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Identification of a Rare Splice Site Mutation in a Palestinian Family With West Syndrome

By

Hamza Ma'rouf Dweik

In Partial Fulfillment of the Requirements for the Degree
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"Identification of a Rare Splice Site Mutation in a Palestinian Family With West Syndrome"

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ABSTRACT

West Syndrome is a convulsive disorder consists of a triad of a unique seizure type (spasms), hypsarrhythmia, and developmental arrest or regression. It begins in infancy, mostly between 4 and 6 months of life. Its incidence worldwide is approximately 2-5 per 10,000 live births; however, in Palestine there is no statistical data available. The syndrome is classified according to etiology into symptomatic, cryptogenic and idiopathic. WS has many causes, and their mechanisms are still unclear. It is a genetically heterogeneous condition, and the number of the discovered genes are increasing.

In this research the DNA of a Palestinian family were sequenced by next generation exome sequencing analysis. Validation of genotype to phenotype segregation was done using classical Sanger Sequencing method, also a 100 healthy individuals were analyzed to identify the carrier frequency among the healthy Palestinian population.

The results of exome sequencing revealed a substitution mutation (G>A) at the 5`splice donor site of the *ARFGEF2* gene in the locus chr20:47,592,737 (c.1985+ 1G>A). The substitution activated cryptic splice site at the previous exon (exon 14) that leads to deletion of 44 base pairs of that exon as was shown by sanger method. DNA sanger sequencing results indicates that this *ARFGEF2* c.1985+ 1G>A mutation segregates perfectly with the phenotypes in the family under an autosomal recessive mode of inheritance. Using 100 Palestinian healthy controls, we could not find this mutation either in homozygous or heterozygous state.

Conclusion

Our study revealed that the mutation in the *ARFGEF2* gene seems to be the cause of West Syndrome phenotype in this family, with (0 %) carrier frequency in the tested healthy population.

Key Words

West syndrome, Infantile Spasms, Pathophysiology. Periventricular Heterotopia/PNH, A*RFGEF2*.

تحديد طفرة وراثية نادرة في موقع ربط الجينات في عائلة فلسطينية مصابة بمتلازمة WS

إعداد

حمزة معروف دويك

ملخص الدراسة

متلازمة West أو التشجنات الطفولية هو إضطراب تشنجي يتكون من ثالوث، نوبة مرضية فريدة (تشنجات) ، إضطراب النظم المترافع، وتوقف أو تراجع النمو، تبدأ في سن الطفولة غالبا ما بين عمر 4 و 6 أشهر.عدد حالات المصابين بهذا المرض في فلسطين غير معروف. وتصنف متلازمة West الى : أعراضية، مجهولة الأصل، مجهولة السبب. لدى متلازمة West العديد من الاسباب وآلية حدوثها لا تزال غير معروفة, ويعتبر هذا المرض من الحالات الغير متجانسة وراثيا.وان عدد المورثات المسؤولة عن هذا المرض في ازدياد ملحوظ.

في هذه الدراسة استخدمت تقنية (Next Generation Sequencing) على الحامض النووي الرايبوزي (DNA) لعائلة فلسطينية, وكذلك تم استخدام تقنية (Sanger Sequencing) لتقييم مدى علاقة الطفرة المكتشفة بالأعراض الظاهرة على أفراد العائلة , وكيفية انتقالها من جيل لأخر. كما تم تحليل عينات 100 فرد سليم لاكتشاف نسبة الحاملين لهذا المرض في المجتمع الفلسطيني.

كشفت تقنية (Next Generation Sequencing) عن وجود طفرة إستبدال نادرة الحدوث في الموقع`5 الجهة المانحة في عملية ربط الجينات في الجين (*ARFGEF2*) *في الموقع* (c.1985+ 1G>A) chr20:47,592,737 عند هذه العائلة ، وهي عبارة عن تغير في قاعدة نيتروجينية واحدة من Guanine (G) الى Adenine (A) ، ما أدى الى تحفيز موقع خفي جديد لربط الجينات داخل ال Exon السابق لهذا الموقع (Exon 14)، وبالتالي شطب 44 قاعدة نيتروجينية من نهاية هذا ال (Exon), كما أظهرت نتائج تقنية (Sanger Sequencing), و كشفت نتائج تقنية (Sanger Sequencing) أن هذه الطفرة المتنحية في الجين *(ARFGEF2)* تنتقل من الآباء الحاملين لها إلى الأبناء المصابون في العائلة.

وأوضحت الدراسة انه لا أحد من ال 100 فرد الاصحاء من المجتمع الفلسطيني يحمل هذه الطفرة دون ظهور الأعراض لديهم.

DECLARATION

I declare that the Master Thesis entitled " Identification of a Rare Splice Site Mutation in a Palestinian Family With West 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 taken from the work of others and to the extent that such work has been cited within the text of my work, 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 work to the soul of my father (God bless his soul) who taught me to trust on Allah, believe in hard working and nothing is impossible, his support and encouragement in every possible way and every step of the way reach me to get this honor.

All the efforts that went into the completion of my Masters degree I dedicate to my mother, brothers and sisters.

A special feeling of gratitude to my wife whose sacrificial care for me and our children made it possible for me to complete this work.

Finally, I dedicate this thesis to every stone of my beloved city; Jerusalem.

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Thank you all.

Abbreviations

ACTH	Adrencorticotropic hormone
ARFGEF2	ADP-ribosylation factor guanine exchange factor 2
ARFs	ADP-ribosylation factors
ARX	The Aristaless
BIG2	Brefeldin A inhibited guanine nucleotide exchange factor 2
BLAST	Basic Local Alignment
CDKL5	Cyclin-dependent kinase-like 5
CNS	Central Nervous System
CRH	Corticotropin-releasing hormone
CSF	Cerebrospinal fluid
EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalograph
FLAIR	Fluid-attenuated inversion recovery
GEFs	Guanine Exchange Factors
ILAE	International League Against Epilepsy
IS	Infantile spasms
MCD	Malformations of cortical development
MRI	Magnetic Resonance Imaging
MRS	Magnetic Resonance Spectroscopy
PCR	Polymerase chain reaction
PNH	Perinodular heterotopia
REM	Rapid eye movement
SDS	Sodium dodecyl sulfate
SMART	Simple Modular Architecture Research Tool
TGN	Trans-Golgi network
TSC	Tuberous sclerosis
VGB	Vigabatrin
WS	West Syndrome

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CHAPTER ONE

1.1 General Introduction

West Syndrome is a convulsive disorder include both a differential seizure type and an age specific epilepsy syndrome. It is characterized by symmetric, Salaam like contractions of the trunk, with stretching and rising the arms, and tonic extension of the legs (Kellaway P, 1979b) , the attack lasts between 1 second up to 5 seconds, and with clusters of 3-20 spasms per day (DulacO, 2001). As the name implies, IS occur mostly during the first year of life with a peak between 4-7 months, male to female ratio of 4:6, and with incidence of approximately 2-5 per 10,000 live births (Pavone P, 2014), (Figure 1.1). Infantile spasms are considered the essential signs of West Syndrome (Paciorkowski AR, 2011), so many physicians don't differentiate between IS and WS, taking in mind that the two terms are equivalent, and are often used interchangeably (Wong M, 2001).

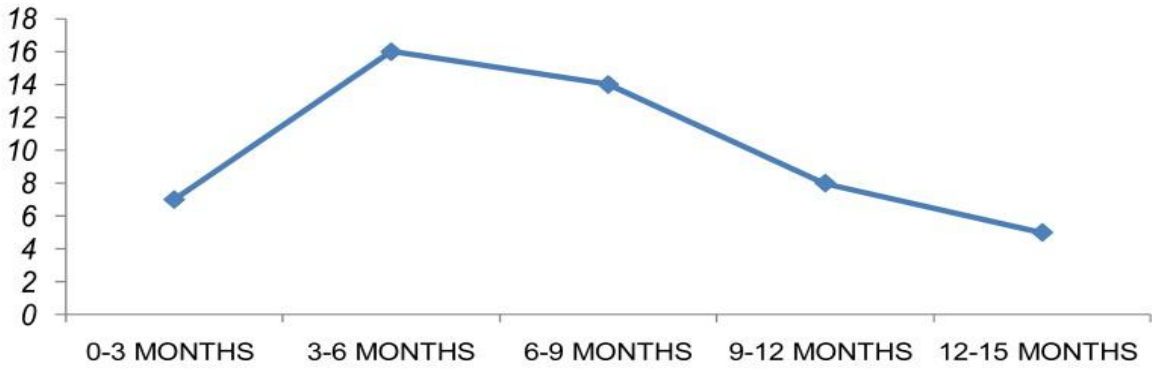


Figure 1.1 Distribution of infantile spasm according to age of onset.(Khreisat, 2011)

West Syndrome was first described in 1841, by Dr. William James West (1793-1848) in the scientific journal "The Lancet", his son was the affected person he described (West WJ, 1841). Since that time, many aspects including basic definitions and classifications of seizure, etiology, pathophysiology, and the effect of treatment, remain mysterious (Wong M, 2001). Through the past decade many of these aspects have been resolved, but many questions remain. When infantile spasms defined as an epileptic syndrome, that consists of the clinical electroencephalographic triad of spasms, hypsarrhythmia, and mental retardation, it is referred to as West Syndrome (Go CY, 2012b). Alternatively, when infants have epileptic spasms and psychomotor delay in the absence of hypsarrhythmia, they are still considered to have epileptic syndrome with infantile spasms, but wouldn't be labeled with West syndrome (Wong M, 2001).

Previously IS and WS have been classified etiologically (Commission on Classification and Terminology of the International League Against Epilepsy, 1989) as:

1) Symptomatic: resulting from an identified underlying disorder, in which neurological features or a clear developmental delay starts before the onset of spasms.

2) Cryptogenic: restricted for cases with neurological symptoms, or developmental delay, where the presumped cause or etiology is not proven.

3) Idiopathic: include cases in which WS occur without any identifiable underlying cause, or other neurological signs or symptoms.

Currently, recent recommendations by the International League Against Epilepsy (ILAE) suggest to replace these classification, since the recent genetic facts imply that all WS may be symptomatic, and result from disturbances in key genetic pathways of brain development (Berg AT, 2010). And more recently, genetic and biological classifications of WS have been proposed by Paciorkowski AR, et al. in (2011).

WS have many causes, and their mechanism is still unknown, little explanations have been established about the pathophysiology of infantile spasms, and it seems likely that a wide range of factors affecting the cortical development process can produce spasms.

Causes of IS include:

- Congenital infectious diseases such as toxoplasmosis, herpes simplex and Rubella.
- Perinatal brain damage (15%), such as cystic encephalomalacia.
- Postnatal causes such as anoxia, encephalitis, meningitis and trauma
- Neurocutaneous syndromes, including tuberous sclerosis (TSC), neurofibromatosis, and incontinentia pigmenti.
- Inborn errors of metabolism.
- Tumors.
- Chromosomal abnormalities and genetic abnormalities, such as Down syndrome, Miller–Dieker syndrome, or ARX mutation, and Aicardi's syndrome.
- Malformation of cortical development, usually in the posterior quadrants of the brain such as lissencephaly, Perventricular Heterotopia, focal cortical dysplasia, or hemimegalencephaly.
- Idiopathic 5%.

1.2 Problem Statement

The disease is considered "one of the catastrophic childhood epilepsies" (Fois A, 2010). The diagnosis, evaluation, and management of the syndrome, are still posing great challenges for health care professionals, pediatric neurologists and affected families. However, complete understanding of the etiology and genetic factors contributing to cause infantile Spasms (West Syndrome), will be of great importance in the prognosis and treatment of this syndrome, and will help in controlling the seizure and improve the level of normal development in the affected children. Overall, reducing the incidence of this condition especially in communities that have high percentage of consanguineous marriages such as Palestine. In this research we identify a rare autosomal recessive mutation in the *ARFGEF2* (ADP-ribosylation factor guanine exchange factor 2) gene in a consanguine Palestinian family, having two siblings sharing the same phenotype (Mental Retardation, Convulsive Disorder, Microcephaly). It has been argued whether this mutation in the *ARFGEF2* is the causative mutation of the syndrome. Two reports have

mentioned the association of *ARFGEF2* mutation in the pathophysiology of West Syndrome (Banne E, 2013, Sheen VL, 2004a) in different families. Thus, the study results add a supportive evidence that *ARFGEF2* gene mutation is indeed the causative mutation of this phenotype.

1.3 Objectives of the study

The objectives of this study were:

1- To identify disease causing mutation in the children of Palestinian family diagnosed with infantile spasms (West Syndrome) phenotype.

2- To verify the segregation of this mutation among the family member.

3- To raise the consideration of *ARFGEF2* gene mutation in the diagnosis of Infantile spasms (West Syndrome).

4- To determine the carrier frequency within the Palestinian healthy population.

CHAPTER TWO

2. Literature Review

2.1 Historical Overview of West Syndrome

It was in 1841 when the medical world was introduced to the first case of infantile spasms, a letter published in the scientific journal 'The Lancet' wrote by a British physician from Tunbridge, United Kingdom, called Dr. William James West (West WJ, 1841) describing a strange form of convulsions that he hadn't seen before, the letter was describing his son's (James Edwin West) illness, and it described the infantile spasms phenotype features precisely, Dr. West aimed to inform the medical world about his son's problem and get help in treating him.

The letter of Dr. West as he wrote (West WJ, 1841) :

"Sir: I beg…to call the attention of the medical profession to a very rare and singular species of convulsions peculiar to young children. As the only case I have witnessed is in my own child, I shall be grateful to any member of the medical profession who can give me information on the subject….., It was at this time that I first observed slight bobbing of the head forward, which I then regarded as a trick, but were, in fact, the first indications of disease; for these bobbings increased in frequency, and at length became so frequent and powerful, as to cause a complete heaving of the head forward towards his knees, and then immediately relaxing into the upright position, something similar to the attacks of emprosthotonos: these bowings and relaxings would be repeated alternately at intervals of a few seconds, and repeated from ten to twenty or more times at each attack, which attack would not continue more than two or three minutes; he sometimes has two, three, or more attacks in the day; they come on whether sitting or lying; just before they come on he is all alive and in motion, making a strange noise, and then all of a sudden down goes his head and upwards his knees; he then appears frightened and screams out: at one time he lost flesh, looked pale and exhausted, but latterly he has regained his good looks, and, independent of his affections, is a fine grown child, but he neither possesses the intellectual vivacity or the power of moving his limbs, of a child of his age; he never cries at the time of the attacks, or smiles or takes any notice, but looks placid and pitiful, yet his hearing and vision are

good; he has no power of holding himself upright or using his limbs, and his head falls without support".

Dr. West made great efforts in treating his son, and consulted other expert physicians Drs. Clarke and Locock from London about his son's illness, they recognized the symptoms of the disease in other cases, but they couldn't help Dr. West in treatment. The boy died from tuberculosis, on 27th September, 1860 at age 20 years (Norbert J. Piesa, 2003). Soon after the description of Dr. West, Dr. William Newnham described four cases of infantile spasms in 1849, one of them was Dr. West's son (Newnham, 1849), but he used different term "eclampsia nutans". After that a report by Barnes in 1873 describing infantile spasms was published (Barnes H, 1873). Nevertheless, a 100 years have passed since Dr. West's letter, the disease wasn't mentioned in the united kingdom except these two papers (Barnes, Newnham) according to Jeavons report in 1964 (Jeavons PM, 1964), until 1955 when a report of Illingworth describing 12 cases of infantile convulsions was published (Illingworth RS, 1955).

