THE HONG KONG POLYTECHNIC UNIVERSITY

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Mutational analysis of *RHO*, *RDS* and *PRPF31* genes in Chinese patients with retinitis pigmentosa

LIM King-Poo

A thesis submitted in partial fulfillment of the requirements for the Degree of Master of Philosophy

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CERTIFICATE OF ORIGINALITY

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

LIM King-Poo

Dedication

To God.
His encouragement is the motivation
for finishing this project.

To my beloved wife and daughter.
None of this would
be possible without their love and support.

In memory of my cat Lan Jai.
Lonely time was accompanied by
his being.

Abstract

Retinitis pigmentosa (RP) is a group of genetically heterogeneous eye diseases. A characteristic feature of RP is the progressive degeneration of photoreceptors in the retina and eventual blindness. It is one of the most common genetic eye diseases with a prevalence of about 1 in 4000. More than one thousand people are estimated to have RP in Hong Kong. Genetic mutations play a definitive role in RP.

In order to identify the genetic alterations underlying RP in Hong Kong Chinese, DNA samples from 79 RP subjects were screened for mutations by single strand conformation polymorphism (SSCP) analysis of polymerase chain reaction (PCR) amplified fragments (PCR-SSCP). Seventy five samples from anonymous healthy blood donors were used for determining the frequencies of base changes in normal subjects. Three genes were investigated in this study: rhodopsin (***RHO***), retinal degeneration, slow (***RDS***) and PRP31 pre-mRNA processing factor 31 homolog (yeast) (***PRPF31)***. All exons and their flanking regions of these three genes were screened and all sequence variations were characterized by cycle sequencing. A putative promoter region of ***RHO*** was also investigated.

In total, 37 sequence variations were found in this study. Of these variations, 14 have been reported previously while 23 were novel. Sequence variations were scattered throughout the genes. Nine sequence variations were identified in ***RHO***, 10 in ***RDS*** and 18 in ***PRPF31***.

Five significant findings can be summarized as follows. First, a 3-T deletion of ***RHO*** located in the 5' negative regulatory element was identified in a subject with Usher syndrome (RP plus inherited hearing impairment). In sequence comparison

between organisms, 3-T was found conserved among human, cow, mouse and rat. The consequences of this variation should be further confirmed by functional assays. Second, the well-known ***RHO*** missense mutation P347L and a novel +6307delT (also denoted as F262***fsX***320) frameshift mutation of ***PRPF31*** were identified in an autosomal dominant RP (adRP) family. Affected subjects were found to carry either one or both of these two mutations: homozygous P347L ***RHO*** mutation with wild type ***PRPF31*** (P347L +/+; F262***fsX***320 -/-), wild type ***RHO*** mutation with heterozygous F262***fsX***320 ***PRPF31*** mutation (P347L -/-; F262***fsX***320 +/-), and homozygous P347L ***RHO*** mutation with heterozygous F262***fsX***320 ***PRPF31*** mutation (P347L +/+; F262***fsX***320 +/-). Third, a novel S2C missense of ***PRPF31*** was also identified in two adRP families. Incomplete penetrance was suspected in one of the families that had an asymptomatic carrier of this mutation. Fourth, a novel complex ***RDS*** mutation, +232G>C;+232_+233insT, was found in an RP subject. These base changes caused a frameshift (A78***fsX***176) and were predicted to produce a truncated protein. Finally, the frequencies of haplotypes consisting of three polymorphism alleles in ***RDS***, E304Q, K310R and G338D, showed a statistical significant difference (***p=0.0131)*** between the RP and the control groups. This indicated that there might be an association between the haplotype and RP.

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List of abbreviations

µl	Micro liter
ad	Autosomal dominant
ar	Autosomal recessive
ATP	Adenosine triphosphate
BPS	Branchpoint sequence
CERK	Ceramide kinase
CERKL	Ceramide kinase-like
cGMP	Cyclic guanosine monophosphate
CRA	Central retinal artery
CRALBP	Cellular retinaldehyde binding protein
Da	Dalton
dNTPs	Deoxyribonucleoside triphosphates
DM	Myotonic dystrophy
ERG	Electroretinogram
GC	Guanylate cyclase
GDP	Guanine diphosphate
GPCR	G protein coupled receptor
GTP	Guanine triphosphate
ml	Milliliter
mRNA	Messenger RNA
MSS	Mutant steady state
NCBI	National center for biotechnology information
NLS	Nuclear localization signal
PDE	Phosphodiesterase
PRPF31	Precursor mRNA-processing factor 31 homolog (yeast)
RDS	Retinal degeneration gene, slow

RHO	Rhodopsin
ROS	Rod outer segment
RP	Retinitis pigmentosa
RPE	Retinal pigment epithelium
rRNA	Ribosomal RNA
SNP	Single nucleotide polymorphism
snRNAs	Small nuclear RNAs
snRNP	Small nuclear ribonucleoprotein
SSCP	Single strand conformation polymorphism
ssDNA	Single-strand DNA
UTR	Untranslated region
xl	X-linked

Chapter 1 Introduction

1. Introduction

1.1 Background

Light not only gives us energy, but also acts as a medium for perceiving our environment. Images are formed by the reflection of light from an object to the eye. Therefore, the loss of eyesight would change one's lifestyle drastically. Genetic eye diseases are the leading causes of blindness in the world. Retinitis pigmentosa (RP) is one of the most common genetic eye diseases with a world prevalence of 22.4 per 100,000 (Haim et al, 1992). There is no evidence of extensive variation in prevalence in different ethnic groups: 1:4016 in China (Hu, 1982), 1:5200 in Maine (Bunker et al, 1984), 1:7000 in Switzerland (Ammann et al, 1965), etc. Therefore, 1.5 million people are estimated to be affected by RP worldwide.

In the course of RP, progressive degeneration of retinal cells, and rod photoreceptors in particular, results in night blindness at early stage and total blindness at late stage. The clinical expression of the disease phenotype is heterogeneous both within and between families. The mode of inheritance of RP also shows heterogeneity in that it can be autosomal dominant, recessive or X-linked. Presently, there is no effective treatment or cure for the disease.

Since the first mutation was detected in 1990, at least 38 genes have been identified that are associated with RP, and 30 of them have been cloned (http://www.sph.uth.tmc.edu/RetNet). Research delineating genetic mutations in RP is being conducted worldwide. Identification of the mutations is crucial to genetic counseling, disease management and the development of treatments such as gene therapy, and is also necessary for the understanding of the physiological and disease mechanisms.

According to prevalence, there are more than 1000 people affected by RP in Hong Kong. However, no systematic and familial study of this disease in Hong Kong has been carried out. This project was initiated by the Retina Hong Kong (formerly known as "Hong Kong Retinitis Pigmentosa Society", http://www.retina.org.hk) as a part of the Hong Kong Patients Register of Retinal Degenerations. The aim of the Register is to establish a territorial database with clinical and genetic information of patients with inherited retinal degenerative diseases. Candidate gene mutation screening is the basis of the present study. Genetic information from this screening will promote clinical data analysis, further research study and the development of potential therapies.

1.2 Project objective

Three RP related genes, ***RHO, RDS*** and ***PRPF31,*** were selected for mutational analysis in 79 Chinese patients with RP. The patients came from 59 unrelated families. The exons and their flanking intron sequences of these genes were screened for mutation by single strand conformation polymorphism (SSCP) analysis. The putative regulatory region of the rhodopsin gene, from just upstream of the transcription start site to +250-bp, was also included in the screening. Sequence variations detected by SSCP were characterized by DNA sequencing. In order to verify mutations found and establish the allele frequency of these sequence variations in the Hong Kong Chinese population, 75 control samples from healthy individuals were genotyped for each variation.

1.3 The retina: structure and physiology

Light is indispensable to us and our eyes are its fabulous partners. RP affects the retina, the most complex and vital structure in our eyes. The retina is similar to the film of a camera with a thickness of approximately 0.5 mm, on which light rays are focused and then transduced to the photoreceptors then to the optic nerve that generates signals to be transmitted to the brain. The retina is a part of the central nervous system and develops from the neuroectoderm (Edward & Kaufman, 2003). It can be divided into five basic functional layers ***(Figure 1)***: 1) retinal pigment epithelium (RPE), 2) photoreceptor layer, 3) intermediate neuronal layer, 4) ganglion cell layer and 5) nerve fiber layer. The ganglion cells are the earliest to differentiate and the photoreceptors are the latest.

The arrangement of the layers occurs in an inside-to-outside manner such that the ganglion cell layer faces the vitreous body and the retinal pigment epithelium is connected to Bruch's membrane of the choroid. Therefore, light must first pass through other layers before it reaches the photoreceptors. After the signal is transduced by the photoreceptors, it is transmitted in the opposite direction towards the ganglion cell layer and finally to the brain by the cells of nerve fiber layer. There are two different blood supply systems in the retina, the central retinal artery (CRA) and the choroid. The CRA is responsible for the blood supply to the inner two-thirds of the retina (e.g. ganglion cells and bipolar cells). The outer one-third of the retina is nourished by the choroidal blood vessels (e.g. RPE, photoreceptors). Each of the layers is described briefly in the following paragraphs.

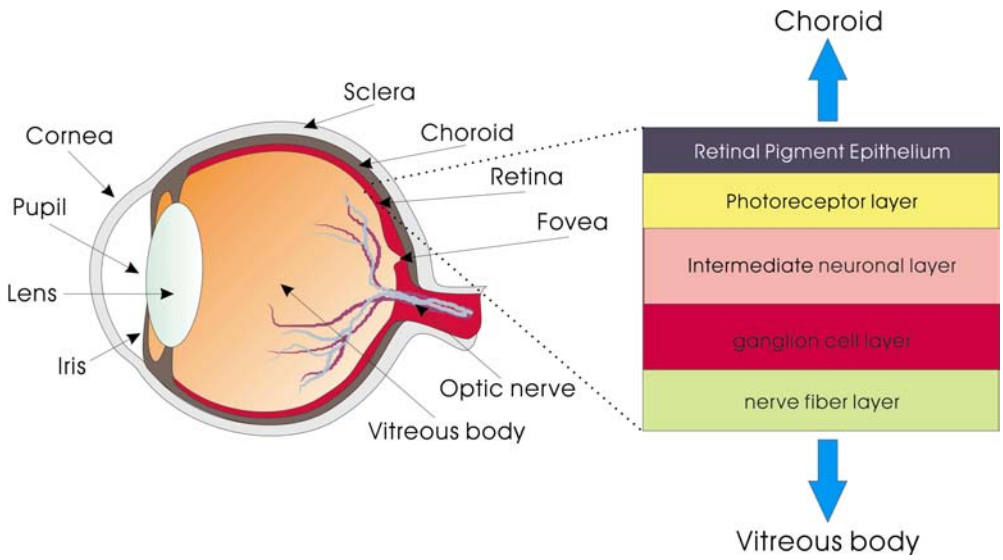


Figure 1 Structure of the eye and the retina

1.3.1 Retinal pigment epithelium

RPE is a highly pigmented monolayer of cells that lies between the photoreceptors and the vascular bed of the choroid. The RPE serves several important functions (Schraermeyer & Heimann, 1999): 1) regulates the transport of nutrients and waste, 2) renews the shed outer segment of photoreceptors by phagocytosis, 3) contains melanosomes, which absorb harmful high-energy light to protect the outer retina, 4) contributes to the regeneration of visual pigments, 5) maintains retinal homeostasis, and 6) builds up the blood-retina barrier.

1.3.2 Photoreceptor layer

The photoreceptor layer is the primary site for phototransduction that converts light energy in the visible light range (400-700 nm) to nerve impulses. There are two types of photoreceptor cells, rods and cones at a ratio of about 20:1. There are approximately 130 million rods and 6 million cones. The rod primarily deals with dim illumination vision (scotopic vision) and is most sensitive to blue light (500 nm). Three different kinds of cone cells (red/green/blue) are present to detect the photopic vision in full illumination and color perception. However, the distribution of rods and cones is different in the retina (Curcio et al, 1990). Rods are localized mainly in the periphery of the retina but cones are clustered predominantly in the macula.

The intermediate neuronal layer contains several types of cell bodies in the middle layer of the retina that act as a connection layer between the photoreceptors and the ganglion cells. The signals generated from the photoreceptors are conveyed to the bipolar cell by synaptic contacts. The axon terminal end of the bipolar cell is presynaptic to another neuron, a ganglion cell, in several possible ways: one-to-one,

divergent or convergent wiring (Clyde, 1999). Another pathway for signal transduction is the lateral pathway mediated by the horizontal cell and the amacrine cell. Horizontal cells and amacrine cells provide feedback to modulate signals transferred across these synapses (Dacey, 1999). The Muller cell is one of the glial cells present in the retina that extends through the full thickness of the retina. One of its functions is to regulate the microenvironment, such as potassium homeostasis, in the retina (Newman, 1993).

1.3.3 Ganglion cell layer

The ganglion cell layer is the last neural connection for the nerve impulse in the eye. The axons of ganglion cells are grouped together to form the nerve fiber layer and converge at the optic nerve head to form the optic disk. There are six regions in the brain to receive the signals from the ganglion cells (Rodieck, 1999). Each region has a different function and receives signals from different subsets of ganglion cell types. The receptive field of ganglion cells in the retina has been studied by Brown et al (2000). The group demonstrated that the sensitivity across the receptive field is not uniform and appears dome-shaped, peaking at the center and declining toward the edge. This pattern of sensitivity profile is known as the difference of Gaussians (Donner & Hemila, 1996). One advantage of this pattern is to improve the signal-to-noise ratio of the ganglion cell and hence increase contrast sensitivity (Tsukamoto et al, 1990).

1.4 Rod photoreceptors and phototransduction

1.4.1 The structure of photoreceptors

Both photoreceptor rods and cones are responsible for signal transduction in the retina. However, abnormal rod photoreceptors are the main cause of RP. The rod photoreceptor is rod-shaped and usually contains four distinctive compartments: outer segment, inner segment, nucleus and synaptic ending ***(Figure 2)***. The rod outer segment (ROS) comprises a stack of flattened membranous discs, highly specialized for photon absorption and energy conversion. These discs undergo disc morphogenesis so that the outer segment is completely renewed about every two weeks (Steinberg et al, 1980; Young, 1976). Proteins incorporated into the ROS are grouped into two general categories: structural proteins and those responsible for phototransduction. In addition, they are distributed into four distinct structural domains, namely, plasma membrane, cytosolic space, disc membrane and intradiscal space. The disc membranes are studded with thousands of photosensitive rhodopsin (RHO) pigments that are the key elements in the phototransduction pathway. Other proteins, such as RDS, are structural proteins that are associated with the rod outer segment membrane protein-1 (ROM1) where they act as adhesion molecules to interconnect and stabilize the disc (Kedzierski et al, 1999).

The ROS is connected to the inner segment by the connecting cilium. This is the channel for transporting proteins and other substances from the inner segment to the ROS. In addition to protein synthesis, the inner segment also provides energy for the photoreceptor. The clustering of 55-65% of retinal mitochondria and higher retinal cytochrome c oxidase activity in the inner segment indicate that the photoreceptors have a high metabolic rate (Medrano & Fox, 1995). The light regulation of rod sensitivity also takes place in the inner segment (Nakano et al,

2001). The nucleus is the site for genome storage and processing of photoreceptors. The synaptic terminal is connected to the horizontal and the bipolar cells as mentioned before.

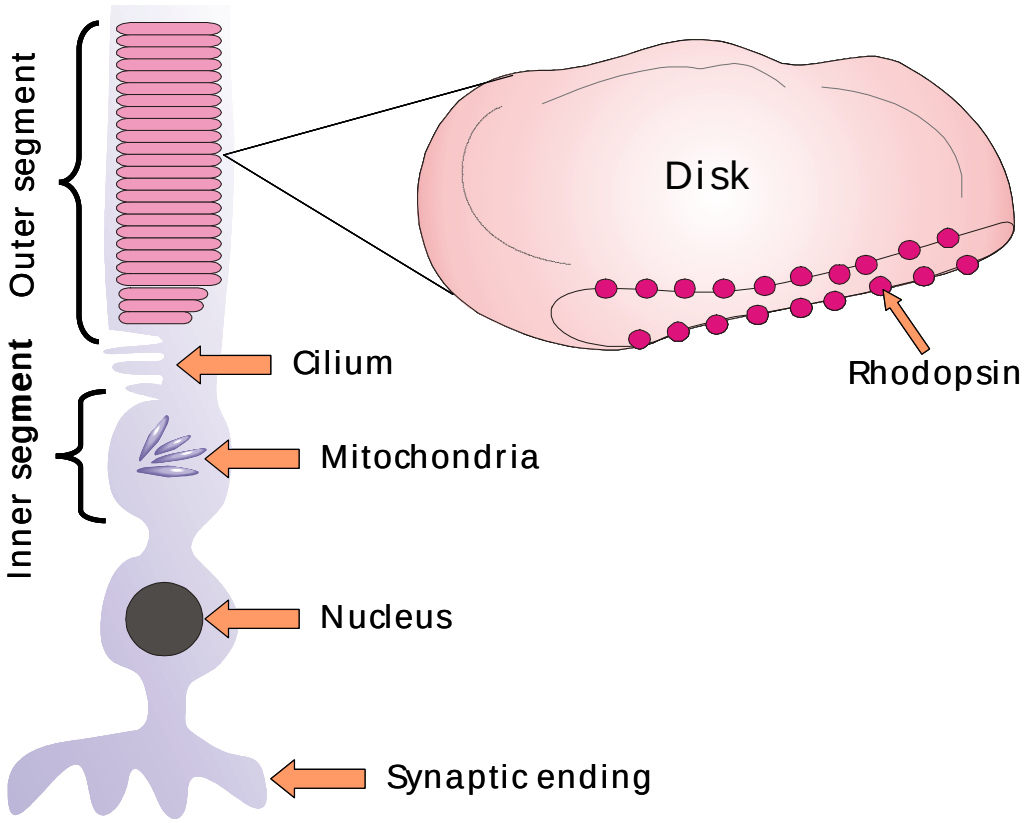


Figure 2. The structure of a rod photoreceptor.

1.4.2 Phototransduction pathway

Activation

Phototransduction and visual cycles are the beginning of vision, in which the visual pigments absorb light energy and generate signal through numerous molecules and mechanisms. The process can be divided into three main steps ***(Figure 3)***: activation, deactivation and adaptation. The activation step begins when rhodopsin (RHO) absorbs a photon. RHO contains a chromophore, 11-cis-retinal, which is activated by photon-induced cis-trans isomerization. The resulting rhodopsin is changed to an active form called metarhodopsin II or Rh* (Farahbakhsh et al, 1993). This conformational change enables the cytoplasmic part of RHO to activate the G protein transducin (Hamm, 2001). The inactive form of transducin consists of three subunits (T_αGDP, T_β and T_γ). Guanine diphosphate (GDP) is released from the T_α subunit and guanine triphosphate (GTP) binds to it. The affinity of T_αGTP to the $T_{\beta\gamma}$ subunit is reduced and thus is free to activate the next effectors. The photoreceptor-specific phosphodiesterase (PDE) then is activated by T_αGTP. PDE consists of two catalytic and two inhibitory units. Each of the catalytic subunits, α and β in rods, is associated with an inhibitory subunit called γ. The activation of PDE occurs through the removal of the γ subunit by the binding of T_αGTP to it (Artemyev et al, 1998). In the subsequent step, cyclic guanosine monophosphate (cGMP) is hydrolyzed to 5'-GMP by the catalytic units and the cytoplasmic cGMP concentration is decreased. The cyclic-nucleotide-gated channel is closed when the concentration of cGMP is decreased (Broillet & Firestein, 1999). Finally, the cell is hyperpolarized, which leads to the release of the neurotransmitter glutamate from the presynaptic terminal.

Deactivation

Deactivation of the photoreceptor is necessary for maintaining its high sensitivity and responsiveness to subsequent light signals. There are three main steps: 1) rhodopsin deactivation, 2) deactivation of transducin and phosphodiesterase, and 3) restoration of cGMP concentration. First, activated RHO is phosphorylated by rhodopsin kinase at the carboxyl terminus (Mendez et al, 2000). Subsequently, arrestin binds to RHO to quench its remaining catalytic activity (Pulvermuller et al, 2000). Other important proteins to be deactivated are transducin and PDE. The GTP bound to transducin is hydrolyzed to GDP and this causes transducin to dissociate from PDE and allow the re-binding of the γ subunit inhibitor (Wensel & Stryer, 1986). The key factors in the hydrolysis of transducin are the γ subunit (Tsang et al, 1998), intrinsic GTPase (Angleson & Wensel, 1993) and GTPase accelerator protein RGS9-1 (He et al, 1998). Finally, the cGMP concentration is restored by guanylate cyclase enzymes (GC) (Dizhoor, 2000). The GC changes GTP to cGMP, and is regulated by guanylate cyclase activating proteins.

If the sensitivity (ratio of response amplitude to light intensity) of the photoreceptors is constant, the maximal level of response amplitude would be reached at relatively modest light levels. In this situation, the photoreceptors could not respond to increasing light intensities. On the contrary, the eye not only detects light stimulus in “ON” and “OFF” states, but also adapts to the ambient light level. This ability of the photoreceptors to adapt to the ambient level of illumination is called “background adaptation”. Two important factors of photoreceptor background adaptation are the reduction in sensitivity and the acceleration in response kinetics (Nikonov et al, 2000).

Adaptation

Three Ca^{2+}-dependent mechanisms are associated with background adaptation and are involved in the phototransduction pathway. The Ca^{2+}-dependent modulation of the light-stimulated PDE shows a progressively important influence on the light response (Koutalos et al, 1995b). The GC activity is also regulated by calcium, which then affects the rate of synthesis of cGMP (Koutalos et al, 1995a). Another target is the cGMP-gated channel, which is modulated by the Ca^{2+}-calmodulin complex (Pugh et al, 1999).

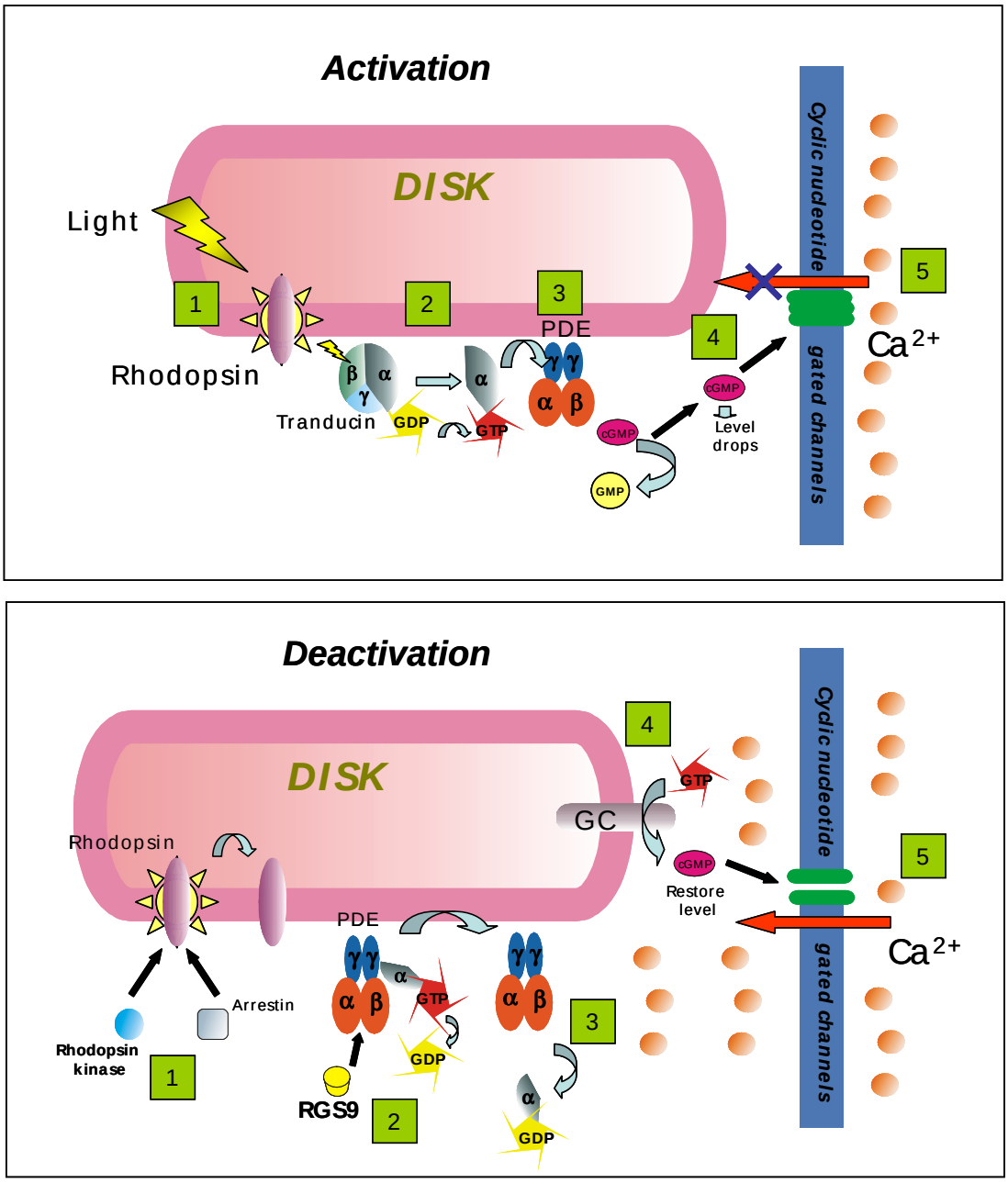


Figure 3 Activation and deactivation of the phototransduction cascade. Activation: 1) Photon activates rhodopsin molecule to metarhodopsin, which in turn activates transducin to cause the bound GDP to be replaced by GTP. 2) The alpha subunit detaches from the beta and gamma subunits. 3) The released alpha subunit activates phosphodiesterase (PDE) and causes the conversion of cyclic GMP (cGMP) to GMP. 4) Reduction of cGMP level causes the cyclic nucleotide gated channel to close. 5) Decline of Ca^{2+} level causes hyperpolarization of the photoreceptor. Deactivation: 1) Rhodopsin kinase causes the phosphorylation of metarhodopsin, and arrestin binds to metarhodopsin to quench the remaining activity of rhodopsin. 2) GTPase accelerator protein (RGS9) binds to the activated PDE and promotes the hydrolysis of GTP to GDP. 3) The alpha subunit is inactivated and dissociates from the PDE. 4) Guanylyl cyclase (GC) synthesizes cGMP from GTP and restoration of cGMP level causes the channel to open. 5) Ca^{2+} reenters the cytoplasm.

1.5 RP: Symptoms, diagnosis and disease management

1.5.1 Symptoms

Typical symptoms of RP are night blindness and progressive loss of mid-peripheral visual field and central vision at the late stage. Hu (1982) reported that in people of Chinese origin the average age of onset was 24.7 years in the autosomal dominant type, 22.9 years in the autosomal recessive type and five years in the X-linked type. However, patients also show clinical heterogeneity in that the age of onset, severity, topographic pattern of visual loss, cone involvement, ophthalmoscopic findings and family history are variable both within and between families (Kaplan et al, 1990). In some cases, the central vision is also affected in the early stage (Foxman et al, 1985). The central vision defect usually follows the peripheral abnormality. In addition, photopsia (the perception of light flashes) was found in 31% of RP patients (Heckenlively & Krauss, 1988).

Some forms of RP can be syndromic in that other systems or organs are also affected. Usher syndrome is RP accompanied by congenital hearing loss. It is the most common form of syndromic RP accounting for about 14% of RP cases where 50% of patients are both deaf and blind (Boughman et al, 1983). It is an autosomal recessive condition of congenital hearing loss and retinitis pigmentosa. It is classified into three major groups where type 1 and type 2 are more prevalent than type 3 (Hope et al, 1997). Bardet Biedl syndrome is another type of syndromic RP that is less frequent than Usher syndrome with a prevalence of 1/150,000 (Beales et al, 1999). However, it is a clinically complex syndrome associated with retinopathy, obesity, hypogonadism, renal dysfunction, postaxial polydactyly and mental retardation (Koenig, 2003).

1.5.2 Diagnosis

The diagnosis of RP has been based traditionally on clinical examination and tests. When the following criteria are satisfied, the diagnosis of RP is established (Marmor et al, 1983): 1) rod dysfunction as measured by dark adaptation and electroretinogram (ERG), 2) progressive loss of photoreceptor function, 3) loss of peripheral vision, and 4) bilateral involvement. The retina is examined by the following methods for the presence of these features.

Ophthalmoscope

An ophthalmoscope is an instrument used to examine the retina and the vitreous. The image of the fundus can be surveyed by an ophthalmoscope to localize the lesions of RP. The pathological changes of RP always progress through a series of stages (Pagon, 1988). The earliest features are the arteriolar narrowing and deposition of fine granularity in the RPE. Bone spicule pigmentation appears in the mid-periphery ***(Figure 4)***. The pigmentary changes become denser and gradually spread both posteriorly and anteriorly to form a ring-like scotoma. When the visual field shows progressive contraction, a waxy pallor appears in the optic disc. In the vitreous, dust-like particles are present that are mainly the cells released from the RPE due to degeneration (Albert et al, 1986). The formation of cataract is also common in RP patients (Merin, 1982).

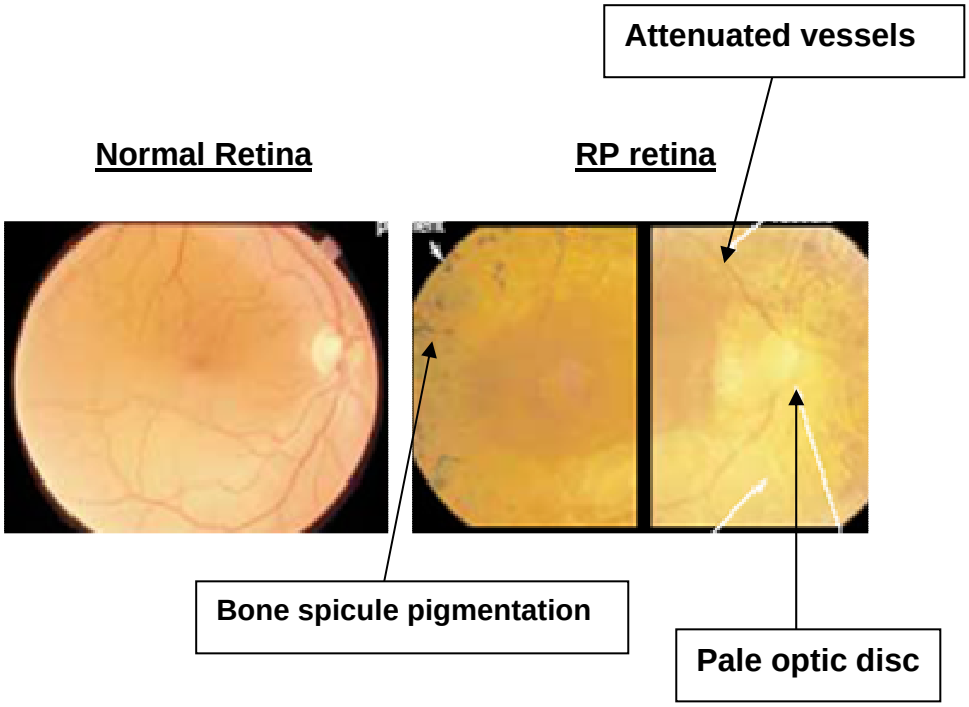


Figure 4 Ophthalmoscopic views of the retina in normal and RP subjects (Modified from Chapple et al (2001)).

Perimetry

Perimetry (or visual field testing) refers to the measurement of the area of functional retina by a perimeter. Visual field is diminished in RP due to loss of functional photoreceptors. The process usually begins in the mid-peripheral field where an annular scotoma is formed. As the disease progresses, a residual central and a temporal or nasal peripheral field island remain. In advanced stages, only the small central field remains (Heckenlively & Krauss, 1988). The average half-life of the visual field loss, independent of the field phenotype, was 7.3 years for target V_{4e} and 6.8 years for target II_{4e} (Grover et al, 1997).

Electroretinography

Electroretinography (ERG) is an objective electrophysiological test to evaluate retinal response to light stimulation (Sunness, 1999). Contact lens-type electrodes are placed on the cornea of a patient. The potential differences are recorded from the responses of light-adapted (photopic) and dark-adapted (scotopic) stimulation of the eye. An electroretinogram is generated and consists of two wave forms: a-wave and b-wave. The a-wave is the initial negative deflection generated from the photoreceptors. The b-wave is the positive deflection generated by Muller cells and represents the processes occurring in the bipolar region. In general, the photopic response is used to measure cone function, and the scotopic response determines rod function. A decline in ERG is observed in RP (Birch & Hood, 1995) and is due to: 1) shortened ROS, 2) random loss of photoreceptors throughout the retina, 3) death of photoreceptors locally, 4) defect in phototransduction pathway, or 5) any combination of the above. Birch et al (1999) studied the declining rate of ERG in RP

over a period of four years where the decline in cone and rod ERG amplitude was 60% and 64% respectively.

Disease management

Although RP is the best understood retinal disease, it is incurable. However, the progressive vision deterioration can be minimized and symptomatic relief given. For example, the pathological changes are accelerated in light-exposed conditions (Wang et al, 1997). Therefore, patients are recommended to wear protective glasses while outdoors. Some studies indicated that vitamin A supplementation helps to decrease the rate of loss of photoreceptors (Berson et al, 1993; Li et al, 1998). Cataract and macular edema are the most frequent complications associated with RP. Cataract extraction is recommended to improve visual symptoms (Jackson et al, 2001). A carbonic anhydrase inhibitor is given to patients to relief the macular edema (Giusti et al, 2002).

On the other hand, some promising new treatment regimes are under investigation. Apoptosis is the common final pathway for photoreceptor degeneration. Some anti-apoptotic agents have been shown to control the process (LaVail et al, 1998; Chong et al, 1999; Liang et al, 2001). The discovery of the genes associated with RP offers targets for gene therapy by either delivery of a functional gene or disruption of the mutant transcripts (Acland et al, 2001; LaVail et al, 2000). Retinal transplantation by introduction of cells into the sub-retinal space was suggested to limit the disease process and functional deterioration (Mohand-Said et al, 2000; Mizumoto et al, 2001). Transplantation of artificial devices instead of cells has also been considered (Chow et al, 2001).

1.6 Pathological changes in the retina

The term “retinitis pigmentosa” was first used to describe the fundus findings in complicated night blindness (Donders, 1855). Although used for many years, the term is a misnomer. The term “retinitis” indicates retinal inflammation, which is not associated with RP. The term “pigmentosa” refers to the melanin pigment released by the RPE.

