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Screening for three mutations of genetic disorders in consanguineous Palestinian families with retinopathy

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Screening for three mutations of genetic disorders in consanguineous Palestinian families with retinopathy

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Thesis Approval

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Dedication

This thesis is dedicated to my father, my mother, my brother, my sisters and to Dr. Ghassan Balousha who supported me all the way since the beginning of my studies and was a great source of motivation and inspiration.

Great thanks for your support

Declaration

I certify that this thesis submitted for the degree of Master is the result of my own research, except where otherwise acknowledged, and that this thesis (or any part of it) has not been submitted for a higher degree to any other university or Institution.

Signed: _____

Mohammed Mousa Mostafa Falana

Date: 15 / 5 / 2017

Acknowledgment

I dedicate my thesis to all those who offered me their time, effort and support.To who have lighted a candle in my life my father and my mother. Special thanks to my mother who gave me hope and strength in my studies has stayed up nights for me.

Not forget my father, who gave me every minute of his time to make me happy.To all my family members who have been a source of endless support and encouragement.

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Abstract

Consanguinity is a deeply rooted cultural trait in Middle Eastern societies, especially in the Arab rural populations due to socio-cultural factors like maintenance of family structure, property or ease of marital arrangements.Thus Autosomal recessive retinitis pigmentosa genes are highly associated with consanguineous families.

The prevalence of autosomal hereditary recessive diseases is high in Middle Eastern Arab societies, mainly due to the high rate of consanguineous marriages. In clinical genetics, this includes relationships of second cousins or closer.

we designed an ARMS (Amplification - Refractory Mutation System) technique for the three autosomal recessive genes FAM161, CNGA3, and CRB1.In this study we developed an ARMS technique for the three autosomal recessive genes FAM161, CNGA3, and CRB1 in 18 Palestinian families with consanguinious marriages.We succeeded in designing cheaper methods for detecting these genes.

ARMS is a simple method for detecting any mutation involving single base changes or small deletions. ARMS is based on the use of sequence-specific PCR primers that allow amplification of target DNA sequence only when the target allele is contained within the sample. ARMS allows genotyping solely by inspection of reaction mixtures after agarose gel electrophoresis. The system is simple, reliable and non-isotopic. It will clearly distinguish heterozygotes at a locus from homozygotes for either allele. It is a economic approach that can use standard laboratory equipment.

Another use is the identification of SNPs restriction fragment length polymorphism (RFLP), in which a difference in homologous DNA sequences can be detected by the presence of fragments of different lengths after digestion of the DNA samples in question with specific restriction endonucleases. The presence or absence of the restriction site can be determined by agarose gel electrophoresis of the digested fragments and visualization using ethidium bromide staining and ultraviolet (UV) illumination .

During the study period, 18 families with ophthalmological diseases on a consanguineous background in Arab villages of Palestine were recruited , and ARMS technique used to identify genetic mutations.

Two mutations out of the three propsed could be identified : FAM161 (c.1003C>T/p.R335X), CNGA3 (c.985G>T - Gly329Cys) and CRB1 gene wasn‘t identifed by this technique. The results demonstrate that we succeeded in designing a cheap and timesaving method for detecting these genes.

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Table of Abbreviations

Abbreviation	Full Word
ARMS	Amplification Refractory Mutation System.
FAM161A	Family With Sequence Similarity 161 Member A.
CNGA3	Cyclic Necleotide Gated Cation Channels A3.
CRB1	Human Cyclic Sapiens Crumbs Family Member1.
RFLP	Restriction Fragment Length Polymorphism .
F	Inbreeding Coefficient.
CTGA	Catalogue For Transmission Genetic In Arabs.
RBE	Retinal Pigment Epithelium.
FA	Fundus Albipunctatus.
RP	Retinitis Pigmentosa.
LCA	Leber Congenital Amaurosis.
CNG	Cyclic Nucleotide Gated.
ACHM	Achromatopsia.
AQU	Al-Quds University.

Chapter 1

1. Introduction

Consanguinity is derived from two Latin words "con" meaning common, or of the same and "sanguineous" meaning blood, hence, referring to a relationship between two people who share a common ancestor. In other words, consanguineous marriage refers to the union contracted between biologically related individuals; union between couples who are related as second cousins or closer (Alwan and Modell, 1997; Modell and Darr, 2002).

1.1 Consanguinity in World Populations

Consanguineous marriages have been practiced since the early existence of modern humans. At present, about 20% of world populations live in communities with a preference for consanguineous marriage (Modell and Darr, 2002). Consanguinity rates vary from one population to another depending on religion, culture and geography. Noticeably, many Arab countries display some of the highest rates of consanguineous marriages in the world ranging around 20-50% of all marriages, and specifically favoring first cousin marriages with average rates of about 20-30% as showed in (Fig. 1.1).

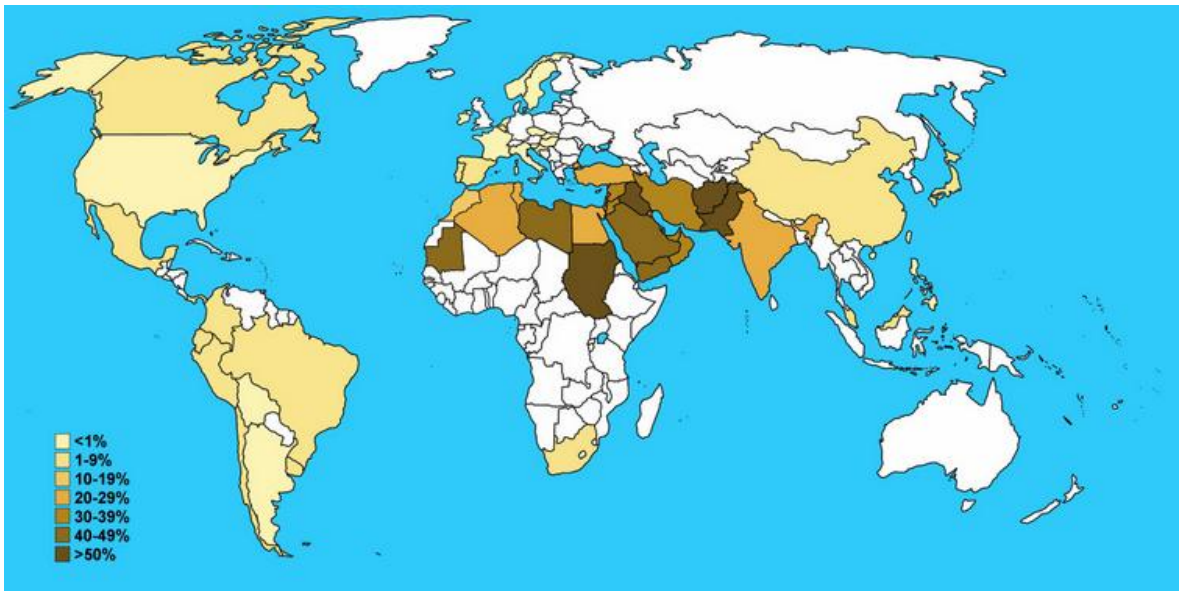

Figure 1.1: Schematic representation of consanguineous marriage rates worldwide (Tadmouri, 2008).

1.2 Consanguinity in Arab Populations

Consanguinity rates show wide variations among Arab countries, as well as within the same country as showed in (Table 1.1). However, reports from Arab countries on consanguinity rates may sometimes include marriages between third cousins or far relatives within the consanguineous category. Although this discrepancy affects the total consanguinity rate, it does not markedly alter the average inbreeding coefficient (Tadmouri, 2008).

Therefore, for comparison of consanguinity rates among populations, two parameters are best used; the mean inbreeding coefficient (F) and marriages between first cousins. However, Arab societies have a long tradition of consanguinity, and the cumulative estimate of (F) may exceed the estimated value which is calculated for a single generation (Bittles et al., 1993).

Table 1.1: Consanguinity rates in arab population (Tadmouri, 2008). IC: First cousin, >IC : Second cousin

Country	>1C, 1C	Overall consanguinity
Algeria	11.3	22.6-34
Bahrain	24.5	39.4-45.5
Egypt	14.3-23.2	20.9-32.8
Egypt (Nubia)	39-47.2	60.5-80.4
Iraq	29-33	47-60
Jordan	19.5-39	28.5-63.7
Kuwait	16.9-31.7	22.5-64.3
Lebanon	6.7-31.6	12.8-42
Libya		48.4
Mauritania		47.2
Morocco	8.6-10	19.9-28
Oman	24.1	56.3
Palestine	13.6-34.2	17.5-66.3
Qatar	34.8	54

Consanguineous marriages are generally thought to be more stable than marriages between non-relatives, though there are no studies to compare divorce rates of consanguineous and non-consanguineous marriages among Arabs. It is generally believed that the husband's family would side with the consanguineous wife in marital disputes since she is considered part of the extended family. When there are children with disabilities, more family members share in caring for these children (Khlat, 1988).

1.3 Consanguinity in Palestine:

Many Arab countries display some of the highest rates of consanguineous marriage in the world ranges between 20-50% of all marriages (Bittles, 2008). Among Palestinian Arabs the rate of consanguinity is very high and some 44.3% of the marriages are between relatives (22.6% of them between first cousins). Secular changes in the consanguinity rates have been noticed in some Arab populations. In Jordan (Hamamy et al., 2005), Palestine (Sharkia et al., 2008), Egypt (Hafez et al., 1983) as showed in (Table 1.1),the frequency of consanguineous marriage is decreasing. Several factors may be playing a role in the decrease of consanguinity rates in Palestine . Amongst these factors are higher female education levels, declining fertility resulting in lower number of suitable relatives to marry, more mobility from rural to urban settings, and the improving economic status of families (Bittles et al., 1993).

Genetic analysis shows that Palestinians are of mainly of Levantine ancestry, most similar to Syrians, Lebanese, Jordanians, Cretans and Egyptians. The Palestinian population was 3,761,646 in 2007; of these, 2,345,107 are residents of the West Bank, including 208,000 in East Jerusalem. High consanguinity rates have been reported in Muslim Arabs (44 %) and Druze (47 %). There is little available data regarding genetic disorders in the Palestinian territories; however, there are hundreds of genetic disorders among Palestinians living in the West Bank and Gaza Strip (Hussein et al., 2010).

The Palestinian population is characterized by high consanguinity rates (about 44 %), large family size (5.5 children per family), and pregnancies in women over the age of 45. These factors result in the high incidence of genetic disorders, particularly the increase in rare recessive genetic disorders (Hussein et al., 2010).

1.4 Negative Effects of Consanguinity on Reproductive Health

1.4.1 Consanguinity and Congenital Malformations:

Approximately 3-5% of all live newborns have a medically significant birth defect. The recent report by March of Dimes estimated birth defects to be >69.9/1000 live births in most Arab countries, as opposed to <52.1/1000 live births in Europe, North America and Australia (Christianson et al., 2006) and lower observed rates of 12.5/1000 defects births was registered in the Kuwait (Madi et al., 2005). A strong association of consanguinity with poverty and low education points to the need to avoid exposure to environmental hazards in these families (Harlap et al., 2008). In addition, the risk of birth defects in first cousin marriages may be estimated to be 2-2.5 times the general population rate, mainly due to the expression of autosomal recessive disorder (Bennett et al., 2002; Murshid et al., 2000; Murshid, 2000). Consanguinity rates were noted to be higher among parents of newborns with congenital hydrocephalus and neural tube defects than in the general population in some studies, but not in the others (Khlat and Khoury, 1991). A positive association of consanguinity with cleft lip and palate was reported in Palestine (Bittles and Black, 2010).

1.4.2 Consanguinity and Postnatal Mortality:

Countries with high rates of consanguineous marriages generally report smaller effects of consanguinity on mortality than populations with low rates of consanguineous marriages. This finding is not surprising, given the limited control for concomitant variables such as socioeconomic status, maternal education, birth intervals, and public health facilities and practices in most consanguinity studies (Khlat and Khoury, 1991).

The most recent mortality estimate derived from a multinational study of over 600,000 pregnancies and live births is that first cousin progeny experience 4.4% more pre-reproductive deaths than the offspring of non-consanguineous unions (Bittles and Neel, 1994).

Most studies among Arabs have indicated that postnatal mortality is higher among offspring of consanguineous parents than among non-related parents.The increased postnatal mortality among the offspring of consanguineous parents may be related to the effect of deleterious recessive genes and multi-gene complexes inherited from a common ancestor (Khlat, 1988).

1.4.3 Consanguinity and Autosomal Recessive Disorders:

In offering preconception counseling for consanguinity, it is crucial to distinguish between families with a known genetic or inherited disorder and those with unknown disorder by taking a detailed family history and constructing a four-generation pedigree (Bennett et al., 2002).

Reports have shown that in certain clinical settings, practice guidelines regarding collecting information on consanguinity as part of family history are not available despite the relevance of such history in identifying at risk pregnancies. Specific questions addressed to the couple could help in eliciting the presence of a genetic or hereditary disorder in the extended family (Bishop et al., 2008). These could include inquiry about the presence of any of the following in blood relatives:

- Birth defects or congenital anomalies.
- Early hearing impairment.
- Early vision impairment.
- Mental retardation or learning disability.
- Developmental delay or failure to thrive.
- An inherited blood disorder.
- Unexplained neonatal or infant death in offspring.

Inbreeding depression in human health is the major focus of interest in hereditary and medical genetics. Inbreeding increases the coefficient of autosomal recessive genes, which in turn increases the probability of the expression of pathogenic mutations in a homozygous state. Autosomal recessive disorders occur in individuals who have two copies of an allele for a particular recessive genetic mutation (Zhang, 2005).

An analysis of data in the Catalogue for Transmission Genetics in Arabs (CTGA), a database on genetic disorders in Arab populations maintained by the Centre for Arab Genomic Studies, indicates that in contrast to international databases, the overwhelming proportion of the disorders in the CTGA Database follow a recessive mode of inheritance (63%) compared to the smaller proportion of dominantly inherited traits (27%). A detailed study of countries for which surveys on the occurrence of genetic disorders have been completed (United Arab Emirates, Bahrain, and Oman) indicates that recessive disorders are more in number than the dominant ones (Tadmouri, 2008).

As explained above, given the high rates of consanguinity in these countries, this pattern is not entirely surprising. In a study from Jordan, the consanguinity rate among parents affected by autosomal recessive conditions was around 85%, while it was 25-30% among parents affected by other genetic conditions such as X-linked recessive, chromosomal and autosomal dominant (Hamamy et al., 2005).

1.5 Autosomal Recessive disorder and Ophthalmic Disease in Palestine

As a consequence of the high consanguinity rate among the Palestinian, many recessive disorders are present with a relatively high frequency. In 1997, a survey of 2000 different Palestinian Arab families who visited genetic clinic in palestine, 601 an autosomal recessive disease were diagnosed. The distribution of these disorders was not uniform and some disorders such as Krabbe disease were found at high frequency in only a small part of the population. For other disorders, a high prevalence was also reported among Palestinian Arabs living in other regions, for example, thalassaemia, Bardet-Biedl syndrome, Meckel syndrome, retinopathy, and recessive osteopetrosis (Genet, 1997).