In Europe especially France and Germany, more articles have been published on this syndrome (Capovilla G, 2013). In 1883 a French physician called Féré described the disease 'tic de Salaam or salutation' and he used several references one of them was West's letter (Féré C, 1883), In Germany the study of Vazquez and Turner describing 10 cases with flexion spasms, mental retardation, and an abnormal electroencephalograph (EEG), was published (Vazquez HJ, 1951), the authors thought it was a new syndrome and called it 'epilepsia generalizada en flexion'. After the year 1951 a significant increase in the articles published on West Syndrome were noticed and reached to more than a dozen per year.

Obviously, the term 'West syndrome' hasn't been used all the period from West's letter in 1841 until 1960. At 1960; the 9th Marseille Colloquium conference focused on infantile spasms was organized by Gastaut (Eling P, 2002), in that conference he proposed to use the term 'West syndrome' in respect to his discoverer Dr. West. Later on he and his colleague Poirier published a comprehensive study on infantile spasms carries a subtitle "syndrome de West" (Fukuyama Y, 2001), so they were considered officially the first to use the term 'West syndrome' which is used largely to our date.

2.2 Clinical Features of Infantile Spasms (West Syndrome)

West Syndrome consists of a triad of a unique seizure type (spasms), hypsarrhythmia, and developmental arrest or regression. The hallmark manifestations of the syndrome are the spasms. In 90% of cases the spasms begin before the age of 12 months (Go CY, 2012b). However, the workshop of the International League Against Epilepsy (ILAE) in 1991 proposed that epileptic spasms might occur also in childhood, not only in infancy (Commission and Classification and Terminology of the International League Against Epilepsy, 1992), and a rare cases of late occurrence of epileptic syndrome up to 14 years of age have been reported (Rantala H, 1999)

2.2.1 Features of the Spasms

Spasms appear in cluster (the period between sequential spasms is less than 60 seconds), accompanied by irritability or crying during or after the attack. During the seizure, arrest, deviation of the eyes and/or changes in respiratory pattern may be seen. After the crisis, children may show irritability or transient hyporeactivity. In many times the spasms occur throughout the day and night but rarely during sleep. Frequently, they occur immediately after awakening, and with frequency varies from only a few times a day to several hundred a day (Kellaway P, 1979a). Usually the spasms cause contractions of the axial muscle groups either symmetrically, or bilaterally, or briefly, and suddenly.

The spasms are categorized according to the type, distribution, and the number of the muscle groups affected into flexor, extensor, and mixed flexor-extensor spasms (Fusco L, 1993). The flexor spasms involve flexion of the neck, trunk, and extremities, resulting in jack-knifing movement, also it may include adduction of the arms occur in a self-hugging motion, and together they form the ritual of salaam, thus, the term "salaam attacks". When only the neck flexor muscles are involved, the spasms may be a head nod, and if the shoulder girdle involved a shrug-like movement appears (Kellaway P, 1979a). Extensor spasms, involve the extension of the neck, trunk, and extremities. Mixed flexor-extensor spasms, involve combinations of neck, trunk, arm flexion and leg extension, or leg flexion and arm extension. (Figure 2.1). One third to

one half of patients with epileptic spasms have other seizure types include partial, myoclonic, tonic, and tonic clonic seizures. (Koo B, 1993).

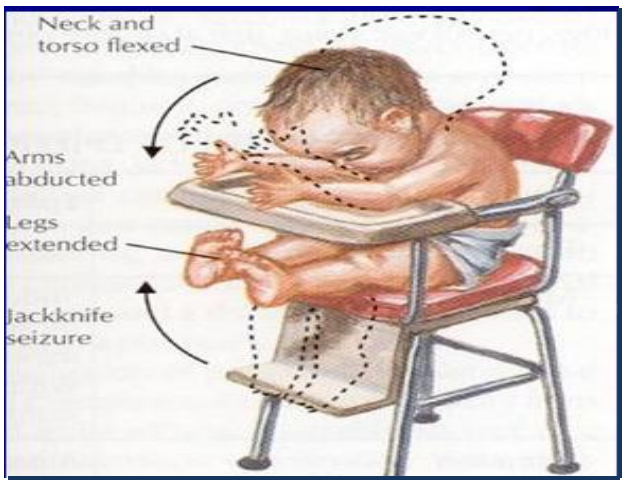


Figure 2.1 Muscle groups involved in the spasms attack. (Jones et al., 2013)

2.2.2 EEG Pattern Features

The classical electroencephalograph (EEG) pattern of patients with infantile spasms (West Syndrome) is hypsarrhythmia (mountainous arrhythmia); (Figure 2.2). It is remarkably abnormal pattern composed of completely messy and disorganized background, a very high voltage, random, slow waves and spikes in all cortical areas that are asynchronous, nonrhythmic, and variable in duration and topography (Gibbs EL, 1954). The hypsarrhythmia pattern is common during early infancy and rare after the age of 5 years, and it is present in approximately 66% of the cases (Gibbs EL, 1954). Although it was first described by Gibbs in the 1950s in 237 cases of infantile spasms children, recent reports indicated that hypsarrhythmia is not found in all the cases of epileptic spasms, or throughout the clinical course of the spasms itself (Lux AL, 2007, Caraballo RH, 2011).

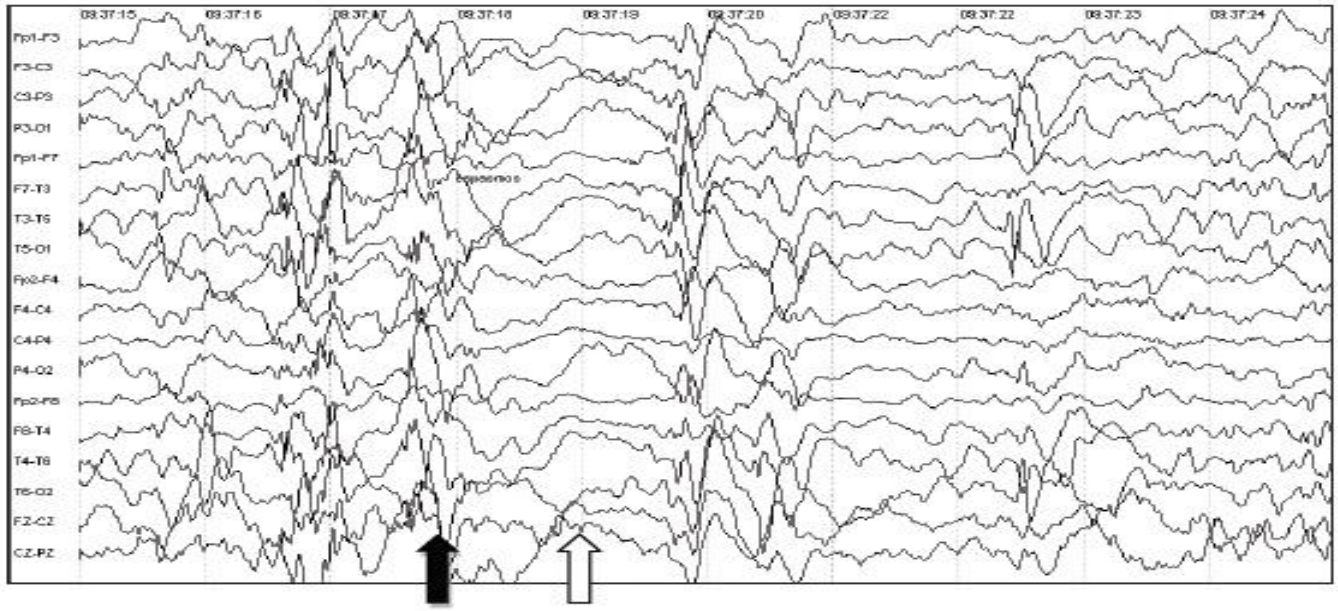


Figure 2.2. EEG of ten month old patient with West Syndrome, dark arrow show high amplitude slow waves, followed by diffuse attenuation (clear arrow).(Spinosa M, 2011)

2.2.3 Developmental Delay or Regression

Although 75% of children with IS have Mental retardation (Kurokawa T, 1980). The proposal of West Delphi Group proposed that the including of developmental delay in the clinical features of West Syndrome has no reason (Lux AL, 2004b), because it is difficult to evaluate developmental delay at early infancy or at the time of the spasms start. In addition the spasms are subtle, and it is hard to recognize their true starting time.

2.2.4 Other clinical Features

Neuropsychological impairment could be arise before the onset of spasms, Axial hypotonia and loss of hand grasping, loss of eye contact, motor and visual deficits may be also present.

2.3 Diagnostic Criteria of West syndrome

The base line in the diagnosis of West Syndrome is the parents or physician observation of the spasms and its features [occur in cluster, type of muscles that are involved (flexor, extensor, or mixed)] (Kellaway P, 1979a). Generally the spasms are variable in their occurrence, they may be sudden, brief, or missed. So parents should estimate the number of spasms that their children have, it is recommended to use a home video recording, especially in cases of subtle spasms. As the spasms are detected it is recommended to do EEG evaluation as soon as possible, the best way for the evaluation of EEG pattern is an overnight 24hr video EEG (Lux AL, 2004b), this will help in detecting and documenting EEG during awakens of the patient and all stages of sleep. If the hypsarrhythmia isn't detected the EEG should be repeated within one week, and if the video isn't available a prolonged EEG 4 to 6 hours during the awaken condition and the sleep period is enough for capturing the hypsarrhythmia pattern (Watanabe K, 1993).

Once the hypsarrhythmic pattern and the spasms are identified, the etiological diagnosis becomes essential for appropriate evaluation of WS cases (Shields WD, 2002). More additional important factors in the diagnosis of WS are patients history, physical examination, and the age of the child (the spasms typically begin in the first year of life, most commonly between 4 and 8 months of age) (Hrachovy R, 2002) (Figure 2.3). Taking in consideration these factors, the diagnosis of a wide majority of West Syndrome cases will be conducted with certainty.

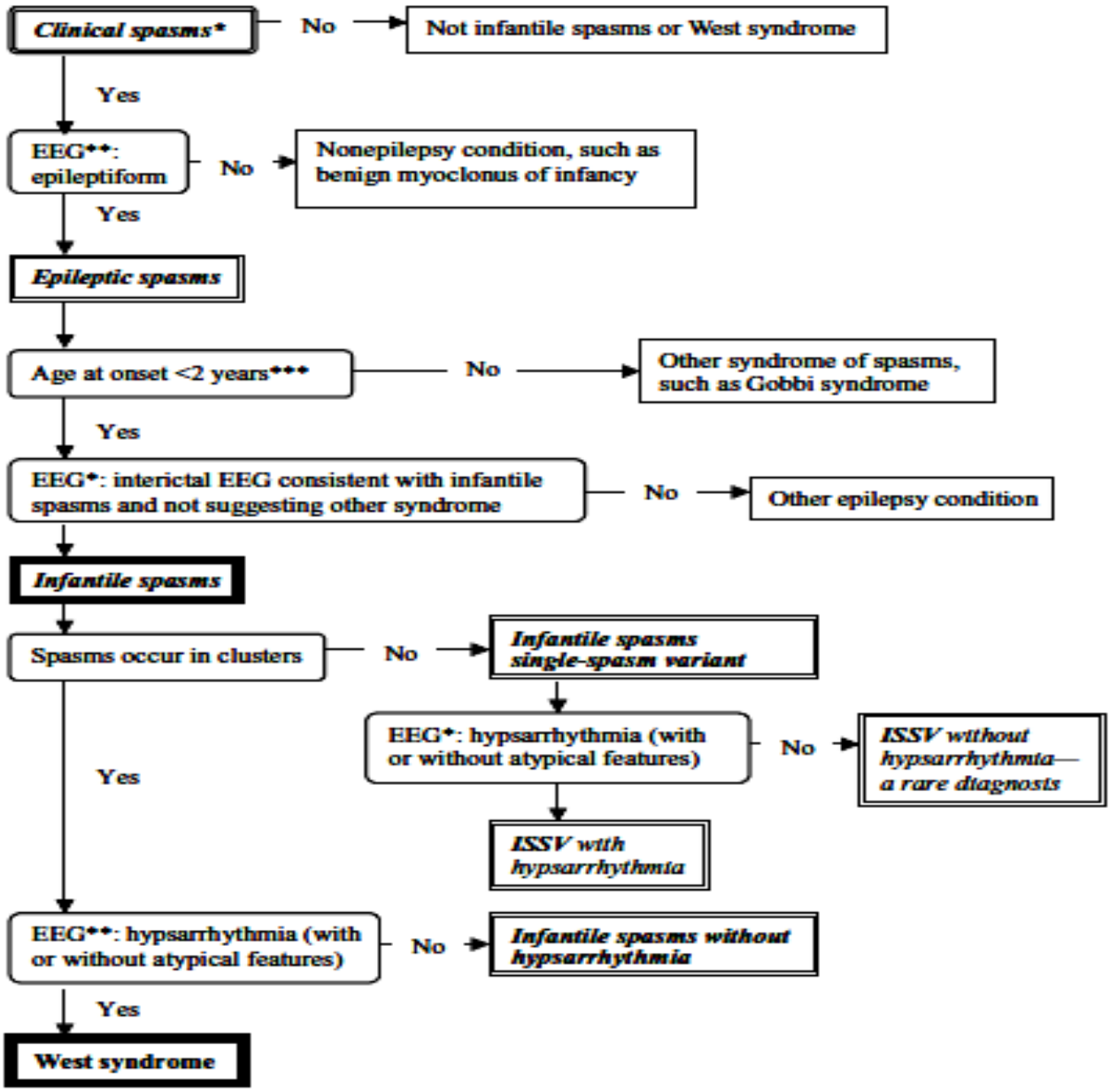


Fig 2.3. This figure illustrates steps in diagnosis of West Syndrome.(Lux AL, 2004b)

2.4 The Etiological Diagnosis of West Syndrome

Establishing the etiological diagnosis of WS has a great importance and considered essential, it has a deep effect on treatment and prognosis of the disease (Shields WD, 2006). It also helps in initiation of a specific therapy procedure that improves the developmental outcome of the affected infants. As an example, patients that are diagnosed to have IS secondary to TSC it is recommended to be treated with Vigabatrin (VGB) as the drug of choice, while patients that have IS without TSC, it is better for them to receive Adrencorticotropic hormone (ACTH) as first line drug, and those that imply brain malformations of cortical development, surgery is the best choice for them (Wheless JW, 2007). Another important factor for the etiologic diagnosis is that West Syndrome might be genetic in nature and can be transmitted to offspring, so it is important to evaluate the etiologic diagnosis in every patient and conduct a genetic counseling (Shields WD, 2006). However, as noted before there are some cases where the etiology can't be determined (Cryptogenic group), which forms 30% of cases, while in the remaining 70% of cases the etiology can be identified (Symptomatic group). The criteria of etiologic diagnosis are summarized as follow.

2.4.1 Patient History

The history of patients with West syndrome is considered fundamentally in the diagnosis criteria as most other neurologic disorders, but here special attention should be paid when taking the history of children with infantile spasms, especially there are many disorders that are associated with WS, two disorders have been reported to be the most important among others (Shields WD, 2006):

(i) A history of hypoxic ischemic encephalopathy (e.g., perinatal hypoxic ischemic encephalopathy, postcardiac arrest).

(ii Prenatally Central Nervous System (CNS) infections such as (transplacental infections), or perinatally (herpes simplex virus), and postnatally (e.g., meningitis or encephalitis).