Shortening of the rod ROS is the earliest histological abnormality ***(Figure 5)*** (Milam et al, 1996). This signifies the death of rods, which usually begins in the mid-peripheral retina and progresses to the far periphery and macula. However, some cases have been reported where cell death is seen initially in the inferior retina (Ayuso et al, 1996). Using immunocytochemical methods, the cytopathologic abnormalities can be identified. The abnormal localization of mutant rhodopsin has been seen in some RP patients (Li et al, 1994) and in transgenic mice (Li et al, 1996). The accumulation of mutant rhodopsin in the endoplasmic reticulum or the Golgi complex in the inner segment was proposed to interfere with the function of the organelles and lead to cell death (Manoil & Traxler, 1995). In some studies, abnormal rod sprout neurites have been found (Li et al, 1995; Milam et al, 1996). These neurites extend and bypass the horizontal and bipolar cells to the inner limiting membrane. This abnormality would likely impede the normal functional integration of photoreceptors. Pathological changes in cone cells usually follow the death of rods. These changes include shortened outer segment, axonal elongation, abnormal synapse and death (Li et al, 1994; Milam et al, 1996). The RPE is detached from the Bruch's membrane after the death of the photoreceptors, migrating to and accumulating in the inner retina to form the bone spicule pigmentation (Li et al, 1995). Some studies have also reported the thickening of

Bruch's membrane (Duvall et al, 1986; Kuntz et al, 1996). Tissue inhibitor of metalloproteinase-3 was suggested to be associated with this abnormality but the exact mechanism is unknown (Fariss et al, 1998). The Muller cells undergo reactive gliosis including cellular hypertrophy and migration of enlarged nuclei to the outer retina (Li et al, 1994; Li et al, 1995). Amacrine and horizontal cells also undergo neurite sprouting in the retina of RP patients (Fariss et al, 2000). The elevation in glutamine and arginine contents within Muller cells was suggested to contribute to photoreceptor degeneration (Fletcher, 2000). Retinal blood flow is decreased in RP patients (Grunwald et al, 1996) and vascular modeling occurs in response to the metabolic changes. The retinal vessels encircled by RPE undergo fenestrations that lead to the leakage of albumin into the perivascular matrix (Li et al, 1995). Other rare abnormalities have also been found in RP patients, including exudative retinal detachment (Khan et al, 1988), peripheral retinal vasculopathy (Grizzard et al, 1978) and retinal neovascularization (Uliss et al, 1986).

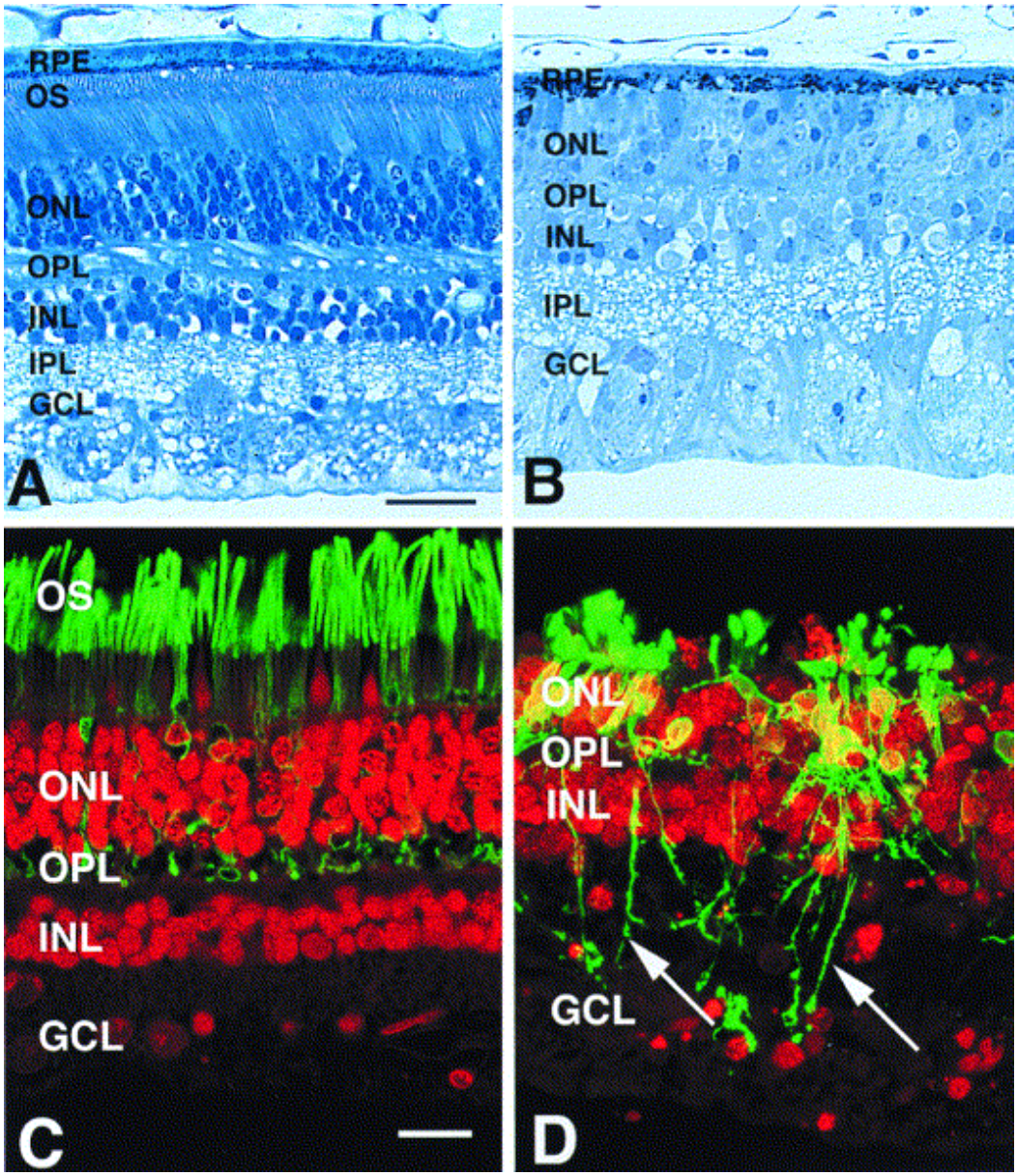


Figure 5. The histological sections show the pathological changes in the retinitis pigmentosa retina (B and D) compared to normal retina (A and C). Degeneration of the retina is obvious from the observations that some photoreceptors have died and some lack outer segments (B). Immunostaining of photoreceptors also shows the neurite sprouting in the retina of RP (Indicated by white arrows in D). (Photos were taken from Fariss et al (2000)). RPE = retinal pigment epithelium; OS = outer segments; ONL = outer nuclear layer; OPL = outer plexiform layer; INL = inner nuclear layer; IPL = inner plexiform layer; GCL = ganglion cell layer

1.7 Pathogenesis

Although photoreceptor degeneration occurs in different RP phenotypes, apoptosis is the final common pathway leading to cell death (Li & Milam1995; Reme et al, 1998). However, the exact mechanisms leading to cell death are unknown. Our knowledge in this area has been obtained mainly from experimental animal models. There were two competing views arising from these experiments. One of them suggested some possible mechanisms including faulty disc morphogenesis, calcium toxicity/metabolic overload, RPE dysfunction, and constitutive activation of photoreceptors (Travis, 1998; Pierce, 2001) .

Faulty disc morphogenesis refers to the disruption of the outer segment formation due to the genetic mutations. Either the outer segment is completely omitted or the photoreceptor proteins are misrouted. ***rho***-/- mice generate no outer segment and lose their photoreceptors (Humphries et al, 1997). Accumulation of mutant proteins in transport vesicles is also common. Transgenic mice carrying the proline-347 to serine (P347S) mutation fail to renew outer segment because of the accumulation of extracellular vesicles near the junction between the inner and outer segments (Li et al, 1996). Another type of defective morphogenesis is caused by the misfolding of mutant P23H rhodopsin, which accumulates in inner segments (Liu et al, 1997).

Why does a defective outer segment lead to cell death? Travis (1998) proposed that oxygen toxicity was a possible reason for cell death after the loss of the outer segments. A two-stage model has been proposed to support this hypothesis (Stone et al, 1999). In the first stage, lethal mutation and/or environmental factors cause depletion of the photoreceptor population and lead to a reduction in oxygen

consumption and a rise of oxygen level. Hyperoxia is toxic and causes further depletion in the cell population. Photoreceptors would be expected to up-regulate the expression of protective factors to counteract this toxic effect.

The γPDE-deficient ***rd*** mice are models for studying calcium toxicity/metabolic overload. The retinal degeneration in γPDE-deficient ***rd*** mice is due to the lack of hydrolytic activity of PDE (Tsang et al, 1996). The concentration of cGMP in the mice is maintained at a high level so that massive influxes of Na^+ and Ca^{2+} occur. The resulting metabolic overload and direct toxicity have been suggested as the cause of cell death (Travis, 1998). The increase in Ca^{2+} concentration may also activate apoptosis directly (Chang et al, 1993).

Sometimes, the neighboring cells could contribute to cell death. The RPE plays a role in the renewal of ROS by phagocytosing dead cells. In Rpe65-/- mice, all-trans-retinyl ester accumulates in the RPE (Redmond et al, 1998). As a result, the RPE is damaged, which leads to death of the rods (Hamel et al, 2001).

Mutations in genes responsible for phototransduction result in the constitutive activation of photoreceptors (equivalent-light hypothesis) (Fain & Lisman, 1993; Lisman & Fain, 1995). The triggering of apoptosis in RP is Ca^{2+} dependent (Fain & Lisman, 1999). For example, the mutation in arrestin would lead to low levels of cGMP and channel closure. Consequently, the entry of Ca^{2+} would be blocked and intracellular Ca^{2+} concentration reduced.

As opposed to these mechanisms, Clarke el al. (2000) proposed a novel principle for the underlying mechanisms, the one-hit or constant risk model. They suggested

that other proposed mechanisms explained the induction of apoptosis as a progressive accumulation of cellular damage. These cumulative damage hypotheses generally fit sigmoidal cell-death kinetics, in which the risk of death increases with time. The one-hit model was concluded from the study of cell-death kinetics in eleven animal models of inherited retinal degeneration. In contrast, the kinetics of these models showed a constant or exponentially decreasing risk of death but not sigmoidal decline. The features of the one-hit model are that each mutant photoreceptor has a constant risk of death throughout life and is at the same risk of death as every other mutant. The time of death is random and independent of other factors. The clinical observation of photoreceptor function in patients by ERG also showed the exponential decay of response (Birch et al, 1999).

How can this phenomenon be explained? Clarke el al. (2000) proposed the mutant steady state (MSS) hypothesis to explain the underlying biochemical mechanism. First, the mutation is not lethal to the cell, otherwise, the cell would die immediately and the phenotype would be congenital blindness. The constant risk of death is given by subtle changes in the biochemistry of the cell. The molecules responding to the mutation may come from mutant response genes (MuRGs), mutant response proteins (MuRPs) or mutant response metabolites (MuRMs), but their nature is unknown. As illustrated in ***Figure 6***, every cell will enter apoptosis when the normal fluctuation in concentration of a compound X reaches the apoptotic threshold. For the mutant one, the mean concentration of X is increased and higher than normal. Thus, the change exceeding the threshold is more probable. In a study of Huntington's disease, the results were consistent with this model with an exponential decline in cell number (Perutz & Windle, 2001).

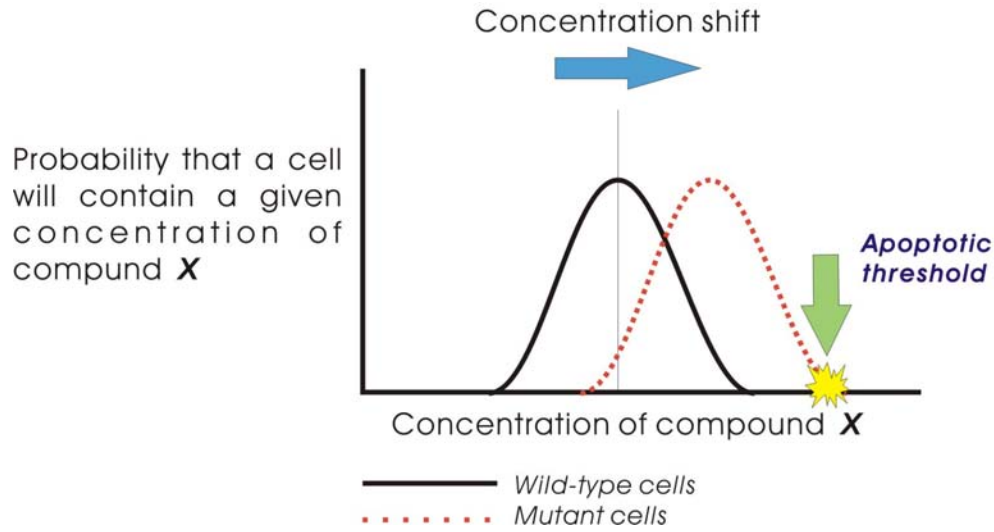


Figure 6 Mutant steady state (MSS) hypothesis. (Diagram modified from Pacione et al (2003))

Further, Clarke et al (2000) explained that the exponential decrease in the risk of death might be due to diffusible factors. Two possible mechanisms may account for this phenomenon: more survival factor is released when the photoreceptors shrink, or there is a reduction in the quantity of toxic factors released from dying cells when the mutant population falls. The influence of diffusible factors on the risk of death was supported by at least one animal study (Kedzierski et al, 1998). In hemizygous transgenic female mice (only carrying the transgene on one X-chromosome) on the rds-/- genetic background, both the transgene-expressing (the transgene expressed from the normal rds allele) and non-expressing photoreceptors underwent degeneration. Release of a toxic factor or factors from the mutant allele was suggested to trigger apoptosis in neighboring wild type cells.

This intercellular communication explains not only the exponential kinetics of cell death, but also the spread of disease from rod to cone cells in RP via a so-called 'bystander effect' hypothesis, as suggested by Ripps (2002). Gap junction channels are present in many types of cells including the photoreceptors and other neurons in the retina (Zahs et al, 2003; Lee et al, 2003). Gap junctions consist of two multimeric connexons that reside in the plasma membranes of closely apposed cells. Ions or small molecules (<1 kDa) pass through these junctions as part of intercellular communication. It is believed that dying rod cells either trigger cell death in neighboring cones by releasing toxic factors, or are eliminated so that supply of vital factors to neighboring cones is depleted. Therefore, the degeneration of photoreceptors spreads progressively from rod to cone.

No matter how elegant these models or hypotheses are, the key molecules involved in the regulation of cell death need to be identified. Recently, ceramide, a

sphingolipid, was found to be important for the viability of photoreceptor cells (Acharya et al, 2003). Ceramidase is the enzyme that hydrolyses ceramide to sphingosine. Cloning and expression of the ceramidase gene in ***arr2***^{3} mutant photoreceptors of Drosophila produced a near-wild type level of rhodopsin, and the photoreceptors were rescued from death. The ***arr2***^{3} mutants are defective in clathrin-mediated endocytosis of metarhodopsin. (Metarhodopsin is the active form of rhodopsin employed in the visual signaling cascade.) In addition, the accumulation of ceramide in the cells was observed. Therefore, it is believed that the ceramide level is tightly coupled to the clathrin-mediated internalization of rhodopsin and critical to the survival of photoreceptors. The role of ceramide in apoptosis was elucidated by studying the response of the Hep G2 cell line to ceramides (Gentil et al, 2003). Ceramide was found to inhibit the mitochondrial respiratory chain reaction and this resulted in apoptotic cell death. This apoptotic mechanism was not discovered in RP until recently. Tuson et al (2004) refined the RP26 locus to a 12.5 Mb interval by homozygosity mapping in an RP26 family. This interval contains several annotated genes and partially characterized mRNAs. A 532 amino acid protein was predicted by retinal cDNA library screening and ***in silico*** assembly. This protein showed the highest similarity (29% identity; 50% similarity) with human ceramide kinase (CERK) and the gene was named human ceramide kinase-like (***CERKL***). CERK converts ceramide to its metabolite. Through mutation screening of ***CERKL*** in the RP26 family and normal control individuals, all the RP patients had the homozygous nonsense mutation (R257X) in exon 5 while control subjects did not. Again, ceramide metabolism was correlated with RP and could be the element to trigger retinal degeneration by apoptosis. Although there are no data directly supporting the role of ceramide in neighboring cell death, ceramide may be the unknown compound X in the one-hit cell death model and the toxic factor in the bystander effect model.

1.8 Genetic aspects of RP

1.8.1 Mode of inheritance

RP is inherited in a classical Mendelian fashion: autosomal dominant (ad), autosomal recessive (ar) or X-linked (xl). The lack of a family history is reported as simplex or sporadic. The proportion of each type varies from study to study. In the US, the inheritance patterns consisted of approximately 84% arRP, 10% adRP and 6% X-linked RP (Boughman et al, 1980). In this study, simplex cases were not included in the calculation. However, in one Danish study (Haim, 1992), the distribution was 6.9% for adRP, 22.6% for arRP, 10.8% for X-linked RP and 43.2% for simplex cases. In China, 209 cases of RP in Shanghai were studied and the proportions for adRP, arRP, X-linked RP and simplex cases were 33.1, 11.0, 7.7, and 48.3% respectively (Hu, 1982).

Clinical subtypes can be classified by the mode of inheritance (Kaplan et al, 1990). For adRP, the clinical subtypes are divided into two forms according to the delay in macular involvement. Four subtypes could be recognized in arRP according to the age of onset and severity. In a similar manner, X-linked RP could be divided into two subtypes: night blindness or myopia involvement.

The two forms of adRP are type I (or diffuse) and type II (or regional). Type I RP is characterized by an early diffuse loss of rod sensitivity followed later by cones with an early onset of night blindness (Massof & Finkelstein, 1981). Type II is characterized by a compartmentalized and combined loss of rod and cone sensitivity with adult onset of night blindness. In addition, adRP can also be classified into four subtypes by the ERG findings and fundus (Fishman et al, 1985).

1.8.2 Genetic Heterogeneity

Locus heterogeneity

Genetic heterogeneity is one of the probable reasons behind variable clinical presentations among RP patients. One type of genetic heterogeneity is termed locus heterogeneity. The advent of molecular technology has opened up the possibility of detecting the molecular defects in RP. Presently, there are at least 38 genes known to be associated with RP, of which 30 have been cloned ***(Table 1)***. These numbers do not include the various syndromic forms of RP that involve 33 other genes. Recent updates of these genes can be found at the RetNet (http://www.sph.uth.tmc.edu/RetNet).

Thirteen genes have been found to cause adRP and 12 of them have been cloned. The first locus was identified on chromosome 3q21-24 (McWilliam et al, 1989). Thereafter, a mutation was identified in the rhodopsin (***RHO***) gene, causing adRP (Dryja et al, 1990a). Mutations in three cloned genes, ***RHO***, ***RDS*** and ***RP1*** account for over 50% of adRP cases (http://www.sph.uth.tmc.edu/RetNet/). Although 21 genes have been found to cause arRP (at the time of writing), each of the genes individually accounts only for a small number of arRP cases. For example, only about 2% of arRP cases occur that have mutations in the ***RPE65*** gene (Morimura et al, 1998).

Table 1. The genes found in non-syndromic forms of RP.

Mode of Inheritance	No.	Gene	Location	Protein	Percent within the Group
Autosomal Dominant					
Mapped & Cloned	1	***PRP3***	1q21.2	Human homolog of yeast pre-mRNA splicing factor 3	Unknown
	2	***RHO (RP4)***	3q21-q24	Rhodopsin	30-40%
	3	***RDS (RP7)***	6p21.2-cen	Peripherin/RDS	5%
	4	***PIM1K (RP9)***	7p14.3	PIM1-kinase associated protein 1	Unknown
	5	***IMPDH1 (RP10)***	7q32.1	Inosine monophosphate dehydrogenase 1	Unknown
	6	***RP1***	8q11-q13	RP1 Protein	5-10%
	7	***ROM1***	11q13	Retinal Outer Segment Membrane 1	Rare
	8	***NRl (RP27)***	14q11.2	Neural retina leucine zipper transcription factor	Rare
	9	***PRPC8 (RP13)***	17p13.3	Precursor mRNA processing protein C8	Unknown
	10	***FSCN2***	17q25	Retinal fascin	3.3% in Japanese
	11	***CRX (CORD2)***	19q13.3	cone-rod otx-like photoreceptor homeobox transcription factor	Rare
	12	***PRPF31 (RP11)***	19q13.4	Human homolog of yeast pre-mRNA splicing factor	15-20%
Mapped but not cloned	1	***RP17***	17q22		Unknown
Autosomal Recessive					
Mapped & Cloned	1	***ABCA4 (RP19)***	1p21-p22	ATP-binding cassette transporter - retinal	Rare
	2	***RPE65 (RP20)***	1p31	Retinal pigment epithelium-specific 65 kD protein	2%
	3	***CRB1 (RP12)***	1q31-q32.1	Crumbs homolog 1	Rare
	4	***USH2A***	1q41	Usherin	4-5%
	5	***MERTK***	2q14.1	c-mer protooncogene receptor tyrosine kinase	Unknown
	6	***CERKL (RP26)***	2q31.2-q32.3	Ceramide kinase-like protein	Unknown
	7	***SAG***	2q37.1	Arrestin (s-antigen)	In Japanese

Table 1. The genes found in non-syndromic forms of RP. (Continued)

Mode of Inheritance	No.	Gene	Location	Protein	Percent within the Group
	8	***RHO (RP4)***	3q21-q24	Rhodopsin	Rare
	9	***PDE6B***	4p16.3	Rod cGMP phosphodiesterase subunit	3-4%
	10	***CNGA1***	4p12-cen	Rod cGMP-gated channel subunit	Rare
	11	***LRAT***	4q31.2	Lecithin retinol acyltransferase	Unknown
	12	***PDE6A***	5q31.2-q34	cGMP phosphodiesterase subunit	3-4%
	13	***TULP1 (RP14)***	6p21.3	Tubby-like protein 1	Rare
	14	***RGR***	10q23	RPE-retinal G protein-coupled receptor	Unknown
	15	***NR2E3***	15q23	Nuclear receptor subfamily 2 group E3	Unknown
	16	***RLBP1***	15q26	Cellular retinaldehyde-binding protein	Unknown
	17	***CNGB1***	16q13-q21	Rod cGMP-gated channel subunit	Unknown
Mapped but not cloned	1	***RP28***	2p11-p16		One family
	2	***RP29***	4q32-q34		Unknown
	3	***RP25***	6cen-q15		10-20% in Spain
	4	***RP22***	16p12.1-p12.3		Unknown
X-Linked					
Mapped & Cloned	1	***RP2***	Xp11.3	Novel protein with similarity to human cofactor C	10%
	2	***RPGP (RP3)***	Xp21.1	Retinitis pigmentosa GTPase regulator	25-30%
Mapped but not cloned	1	***RP6***	Xp21.3-p21.2		Unknown
	2	***RP23***	Xp22		Unknown
	3	***RP24***	Xq26-q27		Unknown

Bhattacharya et al (1984) were the first to use molecular techniques to study RP. The probe L1.28 of restriction fragment length polymorphisms was shown to be closely linked to X-linked RP. This locus is known as ***RP2*** and accounts for about 10% of X-linked RP (Hardcastle et al, 1999). ***RPGR*** is the most commonly mutated X-linked gene accounting for about 20% of X-linked RP (Buraczynska et al, 1997).

Although the retina develops from the nervous system, it is unlike other inherited degenerative diseases (e.g. Alzheimer's disease) where the mutations are found in a group of functionally related proteins. Identified mutant proteins show great functional diversity and can be classified into several functional classes. However, most of the functions and the pathological consequences of these gene products are still not well understood. Especially, the roles of two recently identified genes, ***IMPDH1*** and ***PIM1K (Table 1)***, remain to be defined by further functional assays. Classification of the genes could provide a clue to the understanding of the mechanisms contributing to RP. Until now, most of the identified genes are involved in the phototransduction cascades. Mutations in ***RHO*** (al-Maghtheh et al, 1993), ***PDE*** α and β (Huang et al, 1995; McLaughlin et al, 1995) and ***CNGA1*** (Dryja et al, 1995) have been found in RP. Mutated genes whose proteins are involved in the deactivation steps have also been identified. Arrestin suppresses the activity of metarhodopsin via binding to it. A mutation in ***SAG***, the arrestin gene, was found in a Japanese arRP patient (Nakazawa et al, 1998).

RPE65, ***RLBP1*** and ***ABCA4*** genes encode the proteins responsible for recycling all-trans retinal to 11-cis retinol and transporting the 11-cis retinal from RPE to ROS for re-entry into the visual transduction cycle. The ATP-binding cassette transporter is the product of the ***ABCA4*** gene, which is responsible for transporting all-trans retinal from ROS to RPE (Sun et al, 1999). RPE65 is a microsomal protein that is expressed specifically in the RPE (Bavik et al, 1993) and is suggested to play a critical role in the isomerization of all-trans retinol to 11-cis retinol (Van Hooser et al, 2000; Redmond et al, 1998). Mutations in ***RPE65*** account for about 2% of arRP (Morimura et al, 1998). Cellular retinaldehyde binding protein (CRALBP), encoded by the ***RLBP1*** gene, binds to the 11-cis retinol and promotes the oxidation of 11-cis retinol to 11-cis retinal (Stecher et al, 1999). The mutation in ***RLBP1*** is associated with arRP (Maw et al, 1997).

RDS and ROM1 are not directly involved in phototransduction but are thought to provide structural support to the flattened discs of the ROS. Mutations in ***RDS*** have been found in a variety of inherited retinal dystrophies including RP (Bareil et al, 2000; Weigell-Weber et al, 1996; Trujillo et al, 2001). ROM1 interacts with RDS to form a mixture of homo- and heterotetrameric core complexes (Loewen & Molday, 2000). For the ***ROM1*** mutation, one single case of mutation was suspected as evidenced by a pathological change (Reig et al, 2000).

There is a proximal sequence from -505 to +41 bp in the rhodopsin gene, which is believed to mediate its transcription (Mani et al, 2001). Neural retina leucine zipper and cone rod homeobox proteins are the trans-acting elements regulating gene transcription in this region (Mitton et al, 2000). ***NRL*** and ***CRX*** are the genes encoding these two proteins and were recently identified to be associated with RP (Martinez-Gimeno et al, 2001; Sohocki et al, 2001) .

Mutations in ***RP1*** account for approximately 6% to 10% of adRP cases (Liu et al, 2002). RP1 is located in the connecting cilia, beginning in the apical inner segment and extending into the proximal outer segment. Its functions were suggested to be protein transportation and correct stacking of outer segment discs (Gao et al, 2002; Liu et al, 2003).

Normal phagocytosis in RPE is a receptor-mediated process. MERTK is a receptor tyrosine kinase and is involved in the phagocytosis of outer segments (Feng et al, 2002). Mutations in ***MERTK*** were identified in RP patients, suggesting that the RPE phagocytosis pathway is a possible disease target for RP (Gal et al, 2000). In the past, mutations in RP were always thought to occur only in the retina-specific genes. Detection of mutations in genes encoding ubiquitously expressed splicing factors draws our attention to other possibilities (McKie et al, 2001; Vithana et al, 2001; Chakarova et al, 2002). These genes include ***PRPF8***, ***PRPF31*** and ***HPRP3,*** which encode small nuclear ribonucleoproteins for spliceosomes.

Generally, allelic heterogeneity is defined in two broad senses: either different mutations in the same gene causing the same phenotypes or different mutations in the same gene causing different phenotypes. The genes identified in RP have both characteristics. For example, over 100 different mutations have been identified in the ***RHO*** gene (Gal et al, 1997) and over 70 in the ***RDS*** gene (Kohl et al, 1998). These different mutations in the same gene can cause RP as well as other diseases. Indeed, clinical presentations were different for each of the mutations in RP.

The Pro23His mutation of rhodopsin was the first reported mutation in RP (Dryja et al, 1990a). However, patients with another point mutation in rhodopsin, Pro347Leu, showed more rapid progression than patients with the Pro23His mutation (Oh et al, 2003). Variability in clinical outcome was demonstrated for a different mutation (Pro23Ala) on the same codon (Oh et al, 2000). Most of the rhodopsin mutations caused adRP although arRP was also reported (Rosenfeld et al, 1992). Mutations in rhodopsin also give rise to congenital stationary night blindness (Rao et al, 1994).

Non-Mendelian inheritance

The genetic complexity of RP does not end with these heterogeneities. Non-Mendelian inheritance is also a feature of RP. Like other cell types, photoreceptors also contain mitochondria. The extra-nuclear genome in mitochondria provides another chance for mutation. A mutation in the mitochondrial ***MTTS2*** gene was first reported in syndromic RP with progressive sensorineural hearing loss (Mansergh et al, 1999). Digenic inheritance was first reported in three RP families (Kajiwara et al, 1994). The family members with a heterozygous mutation in either ***RDS*** or ***ROM1*** (but not both) were not affected. The affected members had double heterozygous mutations in both ***RDS*** and ***ROM1***. The same mutation in ***RDS*** (missense mutation Leu185Pro) in combination with different ***ROM1*** mutations was found in all digenic patients reported to date (Dryja et al, 1997).

These observations indicate that the ***ROM1*** mutations in digenic RP could be different. However, not just any mutation in ***RDS*** together with the ***ROM1*** mutation can produce a disease phenotype. Jacobson et al (1999) found that double heterozygous mutations in the Arg13Trp ***RDS*** and the Arg16His ***ROM1*** were not sufficient to cause RP in all cases. Recently, a more complex and intriguing inheritance pattern was found in RP. In a family with Bardet–Biedl syndrome, triallelic inheritance was present (Katsanis et al, 2001). This syndrome is caused by mutations in at least seven loci (***BBS1 to BBS7***) where three mutations in two ***BBS*** loci are required to manifest the disease.

Variable expressivity

Variable expressivity introduces an additional level of complexity in RP. Several studies found variable expressivity in adRP (Jay, 1982; Nordstrom, 1993; Kim et al, 1995). ***RP9*** was identified in adRP families that display phenotypes varying from nearly normal and asymptomatic to severe with extinguished electroretinograms and barely detectable dark-adapted static threshold sensitivities. Clinical variability is also found in arRP and X-linked RP (Nakazawa et al, 1998; Keith et al, 1991; Mashima et al, 2000). In addition, some heterozygous female carriers showed extreme variability in chromosome X inactivation in X-linked RP families (Jacobson et al, 1997; Souied et al, 1997).

In the past, variable expressivity in the ***RP11*** locus was believed to be due to the incomplete penetrance of an unknown gene (Moore et al, 1993). More detailed family studies and clinical analysis showed that the risk of being symptomatic during a working lifetime was only 31% among the offspring carrying the mutation (Evans et al, 1995; Al-Maghtheh et al, 1996). In contrast to mutations in ***RP9***, the RP11 family showed “all-or-none” incomplete penetrance, i.e. the mutation carriers were either symptomatic or asymptomatic. This form of inheritance is termed bimodal expressivity. This phenomenon is explained by another silent ***RP11*** allele or a closely linked locus influencing the ***RP11*** mutation in trans. This suggestion was supported by finding asymptomatic patients who inherited a different wild type allele (Vithana et al, 2001). Vithana et al (2001) also determined that the ***RP11*** locus was the same as the pre-mRNA splicing gene ***PRPF31***. Vithana et al (2003) further demonstrated that mRNA expression level of wild type ***PRPF31*** was significantly different between symptomatic and asymptomatic carriers.

1.9 Genes and proteins

Studying the molecular genetic basis of RP allows us to understand the pathogenesis of RP and also enhance our knowledge of the encoded proteins and normal retinal functions. New genes expressed in the retina have often been discovered in this manner, a process referred to as reverse genetics. For example, the RP1 protein was recently discovered through linkage and mutational analysis in RP patients (Sullivan et al, 1999). ***RHO***, ***RDS*** and ***RPRF31*** are the genes studied in this project.

1.9.1 Rhodopsin

The rhodopsin gene was isolated and sequenced by Nathans & Hogness (1984). ***RHO*** consists of five exons consisting of 1044 base pairs (bp) in length and encoding a protein of 348 amino acid residues ***(Figure 7)***. The gene was mapped to 3q21-3q24 using in situ hybridization techniques with a DNA probe (Sparkes et al, 1986). Over 100 different mutations have been identified in the rhodopsin gene and these are mainly associated with adRP (Gal et al, 1997). Rhodopsin is a highly specialized G protein coupled receptor (GPCR) comprising about 70% of the total protein in ROS (Hamm & Bownds, 1986). It consists of seven transmembrane domains that form a compact bundle to hold the 11-cis retinal chromophore via Schiff base linkage (Menon et al, 2001). The common post-translational modifications are N-glycosylation at the N-terminal region, disulfide bridges and thiopalmitoylation at the C-terminal tail. The transmembrane domains, N-terminal and C-terminal tails form three topologically distinct regions: the cytoplasmic domain, the transmembrane domain and the extracellular domain. The cytoplasmic domain is composed of three cytoplasmic loops and the carboxy-terminal tail that provide binding and

activation sites for proteins in phototransduction. The transmembrane domains may be important in stabilizing the ground state structure and holding the 11-cis retinal chromophore. The amino acids Lys-296 and Glu-113 are found to be the key amino acids for chromophore binding. The extracellular domain was found to be important for proper folding of the RHO that allows cellular processing and chromophore binding. Obviously, the structure and conformation of RHO is a highly sophisticated arrangement where the native 3-dimensional rhodopsin molecule provides physicochemical interactions with other molecules.

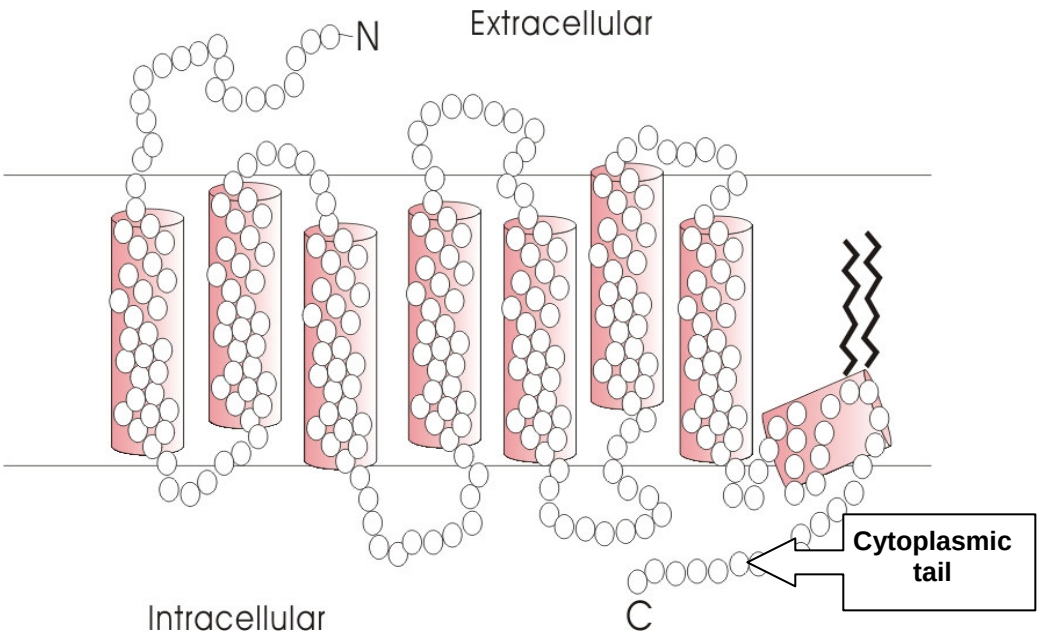


Figure 7 The structure of the rhodopsin molecule.

