrophoresis where agarose powder (Lonza SeaKem LE Agarose, Cat# 50004) was melted in 1X TAE buffer (2 M Tris pH=0, 0.05 M EDTA pH =8, 1M Acetic acid) with the addition of few drops of Ethidium bromide (Amresco, Cat # E405-5ML) so that DNA will be visualized when it's exposed to UV. The percentage of agarose gel depends on the molecular weight of the product/element being analyzed; typically, 1% for genomic DNA and 1.5% for PCR products accompanied with the usage of an appropriate DNA ladder (usually 100 bp DNA ladder (GeneRuler, Thermo Scientific, Cat# SM0241)). Gel electrophoresis was done in 1X TAE running buffer for 20minutes at around 120V. Genomic DNA was visualized and photographed by the use of Molecular Imager Gel Doc^{TM} XR+ Imaging System (Bio- Rad Laboratories, Serial # 721BRO3428).

3.3 Whole Exome Sequencing (WES)

Whole-exome sequencing (WES) method is a NGS analysis, which has been designed to detect thousands of distinct exonic locations of protein-coding loci and especially those located near segmental genomic CNVs within the human genome. Despite the fact that exonic loci constitute only 1% of the genomic regions, but they are responsible of 85% of disease-causative mutations which are located within these protein- coding regions (Choi *et al.* 2009). Nextera Flex Illumina sequencing method has been used to enhance the effective sequencing of the complete coding regions "whole exome" with high sensitivity and specificity which reach around 95% of the targeted protein- coding regions. Major steps for WES analysis include library preparation and enrichment of the exonic regions of the genome by the hybridization of probes which bind specifically to exonic loci. The library preparation includes the tagmentation of the genomic DNA then its fragmentation and ligating specific adapters to the desired exonic loci to be sequenced followed by PCR amplification step. Afterwards, the biotinylated probes which are uniquely specific for exons are hybridized to the libraries, then enrichment step is performed through the addition of avidin capture beads followed by further cycle of PCR amplification so that the library is ready to be sequenced. Then the obtained data are aligned

against the reference genome to detect any abnormal variants within the tested sample (Katsanis *et al.* 2013).

Whole exome sequencing was accomplished using Nextera Flex for Encrihment as indicated by Illumina, Inc (Cat #1000000048041v00). DNA was fragmented to the appropriate size 300bp by using Illumina DNA Prep Tagmentation (S) Beads (Cat# 20026214) which facilitate the preparation of genomic DNA library in which a transposase enzyme directly fragments and inserts specific adapter sequences to both ends of each fragmented dsDNA in a single-tube reaction termed "tagmentation" combining DNA fragmentation, end-repair, and adaptor-ligation steps into a single step to prevent chimera formation. Afterwards, multiple index (barcode) sequences IDT for Illumina (Nextera DNA UD Indexes Set A, Cat#20026121) were ligated to each end of the fragmented DNA to amplify the insert DNA by PCR. Also, those adapters have specific sequences designed to hybridize with the surface of the flow-cell (Illumina). The DNA fragments were afterwards amplified by PCR using Nextera DNA Flex Pre-Enrichment Library Prep- PCR reagents (Cat #20026216) this step specifically amplify fragmented DNA with ligated adapters on both ends. A post-library clean-up step is used to refine library size and remove adaptor dimers or other library preparation artifacts by the use of Agencourt AMPure XP beads (Cat#A63880) clean up. While the quality of the pre-enriched libraries was checked on the Agilent Technologies 4200 Tape Station Bioanalyzer (Agilent Technologies, Cat# G2991AA) using a high sensitivity DNA kit. DNA libraries were then combined into a single 12-plex pool, subsequently the hybridization of the targeted exonic regions is made possible by the use of capture probes which are Coding Exome Oligos (Exome Panel (45Mb), Cat#15050026). Afterwards, streptavidin beads (Nextera DNA Flex Enrichment Beads + Buffers, Cat#20026212) were used to capture the hybridized probes on the exome from the previous step, followed by two steps of washing to get rid of non-exome products. Also, the hybridization, capture of the hybridized probes were repeated to assure exome-specific products, followed by a final PCR amplification step. All the PCR amplification steps were performed on the thermal cycler (VeritiTM 96-well Thermal Cycler by Applied Biosystems- Serial# 2990213940) according to the manufacturer's instruction, to enrich for the DNA products that have adapters ligated to both their ends, so that the library will be ready for quantitation and loading onto the flow cell.

The concentration of the enriched libraries was quantified by Qubit v.3 Fluorometer using the Qubit dsDNA HS Assay kit. While the quality of the enriched libraries was checked on the

4200 TapeStation Bioanalyzer (S/N: DEDAA00152). Then each captured library was normalized and loaded onto Illumina NextSeq 500 platform (Illumina, Inc. serial # NB501033) to perform high- throughput sequencing for a target of ~ 100 million 2x76 paired-end reads per sample, by using NextSeq™ 500 Wash Flow Cell (Illumina, Cat# 15050205) and NextSeq 500/550 High Output Reagent Cartridge v2 (Illumina, Cat# 15057931).

After sequencing on the NextSeq500, bioinformatics analysis was performed to utilize sequencing data generated from the sequencing platform. The base-calling software BaseSpace Sequence Hub received data from the imager after each reaction cycle to form raw read data. Then, the obtained data was uploaded onto Bethlehem University's server where the resultant reads saved as FASTQ files type (raw data) were aligned to the reference genome UCSC, Human Hg19/GRCh37 using Burrows-Wheeler Aligner (BWA) (http://bio-bwa.sourceforge.net/). Before obtaining variant calling files VCF by Genome Analysis Toolkit GATK (http://gatk.broadinstitute.org/hc/en-us), the BAM file formats (mapped reads to the reference genome) underwent preprocessing steps of quality-control procedure by the removal of potential PCR duplicates with Picard v1.59, the realignment around indels, and the recalibration of bases' quality. Afterwards, the final acquired variants were annotated by ANNOVAR tool (http://annovar.openbioinformatics.org) through applying several filtering criteria to detect potential pathogenic variants by the prioritization of nonsynonymous, nonsense, frameshift, and splice-site variants with a minor allele frequency of <1% in ExAC (http://exac.broadinstitute.org) and gnomAD (https://gnomad.broadinstitute.org) databases, and by using variant prediction tools such as PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), REVEL (rare exome variant ensemble learner) and SIFT (http://sift.jcvi.org/) tools. Also, by checking the databases of genetic diseases DECIPHER v10.2 (https://decipher.sanger.ac.uk/), OMIM (https://omim.org/), GeneReviews (https://www.ncbi.nlm.nih.gov/pubmed/) tools. If neither point mutations nor indels less than 7bp are detected, XHMM (eXome-Hidden Markov Model, atgu.mgh.harvard.edu/xhmm) tool was utilized to recover information on Copy Number Variants CNVs from targeted raw exome sequence data files. All detected CNVs were compared to known CNVs in publicly available databases, including OMIM (https://omim.org/), GeneReviews, Decipher v10.2, ClinVar (https://www.clinicalgenome.org/), and DGV (https://dgv.tcag.ca/ dgv/app/home). Variants with low coverage rate, predicted benign or likely benigen by SIFT and PolyPhen,

synonymous, 1000 Genomic Project and minor allele frequencing > 0.1% on ExAC were filtered out.

3.4 Quantitative PCR Validation and Family Segregation

WES identified a heterozygous deletion of 737,067bp within chromosome 16. Thus, primers were designed from within the identified deleted region using Primer3 Input (primer3.ut.ee). While the internal control primers were designed for the *ABL* gene. Primer sequences and product size for real-time PCR are listed in Table 3.1.

Table 3.1 Primer sequences: The primer's sequences and the expected product sizes used for qPCR validation.

Primer Name/ Product sizes	**Sequence**
chr16: 29469235-30206302 deletion/ 182bp	**Forward:** 5' GCC CAG GAT TAC TAA AGC AT3' **Reverse:** 5' GCA GCC CTT TAT TTC AGA GA 3'
ABL internal control/ 184bp	**Forward:** 5' TGG GTC CCA AGC AAC TAC AT 3' **Reverse:** 5' CCC TCC CTT CGT ATC TCA GC3'

Hair sample and buccal cells swab from the suspected mosaic mother were taken at Istishari Arab Hospital IAH, Ramallah. PrepFiler BTA Forensic DNA lysis and extraction from those samples was achieved by using PrepFiler LySep Columns (Applied Biosystems, Cat# 4442889) and PrepFiler Sample Tubes (Applied Biosystems, Cat# 4443225) according to the manufacturer's instructions. The DNA extraction was performed upon AutoMate Express DNA Extraction System (Applied Biosystems, Serial# PFX1702B1120).