In this study, we were interested in inherited ophthalmic disorders observed in consanguineous marriage. However, a special attention was paid to ophthalmic retinal disorders "retinopathy" , many recent publications illustrating novel mutations in genes expressed in retinal epithelium cells that eventually leads to blindness and loss of vision in consanguineous Palestine family.

1.6 Anatomy of the Eye

Eyes are sense organs that providing organisms with vision. The ability to process visual details, as well as enabling several photo response functions that are independent of vision. Eyes detect light and convert it into electro-chemical impulses in neurons. In higher organisms, the eye is a complex optical system which collects light from the surrounding environment, regulates its intensity through a diaphragm, focuses it through an adjustable assembly of lenses to form an image, converts this image into a set of electrical signals, and transmits these signals to the brain through complex neural pathways that connect the eye via the optic nerve to the visual cortex and other areas of the brain (Land et al.,1992).

The eye has three layers or coats and three fluid containing structures as shown in (Fig. 1.2). The outer layer is the fibrous layer, consisting of the cornea, sclera and lamina cribrosa. The middle vascular layer consists of the iris, ciliary body (consisting of the pars plicata and pars plana) and choroids. Finally, the inner nervous layer consists of the pigment epithelium of the retina, retinal photoreceptors and retinal neurons (Galloway et al., 2006).

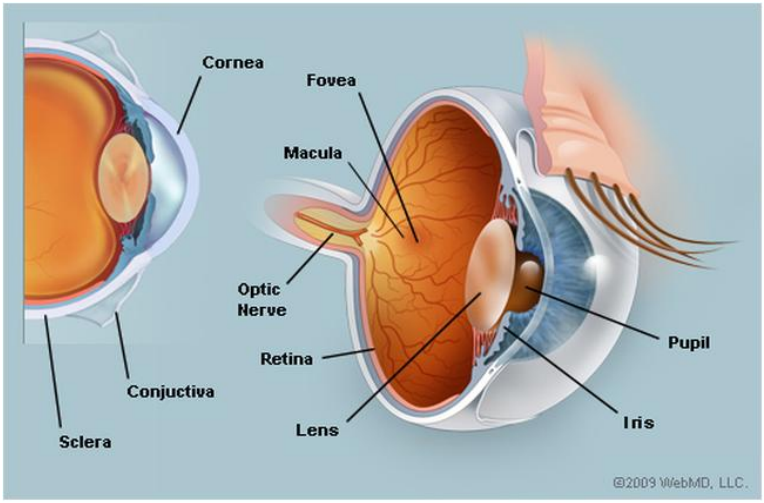

Figure 1.2: Anatomy of eye. http://www.webmd.com/eye-health/picture-of-the-eyes#1

1.7 Anatomy of the Retina

Normal retina contains neuroretina and retinal pigment epithelium. The neuroretina consists of outer and inner segments of photoreceptors (rods and cones), external limiting membrane, outer nuclear layer, outer plexiform layer, inner nuclear layer, inner plexiform layer, ganglion cell layer, nerve fiber layer and internal limiting membrane as in (Fig. 1.3) and (Fig. 1.4) (Melloni, 1971; Gupta et al., 2015).

There is a broad spectrum of retinal pathology including congenital abnormalities, dystrophies, degenerations (notably age-related macular degeneration), retinal vascular diseases, toxicities, inflammatory diseases, neoplasms, trauma and retinal involvement of systemic diseases (Gupta et al., 2015).

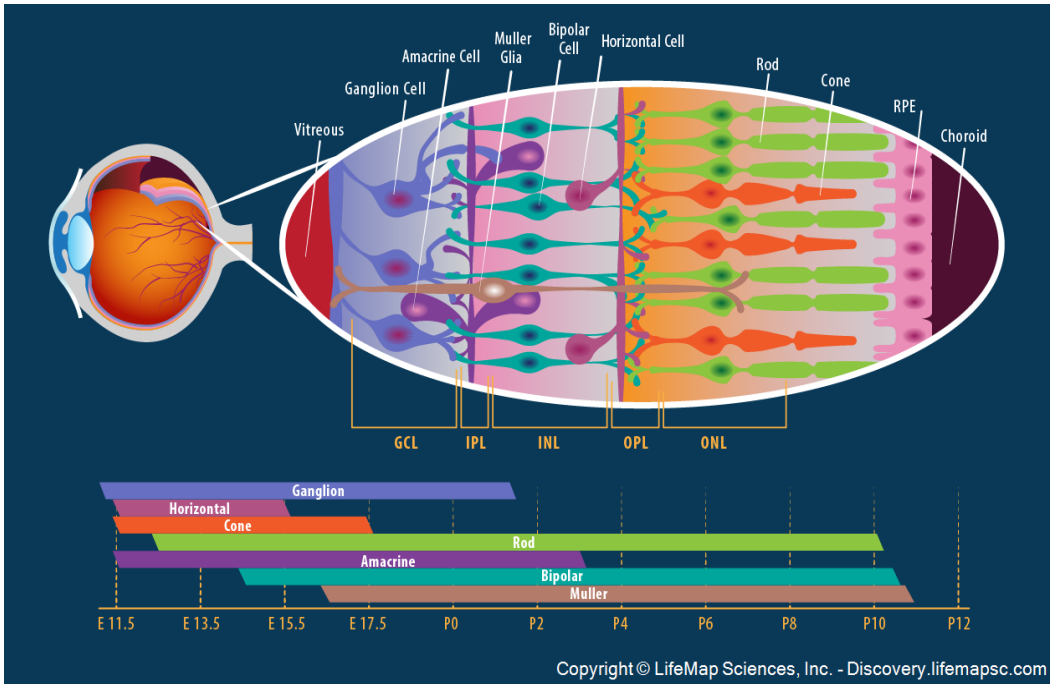

Figure 1.3: A schematic section through the human eye with a schematic enlargement of the retina (Melloni, 1971).

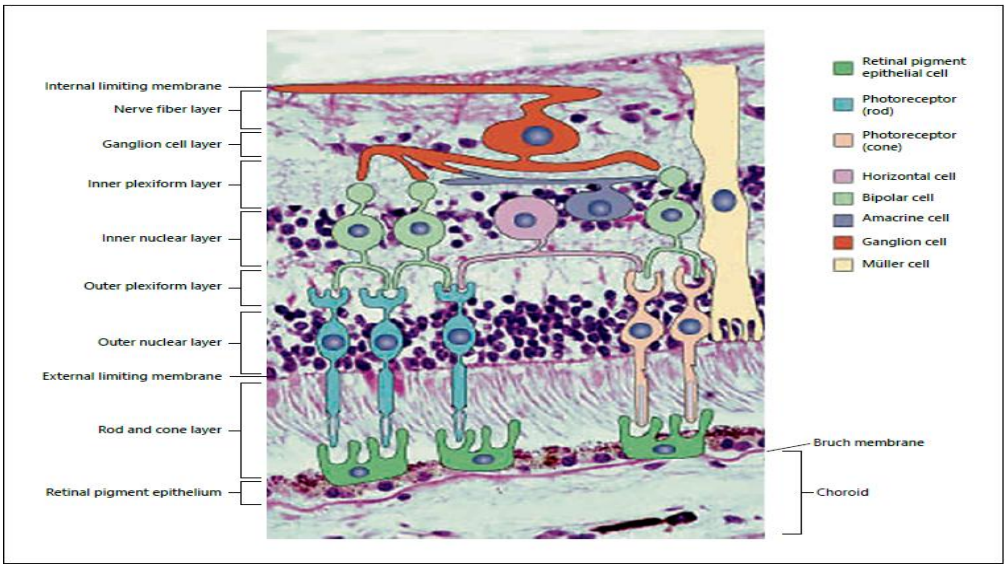

Figure 1.4 : Photomicrograph of the retina combined with a diagram of pertinent retinal cells (Gupta et al., 2015).

The fundus can be examined by ophthalmoscopy and/or fundus photography (Cassin and Solomon, 1990). The color of the fundus varies both between and within species.It is the interior surface of the eye opposite the lens and includes the retina, optic disc, macula. In one study of primates the retina is blue, green, yellow, orange, and red; only the human fundus (from a lightly pigmented blond person) is red as shown in (Fig. 1.5). The major differences noted among the primate species were size and regularity of the border of macular area, size and shape of the optic disc, and pigmentation of the retina (Wolin and Massopust, 1967).

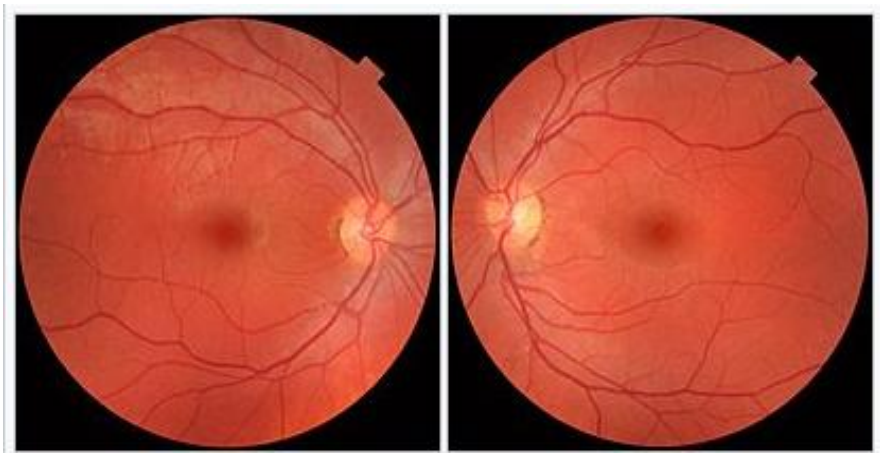

Figure 1.5: Fundus photographs of the right eye (left image) and left eye (right image). http://www.2c.ie/services/digital-retinal-photography.

Eye development and photoreceptor maintenance are dependent on the retinal pigment epithelium (RPE), a thin layer of cells that underlie the neural retina (Leung et al., 2007). The (Fig. 1.6) is showing the structure of fundus.The optic disc is the anatomical location of the eye's "blind spot", the area where the optic nerve and blood vessels enter or leave the retina. The macula is an oval-shaped pigmented area near the center of the retina of the human eye that contain high density of cones (Cassin and Solomon, 1990).

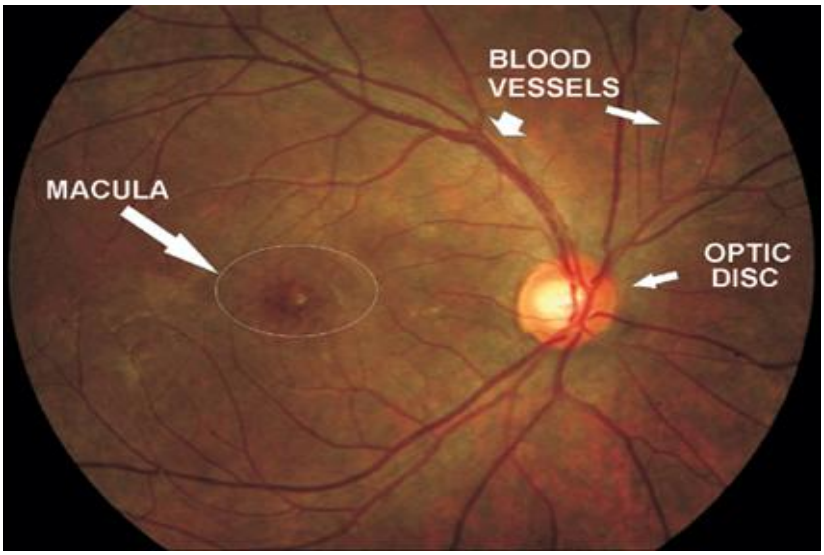

Figure 1.6 : Normal fundus image. http://www.rpfightingblindness.org.uk/

1.8 Inherited ophthalmic Retinal Disorder

Genetic disorders are diseases that result from a change in the normal DNA sequence. Most genetic disorders are caused by multifactorial means involving a combination of genetic and environmental factors. Thousands of human diseases are now known to be caused by single gene disorders and chromosomal abnormalities, many of which involve the eye (Al-Enezi et el., 1998).

The human eye is a complex and unique organ in a sense that it allows for many of the clinical features to be characterized with an unaided bare eye or simple ophthalmic examination tool. Thus the phenotypes of many ophthalmic genetic conditions had been well characterized, and the ophthalmic exam may offer important information in detecting the underlying genetic disorder (Yanoff and Duker, 2004).Hereditary retinal dystrophies are a broad group of genetic retinal disorders of varying severity and with differing inheritance patterns (Moore et al., 2008) .

During the past twenty years, there has been an exponential increase in the knowledge and understanding of ocular genetic diseases and syndromes. The number of human eye diseases that have a known genetic or hereditary component continues to increase (Mathebula, 2012).

Approximately 4000 genetic diseases and syndromes are known to affect humans, and at least one-third involve the eye. In addition, genetic diseases are the most common cause of blindness in infants and children in developed countries. With the development and progress of science at the molecular level, it becomes possible to seek and investigate of the occurrence and existence of the disease in patients to confirm their clinical diagnosis (Hurst, 1992).

The ocular pathology can be classified into non-genetic and genetic diseases. The non-genetic diseases are those caused by trauma, inflammation due to foreign body or by pathogens such as viruses, bacteria or fungi. However, the genetic alterations that affect genes involved in the development and functionality of the eye can include somatic as well as inherited changes.

Examples of somatic mutations that can affect the eye function include a mutation in the retinoblastoma gene (RB1 gene) that leads to an eye tumor called retinoblastoma. Inherited mutations that affect the normal physiology of the eye can occur in many genes involved in the vision process. Examples of these genes include MYOC gene that causes glaucoma, CHM gene that encodes Rab escort protein-1, and FBN1 gene which is responsible for Marfan syndrome. Mutations in RP38, MERTK, PDE6A, RP35, PGR, FAM161, CNGA3 and CRB1 are known to be the cause of the Retinitis pigmentosa (RP) (Mathebula, 2012).

Fundus albipunctatus (FA) is an autosomal recessive congenital night blinding disorder characterized by the presence of retinal white dots caused by mutations in many genes such as FAM161A , CRB1 and RDH5 . It is characterized by numerous small, subretinal, white or place-yellow spots in the perimacula and the periphery of the retina as in (Fig. 1.7) (Simon et el., 1999).

