In-frame Three Base Pair Deletion of Achromatopsia disease in Six Palestinian Families

Abstract

Background:

Achromatopsia is a rare autosomal recessive disease that affects vision and leads to total color blindness. Achromatopsia patients suffer from photophobia, nystagmus, cataracts, reduced visual acuity, and eccentric fixation. The disease has been demonstrated to result from mutations in four different genes, CNGB3, CNGA3, GNAT2, and PDE6C. Although achromatopsia is very rare disorder, it is frequently found in some areas of the world such as Iraq, Morocco, Iranian Jews(Zlotogora, 1995), it also is present throughout the world with varying incidence according to the geographical area. From the last decade till today, extraordinary research studies have been applied to discover the genetic basis of the disease. The purpose of our study is to discover the genetic causes of achromatopsiain the Palestinian families suffering from disease.

Methods:

Six Palestinian families with achromatopsia were recruited for our study; the affected individuals, their parents and their unaffected siblings were ascertained. Linkage exclusion approach was undertaken to identify the causative gene/s in our families. Sequence analysis of the coding region of the gene/s that we failed to exclude its linkage to the disease in a particular family to pinpoint the causative mutation(s) was performed. All identified mutations were tested for segregation with the phenotype in the families and screened in other families with the same phenotype.

Results:

The initial results of linkage exclusion in an extended family revealed that *CNGA3*, which codes for a cyclic nucleotide-gated cation channel alpha 3 in cone photo receptors in retina, is most likely the responsible gene for achromatopsia. Sequencing of all coding exons of this gene revealed an in-frame three base pair deletion, resulting in deletion of isoleucine 312, which seems to be critical for the integrity of transmembrane domains of this photoreceptor. Sanger sequencing results revealed that this deletion perfectly segregates with the phenotype in all of the six families in autosomal recessive mode of inheritance.

Conclusion:

An in-frame three base pair deletion in exon 8 of CNGA3 gene was identified in a homozygous form in all affected members in all six Palestinian families with achromatopsia in our study. This gene has been shown to cause achromatopsia in people from different ethnic backgrounds.

Key words:

Achromatopsia, linkage exclusion, homozygous, cyclic nucleotide gated channel alpha 3 (CNGA3).

ملخص الدراسة

مرض عمى الألوان، هو مرض وراثي متنحي نادر، حيث انه يؤثر على النظر و يؤدي الى عمى الوان كلي. يعاني مرضى عمى الالوان من عدة اعراض تشمل،فوبيا الضوء، و الرأرأة (تذبذب مقلتي العين اللارادي)، و اعتدام عدسة العين، و انخفاض حدة البصر. اظهرت الدراسات على ان هذا المرض ناتج عن طفرات في اربع جينات في الانسان وهي **CNGA3**، **CNGB3, PDE6C, GNAT2**. على الرغم من ان مرض عمى الالوان هو مرض نادر جدا، الا انه يوجد في بعض المناطق في العالم بشكل متكرر مثل العراق، و المغرب، و يهود ايران (**Zlotogora, 1995**)، و هو ايضا موجود في جميع انحاء العالم بنسب متراوحة تعتمد على المنطقة الجغرافية.

في العقد الماضي و حتى يومنا هذا، أجريت دراسات مكثفة على هذا المرض محاولة الكشف عن الاسباب الجينية لهذا المرض. الهدف من هذه الدراسة هو كشف المسببات الجينية لمرضى فلسطينيين يعانون من مرض عمى الالوان (الاكروماتوبسيا).

الطرق والأساليب المستخدمة في الدراسة:

استنادا الى ان هناك اربع جينات مسببة للمرض، تم استخدام تقنية (**Linkage exclusion approach**) أولا لتحديد الجين أو الجينات المسؤولة عن المرض في أربع عائلات فلسطينية يعاني بعض أفرادها من المرض. أيضا تم للكشف عن اي طفرة في اكسونات الجين المسؤول عن المرض تم استخدام (**sequencing Sanger)** . ثم تم فحص امكانية و كيفية انتقال الطفرة المكتشفة من جيل الى آخر، بالاضافة الى فحص علاقة هذه الطفرة بالاعراض الموجودة لدى الأفراد المصابين بالعائلة.

النتائج:

أظهرت تقنية(**Linkage exclusion approach**) ان الجين المسؤول عن المرض هو **CNGA3**. و هذا الجين مسؤول عن ترجمة بروتين خاص في المستقبلات الضوئية المخروطية في شبكية العين و هو (**cyclic nucleotide gated cation channel alpha3**). و أيضا كشفت تقنية (**Sanger sequencing**) عن وجود طفرة متنحية في الاكسون الثامن لهذا الجين تسبب حذف لثلاثة قواعد نيتروجينية (كودون) و بالتالي ادت الى حذف الحمض الاميني (**Isoleucine**) رقم ٣١٢، و هذا الحمض مهم جدا لشكل و وظيفة احدى اجزاء هذا البروتين. و تبين ان هذه الطفرة المتنحية تنتقل من الآباء الى الأبناء في هذه العائلات الفلسطينية و تسبب مرض عمى الألوان.

تم الكشف في دراسات اخرى ان هذا الجين يسبب مرض عمى الألوان لدى اشخاص في عدة مناطق في العالم من اصول عرقية مختلفة.

DECLARATION

I declare that the Master Thesis entitled "**In-frame Three Base Pair Deletion Causes Achromatopsia in Six Palestinian Families**" is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

Name and signature: Fadwa Bajes Shiebat

Date: 2015

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Date: 2015

Dedication

I would to dedicate this thesis to my father Bajes Zawahreh Who supported me and encouraged me to complete my master work.

I would to dedicate this thesis to my mother who encouraged me to complete the master degree and support me and gave her time and efforts to take care of my daughter.

I would to dedicate this thesis to my husband Muhand who support me and encouraged me to complete my master work.

I would to dedicate this thesis to my daughter Miral.
I would to dedicate this thesis to my sister Khadeja who helped me to complete my master degree, and gave me her support.

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List of Abbreviations

ACHM	Achromatopsia
ERG	Electroretinigram
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CNBD	Cyclic nucleotide binding domain
CLZ	Carboxy-trminal leucine zipper
CNGA3	Cyclic nucleotide gated channel alpha 3
CNGs	Cyclic nucleotide gated channels
CNGB3	Cyclic nucleotide gated channel beta 3
cNMPs	Cyclic nucleotide monophosphates
DNA	Deoxy ribonucleic acid
EDTA	Ethelynediaminetetraacetic acid
GNAT2	Guanine nucleotide binding protein G(T) subunit alpha-2
PCR	Polymerase chain reaction
PDE6C	Phosphodiesterase 6C
PDE6H	Phosphdiesterase 6H
OCT	Optical coherencetomography
R	Rhodopsin
R*	Active rhodopsin
G	Transducin

GTP	Guanocine-5’- triphosphate
PDE	Phosphodiesterase
OSNs	Olfactory sensory neurons

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CHAPTER ONE

1.1 Introduction

Achromatopsia(ACHM) also referred to as rod monochromay or total color blindness is a rare hereditary disease that affects the retina of the eyes and causes complete color blindness and affects approximately 1 out of 30.000 individuals in the general population (Sharpe and Nordby., 1990; Sharpe et al.,1999). The clinical features of the disease including nystagmus, low visual acuity (ampylopia), photophobia, hyperopia, eccentric fixation, small central scotoma, and reduced or lack of color recognition, and some of these symptoms can develop within the first months after birth and increase over time. The disease is inherited in recessive mode of inheritance (Ahuja et al., 2008; Kohl et al., 2002; Kohl et al., 1993; Kohl et al., 1998).

Diagnosis of achromatopsia is via color vision tests, electroretinogram (ERG) which measures the degree of cones response to light, and optical coherencetomography (OCT) which detects any foveal hypoplasia and other photoreceptors abnormalities (Bijveld et al., 2011; Thomas et al., 2011). Now we are also can perform genetic testing that detects mutations responsible for achromatopsia.

1.1.1 Types of achromatopsia

Cerebral achromatopsia:

Cerebral achromatopsia is acquired condition since it is caused by injury or illness that cause infarction of ischemia in specific area in the ventral occipitotemporal cortex of brain and leads to cortical damage and consequently color loss. Patients of cerebral achromatopsia have experience of colors because they are born with normal color vision unlike congenital achromatopsia patients who are born with no color vision (Bouvier and Engel, 2006; Jaeger et al., 1988).

Congenital achromatopsia

Congenital achromatopsia is inherited as autosomal recessive phenotype and divided into two forms:

Complete achromatopsia, is the typical form of achromatopsia in which the patients have total color blindness due to the impaired function of the three normal types of the cone photoreceptors which are responsible for color discrimination and suffer from all symptoms of the disease. Parental consanguinity is noted in many cases of this hereditary disease (Kohl et al., 1993).

Incomplete achromatopsia is an atypical form of achromatopsia in which the patients have one or more of three normal types of cones. The severity of the disease is less than in complete form, and the patients keep recognition of some colors, also they have reduced visual acuity(Kohl et al., 1993).

1.1.2 A short overview of the eye anatomy and the process of vision:

The eye is sophisticated, soft and so small spherical shaped organ, with diameter ranging from 24 to 25 mm. The human eye is composed of six regions as following:

The cornea, which is transparent dome shaped part and exposed to the environment with 78 % water consistent. The main function of the cornea is to refract and transmit light, and it is covered by the tear films that acts as lubricator for eyelids and protect the cornea itself. The cornea arranged in five layers, the epithelium, Bowman's layer, stroma, Descemet's membrane and endothelium. The aqueous humor is the chamber that contains aqueous fluid to nourish the cornea and lens which are lacks the blood vessels, and the majority of the fluid is water and it is secreted to the chamber by the ciliary process and so creates the intraocular pressure. The iris region contains pigmented fibro vascular tissues (stroma). The iris function is to control the pupil size according to the intensity of the light and it also determines the color of the individual's eyes, furthermore it divides the eye into posterior and anterior, regions located at the front of iris is known as anterior ocular region, while region at the back of the iris are known as the posterior

region. The lens is a biconvex part of the eye and contains no blood vessel, and its role in the visual pathway is to focus the light onto the retina. The vitreous humor is the area between the lens and the retina, and it is filled by clear solution and supplies the retina and lens with metabolites. The sclera is the last layer of the eye and it is connected to the choroidal and retinal layers (Acharya et al, 2008) (Figure 1.1).

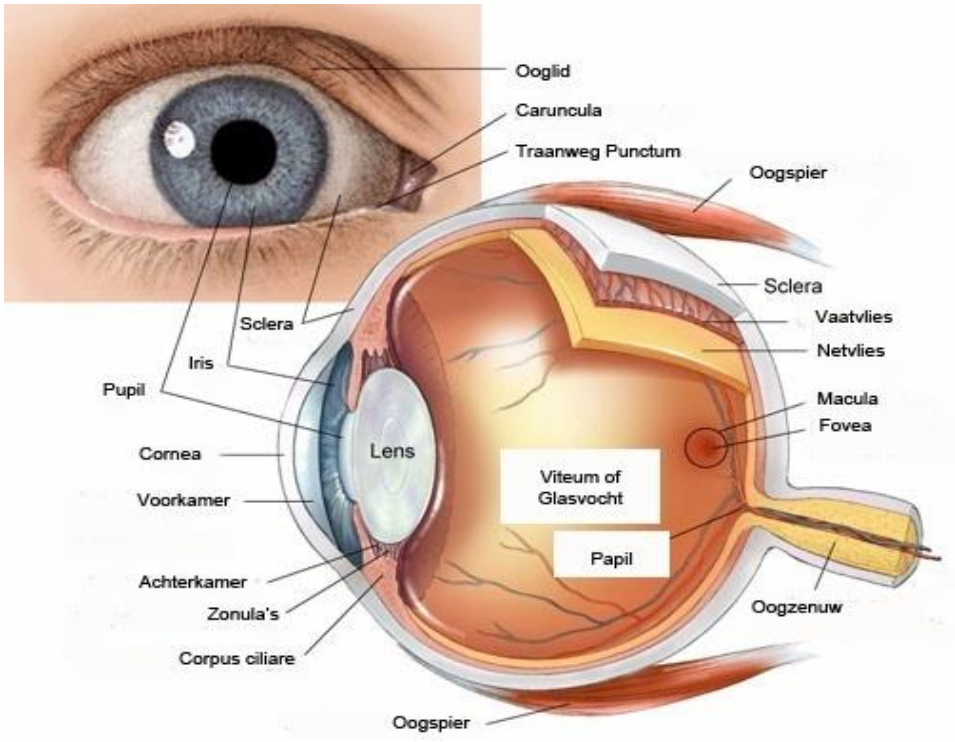


Figure 1.1: Anatomy of the human eye (figure is from: http://www.drpion.be/bouw-van-het-oog.htm)

Other important parts of the eye are retina and choroid. The retina is the layer that made up of several neuronal layers. The function of the retina is to convert light energy into neural signals, so it sends the visual information which enters the eye to the central nervous system to analyze and interpret that information. The neural cells that mediate this function and located at the retina are three types: the photoreceptors (rods and cones), the bipolar, and the ganglion cells (Figure 1.2).

The sensitivity of the cones to light is low; as a result it is responsible for vision in bright light and provides color vision and high resolution vision (photopic). In contrast, the rods sensitivity to light is high and so they are specialized for vision in dark and provide low resolution; black and white vision (scotopic) (Acharya et al, 2008; Mustafi et al., 2009). Rods and cones have specialized part known as outer segment, which is longer in rods with discs separated from the ciliary plasma membrane and shorter in cones with discs attached to the cilium membrane along the outer (Arikawa et al., 1992; Mustafi et al., 2009). Another distinguishable feature in cone is that it has three types, each has a single photo pigment (Marc and Sperling, 1977). The three cone types are divided according to the pigment in each of them that respond to specific wavelengths: S cones mostly respond to short wavelengths (415-430 nm), M cones to middle wavelengths (530-537 nm), L cones to long wavelengths (555-565 nm) (Mustafi et al., 2009).