2.4.2 Physical/Neurological Examination

Careful examination of patients leads to the diagnosis of WS etiology in many cases. The examination of skin abnormalities is of great important, like the presence of "ash leaf" spots is an evidence of tuberous sclerosis (TSC), which is the most common neurocutaneous disorder (Ohtahara S, 1993), and the presence of caf´e au lait spots is associated with neurofibromatosis, the presence of swirling pigmented skin indicates incontinentia pigmenti (Shields WD, 2006). In patients with dysmorphic features, Down syndrome or Miller–Dieker syndrome are suggested, and patients with developmental delay show evidence for the presence of cerebral palsy disorder (Trevathan E, 1999).

2.4.3 Magnetic Resonance Imaging (MRI)

It is recommended to do early imaging to help with the etiologic differential diagnosis of patient with West Syndrome. Magnetic Resonance Imaging (MRI) has a high sensitivity for detecting abnormalities in West syndrome patients compared to other neuroimaging techniques, and leads to confirmation of etiology in approximately 70% of cases (Van Bogaert P, 1993), Imaging studies showed that 40-50% of patients have clear migration abnormalities or syndromes, and 20% showed nonspecific abnormalities (e.g., atrophy) (Saltik S, 2003). Practically, MRI should be obtained before the initiation of treatment, because ACTH treatment and VGB treatment may cause transient abnormalities that lead to false interpretation of the images (Konishi Y, 1992). It is recommended to repeat imaging if the patient does not respond to treatment, and in cases where there is clinical deterioration (Gaillard WD, 2009).

2.4.4 Metabolic Etiology Workup

The etiologic diagnosis can be assessed for 70% of the cases, so no need for the metabolic work up, but in the remaining 30%, if the history, physical, and neurologic examinations, and analysis of EEG and MRI, couldn't be helpful, a metabolic etiology should be conducted including

pyridoxine challenge (100-mg pyridoxine is administrated intravenously to identify pyridoxine-dependent seizure disorder), urine for organic acids, serum for amino acids, lactate, pyruvate, and ammonia, biotinidase determination, lumbar puncture to include neurotransmitters, amino acids, and folate metabolites, cerebrospinal fluid (CSF) glucose and glycine studies (Pellock JM, 2010). At the end if the etiology hasn't been identified after all these tests then the patient is labeled as cryptogenic and targeted genetic testing may be suggested.

2.5 Pathophysiology of West Syndrome

West Syndrome has a large number of etiologies, and the causes are considered extremely variable (Jeavons PM, 1964). This variability in the etiology of WS make the scientists and researchers conclude that there should be a dominant pathway shared by all patients of infantile spasms that produces this type of seizure, or there are a multiple pathophysiological mechanisms that produce WS (Baram TZ, 1993, Marsh ED., 2009, Frost JD, 2005). The past 50 years have witnessed an intensive research efforts to identify and explain the pathologic features and processes associated with infantile spasms, but none of them have completely explained the pathophysiology of this syndrome (Frost JD, 2005). A number of hypothesis have been proposed, but unfortunately they are still not proved yet and animal models are necessary to clarify the pathophysiology of this syndrome.

2.5.1. Brain Stem Dysfunction Hypothesis

The model suggested that the initial defect arises from losing the relationship between the inhibitory neurons (noradrenergic and serotonergic) at ceruleus and dorsal raphe region of brain stem, and the excitatory cholinergic neurons in the adjacent pontine regions (Hrachovy RA, 1989a). This defect was assumed to cause a decrease in the output of the cholinergic system, and thus leads to an abnormal activity in the descending pathways (control spinal reflex activity), and the ascending tracts from the same pontine regions. Despite many studies reported an evidences for this hypothesis (Nausieda PA, 1982, Langlais PJ, 1991, Pranzatelli MR, 1989), the

model forces a fact that brainstem dysfunction might be secondary to a primary disturbance located in some other region of the brain.

2.5.2 Immune System Dysfunction Hypothesis

Studies in IS patients (Hrachovy RA, 1985, Suastegui RA, 2001, Mota NG, 1984, Reinskov T, 1963) indicated an alteration in the immune system of some patients and revealed the presence of antibodies to brain tissue, increased numbers of activated B and T cells, abnormal human leukocyte antigen (Hrachovy RA, 1985) in the peripheral blood of these patients, these observations made many investigators hypothesize that IS may result from a defect of the immune system (Hrachovy RA, 1989a, Martin F, 1964). But no casual relationship had been established, and the alteration may simply reflect responses to underlying brain damage.

2.5.3 Cortical–Subcortical Interactions Hypothesis

Several studies introduced an evidence on the association of cortical lesions dysfunction in WS patients. Chugani et al. (1992) proposed a theory indicated that "at a critical maturational stage, the abnormal activity within the serotonergic raphe nuclei is triggered by a diffuse or focal cortical abnormality, and the observed hypermetabolism within the lenticular nuclei is produced by the synchronous raphe-striatal pathway activation." (Chugani HT, 1992).

The hypsarrhythmic EEG pattern is produced by the projections of raphe-cortical, and cortico-cortical, while the epileptic spasms result from projections from brainstem regions to spinal cord neurons. Another researchers such as Dulac et al. (1994) proposed that "the abnormal EEG pattern resulted from cortical dysfunction, and the spasms result from the functional differentiation of sub-cortical structure like the basal ganglia which result from continuous abnormal cortical activity" (Dulac O, 1994), more recent hypothesis has been demonstrated by Lado and Moshe (2002) which suggest that the disorder develop when a supportive changes for convulsant occur in both cortical and brainstem regions; ie; when epilptogenicity increases in

one area (either cortex or brainstem), the other region (brainstem or cortex) fail to suppress the epileptic activity at that time, so the disorder appears (Lado FA, 2002).

2.5.4 Hypothalamic-Pituitary-Adrenal Axis Dysfunction Hypothesis

The hypothesis emerged from the fact that low ACTH hormone and cortisol levels have been detected in the CSF of WS patients (Baram TZ, 1992a, Baram TZ, 1995), in addition the effectiveness of ACTH and corticosteroids treatment for WS patients implies the role of the hypothalamo-pituitary-adrenal axis in the pathogenesis of WS. The hypothesis suggests that stress conditions in the developing brain, leads to excessive releasing of corticotropin-releasing hormone (CRH) hormone which in turn results in seizures and neuronal structural abnormalities (Chen Y, 2008). The key element of the hypothesis that CRH is necessary for neuronal excitability in the normal developing brain, and when there is abundance of CRH-receptors meet with high levels of CRH this lead to a permanent epileptogenic changes in brainstem, which then become the site of spasm origin (Stafstrom CE., 2009).

2.5.5 Developmental Desynchronization Hypothesis

Normally the brain function is supposed to be dependent on a balanced maturation pattern, but in WS this pattern become unbalanced and so certain brain systems become dysfunctional (Frost JD, 2005). A general hypothesis has been proposed by Dr. James D. Frost, Jr. in 2005, suggested that WS may result from desynchonization during normal developmental processes of two or more central nervous systems (e.g. Synaptogenesis, Myelination, Neuronal migration) lead to disturbance in brain function and WS. A key point of this hypothesis is that the disturbed function results from multiple causative factors, which makes it compatible with the variety of pathological findings and etiologies associated with infantile spasms.

2.6 Genetic Etiologies of WS

West Syndrome are considered genetically heterogeneous conditions, result from disturbances in the genetically regulated pathways of brain development, and abnormalities in the gene expressed at the synapse (Paciorkowski AR, 2011). It has been proven that mutations of several genes regulating brain development are associated with specific brain malformations (Barkovich AJ, 2001), and during the last two decades the number of genes and proteins related to clinical phenotypes associated to infantile spasms, has been largely expanded. The Aristaless (ARX) and the cyclin-dependent kinase-like 5 (CDKL5) genes were found to be associated to children having infantile spasms, hypsarrhythmia and developmental delay (Russo S, 2009, Kato M, 2003).

More recently, mutations in other well-known and new genes have been postulated to cause phenotypes with infantile spasms such as SLC25A22, STXB1, SPTAN1, SCN2A, PLCB1, ST3GAL3, FOXG1, MEF2C, DCX, PAFAH1B1, TUBA1A, TSC1, TSC2, NF1, NSD1, KCNQ2, GLYCTK,GRIN1, GRIN2A, and MAGI2 genes (Paciorkowski AR, 2011). In addition, infantile spasms have been occasionally reported with several other well-characterized predisposing genotypes, including autosomal recessive severe microcephaly with perinodular heterotopia/PNH (ARFGEF2 gene), Freeman–Sheldon syndrome (MYH3 gene), mitochondrial encephalomyopathy with elevated methylmalonic acid (SUCLA2 gene), neurogenic muscle weakness, ataxia, retinitis pigmentosa/NARP (MT ATP6 gene), Shinzel– Giedion syndrome (SRTBP1 gene), Smith–Lemli–Opitz syndrome (DHCR7 gene), Smith–Magenis syndrome (deletion 17p11.2), Sotos syndrome (NSD1 gene) and X-linked perinodular heterotopia/PNH (FLNA gene) (Paciorkowski AR, 2011).

Moreover, the early classification of Paciorkowski AR et al. in 2011 demonstrated a growing group of developmental disorders that have a clear association with infantile spasms, which include Aicardi syndrome, PEHO (progressive encephalopathy with edema, hypsarrhythmia and optic atrophy and PEHO-like syndromes, isolated hemimegalencephaly (HMEG), mythocondrial dysfunction, neonatal hypoglycemia, the pyridoxine-dependent/ responsive epilepsies, some of the epidermal nevus syndromes (e.g., nevus sebaceous syndrome), schizencephaly, and the groups of perinatal stroke or other cerebral hypoxic events or post-infectious injuries (e.g., TORCH, other viruses or bacterial meningitides).

2.7 Cortical Development and Periventricular Heterotopia

Normal developmental process of cerebral cortex involves a critical stages that occur in a series of highly overlapped organized events, including neural stem cell proliferation, migration, and finally neuronal differentiation (Pang T, 2008). Malformations of cortical development process (MCD) are produced by disruption of these stages. MCD involves a wide range of syndromes with varied genetic etiologies, anatomic abnormalities, and clinical manifestations. Increasingly these malformations are found to be an important cause of epilepsy and mental retardation which are features of West Syndrome (Kuzniecky RI, 1994). The neuronal progenitor cells that establish the cerebral cortex pass through different pattern of differentiation and migration pathways Initially, these cells originate from the periventricular and subventricular zones undergo symmetric cell division that produces two identical daughter cells continue to divide and renew the population of the ancestor cells, at the same time they undergo asymmetrical cell division that produces two types of cells, progenitor cells that re-enter the cell cycle, and neuroblasts (immature neuronal cells) that have the ability to adhere to the scaffolding created by the radial glia and migrate into the cortical plate (Figure 2.4). (Chenn A, 1995).

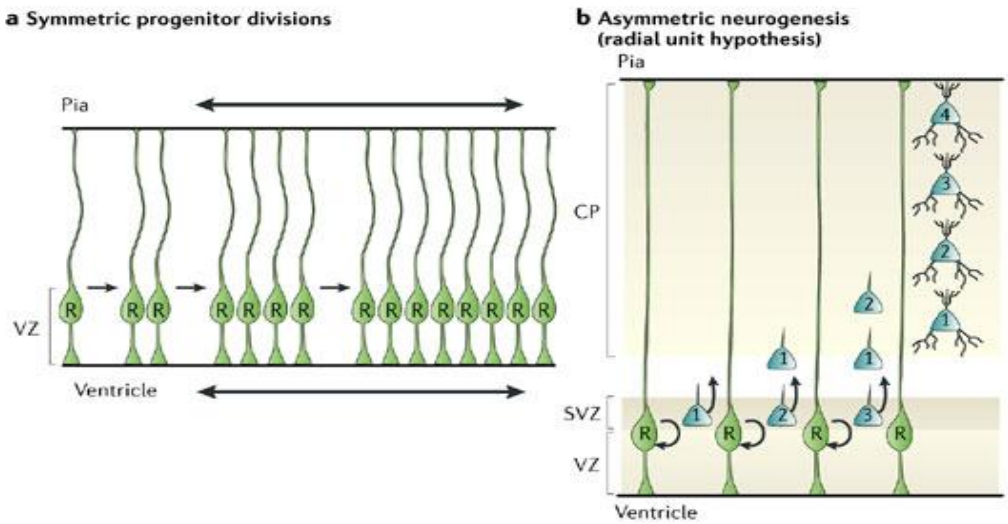


Figure 2.4. Schematic drawings illustrating division patterns observed in the embryonic cortex during development. (a) Symmetric progenitor divisions in the ventricular zone (VZ) increase the founder cell (radial glia (R), green) population. (b) Asymmetric neurogenic divisions in the VZ will yield self-renewal of radial glial cells and produce neurons (blue) destined for different layers in the corticalplate (CP) (Kriegstein et al., 2006).

The neuroblasts start to migrate and departure from the ventricular zone toward the cortical plate radially in the form of retrograde fashion movement and contact with ventricular surface. The excitatory or glutamatergic cortical neurons migrate radially from the ventricular zone to the cerebral cortex by adhering to the radial glial fibers, while the inhibitory or GABAergic cortical interneurons originate from the ventricular zone and the germinal zone of the basal ganglia. Once these neurons reach their appropriate position of the cortical plate, they arrest there, and the cerebral cortex layers started to be developed, the deeper layers (V/VI) are formed from the earlier generated neurons, while the superficial layers (II/III) are formed from the later generated neurons (Figure 2.5). Finally, the neurons undergo differentiation during gestation and infancy period, into specific neuronal cell types that form the cortical connections locally and long-distance projections.