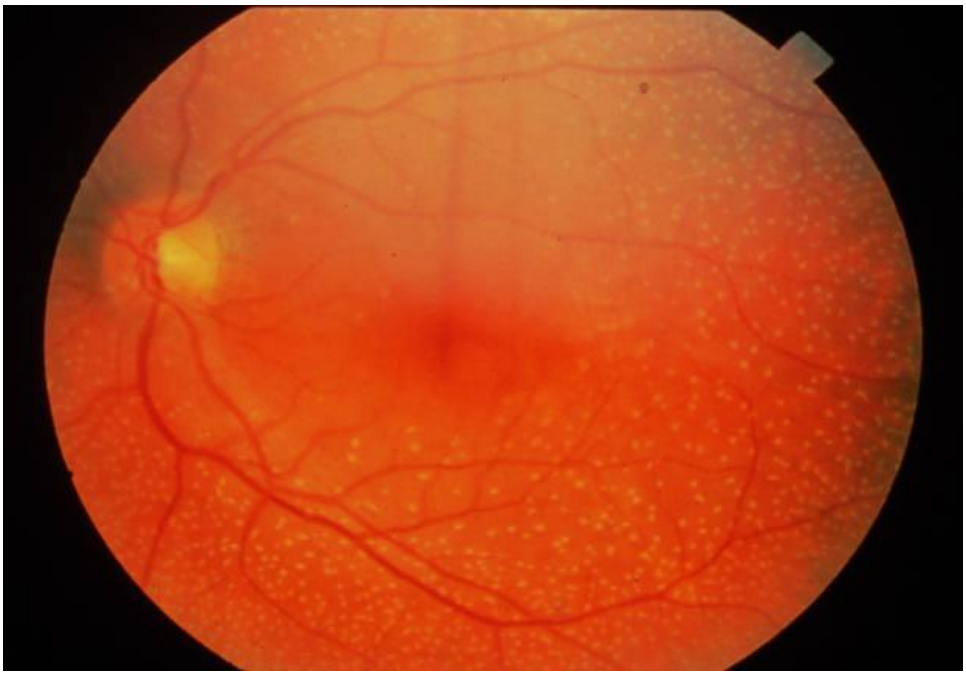

Figure 1.7 : Fundus Albipunctatus , there are multiple yellow-white lesions at the level of the RPE. http://www.djo.harvard.edu/files/3697_493.jpg

1.9 Retinitis Pigmentosa as an Autosomal Recessive Ophthalmic Disorder

Retinitis pigmentosa (RP) is an inherited degenerative eye disease that causes severe vision impairment due to the progressive degeneration of the rod photoreceptor cells in the retina (Busskamp et al., 2010). This form of retinal dystrophy manifests initial symptoms independent of age; thus, RP diagnosis occurs anywhere from early infancy to late adulthood (Robert, 2003).

Patients in the early stages of RP first notice compromised peripheral and dim light vision due to the decline of the rod photoreceptors. The progressive rod degeneration is later followed by abnormalities in the adjacent retinal pigment epithelium (RPE) and the deterioration of cone photoreceptor cells. Affected individuals may additionally experience defective light-dark adaptations, nyctalopia (night blindness), and the accumulation of bone spicules in the fundus (Ferrucci et al., 1998).

RP is a clinically and genetically heterogeneous group of hereditary retinal disorders, being one of the most common types of retinal degenerations worldwide with a prevalence of 1:4000. Up to now, 53 genes have been associated with RP, whose defects cause a progressive loss of rod photoreceptor function, followed by cone photoreceptor dysfunction often leading to complete blindness (Ditta Zobor, 2012). An autosomal dominant retinitis pigmentosa is caused by mutations in RP1 gene (Ziviello, 2005). Mutations in the RPGR gene are the most frequent cause of X-linked retinitis pigmentosa (Meindl, 1996). Mutation in FAM161, CNGA3, and CRB1 are examples of autosomal recessive retinitis pigmentosa.

This descriptive study was used to investigate the presence of three gene
(FAM161A, CNGA3 and CRB1) in consanguineous Palestinian families with RB.

1.10 Autosomal recessive genetic disorders causing RP

This study is unique in that it incorporates both clinical and molecular aspects of the disease in the target families. This study highlights and demonstrates the target genes (FAM161, CNGA3, and CRB1) that cause RP, leading to proper management of ophthalmic patients in the setup of diagnosis and treatment.

1.10.1 Family with Sequence Similarity 161, Member A Gene (FAM161A):

Family with sequence similarity 161, member A (FAM161A) is a gene expressed at the base of the photoreceptor connecting cilium in humans, mice and rats. Furthermore, it is also present at the ciliary basal body in ciliated mammalian cells, both in native conditions and upon the expression of recombinant tagged proteins (Di Gioia, 2012). Thus FAM161A is another member of the growing list of ciliary proteins implicated in human disease and FAM161A-associated RP must be considered as a ciliopathy (Mockel, 2011).

In 2010, Langmann et al. analyzed the candidate gene FAM161A and identified a homozygous mutation (R229X; 613596.0001) that was found to co-segregate with disease in the family. Screening of FAM161A in 118 patients from Germany with recessive or sporadic forms of RP revealed the presence of another homozygous mutation (R437X) in 3 patients (Langmann, 2010). A new study on this gene using homozygosity mapping revealed a new nonsense mutation (c.1003C>T/p.R335X) that is responsible in causing autosomal recessive retinitis pigmentosa in a Palestinian family (Ditta Zobor, 2014).

1.10.2 Cyclic Nucleotide Gated Channel Alpha 3 Gene (CNGA3):

Cyclic nucleotide gated channel alpha 3 gene causes autosomal recessive achromatopsia; a congenital autosomal recessively inherited disorder characterized by a lack of color discrimination, low visual acuity (<0.2), photophobia, and nystagmus (Kohl S, 2005 Mar).

CNGA3 gene encodes one of a family of alpha subunits that form CNG ion channels required for sensory transduction in rod photoreceptors and in olfactory neurons. The CNG3 channel consists of CNGA3 and CNGB3 in a heterotetrameric structure of 2 alpha and 2 beta subunits (Sundin, 2000). In 2001, Wissinger et al. explored the results of a first comprehensive screening for CNGA3 mutations in a cohort of 258 additional independent families with hereditary cone photoreceptor disorders including not only patients with the complete form of achromatopsia as in (Fig. 1.8), but also patients with incomplete achromatopsia and even a few patients diagnosed with severe progressive cone dystrophy. Four mutations (R277C, R283W, R436W, and F547L) accounted for 41.8% of all the detected mutations (Wissinger, 2001).

Figure 1.8 : A photograph of the central retina of a "normal" on the left side and a young patient with achromatopsia. http://www.achromatopsia.info/reduced-visual-acuity/.

Recently study on CNGA3 gene using genetic screening in the Medical College of Wisconsin and the University of Washington reported a mutation c.985G>T - Gly329Cys in two Jordanian patients diagnosed with achromatopsia (Mohamed and Genead, 2011).

1.10.3 Homo sapiens Crumbs Family Member 1 Gene (CRB1):

Homo sapiens crumbs family member 1 (CRB1) mutations were a relatively frequent cause of autosomal recessive early onset retinal degeneration in Palestinian populations (10% of families with Leber congenital amaurosis LCA), and caused severe retinal degeneration at an early age (Beryozkin, 2013). LCA (the term congenital refers to a condition present from birth and amaurosis refers to a loss of vision) is a severe form retinal dystrophy with early childhood blindness (Hollander, 2008).

CRB1 is positioned in a belt-like structure of the inner segments where it is believed to function in the maintenance of cell polarity of the photoreceptors. In a previous study on this gene, the gene c.3307G>A/p.Gly1103Arg mutation was detected in a patient diagnosed with LCA (Hanein, 2005). Moreover, the same mutation was identified in two families from the vicinity of Jerusalem in new recent study on this gene in Palestinian populations on retinal degeneration (Avigail Beryozkin, 2013).

Detection of different mutations in a DNA sample, by using many techniques in clinical diagnostic laboratories, can lead to a diagnosis, possible prognosis, and prospective therapy treatments. For example, single-nucleotide polymorphism arrays (SNP array) is a technique used in detection of mutations, while Sanger sequencing is a technique used to detect unknown mutation by amplifying PCR products of a particular region of interest (Wong, 2013)

The Objectives and aims

The study aim to:

- Screening for specific genetic mutations in ophthalmic patients for consanguineous families.
- Highlight the role of molecular technique in retinopathies diagnosis.
- Develop a molecular technique for ophthalmic genetic mutation screening.

The Significance of the study

The Palestinian population is known to have a relatively high level of consanguineous marriages, leading to a relatively high frequency of autosomal recessive (AR) diseases. Our purpose was to use the homozygosity mapping approach, aiming to prioritize the set of genes and identify the molecular genetic causes underlying AR retinal degenerations in the Palestinian population .

Inclusion and Exclusion Criteria

The inclusion criteria of our study is more than one Palestinian ophthalmic patient suffering from reduced vision, retinopathy, or night blindness derived from parents of consanguineous marriage, the ophthalmic disorder with no gender or age preference.

The exclusion criteria include ophthalmic patient from non consanguineous families, one only affected patient from consanguineous family, and other non inherited ophthalmology disorders.

1.11. Literature Review

1.11.1 Family with Sequence Similarity 161 Member A:

Retinitis pigmentosa (RP) is a hereditary disease that leads to the progressive degeneration of retinal photoreceptor cells and to blindness. It is caused by mutations in several distinct genes, including the ciliary gene FAM161A, which is associated with a recessive form of this disorder (Berson, 1993). Mutations in FAM161A have been found to represent the cause of RP28-associated autosomal recessive RP (arRP) in the initial Indian family in which the RP28 locus was mapped (Gu et al., 1999).

By searching for genes within the retinitis pigmentosa-28 locus on chromosome 2,the researcher identified the FAM161A gene. The most abundant FAM161A transcript encodes a deduced 660-amino acid protein. Quantitative RT-PCR detected both FAM161A variants in all tissues examined. The short variant showed highest expression in testis, followed by retina, heart, and liver. The long variant showed highest expression in retina, followed by testis, liver, heart, and muscle (Langmann et al., 2010). In previously studied, Using traces of DNA from a patient with retinitis pigmentosa mapping to chromosome 2p15-p11,the reasrcher analyzed the candidate gene FAM161A and identified a homozygous mutation that was found in families with the disease. Screening of FAM161A in 118 patients from Germany with recessive or sporadic forms of RP revealed the presence of another homozygous mutation in 3 patients (Gu et al., 1999; Langmann et al., 2010).

In 2014, the researcher performed a clinical and molecular genetic study of a consanguineous Palestinian family with two three siblings affected with retinitis pigmentosa. DNA samples were collected from the index patient, his father, his affected sister, and two non-affected brothers. Funduscopic examination revealed a typical appearance of advanced RP with optic disc pallor, narrowed retinal vessels, bone-spicule like pigmentary changes in the mid-periphery and atrophic changes in the macula.

The younger affected brother (37 years) was reported with overall milder symptoms, while the youngest sister (21 years) reported problems only with night vision. Sequencing analysis revealed the presence of a novel homozygous nonsense mutation, c.1003C>T/p.R335X in the index patient and the affected sister (Zobor et al., 2014).

1.11.2 Cyclic Nucleotide-Gated (CNG) Cation Channels A3:

Cyclic nucleotide-gated (CNG) cation channels are essential in visual and olfactory signal transduction. These proteins, CNG1 and CNG2, are encoded by 2 different genes. The CNG1 channel is activated at 40-fold higher cGMP concentrations than the CNG2 channel (Biel et al., 1994) .Complete achromatopsia or rod monochromatism is a stationary cone dystrophy, with an incidence of 1 in 30 000, in which functional cones are absent from the retina (Sharpe et al., 1999). Studies of patients with achromatopsia have revealed more than 40 disease causing mutations in CNGA3, the majority being missense mutations. In the patient groups studied, four CNGA3 mutations (Arg277Cys, Arg283Trp, Arg436Trp, and Phe547Leu) accounted for 40% of all mutant CNGA3 alleles (Wissinger et al., 2001).

Total colorblindness, also referred to as rod monochromacy or complete achromatopsia, is a rare congenital autosomal recessive disorder characterized by photophobia, reduced visual acuity, nystagmus, and the complete inability to discriminate between colors. Electroretinographic recordings show that in achromatopsia the rod photoreceptor function is normal, whereas cone photoreceptor responses are absent (Kohl et al., 1998).In complete achromatopsia, cone photoreceptors, the retinal sensory neurons mediating color vision, seem viable but fail to generate an electrical response to light. The CNGA3 gene encodes one of a family of alpha subunits that form CNG ion channels required for sensory transduction in rod photoreceptors and in olfactory neurons. The CNG3 channel consists of CNGA3 and CNGB3 in a heterotetrameric structure of 2 alpha and 2 beta subunits (Sundin et al., 2000).

In 1998, the researcher performed linkage analysis in 8 families with total colorblindness, also known as rod monochromacy or achromatopsia, an autosomal recessively inherited condition. Linkage was found with markers located at the pericentromeric region of chromosome 2. Further homozygosity mapping refined the locus to an interval of approximately 3 cM covering the locus, which they designated ACHM2 (Wissinger et al., 1998).

The researcher screened for CNGA3 mutations in 258 independent families with hereditary cone photoreceptor disorders and found CNGA3 mutations not only in patients with the complete form of achromatopsia, but also in patients with incomplete achromatopsia and even in a few patients diagnosed with severe progressive cone dystrophy (Wissinger et al., 2001). In 2007,the researcher analyzed the CNGA3, CNGB3, and GNAT2 genes in 16 unrelated patients with autosomal recessive ACHM: 10 patients had mutations in CNGB3, 3 had mutations inCNGA3, and no coding region mutations were found in 3 patients. The authors concluded that CNGA3 and CNGB3 mutations are responsible for the substantial majority of achromatopsia. In a study of 15 Chinese patients from 10 unrelated families with ACHM, Liang et al. (2015) identified CNGA3 mutations in 13 patients from 8 families (Wiszniewski et al., 2007) .

In a female patient from a consanguineous Saudi Arabian family who had been diagnosed with Leber congenital amaurosis, the researcher identified homozygosity for a missense mutation in the CNGA3 gene that segregated with disease in the family. The mutation, located at a conserved residue in the cGMP-binding domain, was not found in 200 controls or in the dbSNP130 or 1000 Genomes databases. The female patient had nystagmus noted shortly after birth, and electroretinogram was nonrecordable at 10 months of age. At 2 years of age she was noted to have very sluggish pupils and no visual responses were elicited (Wang et al. 2011).

1.11.3 Human sapiens Crumbs Family member 1 Gene:

CRB1 is a human homologue of the Drosophila melanogaster gene coding for protein crumbs (crb), which is expressed in the retina and the brain (Hollander et al., 1999). Mutations in the CRB1 gene are associated with variable phenotypes of severe retinal dystrophies, ranging from Leber Congenital Amaurosis (LCA) to rod-cone dystrophy (also called retinitis pigmentosa (RP)) (Benayoun et al., 2009). In 2004,the researcher stated that 71 different sequence variants had been identified in the CRB1gene in patients with retinal dystrophies. They provided an overview of currently known CRB1variants and discussed their effects (Hollander et al., 2004) .