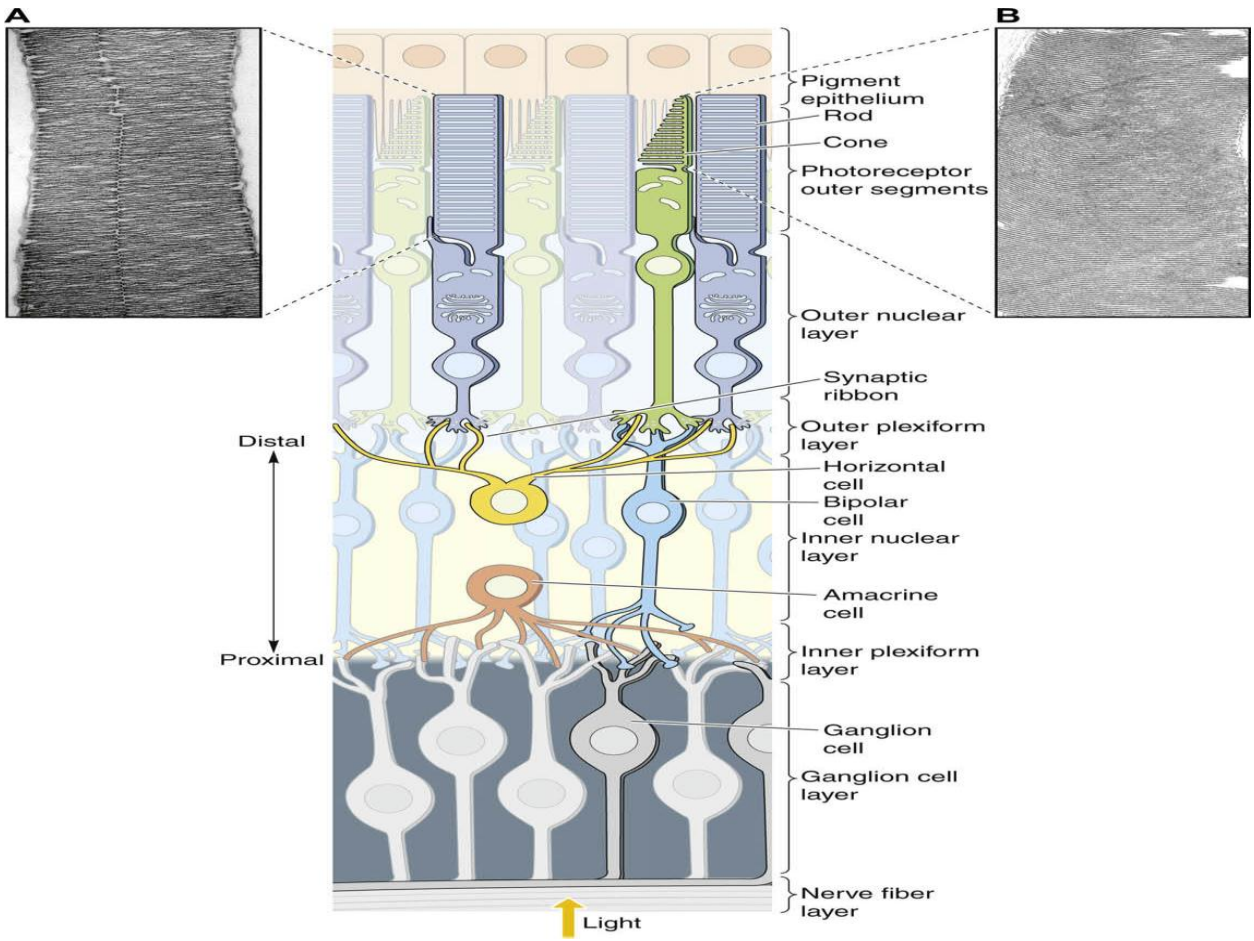


Figure 1.2: diagram showing the retina structure and differences in photoreceptors and their arrangement in the retina. (A) The image of the rod outer segment taken by electron microscope. (B) The image of the cone outer segment (image provided by Dr. Steven K.Fisher). (Mustafi et al., 2009)

Visual process begins when a light beam is caught by the cornea of the eye and passes through the pupil to the lens which refracts it together with cornea inverted image onto the retina where it is detected and absorbed by cones or rods depending on the wavelength of the light and converted into electrical signals, and then passes these signals to bipolar cells into ganglion cells which sends axons through the optic nerve to the brain (visual cortex) where it processed and interpreted to an image which has known as vision. Other cells found in the retina support the process and modify the signals before leaving the eye such as amacrine cells and horizontal cells (Acharya et al, 2008)

1.1.3 Visual phototransduction

Phototransduction is the biochemical process that begins when a light is absorbed by a photoreceptor pigment molecule (Lamb and Pugh, 2006). The process is an example of G-protein-coupled receptor signal transduction and involves three biochemical episodes.

- In the disk membrane of photoreceptor, rhodopsin (R) is the visual pigment which in its inactive form composed of chromophore 11-cis retinal and opsin. By absorption of a single photon; 11-cis retinal is isomerized to its all-trans configuration to induce a conformational change in opsin to be able to bind to other protein and be activated rhodopsin (R*).

- Each of the activated molecules of rhodopsin attach molecule of transducin (3-subunit G protein: beta, alpha, and gamma) in order to activate it by binding alpha subunit to GTP and form R*-G releasing the bounded molecule of GDP from alpha subunit of G protein,

then a GTP molecule can bind to G protein, the activated form is denoted by G*. Next step is the activation of phosphodiesterase (PDE) by G*by separating the inhibitory gamma subunit. Then the activated PDE breaks down cyclic GMP (cGMP) to 5'-GMP. As a result, the concentration of cGMP declines.

- Dropped levels of cGMP, leading to closure of the cyclic nucleotide gated channels (CNGA3/CNGB3) in the plasma membrane that adjusts the flow of electrical current to the outer segment of the plasma membrane and so the sodium and calcium influx is prevented and thereby creating electrical response in the cell, leading to hyperpolarization of the membrane. As a consequence of low concentration of calcium in the cell, the amount of the neurotransmitter glutamate that released from the synaptic terminals is lowered (Kramer and Molokanova, 2001; Lamb and Pugh, 2006; Muller and Kaupp, 1998).

In cones, the cascade proceeds in a similar manner as rods except for small differences in the subtypes of the signaling proteins, cGMP sensitivity and calcium permeation (Picones and Korenbrot, 1995; Rebrik and Korenbrot, 1998). In cones the visual pigment is opsin which is similar to rhodopsin in rods and from the same G-protein coupled receptor super family. The three types of cone opsins (L, M, and S) have the same 11-cis retinal as rhodopsin but differ in their spectral sensitivity to light in cones which range from 360 nm (UV) to 575 nm (red), so each type of opsins has to absorb light with specific wavelength to be activated.

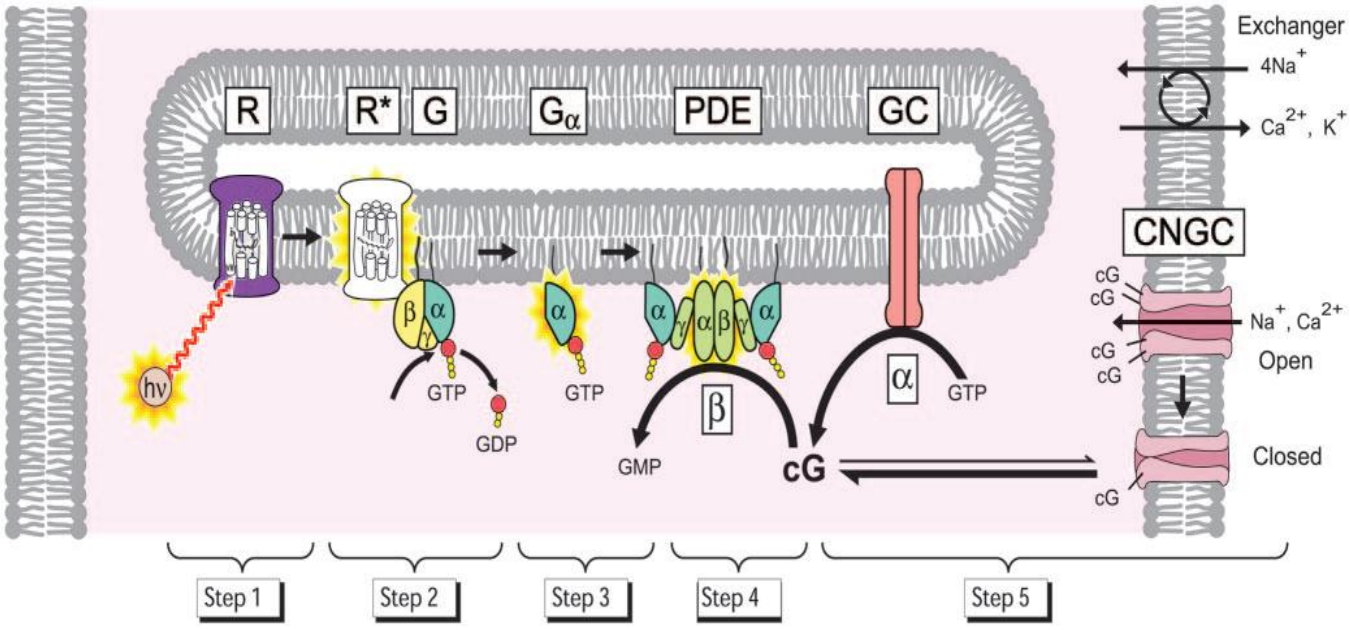


Figure 1.3: The G-protein cascade of phototransduction after absorbtion of a photon (Lamb and Pugh, 2006)

1.1.4 Heterogeneity of autosomal recessive achromatopsia

It has been shown that mutations in one of four genes CNGB3, CNGA3, GNAT2, and PDE6C can cause the complete form of autosomal recessive achromatopsia. Some mutations in PDE6H, GNAT2, and CNGA3 have been implicated in incomplete achromatopsia (Kohl et al., 2012; Rosenberg et al., 2004; Trankner et al., 2004) more data about these genes are summarized in table 1.1. It is known that all of these genes are expressed in the cone photoreceptors of the eye, so mutations in these genes will affect their encoded proteins and eventually affect the transduction cascade. The detected mutations were missense, nonsense, splicing site, and frame shift insertions and deletions.

Many studies utilized animal models to prove the effects of these mutations and understand the role of these proteins in the retina. A knockout mouse model for CNGA3 displayed a complete loss of function and abnormalities in structure of cone photoreceptors in retina (Biel et al., 1999a; Michalakis et al., 2005), other study using CNGB3 canine models concluded that mutations in CNGB3 gene leads to cone degeneration in those canines and arising of

achromatopsia like symptoms such as photophobia and undetectable cone function (Sidjanin et al., 2002). Moreover, a mouse model for GNAT2 for achromatopsia that had a missense mutation in GNAT2 showed nonfunctional cones in retina (Chang et al., 2006), another mouse model was cpfl1 mouse mutant which had insertion and deletion mutations in PDE6C that resulted in damage of cones (Chang et al., 2009)

Table 1.1: Summary of the causative genes in achromatopsia

Gene symbol	Location	Encoded protein	Locus name	Proportion in achromatopsia	OMIM number	References
CNGA3	2q11.2	Cyclic nucleotide-gated cation channel alpha 3	ACHM2	~25%	OMIM 216900	(Kohl et al., 1998; Wissinger et al., 2001)
CNGB3	8q21.3	Cyclic nucleotide-gated cationchannel beta 3	ACHM3	~40-50%	OMIM 262300	(Kohl et al., 2000)
GNAT2	1p13.3	Guanine nucleotide – binding protein G(T) subunit alpha 2	ACHM4	<2%	OMIM 613856	(Aligianis et al., 2002)
PDE6C	10q23.33	Cone cGMP specific 3',5'-cyclic phosphodiesterase subunit alpha	ACHM5	<2%	OMIM 613093	(Chang et al., 2009; Thiadens et al., 2009)
PDE6H	12p12.3	Retinal cone rhodopsin –sensitive cGMP 3',5'-cyclic phosphodiesterase subunit gamma	ACHM6	~0.3%	OMIM 610024	(Chang et al., 2009; Thiadens et al., 2009)

Data about genes, protein name, locus name, chromosomal location, and gene symbol are collected from databases: OMIM.

1.2 Problem statements and objectives

Achromatopsia, whether genetic or acquired, is a serious and critical health issue since it is accompanied with vision loss and many other visual problems. Patients with ahromatopsia requires special care, they are sensitive to light and have low visual acuity, they also face challenges in various fields of life such as education, social life, and routine daily skills requirement.

Correct diagnosis of achromatopsia is important to cope with the disease and treat its manifestations by supplying the patients with special glasses with filters or the red tinted contact lenses that decrease the sensitivity for light and promote the visual acuity (Park and Sunness, 2004). Genetic studies and counseling especially in case of inherited achromatopsia aid in understanding its basics and causes, and most importantly determine the carriers among families that have patients with achromatopsia.

Carrier testing for members of family can be performed after identifying the mutation that causes the disease in that family. The main aim of the test is the prevention of the disease. To reach this goal and lower the incidence of the disease, elucidating the genetic determinants of the disease is required especially in societies that are known to suffer from high rate of consanguineous marriages, and the Palestinian society is one of them.

Achromatopsia is very rare disorder and frequently found in some areas of the world such as Iraq, Morocco and Iranian jews (Zlotogora, 1995), it also present throughout the world with varying incidence according to the geographical area. Acromatopsia is found among Palestinian families which suffer from high percentages of consanguineous marriages, and due to the special care that is required for patients; we decided to discover the genetic causes of achromatopsia in consanguineous Palestinian families.

The aim of the work in this thesis is to identify the mutation responsible for achromatopsia phenotype in six Palestinian families having congenital achromatopsia. The symptoms of the affected members of our families include nystagmus, photophobia, and complete color blindness.