1.9.2 RDS

The ***RDS*** gene is located on chromosome 6 and consists of three exons (Travis et al, 1991). The mRNA has 1038 bases and codes for a protein of 346 amino acids. Retinal degeneration gene, slow (RDS, also known as peripherin 2) is a 39-kDa integral membrane glycoprotein that is a member of the tetraspanin superfamily. It consists of four transmembrane domains and a large intradiscal domain (D2 loop/EC2 domain) located at the rod and cone photoreceptor outer segment discs ***(Figure 8)*** (Arikawa et al, 1992; Connell & Molday, 1990). RDS forms a homo-oligomeric structure with itself by intermolecular disulfide bonds at the D2 loop. This loop also interacts with another tetraspanin membrane protein (ROM1) to form a mixture of homo- and heterotetrameric core complexes (Loewen & Molday, 2000). This subunit assembly was suggested to be important in disc morphogenesis and stabilization. Mutations in ***RDS*** have been found in a variety of inherited retinal dystrophies including RP (Trujillo et al, 2001; Bareil et al, 2000; Weigell-Weber et al, 1996). The first ***RDS*** mutation of autosomal dominant RP was reported in 1991 (Kajiwara et al, 1991). Subsequently, numerous mutations have been identified, including missense and nonsense mutations (Lam et al, 1995), 3-base deletions (Farrar et al, 1991), small insertion/deletions causing a frameshift (Jacobson et al, 1996a) and large in-frame deletions (Jacobson et al, 1996b).

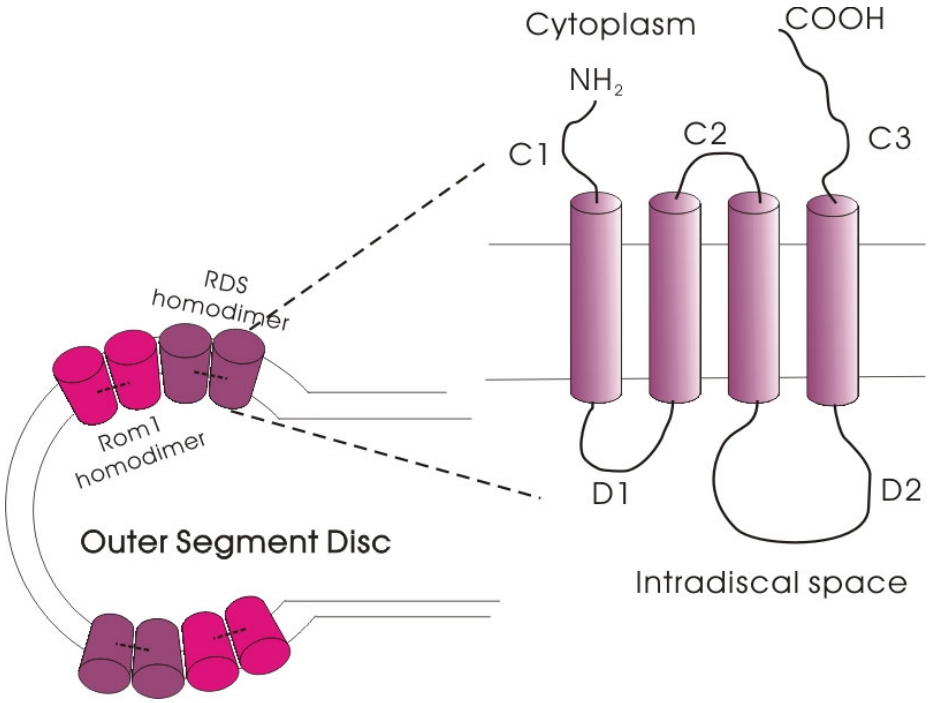


Figure 8 The structure of the RDS molecule and its association with ROM1 in the outer segment disc.

1.9.3 PRPF31

PRPF31 is a recently identified gene related to RP. Only limited information about the effect of the gene and its mutations on RP is available. By refined linkage mapping and ***in silico*** gene prediction, mutations in ***PRPF31*** were identified in five previously reported British ***RP11*** families (Vithana et al, 2001). The sequence has 20% overall sequence identity with yeast ***S. cerevisiae*** Prp31p protein and 38% with ***S***. ***pombe*** prp31+ (the score is modest). In particular regions, certain domains share a much higher identity. The Prp31p is a pre-mRNA splicing factor in yeast (Weidenhammer et al, 1996). Interestingly, two other splicing associated genes have also been identified: ***PRPC8*** from previously reported ***RP13*** families and ***HPRP3*** from ***RP18*** families (McKie et al, 2001; Chakarova et al, 2002). The splicing machinery involves a complex and poorly understood process although its main purpose is to remove introns from the pre-mRNA. Several factors are prerequisites for this process: pre-mRNA containing a 5' splice site, branchpoint sequence (BPS), pyrimidine tract and 3' splice site, spliceosome complex and splicing factors.

The spliceosome is the key machine that acts as a catalyst in splicing, forming a collection of small nuclear RNAs (snRNAs) and proteins (Jurica & Moore, 2003). The components of the spliceosome include five snRNAs U1, U2, U4, U5 and U6 each bound by several proteins to form small nuclear ribonucleoproteins (snRNP), and some less stably-associated splicing factors. When an intron excision is completed, a newly reassembled spliceosome is needed for the next excision event. It is estimated that about 300 proteins participate in splicing. Briefly, the splicing process occurs in two main steps (Burge et al, 1999). Initially, the U1 snRNP binds to the 5' splice site and the U2 snRNP engages the BPS. Then, U4, U5 and U6

snRNPs are recruited to form a tri-snRNP complex and conformational rearrangement occurs. The 2' hydroxyl of the adenosine of BPS attacks the 3' hydroxyl group of 5' splice site to generate a 2'-5' bond. The second step starts after another rearrangement takes place. The free 3' hydroxyl group of 5' exon attacks the 3' splice site and forms a new phosphodiester bond with the 3' exon. As a result, the two exons are joined. The breaking and forming of phosphodiester bond is mediated by transesterification.

The ***PRPF31*** is a 14-exon gene spanning about 18kb and encoding a protein of 499 amino acids. Later, this protein was identified as a 61kDa protein (61K) in the spliceosome complex (Makarova et al, 2002). It was demonstrated that protein 61K interacts with the U5 snRNP and promotes the tri-snRNP complex formation. Similar to ***PRPF31***, other two mutated splicing factor genes in RP, ***PRPC8*** and ***HPRP3***, encode the U5 snRNP and a U4/U6 associated protein (Hprp3p) in yeast, respectively.

Chapter 2 Materials & Methods

2 Materials & Methods

2.1 Subjects and samples

Fifty nine families were included in this study. From these families, 79 subjects were diagnosed as having RP or Usher syndrome. There were 18 adRP, 18 arRP, 34 simplex cases, 9 other RP cases with uncertain mode of inheritance, and 2 cases of Usher syndrome (autosomal recessive). These RP patients were examined under the Hong Kong RP Patients Register Scheme with written informed consent from subjects participating in the study. Eye examination was performed at the Optometry Clinic of The Hong Kong Polytechnic University and then diagnosed by Dr. Leung Kam-wah at the Hong Kong Society for the Blind. Clinical genetic counseling was provided by Dr. Stephen T. S. Lam and his colleagues at the Clinical Genetic Service, Department of Health. Blood samples were taken for molecular genetic analysis. It should be noted that this collection of RP patients had never been screened for mutations in any gene implicated for RP. Seventy five anonymous blood samples were obtained from healthy blood donors to serve as normal controls.

2.2 DNA extraction

A modified salting-out method was used for DNA extraction (Miller et al, 1988). Two milliliters (ml) of whole blood were mixed with six ml of erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA, pH7.4). The mixture was incubated on ice for 30 minutes and then centrifuged for 10 minutes at 2000 ***g***. The supernatant was discarded carefully without disturbing the pellet, and the pellet was resuspended in two ml of nucleus lysis buffer (10 mM Tris-HCl, pH 8.2, 400 mM NaCl, 2mM Na_2EDTA, 1% SDS, 200 µg/ml proteinase K). The reaction mixture was incubated at 55°C overnight or until the proteins were completely digested. Subsequently, 0.67 ml of saturated NaCl was added and mixed thoroughly. The cellular proteins were pelleted by centrifugation for 15 minutes at 2500 ***g***. The supernatant was collected in a new tube and 2.6 ml of isopropanol were added followed by inversion of the tube until the DNA precipitated out as threads. The precipitated DNA was pelleted by centrifugation for five minutes at 2000 ***g***. The pellet was washed briefly in 70% ethanol and allowed to air-dry at 37°C in an oven. The pellet was allowed to dissolve overnight in 0.5 ml of TE buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA). The quantity and purity of the DNA was measured at 260nm using an MBA 2000 spectrophotometer (PerkinElmer, Inc.) and stored at -20°C until use.

2.3 Primer Design

PCR primers were designed for the rhodopsin, ***RDS*** and ***PRPF31*** genes ***(Tables 2-4)*** using the software Oligo (Version 6; Molecular Biology Insights, Inc., Cascade, USA). Primer sequences were selected to minimize cross-complementarity and self-complementarity. The GC content was between 45% and 60% and the estimated annealing temperature was approximately 60°C. To increase efficiency, two PCR fragments of different lengths were run simultaneously in each lane during

SSCP analysis. The maximum length of PCR fragments was around 300 bp. The primers were designed to generate overlapping fragments of a maximum length of 300 bp so that each nucleotide of the studied regions was included. Finally, specificity and product yield were optimized by titration of magnesium and primer concentrations.

2.4 Polymerase Chain Reaction

PCR was performed in a 25 µl mixture containing 1x PCR Buffer II (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 50 ng human genomic DNA template, 0.2 mM of each deoxyribonucleoside triphosphate (dNTP), and 0.5 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, USA). The concentrations of primers and magnesium were specific to the template ***(Tables 2-4)***.

Reactions were initiated by heating at 95°C for 10 minutes followed by 38 cycles of amplification. Each cycle consisted of 20 seconds (s) at 95°C, 25 s at 60°C, and 25 s at 72°C. Final extension was carried out for 10 minutes at 72°C. DNA amplification was carried out in a 96-Well GeneAmp PCR System 9700 (Applied Biosystems, Foster City, USA).

Table 2 PCR for the ***RHO*** gene.

Fragments (Location)	***Primers***	***Sequences* (5'→3')***	***Product length (bp)***	***PCR conditions#***	
				Primer (μM)	***$MgCl_2$ (mM)***
RPPR1-1@ (Regulatory)	RPPR1F1	CCC ATC GTC CCT ATT TCA AAC C	297	0.5	2.5
	RPPR1R1	TGA CCC CCC CAG ACC CTT AT			
RPPR1-2@ (Regulatory)	RPPR1F2	AGG AAC TTA GGA GGG GGA GGT	172	0.5	2.5
	RPPR1R2	GTT AGA GCC TTC TGT GCC ATT C			
RHOe1-1@ (Exon 1)	RHOe1F1	AGC ATT CTT GGG TGG GAG CA	292	0.5	1.5
	RHOe1R1	CAC GGC TAG GTT GAG CAG GAT			
RHOe1-2 (Exon 1)	RHOe1F2	CAA GAA GCT GAG CAC GCC TCT	197	0.5	1.5
	RHOe1R2	CTG CAC ACC CCA CCC ACA C			
RHOe2 (Exon 2)	RHOe2F1	GTG CTG ACC GCC TGC TGA C	231	0.5	1.5
	RHOe2R1	CGG AGC TTC TTC CCT TCT GCT			
RHOe3@ (Exon 3)	RHOe3F1	TGT TCC CAA GTC CCT CAC AGG	259	0.5	1.5
	RHOe3R1	CTG GAC CCA CAG AGC CGT GA			
RHOe4@ (Exon 4)	RHOe4F1	GGA GGA GCG ATG GTC TGG AC	300	0.5	1.5
	RHOe4R1	CTC CCA CCC GCA GTA GGC			
RHOe5@ (Exon5)	RHOe5F1	TGC CCC TGA CTC AAG CCT CT	172	0.5	1.5
	RHOe5R1	CTT TAG TCG GCC ACA GAG TCC TAG			

* ***Underlined bases indicate mismatches with the reference sequence (see Table 5 for details).***
\# ***See Section 2.4 for details.***
@ ***Fragments which show sequence variations. See Chapter 3 for details.***

Table 3 PCR for the ***RDS*** gene.

Fragments (Location)	***Primers***	***Sequences* (5'→3')***	***Product length (bp)***	***PCR conditions#***	
				Primer (μM)	***$MgCl_2$ (mM)***
RDSe1-1 (Exon 1)	RDSe1F1	TTA AGG TTT GGG GTG GGA GCT	199	0.5	2.5
	RDSe1R1	CTT TCG GAG TTC AAT CTT CAG GA			
RDSe1-2@ (Exon 1)	RDSe1F2	GTG TTG GCT GGC ATC ATC ATC	289	0.5	2.5
	RDSe1R2	CCC GAA GCA GAA AGC AGC AG			
RDSe1-3@ (Exon 1)	RDSe1F3	TCC TCT TCA ACA TCA TCC TCT TC	297	0.5	2.5
	RDSe1R3	TAG CTC TGA CCC CAG GAC TG			
RDS2-1 (Exon 2)	RDSe2F1	GGA AGC CCA TCT CAA GCT GTC	177	0.5	2.5
	RDSe2R1	CTC CGT CTG GTG GTC GTA AC			
RDS2-2 (Exon 2)	RDSe2F2	CAG ATC ACC AAC AAC TCA GCA C	186	0.5	2.5
	RDSe2R2	CTT ACC CTC TAC CCC CAG CT			
RDSe3@ (Exon 3)	RDSe3F1	ATT GCC TCT AAA TCT CCT CTC C	295	0.5	2.5
	RDSe3R1	CTT GGA GTG AAC TAT TTC TCA GTG			

* ***Underlined bases indicate mismatches with the reference sequence (see Table 5 for details).***
\# ***See Section 2.4 for details.***
@ ***Fragments which show sequence variations. See Chapter 3 for details.***

Table 4 PCR for the ***PRPF31*** gene.

Fragments (Location)	***Primers***	***Sequences* (5'→3')***	***Product length (bp)***	***PCR conditions#***	
				Primer (μM)	***$MgCl_2$ (mM)***
PRPF5P (5'UTR)	PRPF5PF1	GCA TCC AGT CCT CTG AGT TG	288	0.3	2.5
	PRPF5PR1	GCA CCA CTG TTT CTA GCG TTA G			
PRPFe1@ (Exon 1)	PRPFe1F1	GGG GAC CAA TCA GAG AGT AGC	179	0.3	2.5
	PRPFe1R1	CGC CTT TTT CCT CAC ACA CTT C			
PRPFe2@ (Exon 2)	PRPFe2F1	GGG GAG AAT CAT CGC TCA GTA A	291	0.3	2.5
	PRPFe2R1	CCT GCT AGG AAC ACC TCT TGT C			
PRPFe3 (Exon 3)	PRPFe3F1	AGA GTG CTG GAT TCT GAC TGT CT 3	209	0.3	2.5
	PRPFe3R1	CTC TGG AAA AGG CTG AGA AG 3			
PRPFe4 (Exon 4)	PRPFe4F1	ACC CAT GCC TCC GTG TCC T	231	0.1	2.5
	PRPFe4R1	GCA GGT CAA CCT TGA TCT GAG C			
PRPFe5 (Exon 5)	PRPFe5F1	CCA GCA AAG TCT ACC TTC CAT C	180	0.3	2.5
	PRPFe5R1	GAA GCA CCC CAC CTT CTC TG			
PRPFe6 (Exon 6)	PRPFe6F1	CGA GCC TCC CCT ATC TTC TC	193	0.3	2.5
	PRPFe6R1	AGC GTA ATC CCC AAT CCC ATT AG			
PRPFe7@ (Exon 7)	PRPFe7F1	ACA ACA GGC AGG CGG GAG A	307	0.1	2.5
	PRPFe7R1	CCC CCA TAC TAC AGA AAA GGA TGT			
PRPFe8@ (Exon 8)	PRPFe8F1	CCC ACC TCT CTG CTT TCT TCT	290	0.3	1.5
	PRPFe8R1	TGG CTG CTC AGG CTG TCT G			

Fragments (Location)	*Primers*	*Sequences* (5'→3')*	*Product length (bp)*	*PCR conditions#*	
				Primer (µM)	*$MgCl_2$ (mM)*
PRPFe9 (Exon 9)	PRPFe9F1	GCT TTG CTG TTA CCT CTG TCT G	174	0.3	2.5
	PRPFe9R1	CCT CAC CTT TCC CTC CTC AC			
PRPFe10@ (Exon 10)	PRPFe10F1	GTG GCG GTG AGG CAG CAT TA	308	0.3	1.5
	PRPFe10R1	GGC TTC TCC CCT CCA TGA C			
PRPFe11@ (Exon 11)	PRPFe11F1	CCG ATA GGC ATG GGG GTC AT	197	0.3	1.5
	PRPFe11R1	TGG CTG TGG GGT TGA GGA G			
PRPFe12@ (Exon 12)	PRPFe12F1	AGG TCC TGG TCG CTG AAC TG	228	0.3	2.5
	PRPFe12R1	GCT CCC CCC ACC TTG TGT C			
PRPFe13 (Exon 13)	PRPFe13F1	GTG CGG CAG ACA CAG GTA AAC	279	0.3	2.5
	PRPFe13R1	CTC AGG GCT GGG GAC AGA G			
PRPFe14 (Exon 14)	PRPFe14F1	CTC ACC TAT CCC ATC ATC CTC T	272	0.3	1.5
	PRPFe14R1	CCA GTG GCA GGG CAG GTT CT			
PRPF3P@ (3' UTR)	PRPF3PF1	GGA AAC AGA GGT CCA GTC CT	310	0.3	2.5
	PRPF3PR1	CTC CAC AGT TCT GAT TCT TCT AGT			

* ***Underlined bases indicate mismatches with the reference sequence (see Table 5 for details).***
\# ***See Section 2.4 for details.***
@ ***Fragments which show sequence variations. See Chapter 3 for details.***

2.5 Single strand conformation polymorphism analysis

Two µl each of the paired PCR products were mixed with five µl of SSCP loading solution (95% formamide, 20mM Na_2EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) and one µl of water. This mixture was denatured at 95°C for seven minutes and then cooled in ice immediately. Electrophoresis was performed as follows: 10 µl of the mixture were loaded per well in 10%T/1%C polyacrylamide gel with or without 5% glycerol. The gels were run for three hours at 4°C or 20°C and 400 V. Tris-borate-EDTA (TBE) buffer (1x; 89 mM Tris, 89 mM boric acid, 2.5 mM Na_2EDTA, pH 8.3) was used in the gels and the tank, and the tank buffer was mixed continuously during electrophoresis.

It is worth noting that the strategy of pairing two PCR products in a single lane reduced the number of SSCP gels by half during the screening stage. The short fragment was about 170-200 bp, and the long fragment about 270-300 bp.

Note that ***every*** single fragment amplified from the ***patients'*** samples was analyzed with SSCP under four different combinations of conditions. These four conditions were gel without glycerol / 4°C, gel with glycerol / 4°C, gel without glycerol / 20°C, and gel with glycerol / 20°C. However, when ***control*** samples were genotyped for the sequence variations identified, SSCP was performed under the single condition giving the best resolution for the sequence variation concerned, as found during the screening stage. This optimal SSCP genotyping condition varied between sequences.

After electrophoresis, the gels were stained using the procedure described by Yip (2000). Briefly, the gels were fixed in two changes of 10% ethanol and 0.5% acetic

acid for two minutes each, stained in two changes of freshly prepared 1% silver nitrate solution for seven minutes each. The silver nitrate solution was decanted and the gel was developed in 1.5% NaOH containing 0.01% sodium borohydride and 0.4% (v/v) formaldehyde. The stained gel was rinsed in three changes of water and dried at 83°C for 30 minutes. The dried gel was stored permanently for later review.

2.6 DNA sequencing

Genomic DNA was amplified using the same primers and conditions described in Section 2.4. PCR products were prepared for sequencing by enzyme digestion. Briefly, five µl of PCR product were mixed with one µl of shrimp alkaline phosphatase (1 U/µl) and one µl of exonuclease I (10 U/µl). The mixture was incubated at 37°C for 15 minutes followed by inactivation by heating at 80°C for 15 minutes. The prepared PCR products were sequenced using the ABI PRISM BigDye Terminators (ver. 2.0 or ver. 1.1) Cycle Sequencing Ready Mix Kit (Applied Biosystems, Foster City, USA) according to the manufacturer's instructions. Cycle sequencing was performed by incubation at 96°C for two minutes to denature the template, followed by 38 cycles each consisting of 96°C for 10 s, 50°C for 10 s and 60°C for four minutes. The sequencing mixture was purified using the DyeEx Spin Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Template suppression reagent (13µl) was added to the dried product and denatured at 96°C for five minutes. Finally, the cycle sequencing products were analyzed using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, USA).

2.7 PCR cloning

PCR cloning was performed using the TOPO TA Cloning® kit (Invitrogen). Freshly amplified PCR products were added to a ligation mixture (1µl 50 mM NaCl and 2.5 mM $MgCl_2$ salt solution; 2µl sterile water; 1µl TOPO vector) to a total volume of 6µl.

The reaction was incubated for five minutes at room temperature. Two µl of the ligation reaction were added to 50 µl electrocompetent ***E. coli*** and electroporated using a Bio-Rad electroporator (Bio-Rad Laboratories, Inc., Hercules, USA). Following electroporation, 250 µl SOC medium were added and the mixture was incubated for 1hr at 37°C. Ten microliters of the solution were plated on LB agar containing 50 µg/ml ampicillin and X-gal. Individual white colonies were picked and grown overnight at 37°C in LB broth with agitation. Plasmid DNA was extracted using the NucleoSpin® Plasmid DNA extraction kit (MACHEREY-NAGEL). DNA cycle sequencing was performed using the M13(+) primers.

2.8 Statistical analysis

In the present study, allele frequencies of sequence variations were calculated and analyzed. These calculations were based on the number of ***unrelated*** RP patients (n=59) and controls (n=75). The consistency of the observed genotype distribution according to the Hardy-Weinberg equilibrium was tested by chi-squared analysis. Comparison of genotype distribution between patient and control groups was also analyzed by Fisher's exact test for variations that had heterozygosity of at least 0.1. All statistical analysis was performed using PowerMarker (Version 3.21, Jack Liu, http://www.powermarker.net).

Chapter 3 Results

3 Results

3.1 Nomenclature of sequence variations

The numbering of the positions of all mutations and genetic variants starts from the first translated base of the genomic sequences of GenBank entries ***(Table 5)***. The nucleotide +1 corresponds to the A of the ATG translation initiation codon and -1 is the first 5' nucleotide upstream of ATG initiation codon. For the intronic variations, the nomenclature begins with IVS followed by intron number, the direction (+ means start from 5' end and – means start from 3' end) and the specific variations. For example, IVS3+4C>T refers to the fourth nucleotide changed from C to T, counting from the 5' end of intron 3. A sequential variation number is also assigned to each sequence variation e.g. ***V1, V2, V3, etc.*** for the sake of easy discussion. Any variation at the protein level is indicated with the standard one-letter codes ***(Appendix)***. For example, V209M means that the valine at codon 209 was changed to methionine. For frameshift mutations, the first amino acid that is actually changed as a result of the frameshift, and the codon that is changed to stop codon are indicated. For example, alanine at codon 78 was the first amino acid changed and a stop codon was created at codon 176 in A78***fsX***176.

Table 5 Summary of the information for genes studied.

Gene	Rhodopsin (*RHO*)	retinal degeneration, slow (*RDS*)	PRP31 pre-mRNA processing factor 31 homolog (yeast) (*PRPF31*)
Chromosome	3q21-24	6p21.1-cen	19q13.4
Structure	5 exons Transcript length: 2673 bp Translation length: 348 residues	3 exons Transcript length: 2971 bp Translation length: 346 residues	14 exons Transcript length: 1834 bp Translation length: 499 residues
Reference sequence in this study	GeneBank: GI: 37550867 NT_005612.14 REGION: 35742235..35749740	GeneBank: GI: 29804415 NT_007592.13 REGION: complement (33461066..33487883)	GeneBank: GI: 29800594 NT_011109.15 REGION: 26886626..26903732

3.2 Results of SSCP analysis and cycle sequencing

In total, 37 sequence variations of ***RHO, RDS*** and ***PRPF31*** were found in this study. Of these variations, 14 had been reported previously while 23 were novel findings. The information on sequence variations found is shown in ***Figures 9-11***. SSCP patterns and cycle sequencing results are shown in ***Tables 6-8***.

Three PCR fragments each contained two or more sequence variations and hence produced complex SSCP patterns, as shown in ***Figures 12-14***. For the fragment carrying V3 (-51G>A) and V4 (-26A>G) in ***RHO***, three haplotypes were obtained (G-A, G-G and A-G). For the fragment carrying V14 (+23909C>G), V17 (+24012G>A) and V18 (+24053C>T) in ***RDS***, three haplotypes were identified across these three sites (G-G-T, G-G-C and C-A-C). Finally, for the fragment carrying V27 (IVS9-72G>T), V28 (IVS9-70T>C and V29 (IVS9-67G>A) in ***PFPF31***, three haplotypes were identified across these three sites (G-C-G, G-T-G and T-C-A). Theoretically, there are four possible 2-site haplotypes and eight possible 3-site haplotypes. Here, only three out of four possible 2-site haplotypes, and three out of eight possible 3-site haplotypes were obtained. This indicated that the two or three sequence variations carried by each PCR fragment were in strong linkage disequilibrium. This was not unexpected because of the physical proximity of these variations.

The ability to define haplotypes is one advantage offered by SSCP. However, some variations carried in the same PCR fragment could be detected only under different PCR conditions. One example was the fragment carrying V24 and V26 (data not shown). It is interesting to note that one of the sequence variations, V34 ***(Table 8, p.73)*** could not be resolved by SSCP under any studied conditions. This variation

was detected only when V35 and V36 on the same fragment were characterized by cycle sequencing.

For the complex mutation V10 (+232G>C;+232_+233insT) found in one RP sample, both the +232G>C and +232_233insT variations were found on the same chromosome. Thus, the other chromosome was wild type. This combination was suggested by the variant SSCP pattern ***(Table 7, p.69)*** and confirmed by sequencing of the cloned insert ***(Figure 15)***.

For V22 (+5439T>C) in ***PRPF31***, only homozygous genotypes (T/T and C/C) were found initially ***(Table 8, p.71)***. Theoretically, this was impossible for a total sample size of 154. In another fragment carrying this variation but amplified by a second primer pair, all three possible genotypes were obtained ***(Figure 16)***. The reason for the failure to detect heterozygotes in the first fragment remains to be determined (see Section 4.1.2 for discussion).

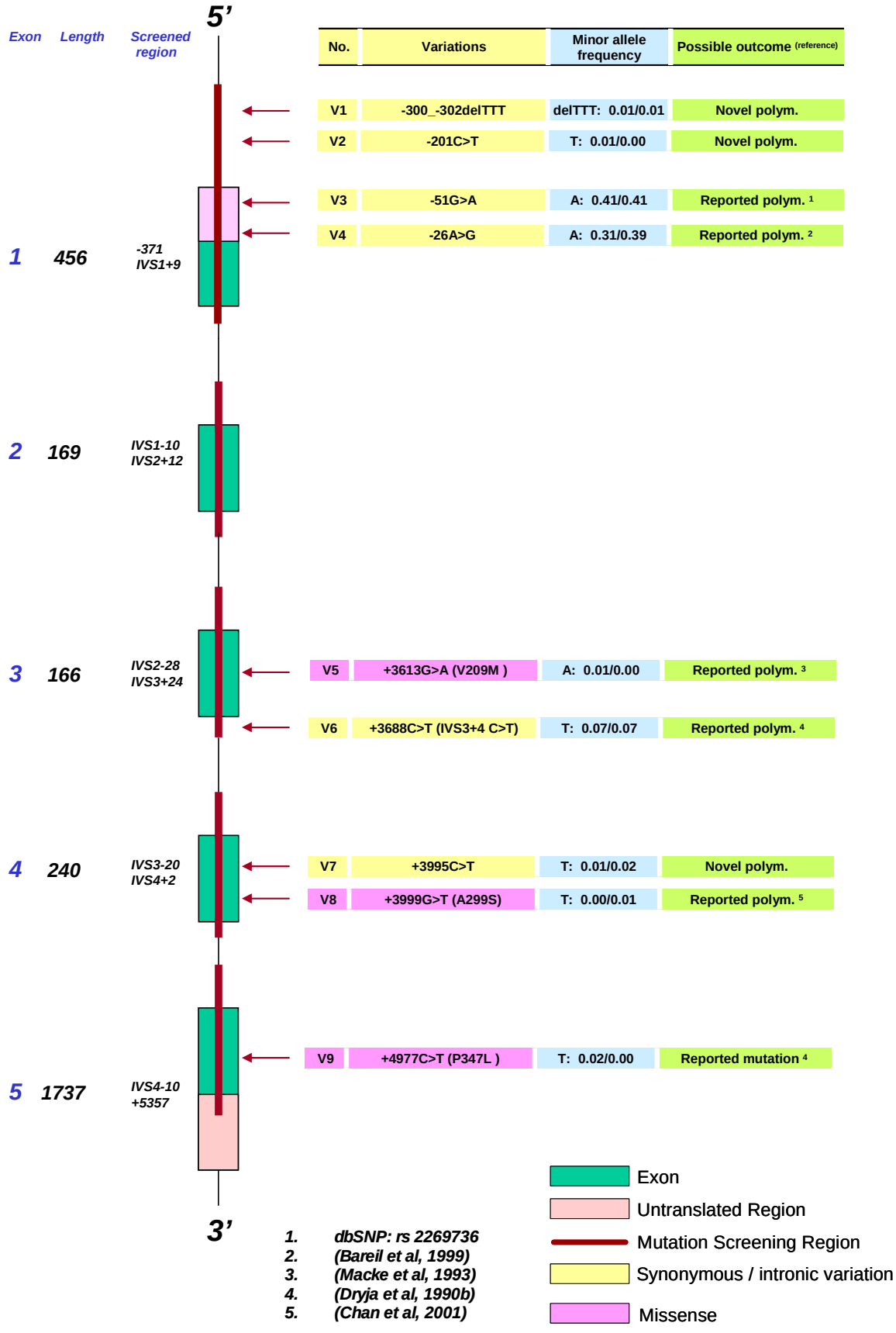


Figure 9 Summary of sequence variations found in ***RHO.*** The minor allele frequency is for the RP patient group (before the slash) and the control group (after the slash).

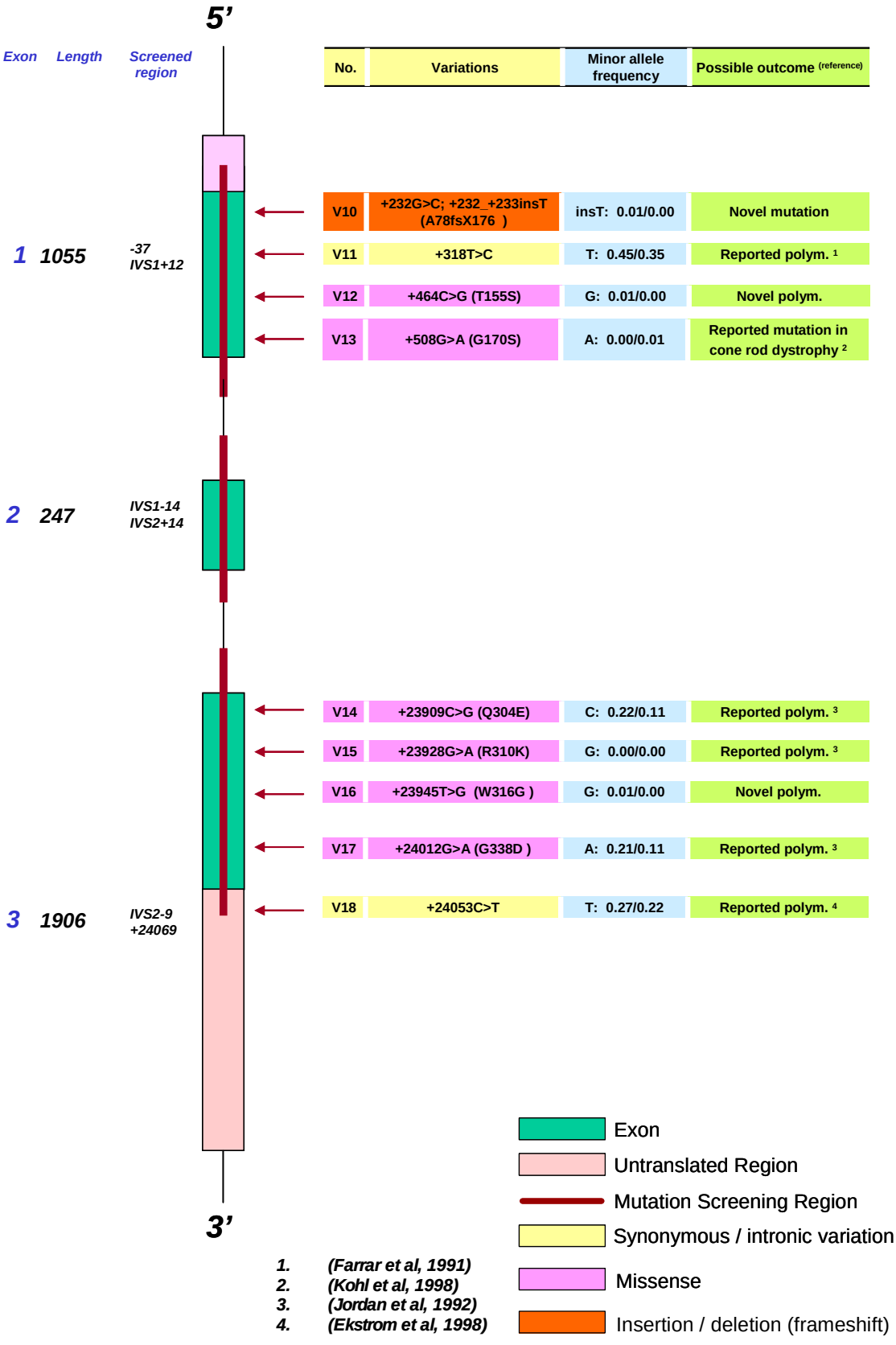


Figure 10 Summary of sequence variations found in ***RDS.*** The minor allele frequency is for the RP patient group (before the slash) and the control group (after the slash).