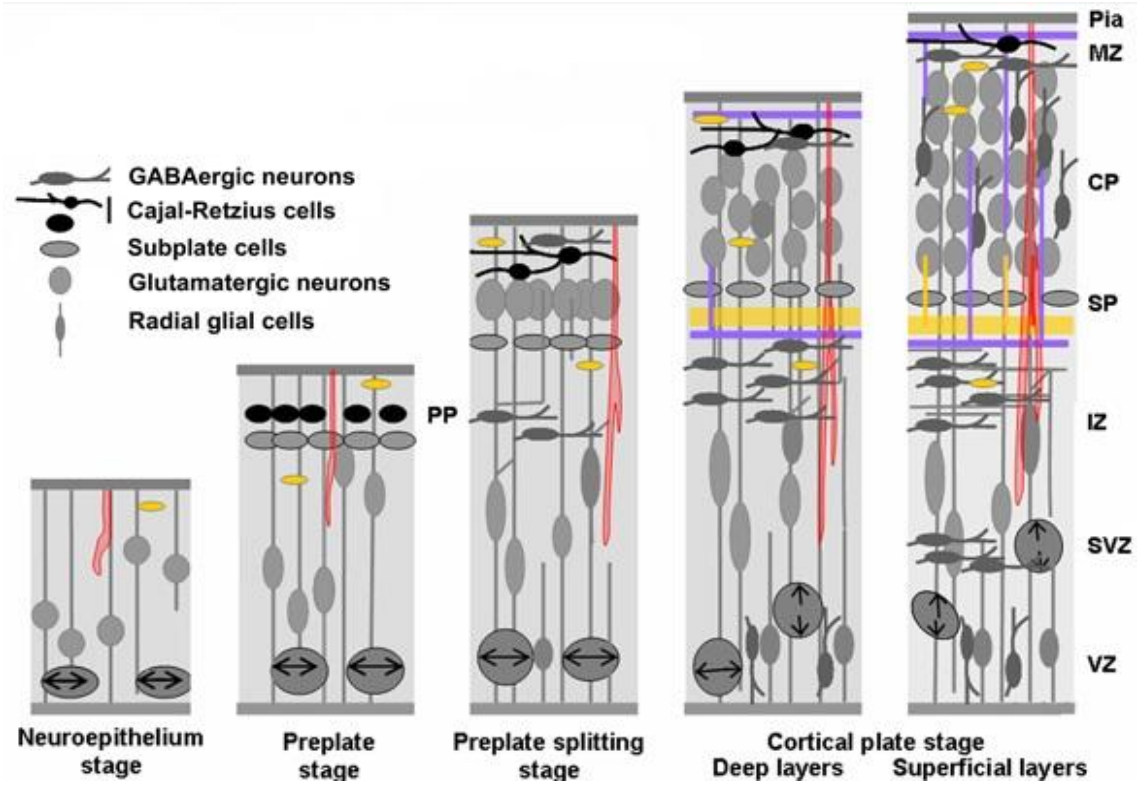


Figure 2.5. Cortical neurogenesis in the mouse neocortex begins with an intense proliferation of the progenitor cells located in the ventricular zone (VZ). These populations of cells give rise to most of the GABAergic neurons (subpallium) and glutamatergic neurons and glial cells (pallium) of the cerebral cortex. Once generated, neurons migrate toward the pial surface and complete their differentiation in the cortical plate (CP). Glutamatergic neurons destined to populate the deeper layers of the cortex are generated and then migrate away from the VZ earlier than the neurons destined for progressively more superficial layers. GABAergic neurons arise from more ventral structure and migrate tangentially in the developing CP.(Tania et al., 2013)

2.7.1 Periventricular Heterotopia (PH)

When the neurons fail to migrate from the ventricular zone toward the cortical plate, neuronal nodules of the gray matter accumulate along the walls of the lateral ventricles leading to abnormal positioning of the post mitotic neurons (Periventricular Heterotopia), (Figure 2.6) (Guerrini R, 2001). Sometimes it is referred as "Subependymal heterotopia", as the heterotopic gray matter situated beneath and adjacent to the epndyma. This disorder is characterized by a wide spectrum of clinical presentations, epilepsy is the most common with 90% estimation of patients to develop at the first year of their life (Leventer RJ, 2008). Although it has been suggested in several studies that there is a functional and anatomic correlation between the heterotopic nodules and the cortex that participate in epileptogenesiss and higher cortical functions (Passarelli V, 2014), the mechanism of epileptogenesis in PH remains controversial and more studies are needed to confirm these findings. However, studies on focal cortical dysplasia, including subcortical heterotopia revealed that epilepsy is considered a final common pathway of malformations in the cortex, in which an imbalance between excitatory and inhibitory tone leads to seizure (Crino PB, 2002), another pathological study on children with epilepsy indicated that intranodular GABAergic pathways has more excitatory neurons than inhibitory in PH (Hannan AJ, 1999).

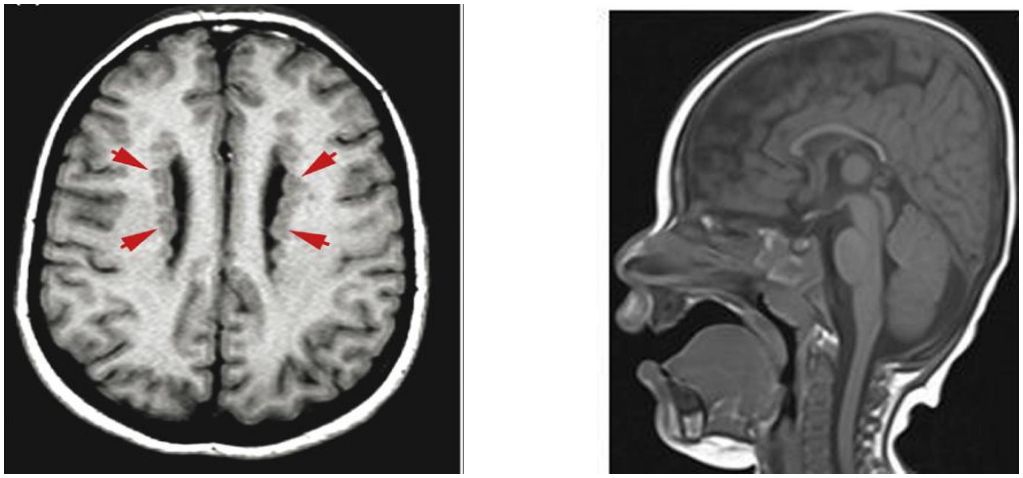


Fig 2.6. Brain MRI of patients with a homozygous *ARFGEF2* mutation show periventricular heterotopia with thin corpus callosum. Left: horizontal section-T2 weighted. Periventricular heterotopia seen in the lateral ventricles of patient (Sarkisian M, 2008) . Right: thin corpus callosum in a T1-weighted MRI sagittal section (Banne E, 2013).

2.7.2 Periventricular Heterotopia Pathogenesis

Several factors involved in causing PH, they could be acquired during the stages of brain development in the uterus, or after corticogenesiss prenatally or postnatally (Pang T, 2008) These factors include: genetic mutations (e.g., FilaminA, ARFGEF2 genes), or extrinsic factors (e.g., irradiation, infection, injury) (Passarelli V, 2014). Many phenotypes and clinical disorders associated with PH have been identified, caused by other genes, such as: Fragile X syndrome, Ehlers-Danlos syndrome, Megalencephaly, Polymicrogyria, Dyslexia, Hydrocephaly, Oro-facio-digital anomalies + microphthalmia, Mental retardation.

Genetically PH is considered a heterogeneous disorder, many genes have been identified causing PH, (Table 2.1), but FLNA and ARFGEF2 genes are the main causes genes, they are highly expressed in the ependymal lining of the ventricular zone, and involved in cell adhesion (Lu J, 2005).

Table 2.1 Genes associated to PH.(Sarkisian M, 2008)

Gene Name	Chromosomal Location
FLNAa	Xq28
ARFGEF2	20q13.13
STS	Xp22.3
MLYCD	Q6.q24.3
ASXL2 ***KIAA1803***	T(2;9)(p24;q31)
MAN1A2 ***GSTA2***	t(1;6)(p12;p12.2)
---	Duplications of the distal region of chromosome 5p
---	Translocation involving chromosomes 2p24 and 9q32

2.7.2.1 *FLNA* Gene

FLNA mutation causes The X-linked dominant form of PH, where the heterozygous females show bilateral and symmetrical PH, normal intellectually, focal epilepsy, while mutations in males show early lethality (Mochida GH, 2004). 80% of inherited cases with PH are caused by mutations in the *FLNA* gene (Passarelli V, 2014). The protein encoded by *FLNA* gene has a critical function in cortical development, it has been found that this protein is highly expressed at the neuroependyma along the ventricular zone, the endothelial cells lining the blood vessels, and lesser expression in early postmitotic neurons of cortical plates (Fox JW, 1998), *FLNA* is an actin-binding phosphoprotein, it helps the migrating neurons to attach onto the radial glial scaffolding before they leaving the ventricular zone toward the cortex, mutations in *FLNA* gene cause disruption in the neuroepithelium which leads to weaken the attachment of migratory neurons and cause PH (Tu Y, 2003).

2.7.2.2 *ARFGEF2* Gene

ARFGEF2 gene encodes brefeldin A inhibited guanine nucleotide exchange factor 2 (BIG2) protein, which is one of the three Guanine Exchange Factors (GEFs) expressed in the brain, especially during the period of neuronal migration and proliferation (Sheen VL, 2004a). GEFs are activated by the replacement of bound GDP with GTP which is mediated by BIG2/Sec7 Arf1 GEF domain, that is responsible for its guanine exchange activity (Banne E, 2013), consequently the activated GEFs activate the ADP-ribosylation factors (ARFs), which regulate vesicle trafficking and the transport of molecules from the interior of the cell to its surface (Pacheco-Rodriguez G, 2002).

ARFGEF2 gene mutations cause the autosomal recessive form of PH, individuals with this mutations also develop microcephaly, epilepsy, and severe developmental delay (Passarelli V, 2014), these features are associated with West Syndrome as discussed earlier. The gene has a developmental role in cortical development, affecting neuronal progenitors migration and proliferation, the study of JIE LU, et al. in 2006 revealed that high levels of *ARFGEF2* mRNA and BIG2 protein expression were detected in the ventricular (VZ) and subventricular zones (SVZ) of the brain, also at the periventricular region, during the embryonic day E11-E17 of mice embryonic cortical neurogenesis (JIE LU, 2006), these observations support the idea that disruption of the neural progenitor proliferation and migration along the neuroependyma may contribute to PH formation and microcephaly. In addition the study of Volney L Sheen, et al. 2004 demonstrated that *ARFGEF2* function is required for neuronal migration, and the BIG2 protein regulates vesicle trafficking from the trans-Golgi network (TGN) to the cell surface, these vesicles could be adhesion molecules such as ß-catenin and E-cadherin which are required for the departure of neuronal precursors from the ventricular zone (Sheen VL, 2004a). So the disruption in *ARFGEF2* gene and it's encoded protein BIG2 could impair and prevent the postmitotic neurons from exiting the ventricular zone and disrupt cell-cell adhesion causing PH.

Two mutations in *ARFGEF2* have been reported in four patients from two Turkish families in 2004 with severe developmental delay, microcephaly, epilepsy, and periventricular heterotopia. (Sheen VL, 2004b), more recently another mutation in the *ARFGEF2* gene affecting the first base of intron 15 chr20: 47592737, c.1958(+1) G>A has been described in five patients from

Palestinian family having PH, microcephaly, infantile spasms and severe mental retardation (Banne E, 2013). Despite these findings the mutations in the *ARFGEF2* is considered a rare cause of PH (Liu JS, 2011). The present study also reported the same mutation chr20: 47592737, c.1958(+1) G>A in the *ARGEF2* gene in different Palestinian family having West Syndrome features.

2.8 West Syndrome Treatment

The treatment of West Syndrome should starts immediately after diagnosis. The ultimate goal in the treatment strategy is to stop the epileptic attacks, normalize the EEG abnormalities pattern and to improve or avoid neurodevelopmental delay, with the lowest side effects.

The American Academy of Neurology and the Child Neurology Society proposed a definition of an effective treatment is the one that leads to cessation of spasms and deletion of the hypsarythmic pattern after two weeks of treatment starting, and a continuously 28 days from the last spasms (Mackay MT, 2004a). For this purpose, the use of ACTH and oral prednisone, and gamma-vinyl GABA (Vigabatrin) have been considered the most effective drugs for West Syndrome patients, other antiepileptic drugs and surgical option in anticonvulsant resistance patients are also an option (Fois A, 2010). Several studies and surveys have been conducted proposing and comparing the various options for the drug of choice and the amount of dosages (Table 2.2) .

The European expert opinion survey 2007 demonstrated that ACTH and prednisone are the first line therapy in WS patients (Wheless JW, 2007); various prospective studies indicated that ACTH or prednisone lead to cessation of spasms and improvement in the EEG within a couple of weeks for about 60-80% of IS patients (Lombroso CT, 1983, Lux AL, 2004a), further studies reported that ACTH is more effective than prednisone, and some concluded that patients who don't respond to ACTH may respond to prednisone and vice versa (Snead OC, 1983, Baram TZ, 1996). Regarding the amount of dosages and time of treatment for the hormonal therapy, studies have reported that low doses of ACTH effect are equivalent to high doses (Hrachovy RA, 1994), some experts thought that high dosages of ACTH (150 units/m2/d) is presumably more effective than low dosages (20–40 units/m2/d) (Pavone P, 2014). Recently, a special report of the

Guideline Development Subcommittee of the American Academy of Neurology and the Practice Committee of the Child Neurology Society provides an evidence-based guideline update stated that ACTH is probably effective, with low doses for short treatment of IS. Vigabatrin is also possibly effective in the cessation of spasms and abolition of hypsarrythmia specially in childrens with TSC and IS (Go CY, 2012a). The practice also concluded that ACTH or prednisolone may be used preferably Vs. Vigabatrin in infants with cryptogenetic IS, as it can improve developmental outcome. It has been reported that long time treatment with ACTH cause several side effects such as irritability, increased appetite and cushingoid features, hypertension and hypokalemia, immunosupression, cataract, gastritis (Riikonen R, 2005).

The mechanism of action for ACTH remain elusive and several hypothesis have been proposed: ACTH bind to steroid receptors in the brain and thus altering excitability, reduction of cortico tropin hormone (CRH), ACTH may increases the inhibitory neurotransmitters GABA, ACTH may act as neurotransmitter and interfere with the epileptogentic prosperities of the opiate receptors (Zupanc ML, 2003). The use of vigabatrin for short time treatment should be done under careful examination of the visual field; because it has been reported that vigabatrin has a side effect on peripheral visual field and retinopathy with loss of peripheral vision in both eyes (Brodie SE, 2011). Previous studies comparing Vigabatrin Vs ACTH have demonstrated similar efficacy of both of them, with superiority of ACTH (Vigevano F, 1997), and other reports indicated that Vigabatrin has a lower tolerated side effects than ACTH (Cossette P, 1999). In a summary ACTH is considered the drug of choice for WS patients in the United States, and vigabatrin is considered the first line treatment in Europe.

Table 2.2 Summary of recommendations of major reviews and meta-analysis of IS literature (Mackay MT, 2004b, Wheless JW, 2007, Wheless JW, 2005,, Hancock E, 2001).

Review/Parameter	Recommendations
American Academy of Neurology and Child Neurology Society (2004)	ACTH "probably" effective, vigabatrin "possibly." Vigabatrin best for tuberous sclerosis. Low-dose steroids not effective. All other treatments inconclusive.
National Institute for Health and Clinical Excellence (NICE) (2004)	Both corticosteroids and vigabatrin are effective.
United States Expert Consensus (2005)	ACTH and topiramate "usually" appropriate; zonisamide, vigabatrin, valproate, prednisone "sometimes" appropriate. Vigabatrin and ACTH "usually" appropriate for tuberous sclerosis.
European Expert Consensus (2007)	Vigabatrin (best), ACTH, and prednisone "usually" appropriate, valproate "sometimes" appropriate. Vigabatrin the only "usually" appropriate treatment for tuberous sclerosis.
Cochrane Review (2008)	Hormonal treatment (either high dose oral or ACTH) leads to a more rapid response than vigabatrin.

Conventional antiepeliptic drugs also have been considered effective for the treatment of West Syndrome patients, and a wide studies reported their effectiveness in treatment (Table 2.3) (Pavone P, 2014), these drugs include Sodium Valproate, Zonisamide, Topiramate, Nitrazepam, Pyridoxine, Sulthiame, Benzodiazepines (clonazepam). However, In drug resistance patients surgery and ketogenic diet is proposed to the treatment criteria. Ketogenic diet has been used since 1921 for the treatment of childhood epilepsies (Nieh S & Sherr E, 2014), it consists of low carbohydrate, high fats and adequate protein with a ratio of 3:1 or 4:1, so the body relies on fats to produce energy, many clinical studies have demonstrated good efficacy of ketogenic diet in the treatment of WS patients (Li B, 2013), the mechanism of the diet is unknown (Freeman J, 2006). Surgery is the last option for WS patients treatment specially those who have failed drugs treatment, and have been identified with focal cortical lesions by MRI or PET, surgery treatment has been reported to be the best option for intractable WS patients and anticonvulsant resistance patients (Fois A, 2010).