The researcher sought to identify CRB1 mutations in a large cohort of patients with recessive retinal dystrophies and to document the retinal phenotype and visual prognosis. They recruited 306 patients with Leber congenital amaurosis (LCA), early-onset childhood retinal dystrophy, or juvenile-onset retinitis pigmentosa to the study. Mutations in CRB1, including 17 novel mutations, were identified in 41 patients from 32 families, and those patients underwent detailed phenotyping. Common phenotypic features included hypermetropic refractive error, nummular pigmentation at the level of the retinal pigment epithelium (RPE), and increased retinal thickness on optical coherence tomography (OCT) (Henderson et al., 2011).

In 2012, the recearcher analyzed the CRB1 gene in 400 index patients with a provisional diagnosis of retinitis pigmentosa and identified 11 patients carrying likely pathogenic variants of CRB1. Analysis of the more than 150 previously reported CRB1 mutations and the clinical features of the respective patients showed that no specific allele combination could be assigned to a particular phenotype (Bujakowska et al., 2012). Because of the early onset of disease in patients who have retinal pigmentosa with preserved paraarteriolar retinal pigment epithelium (RP12), with severe loss of vision at ages of less than 20 years, den Hollander et al. considered CRB1 to be a good candidate gene for Leber congenital amaurosis. They detected mutations in CRB1 in 7 (13%) of 52 patients with LCA from the Netherlands, Germany, and the United States (Hollander et al., 2001).

Chapter 2

Materials and Methods

2.1 Study design

This descriptive study was aimed to recruit consanguineous Palestinian families with more than one family member suffering from clinical signs of reduced vision, night blindness and retinopathies. The target individuals were selected among patients attending ophthalmological clinics in the West-Bank. Venous blood sample of 5 ml. Samples were collected from whole blood samples using QIAamp® DNA Mini Kit, DNA was extracted from all family members (affected and phenotypic healthy individual) including the parents. Review of the achieved clinical data and the literature review were need to guide us to detect the possible mutation causing such ophthalmic disorders in each family.

2.2 Preliminary Pilot study

A preliminary work was conducted on patients with reduces vision from consanguineous families. There clinical examination showed various degree of reduced vision with night blindness. A fundoscopic examination revealed Fundus Albipunctatus characteristic for retinitis pigmentosa. Genetic examination of the DNA samples extracted from the collected whole blood samples from all family members revealed the presence of gene mutation in the affected individual and others with healthy phenotype.

Samples were collected after signing a written consent from all individuals participating in the study. A pedigree drawing software (http://www.cegat.de/en/for_physicians/pedigree-chart-designer/) was used to draw the pedigree and to identify the level of cousinship and consanguinity in the target family. Clinical investigation was performed with the assistance of ophthalmologist in Musallam Specialty Hospital and private clinics with review of family history of consanguinity. Laboratory part (blood sample collection delivery , extraction of DNA from blood was performed using QIAamp® DNA Mini Kit, and then the quantity and quality of DNA at the medical research lab AQU was determine .

2.3 Samples collection

A total of 129 individuals (18 Palestinian consanguineous families) were used in this study. Target families are residents of different Palestinian Governorates; Ramallah, Hebron , Bethlehem, Gaza, Nablus and Jericho during the period between November 2014 to November 2016. Attending ophthalmological clinics in private and governmental hospital. 5 ml of blood were collected from each family members (affected and phenotypic healthy ones) including the parents in EDTA tube. Examinations were carried out respecting the Code of Ethics of the World Medical Association (Declaration of Helsinki). Informed consent was obtained from all family members. Blood was delivered in a cold box to Al-Quds Medical research laboratory - Al-Quds University and stored at −20˚C for future analysis.

2.4 DNA extraction

Genomic DNA was prepared from peripheral venous blood using QIAamp® DNA Mini Kit and stored in TE buffer in Palestine. Whole genome SNP genotyping was done using Affymetrix 250k_NspI SNP arrays (Affymetrix, Santa Clara, CA) in Germany (Ditta Zobor, 2014). SNP chip genotypes were used for mapping of homozygous regions applying the online version of the Homozygosity Mapper software (Seelow D, 2009).

DNA extracted from all family members(affected and phenotypic healthy ones) including the parents.The procedures this kit were Pipet 20 µl QIAGEN Protease (or proteinase K) into the bottom of a 1.5 ml microcentrifuge tube.Then was added 200 µl sample to the microcentrifuge tube and was added 200 µl Buffer AL to the sample. The mix was incubated at 56°C for 10 min. Briefly centrifuge the 1.5ml microcentrifuge tube to remove drops from the inside of the lid. Thin was added 200µl ethanol (96–100%)to the sample, and mix again by pulse-vortexing for 15s. After mixing, briefly the sample was centrifuged to remove drops from the inside of the lid. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. QIAamp Mini spin column was opend and was added 500 µl Buffer AW1 without wetting the rim. The cap was closed and was centrifuged at 6000 x g (8000 rpm) for 1 min.

The QIAamp Mini spin column was opend and was added 500 µl Buffer AW2. The sample was centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min. The QIAamp Mini spin column was placed in a clean 1.5 ml microcentrifuge tube and was discard the collection tube containing the filtrate. It was opend the QIAamp Mini spin column and was added 200 µl Buffer AE or distilled water. The sample was incubated at room temperature (15–25°C) for 1 min, and then was centrifuged at 6000 x g (8000 rpm) for 1 min.Finally , DNA was stored at -20c . DNA samples were amplified as in Table (2.2) and loaded into the gel. DNA marker ladder of 100bp (Thermo scientific Lithuania) was used in each run.

2.5 Amplification of DNA

2.5.1 Primers design and DNA amplification:

In this study, new primers were designed based on the conserved DNA sequences of three genes references retrieved from the Gene Bank (FAM161A, CNGA3 and CRB1). All sequences were aligned to each other using the program (http://multalin.toulouse.inra.fr/multalin/) to detect nucleotide variations within these sequences which can be used for further mutation identification as shown in appendix A.6 and B.6.

The primer3 website program was used for primer design (http://www.primer3plus.com/cgi-bin/dev/primer3plus.cgi) as shown in appendix A, B and C. Three sets of primers (forward and reverse) were used as shown in Table (2.1).

Polymerase chain reaction (PCR) was used to amplify three genes (FAM161A, CNGA3 and CRB1).

FAM161A mutation was mentioned as a novel mutation in new study (c.1003C>T/p.R335X) as a nonsense mutation (Reference Sequence: NM_001201543) (Zobor, 2014). This mutation was identified on its reference sequence comparing with the normal sequence without mutation starting from the coding sequence after 103 bases.

According to a previous study in two Jordanian patients with a diagnosis of achromatopsia have a mutation on GNGA3 gene c.985G>T - Gly329Cys (Genead, 2011). We followed this mutation on its Reference Sequence: NM_001298.2, then we located the normal sequence starting from the coding sequence after 418 bases.

Recent study in the Palestinian populations on retinal degeneration showed a mutation on CRB1 gene c.3307G>A/p.Gly1103Arg (Avigail Beryozkin, 2013). we had identified this mutation in its Reference Sequence: NM_201253.2, then we located the normal sequence without the mutation starting from the coding sequence after 210 bases, and then we did many steps to get the ARMS technique.

Table 2.1: The main properties of the primers used in this study.

Gene name	Primer	Primer sequence	Primer size bp	Amplicom size	Annealing temperature °C
FAM161A	(F) Normal (F) Mutant	5'-CAAGGGAGGAACAGAAGC-3' 5'–CAAGGGAGGAACAGAAGT-3'	18	172	
	(R) Common	5'-CAGCTGTGTCCTAAGGTT-3'	18		58
CNGA3	(F) Normal (F) Mutant	5'-GCCATTTCCAAGTTCATTG-3' 5'-GCCATTTCCAAGTTCATTT-3'	19	126	
	(R) Common	5'-TAAGGGTCAAGGTGGACCAG- 3'	20		54
CRB1	(F) Normal (F) Mutant	5'-CTAAGTACAATAGAAATCG-3' 5'-CTAAGTACAATAGAAATCA-3'	19	142	54
	(R) Common	5'-TTGCAGACATTTAACTGC- 3'	18		

The final concentration of working primers was 10 µM/ml. We mixed primers from forward and reverse (10 µM F +1 0 µM R) with 80 µM ultrapure water. For DNA amplifications, PCR reactions were performed in 25-µl PCR .We had prepared PCR mix that contained 2.5 µM of mix primers , 2.5 µM of Red master mix , 15.5 µM of DW and 2µl of the extracted DNA. The DNA samples were amplified using Bio Rad thermal cycler according to conditions in Table (2.2). Nuclease-free water was used as negative control in each run.

Table 2.2 : PCR conditions used for amplifying DNA samples.

	Step	Temperature °C	Time, Min	Number of Cycles
1	Initial Denaturation	94	4	1
2	Denaturation	94	0.5	34 for *FAM161* 34 for *CNGA3* 35 for *CRBI.*
3	Annealing	58 for *FAM161* 54 for *CRB1* and *CNGA3*	0.5	
4	Extension	72	0.5	
5	Final Extension	72	5	1

2.5.2 Gel electrophoresis:

All PCR products were separted on 2 % agarose gel (Agarose LE, Analytical gradient, Promega, Spain). The gel was prepared by dissolving 2g of agarose in 100 ml solution of 1X Tris-acetate EDTA buffer (TAE) (40 mM of Tris acetate and 1mM EDTA). The agarose was dissolved in Erlenmeyer flask using microwave for about 1min till completely dissolved, and then 3.5µl of 10 mg/ml (0.35µg/ml) of Ethidium Bromide was added for DNA staining. The gel was poured in the gel tray in the casting chamber (Bio-Rad Laboratories Inc., USA). PCR products were loaded onto the gel. DNA marker ladder of 100bp (Thermo scientific Lithuania) was used in each run.

2.5.3 DNA sequencing:

The PCR products of successfully amplified samples (n= 4) were sent for sequencing in both directions using forward and reverse primers as shown in appendix (D). At HyLab sequencing service (Rehovot, Israel) the PCR reactions and conditions were performed as described above.

Chapter 3

Results

3.1 Sample collection

A total of 129 samples were collected from 18 consanguineous Palestinian families residing in different regions of Palestine: 43 samples were obtained from Ramallah , 34 from Hebrew, 19 from Bethlehem, 16 from Gaza, 9 from Nablus and 8 from Jericho as in (Fig. 3.1).Overall, samples collected from individuals with affected phenotypes (night blindness and loss vision) (n= 39/129; 30%) and from individuals with healthy phenotype (n= 90/129; 70%) as in (Table 3.1).

To determine the severity of disease (39 affected individual) , the retina of the patient was examined use Slit Lamp Examination with (90),(78) lens . The severity depends on the locatopn of spots (peripheral v.s central , macula and optic disk)as in (Table 3.2).Table 3.3, shows the Distribution of onset of disease for affected individuals.

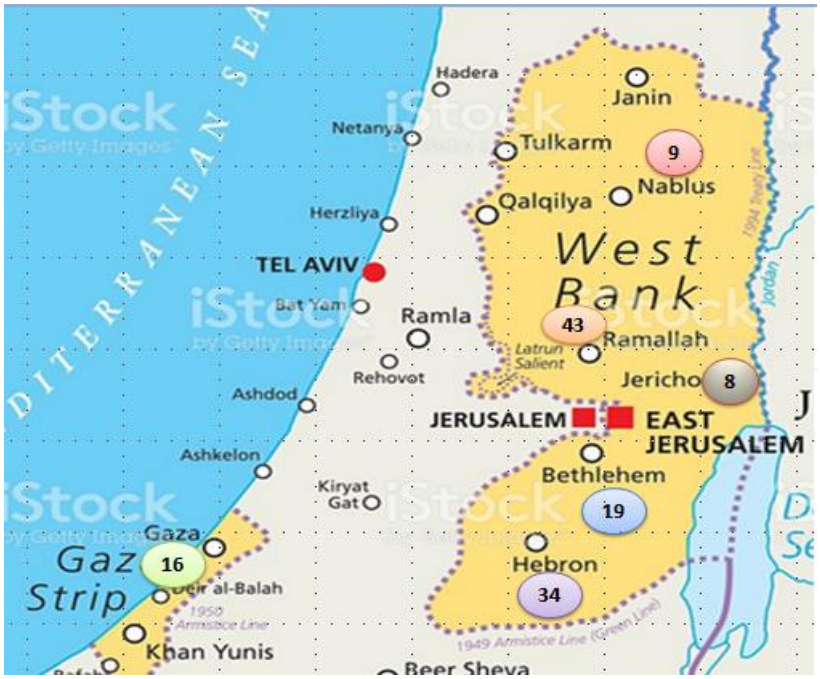

Figure 3.1: The geographic distribution of the blood sample collected from Palestine, the number of collected blood per district was indicated. http://www.istockphoto.com/vector/state-of-palestine-west-bank-and-gaza-strip-political-map.

Table 3.1: Distribution of samples (affected and unaffected phenotype) by region.

Region	Number of family	Number of Affected phenotype	Number of unaffected phenotype
Ramallah	5	14	29
Hebron	4	9	25
Bethlehem	3	7	12
Gaza	2	4	12
Nablus	2	2	7
Jericho	2	3	5
Total	18	39 (30 %)	90 (70 %)

Table 3.2: Distribution of retinal spots location on fundoscopy and severity severity .

Severity	Location of spots	Valid Percent
Peripheral	15	38.5 %
Central	10	25.7 %
Macula	2	5 %
Optic disk	0	0 %
Not tested	12	30.8 %
Total	39	100 %

Table 3.3: Distribution of onset of disease for affected individuals.

Onset of disease	Number of patient	Valid Percent
At birth	22	56.3 %
2 – 15 years	11	28.3 %
15 – 25 years	6	15.4 %
More than 25	0	0 %
Total	39	100 %

3.2 Three mutatons detected in index patients

To determine the target gene for each family member, after DNA extraction from all family members ,the index patient in each family had been considered as a target for gene mutation search. PCR products prepared and DNA samples were amplified as in Table (2.2) and loaded into the gel. DNA marker ladder of 100bp (Thermo scientific Lithuania) was used in each run. The gel was run at 100V for 45min. In (Fig. 3.2),(Fig. 3.3) and (Fig. 3.4), Respectively, PCR products of FAM161A, CNGA3 and CRB1 genes for the index patient in each family (n = 18) .