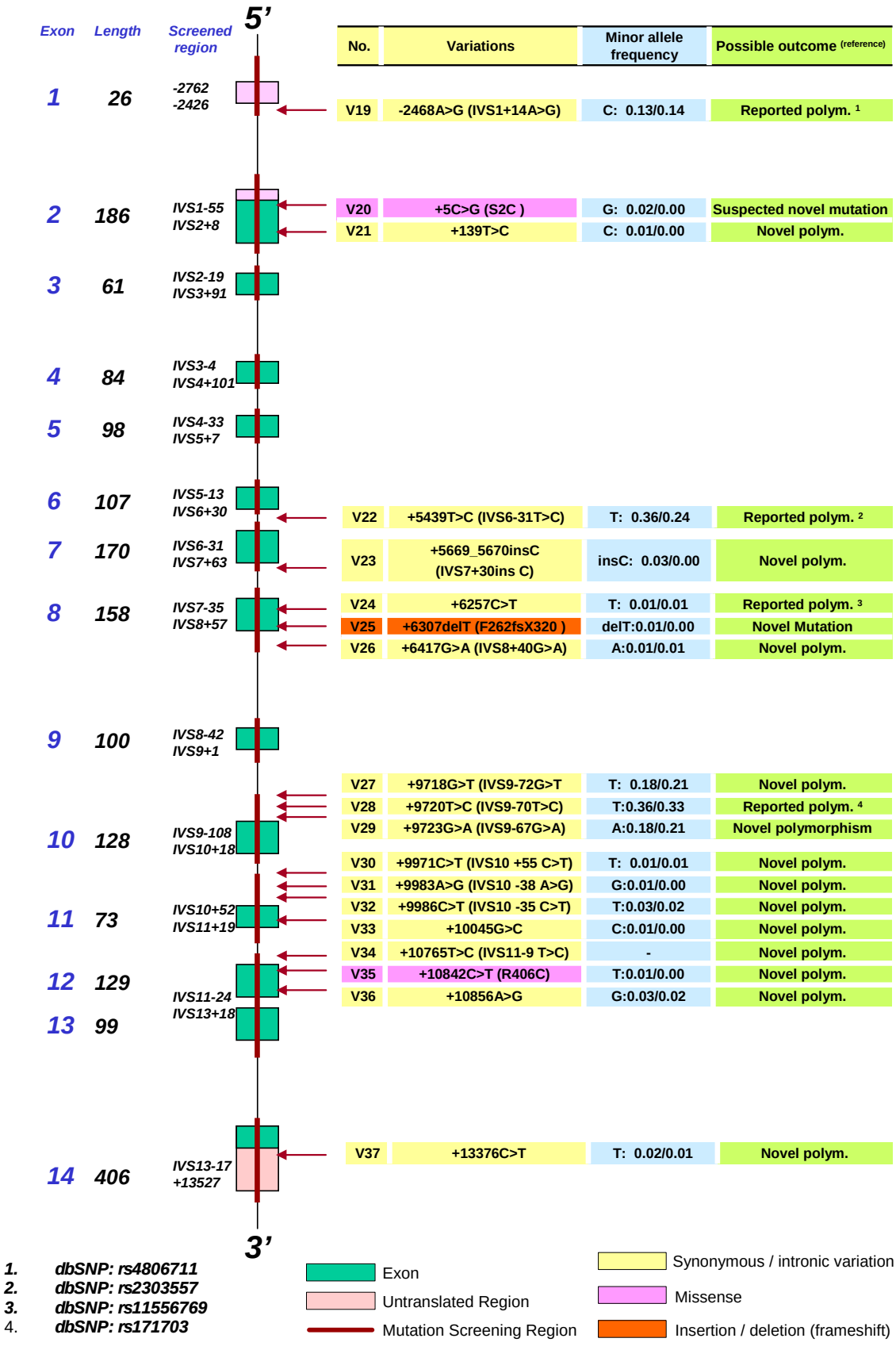


Figure 11 Summary of sequence variations found in ***PRPF31***. The minor allele frequency is for the RP patient group (before the slash) and the control group (after the slash).

Table 6 SSCP patterns and sequencing results of sequence variations in ***RHO***.

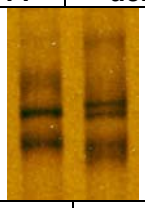
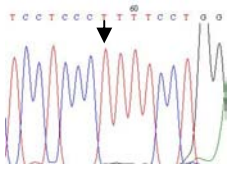
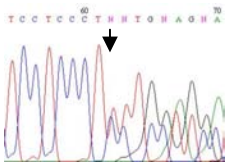
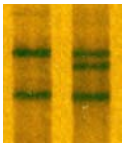
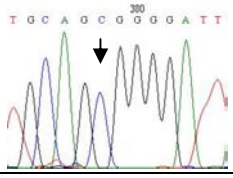
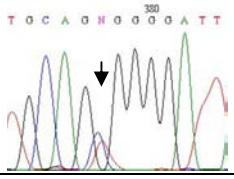
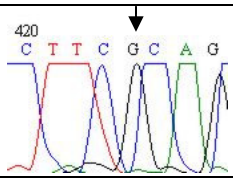
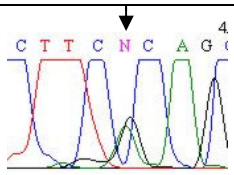
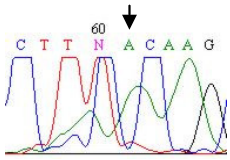
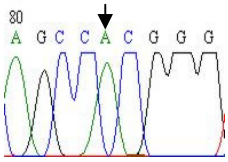
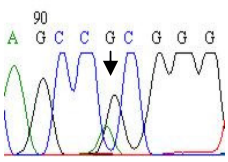
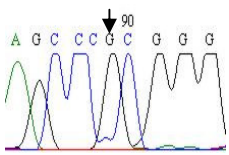
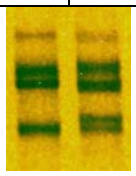
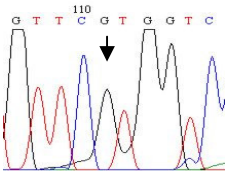
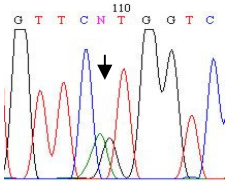
Variation No.	SSCP Patterns			Sequencing Results (Genotypes)		
V1	**TTT/ TTT**	**TTT/ del**		**TTT/TTT**	**TTT/del**	
						
V2	**C/C**	**C/T**		**C/C**	**C/T**	
						
V3	**G/G**	**G/A**	**A/A**	**G/G**	**G/A**	**A/A**
	Refer to Fig. 12					
V4	**A/A**	**G/A**	**G/G**	**A/A**	**G/A**	**G/G**
	Refer to Fig. 12					
V5	**G/G**	**G/A**		**G/G**	**G/A**	
						

Table 6 SSCP patterns and sequencing results of sequence variations in ***RHO*** (continued).

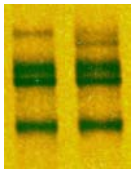
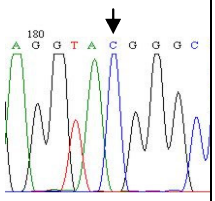
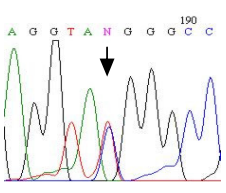
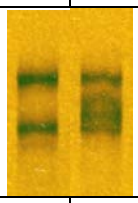
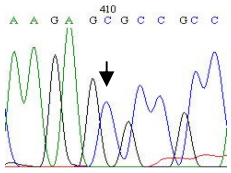
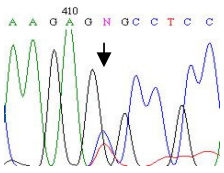
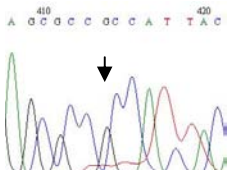
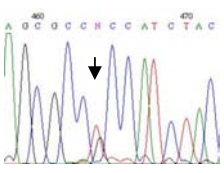
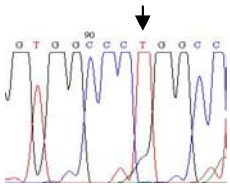
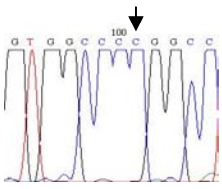
Variation No.	SSCP Patterns		Sequencing Results (Genotypes)		
V6	**C/C**	**C/T**	**C/C**	**C/T**	
					
V7	**C/C**	**C/T**	**C/C**	**C/T**	
					
V8	**G/G**	**G/T**	**G/G**	**G/T**	
					
V9	**T/T**	**C/C**	**T/T**	**C/C**	
					

Table 7 SSCP patterns and sequencing results of sequence variations in ***RDS***.

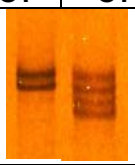
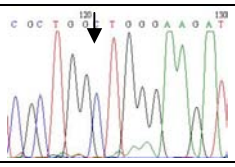
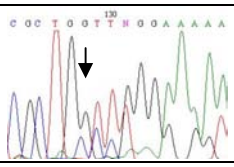
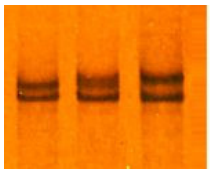
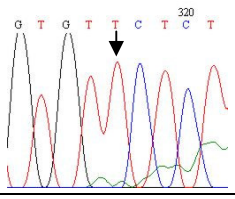
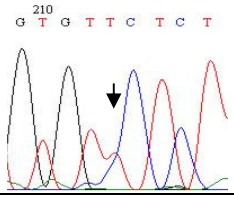
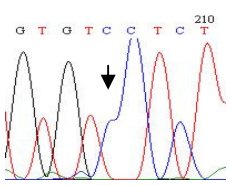
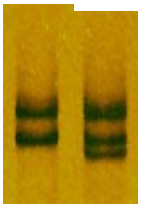
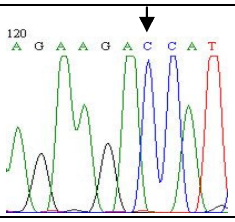
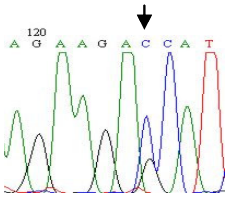
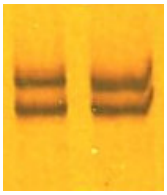
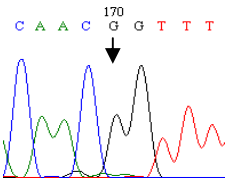
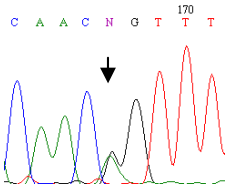
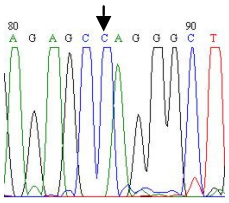
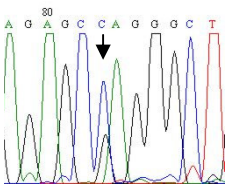
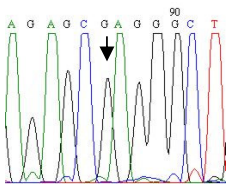
Variation No.	SSCP Patterns			Sequencing Results (Genotypes)		
V10	GCT/ GCT	GCT/ CTC		GCT/GCT	GCT/CTC	
						
V11	T/T	T/C	C/C	T/T	T/C	C/C
						
V12	C/C	C/G		C/C	C/G	
						
V13	G/G	G/A		G/G	G/A	
						
V14	C/C	C/G	G/G	C/C	C/G	G/G
	Refer to Fig. 13					

Table 7. SSCP patterns and sequencing results of sequence variations in ***RDS***, (continued).

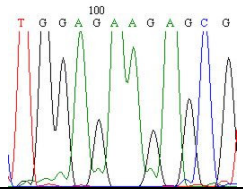
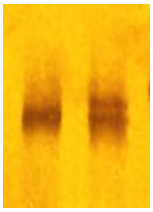
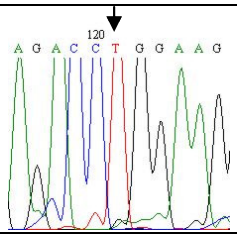
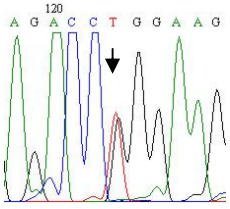
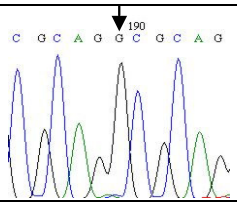
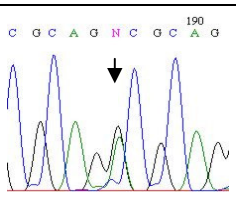
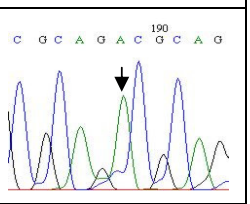
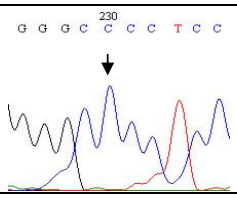
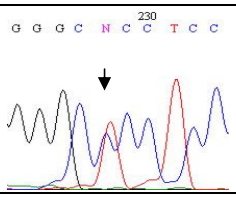
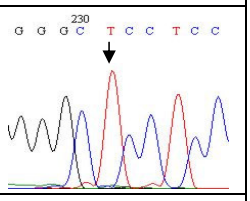
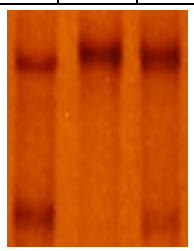
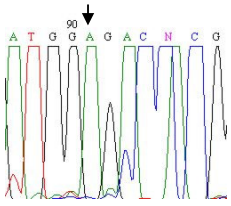
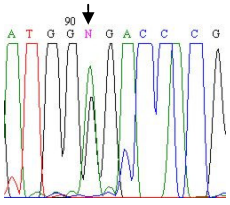
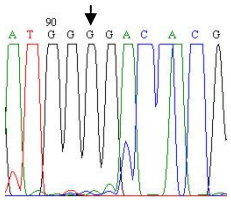
Variation No.	SSCP Patterns	Sequencing Results (Genotypes)		
V15	**A/A**	**A/A**		
				
V16	**T/T** **T/G**	**T/T**	**T/G**	
				
V17	**G/G** **G/A** **A/A**	**G/G**	**G/A**	**A/A**
	Refer to Fig. 13			
V18	**C/C** **C/T** **T/T**	**C/C**	**C/T**	**T/T**
	Refer to Fig. 13			
V19	**A/A** **G/G** **A/G**	**A/A**	**A/G**	**G/G**
				

Table 8. SSCP patterns and sequencing results of sequence variations in ***PRPF31***.

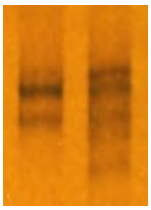
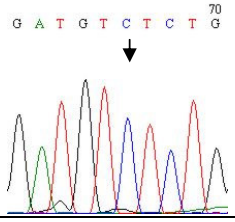
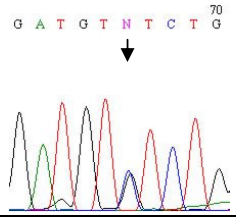
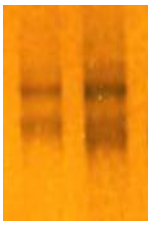
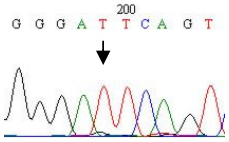
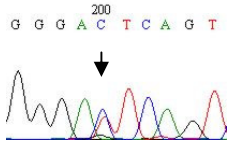
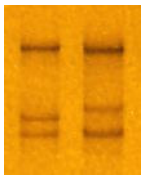
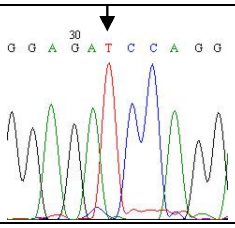
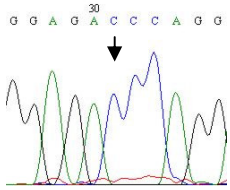
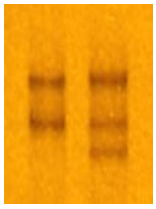
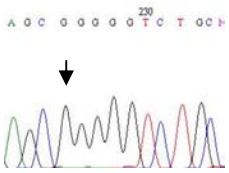
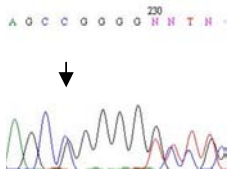
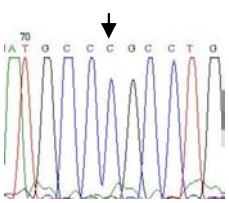
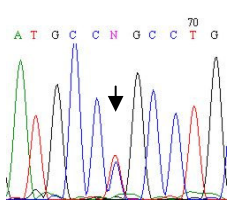
Variation No.	SSCP Patterns		Sequencing Results (Genotypes)		
V20	**C/C**	**C/G**	**C/C**	**C/G**	
					
V21	**T/T**	**T/C**	**T/T**	**T/C**	
					
V22	**T/T**	**C/C**	**T/T**	**C/C**	
					
V23	**CGG/ CGG**	**CGG/ CCG**	**CGG/ CGG**	**CGG/ CCG**	
					
V24	**C/C**	**C/T**	**C/C**	**C/T**	
					

Table 8. SSCP patterns and sequencing results of sequence variations in ***PRPF31*** (continued).

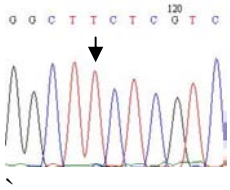
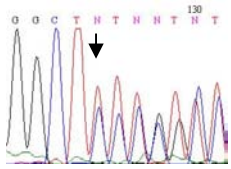
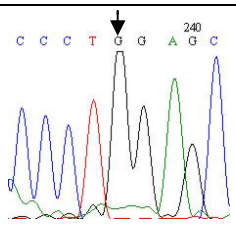
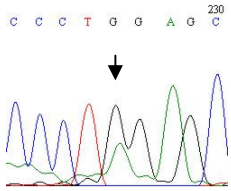
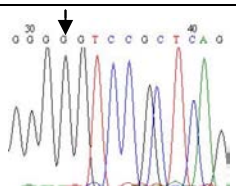
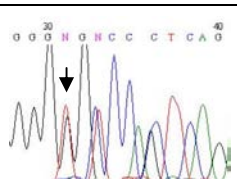
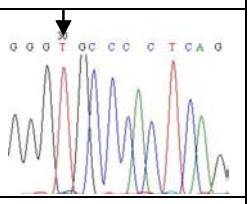
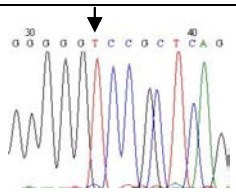
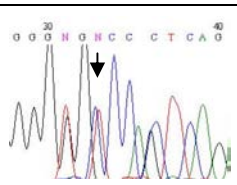
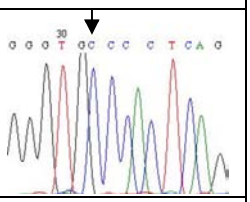
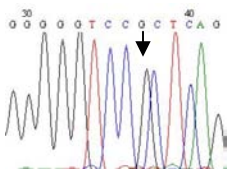
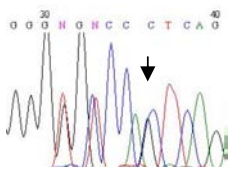
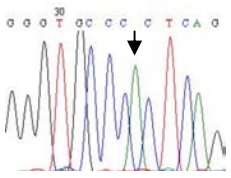
Variation No.	SSCP Patterns			Sequencing Results (Genotypes)		
V25	**TTC/ TTC**	**TTC/ TCT**		**TTC/TTC**	**TTC/TCT**	
						
V26	**G/G**	**G/A**		**G/G**	**G/A**	
						
V27	**G/G**	**G/T**	**T/T**	**G/G**	**G/T**	**T/T**
	Refer to Fig. 14					
V28	**T/T**	**T/C**	**C/C**	**T/T**	**T/C**	**C/C**
	Refer to Fig. 14					
V29	**G/G**	**G/A**	**A/A**	**G/G**	**G/A**	**A/A**
	Refer to Fig. 14					

Table 8. SSCP patterns and sequencing results of sequence variations in ***PRPF31*** (continued).

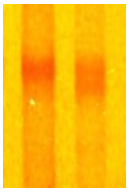
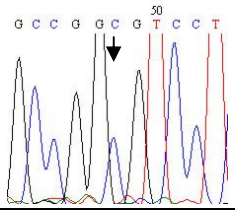
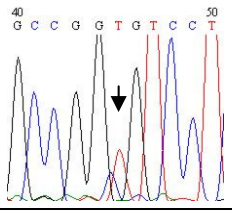
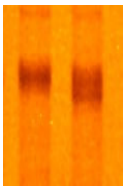
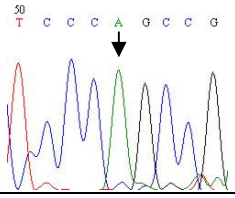
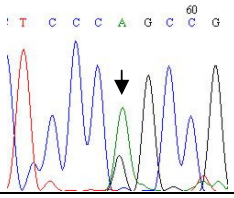
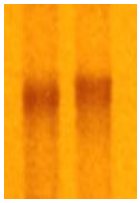
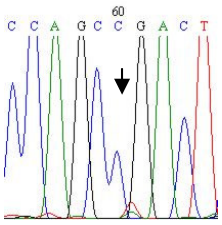
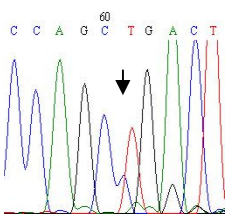
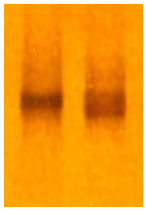
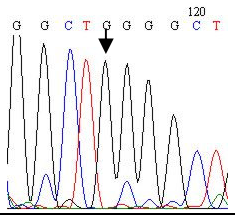
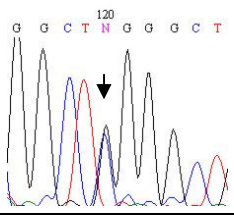
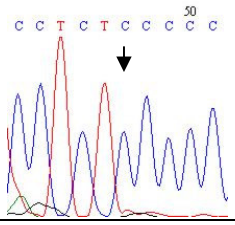
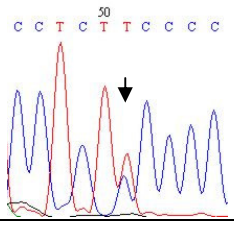
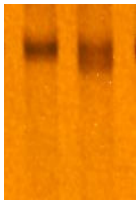
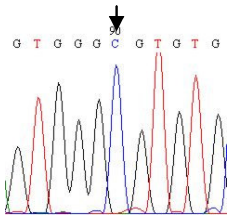
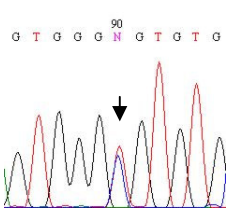
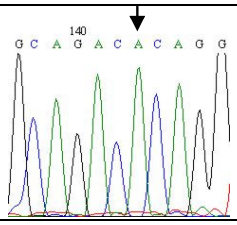
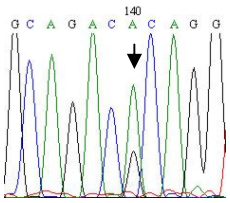
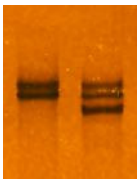
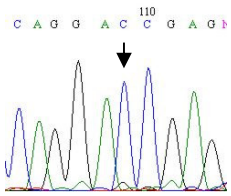
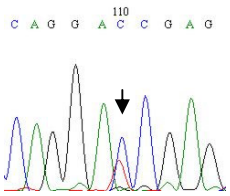
Variation No.	SSCP Patterns		Sequencing Results (Genotypes)		
V30	**C/C**	**C/T**	**C/C**	**C/T**	
					
V31	**A/A**	**A/G**	**A/A**	**A/G**	
					
V32	**C/C**	**C/T**	**C/C**	**C/T**	
					
V33	**G/G**	**G/C**	**G/G**	**G/C**	
					
V34	**C/C**	**T/C**	**C/C**	**T/C**	
	Could not be resolved in SSCP. **Refer to *Figure 34*, *p.104***				

Table 8. SSCP patterns and sequencing results of sequence variations in ***PRPF31*** (continued).

Variation No.	SSCP Patterns		Sequencing Results (Genotypes)		
V35	**C/C**	**C/T**	**C/C**	**C/T**	
					
V36	**A/A**	**A/G**	**A/A**	**A/G**	
					
V37	**C/C**	**C/T**	**C/C**	**C/T**	
					

SSCP Lane		1		2		3		4		5		6	
Variation No.		V 3	V 4	V 3	V 4	V 3	V 4	V 3	V 4	V 3	V 4	V 3	V 4
Band 1	→					G	G	G	G	G G	G G		
Band 2	→	G G	A A	G	A	G	A						
Band 3	→			A	G			A	G			A A	G G

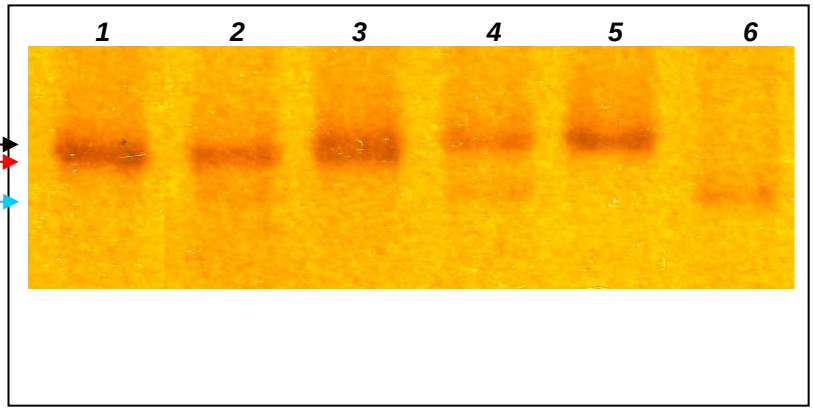


Figure 12 SSCP patterns of common ***RHO*** sequence variations V3 (-51G>A) and V4 (-26A>G) in different samples. The results indicate that three haplotypes (G-A, G-G and A-G) and six 2-site genotypes are observed across these two sites V3 and V4.

SSCP Lane		1			2			3			4			5			6		
Variation No.		V14	V17	V18	V14	V17	V18	V14	V17	V18	V14	V17	V18	V14	V17	V18	V14	V17	V18
Band 1	→	G G	G G	T T							G	G	T				G	G	T
Band 2	→				G G	G G	C C							G	G	C	G	G	C
Band 3	→							C C	A A	C C	C	A	C	C	A	C			

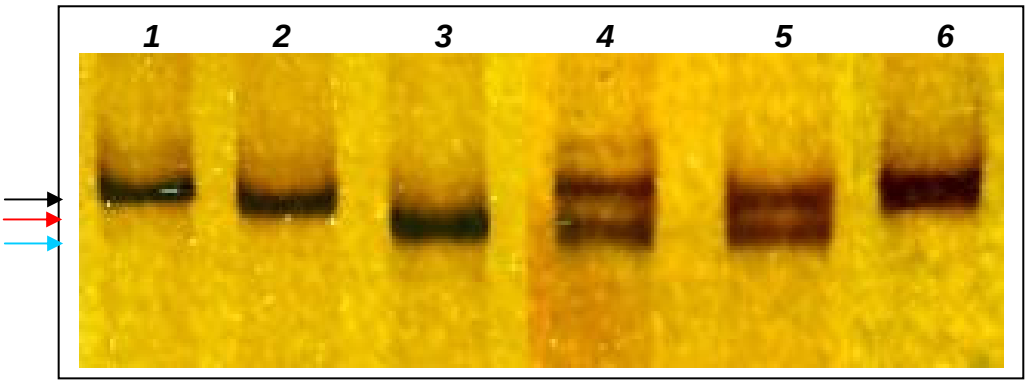


Figure 13 SSCP patterns of common ***RDS*** sequence variations V14 (+23909C>G), V17 (+24012G>A) and V18 (+24053C>T) in different samples. Across these three sites (V14, V17 and V18), three haplotypes (G-G-T, G-G-C and C-A-C) and six 3-site genotypes were identified.

SSCP Lane	1			2			3			4			5			6		
Variation No.	V27	V28	V29	V27	V28	V29	V27	V28	V29	V27	V28	V29	V27	V28	V29	V27	V28	V29
Band 1 →	T		A							T		A				TT		AA
Band 2 →	G		G	GG		GG	GG		GG	G		G	GG		GG			
Band 3 →					T						T			TT				
Band 4 →		CC			C			CC			C						CC	
Possible Combination	TG	CC	AG	GG	TC	GG	GG	CC	GG	TG	CT	AG	GG	TT	GG	TT	CC	AA

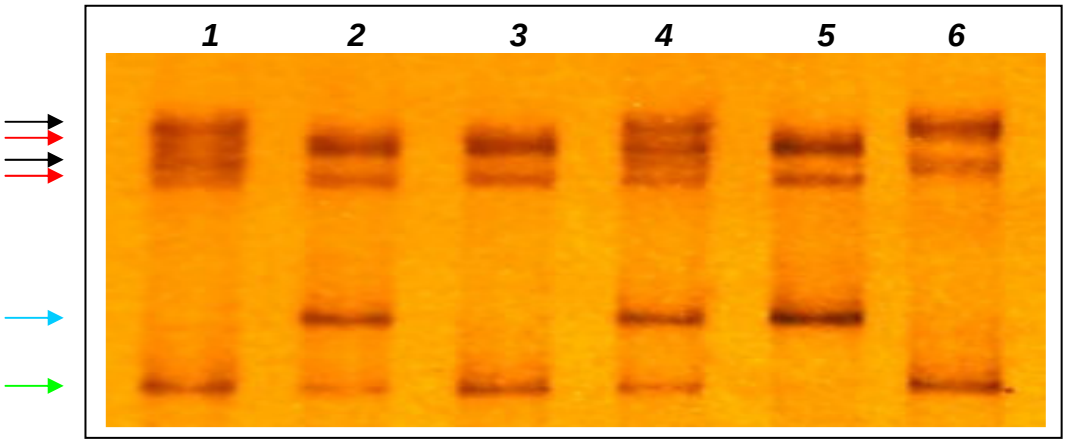


Figure 14 SSCP patterns of common ***PRPF31*** sequence variations V27 (IVS9-72T>C), V28 (IVS-70T>C) and V29 (IVS-67G>A) in different samples. Across these three sites, three haplotypes (G-C-G, G-T-G and T-C-A) and six 3-site genotypes were observed.

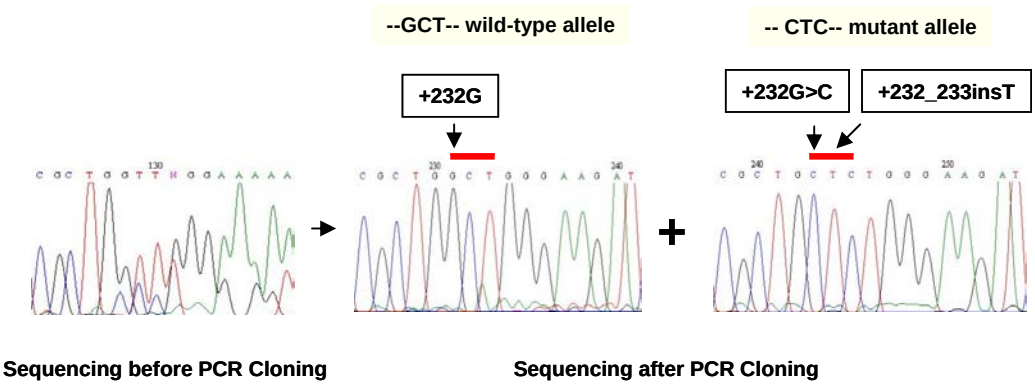


Figure 15 Cycle sequencing results of V10 (+232G>C;+232_+233insT) mutation after PCR cloning. Both ***RDS*** sequence variations are on the same chromosome.

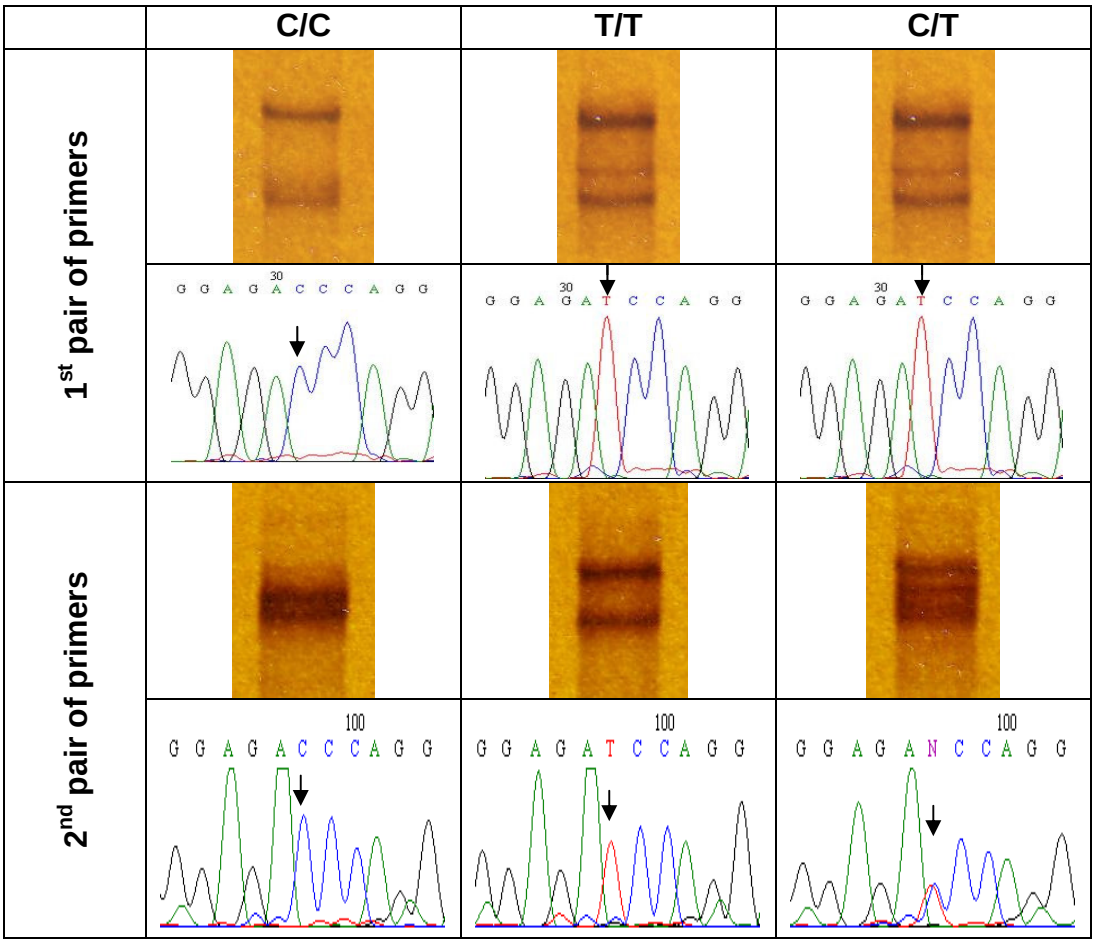


Figure 16 Heterozygous C/T samples of V22 (+5439T>C or IVS6-31T>C) variation in ***PRPF31*** were only detected by a 2nd pair of PCR primers.

3.3 Mutation screening in rhodopsin

Nine sequence variations were identified in the rhodopsin gene: deletion (-300_-302delTTT) in the 5' regulatory region; single nucleotide changes in the 5' regulatory region and intron 3 (-201C>T, -51G>A, -26A>G and IVS3+4C>T); and synonymous (+3995C>T) and missense substitutions (V209M, A299S and P347L) in the coding sequences ***(Figure 9)***.

In the 5' regulatory region, a heterozygous deletion of three T's (V1: -300_-302delTTT) was found in one of the RP patients with Usher syndrome. This variant was also found in one control sample. Only the proband's sample (II-1) was available in this study so that no co-segregation information could be obtained ***(Figure 17)***. A single nucleotide substitution (V2: -201C>T) was another sequence variation found in the 5' regulatory region. In family C7343, proband III-5 was heterozygous for this variation but other family members' samples were not available for screening ***(Figure 18)***. This variation was not found in the controls. These two variants, V1 and V2***,*** have not been reported previously.