Table 2.3 The table below summarizes the options for WS treatment and their possible side effects.

Treatment	Mechanism of action	Dose	Side effects	Percentages spasm free
Valporate	Enhancement of GABA, reduction of T-type Ca^2 channel current	20-300 mg/kg/day	Hepatotoxicity Irritability Tremor Weight gain Alopecia	72% - 73%
Topiramate	Inhibition of voltage sensitive Na channels blocker, glutamate-receptor antagonist, enhancement of GABA agonist	10 - 40 mg/kg	Renal stones Sedation Anorexia Cognitive impairments	20% - 30%
Zonisamide	Inhibition of T-type Ca channels and Na channels	4 - 22 mg/kg/day	Sedation Anorexia Renal stones Leucopenia	33%- 36%
Benzodiazepines	Enhancement of GABA	0.01 mg/kg/day	Sedation Irritability Increased secretion tachyphylaxis	----------
Pyridoxine	Enhancement of GABA	30 - 400 mg/day	Minor	0% - 29%

CHAPTER THREE

3. Patients, Materials and Methods

3.1 Patients

The story started when a consanguine Palestinian family having two male brothers suffered from mental retardation associated with some kind of epilepsy referred to our lab for genetic testing following consultation with their pediatric neurologist . At the time, our lab was collecting families with mental retardation phenotype, for research study purpose, we explained to this family about our research and the purpose of the study, so they agreed to participate in this research, and a consent form was assigned by the parents (since their children were less than 18 years old). The family members consist of first degree related healthy parents and three children; two male brothers show clinical features similar to each other (Convulsive Disorder, Mental Retardation), and a healthy sister that is developing normally. The family pedigree shows an X-linked or an autosomal recessive mode of inheritance, (Figure 3.1), but after validation of the results we concluded that it is an autosomal recessive. We were interested in this family and expected to find out an interesting genome variation, since the two brothers have the same phenotype.

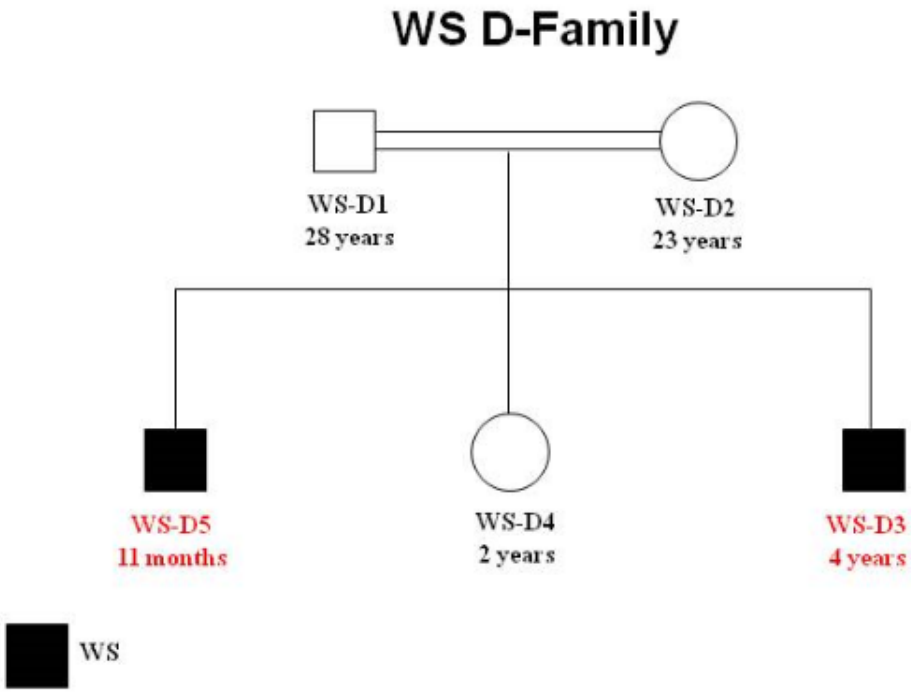


Figure 3.1

WS-D5 and WS-D3: have West Syndrome with microcephaly and global developmental delay.

3.1.1 Clinical Evaluation of the Affected Siblings

(According to the Pediatric neurologist)

The proband is a third child (male) for first degree related healthy parents with full term spontaneous delivery, weight at birth 3 kg, hearing test normal, the postnatal period reported to be normal until the age of 10 months, he started to suffer from short episodes of convulsions in the form of tonic limb movements and clusters of spasms with up rolling of the eyes upon awakening from sleep. The patient treated promptly with Valporic acid, and the episodes of convulsions were successfully stopped.

- Physical examination showed:

Axial hypotonia and peripheral spasticity and hyper-reflexia, poor eye contact, positive Babiniski, Microcephaly <<2p, FTT. There were several small hyperpigmented patches on the skin, no abnormality detected in other organs (heart, lungs and abdomen).

- The first EEG showed generalized slow background with multiple episodes polyspikes dominant mainly on the left hemisphere suggesting the hypsarrythmic pattern.

By the age of one year the brain MRI images showed preserved mid line structures, sub-epindymal heterotopia with periventricular thick cortical material, dialated subarachnoid space especially in the temporal and frontal areas, mega cysterna magna and thin corpus callosum.

- Metabolic work up and lab tests:

Acylcarnitine, total and free carnitine, pyruvate and ammonia in blood, all were normal. Organic acids in urine showed metabolites of valporic acid and 3-OH Isovaleric acid, lactate in blood 3.23 {nl up to 2.5}.

The boy suffers from global developmental delay, until now he can't sit alone, can't stand, no words or social eye contact. His MDI is below 50 by Bailly test.

His older brother has the same phenotype with similar findings in physical examination; he is also suffering from severe global developmental delay MR, FTT, axial hypotonia, pyrammidal signs, seizures and microcephaly. Amino acids in blood, acylcarnitine, VLFA - all are normal. Organic acids in urine- normal. His brain MRI and MRS were normal except for thin corpus callosum. He is dependent on the mother in nearly all daily activities. He did an EMG testing

that showed myopathic changes without neuropathy, the muscle biopsy didn't show any structural or histochemical changes, and the enzymatic activities of the 5 mitochondrial respiratory chain complexes- normal.

According to the above clinical findings with EEG and MRI images, and our literature review the patient has a typical phenotype of West Syndrome diagnosis.

3.2 Materials

3.2.1 Buffers, Gels and Solutions

- **Ethidium Bromide**
  Ethidium bromide was dissolved in the double distilled sterile water to a final concentration of 1mg/ml. (or bought premade to this concentration)

- **Agrose gel**
  0.8%, 1.5%, 3% agarose
  1X TBE buffer
  Final concentration of 0.01% ethidium bromide

- **Red blood cell lysis buffer**
  155 mM NH_4Cl
  10m NH_4HCO_3
  0.1 mM EDTA with (PH=7.4)

- **1X lysis buffer**
  50 M Tris HCL with (PH=7.5)
  100 mM NaCl
  mM EDTA with (PH=8)

- **5X loading buffer**

  0.25% bromophenol blue

  0.25% Xylene cyanol FF

  30% Glyserol in water

- **50X TAE Buffer**

  2M Tris ph 8.0

  1M Acetic acid

  0.05M EDTA

  Adjust to PH=8.0

- **Proteinase K**

  Proteinase K was dissolved in double distilled sterile water to a 5mg/ml final concentration.

- **64X Buffer**

  Enhancing buffer.

  Up to 64 (0.12 µl bigdye in 10µl reaction).

3.2.2 Reagents, Instruments and Kits

Reagents

Reagent	Supplier	Product specifications
Agarose	ORNAT	SeaKem® LE Agarose
Oligonucleotide primers	Hylabs	
Super therm polymerase	Eisenberg Bros	CAT# JMR-80
10x polymerase Buffer	Eisenberg Bros	CAT# JMR-420
dNTPs 2.5mM	TAMAR	CAT# R0181,4X0.25mM
100bp plus DNA ladder	TAMAR	ThermoScientific, GeneRuler™
Exonuclease I	BioLabs	CAT# M0293L,15000 units
Antaractic Phosphatase	BioLabs	CAT# M0289L, 5000 units
Q solution	Qiagen	
Proteinas K	aMReSCO®	LOT# 1311C384

Reagent	Supplier	Product specifications
Hi Di Formamide	Applied Biosystems	CAT# 4311320
Chloroform	Biolabs	CAT# 67-66-3
Isopropanol		CAT# K24879734809

Kits

Kit	Supplier	Product specification
BigDye™ Terminators V1.1 Cycle Sequencing Reaction Kit	Applied Biosystems	CAT# 4337451-100
Msp1 RE Kit	BioLabs	CAT# R0106L
qScript™ cDNA Synthesis Kit	Quanta Biosciences	CAT# 95047-025 95047-100 95047-500
RNA Isolation Kit using Trisol	Invitrogen	CAT # 15596-018

Instruments

Instruments	Supplier	Instrument Specification
NanoDrop®		
Agarose gel electrophoresis apparatus	BioRad	SUB-CELL® GT
Agarose gel electrophoresis power supplier	BioRad	Power PAC 300
Gel DOC Gel DOC™	BioRad	Molecular Imager
PCR machine	Applied Biosystems	PCR System 9700 GeneAmp®
Sanger Sequencing Genetic Analyzer Machine	Applied Biosystems	ABI 3130XL

3.3 Methods

3.3.1 Blood Collection

Blood samples were collected from all the family members (affected and non- affected individuals) for DNA extraction. Peripheral whole blood was taken by a phlebotomy vacuum system and mixed with 9 ml K3EDTA tube. A consent form was assigned by each member of the family, for those who are under the age 18, one of the parents assigned instead.

3.3.2 Genomic DNA Extraction (Salt based Method)

Each blood sample was mixed with about 20 ml of the red blood cells lysis buffer, and kept on ice for bout 20-30 minutes (min), being shaken by hand from time to time, until blood becomes transparent. Centrifugation was done at 2000 round per minute (rpm) for 10min. at 4C°. The supernatant was carefully removed and the pellet was re-suspended in 3 ml red blood cell lysis buffer and centrifugation was repeated. The pellet was then suspended in a mix of 1x lysis buffer, 100 µl of 20%SDS and proteinase K, and incubated at 37C° overnight (it can be incubated at 55C° for 3 hours). At this point the tubes are clear with no pellet (3 ml total volume). One ml of 6M NaCl was added and vigorously vortex until the solution appeared foamy. Centrifugation was done at 3000 rpm for 20 min. at 25C°, followed by gently transferring the upper phase into a clean 15 ml tube, avoiding the salt protein deposit. Twice the volumes of 100% cold ethanol (EtOH) were added to the upper phase followed by gentle inversion until the DNA appears in the tube. DNA was removed with a glass Pasteur pipette, followed by washing in 70% EtOH (in eppendorf tube) and then air drying for a few minutes on Pasteur pipette. DNA was then dissolved in DDW (200- 600 µl depending on the amount of DNA) and left at room temperature (RT) overnight, before long term storage.

3.3.3 Next Generation Sequencing (Exome Sequencing)

Next generation sequencing (NGS) was used to determine the variations of all coding regions, or exons, of ARFGEF2 gene, It provides coverage of more than 95% of the exons. NGS is often referred to as massively parallel sequencing, which means that millions of small fragments of DNA can be sequenced at the same time.

Exome sequencing was used in this research to detect the mutations that is responsible for the WS phenotype in WS-D family. It was done by Dr. Hashem Shahin, on all the members of WS-D family (both parents, the 2 affected children, and their normal sister) in Prof. Mary-Claire King's Laboratory at the University of Washington in – Seattle, USA.

3.3.4 Mutation Detection By Direct Sequencing

3.3.4.1 RNA Isolation (Trizol Reagent Protocol)

From the results of Exome Sequencing, there was a need to extract RNA from the blood samples, since the mutation was in a splice site. So we collected blood samples another time from all the family members, as described in section (**3.3.1**), and extracted RNA using Trizol reagent protocol from three samples of them; (one of the parents {WS-D1}, the normal sister {WS-D4}, one of the affected brothers {WS-D5}) . The blood samples went through DNA extraction steps (salt based method, section **3.3.2**), until reach to the step were the pellet was washed with 3 ml RBCs lysis buffer, then the pellets were resuspended in 100 µl of the RBCs lysis buffer and transferred into 1.5 ml DANase, RNAase free tubes. 1ml of Trizol was added and shook vigorously by hand for 1 min and incubated 5 min at RT, 200 µl of Chlorophorm were added to each tube and shook vigorously by hand for 15sec. and incubated 3 min at RT. Then centrifugation was done at full speed (14000 rpm) for 5min at 4C°. Three layers appear Pink, Milky, and Colorless. The transparent layer (RNA) was transferred into a new 1.5 ml tube carefully, avoid touching the interphase layer (DNA sits there), and incubated for 10 min at RT after adding 500 µl of Isopropanol. Centrifugation was done for 10 min at 14000 rpm, 4C°. The pellet then was washed once with 1 ml 75% ethanol and centrifugation was repeated. The supernatant was removed completely and the pellet was dried for 5 min at RT (tubes stay open). The pellet was redissolved in 25 µl RNAase free water. RNA concentration was measured by NanoDrop taking 1 µl from the suspension.

3.3.4.2 cDNA Synthesis

RNA samples were subjected to cDNA synthesis using the q-Script cDNA synthesis Kit, following the manufacture instructions. A total volume of the reaction was 20 µl, with RNA concentration of 50 ng/µl. The reaction mix composed of 4 µl of qScript Reaction Mix (5X), 1µl of qScript RT, RNA sample, Nuclease free water to complete the volume of the reaction to 20µl. The mixture was vortex gently and centrifuged 10 sec to collect contents, all the components of the kit were put on ice while use.

cDNA Synthesis Cycle Conditions

22 C°	5 min
42 C°	30 min
85 C°	5 min
4 C°	hold

3.3.4.3 Polymerase Chain Reaction (PCR)

A 373bp PCR fragment product including exon13, exon14, and exon15 of the cDNA encompassing the *ARFGEF2* gene mutation was amplified using pair of primers designed using primer3 software available at (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) (Figure 3.2) .

The Amplification process was done using our PCR machine (GeneAmp-PCR system 9700) from Applied Biosystem.

Primer	Primer Sequence	Tm
Forward Primer	5'- GAGGAAGAAAGGCCTGGAGT -3'	59.82 C°
Reverse Primer	5'- GTGCAGGAATTGGGCTATGT -3'	59.96 C°

Figure 3.2 Location of primers on the amplified exons

cDNA: Exons 13+14+15

GAGCTCAGCCTGAGGAAGAAAGGCCTGGAGTGCCTCGTGTCCATTCTCAAGTGCATGGTG

F ⟶

GAGTGGAGCAAAGACCTGTATGTGAATCCCAACCACCAGACCAGCCTCGGTCAGGAGAGG

CTCACGGATCAGGAAATAGGGGATGGGAAAGGCCTTGACATGGCAAGACGGTGTAGTGTG

ACGTCCATGGAGTCCACAGTGTCCTCGGGGACCCAGACAACTGTTCAGGATGACCCTGAG

CAATTTGAGGTCATCAAGCAACAAAAAGAAATCATTGAACACGGCATCGAGCTGTTCAAC

AAGAAACCCAAGAGGGGGATCCAGTTTCTCCAGGAGCAGGGCATGCTGGGAACGTCAGTT

GAAGACATAGCCCAATTCCTGCACCAGGAGGAGCGCCTGGATTCC

R ⟵

Table.3.1 Standard PCR Reaction Mix per 25µl of total volume

Reagent	Volume In µL
10X Buffer	2.50
Polymerase (supertherm polymerase)	0.25
Q-Solution	5.00
dNTPs (2.5Mm)	2.00
Forward primer	0.50
Reverse primer	0.50
Nuclease free H_2O	13.25
cDNA Template	1.00

PCR Cycling Conditions (T.D 55 °C)

94 C° 5 min

94 C° 30 sec
63 C° 30 sec * } X2
72 C° 45 sec **

94 C° 30 sec
61 C° 30 sec } X2
72 C° 45 sec

94 C° 30 sec
59 C° 30 sec } X2
72 C° 45 sec

94 C° 30 sec
57 C° 30 sec } X2
72 C° 45 sec

94 C° 30 sec
55 C° 30 sec } X35
72 C° 45 sec

72 C° 5 min

4 C° ∞

* Annealing temperature depends on the Tm of the primers used for PCR amplification.