1.2.1 Specific objectives

- Carry out linkage exclusion analysis to determine the linkage to any of the causative genes
- Detect the possible mutation/s using Sanger sequencing and bioinformatics tools
- Confirm the mutation/s by determining the segregation of the mutation in the gene of achromatopsia in all family members.
- Genotyping of 200 healthy, unaffected Palestinian controls from the same geographical region as our families, if possible.

CHAPTER TWO

Review of literature

Since the basis of achromatopsia disease is the loss of the functioning cones; rods mediates the vision process alone in patients, and this explains the disease clinical features, as cones provides color and sharp vision. In recent years it has been elucidated that mutations in genes coding for proteins involved and play important roles in phototransduction cascade in cones resulting in dysfunction or loss of these proteins which cause achromatopsia. So far, five genes, CNGA3, CNGB3, GNAT2, PDE6C and PDE6H have been characterized to be causative genes for achromatopsia, and accordingly achromatopsia is divided into five forms depending on the locus: ACHM2, ACHM3, ACHM4, and ACHM5 and all of these forms are inherited in a recessive mode of inheritance (OMIM 216900, OMIM 262300, OMIM 613856, OMIM 613093, and OMIM 610024).

2.1 Forms of achromatopsia

Achromatopsia-2 (ACHM2)

Mapping studies for achromatopsia revealed that the locus of achromatopsia-2 is on chromosome 2q11where the CNGA3 gene is located (Arbour et al., 1997; Wissinger et al., 1998). The first reported mutations in CNGA3 associated with achromatopsia were in 1998 (Kohl et al., 1998). Later on, more studies investigated CNGA3 gene associated with achromatopsia. Up to now, more than eighty mutations have been detected, and the large proportion of these mutations are missense mutations (Kohl et al., 1993). Moreover, mutations in CNGA3 gene have been implicated in nearly 25% of achromatopsia cases (Kohl et al., 1998; Wissinger et al., 2001).

Achromatopsia-3 (ACHM3)

Mutations in CNGB3 gene on chromosome 8q21 have been found to be associated with ACHM3. The gene is composed of 18 exons and encodes the cyclic nucleotide gated cation channel beta 3, which forms the beta subunit of the CNG channels in cone photoreceptors that mediates a central role in phototransduction process (Kohl et al., 2000; Sundin et al., 2000). The length of the resulted polypeptide is 809 amino acids.

Thus far, more than 40 mutations of diverse types in CNGB3 have been detected in patients of achromatopsia including nonsense mutations, frame shift deletions and insertions, and stop codon mutations (Kohl et al., 1993). c.1148delC is the most common mutation among achromatopsia cases with proportion of (70%) of CNGB3 alleles that cause achromatopsia and 40% of alleles that cause achromatopsia in general (Wiszniewski et al., 2007). Mutations in CNGB3 gene play an etiological role for 40-50% of achromatopsia cases (Kohl et al., 2000; Kohl et al., 2005).

Achromatopsia-4 (ACHM4)

ACHM4 is caused by mutations in the GNAT2 gene accounting for <2% of achromatopsia cases (Aligianis et al., 2002; Kohl et al., 2002; Michaelides et al., 2003; Pina et al., 2004). GNAT2 gene is specifically expressed in cone photoreceptors and known to be functionally important in the phototransduction cascade. The total number of mutations in this gene that have been elucidated till now is only ten.

The gene consists of 8 exons, and its polypeptide is 354 amino acids and is located at chromosome 1p13.3. The gene encodes for the (guanine nucleotide binding protein G) and functions in transducin alpha-2 subunit of cone photoreceptors.

Achromatopsia-5 (ACHM5)

ACHM5 is caused by mutations in PDE6C gene (Thiadens et al., 2009). The mouse mutant cpfl1 is considered to be a mouse model for mutated PDE6C in achromatopsia. Other four missense mutations, one frame shift mutation, and 5 bp deletion were revealed in four families (Thiadens et al., 2009), and eleven different mutations in PDE6C were also found by Grau et al (Grau et al., 2011). In addition, the gene is considered to be responsible for nearly <2% of achromatopsia cases (Chang et al., 2009) making this form rather rare.

The gene consists of 22 coding exons and is located on chromosome 10q23.33 (Piriev et al., 1995). The polypeptide produced by the gene is 858 amino acids, which is the alpha subunit of cyclic guanosine monophosphate (cGMP) phosphodiesterase in retinal cone photoreceptors.

Achromatopsia-6 (ACHM6)

ACHM was added to the list when a homozygous nonsense mutation (c.35C>G) was revealed in PDE6H gene, which encodes the inhibitory gamma subunit of the cyclic guanosine monophosphate (cGMP) phosphodiesterase that is specifically expressed in the three types of cone photoreceptors of retina (Kohl et al., 2012).

2.2 Cyclic nucleotide gated ion channels (CNGs)

Cyclic nucleotide-gated ion channels are nonselective cation channels that are activated by the binding of the cyclic nucleotide monophosphates (cNMPs) to binding site on the channel and leads to opening the channels in the photoreceptor cells and olfactory transduction cascades (Burns and Baylor, 2001; Fesenko et al., 1985; Zufall and Munger, 2001). CNG channels are member of the pore-loop cation channels which contains cyclic-nucleotide binding domain (Kaupp and Seifert, 2002; Zagotta and Siegelbaum, 1996), and allow the passage of k+, Na+, and Ca++ (Kaupp and Seifert, 2002).

CNG channels are expressed in neuronal tissue such as photoreceptors and OSNs (Olfactory neurons that carry the sensory information for smell sense) and nonneuronal tissue such as liver, spleen, testes, heart, pancreas, kidney, lungs. In general CNG channels respond to cAMP and cGMP, while in photoreceptor cells (cones and rods), the CNG channels respond only to cGMP. When the membrane potential equals -60mV and the medium is ionic, CNG channels conducts currents of Na+ and Ca++ inside the cell which occurs by binding the ions to sites in the channels (Kaupp and Seifert, 2002). CNG channels are composed of principal alpha subunits and modulatory beta subunits. The types of the subunits depend on the tissue where the CNG channels are expressed. CNG channels are tetramers (Liu et al., 1996; Varnum and Zagotta, 1996) composed of three CNGA1 subunit and one CNGB1 subunits that form around a central pore in rods, and two CNGA3 subunits and two CNGB3 subunits in cone photoreceptors, while other studies reported the stoichiometry of cones CNG channels 3A:1B (Gerstner et al., 2000; Peng et al., 2004; Weitz et al., 2002; Zhong et al., 2002). On the other hand, CNG channels in olfactory sensory neurons are made of three types of subunits: one CNGB1, two CNGA2, and one CNGA4 subunits (Bradley et al., 1994; Zheng and Zagotta, 2004).

In the mammalian genomes, there are six different genes encoding CNG channels, four genes encode alpha subunits and two genes encode beta subunits (Bradley et al., 2001; Pifferi et al., 2006). Moreover, these six genes are divided into two genes subfamilies. Genes of the first subfamily include (CNGA1, CNGA2, CNGA3), and CNGA4 encode the principal alpha subunits and the second subfamily consists of (CNGB1 and CNGB3) (Kaupp and Seifert, 2002). In mammalian subunits, the regions from NH2 terminal till S2 is encoded by different small exons, while the area from S2 to the COOH terminus is encoded by one large exon which is conserved

in A1 to A3 subunits in various variety of species including human (Bonigk et al., 1996; Dhallan et al., 1992), while the region between S2 and COOH is encoded by twelve exons.

2.2.1 CNG channels structure

Each CNG channel consists of alpha and beta subunits which are considered to be members of the six transmembrane channel superfamily S1 to S6 which are flanked by cytosolic NH2 and COOH terminal coiled coil domains and the pore is composed of 20 to 30 amino acids situated between the S5 and S6 segments. Furthermore, it has been shown that the alpha subunits in CNG channels forms the primary subunits since it is the ion conducting units, while the beta subunits are considered to be modulatory (Gerstner et al., 2000; Zagotta and Siegelbaum, 1996). Also, alpha and beta subunits homologues have been found in *Drosophila* (Baumann et al., 1994) and *Caenorhabditis elegans* (Coburn and Bargmann, 1996*).*

CNG channels contains many structural sequence motifs and functional domains (Figure 2.1) including the cyclic nucleotide binding domain (CNBD) which is located in the C terminus region, the cyclic nucleotide binding domain is considered to be the activation domain (Kaupp et al., 1989; Pifferi et al., 2006). The domain is composed of a beta sheet which is made of eight beta strands and two alpha helices. The cyclic nucleotide binds to the beta sheet, resulting in the movement of the alpha helix towards the beta sheet. The interaction between the cyclic nucleotide and the protein occurs through many hydrogen bonds and electrostatic regulations. Other structural motifs in CNG channels include voltage sensor motif which is a charged sequence motif in S4 segment, the C-linker part that attaches the CNBD to the S6 segment, the P-loop which connects the S5 and S6 segments, and the S6 region (Kaupp and Seifert, 2002).

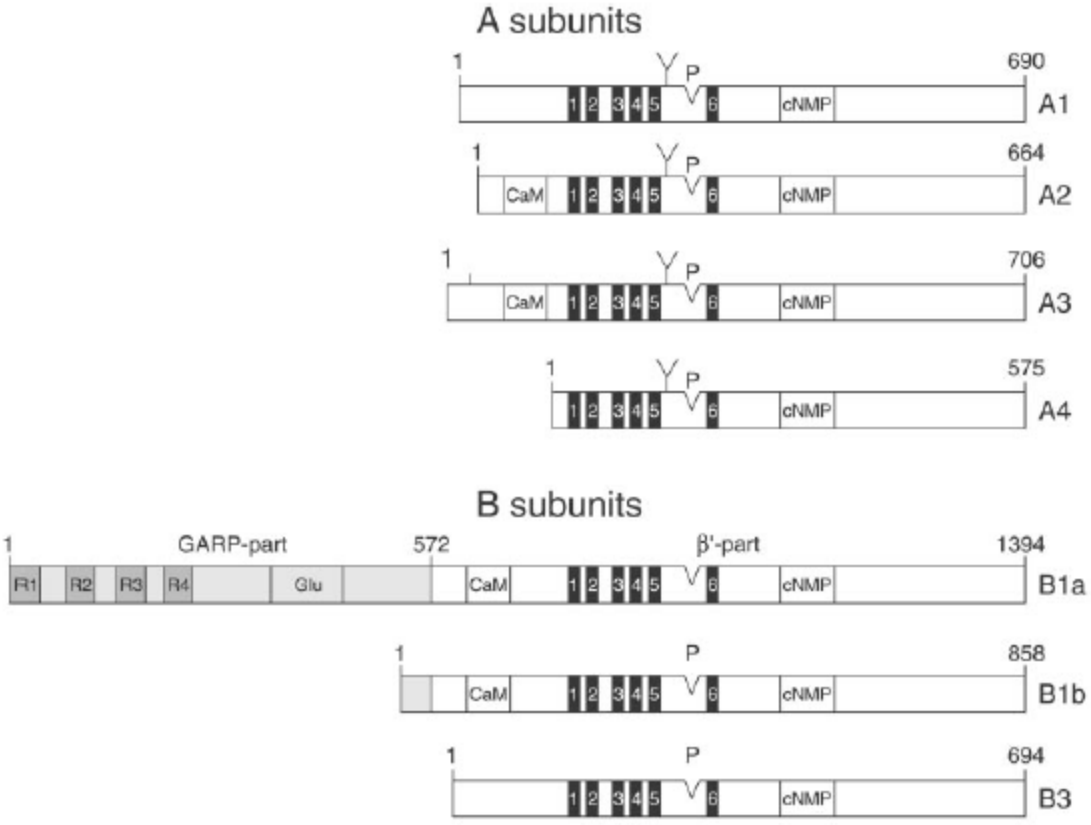


Figure 2.1: Organization of functional domains, Transmembrane segments are indicated by numbers 1-6. CaM, calmodulin-binding site; cNMP, binding site for cyclic nucleotides; P pore region; Glu, glutamic acid-rich part (Kaupp and Seifert, 2002).

2.2.2 CNG channels function in photoreceptors

The function of the CNG channels has been identified in signal transduction in ciliary photoreceptors rods and cones. Rods and cones respond to light by cGMP signaling pathways with hyperpolarization or depolarization. CNG channels are located in the surface membrane of the outer segment of the rods, and since these channels are nonselective and permit for many alkali ions to pass into or out the cell through the membrane, hyperpolarization or depolarization can be created. This can result from the activation of CNG channels by direct binding to cAMP or cGMP (Biel et al., 1999b). In the dark cGMP binds to CNG channels, as a result cations flow into the outer segment, and channels open and the cell depolarizes. When light is detected by photoreceptors, a series of enzymes include the photopigment rhodopsin, the G protein

transducin, and the PDE are activated and interacted with each other leading to the hydrolysis of cGMP, and closing of CNG channels. When CNG channels close, cations entry will be stopped and the cell is hyperpolarized (Kaupp and Seifert, 2002) and the cytoplasmic CA++ concentration will be lowered which acts as intracellular transmitter of this light adaptation and activates guanylate cyclase and regulates the sensitivity of the transduction cascade and restart the recovery from the light response by triggering new cGMP molecules production (Koch, 1992).

2.3 Cyclic nucleotide gated channel alpha 3

Cyclic nucleotide gated channel alpha 3 (gene symbol CNGA3) is located on human chromosome 2 q11.2 (Wissinger et al., 1997). The gene encodes the alpha-subunit of the cyclic nucleotide gated channel and it contains eight coding exons (Wissinger et al., 2001). It was cloned in mouse, rat, bovine, chicken, and human (Biel et al., 1994; Bonigk et al., 1993; Hirano et al., 2000; Misaka et al., 1997; Wissinger et al., 1997) which helped in elucidating the functional and structural properties of the CNGA3.