SYBR green fluorescent mix uses the fluorescent dye in real time polymerase chain reaction (RT- PCR) which intercalates to the double-stranded DNA bases, as at the end of each amplification cycle, the amount of emitted fluorescence is correlated directly with how much DNA has been amplified. The more the concentration of the DNA found within the reaction the more the fluorescence emission increases along each amplification cycle. If any reduction in the patient's DNA hybridization intensity was detected when compared to the internal reference *ABL* this would be considered as sign of a microdeletion existence.

Quantitative PCR (qPCR) was performed on Applied System ABI 7300 Real Time PCR System (Applied Biosystems, Serial# 273004050) using Power SYBR Green PCR Master Mix (Applied Biosystems, Cat#4367659) according to the manufacturer's protocol. qPCR Thermo-cycling conditions on ABI 7300 System were as follows: touch down 50 C for 2 min, 95 C for 10 min; 40 cycles of 95 C for 15 sec, annealing temperature 60 C for 1 min. The *ABL* housekeeping gene was used as an internal control for data normalization. Triplicate reactions were performed for each the tested deletion and the *ABL* gene, and by comparing the

differences in the amplification of the targeted deletion and the reference internal gene, the copy number variation were assessed. Thus, the average DNA content of the 'tested' triplicate Cycle Threshold CT was normalized against the average CT values of the internal *ABL* gene in order to measure the fold change in copy number loss through using the formula $2^{-\Delta\Delta ct}$; where ΔΔCt = (average Ct value for the tested aberration − average Ct value for *ABL* control) for the tested individual − (average Ct value for the tested aberration − average Ct value for *ABL* control) for a normal individual.

Chapter 4

Results

4.1 Clinical Assessment of the 16p11.2 Deletion Patients

Patient II- 8 was a 25-year-old female with normal development. She had several tremor attacks since her marriage, during pregnancy and after her delivery. The attacks manifested as trembling in the upper limbs, accompanied by loss of consciousness lasting for 10 seconds. Since then, the attacks have occurred about 1–2 times per month and are usually aggravated by tiredness or anger. The patient had no history of seizures or epileptic attacks. The patient suffered from aggression and behavioral problems such as harming herself or her infant (proband III- 8). She was taken to a psychiatric clinic by individual I-2, where Lustral 50mg was prescribed to her (50mg/day) ahead with Calmanervin tablets once per night, which the patient has declined to take them. The patient had taken few speech therapy sessions as she had poor articulation of words in the past. Furthermore, she had learning difficulties however she graduated from a normal school with adequate performance. No autistic features were found. Her father I-3 revealed autistic behaviors, but he declined neuropsychological evaluation. However, he was described by his relatives as being non talkative, timid and having few social relationships.

Patient II- 11 is a 21 years old female who was born through normal birth and uncomplicated pregnancy, her developmental milestones (motor function, speech and cognitive function) were severely delayed. She was diagnosed with severe microcephaly and suffered from seizures which resolved once she turned three years old. Her mother said that she sat at 18 months and did not walk or say ‘‘Mama’’ and ‘‘Dada’’ until 5 years of age where she was able to speak a few single words, with poor articulation and prolonged repetitiveness. She was enrolled in elementary school at the age of 8 years, and was noticed to have significant learning difficulty. She did not play with her peers at school. She played only with her older brothers (one II-9 of them was suffering from learning difficulties while the other II- 10 suffered from behavioral problems) and she was incapable of handling conflict between them. During her whole childhood, she can be easily irritated and was frequently in an unhappy mood suffering from sleep disorders and having serious behavioral problems such as self-harming, and in cases of nervosas she pulls her hair. Moreover, despite intensive speech therapy, she did not achieve

scholarly skills such as reading, writing and calculating. At the age of 21 years she still has poor expressive verbal skills with stuttering. Physical examination identified the following features: she suffered from short stature; her height was 1.36 m, and severe microcephaly; her head circumference (HC) was 52, her weight was 65 kg (Body Mass Index BMI of 35.1 considered as overweight). She has mild dysmorphic features: short and down-slanted palpebral fissures, anteverted nares, simple external ears, retrognathia, exotropia, clumsy walking, a broad neck, and sloping shoulders. Patient II- 11 revealed signs of echolalia (repetition of heard speech), hyperphagia and she is addicted to soda drinks. She was noted as having impairment in social interaction and communication and exhibited repetitive motor mannerisms by sitting and standing up more than 10 times within fifteen minutes. The patient continued to be severely handicapped; not fully toilet-trained and required assistance for most basic needs, but was able to feed and dress herself. Most findings suspected by family were confirmed by a medical professional. The father (individual I-3) is a non-communicative man. He had learning difficulty during his childhood as indicated by individual I- 4 who suffers from tremor in her upper limbs ahead with individuals I-5 and II- 12. It was noted that during every visit, individual I-3 did not inquire or offer any information related to his children. Also he has repetitive motor mannerisms like patient II- 11. It was not accessible to test any of these individuals as they refused to perform any molecular tests showing an exaggerated aggressive refusal response.

Patient III- 8 is a one and a half year old male of non-consanguineous parents. He was normally born at full term after uncomplicated pregnancy, with a weight of 3.53kg, a height of 52cm and HC of 31cm (Third percentile) diagnosed as severe microcephaly. At one week old, an MRI has revealed the presence of pachygyria formation ahead with mild to moderate atrophy of the brain associated with ventriculomegaly, particularly of the temporal horns of the lateral ventricles and the brain imaging has confirmed the presence of severe microcephaly (Figure 4.2). At one month old, his HIP ultrasound report was normal, his CBC analysis was normal with Hg=15.7. A CT scan of the brain at the age of two months, has confirmed the microcephaly with partial agenesis of the corpus callosum, associated with ventriculomegaly and moderate brain atrophy.

On December, 2019, it was recommended that WES analysis to be performed for him ahead with both ophthalmic exams and EEG in case of seizures occurrence. At four months old the

child has developed infantile spasms and severe developmental delay, while his EEG test revealed a hypsarrhythmia pattern, thus he was clinically diagnosed with WEST Syndrome. As a result Sabrilan 500mg/12hours and hydrocortisone 10mg was prescribed for him. A CT scan of the brain at six months old, has revealed an increase in ventriculomegaly of the temporal horn associated with partial agenesis of corpus callosum accompanied with frontal and temporal lobes atropies. He had his first tooth at seven months, and at nine months he had his second tooth. Also at seven months old, a VFSS has revealed that on the oral phase, the patient has decreased posterior lingual movement on liquid consistency, and on the pharyngeal phase the delay in the posterior lingual elevation led to a delay in triggering the swallow reflex. Thus, swallowing therapy was continued. At eight months, an ophthalmic exam revealed poor eye contact and cortical visual impairment. At 12 months old, his HC has reached 39cm with a height of 81cm and weight of 11kg. He was given Rivotril 2.5mg/ml one drop/day to treat his repeated and prolonged infantile spasms without any recovery between attacks accompanied with hydrocortisone 10mg.

The proband has severe motor and developmental delay as he cannot crawl, nor roll over, nor pull himself up to an upright position, neither could stand unsupported despite being 18 months old. Also, his mouth was always open with drooling and has poor eye contact and weak interactions with his surroundings. He has a disturbed sleeping pattern, with difficulties falling asleep as well as waking up in the middle of the night crying nonstop for no apparent reason. His family history is positive for developmental delay: his maternal aunt was severely developmentally delayed. Only his parents and his maternal aunt could be examined. Other family members were not accessible. Clinical findings are summarized in table 4.1.

Table 4.1 The clinical findings of the 16p11.2 microdeletion carriers.