** Extension time depends on the size of amplified product ~1Kb = 1min.

We used the Touch Down PCR method in order to increase the specificity of the PCR reaction and avoid amplification of nonspecific sequences by the primers. The PCR cycle starts with annealing temperature several degrees above the estimated T_m of the primers and then it is decreased gradually for the subsequent set of cycles until it reaches the calculated annealing temperature of the primers, at the highest annealing temperature is the more specific binding of the primers to the desired sequence, and the subsequent cycles of PCR can be performed upon the product to amplify those fragments.

3.3.4.4 Gel Electrophoresis

2% of agrose gel was prepared (depending on the size of PCR product) by dissolving the agrose in TAE buffer, then boiled until it became transparent, after short cooling Ethidium Bromide was added to a final concentration of 0.5 ug/ml. Three µL of PCR product were mixed with 3µL of loading buffer and were loaded onto the gel and run in 1X TAE running buffer at a constant voltage of 120V for 20-30 minutes, depending on the fragment size. The PCR products were then visualized under ultraviolet light and photographed using the (Molecular Imager®, Gel DC° TM Imaging System, BioRAD). Approximate PCR product size was determined by matching it to known sizes of the ladder bands (ThermoScientific, GeneRuler™, and 100bp plus DNA ladder) which were loaded in the first well (6µL).

3.3.4.5 Cleaning of PCR Products

Amplified fragments by PCR were purified using 2 enzymes (Exonuclease I and Antaractic Phosphatase). Exonuclease I digests single stranded DNA, and eliminates leftover primers that would otherwise interfere with the sequencing reaction, but leaves double-stranded PCR product intact. Antarctic Phosphatase removes 3' phosphate groups from single nucleotides, thus making leftover dNTPs from the PCR and Exonuclease I primer digestion inert during the sequencing reaction. The PCR products were purified directly from PCR reaction tubes (can be purified from the gel after electrophoresis) using the two enzymes in a total volume of 7 µl for the whole reaction (Table), following the program:

37 C°	30 min
80 C°	20 min
4 C°	∞

Table 3.2 :- Standard Master Mix of PCR products purification per 7 µl of total Volume

Reagent	Volume in µl
Exonuclease I	0.25
Antaractic Phosphatase	0.25
Nuclease free H_2O	1.5
PCR Product	5.0

3.3.4.6 DNA Sanger Sequencing

All PCR-amplified fragments were subjected to direct Sanger sequencing. Sequence amplification of PCR products were performed using GeneAmp-PCR system 9700 (Applied Biosystem) using either the forward or the reverse primer, according to the reaction mix shown in table 3.3.

Table 3.3:- Standard Sanger sequencing reaction mix per 16µl total volume

Reagent	Volume in µl
BigDye Terminator V1.1	0.3
64X Buffer	0.75
5X Buffer	1.5
Forward Primer	0.5
PCR Product	2.0
NF H_2O	10.95

Sequencing PCR Products Conditions

96 C°	1 min	
96 C°	10 sec	X25
50 C°	5 sec	
60 C°	4 min	
4 C°	10 min	

3.3.4.6.1 EDTA/ Ethanol Precipitation of Cycle-Sequenced PCR Products

Sequenced PCR reaction products were cleaned using EDTA/ Ethanol Precipitation method, while EDTA helps to stabilize extension products during precipitation, and helps to wash out unincorporated dyes from the completed reaction. We do this step in order to clean up the sequence PCR products from primers, and remove excess dNTPs, and unincorporated dyes. For each 20 µl of sequencing reactions 5 µl of 125 mM EDTA and 100 µl of absolute ethanol were added and mixed gently. The mixture was incubated in -80 C° freezer for 10min (or in -20C° for 30min). Then samples were centrifuged at 3800 RPM for 30 min at 4C°. The supernatant was discarded and 60 µl of 75% ethanol were added to each sample and then centrifuged directly at 3800 RPM for 20 min at 4C°. The supernatant was discarded and the PCR tubes or plate was inverted and spin up to 500 RPM for 1min to evaporate the ethanol. Then samples were put on 95C° hot plate for 5min in order to dry and remove any residual ethanol. Finally, 16 µl of super-diformamide were added on each sample and were put in 95C° hot plate again for 5 minutes in order to denaturize the two strands from each other, then placed directly on ice for at least 5min. Samples were loaded on the 96–well Optical Reaction Plate (Applied Biosystems) and run on 3130XL Genetic Analyzer (Applied Biosystems) for capillary electrophoresis.

CHAPTER FOUR

4. Results

4.1 Next Generation Sequencing Results

Exome sequencing was carried out on all the family members (both parents, the two affected brothers, and the normal sister). The results revealed a number of variants in the family.

First, C>T substitution in chr1: 1,560,564 C>T, yields MIB2 c.C1065T, however it was excluded because it wasn't present in the second affected brother (Figure 4.1).

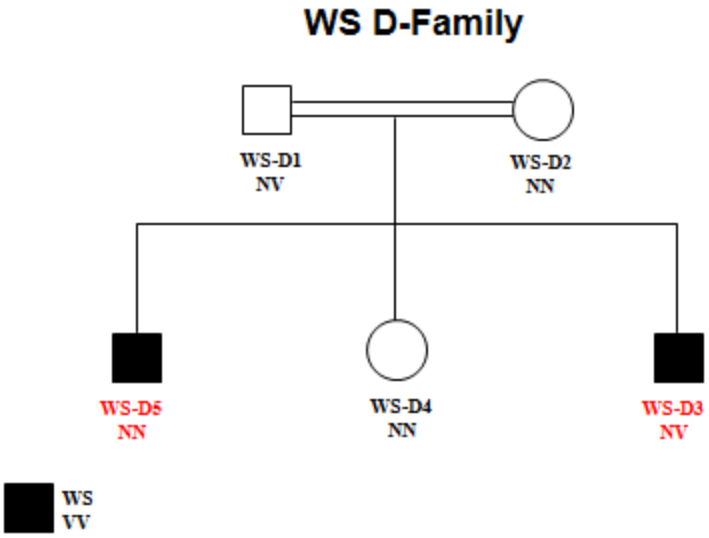


Figure 4.1. Segregation of the mutation chr1: 1,560,564 C>T, yields MIB2 c.C1065T within the family, one of the affected brother carrying the mutation while the other affected brother is wild type.

Second, a substitution variant was found in chromosome one chr1: 1,563,171 C>T, yields MIB2_R483W. (Figure 4.2), however it was excluded because all the family members considered normal for this mutation since the degree of certainty of the variant to cause the disease was very low.

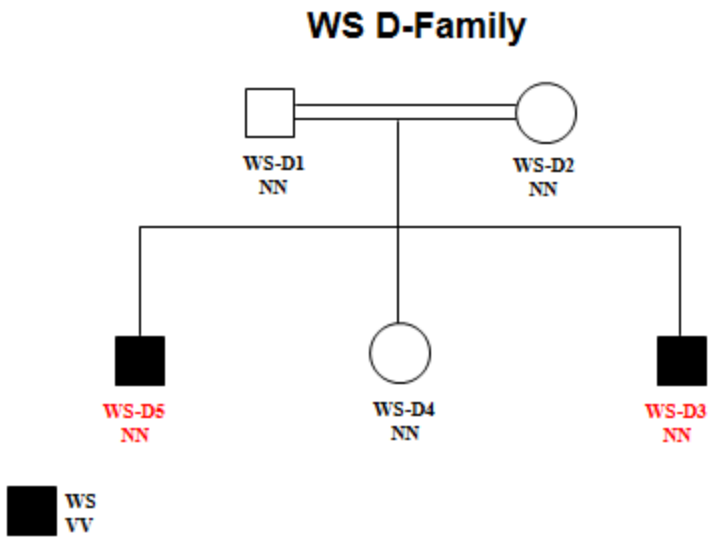
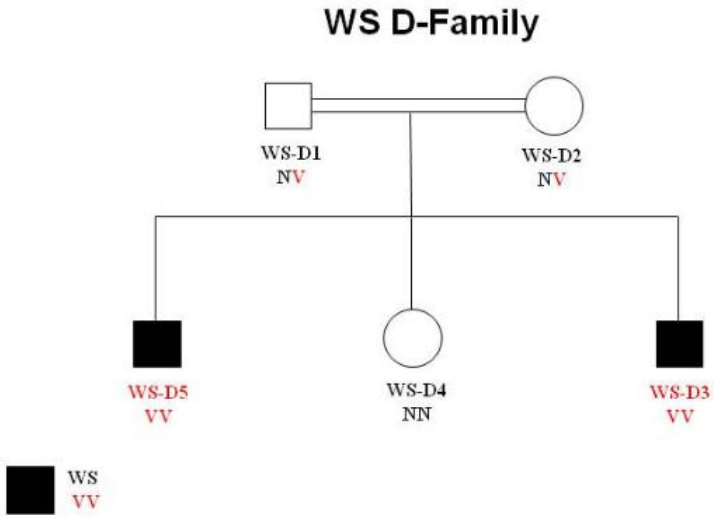


Figure 4.2. Segregation of the mutation chr1: 1,563,171 C>T, yields MIB2_R483W.

Third, A splice site mutation in chr20:47,592,737 G>A, yields *ARFGEF2* c.1985+ 1G>A, had been detected by Next generation sequencing, the degree of confidence for this mutation to be the causative of the disease was high, so we validated this mutation by Sanger sequencing. (figure 4.3).

Figure 4.3. Segregation of the mutation chr20:47,592,737 G>A, yields *ARFGEF2* c.1985+ 1G>A. The parents were in the heterozygous state for this mutation, and the two brothers were in the homozygous state while the sister was wild type for this mutation (based on Exome Sequencing results).

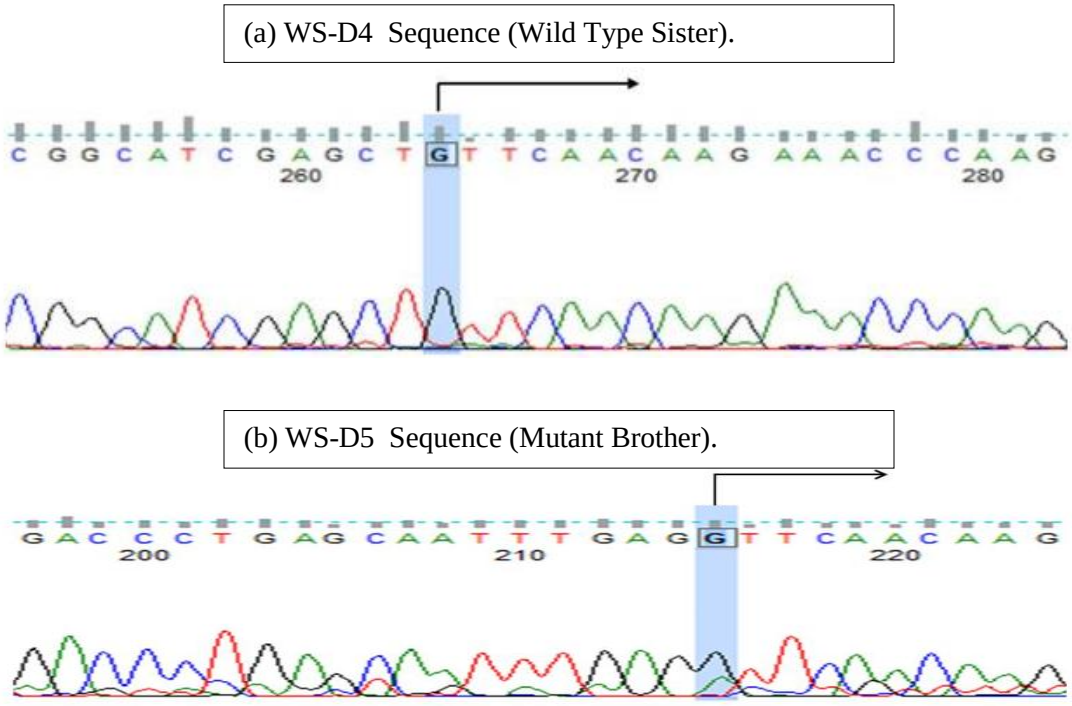


A large deletion 10kb located in chromosome X: 135,292,208- 135,302,713, had been detected but we didn't consider it as a pathogenic mutation of our family phenotypes, because X-linked periventricular heterotopia is associated with mutation located in FLNA gene, males having FLNA mutation experience much more severe symptoms of the disorder than females, but in most cases they die before birth.