CNGA3 is expressed in cone photoreceptor in retina and mediates the electrical response to light in red, green and blue cones of these receptors (Sundin et al., 2000). CNGA3 was also found to be expressed in the hair cells of the inner ear and cochlear outer hair cells (Selvakumar et al., 2012; Selvakumar et al., 2013)

2.3.1 CNGA3 protein structure

CNGA3 is a 78.8 kd molecule protein with length of 694 amino acids. It has a cytoplasmic amino (NH2) terminus, carboxyl (C) terminus where the cyclic nucleotide binding domain is located (CNBD), six transmembrane segments (S1-S6) which have been divided into two parts; the first is the voltage sensor domain which consists of the first four segments (S1 to S4), and the second part is the central pore which consists of the S5 and the S6 connected by pore forming loop transmembranes (Gofman et al., 2014; Matveev et al., 2010). Carboxy-terminal leucine zipper CLZ is another domain which was identified in the carboxyl (C) terminus and reported to

be the responsible for trimeric interaction (Zhong et al., 2002). Figure 2.2 shows the topological model of the CNGA3 protein in addition to the distribution of mutations within the transmembranes of protein which will be discussed later.

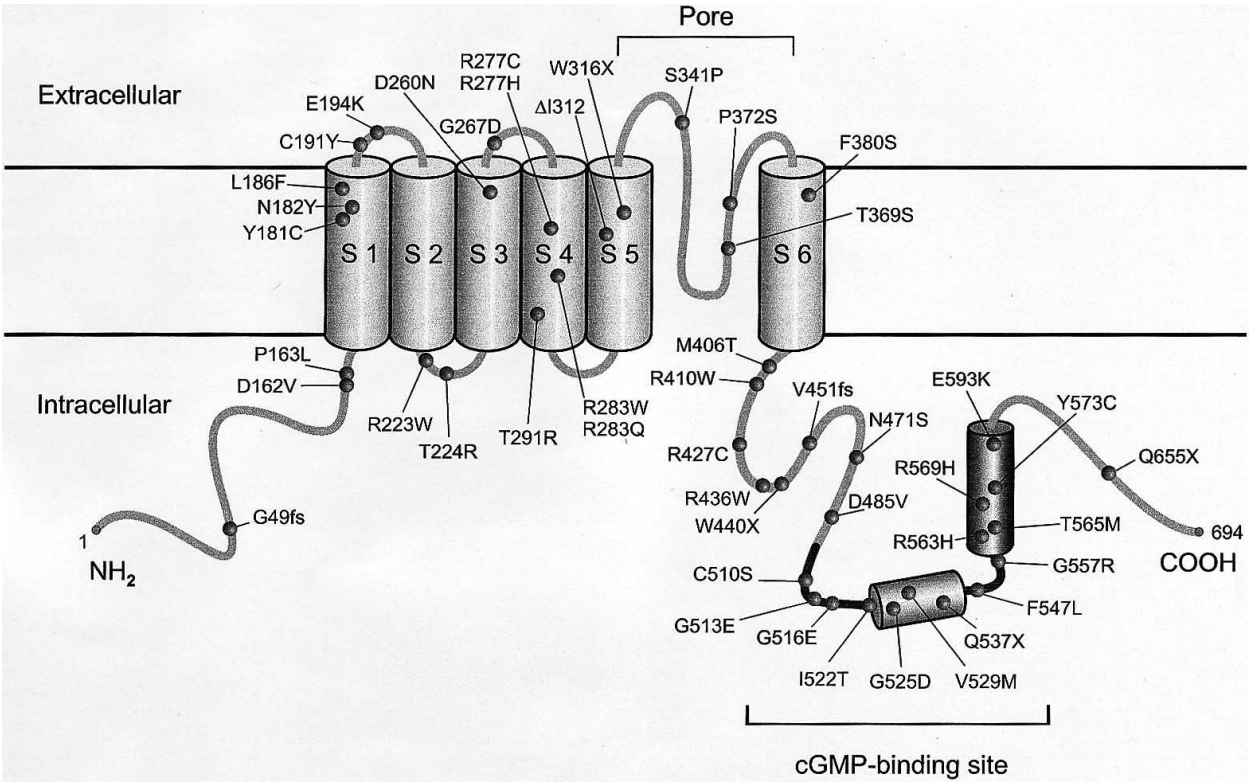


Figure 2.2: Location of the mutation with respect to the proposed topological model of the CNGA3 polypeptide including the six transmembrane helices (S1-S2), the ion pore, and the cGMP-binding domain (Wissinger et al., 2001). The p.Il312del is located in the fifth transmembrane helix.

To date, more than eighty different mutations were identified in CNGA3, and most of these mutations were missense (Kohl et al., 1993). Table 2.1 summarizes the mutations of CNGA3 in human. In 2001 Wissinger et al identified in a study on 58 patients from different nationality 38 new mutations and 8 previously reported mutations in CNGA3, most of these mutations were found to be located at the central and terminal regions of the gene which consists the six

transmembrane helices (S1-S6), cGMP binding domain and the ion pore which are considered to be functionally and structurally important, moreover, most of the mutations were amino acid substitutions (Wissinger et al., 2001).

Table 2.1: **Summary of CNGA3 mutations**

Amino acid alteration	Nucleic acid change	Reference
P163L	488C→ T exon5	
R283W	847C→ T exon7	
R283Q	848G→ A exon7	
T291R	872C→G exon7	
R410W	1228C→T exon7	
V529M	1585G→ A exon7	
F547L	1641C→ A exon7	
G557R	1669G→ A exon7	(Kohl et al., 1998)
G49fs	148insG→ exon2	
D162V	485A→ Texon5	
Y181C	542A→ Gexon5	
N182Y	544A→T exon5	
L186F	566C→ Texon5	
C191Y	572G → A exon6	
E194K	580G→ Aexon6	
R223W	667C→ T exon6	

T224R	671C→ G exon6
D260N	778G→ A exon7
G267D	800G→A exon7
R277C	829G→ T exon7
R277H	830G→A exon7
I312del	934-936delATC exon8
W316X	947G →A exon8
S341P	1021T→C exon8
T369S	1106C→G exon8
P372S	1114C→T exon8
F380S	1139T→C exon8
M406T	1217T→ C exon7
R427C	1279C→T exon7
R436W	1306C→ T exon7
W440X	1320G→ A exon7
V451fs	1350insG exon7
N471S	1412A→ G exon7
D485V	1454A→ T exon7
C510S	1529G→ C exon7
G513E	1538G→A exon7
G516E	1547G→ A exon7

I522T	1565T→C exon7	
G525D	1574G→A exon7	
Q537X	1609C→T exon7	
R563H	1688G→A exon7	
T565M	1694C→T exon7	
R569H	1706G→A exon7	
Y573C	1718A→G exon7	
E593K	1777G→A exon7	
Q655X	1963C→T exon7	(Wissinger et al., 2001)
Arg23Stop	67C→T	
Gln196Stop	58C→T	
Arg221Stop	661C→T	
Arg223Trp	667C→T	
Arg436Trp	1306C→T	
Ile482fs	1443insC	
Phe547Leu	1641C→A	
Gly548Arg	1642G→A	(Johnson et al., 2004)
T224R	671C→G	
T369S	1106C→G	(Trankner et al., 2004)
Pro163Leu	c.488C→T exon5	
Arg283Trp	c.847C→T exon7	

Phe547Leu	c.1641G→ T exon7	(Varsanyi et al., 2005)
Arg283Trp	c.847C→T	
Gly397Val	c.1190G→ T	(Ahuja et al., 2008)
E228K	682G→A	
R439W	1315C→T	
A469T	1405G→A	(Reuter et al., 2008)
p.Arg221Stop	c.661C>T exon6	
Arg277His	c.830G>A exon7	
Ser570Ile	c.1709G>T exon7	
Gly329Cys	c.985G>T exon7	
Arg436Trp	c.1306C>T exon7	
Arg410Trp	c.1228C>T exon7	
Asp514Val	c.1541A>T exon7	
Arg283Gln	c.3848G>A exon7	(Genead et al., 2011)
Tyr357Cys	c.1070A>G	
Thr565Met	c.1694C>T	(Vincent et al., 2011)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials:

3.1.1 Buffers, Gels, and Solutions

- **Proteinase K**

5mg/ml Proteinase K was prepared by dissolving in double distilled sterile water.

- **50X TAE buffer**

2M Tris pH=8.0

1M Acetic acid

0.05M EDTA

Adjust to pH=8.0

- **5X loading buffer**

0.25% Bromophenol blue

0.25% Xylene cyanol FF

30% Glyserol in water

- **1X lysis buffer**

50M Tris HCL with (pH=7.5)

100mM NaCl

mM EDTA (pH=8.0)

- **Red blood cell lysis buffer**

155mM NH_4Cl

10m NH_4HCO_3

0.1mM EDTA with (pH=7.4)

- **Agarose gel**

1.5% agarose

1X TBE buffer

Final concentration of 0.01% Ethidium bromide

- **Ethidium Bromide**

  Ethidium bromide was dissolved in the double distilled sterile water to final concentration of 1mg/ml.

3.1.2 Reagents, Instruments, and Kits

Reagents

Reagent	Supplier	Product specification
20% SDS	aMReSCO®	CAT # 083754-500ml
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, 5000units
Q solution	Qiagen	
Proteinas K	aMReSCO®	LOT# 1311C384
Hi Di Formamide	Applied Biosystems	CAT# 4311320

Kits

Kit	Supplier	Product specification
BigDye™ Terminators V1.1 Cycle Sequencing Reaction Kit	Applied Biosystems	CAT# 4337451-100

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	BioRad	Molecular Imager, Gel DOC™XR+ Imaging System
PCR machine	Applied Biosystems	GeneAmp® PCR System 9700
Sanger Sequencing Machine	Applied Biosystems	ABI 3130XLGenetic Analyzer

3.2 Methods

3.2.1 Samples collection

Venous blood samples were collected from affected and unaffected members of six families. We visited each family at house and clarified the importance of the research for the patients and the aims of the study.

The blood samples were collected from thirty four subjects within which 12 affected subjects and 22 unaffected subjects. Informed consent in accordance to guidelines with the rules of Bethlehem University IRB committee was obtained from each family member up 18 years. Family members under 18 years had one of the parents sign their consent forms on their behalf.

The blood was put in sterile vacutainer tubes containing EDTA (Ethylinediaminetetra acetic acid) to prevent clotting in ratio of 0.1 ml 0.5M EDTA for 20 ml blood [Miller 1988].

3.2.3 DNA extraction by salting out technique

Five to ten ml of blood was used to isolate the DNA of the participated members. 40 ml of red blood cell lysis buffer was added with gentle mix, the tubes then were left on ice for 30 minutes with shaking the tubes from time to time. When the blood appeared as transparent, the tubes were centrifuged at 2000 round per minute (rpm) at 4C for 15 minutes. Then the supernatant was gently discarded and 3 ml of the red blood cell lysis buffer was added on the pellet and again the tubes were centrifuged at the same parameters. Followed by breaking the pellet, which was then suspended in 3 ml of 1x Lysis buffer, 100ul of 20% SDS and 100ul of 5 mg/ml Proteinase K. Tubes was then incubated at 37°C overnight. Then one ml of 6 M NaCl was added to the lysate and strongly vortexed and centrifuged at 3000 rpm for 20 minutes at room temperature. The supernatant was gently transferred to 15 ml tubes, avoiding the salt protein deposit. Two volumes of cold absolute ethanol (EtOH) were added to the supernatant and the tubes were inverted to condense the DNA which was removed by glass Pasteur pipette and directly washed in 70% ethanol. DNA sticked on the Pasteur pipette was left for a few minutes for air drying. Then DNA was dissolved in 200-100ul (depending on the DNA amount) of 0.02% sodium azide and left at room temperature overnight.

3.2.4 Linkage exclusion analysis

Since there are four genes associated with achromatopsia disease, we performed linkage exclusion analysis to determine if we can't exclude linkage to any of the genes. Four families Achro-A family, Achro-B family, Achro-C family, Achro-D family were available for linkage analysis at the time.

3.2.4.1 Microsatellite:

Fluorescently labeled microsatellite markers flanking each of the four known genes CNGA3, CNGB3, GNAT2, and PDE6C were designed and run on all members of the four families using UCSC genome browser. Primers were designed to amplify those regions for genotyping (see Table 3.1 for T.D and primer sequences).

Table3.1: .Microsatellite markers used to study linkage to achromatopsia in families

Gene	Microsatellite	F-primer	R-primer	T.D
CNGA3	18GT	5'-ACTTGGGGAGACCCAAAGAG-3'	5'-GTTTCTTTGCCAAATGTACCCACGTAA-3'	55
CNGA3	21AC	5'-TGTCTTGTAGTGCCGGAAAG-3'	5'-GTTTCTTTTGGGAGCTCTTTCAGGTTG-3'	55
CNGB3	17AT	5'-TGACCCTTATGGACCTTTGC-3'	5'GTTTCTTTGTTAAGGGACATATATAAGGGAGA-3'	56
GNAT2	22AC	5'-AAAGGGGGTGTTTGGTGAG-3'	5'-GTTTCTTTTTCGGACTTTCCAGGAATG-3'	
GNAT2	20TG	5'GGTTCCCTCCTTAGGGCTATC-3'	5'-GTTTCTTAGATGGAGGGTCCCTGSCTT-3'	
PDE6C	19TG	5'-GGCGAGCAAGTGGATATTTG-3'	5'-GTTTCTTCTCACCAAAACCAACCCACT-3'	65
PDE6C	17AC	5'-CTAATGAAGCCAGCCCAGAG-3'	5'-GTTTCTTGGGAAGTCTTCACGGTCAAT-3'	

F-primers fluorescently labeled (FAM)

Extra tail on R-primers for GNAT2 and one F- primer fluorescently labeled (HEX) and the other labeled with FAM.

3.2.4.2 Genotyping PCR program

The sequences of the DNA for genotyping were amplified using primers

Step 1 **95 C** for 4 min

Step 2 (3 cycles) **94** C for 30 sec, **68** C for 30 sec, **72**C for 30 sec

Step 3 (3 cycles) **94**C for 30 sec, **66** C for 30 sec, **72** C for 30 sec

Step4 (3 cycles) **94** C for 30 sec, **64** C for 30 sec, **72** C for 30 sec

Step5(3 cycles) **94** C for 30 sec, **62** C for 30 sec, **72** C for 30 sec

Step 6 (35 cycles) **94** C for 30 sec, 60 C for 30 sec, 72 C for 30 sec

Step7 **72** C for 5 min, **4** C ∞

Annealing temperature depends on the melting temperature of the primer used.