Subject	Onset of Symptoms	Age Studied	EEG (if found)	Clinical Features
III-8	Since birth	2months	Hypsarrhythmia	Severe microcephaly HC of 31cm at birth Pachygyria associated with ventriculomegaly Infantile spasms Hypsarrhythmia Poor eye contact and cortical visual impairment At 12 months, HC of 39cm Severe motor and developmental delay Sleep disorders
II-8	Approximately 7 years	25 years	Normal	Tremor attacks No history of seizures / Aggression and behavioral problems Poor articulation of words Mild learning difficulties No autistic features
II-11	Since birth	21 years	Unknown	Severe delay in motor function, speech and cognitive function Severe microcephaly /Seizures Significant learning difficulty Autistic behaviors Sleep disorders Serious behavioral problems Poor expressive verbal skills with stuttering/ Echolalia (repetition of heard speech) At 21 years: short stature (1.36 m), severe microcephaly (HC was 52cm), weight of 65 kg (BMI of 35.1 overweight). Mild dysmorphic features Hyperphagia and addiction to soda / Impairment in social interaction and communication Repetitive motor mannerisms Severely handicapped

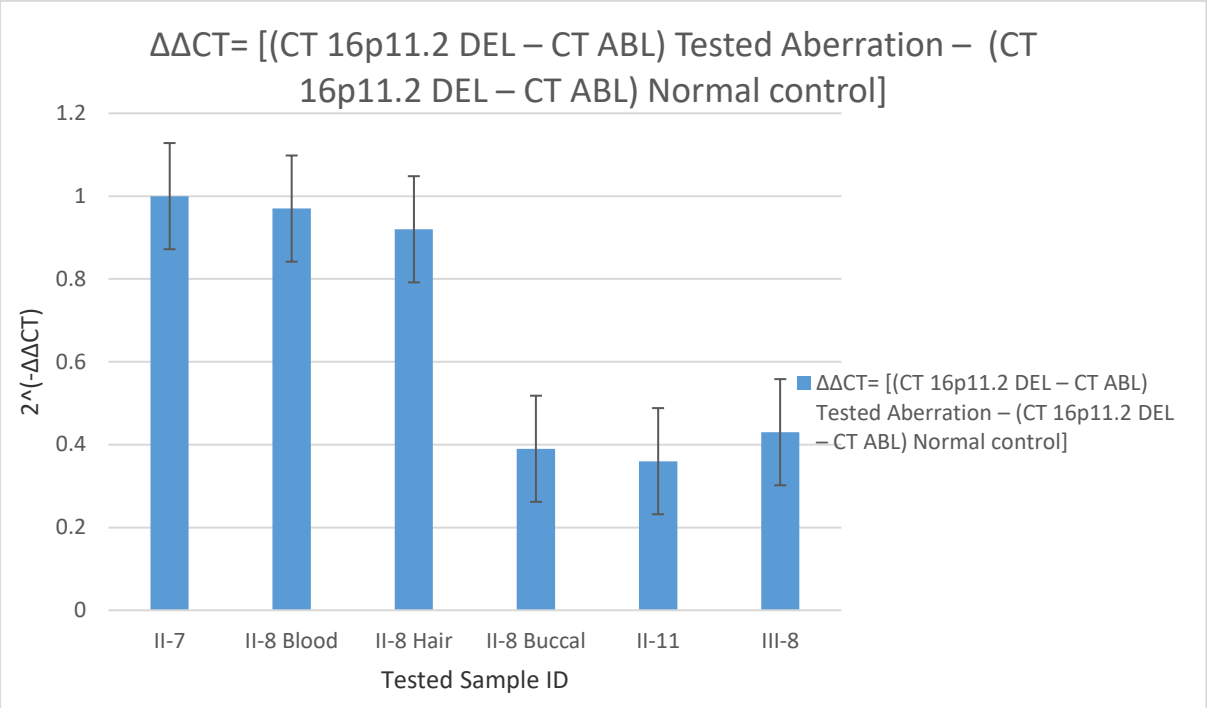

Figure 4.1 Bar chart displaying tested subject IDs on the x-axis, and the fold change in copy number calculated using the formula $2^{(-\Delta\Delta Ct)}$ on the y-axis. Where ΔΔCT= [(CT 16p11.2 Deletion (DEL) – CT ABL) Tested Aberration – (CT 16p11.2 DEL – CT ABL) Normal control]. Black lines represent error bars.

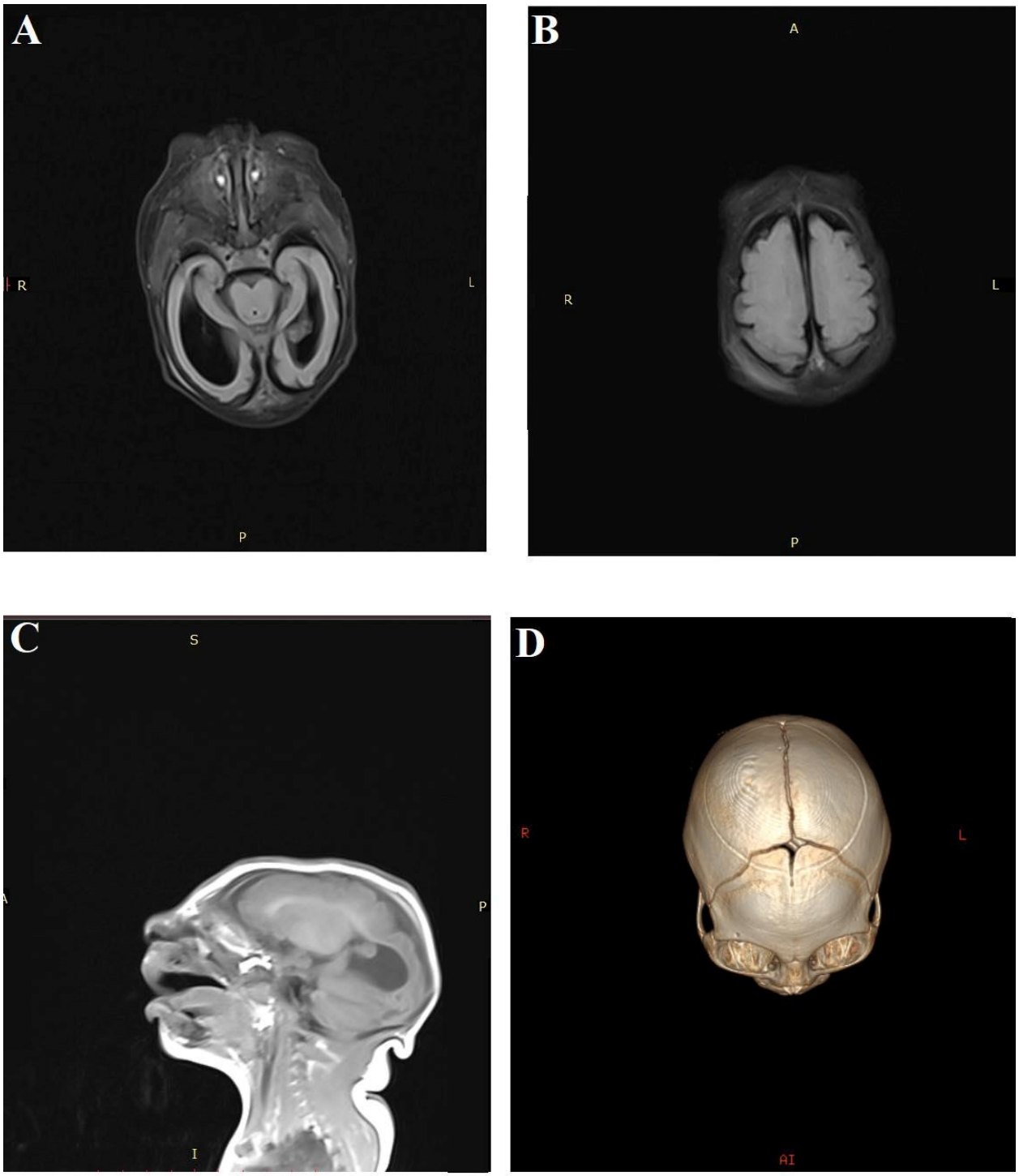

Figure 4.2 Brain MRI images of individual III-8 suggestive of ventriculomegaly and pachygyria where there are few gyri (the ridges between the wrinkles in the brain) (A), (B) Transverse sections T2, (C) sagittal sections T2 and (D) CT scan revealing the absence of crainosyntosis.

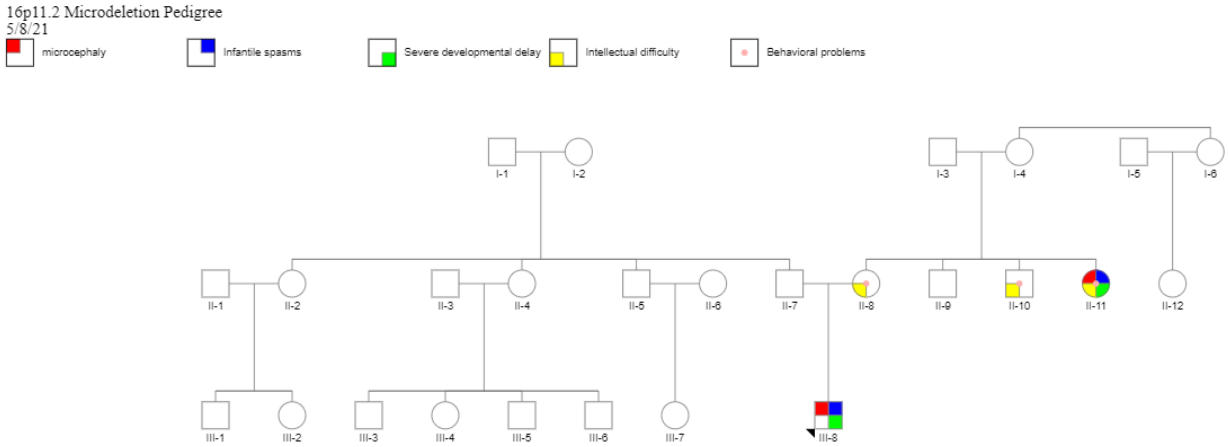

Figure 4.3 The pedigree of the patients with 16p11.2 Microdeletion.