4.2 Sanger Sequencing Results

Validation of the results by Sanger sequencing for the generated cDNA samples of the WS-D family (the father D1, one of the affected brothers D5, the normal sister D4), revealed that this mutation in *ARFGEF2* c.1985+ 1G>A cause change in the normal splicing site (first base in intron 15, G>A), that leads to activation of a new splice site inside the previous exon (exon14) and cause deletion of 44bp from it. The deletion of the nucleotides cause a change in the start locus of exon 15. (Figure 4.4)

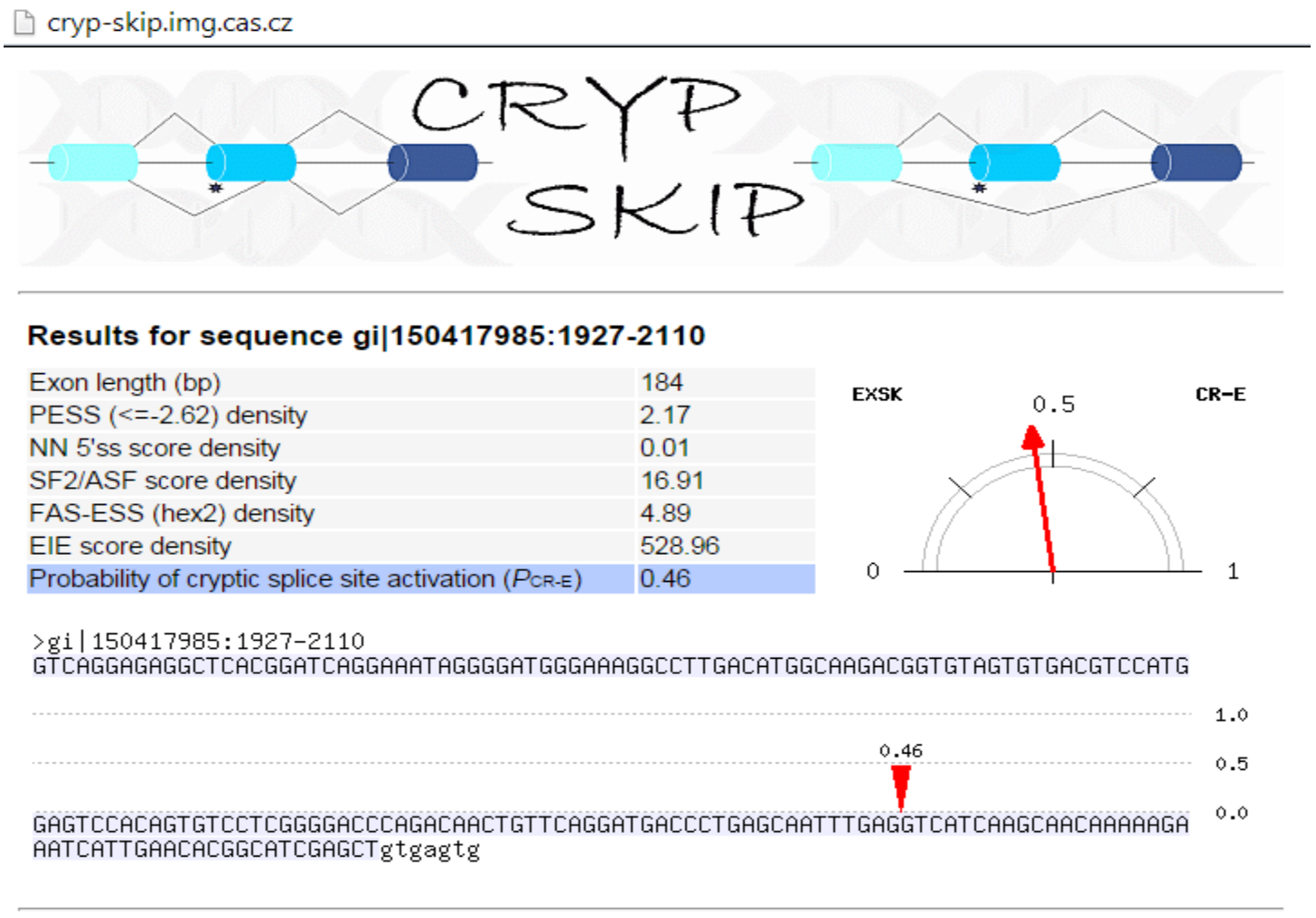
Figure 4.4 Sanger Sequencing Results:
(a) WS- D4: Wild Type Sister, Exon 15 starts GTTCA, and the bases before are for Exon 14 ends with AGCT. (b) WS- D5: Mutant Brother, Exon 15 starts GTTCA, Exon 14 ends with TGAG, so there is a missing base pairs cause change in Exon14 end.



To be more sure about our results and the activation of the cryptic splice site, a prediction tool available on line (http://cryp-skip.img.cas.cz/) was used and the results show a 0.46 probability of cryptic site activation inside exon 14, and show the location of the new splice site that leads to deletion of 44bp from exon 14. (Figure 4.5)

Figure 4.5. The probability of cryptic splice site activation and the location of it.

The upper case letters are for exon14 sequence, the lower case letters are for the intronic region.



4.3 Sanger sequencing for Healthy Controls Results

The identified mutation was subsequently verified and screened in 100 healthy, unaffected Palestinian controls from the West Bank using Sanger sequencing technique. The results showed no one of these individuals carrying the mutation either in homozygous or heterozygous state.

4.4 Sequence Analysis

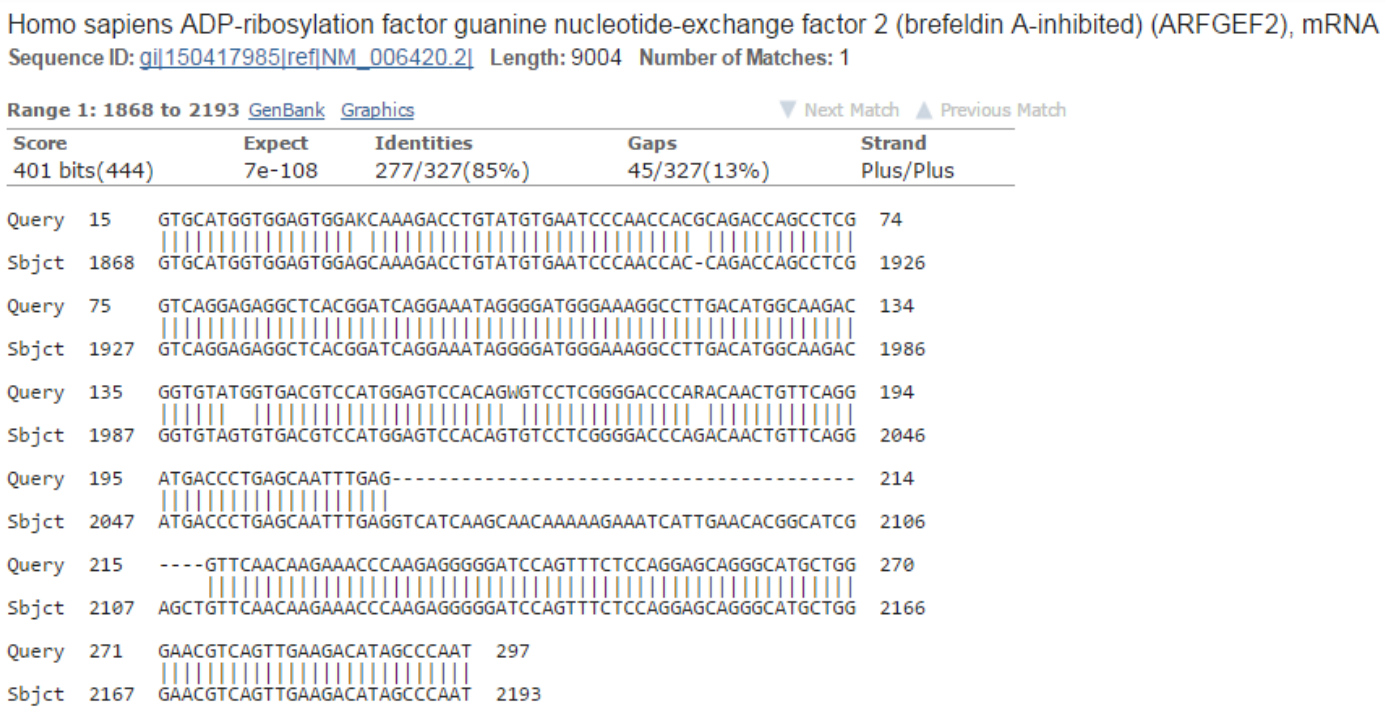
The sequences were analyzed using different online bioinformatics tools.

4.4.1 Basic Local Alignment (BLAST)

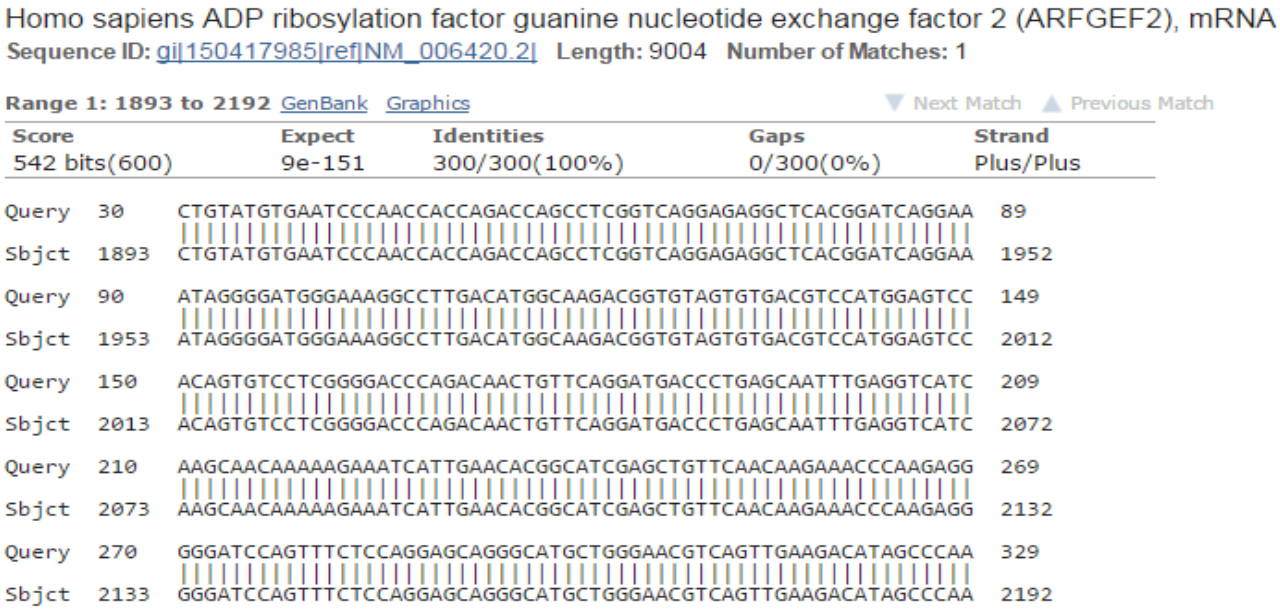
was done using ChromasPro software version 1.34 available online (http://www.technelysium.com.au/ChromasPro.html). The Ref Seq was obtained from NCBI web site (http://www.ncbi.nlm.nih.gov) and aligned with sequencing electropherograms. The results show the deletion of 44 nucleotide from exon 14 for the affected individual, whereas the wild type sister show no deletion at all (Figure 4.6).

Figure 4.6 Comparison of Basic Local Alignment (BLAST), between the sequences of the affected child (WS-D5) and the normal sister (WS-D4), versus the Refseq NM_0064202.2.

(a) BLAST between the obtained sequence of the affected child WS-D5 and the Refseq NM_006420.2, showing a gap of 44bp that are deleted because of the mutation.



(b) BLAST between the obtained sequence of the normal sister WS-D4 and the Refseq NM_006420.2.



4.4.2 SMART Analysis (Simple Modular Architecture Research Tool)

available online at (http://smart.embl-heidelberg.de), it was used to identify the domains that occur within the BIG 2 protein (encoded by the *ARFGEF2* gene) that can provide insights into the protein function. The analysis show that the normal sequence contains the sec7 domain which is required for proper protein transport through the Golgi, (Figure 4.7). The domain facilitates guanine nucleotide exchange on the small GTPases. Whereas the mutated sequence after the deletion of the 44 base pairs show that this domain is completely eliminated from the protein sequence (Figure 4.8), thus the protein seems to lose its normal function, and the disorder appears.

Figure 4.7 SMART analysis for the wild type sequence (WS-D4), showing the functional domains in the normal protein.

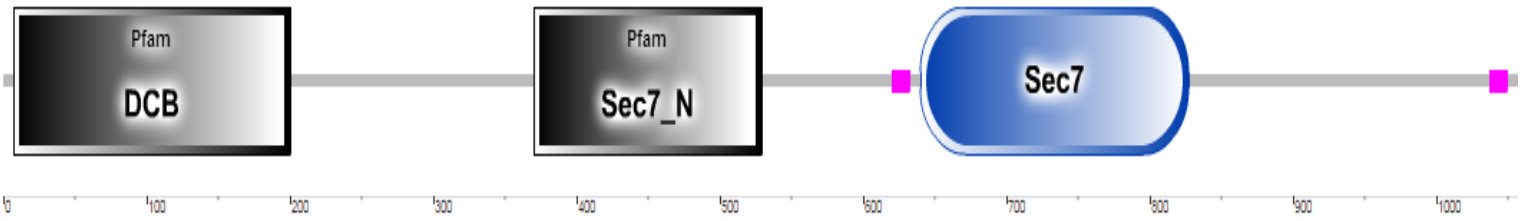
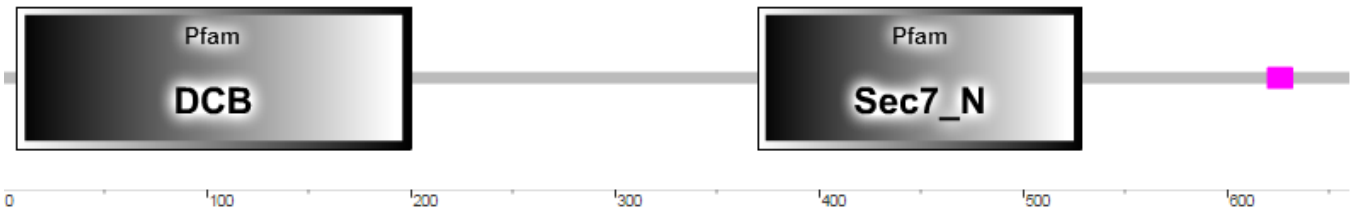


Figure 4.8 SMART analysis for the mutated sequence (WS-D4), showing elimination of the sec7 domain from the protein.



4.4.3. Open Reading Frame Changes

The sequences also were checked for the open reading frame changes as a result of the mutation, the analysis shows a frame shift change and a creation of premature stop codon in the mutated sequence WS-D5 that leads to shortened the normal protein sequence, and as a result a truncated protein appears with unknown function. (Figure 4.9). The whole sequence is in the appendix.

Figure 4.9. (a) Open reading frame for the wild type sister (WS-D4) sequence.

The protein contains (1785aa), the frame starts from position 153 and the stop codon is at location 5508 in the sequence.

```
153 atgcaggagagccagaccaagagcatgttcgtgtcccgggccctg
    M  Q  E  S  Q  T  K  S  M  F  V  S  R  A  L
198 gagaagatcctagccgacaaggaggtgaagcggccccagcactcc
    E  K  I  L  A  D  K  E  V  K  R  P  Q  H  S
243 cagctgcgcagggcctgccaggtggcgctcgatgaaattaaagca
    Q  L  R  R  A  C  Q  V  A  L  D  E  I  K  A
288 gaaatagaaaagcagaggcttggcactgctgcaccaccaaaggca
    E  I  E  K  Q  R  L  G  T  A  A  P  P  K  A
```

```
5373 gaaattatgcagtttgacctgatccctgagctccgagcagttctg
     E  I  M  Q  F  D  L  I  P  E  L  R  A  V  L
5418 cggaagttcttcctacggataggtgttgtgtataagatatggata
     R  K  F  F  L  R  I  G  V  V  Y  K  I  W  I
5463 ccagaagagccatcacaggtaccagcagcactgtcaccagtgtgg
     P  E  E  P  S  Q  V  P  A  A  L  S  P  V  W
5508 tag 5510
```

(b) Open reading frame for the mutant sequence (WS-D5).