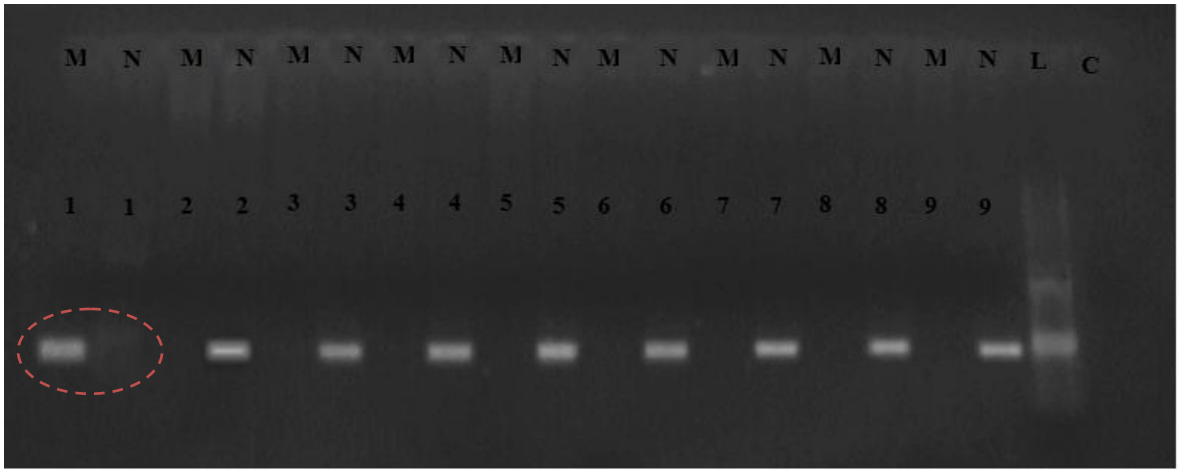

Figure 3.2: PCR products of FAM161A gene. M: Mutant primer , N: Normal primer, Lanes (1, 2, 3,4,5,6,7,8 and 9) are number of sample for 9 families , L: DNA ladder (100 bp), and C : negative control .

In (Fig. 3.2) , The first band (Red Colure) on the family number one appeared on the mutant sample which means that the sequence of the allele bonded at the mutant primer (5’-caagggaggaacagaagt-3) and no band in normal sample. This mean, the index patient in family number one has mutation in FAM161A gene because the band appeared in mutant column. Bands appeared in normal samples, this mean normal allele attached to the normal primer (5’-caagggaggaacagaagc-3’) and no bands appeared in mutant samples (M2,M3,M4,M5,M6,M7,M8 and M9),.

(Fig. 3.3) explains same results, No band appeared in mutant samples (M11,M12,M13,M14,M15,M16,M17 and M18), just appeared in normal samples.The result showed no FAM161A mutation in these families.

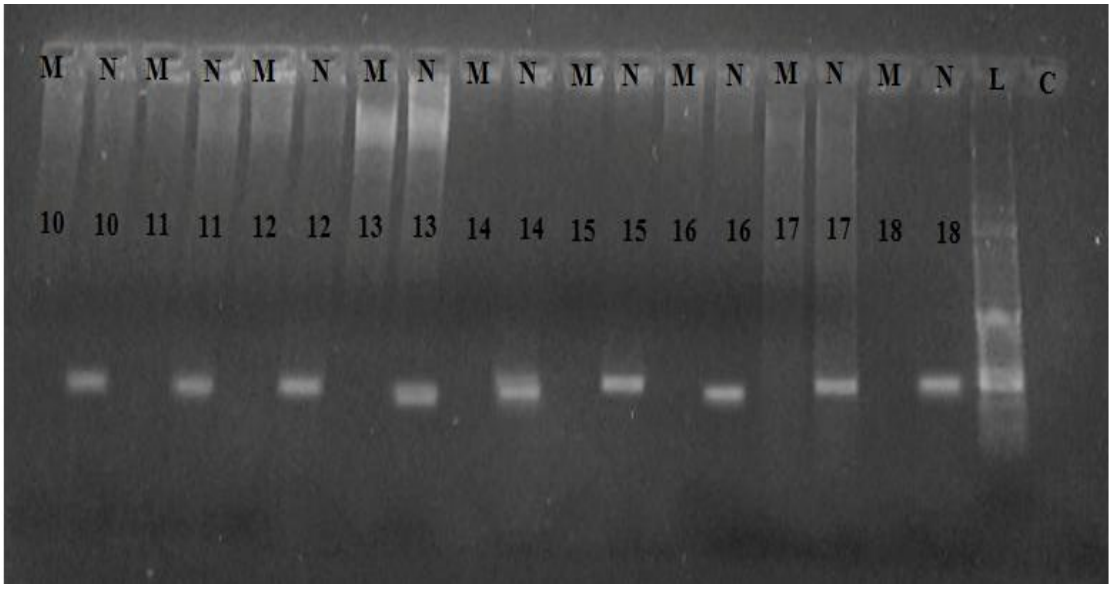

Figure 3.3: PCR products of FAM161A gene. M: Mutant primer , N: Normal primer, Lanes (10, 11, 12,13,14,15,16,17,18 and 19) are number of sample for other 9 families , L: DNA ladder (100 bp), and C : negative control .

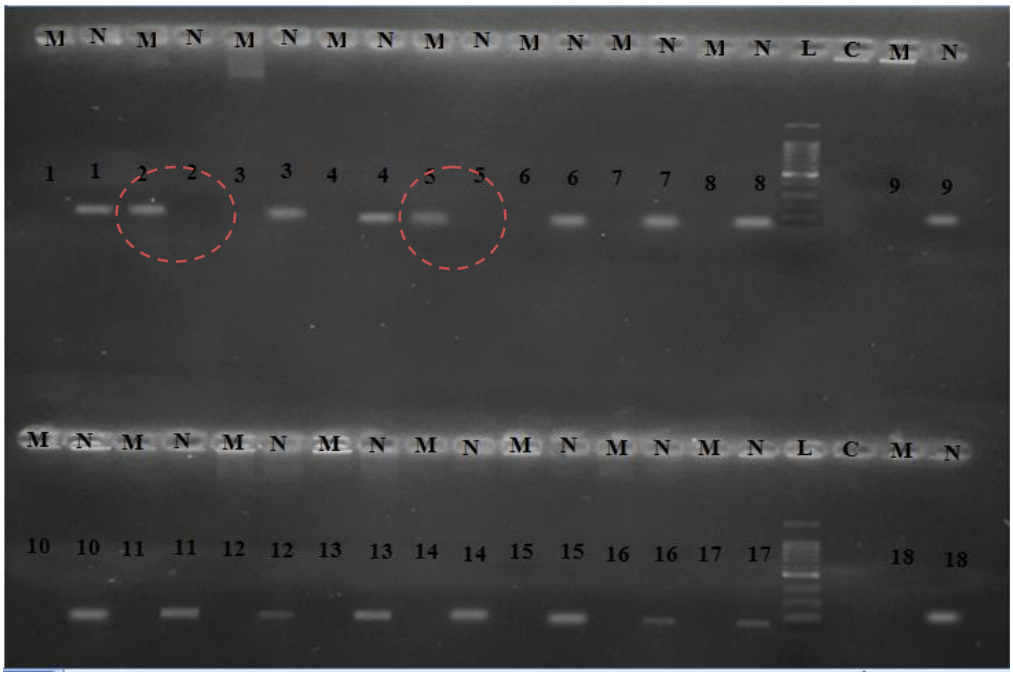

Figure 3.4: PCR products of CNGA3 gene. M: Mutant primer , N: Normal primer, Lanes (from 1 to 18) are number of sample for 18 families , L: DNA ladder (100 bp), and C : negative control .

(Fig. 3.4) is showing the present of CNGA3 mutation among selected 18 index patient. The bands appeared on the mutant sample number 2 and 5, which means that the sequence of the allele bonded at the mutant primer (5'-gccatttccaagttcattt-3') and no band in normal sample. This mean , index patient in families number 2 and 5 have mutation in CNGA3 gene. In samples (M1,M3,M4,M6,M7,M8,M9 and to M18), No bands appeared in mutant samples and appeared in normal samples, this mean normal allele attached to the normal primer (5'-gccatttccaagttcattg -3') so no mutation in CNGA3 in this families.

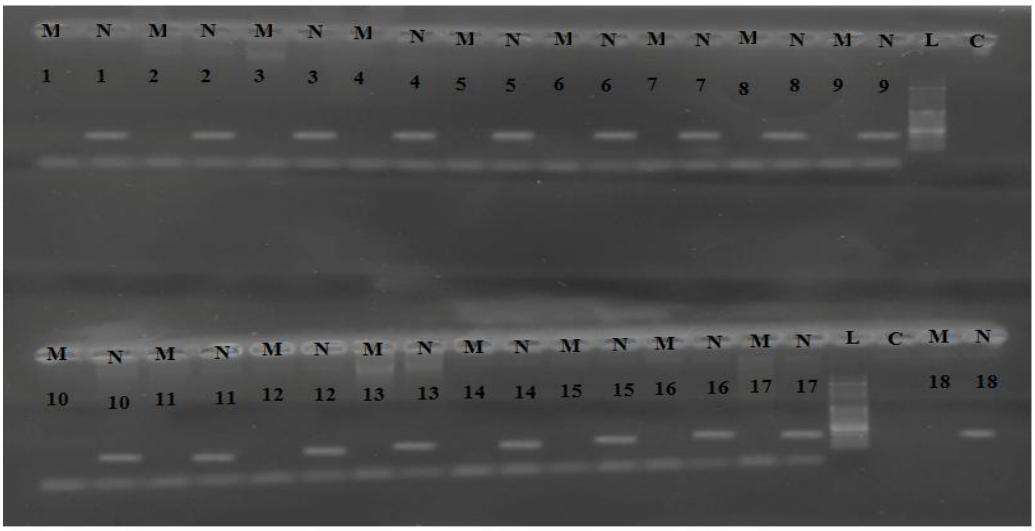

Figure 3.5 : PCR products of CRB1 gene. M: Mutant primer , N: Normal primer, Lanes (from 1 to 18) are number of sample for 18 families , L: DNA ladder (100 bp), and C : negative control .

No bands appeared in all mutant samples (M1 to M18), bands appeared in normal samples, this mean normal allele attached to the normal primer (5’ ctaagtacaatagaaatc 3’),
so no CRB1 mutation was detected in all index patient.

Finally based on this approached the presence of FAM161A mutations and CNGA3 mutations in families (AQ 01, AQ 02 and AQ 05), respectively, is guiding us to search for the given mutation despite the healthy or affected phenotype of the individual.

3.3 Constructing of pedigree of target families

During the recruitment of families and in order to generate pedigree for the target families, the cegat pedigree chart designer software was used. The recruitment familes carried the codes AQ n-1-2 (AQ refers to Alquds University, n:number of the case, 1: number of generation (first ,second ,… etc), 2: order of each person in the generation) to consider the privacy of participating families. The pedigrees of the consanguineous families are shown in (Fig. 3.6) and (Fig. 3.8) .

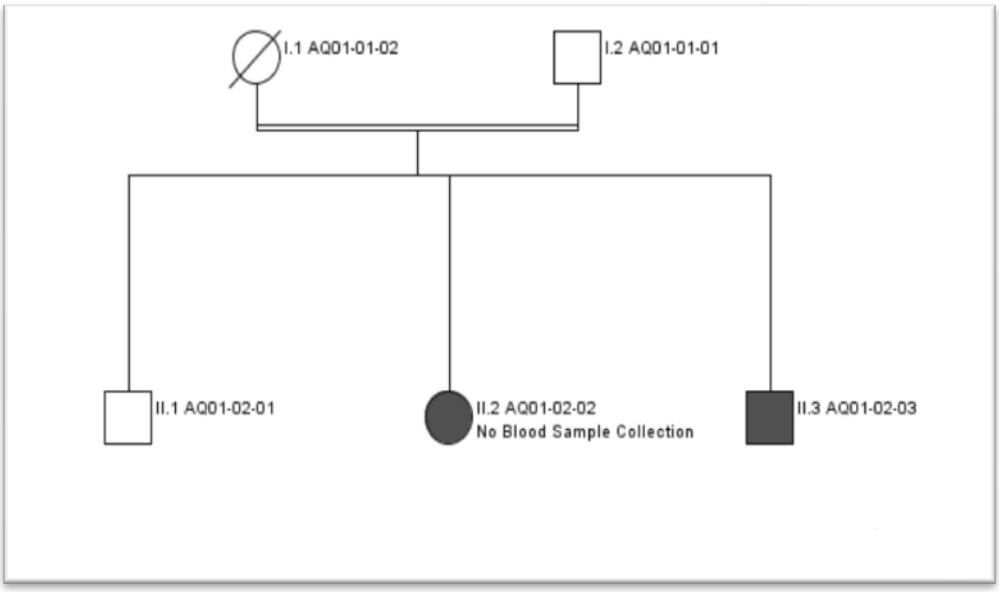

Figure 3.6 : Pedigree of AQ 01 (The family had FAMA161A mutation).

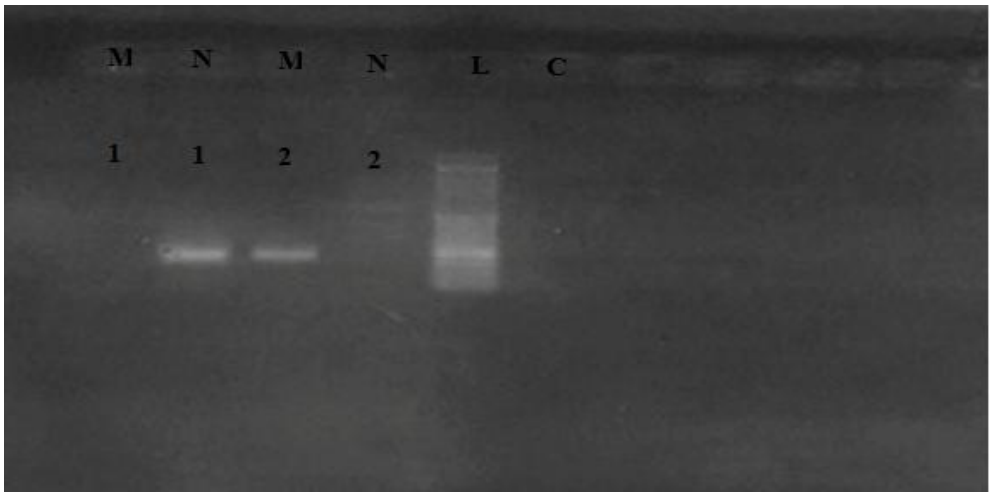

Figure 3.7: PCR products of FAMA161A gene for the first family (AQ 01). M: Mutant primer , N: Normal primer, Lanes (from 1 to 2) are number of samples for two families , L: DNA ladder (100 bp), and C : negative control).

Table 3.4 : Genotypes of AQ 01.