4.2 Identification of Deletion by WES and qPCR validation

Affected Individual III-8 underwent whole-exome sequencing. Variants were filtered by selecting nonsense, splice-site, coding indel variants and nonsynymous variants with PolyPhen2 > 0.5 / unknown and with a minor allele frequency of less than 0.001% in ExAC browser. However, no pathogenic variants have been detected. Thus, according to the filtering criteria, copy number variation calling from raw exome data using XHMM revealed a heterozygous deletion of 737,067bp corresponding to the following chromosomal coordinates (chr16: 29469235- 30206302) as shown in (table 4.2). The heterozygous deletion was absent in Palestinian in-house exome data base, comprising 1000 Palestinians constructed in Hereditary Research Laboratory, Bethlehem University. The identified deletion was confirmed using quantitative polymerase chain reaction (qPCR) using two primer pairs which were designed inside the identified 16p11.2 region which corresponds with the typical recurrent 16p11.2 BP4-BP5 deleted region which extends at genomic location from 29.5 to 30.1 Mb on short arm of chromosome 16 (hg19). The range of the deletion in patient III-8 demonstrated a 0.7Mb deletion in chromosome 16 [16p11.2 (29469235-30206302) × 1] (Table 4.2), which encompassed 37 genes and transcripts, including five Mendelian disease genes (*PRRT2, KIF22, ALDOA, TBX6, and CORO1A*).

In qPCR assay a positive reaction is detected by accumulation of a fluorescent signal. The Cycle Threshold (Ct) is defined as the number of cycle needed for the fluorescent signal to cross the threshold (exceeding the background level). As for calculating the average cycle threshold (Ct) values of the triplicate reactions obtained for the deleted region in 16p11.2 and

the average of the triplicate Ct measurements of the internal control gene *ABL*; the relative fold change in copy number was calculated using the formula $2^{-\Delta\Delta Ct}$ / where ΔΔCt = (average Ct value for the tested aberration − average Ct value for *ABL* control) for the tested individual − (average Ct value for the tested aberration − average Ct value for *ABL* control) for a normal individual, the calculated CT values of the tested 16p11.2 deletion versus *ABL* internal control and fold change in copy number using the $2^{\wedge-\Delta\Delta Ct}$ formula are shown in table 4.3. Based on the $2^{-\Delta\Delta Ct}$ results (Table 4.3), a graph showing the relative fold change in copy number in all tested family members was constructed (Figure 4.1). The amplification plot presented in (Figure 4.4) of a normal versus a heterozygous individual shows fluorescence emission of the triplicate reactions (16p11.2 deleted region vs *ABL* internal control) and is proportional to the synthesized DNA.

Table 4.2 Genomic location of the ~737,067bp deletion encompassed 37 genes and transcripts, including five Mendelian disease genes (PRRT2, KIF22, ALDOA, TBX6, and CORO1A).

Chr	***Cytoband***	***State***	***Start***	***End***	***Size (bp)***	***Genes***
16	p11.2	Loss	29,469,235	30,206,302	737,067	*PRRT2, KIF22, ALDOA, TBX6, and CORO1A*

Table 4.3 CT values of the tested 16p11.2 deletion versus *ABL* internal control and fold change in copy number calculations were performed using the $2^{-\Delta\Delta Ct}$ formula.

Detector	**Reporter**		**Detector**	**Reporter**		
***16p11.2* deletion SYBR**	**SYBR Green**		***ABL* SYBR**	**SYBR Green**		**Fold change**
Sample Name	**Ct Value**	**Average Ct (16p11.2 deletion)**	**Ct Value**	**Average Ct (*ABL*)**	**ΔΔCt**	**2^(-ΔΔCt)**
II-7	18.81	18.39	18.00	17.93	0.00	1.00
	18.32		17.86			
	18.04		17.93			
II-8 Blood Sample	19.52	19.24	18.94	18.74	0.04	0.97
	19.13		18.73			
	19.06		18.55			
II- 8 Hair Sample	18.44	18.4	18.05	17.82	0.12	0.92
	18.33		17.82			
	18.42		17.58			
II-8 Buccal cells	23.05	23.13	21.24	21.31	1.36	0.39
	23.18		21.39			
	23.08		21.16			
	23.15		21.33			
	23.17		21.41			
	23.13		21.35			
II- 11	21.24	21.21	19.25	19.28	1.47	0.36
	21.17		19.39			
	21.22		19.21			
III-8 Proband	20.37	20.27	18.56	18.6	1.21	0.43
	20.17		18.79			
	20.28		18.44			

4.3 Real- Time PCR Validation and Family Segregation

Family segregation analysis was performed on all available family members (III-8, II-7, II-8 blood sample, II-8 Hair sample, II-8 Buccal mucosal cells swab, II-11) using SYBR Green quantitative polymerase chain reaction (qPCR) on an ABI 7300 real-time PCR system (Applied Biosystems). Upon performing qPCR to see if the 16p11.2 microdeletion is segregating within the family; 16p11.2 microdeletion has tested positive in the following patients: patient III-8, patient II-8 Buccal mucosal swab sample and patient II- 11 (Figure 4.1). qPCR analysis revealed the familial transmission of the 16p11.2 microdeletion by confirming that the deletion in the proband III- 8 was transmitted through maternal germline mosaicism, as both the II-8 hair and blood samples were negative for the detected 16p11.2 microdeletion, unlike II- 8 buccal mucosal cells' swab which was positive for the recurrent deletion. Hence, confirming the clear-cut manifestation of the mosaic 16p11.2 microdeletion pattern. Also, qPCR analysis has approved the affected state of the micro-cephalic maternal aunt II- 11 and the proband III-8, thus a pedigree was drawn in Figure 4.3. Other maternal family members were not available for further segregation analysis.

Within this study, it was confirmed that patients carrying the 16p11.2 BP4-BP5 deletions have a wide range of clinical features, and even siblings (patient II-8 and patient II-11) carrying the same microdeletion displayed two unique carrier status: one (II-11) being an affected heterozygous carrier for the 16p11.2 microdeletion while the other (II-8) being a mosaic carrier for the 16p11.2 microdeletion and both have diverse symptoms. Thus, this study has demonstrated an extreme example of a two generation family with three affected members manifesting wide range of heterogeneous phenotypes.