In the 5' untranslated region of exon 1, two sequence variations were found. They were V3 (-51G>A) and V4 (-26A>G). Both variations were transitional changes (A to G or G to A) and were previously reported as polymorphisms (Haga et al, 2002; Bareil et al, 1999). A single nucleotide polymorphism, V6: IVS3+4C>T, was also identified in intron 3. This same polymorphism was also found in control samples and had been reported as a polymorphism in a previous study (Dryja et al, 1990b).

Other variations were located in the exons of ***RHO***. One was synonymous (+3995C>T) and three were missense substitutions (V209M, A299S and P347L). The individual I-2 in the family C8423 ***(Figure 19)*** was heterozygous for the missense substitution V5: V2909M, which was not found in control samples. One of this individual's affected sons (II-3) did not carry the variation. This had also been reported in a previous study without the effect being determined (Macke et al, 1993). Thus, this variation did not co-segregate with the disease phenotype and most probably was a rare polymorphism. In exon 4 of ***RHO***, the synonymous variation +3995C>T (V7) and the missense substitution A299S (V8***)*** were found. Both were found in control samples, and did not co-segregate with the disease phenotype.

The missense mutation P347L (V9) was a reported mutation in adRP (Dryja et al, 1990b). This homozygous mutation was only found in the two affected individuals (II-10 and II-11) in an adRP family, C8289 ***(Figure 20)***. However, other affected individuals studied (II-3, II-8 and III-3) did not carry the mutation. Although it was not detected in control samples, the mutation did not segregate with disease phenotype. Another mutation of ***PRPF31*** was also found in this family. This mutation will be discussed later (Sections 3.5 and 4.5).

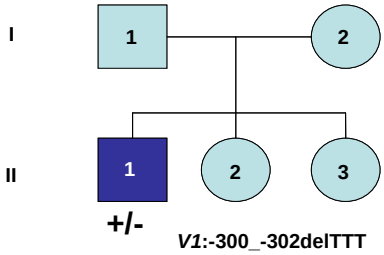


Figure 17 Pedigree diagram of family C8568

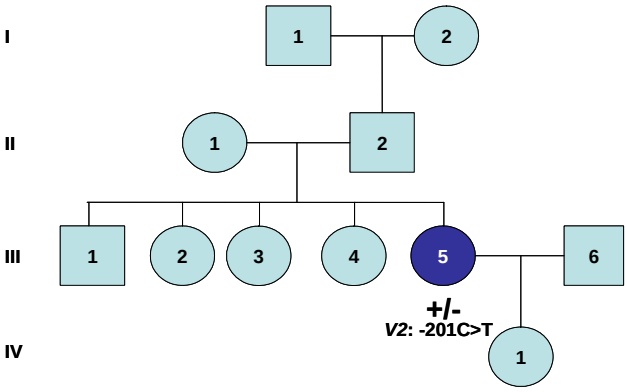
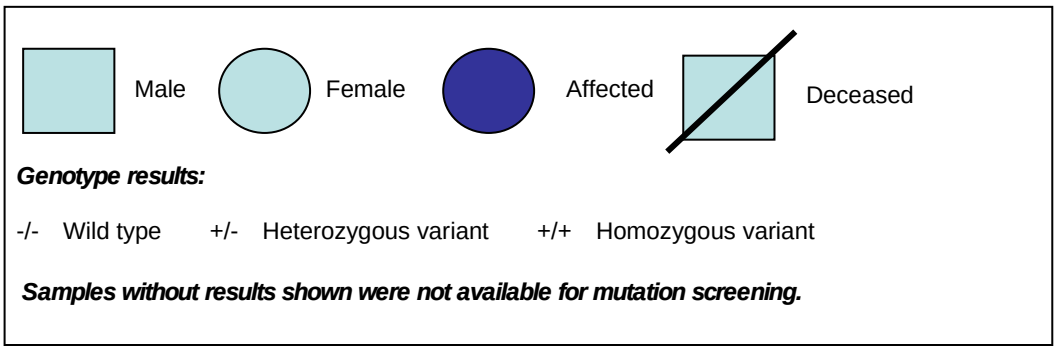


Figure 18 Pedigree diagram of family C7343



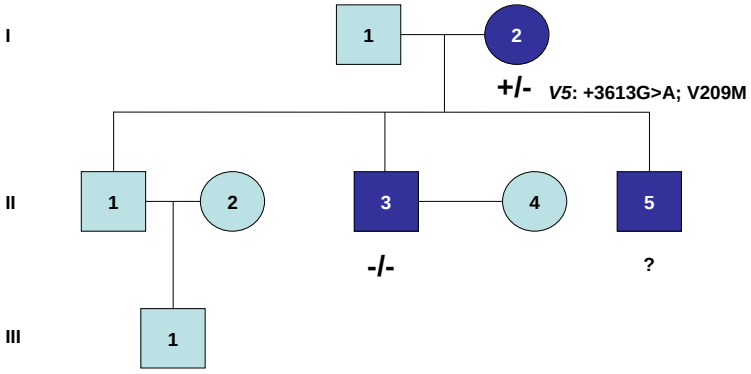


Figure 19 Pedigree diagram of family C8423

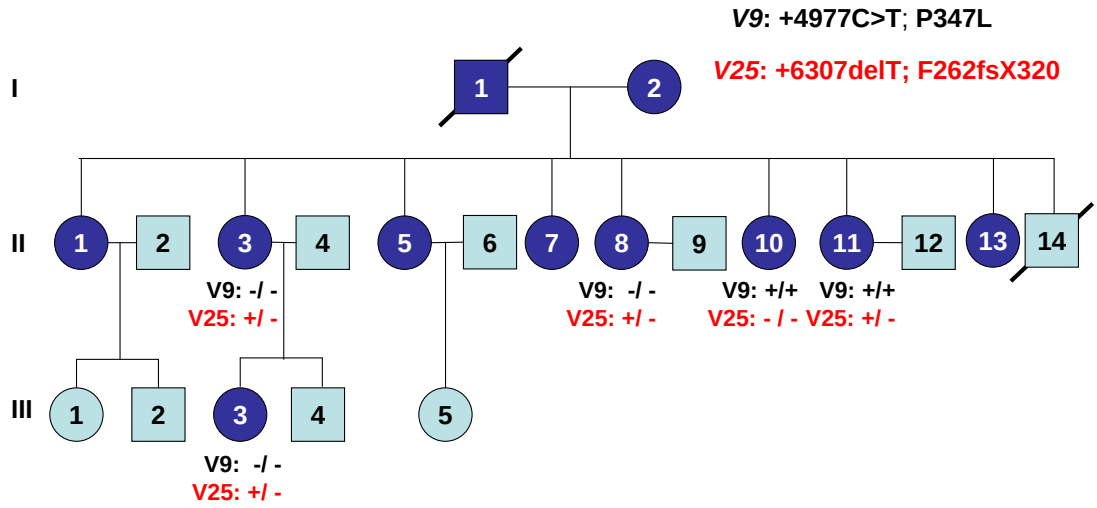
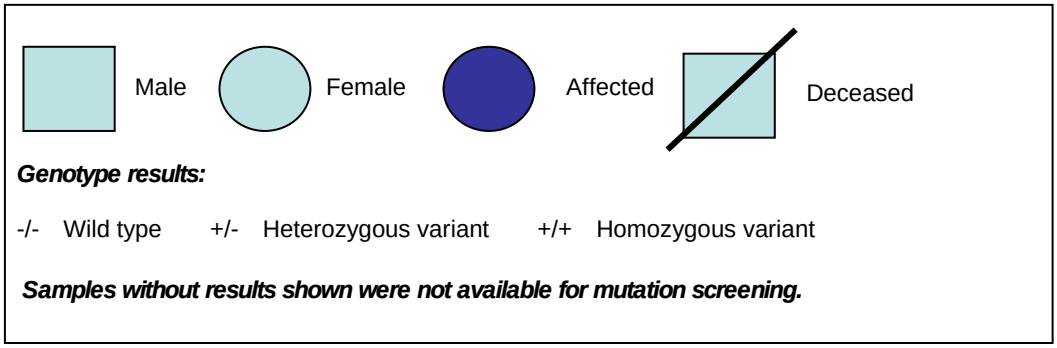


Figure 20 Pedigree diagram of family C8289



3.4 Mutation screening in *RDS*

Ten sequence variations were identified in the ***RDS*** gene: complex changes (+232G>C;+232_+233insT); single nucleotide changes in the 3' UTR (+24053C>T); and synonymous (+318T>C) and missense substitutions (T155S, G170S, Q304E, R310K, W316G and G338D) in coding sequences ***(Figure 10)***. Only the +24053C>T (V18***)*** variation was found in the untranslated region and all other variations were located in the coding region of ***RDS***. V18 was also present in control samples and was reported as a polymorphism in a previous study (Ekstrom et al, 1998b).

A complex mutation (V10:+232G>C;+232_+233insT) was found in one family ***(Figure 22)***. Only the affected individual II-7 was available for screening and this mutation was not found in control samples. Sequencing after PCR cloning revealed that there were two sequence variations. One was a G to C substitution at +232. Another was a T nucleotide insertion between +232 and +233. These two variations were on the same chromosome of the affected individual ***(Figure 15)***. As a consequence, these base changes caused a frameshift (A78***fsX***176) starting from codon 78, and created a premature stop codon after 98 codons ***(Figure 21)***.

A synonymous variation (V11:+318T>C) was found in both RP and control samples and had been reported previously as a polymorphism (Farrar et al, 1991). The +23909C>G (V14) and +24012G>A (V17) variations produced missense Q304E and G338D that had also been reported as polymorphisms (Jordan et al, 1992). Although +23928G>A (V15) also caused the missense substitution R310K, all the RP and control samples were homozygous for this variation. Thus, it was just a sequence difference between the reference sequence and samples within the population. The missense substitutions (V12:+464C>G and V16:+23945T>G) led

to the change of amino acid from threonine to serine at codon 155 and tryptophan to glycine at codon 316, respectively. These two variations had not been reported previously. No control samples had these variations, however the respective variation did not co-segregate with the disease phenotype in families C8445 ***(Figure 23)*** and C8184 ***(Figure 24)***. The missense substitution +508G>A (V13: G170S) was only found in one of the control samples.

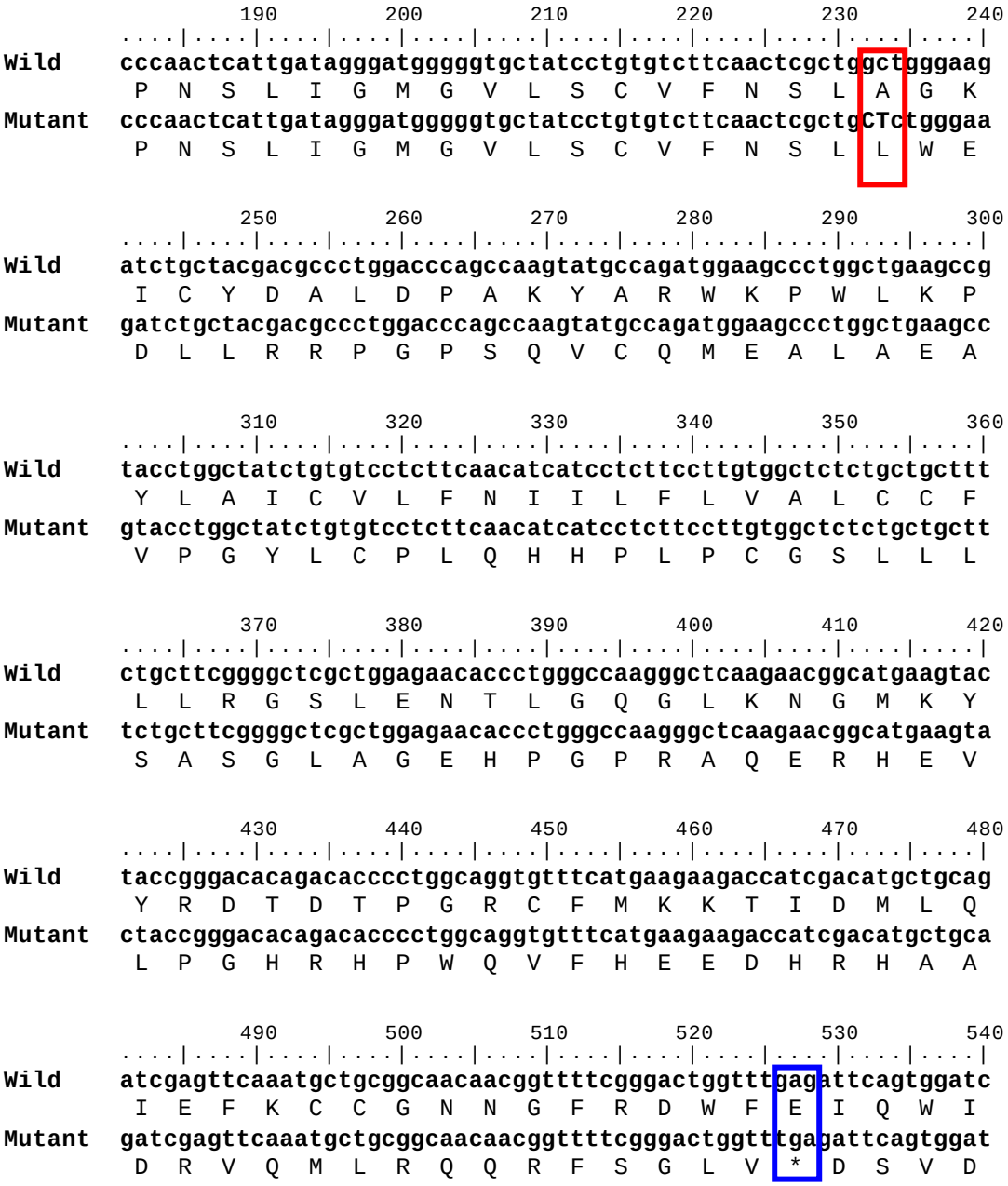
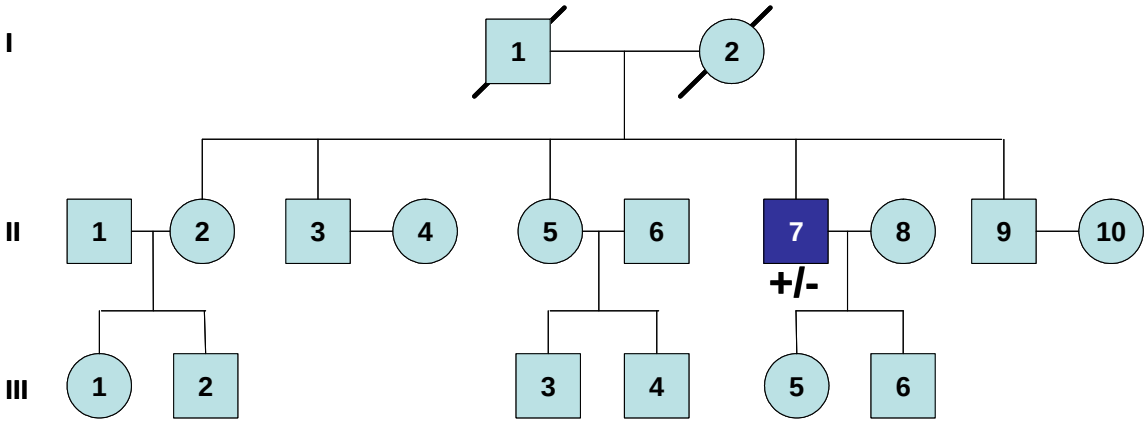


Figure 21 The +232G>C;+232_+233insT complex mutation (V10: A78fsX176) in the ***RDS*** gene. This mutation causes frameshit starting from alanine at codon 78 (red box) and creates a premature stop codon at position 176 (blue box).



***V10:* +232G>C; +232_+233insT; A78fsX217**

Figure 22. Pedigree diagram of family C7765

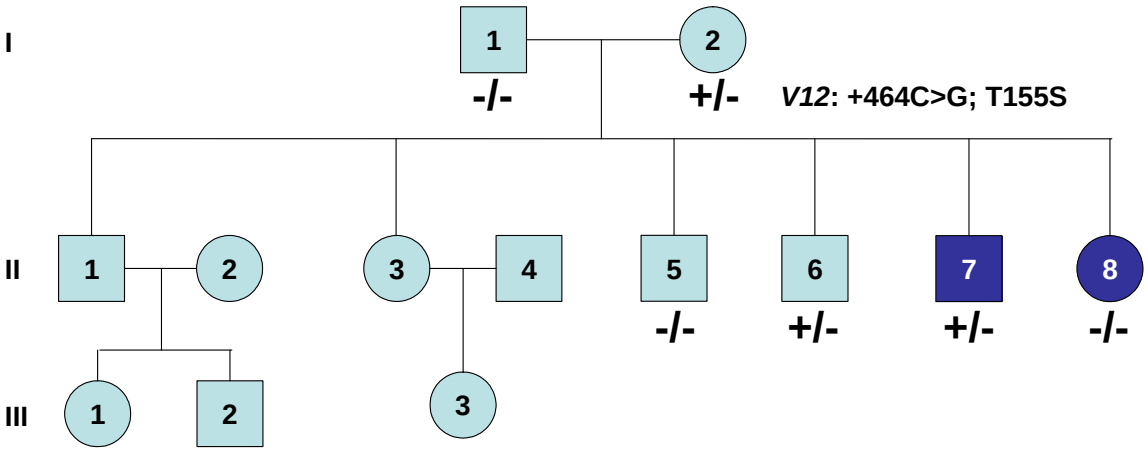
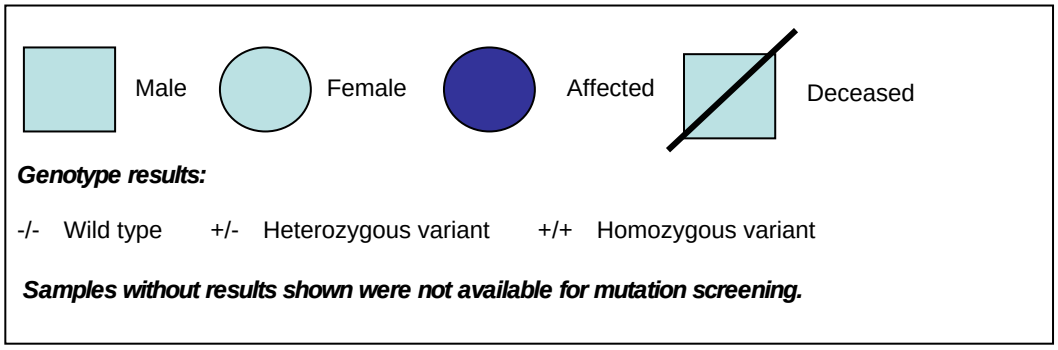


Figure 23 Pedigree diagram of family C8445



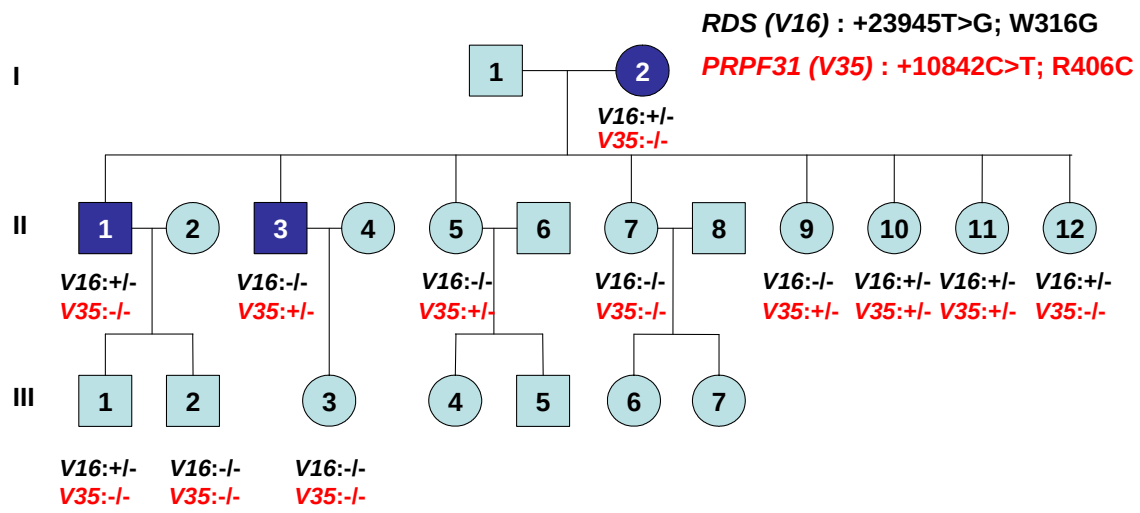
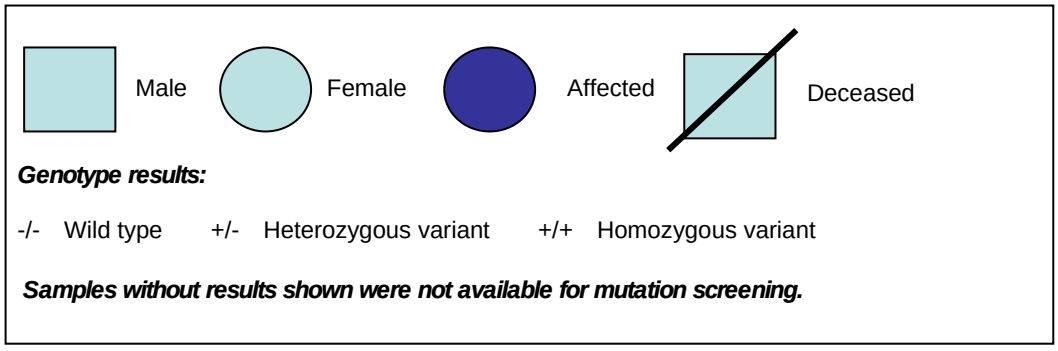


Figure 24 Pedigree diagram of family C8184



3.5 Mutation screening in *PRPF31*

Nineteen variations were identified in ***PRPF31 (Figure 11)***: two missense (S2C and R406C) and four synonymous substitutions (+139T>C, +6257C>T, +10045G>C, +10856A>G); one deletion in exon 8 (+6307delT); one insertion in intron 7 (+5669insC); nine single nucleotide changes in introns (IVS1+14A>G, IVS6-31T>C, IVS8+40G>A, IVS9-72G>T, IVS9-70T>C, IVS9-67G>A, IVS10+55C>T, IVS10-38A>G, IVS10-35C>T and IVS11-9T>C) and one single nucleotide change in 3'UTR (+13376C>T).

Except for IVS1+14A>G (V19), IVS6-31T>C (V22), IVS9-70T>C (V24) and +6257C>T (V28), all other findings were novel. The known variations were reported in dbSNP of NCBI (NCBI SNP ID: rs4806711, rs2303557, rs11556769 and rs171703, respectively). In this study, both RP patients and controls had these variations, which were considered as polymorphisms.

For the synonymous variations, +139T>C (V21) was found in the proband of family C6647 but not in control samples ***(Figure 25)***. The co-segregation of this variation with disease phenotype could not be determined because other family members were not available for testing. The +10045G>C (V33) was a synonymous variation that was not found in control samples. In family C7851, both the proband and her unaffected mother carried the heterozygous variation ***(Figure 26)***. However, the heterozygous +10856A>G (V36) synonymous variation was found in both RP and control samples. These three sequence variations were likely polymorphisms.

There were two missense substitutions in this gene. The +5C>G (V20) variation produced an amino acid change from serine to cysteine at codon 2 (S2C). This

variation was found in family C8062 and family C8734 ***(Figures 27 and 28)***, but not in control samples. In family C8062, both affected individuals III-1 and III-7 carried this variation. However, other family members were not available for segregation analysis. In family C8734, one affected individual (III-5) and one unaffected individual (III-4) were heterozygous. Therefore, S2C did not segregate with the disease phenotype in this family.

On the other hand, the +10842C>T (V35) variation caused an amino acid change from arginine to cysteine (R406C). This variation was found in both affected (II-3) and unaffected (II-5, II-9, II-10 and II-11) individuals of family C8184 ***(Figure 24)***. Two affected members (I-2 and II-1) did not have this variation. Although no control samples had this variation, it did not segregate with the disease phenotype. This indicates that R406C is mostly likely a rare polymorphism. This family also carried another ***RDS*** sequence variation (V16).

An insertion of a C nucleotide (V23:+5669insC) in intron 6 was found in three families (C7970, C8354 and C8745) but not in control samples. In family C7960, only the proband was available for screening and was heterozygous for this variation ***(Figure 29)***. In family C8354, this variation was only found in one affected (II-1) but not in two other affected (II-3 and II-5) individuals ***(Figure 30)***. Both affected (II-1) and unaffected (III-1 and III-2) individuals carried this variation in family C8745 ***(Figure 31).*** Therefore, this intronic variation did not segregate with the disease phenotype in these families. It also is a rare polymorphism.

A heterozygous deletion (V25:+6307delT) was found in family C8289 ***(Figure 20)***, but not in control samples. The affected individuals II-3, II-8, II-11 and III-3 carried this mutation. The mutation (F262***fsX***320) causes a frameshift and creates a downstream premature stop codon leading to a truncated protein product ***(Figure***

33). However, the affected II-10 did not have this mutation. The disease phenotype did not segregate with this mutation alone. See Section 4.5 for discussion.

All other single nucleotide changes were in the intron or 3'UTR regions and have not been reported previously. Except for IVS10-38A>G (V31), both RP and control samples had these variations (V26:IVS8+40G>A, V27:IVS9-72G>T, V29:IVS9-67G>A, V30:IVS10+55C>T, V32:IVS10-35C>T, V34:IVS11-9T>C and V37:+13376C>T), which were most likely polymorphisms. The IVS11-9T>C (V34) variation was detected only by cycle sequencing, but not by SSCP under any studied conditions. In control samples, this variation was also found by sequencing. The IVS10-38A>G (V31) variation was found in family C8998 ***(Figure 32)*** but not in control samples. Both the proband I-1 and his unaffected son II-1 were heterozygous for this variation. Thus, V31 did not segregate with the disease phenotype and was most likely a rare polymorphism.

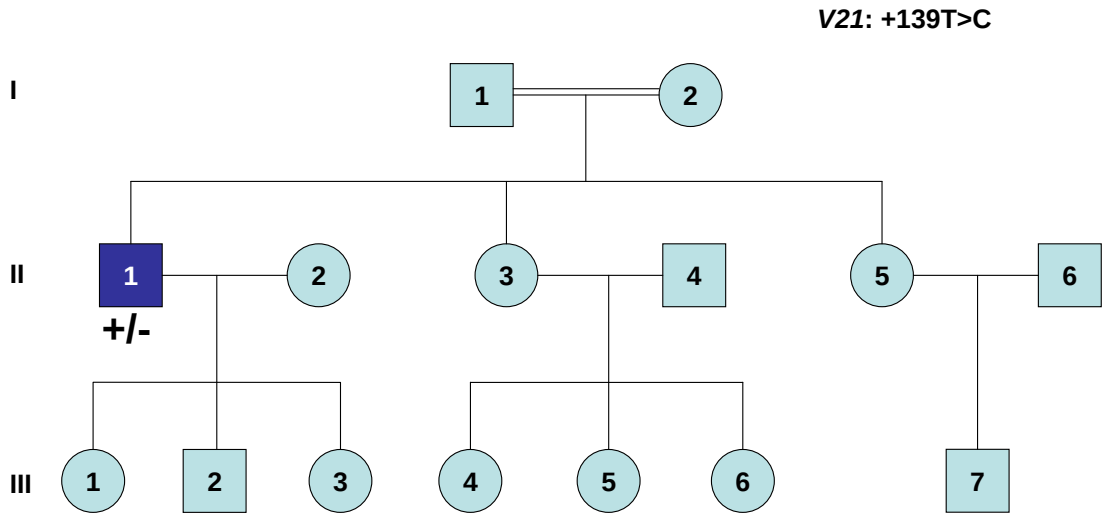


Figure 25 Pedigree diagram of family C6647

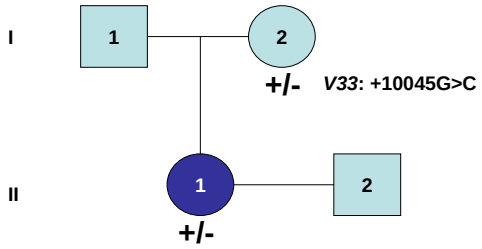
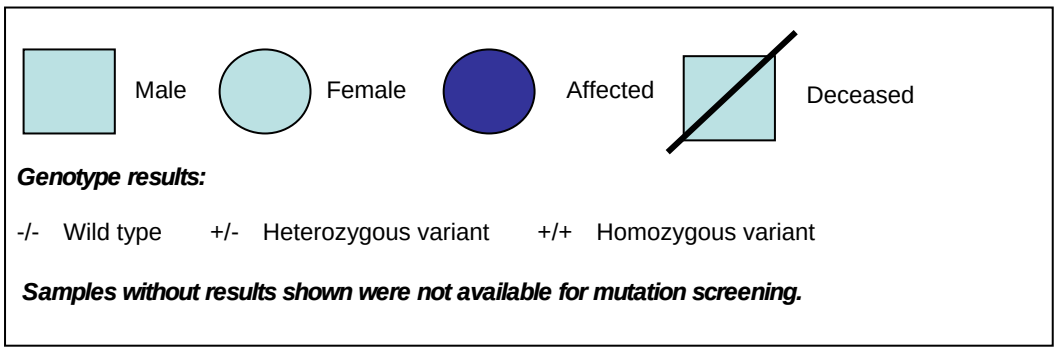


Figure 26 Pedigree diagram of family C7851



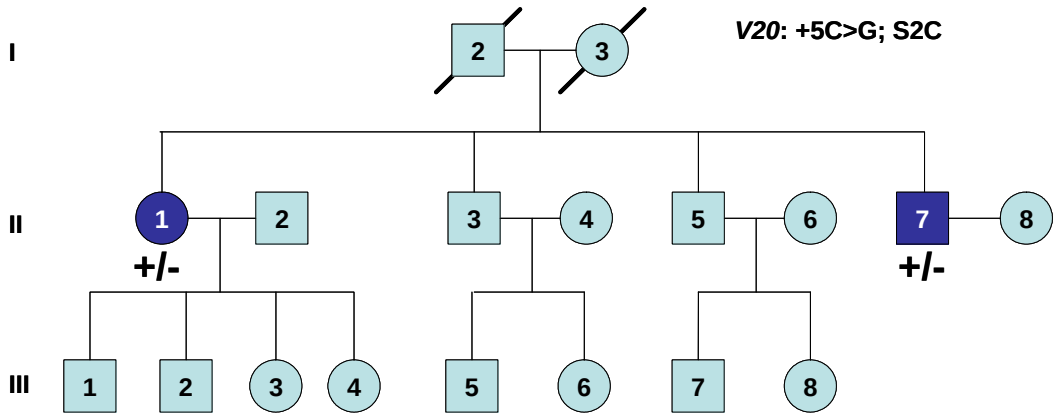


Figure 27 Pedigree diagram of family C8062

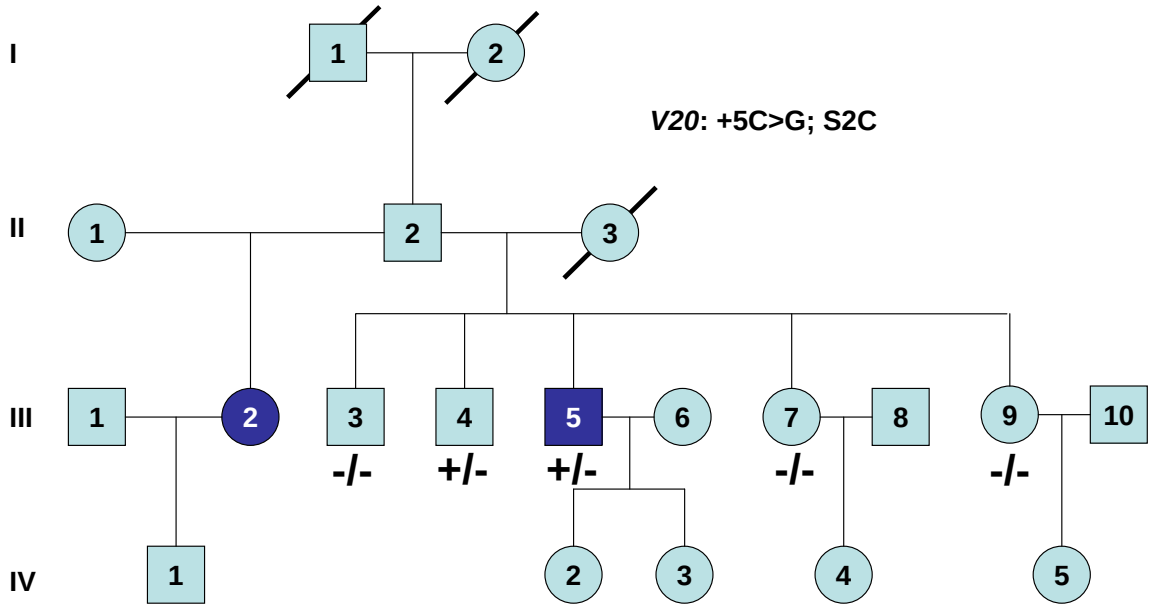
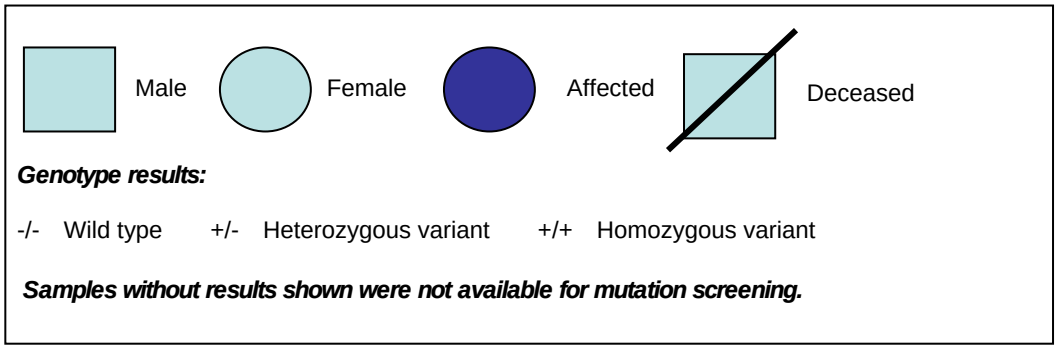


Figure 28 Pedigree diagram of family C8734.