3.2.4.3 Genotyping

The PCR product was diluted 1:50 (1 µl of PCR product + 50 µl of Nuclease free water (N.F)), and then 0.3 µl of 400 X-Rhodamine (Rox) and 11.7 µl Hi Di (Highly deionized formeamide) were added to each PCR product. Then the mix was incubated for 2 minutes on 95 C in order to denature DNA strands, followed by incubation for five minutes on ice. Samples were run on gene mapper version.4 (3130XL Genetic Analyzer from Applied Biosystem) after loading them on the 96-well optical ReactionPlate from Applied Biosystem at Bethlehem University. The results were analyzed using the genemapper.

Table 3.2 Reaction mix per 13 µl of total volume:

Reagent	**volume in µl**
GS-400HD ROX	0.3 µl
Hi Di	11.7 µl
PCR product	1 µl

3.2.5 Mutation analysis

3.2.5.1 Detection of mutation by direct sequencing

3.2.5.1.1 Polymerase Chain Reaction (PCR)

The seven coding exons and flanking intronic sequences of CNGA3 gene were amplified using a primer pair for each as following:

Exon-2 primers (product size is 159 bp)

Primer	**Primer sequence**
Forward primer	**5'-AACCGAGAAGATGGCCAAGA-3'**
Reverse primer	**5'-TGACCAGAGCCAGGCATAAA-3'**

Exon-3 primers (product size is 196 bp)

Primer	Primer sequence
Forward primer	**5'-CAGGAGGTAGATGGGCTTGA -3'**
Reverse primer	**5'-AGGTAGGGACTGAGGCTG-3'**

Exon-4 primers (product size is 278 bp)

Primer	Primer sequence
Forward primer	**5'-TCTGGCTCAGTGCTTGTGTC-3'**
Reverse primer	**5'-AGCAAAGCACAGGCTTCCT-3'**

Exon-5 primers (product size is 1816 bp)

Primer	Primer sequence
Forward primer	**5'-CCCAAGGAATGGAAACAGAG-3'**
Reverse primer	**5'-CCAAGCTGG-3'GGCTTCTATG-3'**

Exon-6 primers (product size is 200 bp)

Primer	Primer sequence
Forward primer	**5'-GGCTCTCTAAAACCCTCCAAA-3'**
Reverse primer	**5'-CCTGTCGCTTACCTGCAAAT-3'**

Exon-7 primers (product size is 200 bp)

Primer	Primer sequence
Forward primer	**5'-TCCCACATGGCTTCTTTAGG-3'**
Reverse primer	**5'-ATCCACCATGCTGGGTCTC-3'**

Exon-8-1 primers (product size is 596 bp)

Primer	Primer sequence
Forward primer	**5'-CCTCTGTGATGCCCAATGAC-3'**
Reverse primer	**5'-CCCGTGAGGCATTCATATTC-3'**

Exon8-2 primers (product size is 601 bp)

Primer	Primer sequence
Forward primer	**5'-CTGGTCCACCTTGACCCTTA-3'**
Reverse primer	**5'-CCCCTTGATGTTCAGAATGC-3'**

Exon-8-3 primers (product size is 595 bp)

Primer	Primer sequence
Forward primer	**5'-GAAGGGAGATATTGGGAAGGA-3'**
Reverse primer	**5'-CTGACAGTCGACCCTGTGAA-3'**

Table 3.3 PCR reaction mix per 25µl of total volume:

Reagent	**volume in µl**
10X Buffer	2.50
Polymerase (super therm polymerase)	0.25
Q-Solution	5.00
dNTPs (2.5mM)	2.00
Forward primer	0.50
Reverse primer	0.50
100ng/µl DNA template	1.00
Nuclease free water	13.25

PCR program (T.D 55 programs)

The PCR program was:

Step 1: **95** C for 4 min.

Step 2 (3cycles): **94**C for 30sec, **68** C for30 sec*, **72** C for 30sec**

Step 3 (3cycles): **94**C for 30sec, **66** C for 30 sec, **72** C for 30sec

Step 4 (3cycles): **94**C for 30sec, **64** C for 30 sec, **72** C for 30sec

Step 5 (3cycles): **94**C for 30sec, **62** C for 30 sec, **72** C for 30sec

Step 6 (35 cycles): **94**C for 30sec, **55** C for 30 sec, **72** C for 30sec

Step 7:72 C for 5 min, **4** C ∞

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

** Extension time was determined according to the size of amplified product (each 1KB= 1min)

Amplification processes were performed by our PCR machine GeneAmp-PCR system 9700 from Applied Biosystem.

3.2.5.1.2 PCR products Electrophoresis.

Agarose gel was used for electrophoresis of PCR products. The concentration of agarose gel was 1.5% and it was determined depending on the sizes of the PCR products. 1X TAE was added to the prepared agarose and then boiled to completely dissolve in TAE, and ethidium bromide was added to agarose solution after mild cooling. Three ul of PCR products were loaded onto the gel after complete cooling and for size estimation three ul of DNA size ladder was loaded to the first well, then the gel run in 1X TAE running buffer at 120V for 20 to 30 minutes depending on the size of the fragment. DNA fragments were documented and spotted using ultraviolet light and photographed using the (Molecular Imager®, Gel DOC TM Imaging System, BioRAD).

3.2.5.1.3. Cleaning of PCR products

The cleanup reaction is a critical step prior to sequencing to remove unincorporated primers, enzymes, salts and unincorporated nucleotides. The fragments can be purified from agarose gel after electrophoresis or from the PCR reaction. In our study, since PCR products showed clear bands on agarose gel, the products were directly purified from PCR reaction using Exonuclease I and Antaractic Phosphatase enzymes. The role of Exonuclease is to degrade the remaining primers. Antaractic Phosphatase removes the leftover nucleotides.

Table 3.4 Master mix of enzymes per 5 µl of PCR

Reagent	**volume**
Exonuclease I	0.25 µl
Antaractic Phosphatase	0.25 µl
Nuclease free H2O	1.5
PCR Products	5

The total volume for whole reaction is 7 µl

PCR clean program for enzyme purification

37 C 30 min

80 C 20 min

4 C ∞

3.2.5.1.4. Sequencing of the purified PCR products

10ng of PCR product per 100 bp of the length of PCR fragment were used for DNA sequencing with 10pm of a single primer was added to 1ul of BigDyeTM Terminators V1.1 Cycle Sequencing Reaction Kit (Applied Biosystems) and 4 uls of BigDye reaction buffer and the reaction was completed to 20µls of volume using nuclease free water. Sequences of samples were amplified using GeneAmp-PCR system9700 from Applied Biosystem. Purification of the products is shown in section 3.2.5.1.5.

Table 3.5 Standard Sanger sequencing reaction mix per 20µl total volume

Reagent	**volume**
5X buffer 4.00 µl	
BigDyeTerminater V1.1	1.00µl
Forward primer	0.50µl
DNA template	1.00µl
Nuclease Free H2O	13.50µl

Sequencing PCR reaction program:

The PCR program was:

Step1: **96 C** for 1 min.

Step2 (25 cycle): **96 C** for 10 sec, **50 C** for 5 sec, **60 C** for 4 min.

Step3: **4 C** for 10 min.

3.2.5.1.5. Cleaning of the cycle-sequenced products using EDTA/Ethanol precipitation method

Cycle-sequenced products were purified using EDTA/Ethanol precipitation method which removes unincorporated primers and dyes and dNTPs before performing sequencing by the machine.

Precipitation and cleaning of each sequenced PCR reaction was done by adding 100µl of absolute ethanol and 5 µl of 125mM EDTA and gently mixing. The reactions were incubated at -20C for 30 minutes. This was followed by centrifugation for 30 minutes at 3800 RPM at 4^{o}C. Then supernatant was discarded and 60µl of 70% ethanol were added to each sample, and wereagain centrifuged for 20 minutes using the same parameters. Then supernatant was discarded and the samples were put upside down on tissue paper and centrifuged at 500 RPM for 1 minute. The samples were dried at 95^{o}C for 5 minutes to remove any remaining ethanol. 16µl of Hi-Di Formamide were added, and the samples were denatured at 95^{o}C for 2 minutes. At last, the samples were incubated in ice for 5 minutes. Samples were run on sequencing machine (3130XL Genetic Analyzer from Applied Biosystem) after loading them on the 96-well optical Reaction Plate from Applied Biosystem at Bethlehem University. The results were analyzed on FINICH TV software.

3.2.6 Genotyping of healthy controls

200 healthy, unaffected Palestinian male controls from Hebron were collected, with age ranging from 20- 35 years old, DNA were extracted from their whole blood samples, and the sequence that contain the mutation was amplified using the same primer set, and then sequenced and analyzed to detect the presence of the mutation.

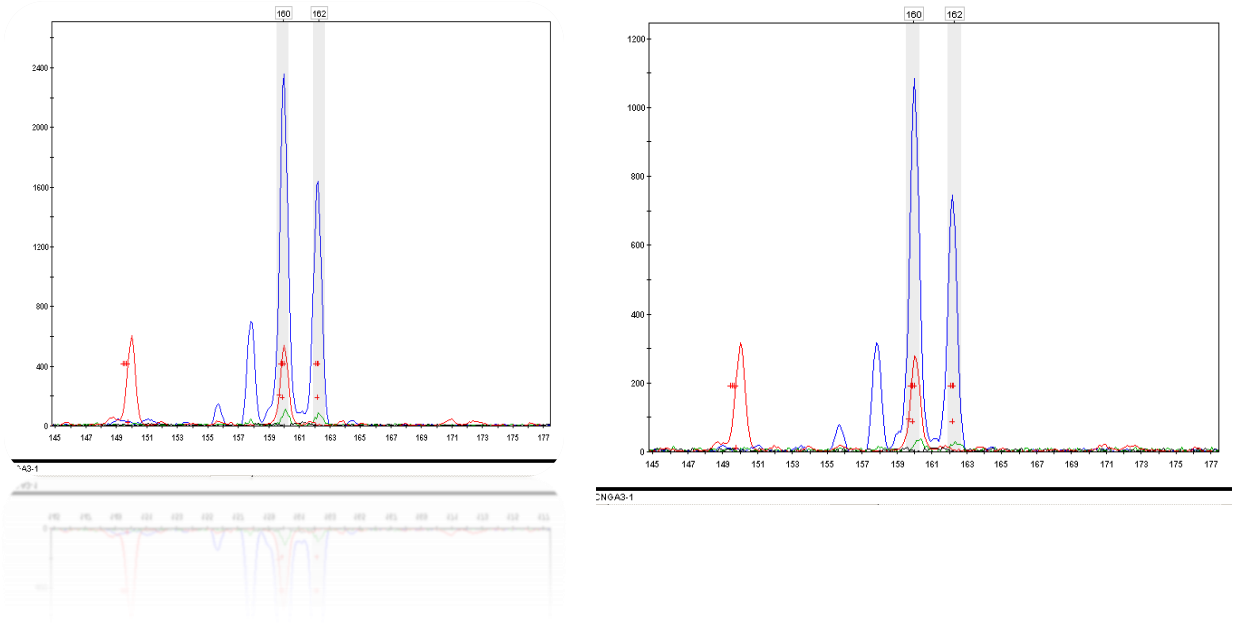
CHAPTER FOUR

RESULTS

4.1 Linkage Exclusion Analysis Results

Haplotypes construction for the causative genes was based on the genotypes of the two microsatellites for each gene. All markers were informative in all families and provided initial results that excluded CNGB3, GNAT2, and PDE6C genes, and we could not exclude linkage to CNGA3 gene. So markers flanking CNGA3 were homozygous in affected members in all families. Fragment analysis of CNGA3 microsatellite markers of unaffected parents and one affected individual and one unaffected individual of Achro-A family is shown in figure 4.1, and figure 4.2; unaffected parents and the unaffected member were heterozygous, while the affected one was homozygous, and the same informative results were obtained by the second CNGA3 microsatellite. Pedigree of family Achro-A used for linkage analysis with haplotypes are shown in figure 4.3.