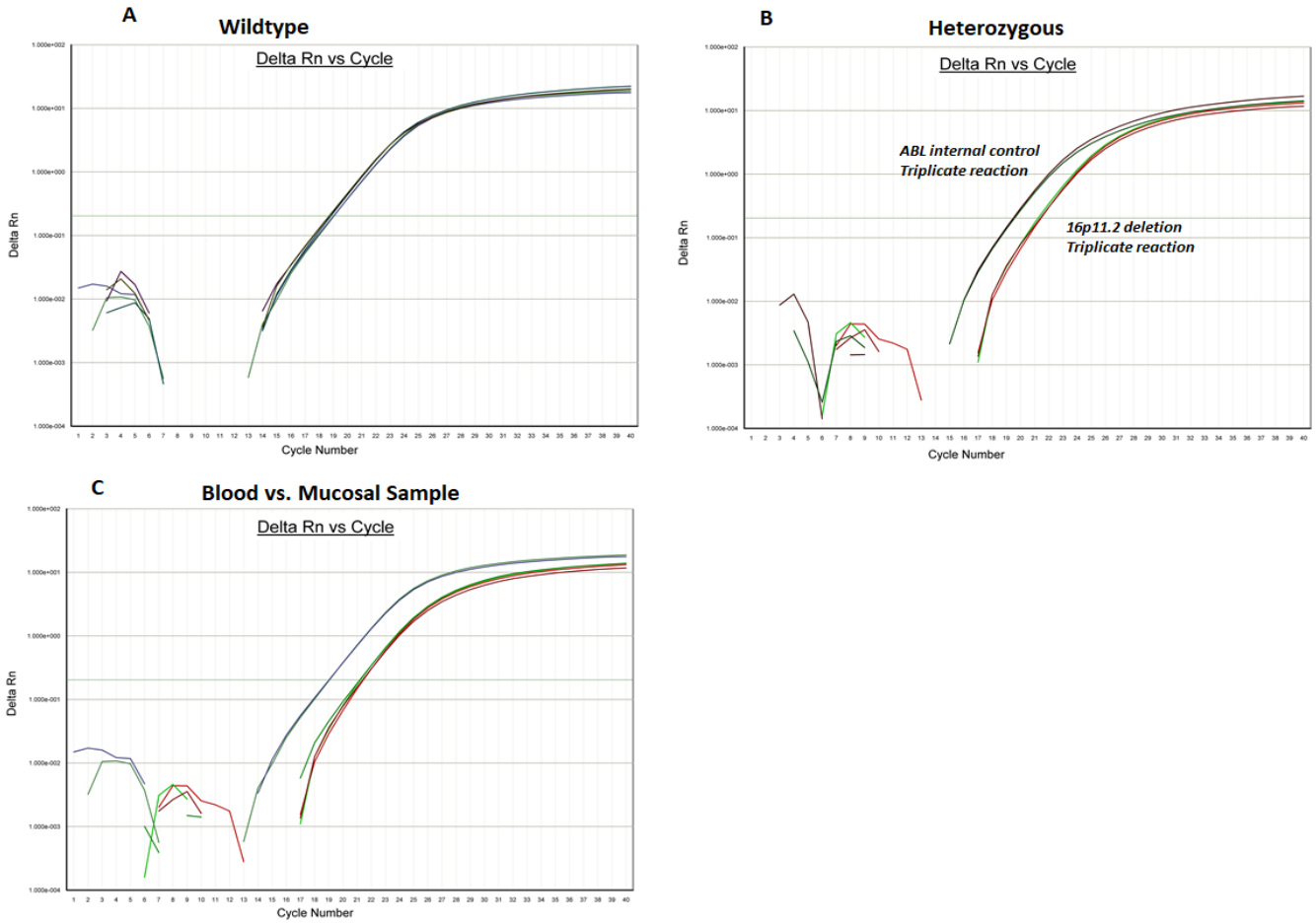

Figure 4.4 Amplification plot of the qPCR reaction displaying cycle number on the x-axis and "Delta Rn" value (normalized reporter fluorescent signal) on the y-axis. Amplification plot of the triplicate reactions (tested *16p11.2* deletion and the *ABL* reference gene) of a (A) wildtype individuals patient II-7 and the blood & hair samples of patient II-8 versus a (B) heterozygous individual who is affected and a carrier of 16p11.2 deletion such as patient II-11, patient III-8 and the buccal mucosal swab of patient II-8 (C) an amplification plot of blood sample versus buccal mucosal cells swab of patient II-8 as a confirmation of the presence of clear-cut somatic mosaicism of 16p11.2 deletion.

4.4 Genomic Region of 16p11.2 BP4-BP5 and Associated Genes

The deletion range of patient III-8 with 16p11.2 microdeletion is shown in Figure 4.5. The range of the deletion in patient III-8 covered a large region of 737,067bp corresponding to the following chromosomal coordinates (chr16: 29469235- 30206302). This range includes 32 protein- coding genes, seven RNA genes, and four pseudogenes.

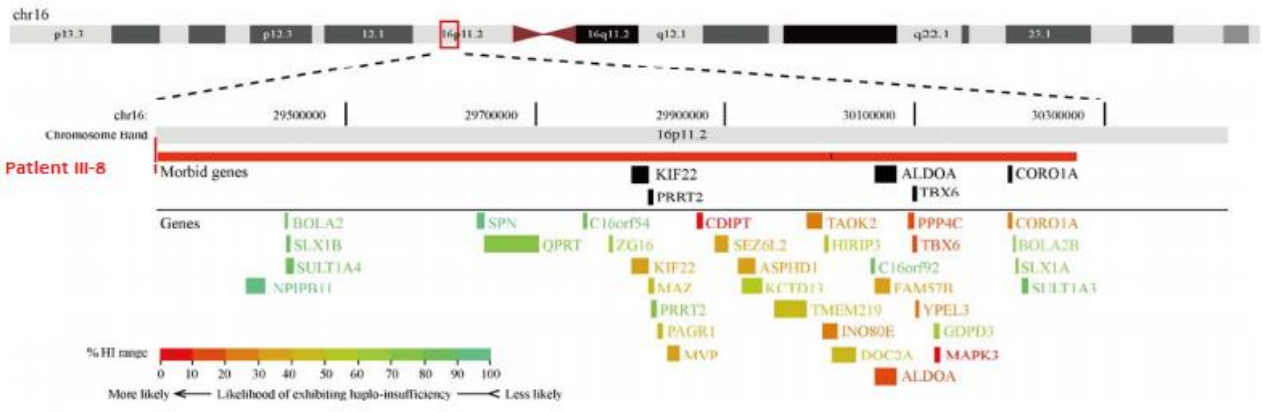

Figure 4.5 Genomic positions of the 16p11.2 BP4-BP5 deletion of the patient III-8 are marked in red bar. The involved genes are produced using the Ensemble Genome Browser (https://grch37.ensembl.org/Homo_sapiens/Location) and listed below the bars. The morbid genes are indexed using black fonts. The haploinsufficiency scores of the involved genes are produced using the DECIPHER database (https://decipher.sanger.ac.uk/) and indexed by colored fonts.

Chapter 5

Discussion

Within the human genome chromosome 16 is considered one of the gene-rich chromosomes. The short arm (p) of chromosome 16 is rich in intrachromosomal segmental duplications which predispose this region of 16p11.2 to rearrangements caused by NAHR which leads to instability of the genomic architecture. Thus, rendering the 16p11.2 region into a hotspot for genomic disorders since it is flanked by 147Kb duplicated blocks of LCRs at its ends.

The main purpose of this project was to identify the underlying molecular cause of Palestinian individuals with various combinations of: severe microcephaly, severe developmental delays, language impairment disorders, behavioral disorders and seizures. Those patients have manifested heterogeneous phenotypes even among siblings (patients II-8, II-11) despite carrying the same recurrent 16p11.2 microdeletion.

After the analysis of the WES, the causative molecular cause behind the manifested phenotypes was 16p11.2 deletion syndrome as the deletion was a 737,067bp in chromosome 16 [16p11.2 (29469235-30206302) × 1] which segregated within the familial pedigree as confirmed by the qPCR analysis. The heterozygous deletion was absent in Palestinian in-house Exome data base, comprising 1000 Palestinians. The typical recurrent 16p11.2 deletion and duplications are usually 600kb occurring at BP4- BP5 region. While in proband III -8 the size of the 16p11.2 deletion was approximately 737kb, which was overlapping with the frequently reported 600Kb 16p11.2 BP4-BP5 CNV deleted region.

Confirmation of the detection of 16p11.2 deletion region was performed by quantitative polymerase chain reaction (qPCR) analysis in this research study, while in other studies they confirm the microdeletion's presence by either using qPCR or multiplex ligation-dependent probe amplification, or array analysis (Kaminsky *et al.* 2011). qPCR analysis was performed firstly, to confirm the presence of 16p11.2 microdeletion and secondly to reveal the mode of inheritance of this CNV. Upon analyzing blood samples, both parents tested negative, however the maternal aunt patient II-11 manifested typical phenotypes of the recurrent 16p11.2 deletion carriers. As a result, even if the deletion is not detected in either parent, then there is chance of transmission due to presumed maternal germline mosaicism (Dehainault *et al.* 2016). Therefore, hair and buccal mucosal cells' swab were collected from both parents. Analysis of the 16p11.2 region using hair and buccal mucosal swabs of both parents by qPCR revealed that the father is negative while the mother II-8 carried the same microdeletion only within her buccal mucosal cell line (7 out of the

9 replicates have revealed the 16p11.2 microdeletion- data not shown) while her blood and hair samples were negative for the microdeletion revealing a somatic mosaicism form of the deletion. The 16p11.2 microdeletion was also confirmed in the blood of the maternal aunt II- 11 which is overlapping the same microdeletion detected in the proband II-8. The segregation in family reveals that the deletion was inherited as autosomal dominant trait, due to the presence of a presumed germline mosaicism. Due to the inaccessibility of female germ cells, the molecular analysis in such cases remains incomplete. However, since the deletion was transmitted to an offspring proband III-8 confirms a clear-cut presence of germline mosaicism. Moreover, manifesting that her sister patient II-11 is a carrier of the 16p11.2 microdeletion further confirms that the deletion was transmitted and not a de novo case. Therefore, those results confirm the presence of both somatic and germline mosaicism within individual II-8. Several studies have detected mosaic forms of the 16p11.2 microdeletion by the use of several methods such as Array comparative genomic hybridization aCGH, Chromosomal Microarray Analysis CMA and the Fluorescence in situ hybridization FISH analysis (Bijlsma *et al.* 2009; Fernandez *et al*. 2010; Iossifov *et al.* 2012; Kumar *et al.* 2008; Shinawi *et al.* 2010; Steinman *et al.* 2016; Weiss *et al.* 2008; Zufferey *et al.* 2012). This research was the first of its kind to detect a mosaic status of this 16p11.2 deletion by the use of qPCR. The deleterious impact of the deletion is further highlighted by its low fitness reflected by the rarity of multigenerational carrier families, which reveals why most cases of 16p11.2 recurrent deletion occurs de novo. Our study is the first to detect a Palestinian family carrying this 16p11.2 deletion and one of the first multigenerational families with unique inheritance pattern of this deletion.