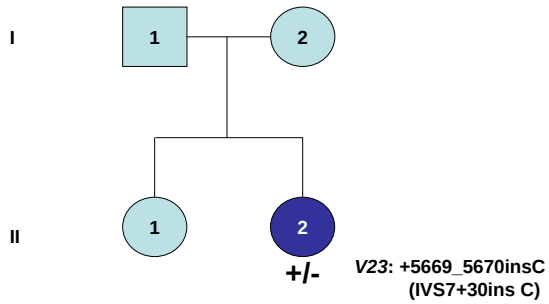


Figure 29 Pedigree diagram of family C7970

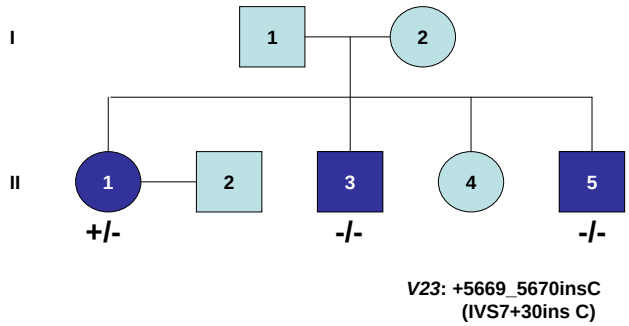


Figure 30 Pedigree diagram of family C8354

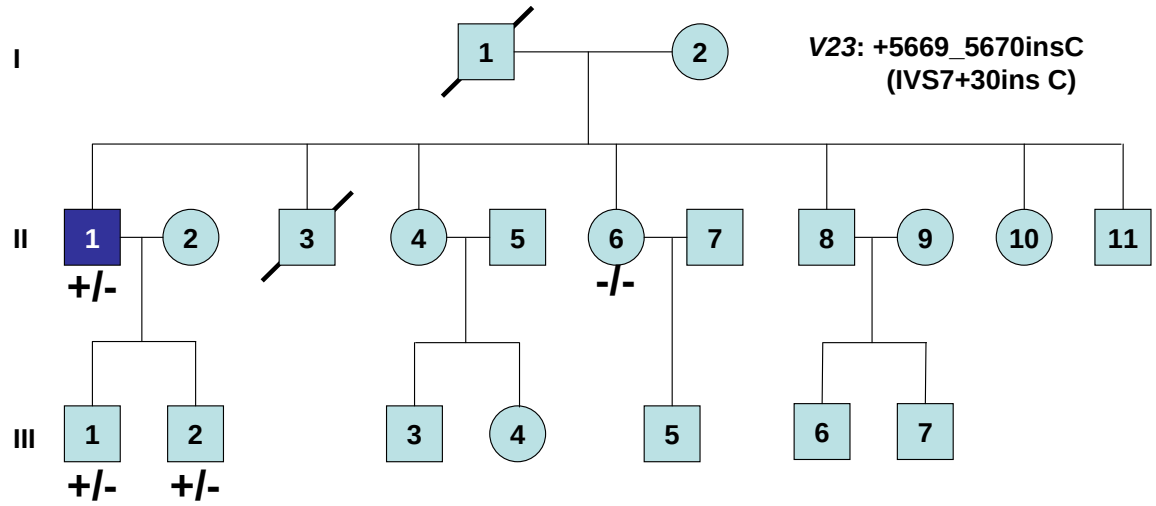
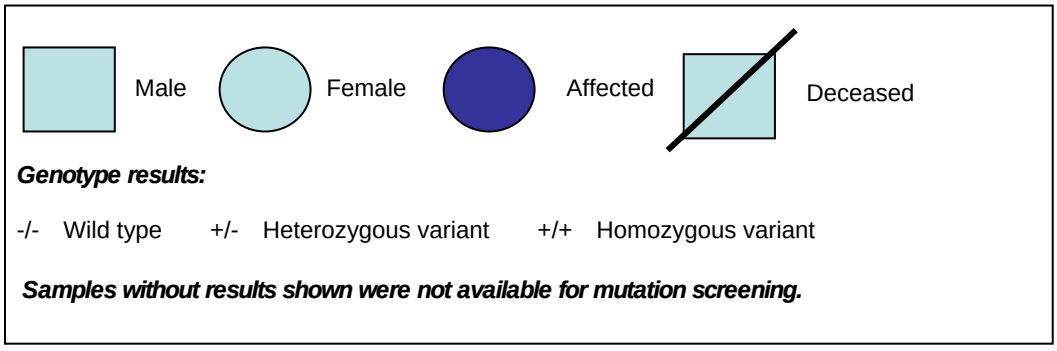


Figure 31 Pedigree diagram of family C8745



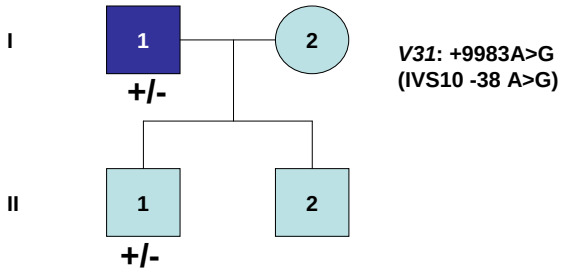
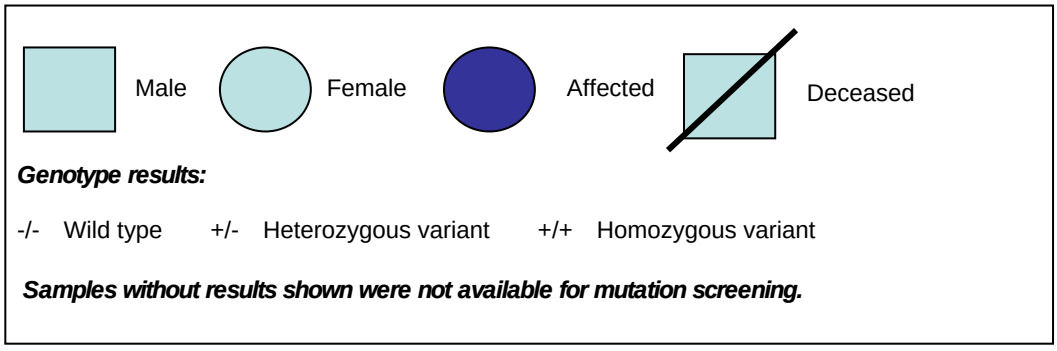


Figure 32 Pedigree diagram of family C8998



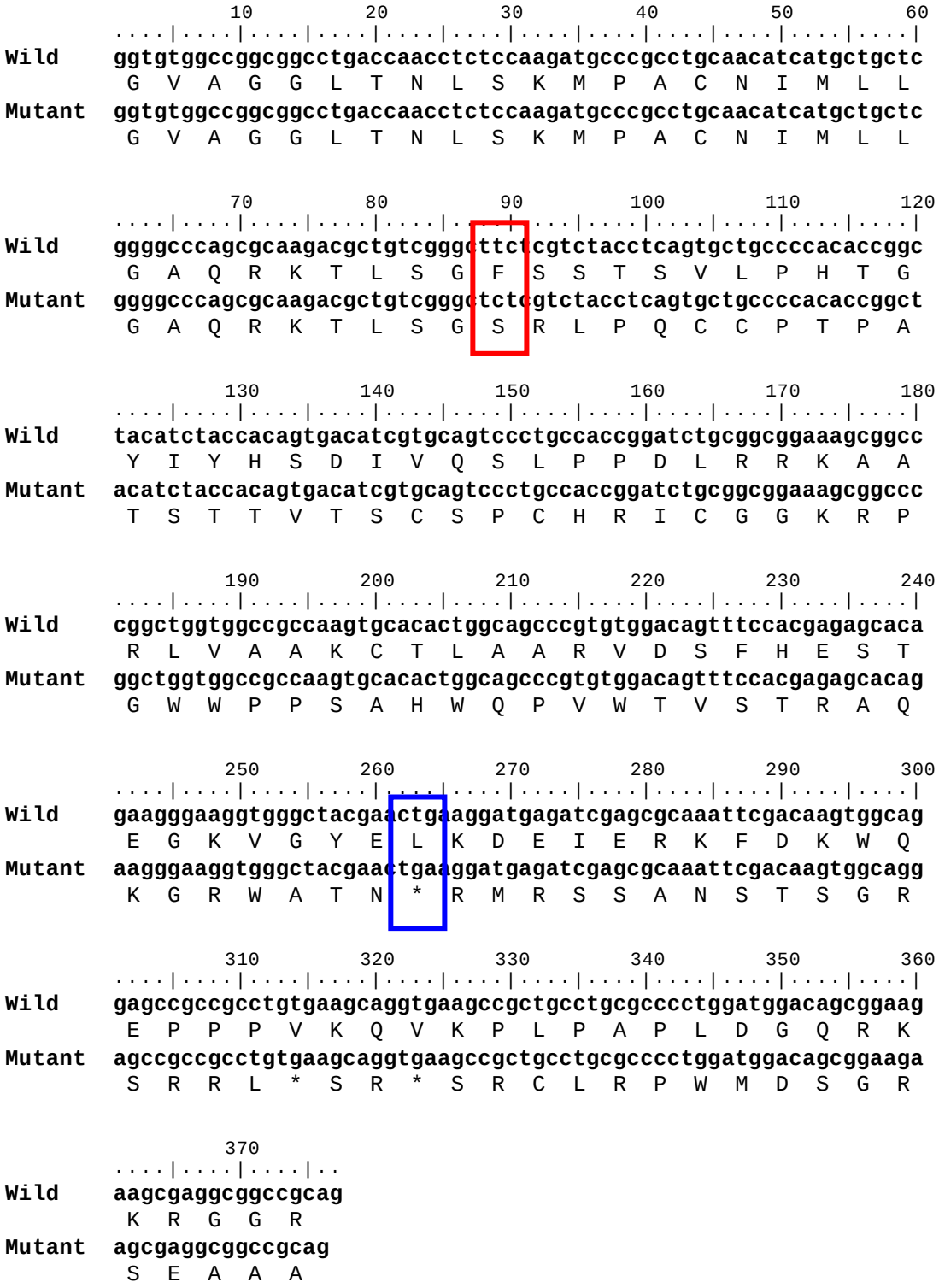


Figure 33 The +6307delT mutation (V25: F262fsX320) in the ***PRPF31*** gene. This deletion causes a frameshift starting at phenylalaine at codon 262 (red box) and creates a premature stop codon at codon position 320 (blue box).

3.6 Allele frequencies of the variations

The genotype counts and frequencies of the common sequence variations (heterozygosity ≥ 0.1) are listed in ***Table 9***. All sequence variations were shown to be in Hardy-Weinberg equilibrium. Except for the missense variations V14 (Q304E) and V17 (Q338D) of ***RDS***, the genotype distributions of these variations did not show statistically significant differences between RP and control groups.

Both V14 (Q304E) and V17 (Q338D) were found in the same PCR fragment and showed a significant difference (***p***<0.05) in genotype distribution between RP and control groups. Haplotypes of these variations together with V18 were revealed by the SSCP patterns of the fragment ***(Figure 13)***. The haplotype counts were calculated and the difference between the two groups was analyzed with the chi-squared test ***(Table 10)***. In this case, the difference was statistically significant (***p = 0.0131***).

Table 9 Fisher's exact test results for the differences in sequence variations between patients and controls.

*Variations/Groups**	*Count of genotype (frequency %)*			*Heterozygosity*	*Fisher's exact test (p value)*
v3	*G/G*	*A/A*	*A/G*	*0.51*	*0.3603*
RP	*18 (31)*	*7 (12)*	*34 (58)*		
Controls	*26 (36)*	*14 (18)*	*35 (46)*		
v4	*A/A*	*G/G*	*A/G*	*0.42*	*0.1969*
RP	*8 (14)*	*31 (53)*	*20 (34)*		
Controls	*11 (15)*	*28 (39)*	*36 (48)*		
v11	*T/T*	*C/C*	*T/C*	*0.54*	*0.1584*
RP	*10 (17)*	*16 (27)*	*33 (56)*		
Controls	*6 (8)*	*28 (38)*	*41 (54)*		
v14	*G/G*	*C/C*	*C/G*	*0.29*	*0.0253*
RP	*35 (60)*	*2 (3)*	*22 (37)*		
Controls	*58 (77)*	*0 (0)*	*17 (23)*		
v17	*G/G*	*A/A*	*G/A*	*0.29*	*0.0253*
RP	*35 (60)*	*2 (3)*	*22 (37)*		
Controls	*58 (77)*	*0 (0)*	*17 (23)*		
v18	*C/C*	*T/T*	*C/T*	*0.40*	*0.5704*
RP	*30 (51)*	*3 (5)*	*26 (44)*		
Controls)	*45 (60)*	*3 (4)*	*27 (36)*		
v19	*A/A*	*G/G*	*A/G*	*0.24*	*0.1011*
RP	*46 (78)*	*2 (3)*	*11 (19)*		
Controls	*54 (72)*	*0 (0)*	*21 (28)*		
v22	*T/T*	*C/C*	*T/C*	*0.34*	*0.0621*
RP	*12 (20)*	*26 (44)*	*21 (36)*		
Controls	*6 (8)*	*45 (60)*	*24 (32)*		
v27	*G/G*	*T/T*	*G/T*	*0.27*	*0.8721*
RP	*41 (69)*	*3 (5)*	*15 (25)*		
Controls	*49 (65)*	*5 (7)*	*21 (28)*		
v28	*T/T*	*C/C*	*T/C*	*0.40*	*0.3592*
RP	*11 (19)*	*27 (46)*	*21 (36)*		
Controls	*8 (11)*	*34 (45)*	*33 (44)*		
v29	*G/G*	*A/A*	*G/A*	*0.27*	*0.8751*
RP	*41 (69)*	*3 (5)*	*15 (25)*		
Controls	*49 (65)*	*5 (7)*	*21 (28)*		

*Sample size: n = 59 for RP and n = 75 for controls except for V22 where n = 72 for controls

Table 10 Haplotype frequencies of ***RDS*** variations ***V14, V17 and V18***.*

Groups	***Haplotype counts (frequency %) V14-V17-V18***			***Chi-squared value (p value)***
	G-G-T	**G-G-C**	**C-A-C**	
RP	***39 (33)***	***55 (47)***	***24 (20)***	***8.67, df=2 (0.0131)***
Controls	***37 (25)***	***96 (64)***	***17 (11)***	

* Note that all three variations were found in the same PCR fragment.

Chapter 4 Discussion

4 Discussion

4.1 Mutation Detection

Mutation scanning methods are designed to scan sequences of DNA for ***unknown*** mutations to avoid sequencing a whole stretch of DNA that may differ by only a single base from the wild type sequence. Single strand conformation polymorphism (SSCP) analysis is a scanning method and was used in this study to detect base changes in the DNA samples. Briefly, DNA is amplified by polymerase chain reaction (PCR) using specific primers for the target fragments. Denaturation separates the double-stranded PCR product into single-strand (ss) DNA fragments. These ssDNA fragments are loaded onto a non-denaturing polyacrylamide gel and separated by electrophoresis. During electrophoresis, the ssDNA will form folded conformations that are dependent on intramolecular interactions (hydrogen bonds) that in turn are dependent on its base sequence. Therefore, any change in DNA sequence would be detected as a mobility shift and give different banding patterns.

SSCP was introduced in 1989 (Orita et al, 1989) and soon became a commonly used technique for mutation detection. A PubMed search for the term ***SSCP*** yielded over 7000 entries since 1989. Recently, some new automatic mutation detection systems, e.g. denatured high performance liquid chromatography, have been introduced. The trend of the usage of SSCP has been declining. However, SSCP analysis is still a simple method that is easy to set up and that does not require any special equipment or reagents. It is also commonly used for the study of RP (over 90 entries in PubMed) and even in some recent studies of RP (Greenberg et al, 2003; Wang et al, 2003; Bernal et al, 2003).

In this study, the SSCP running conditions were based on previous studies, thereby minimizing the time spent in optimization. The sensitivity of SSCP is about 80-90% under optimal conditions (Sheffield et al, 1993). Several modifications have improved the overall sensitivity to close to 100% (Kukita et al, 1997; Orita et al, 1989). Virtually all possible mutations can be detected by running SSCP under a combination of three or more different conditions (Hayashi, 1991; Hayashi & Yandell, 1993; Cotton, 1997). Although several improvements were implemented in SSCP, there remain several factors that hamper mutation detection. While some of these factors were technical problems inherent to SSCP, others were not. These factors were recognized in this study and will be discussed in the following sections.

4.1.1 Sensitivity of mutation detection by SSCP

Four electrophoretic conditions were used for mutation screening in this study: 10%T/1%C at 20°C and 4°C, and 10%T/1%C with 5% glycerol in the gel at 20°C and 4°C. These combinations were based on the successful detection of all mutations reported previously (Yip, 1997). However, one variation, +10765T>C (V34) of ***PRPF31***, could not be detected under all four conditions in the present study. Three variations were carried by the same PCR fragment, +10765T>C (V34), +10842C>T (V35) and +10856A>G (V36) ***(Figure 34)***. For samples 2254 and 2798, the genotypes for +10842C>T and +10856A>G were the same. However, the genotypes for +10765T>C were different, T/C for sample 2254 and T/T for sample 2798. Yet, no obvious band shift was seen between them. For samples 2440 and 2304, the genotypes for +10842C>T and +10856A>G were also the same and the genotypes for +10765T>C were different. Again, there was no band shift detected. Analysis of band patterns showed that band shifts were produced separately by the +10856A>G and +10842C>T under different electrophoretic conditions. Therefore, no band pattern was unique to +10765T>C, which was only detected by cycle sequencing.

In this study, there were a total of 37 variations detected in the three genes studied. If these variations were assumed to encompass all sequence variants existing in the samples, only one was not detected by SSCP. Therefore, the detection rate of SSCP using these combinations was 97.3%, which was still reasonable and acceptable.

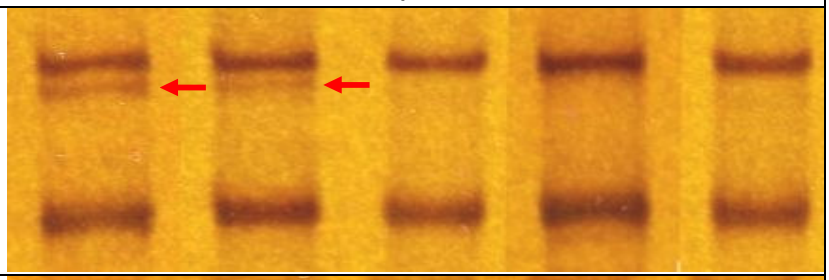
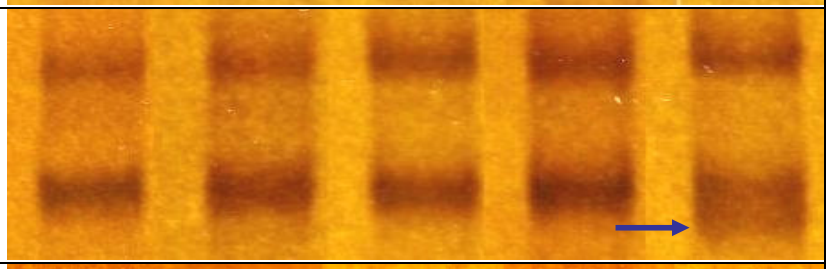
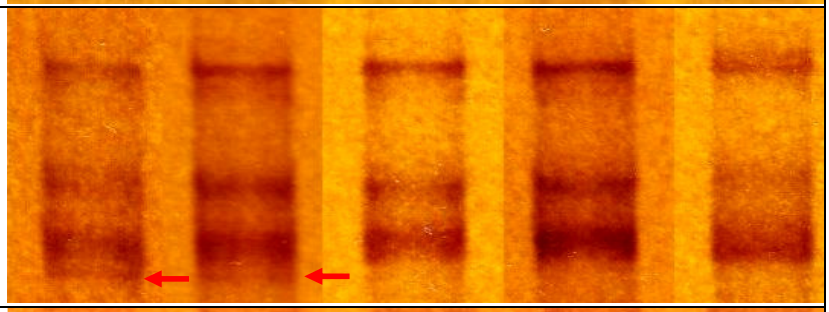
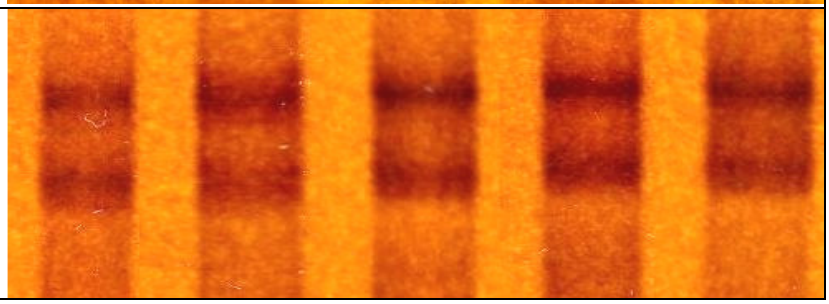
	Sample number				
	2254	**2798**	**2440**	**2304**	**2443**
Variations	Sequencing results				
+10765T>C (V34)	T/C	T/T	C/C	T/C	C/C
+10842C>T (V35)	C/C	C/C	C/C	C/C	**C/T**
+10856A>G (V36)	**A/G**	**A/G**	A/A	A/A	A/A
SSCP Conditions	SSCP patterns				
20°C					
20°C + 5% Glycerol					
4°C					
4°C + 5% Glycerol					

Figure 34 SSCP under four different electrophoretic conditions did not reveal the +10765T>C sequence variation (V34).

4.1.2 PCR dropout

Presently, most molecular technologies are PCR-based although PCR is not perfect. The V22 variation (IVS6-31T>C) in ***PRPF31*** was found in both RP and control groups. However, only the two types of homozygotes (T/T and C/C) were found using the first pair of primers while no heterozygote (T/C) was identified at all. Cycle sequencing confirmed the genotypes of these PCR products ***(Figure 16)***. Repeated testing with the first pair of primers gave identical results, which were obvious violation of the Hardy-Weinberg law. Note that V22 is a known sequence variation (rs2303557) documented in dbSNP of NCBI.

A second pair of primers was designed ***(Figure 35)*** that produced both homozygous and heterozygous genotypes ***(Figure 16)***. In particular, the frequency of the minor allele (T) was 0.36 for RP patients and 0.24 for controls ***(Figure 11)***, which was comparable to that (0.26) reported in dbSNP.

Comparison of results from these two pairs of primers indicated that the first pair of primers always failed to amplify the C allele in the presence of the T allele. This phenomenon is known as allelic dropout. Genotyping error caused by disruption of the annealing sites for primers has been reported previously (Keicho et al, 2001). Cycle sequencing of PCR products amplified by the second and the third pairs of primers ***(Figure 35)*** did not, however, reveal any sequence variation in the annealing sites for the first pair of primers.

Another cause of allelic dropout is low copy number DNA samples as seen in single cell analysis for pre-implantation genetic diagnosis (Gill, 2001; Thornhill et al, 2001). However, this was not likely the cause in this case. Although a minute amount of

DNA commonly causes allelic dropout, this problem also occurs in samples with sufficient amounts of DNA (Tsai et al, 2001; Schulze et al, 1998). Secondary structures in the amplified region may also prevent efficient amplification although this could be alleviated by altering the PCR conditions in some instances (Wedell et al, 1994). Site- and nucleotide-specific mis-incorporation by ***Taq*** DNA polymerase has also been reported (Ruiz-Opazo et al, 1994; Lee-Jackson et al, 1993). However, the reason for PCR dropout in this study remains to be determined.

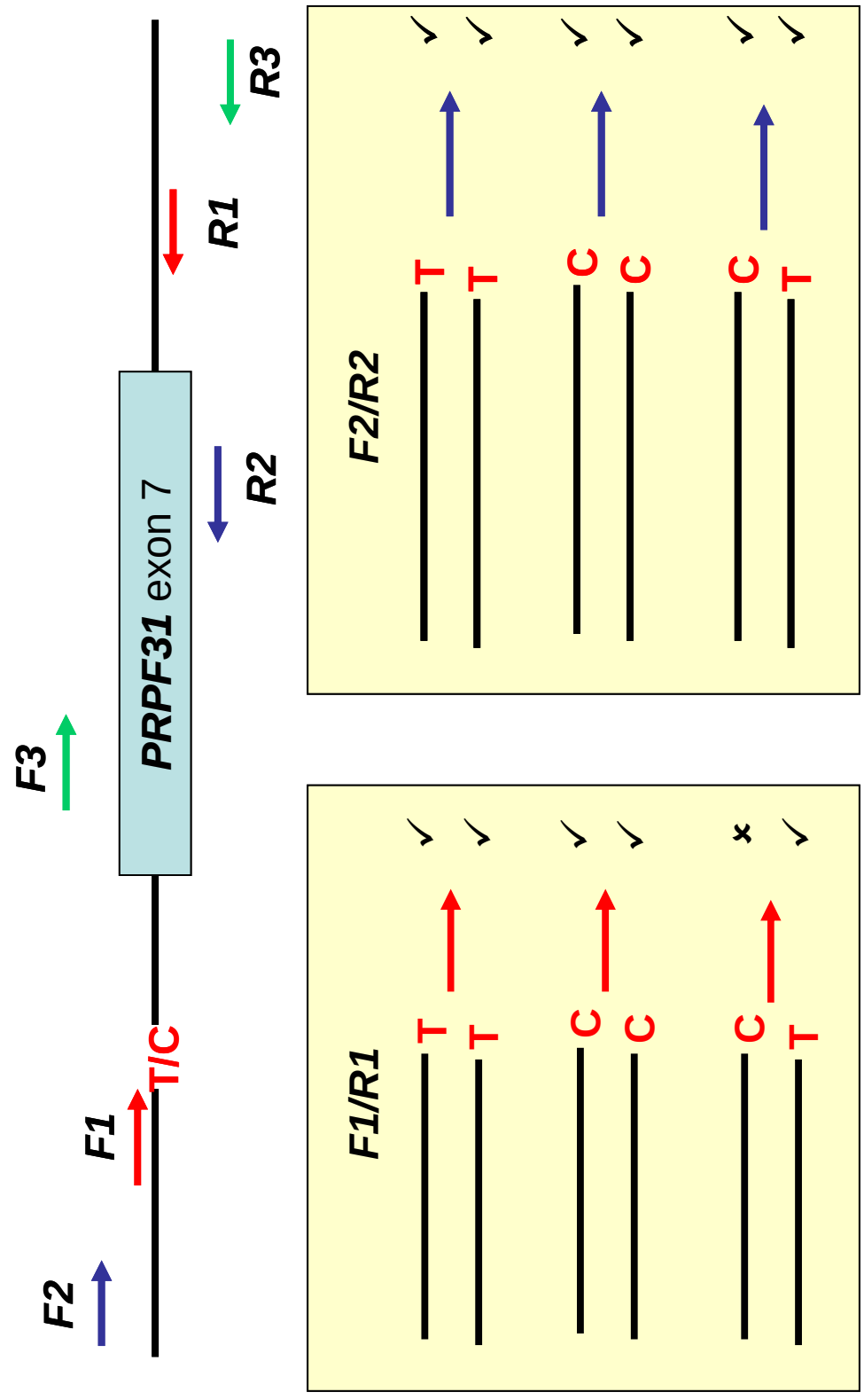


Figure 35 The primers used for the detection of IVS6-31T>C (V22) variation in ***PRPF31***. The heterozygous T/C was detected only using second pair primers (F2/R2) and no variation was found in the priming sites of the primers (F1/R1) by the second and third pairs of primers (F2/R2 and F3/R3).

4.2 Mutations of *RHO*

Our collection of RP patients had never been screened for mutations in any gene implicated in RP. Therefore, a systematic screening for mutations in these genes was necessary. Three genes were selected for this study: two genes that had been extensively studied before (***RHO*** and ***RDS***) and one gene that was recently cloned (***PRPF31***).

Mutational analysis of the rhodopsin gene in RP patients has been conducted worldwide (Rakoczy et al, 1995; Bareil et al, 1999; Dikshit & Agarwal, 2001; Trujillo et al, 2000). Over one hundred mutations have been found since the first rhodopsin mutation was discovered (Dryja et al, 1990a). However, the discovery of novel rhodopsin mutations in RP is ongoing. According to PubMed, new rhodopsin mutations have been published every year since 1990.

4.2.1 Regulatory regions

Although many mutational analyses of ***RHO*** have been carried out, no mutation has been found in the 5' regulatory region of ***RHO***. From the analysis of gene mutation databases, it was believed that over 150 promoter mutations were associated with diseases (Krawczak et al, 2000). For example, two transversion mutations in the factor VII gene promoter were found in Finnish female patients with moderate bleeding tendency (Kavlie et al, 2003). A thymidine insertion was detected in cells derived from patients deficient in the encoded enzyme at the 5' end of the fatty acid delta-6-desaturase gene (Nwankwo et al, 2003).

Regulation of gene transcription is mediated by the interaction of DNA motifs, transcription factors and/or RNA polymerase. Mutations in the promoter regions

could alter the binding of these factors and hence affect gene expression. DNA polymorphisms in the promoters were also believed to influence these processes (Knight, 2003). The finding of RP mutations in the retinal cell transcription factor NRL indicated that this regulatory mechanism could be involved in the disease processes leading to RP (Bessant et al, 1999). A region from just upstream of the transcription start site to -250 bp (equivalent to -345 bp when +1 starts at the translation site) of the rhodopsin gene was demonstrated to have promoter activity by functional assays (Mani et al, 2001; Zhang et al, 2003). In addition to exons and their flanking intron sequences, this putative regulatory region of the rhodopsin gene was also screened for mutations in the present study. Four sequence variations in the 5' regulatory region were identified in this study. Two were novel and will be discussed in the following sections.

-300_-302delTTT

The -300_-302delTTT (V1) variation was a novel sequence variation in the 5' regulatory region of ***RHO***. It was detected in the proband of a simplex Usher family (C8568) ***(Figure 17)*** and also in one control sample. No other samples from this family were available for investigation. Thus, co-segregation analysis could not be performed to verify the association of this variation with disease phenotype. The status of the "healthy" control was unknown because the collection of control samples was anonymous. Therefore, the possibility of the control being a late-onset or asymptomatic RP case could not be ruled out completely. However, no rhodopsin mutation has been found in the Usher form of RP until now. Since most of the genetic mutations (73-83%) of Usher syndrome type II were identified in ***USH2A*** (Weston et al, 2000), mutational screening of ***USH2A*** could be carried out for this family. If mutation is found in ***USH2A***, this ***RHO*** variation is most likely a polymorphism.

Functional analysis of the ***RHO*** promoter in the region from -233 to -203 (equal to -328 to -298 when the A of the initiation codon is +1) has been reported (Mani et al, 2001). A targeted disruption of the region -233 to -203 in the ***Xenopus*** rhodopsin promoter was generated. The resulting construct was transfected into ***Xenopus*** embryos and the reporter activity was measured. The disruption of this region resulted in a 3-fold increase in reporter gene expression. A similar study was conducted and the results also indicated that the reporter activity was increased with the truncated rhodopsin promoter (May et al, 2003). Therefore, a negative regulatory element may lie within this region.

The -300_-302delTTT deletion is located within this putative negative regulatory element ***(Figure 36)***. Further, the sequences were conserved between human, cow,

mouse and rat. Three possible outcomes could arise from the deletion of 3-T in this region: 1) increased rhodopsin expression by disruption of the negative regulatory element; 2) decreased rhodopsin expression by alteration of the binding affinity of a transcription factor in this domain; or 3) no remarkable change. Either over- or under-expression of rhodopsin could cause retinal degeneration in transgenic mice (Olsson et al, 1992). Therefore, photoreceptors could probably degenerate as a result of altered gene expression due to this deletion. However, the consequence of this deletion should be verified by functional assay.

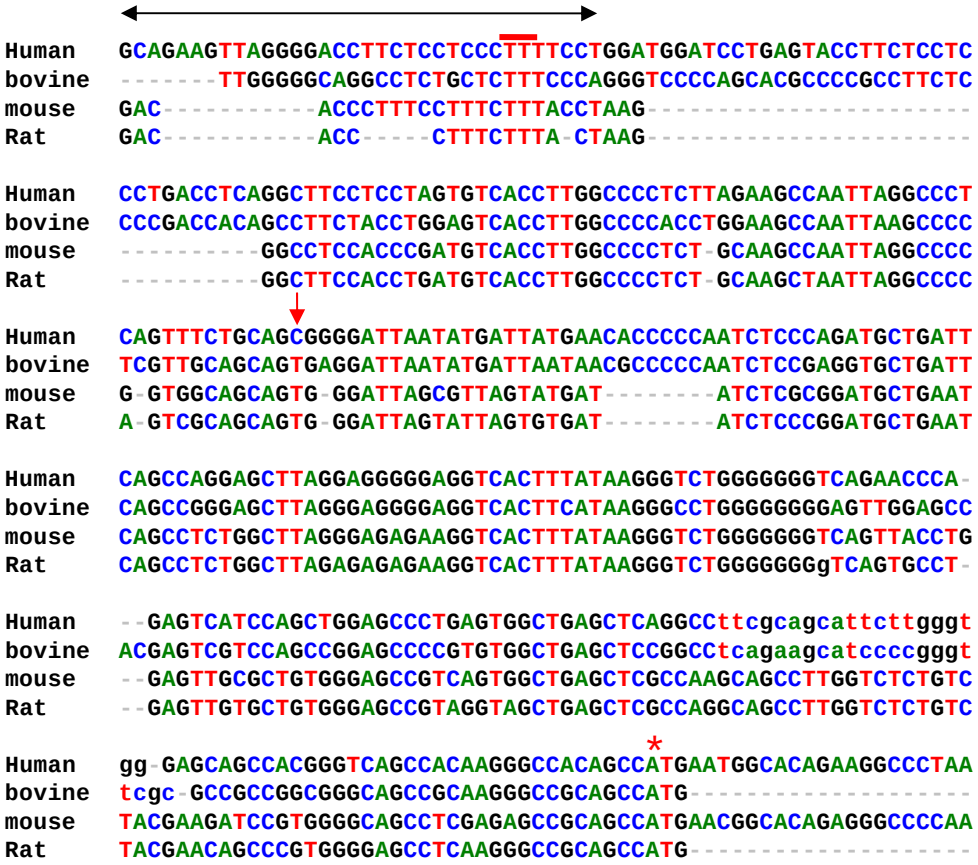


Figure 36 Sequence comparison of the human, bovine, mouse and rat rhodopsin promoter DNA sequences.

*(◄——► is the putative regulatory region; — is the deleted sequence -300_-302delTTT; ▼ -201C>T variation; * is the A of the ATG initiation codon)*

-201C>T

The -201C>T (V2) variation was also a new finding identified in a simplex RP family ***(Figure 18)***. This variation was not found in the control samples. However, only the affected sample was available so that segregation analysis of the variation with disease phenotype was not feasible. Previous functional analysis of the rhodopsin promoter revealed that some sequence elements present in the promoter played a role in the regulation of gene expression. BAT-1 is a DNA sequence in the rhodopsin gene, and the Crx nuclear protein binds to the BAT-1 sequence in order to activate gene expression (Chen et al, 1997). Using a DNase I footprint assay, the BAT-1 region (from -109 to -80) of the bovine rhodopsin gene was protected from digestion by binding with the Crx fusion protein. The -201C>T variation in human ***RHO*** was located within this promoter region ***(Figure 36)*** and may affect the binding of Crx to the BAT-1 site and hence expression.

The nucleotide at the position corresponding to -201 in human ***RHO*** was not conserved ***(Figure 36)***. In addition, Crx is a member of the Otx homeodomain protein family. For the Otx family, a specific amino acid-base pair contact was identified (Hanes & Brent, 1991). The sequence motifs TAATCC/T and TAATCA were found in photoreceptor-specific upstream regions of several species (Furukawa et al, 1997). Therefore, only sequence variations within these sequence motifs could alter the binding of Crx. However, the -201C>T variation did not fall within this motif and, therefore, was less likely to affect gene regulation.