1. Achr-A1 (unaffected father)2.Achro-A2 (Unaffected mother)



3.Achro-A6 (Affected individual) **4. Achro-A7 (Unaffected individual)**

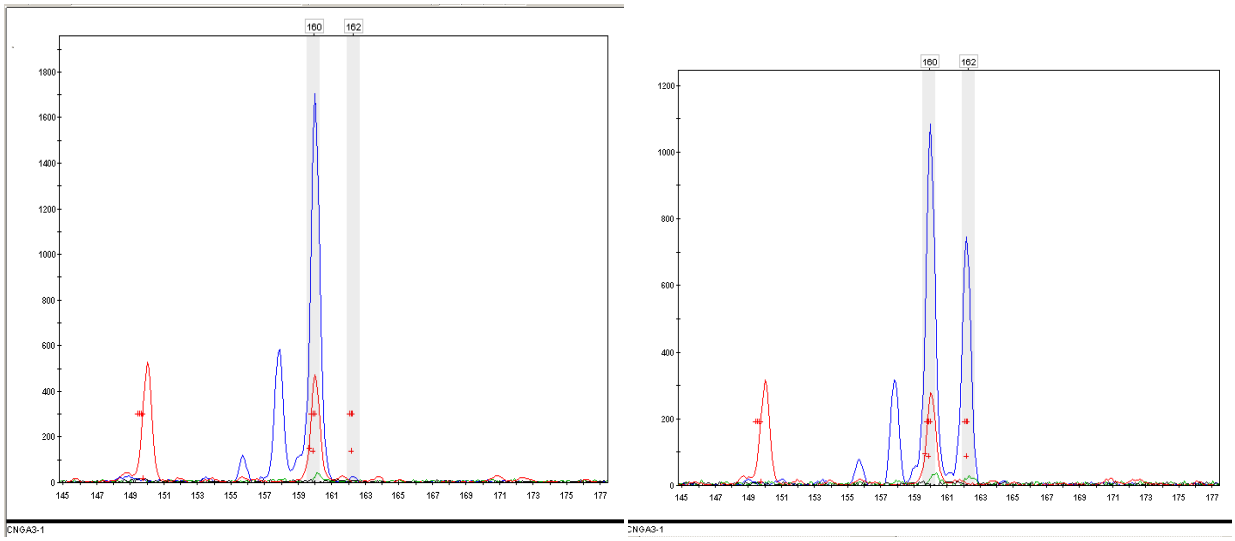
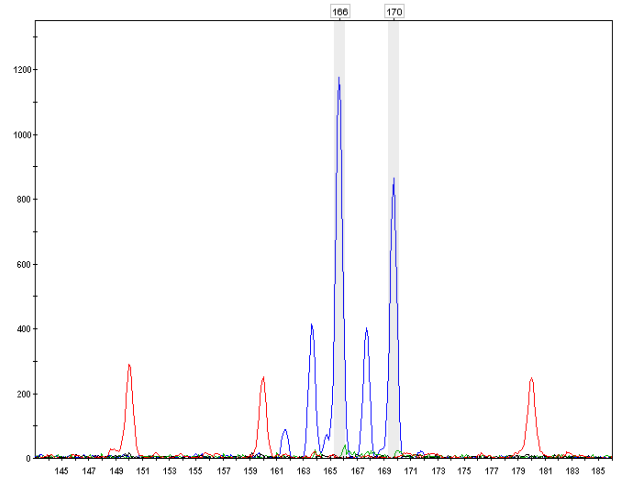
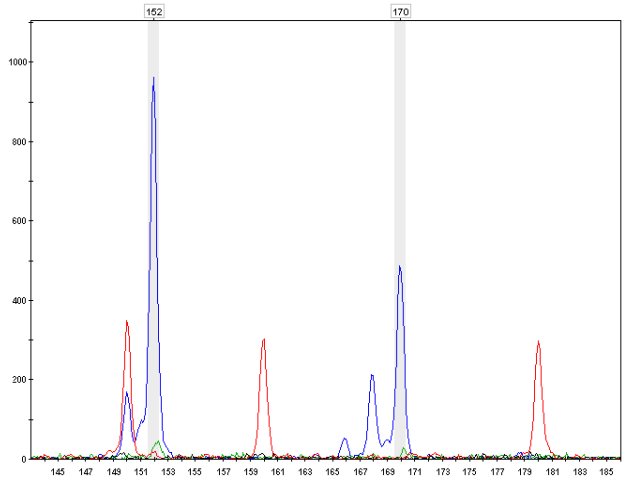


Figure 4.1 Fragment analysis of CNGA3-1 microsatellite markers

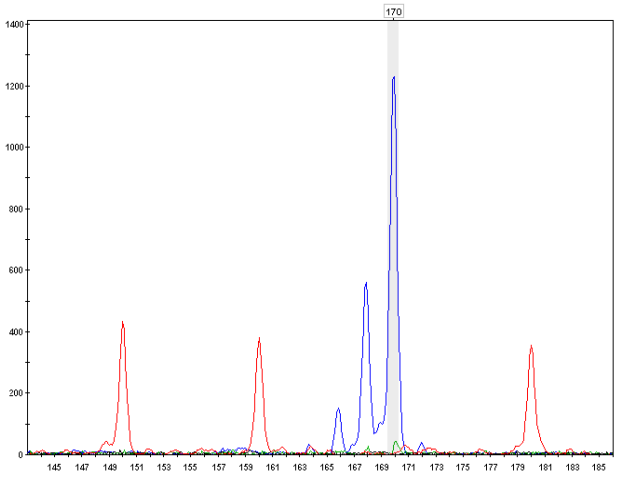
1. Achro-A1



2.Achro-A2



3.Achro-A6



4.Achro-A9

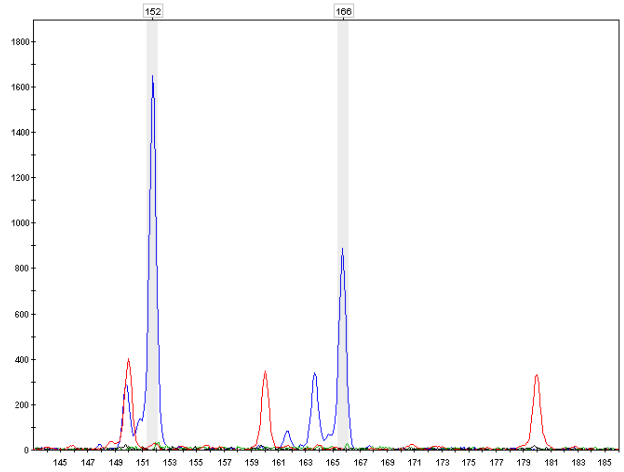
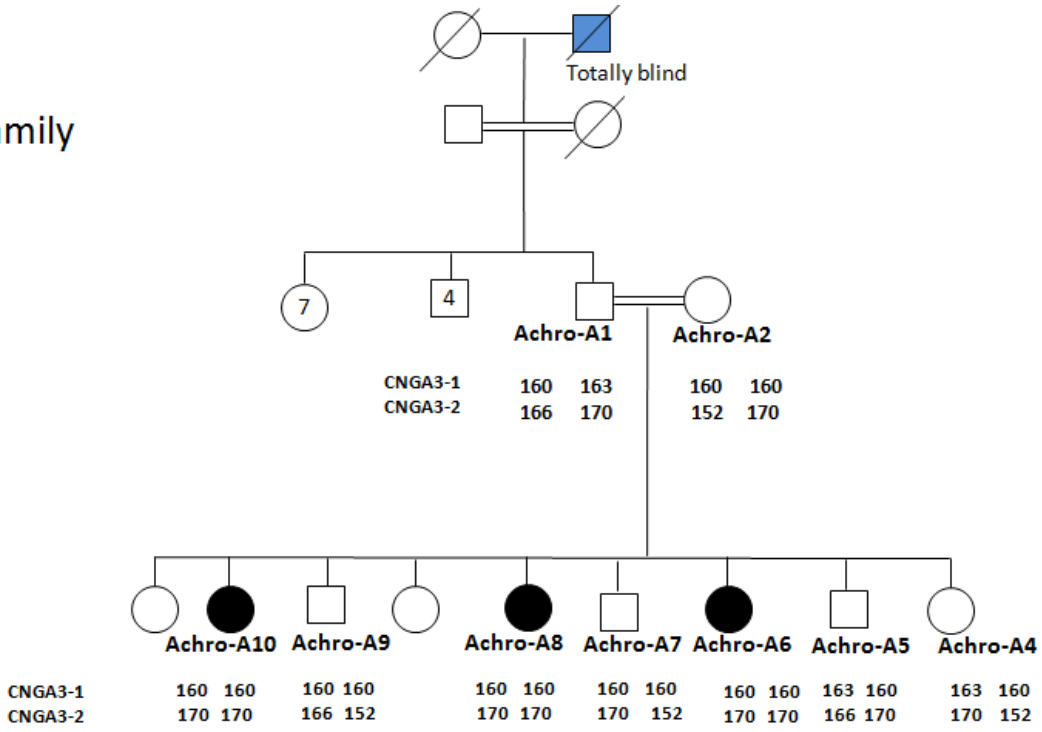
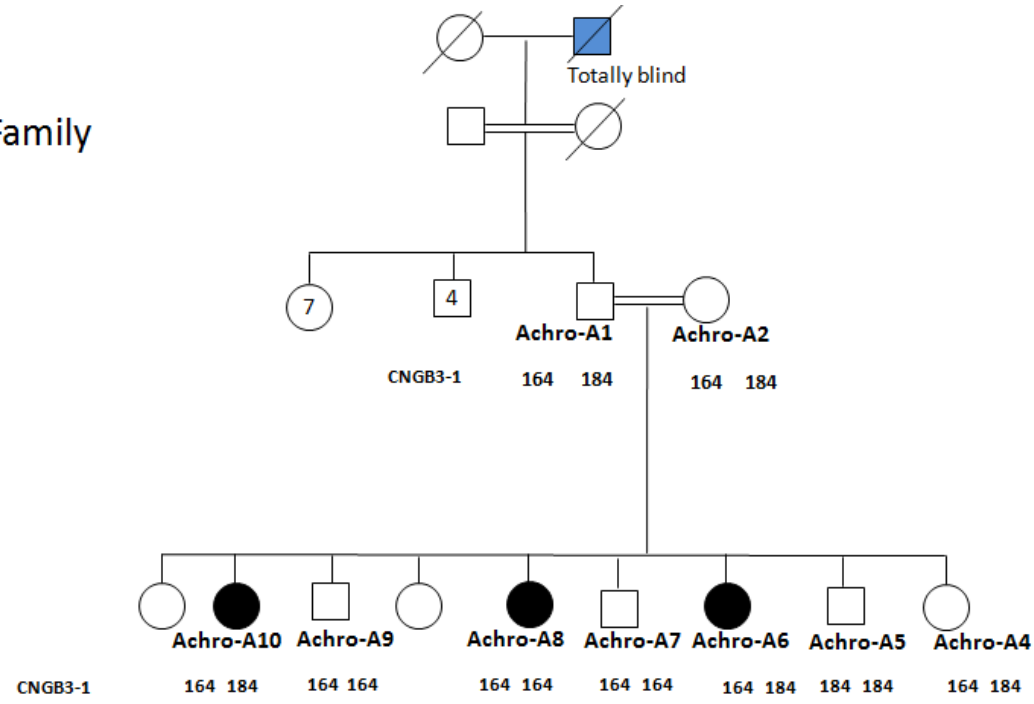


Figure 4.2 Fragment analysis of CNGA3-2 microsatellite markers

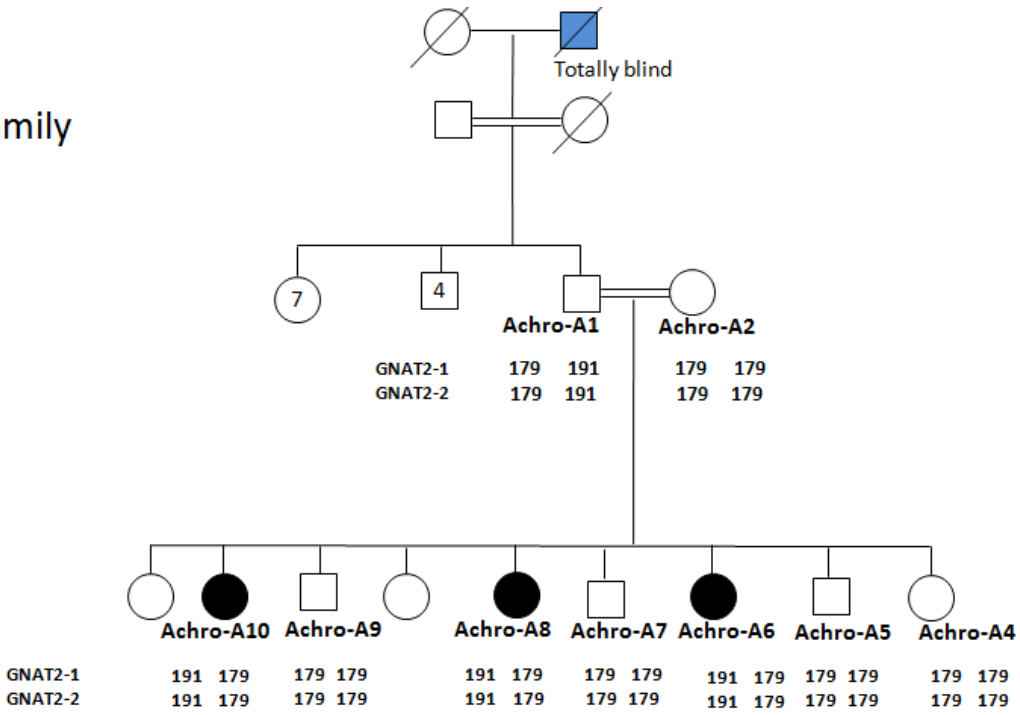
(1)
Achro-A Family



(2)
Achro-A Family



(3)
Achro-A Family



(4)
Achro-A Family

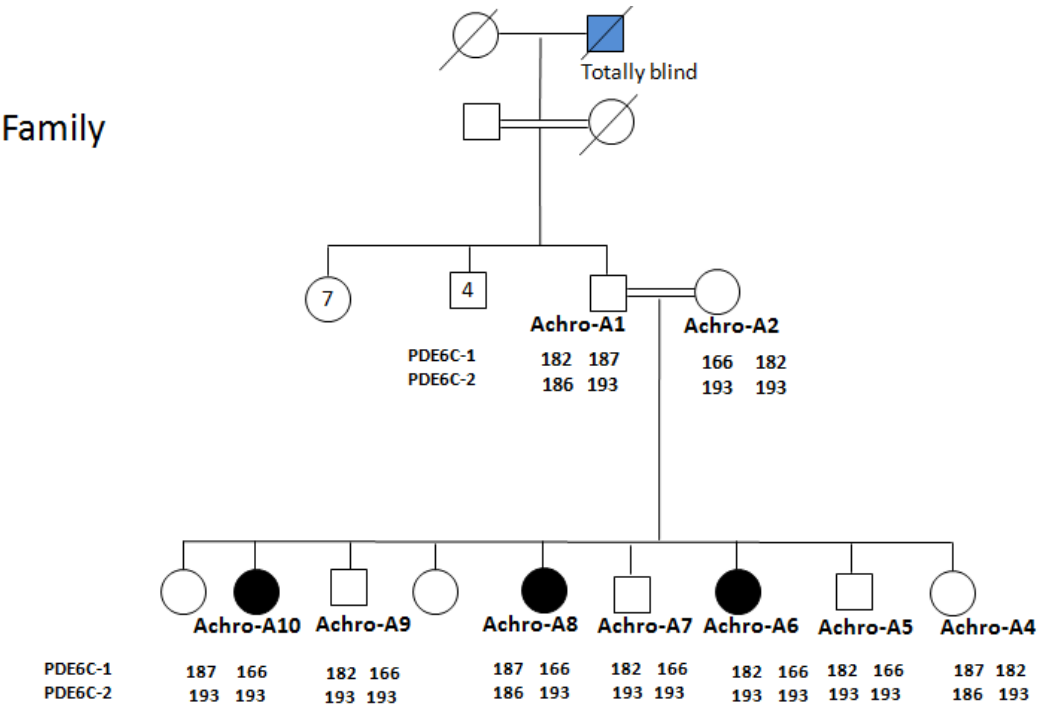


Figure 4.3 Pedigrees of Achro-A family with haplotypes used for linkage exclusion analysis

1. Haplotypes of CNGA3 in Achro-A family
2. Haplotypes of CNGB3 in Achro-A family
3. Haplotypes of GNAT2 in Achro-A family
4. Haplotypes of PDE6C in Achro-A family

4.2 Sanger sequencing of the CNGA3 coding exons results

CNGA3 gene is composed of eight exons. The first exon was excluded at this point, since it is noncoding exon. The remaining seven coding exons were sequenced in one affected individual (Achro-A6) and the parents of Achro-A family (Achro-A1 and Achro-A2). Sequencing results revealed that there is an in-frame three base pair ATC deletion at chr:99,012,573-99,012,576 del ATC, leading to the deletion of the codon of Isoleucin (p.Ile312del). The deletion is predicted to disrupt the structure of the transmembrane of the cone photoreceptors which is specifically expressed in cones of retina.