The pathogenesis of the 16p11.2 microdeletion is unquestionable, since there are various reported cases of 16p11.2 carriers manifesting variable clinical phenotypes and heterogeneity in this genomic disorder (Bijlsma *et al.* 2009; Ciuladaite *et al.* 2011; Ghebranious *et al.* 2007; Glessner *et al.* 2009; Li *et al.* 2018; Shen *et al.* 2010). This research has revealed the wide diversity in clinical phenotypes and complex heterogeneity of the recurrent microdeletion at 16p11.2 region within the same family of two generations as has been demonstrated by several studies which had shown that the clinical variability is detected in familial cases and unrelated patients alike. As it has been demonstrated that within this research the 16p11.2 microdeletion was presented with learning difficulties, language impairment, obesity, motor and developmental delay, behavioral problems, microcephaly, sleep disorders, and seizures which has been described by several studies

(Hernando *et al.* 2002; Ghebranious *et al.* 2007; Bijlsma *et al.* 2009; Shinawi *et al.* 2010; Shiow *et al.* 2009; Schaaf *et al.* 2011; Shen *et al.* 2010).

The phenotype of each member was ranging from seemingly "asymptomatic" in individual II-8 reaching to the bizarre phenotype noticed in individuals' III-8, II-11 which both of them revealed signs of microcephaly. Despite there is not any constant pattern of phenotypes detected in 16p11.2 microdeletion carriers, but macrocephaly is the most common feature in deletion carriers (Steinman *et al.* 2016). Moreover, as revealed by Steinman *et al.* 2016 only 2% of 16p11.2 deletion carriers are microcephalic just like individual II-11 and III- 8, while most of the reported 16p11.2 microdeletions carriers are macrocephalic (36%) (Steinman *et al.* 2016). Furthermore, MRI of patient III-8 has revealed the presence of pachygyria formation ahead with moderate atrophy of the brain associated with white matter and/or corpus callosum abnormalities and ventricular enlargement those bizarre findings are normally associated with 16p11.2 microduplication rather than the microdeletion carriers. The presence of those abnormalities means that 16p11.2 deletion carriers will have more difficulties in communication, social life, reduced verbal IQ and in general a worse life than microdeletion or microduplication carriers without any structural brain aberrations (Li *et al.* 2013; Hinkley *et al.* 2019, Owen *et al.* 2018).

According to literature, 20% of 16p11.2 deletion carriers are diagnosed with ASD. However, some 16p11.2 deletion individuals do not meet the full criteria of ASD, but they reveal significant rate of autistic features such as insistence on sameness, restricted range of interest, stereotyped/repetitive behavior manners, social and behavioral difficulties in contrast with other family members and those signs are detected in both individual II-11 and her father I-3 (Bijlsma *et al.* 2009; Glessner *et al.* 2009; Hanson *et al.* 2015; Zufferey *et al.* 2012). Which further confirms the association of 16p11.2 locus with these phenotypes is that in mice models when the homologous 16p11.2 region was deleted, the mice exhibited symptoms of repetitive behaviors, hyperactivity, reduced weight and recognition memory insufficiencies (Arbogast *et al.* 2016). Moreover, it was reported that de novo deletion carrier had higher IQ value than familial deletion carriers (Zufferey *et al.* 2012) as it was demonstrated that patient II-11 suffers from intellectual difficulties in contrast with control members (individual II-9). Furthermore, patient II-8 have normal IQ compared to her sister individual II-11, which is due to the fact that mosaic carriers of genomic disorders inclined to have higher IQs than those with constitutional ones (D'Gama *et al.* 2018). In addition, it was noticed that within this family the eldest sister II-8 had less severe form of the disease compared with her younger sister II-11, which is due to the difference in the genetic

background of each individual in the same family as patient II-8 has a mosaic pattern of the 16p11.2 microdeletion unlike her sister II-11 who is a heterozygous carrier of the disorder.

Another characteristic that patient II-11 had manifested is her short stature as a result of the microdeletion. As it was demonstrated that some individuals with the microdeletion show short stature compared to the general population as the recurrent microdeletion reduces final adult height by 1 Standard Deviation (SD) (Bijlsma *et al.* 2008; Ghebranious *et al.* 2007; Shen *et al.* 2010; Weiss *et al.* 2008). Regarding the behavioral difficulties detected in microdeletion carriers, several studies have displayed that majority of 16p11.2 microdeletion carriers develop several behavioral disorders including aggression, overactivity and ADHD and those symptoms are demonstrated within patients II-8 and II-11 (Bijlsma *et al.* 2009; Christian *et al.* 2008; Fernandez *et al.* 2009; Kumar *et al.* 2009). It was reported that speech and language impairments may be the cause to behavioral problems (Shinawi *et al.* 2010).

The most common clinical characteristics for 16p11.2 deletion according to previous studies are language delays, phonologic processing disorders, expressive language disorders and cognitive impairment (Bijlsma *et al.* 2009; Fernandez *et al.* 2010; Ghebranious *et al.* 2007; Kumar *et al.* 2008; Marshall *et al.* 2008; Steinman *et al.* 2016; Weiss *et al.* 2008; Zufferey *et al.* 2012). In agreement with these studies, speech delay was found in the mother II-8 and the maternal aunt II-11 who did not say ‘‘Mama’’ and ‘‘Dada’’ until 5 years of age where she was able to speak a few single words, with poor articulation and prolonged repetitiveness. Additional symtomps that individual II-8 suffered from are tremors that are commonly not noted until school age or later (Steinman *et al.* 2016) which manifested itself in individual II-8 after marriage, these symptoms are normally demonstrated in microduplication carriers.

Also, various studies have revealed that carrying 16p11.2 microdeletion predispose its carrier to higher risk of obesity in comparison to the general population (Bijlsma *et al.* 2009; Bochukova *et al.* 2010; Fernandez *et al.* 2010; Ghebranious *et al.* 2007; Maillard *et al.* 2015; Shinawi *et al.* 2010; Tabet *et al.* 2012; Walters *et al.* 2010). Most of these studies have shown that once 16p11.2 microdeletion carriers reach seven years of age, they gain a lot of weight and become severely obese which is the same case that occurred in the maternal aunt II-11 who has gained a lot of weight and became severely obese with a BMI of 35.1 (considered as overweight) (Yu *et al.* 2011). According to another study by Yu *et al.* 2011 has revealed that deletion carriers -such as the case of the proband III-8 (no information known about maternal aunt II-11) - manifest early feeding difficulties due to poor coordination between sucking and swallowing (Kumar *et al.* 2009).

Moreover, it is expected that male 16p11.2 deletion carriers have more severe obesity than their fellow female carriers which reveal that proband III-8 will be more severely obese than his maternal aunt (individual II-11). Furthermore, the change in satiety is detected in pediatric 16p11.2 CNV carriers before the appearance of obesity, since response to satiety depends upon gene dosage. Therefore, the morbid obesity and hyperphagia observed in maternal aunt II-11 shall be different than the obesity that will manifest itself in her nephew patient III-8 later in life, as it was reported that obesity is an early-onset revealing an age and gender-dependent trait in 16p11.2 microdeletion carriers (Bijlsma *et al.* 2009; Fernandez *et al.* 2010; Ghebranious *et al*. 2007; Kumar *et al.* 2009; Maillard *et al.* 2015; Shinawi *et al.* 2010; Walters *et al.* 2010; Yu *et al.* 2011). In addition, it was demonstrated that patient II-8 suffered from anxiety disorders which was detected in various studies (Fernandez *et al.* 2009; Hanson *et al.* 2015; Kumar *et al.* 2008; Niarchou *et al.* 2019; Zufferey *et al.* 2012). Furthermore, both maternal aunt II-11 and her nephew III-8 have manifested sleep disorders as have been reported by several documented cases (Bijlsma *et al.* 2009; Fernandez *et al.* 2009; Horev *et al.* 2011).