4.2.2 P347L

Mutation findings in family C8289

The sequence variation +4977 C>T (V9) caused an amino acid substitution of leucine for proline at codon 347. This variation was a previously reported mutation and it was also identified in an adRP family (C8289) in this study ***(Figure 20)***. The C8289 family consisted of three generations. Individuals I-1 and I-2 were suspected to have eye disease but no blood samples were taken. In the second generation, these parents gave birth to nine offspring: eight females and one male. All females were diagnosed with RP and the male died at a very young age for unknown reason. Only blood samples from individuals II-3, II-8, II-10 and II-11 were available. III-3 was also diagnosed to have RP and her sample was tested in this study. The P347L mutation of rhodopsin was detected only in the II-10 and II-11 individuals who were both homozygous for the P347L mutation. However, a ***RHO*** mutation was not found in individuals II-3, II-8 and III-3. This issue will be discussed later (Section 4.5).

Pathological consequences of the P347L mutation.

Rhodopsin is an integral membrane protein composed of three distinct domains: extracellular domain, transmembrane domain and intracellular domain. Photon capture is the primary role of rhodopsin in the phototransduction pathway. This is initiated by isomerization of the 11-***cis***-retinal chromophore moiety. The 11-***cis***-retinal molecule covalently binds to the lysine residue at position 296 and is stabilized by glutamic acid 113 within the transmembrane domain of rhodopsin. Obviously, mutations in these or other associated amino acids would disrupt the conformational stability of rhodopsin.

Class II mutations

Several functional studies on rhodopsin mutations have been conducted. L125R was a severe mutation detected in adRP patients (Dryja et al, 1991). Studies revealed that the mutant protein was misfolded due to the formation of an abnormal disulfide bond (Hwa et al, 2001). As a result, the mutant rhodopsin was defective in 11-cis-retinal binding and accumulated in the endoplasmic reticulum of photoreceptors (Sung et al, 1993). The expression level of the mutant protein was also lower than that of the wild type (Garriga et al, 1996). Sung et al (1991) classified this type of mutation in rhodopsin as class II, and most mutations of this type are found in the intradiscal and the transmembrane domains.

The role of proline 347 at the C-terminus

Proline 347 is the penultimate amino acid located at the carboxy-terminal tail of the intracellular domain. The carboxy-terminus stretches from glutamic acid 332 to alanine 348. Both cysteine 322 and cysteine 323 contain palmitoyl groups. Amino acid sequence comparison of vertebrate visual pigments shows high sequence conservation in this tail (Macke et al, 1995). The high degree of conservation of amino acid sequences in the carboxy-terminus is also found within the G-protein-coupled receptor family (Mirzadegan et al, 2003), which indicates that the carboxyl-terminus region is important for the function of rhodopsin. Approximately one-fifth of the adRP RHO mutations were located within this tail. For proline 347, seven different mutations have been found: Pro347Ala, Pro347Arg, Pro347Gln, Pro347Leu, Pro347Ser, Pro347Thr and Pro347Cys. This shows that Pro347 is a key amino acid in rhodopsin that has a strong tendency to be mutated.

In vitro studies of the Pro347 mutant revealed normal function. There were no abnormalities in protein folding, binding to 11-cis-retinal and expression level, and no accumulation of protein in endoplasmic reticulum (Sung et al, 1991; Sung et al, 1993; Weiss et al, 1995). Thus, the P347L mutation induced functional defects other than these. In transgenic mice carrying Q344ter of rhodopsin, immunofluorescence staining of the retina showed that the Q344ter mutant protein was found at high concentration both in the plasma membrane of the cell body and in the outer segment (Sung et al, 1994) while wild type rhodopsin was localized only in the outer segment. This unusual distribution of mutant rhodopsin was also found in transgenic mice expressing P347S and transgenic pigs expressing P347L (Li et al, 1996; Li et al, 1998). The abnormal distribution was believed to be the cause, rather than the result, of photoreceptor death because it occurred prior to the degeneration of the outer segment. Sung et al (1991) classified this type of rhodopsin mutation as class I, most of which are found in the carboxy-terminal tail of the intracellular domain.

Mutations at the C-terminus affect protein trafficking

Accumulation of mutant protein indicated that there was a defect in protein trafficking. The photoreceptor shows three distinct structures: outer segment, inner segment and synaptic terminal. Rhodopsin and other essential substrates are synthesized in the inner segment and then transported vectorially to the outer segment through a connecting cilium. Transportation is mediated by specialized post-Golgi vesicles that bud from the trans-Golgi network (Deretic & Papermaster, 1991). A study on post-Golgi trafficking indicated that the carboxyl-terminus played a critical role in regulating rhodopsin sorting into these vesicles (Deretic et al, 1998). A synthetic peptide corresponding to the carboxyl-terminus (short peptide composed of amino acids 330 to 348) was added to a cell-free post-Golgi membrane formation assay. The addition of this peptide inhibited post-Golgi trafficking and this was likely due to

competition for sorting factors between the rhodopsin carboxyl-terminus and the synthetic peptide. Elimination of the inhibitory effect on rhodopsin trafficking was found with a synthetic peptide containing a deletion of the last five amino acids. A similar effect was demonstrated for peptides that were substituted with amino acids corresponding to mutations P347S and V345M found in adRP patients. This experiment indicated that the last five amino acids of rhodopsin are important for rhodopsin trafficking. In particular, valine 345 and proline 347 are absolutely required for signal recognition.

In photoreceptors, axonemal and cytoplasmic microtubular systems were found (Chaitin et al, 1989; Troutt & Burnside, 1988). Cytoplasmic microtubules are oriented with two ends: plus and minus. Plus ends are directed towards the synapse and minus ends directed towards the basal body at the base of the outer segment in a longitudinally arrayed manner. Dynein is a large macromolecular complex consisting of heavy chains, intermediate chains and light chains. It is a molecular motor moving along the microtubule by using the energy liberated from ATP hydrolysis (Mallik et al, 2004). Using ***in vitro*** fusion protein binding assay, a cytoplasmic dynein light chain, Tctex-1, was found to interact with the carboxyl-terminus of rhodopsin (Tai et al, 1999). These results further showed that the binding affinities of the carboxyl-terminus of rhodopsin mutants (Q344ter, V345M, P347S and P347L) to Tctex-1 were significantly reduced.

Apoptosis caused by misrouted mutant

The evidence discussed above suggested that the P347L mutation in rhodopsin affected a signal recognition site present on the carboxyl-terminus. As a result, cytoplasmic dyneins could not bind to the rhodopsin-bearing vesicles and carry them towards the outer segment efficiently. Therefore, mutant proteins were improperly transported and accumulated in the inner segment of photoreceptors. How did the

improper accumulation of mutant protein induce apoptosis? Two different mechanisms have been suggested. First, accumulation of mutant vesicles was always accompanied by defective disc morphogenesis. Shortened outer segments were found in a study of transgenic mice carrying P347S or P334ter mutations (Li et al, 1996). Insufficient rhodopsin transportation to the outer segment for disc renewal may induce it to shorten and hence trigger apoptosis in the photoreceptors (see section 1.7 for details).

The second mechanism suggested that apoptosis was due to the accumulated mutant vesicles themselves (Sung et al, 1994). The presence of high levels of mutant in improper locations may interfere with normal cellular processes. In addition, apoptosis may be associated with the excessive metabolic burden of destroying the mutant protein accumulated in the photoreceptors. This mechanism was supported by the observation that the rate of cell death was correlated with the amount of accumulated mutant protein (Green et al, 2000). However, direct evidence has not been found and the precise mechanism of triggering apoptosis by the mutant protein remains a mystery.

Table 11 Comparison of ophthalmologic findings between several RP families shows that clinical expression of RP is variable.

Families	Age	Onset Age	VA (logMAR)	VF (degree)	Bone-spicule Pigmentation	Macular lesion
C8568	31	3	1	18	Y	N
C7343	42	15	0.6	6	Y	N
C8289	38	15	1	12	Y	N
C7765	62	25	0.3	6	Y	N
C8184	49	8	0.6	3	Y	N
C8062	65	20	LP	0	Y	Y
C8734	36	10	0.08	24	Y	N

VA = Visual acuity; VF = Visual Field; LP = Light perception; Y = Yes; N = No

Clinical expression of the P347L mutation

Comparison of clinical data from different RP families of our collection demonstrates variations in disease expression ***(Table 11)***, as has been reported in other studies. For instance, a study of 148 patients with adRP revealed that patients with the P347L mutation were more severely affected than those without a rhodopsin mutation (Berson et al, 1991).

Among patients with ***RHO*** mutations, differences in severity were also observed. One systematic study showed that the severity of the disease depended on the location of the mutation within rhodopsin (Sandberg et al, 1995). Patients with mutations in the intradiscal domain tended to have better visual function than patients with mutations in the cytoplasmic domain. There could be two possible outcomes from these observations. First, the disease prognosis is different for the different mutations. The second possibility is that the mutations induce a similar rate of retinal degeneration, with severity correlated with the time of onset. The latter possibility came from the observation that patients with mutations in the cytoplasmic domain appeared to have an earlier time of onset than patients with mutations in the intradiscal domain.

To eliminate the age factor, a study based on longitudinal follow-up examinations was performed to estimate the disease progression rate (Berson et al, 2002). Again, the patients were grouped by the location of mutations within rhodopsin intradiscal, transmembrane or cytoplasmic domains. The disease progression rate with respect to visual field loss and ERG amplitude was faster in patients with mutations in the cytoplasmic domain than in patients with mutations in other domains.

The P347L mutation is located in the cytoplasmic domain. Patients with the P23H mutation of rhodopsin had better visual function than those with the P347L mutation of rhodopsin (Dryja et al, 1990b). This observation was supported by another study comparing the clinical expression between the proline 23 and 347 mutations (Oh et al, 2003). Thus, it was believed that patients II-10 and II-11 ***(Figure 20)*** with the P347L mutation had a poorer prognosis. However, the clinical data for the present study were recorded only at the first visit and no follow-up was performed.

4.3 Mutations of *RDS*

4.3.1 Complex mutation (+232G>C; +232_+233insT)

The complex sequence variation V10 (+232G>C; +232_+233insT) was found in one of the RP patients but not in controls ***(Figure 22)***. Sequence analysis of PCR cloning products revealed that the base substitution (+232G>C) and the single base insertion (+232_+233insT) were on the same chromosome ***(Figure 15)***. If +232G>C was the only missense substitution, the amino acid would change from alanine (GCT) to proline (CCT) at codon 78. However, the T nucleotide insertion following the missense substitution would change the amino acid to leucine (CTC). As a consequence, these base changes caused the frameshift A78***fsX***176 starting from codon 78 and created premature termination after 98 codons. The effect of the missense substitution Ala78Leu could not be predicted simply from this study, however, a truncated protein would be expected from the frameshift mutation. The truncated protein is predicted to be 175 amino acids long and is only about half of the full-length functional protein (346 amino acids). The resulting protein will lose the intradiscal D2 loop, the transmembrane domains M4, and the cytoplasmic C3 carboxyl terminus. Approximately 72% of the ***RDS*** mutations clustered in these regions of ***RDS*** (Kohl et al, 1998).

Effects of mutations on the function of RDS

RDS has important roles in disc morphogenesis and stabilization. Velocity sedimentation experiments showed that RDS exists as a mixture of homotetramers, heterotetramers and oligomers (Loewen & Molday, 2000). The basic unit of the complexes is the core tetramer made up of dimers of RDS and/or ROM1 protein molecules. Dimerization of the molecules is mediated by the D2 loop domain of RDS (Loewen et al, 2001). Functional studies on the mutant C214S RDS protein revealed that the formation of tetramers of RDS/RDS and RDS/ROM1 was abolished. The interaction between mutant and wild type molecules was also affected. The mutant protein formed aggregates, which was likely due to protein misfolding and aberrant intermolecular disulfide bonding caused by the mutation.

Protein trafficking of the RDS expressed in transgenic ***X***. ***laevis*** showed that wild type protein was transported efficiently from the endoplasmic reticulum of the rod inner segment to the outer segment, but the P216L mutant was retained in the inner segment (Loewen et al, 2003). This study indicated that correct subunit assembly was required for RDS targeting and incorporation into the disk membrane. A checkpoint between the inner and outer segments was suggested to allow only correctly assembled RDS/ROM-1 to be incorporated into the disk membrane.

These findings suggested that the disease phenotype would likely be caused by the defects in these critical regions of RDS. The truncated protein resulting from the complex mutation found in this study is expected to lose these regions and thus produce the disease phenotype. Although the relationship between the misrouting of mutant protein and retinal degeneration is unknown, several different frameshift mutations have been found in ***RDS*** of RP patients, that are associated with disease phenotypes (Wroblewski et al, 1994; Lam et al, 1995; Jacobson et al, 1996a). Thus,

this variation (+232G>C; +232_+233insT) could be a pathological mutation causing a similar disease phenotype in the RP subjects. The son and the daughter of the proband did not exhibit RP. However, these individuals have not been examined and their blood specimens were not available for the present study. This mutation is the first novel mutation of the ***RDS*** gene in Chinese RP patients.

4.3.2 +464C>G and +508G>A missense substitutions

The missense substitution +464C>G (V12: T155S) was found in three members of one arRP family ***(C8445; F.23)***: the proband (II-7), his brother (II-6) and his mother (I-2). However, both II-6 and I-2 were unaffected. This substitution was not found in the controls. In addition, the amino acid was highly conserved ***(Figure 37)*** and was located in the D2 loop domain. However, the substitution did not segregate with the disease phenotype. There was only one missense variation suspected to be a polymorphism within this region of ***RDS*** (Dryja et al, 1997). Of all other sequence variations within this region, about 50 mutations (72% of all ***RDS*** mutations and 96% of variations found in the D2 loop region) were disease-associated mutations. If it was merely a polymorphism, it could be a novel and rare polymorphism within this region.

The sequence variation +508G>A (V13: G170S) was also conserved ***(Figure 37)***, but was found only in one control sample. The particular variation would cause the change of amino acid from glycine to serine at codon 170. This mutation was also located in the D2 loop region. The same mutation at this position was reported in cone-rod-dystrophy but not in RP (Kohl et al, 1998). The consequence of this variation could not be deduced in the present study because its corresponding control was anonymous.

Figure 37 Alignment of ***RDS*** sequences from several organisms.

4.3.3 Q304E, R310K and G338D variations and their association with RP

Five sequence variations were found in the C-terminus of ***RDS (Figure 10)***. The genotype distributions of the missense variations, +23909C>G (V14: Q304E) and +24012G>A (V17: G338D), in RP showed statistically significant differences from those of the control group (p=0.0252, Fisher's exact test) ***(Table 9)***. The mutations were on same PCR fragment detected under the same SSCP conditions. Haplotypes of these variations were revealed by the SSCP patterns of the fragment ***(Figure 13)***. The SSCP patterns and sequencing results showed that these variations, Q304E (G>C) and G338D (G>A) were in complete linkage disequilibrium. Haplotype counts were calculated for V14, V17 and V18 and were statistically different (p=0.0131, chi-squared test) **(*Table 10)***.

Table 12 Allele frequencies of three ***RDS*** C-terminus polymorphisms in different studies.

Populations /Studies	Patient's group*	References	Allele frequencies of the polymorphisms#					
			Allele C (V14, E304Q)		Allele G (V15, K310R)		Allele A (V17, G338D)	
			Controls	Patients	Controls	Patients	Controls	Patients
Ireland	RP	Jordan et al, 1992	0.51	0.58	0.53	0.45	0.59	0.70
Germany	AVMD	Felbor et al, 1997	0.09	0.30	0.06	0.07	0.09	0.30
Lithuania	RP	Kucinskas et al, 1999	-	0.36	-	0.15	-	0.36
US	-	Dryja et al, 1997	0.22	-	0.11	-	0.23	-
Japan	RP	Budu et al, 2001	0.19	0.19	-	0.00	0.17	0.18
Hong Kong (Chinese)	RP	This study	0.11	0.22	0.00	0.00	0.11	0.22

***AVMD = adult vitelliform mucular dystrophy**
RP= retinitis pigmentosa
 # "-" indicates that data were not available

Comparison of these variations to other studies

The allele frequencies of these sequence variations varied in different populations ***(Table 12)***. In Ireland, E304Q was 0.49/0.51 (G/C) in normal controls (n=160) and 0.42/0.58 in adRP patients (n=60) (Jordan et al, 1992). In Lithuania, it was 0.64/0.36 in adRP patients (n= 33) although data for normal controls were not available (Kucinskas et al, 1999). In the USA, it was 0.78/0.22 in normal controls (n= 60) however, no patient data were reported (Dryja et al, 1997). In Germany, it was 0.91/0.09 in normal controls (n= 39) and 0.70/0.30 in patients with adult vitelliform macular dystrophy (n=28) (Felbor et al, 1997). In Japan, it was 0.81/0.19 in normal controls (n=39) and 0.81/0.19 in patients with adRP, arRP and other retinal dystrophies (n=80) (Fujiki et al, 1998).

The V15: +23928G>A (R310K) sequence variation was a previously reported polymorphism (Jordan et al, 1992). The allele frequencies of A and G were 0.55 and 0.45, respectively, in Irish RP patients. In other populations, the allele frequency of G was much lower. The nucleotide in the reference sequence ***(Table 5)*** was G. However, nucleotide A was detected in all RP and control samples in this study.

The findings of Jordan et al (1992) were quite different from other observations. First, the variant alleles were more prevalent in the Irish population. For G338D alone, the distribution of allele frequencies was different between controls and RP in Ireland. However, the frequencies of E304Q and G338D were similar in other populations and linkage disequilibrium was evident between these two variations. There was no indication of an association between these alleles and RP in these studies. Only the study of adult vitelliform macular dystrophy in Germany showed

that the comparison of the allele frequencies between control and RP was statistically significant (χ^2 = 8.8 and ***p*** = 0.003). Linkage disequilibrium of these alleles (E304Q and G338D) was also demonstrated. The allele frequencies of E304Q and G338D in Chinese were similar to those in the German population and the frequencies of the C and A alleles of these two sequence variations were lower in controls. However, the allele frequency of K310R in Chinese was similar to that in Japanese. Since both E304Q and G338D were found at appreciable frequencies in normal individuals, they were both reported as neutral polymorphisms (Jordan et al, 1992). E304 was conserved among human, mouse, rat and cow. For G338D, however, glycine only existed in humans and aspartate existed in other species.

Possible effects of genetic variants

A positive association between RP and a given haplotype can be explained by three reasons. First, the haplotype itself is functional and directly affects the expression of RP. Second, an unknown allele at another locus affects the expression of RP, which is in linkage disequilibrium with the haplotype. Finally, this result could be due to artifact or error.

The functional roles of the RDS C-terminus have been studied. Although the D2 loop of RDS is critical for subunit assembly, expression of a chimeric protein consisting of ROM and a segment of the D2 loop in ***rds***−/− mice could not remedy the disease phenotype (Kedzierski et al, 1999). It was suggested that a second domain of RDS might be required in ***trans*** for its normal function. The discs in ROS undergo disc morphogenesis through phagocytosis. These processes require membrane fusion to maintain the normal structure and physiological function of the photoreceptor (Boesze-Battaglia, 2000). Complete inhibition of fusion activity was caused by an insertion in the C-terminus but not in the D2 loop (Boesze-Battaglia & Stefano, 2002). Furthermore, this was supported by the study of the C-terminal peripherin/rds polypeptide fusion protein (Boesze-Battaglia et al, 2003). The results indicate that the C-terminus is essential for mediating membrane fusion and producing the conformational change in structure. It was suggested that fusion competency requires a tetrameric arrangement of amphiphilic α-helical peptides.

Although the functional roles of the C-terminus have been illustrated in these studies, no mutagenesis study of missense mutations has yet been conducted for the C-terminus. In addition, only two mutations have been reported in this region and both were nonsense mutations. One was a 2-bp deletion in codons 299-300

associated with butterfly-shaped pigment dystrophy (Nichols et al, 1993). Another was a 1-bp deletion in codon 307 associated with RP (Gruning et al, 1994). Therefore, we could not predict the functional effects of these variations on ***RDS*** from these results. Three sequence variations, E304Q, G338D and +24053C>T (i.e., V14, V17 and V18), create the three different haplotypes G-G-T, G-G-C and C-A-C ***(Figure 13)*** found in this study in the Chinese population. As a result, three functionally different proteins might be produced by these haplotypes. The effects of these variations might not be dramatic enough to cause disease but their functional activities may be different. Thus far, no study has been conducted in this aspect.

4.4 Mutations of *PRPF31*

4.4.1 The disorders of pre-mRNA splicing

Splicing of pre-mRNA is an essential process in our body, that makes the pre-mRNA molecule become a correct sequence for protein translation. The precision of splicing also affects which protein is to be translated by alternative splicing. Disruption of this process may lead to qualitative or quantitative changes in the protein. The components of the splicing machinery consist of the classical splice site sequences (3' splice site, 5' splice site and branch site) and the spliceosome complex. In addition, some auxiliary control elements such as SR binding factors, exonic splicing enhancers/silencers (ESEs/ESSs) and intronic splicing enhancers/silencers (ISEs/ISSs) have been identified (Black, 2003). Splicing defects can be classified into two main categories, ***cis***- or ***trans***-disruption, according to which target is disrupted.

Cis-disruption is the error on the sequence itself so that it is a gene-specific effect. The most common mutation is in the classical splice sites. The transitional substitution of 3' acceptor splice site of ***RHO*** was found in an adRP family (Whitehead et al,1998). The mutation resulted in the deletion of exon 5 and affected the transcript level. The disruption of these splice sites most likely introduces premature stop codon and the mutant is typically degraded by nonsense-mediated decay.

Cis-disruption mutations sometimes result in the use of alternative splice sites. For example, the frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) is caused by the mutation of ***MAPT*** gene that encodes tau protein. Tau is required for the microtubule-dependent transport of axons. It is expressed in six

isoforms from a single gene by alternative splicing (Goedert & Jakes, 1990). The alternative splicing in exon 10 results in two different forms of protein: three microtubule binding repeats (3R tau) or four microtubule binding repeats (4R tau). The ratio of expression of these two forms (4R tau/3R tau) is balanced at about one in human adult brain. Some mutations in FTDP-17 altered this ratio to about two to three that increased the level of protein with inclusion of exon 10 (Hutton et al, 1998). The alteration of this ratio was correlated with the insoluble tau aggregates in patients with FTDP-17.

The ***trans***-disruption affects the components of spliceosome or the binding factors that regulate alternative splicing. Mutations in ***DMPK*** gene, CTG expansion in the 3' UTR of the gene, cause the myotonic dystrophy (Brook et al, 1992). These CUG expansions in the RNA alter the regulation or localization of the RNA-binding proteins which were essential to the correct alternative splicing, e.g. CUG-BP (Timchenko et al, 2001). At least five genes were found to be in aberrant splicing in myotonic dystrophy (DM) (Ranum & Day, 2004). Huntington's disease is similar to DM in the expansion of trinucleotide repeats. However, the CAG repeats are translated and encode glutamine to form poly-glutamine tracts of the huntingtin gene (***HD***) (Ambrose et al, 1994). Several huntingin-interacting proteins were identified and some are RNA splicing factors, e.g. HYPA/FBP-11, HYPB and HYPC (Faber et al, 1998). A study indicated that these factors are associated with nuclear inclusions of HD (Passani et al, 2000). However, no further study has revealed that splicing mechanism is affected and aberrant form of protein produced.

The PRPF31 is a component of the spliceosome complex. Mutations of ***PRPF31*** are believed to disrupt the formation of spliceosome complex and hence belong to the ***trans***-disrupting splicing disorder. The mutations found in this study and their details will be discussed in the following sections.

4.4.2 S2C mutation, incomplete penetrance and allele-specific expression

The +5C>G (V20: S2C) missense variation was found in two autosomal dominant RP families ***(Figures 27 and 28)***, but not in controls. In family C8062, only two affected individuals were available for this study and they were both heterozygous for this variation. The same variation was also detected in two individuals of the C8734 family. One individual (III-5) was RP but the other (III-4) was not. Detailed segregation analysis could not be performed in these families because other samples were unavailable.

Functional significance of S2C

Serine is the second amino acid of the PRPF31 protein. The nucleotide change from C to G at the +5 position (+1 being the A of the start codon) led to the change of amino acid from serine to cysteine. Comparison of the amino acid sequences of the PRPF31 proteins from different species showed that this serine (S) residue was highly conserved ***(Figure 38)***, indicating the functional importance of this serine residue in PRPF31.

On the basis of yeast mutant complementation analysis and in ***vivo*** functional analysis of the PRPF31 missense mutant, it was suggested that the A194E and A216P mutant proteins functioned normally in their splicing activities (Deery et al, 2002). However, the mutations rendered the proteins insoluble and also resulted in misrouting of the proteins so that the translocation of PRPF31 protein from cytoplasm to nucleus was impeded. Functional assays for the S2C mutation should be carried out to investigate if its effect is similar to that of A194E and A216P or not, or if there is any alteration in splicing activity.

Figure 38 Sequence comparison of PRPF31 from several species. The second amino acid serine is conserved among them.

The second codon is highly conserved

The conservation of the amino acid at codon 2 also was demonstrated in other genes when several eukaryotic genomes were compared (Niimura et al, 2003). The second codon was the most biased among all positions for almost all eukaryotes examined. The second C nucleotide of the second codon was a highly preferential nucleotide in all eukaryotes examined. This indicated that the alteration of amino acid and/or the C nucleotide might affect the regulation of translation to a certain extent. The sequence variation at position +5 was very close to the start codon ATG. Traditionally, the "GCCACC**aug**G" sequence is known as the Kozak sequence motif that is a recognition motif used by the ribosome to initiate translation (Kozak, 1984). The sequence context of the ***PRPF31*** gene corresponding to this region is “CTCGGG**aug**TCT”. The underlined C was the nucleotide changed to G in this sequence variation. The experiment conducted by Kozak (1997) showed that variation at the +5 position did not obviously affect the recognition initiator codon. However, other studies demonstrated that translation initiation was influenced by the context of the second codon (Stenstrom et al, 2001a; Stenstrom et al, 2001b; Stenstrom & Isaksson, 2002). There was up to a 20-fold difference produced by the variations in the context of the second codon. The underlying mechanism of how the second codon affects gene expression is unclear. The effect may result from the involvement of the respective decoding rRNA, or the interaction with the 16S rRNA or other initiation factors. It is possible that the S2C variation could affect translation efficiency and hence the level of translation.

Post-translational modification is mediated by the second codon

The second codon not only affects the initiation of translation but also the post-translational modification processes. Removal of the N-terminal methionine and N-acetylation are two important modification steps necessary for a functional

protein. During protein synthesis, methionine is added at the N-terminus of a polypeptide chain. Removal of N-terminal methionine is catalyzed by methionine aminopeptidases (MetAP). Two classes of methionine aminopeptidases (MetAP1 and MetAP2) have been characterized (Chen et al, 2002). Their activities are specific to highly conserved substrates dictated by the amino acids adjacent to the N-terminal methionine of the polypeptide chain (Huang et al, 1987; Chen et al, 2002).

N-acetylation is another important co-translational modification. N-acetyltransferases are the enzymes responsible for the addition of an N-acetyl group to the N-terminus of polypeptides. These enzymes are also substrate-specific in that amino acid residues adjacent to methionine also affect their activity (Boissel et al, 1988). Although there has been no report of PRPF31 having these modifications, the S2C missense variation could affect the enzyme activities required for these processes. It is tempting to speculate that the effects of the PRPF31 S2C variation could affect protein trafficking, translational efficiency and posttranslational modification of PRPF31. Again, functional assays need to be performed to elucidate the effects of the S2C variation.

Allele-specific expression

The different expression levels of ***PRPF31*** were shown to be allele-specific in a previous study (Vithana et al, 2003). This phenomenon deviates from the classic Mendelian inheritance which assumes that both maternal and paternal genes express equal amounts of mRNA. Recently, a genome-wide analysis of gene expression was studied by SNP chip (Lo et al, 2003). The expression level of each allele of 1063 genes was determined in fetal RNA samples that were heterozygous.

It was found that differences in allelic expression are common, affecting about 20% to 50% of human genes. Some showed greater than a fourfold difference in

expression between the two alleles. These findings were supported by another study (Bray et al, 2003). Therefore, a sequence that directs expression and affects transcription could contribute to differential gene expression. These ***cis***-acting variations were believed to be located at the promoter regions of the genes (Hoogendoorn et al, 2003). This phenomenon was in agreement with the initial analysis of the human genome project in that the total number of genes found was lower than expected. There must be something to make up the differences within and among species. Variation of gene expression might be a reasonable explanation.

Allelic expression and disease mechanisms of RP

These findings prompted us not only to rethink the underlying mechanisms of complex diseases resulting from subtle variations in gene expression, but also to explore the mystery of heterogeneity and disease mechanisms of RP. In familial adenomatous polyposis (FAP), allelic expression of the tumor suppressor gene ***APC*** was shown to be different at an allele ratio of about 66% (Yan et al, 2002). No variation was found in the coding regions of ***APC*** in these patients except for the existence of polymorphisms. Linkage analysis was performed with these SNPs and the allele with lower mRNA expression was linked to the disease.

Differential expression was found in all affected individuals but not in normal individuals. Interestingly, loss of heterozygosity of ***APC*** was found in tumor cells, and the lost allele was the one with higher expression. Thus, FAP developed in these patients due to two events: loss of a copy of ***APC*** and inheritance of a lower expressing wild type allele. As a result, the physiological level of ***APC*** could not be reached and tumor suppressor activity was reduced. This situation was similar to that observed in RP with the ***PRPF31*** mutation predisposing to disease because of

the inheritance of a low expression wild type allele. Incomplete penetrance due to the mutation of ***PRPF31*** alone could not cause disease phenotype.

If this mechanism was truly associated with RP, the strategy of disease study in RP would be revolutionary, where the measurement of the gene expression should be included in RP study. Allelic expression could not only cause disease directly, but also modify the degree of severity so that clinical heterogeneity would exist within and between families harboring the same mutation. Moreover, other parts of the target genes should also be investigated in the patients without mutations in coding regions because disease may arise from the variations in these regions.

Therefore, the asymptomatic S2C carrier (III-4) of family C8734 ***(Figure 28)*** could inherit the mutation, but probably showed incomplete penetrance of RP. Analysis of other polymorphisms within the same gene revealed a hint of this possibility. The IVS6-31T>C (V22), IVS9-72 G>T (V27), IVS9-70 T>C (V28) and IVS9-67 G>A (V29) variations ***(Figure 11)*** found in this study were polymorphic markers close to the S2C variation. The IVS9-72 G>T, IVS9-70 T>C and IVS9-67 G>A variations were detected in the same PCR fragment under the same SSCP condition. The genotypes and haplotypes of these variations were different in individuals III-4 (unaffected) and III-5 (affected) ***(Table 13)***. Individual III-4 was heterozygous for IVS6-31T>C, but III-5 was homozygous. In addition, III-4 was heterozygous (T-C-A / G-T-G) for haplotypes containing IVS9-72 G>T, IVS9-70 T>C and IVS9-67 G>A variations, but III-5 was homozygous for the haplotype G-T-G. However, these sequence variations were not detected with S2C on the same PCR product. Which of the alleles and haplotypes were on the wild type chromosome of III-4 could not be determined unambiguously at the present moment. This analysis raised a possibility for further evaluation. A nearby polymorphism that is on the same PCR

fragment of S2C variation should be used for confirmation of this possibility and allele-specific RT-PCR could be done to confirm this hypothesis.

Allele-specific expression of ***PRPF31*** could be a possible reason for incomplete penetrance in RP. However, the different levels of wild type expression of ***PRPF31*** could occur in conjunction with other factors in the asymptomatic carriers. The wild type allele in asymptomatic carriers could be in linkage disequilibrium with other genes. Mutations or allele-specific expression of other closely linked genes could also explain the results. These imply that the effects of the ***PRPF31*** mutation might be synergistic or modified by other genes.

Table 13 Genotypes of flanking sequence variations in asymptomatic and symptomatic carrier of S2C of the ***PRPF31*** gene in family C8734.*

Sample	RP	Variations		Haplotypes			
		S2C (V20)	IVS6-31T>C (V22)		IVS9-72 G>T (V27)	IVS9-70 T>C (V28)	IVS9-67 G>A (V29)
III-4	No	C/G	T/C	1	T	C	A
				2	G	T	G
III-5	Yes	C/G	T/T	2	G	T	G
				2	G	T	G

**** See Figure 28 for the pedigree diagram.***

4.4.3 F262*fs*X320

Only two affected individuals in family C8289 had the P347L rhodopsin mutations ***(Figure 20)***. Three other affected individuals (II-3, II-8 and III-3) available did not have mutations in the screened regions of rhodopsin. Although P347L is a well-known mutation, it did not seem to segregate with the disease phenotype. Mutational analysis of ***PRPF31*** in this family revealed that a T nucleotide deletion was found in the samples. This deletion causes a frameshift mutation (V25: F262***fs***X320) and creates a downstream premature stop codon leading to a truncated protein product ***(Figure 33)***. The affected individuals II-3, II-8, II-11 and III-3 were heterozygous for this mutation. When these results were combined with the P347L mutation of rhodopsin, all of the affected individuals available were confirmed to carry either one or both of these two mutations.

Thus far, a total of eleven mutations of ***PRPF31*** have been reported in four published RP studies ***(Table 14)***. With the exception of the two missense mutations, other mutations caused obvious protein sequence changes. The predicted protein carrying the F262***fsX***320 mutation would have 180 amino acids truncated when compared with the full-length wild type protein of 499 amino acids. Functional study of PRPF31 protein targeting showed that there was a nuclear localization signal (NLS) peptide sequence located between residues 351 and 364 of the protein (Deery et al, 2002). The peptide was believed to be a recognition signal for protein transportation to nucleus (Jans et al, 2000). The mutant protein with the NLS deletion accumulated in the cytoplasm but not in the nucleus. This experiment demonstrated that the NLS was important for PRPF31 nuclear transportation. The truncated protein of mutant F262***fsX***320 PRPF31 does not contain this NLS, as expected and, consequently, is expected to accumulate in cytoplasm and not enter

the nucleus. Whether protein accumulation in the cytoplasm would lead to pathological changes or not, the amount of functional protein in the nucleus is expected to be reduced.

Heterozygous carriers most likely lose 50% of the functional protein. Under normal circumstances, the remaining normal allele can cope with the required cellular physiology and, therefore, the inheritance is recessive. However, loss of one allele resulted in autosomal dominant inheritance for this family. PRPF31 is a ubiquitously expressed protein, but the disease phenotype only appears in retinal cells. It was suggested that one functional copy of ***PRPF31*** is insufficient because of the high metabolic demand of photoreceptors. The functional loss of one allele resulting in haplo-insufficiency was suggested as the underlying disease basis of RP11 adRP (Baehr & Chen, 2001). In a functional study of a yeast homolog, the tri-snRNP spliceosome complex formation was inhibited by the absence of the 61K protein product of ***PRPF31*** (Makarova et al, 2002). Consequently, the concentration of functional tri-snRNP in the cell was decreased by ~50%. This reduction in activity may affect the synthesis of other key proteins in the cell. This haplo-insufficiency existed only when the wild type allele could not compensate for the loss of functional activity in symptomatic carriers, which was not the case for asymptomatic carriers. An asymptomatic carrier was not found in this study, but might exist in the unavailable individuals of the third generation.