4.3 Sanger sequencing results. Segregation testing of the deletion with achromatopsia phenotype in all families.

Results of sequencing of all affected and unaffected members in all families Achro-A, Achro-B, Achro-C, Achro-D, Achro-E, and Achro-F showed that the deletion segregates perfectly in a recessive mode of inheritance in all families. It also revealed that the unaffected parents were heterozygous for the deletion and the unaffected siblings were wild type or heterozygous, while the affected members were homozygous for the mutation.

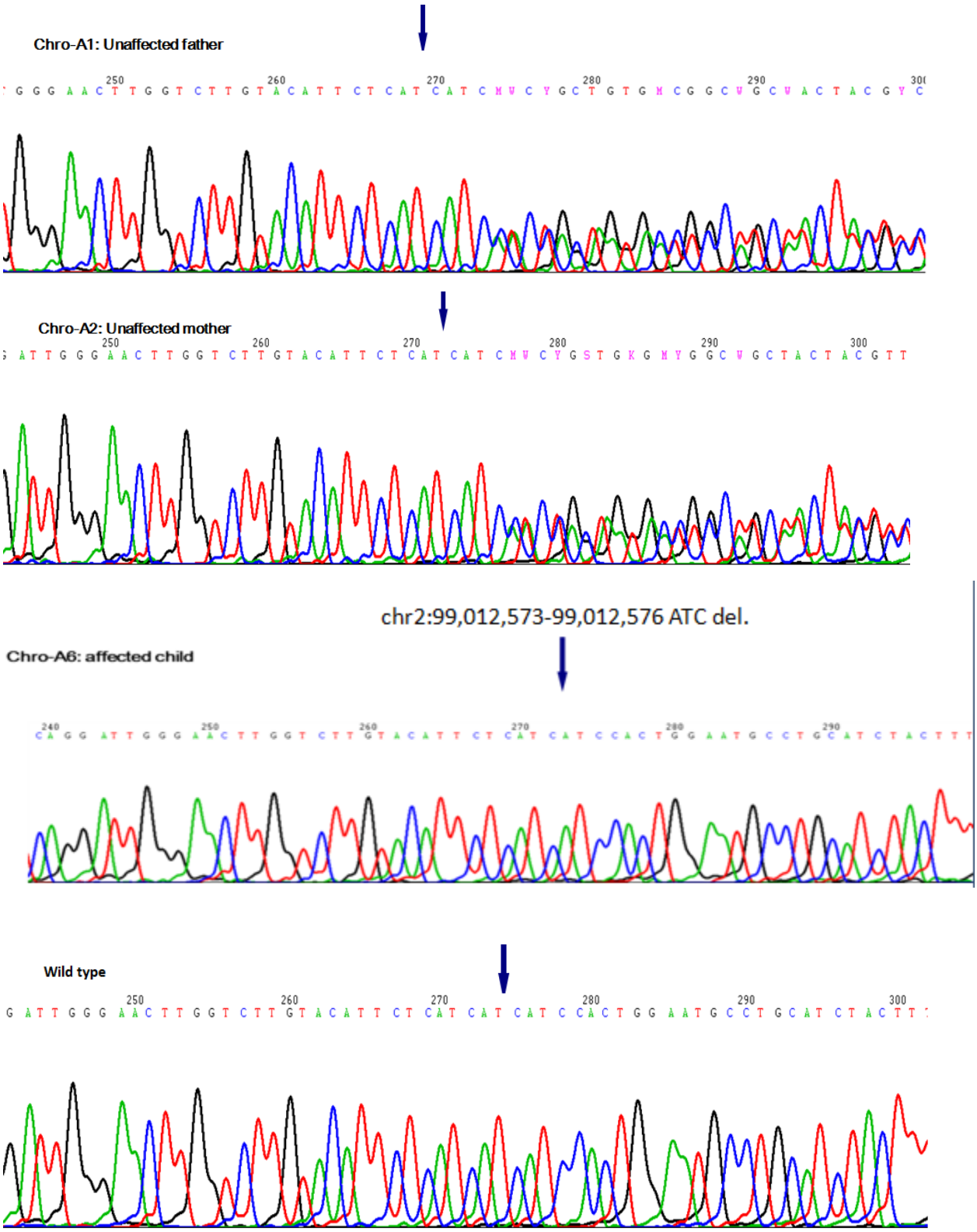
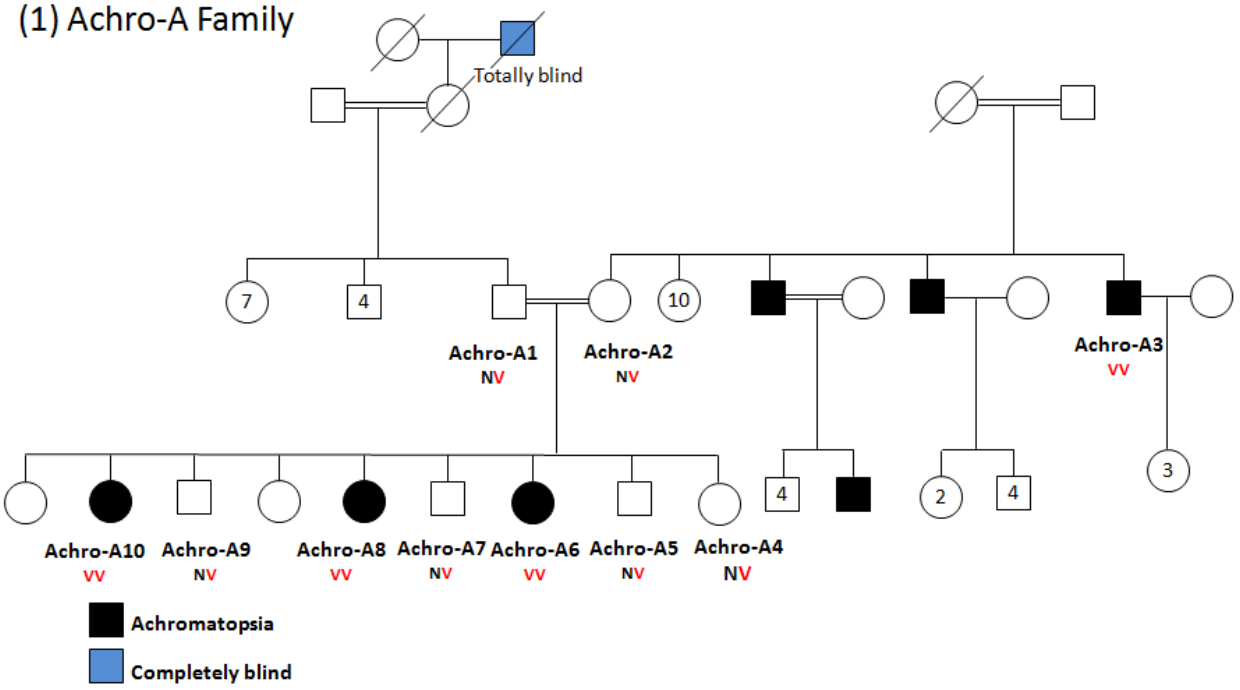
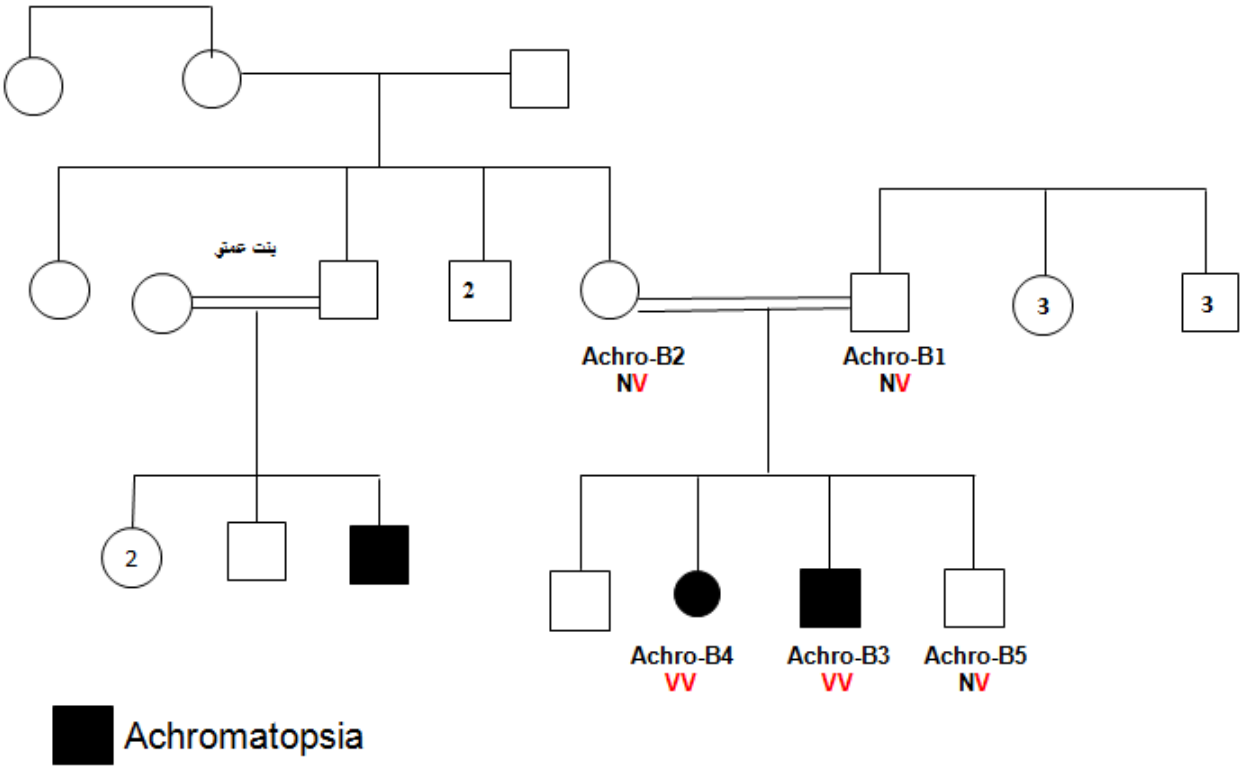


Figure 4.4: electropherogram of the parents and affected child and wild type of family Achro-A**.**