Code	**Gender**	**Status (Phenotype)**	**Genotype**
AQ1-01-01	Male	Unaffected father	NO Blood sample
AQ1-01-02	Female	Unaffected mother	Died
AQ1-02-01	Male	Unaffected	FAM161A: Normal
AQ1-02-02	Female	Affected	No blood sample
AQ1-02-03	Male	Affected	FAM161: Homozygous mutation

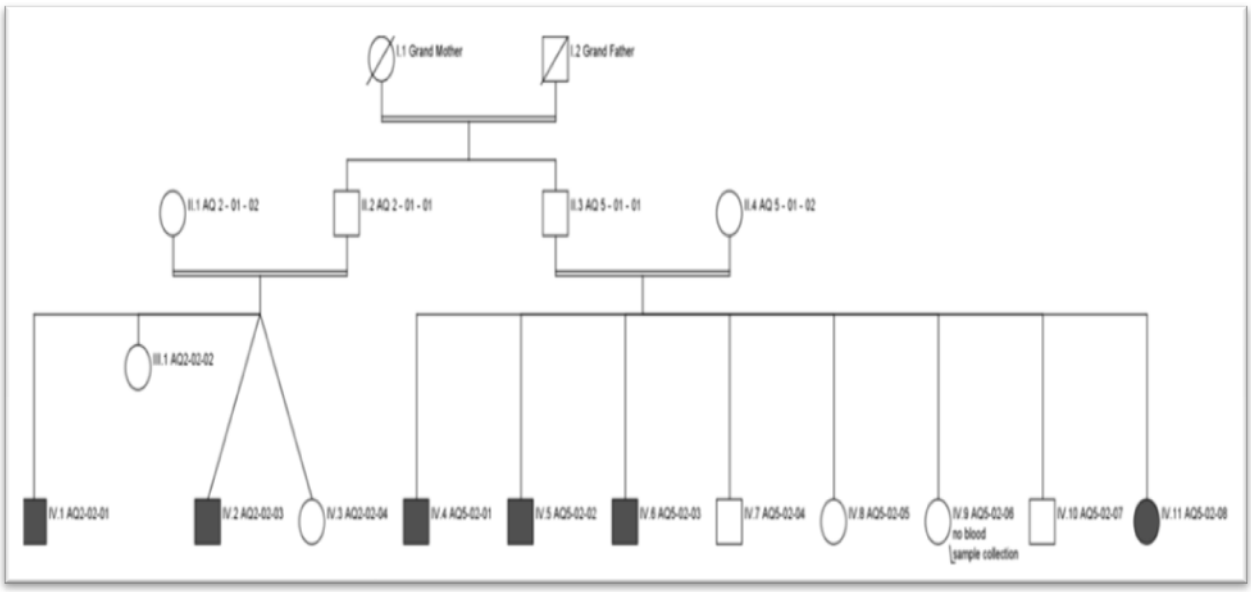

Figure 3.8 : Pedigree of AQ 02 and AQ 05 (The families had CNGA3 mutation).

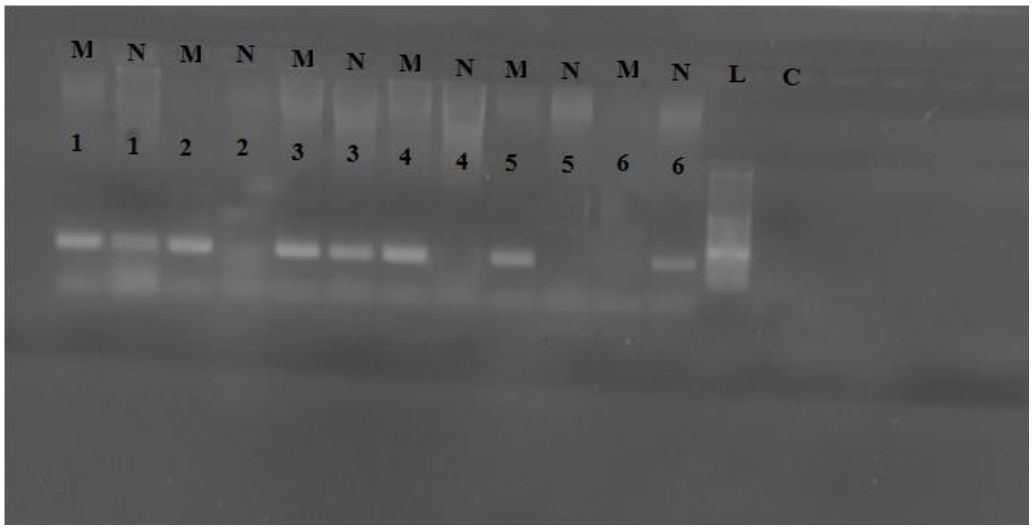

Figure 3.9: PCR products of CNGA3 gene for the second family (AQ 02). M: Mutant primer , N: Normal primer, Lanes (from 1 to 6) are number of samples for two families , L: DNA ladder (100 bp), and C : negative control).

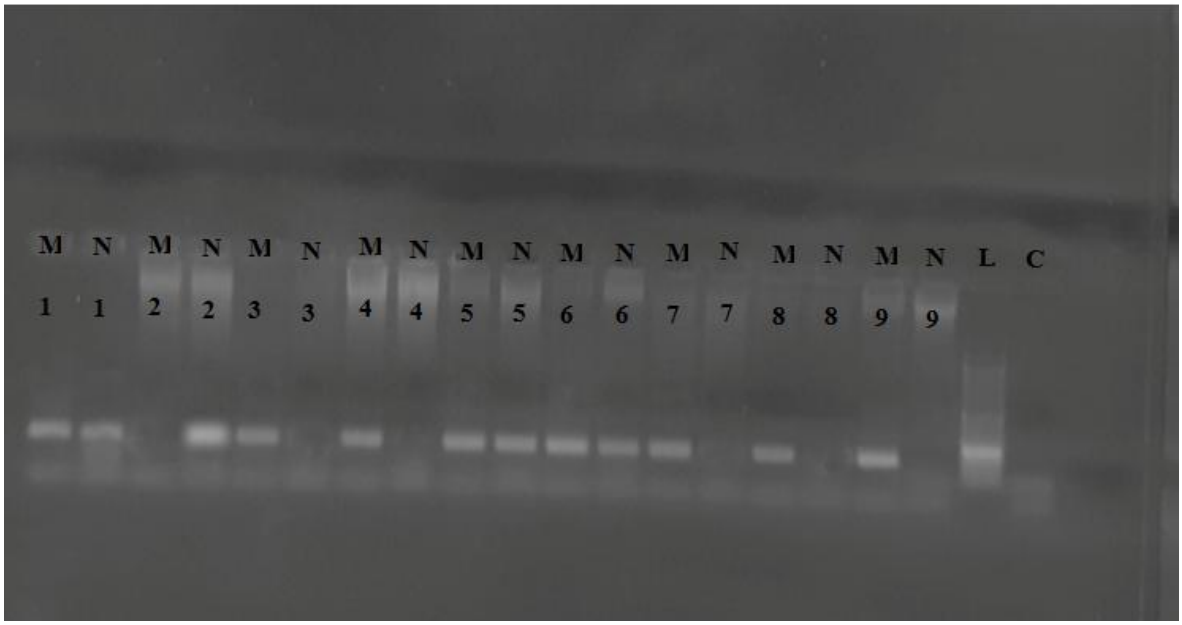

Figure 3.10: PCR products of CNGA3 gene for the third family (AQ 05). M: Mutant primer , N: Normal primer, Lanes (from 1 to 9) are number of samples for two families , L: DNA ladder (100 bp), and C : negative control).

Table 3.5: Genotypes of AQ 02 family .

Code	Gender	Status	Genotype
AQ2-01-01	Male	Unaffected father	CNGA3: Heterozygous mutation
AQ2-01-02	Female	Unaffected mother	CNGA3: Homozygous mutation
AQ2-02-01	Male	Affected	CNGA3: Heterozygous mutation
AQ2-02-02	Female	Unaffected	CNGA3: Homozygous mutation
AQ2-02-03	Male	Affected, twin to AQ2-02-04	CNGA3: Homozygous mutation
AQ2-02-04	Female	Unaffected, twin to AQ2-02-03	CNGA3: Normal
AQ5-01-01	Male	Unaffected father	CNGA3:Heterozygous mutation
AQ5-01-02	Female	Unaffected mother	CNGA3: Normal
AQ5-02-01	Male	Affected	CNGA3:Homozygous mutation
AQ5-02-02	Male	Affected	CNGA3:Homozygous mutation
AQ5-02-03	Male	Affected	CNGA3:Heterozygous mutation
AQ5-02-04	Male	Unaffected	CNGA3:Heterozygous mutation
AQ5-02-05	Female	Unaffected	CNGA3:Homozygous mutation
AQ5-02-06	Female	Unaffected	No blood sample
AQ5-02-07	Male	Unaffected	CNGA3:Homozygous mutation
AQ5-02-08	Female	Affected	CNGA3:Homozygous mutation

Chapter 4

Discussion

Consensual marriage is present in palestine with high rate, but the highest rates of marriages to close relatives are consistently reported in the more traditional rural areas and among the poorest and least educated society, these findings were expected in all villages of the study area, which gives a rise to so many genetic disorders related to this marriage, Especially rare autosomal recessive disease genes.

One opthalmic disorder considered to be the most important disorder assocaiste with retinopoahty as autosomal recessive disease “Rhinitis pigmentosa”. In target familles, all family members were screened regaedless of their phenotype.

For this purpose , we had developed a simple , cost effective, fast method to detect the presece of one or more of the three studied mutations. In this study, the aim of the project was accomplished to design a new and test by using amplification-refractory mutation system (ARMS). This method is characterized cheap and timesaving that detecting the presence of FAM161A (c.1003C>T/p.R335X), CNGA3 (c.985G>T, Gly329Cys). One of the drawback of this method to show similar achievements in CRB1 gene detection,

Therefore, the ARMS method for deteced FAM161A gene in AQ (01) family was very successful (Fig. 3.7). The first band (AQ 01-02-01) appeared at the normal site meaning that the normal allele attached to the normal, the second band (AQ 01-02-03) appeared at the mutant primer meaning that the sequence of the allele linked to the mutant primer, this means,that sample (AQ 01-02-03) had homozygous mutation .

The ARMS method for CNGA3 gene scaning was effective in all samples for the two family examined in the study (AQ 02 and AQ 05) (Fig. 3.9) and (Fig. 3.10).In AQ 02, all family members had mutation in CNGA3 gene except last member (AQ02-02-04). In two members (AQ02-01-01 and AQ02-02-01), bands appeared at the normal and mutant site meaning that the sequence of the allele linked to forward and reverse primers , meaning that the mutation is heterozygous .

In other mutant family members, bands appeared at the mutant primer, that means that the mutation is homozygous mutation .In AQ (05), all family members had mutation in CNGA3 gene except one member (AQ05-01-02). In three members (AQ05-01-01, AQ05-02-03 and AQ05-02-04), bands appeared at the normal and mutant site meaning that the sequence of the allele linked to reverse and forward primers, meaning they had a heterozygous mutation . In other mutant family, have members (AQ05-02-01, AQ05-02-02, AQ05-02-05, AQ05-02-07 and AQ05-02-08), bands appeared at the mutant primer (5'-gccatttccaagttcattt-3'), that means the mutation is a homozygous. Finally, CRB1 gene mutation wasn't detected in all familes as shown in (Fig. 3.5), bands appeared at the normal site which means that the normal allele attached to the normal primer .

The ARMS technique worked well in FAM161A and CNGA3 genes detection, but it didn't function with CRB1 gene. These results can lead us to search more in this field in the future researches since the results of this research confirm the outcomes of ARMS technique which were positive.

Finally, we show a very high diagnostic yield of DNA sequencing for a highly diverse spectrum of ophthalmic disorders in patients from Arab families of the Palestinian territories, even with limited prior clinical history. Applying targeted genes, we were able to identify the mutations causing diseases in three out of 18 consanguineous families (= 17%) and thus providing a molecular diagnosis for the families and physicians.

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Appendix A

Primer design , aligment and DNA sequencing of FAM161A gene

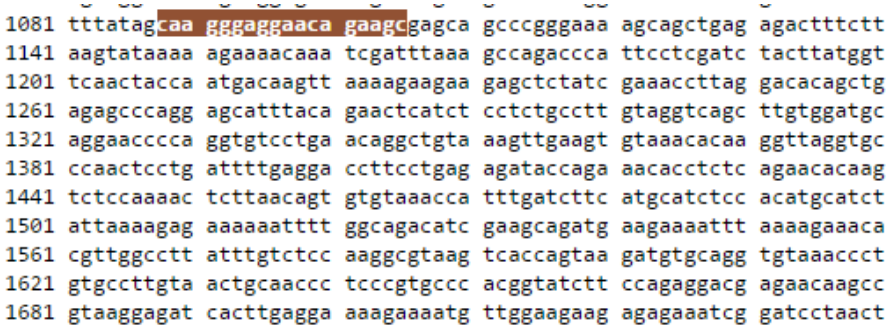

Figure A.1 : The sequence location of forward primer (on label) that we design for FAM161A gene from NCBI site.

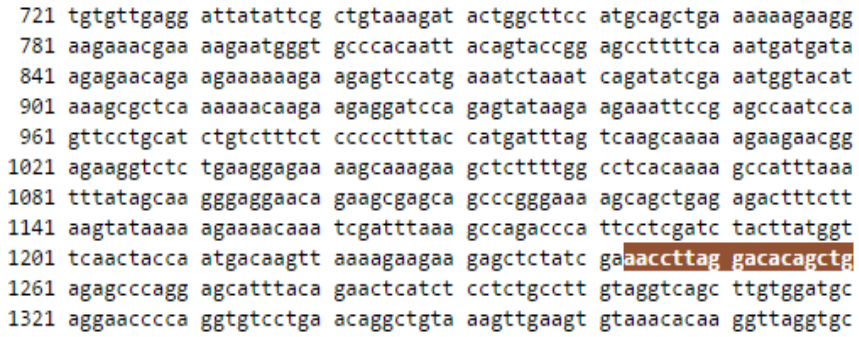

Figure A.2 : The sequence location of reverse primer (on label) that we design for FAM161A gene from NCBI site.

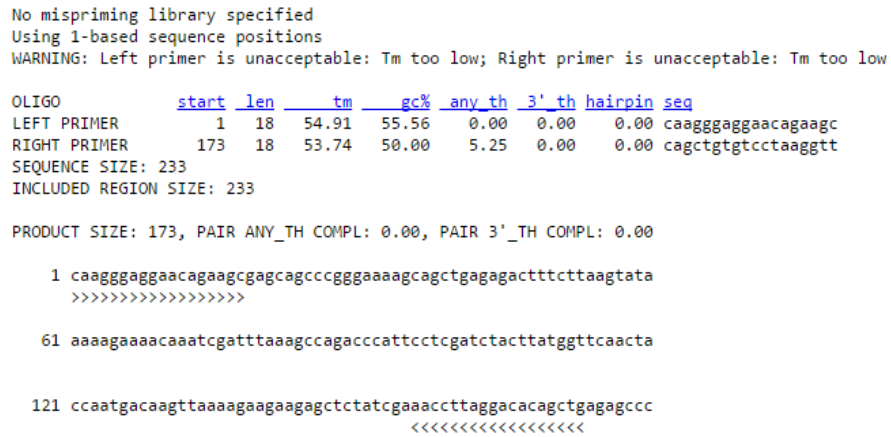

Figure A.3 : The result that obtained from primer 3 site when we design the forward and reverse primers for FAM161A gene.