Moreover, patient III-8 who inherited the deletion maternally has a more severe clinical manifestation than the maternal aunt II-11. However, his clinical phenotype is quite diverse that his aunt II-11, despite that both of them carry the same genotype. Maybe first of all the reason behind the complexity in the manifestation of phenotypes and heterogeneity is due to different breakpoints occurring at the deletion site in each 16p11.2 microdeletion carrier. Or it could be due to the existence of a mosaicism form which leads to the manifestation of wide range of diverse phenotypes in first generation carriers (Glessner *et al.* 2009). In addition, according to Eichler *et al.* 2008 the wide diversity of phenotypes detected in 16p11.2 rearrangements is dependent upon the genetic background of the short arm (p) of chromosome 16 which is unique by the crowded duplicated segments that evolved within it in less than 15 million years ago. Moreover, chromosome 16 carries multiples copies of one of the fastest evolving gene families within the human species which further adds to the heterogeneity of phenotypes manifested in each microdeletion carrier. Furthermore, the difference in the genetic background of individuals leads to the manifestation of a wide range of phenotypes which could extend from severely affected carrier to seemingly normal phenotype (Eichler *et al.* 2008; Niarchou *et al.* 2019; Shen *et al.* 2010). However, it has been displayed that the existence of 'asymptomatic' normal 16p11.2 microdeletion carriers is uncommon, as this genomic region is conserved and unique for the human species, indicating that the seemingly 'normal' deletion carriers haven't been correctly assessed

which is the case of the mother II-8 who was presumed to be apparently "normal" but in fact she is affected due to carrying the 16p11.2 microdeletion in its mosaic form (Zufferey *et al.* 2012). Furthermore, another reason why there are a wide range of phenotypes may be due to the mode of inheritance of this genomic disorder which does not follow the Mendelian rules of inheritance, revealing a complexity of transmission of this autosomal dominant disorder, as it could be transmitted (from a carrier of the 16p11.2 microdeletion, or from a mosaic "asymptomatic" parent) or it could occur de novo. As it was revealed that both patients maternal aunt II- 11 and proband III- 8 manifest the hereozygous affected form of the 16p11.2 rearrangement within their analyzed blood, unlike the third patient the mother II- 8 who revealed a mosaic state of the deletion indicating the presence of both somatic and germline mosaicism as it was confirmed that somatic and germ-line mosaicism can coexist in the same individual (Youssoufian *et al.* 2002).

Despite the fact that there are a large range of phenotypes. However, the majority of these abnormalities are infrequent and unique, which is documented through the wide range of different phenotypes manifested in individuals II-8, II-11 and III-8. In summary, 16p11.2 microdeletion carriers exhibit a variety of neurologic abnormalities, some shared and some unique.

Various studies have revealed that structural brain abnormalities (Owen *et al.* 2018) detected by pathological and imaging studies (Hinkley *et al.* 2019) ahead with the abnormal neural connectivity (Li *et al.* 2013) trigger the manifestation of 16p11.2 microdeletion's phenotypes. Thus, it is hypothesized that the deletion directly affects the neural polarity involved in all phenotypes, including energy balance. Neuronal polarity is known as the highly polarized neuronal cells which consists of two distinct compartments, single axon and multiple dendrites, which is crucial for the structure and function of neurons. Axons release neurotransmitters from their synaptic vesicles in response to electrical signals release from the cell body, which are bound to the receptors of the dendritic spines. It is possible that neurological phenotypes are related to haploinsufficiency of distinct genes which control brain development and function (neural polarity). It was revealed that manifestation of a specific phenotype relies upon the change in gene dosage at the 16p11.2 BP4-BP5 CNVs region (Steinman *et al.* 2016). As demonstration of overlapping phenotypes are suggested to result from "failure of homeostatic regulation of synaptic function." Since as Zoghbi and Bear [2012] proposed that "optimal synaptic function occurs within a limited dynamic range".

It has been demonstrated that 22 genes with the 16p11.2 deleted region are expressed in the developing human fetal nervous system (Ciuladaite *et al.* 2011; Li *et al.* 2013). Moreover, within

the 16p11.2 locus there is a high expression of brain- specific genes which are crucial for nervous system development (Blaker- Lee *et al.* 2012; Tabet *et al.* 2012). Furthermore, several studies have classified the region of 16p11. 2 as a novel gene domain which is tightly associated with intellectual disability, neural developmental disorders and abnormal brain function (Arbogast *et al.* 2016; Blaker- Lee *et al.* 2012; Li *et al.* 2013; Weiss *et al.* 2008).

Several studies have displayed that there is a strong association between several candidate genes (*Kif22, Aldoa, SEZ6L, QPRT, PRRT2, KCTD13, MAPK3, DOC2A MAZ and TBX*) and neurodevelopmental/seizure disorders demonstrated in 16p11.2 microdeletion carriers. *Kif22* gene has not been related to brain function disorders, but it was reported that heterozygous mutation of *Kif22* has been associated with epilepsy, and patient III-8 suffers from infantile spasms (WEST syndrome) which are a rare type of epilepsy in children which is confirmed by the detection of hypsarrythmatic pattern in his EEG test (Figure 8.1 in appendix) (Blaker – Lee *et al.* 2012). Moreover, *Kif22* has a role in the transport of synaptic vesicles to the axon and of transmitter receptors to the dendrite (Li *et al.* 2013). Further, confirming its essential role in the establishment of neuronal polarity. Another candidate gene is the *Aldoa* gene which its reduced expression has been accompanied with mental retardation, microcephaly (patients III-8 and II-11) and language delay (patients II-8 and II-11) (Blaker- Lee *et al.* 2012). On the other hand, its overexpression has been detected in schizophrenic and depressed patients (patient II-8), associating this gene with neuropsychiatric disorders. While another reported gene is *SEZ6L2* that has high specific expression within the central nervous system, and it has been associated with neurodevelopmental disorders such as seizures (patient III-8), autism and motor coordination delay (patients II-11 and III-8) (Ciuladaite *et al.* 2011; Crepel *et al.* 2011; Kumar *et al.* 2009; Miyazaki *et al.* 2006; Shinawi *et al.* 2010).

Inside the 16p11.2 BP4-BP5 region, there is a reported smaller deletion of around 118kb that encompasses the following genes: *MVP, CDIPT, SEZ6L2, ASPHD1, and KCTD13* (Golzio *et al.* 2012; Zufferey *et al.* 2012) which identified the *KCTD13* gene as a key element behind the neurodevelopmental aberrations of the 16p11.2 micodeletion (Golzio *et al.* 2012). As it was revealed that overexpression of *KCTD13* in embryos of zebrafish models has led to microcephaly (patient II-11 and III-8) and significant increase in apoptosis of the developing brain and a decrease in proliferation of neuronal progenitor cells, while on the other hand knockdown of *KCTD13* gene at the same locus induced macrocephaly and obvious increase in proliferative cells with no change in apoptosis, revealing the same mirror phenotype of different head size detected

in human 16p11.2 CNV losses and gains (Crespi *et al.* 2009), such critical changes in head circumference are due to changes in the numbers of mature neurons since *KCTD13* has a role in neurogenesis (Golzio *et al.* 2012; Steinman *et al.* 2016).

Another suspected gene is the *MAPK3* which phosphorylates a number of transcription factors which recruits specific genes crucial for synaptic remodeling, maturation and synaptic plasticity during early periods of brain development (Ciuladaite *et al.* 2011; Kumar *et al.* 2009). Presynaptic NMDA receptors have a key role in excitatory neurotransmission and synaptic plasticity. Moreover, NMDA receptors were revealed to be involved in long term potentiation (LTP), which is vital for learning and memory and it has been detected to be reduced in *MAPK3* deficient mice displaying hyperactivity, abnormal avoidance behaviors, low LTP, difficulty adapting to change and sleeping abnormalities further explaining why both patient III-8 and his maternal aunt II-11 suffered from sleep disorders and have learning and memory impairment disorders (Barnby *et al.* 2005; Horev *et al.* 2011; Mazzucchelli *et al.* 2002; Shinawi *et al.* 2010). MAPK3 has been associated with learning difficulties found in 16p11.2 micodeletion carriers (patients II-8, II-11) as the *MAPK* pathway is a key regulator of neural progenitor biogenesis, learning and memory, which was confirmed by other studies where an upregulation of *mTOR* and *MAPK* pathways was detected in post-mortem brains of mild and severe idiopathic ASD patients (Blaker- Lee *et al.* 2012; Rosina *et al.* 2019).

Other candidate gene is *Doc2a* gene which is one of the post-synaptic density genes that are uniquely expressed in brain and neuronal cells, located at synaptic vesicles as a calcium sensor involved in neurotransmission release (Arbogast *et al.* 2016; Blaker- Lee *et al.* 2012; Kumar *et al.* 2009; Li *et al.* 2013; Mazzucchelli *et al.* 2002; Marshall *et al.* 2008; Shinawi *et al.* 2010). It has been shown that *Doc2a* has a role in controlling neural polarity “structure” and synaptic transmission “function” to ease the informational flow by regulating synaptic release when calcium concentration is high in presynaptic terminal. Thus, *Doc2a* regulates the neurotransmission during repetitive synaptic activation which is critical for learning and memory formation. It has been demonstrated that homologs of 16p11.2 micodeletion region in *Doc2a* heterozygous mutant mice and zebrafish models developed defects in excitatory synaptic transmission, impairing long-term potentiation, hyperactivity, seizures, displaying learning and behavioral issues and those phenotypes are manifested in 16p11.2 micodeletion carriers (patients II-8, II-11 and III-8) (Arobogast *et al.* 2016; Kumar *et al.* 2009; Li *et al.* 2013; Mazzucchelli *et al.* 2002; McCammon *et al.* 2017; Sakaguchi *et al.* 1999).