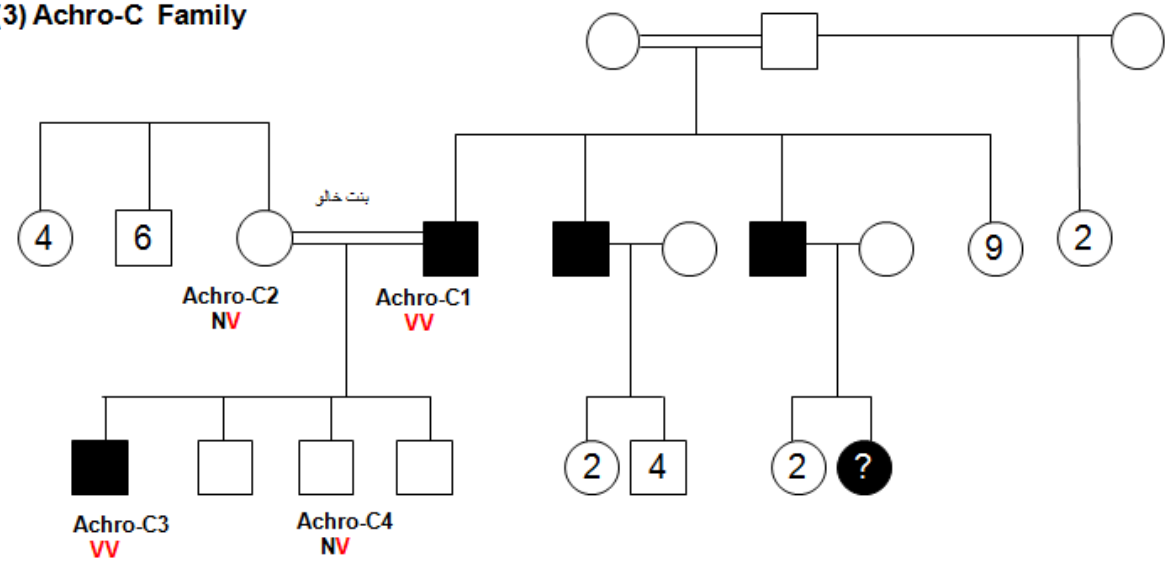
(1) Achro-A Family



(2) Achrom-B Family

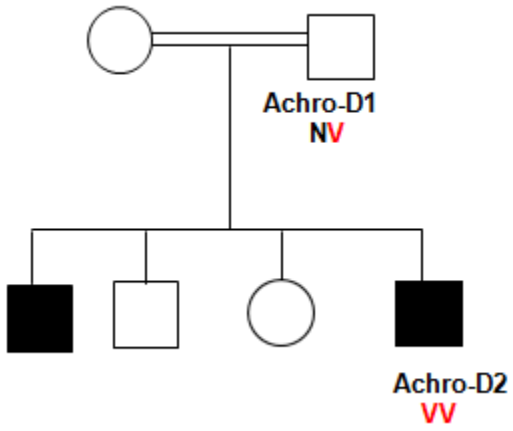


(3) Achro-C Family



Achromatopsia

(4) Achro-D Family



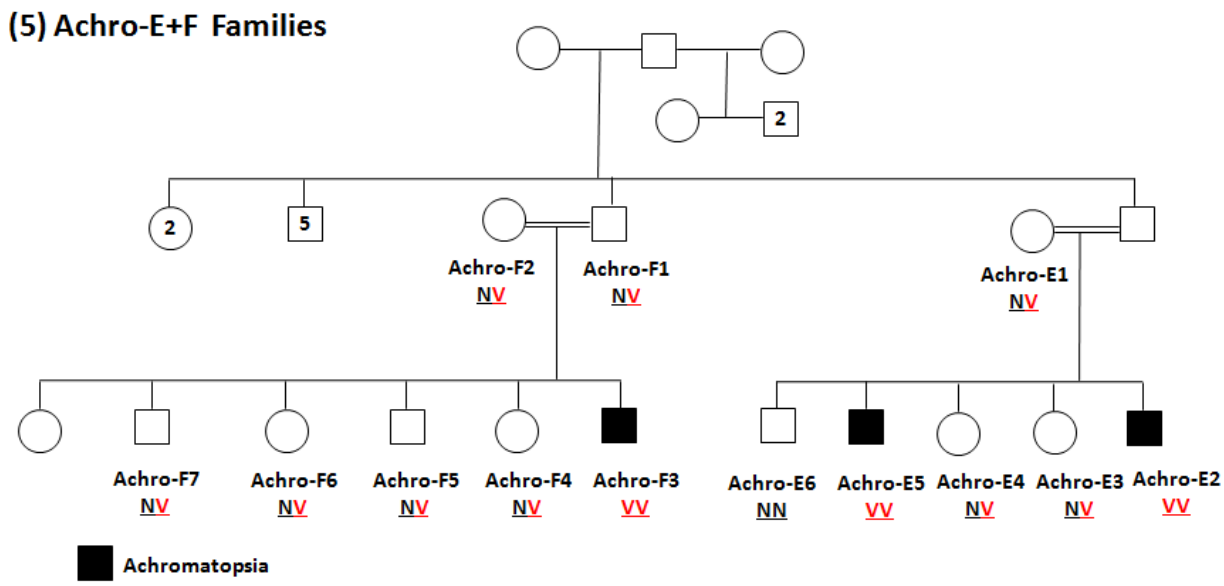


Figure 4.5: segregation ofthe ATC deletion with achromatopsia in Achro-A, Achro-B, Achro-C, Achro-D, Achro-E, and Achro-F families.

(1) Segregation of ATC deletion with the phenotype in Achro-A family.

(2) Segregation of ATC deletion with the phenotype in Achro-B family.

(3) Segregation of ATC deletion with the phenotype in Achro-C family.

(4) Segregation of ATC deletion with the phenotype in Achro-D family.

(5) Segregation of ATC deletion with the phenotype in Achro-E+F families.

4.4 Bioinformatics Tools Results

We identified that phenotype in the six families with members affected with achromatopsia resulted from an in-frame three base pair deletion (ATC) that leads to deletion of the codon for Isoleucin residue 312 in the cyclic nucleotide gated cation channel alpha subunit 3 (CNGA3) protein. Using the UCSC genome browser, we found that this amino acid is highly conserved among 45 vertebrate genomes. So mutation or deletion in this amino acid is most likely to be harmful and will leads to nonfunctional CNGA3 protein by damaging the integrity of the transmembrane domain of the protein.

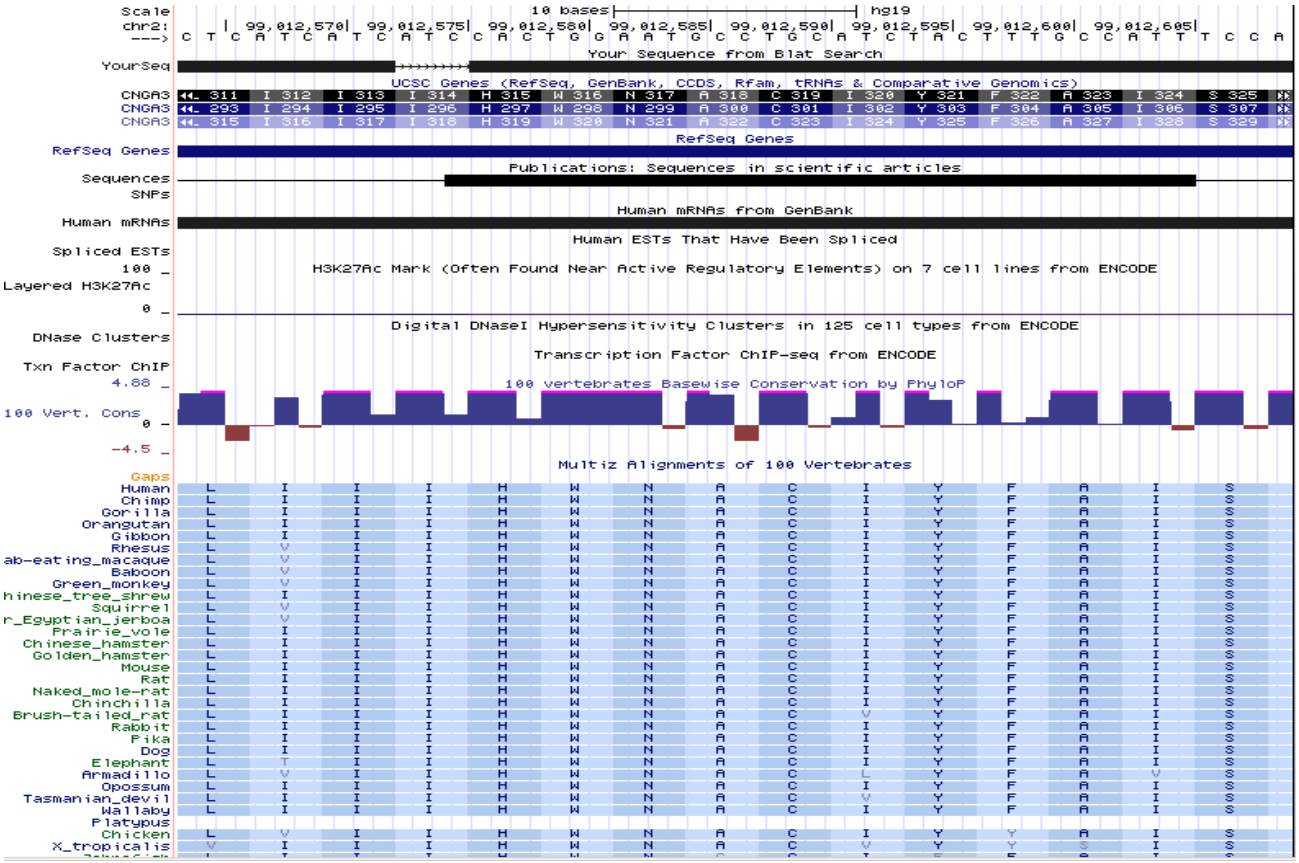


Figure 4.6: Alignment of the amino acid residue of CNGA3 in human with other 45 vertebrate genomes. As noticed in the figure that the amino acid (I) Isoleucin with red color is totally conserved among these species.

The mutation in CNGA3 gene; c1357delATC (p.Ile312del) perturb the integrity of the fifth transmembrane of the CNGA3 protein that plays important role in the light transduction cascade and consequently color discrimination.

4.5 Sanger Sequencing Results of Healthy Controls

200 healthy, unaffected Palestinian controls from Hebron were genotyped. Genotyping revealed that 0.5% of these controls were heterozygous for c1357delATC (p.Ile312del) while none of them was homozygous.

CHAPTER FIVE

Discussion and Conclusion

5.1 Discussion

Mutations in CNGB3, CNGA3, and GNAT2 genes have been described to be associated with the most of studied achromatopsia cases. These genes encoding essential proteins which play crucial roles in the color vision in the three types of cone photoreceptors. CNG channels in cones composed of CNGA3 and CNGB3 subunits (Kaupp and Seifert, 2002). As it is described; that the CNGA3 subunits able to form homotetramers if expressed alone while CNGB3 subunits needs coexpression of CNGA3 (Wissinger et al., 1997).

To date, most of mutations in CNGA3 that were identified are missense mutations (<80%) suggesting that there is no chance for mistakes in this gene, even if it was a substitution in this gene, in contrast, only few of CNGB3 mutations are missense (Kohl et al., 1993). Furthermore, this gene is highly conserved in CNG channel alpha subunits; which indicates its functional and structural importance.

Some previous studies reported the estimation of the CNGA3 mutations that contributed to achromatopsia as 25% of cases (Wissinger et al., 2001), while other studies reported the estimation to be 41% of cases (Johnson et al., 2004). Till now, the classifications and frequencies of the mutations associated with achromatopsia among Arabs have not been determined (Ahuja et al., 2008).

CNGA3 knockout mouse model study revealed morphological and functional abnormalities of cones (Biel et al., 1999a). Mutations in this gene have been concluded to cause achromatopsia (Matveev et al., 2008).

In our present study, we identified six Palestinian families with achromatopsia. All of the affected siblings have potophobaia, nystagmus, and color blindness. In all six families; we identified an in-frame three base pair deletion c.1357del ATC (p.Il312del) that resulted in loss of codon (ATC) that codes for the amino acid Isoleucine in exon eight of CNGA3 gene which

encodes for the alpha subunit of the CNG channels in retina. The deletion segregates with achromatopsia in all tested families Achro-A, Achro-B, Achro-C, Achro-D, Achro-E, Achro-F which involved in the study. We reported this deletion as responsible for the achromatopsia in those Palestinian families. All twelve affected individuals who were tested in this study were homozygous for the deletion and all twenty two unaffected individuals were either wild type or heterozygous. Our results propose that CNGA3 gene is the causative gene of achromatopsia in the six Palestinian families.

In our study, we used the direct sequencing for determination the mutation carriers since it is available in the laboratory, and also by using bioinformatics tools, we did not find restriction enzymes that discriminate mutant and wild type. In addition, we could not use fragment analysis because it is so expensive.

In CNGA3, the p.Il312del is present in a conserved region and this region is part of the fifth transmembrane helix (S5) (Wissinger et al., 2001) and it is predicted by bioinformatics tools that this mutation perturbs the transmembrane structure of CNGA3 protein. The mutation in this transmembrane affects the ligand sensitivity, also it causes impaired protein folding and may cause disturbed surface expression and so instable protein which means that the fifth transmembrane is critical for the functional and structural integrity of the cone CNG channels (Shaikh et al., 2014).

The p.Il312del mutation that has been reported in our study has been previously identified in achromatopsia patients from different origins, Italy and Haiti (Wissinger et al., 2001).

The six families in our study carry the same in frame deletion, and this finding proposes that these families have the same ancestor, and the point that support our suggestion is that all of the families from the same geographical region of Hebron, and five families Achro-A, Achro-B, Achro-C, Achro-E, Achro-F carry the same family name, while Achro-D family belongs to different family but from the same geographical region and has the same mutation as other families, which also propose that this family derived from the same ancestor of the other families.

We noticed that one of the families has affected father Achro-C1, and this is due to the first cousin marriage of his parents. Heterozygotes for the p.Il312del mutation don't have any visual problems and their photoreceptors keep their normal function, which means that this mutation acts in a recessive manner.

Consanguineous marriages are common among the Palestinian population and it is distributed for 40% of all marriages in west bank {Palestinian central Bureau, 2009}. Many of genetic diseases such as autosomal recessive diseases can be highly increased in occurrence as a result of consanguineous marriages. All of the families here have first cousin marriage which explains presence of achromatopsia affected offspring.

Other critical components involved in the phototransduction cascade such as phosphodiesterase subunits and the beta and gamma subunits of transducin may be also candidates for the disease.

It is important to determine the genetic background and causative mutations which leads to correct diagnosis of the achromatopsia in these Palestinian families, and consequently; right dealing with the manifestations and treatment will highly help the patients to coexist with the disease, for example, using of dark colored pink glasses as supportive therapy decreases the light sensitivity to some extent and increase the scotopic vision.Genetic counseling in Palestinian families with achromatopsia disease, in addition to CNGA3 mutation screening in population might be done to detect the carrier individuals in order to prevent more achromatopsia cases.

Pre-marriage testing for this mutation in the CNGA3 gene in consanguineous Palestinian families having members suffers from the disease is highly recommended to prevent the occurrence of more cases of achromatopsia among our population.

5.2 Conclusions

Finally, we conclude that:

- The identified frame shift mutation in this study p.Il312del that cause deletion of the codon for Isoleucine at amino acid 312 of the fifth transmembrane helix of CNGA3; is responsible for achromatopsia disease in these six Palestinian families which are consanguineous.
- The mutation in CNGA3 was not detected in 200 Palestinian healthy controls from Hebron using Sanger sequencing.
- The mutation segregates perfectly with the phenotype in all families in a recessive mode of inheritance.
- All affected individuals in all families are homozygous for the mutation while the unaffected are heterozygous or wildtype.
- CNGA3 gene has been shown to cause achromatopsia disease in people from different ethnic backgrounds.

CHAPTER SIX

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