Table 14 Summary of ***PRPF31*** mutations reported to date.*

Locations	Mutation[#]	Protein alteration/Predicted change	References
Exon 5	402-414 del	H111_I114 del	Wang, 2003
Intron 5	IVS5-1G>A	S141fsX197	Xia, 2004
Intron 6	IVS6+3A>G	Inactivation of donor splice site	Vithana, 2001
Intron 6	IVS6-3 to –45 del	Inactivation of splice acceptor site	Vithana, 2001
Exon 7	581C>A	Ala194Glu	Vithana, 2001
Exon 7	580-581 dup 33 bp	Frameshift and insertion of 11 aa inframe	Vithana, 2001
Exon 7	646G>C	Ala216Pro	Vithana, 2001
Exon 8	732-737 indel 20bp	M244fsX248	Martinez-Gimeno, 2003
Exon 8	769-770 ins A	K257fsX277	Vithana, 2001 / Martinez-Gimeno, 2003
Exon 8	785 del T	F262fsX320	This study
Exon 8	828-829 del CA	H276fsX277	Martinez-Gimeno, 2003
Exon 11	1115-1125 del	Frameshift, 98 novel aa then STOP	Vithana, 2001

**** aa = Amino acid***
Indel = Insertion/deletion

[#]***Position 1 corresponds to the position 36 of sequence of GenBank accession number AL050369***

4.5 Mutations of *RHO* and *PRPF31* in family C8289

The most interesting finding was the combination of the two mutations in the same family of C8289 ***(Figure 20)***. The results of the mutational study of ***RHO*** and ***PFPF31*** revealed that only two genotypes of each gene were found in the available individuals. For ***RHO***, it was either normal or homozygous P347L. Therefore, both parents, I-1 and I-2, should be heterozygous for P347L. Again, there were four heterozygous carriers of F262***fsX***320 and one homozygote of wild type ***PRPF31*** among the five affected individuals available. The chance of two unrelated individuals having the same mutation of ***PRPF31*** is very low. Thus, the genotypes of the parents could probably be predicted as one being heterozygous for F262***fsX***320 and another being normal for ***PRPF31***.

Theoretically, the genotypes of I-1 and I-2 could give six possible genotypes to their offspring ***(Figure 39)***. However, heterozygotes for the ***RHO*** mutation (+/-), irrespective of the ***PRPF31*** genotype (+/- or -/-), and wild type homozygotes for both genes (P347L -/-; F262fsX320 -/-) were not observed in the affected individuals available, but might be found in other unavailable individuals. The combination of the homozygous P347L rhodopsin mutation with the wild type ***PRPF31*** (P347L +/+; F262***fsX***320 -/-) was identified in II-10. The combination of the heterozygous F262***fsX***320 ***PRPF31*** mutation with the wild type rhodopsin (P347L -/-; F262***fsX***320 +/-) was identified in II-3, II-8 and III-3. The combination of the homozygous P347L rhodopsin mutation with the heterozygous F262***fsX***320 ***PRPF31*** mutation (P347L +/+; F262***fsX***320 +/-) was identified in II-11. It is intriguing that II-11 inherited both mutations and was homozygous for the P347L mutation. Different combinations of these genotypes might produce different clinical manifestations among these RP patients.

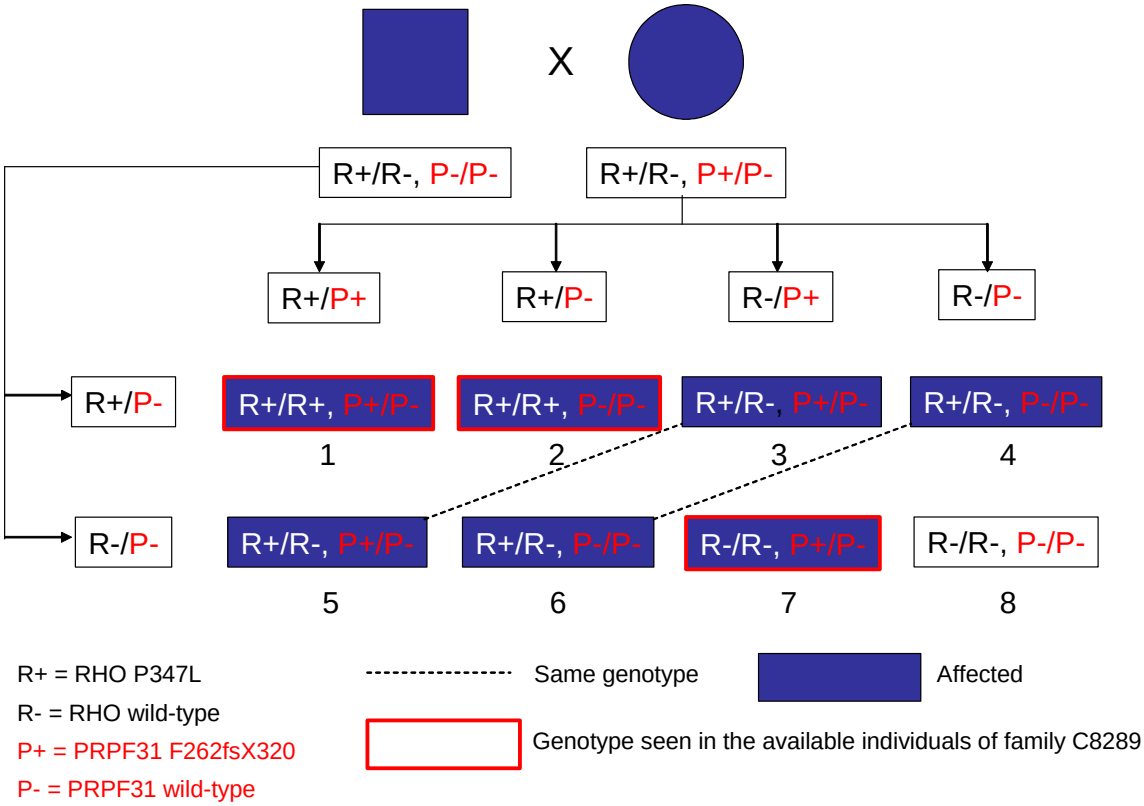


Figure 39 The six possible genotypes inherited from I-1 and I-2 individuals of family C8289. The same results are obtained if the genotypes of the two parents are exchanged.

4.5.1 Homozygosity of autosomal dominant mutation is rare

In this family ***(Figure 20)***, two of the affected sisters in the second generation were homozygous for the P347L mutation and two of them did not have this mutation. Therefore, their parents (I-1 and I-2) should be heterozygous for this autosomal dominant mutation. Theoretically, 75% of the offspring would inherit at least one copy of the mutation and hence be affected. In fact, this situation was quite rare under conditions of random mating. Both previously reported homozygous mutations in ***RHO***, V137M and G188R, were found in consanguineous families (Ayuso et al, 1996; Reig et al, 2000). However, I-1 and I-2 came from different families and cities of Mainland China. This made homozygous mutation due to consanguineous marriage in this family unlikely. When the homozygous mutation was considered, especially in the autosomal dominant mutation, other possibilities should be eliminated first. In addition, samples of other family members should be obtained for confirmation of this finding.

Deletion of a DNA segment in one chromosome could be one reason for the detection of apparent homozygosity. We could eliminate this possibility by measuring the gene dosage by quantitative methods using the same primers or by assessing neighboring polymorphic markers for heterozygosity. Single nucleotide polymorphism or microsatellite markers could be used for testing heterozygosity. For II-10 and II-11, the nearest SNP in the heterozygous state was -51G>A (V3) in this study. II-10 was heterozygous G/A, but II-11 was homozygous G/G at this position. Gross gene deletion seemed unlikely based on these results. However, other possibilities could not be eliminated, e.g., deletion of a segment of DNA or sequence variation in the PCR priming sites. Deletion of a DNA segment would most likely be a pathological event. Under this circumstance, irrespective of whether the patients are homozygous for the P347L mutation or are compound

heterozygous (heterozygous P347L mutation and heterozygous deletion of DNA segment), they would all have the disease phenotype.

Uniparental disomy occurs if one individual inherits the same two copies of DNA segment from one parent. As a result, the individual also becomes homozygous for this mutation. This situation was reported in one case of RP with a ***USH2A*** mutation (Rivolta et al, 2002)**.** To investigate this possibility, DNA samples from these patients should be analyzed with some closely linked microsatellite markers.

4.5.2 Homozygous vs heterozygous mutation

Are the disease outcomes of homozygous inheritance of an autosomal dominant mutation different from those of heterozygotes? To date, there have been only two reported mutations of rhodopsin found in homozygous RP patients, the V137M and G188R mutations in rhodopsin (Ayuso et al, 1996; Reig et al, 2000). However, there were no obvious differences between homozygous and heterozygous patients in disease severity. Indeed, the gene dosage effect of a mutant allele depends on the disease mechanism of that mutation. There were two main types of dominant mutations of rhodopsin, gain-of-function or dominant-negative mutations, and their outcomes were totally different. For a gain-of-function mutation, the mutant allele created a novel activity that stimulated an inappropriate process in the rod cell that was not affected by the wild type protein.

4.5.3 Gain-of-function mutations in rhodopsin

The class II mutations of rhodopsin always cause changes in protein folding and protein transportation. The P23H mutation was one example of a mutant protein that accumulated in the endoplasmic reticulum of the inner segment. This situation was similar to other degenerative diseases, e.g., Alzheimer's disease, characterized by

protein misfolding and aggregation (Stefani & Dobson, 2003). Protein aggregation was demonstrated to inhibit the ubiquitin-proteosome system that functions to degrade misfolded proteins inside the cell as a cellular quality control mechanism (Bence et al, 2001). A recent study revealed that the P23H mutant was prone to protein aggregation and was linked to inhibition of the ubiquitin-proteosome system that is toxic to the cell (Illing et al, 2002). This mechanism represents a toxic gain-of-function mutation. The G188R mutation also caused misfolding of rhodopsin and accumulation of the mutant protein; therefore, it was also a gain-of-function mutation (Liu et al, 1996).

On the other hand, the V137M mutation was shown to increase the initial activation rate of transducin (Andres et al, 2003). Excessive phototransduction signaling was suggested to play a role in retinal degeneration (Lem & Fain, 2004) and this inappropriate activity was the result of a gain-of-function mutation. The outcome of a gain-of-function mutation was that the dominant phenotype remained unchanged even though the ratio of mutant to wild type protein was changed (Bruijn et al, 1998). Therefore, gene dosage did not affect the phenotype and the clinical expression between homozygous and heterozygous states was similar.

4.5.4 P347L is a dominant-negative mutation

In contrast, dominant-negative mutations showed variability in effect, dependent on the amount of mutant proteins (Herskowitz, 1987). It was believed that the mutant protein disrupted the activity of the wild type. In studies of transgenic mice carrying the P347S mutation, the severity of retinal degeneration was shown to correlate with the level of transgene expression. The severity of degeneration was greater in mice homozygous for the transgene and in mice with a higher ratio of transgenic mutant protein (Li et al, 1996). Another study also demonstrated that post-Golgi

transportation was dependent on the concentration of the wild type rhodopsin (Deretic et al, 1998). Therefore, a gene dosage effect would be expected to be exhibited by patients carrying the homozygous and heterozygous P347L mutation.

However, these findings were from ***in vitro*** or transgenic mouse models. Thus far, all previous studies on the P347L mutation were based on patients with the heterozygous mutation. This finding was the first reported case for the P347L mutation in the homozygous form. The II-10 subject was homozygous for the P347L mutation but homozygous for wild type ***PRPF31 (Figure 20)***. Her clinical findings were typically early-onset, bone-spicule pigmentation, constricted visual field and abolished ERG in the second decade ***(Table 15)***. These signs appeared as a type I mutation of the rhodopsin gene. However, there was no heterozygous carrier found in this family. As mentioned before (Section 4.2.2), Oh et al (2003) showed that patients with the heterozygous P347L mutation had a poorer prognosis than those with the P23H mutation. They also agreed that the homozygous form would be more severe than the heterozygous form (Oh, personal communication). Although the actual clinical differences between homozygous and heterozygous forms could not be compared, all the offspring of II-10 and II-11 would be affected as expected. Clinical genetic counseling would be helpful for these individuals.

Table 15 Clinical findings in family C8289.

Samples	Mutation	Age	Onset Age	VA (logMAR)	VF (degree)	ERG	Bone-spicule Pigmentation	Macular lesion
II-1*	?	?	?	?	?	?	?	?
II-3	F262fsX320	48	5	1.08	6	NR	Y	N
II-5*	?	44	20	0.5	12	?	Y	N
II-7*	?	42	10	0.5	18	?	Y	N
II-8	F262fsX320	40	10	FC	8	NR	Y	Y
II-10	P347L	38	15	1	12	NR	Y	N
II-11	F262fsX320 & P347L	36	25	0.1	12	NR	Y	N
II-13*	?	33	15	0.18	48	NR	N	N
III-3	F262fsX320	25	4	0.3	18	NR	N	N

Sample not available for mutation screening; VA = Visual acuity; VF = Visual Field; FC = Finger count; ERG = Electroretinogram; NR = non-recordable; Y = Yes; N = No; ? = test not performed

4.5.5 Clinical heterogeneity

The clinical expression of these two mutations was variable. The age of onset ranged from 4 to 25 years. Individual II-8 was severely affected. For example, her visual acuity was markedly reduced and macular lesions were observed. However, II-13 had good residual visual field and no bone-spicule pigmentation. Intrafamilial clinical variability had been reported previously for the P347L and ***PRPF31*** mutations separately (Berson et al, 1991; Vithana et al, 2001). The existence of two mutations in this family could also explain the differences. Prediction of genotype-phenotype correlation was difficult because five other affected individuals were not available for mutational analysis.

Two categories of clinical expression could be classified by analysis of the available data. Except for II-11, the age of onset for the F262***fs***X320 mutation (≤10 years of age) seemed to be earlier than that for P347L. The residual visual field of II-3 and II-8 was less than 9° while that of II-10 was 12°. Individual II-8 was the most severely affected in that her visual acuity was the worst and the macula was involved. Therefore, the F262***fs***X320 mutation seemed to have a more dramatic effect than the P347L mutation. If more data could be obtained from the mutational analysis of the remaining members, this observation could be substantiated. On the other hand, the clinical data of II-11 were quite different from those of others. The age of onset of II-11 was quite late and her visual acuity was better preserved.

4.5.6 Unexpected findings

The presence of digenic RP and Bardet-Biedl syndrome (triallelic syndromic RP) suggests that gene-to-gene interactions probably play a role in disease development. In particular, the mutational analysis in an Usher family revealed that combinations of

mutations in the ***USH1B*** and ***USH3*** genes produced different phenotypes (Adato et al, 1999). A more severe form of Usher syndrome was found in a person harboring mutations from both genes. Subject II-11 of family C8289 also carried mutations of both ***RHO*** and ***PRPF31***.

Therefore, it would seem intuitive to expect that the additive effect of the two mutations would make the clinical outcome of II-11 more severe than others. However, this was not the case. From the clinical data of this family, the severe phenotype was not noticeable in II-11. What are the possible causes of this phenotypic expression from two mutations? There are four possibilities: 1) no additive effect of these two mutations; 2) the effects of the ***RHO*** or ***PRPF31*** mutation override the other; 3) incomplete penetrance of the ***PRPF31*** mutation in II-11; or 4) unknown mechanisms.

The compound effects from these two gene mutations were expected for two reasons. First, mutations in any one of these genes should lead to the same disease phenotype, such that the two proteins may act in the same functional pathway of the photoreceptor. In addition, the functional properties of PRPF31 are mainly for mRNA splicing and rhodopsin is a massively produced key protein in the photoreceptor (93% of total protein of ROS) (Makino et al, 1977). The subsequent effect of reducing splicing efficiency due to ***PRPF31*** mutations would be a reduced amount of functional rhodopsin. Therefore, the disease phenotype should be more severe. However, the results did not show that the disease phenotype was more severe with these two mutations combined. Thus, they might not act in the same pathway of disease development, or their effects might not be additive.

Alternatively, a “two-threshold nondiscrete model” may explain this phenomenon (Dipple & McCabe, 2000). In this model, a uniform phenotype will be observed when the upper or lower level of mutant protein function is reached. An indeterminate range is between these two levels, in which the phenotype is variable and is affected by additional unlinked genes and/or environmental factors. From this proposal, either the level of ***PRPF31*** or ***RHO*** mutant or both reached the upper range so that the phenotype was uniform and was not affected by each other. There was only one individual who was homozygous for the ***RHO*** mutation with wild type ***PRPF31*** in this family. In addition, the homozygous form of the P347L mutation was not reported before. Clinical variability in the homozygous form could not be substantiated. The variability of clinical expression in this family was obvious only in the individuals with the ***PRPF31*** mutation. Therefore, this explanation was true only if homozygous ***RHO*** mutation reached the upper level.

Finally, the clinical expression of the ***PRPF31*** mutations might show an incomplete penetrance so that asymptomatic carriers might be present in affected families. There was no asymptomatic carrier found in the C8289 family. All mutations were expressed fully and caused RP. However, there might be a masking effect if two mutations co-existed in the same individual. Individual II-11 could be an asymptomatic carrier for the ***PRPF31*** mutation but symptomatic for the ***RHO*** mutation. Overall, II-11 was symptomatic. Under this assumption, there was no additive effect because the ***PRPF31*** mutation did not exert any effect in the II-11 individual.

4.5.7 The complexity of RP

Unveiling of genotype-phenotype correlation is one of the ultimate goals in studying genetic disease. RP is no exception. As with other complex diseases, the genotype-phenotype correlation of RP cannot be explained simply by Mendelian principles alone. To solve this problem, defining the RP phenotype and its cause is critical. RP is in fact a group of diseases exhibiting a similar disease phenotype in that retinal degeneration happens primarily in the rod photoreceptor. Although RP can be caused by mutations in many different genes, their final ultimate pathway is the same - apoptosis. Apoptosis is the cause of retinal degeneration. Therefore, the question is how the mutations trigger apoptosis and lead to degeneration.

For the P347L mutation, the pathological effect of mutation was cell death caused by a reduced amount of mutant protein in outer segments or accumulation of mutant protein in the inner segment. For the F262fsX320 mutation, there were also two possible mechanisms leading to cell death: reduction of key protein production such as rhodopsin, or other unknown mechanisms such as accumulation of mutant protein in the cytoplasm due to the loss of the nuclear localization signal peptide.

Based on these observations and assumptions, two scenarios are proposed ***(Figure 40)***. **(A)** In the first scenario, if II-11 is asymptomatic and has the F262fsX320 mutation, the overall effect would be caused only by the P347L mutation. If clinical variability is shown in the homozygous P347L mutation, it could be contributed to by other factors. However, no additional effect would be observed if the P347L mutation reaches the uniform level of “two-threshold nondiscrete model” as explained above.

(B) In second scenario, the overall outcome is either reduced apoptosis or no change. For ***PRPF31***, it is assumed that the F262fsX320 mutation demonstrates complete penetrance in II-11 and F262fsX320 affects the amount of rhodopsin production due to haplo-insufficiency of PRPF31. At the same time, the apoptosis caused by P347L is triggered by the accumulation of mutant protein in the photoreceptors. Because all rhodopsin protein would be mutant in this individual, the amount of accumulated mutant in the photoreceptors would be decreased by the F262fsX320 mutation. Consequently, the level of accumulated mutant protein necessary to trigger apoptosis is also decreased. As a result, the two opposite effects would cancel out so that no overall change would be observed. Alternatively, the overall effect would be reduced if one side was more dominant. These scenarios are only simplified models to explain the effects of combinations of two mutations in the same individual. The actual situation will be more complex and should be further investigated by other studies. However, the significance of these findings is a way to elucidate the molecular basis of RP.

4.5.8 Significance of findings in family C8289

This family will provide an excellent “model” for studying RP because it includes individuals with the first reported homozygous P347L mutation. Although the accumulation of mutant protein was found in the P347L mutation, most of the mutant proteins could be located in the outer segments and could function normally in the heterozygous form and animal models. The pathway of protein transport and the triggering of apoptosis by the mutant protein are still unclear. The homozygous form of the P347L mutation provides a genetic background without the interference of the wild type protein to study the mechanism of disease. The ability to study individuals

with this mutation moves the study of RP from the animal model to the real-life situation.

It is worth emphasizing that the novel finding of a family harboring two different mutations is helpful in the study of disease mechanism. It will be interesting to discover the interactions between two autosomal dominant mutations in the same individuals. In particular, the underlying disease mechanism of the mutation of the ubiquitously expressed ***PRPF31*** remains a mystery. The effects of mutations in ***PRPF31*** were found only in the photoreceptor probably because there is a high metabolic demand. Rhodopsin is a major protein and has a high turnover in the photoreceptor. Therefore, it is intuitive that the production of rhodopsin affected by mutation of ***PRPF31*** would be the major cause of the disease. Further investigation of the effects of the mutations in these two key proteins in the same individual could provide an answer.

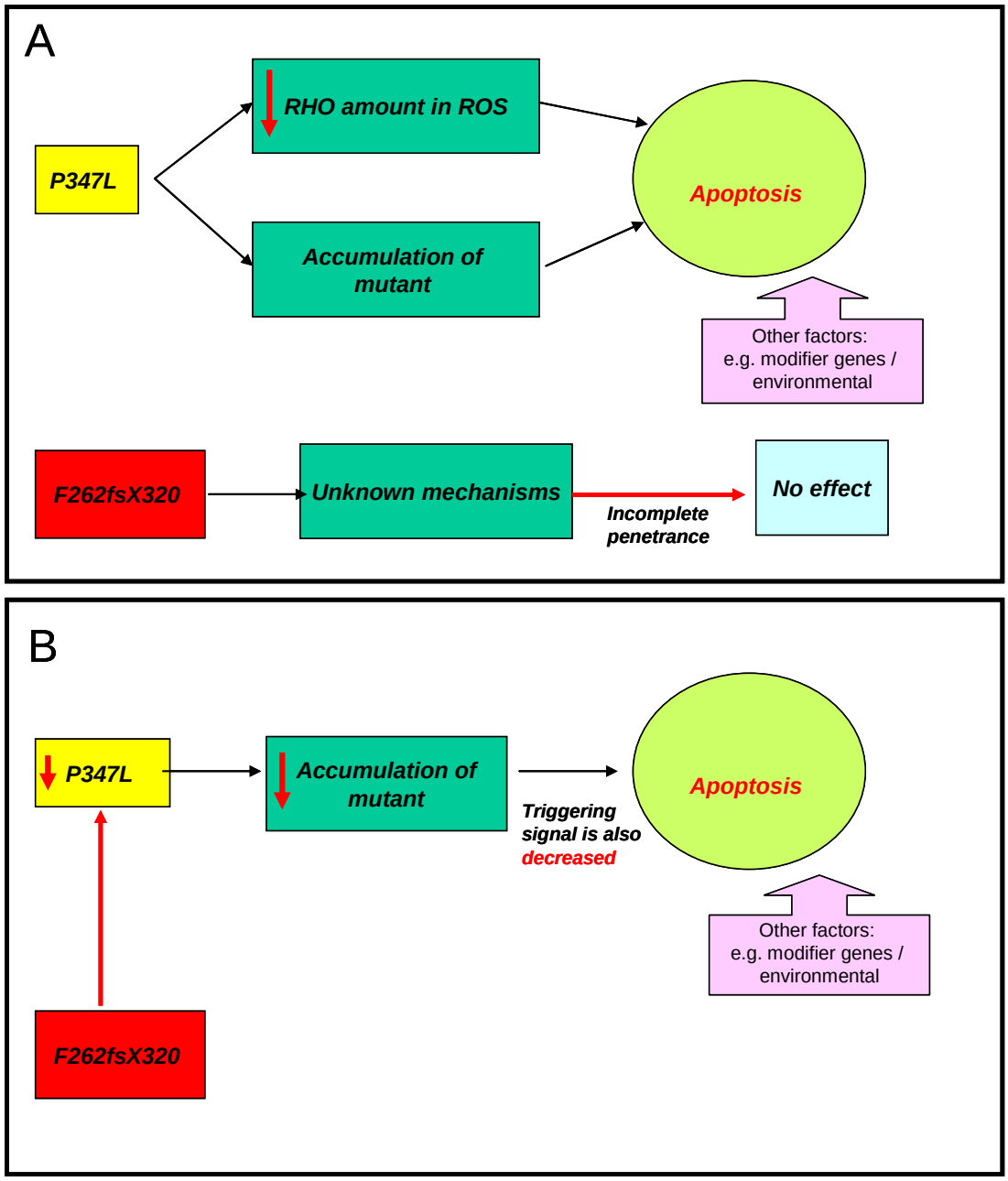


Figure 40 Two proposed scenarios to explain the possible effects of combination of P347L and F262***fs***X320 mutations in the same individual. ROS = rod out segment. See text for explanation.

4.6 Mutation frequencies of studied genes

4.6.1 RHO

In RP patients, the prevalence of ***RHO*** mutations varies from population to population: 25-30% in British or American, 7% in German and 2.5% in Japanese (Inglehearn et al, 1992; Sung et al, 1991; Bunge et al, 1993; Budu et al, 2000). The reported frequency in a study of Chinese RP was about 3.6% (Zhang et al, 1999). In the present study of Chinese RP in Hong Kong, the frequency of ***RHO*** mutation was about 1.69% (1/59 cases), which was similar to the finding of another study in Hong Kong Chinese RP (Chan et al, 2001). Therefore, the prevalence of this mutation is lower in RP cases in Hong Kong Chinese than in other western populations.

The P23H missense substitution was the first mutation identified in RP (Dryja et al, 1990a). It was estimated that about one third of rhodopsin mutations consisted of P23H in the USA (Dryja et al, 1991). Although the prevalence of ***RHO*** mutations was also high in the British RP population, the P23H mutation was not found in RP in the British population (Inglehearn et al, 1992). Similar findings that P23H was a rare mutation in other populations have been reported (Bunge et al, 1993; Wada & Tamai, 2003). A “founder effect” is the most likely explanation of these observations (Inglehearn et al, 1992). In contrast, mutations of codon 347, which were the second most common in the USA, were also identified in other populations. The results of mutation screening in this study and another similar study in Hong Kong were consistent with these observations (Chan et al, 2001). Thus, the P23H mutation was extremely rare but the P347L mutation was found in Hong Kong RP patients. It is probable that there is less selective pressure on the P23H mutation because it causes a mild and late onset of RP than the mutations involving P347.

4.6.2 RDS

On the other hand, the frequency of the ***RDS*** mutations in RP was quite similar between different populations, with prevalence of about 3.5% in the USA and Canada, 3% in Spain and 2.2% in Japan (Dryja et al, 1997; Trujillo et al, 1998; Budu et al, 2001). To date, only two mutational analyses of ***RDS*** have been performed in China. One study did not find any mutation in a group of 83 RP patients while another study identified a previously reported mutation in a single family (Yang et al, 2000; Zhang et al, 1998). Therefore, this study is the first report of a novel ***RDS*** mutation in Chinese RP patients and a prevalence of 1.7% of the RDS mutation in Chinese RP is estimated from this study. This frequency indicates that the ***RDS*** mutation is rare in Chinese RP patients. Digenic inheritance of human disease was initially discovered in RP due to ***RDS*** and ***ROM1*** mutations. In a digenic RP family, only the individual with double heterozygous mutations in both ***RDS*** and ***ROM1*** is affected. Therefore, an asymptomatic individual may carry at least one of the mutations and a symptomatic individual would carry both of the mutations. There was no RP family observed having this inheritance pattern in the present study. Therefore, digenic RP is not commonly found in Chinese RP.

4.6.3 PRPF31

There have been very few mutational studies of the ***PRPF31*** gene in RP. Approximately 6% of sporadic RP in the UK and 2% of adRP in Spain were identified with mutations in this gene (Vithana et al, 2001; Martinez-Gimeno et al, 2003). Other studies were conducted in the Chinese RP population but only a single family was studied in each case (Xia et al, 2004; Wang et al, 2003). The present study is the first mutational study of ***PRPF31*** in Chinese RP across a number of RP families. The frequency of the ***PRPF31*** mutation in Chinese RP was about 1.7% if only the F262***fs***X320 mutation was counted, but it was about 5% if both F262***fs***X320 and S2C mutations were included. This frequency should be confirmed by further investigation of the role of S2C in RP and other mutational studies of ***PRPF31*** in Chinese.

4.7 Further investigations

The designation of mutations was based on the criteria suggested by Cotton and Scriver (1998) as a guideline to distinguish between disease-causing mutations and neutral polymorphisms. Three mutations found in this study are most probably disease-causing. They all co-segregated with the disease phenotype and produced malfunctional proteins. The P347L mutation was a previously reported mutation and its effects on rhodopsin have been well studied in several animal models. Therefore, it undoubtedly caused the disease phenotype in the affected patients of this study.

The other two mutations, A78fsX176 of ***RDS*** and F262fsX320 of ***PRPF31***, are novel findings. As discussed before, the frameshift alterations would cause the truncated proteins to lose some important protein domains. Although this feature results in a significant alteration, the deleterious effects of these mutations should be confirmed by functional studies. The benefits of studying their functional changes are to not only provide a proof of mutation but also unveil the disease mechanisms of these mutations. In particular, the primary effects of mutations of ***PRPF31*** on retinal degeneration are still unclear.

Animal models provide a useful tool for studying mutations in RP. The well-known P347L mutation has been investigated in transgenic pigs (Mahmoud et al, 2003). To determine the effect of the A78fsX176 of ***RDS*** and F262fsX320 of ***PRPF31*** mutations, transgenic animal models could also be used. After the mutations are induced in the animals, the physiological and pathological changes of the retina can be studied to see whether the mutations would cause retinal degeneration or not. In addition, the effects of the combination of two mutations, P347L of ***RHO*** and

F262fsX320 of ***PRPF31***, could be investigated by studying animals carrying both mutations.

To confirm the two ambiguous mutations, -300_-302delTTT of ***RHO*** and S2C missense of ***PRPF31***, other strategies can be used. The alteration of ***RHO*** expression is predicted from the variation -300_-302delTTT because the change may disrupt the binding site of the putative regulatory region. Therefore, the comparison of gene expression between mutant and wild type can confirm the effect of the alteration. The gene reporter assay and mobility shift assay are two useful techniques to study the effects of deletions on regulation of gene expression. When the mutation abolishes the binding site of a transcription factor in the promoter, the signal production in a gene reporter assay would be increased due to the negative control of this region of rhodopsin promoter. There may also be differences in the patterns of mobility shift assay between mutant and wild type sequences. However, signal production would be decreased as a result of the mutant sequence motif increasing the binding affinity to the transcription factor.

Indirect evidence supports the premise that the second amino acid of PRPF31 is an important amino acid in protein function. However, S2C did not appear to segregate with disease phenotype in the C8734 family, which may be explained by incomplete penetrance. To prove that S2C is a disease-causing mutation, two approaches can be used. First, recruitment and genotyping of the remaining family members would further clarify the incomplete penetrance in these families. In addition, a more comprehensive study of the allelic expression of ***PRPF31*** would support the view that the disease phenotype may be due to the insufficient compensation of wild type alleles in affected individuals.

Subsequently, the effect of missense mutations on protein function can be studied by functional assays. To investigate the effect of a missense mutation on gene expression, a reporter gene assay containing the plasmid contruct of the mutant can be studied. On the other hand, the effect of a missense mutation on co-translational processing can be investigated by ***in vitro*** synthesis assays of site-directed mutants of ***PRPF31***.

Chapter 5 Conclusions

5 Conclusions

In conclusion, three mutations of the studied genes were found in the 59 RP families: the previously reported P347L mutation of ***RHO***, a novel frameshift mutation (A78fsX176) of ***RDS*** and a novel frameshift mutation (F262fsX320) of ***PRPF31***. The mutations of ***RHO*** and ***PRPF31*** were found in the same family with different combinations of genotypes. The most intriguing result was that one of the affected family members inherited both mutations. This individual was homozygous for the P347L mutation and heterozygous for F262fsX320, but no additive effects of these mutations were observed from the clinical manifestations. It was suspected that this outcome was due to the incomplete penetrance of F262fsX320 in this patient and/or was caused by some unknown mechanisms.

Two suspected mutations were also identified in this study. A novel deletion of 3 bp (-300_-302delTTT) in the promoter of ***RHO*** was found in a patient with Usher syndrome. This sequence variation was located within the putative negative regulatory element. It was suspected to interfere in the expression of ***RHO*** and cause disease phenotype because either over- or under-expression of ***RHO*** would cause degeneration of photoreceptors. The S2C missense variation is also a novel finding of ***PRPF31***. It is a highly conserved amino acid and its position in amino acid sequence indicates it is a functionally important amino acid. This variation was found in two RP families. Although it existed in both affected and normal members of one family, incomplete penetrance of the mutation could be put forward to explain the individual unaffected by carrying the S2C mutation. However, other possibilities cannot be completely excluded.

When only the three mutations (***RHO***: P347L; ***RDS***: A78fsX176, and ***PRPF31***: F262fsX320) were included in the calculation, the frequency of ***RHO*** mutation was about 1.7% (1/59 cases), which was similar to the finding of another study in Hong Kong Chinese RP patients (Chan et al, 2001). Therefore, the prevalence was lower in Hong Kong Chinese RP than in other western populations of RP. Mutation screening of the ***RHO*** promoter in RP revealed that the ***RHO*** promoter did not harbor common mutations leading to RP. Similar frequencies of ***RDS*** and ***PRPF31*** mutations (1.7%) were also found in this study, indicating that mutations of these genes were not prevalent in Hong Kong Chinese RP patients. In addition to mutations, 18 novel sequence variations were identified and their allele frequencies were established.

Although there are still unknown disease mechanisms to be elucidated and suspected mutations to be confirmed, the well-known feature of the P347L mutation and malfunction of the truncated proteins caused by frameshift mutations are probably the cause of RP in the patients under study. Therefore, genetic counseling is an immediate action that should be provided for the affected families.

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Appendix

Amino acid	One letter code	Three letter code	Possible codons
Alanine	**A**	Ala	GCA, GCC, GCG, GCT
Asparagine or Aspartic acid	**B**	Asx	AAC, AAT, GAC, GAT
Cysteine	**C**	Cys	TGC, TGT
Aspartic acid	**D**	Asp	GAC, GAT
Glutamic acid	**E**	Glu	GAA, GAG
Phenylalanine	**F**	Phe	TTC, TTT
Glycine	**G**	Gly	GGA, GGC, GGG, GGT
Histidine	**H**	His	CAC, CAT
Isoleucine	**I**	Ile	ATA, ATC, ATT
Lysine	**K**	Lys	AAA, AAG
Leucine	**L**	Leu	CTA, CTC, CTG, CTT, TTA, TTG
Methionine	**M**	Met	ATG
Asparagine	**N**	Asn	AAC, AAT
Proline	**P**	Pro	CCA, CCC, CCG, CCT
Glutamine	**Q**	Gln	CAA, CAG
Arginine	**R**	Arg	AGA, AGG, CGA, CGC, CGG, CGT
Serine	**S**	Ser	AGC, AGT, TCA, TCC, TCG, TCT
Threonine	**T**	Thr	ACA, ACC, ACG, ACT
Valine	**V**	Val	GTA, GTC, GTG, GTT
Tryptophan	**W**	Trp	TGG
stop codon	**X**	X	TAA, TAG, TGA
Tyrosine	**Y**	Tyr	TAC, TAT
Glutamine or Glutamic acid	**Z**	Glx	CAA, CAG, GAA, GAG