The protein after the deletion of 44bp has (1140 aa), the frame starts from position 2047, and the stop codon is at position 5466 of the sequence.

```
2047 atgaccctgagcaatttgaggttcaacaagaaacccaagaggggg
     M  T  L  S  N  L  R  F  N  K  K  P  K  R  G
2092 atccagtttctccaggagcagggcatgctgggaacgtcagttgaa
     I  Q  F  L  Q  E  Q  G  M  L  G  T  S  V  E
2137 gacatagcccaattcctgcaccaggaggagcgcctggattccacc
     D  I  A  Q  F  L  H  Q  E  E  R  L  D  S  T
2182 caagtaggcgattttctgggagatagcgcaaggttcaacaaggag
     Q  V  G  D  F  L  G  D  S  A  R  F  N  K  E
```

```
5287 aagttcaaagcacatgcttcaatgtactacccctacttgtgtgaa
     K  F  K  A  H  A  S  M  Y  Y  P  Y  L  C  E
5332 attatgcagtttgacctgatccctgagctccgagcagttctgcgg
     I  M  Q  F  D  L  I  P  E  L  R  A  V  L  R
5377 aagttcttcctacggataggtgttgtgtataagatatggatacca
     K  F  F  L  R  I  G  V  V  Y  K  I  W  I  P
5422 gaagagccatcacaggtaccagcagcactgtcaccagtgtggtag 5466
     E  E  P  S  Q  V  P  A  A  L  S  P  V  W  *
```

CHAPTER FIVE

5. Discussion and Conclusion

5.1 Discussion

West Syndrome and related phenotypes are an age-dependent epilepsy syndromes that begin in infancy, mostly between 4 and 6 months of life (Mackay MT, 2004b), and with incidence of approximately 2-5 per 10,000 live births (Pavone P, 2014). West Syndrome was first described in 1841, by Dr. William James West (1793-1848) in the scientific journal "The Lancet" (West WJ, 1841). Many aspects of infantile spasms regarding the etiology, and pathophysiology, and the effect of treatment, remain mysterious.

Recently an increase evidences revealed that WS results from disturbances in the genetic pathways of brain development, specifically malformation in the cortical development stages (Berg AT, 2010). It has been found that these malformations cause epilepsy and mental retardation. Periventricular Heterotopia is one of these malformations, which is characterized by faulty migration of the postmitotic neurons from the ventricle zones toward the cortex leading to accumulation of abnormal nodules of neurons along the ventricle walls. Several factors involved in causing PH, these factors include: genetic mutations (e.g., FilaminA, *ARFGEF2* genes), the main causes genes, or extrinsic factors (e.g., irradiation, infection, injury) (Passarelli V, 2014).

Here we identified a rare splice site mutation in the *ARFGEF2* gene located on chromosome 20 chr20: 47592737, c.1958(+1) G>A in a Palestinian family, the family consists of healthy normal first degree parents, and a 11 months son affected with (sub-epindymal heterotopia, thin corpus callosum, global developmental delay, and hypsarrythmia), these features are all have been reported with WS. The substitution of a single base pair (G>A) at the 5`splice donor site of the *ARFGEF2* gene activated cryptic splice site at the previous exon (exon 14) that leads to deletion of 44 base pairs of that exon. It has been suggested that the deletion includes elimination of an important functional domain from the encoded protein called "Sec7", and creation of premature stop codon that leads to release of non functional truncated protein, as a result disturbing the function of the normal protein BIG2. Sec7 domain facilitates the function of BIG2 protein in

transporting the postmitotic neurons and other molecules from the interior of the cell to its surface through the Golgi apparatus complex.

So, following Sanger Sequencing and genotype segregation with the phenotype in the family, we came out to a conclusion that this mutation is the most likely cause the phenotype in the affected individuals in the family. Previous reports indicated that this mutation is rarely associated with WS patients, for our knowledge only two reports discussed the mutation (Banne E, 2013, Sheen VL, 2004a), the results of this research added a supportive evidence and make us more confident on the causality of this mutation to the disease and solved the genetic problem within a new Palestinian family having the same phenotype WS.

The pathophysiology of WS have been a complicated issue for scientists to explain, many hypothesis have been proposed but no clear evidence was introduced, one of these hypothesis proposed the association of cortical lesions and abnormal activities in the subcortical regions that show primary cortical dysfunction in WS patients. However, The process of cerebral cortex development is very critical in the brain development journey, it includes a various stages starting from neural stem cell proliferation, migration, and finally neuronal differentiation. The study of (JIE LU, 2006) proved that *ARFGEF2* mRNA and BIG2 protein is highly expressed in the ventricular (VZ) and subventricular zones (SVZ) of the brain, also at the periventricular region, during the embryonic brain development process, and (Sheen VL, 2004a) functional study demonstrated that *ARFGEF2* function is required for neuronal migration, and the BIG2 protein regulates vesicle trafficking from the trans-Golgi network (TGN) to the cell surface.

ARFGEF2 gene encodes brefeldin A inhibited guanine nucleotide exchange factor 2 (BIG2) protein, which is one of the three Guanine Exchange Factors (GEFs) expressed in the brain, especially during the period of neuronal migration and proliferation (Sheen VL, 2004a).
GEFs are activated by the replacement of bound GDP with GTP which is mediated by BIG2/Sec7 Arf1 GEF domain, that is responsible for its guanine exchange activity (Banne E, 2013), consequently the activated GEFs activate the ADP-ribosylation factors (ARFs), which regulate vesicle trafficking and the transport of molecules from the interior of the cell to its surface through the Golgi apparatus complex (Pacheco-Rodriguez G, 2002). These molecules could be adhesion molecules such as ß-catenin and E-cadherin which are required for the departure of neuronal precursors from the ventricular zone toward the cortex (Sheen VL, 2004a).

The facts emphasize the importance of neuronal cell migration stage in the development process of the embryo brain, and development of normal cerebral cortex, emphasize the importance of our findings in this study. The study solved the genetic bases of WS inheritance in this new Palestinian family and gave them a chance to get more healthy children's, which indeed had been adapted and the family succeeded by In Vitro Vertelization and PGD testing to get two healthy female babies. This successful trial add a strong evidence for the precise and correctness results of our study, and increase the valuable work of Bethlehem University researches, specially the Hereditary Genetic Labs.

Consanguineous marriages are traditional and respected in the Palestinian community and represent about 43% of all marriages in West Bank according to the latest statistics of the Palestinian Central Bureau of statistics (2009). It is recognized that the consanguineous unions lead to increased incidences of autosomal recessive disorders. The closer biological relationship between parents, the greater is the probability that their offspring will inherit identical copies of one or more detrimental recessive genes.

5.2 Conclusion

- Periventricular Heterotopia (PH), Microcephally, Mental Retardation phenotype in the WS-D family are a result of single base pair substitution (G>A) at the 5`splice donor site of the *ARFGEF2* gene which activated cryptic splice site at the previous exon (exon 14) that leads to deletion of 44 base pairs of that exon, and disturb the function of the encoded protein BIG2.
- The mutation segregates perfectly with the phenotypes in this family under an autosomal recessive mode of inheritance.
- The carrier frequency for this mutation in the tested healthy Palestinian population were zero%.

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Appendices

Appendix A

Protein Sequences (Wild Type and Mutated)

> normal

MQESQTKSMFVSRALEKILADKEVKRPQHSQLRRACQVALDEIKAEIEKQRLGTAAPPKANFIEA
DKYFLPFELACQSKSPRVVSTSLDCLQKLIAYGHITGNAPDSGAPGKRLIDRIVETICSCFQGPQTD
EGVQLQIIKALLTAVTSPHIEIHEGTILQTVRTCYNIYLASKNLINQTTAKATLTQMLNVIFTRMEN
QVLQEARELEKPIQSKPQSPVIQAAAVSPKFVRLKHSQAQSKPTTPEKTDLTNGEHARSDSGKVS
TENGDAPRERGSSLSGTDDGAQEVVKDILEDVVTSAIKEAAEKHGLTEPERVLGELECQECAIPP
GVDENSQTNGIADDRQSLSSADNLESDAQGHQVAARFSHVLQKDAFLVFRSLCKLSMKPLGEGP
PDPKSHELRSKVVSLQLLLSVLQNAGPVFRTHEMFINAIKQYLCVALSKNGVSSVPDVFELSLAIF
LTLLSNFKMHLKMQIEVFFKEIFLNILETSTSSFEHRWMVIQTLTRICADAQCVVDIYVNYDCDLN
AANIFERLVNDLSKIAQGRSGHELGMTPLQELSLRKKGLECLVSILKCMVEWSKDLYVNPNHQT
SLGQERLTDQEIGDGKGLDMARRCSVTSMESTVSSGTQTTVQDDPEQFEVIKQQKEIIEHGIELFN
KKPKRGIQFLQEQGMLGTSVEDIAQFLHQEERLDSTQVGDFLGDSARFNKEVMYAYVDQLDFCE
KEFVSALRTFLEGFRLPGEAQKIDRLMEKFAARYIECNQGQTLFASADTAYVLAYSIIMLTTDLHS
PQVKNKMTKEQYIKMNRGINDSKDLPEEYLSSIYEEIEGKKIAMKETKELTIATKSTKQNVASEK
QRRLLYNLEMEQMAKTAKALMEAVSHAKAPFTSATHLDHVRPMFKLVWTPLLAAYSIGLQNC
DDTEVASLCLEGIRCAIRIACIFGMQLERDAYVQALARFSLLTASSSITEMKQKNIDTIKTLITVAH
TDGNYLGNSWHEILKCISQLELAQLIGTGVKTRYLSGSGREREGSLKGHTLAGEEFMGLGLGNL
VSGGVDKRQMASFQESVGETSSQSVVVAVDRIFTGSTRLDGNAIVDFVRWLCAVSMDELASPH
HPRMFSLQKIVEISYYNMNRIRLQWSRIWHVIGDHFNKVGCNPNEDVAIFAVDSLRQLSMKFLEK
GELANFRFQKDFLRPFEHIMKKNRSPTIRDMAIRCIAQMVNSQAANIRSGWKNIFAVFHQAASDH
DGNIVELAFQTTCHIVTTIFQHHFPAAIDSFQDAVKCLSEFACNAAFPDTSMEAIRLIRFCGKYVSE
RPRVLQEYTSDDMNVAPGDRVWVRGWFPILFELSCIINRCKLDVRTRGLTVMFEIMKSYGHTFE
KHWWQDLFRIVFRIFDNMKLPEQLSEKSEWMTTTCNHALYAICDVFTQFYEALNEVLLSDVFAQ
LQWCVKQDNEQLARSGTNCLENLVISNGEKFSPEVWDETCNCMLDIFKTTIPHVLLTWRPVGME
EDSSEKHLDVDLDRQSLSSIDKNPSERGQSQLSNPTDDSWKGRPYANQKLFASLLIKCVVQLELI
QTIDNIVFYPATSKKEDAEHMVAAQQDTLDADIHIETEDQGMYKYMSSQHLFKLLDCLQESHSF
SKAFNSNYEQRTVLWRAGFKGKSKPNLLKQETSSLACCLRILFRMYVDENRRDSWEEIQQRLLT
VCSEALAYFITVNSESHREAWTSLLLLLLTKTLKINDEKFKAHASMYYPYLCEIMQFDLIPELRAV
LRKFFLRIGVVYKIWIPEEPSQVPAALSPVW*

>mutated

MQESQTKSMFVSRALEKILADKEVKRPQHSQLRRACQVALDEIKAEIEKQRLGTAAPPKANFIEA
DKYFLPFELACQSKSPRVVSTSLDCLQKLIAYGHITGNAPDSGAPGKRLIDRIVETICSCFQGPQTD
EGVQLQIIKALLTAVTSPHIEIHEGTILQTVRTCYNIYLASKNLINQTTAKATLTQMLNVIFTRMEN
QVLQEARELEKPIQSKPQSPVIQAAAVSPKFVRLKHSQAQSKPTTPEKTDLTNGEHARSDSGKVS
TENGDAPRERGSSLSGTDDGAQEVVKDILEDVVTSAIKEAAEKHGLTEPERVLGELECQECAIPP
GVDENSQTNGIADDRQSLSSADNLESDAQGHQVAARFSHVLQKDAFLVFRSLCKLSMKPLGEGP
PDPKSHELRSKVVSLQLLLSVLQNAGPVFRTHEMFINAIKQYLCVALSKNGVSSVPDVFELSLAIF
LTLLSNFKMHLKMQIEVFFKEIFLNILETSTSSFEHRWMVIQTLTRICADAQCVVDIYVNYDCDLN
AANIFERLVNDLSKIAQGRSGHELGMTPLQELSLRKKGLECLVSILKCMVEWSKDLYVNPNHQT
SLGQERLTDQEIGDGKGLDMARRCSVTSMESTVSSGTQTTVQDDPEQFEVQQETQEGDPVSPGA
GHAGNVS*

Appendix B

WS-D Family Sequences

D1- Father

CCGCAGTGTACTTCTAGTGCATGTGGAGGGTCAAAGCCCTGTTGAATCCCTTACTGC
TTCCACCTCTGTCAGACCTGGCTCACGTGTCAKGACTATAGATGGAAAGCCCTTGAC
ATTGATAGACGGTGTATGGTGACGTCCATGTACCCACAGTGTCCTCGTATCCCAACA
ACTGTAAGGATGACCCTGASCATTTGAGGCTTTCTTTCTGACAAATAAATAATTGAA
CACGGCATCTAGCTGTCTATAAGAACCCAAGAGGGGGATCCGGTTTCTCCAGAGCA
GCCATGCTGAAACGTCAGGAAGACATAGCCCAATCGGCAAAGGCCTTTTACGGCTG
ATATTGTAAGCAATCTGTGTGCATGTGTACATATGCATCTGTTTTCAGGGAAAGAAA
AGCTGTTAAAGCAATTAAGTATGAATTCTTGCTACTACTTAAWATAAACCTTTAGAA
ATTATTGACCTATTAAAGGGAACTGACTTAGCCAATCCA

D4- Sister

CACATGTACTTGTCTTGATGTCGGACAAGCTGTATGTGAATCCCAACCACCAGACCA
GCCTCGGTCAGGAGAGGCTCACGGATCAGGAAATAGGGGATGGGAAAGGCCTTGAC
ATGGCAAGACGGTGTAGTGTGACGTCCATGGAGTCCACAGTGTCCTCGGGGACCCA
GACAACTGTTCAGGATGACCCTGAGCAATTTGAGGTCATCAAGCAACAAAAAGAAA
TCATTGAACACGGCATCGAGCTGTTCAACAAGAAACCCAAGAGGGGGATCCAGTTT
CTCCAGGAGCAGGGCATGCTGGGAACGTCAGTTGAAGACATAGCCCAACCCTACAC
GGTGAAATGAAAGCAATCTGTGTGCATGTGTACATATGCATCTGTTTTCAGGGAAAG
AAAAGCTGTTAAAGCCAATACGAATTCTTGCTACTACTTTAATATTAACCTTTAAGT
GAAATTTATTGACACTATTACAAATGTGGCAACTGACATAGCCCAATTCCGCA

D5- Affected child

CCAGTCTTCTGTGCATGGTGGAGTGGACAAAGACCTGTATGTGAATCCCAACCACCA
ACCAGCCTCGGTCAGGAGAGGCTCACGGATCAGGAAATAGGGGATGGGAAAGGCCT
TGACATGGCAAGACGGTGTATGTGACGTCCATGGAGTCCACAGGTCCTCGGGGACC
CAACAACTGTTCAGGATGACCCTGAGCAATTTGAGGTTCAACAAGAAACCCAAGAG
GGGGATCCAGTTTCTCCAGGAGCAGGGCATGCTGGGAACGTCAGTTGAAACATAGC
CCAATCCGAAAAAAAAAAAAAGGCCCAAAGCGGCCGACTTCAATGTGCAAAGGCTTA
GGTGTGGATATTGTAAGCAATCTGTGTGCATGTGTACATATGCATCTGTTTTCAGGG
AAAAGAAAAAGCTGTTAAAAGCCAATTACTTGAATTTTGCTACTACTTTTATAATTA
ACCTTTAAGGAAAATTTATTGGACCCTTATACCAAGGGGAACTGGCTAACCAAAGA
AAATGGATTGTTTTGGGGGCTTKTCTGGCCGATGAATACTTTTTTGGAACGTCCCTTG
CCCGCCTGGTTTAAAAAGT