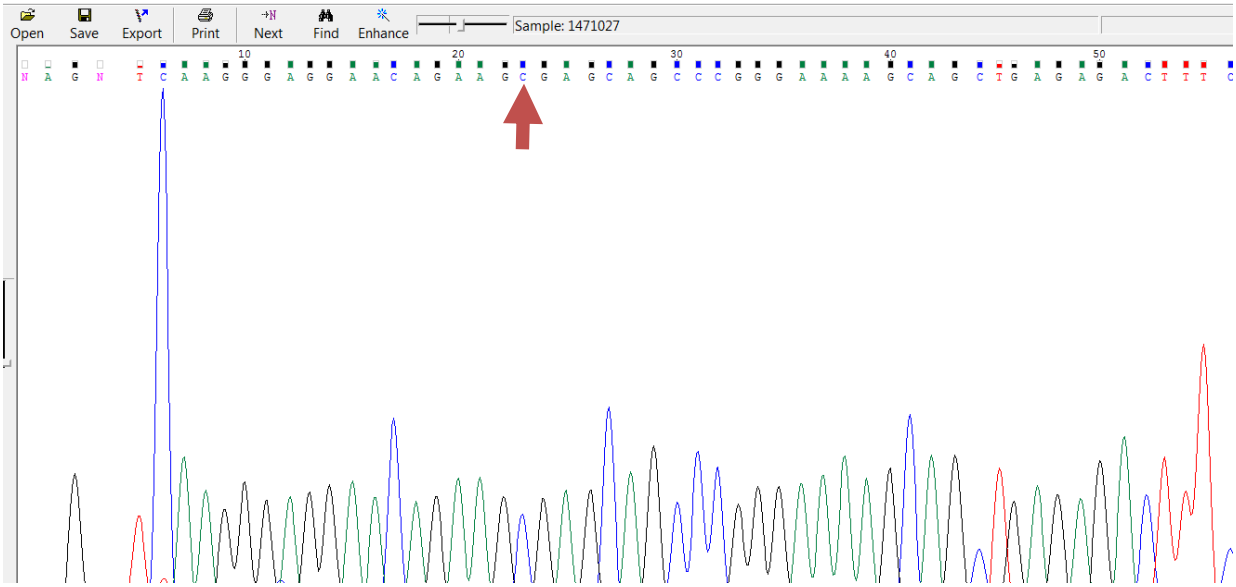

Figure A.4 : BioEdit software for analysis of the obtained DNA sequences for normal FAM161A gene (Red marker). The DNA sequences were analyzed and arranged to prepare them for BLAST analysis step.

Figure A.5 : BLAST of FAM161A sequence againts refernce strains sequence.

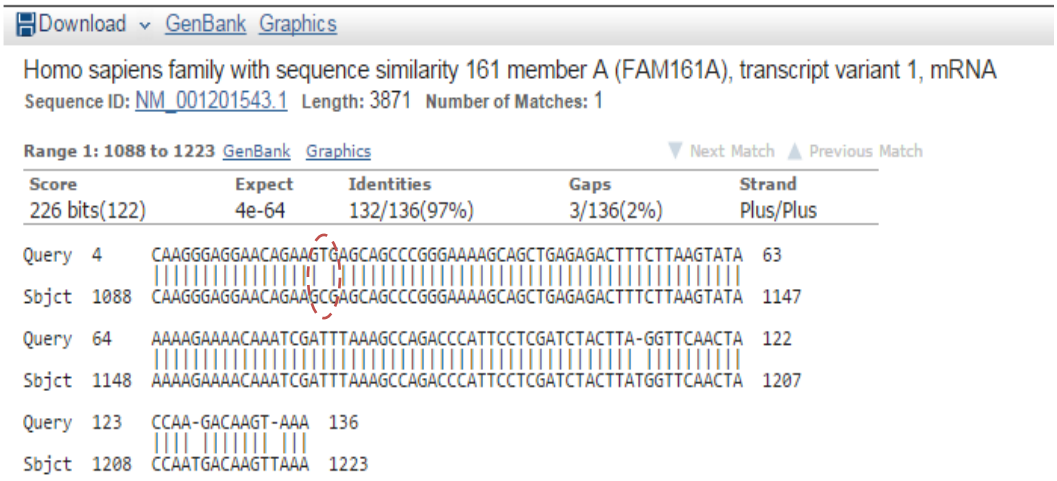

Figure A.6 : The result of aligment from BLAST for FAMA161A gene (Red marker is site of mutation).

Appendix B

Primer design , aligment and DNA sequencing of CNGA3 gene

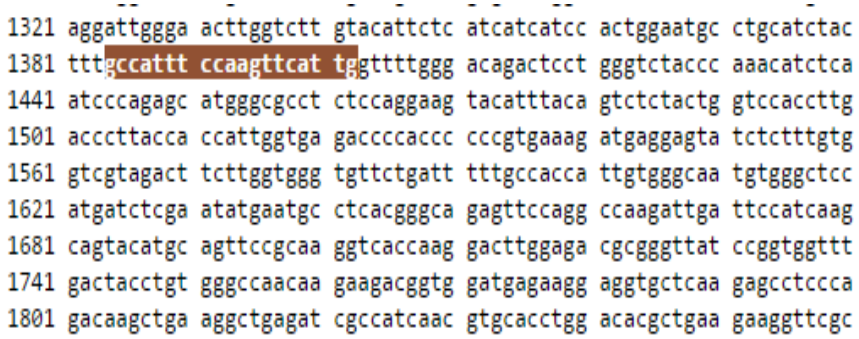

Figure B.1: The sequence location of forward primer (on label) that we design for CNGA3 gene from NCBI site.

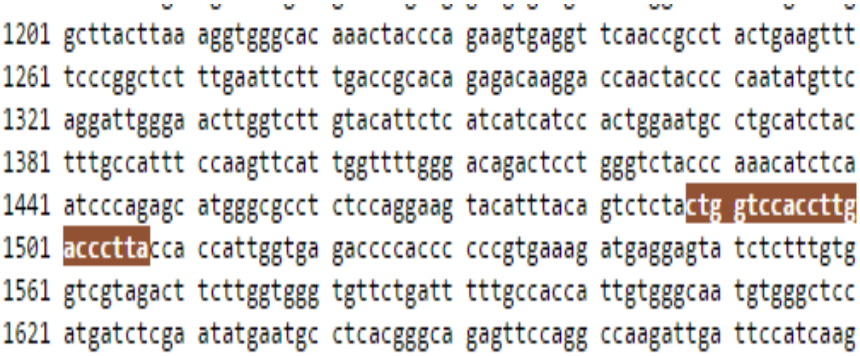

Figure B.2: The sequence location of reverse (on label) that we design for CNGA3 gene from NCBI site.

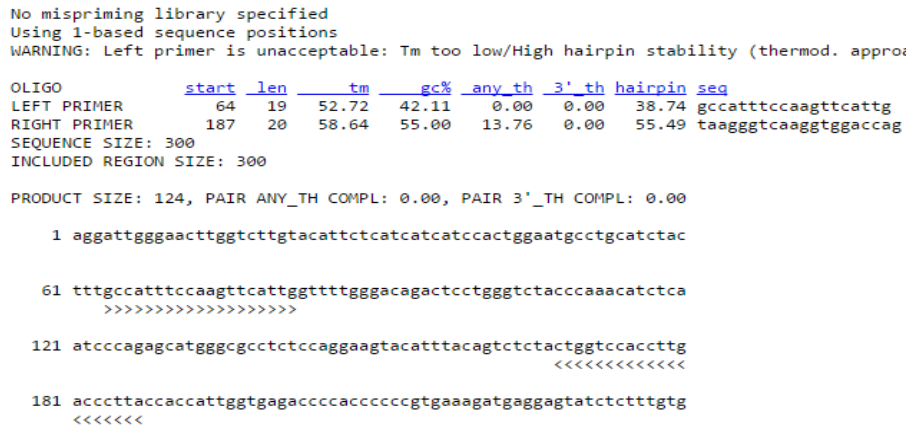

Figure B.3: The result that obtained from primer 3 site when we design the forward and reverse primers for CNGA3 gene.

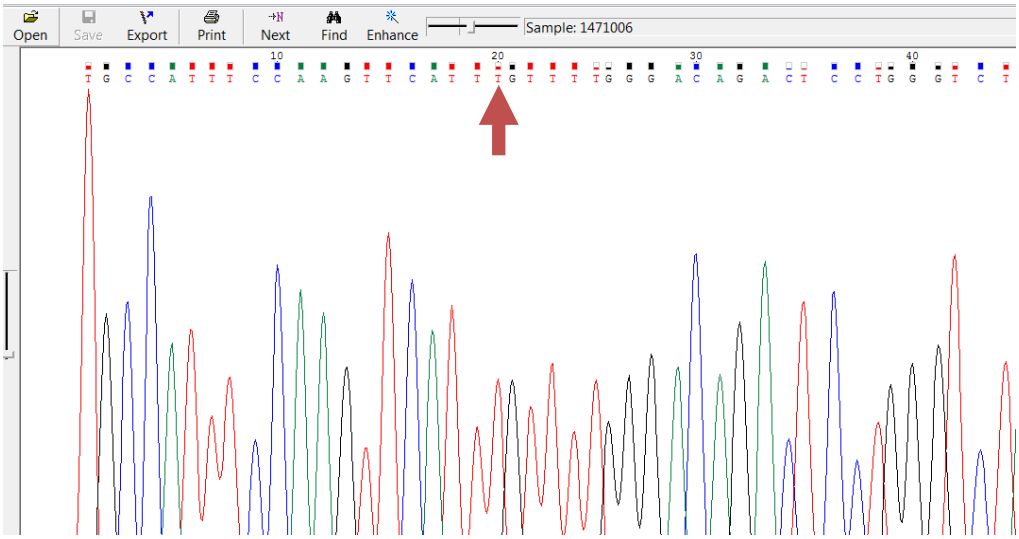

Figure B.4 : BioEdit software for analysis of the obtained DNA sequences for mutant CNGA3 gene (Red marker). The DNA sequences were analyzed and arranged to prepare them for BLAST analysis step

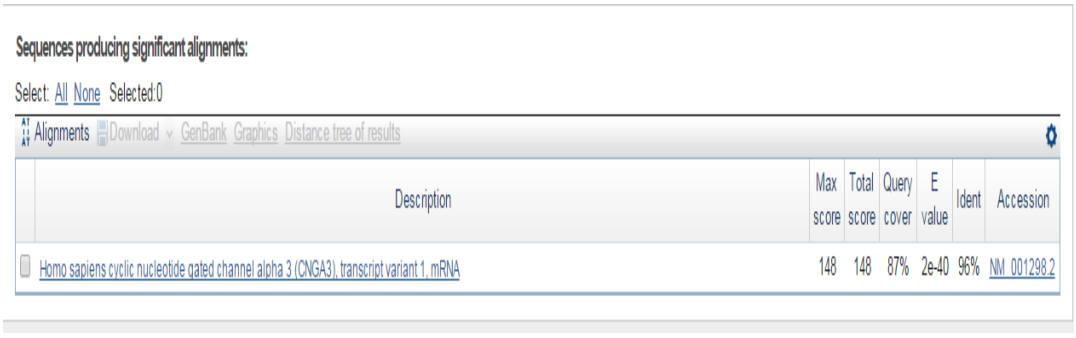

Figure B.5: BLAST of CNGA3 sequence againts refernce strains sequence.

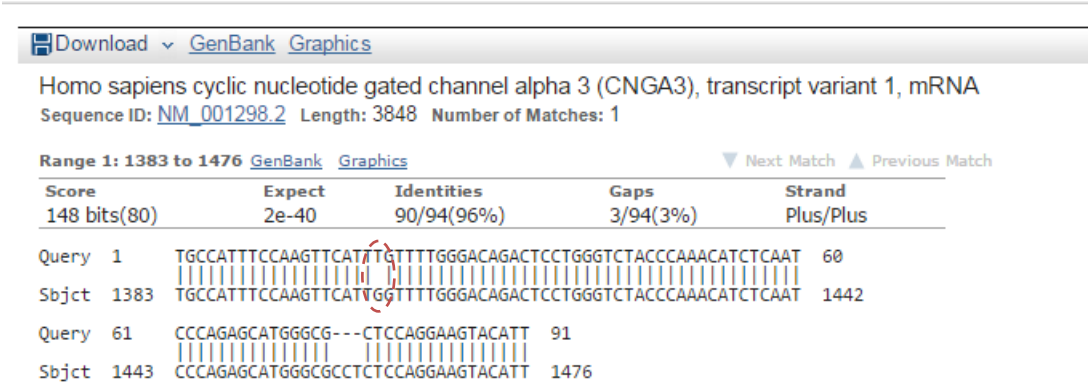

Figure B.6: The result of aligment from BLAST for CNGA3 gene(Red marker is site of mutation).

Appendix C

Primer design of CRB1 gene

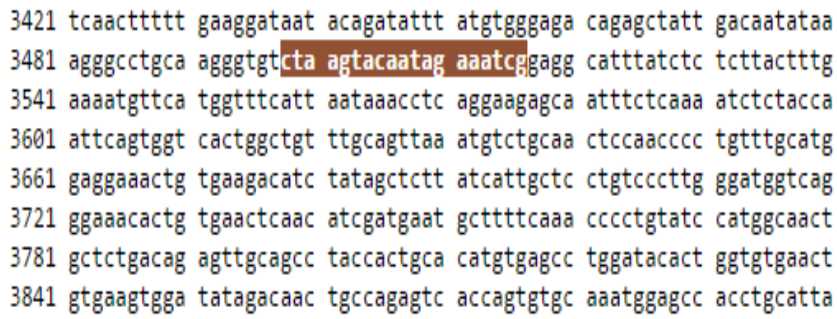

Figure C.1: The sequence location of forward primer (on label) that we design for CRB1 gene from NCBI site.

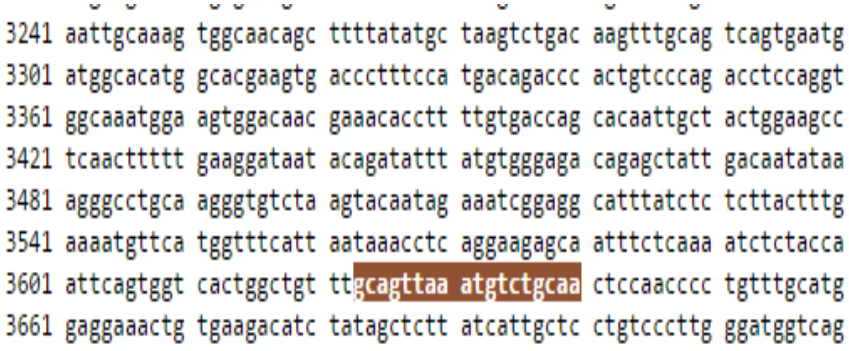

Figure C.2 : The sequence location of reverse primer (on label)that we design for FAM161A gene from NCBI site.