Another gene associated with 16p11.2 micodeletion's phenotypes is *MAZ* gene encodes the regulator gene *MYC* which improves the activity of NMDA receptor subunit type 1 during neuronal differentiation and serotonin pathway which are key elements in long term potentiation which is critical for learning and memory, which further confirms the association of 16p11.2 BP4-BP5 CNVs with language impairment disorders detected in patient II-11 and II-8 (Barnby *et al.* 2005; Kumar *et al.* 2009; Shinawi *et al.* 2010).

Although the role of some genes have been identified to be associated with specific phenotypes, but the mechanism of each gene and its associate genes leading to neurodevelopmental abnormalities needs further studies (Arbogast *et al.* 2016; Kumar *et al.* 2009; Marshall *et al.* 2008; Weiss *et al.* 2008).

Detection of the 16p11.2 microdeletion provides families with information about the expected phenotypes and neurological problems that their offspring may manifest. Furthermore, it allows parents to contact other families with children carrying the same genotype as a sense of creating a community where they share some of the challenges, problems and struggles faced by the 16p11.2 microdeletion carriers. Moreover, prenatal diagnoses for each pregnancy is required once the 16p11.2 microdeletion is manifested within a family so as to prevent any recurrence of the disorder (Zufferey *et al.* 2012). Those results will give a chance for the family to arrange future marriages and to perform Preimplantation Genetic Diagnoses (PGD) and In-Vitro Fertilization (IVF) to enable them of having healthy offspring. However, due to the presence of wide diversity of phenotypes manifested within this monogenic disorder, genetic counseling may face a problem in interpreting the results of prenatal testing and accurately predicting the expected phenotype (Dehainault *et al.* 2016).

Some of the strengths of this study include the demonstration of a germline mosaicism as the culprit cause underlying the mode of transmission of such a monogenic disorder which occurs typically as de novo. Furthermore, it has been the first of its kind to detect a mosaic pattern of the disease by the use of qPCR analysis in a two generation family. However, there are still some limitations that should be acknowledged which are the small number of 16p11.2 microdeletion carriers within this study, also the variability in the historical information provided by the parents and grandparents, and the age of the subject in which he/she manifested a symptom (less medical information recalled or known about earlier life in older patients II-8 and II-11). Further drawback is that several individuals in the study have declined to perform any genetic tests for detection of the 16p11.2 microdeletion.

Chapter 6

Conclusion

It has been demonstrated through several prior studies ahead with this thesis study that 16p11.2 CNV rearrangements are associated with phenotypic heterogeneity among closely related individuals despite their acquisition of half of the same genetic and environmental background (Li *et al.* 2013; Shen et al. 2010). As it has been displayed there is a diverse variability in phenotypes manifested in the three deletion carriers in this Palestinian family which further confirms the phenotypic complexity and heterogeneity of 16p11.2 BP4-BP5 deletion disorder associated with copy number imbalances at this genomic locus. Furthermore, the mode of inheritance of this genomic disorder does not follow the Mendelian rules of inheritance, revealing a complexity of transmission of this autosomal dominant disorder, as this research was the first of its kind to detect a mosaic status of a transmitting parent carrying this 16p11.2 micodeletion by the use of qPCR analysis.

In this thesis, we report a two generation Palestinian family diagnosed with severe microcephaly, developmental delay, WEST syndrome and speech impairment. Molecular screening for a genetic cause underlying their phenotypic manifestation has required Whole Exome Sequencing and family segregation through qPCR analysis. The results revealed; the presence of a 0.7Mb microdeletion at 16p11.2 which is overlapping with the recurrent 16p11.2 BP4-BP5 microdeletion, further confirming this microdeletion as the culprit cause behind the manifested phenotypes. The absence of this deletion within the normal healthy Palestinians controls further confirm the pathogenicity of this 16p11.2 microdeletion. This study confirms previous observation that 16p11.2 micordeletion is associated with severe motor delay, speech impairment, obesity, short stature and infantile spasms. The 16p11.2 microdeletion was reported before, but the manifestation of both germline and somatic mosaicism observed in individual II-8 in a Palestinian family is the first of its kind to be known in West Bank, although it has reported in various studies across the globe (Bijlsma *et al.* 2009; Fernandez *et al.* 2010; Iossifov *et al.* 2012; Kumar *et al.* 2008; Shinawi *et al.* 2010; Steinman *et al.* 2016; Weiss *et al.* 2008; Zufferey *et al.* 2012). This research was the first of its kind to detect a mosaic status of this 16p11.2 microdeletion by the use of qPCR analysis. Further investigations should be performed to understand the variable phenotypic heterogeneity underlying this CNV disorder in Palestine to offer an appropriate genetic counselling and potential therapeutic interventions.

Chapter 7

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Appendices:

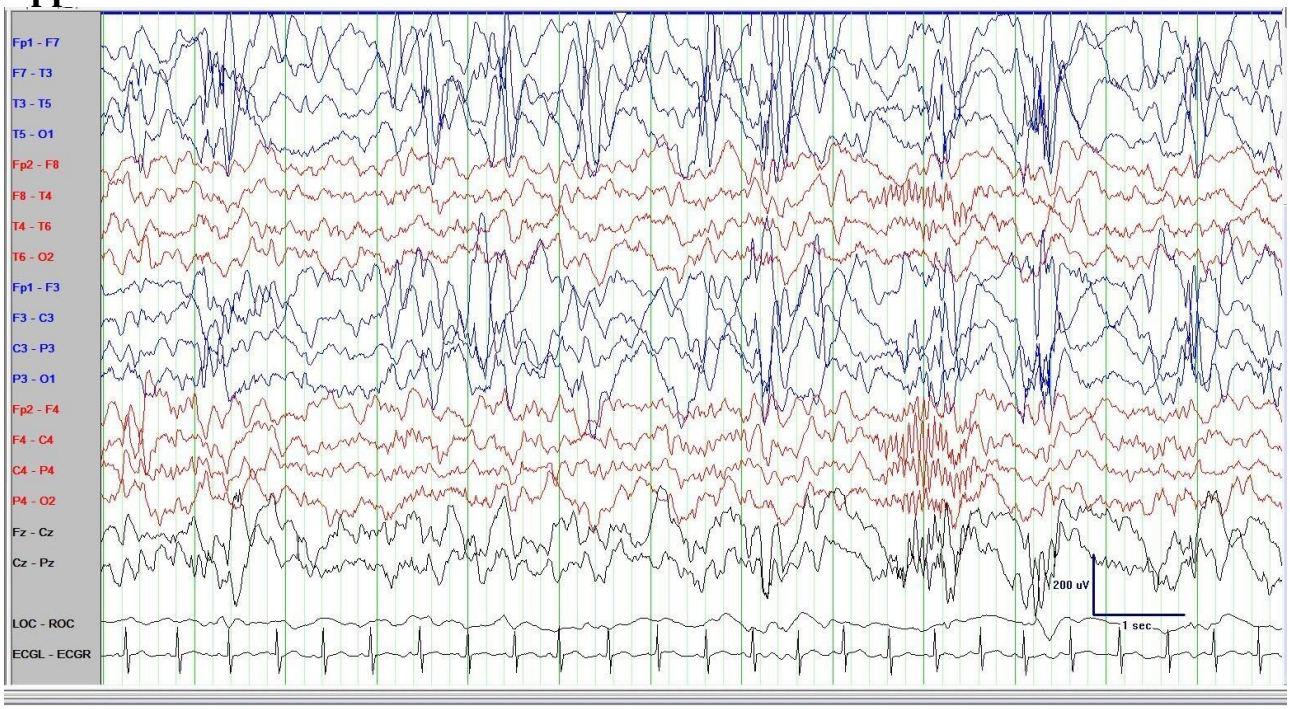

Figure 8.1 Infantile spasms (IS), also called West Syndrome, is a rare type of epilepsy in children. The seizures with this type of epilepsy (called "spasms") look like quick body jerks, sometimes with head drops or extended arms. These seizures are brief, but can occur in clusters. The figure reveals the EEG test of patient III-8. His electroencephalogram (EEG) test reveals an abnormal chaotic non-rhythmic brainwave pattern called hypsarrhythmia, obtaining this pattern confirms the diagnosis of infantile spasms. Hypsarrhymatic pattern reveals high amplitude waves and spikes which are randomly appearing and with no topographical distribution identified, also, there is no frequency nor amplitude gradient, indicating a highly disorganized brain activity.