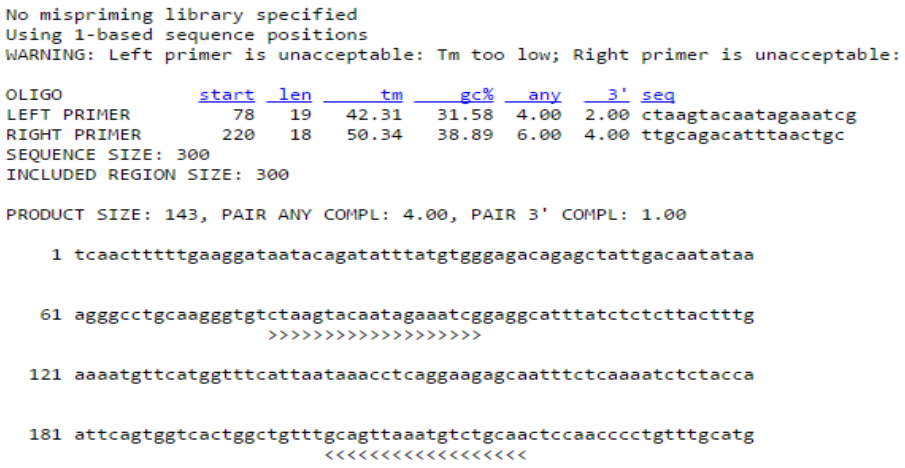

Figure C.3 : The result that obtained from primer 3 site when we design the forward and reverse primers for CRB1 gene.

Appendix D

DNA sequencing for FAM161A and CNGA3 gene

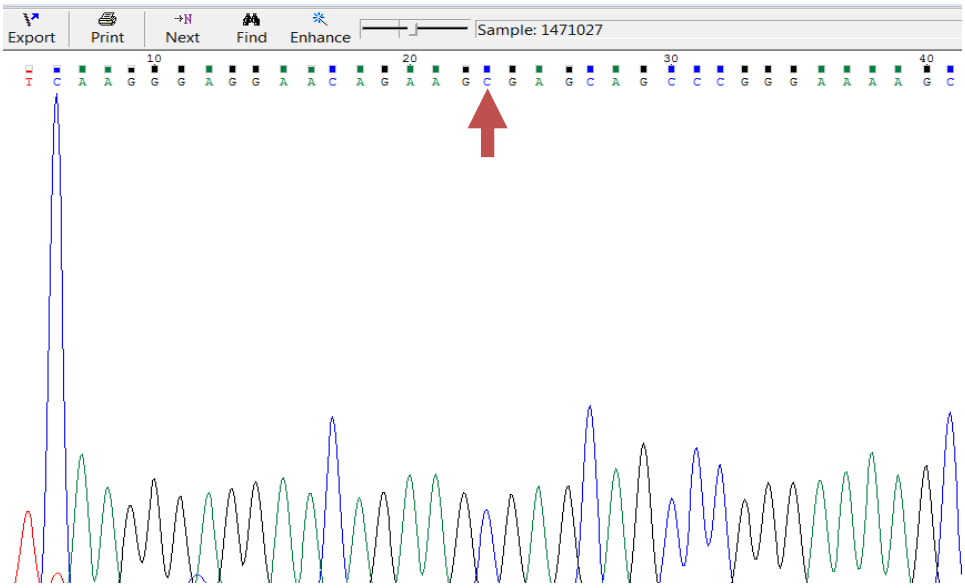

Figure D.1: BioEdit software for analysis of the normal DNA sequences for FAM161A gene (Red marker was normal).

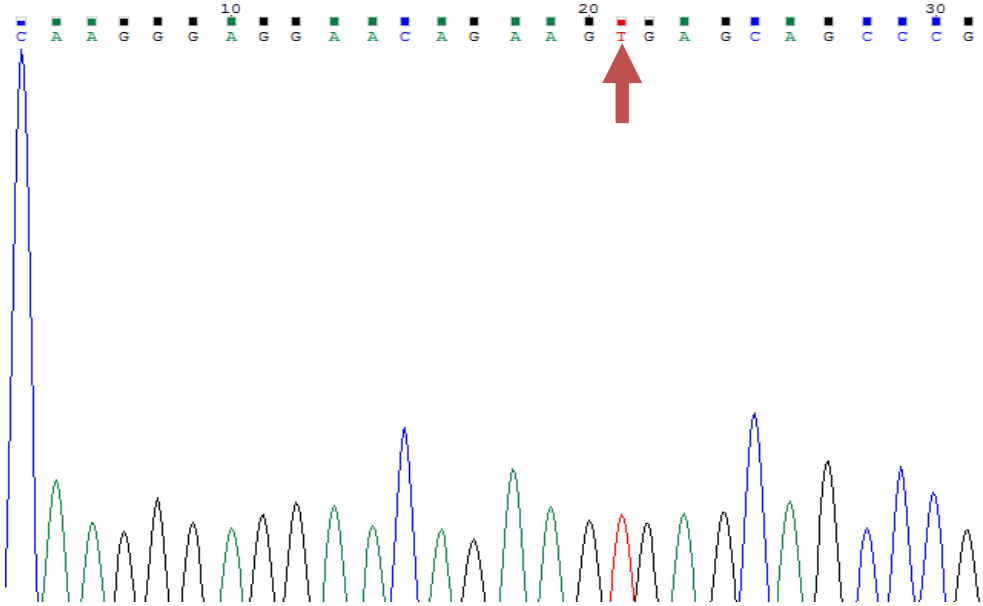

Figure D.2 : BioEdit software for analysis of the mutant DNA sequences for FAM161A gene (Red marker was mutant).

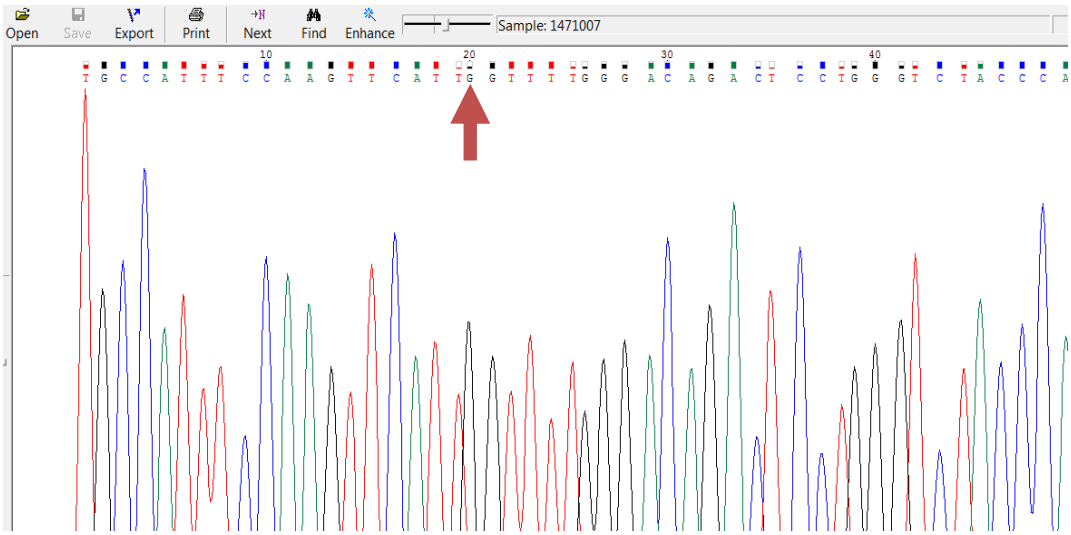

Figure D.3 : BioEdit software for analysis of the normal DNA sequences for CNGA3 gene (Red marker was normal).

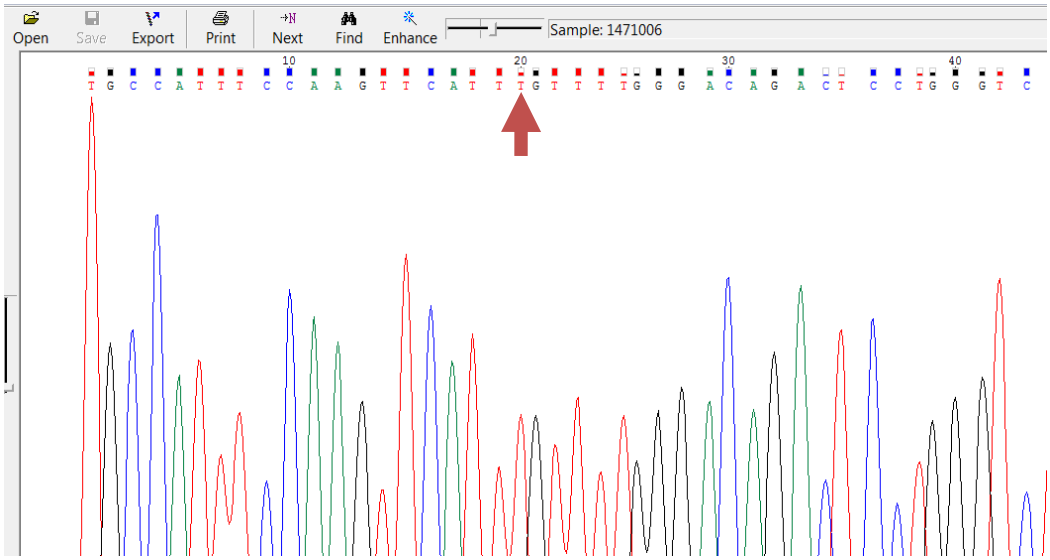

Figure D.4: BioEdit software for analysis of the mutant DNA sequences for CNGA3 gene (Red marker was mutant).

Appendix E

Consent form for all family members

الموافقة المسبقة للمرضى

(الاسم)_____(تاريخ الميلاد)_____

- لقد تلقيت فكرة واضحة عن المشروع البحثي ، والمتعلق بالتشخيص الوراثي وآثاره.
- لقد تلقيت معلومات كاملة من الطبيب في بلدي بشأن التفسيرات الممكنة والقيود المفروضة على اختبار الوراثية منها.
- ليس لدي المزيد من الأسئلة.
- أقر بأن مشاركتي طوعية تماما. وأنا حر لسحب مشاركتي دون إبداء أي سبب. وفي هذه الحالة ، سيتم تجاهل البيانات الخاصة بي وعيناتي من الحمض النووي. ولا يشكل هذا الانسحاب أي خسارة في الاستحقاقات بالنسبة لي.
- أنا اوكد الموافقة على سحب عينة الدم الوريدي ، و بالتالي استخراج الحمض النووي والاختبارات الجينية.
- وأنا اوافق على ارسال عينة الحمض النووي من بلدي الى معهد أبحاث الدماغ السريرية في توبنغن (ألمانيا) لعمل التحليلات الجينية ضمن هذا المشروع و لتخزينها لتحديد الأساس الجيني للمرض لدي.

وبالإضافة إلى ذلك ، لقد قررت أنه بالامكان استخدام عينتي كالتالي:

يرجى الأجابة : نعم أو لا

لا	نعم	
☐	☐	أن يتم تخزين الحمض النووي الخاص بي في معهد أبحاث الدماغ في توبنغن ، ألمانيا لاستخدامه في مشاريع أخرى حول مرضي حتى أعلن انسحابي
☐	☐	أتمنى أن أكون على إطلاع حول النتائج. لقد تم إبلاغي بأن النتائج تستند على مشروع بحثي ولا تلبي معايير التشخيص الجينية الروتينية.
☐	☐	أوافق على أن نتائجي الجينية سيتم تخزينها مع البيانات لأكثر من 10 سنوات في توبنغن ، ألمانيا.

ملاحظات إضافية

توقيع الطبيب	تاريخ التوقيع المكان	الطبيب المحول
المريض / الوصي القانوني	تاريخ التوقيع المكان	المريض / الوصي القانوني

تحديد الجينات الوراثية البصرية المتعلقة بأمراض شبكية العين (*Retinopathy*) الناتجة من زواج الاقارب بواسطة التحليل الجيني الجزيئي من عدة محافظات فلسطينية مختلفة.

إعداد: محمد موسى مصطفى فلنة

إشراف: د. غسان بعلوشة

الملخص:

يعد زواج الأقارب من العادات المتجذرة في مجتمعات الشرق الأوسط وبالأخص في المناطق القروية من المجتمعات العربية ويرجع ذلك للمورث الثقافي والاجتماعي لهذه المجتمعات. ان الطفرات الذاتية المتنحية والمورثة لمرض التهاب الشبكية الصباغي لها علاقة كبيرة بزواج الاقارب.

وعادة ما يكون زواج الاقارب من الدرجة الاولى وفي حالات اخرى يكون من درجات ابعد داخل العائلة. ان انتشار الامراض الوراثية الناتجة عن طفرات متنحية منتشرة انتشار كبيرا في المجتمعات العربية نظرا لتكرار زواج الاقارب من جيل الى جيل.

ان الهدف من هذه الدراسة هو تطوير طريقة سهلة بسيطة التكاليف للكشف عن وجود ثلاث طفرات اساسية ومسببة لمرض التهاب الشبكية الصباغي FAM161A , CNGA3 and CRB1 . وذلك عن طريق تكنولوجيا ARMS .

قمنا بتصميمه للطفرات الثلاث وتشمل الدراسة 18عائلة فلسطينية لازواج اقارب ويوجد في كل عائلة اكثر من شخص يعاني من مشاكل بصرية. لقد نجحنا في هذه الدراسة وباستخدام هذه الطريقة في طرح طريقة رخيصة التكاليف وسهلة الاداء للكشف عن أي تغيير في القاعدة للحمض الاميني (طفرة). لقد حددت هذه الطريقة الانماط الجينية للمرض بتحديد نوع الطفرة لديهم وما اذا كان المريض متماثل اوغير متماثل (Homozygous or heterozygous) في الطفرات الثلاث المقترحة.

وذلك باستخدام أليات مخبرية بسيطة لتنفيذ هذه الطريقة وبأقل التكاليف. ان من استخدامات هذه الطريقة هو تحديد RFLP حيث يتم تحديد الفرق في عينات الحمض النووي المتماثلة جينيا بوجود قطع بأطوال مختلفة بعد تذويب الحمض النووي بمادة (Specific restriction endonuclease) .

ان وجود او عدم وجود (Restriction site) يمكن تحديده على مادة الهلام (Agarose gel electrophoresis)

ان لمرضى التهاب الشبكية الصباغي وعائلاتهم احتياجات معقدة تشمل التكلفة الباهظة للتشخيص والعلاج وقد جاءت هذه الطريقة لتخفف تكلفة تشخيص المريض وتحديد الطفرة لديه خصوصا وان معظم المرضى ينحدرون من مجتمعات قروية فقيرة.

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ان اثنان (FAM161 , CNGA3) من اصل ثلاث طفرات يتم البحث عنها بواسطة هذه التكنولوجيا المقترحة تم الكشف عنها في 3 عائلات من اصل 18عائلة مشتركة في الدراسة. اما بالنسبة للطفرة الثالثة (CRB1) فلم يتم الكشف عنها.

تظهر النتائج النجاح في الكشف عن الطفرات المذكورة اعلاه باستخدام هذه التكنولوجيا البسيطة وبتكاليف رخيصة اقارب لكن يتم تحديد HGمما يعزز اعتماد هذه الطريقة في فحص مرض العيون المنحدرة من عائلات بزواج النمط لجيني لمرضهم باجراء روتيني بسيط وسريع وسهل.